DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MD 21702-5012

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ERRATA - AD-B148458

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SGRD-RMI-S (70-1y)

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DEVELOPMENT OF METHODS FOR CARRIER-MEDIATED TARGETED DELIVERY OF ANTIVIRAL COMPOUNDS USING MONOCLONAL ANTIBODIES

Annual/Final Report

Marcia I. Dawson, Ph.D.
Robert W. Sidwell, Ph.D.
Bill B. Barnett, Ph.D.

January 1, 1990

Supported by

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-86-C-6120

Submitted by

SRI International
333 Ravenswood Avenue
Menlo Park, California 94025

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No 0704-0188

1a. REPORT SECURITY CLASSIFICATION: UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY: N/A	3. DISTRIBUTION/AVAILABILITY OF REPORT: Distribution authorized to U.S. Government agencies only; Proprietary information, 11/16/86
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE: N/A	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): SRI LSU-1956-4	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: SRI International; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): 333 Ravenswood Avenue, Menlo Park, California 94025	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable): SGRD-RMI-S	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-86-C-6120
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21702-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 63002A; PROJECT NO 3M263002D807; TASK NO. AD; WORK UNIT ACCESSION NO 032

11. TITLE (Include Security Classification)
DEVELOPMENT OF METHODS FOR CARRIER-MEDIATED TARGETED DELIVERY OF ANTIVIRAL COMPOUNDS USING MONOCLONAL ANTIBODIES

12. PERSONAL AUTHOR(S)
Marcia I. Dawson, Ph.D.; Robert W. Sidwell, Ph.D.; Bill B. Barnett, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual*/Final	FROM 04/01/86 TO 12/31/89	1990 January 1	237

16. SUPPLEMENTARY NOTATION
Prepared with the cooperation of Utah State University, Logan, Utah
*Annual covers the period of time April 1, 1989 - December 31, 1989

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	03	
06	01	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Synthesis and biological evaluation of antiviral drug (ribavirin)-monoclonal antibody conjugates for treatment of Pichinde virus infections: RA1.

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

SRI International and Utah State University developed methodology to assess the effectiveness of antiviral drug-monoclonal antibody conjugate therapy for the treatment of viral infections. Specifically, monoclonal antibodies to Pichinde virus surface antigens were prepared and conjugated to the antiviral drug ribavirin, and the resulting conjugates were screened for antiviral activity in vitro. Immunoconjugates were inactive because the monoclonal antibody did not internalize. Subsequent work using a polyclonal antibody produced an immunotoxin that had antiviral activity. Animal model systems were developed for in vivo evaluations.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Virginia M. Miller	22b. TELEPHONE (Include Area Code): 301/663-7325; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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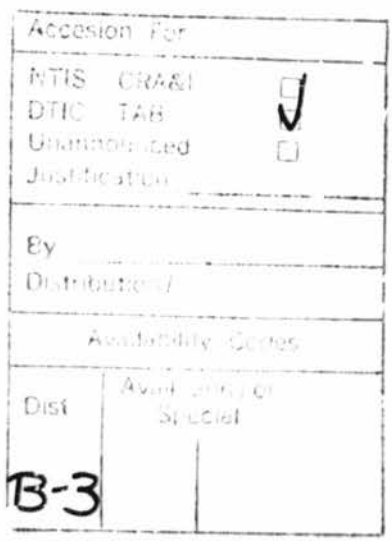

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OBJECTIVE AND SPECIFIC AIMS

The overall objective of this research was to improve the antiviral efficacy of virus inhibitory compounds by targeting their delivery to virally infected cells. This delivery was to be facilitated by means of monoclonal antibodies specific for the surface-expressed viral antigens. To accomplish this goal we proposed to:

(1) Develop tethering methodologies for the conjugation of ribavirin to monoclonal antibodies, using tether groups of various stabilities, such as ketals, esters, dialkyl phosphates and disulfides.

(2) Raise monoclonal antibodies against specific Pichinde virus antigenic determinants expressed on cells infected with Pichinde virus. These antibodies were to be nonneutralizing or only weakly neutralizing.

(3) Prepare, purify and characterize monoclonal antibody-drug conjugates.

(4) Evaluate the efficacy of monoclonal antibody-drug conjugates against Pichinde virus infections in an in vitro system.

(5) Evaluate the efficacy of conjugates antivirally active in vitro against animal infections with Pichinde virus. The efficacy, expressed as therapeutic index, to be compared with that of ribavirin without antibody, the antibody without ribavirin, and a mixture of the antibody and ribavirin.

The responsibility for performing these tasks was divided between SRI International and Utah State University investigators as follows:

SRI International investigators were to develop the methodologies described in the first specific aim and prepare and purify the antibody-ribavirin conjugates described in specific aims 3, 4, and 5.

Utah State University investigators were to derive, produce, and characterize the monoclonal antibodies described in the second specific aim and to perform the evaluations of the ribavirin derivatives and the antibody-ribavirin conjugates as described in specific aims 3, 4, and 5.

RESEARCH SUMMARY

The hemorrhagic fevers are debilitating lethal diseases for which no vaccine is available. The antiviral drug ribavirin has demonstrated therapeutic efficacy for the treatment of Pichinde virus-induced hemorrhagic fever in laboratory animals. Unfortunately, its systemic side effects preclude therapeutic use in humans. Targeted delivery of ribavirin to virally infected cells alone would reduce side effects, improving the therapeutic index to a clinically useful level. Targeted delivery of antiviral drugs using monoclonal antibodies (MAbs) was explored as a method for achieving this goal.

In the 3.5 years of USAMRDC Contract No. DAMD17-86-C-6120 (Development of Methods for Carrier-Mediated Targeted Delivery of Antiviral Compounds Using Monoclonal Antibodies) considerable progress was made to develop methodology for coupling ribavirin to a MAb directed against an antigen expressed on the surface of Pichinde virus-infected cells.

Research was conducted to: (1) synthesize derivatives of ribavirin that would permit attachment of this antiviral drug to MAbs, (2) covalently link these ribavirin derivatives through their tether groups to MAbs directed against the viral antigens expressed on the surface of Pichinde virus-infected cells, and (3) develop in vitro and in vivo Pichinde virus models for use as test systems for evaluating the targeted delivery of antiviral compounds using MAbs against Pichinde virus-coded antigens expressed on the surface of virally infected cells.

SYNTHETIC PROGRAM

Task I. Synthesis of Ribavirin Derivatives Having Tether Group Functionality and Their Conjugation to Anti-Pichinde Virus Monoclonal Antibodies

The synthetic accomplishments were:

(1) Synthesis of modified ribavirin derivatives for conjugation to MAb PC4.9A6 directed against an antigen expressed on the surface of Pichinde virus-infected cells. The following were prepared: (a) ribavirin-5'-*O*-hemisuccinate, (b) ribavirin-5'-*O*-(2,3-dimethyl)hemisuccinate, (c) 2',3'-*O*-ketal of ribavirin with 4-ketopentanoic acid, (d) 2',3'-*O*-ketal of ribavirin with 5-ketohexanoic acid, (e) ribavirin-5'-*O*-phosphate, (f) ribavirin-5'-*O*-diphosphate, (g) ribavirin-5'-*O*-triphosphate, (h) ribavirin-5'-*O*-(5-carboxypentyl)phosphate, and (i) ribavirin-5'-*O*-(6-aminohexyl)phosphate. The 5'-*O*-hemisuccinate ester was found to be too hydrolytically labile for stability in the plasma. The 5'-*O*-(2,3-dimethyl)-hemisuccinate was too stable because insufficient ribavirin was released, as determined by its antiviral activity in an immunofluoresence assay against Pichinde virus infection in Vero 76 cells. The same was true of the two 2',3'-*O*-ketals. Ribavirin-5'-*O*-phosphate, and the corresponding 5'-*O*-diphosphate and 5'-*O*-triphosphate displayed antiviral activity, indicating that phosphate esters could be possible tethering functions. Various routes were explored for linking ribavirin-5'-*O*-(5-carboxypentyl)phosphate to MAbs and polymeric carrier molecules; none was successful although the reaction could be readily performed on analogs lacking a 3'-hyroxyl group. Evidently the hydroxyl group prevented activation of the phosphate and its linkage. Therefore, the functional groups were reversed and the 6-aminohexyl tether was introduced onto the 5'-*O*-phosphate.

(2) Because ribavirin has such a weak UV chromophore, methods were investigated to establish loading of the drug onto polymeric carriers and MAbs. This necessitated the synthesis of radiolabeled ribavirin from [1'-^{3}H]- and [3'-^{14}C]ribose. This material was

subsequently converted to the labeled 5'-*O*-hemisuccinate and 5'-*O*-(5-carboxypentyl)-phosphate derivatives. The former was used to prepare an immunoconjugate with MAb PC4.9A6. The latter could not be tethered through an amide bond to either a carrier or MAb. Other assay methods investigated were determination of amino groups removed from the MAb after conjugation and a radioimmunoassay for ribavirin. Both of these methods were inaccurate compared with radiolabel determination. A phosphorus assay was developed to establish the loading of the ribavirin-5'-*O*-phosphate derivatives onto polymeric carriers, obviating the need for using radiolabeled material.

(3) Methods were first investigated for direct loading of the functionalized ribavirin derivatives onto the MAb. The 5'-*O*-hemisuccinate was investigated first. Unfortunately, direct conjugation of this derivative of ribavirin to MAb PC4.9A6 afforded a loading of only four drugs per antibody--a loading deemed insufficient for antiviral activity because of the high ED_{50} value (3.2-10 μg/mL) of ribavirin. Loading could be enhanced using a polymeric carrier. (Aminohexyl)dextran was prepared. It was not possible to produce any adduct of this polymer with ribavirin-5'-*O*-(5-carboxypentyl)phosphate because of the presence of the 3'-hydroxyl group on the latter. Therefore, (carboxymethyl)$_{55}$dextran was used as the carrier to which 4.6 to 9.6 ribavirin-5'-*O*-(6-aminohexyl)phosphate molecules were linked. The ribavirin-dextran polymer having a loading of 4.6 ribavirins per carrier was linked to MAb PC4.9A6, giving a conjugate having 33 ribavirin molecules per antibody--a ninefold higher loading than by the first approach. The level of loading of a ribavirin-dextran adduct onto the MAb and therefore that of the drug was established using a fluorescein hydrazide tethered to the (carboxymethyl)$_{55}$dextran. A variety of methods were investigated for separating the ribavirin-dextran-antibody conjugate from free antibody and ribavirin-dextran polymer. Usual purification methods used for the preparation of immunoconjugates failed; however, sucrose density centrifugation purified the ribavirin-5'-*O*-(6-aminohexyl)phosphate-(carboxymethyl)dextran-MAb PC4.9A6

conjugate. This material was inactive against Pichinde virus infection in Vero 76 cells. The lack of activity may have been caused by the fact that MAb 4.9A6 is not internalized by the infected Vero 76 cell.

(4) Methods were investigated for synthesizing a conjugate having higher loading. A pentadecamer of ribavirin-5'-*O*-phosphate [5'-*O*-(6-aminohexyl)phosphate of poly-(ribavirin-3',5'-*O*-phosphate)$_{15}$-thymidine-5'-*O*-phosphate] was synthesized in 14% overall yield. This oligoribonucleotide analog had a 6-aminohexyl group at its 5'-*O*-phosphate ester terminus for linkage to a polymeric carrier and a thymidine at the 3'-terminus in order to determine loading by spectroscopic methods. Biological evaluation indicated that this polymer was inactive against Pichinde virus infection in Vero 76 cells. The lack of activity may be due to lack of uptake by the cells--a situation that would be corrected using a conjugate prepared from an antibody that is internalized.

(5) Model studies were conducted to develop methodology for attachment of the ribavirin oligomer to a carrier protein. The 5'-*O*-(6-aminohexyl)phosphate of poly-(thymidine-3',5'-*O*-phosphate)$_{12}$-thymidine-5'-*O*-phosphate was used for this purpose. Loadings of 1.5 oligomers per protein G as carrier protein were achieved. This technology was not transferred to the ribavirin oligomer after it was established that MAb PC4.9A6 did not internalize.

In summary, in this synthetic program we successfully developed methodology for tethering the antiviral drug ribavirin to MAbs at loadings of 4 to 33 ribavirin molecules per protein molecule.

BIOLOGICAL ASSAY DEVELOPMENT AND EVALUATION

One of the primary tasks of the USU investigators was to produce MAbs to be used for the production of the antibody-drug conjugates. The fusion procedure described in this report was used to derive more than 50 antibody producing hybridoma cell lines. Four of

these cell lines, PC4.7C2, PC4.8D2, PC4.9D3, and PC4.9A6, produced antibodies that recognized Pichinde virus antigens expressed on the surface of Pichinde virus infected cells in vitro and in vivo. None of the MAbs displayed Pichinde virus neutralizing activity in vitro or in vivo. This lack of protective activity was a desired characteristic since any antiviral activity would then be attributable to the antibody-drug conjugates and not an antiviral activity inherent in the antibody. Fluorescein conjugates of MAbs PC4.9A6 and PC4.9D3, administered intraperitoneally, were shown to concentrate in the virus target tissues of Pichinde virus-infected hamsters reaching at least 10-fold greater levels in the livers of infected animals than in the livers of noninfected animals.

USU investigators developed an immunofluorescence assay and used it to determine the antiviral activities of a series of ribavirin derivatives, produced by SRI investigators. A particularly promising candidate for drug tethering to antibodies, ribavirin 5'-hemisuccinate, was found to have an ED_{50} of 10 to 32 µg/mL and an MTD of 1,000 µg/mL, while a more stable derivative, ribavirin 5'-*O*-(2,3-dimethyl)hemisuccinate, had an ED_{50} of 100 to 320 µg/mL and an MTD of 32 µg/mL.

Through the course of producing ribavirin-antibody conjugates, SRI International investigators noted that 3 to 6 ribavirin molecules could be attached to a single MAb molecule, but the yields on these conjugations were low and the loading of ribavirin onto the conjugates was too low to be evaluated in in vitro antiviral assays. Strategies for multiple loading of ribavirin to carrier molecules were initiated by SRI investigators. One of the approaches for multiple loading of ribavirin was to synthesize ribavirin polymers linked through phosphodiester linkages. Several phosphorylated forms of ribavirin, corresponding to ribonucleoside phosphates, were synthesized and shown to retain in vitro antiviral activity against Pichinde virus. Another SRI International approach to multiple loading was based on dextran polymers. Ribavirin derivatives suitable for multiple loading onto dextran were synthesized and used to produce a (carboxymethyl)dextran-ribavirin

conjugate. The initial ribavirin derivative retained antiviral activity. The dextran multiply loaded with ribavirin was targeted by conjugation to a Pichinde virus specific antibody.

A problem that continually confronted our efforts with ribavirin immunoconjugates was that ribavirin must be present in relatively high concentrations to exert an antiviral effect. Therefore multiple loading technologies and compatible delivery systems were developed for ribavirin so that the application of antibody-mediated delivery of ribavirin would be more likely to yield strong virus inhibitory activity. SRI International investigators produced high-density ribavirin immunoconjugates and provided such a conjugate to USU investigators.

Until immunodrugs for evaluating antibody mediated delivery to arenavirus-infected cells were available, USU investigators produced and studied several immunotoxins as potential anti-Pichinde virus therapies. The rationale behind the immunotoxin approach was to utilize the targeted delivery of a peptide cytotoxin to kill the virus-infected cells in culture or in an animal, thereby sparing the remaining noninfected cells from being overwhelmed by the viral infection.

MAb PC4.9A6 was conjugated to gelonin through a disulfide linkage. The PC4.9A6-gelonin immunotoxin retained the antigen-binding activity of MAb PC4.9A6 and the anti-ribosomal toxicity of gelonin, but lacked specific cytotoxicity towards Pichinde virus-infected cells. Apparently the PC4.9A6-gelonin immunotoxin was not internalized by target cells. Because the lack of antiviral activity appeared to be a characteristic of MAb PC4.9A6, polyclonal antibodies isolated from hyperimmune rabbit sera (HRS) were subsequently conjugated to gelonin.

Strong antiviral activity against Pichinde virus was demonstrated using polyclonal antibodies conjugated with gelonin. A single treatment with HRS-gelonin at 20 h p.i. reduced the virus yield by over 1,000-fold with no detectable nonspecific cytotoxicity. The ED_{50}, MTD and therapeutic index for HRS-gelonin immunotoxin were each more

favorable than the corresponding parameters for ribavirin. The ED_{50} for HRS-gelonin was 0.016 μM compared with 40 μM for ribavirin, indicating that one molecule of the HRS-gelonin immunotoxin mediated an antiviral effect equivalent to that of 2,500 molecules of ribavirin. The anti-Pichinde virus immunotoxins offer a promising avenue for the development of antiviral therapies utilizing antibody-mediated delivery of antiviral substances. Alternately, this work indicates that MAbs could be produced that would effectively deliver antiviral drugs within Pichinde virus-infected cells.

Task II. In vitro Antiviral Screening of Drugs Against Pichinde Virus

(1) The antiviral drug assay based on inhibition of immunofluorescence (Burns et al. 1988) is as sensitive as Pichinde virus antiviral assays based on inhibition of cytopathic effect (CPE) or plaque reduction (PR) and offers the advantages of economy of reagents, rapidity, and a less ambiguous endpoint.

(2) Ribavirin 5'-*O*-hemisuccinate may be a suitable derivative to be used for tethering to antibodies. A more hindered ester, ribavirin 5'-*O*-(2,3-dimethyl)hemisuccinate, while more stable than the ribavirin 5'-*O*-hemisuccinate, displayed lower antiviral activity and was more toxic. The antiviral assays were conducted in vitro using the ribavirin adducts without conjugation to MAb. It is quite likely that the 5'-*O*-ester linkage must be cleaved before the dimethylhemisuccinate or hemisuccinate derivatives can express antiviral activity. Thus, although the ribavirin 5'-*O*-(2,3-dimethyl)hemisuccinate is less active in the in vitro antiviral assay, the compound may be active when conjugated to MAb.

(3) Multiple loading of ribavirin onto virus-specific antibodies via either phosphate ester linkages [poly(ribavirin phosphate)] or ribavirin-substituted dextran polymers is a promising approach for carrier-mediated targeted delivery. In the future, both polyribavirin

and dextran multiply loaded with ribavirin should be targeted by attachment to virus-specific antibodies that internalize.

Task III. Derivation, Production and Purification of Monoclonal Antibodies Specific for Antigens Expressed on the Surface of Pichinde Virus Infected Cells

(1) Several modifications of previous procedures included in the final hybridoma derivation protocol should be included in future derivations. All of these modifications were evaluated independently and each had a positive effect. Peritoneal macrophages in the fusion wells served to condition the medium; they also phagocytized dead cells and debris, thereby reducing the toxicity of the fusion culture medium. Other modifications included an antigen boost administered to the spleen donor mice 5 days prior to the fusion, the avoidance of HEPES when dimethyl sulfoxide was present in the medium, only very gentle manipulation of the fusion cell mixture during the critical fusion steps, and not adding the aminopterin to the fusion cell cultures until 24 h postfusion.

(2) Ammonium sulphate precipitation followed by affinity chromatography on protein A is a suitable procedure for isolating and purifying the MAbs developed in these studies. The IgG recoveries from murine ascites fluids ranged from 9% to 40%. The purification procedure did not adversely affect antigen binding activity. MAbs PC4.9A6 and PC4.9D3 either as crude ascites preparations or as highly purified immunoglobulins were not adversely affected by lyophilization.

Task IV. Anti-Pichinde Virus Monoclonal Antibodies. Characterization: Binding to Pichinde Virus Antigens Expressed on the Surface of Living Cells, Isotype Analysis, Epitope Specificity and Viral Protein Specificity

(1) MAbs PC4.7C2, PC4.8D2, PC4.9D3, and PC4.9A6 bind to Pichinde virus antigens expressed on the surface of cells infected with Pichinde virus. Based on binding characteristics these MAbs are suitable candidates as drug delivery vectors for the study of antibody-mediated targeted delivery of antiviral compounds.

(2) MAbs PC4.7C2 and PC4.9A6 bind to the same epitope, while MAbs PC4.8D2 and PC4.9D3 bind to two other epitopes. Competitive binding assays with anti-LCMV MAb WE33.6, which cross reacts with the GP2 glycoprotein of Pichinde virus, indicated that MAbs PC4.9A6 and PC4.9D3 bind to epitopes on the GP_2 glycoprotein of Pichinde virus.

Task V. Ascites Tumor Formation Using Hybridomas Derived from the Fusion of FOX-NY Myeloma Cells with Spleen Cells from RBF/Dn Mice

(1) By increasing the pristane dosage to 1.0 to 2.0 ml/mouse the efficiency of ascites tumor formation can be increased. However, this is not satisfactory for all RBF/DnJ x FOX-NY hybridoma cell lines. In these studies two out of four cell lines failed to produce ascites even in recipient mice that had received 2.0 mL of pristane.

(2) Using hybrid (RBF/Dn x BALB/c)F_1 mice as RBF/DnJ x FOX-NY hybridoma cell line recipients, even with increased pristane dosage levels, does not dramatically increase the ascites tumor yields over those obtained using BALB/c mice.

(3) An aggressive immunosuppression regimen of pristane priming (1 to 2 mL) followed by total body irradiation (350 rad) in hybrid (RBF/Dn x BALB/c)F_1 mice dramatically enhances the propagation of RBF/DnJ x FOX-NY hybridoma cell lines as ascites tumors.

Task VI. In vivo Targeting with Anti-Pichinde Virus Monoclonal Antibodies

(1) The main target organ for Pichinde virus in the MHA hamster is the liver. Pichinde virus titers in the livers were a thousand-fold higher than titers observed in any other organs.

(2) MAbs PC4.9A6 and PC4.9D3 after i.p. administration to Pichinde virus infected hamsters concentrate in the livers of the infected animals. MAbs PC4.9A6 and

PC4.9D3 reach at least 10-fold greater levels in the livers of infected animals than in livers of noninfected animals. These MAbs have potential for evaluating targeted delivery of antiviral drugs.

Task VII. Weanling Hamsters for In Vivo Antiviral Evaluations.

(1) Weanling MHA hamsters, weighing 30 grams were as sensitive to Pichinde virus challenge as were young adult (90 gram) MHA hamsters. Weanling hamsters could be used in place of young adult hamsters.

Task VIII. Preparation and Evaluation of Immunotoxins as Therapies for Pichinde Virus Infections.

(1) The PC4.9A6-gelonin immunotoxin produced and described in this report retained the antigen-binding activity of MAb PC4.9A6 and the anti-ribosomal toxicity of gelonin, but lacked specific cytotoxicity towards Pichinde virus-infected cells. Apparently the PC4.9A6-gelonin immunotoxin was not internalized by target cells.

(2) Indirect immunotoxins consisting of protein G linked to gelonin through a disulfide bond are potent immunotoxins, which can be used to screen for virus-specific antibodies to be used for antibody-mediated targeted delivery of antivirals.

(3) The experiments utilizing polyclonal antibodies for the targeted delivery of antivirals demonstrated a very strong antiviral effect against Pichinde virus. A single treatment with HRS-gelonin at 20-h p.i. reduced the virus yield by over 1,000-fold with no detectable nonspecific cytotoxicity. The experiments to determine the time course for the expression of immunotoxin activity indicated that multiple treatments with immunotoxin might produce even more dramatic reductions in virus yields.

(4) The ED_{50}, MTD, and therapeutic index for HRS-gelonin immunotoxin were each more favorable than the corresponding parameters for ribavirin. The ED_{50} for HRS-gelonin was 0.016 μM compared to 40 μM for ribavirin. In other words, one molecule of

the HRS-gelonin immunotoxin mediated an antiviral effect equivalent to that of 2,500 molecules of ribavirin. Thus, the anti-Pichinde virus immunotoxins described in this report appear promising for use in developing antibody-mediated delivery of antiviral substances. It would have been interesting if similar studies could have been performed with HRS-ribavirin conjugates. Unfortunately, HRS was not available in sufficient time to SRI for any conjugate work.

STATEMENT OF PROBLEM UNDER STUDY

Under USAMRIID Contract No. DAMD17-86-C-6120, SRI International and Utah State University developed methodology to assess the effectiveness of antiviral drug-MAb conjugate therapy for the treatment of viral infections. Specifically, we undertook the preparation of MAbs directed against viral antigens expressed on the surface of Pichinde virus-infected cells, the conjugation of the antiviral drug ribvavirin to these MAbs, the development of in vitro and in vivo models to assess drug efficacy, and the biological assessment of the antiviral activity of the conjugates compared with that of the free drug in virally infected cells in culture and in hamsters.

BACKGROUND

Approximately 60% of human illnesses are caused by viral infections (Horsfall, 1965). In particular, military personnel may be exposed to many different virulent pathogens when deployed abroad. The spread of these pathogens is exacerbated by inadequate sanitation, poor health conditions, and crowded living and working conditions. The arenaviruses (Junin, Lassa, and Machupa) cause hemorrhagic fevers that have high mortality rates in humans. These diseases are characterized by high fever, leucopenia, gastrointestinal hemorrhagic manifestations, shock, and, in some instances, a neurologic syndrome (Johnson et al., 1967). The duration of illnesses is generally two to three weeks, and complete recovery requires a long convalescence. The mechanism of pathogenesis for these viruses in humans appears to be direct damage to the cells. Viruses cause extensive capillary damage (either directly or indirectly), which has been proposed as the causative factor of the organ damage resulting from these diseases (Casals, 1982).

Because vaccination therapy or drug therapy for many of the infections caused by members of the Arenaviridae is not available or effective, new methods of treatment must be developed. In addition, in those cases where antiviral drugs have been shown to inhibit the virus in vitro, effective therapy in vivo may require the administration of such high drug doses that toxic side effects occur. Therefore, by targeting the delivery of antiviral drugs to the virus or the virally infected cell, lower concentrations of drugs could be administered, thereby reducing their toxic side effects.

RATIONALE

Targeted drug therapy using antiviral drug-MAb conjugates has potential value for the treatment of viral infections. MAbs to specific antigens expressed on the viral surface or on the surface of virally infected cells would specifically deliver the conjugated drug to the virus or the infected cell. Therefore, drug efficacy would be enhanced and systemic side effects would be reduced relative to standard treatment modalities using unbound drugs.

The drug-MAb method of targeted drug delivery is currently being investigated as a new therapeutic approach for the treatment of cancer (Arnon and Sela, 1982; Bjorn et al., 1986; Dillman et al., 1986; Embleton et al., 1986; Forrester et al., 1984; Gilliland et al., 1980; Hurwitz, 1982; Hurwitz et al., 1983, 1985; Kato et al., 1984; Kulkarni et al., 1981; Pimm et al., 1982; Seeger et al., 1982; Tsukada et al., 1982, 1983; Youle and Neville, 1980). MAbs that are specific for antigenic determinants on tumor cells have been developed (Hurwitz et al., 1985; Seeger et al., 1982). The chemotherapeutic drugs doxorubicin (Dillman et al., 1986; Hurwitz et al., 1983; Pimm et al., 1982), daunomycin (Kato et al., 1984; Tsukada et al., 1982, 1983), and methotrexate (Kulkarni et al., 1981), the toxin ricin (Forrester et al., 1984; Youle and Neville, 1980), other toxins (Bjorn et al., 1986), and the protein A chain of ricin (Embleton et al., 1986; Gilliland et al., 1980) have been covalently linked to antibodies against antigens on tumor cell lines. The resultant conjugates have been screened in both in vitro and animal model systems. Conjugation of both drugs and toxins did not interfere with the binding of antibodies to their antigenic determinants. The conjugates had increased specific cytotoxicity to cancer cells in culture compared with control cells lacking the antigens (Kato et al., 1984; Tsukada et al., 1982). Most important, the conjugates prolonged the survival times of animals infected with the

cancer cells over those of infected animals treated with the unbound drugs or antibodies alone or with combinations of free drugs with the antibodies (Tsukada et al., 1983).

TASK REPORTS

This final report describes research accomplished in the 3.5 years (April 1, 1986 to December 31, 1989) of USAMRIID Contract No. DAMD17-86-C-6120. Because of the wide scope and diversity of this multidisciplinary project, this report has been subdivided into particular task reports, which are presented in the order set forth in the Research Summary.

TASK I. SYNTHESIS 'IBAVIRIN DERIVATIVES HAVING TETHER GROUP FUNCTIONAI 'D THEIR CONJUGATION TO ANTI- PICHINDE VIRUS MO. NAL ANTIBODIES

A. Introduction

Because of its antiviral activity, ribavirin (Witkowski et al., 1972; Witkowski et al., 1973) was selected as the drug to determine the potential of targeted drug delivery using MAbs. Ribavirin has antiviral efficacy against Pichinde virus infections in animals and therefore has potential for treatment of this infection in humans if its therapeutic index can be enhanced either by reducing its toxic side effects or by selectively increasing its concentration in the infected cells. Selective delivery of ribavirin could be achieved using a ribavirin-MAb conjugate with a MAb that binds specifically to an antigen expressed on the surface of Pichinde virus-infected cells and, once bound, is internalized to the lysozomes where the conjugate would be cleaved to release an active species of the drug. Therefore, the most effective conjugate must (1) employ a MAb that binds effectively to a viral antigen expressed on the surface of the infected cell and is subsequently internalized, (2) have sufficient loading of drug to achieve an effective drug concentration within the cell, and (3) have a tether bond of sufficient stability to deliver the intact conjugate to the infected cell but sufficient lability that the bond is cleaved by either an enzymatic or hydrolytic

mechanism in the cell. Alternately, if the conjugate were not internalized, the linkage must have sufficient stability so that an intact conjugate is delivered to the surface of the virally infected cell but sufficient lability so that its chemical or enzymic hydrolysis at the cell surface occurs to release a high local concentration of the drug, which would then enter the cell by the usual active or passive transport process.

The 2', 3', and 5'-*O*-positions of the ribofuranose ring of ribavirin were selected as positions for introduction of tether functionality by which the drug would be linked to the MAb. Ketals, and carboxylate and phosphate esters were investigated as potential tether functional group and various strategies were investigated to effect the tethering. Two approaches were used for drug loading: (1) direct attachment of the functionalized drug on the Mab, and (2) attachment of the functionalized drug to a carrier, which would then be tethered to the Mab. The first approach would permit only low drug loadings unless a polymeric version of the drug were used. The second approach would permit higher loading levels.

To accurately assess the biological activity of the MAb-drug conjugates, it was necessary to determine the number of drug molecules attached to each antibody. Because ribavirin has a very weak ultraviolet chromophore, conventional loading determinations using spectroscopic absorption were not possible. Therefore, we investigated the following alternate methods: (1) determination using a radioimmunoassay, (2) determination using radiolabeled ribavirin, (3) phosphorous analysis for phosphate analogs, (4) use of an ultraviolet chromophoric group attached to ribavirin, and (5) use of a chromophore bound to a carrier molecule.

B. Experimental Methods

In this section, full experimental procedures are presented for those compounds and conjugates reported in Quarterly Reports No. 13 and No. 14 (Dawson et al., 1989).

Procedures for the remainder of the target compounds are presented in full in Annual Reports No. 1 to No. 3 (Dawson et al, 1987; Dawson et al., 1988; Dawson et al., 1989) and will not be repeated in this report.

General Procedures and Instrumentation. When required, reactions and purifications were conducted with deoxygenated solvents and under inert gas (argon) and subdued light. Solvents were dried or distilled before use. Melting points were uncorrected. TLC analyses were performed on Analtech silica gel analytical plates, using detection by UV or anisaldehyde spray (0.5 mL of anisaldehyde in 0.5 mL of concentrated sulfuric acid added to 9 ml of 95% ethanol containing a few drops of acetic acid, with the sprayed plate heated to 100-110°C for 20 to 30 min). Silica gel 60 (E. Merck No. 9385) for column chromatography was obtained from Brinkman. A Pharmacia Ultrarac fraction collector equipped with a UV detector (280 nm) and a peristaltic pump was used to collect column fractions where required. Lyophilizations were performed using a Labconoco lyophilizer. HPLC analyses were performed on a Bio-Rad MAPS instrument. IR spectra were recorded with a Perkin-Elmer 710B infrared spectrometer. NMR spectra were obtained with a JEOL FX90Q or Varian 400 MHz spectrometer, using tetramethylsilane as an internal standard (δ 0). UV spectra were taken on a Perkin-Elmer 575 spectrophotometer. Elemental analyses were conducted by Galbraith, Knoxsville, Tennessee. Mass spectral analyses were performed by Dr. David Thomas, SRI International, using a Ribermag Model R10-10C mass spectrometer. Samples were introduced using the desorption probe for both electron-impact and chemical-ionization studies. The number in parentheses appearing at the end of each experimental procedure refers to the SRI notebook number and page number for the start of that experiment. Only procedures for those synthetic steps in the successful routes to the target compounds are described.

Synthesis of Fluoresceinated (Carboxymethyl)$_{50.2}$[6-(ribavirin-5'-*O*-phosphato)hexylcarboxamido]$_{4.8}$dextran-MAb PC4 9A6 Immunoconjugate (I-19). To a solution of 10 mg of fluoresceinated (carboxymethyl)$_{50.2}$[6-(ribavirin-5'-*O*-phosphato)hexylcarboxamido]$_{4.8}$dextran (I-18, Mw 12,200) in 0.5 mL of water was added a freshly prepared solution of 20 mg (0.1 mmol, 2.3 equivalents based on carboxyl groups) of 3-ethyl-1-(3-dimethylaminopropyl)carbodiimide hydrochloride (35) in 0.5 mL of water, and the mixture was stirred for several minutes (pH 5.3). This solution was added to a 4°C solution of 10 mg of MAb PC4.9A6 in 1.8 mL of 0.15 M phosphate-buffered saline (PBS). The pH of this solution was 7.75. After stirring for 5 h at 4°C (pH 7.2), the reaction mixture was dialyzed (Mw 14,000 cutoff) against PBS (2 x 2000 mL) for 40 h. The retentate was centrifuged to remove precipitated protein, and the solution was concentrated (Mw 30,000 cutoff) to 2 mL. Next, 150-µL aliquots of the concentrated solution were layered onto each of twelve 5-20% sucrose gradients prepared in PBS. The gradients were centrifuged for 21 h at 39,000 x *g* (vacuum, 4°C). After centrifugation, the gradients showed a deeper yellow band in the upper half of the gradient and a flocculent yellow precipitate. The gradients were fractionated (0.5-mL fractions) in the cold. However, approximately 0.1 mL of material was left in the bottom of each tube. The UV absorbance at 280 and 492 nm was monitored for each fraction. In Figure I-1, the absorption profile of the fractions is depicted for these two wavelengths. The precipitate in each tube was solubilized in 0.4 mL of 0.6 M PBS, pH 7.2, with gentle mixing for 0.5 h. The solubilized protein fractions were pooled (4.8-mL volume) and centrifuged (3000 rpm, 30 min, 4°C) to give a supernatant solution with A_{280} = 0.902 and A_{492} = 0.775. If the absorbance at 280 nm of the modified dextran is considered, the solubilized precipitate contained 1.1 mg of protein having a loading of 6.8 moles of the fluoresceinated derivatized (carboxymethyl)dextran per mole of protein or 33 moles of ribavirin per mole of protein. This material was dialyzed against 0.15 M PBS to remove the salt and

concentrated to 1 mL. The ninth fraction from the collection tubes was dialyzed against 0.15 M PBS and concentrated to give 0.9 mL of solution containing 0.4 mg of protein having a loading of 4.0 moles of fluoresceinated derivatized (carboxymethyl)dextran or 19 moles of ribavirin per mole of protein.

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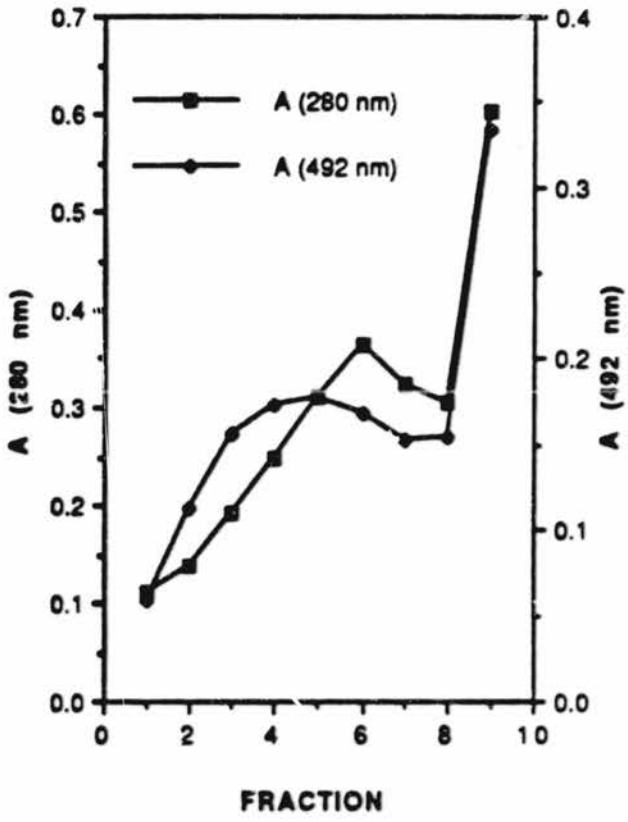

Figure I-1. Absorbance profile at 280 and 492 nm of fractions obtained by sucrose density centrifugation of reaction mixture of MAb PC4.9A6 and fluoresceinated (carboxymethyl)$_{50.2}$[6-(ribavirin-5'-*O*-phosphato)$_{4.8}$hexylcarboxamidomethyl]dextran on 5-20% sucrose density gradient in 0.15 M PBS.

5'-*O*-(6-Aminohexyl)phosphate of Poly(ribavirin-3',5'-*O*-phosphate)$_{15}$-thymidine-5'-*O*-phosphate Triethylammonium Salt (I-20).

1. Polymer Synthesis. A literature procedure (N. Usman et al., 1987) was adapted. The oligomer was assembled in a continous process using an Applied Biosystems Model 380A DNA Synthesizer by the stepwise addition of nucleoside units beginning with a thymidine attached to the support for the synthesis, followed by 15 ribavirin-5'-*O*-phosphate units, and was terminated by a protected 6-aminohexyl phosphate. The cycle modified for the synthesis of an RNA analog is given in Figure I-2. The solid-phase synthesis employed 12 columns, each loaded with 1 mol of a protected thymidine. The solid support used was a long-chain alkylamine functionalized controlled pore glass (CPG) to which 5'-*O*-tritylthymidine-3'-*O*-succinate had been linked through an amide bond.

Step	Reagent	Time (sec)
1	3% trichloroacetic acid/methylene chloride	150
2	acetonitrile	90
3	0.1 M ribavirin phosphoramidite/0.45 M 1*H*-tetrazole	12
4	wait	420
5	0.5 M acetic anhydride/0.5 M 4-dimethylaminopyridine in 2,6-lutidine/tetrahydrofuran 20:80	45
6	0.1 M iodine in tetrahydrofuran/pyridine/water 7:2:1	45

Figure I-2. Automated synthesis cycle.

The trityl protecting group was removed from the 5'-position of the nucleoside by treatment with 3 mL of 3% trichloroacetic acid (79) in methylene chloride. A 0.1 M solution of 2'-*O*-*t*-butyldimethylsilyl-5'-*O*-(4,4'-dimethoxytrityl)ribavirin-3'-*O*-[(*N*,*N*-diisopropyl-

amino)(methyl)phosphoramidite] (77) in 0.18 mL of acetonitrile (0.162 g of 77/2.00 mL of acetonitrile) was activated by 0.18 mL (0.081 mmol) of 0.45 M 1*H*-tetrazole (49) in acetonitrile and then allowed to react with the deprotected 5'-hydroxyl group, adding the second unit to the oligomer chain. A binary capping system of 0.9 mL of 0.5 M acetic anhydride/0.5 M 4-dimethylaminopyridine (DMAP, 76) in 2,6-lutidine-tetrahydrofuran (THF) 20:80 was used to acetylate any free hydroxyl groups, preventing formation of lower-molecular-weight and/or branched oligomers. This step was followed by oxidation of the phosphite group with 0.1 M iodine in THF-pyridine-water 7:2:1 to generate the 3',5'-*O*-phosphate interribonucleotide linkage. Next, the 4,4'-dimethoxytrityl group was removed from the 5'-terminus of the polymer with 1.0 mL of 3% trichloroacetic acid (79) in methylene chloride. The dimethoxytrityl cation released was quantitated by measuring the absorbance at 498 nm of a mixture of a 3.0-mL aliquot of column effluent from this step that had been diluted to 50 mL with 0.1 M *p*-toluenesulfonic acid in acetonitrile. This colorimetric assay is water-sensitive. The coupling was repeated to generate an oligomer containing 15 ribavirin units. The oligomer was terminated by reaction with 0.18 mL of 0.15 M of [6-(*N*-4-methoxytritylamino)hexyl](2-cyanoethyl)(*N*,*N*-diisopropylamino)-phosphoramidite (81) in acetonitrile. The monomethoxytrityl group was retained as an aid for visualization during purification on polyacrylamide gels. The average coupling efficiency, as measured by trityl cation release, was 96.5-98%. Twenty-seven 1-μmol columns were used for the synthesis.

2. Removal of the Oligoribonucleotide from the Solid Support and Deprotection. The columns were removed from the instrument and dried at reduced pressure. The support with its tethered oligoribonucleotide (82) was removed from the columns and placed in 10 BioRad Econo-Columns connected to stopcocks. The methyl group protecting the phosphate functions was removed by treatment of each column with 3.0 mL of thiophenol (83)-triethylamine-dioxane 1:2:2 for 1 h at room temperature. After

draining off the thiophenol solution, each column was washed with 15 mL of 95% ethanol. The support was washed three times with ethanol. The oligomer chain was then cleaved from the support by treatment with 3.0 mL of concentrated ammonium hydroxide in 95% ethanol 3:1 for 1 h. The cleavage step was repeated. The support was washed three times with ethanol. The oligomer-containing eluant was transferred to a 250-mL round-bottomed flask, one drop of triethylamine was added to maintain basicity in order to stabilize the trityl group, and the solution was concentrated and lyophilized. The residue was treated with 7.0 mL of 1 M tetra(*n*-butyl)ammonium fluoride (23) in THF overnight to remove the 2'-*O*-silyl protecting groups. The reaction mixture was quenched with 7.0 mL of 0.05 M ammonium acetate. This solution was applied to a 2.5 x 40-cm Sephadex G-15 column (40 g dry weight, 90-mL blue dextran void volume) for desalting and removal of organic impurities. Elution with 0.05 M ammonium acetate at 1 mL/0.5 min afforded the fully deprotected oligomer in the void-volume, as detected by 260-nm absorption. The void-volume fraction was transferred to a 250-mL round-bottomed flask, one drop of triethylamine was added, and the solution was lyophilized. The residue was dissolved in 12 mL of 0.05 M triethylammonium (TEA) acetate containing one drop of triethylamine and apportioned among eight 1.5-mL Eppendorf tubes for evaporation on the Savant centrifuge to give a yellowish gum.

3. Purification by Polyacrylamide Gel Electrophoresis (PAGE). Each Eppendorf tube was treated with 80 µL of 90% deionized formamide and the contents of each was apportioned among eight PAGE wells (1.2-mm x 1.5-mm, 1.5-mm slab of 20% polyacrylamide containing 7M urea). Therefore, the contents of three CPG columns were run on one 10-well plate, with two wells being reserved for the marker dyes bromophenol blue and xylene cyanol. To ensure ease of visualization by UV, the trityl groups were retained during this purification step. Electrophoresis was conducted at 30 mAmps constant current for 1.5-2 h, at which time the xylene cyanol band had migrated half-way

down the gel. The oligomer was visualized under short wavelength UV light by removing the gel from its glass support and laying it on a plastic-wrapped, preparative thin-layer chromatography (TLC) plate containing a fluorescent indicator. The product bands (R_f 0.60) were excised from the gel using a clean razor blade. The gel slices were transferred to a BioRad Econo-Column (1 column/gel) attached to a stopcock, covered with 0.05 M TEA acetate, and extracted for 24 h at room temperature to elute the oligomer. This extraction process was repeated twice. The gels were then washed with the same buffer. The extracts were combined and lyophilized to give a white residue containing the product, salts, and acrylamide.

4. Removal of Acrylamide, Detritylation, and Purification. The lyophilized residue from the 27 CPG columns was dissolved in 5 mL of 0.05 M ammonium acetate and applied to the same Sephadex G-15 column that had been used after the desilylation step and had been thoroughly washed. The oligoribonucleotide was eluted in the void volume (90 mL) using 0.05 ammonium acetate. The eluant was transferred to a 250-mL round-bottomed flask and lyophilized to give a white gum. The residue was diluted with 10 mL of 80% acetic acid (v/v) and stirred for 3 h at room temperature. Water (50 mL) was added and the aqueous layer was washed with ether (3 x 25 mL) to remove nonpolar organic compounds. The aqueous layer was evaporated for a brief period to remove traces of ether and then lyophilized to give the detritylated product as a yellowish gum. This residue was diluted with 12 mL of 0.05 M TEA acetate for purification by reverse-phase column chromatography on 27 Sep-Pak cartridges (Waters Assoc.), which had been pretreated by washing with 10-mL volumes of methanol and water. A 0.50-mL aliquot of oligomer solution was transferred by syringe to each cartridge. The eluant was collected and passed through the cartridge again. Salts were removed from the contents of the cartridge by washing with 20 mL of water. The absorbance of this rinse was measured at 260 nm to determine that no oligomer had been eluted from the column during the wash

step. The product was isolated by elution with 3.0 mL of 50% 0.05 M TEA acetate in methanol followed by a 1.0-mL rinse. The rinse was monitored at 260 nm and showed no absorption. The combined elutes were evaporated and lyophilized to constant weight to give 24.7 mg of **I-20** as a white solid.

5. Oligomer Characterization. This residue was dissolved in 50 mL of water for spectrophotometric analysis based on thymidine concentration. The absorbance at 260 nm was 0.647. Based on an extinction coefficient for thymidine of 8.8×10^3, the overall yield was 25.0 mg (13.6%). Based on the 96.5-98% coupling yield calculated from detritylation, the oligomer yield should be 60%; however, this does not account for mechanical losses and incomplete reactions.

^{1}H NMR (D_2O) δ 1.25 (t, J = 7.5 Hz, 144, NCH_2CH_3), 1.2-1.7 (m, 8, $(CH_2)_4$), 1.89 (s, 3, thymidine CH_3), 2.02 (s, 3, CO_2CH_3), 2.32 (t, J = 6 Hz, 2, 2'-thymidine H), 2.92 (t, J = 8 Hz, 2, NCH_2), 3.17 (q, J = 7.5 Hz, 96, NCH_2CH_3), 3.34 (t, J = 8 Hz, 2, CH_2OP), 4.09 (m, 32, 5'-H), 4.22 (m, 1, 4'-thymidine H), 4.33 (broad s, 15, 4'-ribavirin H), 4.51 (m, 1, 3'-thymidine H), 4.61 (t, J = 4 Hz, 15, 3'-ribavirin H), 4.81 (m, 15, 2'-ribavirin H), 5.95 (d, J = 4 Hz, 15, 1'-ribavirin H), 6.27 (t, J = 6 Hz, 1, 1'-thymidine H), 7.63 (s, 1, thymidine 6-ArH), 8.69 (s, 15, ribavirin 5-ArH). The NMR was analyzed to determine whether any bases were lost during the synthesis. The integration of the ribavirin 5-ArH signal was 99% of that of the 4'-H signal, indicating that the amount of depurination was very low. The ratio of thymidine to ribavirin units in this oligomer was determined to be 1:15 using the integrations of the 1'-H of each. A sharp singlet was also found at 0.92 ppm from unhydrolyzed *t*-butyldimethylsilyl groups. Integration indicated that this group constituted 0.33 of the 15 2'-*O* ribavirin residues, or 2% of the protecting group remained. Overnight reaction of the oligomer with tetra(*n*-butyl)ammonium fluoride should drive this cleavage to completion.

HPLC (BioRad MA7Q anion exchange column, 20 mM Tris•HCl, pH 7.5, to 20 mM Tris•HCl, pH 7.5, 500 mM NaCl over 10 min at 1.0 mL/mir., 260 nm) t_R 10.0 min (96%).

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Synthesis of the 5'-*O*-(6-Aminohexyl)phosphate of Poly(thymidine-3',5'-*O*-phosphate)$_{12}$-thymidine-5'-*O*-phosphate Triethylammonium Salt (I-21).

1. Oligomer Synthesis. The solid-support synthesis used for poly-(ribavirin-3',5'-*O*-phosphate) was modified for the synthesis of the 5'-*O*-(6-aminohexyl)-phosphate of poly(thymidine-3',5'-*O*-phosphate)$_{12}$-thymidine-5'-*O*-phosphate triethyl-ammonium salt (**I-21**) by shortening Step 4 (wait step) to 30 sec because of decreased steric hindrance at the 3'-*O*-position of the deoxyribose ring . A 0.1 M solution of 5'-*O*-(4,4'-dimethoxy-trityl)thymidine-3'-*O*-[(*N*,*N*-diisopropylamino)(methyl)phos-phoramidite] (**87**) in 0.15 mL of dry acetonitrile was activated with 0.15 mL of 0.45 M 1*H*-tetrazole in acetonitrile and then allowed to react with the deprotected 5'-hydroxyl group of thymidine attached via a 3'-*O*-succinate group to alkylamino-functionalized CPG on three 1-μmol columns. The capping of truncated sequences, the oxidation of the trivalent phosphorus, and the removal of the dimethoxytrityl groups proceded analogously to the synthesis of poly(ribavirin-3',5'-*O*-phosphate). The reaction cycle was repeated to generate an oligomer of 13 thymidine units. The addition of the last unit in the sequence again required the reaction of the amino-linker phosphoramidite [6-(*N*-4-methoxytritylamino)hexyl](2-cyanoethyl)(*N*,*N*-diisopropylamino)phosphoramidite (**81**) with the deprotected 5'-hydroxyl terminus of the oligomer chain, which had been deblocked by treatment with 3% trichloroacetic acid in methylene chloride. The average coupling efficiency, as determined by trityl cation release, was 99%.

2. Removal of the Oligonucleotide from the Solid Support and Deprotection. The columns were removed from the DNA synthesizer and dried briefly at reduced pressure. The column contents were placed in one BioRad Econo-Column equipped with a stopcock. The methyl protecting groups on the phosphates were removed by treatment with 3.0 mL of thiophenol-triethylamine-dioxane 1:2:2 for 1 h. The thiophenol was drained off and the column was rinsed with 15.0 mL of 95% ethanol. The tethered oligomer was then treated with 3.0 mL of concentrated ammonium hydroxide/95% ethanol 3:1 for 1 h to cleave the tether linkage. The cleavage step was repeated. The column was then washed with three 15-mL portions of 95% ethanol. The combined column eluants were transferred to a 150-mL round-bottomed flask, evaporated, and then lyophilized to a fluffy white solid.

3. Desalting, Detritylation, and Purification. The residue from lyophilization was dissolved in 3.0 mL of 0.05 M ammonium acetate and passed through a 1.5 x 25-cm Sephadex G-15 column (10 g) using 0.05 M ammonium acetate as the eluant (25-mL void volume). The void-volume peak (260-nm monitoring) was collected, evaporated, and lyophilized to a yellow gum. This residue was diluted with 10 mL of 80% acetic acid and stirred for 3 h at room temperature to remove the trityl protecting group, which was not needed for visualization. Water (50 mL) was added and the solution was lyophilized to dryness.

The residue was dissolved in 3 mL of water and apportioned among three Eppendorf tubes. Each Eppendorf tube was treated with 80 µL of 90% deionized formamide and divided among eight PAGE wells for purification by gel electrophoresis. After electrophoresis, the oligomer was visualized under short-wavelength UV light by placing the gel on a plastic-wrapped preparative TLC plate containing a fluorescent indicator. The product bands were excised and soaked as mentioned in the previous

electrophoresis procedure. After the gel pieces were extracted three times, the extracts were combined and lyophilized to give a white residue.

The lyophilized residue from the three CPG columes was dissolved in 5 mL of 0.05 M TEA acetate and applied to the same Sephadex G-15 column that was previously used for desalting the polymer. The oligonucleotide was eluted in the void volume using 0.05 M ammonium acetate. The desired eluant fractions were pooled and lyophilized to give a white powder. To this residue was added 4.5 mL of 0.05 M TEA acetate. Aliquots (0.50 mL) of this solution were added to each of nine Sep-Pak columns (Waters), which had been rinsed with 10-mL volumes of methanol and water. The column effluent was collected and passed through each cartridge a second time. Salts were then washed from the column with 20 mL of water. The product was eluted from each column with 3.0 mL of 50% 0.05 M TEA acetate/methanol, followed by a rinse with the same solvent. The UV absorbance at 260 nm of this rinse indicated that elution was complete. The column eluants were pooled, evaporated, and lyophilized. Repeated evaporation with 25-mL portions of water removed TEA acetate to give a white solid, which was enriched in the desired oligomer.

4. Oligomer Characterization. The oligomer was dissolved in 100 mL of water. Its absorption at 260 nm was 1.900, indicating that the molarity of the polymer was 1.7×10^{-6} M. If no other UV-absorbing material was present, the yield was 7.2 mg (46%).

(8954-36)

Preparation of the Conjugate I-22 of the 5'-*O*-(6-Aminohexyl)-phosphate of Poly(thymidine-3',5'-*O*-phosphate)$_{11}$-thymidine-5'-*O*-phosphate and Protein G Using *N*-Succinimidyl-3-(2-pyridyldithio)-propionate to Effect Coupling.

1. Reaction of the 5'-*O*-(6-Aminohexyl)phosphate of Poly(thymidine-3',5'-*O*-phosphate)$_{11}$-thymidine-5'-*O*-phosphate with *N*-Succinimidyl-3-(2-pyridyldithio)propionate. To 1.4 mL of 0.10 M sodium bicarbonate, pH 8.25, was added with stirring at 20°C a solution of 36.7 A_{260} nm units (1.86 mg, 3.21 x 10^{-7} mol), measured in 0.05 M triethylammonium acetate (TEA-HOAc), pH 7.0, of the 5'-*O*-(6-aminohexyl)phosphate of poly(thymidine-3',5'-*O*-phosphate)$_{11}$-thymidine-5'-*O*-phosphate (I-21) in water, followed by 0.70 mL of a 1% (3.2 x 10^{-3} M) solution of *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP, 85, 2.25 x 10^{-5} mol) in dimethylsulfoxide in two aliquots over a period of 30 min. The reaction mixture was stirred at ambient temperature for 20 h and then centrifuged. The solution was chromatographed on Sephadex G-25 (39 x 1.2-cm column) using 0.05 M TEA-HOAc, pH 7.0, as the eluant. The product eluted in the 14.9 mL following the void volume of 26.9 mL. The excess SPDP and *N*-hydroxy-succinimide byproduct began to elute after two volid volumes. The combined product fractions were lyophilized, and then similarly rechromatographed on Sephadex G-25 to give 38.5 A_{260} nm units (97% recovery from the first Sephadex column) of the 3-(2-pyridyldithio)propionyl derivative of the oligomer. The product was lyophilized. The extent of reaction of the amino group on the oligomer with SPDP was estimated to be 76% by the method of Carlsson et al. (1978), involving reduction of the disulfide bond with dithiothreitol, and measurment of the pyridine-2-thione (ε 8.08 x 10^3) released at 343 nm. The A_{280} (0.05 M TEA-HOAc, pH 7.0) of the product, after correction for absorbance at 280 nm due to the 2-pyridyldithio substituent, indicated 102% recovery of the oligomer.

2. Reaction of the 5'-*O*-{6-[3-(2-pyridyldithio)propionamido]-hexyl}phosphate of Poly(thymidine-3',5'-*O*-phosphate)$_{11}$-thymidine-5'-*O*-phosphate with Dithiothreitol. The (pyridyldithio)propionyl derivative of the oligomer (total product minus the sample used for estimation of the level of disulfide substitution) in 1.0 mL of 0.05 M TEA-HOAc, pH 7.5, was treated with 0.5 mL of 0.05 M dithiothreitol (DTT, 0.025 mmol), and the resultant solution was allowed to stand for 2.5 h, and then combined with the lyophilized sample used for estimation of 2-pyridyldithio content. The mixture was chromatographed on Sephadex G-25 (35 x 1.2-cm column) using degassed 0.05 M TEA-HOAc, pH 7.0, as the eluant. The product eluted in the 10.2 mL following the void volume. The thiol-containing product was immediately lyophilized. The thiol content was estimated on an identically prepared sample using the Ellman reaction (Deakin et al., 1963). The A_{412} nm, following reaction of the sample with 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.10 M sodium phosphate, pH 8.0, corresponded to 89% of theory for complete thiol functionalization of the oligomer. The product used for protein conjugation was not assayed for thiol group content, but was removed from the argon-filled lyophilization flask and used immediately.

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3. Reaction of Recombinant Protein G with SPDP. Recombinant Protein G (3.3 mg, 1.5 x 10^{-7} mol, Mw 22,000, Zymed, South San Francisco, CA) was dissolved at 4°C in 1.0 mL of 0.10 M sodium phosphate, 0.15 M sodium chloride, pH 7.5. The solution was treated at ambient temperature with 24 µL (3.2 equivalents) of 0.02 M SPDP in ethanol and then stirred for 30 min. The solution was immediately applied to Sephadex G25 (24 x 1.2-cm column) and eluted with 0.10 M sodium phosphate, 0.15 M sodium chloride, pH 7.5. Following a void volume of 14.5 mL, the product (4.49 A_{280} units) eluted in 7.5 mL. The reagents eluted after the second void volume. The product (86) fractions were concentrated to 1.50 g of a clear solution using a Centricon 10 micro-

concentrator (Amicon, MA). Estimation of the 2-pyridyldithio content and the A_{280} nm due to protein A, after correction for the absorbance at 280 nm due to the 2-pyridyldithio group, indicated a 95% recovery of protein G having 2.45 3-(2-pyridyldithio)propionyl groups per protein. A 0.44-g aliquot of this solution, was supplied to F. Szoka, University of California, San Francisco, for liposome conjugation studies.

(7747-80)

4. Conjugation of Thiolated Oligomer to 3-(2-Pyridyldithio)-propionylated Protein G. A 0.75-g aliquot of the 3-(2-pyridyldithio)propionyl-derivatized protein G (**86**) solution in 0.10 M sodium phosphate, 0.15 M sodium chloride, pH 7.5, containing 1.57 mg (7.1×10^{-8} mol) of recombinant protein G, was treated under argon with 1.0 mL (2.85×10^{-7} mol, by Ellman thiol estimation) of a solution of the thiolated oligomer in the same degassed, pH 7.5 buffer. The solution was stirred at ambient temperature under argon with protection from light for 30 h. The clear solution was chromatographed at 4°C on Sephadex G-75 (60 x 1.0-cm column) in the phosphate buffer. Following a void volume of 23.7 mL, the conjugate (16.2 A_{260} units) eluted in 16.2 mL (0.59 void volume), and was immediately followed by unreacted thiol or the disulfide. The conjugate solution was concentrated to 1.5 mL using a Centricon 10 microconcentrator. A total of 0.56 A_{260} units passed through the membrane. The conjugate was then rechromatographed on Bio-Gel P100 (62 x 1.0-cm column) in the same buffer to give 16.3 mL of conjugate (15.1 A_{260} units) following a void volume of 27.0 mL. The solution of product **I-22** was concentrated and the buffer exchanged to 0.01 M potassium phosphate, 0.15 M sodium chloride, pH 7.2, (1.9 mL x five exchanges) using a Centricon 10 microconcentrator. A 0.165-mL aliquot was diluted to 1.99 mL for UV estimation ($A_{280} = 0.463$, $A_{260}/A_{280} = 1.52$). Assuming a complete recovery of

protein and after correction for the absorbance due to protein, the loading of the oligomer onto protein G was established from the A_{260} as 1.9 oligomers per protein G.

(7747-85)

C. Results and Discussion

The target compounds synthesized under this contract are listed in Table I-1. In addition to chemical structures, this table also lists compound name, SRI code number, report number in which the synthetic procedure is located, and, for comparison purposes, the antiviral activity.

1. Ribavirin Synthesis. At the beginning of the contract, ribavirin (**I-1**) was not available from the USAMRDC and was therefore synthesized by the procedure of Witkowski et al. (1973). This route is shown in Scheme I-1. Cyanogen (**1**) was allowed to react with hydrazine (**2**) to give 1-cyanoformidic acid hydrazide (**3**) in 44% yield on recrystallization from isopropyl alcohol-hexane. Hydrazide **3** was heated at reflux temperature with excess triethyl orthoformate (**4**) to give 3-cyano-1,2,4-triazole (**5**) in 78% yield after recrystallization from ethyl acetate-benzene. A mixture of triazole **5** and 1,2,3,5-tetra-*O*-acetyl-ß-D-ribofuranose (**6**) was heated at 150°C (external temperature) in the presence of bis(4-nitrophenyl) phosphate (**7**) as the catalyst, with concomitant removal of the acetic acid byproduct at reduced pressure, to afford a 71% yield of triacetate **8** after recrystallization from ether. Hydrolysis of the three acetate groups and the nitrile group of **8** with ammonium hydroxide at 20°C followed by recrystallization from ethanol gave a 78% yield of ribavirin, the melting point, and infrared (IR) and proton nuclear magnetic resonance (^{1}H NMR) spectra of which were in agreement with those reported in the literature (Witkowski et al., 1973).

2. Radiolabeled Ribavirin. To establish loading using isotopic labeling, it was necessary to prepare radiolabeled ribavirin because it was too costly to purchase in

Table I-1. Ribavirin Derivatives, Carriers and Co. ugates

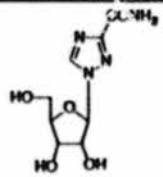
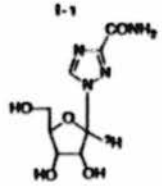
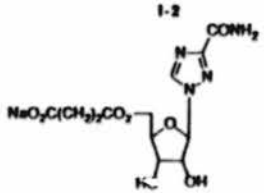
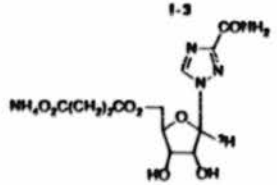
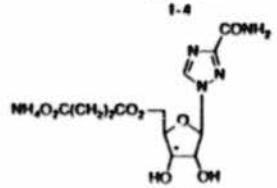
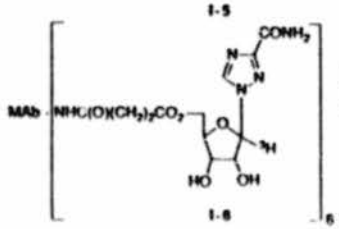
Structure, Name, and Code Number	Report	Biological Activity								
		CPE against Punta Toro Virus in LLC-MK$_2$D Cells			CPE Against Pichinde Virus in Vero 76 Cells			Plaque Redn Assay for Pichinde Virus in Vero 76 Cells	Immunofluorescence Assay for Pichinde Virus in Vero 76 Cells	
		VR	ED$_{50}$ (mM)	MTD (mM)	VR	ED$_{50}$ (mM)	MTD (mM)	ED$_{50}$ (mM)	ED$_{50}$ (mM)	MTD (mM)
 I-1 Ribavirin (1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide) (SRI 7422-34, 56)	Annual No. 1	1.4	0.033	0.13	0.5	0.025	0.41	0.024	0.02	>4.1
 I-2 [1'-^{3}H]Ribavirin (SRI 7421-66)	Annual No. 1									
 I-3 Ribavirin-5'-*O*-hemisuccinate Sodium Salt (SRI 7422-88)	Annual No. 1	1.3	0.04-0.13	0.41	0.4	0.041	1.3	0.05	0.04	0.13
 I-4 [1'-^{3}H]Ribavirin-5'-*O*-hemisuccinate Ammonium Salt (SRI 8144-5)	Annual No. 2									
 I-5 [3'-^{14}C]Ribavirin-5'-*O*-hemisuccinate Ammonium Salt (SRI 8144-5)	Annual No. 2									
 I-6 [1'-^{3}H]Ribavirin-5'-*O*-hemisuccinate-MAb PC4.9A6 Immunoconjugate (SRI 8144-19)	Annual No. 2									

Table I-1. Ribavirin Derivatives, Carriers and Conjugates (continued)

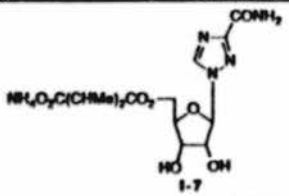
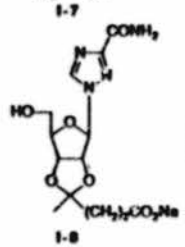
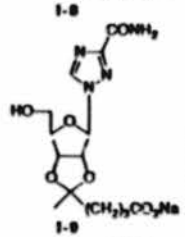
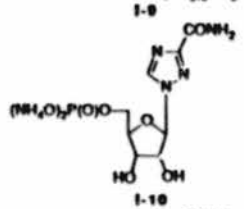
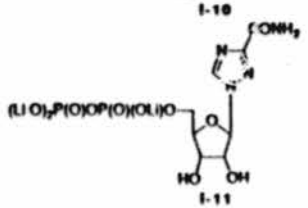
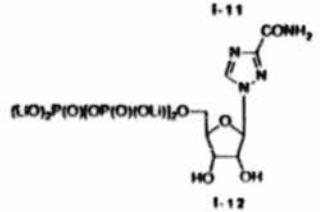
Structure, Name, and Code Number	Report	Biological Activity: CPE against Punta Toro Virus in LLC-MK_2D Cells VR	CPE against Punta Toro Virus in LLC-MK_2D Cells ED_{50} (mM)	CPE against Punta Toro Virus in LLC-MK_2D Cells MTD (mM)	CPE Against Pichinde Virus in Vero 76 Cells VR	CPE Against Pichinde Virus in Vero 76 Cells ED_{50} (mM)	CPE Against Pichinde Virus in Vero 76 Cells MTD (mM)	Plaque Redn Assay for Pichinde Virus in Vero 76 Cells ED_{50} (mM)	Immunofluorescence Assay for Pichinde Virus in Vero 76 Cells ED_{50} (mM)	Immunofluorescence Assay for Pichinde Virus in Vero 76 Cells MTD (mM)
 I-7 Ribavirin-5'-*O*-(2,3-dimethyl)-hemisuccinate Ammonium Salt (SRI 7794-53)	Annual No. 2								0.3-0.8	0.08
 I-8 2', 3'-*O*-Ketal of Ribavirin with Sodium Levulinate (SRI 7422-80)	Annual No. 1	0.0	>4.1	>4.1	0.2	>4.1	>4.1	>4.1		
 I-9 2', 3'-*O*-Ketal of Ribavirin with Sodium 4-Acetylbutyrate (SRI 7422-58, 62, 82)	Annual No. 1	0.0	>4.1	>4.1	0.0	>4.1	>4.1	>4.1		
 I-10 Ribavirin-5'-*O*-phosphate Diammonium Salt (SRI 7421-68, SRI 7747-1)	Annual No. 1								0.08	2.9
 I-11 Ribavirin-5'-*O*-diphosphate Trilithium Salt (SRI 8554-57)	Allen et al., 1978								0.08	2.0
 I-12 Ribavirin-5'-*O*-triphosphate Tetralithium Salt (SRI 8554-58)	Allen et al., 1978								0.35	1.7

Table I-1. Ribavirin Derivatives, Carriers and Conjugates (continued)

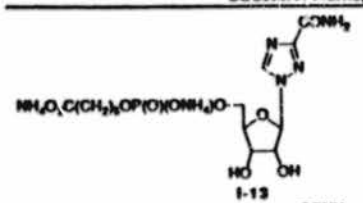
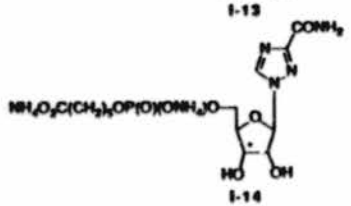
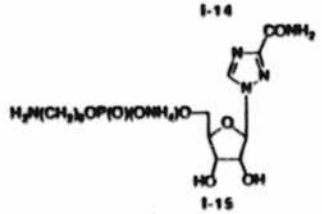
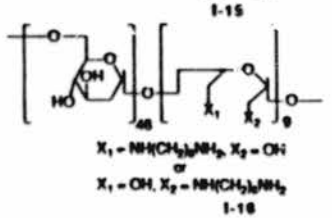
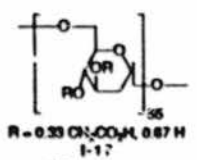
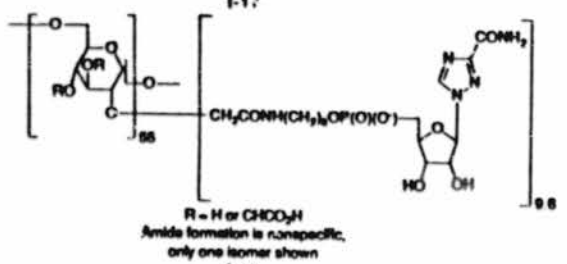
Structure, Name, and Code Number	Report	Biological Activity: CPE against Punta TOro Virus in LLC-MK_2D Cells VR	CPE against Punta TOro Virus in LLC-MK_2D Cells ED_{50} (mM)	CPE against Punta TOro Virus in LLC-MK_2D Cells MTD (mM)	CPE Against Pichinde Virus in Vero 76 Cells VR	CPE Against Pichinde Virus in Vero 76 Cells ED_{50} (mM)	CPE Against Pichinde Virus in Vero 76 Cells MTD (mM)	Plaque Redn Assay for Pichinde Virus in Vero 76 Cells ED_{50} (mM)	Immunofluorescence Assay for Pichinde Virus in Vero 76 Cells ED_{50} (mM)	Immunofluorescence Assay for Pichinde Virus in Vero 76 Cells MTD (mM)
 I-13	Ribavirin-5'-*O*-(5-carboxypentyl)-phosphate Bisammonium Salt (SRI 8315-5)	Annual No. 2								
 I-14	[3'-^{14}C]Ribavirin-5'-*O*-(5-carboxy-pentyl)phosphate bisammonium Salt (SRI 8315-47)	Annual No. 3								
 I-15	Ribavirin-5'-*O*-(6-aminohexyl)-phosphate Ammonium Salt (SRI 8699-28)	Annual No. 3							0.4	>2.3
 I-16	(6-Aminohexylamino)dextran (SRI 8297-61)	Onishi and Nagai, 1986								
 I-17	(Carboxymethyl)dextran (SRI 8554-3)	Annual No. 3							>2.25	72.3
 I-18	Ribavirin-5'-*O*-(6-aminohexyl)phosphate conjugate with (Carboxymethyl)-dextran (SRI 8699-58, SRI 8699-167)	Annual No. 1							>2.3	>2.3

Table I-1. Ribavirin Derivatives, Carriers and Conjugates (continued)

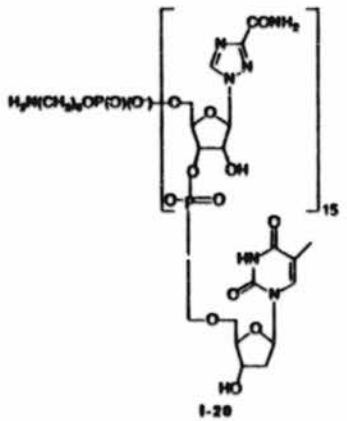
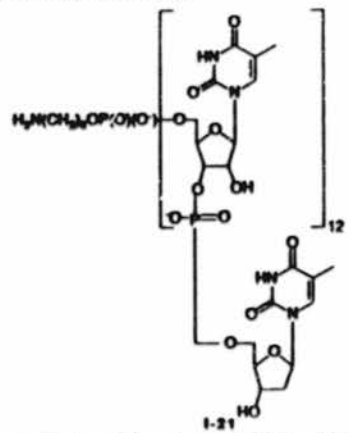
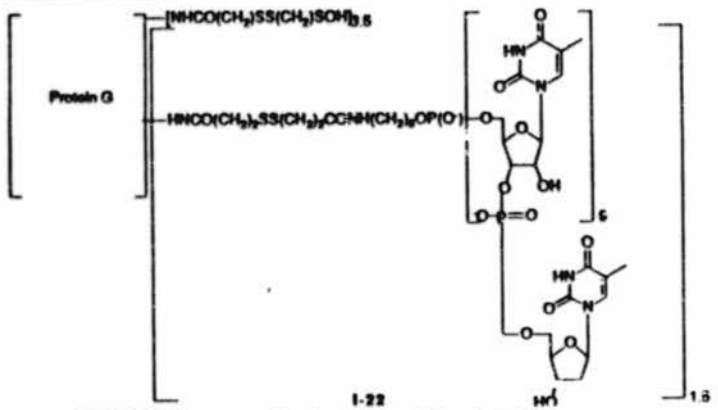
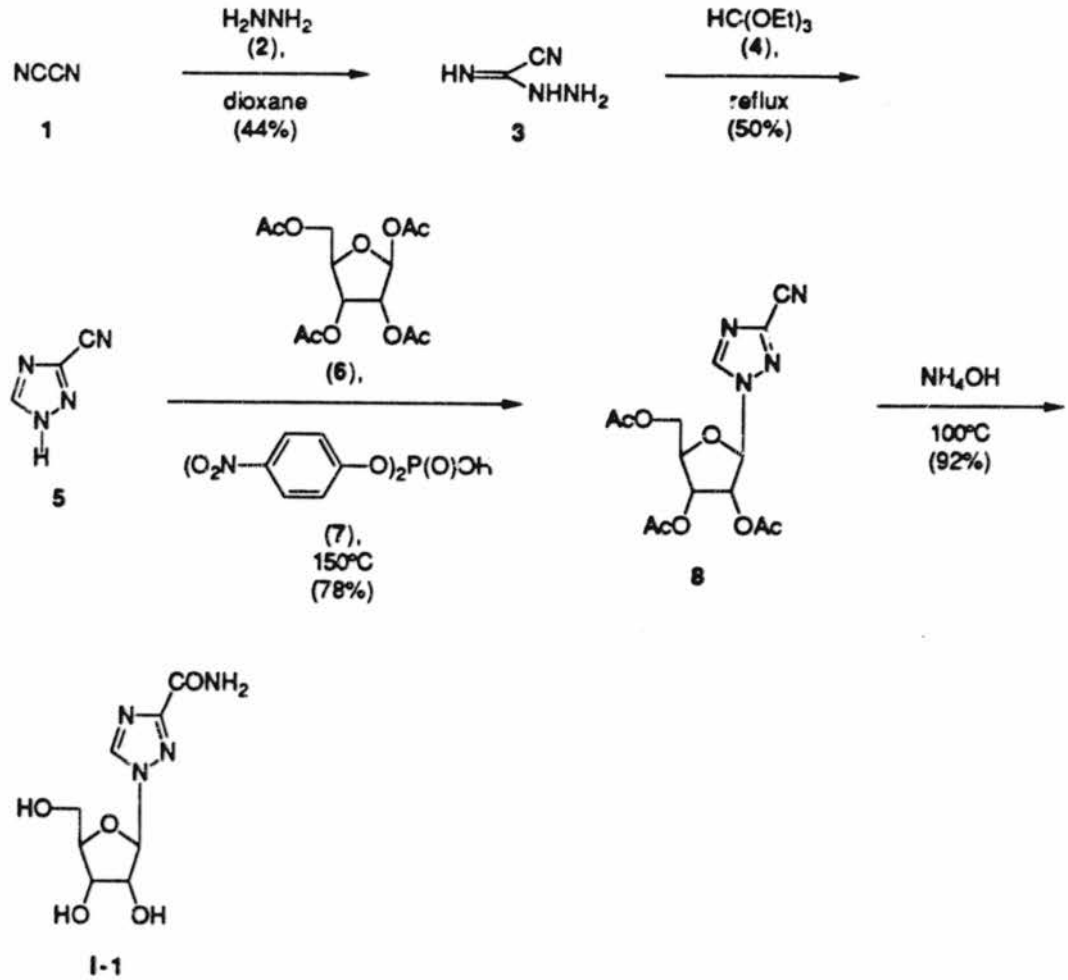
Structure, Name, and Code Number	Report	Biological Activity: CPE against Punta TOro Virus in LLC-MK_2D Cells VR	CPE against Punta TOro Virus in LLC-MK_2D Cells ED_{50} (mM)	CPE against Punta TOro Virus in LLC-MK_2D Cells MTD (mM)	CPE Against Pichinde Virus in Vero 76 Cells VR	CPE Against Pichinde Virus in Vero 76 Cells ED_{50} (mM)	CPE Against Pichinde Virus in Vero 76 Cells MTD (mM)	Plaque Redn Assay for Pichinde Virus in Vero 76 Cells ED_{50} (mM)	Immunofluorescence Assay for Pichinde Virus in Vero 76 Cells ED_{50} (mM)	Immunofluorescence Assay for Pichinde Virus in Vero 76 Cells MTD (mM)
 I-19	Quarterly Report No. 14									
Fluoresceinated (Carboxymethyl)[6-(ribavirin-5'-*O*-phosphato)hexyl-carboxamido]$_4$ gdextran-MAb PC4.9A6 Immunoconjugate (SRI 9047-73)										
 I-20	Annual No. 1								>0.008	>0.008 >0.1 (based on ribavirin)
5'-*O*-(6-Aminohexyl)phosphate of poly(ribavirin-3',5'-*O*-phosphate)thymidine-5'-*O*-phosphate Triethylammonium Salt (SRI 7793-34, SRI 8554-87)										
 I-21	Annual No. 3									
5-*O*-(6-Aminohexyl)phosphate of Poly(thymidine-3'-5'-*O*-phosphate)$_{12}$thymidine-5'-*O*-phosphate Triethylammonium Salt (SRI 8554-[illegible], SRI 8954-36)										

Table I-1. Ribavirin Derivatives, Carriers and Conjugates (concluded)

Structure, Name, and Code Number	Report	Biological Activity							
		CPE against Punta TOro Virus in LLC-MK_2D Cells			CPE Against Pichinde Virus in Vero 76 Cells			Plaque Redn Assay for Pichinde Virus in Vero 76 Cells	Immunofluorescence Assay for Pichinde Virus in Vero 76 Cells
		VR	ED_{50} (mM)	MTD (mM)	VR	ED_{50} (mM)	MTD (mM)	ED_{50} (mM)	ED_{50} (mM) MTD (mM)
 5'-*O*-[6-(3-Mercaptopropionylamido)hexyl]phosphate of Poly(thymidine-3', 5'-*O*-phosphate)$_{11}$thymidine-5'-*O*-phosphate Triethylammonium Salt - Protein G Conjugate (SRI 7747-85)	Final Report								

Scheme I-1. Synthesis of Ribavirin (I-1)

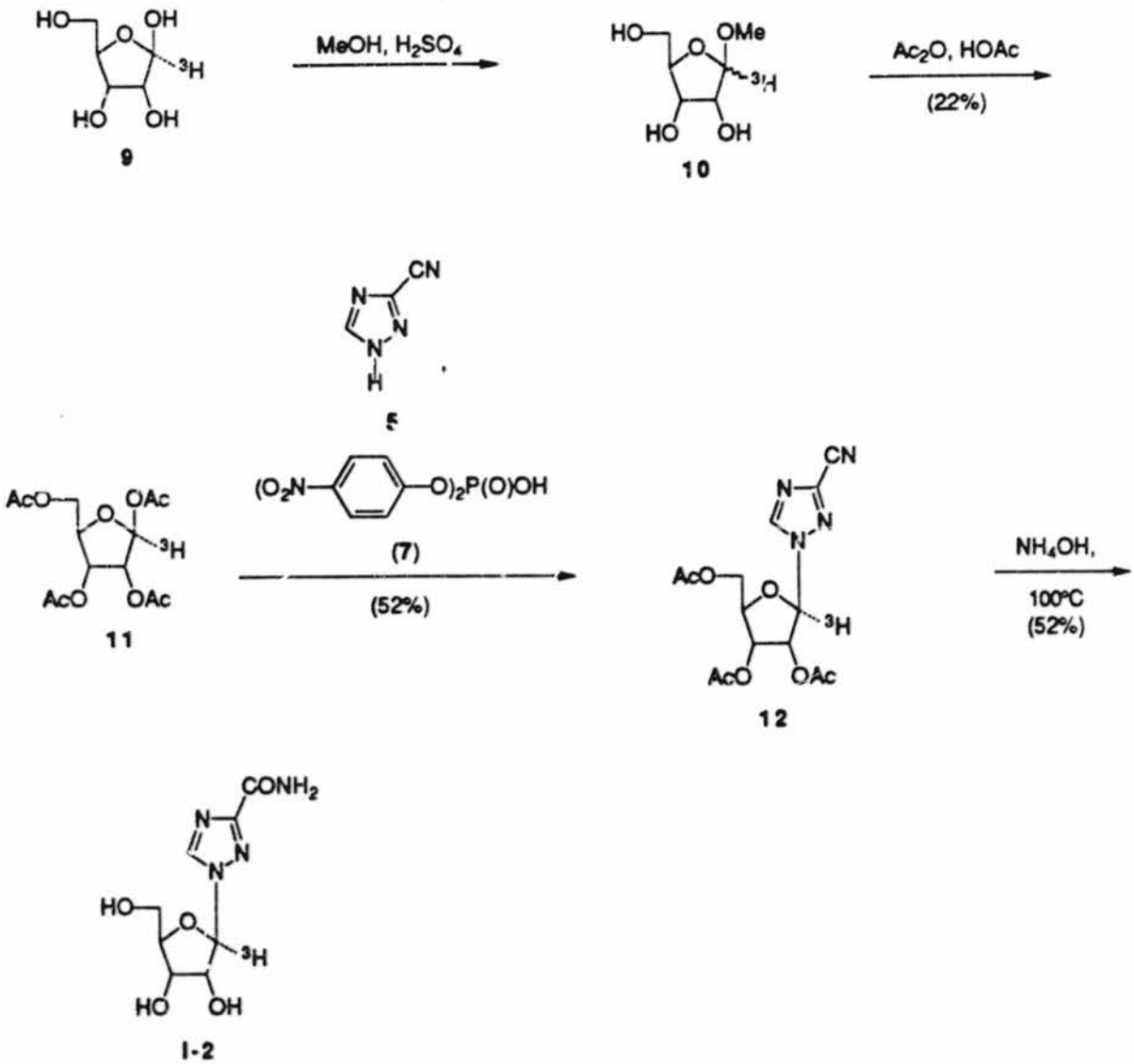


sufficient quantity for conversion to all the functionalized derivatives required. [1'-^{3}H]Ribavirin (**I-2**) was prepared in a fashion analogous to the route used for unlabeled [1-^{3}H]ribose (Scheme I-2). Two cold runs on the scale that would be used for the radiolabeled synthesis were first performed to verify the experimental methods. [1'-^{3}H]Ribavirin of 97.5% radiochemical purity (specific activity 0.82 mCi/mmol) was obtained. [3'-^{14}C]Ribavirin was prepared by the same route.

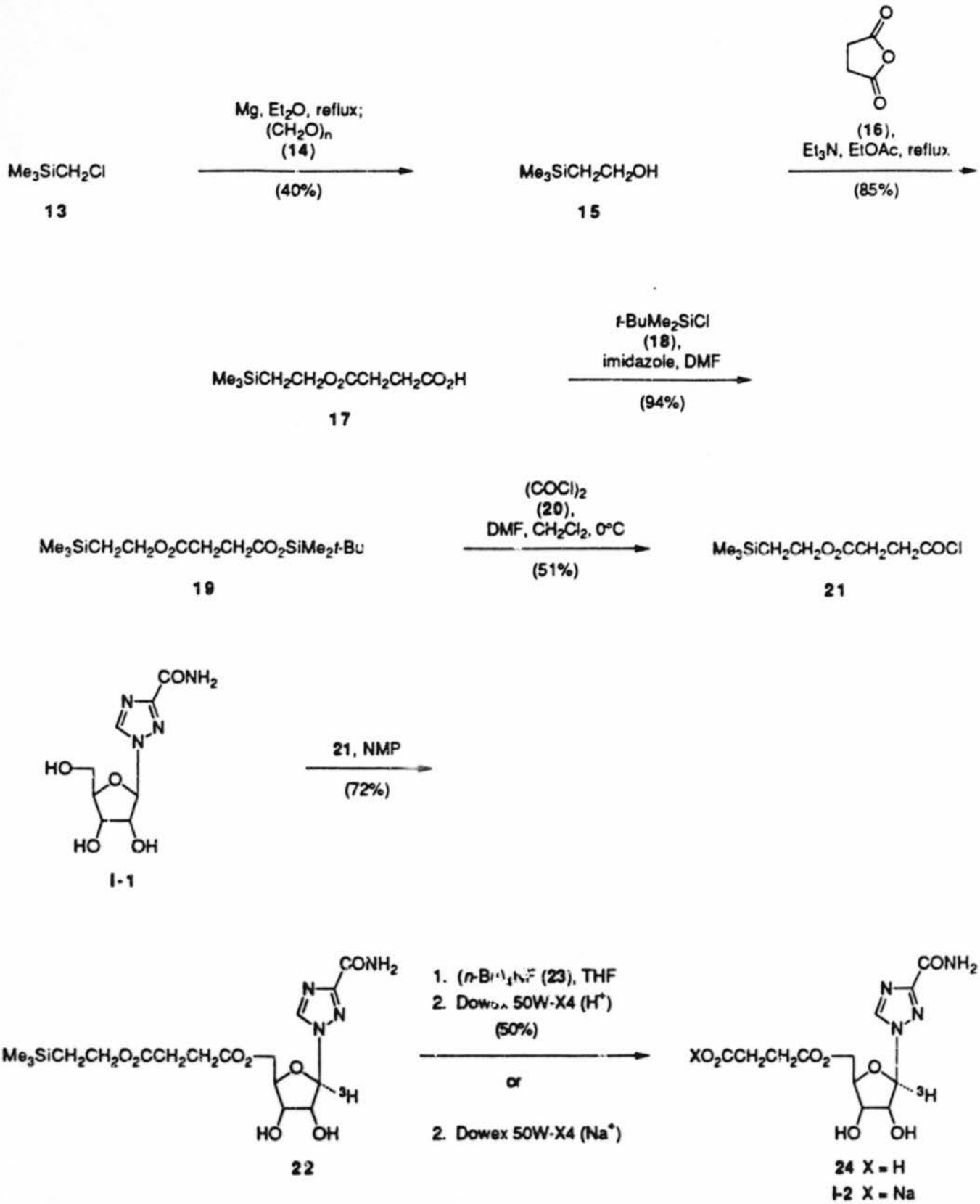
3. **Ribavirin-5'-*O*-carboxyl esters.** The first ribavirin derivative synthesized was the 5'-*O*-hemisuccinate (**I-3**), which was prepared by the sequence of reactions depicted in Scheme I-3. The carboxyl group of this hemisuccinate was to be used for linking to a MAb by formation of an amide bond with the ε-amino groups of the lysines of the Mab. Several routes for the formation of the 5'-*O*-hemisuccinate were explored before success was achieved. Direct reaction of succinic anhydride with ribavirin in dimethylformamide has been reported to give the desired product (Austin et al., 1983). Ribavirin was allowed to react with 1.0 equivalent of succinic anhydride in *N*-methyl-pyrrolidinone at 60°C for 1 h, and the resulting reaction mixture was purified on DEAE-Sephadex A-25 resin (0.0-0.5 M ammonium bicarbonate gradient) to give starting material, succinylated ribavirin, and polysuccinylated ribavirin. The second fraction proved to be a mixture of 2'-, 3'-, and 5'-*O*-hemisuccinates according to the ^{1}H NMR spectrum, which showed three signals at 8.82, 8.87, and 8.90 ppm for the 5-proton of the triazole ring in a ratio of 1.0:1.3:3.6. The low selectivity of this succinylation may have been caused by the temperature at which it was conducted. Unfortunately, lower reaction temperatures did not dissolve ribavirin and therefore no reaction occurred. Because the hemisuccinate mixture could not be further purified efficiently, this approach was abandoned.

The second approach to the 5'-*O*-hemisuccinate involved the selective acylation of the primary 5'-hydroxyl group of ribavirin using an activated succinate ester that had one carboxyl group protected by a readily cleavable ester group. This approach was based on

Scheme I-2. Synthesis of [1'-^{3}H]Ribavirin (I-2)



Scheme I-3. Syn. ases of Ribavirin-5-*O*-Hemisuccinate Sodium Salt (I-3)



the report (Okabayashi et al., 1977) that acid chlorides of succinate half esters react specifically with the 5'-hydroxyl group of nucleosides. The 2,2,2-trichloroethyl ester protecting group was investigated first because this group can be readily cleaved under mild reductive conditions. Reaction of succinic anhydride with 2,2,2-trichloroethanol in refluxing ethyl acetate in the presence of 1.0 equivalent of triethylamine as the base gave the 2,2,2-trichloroethyl hemisuccinate in 80% yield after recrystallization from chloroform-hexane. This hemisuccinate was converted to the acid chloride by treatment with 9.0 equivalents of thionyl chloride at relux for 1 h. The acid chloride was not purified but allowed to react directly with ribavirin in *N*-methylpyrrolidinone to give a 65% yield of the 5'-*O*-hemisuccinate ester of ribavirin, the structure of which was confirmed by IR and ^{1}H NMR spectroscopy and elemental analysis. The next step in the synthesis of ribavirin-5'-*O*-hemisuccinate was the cleavage of the trichloroethyl protecting group, which proved to be problematic. Reductive cleavage with chromous chloride in acetone (Meyers et al., 1979) gave a complex mixture of products, whereas reductive cleavage with zinc in aqueous ammonium acetate-tetrahydrofuran (Just et al., 1976) for three days gave a small amount of the desired hemisuccinate and another compound from which it could not be separated by ion-exchange chromatography on DEAE-Sephadex A-25 (0.0-0.5 M ammonium bicarbonate gradient).

The readily cleavable 2-(trimethylsilyl)ethyl ester was the next protecting group investigated. Use of this group resulted in a successful synthesis of the 5'-*O*-hemi-succinate ester of ribavirin (Scheme I-3). The synthetic sequence was similar to that used for preparation of the 2,2,2-trichloroethyl succinate ester of ribavirin. Trimethylsilyl chloride (**13**) was converted to the Grignard reagent by treatment with magnesium in refluxing ether. Reaction of the Grignard reagent with paraformaldehyde (**14**) followed by an aqueous ammonium chloride workup afforded 2-(trimethylsilyl)ethanol (**15**) in 40% yield. This silylated alcohol was heated for 2 h with succinic anhydride in refluxing ethyl

acetate containing 1.0 equivalent of triethylamine as the base to give an 85% yield of 2-(trimethylsilyl)ethyl hemisuccinate ester (**17**).

In contrast to the 2,2,2-trichloroethyl ester protecting group, the 2-(trimethylsilyl)-ethyl protecting group was too labile to survive the direct conversion of the hemisuccinate to the acid chloride. Both thionyl chloride and oxalyl chloride, even in the presence of triethylamine as the base, cleaved the 2-(trimethylsilyl)ethyl protecting group. Treatment of this hemisuccinate with thionyl chloride gave succinic anhydride, presumably from protodesilylation by the hydrogen chloride produced from formation of the acid chloride. Because direct conversion was not possible, a stepwise procedure was employed. The *t*-butyldimethylsilyl ester (**19**) of **17** was prepared in 94% yield using *t*-butyldimethylsilyl choride (**18**) and imidazole in dimethylformamide at room temperature for 24 h (Corey and Venkateswarlu, 1972). The *t*-butyldimethylsilyl ester was readily converted to the acid chloride **21** in 52% yield under aprotic conditions, using oxalyl chloride (**20**) in methylene chloride at 0°C for 1.5 h (Wissner and Grudzinskas, 1978). Treatment of ribavirin with acid chloride **21** in *N*-methylpyrrolidinone for 16 h at room temperature and purification by chromatography on silica gel with 0-10% methanol-chloroform gave the 5'-*O*-succinate ester **22** in 72% yield. Removal of the 2-(trimethylsilyl)ethyl protecting group with tetra(*n*-butyl)-ammonium fluoride (**23**) in tetrahydrofuran at room temperature for 18 h, followed by DEAE-Sephadex A-25 (0.0-05 M ammonium bicarbonate gradient) and Dowex 50W-X4 resin (H^+ form) chromatographies gave ribavirin-5'-*O*-hemisuccinic acid (**24**) in 50% isolated yield after lyophilization. Unfortuately, this material proved to be very hygro-scopic and in the presence of water gave a solution with a pH lower than 2, leading to hydrolysis of the carboxylate ester at the 5'-position of the ribofuranose ring. To enhance compound stability, the sodium salt (**I-3**) of acid **24** was prepared. Cleavage of the ester protecting group of **23** and purification by ion-exchange chromatography on

Dowex 50W-X4 resin (Na^+ form) gave a 46% yield of the sodium carboxylate **I-3**. This material was hygroscopic also but did not decompose on storage.

To accurately assess the activity of this derivative in biological assays, its stability under the antiviral assay conditions was determined. If ribavirin were released by hydrolysis in the assay medium rather than in the virally infected cells, false positive results would be obtained. Therefore a 4.0 nM solution of the sodium salt of the 5'-*O*-hemisuccinate ester was incubated under physiologic conditions (0.05 M potassium phosphate buffer, pH 7.4, 0.15 M NaCl; 37°C bath) and aliquots were analyzed by high-performance liquid chromatography (HPLC). As shown in Table I-2, the reverse-phase chromatography conditions cleanly separated the derivatives from ribavirin. HPLC analysis indicated that ribavirin was stable to the assay conditions after incubation at physiologic pH at 37°C for 15 days. The hemisuccinate of ribavirin slowly hydrolyzed with 17% being converted to ribavirin after seven days and 30% after 15 days.

Table I-2. Reverse-Phase HPLC[a] of Ribavirin (**I-1**) and Its Derivatives **I-3**, **I-8**, and **I-9**

Compound	Retention Time (min)
Ribavirin (**I-1**)	2.02
I-3	2.27
I-9	3.09 (4.82)[b]
I-8	6.58 (8.62)[b]

[a]C18 Nova-Pak column with elution at a flow rate of 2.0 mL/min with 98% 0.1 M sodium acetate-2% methanol (5 min), then a linear gradient to 80% 0.1 M sodium acetate-20% methanol (6 min), followed by a rinse with the original buffer mixture (1 min) and re-equilibration (4 min), and detection at 254 nm.

[b]Minor diastereomer is in parentheses. Ratio of peak areas was 9:1 for **I-8** and 2:1 for **I-9**.

These results indicated that if the carboxylate ester approach were to be an effective one, our synthetic efforts should be directed to synthesizing more sterically hindered esters that would have a slower rate of hyrolysis. A 5'-*O*-hemisuccinate with an alkyl substituent α to the ester bond would have a much slower rate of hydrolysis for this reason. The 5'-*O*-(2,3-dimethyl)hemisuccinate (**I-7**) of ribavirin was synthesized to test this hypothesis. The synthesis of this derivative followed that of the unsubtituted hemisuccinate **I-3**. meso-2,3-Dimethylsuccinic acid (**25**) was heated at reflux with acetyl chloride (**26**) to afford 2,3-dimethylsuccinic anhydride (**27**) in 89% yield (Scheme I-4). Treatment of anhydride **27** with 2-(trimethylsilyl)ethanol (**15**) gave the hemiester **28** in 82% yield. Reaction of hemiester **28** with *t*-butyldimethylsilyl chloride (**18**) in dimethylformamide afforded mixed diester **29** in 76% yield after vacuum distillation. Reaction of **29** with oxalyl chloride (**20**) produced, in 80% yield, the unstable and extremely moisture-sensitive acid chloride **30**, which was not purified but was immediately allowed to react with ribavirin in *N*-methyl-pyrrolidinone in the presence of 0.1 equivalent of 2,6-di(*t*-butyl)pyridine (**31**). Workup and chromatography on silica gel gave the 2-(trimethylsilyl)ethyl 2,3-dimethyl-succinate 5'-*O*-ester of ribavirin (**32**) in 36% yield. Treatment of **32** with tetra(*n*-butyl)-ammonium fluoride (**23**) in tetrahydrofuran afforded the free acid in 85% crude yield. This material was purified by ion-exchange chromatorgraphy on DEAE-Sephadex A-25 (0.0-0.25 M ammonium bicarbonate gradient) to afford the ammonium salt **I-7** of the 2,3-dimethyl-succinate 5'-*O*-ester of ribavirin in 60% yield.

Hydrolysis studies indicated that **I-7** remained intact after 13 days at 37°C in phosphate-buffered saline (PBS) at pH 7.4, whereas 20% of the unsubstituted hemi-succinate **I-3** underwent hydrolysis over the same period of time (Table I-3). These studies indicated that the 5'-*O*-(2,3-dimethyl)succinate of ribavirin should be a better candidate for tethering to a MAb than the 5'-*O*-hemisuccinate. Ribavirin, ribavirin-5'-*O*-hemisuccinate, and ribvavirin-5'-*O*-(2,3-dimethyl)succinate were screened for antiviral

Scheme I-4. Synthesis of Ribavirin-5'-*O*-(2,3-dimethyl)hemisuccinate (I-7)

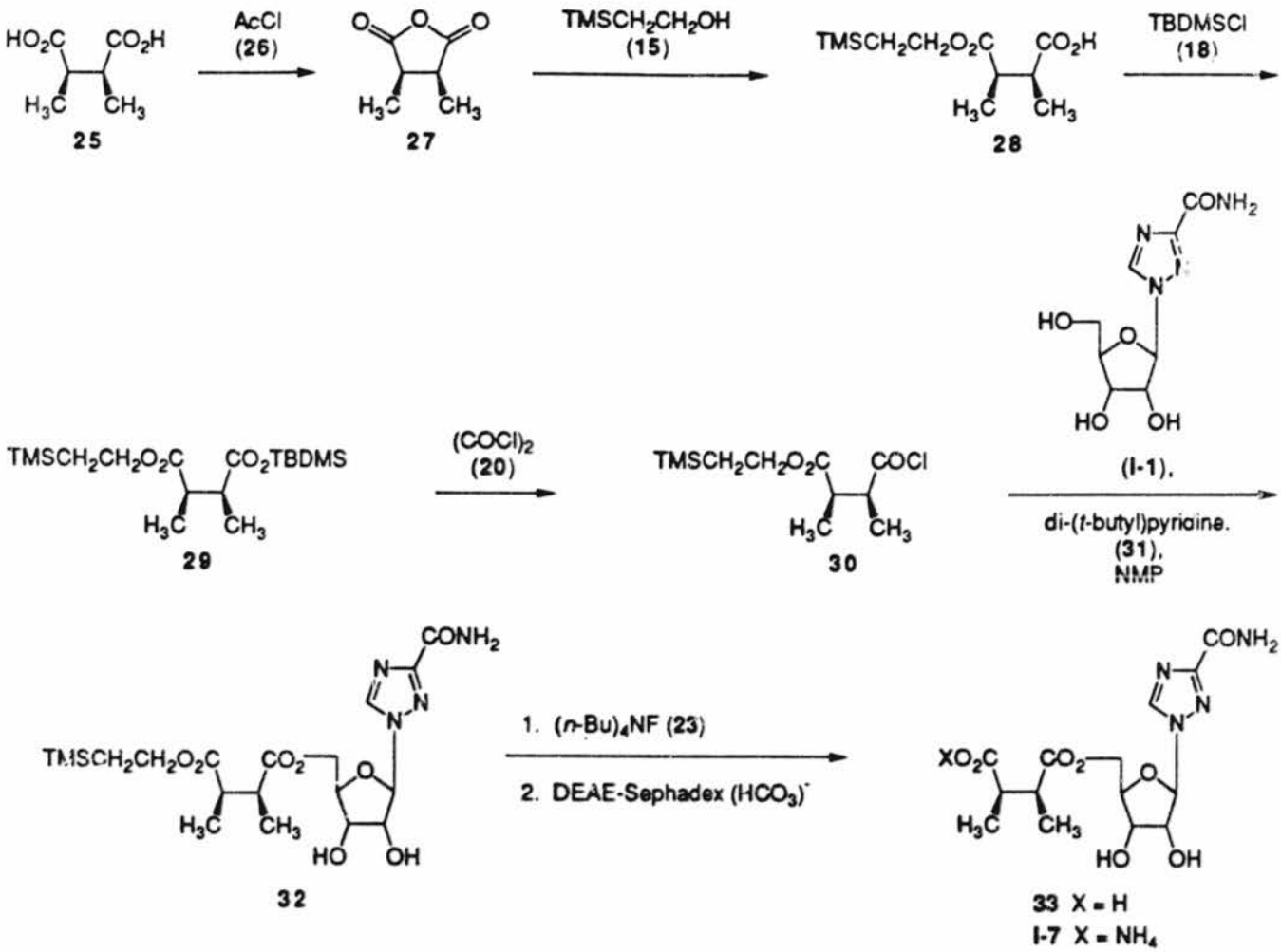


Table I-3. Hydrolysis of the 5'-*O*-Hemisuccinate and 5'-*O*-(2,3-Dimethyl)hemisuccinate of Ribavirin

Tether	Ribavirin Released (%)[a]				
	0 h	48 h	144 h	240 h	312 h
5'-*O*-Hemisuccinate **I-3**	0	7	11	15	20
5'-*O*-(2,3-Dimethyl)hemisuccinate **I-7**	0	0	0	0	0

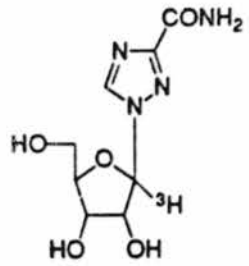
[a]Estimated using high-performance chromatography on a reverse-phase Waters Radialpak A column using 0.1 M ammonium acetate buffer-methanol (98:2) at a flow rate of 2.0 mL/min, detection at 260 nm and benzoic acid as the internal standard. The retention times were: ribavirin, 3.6 min; benzoic acid, 3.9 min; 5'-*O*-hemisuccinate of ribavirin, 4.6 min; and 5'-*O*-(2,3-dimethyl)hemisuccinate of ribavirin, 7.8 min (SRI Experiment 7794-57).

activity against Pichinde virus infection in Vero 76 cells using an immunofluoresence assay. Ribavirin had an ED_{50} between 3.2 and 10 µg/ml and a maximum tolerated dose (MTD) of 320 µg/ml. Ribavirin-5'-*O*-hemisuccinate had an ED_{50} for antiviral activity that was one-log higher and a MTD that was three-fold higher. Therefore, this derivative had a more favorable therapeutic index than did ribavirin. In contrast, the 5'-*O*-(2,3-dimethyl)-hemisuccinate had an ED_{50} that was two-logs higher than that of ribavirin and a MTD that was one-tenth that of ribavirin, indicating a far less favorable therapeutic index than that of either ribavirn or its 5'-*O*-hemisuccinate. Although the therapeutic index of these derivatives may change after conjugation to a MAb, for preliminary experiments we decided to use the ribavirin-5'-*O*-hemisuccinate for preparation of a ribavirin-MAb conjugate because of its greater activity.

4. Conjugate Loading Methodology. Estimation of ribavirin loading on the MAb-ribavirin conjugate required development of a reliable assay procedure. The weak UV chromophore of ribavirin (206 nm, ϵ 1.2 x 10^4 in water), which was coincident with the end absorption of the Mab, precluded spectroscopic drug-loading estimations. Radiolabeling was considered the most accurate method and necessitated the synthesis of radiolabeled derivatives. Process development was initiated using unlabeled ribavirin to prepare ribavirin-5'-*O*-hemisuccinate on a small scale in order to develop methodology for preparing [1'-^{3}H]ribavirin-5'-*O*-hemisuccinate from the tritiated drug. After this methodology had been developed, the radiolabeled derivative (specific activity, 6.778 mCi/mmol) was prepared in two steps in 28% overall yield on a 0.032-mmol scale (Scheme I-5). [3'-^{14}C]Ribavirin-5'-*O*-hemisuccinate was also prepared. The tritiated material was used for the inital conjugation work with MAb PC4.9A6 (**36**). Formation of an amide bond between the MAb and the hemisuccinate was effected using the water-soluble carbodiimide 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDIC, **35**) as the coupling agent between the lysine amine groups of the MAb and the carboxyl group of the hemisuccinate. Protein recovery after conjugation and chromatographic purification was 27%. Loading of ribavirin on the ribavirin-5'-*O*-hemisuccinate-MAb PC4.9A6 conjugate was determined to be 6.7 mol of drug per mol of MAb from the molar concentrations determined by scintillation counting of the labeled drug and by absorbance at 280 nm for the protein.

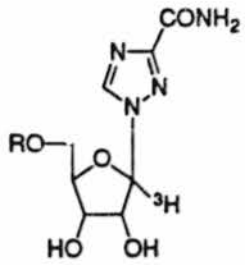
Because the large quantities (10 mg) of conjugate required for antiviral screening required large amounts of the radiolabeled derivatives of ribavirin and because radiolabeled material would be required for every conjugation experiment, we decided to explore alternate methods of preparing the conjugate that would permit us to determine drug loading. The synthetic method selected was to use an activated ester derivative of ribavirin-5'-*O*-hemisuccinate that would release a chromophoric group during the conjugation to the

Scheme 1-5. Synthesis of Radiolabeled Ribavirin-5-*O*-hemisuccinate-MAb PC4.9A6 Conjugate (**I-6**)



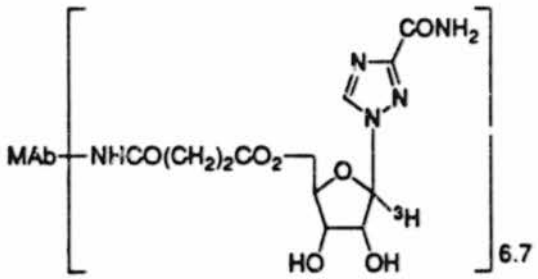
I-2

$TMS(CH_2)_2O_2C(CH_2)_2COCl$ (**21**), NMP



34 R = $TMS(CH_2)_2O_2C(CH_2)_2CO$
I-4 R = $NH_4O_2C(CH_2)_2CO$

1. $(n\text{-}Bu)_4NF$ (**23**), THF
2. EDIC (**35**), MAb PC4.9A6 (**36**), 0.01 M K Phosphate, 0.5 M NaCl, pH 7.6



I-6

protein. The 4-nitrophenyl ester of the hemisuccinate was selected as the active ester species. Release of the 4-nitrophenoxide anion during alkylation of the Mab would afford an indirect method of determining loading of the drug onto the MAb by monitoring the absorbance at 404 nm, which is the absorbance maximum of 4-nitrophenoxide at pH 7.6. Protein recovery after conjugation using this method was 66%. Loading of ribavirin onto the conjugate was determined to be 4.8 mol of drug per mol of MAb from the 4-nitrophenoxide released after the amount formed by hydrolysis was subtracted. Although initial experiments using this method of loading appeared promising, further work indicated that the method was not reproducible. The lack of reproducibility may have been caused by the low solubility of the 4-nitrophenoxide ester in the reaction buffer.

Another loading method studied was the spectrophotometric measurement of the number of free amino groups on the protein. In the immunoconjugation, the drug is linked to the amino groups of the MAb by an amide bond. Therefore, the reacted amino groups are no longer able to react with trinitrophenylsulfonate in the Habeeb primary amino group assay for proteins (Habeeb, 1966) to give a yellow color absorbing at 335 nm. The difference in the number of free amino groups on the protein before [there being approximately 90 amino groups on a mouse antibody (Habeeb, 1966)] and after the conjugation step would give the number of amino groups reacting with the drug and, hence, the number of drugs bound per antibody.

To ascertain the accuracy of the Habeeb method, studies on methotrexate-immunoglobulin conjugates were performed concurrently with the studies on the ribavirin conjugates. Because methotrexate has a UV chromophore at 370 nm (ε 6.64 x 10^3) in PBS, pH 7.6, loadings were readily determined spectrophotometrically as a check for the accuracy of the method. Protein concentrations were determined by the absorbance at 280 nm (ε 2.5 x 10^5), with MTX and ribavirin controls run. MTX had an appreciable absorbance at 280 nm (0.4 of the 370-nm absorption) that had to be subtracted to accurately

assess the protein concentration of the immunoconjugate solutions. At 280 nm, the absorbance of ribavirin was negligible. Protein concentrations were verified by the Lowry procedure (Lowry et al., 1951). Neither MTX nor ribavirin absorbed at 660 nm under Lowry conditions and therefore did not interfere with the assay. There was excellent agreement between the immunoglobulin concentrations determined by the UV spectrophotometric and Lowry methods. Unfortunately, this agreement was not found between the spectrophotometric and Habeeb methods. UV absorption indicated that there was an average of 4.3 MTX molecules bound per molecule of mouse antibody, whereas the Habeeb method indicated 3.9. UV absorption indicated 3.4 molecules of MTX bound per human antibody, whereas the Habeeb method indicated 5.7. These different loading results may reflect the inaccuracy of using this type of modification in the Habeeb method in which two large numbers are subtracted to give a small number. Greater accuracy could perhaps be achieved if far higher drug loadings were obtained. Preliminary experiments indicated a maximum loading of 11 ribavirin molecules per MAb by the 4-nitrophenyl ester method. This level of loading would probably not be sufficient for loading determinations by the Habeeb method.

Some studies were performed to develop a radioimmunoassay (RIA) to quantitate drug loading. A RIA for ribavirin in serum has been developed using rabbit polyclonal serum (Austin et al., 1983). We proposed that the polyclonal antibodies in the serum should recognize the ribavirin derivative terthered to MAb PC4.9A6 because they are directed against ribavirin-5'-*O*-hemisuccinate covalently linked to ovalbumin--a conjugate prepared by the same method of attachment as that used to prepare the MAb conjugate. Rabbit anti-ribavirin serum was obtained from J. D. Connor, M. D., University of California Medical School, San Diego, CA, and [^{14}C]ribavirin of high specific activity (33 mCi/mmol) was obtained from Viratek, Irvine, CA. Unfortunately, we were not able to reproduce the reported assay procedure using unbound ribavirin. For this reason, Dr.

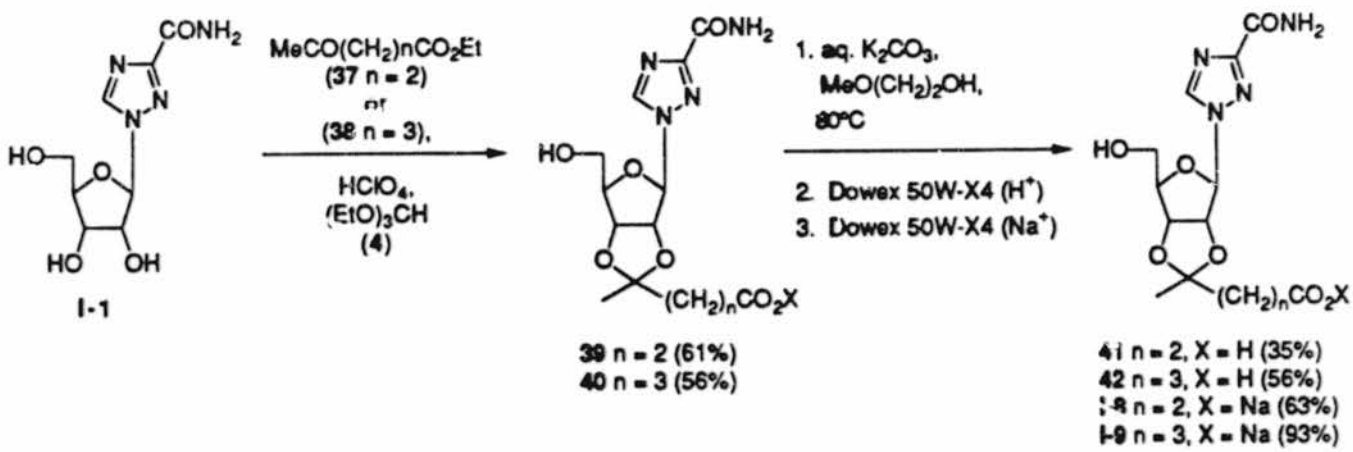
Connor graciously offered to screen the ribavirin-MAb conjugate to determine the amount of ribavirin covalently bound to the conjugate. A loading of 2.00 mol of ribavirin per mol of MAb was found by the RIA assay. Therefore, only 42% of the available covalently linked ribavirin was accessible to the polyclonal antibodies.

Therefore of these four assay methods for loading: (1) radiolabeling, (2) 4-nitrophenoxide anion release from an activated ester, (3) estimation of unreacted amino groups on the conjugate by the Habeeb method, and (4) RIA, the first method was the most accurate and reproducible.

5. Ribavirin-2',3'-*O*-ketals. Because the lability of the unhindered hemisuccinate bond and the lack of activity and the low therapeutic index of the more hindered (2,3-dimethyl)succinate derivative were concerns, we decided to explore alternate methods of tethering. Tethering through the 2'- and 3'-hydroxyl groups of ribavirin was investigated next. It was proposed that a ketal prepared by reaction of a ketone with these hydroxyl groups would be sufficiently stable at physiologic conditions (pH 7.2) to permit the MAb to deliver the drug to the virally infected cell, and that, once in the cell, would be cleaved in the acidic environment of the lysozomes to release ribavirin. The ketones selected for tethering had a carboxylic acid group for linkage to a MAb through an amide bond. The two keto esters selected for ketalization with ribavirin were ethyl levulinate (**37**) and ethyl 4-acetylbutyrate (**38**). Ketalization of ribavirin with ethyl levulinate under standard ketalization conditions using *p*-toluenesulfonic acid as the acid catalyst in refluxing benzene failed because of the low solubility of ribavirin in this solvent. The use of excess *p*-toluenesulfonic acid (10 equivalents) in ethyl levulinate as both the reactant and the solvent--a procedure that is effective at forming the acetonide of some nucleosides in acetone (Hampton and Magrath, 1957)--did not lead to ketalization. Bis(4-nitrophenyl) phosphate in either dimethyl sulfoxide or ethyl levulinate as the solvent also failed. Bis(4-nitrophenyl) phosphate was reported to be effective at forming the acetonide of uridine in

acetone (Hampton, 1961). The ketal **39** was successfully prepared using a catalytic amount (0.14 equivalent) of the much stonger acid, perchloric acid (Fuertes et al., 1974), in ethyl levulinate as the solvent and in the presence of triethyl orthoformate (**4**) to remove the water formed in the reaction (Scheme I-6). Reaction for 2 h at room temperature followed by chromatography on silica gel with 0-10% methanol/chloroform gave a 51% yield of ketal ester **39**. Only one diastereomer was isolated from this reaction and chromatography. The newly formed chiral center at the 2-position of the dioxolane ring had an R configuration. This configuration was established from the Nuclear Overhauser Effect (NOE) between the 1'-proton on the ribofuranose ring of ribavirin and the ß-methylene protons of the 2-propionate group of the dioxolane ring that was observed in the ^{1}H NMR spectrum. The appearance of such an NOE indicated that the 1' and methylene protons were within 3.5 Å of one another, leading to the conclusion that the propionate side chain resided under the ribose ring and the methyl group projected outward. Subsequent preparations afforded both ketals.

Scheme I-6. Synthesis of Ribavirin 2',3'-*O*-Ketals and Their Sodium Salts **I-8** and **I-9**



Ketalization using ethyl 4-acetylbutyrate (**38**) in the presence of perchloric acid as the catalyst afforded a 25% yield of a mixture of two diastereomeric ketals (**40**), which were isolated by chromatography on silica gel with 0-10% methanol-chloroform. The integrals of the ethyl ester quartets at 4.01 and 4.05 ppm in the ^{1}H NMR spectrum of this ketal mixture were in the ratio of 1:4.

The hydrolysis of the ester protecting groups on ketals **39** and **40** was investigated next. Aqueous potassium hydroxide in ethanol proved to be too basic because the carboxamide group at the 3-position of the triazole ring of ribavirin was also hydrolyzed. This side reaction was reduced by using potassium carbonate as the base. Methoxyethanol was employed as the organic solvent to improve solubility of the starting material. Ketal esters **39** and **40** were treated with 3.0 equivalents of 1 M aqueous potassium carbonate in methoxyethanol (1:1/v:v) at 80°C for 2 h. After cooling to room temperature, the reaction mixtures were partially neutralized with Dowex 50W-X4 resin (H^+ form, pH 5) before purification by chromatography on DEAE-Sephadex A-25 (0.0-0.5 M ammonium bicarbonate gradient) and ion exchange with Dowex 50W-X4 resin (H^+ form) to give the ketal acids **41** and **42** in yields of 56% and 25%, respectively, as fluffy white solids. Both acids were very hygroscopic and proved to be unstable in hydrated form, being hydrolyzed to ribavirin and levulinic and acetylbutyric acids. Hydrolysis also occurred on dissolution in water (pH 4). However, the acids were quite stable in anhydrous solvents and in basic solution. Therefore, to circumvent this hydrolysis problem, the sodium salts **I-8** and **I-9** of **41** and **42**, respectively, were prepared. The salts were stable on storage at room temperature.

Because of hydrogen binding of the hydroxyl protons of these derivatives with the polar solvent in which the ^{1}H NMR spectra were run and because the sodium salts of the ketals were diastereomeric mixtures at the 2-position of the dioxolane ring of the ketal, the interpretation of the spectral signals proved to be difficult. Nevertheless, the spectra

indicated that esterification of the ribofuranose ring of ribavirin at the 5'-hydroxyl group shifted the the 4'- and 5'-protons downfield and that ketal formation shifted the 1'-, 2'-, 3'-, and 4'-protons downfield, with the largest shift occurring for the 2'- and 3'-protons. However, these two protons could not be distinguished. The signals for the protons on the methyl group at the 2-position of the dioxolane ring of ketals **I-8** and **I-9** appeared as two singlets whose integrals corresponded to the ratio of the diastereomers (9:1 and 9:2, respectively) found by HPLC analysis. In addition, the signal for the 5-hydrogen on the triazole ring of ketal derived from levulinic acid appeared as two singlets in a ratio of 8:2.

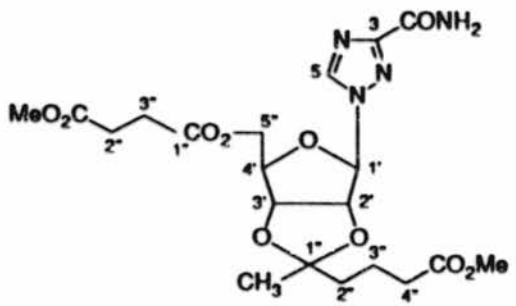
To verify the structures of these ketals, their ^{13}C NMR spectra were compared with that of ribavirin (Table I-4), the spectrum of which in hexafluorobenzene has been reported (Kreishman et al., 1972). The triazole ring and amide carbon signals [146.3 (5), 158.4 (3), and 1621.8 ppm ($CONH_2$)] were identified by comparison with the signals of the carbons of other ribavirin analogs. The corresponding signals for ribavirin were shifted downfield by approximately 8.7 ppm when dimethyl sulfoxide-d_6 was used as the solvent. On derivatization of the ribofuranose ring, the positions for these signals remained constant. Esterification of the 5'-hydroxyl group of ribavirin shifted the 5'-carbon signal 2.3 ppm upfield and the 4'-carbon signal 3.6 ppm downfield but had very little effect on the 2'- and 3'-carbon signals. The signal for the carboxylate carbon of the ketal of 4-acetylbutyrate was assigned downfield relative to that of the ester carbonyl because similar downfield shifts of 2 ppm were observed for the carbonyl carbon on conversion of the carboxylic acids **41** and **42** to the corresponding sodium carboxylates **I-8** and **I-9**, respectively. As in the spectrum of ribavirin, the 2'- and 3'-carbon signals could not be assigned. This was also the case with the methylene carbons adjacent to the carbonyl carbons of the tether group.

The 2'- and 3'-carbon signals in the spectra of ketals **I-8** and **I-9** were shifted downfield by approximately 10 ppm, whereas the 4'-carbon signal was shifted upfield by

Table I-4. ^{13}C Spectra of Ribavirin (I-1) and Its 5'-*O*-Hemisuccinate Sodium Salt I-3 and 2',3'-*O*-Ketal Sodium Salts I-8 and I-9[a]

	Carbon (ppm)[b]													
	Triazole Ring			Ribofuranose Ring					Tether Group					
Cmpd	3	5	$CONH_2$	1'	2'[c]	3'[c]	4'	5'	CH	1"	2"	3"	4"	CO
I-1	167.3	155.0	170.5	101.8	84.6	80.1	95.6	71.3						
I-3	167.4	155.4	170.5	101.8	84.2	80.4	92.0	73.7		183.3	40.8[c]	42.3[c]		185.9
I-8	167.4	155.4	170.4	102.8	94.0	91.5	98.3	71.4	33.4	124.5	30.7	48.3[c]	48.6[c]	187.2
				(94.4)	(92.0)	(98.5)		(34.2)	(124.9)	(31.4)				
I-9	167.3	155.3	170.4	102.9	94.0	91.5	98.3	71.3	33.4	124.5	42.5	46.2		186.8
					(91.8)			(33.7)	(124.9)	(43.3)				

[a]The general numbering system for ribavirin and its derivatives is:



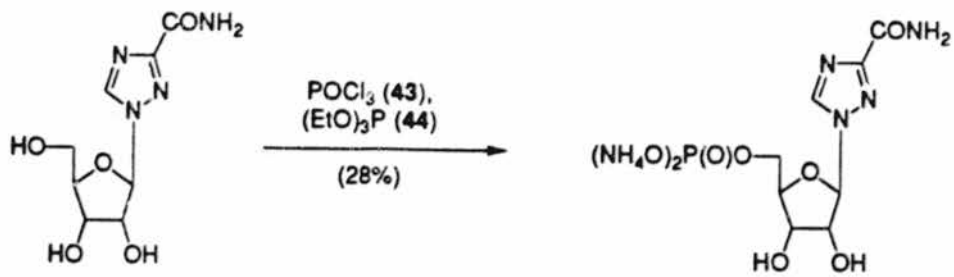
[b]Relative to TMS. Observed signals for carbons of minor diastereomer appear in parentheses.

[c]Signals could be transposed.

2.7 ppm. The spectra of **I-8** and **I-9** clearly indicated that both were diastereomeric mixtures. Two signals were observed for the carbons at the 2-position of the dioxolane ring (1") of the ketal and the two adjacent carbons (CH_3 and 2"). In the spectrum of ketal **I-8** prepared from levulinic acid, two signals were observed for the 2'-, 3'-, and 4'-carbons; in the spectrum of ketal **I-9** prepared from 4-acetylbutyric acid, only one of the two ribofuranosyl carbons making up the dioxolane ring had two distinct signals, whereas the other carbon and the 4'-carbon signals displayed downfield shoulders. The ratio of the heights of the signals for the diastereomeric carbons agreed with the diastereomeric ratios found by ^1H NMR spectroscopy and HPLC. The signals for the minor diastereomer were downfield by ≤ 0.8 ppm relative to those of the major diastereomer. These minor signals and their shift positions were useful in making assignments for the 2'-, 3'-, and 4'-carbons of the sugar ring and the 1"-, 2"-, and methyl group carbons on the tethers. Again, the 2'- and 3'-carbons of the ribofuranose ring of both ketals and the 3"- and 4"-carbons on the tether of ketal **I-8** prepared from levulinic acid could not be identified conclusively. HPLC analysis (Table I-2) indicated that both ketals were stable to incubation at physiolgical pH for 15 days. Unfortunately, both were inactive against Pichinde virus infection in vitro. For this reason, immunoconjugates of these compounds were not prepared.

6. Ribavirin-5'-*O*-phosphate Esters and Conjugate Preparation. We next investigated ribavirin-5'-*O*-phosphate ester derivatives. These phosphate esters would be far more hydrolytically stable than the 5'-*O*-hemisuccinates because they would not hydrolyze under physiological conditions but would be cleaved by lysosomal enzymes (Allen et al., 1978). Esterification of ribavirin-5'-*O*-phosphate with a tether having a carboxylic acid or amino group function would provide a means of tethering the derivative to a MAb. Ribavirin-5'-*O*-phosphate (Streeter et al., 1973) was prepared in 28% yield by reaction of ribavirin with phosphorous oxychloride (**43**) in the presence of triethylphosphite (**44**) (Scheme I-7). The phosphate diester derivative presumably could be

Scheme I-7. Synthesis of Ribavirin-5'-*O*-phosphate (**I-10**)



I-1 **I-10**

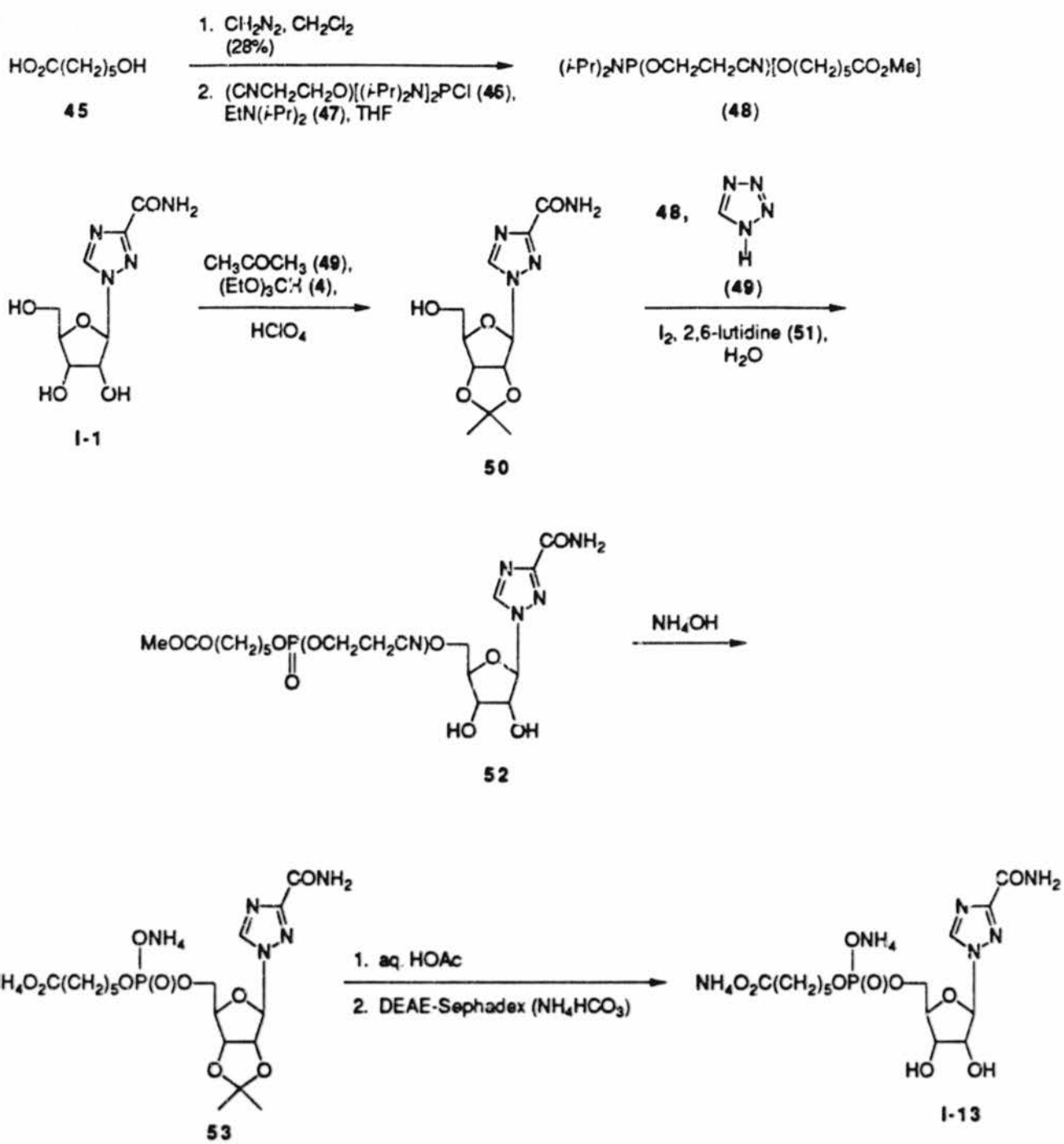
synthesized by reaction of ribavirin-5'-*O*-phosphate with a functionalized alcohol such as car-boxymethymethanol in the presence of a coupling reagent. To prevent formation of a cyclic phosphate between the the 5'- and 3'-*O*-positions of the ribofuranose ring, the hydroxyl groups were protected by acetylation with acetic anhydride in pyridine. Unfortunately, a complex mixture resulted that only afforded a low yield of the desired 2',3'-*O*-diacetate after DEAE-Sephadex chromatography using an ammonium sulfate gradient.

Alternate, more direct methods of preparing the phosphate diesters were investigated. Selective introduction of a phosphate group at the 5'-*O*-position of ribavirin required protection of the other two hydroxyl groups at the 2'- and 3'-positions. The 2'- and 3'-hydroxyl groups were protected by ketalization with acetone (**49**) using perchloric acid as the catalyst in the presence of triethyl orthoformate (**4**) to remove the water formed in the reaction. The acetonide **50**, which was prepared in 92% yield, was allowed to react with (5-carbomethoxypentyl)(2-cyanoethyl)(*N*,*N*-diisopropyl)phosphoramidite (**48**) in the presence of tetrazole (**49**) in acetonitrile. The phosphite ester that formed was oxidized to

the phosphate **52** with iodine in the presence of 2,6-lutidine in water-tetrahydrofuran. The cyanoethyl and methyl ester protecting groups were removed from the protected phosphate ester by treatment with aqueous acid to give ribavirin-5'-*O*-(5-carboxypentyl)phosphate (**I-13**) (Scheme I-8).

Methods were next investigated for coupling ribavirin-5'-*O*-(5-carboxypentyl)-phosphate to MAb PC4.9A6. From the results of direct loading of ribavirin onto this MAb, we concluded that levels would probably be insufficient to exert any appreciable antiviral effects using direct tethering of the ribavirin derivative to the MAb. Loading of the derivative onto a multifunctionalized polymer would permit us to greatly increase the amount of drug delivered. The polymer we elected to derivatize with the carboxypentylated ribavirin phosphate was (6-aminohexylamino)$_{11}$dextran (Mw 9,000) (**I-16**), which was prepared by sodium periodate oxidation of dextran, followed by Schiff base formation with 1,6-diaminohexane, and sodium cyanoborohydride reduction to the dialkylamine. We were unable to form an amide bond between these two species using EDIC under a variety of pH conditions. In contrast, we could readily produce an adduct between 2',3'-dideoxy-cytidine-5'-*O*-(5-carboxypentyl)phosphate and (6-aminohexylamino)$_{11}$dextran having a loading of 6.7 dideoxycytidines per dextran (Dawson et al., submitted). Because the reaction worked on a nucleotide lacking a 3'-hydroxyl group, we propose that the 3'-hydroxyl group interfered in the reaction--perhaps a cyclic species was formed between the carboxylate group and the 5'-*O*-phosphate that was subsequently cleaved by the 3'-hydroxyl group. We also attempted to use the 4-nitrophenyl ester method to form the amide bond. The ammonium counter ion was exchanged for pyridinium to prevent primary amide formation during EDIC coupling, which was conducted in water. The ^{1}H NMR spectrum of the reaction product mixture indicated that the 4-nitrophenyl ester of ribavirin-5'-*O*-(5-carboxylphenyl)phosphate was formed under these conditions. Two preliminary conjugations on MAb PC4.9A6 and albumin using [3'-^{14}C]ribvavirin-5'-*O*-(5-carboxy-

Scheme I-8. Synthesis of Ribavirin-5'-*O*-(5-carboxypentyl)phosphate Bisammonium Salt (**I-13**)

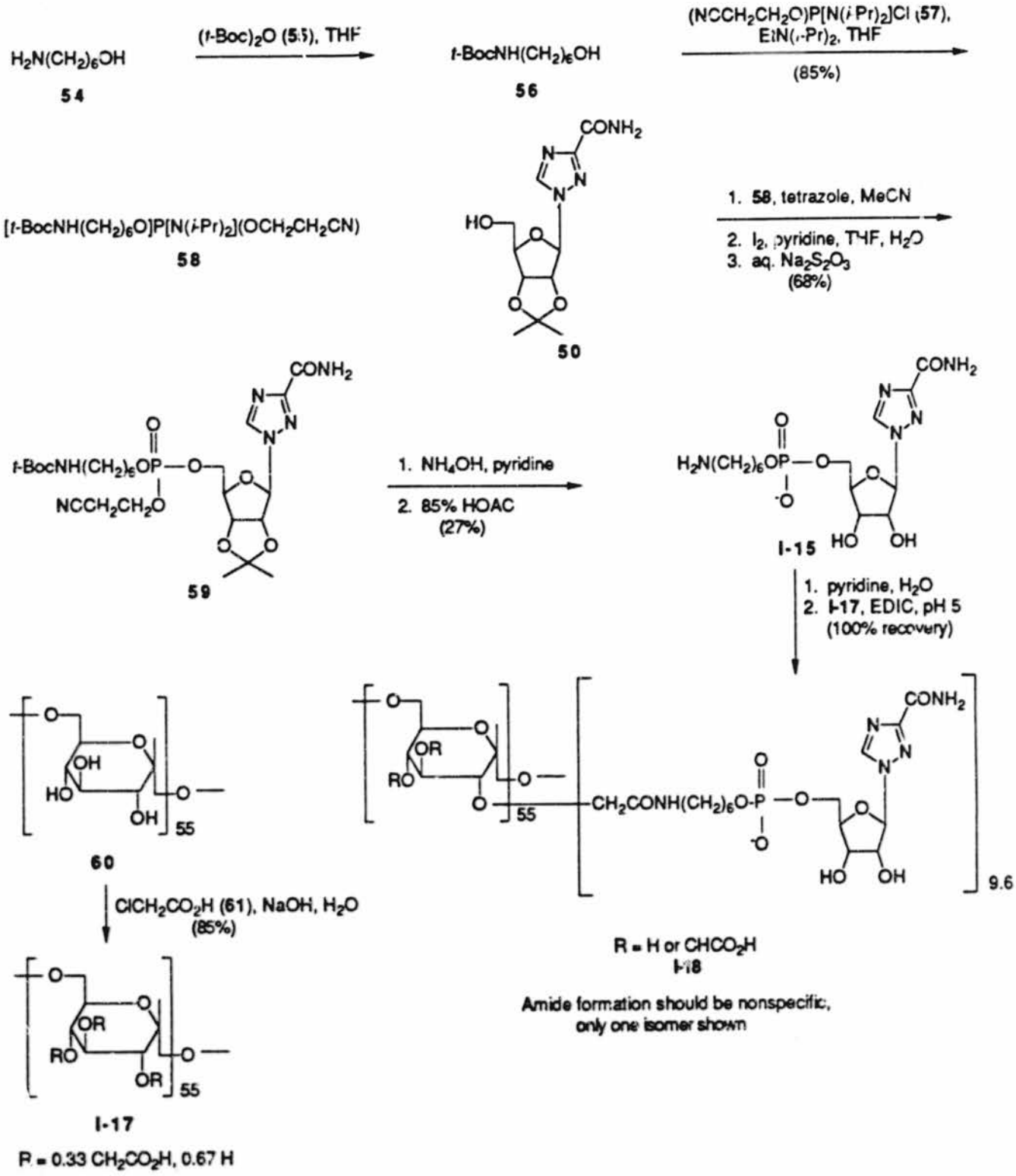


pentyl)phosphate (**I-14**) produced only hydrolysis products and no acylated protein. Perhaps the 4-nitrophenyl ester formed from the 6-substituted hexanoic acid was too lipophilic for nucleophilic attack by the ε-amino groups of the protein lysines and, therefore, only attack by hydroxide occurred. Acylation in mixed solvent systems (water with dimethylformamide, tetrahydrofuran, or dioxane, which could solubilize the pendant group of the tether) also failed. Even in the presence of the organic solvent, hydrolysis of the ester occurred at a faster rate than did acylation of protein, as determined by monitoring the appearance of 4-nitrophenoxide anion.

Because this approach was not promising, we decided to switch functional groups so that an amino group resided on the ribavirin derivative and the polymeric carrier bore the carboxyl groups. (Carboxymethyl)$_{55}$dextran (Mw 12,200) was prepared by alkylation of dextran with chloroacetate in the presence of potassium hydroxide. Titration indicated that 55 carboxymethyl groups (one per glucose residue) were loaded onto the dextran. Ribavirin-5'-*O*-(6-aminohexyl)phosphate (**I-15**) was prepared next and then linked to (carboxymethyl)dextran (**I-17**) by the routes outlined in Scheme I-9. The starting material for this synthesis was rivavirin-2',3'-*O*-acetonide (**50**). The conversion of the acetonide to the 5'-*O*-substituted phorphoramidite employed either (2-cyanoethyl)(*N*,*N*-diisopropyl)[6-(*N*-trifluoroacetylaminohexyl]phosphoramidite or (2-cyanoethyl)(*N*,*N*-diisopropyl)[6-(*N*-*t*-butyloxycarbonylaminohexyl]phosphoramidite (**58**), using a similar sequence of reactions to those used for preparing the 5'-*O*-(5-carboxypenyl)phosphate. The *t*-butyloxycarbonyl group was used to increase the stability of the phosphoramidite **58** to purification on silica gel. The trifluoroacetyl protecting group was removed by treatment with base, whereas the *t*-butyloxycarbonyl group was removed during the acid-catalyzed hydrolysis of the acetonide group.

To ensure that the carboxyl groups on the (carboxymethyl)dextran were not converted to primary amide groups during the coupling with ribavirin-5'-*O*-(6-amino-

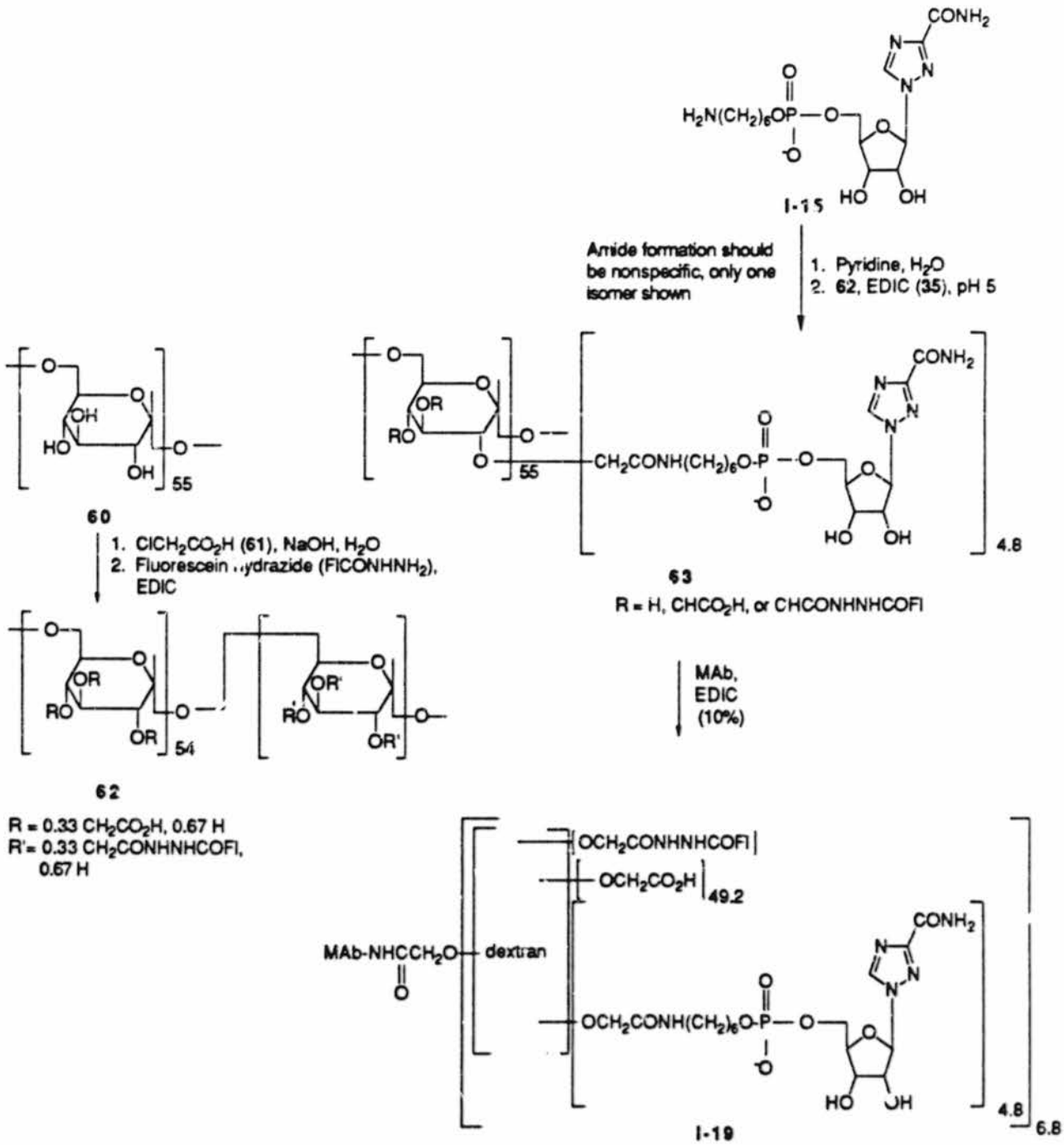
Scheme I-9. Synthesis of Adduct I-18 of Ribavirin-5'-*C*-(6-aminohexyl)phosphate with (Carboxymethyl)$_{55}$dextran



hexyl)phosphate ammonium salt, the ammonium counter ions were replaced by pyridinium ions. Coupling was effected with EDIC. A loading of 9.6 molecules of the ribavirin derivative onto the functionalized dextran was established using a colorimetric assay for inorganic phosphate (Ames, 1966), which was verified by elemental analysis. Although this adduct could be tethered to MAb PC4.9A6, determination of loading onto the immunoconjugate using the inorganic phosphate assay produced inconsistent results. For this reason, we elected to use a fluorescent indicator to establish the level of loading of functionalized dextran onto the MAb. Fluorescein hydrazide ($FlCONHNH_2$) was linked to the modified dextran through a carboxamide bond at a loading of one fluorescein per dextran (Scheme I-10). This fluoresceinated dextran (**62**) was then derivatized with ribavirin-5'-*O*-(6-aminohexyl)phosphate (**I-15**) using EDIC to give a loading of 4.8 ribavirins per modified dextran. Ribavirin levels were determined by the inorganic phosphate assay with controls conducted to establish that the fluorophore did not interfere in the assay, and the modified dextran concentration was established from the fluorescein absorbance.

The unreacted carboxyl groups on the fluoresceinated ribavirin-5'-*O*-(6-amino-hexyl)phosphate-(carboxymethyl)dextran adduct **63** were used for tethering to the terminal amino groups of the lysines of MAb PC4.9A6. Loading onto the MAb was established by determining the ratio of fluorescein absorbance to that of protein at 280 nm. A major problem in this immunoconjugate (**I-19**) preparation occurred during the purification. Standard purification methods using gel exclusion and ion exchange failed to remove the (carboxymethyl)dextran derivatized with ribavirin from the immunoconjugate. The large number of unreacted carboxymethyl groups on the (carboxymethyl)dextran caused this problem. Replusive interactions between the carboxyl groups caused the polymer to assume a rigid-rod conformation that resulted in its elution from a variety of gel-exclusion columns in the same volumes as that of the MAb. Cation exchange (DEAE-Sephadex) also

Scheme I-10. Synthesis of Immunoconjugate I-19 of Ribavirin-5'-*O*-(6-aminohexyl)phosphate-(Carboxymethyl)dextran with MAb PC4.9A6



failed because there was little difference in overall charge between the derivatized dertran and immunoconjugate **I-19**. Schecter et al. (1987) reported the successful purification of cisplatin-(carboxymethyl)dextran antibody conjugates using ammonium sulfate to remove unconjugated cisplatin-(carboxymethyl)dextran (Mw 40,000, 100 carboxyl groups per molecule) followed by DEAE-cellulose chromatography to remove free antibody. This method failed with the ribavirin-(carboxymethyl)dextran immunoconjugate. The immunoconjugate did not precipitate with ammonium sulfate in concentrations up to 90% saturation. Elution of DEAE-Sephadex with 0.6 M NaCl in 0.01 M potassium phosphate failed to elute the conjugate from the column although the free MAb was readily eluted by 0.15 M NaCl in 0.01 M potassium phosphate, pH 7.2.

Protein A is used to purify MAbs from other proteins. Both the unreacted MAb and the immunoconjugate bound to protein A in the loading buffer, whereas the ribavirin derivatized (carboxymethyl)dextran was not retained. The unreacted MAb was eluted readily from the column in pH 3 buffer. Only a small fraction of the immunoconjugate could be eluted from the protein A column at this pH. However, elution was efficient at pH 2.5, Unfortunately, the conjugate precipitated after this treatment even though the pH of the eluate was immediately brought to pH 7.2 and the elution was conducted in the cold. The precipitate could not be solubilized using high salt, indicating that some denaturation had occurred. At pH 3 or below, the carboxyl groups on the immunoconjugate are protonated, producing a high carboxylic acid concentration in the vicinity of the protein that could facilitate denaturation.

It might have been possible to effect a separation if the number of negative charges on the derivatized dextran were neutralized by conversion to uncharged amides by reaction with ethanolamine and EDIC. However, a preliminary experiment using this strategy was unsuccessful--perhaps because the number of charged groups removed through amide bond formation was too low.

Fortunately, sucrose density centrifugation in which molecules are separated on the basis of differences in their radii proved to be successful. In this method, the molecule having the largest radius moves the farthest through the gradient. A 5-20% sucrose gradient proved to be effective in separating unconjugated ribavirin-(carboxymethyl)dextran adduct, MAb, and the immunoconjugate **I-19**, which was found at the bottom of the tube. Fortuitously, the conjugate having the highest loading precipitated out but was solubilized readily in 0.5 M NaCl in PBS, followed by dialysis. This material had a loading of 6.8 molecules of (carboxymethyl)dextran per MAb or approximately 33 molecules of ribavirin per MAb (10% yield). The soluble fraction at the bottom of the gradient had a loading of 4.0 molecules of (carboxymethyl)dextran per MAb or 19 molecules of ribavirin (4% yield). The low recovery of conjugated material was disappointing. At present, we have no explanation other than steric hindrance for the lack of reactivity of the carboxymethyl groups on the dextran with the amino groups on the MAb, We were able to functionalize (carboxymethyl)dextran with low-molecular-weight amines and, correspondingly, we were able to tether low-molecular-weight carboxylic acids to MAbs using EDIC. Unfortunately, in the synthesis of **I-19** the MAb preferentially coupled with itself rather than the modified dextran, leading to very low protein recoveries.

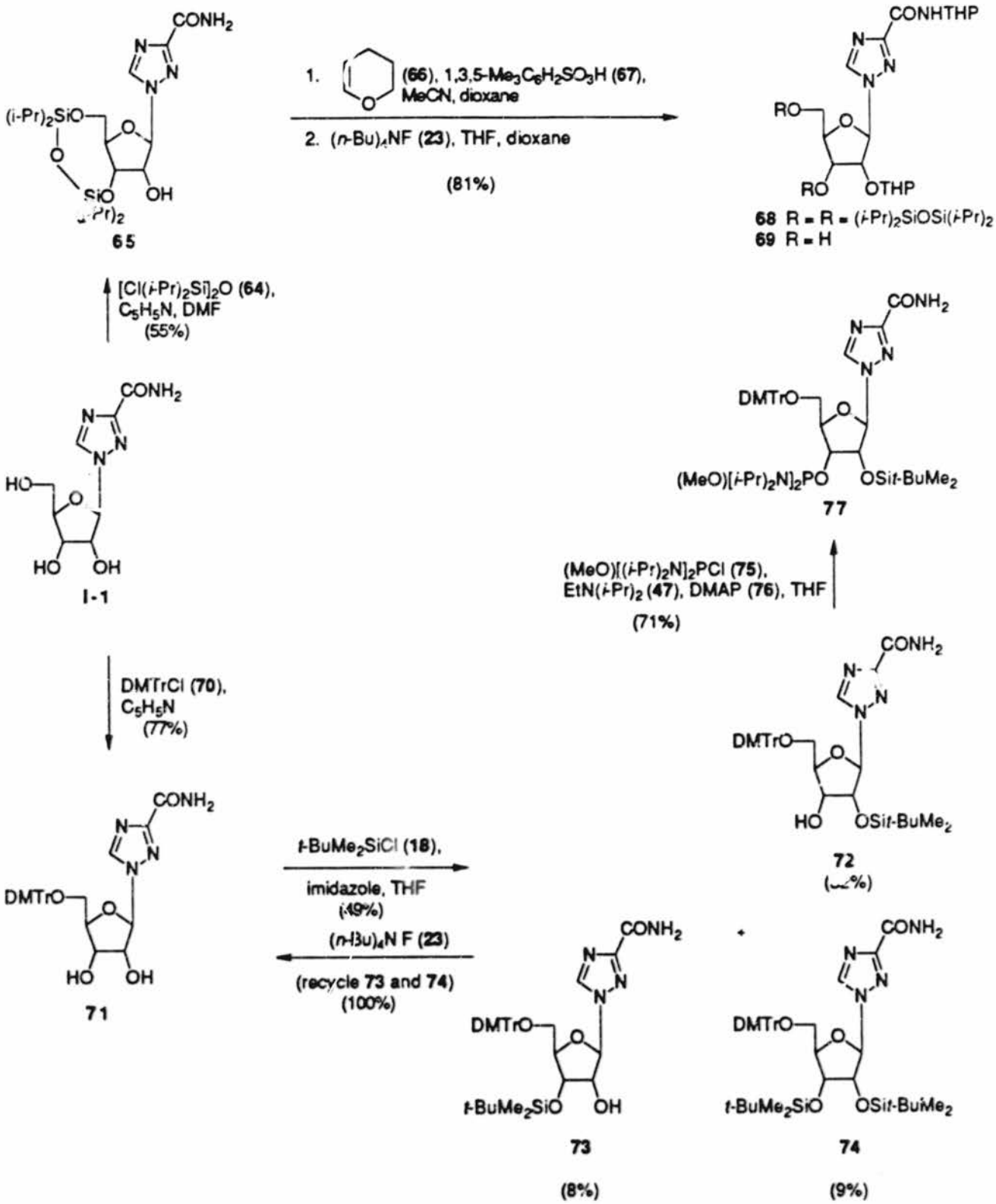
7. **Synthesis of an Oligomer Incorporating Ribavirin.** Another method of increasing ribavirin loading is the linkage of a polymeric version of ribavirin onto the MAb. An oligoribonucleotide analog of ribavirin-5'-*O*-phosphate would serve this purpose. The 5'-*O*-(6-aminohexyl)phosphate of poly(ribavirin-3',5'-*O*-phosphate)$_{15}$-thymidine-5'-*O*-phosphate triethylammonium salt (**I-20**, Scheme I-12) was designed to acheive this purpose. As with ribavirin-5'-*O*-phosphate, phosphatases were expected to cleave the oligomer, releasing ribavirin. The 5'-*O*-terminal aminohexyl tether group of the oligomer was introduced to provide the tether functionality for linkage directly to a MAb or to an intermediate polymeric carrier. The 3'-*O*-terminal thymidine group was used to

initiate the synthesis on the controlled-pore glass solid support and to serve as a chromophore for detection and quantitiation.

The first synthetic target in this synthesis was 2'-*O*-*t*-butyldimethylsilyl-5'-*O*-dimethoxytritylribavirin-3'-*O*-[bis(*N*,*N*-diisopropyl)methylphosphoramidite (**75**), **77**, Scheme I-11), which necessitated the selective protection of the 2'- and 5'-hydroxyl groups of ribavirin. The tetrahydropyranyl ether was the first protecting group investigated for this purpose. The 3'- and 5'-hydroxyl groups of ribvavirin were protected as the 1,1,3,3-(tetraisopropyl)disiloxanyl cyclic ether, to permit selective tetrahydropyranylation at the 2'-hydroxyl position. Unfortunately, under the conditions for the formation of the 2'-*O*-tetrahydropyranyl ether, the amino group of the triazole carboxamide also reacted, giving a bistetrahydropyranyl derivative. Cleavage of the tetrahydropyranyl protecting group from the amide under conditions that removed the 2'-*O*-tetrahydropyranyl ether failed--in fact, this group was even stable to strong acid.

Therefore, the *t*-butyldimethylsilyl ether protecting group was investigated. The 5'-hydroxyl group of ribvavirin was protected as the 4,4'-dimethoxytrityl ether to give **71** (Scheme I-11), which was allowed to react with *t*-butyldimethylsilyl chloride (**18**) in the presence of imidazole (Olgilvie et al., 1978, 1979; Hakimelahi et al., 1981). As in the case of the natural nucleosides, the aromatic ring system of ribavirin permitted the selective protection of the less hindered 2'-hydroxyl group; however, because of the small size of the triazole ring, selectivity was reduced for ribavirin . A mixture of the 2'- and 3'-*O*-*t*-butyldimethylsilyl ethers (**72** and **73**, respectively) and the 2',3'-*O*-bis-*t*-butyldimethylsilyl ether **74** was obtained. The 2'-*O*-*t*-butyldimethylsilyl ether was isolated by chromatography, whereas the 3'-*O*-monosilyl and 2',3'-*O*-bissilyl ethers were recovered and treated with tetra(*n*-butyl)ammonium fluoride (**23**) to cleave the *t*-butyldimethylsilyl ether groups to regenerate **71**. The position for the *t*-butyldimethylsilyl protecting group of **72** was established by ^{1}H NMR spectroscopy in Me_2SO-d_6 and Me_2SO-d_6/D_2O. The signals for

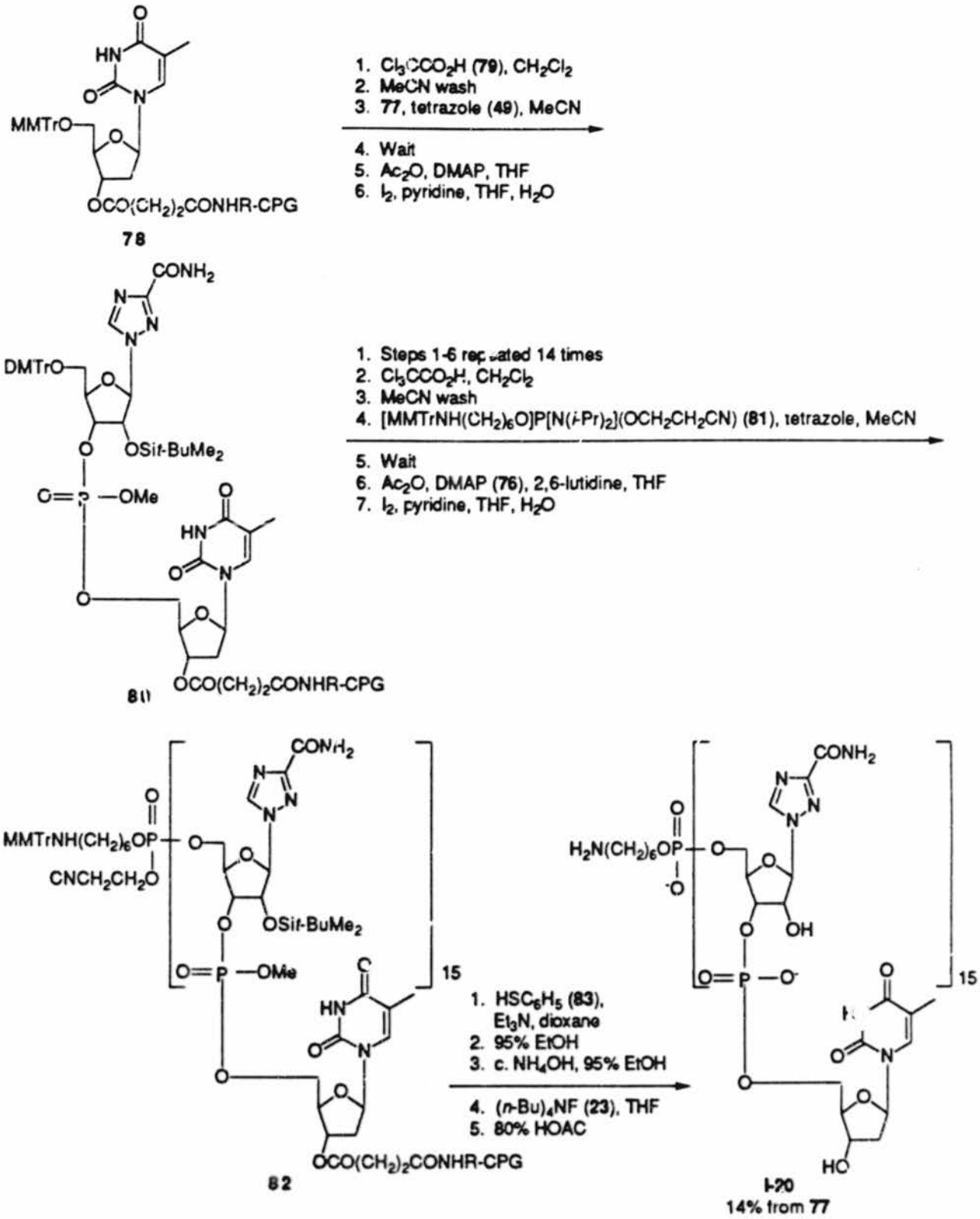
Scheme I-11. Synthesis of a Ribavirin-5'-*O*-Phosphoramidite Reagent **77**



the 3'-proton of **72** and the 2'-proton of **73** collapsed after the addition of D_2O because of exchange with the hydoxyl protons. Ogilvie and coworkers (1979) determined the position of silylation on the ribofuranose ring of nucleosides by comparison of their ^1H NMR with ^{13}C NMR spectra. Silylation of a hydroxyl group produced a downfield shift of the proton to which the silyl group was attached. For example, in the present case, silylation of **72** at the 3'-*O*-position shifted the 3'-proton from 4.32 to 4.38 ppm, whereas silylation at the 2'-*O*-position shifted the 2'-proton from 4.40 to 4.57 ppm. The 3'-*O*-*t*-butyldimethyl-5'-*O*-monomethoxytrityl ether was converted to the phosphoramidite **74** by the *in situ* generation of the phosphatidylating reagent using (*N*,*N*-diisopropyl)methylphosphonamidic chloride (**75**), *N*,*N*-diisopropylethylamine (**47**), and a catalytic amount of 4-dimethylaminopyridine (DMAP, **76**) in tetrahydrofuran. After workup and chromatography, a 71% yield of phosphoramidite **77** was obtained.

Phosphoramidite **77** was used to prepare the 5'-*O*-(6-aminohexyl)phosphate of poly(ribvavirin-3',5'-*O*-phosphate)$_{15}$-thymidine-5'-*O*-phosphate (**I-20**) by the sequence outlined in Scheme I-12. This oligoribonucleotide analog was prepared on a solid support using an Applied BioSystems DNA Synthesizer, with modifications of the program to account for the decreased reactivity of phosphoramidite **77** because of the steric hindrance caused by the *t*-butyldimethylsilyl ether protecting group for the 2'-hydroxyl function. The procedure developed by Ogilvie and coworkers for the synthesis of polyribonucleotides (Usman et al., 1987) was modified. The synthesis was initiated on a thymidine that was attached to controlled-pore glass through a 3'-*O*-hemicuccinate group (**78**). The 5'-*O*-monomethoxytrityl protecting group on the thymidine was first removed by treatment with trichloroacetic acid (**79**) to permit formation of the phosphite ester linkage between the 5'-*O*-position of the thymidine and the 3'-*O*-position of the 2',5'-*O*-protected ribavirin-3'-*O*-phosphoramidite **74**. Unreacted hydroxyl groups on the thymidines were blocked to further reaction by acetylation in the presence of DMAP before the 3',5'-phosphite ester

Scheme I-12. Synthesis of 5'-*O*-(6-Aminohexyl)phosphate of Poly(ribavirin-3',5'-*O*-phosphate)$_{15}$-thymidine-5'-*O*-phosphate Triethylammonium Salt (**I-20**)



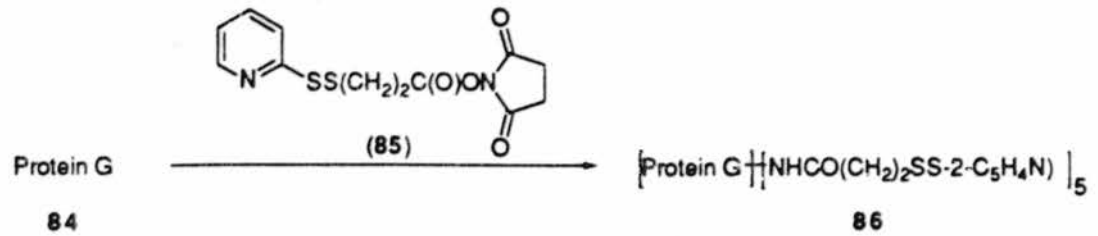
between thymidine and ribavirin was oxidized to the phosphate by treatment with iodine in pyridine-water-tetrahydrofuran. Trichloroacetic acid (**79**) was then used to remove the dimethoxytrityl group from the 5'-*O*-position of the ribavirin, which resided in the 2-position of the nascent polymer chain. This process was repeated 14 times so that 15 ribvarin phosphate groups were incorporated after thymidine. The oligoribonucleotide was terminated by reaction with [(2-cyanoethyl)(*N*,*N*-diisopropyl)-6-(*N*-4-monomethoxytritylamino)hexyl]phosphoramidite (**81**) to introduce amino group functionality on the 5'-spacer arm. Oxidation converted the terminal phosphite ester to the phosphate. The methyl protecting groups were cleaved from the phosphate groups by treatment with thiophenol (**83**)-trimethylamine before the polymer was cleaved from its support by treatment with ammonium hydroxide, which also removed the 2-cyanoethyl protecting group from the terminal 5'-*O*-phosphate group. Next, the *t*-butyldimethylsilyl groups were removed using tetra(*n*-butyl)ammonium fluoride (**23**). The oligoribonucleotide was purified by electrophoresis on a 20% acrylamide gel before the 5'-terminal trityl group was cleaved by treatment with 80% acetic acid. The overall yield for this synthesis was 14% based on thymidine absorbance. This synthesis was accomplished on a 24-mg scale. The oligomer was over 98% pure by HPLC on a MA7Q anion-exchange column. ^{1}H NMR spectroscopy indicated that the fluoride treatment removed 98% of the 2'-*O*-*t*-butyldimethylsilyl protecting groups. A multiplet at 1.2-1.7 ppm was ascribed to the four central methylene groups of the tetramer. A singlet at 1.89 ppm for the methyl group of the thymidine pyrimidine ring integrated for three protons. The anomeric proton of the ribofuranose ring of ribavirin produced a doublet at 5.95 ppm that integrated for 15 protons. The corresponding proton of the furanose ring of thymidine produced a triplet at 6.27 ppm that integrated for one proton. The singlet at 8.69 ppm for the 5-proton of the triazole ring of ribavirin integrated for 15 protons. Comparison of the integral for this proton with that for the 4'-proton of the ribofuranose ring indicated that depurination was

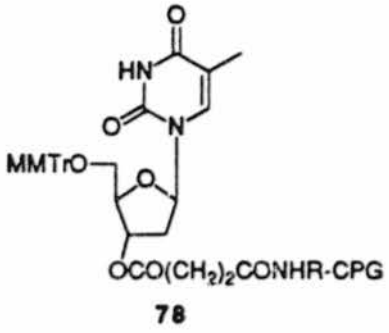
negligible (<1%). The relative integrals for the thymidine and ribavirin heterocyclic ring protons verified the presence of 15 ribavirin units per thymidine.

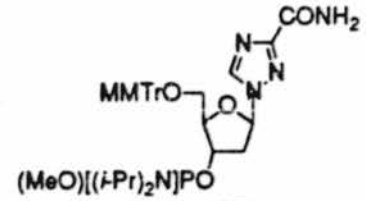
The stability of this ribavirin phosphate polymer to alkaline and enzymatic hydrolysis was determined. Incubation of **I-20** with 0.5 N KOH at 37°C for 1 h resulted in approximately 20% hydrolysis as determined by HPLC (MA7Q anion-exchange). Incubation for an additional 5 h at 37°C led to disappearance of the UV-absorbing peak corresponding to **I-20**. Homoribopolymer **I-20** was resistant to cleavage by ribonuclease A (1.3 mM Tris-HCl, pH 7.5, 1.3 mM $MgCl_2$, 37°C, 15 h) but was cleaved completely by ribonuclease T2 (0.04 mM NaOAc, 37°C, 30 min). These results indicate that, once internalized to the lysosomal compartment, the polymer will be enzymically degraded to release ribavirin-5'-*O*-phosphate within the cell.

Because of the scarity of the ribavirin oligomer, model studies were conducted with the more readily accessible 5'-*O*-(6-aminohexyl)phosphate of poly(thymidine-3',5'-*O*-phosphate)$_{12}$-thymidine-5'-*O*-phosphate triethylammonium salt (**I-21**), which was synthesized in 46% overall yield, to develop methodology for linking the oligomer to carrier proteins (Scheme I-13). The functionalized poly(thymidine) oligomer was tethered to a recombinant protein G (**84**), which binds to the Fc portion of immunoglobulin G, giving a oligonucleotide-protein G-MAb delivery system. Tethering was accomplished by acylating the amino terminus of oligomer **I-21** with *N*-[3-(2-pyridyldithio)propionyloxy]-succinimide (**85**), followed by reaction with dithiothreitol (**89**) to remove the 2-mercapto-pyridine protecting groups. As measured by release of pyridine-2-thione, mercapto group introduction onto the amino tether was 85%. The mercaptopropionylated oligomer was allowed to react with a modified protein G (**86**), which had been pretreated with *N*-[3-(2-pyridyldithio)propionyloxy]succinimide (**85**) so that five 3-(2-pyridyldithio)-propionyl groups were introduced per protein G molecule, to give a loading of 1.5 poly(thymidines) per protein G (**I-22**).

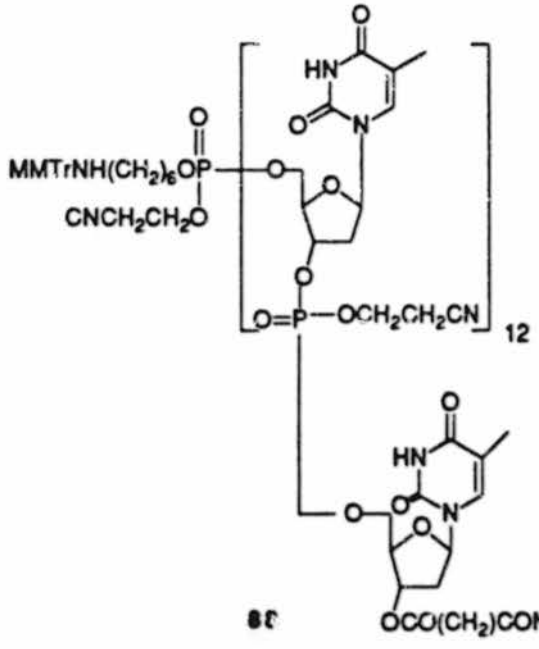
Scheme I-13. Synthesis of 5'-*O*-[6-(3-mercaptopropionamido)hexyl]phosphate of poly(thymidine-3',5'-*O*-phosphate)$_{12}$-thymidine-5'-*O*-phosphate-3-(2-pyridinyldithio)propionylated protein G conjugate (I-22)





1. Cl_3CCO_2H (**79**), CH_2Cl_2
2. MeCN wash
3. 

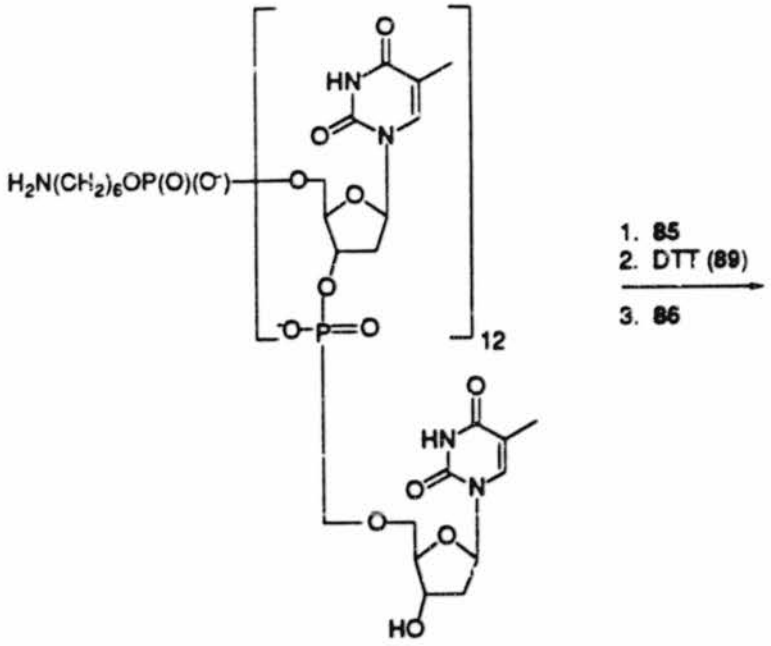
4. Wait
5. Ac_2O, DMAP, THF
6. I_2, pyridine, THF
7. Steps 1-6 repeated 11 times
8. **76**, CH_2Cl_2
9. $[MMTrNH(CH_2)_6O]P[N(i\text{-}Pr)_2](OCH_2CH_2CN)$(**81**), tetrazole, MeCN
10. Wait
11. Ac_2O, DMAP, THF
12. I_2, pyridine, THF, H_2O



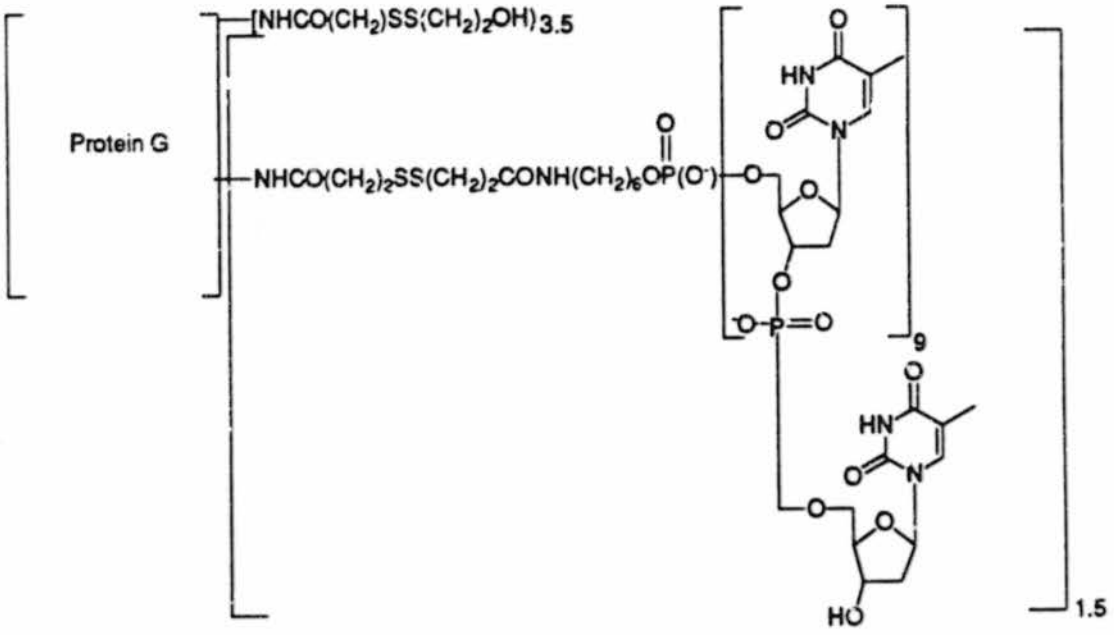
1. conc. NH_4OH, 95% EtOH
2. 80% HOAc

(46% overall from **78**)

Scheme I-13. Synthesis of 5'-*O*-[6-(3-mercaptopropionamido)hexyl]phosphate of poly(thymidine-3',5'-*O*-phosphate)12-thymidine-5'-*O*-phosphate-3-(2-pyridinyldithio)propionylate protein G conjugate (I-22) (continued)



I-21



I-22

D. Conclusions

Therefore, methodology is in hand to prepare immunoconjugates of the functionalized ribavirin oligomer and ribavirin bound to polymeric carriers and establish the loading of ribavirin on these conjugates with MAbs. However, preparation of an immunoconjugate of the ribavirin oligomer with MAb PC4.9A6 was not conducted when experiments with the gelonin-MAb PC4.9A6 immunoconjugate indicated that MAb PC4.9A6 was not internalized. Linkage to a MAb that internalizes should provide a means of delivery of ribavirin to the internal compartment of the cell. Metabolism studies with phosphatases indicated that the ribavirin oligomer is enzymatically degraded. The methodology is therefore in hand to test the efficacy of targeted drug delivery for treatment of Pichinde virus infections once the appropriate MAb is identified.

TASK II. IN VITRO ANTIVIRAL SCREENING OF DRUGS AGAINST PICHINDE VIRUS

A. Introduction

Pichinde virus produces only marginal cytopathogenic effect (CPE) in vitro. This CPE is very slow to develop, requiring 10-14 days in Vero 76 cells. Thus, in the past, antiviral assays against Pichinde virus often utilized plaque reduction (PR), which requires relatively large amounts of drug and, although more rapid than the CPE-based assays, is still slow (5-7 days) to reach an acceptable endpoint. Early in the course of these studies, we developed an immunofluorescence assay (IFA), which can be used in antiviral drug assays against Pichinde virus (Burns et al., 1988). Briefly, antiviral drug activity is determined by measuring the inhibition of Pichinde virus infectivity in Vero 76 cells. The assay is performed using an immunofluorescent cell-count assay to detect Pichinde virus infected cells in 96-well cell culture plates. The presence or absence of fluorescing cells is easy to score; the assay is read after only 24 h; the amount of drug required to perform an assay is reduced by over 80%; and the sensitivity and reproducibility are comparable with that achieved with assays based on plaque reduction. This section describes the immunofluorescence antiviral assay and demonstrates the performance of the assay in the evaluation of the known antiviral drugs, selenazofurin, ribavirin, 3-deazauridine, and (*S*)-DHPA against Pichinde virus. The Pichinde virus immunofluorescence assay was then used to determine the in vitro antiviral activity of many ribavirin derivatives and other compounds and conjugates synthesized during or related to these targeted delivery studies.

SRI chemists synthesized an immunoconjugate of ribavirin-5'-*O*-hemisuccinate with anti-Pichinde virus MAB PC4.9A6. This conjugate, containing 4.8 molecules of ribavirin per molecule of IgG, was evaluated for in vitro activity against Pichinde virus. The low loading of ribavirin achieved led us to redirect our efforts to devising strategies to enhance ribavirin loading. One of the proposed approaches for achieving multiple loading

of ribavirin in a targeted drug delivery system was to synthesize ribavirin polymers linked through phosphate ester linkages. Several phosphorylated forms of ribavirin, corresponding to ribonucleoside phosphates were synthesized by SRI chemists and then evaluated for in vitro antiviral activity against Pichinde virus. This series of compounds included: ribavirin-5'-*O*-phosphate (RMP), ribavirin-5'-*O*-diphosphate (RDP), ribavirin-5'-*O*-triphosphate (RTP), and ribavirin-5'-*O*-(6-aminohexyl)phosphate (SRI 8146-22). Later, poly(ribavirin phosphate) (SRI 7793-34) and derivatives of 2',3'-dideoxycytidine (DDC), DDC-5'-*O*-phosphate (SRI 8554-29), DDC-5'-*O*-(5-carboxypentyl)phosphite (SRI 8699-80), and [6-(DDC-5'-*O*-phosphate)pentylcarboxamido]$_{6.5}$(6-aminohexylamino)$_{4.5}$-dextran (SRI 8880-29), along with DDC (SRI 41) were evaluated for antiviral activity against Pichinde virus in Vero cells using the immunofluorescent cell-count assay.

Another approach to enhancing the effectiveness of targeted delivery is based on using dextran polymers to achieve multiple loading. Towards this end, several ribavirin derivatives were synthesized by Dr. Dawson and then evaluated for in vitro antiviral activity against Pichinde virus. Compounds evaluated were: a ribavirin derivative used in the preparation of the ribavirin-dextran conjugate, namely ribavirin-5'-*O*-(6-aminohexyl)-phosphate bisammonium salt (SRI 8699-28); a ribavirin-dextran conjugate, namely [6-(ribavirin-5'-*O*-phosphate)pentylcarboxamido](carboxymethyl)dextran (SRI 8699-58); and (carboxymethyl)dextran (SRI CD) without ribavirin. One of the potential problems with multiple loading of ribavirin onto a MAb using a dextran polymer is whether or not the dextran-MAb conjugate would retain antigen-binding activity. To address that question, dextran was conjugated to an anti-Pichinde virus MAb by SRI chemists. The resulting conjugate of MAb PC4.9A6 conjugated to (carboxymethyl)-dextran (SRI 9047-34) was evaluated to determine if the modified monoclonal antibody retained binding activity towards Pichinde virus-infected cells. SRI chemists prepared two immunoconjugates of ribavirin-5'-*O*-(6-aminohexyl)phosphate-linked via an amide bond to fluoresceinated

(carboxymethyl)dextran that in turn was conjugated to anti-Pichinde virus MAb PC4.9A6. Immunoconjugate SRI 9047-73-1 had 33 moles of ribavirin per mole of MAb PC4.9A6 and Immunoconjugate SRI 9047-72-2 had 19 moles of ribavirin per mole of MAb PC4.9A6. Both of these immunoconjugates were evaluated for antiviral activity toward Pichinde virus.

B. Materials and Methods

Cells. The Vero 76 line of African green monkey kidney cells obtained from the American Type Culture Collection (ATCC, Rockville, MD) was used as the host cell line for all Pichinde virus assays. Cells were passaged in polystyrene disposable cell-culture flasks using minimum essential medium (MEM, GIBCO Laboratories, Grand Island, NY) containing 9% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT). For antiviral assays, cells were seeded at 6 x 10^4 cells/well in 96-well polystyrene cell-culture plates.

Virus. The An4763 strain of Pichinde virus (obtained from Dr. Joseph D. Gangemi, University of South Carolina School of Medicine, Columbia, SC) was used throughout these studies.

Monoclonal Antibody PC4.9A6. The derivation of the hybridoma cell line that secretes anti-Pichinde virus MAb PC4.9A6 is described under Task III of this report. The characterization of MAb PC4.9A6 is described under Tasks IV and VIII. MAb PC4.9A6 is of the IgG_{2a} isotype, binds to Pichinde virus antigens expressed on the surface of Pichinde virus-infected cells, and displays no Pichinde virus-neutralizing activity.

Antiviral assay. The immunofluorescence assay for evaluation of antiviral drugs against Pichinde virus as recently described by us (Burns et al., 1988) was used for the antiviral evaluations. The assay utilized fluorescein-conjugated anti-Pichinde virus murine MAb prepared in our laboratories. At the end of the 20 to 24 h incubation the media was aspirated and the infected cell cultures were allowed to dry thoroughly at room temperature.

The cells were then fixed in 80% acetone by adding cold deionized water (50 µL/well), followed by addition of 200 µL of cold (-15°C) acetone to the water in each well. After 5 min, the fixative was poured from the plate and the cell sheets were allowed to dry. Cells were either immunostained immediately or stored at -15°C. Fluorescein-labeled MAb towards Pichinde virus was used at a dilution of 1:500 to provide intense specific staining yet only minimal nonspecific background. For immunostaining, 50 µL of the fluorescein-labeled antibody was added to each well and immunostaining was allowed to proceed at 37°C for 1 h before the immunostain was poured from the plate. The plate was inverted on absorbent paper and allowed to drain. Immunostained cells were viewed through the bottom of the plate using a 16X objective and 10X eyepieces on a Zeiss epifluorescence microscope. The number of fluorescing cells was determined and compared to the number of fluorescing cells in wells without drug. Each assay included toxicity controls, as well as virus and normal cell controls, similar to a plaque reduction assay described previously (Huffman et al., 1973). Three virus-containing wells were used for each compound dosage level, with one additional well being used for toxicity controls (cells + sterile virus diluent + compound). Six wells in each panel were used for normal cell controls (cells + sterile virus diluent + drug diluent). The antiviral activity was expressed as the concentration of drug required to reduce the number of infected cells by 50% (ED_{50}). The ED_{50} was determined by plotting the percent inhibition versus compound concentration on semilogarithmic paper, with the ED_{50} level being that concentration causing a 50% reduction in the number of fluorescing cells.

Immunofluorescence assay for antiviral activity of antibody targeted antivirals. The following controls and MAb-ribavirin conjugates were evaluated for antiviral activity against Pichinde virus: MAb PC4.9A6 alone, ribavirin (positive control), ribavirin-5'-*O*-hemisuccinate conjugated to MAb PC4.9A6, ribavirin-5'-*O*-(6-aminohexyl)-phosphate conjugated to MAb PC4.9A6 at two ribavirin loading levels (SRI 9047-73-1 and

SRI 9047-73-2). The evaluation of antibody targeted drugs required some modification of the antiviral assay used for nontargeted drugs. The main modification was that the conjugates were not added to the cell cultures until 18 h after inoculation with virus to allow time for the expression of viral antigens on the infected cells. The second significant modification was to read the assay at 96 h postinoculation (p.i.). Because the conjugate treatment was at 18 hours p. i., infection would already be established in many cells. Therefore, if an antiviral effect were to be manifested, that effect would be demonstrated by an inhibition of spreading of the viral infection to other cells.

For the antiviral evaluations, growth medium was removed from confluent monolayers of Vero cells growing in 96-well culture plates and 100 µL of Pichinde virus diluted in MEM with 2% FBS was added. The virus inoculum was such that there were 5-10 immunofluorescent cell-forming units of Pichinde virus added to each well, resulting in an MOI of approximately 1:10,000. The inoculated cultures were incubated for 18 h at 37°C at which time 100 µL of conjugate in MEM was added. The plates were sealed with plastic wrap and incubated at 37°C until the incubation was terminated at 96 h p. i. by pouring the medium from the cultures and letting the cell sheet air dry prior to fixing with acetone. The fixed cells were immunostained to detect Pichinde virus-infected cells, and the number and size of foci of fluorescing cells were determined. Each assay included toxicity controls, as well as virus and normal cell controls. Four wells were used for each compound dosage level, one for toxicity assessment (cells + sterile virus diluent + compound) and three wells for antiviral assessment (cells + virus+ compound). Six wells in each panel were used for normal cell controls (cells + sterile virus diluent + drug diluent). Pichinde virus infection as detected by immunofluorescence was graded from 0 (normal cells) to 4 (virtually all cells fluorescing). For determination of antiviral activity the scoring parameter was reduction in immunofluorescent foci (IF) determined in a manner

analogous to measuring activity as a reduction in cytopathogenic effect. Antiviral activity was expressed as the concentration of drug required to reduce the IF score by 50% (ED_{50}).

Test compounds. All of the compounds were soluble in the MEM cell culture medium used as compound diluent. Compounds were stored in sealed vials, in the presence of desiccant, at room temperature. Once placed into solution, all compounds were stored at 4°C. Immunoconjugates SRI 9047-73-1 and SRI 9047-73-2 were sterilized by filtration through a 0.2-µm pore-size Gelman acrodisc (product No. 4192) filter using a 10-mL syringe. The filters and syringes were first rinsed with MEM containing 2% FBS. After the conjugate was passed through the filter, sufficient MEM was pushed through the filter to achieve the desired dilution, while rinsing residual compound from the filter. From these sterile filtered solutions, the remaining dilutions of each test compound were made. SRI 9047-73-1 was evaluated in a half log dilution series from 250 µg/mL to 0.25 µg/mL. SRI 9047-73-2 was evaluated in a half-log dilution series from 100 µg/mL to 0.1 µg/mL. The ribavirin-5'-*O*-hemisuccinate PC4.9A6 conjugate was evaluated in half-log dilutions over the range 0.5 to 512 µg/mL.

C. Results

Antiviral evaluations of ribavirin derivatives. The immunofluorescence assay was used to measure the antiviral activity of several compounds against Pichinde virus; data from such an experiment using ribavirin and ribavirin 5'-*O*-phosphate are shown in Table II-1. In the process of verifying the utility and reliability of the antiviral assay using immunofluorescence as the parameter for scoring infection, several other compounds were also evaluated against Pichinde virus and a summary of the results is presented in Table II-2. The in vitro antiviral assay based on immunofluorescence was then used to evaluate the antiviral activity of several ribavirin derivatives produced by Dr. Dawson. Ribavirin 5'-*O*-monophosphate and ribavirin 5'-*O*-hemisuccinate were quite active against Pichinde virus. The results of those assays are listed in Table II-3.

Table II-1. Immunofluorescence assay for antiviral activity against Pichinde virus.

Ribavirin 5'-*O*-phosphate		Ribavirin	
Conc. (µg/mL)	% Inhibition	Conc. (µg/mL)	% Inhibition
1000	100	1600	100
320	100	320	100
100	100	32	65
10	74	10	39
3.2	30	3.2	0
1.0	4	1.0	0

Ribavirin 5'-*O*-phosphate ED_{50} = 3.2-10 µg/mL.

Ribavirin ED_{50} = 10-32 µg/mL.

Table II-2. Activity of various antiviral drugs against Pichinde virus as measured by immunofluorescence assay.

Drug Tested	ED_{50} (µg/mL)
Selenazofurin	1.0
Ribavirin	4.5
3-deazauridine	7.5
(*S*)-DHPA	505.5

Table II-3. Immunofluorescence assay for antiviral activity of ribavirin derivatives against Pichinde virus.

Conc. (μg/mL)	Ribavirin % Inhibition	Ribavirin-5'-*O*-phosphate % Inhibition	Ribavirin-5'-*O*-(2,3-dimethyl)hemi-succinate % Inhibition
1000	100	100	94
320	100	100	65
100	100	100	20
32	100	66	5
10	54	32	0
3.2	15	5	0
1.0	0	5	0

Compound	ED_{50} (μg/mL)	MTD (μg/mL)
Ribavirin	3.2-10	320
Ribavirin 5'-*O*-phosphate	10-32	320
Ribavirin 5'-*O*-hemisuccinate (data not shown)	10-32	1,000
Ribavirin 5'-*O*-(2,3-dimethyl)-hemisuccinate	100-320	32

Since ribavirin 5'-*O*-phosphate had shown activity against Pichinde virus, a series of phosphorylated derivatives of ribavirin were synthesized and evaluated for antiviral activity against Pichinde virus. Data from these evaluations are shown in Table II-4. In the evaluations represented by the data in Table II-1, ribavirin 5'-*O*-phosphate (RMP) was more active than ribavirin, however this was the only time that this degree of activity was observed; in the two subsequent experiments (Tables II-3 and II-4), RMP was slightly less active than ribavirin, and RDP and RTP were progressively less active than RMP.

Antiviral evaluations of poly(ribavirin phosphate), related compounds, and DDC. The results of the evaluation of poly(ribavirin phosphate) and related compounds, as well as DDC, are shown in Table II-5. Of these compounds, only ribavirin demonstrated antiviral activity towards Pichinde virus. The poly(ribavirin phosphate) preparation was quite toxic at all concentrations tested above 50 μg/mL and was partially toxic at 50 μg/mL. Thus, the ED_{50} for poly(ribavirin phosphate) was listed as >50 μg/mL. Subsequently, this toxicity was found to be the result of the counter-ion used in the suspending buffer. The conjugate of DDC-5'-(5-carboxypentyl)phosphate and (6-aminohexylamino)$_{11}$dextran (SRI 8880-29) was also fairly toxic with a maximum tolerated dose (MTD) of 100 μg/mL. Subsequent evaluations of poly(ribavirin phosphate) in a nontoxic suspending buffer failed to demonstrate antiviral activity towards Pichinde virus. The anti-HIV drug DDC or its derivatives exerted no antiviral effect against Pichinde virus.

Antiviral evaluations of compounds related to the dextran polymer approach for multiple loading. The evaluations dealing with the dextran polymer approach were then performed. The antiviral activity of (ribavirin-5'-*O*-(6-aminohexyl)-phosphate bisammonium salt (SRI 8699-28), the ribavirin-(carboxymethyl)dextran conjugate (SRI 8699-58), and (carboxymethyl)dextran (SRI CD) against Pichinde virus are shown in Table II-6. The data in Table II-6 are presented on the basis of molarity in terms of ribavirin content; therefore, Table II-7 shows the corresponding data for the antiviral

Table II-4. Results of immunofluorescence assay for antiviral activity of phosphorylated derivatives of ribavirin against Pichinde virus. Percent of virus controls in cell cultures receiving the indicated compounds at varying concentrations.

	Percent of virus controls and (number of fluorescing cells)				
Conc. (μg/mL)	Ribavirin	RMP	RDP	RTP	SRI 8146-22[1]
1000	0	0	0	0	0
320	0	0	15.1 (7)	36.6 (16)	32.7 (15)
100	0	0	4.3 (2)	63.8 (27)	79.4 (34)
32	0	40.9 (19)	57.3 (27)	81.7 (35)	77.0 (33)
10	11.5 (5)[2]	84.6 (39)	65.9 (31)	94.9 (41)	80.9 (39)
32.2	65.2 (30)	93.2 (43)	67.3 (31)	128.4 (55)	126.8 (54)
1.0	97.0 (45)	101.2 (47)	101.8 (47)	115.2 (49)	>100.0 (>43)
0	100.0 (47)	100.0 (47)	100.0 (47)	100.0 (43)	100.0 (43)
ED_{50}:[3]	5.1	27.4	41	211.6	238.5
MTD:[4]	>1000	1000	>1000	>1000	>1000
TI:[5]	>196	36.4	>24.4	>4.51	>4.2

[1]SRI 8146-22 is ribavirin-5'-*O*-(6-aminohexyl)phosphate.

[2]Average number of fluorescing cells, indicated in parentheses.

[3]ED_{50}: concentration of compound that resulted in a 50% reduction of fluorescent cells as compared to virus controls.

[4]MTD: maximum tolerated dose, greatest concentration of compound that still did not produce cytotoxicity.

[5]TI: Therapeutic index = MTD/ ED_{50}.

Table II- 5. Antiviral activity of DDC, poly(ribavirin phosphate) and related compounds against Pichinde virus.

Compound	ED_{50}[a]	MTD[b]	TI[c]
DDC, SRI 41	>1,000	320	<1
SRI 8966-80	>1,000	>1,000	1
SRI 8554-29	>1,000	>1,000	1
SRI 8880-29	>1,000	100	<1
SRI 7793-34 [poly(ribavirin phosphate)]	>50	50	1
Ribavirin	7.4	1,000	135

[a]ED_{50}: concentration of compound that resulted in a 50% reduction in the number of fluorescing cells as compared to virus controls.

[b]MTD: maximum tolerated dose, greatest concentration of compound that still did not produce cytotoxicity.

[c]TI: therapeutic index = MTD/ED_{50}.

Table II-6. The activity of ribavirin-5'-*O*-(6-aminohexyl)phosphate bisammonium salt (SRI 8699-28), the ribavirin-(carboxymethyl)dextran conjugate (SRI 8699-58), and (carboxymethyl)dextran (SRI CD) as measured by immunofluorescent cell count assay for antiviral activity against Pichinde virus.

	Percent of virus controls (number of fluorescing cells)		
Concentration (mM)[1]	SRI 8699-58	SRI 8699-28	SRI-CD
2.25	94 (27.3)[2]	0	2 (26.7)
0.71	103 (30.0)	13 (3.7)	84 (24.3)
0.225	78 (22.7)	68 (20.0)	57 (16.7)
0.071	85 (24.7)	86 (25.0)	87 (25.3)
0.0225	78 (22.7)	66 (19.3)	69 (20.0)
0.0071	85 (24.7)	80 (23.3)	71 (20.7)
0.00225	92 (26.7)	89 (26.0)	78 (22.7)
ED_{50}[3]:	>2.25	0.38	>2.25
MTD[4]:	>2.25	>2.25	>2.25
TI[5]:	-	>6	-

[1]Concentration (mM): SRI 8699-28 has a molecular weight of 440 and was available in limited quantities; thus 5 mg of SRI 8699-28 was dissolved in 5.05 mL of culture medium to yield a 2.25 mM solution; a similar 2.25 mM solution of SRI 8699-58 was made. The molarity of SRI 8699-58 was in terms of ribavirin content based on the ribavirin incorporation into the polymer at 8.6 µmol/mg; an equivalent weight of SRI CD was evaluated based on 0.7 times the weight of SRI 8699-58 used to make the solutions.

[2]Average number of fluorescing cells.

[3]ED_{50}: concentration (mM) of compound that resulted in a 50% reduction in number of fluorescing cells as compared to virus controls.

[4]MTD: maximum tolerated dose (mM), greatest concentration of compound that still did not produce cytotoxicity.

[5]TI: therapeutic index = MTD/ED_{50}.

Table II-7. Antiviral activity (against Pichinde virus) of ribavirin (concentrations listed on basis of molarity for comparison to data in Table II-6).

Ribavirin Concentration (mM)	Immunofluorescent cell count Percent of virus controls (number of fluorescing cells)
4.1	0
1.3	0
0.41	0
0.13	0
0.041	0
0.013	77 (22.3)[1]
0.0041	92 (26.7)
ED_{50}[2]: 0.023	
MTD[3]: >4.1	
TI[4]: >180.0	

[1]Average number of fluorescing cells.

[2]ED_{50}: concentration (mM) of compound that resulted in a 50% reduction of fluorescent cells as compared to virus controls.

[3]MTD: maximum tolerated dose (mM), greatest concentration of compound that still did not produce cytotoxicity.

[4]TI: therapeutic index = MTD/ED_{50}.

evaluation of ribavirin. The ribavirin-(carboxymethyl)dextran conjugate (SRI 8699-58) displayed no antiviral activity towards Pichinde virus, nor did the nonconjugated (carboxymethyl)dextran; whereas, ribavirin-5'-*O*-(6-aminohexyl)phosphate (SRI 8699-28) had approximately 6% of the activity of the non-derivatized ribavirin (compare data in Tables II-6 and II-7).

Binding of dextran-monoclonal antibody conjugate to Pichinde virus-infected cells. Using the indirect immunofluorescence method, binding of fluoresceinated (carboxymethyl)dextran-MAb PC4.9A6 conjugate (SRI 9047-34) to Pichinde virus-infected cells could be detected out to SRI 9047-34 dilutions of 1:1,000. Using this same detection procedure, anti-Pichinde virus binding activity of nonconjugated PC4.9A6 (ascites fluids) was detectable out through dilutions of 1:50,000. The MAb PC4.9A6 concentration in the ascites fluids was 10 mg/mL and the concentration of derivatized MAb PC4.9A6 in SRI 9047-34 was 0.25 mg/mL. Thus, taking the 40-fold difference in MAb concentration into account, the conjugate SRI 9047-34 retained Pichinde virus-binding activity.

Antiviral evaluations of ribavirin-PC4.9A6 immunoconjugates. The ribavirin-5'-*O*-hemisuccinate conjugated to MAb PC4.9A6 (containing 4.8 moles of ribavirin per mole of IgG) and the fluoresceinated (carboxymethyl)dextran multiply-loaded immunoconjugates SRI 9047-73-1 and SRI 9047-73-2, consisting of ribavirin-5'-*O*-(6-aminohexyl)phosphate linked via (carboxymethyl)dextran to MAb PC4.9A6 were evlauated for antiviral activity toward Pichinde virus. SRI 9047-73-1 contained 33 moles of ribavirin per mole of MAb PC4.9A6 and SRI 9047-73-2 contained 19 moles of ribavirin per mole of MAb PC4.9A6. The antiviral activity was evaluated by inhibition of fluorescing foci-formation in an immunofluorescence assay for Pichinde virus-infected cells. Vero cell monolayers were inoculated with Pichinde virus at a MOI of 1:10,000. The immuno-conjugates and controls were added 18 h later. Four days p. i., the monolayers were fixed,

stained, and examined by immunofluorescence for foci of Pichinde virus-infected cells. In untreated control cultures, large fluorescing foci of several hundred cells were observed. Neither the ribavirin-5'-*O*-hemisuccinate conjugated to MAb PC4.9A6 (evaluated at 0.5-512 μg/mL), the SRI 9047-73-1 (at 0.25-250 μg/mL), nor SRI 9047-73-2 (at 0.1-100 μg/mL) displayed any antiviral activity against Pichinde virus (Table II-8). The positive control in this assay was ribavirin. When ribavirin was added to infected cells at 18 h p. i., there was a dose-dependent inhibition of immunofluorescent foci formation. The concentratoin of ribavirin that reduced the immunofluorescing foci score by fifty per cent (ED_{50}) was 16 μg/mL, the maximum tolerared dose (MTD) was 320 μg/mL, and the therapeutic index (MTD/ED_{50}) was 20.

D. Discussion

The ED_{50} of ribavirin using inhibition of immunofluorescence as the parameter for scoring antiviral activity was consistently between 10 and 32 μg/mL, which is in the same range as the antiviral activity measured using inhibition of cytopathic effect (CPE), reading a marginal end-point or using plaque reduction (PR). The antiviral assay based on inhibition of immunofluorescence was also shown to be applicable to antiviral compounds other than ribavirin.

Ribavirin-5'-*O*-hemisuccinate was found to have an ED_{50} of 10-32 μg/mL and an MTD of 1,000 μg/mL. These values suggested that ribavirin-5'-*O*-hemisuccinate may be a suitable derivative to be used for tethering to antibodies. However, because of concerns over the potential lability of the ribavirin-5'-*O*-hemisuccinate ester bond, a more hindered ester, ribavirin-5'-*O*-(2,3-dimethyl)hemisuccinate, was synthesized by SRI chemists. Although ribavirin-5'-*O*-(2,3-dimethyl)hemisuccinate was much more stable than ribavirin-5'-*O*-hemisuccinate, the addition of the two methyl groups had a deleterious effect on both antiviral activity and cytotoxicity, i.e., ribavirin-5'-*O*-dimethylhemisuccinate had ED_{50} of 100-320 μg/mL and MTD of 32 μg/mL as compared with ribavirin-5'-*O*-hemisuccinate,

Table II-8. Antiviral activity of SRI 9047-73-1, SRI 9047-73-2, and ribavirin against Pichinde virus. Compounds added 18 hours postinoculation and fluorescent foci score read 96 hours postinoculation.

Compound	ED_{50}[a] (µg/mL)	MTD[b] (µg/mL)	TI[c]
Ribavirin	16	320	20
Immunoconjugate SRI 9047-73-1	>250[d]	>250	-
Immunoconjugate SRI 9047-73-2	>100[d]	>100	-
Ribavirin-5'-*O*-hemisuccinate[e] conjugated to MAb PC4.9A6	>512[d]	>512	-
MAb PC4.9A6	>1,000	>1,000	-

[a]ED_{50}: Concentration of compound that resulted in a 50% reduction in fluorescent foci score compared with virus controls.

[b]MTD: Maximum tolerated dose, greatest concentration of compound that did not product cytotoxicity.

[c]TI: Therapeutic index = MTD/ED_{50}.

[d]Maximum concentration evaluated.

[e]Ribavirin-5'-*O*-hemisuccinate conjugated to MAb PC4.9A6 was 0.78% ribavirin by weight, thus the ED_{50} of >512 µg/mL converts to >4 µg/mL based on ribavirin content.

ED_{50} of 10-32 µg/mL and MTD of 1,000 µg/mL. However, these antiviral assays were conducted in vitro using the ribavirin derivatives that were not conjugated to a MAb. It is quite likely that the 5'-*O*-ester linkage must be cleaved before the (2,3-dimethyl)hemisuccinate or hemisuccinate derivatives can express antiviral activity. When an immunoconjugate enters a target cell by endocytosis, the ester linkage between ribavirin and the MAb will be exposed to hydrolytic enzymes in the lysozomes--an environment that may be much different from the conditions experienced by the nonconjugated ribavirin derivatives in our in vitro antiviral assays, which would only enter cells by passive diffusion. Thus, although ribavirin-5'-*O*-(2,3-dimethyl)hemisuccinate is less active in the in vitro antiviral assay, the compound may be active when conjugated to MAb. Over the concentration range 0.5 to 512 µg/mL, there was no activity nor any toxicity for ribavirin-5'-*O*-hemisuccinate conjugated to MAb PC4.9A6. The loading of ribavirin in this conjugate was 4.8 moles of ribavirin per mole of IgG. The most concentrated dosage evaluated based on ribavirin content was 4 µg/mL. In the antiviral assay in which the drug is added at 18 h p. i., the ED_{50} for ribavirin was 16 µg/mL. Although the concentration of ribavirin may have been lower than desired, subsequent studies described under Task VIII of this report indicated that MAb PC4.9A6 was not a suitable antibody for antibody-mediated delivery.

The use of conjugation or multiple loading strategies employing poly(ribavirin phosphate) would conceivably result in various phosphorylated forms of ribavirin within the target cell. Therefore, the following compounds were evaluated against Pichinde virus in Vero cells: ribavirin, ribavirin-5'-*O*-phosphate (RMP), ribavirin-5'-*O*-diphosphate (RDP), ribavirin-5'-*O*-triphosphate (RTP) and ribavirin-5'-*O*-(6-aminohexyl)phosphate (SRI 8146-22). The results indicated that the activity of the ribavirin derivatives was dependent upon the state of phosphorylation of the compound, ribavirin >> RMP > RDP >> RTP. This is most likely due to the inability of the highly charged, phosphorylated

compounds to cross the lipid bilayer of the cytoplasmic membrane. Thus the phosporylated compounds may have trouble entering the cells. The evaluations of poly(ribavirin phosphate) failed to demonstrate antiviral activity, however, concerted efforts to demonstrate or facilitate entry of the poly(ribavirin phosphate) into the cells have yet to be made.

Another strategy for multiple loading of ribavirin would involve attaching a suitably derivatized ribavirin to a dextran polymer. Ribavirin-5'-*O*-(6-aminohexyl)phosphate bisammonium salt (SRI 8699-28) retained antiviral activity; however the activity was 16.5-fold less than that of ribavirin (see Tables II-6 and II-7). Neither the (carboxymethyl)-dextran-ribavirin conjugate (SRI 8699-58) nor the (carboxymethyl)dextran (SRI CD) displayed antiviral activity in the cell culture system. In vitro there may not be a suitable mechanism available for cleaving of the ribavirin from the conjugate and the mechanism (or lack thereof) of conjugate uptake by the cells may be different when comparing the Vero cells in vitro to macrophages and other Pichinde virus target cells in vivo. In order to be useful, multiple loading of ribavirin to MAb via a dextran polymer will require that the conjugate retain antigen binding activity. The fluoresceinated (carboxymethyl)dextran MAb PC4.9A6 conjugate (SRI 9047-34) retained Pichinde virus-binding activity. Thus, this approach to multiple conjugate loading appears promising. Both immunoconjugates consisting of ribavirin linked via (carboxymethyl)dextran to MAb PC4.9A6 (SRI 9047-73-1 and SRI 9047-73-2) displayed no antiviral activity toward Pichinde virus. The most probable explanation for this lack of activity is the inability of the antibody portion of these immunoconjugates to internalize. The studies described under Task VIII of this report indicate that MAb PC4.9A6 is not suitable for drug delivery to Pichinde virus-infected cells. However, immunoglobulins from hyperimmune antisera against Pichinde virus could be used for targeted delivery. Future studies should utilize the dextran polymer approach for multiple loading of ribavirin onto immunoglobulins.

E. Conclusions

1. The antiviral drug assay based on inhibition of immunofluorescence (Burns et al. 1988) is as sensitive as Pichinde virus antiviral assays based on inhibition of CPE or PR and offers the advantages of economy of reagents, rapidity, and a less ambiguous endpoint.

2. Ribavirin 5'-*O*-hemisuccinate may be a suitable derivative to be used for tethering to antibodies. The more hindered ester, ribavirin 5'-*O*-(2,3-dimethyl)hemi-succinate, while more stable than ribavirin 5'-*O*-hemisuccinate, displayed less antiviral activity and was more toxic. The antiviral assays were first conducted in vitro using the ribavirin derivatives without conjugation to MAb. It is quite likely that the 5'-ester linkage must be cleaved before the dimethylhemisuccinate or hemisuccinate derivatives can express antiviral activity. Thus, although the ribavirin 5'-*O*-dimethylhemisuccinate is less active in the in vitro antiviral assay, the compound may be active when conjugated to the appropriate monoclonal antibody.

3. A ribavirin-5'-*O*-hemisuccinate conjugate of MAb PC4.9A6 possessed no antiviral activity against Pichinde virus. The relatively low ribavirin loading in this immunoconjugate may have accounted for the lack of activity, but subsequent studies utilizing gelonin conjugates of MAb PC4.9A6 (described under Task VIII of this report) indicated that MAb PC4.9A6 was not suitable as a carrier antibody for antibody-mediated delivery of compounds to Pichinde virus-infected cells.

4. Multiple loading of ribavirin onto virus specific antibodies via either phosphate ester linkages (polyribavirin) or ribavirin-substituted dextran polymers is a promising approach for carrier-mediated targeted delivery. Poly(ribavirin phosphate) and dextran multiply loaded with ribavirin should be targeted by attachment to virus-specific antibodies if suitable antibodies that internalize are available.

TASK III. DERIVATION, PRODUCTION, PURIFICATION AND EVALUATION OF MONOCLONAL ANTIBODIES SPECIFIC FOR ANTIGENS EXPRESSED ON THE SURFACE OF PICHINDE VIRUS-INFECTED CELLS.

A. Introduction

Hybridoma cell lines were derived through the fusion of FOX-NY myeloma cells with spleen cells from hyperimmunized RBF/Dn mice (Taggart and Samloff, 1983). Four of the resulting (FOX-NY x RBF/Dn) hybridoma cell lines secreted MAbs towards Pichinde virus antigens expressed on the surface of Pichinde virus-infected cells in vitro and in vivo. The production, purification, and characterization of these MAbs is described in this section.

B. Materials and Methods

Derivation of hybridoma cell lines. Polyethylene glycol-induced fusion of FOX-NY myeloma cells to spleen cells from hyperimmunized RBF/Dn mice was used to derive the hybridoma cell lines utilized in these studies. The detailed fusion procedure is described in Appendix A.

Media used in hybridoma derivation. Medium designated as RPMI-1640 consisted of RPMI-1640 containing 15% FBS (in the preparation of media, the FBS was not heat-inactivated as this might be detrimental to the nutrients and growth factors), 10 mM HEPES (Research Organics), 100 U/mL penicillin, 100 µg/mL of streptomycin and 2 mM L-glutamine. Medium designated as RPMI-1640-SF was the same as above, except without serum. Medium designated as AAT consisted of RPMI-1640 containing 4×10^{-7} M aminopterin, 7.5×10^{-5} M adenine and 1.6×10^{-5} M thymidine, whereas medium designated as AT was same as AAT minus aminopterin. The buffer designated as Tris-NH_4Cl consisted of 17 mM Tris base and 140 mM NH_4Cl in distilled water. The "fusion mixture" consisted of polyethylene glycol (PEG) 4000 from Merck (50% solution) and 5%

dimethyl sulfoxide (DMSO) from Sigma in water, sterilized at 121°C on liquid setting for 30 min and aliquoted into polystyrene tubes at 2.0 mL/tube. The fusion mixture was stored at room temperature in the dark, sealed with parafilm. The conditioned medium was prepared by culturing FOX-NY cells in RPMI-1640 in logarithmic phase growth for 48 h, then filtering and sterilizing the medium. Glutamine was added to conditioned medium from a 200 mM working solution that was stored frozen until the time of the cloning experiment.

Cloning. Cells were diluted and plated in each of the various media on separate 96-well cell-culture plates at a dilution calculated to yield 0.5 cell/well. The plates were then placed in a humidified incubator and left undisturbed until observed for clones. The plates were examined 4 days later and a dot was placed over wells containing a single clone and the number of cells in each clone was determined. At 8 days post-seeding, the condition of the cells and the size of the clones was again determined.

Freezing of the hybridoma lines. Cells were in the logarithmic phase of growth at the time of freezing. The freezing medium was a 50/50 mixture of RPMI-1640 with 40% FBS and RPMI-1640 with 20% DMSO, both sterile filtered. Cells were collected by centrifugation, and resuspended in RPMI-1640 without serum. The total number of cells was determined, cells were pelleted again, and resuspended in cold RPMI-1640 with 40% FBS to yield between 2×10^6 to 2×10^7 cells/mL (at this point the volume was one-half the final volume). From this step on the cells and all media were held at 0°C to 4°C. By dropwise addition to the cell suspension, an equal volume of cold RPMI-1640 with 20% DMSO was added. The suspension was dispensed at 1 mL/tube into cryotubes on ice (polypropylene cryotubes, 2 cc with silicone gasket, Nunc, Denmark). Cryotubes were placed in a styrofoam test tube holder (50 tube package of 15-mL disposable centrifuge tubes, item 25319, Corning Glass Works, Corning, NY) and placed in -90°C freezer.

After 12-24 h, the cryotubes were transferred to boxes or canes and stored in liquid nitrogen or ultra-cold freezer.

Recovery of frozen cells. Cells were allowed to partially thaw at 37°C and as soon as pellet of ice was free from the walls of the tube, the contents were poured into a centrifuge tube containing 10 mL of RPMI-1640 with 40% FBS. Cells were collected by centrifugation and medium was removed. Cells were gently suspended in 10 mL of RPMI-1640 with 15% FBS and seeded into T25 flasks.

Cell counting and viability determinations. Cell counting was performed with a hemacytometer. For viability determinations 0.04% trypan blue (Sigma Chemical, St. Louis, MO) was included in the cell suspensions.

Antigen-binding activity of monoclonal antibodies. The antigen-binding activity was determined by an indirect immunofluorescent antibody assay. A half-log dilution series of each antibody sample in PBS was titered by limiting dilution using an indirect fluorescent antibody assay on acetone-fixed Pichinde virus-infected Vero cells in 24-well cell-culture plates. For the assay 200 µL of diluted sample was placed into each well as first antibody and incubated 2 h at 37°C. The first antibody was then removed and the cell sheets were rinsed twice with PBS. The second antibody, 100 µL of fluorescein-labeled anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN), at 1:250 was added and allowed to react at 37°C for 1 h. The second antibody was removed, and the cell sheets were rinsed once with distilled water and examined for fluorescing cells. Cell sheets were rinsed twice with water and examined for fluorescing cells, scoring on a -, +/-, 1+, 2+, 3+, 4+ scale for increasing staining intensity. The reciprocal of the greatest dilution that still produced unmistakable (1+) staining of infected cells was taken as the titer of the sample.

Ascites production. Cells were harvested from cell culture in the logarithmic growth phase by centrifugation at 400 x *g* for 5 min, and then resuspended in RPMI-1640

medium. Cells were injected into mice within 1 h. At the time of inoculation over 95% of the hybridoma cells were viable. Hybrid mice were bred in the USU Laboratory Animal Research Center using RBF/DnJ males from Jackson Laboratories and BALB/c females from Simonsen Laboratories. After weaning, the offspring, (RBF/DnJ x BALB/c)F_1 mice, were housed one sex to a cage. Both male and female mice were used for ascites production. Mice were 15-21 weeks old (18.6 ± 2.2) when inoculated with the PC4.9A6 hybridoma cells. Eight days prior to inoculation with the hybridoma cells, mice received 1.0 mL of pristane (2,6,10,14- tetramethylpentadecane, Sigma Chemical Co., St Louis, MO) by intraperitoneal (i.p.) injection. Seven days after the pristane injection, the mice received a single whole-body 350-rad dose of irradiation. The ^{137}Cs gamma-source delivered 171 rads/min, and the mice were exposed for 2 min and 5 sec. The next day, PC4.9A6 cells, 0.5 mL at 1×10^7/mL were injected, i.p., into the mice.

Collection of ascites fluids. The date of tumor appearance was noted for each mouse, and ascites fluids were collected once every one to two days after the tumors were readily apparent, usually within 3 days of the initial appearance of the tumor. Ascites fluids were collected by inserting an 18 ga. needle (no syringe) into the swollen abdomen and allowing fluids to drain into a 15-mL centrifuge tube. The fluid volume was recorded. At each collection, ascites fluids were clarified by centrifugation at 400 x *g* for 5 min and then stored at -20°C.

Isolation and purification of antibodies from ascites fluids. Immunoglobulins were precipitated from the ascites fluids with 50% ammonium sulphate. While the ascites fluids in an Erlenmyer flask were gently swirled, an equal volume of saturated ammonium sulphate in distilled water was added dropwise. The suspension was allowed to stand overnight at 4°C and then the precipitate was pelleted at 500 x *g* for 15 min. The precipitated immunoglobulins were dissolved in Dulbecco's phosphate-buffered saline [0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, and 140 mM NaCl, pH

7.2; PBS] to the original sample volume, then reprecipitated by the addition of an equal volume of saturated ammonium sulphate. The precipitated immunoglobulins were collected as before by centrifugation. The precipitation was performed one more time. The ascites PC4.9A6 was precipitated a fourth time since there was still some visible hemoglobin in the pellet after the third precipitation. The immunoglobulins recovered from the ammonium sulphate precipitations were dissolved in PBS, and then dialyzed against at least 100 volumes of PBS (e.g., 20 mL of immunoglobulin dialyzed against 2,000 mL of PBS). The dialysis fluids were changed at least four times at intervals of a few hours or more.

The ammonium sulphate-purified fraction from the ascites fluids was further purified by affinity chromatography on protein A using the BioRad Affi-Gel Protein A MAPS II Kit (BioRad Laboratories, Richmond, CA), following the instructions supplied with the kit. The IgG concentration in the final product was determined by optical adsorption at 280 nm (assuming that a solution of IgG at a concentration of 1 mg/mL had an optical density of 1.35 when measured at 280 nm). The IgG_{2a} concentration was determined by radial immunodiffusion. The final buffer exchange was into PBS without Ca or Mg (i.e., 0.01 M Na_2HPO_4, 0.15 M NaCl, pH 7.2).

Determination of IgG_{2a} concentrations. The concentration of immunoglobulin subclass IgG_{2a} in various sample fractions was determined by radial immunodiffusion (RID) with antisera specific for the IgG_{2a} subclass of mouse immunoglobulin. The reagents were purchased as a IgG_{2a} quantitation kit from ICN Biomedicals Inc. (Costa Mesa, CA). The procedures were as described in the supplier's product enclosures.

Storage of purified monoclonal antibodies. The purified MAb pools were dialyzed extensively against PBS without azide. They were then filter-sterilized by filtration through sterile membrane filters with a 0.22-μ pore size. The IgG_{2a} concentration was measured by RID. The purified MAbs were then aliquoted at 5 mL per vial, lyophilized, and stored at -15°C.

Lyophilization of monoclonal antibodies. Ascites fluids and purified MAbs were aliquoted at 5 mL per 25-mL vaccine septum vial, frozen in a -80°C freezer, and then lyophilized using a model FDX-1 34-VP lyophilizer (FTS Systems, Inc. Stone Ridge, NY). Lyophilization to dryness was accomplished in 4-12 h. After lyophilization, the vials were sealed and stored at -20°C.

Preparation of fluorescein-labeled monoclonal antibodies. Purified MAbs were dialyzed against 0.1 M Na_2HPO_4, pH 9.0. Following dialysis, precipitates were removed by centrifugation for 15 min at 550 x *g*. Sufficient fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, MO) was added to result in a protein to FITC wt:wt ratio of 50:1 in the conjugation reaction mixture. The FITC was added as a solution containing 1.0 mg of FITC dissolved in 1.0 mL of 0.2 M Na_2HPO_4, pH 9.0. This FITC solution was prepared just before addition to the immunoglobulin. The conjugation of fluorescein to protein was then accomplished by bringing the pH of the immunoglobulin-FITC mixture to 9.5 by dropwise addition of 0.2 M Na_3PO_4 and allowing the reaction to proceed at 25°C for 2.5 h. The conjugation conditions (Table III-8, i.e., pH and FITC to immunoglobulin ratio) were selected to yield a fluorescein to protein ratio in the final conjugate of approximately 10 fluorescein molecules per molecule of IgG (Hebert et al., 1972). The unconjugated FITC was removed by dialysis against Dulbecco's PBS (0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 and 140 mM NaCl, pH7.2) containing 0.1% sodium azide. The fluorescein-labeled MAb preparations were then further purified by gel-exclusion chromatography on Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, NJ). The FITC anti-Pichinde reagents were aliquoted at 0.25 mL per vial and lyophilized. The fluorescein-labeled MAb preparations were titered by limiting dilution in a direct immunofluorescent cell assay using Pichinde virus-infected Vero cells on coverslips as the targets. The fluorescein-labeled MAbs were diluted in PBS. Staining was at 37°C for 90 min after which the coverslip cell cultures were rinsed once in distilled

water and then mounted in elvanol. The quality and intensity of immuno-fluorescent staining was determined by epifluorescence microscopy. The specific staining intensity was scored on a scale of 0, no visible immunofluorescent staining, through 4, extremely intense immunofluorescent staining.

C. Results

When the fusion procedure described above was used, the yield of hybridoma cell lines was greatly increased over yields obtained with prior procedures. In the fusion termed PC4, more than 50 antibody producing hybridoma cell lines were cultured beyond the 200 cell stage. Four of these, PC4.9A6, PC4.9D3, PC4.8D2 and PC4.7C2, produced antibody to Pichinde virus antigens expressed on the surface of infected cells. These four were each cloned twice.

Effect of using macrophages in the fusion cultures. Mixtures of fusion cells were plated onto two sets of 24-well plates, five plates with macrophage feeder cells and five plates without macrophages. At seven days postfusion there was a difference in sizes of the colonies on the two sets of plates. The colonies in wells with macrophages had an average population of 98 ± 90 cells, whereas those in wells without macrophages averaged 24 ± 28 cells/colony. The percentage of wells yielding hybridoma colonies large enough to warrant antibody screening (at least 200 cells) was 9% in plates without macrophages and 22% in plates with macrophages. All four of the hybridoma cell lines that produced MAb towards Pichinde virus antigens expressed on the surface of infected cells were obtained from the fusion mixtures that had been cultured with macrophages.

Effect of variations in composition of cloning medium. The clones in the plates with conditioned medium reached a maximum size of 30 cells and did not appear healthy; in fact, these clones died out by 8 days at which time 4-20 dead cells were all that remained. The clones in the plate with fresh RPMI-1640 were the most vigorous of the lot;

they were over 200 cells in size by 8 days. There appeared to be some dead cells at the center of the colonies, yet very healthy cells at the edges. It appeared that crowding was a problem with these rapidly growing clones. The clones cultured in a 50:50 mixture of RPMI-1640 and conditioned media did much better than those in conditioned media, but worse than those in fresh RPMI-1640 alone. As shown in Table III-1, many of these colonies were dying out by the time they reached a size of 200 cells.

Evaluation of cell-freezing procedure. FOX-NY cells frozen according to the procedure described in this report were thawed, and cell counts and viabilities were determined at the times indicated in Table III-2. By 96 h after thawing, the FOX-NY cell culture has increased in cell number a factor of ten (just over three doublings) and had returned to >98% viability. The cell freezing procedure described in this report does not adversely affect the viability of the FOX-NY myeloma cell line. Several hybridoma cell lines have subsequently been frozen and revived using this procedure.

Isolation and purification of MAbs from ascites fluids. The purification procedures were initially developed and evaluated using ascites fluids derived from hybridoma cell lines PC4.9A6 and PC4.9D3. MAbs PC4.9A6 and PC4.9D3 were purified from murine ascites fluids using ammonium sulphate precipitation followed by affinity chromatography on protein A. The yields based on IgG_{2a} recovery at each step of the initial purification of MAbs PC4.9A6 and PC4.9D3 are indicated in Table III-3. Following ammonium sulphate precipitation, the product was approximately 75% pure IgG based on examination by SDS-polyacrylamide gel electrophoresis; the purity after the affinity chromatography step was in excess of 95%. The yield of IgG after the ammonium sulphate precipitation was about 85% for MAb PC4.9A6 and 60% for MAb PC4.9D3. The affinity chromatography step appeared to have a recovery of 15-40%. The final products were 110 mg of MAb PC4.9A6 and 12 mg of MAb PC4.9D3. Since the IgG_{2a} content did not necessarily indicate active antibody, the antigen binding activity of the

Table III-1. The effects of different media on cloning of FOX-NY cells.

Medium	No. wells with 1 or more clones four days postseeding	No. wells with mature clones eight days postseeding
RPMI-1640	22	15
Conditioned medium	7	0
Conditioned medium with 2 mM glutamine	13	0
50/50 mix of RPMI-1640/conditioned media (with 2 mM glutamine)	20	10

Table III-2. Viability of FOX-NY cells recovered from frozen cultures.

Time post thawing	Cell count	Viability (%)
4 h	1.8×10^5/mL	82
48 h	3.0×10^5/mL	97
Then split 1:5		
96 h	3.4×10^5/mL	>98

Table III-3. Purification of monoclonal antibodies: Yields and recoveries.

	Starting Ascites		After $(NH_4)_2SO_4$		After Protein A		
Hybridoma	Volume (mL)	$[IgG_{2a}]^1$ (mg/mL)	Volume (mL)	[protein]^2 (mg/mL)	Volume (mL)	$[IgG_{2a}]^1$ (mg/mL)	Yield (%)
PC4.9A6	23	14	25	15	100	1.1	34
PC4.9D3	8	17	10	11	35	0.34	9

[1][IgG_{2a}] mg/mL as determined by radial immunodiffusion with IgG_{2a} specific antisera.

[2][protein] mg/mL as determined by optical density measured at 280 nm.

Table III-4. Antigen binding activity of monoclonal antibodies before and after purification from murine ascites fluids.

	Starting Ascites		After Purification	
Hybridoma	IgG_{2a} (mg/mL)	Binding activity	IgG_{2a} (mg/mL)	Binding activity
PC4.9A6	14	$10^{5.5}$	1.1	$10^{4.5}$
PC4.9D3	17	$10^{5.7}$	0.34	$10^{4.5}$

purified MAbs, measured by indirect immunofluorescence, was determined (Table III-4). The binding activity was $10^{4.5}$ for both MAbs PC4.9A6 and PC4.9D3 preparations having IgG_{2a} contents of 1.1 mg/mL and 0.34 mg/mL, respectively. The antigen binding activity of the raw ascites fluids were $10^{5.5}$ and $10^{5.7}$, respectively, and the corresponding IgG_{2a} contents were 14 mg/mL and 17 mg/mL for MAb PC4.9A6 and MAb PC4.9D3, respectively. The residual binding activity normalized to the IgG_{2a} content was 126% and 300% of the original for MAb PC4.9A6 and MAb PC4.9D3, respectively. Subsequently, a larger pool of purified MAb PC4.9A6 was prepared by this same procedure. MAb PC4.9A6 was purified from 50 mL of ascites fluids. The final product was 21 mL of clear, colorless fluid containing IgG at a concentration of 8.4 mg/mL for an overall yield of approximately 40% through the entire purification procedure. The antigen binding titers determined by indirect immunofluorescent antibody assay were $10^{5.5}$ for the crude ascites PC4.9A6 and $10^{5.5}$ for the purified pool PC4.9A6. The titration data are shown in Table III-5. The purification yields are summarized in Table III-6. The purified MAb PC4.9A6, when analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions, migrated as two sharp bands corresponding to the heavy and light immunoglobulin chains. Based on the electrophoretic analysis, the purity of the purified MAb PC4.9A6 was estimated to be in excess of 95%.

Effect of lyophilization on antigen-binding activity of MAbs PC4.9A6 and PC4.9D3. Lyophilization usually does not grossly affect the activity of polyclonal antisera but some MAbs lose binding activity upon lyophilization. In this study the effect of lyophilization on the anti-Pichinde virus MAbs PC4.9A6 and PC4.9D3 was examined. There was no significant increase or decrease in activity associated with lyophilization (Table III-7).

Preparation and evaluation of fluorescein-labeled MAbs. MAbs PC4.9A6 and PC4.9D3 were conjugated with fluorescein isothiocyanate. These

Table III-5. Titration of starting ascites fluids and purified MAb PC4.9A6 by indirect immunofluorescent antibody assay on Pichinde virus-infected Vero cells.

Sample	Dilution	
Positive control fluorescein-labeled PC4.9A6	10^{-2}	4+
Crude ascites PC4.9A6	10^{-5}	2.5+
Crude ascites PC4.9A6	$10^{-5.5}$	1+
Crude ascites PC4.9A6	10^{-6}	+/-
Crude ascites PC4.9A6	$10^{-6.5}$	-
Crude ascites PC4.9A6	10^{-7}	-
Crude ascites PC4.9A6	$10^{-7.5}$	-
Crude ascites PC4.9A6	10^{-8}	+/-
Purified PC4.9A6	10^{-3}	4+
Purified PC4.9A6	$10^{-3.5}$	4+
Purified PC4.9A6	10^{-4}	3+
Purified PC4.9A6	$10^{-4.5}$	3+
Purified PC4.9A6	10^{-5}	3+
Purified PC4.9A6	$10^{-5.5}$	1+
Purified PC4.9A6	10^{-6}	-
Purified PC4.9A6	$10^{-6.5}$	+/-
Purified PC4.9A6	10^{-7}	+/-
Purified PC4.9A6	$10^{-7.5}$	+/-
Purified PC4.9A6	10^{-8}	-

Table III-6. Purification of monoclonal antibody PC4.9A6. Yields and recoveries.

	Volume (mL)	Protein[1] (mg/mL)	IgG_{2a}[2] (mg/mL)	Antibody titer[3] towards PCV[4]
Starting Ascites PC4.9A6	50	34	10	$1 \times 10^{5.5}$
Purified PC4.9A6	21	8.4	9 (38%)	$1 \times 10^{5.5}$ (42%)

[1]Protein in mg/mL as determined by optical density measured at 280 nm.

[2]IgG_{2a} in mg/mL as determined by radial immunodiffusion.

[3]Antibody titer towards Pichinde virus, measured by indirect immunofluorescence in an end-point dilution assay.

[4]Pichinde virus.

Table III-7. Effect of lyophilization on binding activity of monoclonal antibodies.

	Titer[1]	
Sample	Before lyophilization	After lyophilization
PC4.9A6 purified on protein A	$10^{-4.5}$	$10^{-4.5}$
PC4. 9D3 purified on protein A	$10^{-4.5}$	$10^{-4.2}$
PC4.9A6 ascites fluids	$10^{-5.5}$	$10^{-5.7}$
PC4.9D3 ascites fluids	$10^{-5.7}$	$10^{-5.5}$
PC4.7C2 ascites fluids	$10^{-5.5}$	$10^{-5.5}$

[1]Assayed by limiting dilution in an indirect immunofluorescence assay on Pichinde virus infected Vero cells.

fluorescein-labeled MAbs were used as reagents in Pichinde virus assays, in antiviral assays, as in vivo tracers for MAb distribution studies, and in competitive binding experiments to determine the epitope specificity of anti-Pichinde virus MAbs. All conjugations resulted in fluorescein labeled-antibody preparations with excellent immunostaining characteristics. The volumes and amounts of immunoglobulins and FITC used in the conjugations are shown in Table III-8. The three fluorescein-labeled MAb preparations were titered in a direct immunofluorescent cell assay using Pichinde virus infected Vero cells on coverslips (Table III-9). There was only the slightest nonspecific staining at dilutions of 1:100 or greater, whereas the specific staining was very clear and intense out to dilutions of at least 1:1,600.

Table III-8. Conjugation conditions for producing FITC-labeled monoclonal antibodies.

			Totals	
IgG Source	IgG conc[1] (mg/mL)	Vol (mL)	IgG (mg)	FITC (mg)
Ammonium sulphate-precipitated ascites PC4.9A6	15	5.5	82	1.6
Ammonium sulphate-precipitated ascites PC4.9D3	11	4.5	50	1.0
Protein A affinity-purified PC4.9A6	2.5	6.0	15	0.3

[1]IgG concentration determined assuming protein at 1 mg/mL had OD_{280} of 1.0.

Table III-9. Immunofluorescent staining of Pichinde virus-infected Vero cells with fluorescein-labeled MAbs.

FITC MAb[1]	Dilution FITC-MAb	Specific Staining Intensity[2]
Ammonium sulphate-precipitated ascites PC4.9A6	1:100	4+
Ammonium sulphate-precipitated ascites PC4.9A6	1:400	4+
Ammonium sulphate-precipitated ascites PC4.9A6	1:1,600	2+
Ammonium sulphate-precipitated ascites PC4.9A6	1:6,400	1+
Ammonium sulphate-precipitated ascites PC4.9A6	1:25,600	-
Ammonium sulphate precipitated ascites PC4.9D3	1:100	4+
Ammonium sulphate precipitated ascites PC4.9D3	1:400	4+
Ammonium sulphate precipitated ascites PC4.9D3	1:1,600	4+
Ammonium sulphate precipitated ascites PC4.9D3	1:6,400	2+
Ammonium sulphate precipitated ascites PC4.9D3	1:25,600	-
Protein A affinity-purified PC4.9A6	1:100	4+
Protein A affinity-purified PC4.9A6	1:400	3+
Protein A affinity-purified PC4.9A6	1:1,600	2+
Protein A affinity-purified PC4.9A6	1:6,400	1+
Protein A affinity-purified PC4.9A6	1:25,600	-

[1]The FITC-MAb conjugates are those described in Table III-8.

[2]The specific staining intensity was scored on the 0 to 4+ scale: 0, no visible immunofluorescent staining; 1+ difficult to read, but definitely positive; 2+ easy to read, definitely positive, suitable for competitive binding assays; 3+ very strong staining but not quite maximum; 4+ extremely intense immunofluorescent staining. Note that none of these FITC-MAbs at 1:100 or greater dilutions gave significant nonspecific staining.

D. Discussion

It was determined that cloning in RPMI-1640 medium without feeder cells was generally satisfactory. For cloning hybridoma cells, most procedures call for conditioned medium, however cloning in fresh RPMI-1640 medium prepared with FBS that had not been heat inactivated resulted in a higher yield of mature clones than did cloning in conditioned medium. Conditioned media may be toxic to cells either as a result of toxic products introduced as a result of the conditioning process or through depletion of essential nutrients. On the one hand, efficient cloning may require media with growth factors that are contributed by vigorously growing cells; on the other hand, perhaps the process of conditioning the media may deplete essential ingredients such as glutamine. It may also be that heat inactivation of FBS inactivates essential growth factors. Subsequent experience with hybridoma cells has indicated that most do very well in regular RPMI-1640 medium prepared with FBS that has not been heat inactivated.

MAbs were isolated and purified from murine ascites fluids using ammonium sulphate precipitation followed by affinity chromatography on protein A. The overall yields for the purification of MAb PC4.9A6 and MAb PC4.9D3 were 34-40% and 9%, respectively. From 50 mL of ascites fluids, 21 mL of purified MAb PC4.9A6 with a protein concentration of 840 µg/mL and a very high concentration of active antibody against Pichinde virus was obtained. The residual antigen-binding activity normalized to the IgG_{2a} content was 126% of the original PC4.9A6 ascites fluids, so the purification procedure did not appear to adversely effect the antigen binding activity of MAb PC4.9A6. The high protein concentration of this purified MAb PC4.9A6 should facilitate subsequent conjugation reactions.

MAbs PC4.9A6 and PC4.9D3, either as crude ascites preparations or as highly purified immunoglobulins, were not adversely effected by lyophilization. The MAbs may

be lyophilized and in this form shipped more conveniently than liquid preparations. Lyophilization may also prove useful as a method for further concentrating the MAbs that are diluted during purification and conjugation procedures.

Some of the MAbs produced in the course of these studies were subsequently labeled with fluorescein. These immunofluorescent conjugates proved to be very useful. They have been used in direct immunofluorescent cell-count assays for Pichinde virus where they eliminated the need for a second antibody as is required with indirect immunostaining assays. This has reduced the assay time by about 40%. The fluorescein-labeled MAbs have also been used in comparing the in vivo distribution of anti-Pichinde virus MAbs in infected and noninfected hamsters and in competitive binding assays to determine the epitope binding specificity of several anti-Pichinde virus MAbs.

E. Conclusions

Several modifications of previous procedures were included in the final hybridoma derivation protocol. The peritoneal macrophages in the fusion wells served as feeder cells, conditioned the medium, and phagocytized dead cells and debris, thereby reducing the toxicity of the fusion culture medium. Other modifications included (1) an antigen boost administered to the spleen donor mice five days prior to the fusion, (2) the avoidance of *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) when dimethyl sulfoxide (DMSO) was present, (3) only very gentle manipulation of the fusion cell mixture during the critical fusion steps, and (4) addition of aminopterin to the fusion cell cultures at 24-h postfusion. These modifications were evaluated independently, and each had a positive effect.

1. Several modifications of previous procedures included in the final hybridoma derivation protocol should be included in future derivations. These modifications were evaluated independently and each had a positive effect. Peritoneal macrophages in the

fusion wells served to condition the medium; they also phagocytized dead cells and debris, thereby reducing the toxicity of the fusion culture medium. Other modifications included an antigen boost administered to the spleen donor mice 5 days prior to the fusion, the avoidance of HEPES when dimethyl sulfoxide was present in the medium, only very gentle manipulation of the fusion cell mixture during the critical fusion steps, and finally the addition of aminopterin to the fusion cell cultures 24-h post-fusion.

2. Ammonium sulphate precipitation followed by affinity chromatography on protein A is a suitable procedure for isolating and purifying the MAbs developed in these studies. The IgG recoveries murine ascites fluids ranged from 9% to 40%. The purification procedure did not adversely affect antigen binding activity. MAbs PC4.9A6 and PC4.9D3, either as crude ascites preparations or as highly purified immunoglobulins, were not adversely affected by lyophilization.

TASK IV. ANTI-PICHINDE VIRUS MONOCLONAL ANTIBODIES. CHARACTERIZATION: BINDING TO PICHINDE VIRUS ANTIGENS EXPRESSED ON THE SURFACE OF LIVING CELLS, ISOTYPE ANALYSIS, EPITOPE SPECIFICITY AND VIRAL PROTEIN SPECIFICITY.

A. Introduction

This section describes the characterization of several MAbs derived against Pichinde virus. It was determined that MAbs PC4.7C2, PC4.8D2, PC4.9D3 and PC4.9A6 bind to antigens expressed on the surface of Pichinde virus-infected cells. These four MAbs were selected for further characterization. The epitope and polypeptide binding specificities of the four MAbs were examined using competitive binding assays and radioimmunoprecipitation (RIP). By performing competitive binding assays, it is possible to determine if two MAbs recognize the same or different epitopes. There may be several epitopes on a single polypeptide. For the purpose of targeted delivery with a MAb, some epitopes will be more suitable than others. Therefore, a knowledge of the epitope specificity of candidate MAbs will aid in the assembly of a representative panel of MAbs for studies of antibody-mediated targeted drug delivery. A knowledge of a MAb's polypeptide specificity is also necessary for predicting the suitability of that MAb as a drug delivery vector. Some polypeptides contain epitopes that are displayed on the surface of infected cells, while others do not. The polypeptide binding specificities of the four candidate anti-Pichinde virus MAbs had been examined previously by Western blot analysis, with no apparent reactivity with any of the Pichinde virus protein bands on the electrophoretic blots. However, fluorescein-conjugates of these MAbs had repeatedly been shown to react with Pichinde virus epitopes expressed on the cytoplasmic membrane of Pichinde virus-infected cells. The most likely candidate viral proteins would be GP1 and GP2, both glycoproteins that span the cytoplasmic membrane of Pichinde virus-infected cells. The three-dimensional structure of the hydrophilic and hydrophobic domains of membrane-spanning

glycoproteins is very dependent on the nature of their immediate environment. Thus, the conformation of these glycoproteins following extraction from the virus envelope or cell membrane and SDS-gel electrophoresis may be quite different from the conformation recognized by the MAbs. Binding of MAbs to such glycoproteins may be conformationally dependent and sometimes such MAbs are not detected by Western blot analysis. We therefore examined the protein-binding specificity of the candidate MAbs by RIP using ^{35}S-methionine-labeled Pichinde virus proteins as targets. The cytosols were generated from the Pichinde virus-infected cells without the use of ionic detergents.

B. Materials and Methods

Monoclonal antibodies. The derivation of the hybridoma cell lines that secrete anti-Pichinde virus MAbs was described in a previous section of this report and in Section IV of the Second Annual Report). Most of the work described in this section utilized MAbs PC4.7C2, PC4.8D2, PC4.9D3 and PC4.9A6, which are of the IgG_{2a} subclass and bind to Pichinde virus antigens expressed on the surface of Pichinde virus-infected cells. MAb WE33.6, also of the IgG_{2a} subclass, was obtained from Dr. M. J. Buchmeir (Scripps Clinic, La Jolla, CA). MAb WE33.6 is directed at the GP2 glycoprotein of another arenavirus, lymphocytic choriomeningitis virus (LCMV).

Detection of antibodies that react with antigens expressed on the surface of Pichinde virus-infected cells. Vero cells were infected with Pichinde virus at a multiplicity of infection (MOI) of 0.01. Following an incubation period of 22 h to allow expression of Pichinde virus antigens on the surface of infected cells, the antibody samples were simply added to the media that bathed the cells. After incubation for 1 h at 37°C, the media was removed and cultures were rinsed twice with warm MEM, and then the cultures were dried and fixed with acetone. Cultures were then immunostained to detect murine antibody binding to cells. Immunostaining was performed by adding 100 µL of fluorescein-labeled goat anti-mouse IgG (Boehringer Mannheim Biochemicals,

Indianapolis, IN) to each well and incubating for 3 h. Cell sheets were rinsed twice with water and examined by epifluorescence microscopy for fluorescing cells.

MAb isotype determinations. The isotypes of the MAb were determined by enzyme-linked immunosorbent assay (ELISA) using anti-mouse sub-isotyping reagents (HyClone Laboratories, Inc., Logan, UT), following the supplier's assay instructions.

Cells. Confluent monolayer cultures of Vero cells in 24-well cell-culture plates were seeded 42 h prior to virus inoculation. Cells were grown in MEM with 5% heat-inactivated FBS (HyClone Laboratories, Inc., Logan, UT).

^{35}S-labeled Pichinde virus proteins. Cell culture-grown Pichinde virus strain CoAN4763 was used as the inoculum for producing ^{35}S-labeled Pichinde virus proteins. Vero cells growing in 24-well cell-culture plates were rinsed once with MEM and then inoculated at a MOI of 0.01 with Pichinde virus. Noninfected controls were processed in parallel. Following 1-h adsorption, the inocula were removed and all wells received 2 mL of MEM. At 48-h postinoculation (p.i.) the medium was removed, and the cells were rinsed twice with methionine-deficient MEM. Then 1 mL of methionine-deficient MEM containing ^{35}S-methionine (Trans^{35}S-label™ obtained from ICN Biomedicals, Inc., Irvine, CA) at 60 µCi/mL was added to each well. The cultures were incubated at 37°C an additional 24 h to allow ^{35}S-labeling of proteins. At 72 h p.i. the supernatant fluids were removed, and the monolayers were solubilized.

Solubilization procedure. The cytosol solubilization buffer consisted of 0.05 M NaCl, 0.02 M Tris-HCl, 0.001 M EDTA, 2% Nonidet P-40 (NP-40), and 1 mM phenylmethyl sulfonyl fluoride (PMSF), pH 7.4. Solubilization buffer at 4°C was added to the cell monolayers in the 24-well plate, 0.5 mL/well and incubated at 4°C for 15 min. Wells were rinsed once with an additional 0.5 mL of solubilization buffer/well, the rinse was added to the original lysates, so 1.0 mL of cytosol was derived from each well. The suspensions were vortexed for 15 sec. Nuclei and large debris were removed by

centrifugation for 10 min at 15,000 x *g*. The solubilized cytosols and cell culture supernatant fluids were aliquoted at 1.0 mL/vial and held frozen at -80°C.

^{35}S assays. The amount of radioactivity present in samples was determined by liquid scintillation counting using Beckman Ready Safe™ liquid scintillation cocktail. The extent of ^{35}S-labeled amino acid incorporation into protein was estimated by determining the percentage of the ^{35}S activity that was precipitated by 10% trichloroacetic acid (TCA).

Immunoprecipitation. The immune complexes were formed by mixing 50 µL of ascites fluid or 200 µL of hybridoma supernatant fluid with 100-200 µL of ^{35}S-labeled cytosol containing approximately 10^6 cpm of TCA precipitable material and incubating the mixture for 16 h at 4°C. To this mixture was added 200 µL of a 10% suspension of *Staphylococcus aureus* cells (Sigma Chemical Co., St. Louis, MO), which bind the Fc portion of immunoglobulins due to the presence of protein A on the surface of the bacteria cell membranes. This mixture was further incubated for 2 h on ice, with occasional gentle swirling. The protein A-antibody-antigen complexes were pelleted by centrifugation. The pellets were washed three times with TNE buffer (0.01 M Tris-HCl, 0.1 M NaCl, 0.001 M EDTA, pH 7.5) containing 0.5% NP-40. After the third wash, the complexes were resuspended in electrophoresis sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) and boiled for 2 min. After boiling, the *S. aureus* cells present in the sample were removed by centrifugation and 50-60 µL of supernatant containing the MAb-reactive viral protein was loaded onto polyacrylamide gels for electrophoresis.

Polyacrylamide gel electrophoresis (SDS-PAGE). All SDS-PAGE was performed under the following conditions using a Hoeffer Mighty Small II electrophoresis apparatus. The buffers used were according to Laemmli (1970). The resolving gels were 1.5-mm thick and were 10% total acrylamide (T), with 2.7% crosslinker (C), also with 0.375 M Tris-HCl, pH 8.8, and 0.1% SDS. The stacking gel was 4% T and 2.7% C. The

tank buffer was 0.025 M Tris, pH 8.3, 0.192 M glycine, and 0.1% SDS. The electrophoresis sample buffer was 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol. Gels were run at a constant current of 20 mA per gel for approximately 1.5 h. The gels were cooled by circulating cold tap water through the gel apparatus. Gels were then fixed in a solution of 30% methanol and 10% acetic acid for 15 min. The gels were stained for 1 h in a similar solution with 0.125% Coomassie Blue R-250, and then destained with fixing solution overnight. Molecular weight markers (BioRad Chemical Division, Richmond, CA) were run with each gel and were used to determine the apparent molecular weights of the viral proteins.

Fluorography. Prior to drying, the gels were allowed to equilibrate in double distilled water, and then impregnated with Fluoro-hance™ (Research Products International Corp., Mount Prospect, IL) for 30 min. After this treatment, the gels were dried onto a heavy filter paper backing with a BioRad gel-drying apparatus for 1.75 h at 80°C. The dried gels were placed against Kodak X-OMat X-ray film and exposed for 3 h at -90°C. The films were developed according to the manufacturer's specifications.

Competitive binding assay. Anti-Pichinde virus MAbs PC4.9A6 and PC4.9D3 were labeled with fluorescein isothiocyanate (FITC) as described in this report. These FITC-labeled MAbs were used in competitive binding experiments to determine the epitope binding specificity of the anti-Pichinde virus MAbs. The ability of the sample MAbs to block (competitive binding) the binding of the FITC-labeled MAbs to Pichinde virus-infected Vero cells in an immunofluorescent cell assay was measured. The fluorescein-labeled MAbs were used at a concentration that would yield strong staining. Dilutions of the test MAb were mixed with the fluorescein-labeled MAb and then the mixture was added to an infected culture of Vero cells. Cell sheets were examined for evidence of competition between the two antibodies by comparing intensity of immunostaining when sample diluent only was added to the FITC-labeled MAb.

The nonlabeled MAbs being tested were diluted in Dulbecco's PBS and then 150 µL was added per well to Pichinde virus-infected Vero cells in a 24-well cell-culture plate. These "competing" antibodies were incubated on the cell sheets for 30 min at 37°C prior to adding the FITC-labeled MAb (sufficient FITC-labeled MAb was added to yield very intense immunofluorescent staining of infected cells in the absence of competing antibody). The antibody mixtures on the infected Vero cells were incubated at 37°C for 2.5 h after which the cell sheets were rinsed once in distilled water and then mounted with elvanol. The relative intensity of the specific anti-Pichinde virus staining was then observed using an epifluorescence microscope. Absence of competition indicated that the antibodies were not directed towards the same epitope. Controls included negative control (FITC-labeled MAb competing with diluent only to demonstrate fluorescent staining intensity without competition), and positive control (nonlabeled form of the the FITC-labeled MAb to demonstrate competition).

Animals. Female hamsters, strain MHA, were obtained from Charles River Laboratories (Willmington, MA). The hamsters were 13 weeks old and weighed 150 g when immunized with Pichinde virus.

Hyperimmune hamster sera towards Pichinde virus. A pool of liver homogenates from MHA hamsters that had been infected with Pichinde virus was the source of Pichinde virus antigens for immunization. The pool, a 10% suspension consisting of 1 part liver to 9 parts PBS, was homogenized and assayed for Pichinde virus titer by plaque assay in cell culture. The titer was 3×10^7 plaque-forming units/mL. Since the purpose was to produce antisera and not to cause fatal Pichinde virus infections in the hamsters, the Pichinde virus liver homogenate was heat-inactivated by placing the material in a 60°C water bath for 1 h. The liver homogenate containing Pichinde virus was mixed with an equal volume of Freund's complete adjuvant to generate a stable emulsion. The immunogen was administered by intramuscular injection, 0.25 mL in both hind flanks for a

total volume of 0.5 mL per animal. Booster injections were administered at 21 and 32 days after the primary immunization. Serum was collected 10 weeks after the primary immunization.

Indirect immunofluorescence assay for antibody towards Pichinde virus. Antibody samples diluted in PBS were added to Pichinde virus-infected acetone-fixed Vero cell cultures in 24-well plates. The antibodies on the infected cell cultures were incubated for 2 h then removed. The cell sheets were rinsed twice with PBS. The presence of anti-Pichinde virus antibody was then detected by adding 100 μL of fluorescein-labeled goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) and incubating for 3 h. Cell sheets were rinsed twice with water and examined for fluorescing cells, scoring on a -,+/-, 1+, 2+, 3+, 4+ scale for increasing relative immunofluorescent staining intensity.

C. Results

Detection of antibodies that react with antigens expressed on the surface of Pichinde virus-infected cells. To determine if any of the anti-Pichinde virus MAbs react with antigens expressed on the surface of Pichinde virus-infected cells, the MAbs were added to the cell culture media bathing either infected or noninfected living cells. After a suitable reaction period, the free antibody was rinsed away and the cells were stained with fluorescein-labeled antibody toward mouse IgG. The results of the indirect immunofluorescence assays utilizing viable Vero cells and the MAbs generated towards Pichinde virus indicated that four of the MAbs recognized epitopes displayed on the surface of infected cells. MAbs PC4.7C2, PC4.8D2, PC4.9D3, and PC4.9A6 selectively bound to Pichinde virus antigens expressed on the surface of viable Vero cells infected with Pichinde virus. Antibodies present in normal mouse serum were not selectively taken up by Pichinde virus-infected Vero cells; however, antibodies present in hyperimmune mouse serum did bind to the surface of infected Vero cells. None of the MAbs reacted with

uninfected Vero cells. Neither the hyperimmune hamster serum nor MAb WE33.6 were available for evaluation when these experiments were conducted.

MAb isotype determinations. Twenty-five anti-Pichinde virus MAbs resulting from the PC4-fusion experiment were examined by ELISA using isotype-specific reagents. The majority, 22 out of 25, were of the IgG_{2a} isotype, two were of the IgG_1 isotype and one was of the IgG_{2b} isotype. MAbs PC4.7C2, PC4.8D2, PC4.9D3, and PC4.9A6, the four MAbs that reacted with PCV antigens expressed on surface of infected cells, were of the IgG_{2a} isotype.

Anti-Pichinde virus activity in hyperimmune hamster serum and anti-LCMV MAb WE33.6 by indirect IFA. The hyperimmune hamster serum exhibited a strong response (reciprocal titer of approximately 10^4) to Pichinde virus-infected Vero cells (Table IV-1). In the indirect immunofluorescence assays (IFAs), FITC-labeled goat antimouse IgG was found to be suitable for detecting the hamster IgG. Anti-LCMV MAb WE33.6 , at dilutions of 10^{-3} and 10^{-4}, produced recognizable immunofluorescent staining of Pichinde virus-infected cells. However, there was such a high concentration of IgG in the WE33.6 ascites that the nonspecific staining overwhelmed any specific staining at all dilutions of 10^{-2} or less. This narrow window of staining may be interpreted to mean that MAb WE33.6 has a relatively low specificity for Pichinde virus GP2. The end-point dilution titer by IFA against Pichinde virus-infected target cells was 10^3 for MAb WE33.6 as compared with anti-Pichinde virus IFA titers of $10^{5.5}$ to $10^{6.5}$ for ascites fluids generated from hybridoma cell lines PC4.9A6, PC4.9D3, and PC4.7C2.

Epitope specificities. Competitive binding assays indicated that MAbs PC4.9A6 and PC4.7C2 recognized the same antigenic determinant, whereas PC4.9D3 recognized a distinct nonoverlapping epitope (data shown in Table IV-2). Homologous antisera out to dilutions of 1:1,000 completely blocked binding of MAbs PC4.9A6 or PC4.9D3. Hyperimmune MHA hamster serum out to dilutions of 1:100 (the greatest

Table IV-1. Antibody titers towards Pichinde virus.[a]

Sample	Dilution	IFA Result
Hyperimmune hamster serum	neat	2+, extensive nonspecific staining
Hyperimmune hamster serum	10^{-1}	3+, extensive nonspecific staining
Hyperimmune hamster serum	10^{-2}	4+, minimal nonspecific staining
Hyperimmune hamster serum	10^{-3}	3+
Hyperimmune hamster serum	10^{-4}	2+
Hyperimmune hamster serum	10^{-5}	-
WE33.6 (ascites fluids)	neat	No discernible specific fluorescing cells, extensive nonspecific staining
WE33.6 (ascites fluids)	10^{-1}	No discernible specific fluorescing cells, extensive nonspecific staining
WE33.6 (ascites fluids)	10^{-2}	No discernible specific fluorescing cells, extensive nonspecific staining
WE33.6 (ascites fluids)	10^{-3}	2+
WE33.6 (ascites fluids)	10^{-4}	1+
PC4.9A6 (ascites fluids)	10^{-4}	4+
PC4.9A6 (ascites fluids)	10^{-5}	4+

[a]The antibody titers towards Pichinde virus were measured by an indirect immunofluorescence assay utilizing fluorescein-labeled antibody towards mouse IgG as the second antibody and Pichinde virus-infected Vero cells as targets. Immunofluorescence was graded on a scale of 0 (normal cells with no fluorescence) to 4 (intense specific immunofluorescence in infected cells) and the end-point titer was the reciprocal of the highest dilution yielding immunostaining of at least 2+ intensity. The resulting titers were: 10^4 for the hyperimmune hamster serum, 10^3 for the anti-LCMV MAb WE33.6 (ascites fluids), and $\geq 10^5$ for MAb PC4.9A6, an anti-Pichinde virus MAb (ascites fluids).

Table IV-2. Competitive binding assays for determining epitope specificities.[a]

Labeled antibody	Competing antibody	Dilution	Staining Intensity
PC4.9A6	Diluent (neg control)	-	4+
PC4.9A6	Hyperimmune hamster serum	neat	-
PC4.9A6	Hyperimmune hamster serum	1:10	1+
PC4.9A6	Hyperimmune hamster serum	1:100	2+
PC4.9A6	PC4.9A6 ascites	1:100	-
PC4.9A6	PC4.9A6 ascites	1:1,000	-
PC4.9A6	PC4.9D3 ascites	1:10	4+
PC4.9A6	PC4.9D3 ascites	1:100	4+
PC4.9A6	PC4.7C2 ascites	1:10	-
PC4.9A6	PC4.7C2 ascites	1:100	+/-
PC4.9A6	WE33.6 ascites	1:10	2+
PC4.9A6	WE33.6 ascites	1:100	3+
PC4.9A6	PC4.9A6 supernate	neat	4+
PC4.9A6	PC4.7C2 supernate	neat	4+
PC4.9A6	PC4.8D2 supernate	neat	4+
PC4.9A6	PC4.9D3 supernate	neat	4+
PC4.9D3	Diluent (neg control)	-	3+
PC4.9D3	Hyperimmune hamster serum	neat	-
PC4.9D3	Hyperimmune hamster serum	1:10	-
PC4.9D3	Hyperimmune hamster serum	1:100	-
PC4.9D3	PC4.9D3 ascites	1:100	-
PC4.9D3	PC4.9D3 ascites	1:1,000	-

PC4.9D3	PC4. 9A6 ascites	1:10	3+
PC4.9D3	PC4. 9A6 ascites	1:100	3+
PC4.9D3	PC4.7C2 ascites	1:10	3+
PC4.9D3	PC4.7C2 ascites	1:100	3+
PC4.9D3	WE33.6 ascites	1:10	+/-
PC4.9D3	WE33.6 ascites	1:100	2+
PC4.9D3	PC4.9A6 supernate	neat	3+
PC4.9D3	PC4.7C2 supernate	neat	3+
PC4.9D3	PC4.8D2 supernate	neat	3+
PC4.9D3	PC4.9D3 supernate	neat	+/-

[a]The epitope binding specificity of the anti-Pichinde virus MAbs was examined by competitive binding assays. The ability of the sample antibodies to block (competitive binding) the binding of FITC-labeled anti-Pichinde virus MAbs PC4.9A6 and PC4.9D3 to Pichinde virus-infected Vero cells in an immunofluorescent cell assay was measured. The fluorescein-labeled MAbs were used at a concentration that would yield strong staining; dilutions of the test MAb were mixed with the fluorescein-labeled MAb and then the mixture was added to an infected culture of Vero cells. The presence or absence of competition between antibodies was determined by comparing intensity of immunostaining to that obtained with corresponding controls. Cell sheets were examined for fluorescing cells, scoring on a -, +/-, 1+, 2+, 3+, 4+ scale for increasing relative immunofluorescent staining intensity.

dilution tested) completely blocked the binding of MAb PC4.9D3 and at 1:10 but not at 1:100 blocked the binding of MAb PC4.9A6. MAb PC4.9D3 did not compete with MAb PC4.9A6, and MAb PC4.9A6 did not compete with MAb PC4.9D3 in the reciprocal assay. PC4.7C2 ascites did block the binding of MAb PC4.9A6 out to dilutions of 1:100 (the greatest dilution tested), but did not compete with MAb PC4.9D3. Anti-LCMV MAb WE33.6 partially blocked the binding of both MAbs PC4.9A6 and PC4.9D3 (reagents were not available to test for competition with MAbs PC4.7C2 and PC4.8D2). In contrast to ascites fluids, cell-culture supernates failed to demonstrate competition for binding sites, even in the case of homologous antibodies. Therefore nothing can be claimed about the epitope specificity of MAb PC4.8D2 for which cell culture supernates but no ascites fluids were available.

^{35}S activity in fractions from metabolic labeling experiment. The ^{35}S-labeled cell-culture products were divided among the six fractions described in Table IV-3, each with a total volume of 11-12 mL. The Pichinde virus-infected and noninfected cytosols were used for the RIP experiments.

Polypeptide specificities. All four anti-Pichinde virus MAbs precipitated a single ^{35}S-labeled protein that migrated between the heavy and light chains of the MAbs. The apparent molecular weight of the polypeptide was 38,000 Daltons. This 38K viral polypeptide band was not visible on gels stained with Coomassie blue, but was the most intense viral band on autoradiograms obtained from gels on which total cell lysates from infected cells were run. When mixtures of the immunoprecipitates obtained with all of the MAbs were run in a single lane, the ^{35}S-labeled proteins comigrated, indicating that all four MAbs bind to the same viral protein. When films were overexposed, a minor band, corresponding to a 60K polypeptide, appeared in the lanes containing the MAb immunoprecipitates.

Table IV-3. ^{35}S Activity in fractions from metabolic labeling experiment.[a]

Fraction	Total cpm/20 µL	TCA[b] precipitable ^{35}S
Pichinde virus-infected cytosols	6.36×10^5	18.3%
Noninfected cytosols	6.52×10^5	5.2%
Pichinde virus-infected cell culture supernatants	12.32×10^5	3.1%
Pichinde virus-infected cell culture supernatants (solubilized)	5.66×10^5	1.6%
Noninfected control cell culture supernatants	8.77×10^5	2.9%
Noninfected control cell culture supernatants (solubilized)	1.54×10^5	1.0%

[a]^{35}S-methionine was used to metabolically label polypeptides synthesized in Vero cells, noninfected controls, and cultures infected with Pichinde virus. Cultures were rinsed twice with methionine-deficient MEM. Then 1 mL of methionine-deficient MEM containing ^{35}S-methionine (Trans^{35}S-label™ obtained from ICN Biomedicals, Inc., Irvine, CA) at 60 µCi/mL was added to each well in a 24-well plate. The cultures were incubated at 37°C an additional 24 h to allow ^{35}S-labeling of proteins, the supernatant fluids were removed, and the monolayers were solubilized. The amount of radioactivity present in samples was determined by liquid scintillation counting.

[b]The extent of ^{35}S-labeled amino acid incorporation into protein was estimated by determining the percentage of the ^{35}S activity that was precipitated by 10% trichloroacetic acid (TCA).

When total cell lysates from infected and noninfected cells were electrophoresced in adjacent lanes, autoradiograms demonstrated two intense bands of definite viral origin corresponding to polypeptides with apparent molecular weights of 27,000 and 38,000 Daltons. There were also several, faint bands between 58,000 and 69,000 Daltons. The intensity of the bands indicated that the 38,000 Dalton polypeptide precipitated by the MAbs was the most abundant of the ^{35}S-labeled viral polypeptides. Anti-LCMV MAb WE33.6 did not precipitate detectable levels of ^{35}S-labeled viral polypeptides. When the four anti-Pichinde virus MAbs were were allowed to react with ^{35}S-labeled lysates from noninfected cells, they did not precipitate ^{35}S-labeled polypeptides. When an isotype matched MAb specific for a Friend leukemia virus antigen was allowed to react with the ^{35}S-labeled lysates from Pichinde virus-infected cells, no ^{35}S-labeled proteins were precipitated. This latter observation weights against an interaction between the 38,000 Dalton Pichinde virus polypeptide and murine IgG_{2a} in general or other trivial interactions between the components of the RIP reaction mixture. Polyclonal mouse serum, hyperimmune toward Pichinde virus, precipitated ^{35}S-labeled 27K, 38K, and 58-69K polypeptides. The polyclonal hamster serum was not available for evaluation at the time these experiments were conducted.

D. Discussion

MAbs PC4.7C2, PC4.8D2, PC4.9D3, and PC4.9A6 are specific for Pichinde virus antigens expressed on the surface of viable Vero cells infected with Pichinde virus. This observation indicates that these MAbs may be suitable candidates as drug delivery vectors for the antibody-mediated targeted delivery of antiviral drugs. All were found to be of the IgG_{2a} isotype.

Anti-Pichinde virus MAbs PC4.9A6 and PC4.7C2 recognize the same epitope, whereas MAb PC4.9D3 recognizes a second nonoverlapping epitope. Competitive binding

assays with anti-LCMV MAb WE33.6, which cross reacts with the GP_2 glycoprotein of Pichinde virus, resulted in partial competition with MAbs PC4.9A6 and PC4.9D3. This was one of the indications that anti-Pichinde virus MAbs PC4.9A6 and PC4.9D3 bind to epitopes on the GP2 glycoprotein of Pichinde virus. The anti-Pichinde virus MAbs PC4.7C2, PC4.8D2, PC4.9D3, and PC4.9A6 precipitated a 38,000 Dalton polypeptide that was the major viral polypeptide in the cytosols from Pichinde virus-infected Vero cells. Of the three major polypeptides of Pichinde virus, the 38K polypeptide most likely corresponds to the GP_2 glycoprotein.

Vezza et al. (1977) described three polypeptides obtained from intact Pichinde virus (strain AN3739), GP_1 and GP_2, 64,000 Dalton and 38,000 Dalton glycoproteins respectively, which were found in approximately equal ratios in the viral envelope, and NP (66,000 Daltons), which is a nonglycosylated internal protein comprising about 70% of the protein in purified Pichinde virus. Harnish et al. (1981) examined the viral proteins obtained from purified Pichinde virus (strain AN3739) and cytosols of Pichinde virus-infected BHK-21 cells. They found the virus to contain GP_1 (52K), GP_2 (36K), NP (64K), and a large L protein (200,000 Daltons). However the cytosols contained many additional polypeptides that exhibited a time- and MOI-dependent appearance and abundance. One of these GPC (79K) was a precursor to GP_1 and GP_2. They also found high levels of 48K, 38K, and 28K polypeptides, which were fragments of NP. Buchmeier et al. (1977) found that the major soluble complement-fixing (CF) antigen obtained from cell cultures infected with Pichinde virus was a 20,000-30,000 Dalton fragment of NP. Auperin et al. (1984) determined the nucleotide sequence of the gene coding for NP. According to their analysis, the NP polypeptide from Pichinde virus (strain AN3739) would have a calculated molecular weight of 62,911 Daltons. Buchmeier et al. (1981) used RIP to determine the polypeptide specificities of MAbs towards LCMV and Pichinde virus. They noted that cytosol preparations contained all of the previously reported LCMV

polypeptides, but that the relative proportion of NP was reduced in comparison with intact virions. They also found that a few MAbs precipitated both GP_1 and GP_2 but pointed out that this could have been the result of coprecipitation of a common macromolecular structure. When MAbs that precipitated either GP_1 or GP_2 were allowed to react with cytosols, they often precipitated a small amount of GPC, the precursor of GP_1 and GP_2. We observed a faint band corresponding to a 60K polypeptide on overexposed fluorographs of the immunoprecipitates obtained with the anti-Pichinde virus MAbs PC4.7C2, PC4.8D2, PC4.9D3, and PC4.9A6. This observation is in line with anti-GP_2 specificity.

It must be noted that the Pichinde virus characterized by us was of the strain CoAN4763, whereas the Pichinde virus that has been characterized by others (Auperin et al., 1984; Buchmeier et al., 1977; Buchmeier et al., 1981; Harnish et al., 1981; Vezza et al., 1977) was of strain AN3739. However, it did not appear likely that there would be major polypeptide differences between these two strains of Pichinde virus. We have used the strain CoAN4763 because we found it to be more lethal in the in vivo MHA hamster model than strain AN3739.

LCMV is the prototype of the arenavirus group and shares some antigenic relationship to Pichinde virus. Parekh and Buchmeier (1986) using a panel of MAbs against the GP_1 and GP_2 glycoproteins of LCMV demonstrated the presence of four distinct epitopes on GP_1 and three epitopes on GP_2 of LCMV. They found that none of the MAbs directed at GP_2 were neutralizing, but that MAbs directed against two of the four epitopes on GP_1 did neutralize LCMV. The three epitopes on LCMV GP_2 were overlapping. One designated GP_{2A} defined by several MAbs including WE33.6 partially overlapped the other two epitopes GP_{2B} and GP_{2C}. However, MAbs against epitopes GP_{2B} and GP_{2C} did not compete with each other. MAb WE33.6, generated against LCMV, crossreacts with Pichinde virus (M. J. Buchmeier, personal communication, and

the results presented in this section). In RIP experiments, we found that MAb WE33.6 would not precipitate a discernible polypeptide from ^{35}S-methionine-labeled Pichinde virus cytosols. However, we found that MAb WE33.6 did give positive staining of Pichinde virus-infected Vero cells by indirect immunofluorescence. In competitive binding assays, MAb WE33.6, at a 1:10 dilution, definitely competed for the binding site recognized by MAb PC4.9D3. However this competition was fairly weak, since at a dilution of 1:100, MAb WE33.6 failed to significantly inhibit the binding of MAb PC4.9D3. Homologous MAb completely blocked binding of the FITC-labeled antibody at ascites dilutions of 1:10,000 (data not shown). MAb WE33.6 inhibited the binding of MAb PC4.9A6 only slightly, indicating, at most, only a partial overlap of the recognized antigenic sites. However, the results of these competitive binding assays with MAb WE33.6 indicated that anti-Pichinde virus MAbs PC4.9A6 and PC4.9D3 probably do bind to epitopes on the GP_2 glycoprotein of Pichinde virus.

We also examined the ability of hyperimmune hamster serum to compete for the antigenic sites recognized by anti-Pichinde virus MAbs PC4.9A6 and PC4.9D3. The hamster polyclonal serum at dilutions of 1:10 and 1:100 (highest dilution tested) completely inhibited the binding of MAb PC4.9D3 , but only partially inhibited the binding of MAb PC4.9A6. Apparently the epitope recognized by MAb PC4.9D3 is strongly immunogenic in hamsters, whereas that recognized by MAb PC4.9A6 is less immunogenic.

The SDS-PAGE patterns obtained with the Pichinde virus-infected cytosols described in this report differed in some respects from some of those reported by Harnish et al. (1981). The cell lysis and protein solubilization procedures were patterned after those of Buchmeier and Oldstone (1979) and Smith and Pifat (1982). However, there were some notable differences. Smith and Pifat (1982) included 0.5% sodium deoxycholate, which is an anionic detergent, whereas Buchmeier and Oldstone (1979) did not. We did not include the anionic detergent, but instead used the nonionic detergent NP-40. This may

account for the low recovery of NP, which may have required the ionic detergent in order to be extracted from nucleoprotein complexes and virions. The solubilization buffer used by Buchmeier and Oldstone (1979) did not include EDTA, but contained both $CaCl_2$ and $MgCl_2$. These divalent cations would be chelated by the EDTA that was included in our solubilization buffer. The solubilization buffer used by Smith and Pifat included neither divalent cations nor EDTA. Finally, the pHs of the buffers differed; we used 7.4, Smith and Pifat used 7.5, whereas Buchmeier and Oldstone used 9.0. How the differences in solubilization buffers would account for differences in proportions of the different proteins recovered is not clear, but it seems likely that there would be solubilization buffer-dependent differences. We did not include anionic detergents such as SDS and sodium deoxycholate in the cytosol solubilization buffer because our aim was to determine which proteins were precipitated by the panel of MAbs and we had already determined that the MAbs would not recognize their corresponding epitopes following denaturation by SDS-PAGE buffer. The solubilization buffer used by Harnish et al. (1981) included both SDS and sodium deoxycholate, but their immunoprecipitations were performed with polyclonal sera, which probably would not be as sensitive to epitope conformational changes as would be MAbs.

The diversity and relative amounts of viral polypeptide obtained by RIP will vary depending on the labeling conditions as a result of temporal control of synthesis, incomplete translation, and degradation of polypeptides. Buchmeier and Oldstone (1979) used a 2-h pulse of ^{35}S-methionine at 44-h p.i. and found two major polypeptides, NP (63K) and GPC (75K), in the cytosols from LCMV-infected BHK-21 cells. Using the same labeling procedure, but including high-speed centrifugation to clarify the cytosol supernatant, Buchmeier et al. (1981) observed a substantial reduction in the proportion of NP in the labeled polypeptides and an increase in GP_1 and GP_2. The polypeptide distributions in the cytosols from Pichinde virus-infected BHK-21 cells, reported by

Harnish et al. (1981), were obtained with either 10-min or 1-h pulses of labeled amino acids. In contrast, we included ^{35}S-methionine for 24 h. Another factor leading to variation in RIP distribution patterns is traceable to differences in the hyperimmune sera used to precipitate total viral polypeptides. Harnish et al. (1981) used sera from either convalescent or hyperimmunized hamsters, whereas we used hyperimmune mouse serum from mice that had been immunized with suckling mouse brain homogenates. It is possible that the mouse and hamster sera had different distributions of antigen specificities. We now have a hyperimmune hamster serum pool that is of much higher titer than the mouse serum. In future immunoprecipitation studies, the pattern of polypeptides precipitated by the hamster serum will be compared with the pattern obtained using the mouse serum.

Arenaviruses appear to differentially regulate the expression of NP and the surface glycoproteins. This regulation probably plays a significant role in the ability of arenaviruses to establish persistent infections (Buchmeier and Parekh, 1987). Thus the relative concentrations of NP, GP_1, and GP_2 in infected cells is variable, depending on MOI, time after infection, and the host cell type. Additionally, several NP-related polypeptide fragments and the precursor glycoprotein GPC exhibit a highly variable intensity of expression in cytosols of infected cells (Buchmeier et al., 1977; Harnish et al., 1981). Thus, the apparent level of expression of the various Pichinde virus polypeptides, as reported from different laboratories or from different experiments in the same laboratory, will be difficult to replicate unless the conditions of infection, labeling, and cytosol preparation are carefully reproduced.

In this section we described the binding specificities of four anti-Pichinde virus MAbs as revealed by applying radioimmunoprecipitation techniques, using ^{35}S-labeled lysates from Pichinde virus-infected cells. All four precipitated a 38,000 Dalton polypeptide that was the major viral polypeptide in the cytosols. Since this protein was not precipitated by isotype matched irrelevant MAbs, nor from noninfected Vero cell lysates by

any of the anti-Pichinde virus MAbs, this 38K protein appears to be the Pichinde virus protein recognized by the four anti-Pichinde virus MAbs. That all four MAbs recognized the same protein was not too surprising since each was selected for its ability to bind to Pichinde virus antigens expressed on the surface of Vero cells infected with Pichinde virus. However, competitive binding assays demonstrated that MAbs PC4.9A6 and PC4.7C2 recognize the same epitope and that MAb PC4.9D3 recognizes a second nonoverlapping epitope. Therefore, it appears that the four anti-Pichinde virus MAbs react with at least two different epitopes displayed on the portion of the 38K Pichinde virus protein exposed at the surface of infected cells. At this time it would appear that the most probable candidate is GP_2, however, we are currently performing RIP studies utilizing [^{3}H]-glucosamine to determine if the precipitated polypeptide is GP_2 or perhaps one of the NP fragments. We obtained MAb WE33.6 from Buchmeier. MAb WE33.6 generated toward LCMV and specific for GP_2, also recognized the GP_2 of Pichinde virus (Parekh and Buchmeier, 1986). In RIP experiments utilizing ^{35}S-labeled lysates from Pichinde virus-infected cells, we were unable to detect any glycoproteins precipitated by MAb WE33.6. The polypeptides precipitated by our MAbs should be compared with those precipitated by MAb WE33.6 in RIP experiments utilizing [^{3}H]-glucosamine-labeled lysates.

E. Conclusions

1. MAbs PC4.7C2, PC4.8D2, PC4.9D3, and PC4.9A6 bind to Pichinde virus antigens expressed on the surface of cells infected with Pichinde virus. Based on binding characteristics these MAbs are suitable candidates as drug delivery vectors for the study of antibody-mediated targeted delivery of antiviral compounds.

2. MAbs PC4.7C2 and PC4.9A6 bind to the same epitope, while PC4.8D2 and PC4.9D3 bind to two other epitopes. Competitive binding assays with anti-LCMV MAb WE33.6, which cross reacts with GP_2 glycoprotein of Pichinde virus, indicated that MAbs PC4.9A6 and PC4.9D3 bind to epitopes on the GP_2 glycoprotein of Pichinde virus.

TASK V. ASCITES TUMOR FORMATION, USING HYBRIDOMAS DERIVED FROM THE FUSION OF FOX-NY MYELOMA CELLS WITH SPLEEN CELLS FROM RBF/DN MICE.

A. Introduction

Hybridoma cell lines derived from the fusion of FOX-NY myeloma cells with spleen cells from RBF/Dn mice are more stable in cell culture than are conventionally derived hybridoma cell lines (Taggart and Samloff, 1983). To take advantage of this increased genetic stability, we derived a series of (FOX-NY x RBF/Dn) hybridoma cell lines that secrete MAbs towards Pichinde virus antigens. Four of these MAbs recognized Pichinde virus antigens expressed on the surface of Pichinde virus-infected cells in vitro and in vivo. Because large quantities of these MAbs were required for in vivo antiviral studies, the corresponding hybridoma cell lines were injected into mice for ascites production. However, we found that commonly applied protocols for the production of ascites tumors when applied to these hybridoma cell lines resulted in erratic, inefficient ascites tumor production. Attempts to propagate these (FOX-NY x RBF/Dn) hybridoma cell lines by generally accepted protocols in recipient mice of the strain RBF/Dn, or BALB/c or the (RBF/Dn x BALB/c)F_1 hybrid of the two, resulted in limited ascites production. More aggressive immunosuppression of the recipient mice did increase the success rate for ascites tumor production. By increasing the pristane dose or by including total-body gamma irradiation in the immunosuppression protocols, ascites production was greatly enhanced. This report describes a series of experiments directed at defining conditions for efficient ascites fluid tumor production from FOX-NY x RBF/Dn hybridoma cell lines. Using the optimized ascites production procedure developed in this study, a large scale production of PC4.9A6 ascites fluids was achieved.

B. Materials and Methods

Hybridoma cells. The hybridoma cell lines PC4.7C2, PC4.8D2, PC4.9D3, and PC4.9A6 were derived through the fusion of FOX-NY myeloma cells with the spleen cells from RBF/Dn mice that had been hyperimmunized with Pichinde virus. The hybridoma cell lines had been cloned twice. Hybridoma cells were harvested from cell culture in the logarithmic growth phase, pelleted by centrifugation at 400 x *g* for 5 min, and then resuspended in RPMI-1640 medium. Cells were injected into mice within 1 h. The viability of the cells was determined by trypan-blue exclusion. At the time of inoculation at least 90% of the cells were viable in all of the cultures. The hybridoma cell lines used in these studies secreted anti-Pichinde virus MAb. All four MAbs are of the IgG_{2a} isotype.

Mice. Female BALB/c mice were obtained from Simonsen Laboratories (Gilroy, CA). RBF/Dn mice were obtained from Jackson Laboratories (Bar Harbor, ME). In the initial studies, the mice at the time of receiving hybridoma cells varied from 8 to 13 weeks of age; however, in later studies older mice were used because they produced larger volumes of ascites in the optimized protocol. F_1 hybrid mice were bred in the USU Laboratory Animal Research Center using RBF/Dn males and BALB/c females. After weaning, the resulting (RBF/Dn x BALB/c)F_1 mice were housed one sex to a cage. Both male and female mice were used for ascites production. The final, optimized ascites production protocol was evaluated using a large-scale production of PC4.9A6 ascites. In this study, the mice were 15-21 weeks old (18.6 ± 2.2) when inoculated with PC4.9A6 hybridoma cells.

Pristane priming, irradiation, and hybridoma cell inoculation. The general protocol used in evaluating production parameters was a single i.p. injection (26 ga. needle) of pristane (2,6,10,14-tetramethylpentadecane, Sigma Chemical Co. St Louis,

MO). Six to eight days later, mice received 5 x 10^6 - 1 x 10^7 hybridoma cells in 1.0 mL of RPMI-1640 cell-culture medium without FBS, injected (20 ga. needle) into the peritoneal cavity. The optimized protocol developed in the course of these studies and evaluated through the production of a large pool of PC4.9A6 ascites was as follows. Eight days prior to inoculation with the hybridoma cells, mice received 1.0 mL of pristane by i.p. injection. Seven days after the pristane injection, the mice received a single whole-body 350-rad dose of irradiation. The ^{137}Cs gamma-source delivered 171 rads/min, the mice were exposed for 2 min and 5 sec. The next day, PC4.9A6 cells, 0.5 mL at 1 x 10^7/mL were injected i.p. into 39 mice.

Collection of ascites fluids. The date of tumor appearance was noted for each mouse and ascites fluids were collected every 1-3 days after the tumors were readily apparent, usually within 4 days of the initial appearance of the tumor. The ascites fluids were collected more or less frequently depending on how rapidly the individual tumors grew. Ascites fluids were collected by inserting a 20-ga. needle into the peritoneal cavity and allowing the fluids to drain into a centrifuge tube. The fluids were clarified by centrifugation at 600 x *g* for 5 min and the clear ascites fluids were removed from the pelleted red blood and hybridoma cells and the small amount of residual pristane overlaying the fluids in the tube.

Characterization of ascites fluids. The volume of ascites fluids on a per mouse basis was recorded. Specific antibody titers were determined by limiting dilution in an IFA assay for antibody to Pichinde virus. Antibody samples diluted in PBS were added to Pichinde virus-infected acetone-fixed Vero cell cultures in 24-well plates. The antibodies on the infected cell cultures were incubated for 2 h and then removed. The cell sheets were rinsed twice with PBS. The presence of anti-Pichinde virus antibody was then detected by adding 100 µL of fluorescein-labeled goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) and incubating for 3 h. Cell sheets were rinsed twice with

water and examined for fluorescing cells, scoring on a -, +/-, 1+, 2+, 3+, 4+ scale for increasing staining intensity. The reciprocal of the greatest dilution that still produced unmistakable (1+) staining of infected cells was taken as the titer of the sample.

The effect of pristane on ascites tumor formation. To determine the effect of pristane dosage upon ascites tumor yields, mice (5-6 mice per group) received 0, 0.1, 0.5, 1.0, or 2.0 mL of pristane in a single intraperitoneal injection seven days prior to injection of 5×10^5 PC4.9A6 hybridoma cells.

FOX-NY x RBF/Dn hybridoma cell ascites formation in BALB/c mice primed with high pristane dosage. The (FOX-NY x RBF/Dn) hybridoma cell line PC4.9A6 produced ascites tumors in BALB/c mice if the mice were treated with relatively large doses of pristane. To determine if high pristane dosage would lead to similar results with other (FOX-NY x RBF/Dn) hybridoma cell lines, three cell lines PC4.9D3, PC4.7C2 and, PC4.8D2 were inoculated into mice. Female BALB/c mice (11-12 weeks old) were injected (i.p) with 2.0 mL of pristane. Seven days later each mouse received (i.p.) 5×10^6 hybridoma cells. The production of ascites tumors and resulting ascites fluids was monitored.

Effect of using (RBF/Dn x BALB/c)F_1 mice. Hybrid (RBF/Dn x BALB/c)F_1 mice (6-7 weeks old) were primed with 1.0 mL of pristane (i.p.) and 7 days later were inoculated with 5×10^6 to 1×10^7 PC4.7C2, PC4.8D2, PC4.9D3, or PC4.9A6 hybridoma cells. Mice were examined daily (through 88 days p.i.) for ascites tumor development. As an addendum to this experiment, nonproducing (RBF/Dn x BALB/c)F_1 mice in the PC4.7C2 group received an additional 0.5 mL of pristane 88 days after the original inoculation with PC4.7C2 cells and were irradiated four days later with a ^{137}Cs source, receiving a whole body radiation dose of 350 rad. The irradiation killed one out of the seven mice about 22 h after the radiation exposure. One day after radiation treatment, the mice received hybridoma cells isolated from the ascites fluids of the lone mouse that had

produced a PC4.7C2 ascites tumor. The cells had been cultured in vitro for nine days, the cell viability was 98%, and 3.1 x 10^6 cells were injected into each mouse.

Effect of total body irradiation on ascites production. Hybrid (RBF/DnJ x BALB/c)F_1 mice, 11-12 weeks old received 1.5 mL of pristane (i.p.) followed six days later with 350 rads of total body irradiation delivered by a ^{137}Cs gamma-source. Hybridoma cells were injected the following day. PC4.8D2 hybridoma cells, 8 x 10^6/mouse, were injected (i.p.) into 16 mice (12 irradiated and four not irradiated). PC4.7C2 cells, 7 x 10^6/mouse, were injected (i.p.) into 16 mice (10 irradiated and six not irradiated. All mice had been treated with pristane.

C. Results.

The effect of pristane on ascites tumor formation. When (FOX-NY x RBF/Dn) hybridoma cell line PC4.9A6 was injected (i.p.) into BALB/c mice, increased dosages of pristane resulted in increased efficiency of ascites tumor formation. Without pristane priming, none of the mice developed ascites tumors, and priming with either 0.1 or 0.5 mL of pristane led to a very low percentage of responders, with one out of five mice responding at either pristane dose (Table V-1). In contrast, all of the mice pretreated with either 1.0 or 2.0 mL of pristane produced ascites tumors. The response in the 2.0 mL group was very uniform with an average time to first ascites fluid collection of 8 ± 1 days. The yields of ascites fluids were similar from the groups primed with 1.0 mL of pristane (5.1 ± 1.9 mL/mouse) and 2.0 mL of pristane (6.3 ± 3.5 mL/mouse). The anti-Pichinde virus antibody titers of the ascites fluids from all five mice ranged from 10^5 to $10^{5.5}$ as measured by limiting dilution in an indirect IFA assay.

Table V 1. Effect of pristane dose on ascites production following inoculation of BALB/c mice with FOX-NY x RBF/Dn hybridoma cell line PC4.9A6.

Pristane dose (mL)	% Mice developing tumors (N/T)	Total mL of ascites fluids collected (vol/mouse)	Time from inoculation with hybridoma to first collection (d)		
			Earliest	Latest	Ave ± SD
2.0	100 (6/6)	37.6 (6.3 ± 3.5)	7	9	8.0 ± 1.1
1.0	100 (5/5)	25.6 (5.1 ± 1.9)	9	16	12.6±3.2
0.5	20 (1/5)	1.0	15	-	-
0.1	20 (1/5)	2.8	18	-	-
0.0	0	0	-	-	-

FOX-NY x RBF/Dn hybridoma cell ascites formation in BALB/c mice primed by high pristane dosage. In the previous experiments designed to determine the effect of pristane dosage, hybridoma cell line PC4.9A6, produced ascites tumors in BALB/c mice if the mice were treated with relatively large doses of pristane. To determine if high pristane dosage would lead to similar results with other (FOX-NY x RBF/Dn) hybridoma cell lines, PC4.9D3, PC4.7C2, and PC4.8D2 hybridoma cells were inoculated into mice. All of the mice that were inoculated with the PC4.9D3 cell line developed ascites tumors that were visible by seven days p.i. Fluids were drained from all mice eight days p.i., but the ascites fluids volumes were low (only 3.1 mL/mouse), and the tumors were very virulent, with all the mice dying within four days of developing visible tumors. Two mice that received the PC4.7C2 cell line developed ascites tumors 45 days after receiving the cells (data shown in Table V-2). Pools of both the PC4.7C2 and PC4.9D3 ascites had similar titers by indirect IFA, PC4.9D3 was $10^{-5.7}$ and PC4.7C2 was $10^{-5.5}$. None of the mice that had received cell line PC4.8D2 developed ascites tumors.

Table V-2. FOX-NY x RBF/Dn hybridoma cell ascites formation in BALB/c mice primed by high pristane dosage.

Hybridoma line	% Mice developing tumors (N/T)	Total mL of ascites collected (vol/mouse)	Time from inoculation to first collection (d)		Anti-Pichinde virus titer[a]
			Earliest	Latest	
PC4.9D3	100 (10/10)	31.2 (3.1 ± 2.1)	8	8	$10^{-5.7}$
PC4.7C2	20 (2/10)	8.4 (4.2 ± 0.6)	45	46	$10^{-5.5}$
PC4.8D2	0	0	-		

[a]The anti-Pichinde virus antibody titers of the ascites fluids was determined by limiting dilution in an indirect immunofluorescent antibody assay for antibody to Pichinde virus.

Anti-Pichinde virus ascites production. Effect of using (RBF/Dn x BALB/c)F_1 mice. Of the (RBF/Dn x BALB/c)F_1 mice that received the PC4.9A6 hybridoma cells four out of five developed ascites tumors. The fifth mouse quickly developed a large solid tumor on a hind leg and was killed 22 days after the hybridoma cells were injected without producing ascites fluids (Table V-3). Six of the seven mice that received PC4.9D3 hybridoma cells developed ascites tumors; the only mouse not to produce ascites fluids was killed 22 days p.i. with a large black tumor on the abdomen. One of the eight mice that received PC4.7C2 hybridoma cells developed ascites fluids, the ascites developed in this sole responder long after all of the mice in the PC4.9A6 and PC4.9D3 groups had died. The ascites fluids were collected 78 days after the PC4.7C2 hybridoma cells were injected into the mice. The ascites fluids were assayed for anti-Pichinde virus activity since this late developing ascites tumor could have been a spontaneous tumor rather than a tumor arising from the PC4.7C2 hybridoma cell line. The antibody titer directed against Pichinde virus was over 10^5 as measured by indirect IFA (Table V-4). That concluded the experiment with respect to the effect of using (RBF/Dn x

BALB/c)F_1 mice. However, in an attempt to get ascites from the seven surviving, nonproducing mice in the PC4.7C2 group, the mice were once again primed with pristane 88 days after the original inoculation with PC4.7C2 hybridoma cells. Four days later these mice were irradiated (350 rad), and PC4.7C2 hybridoma cells were injected the next day. One of the irradiated PC4.7C2 mice died 20-22 h after irradiation, apparently as a result of the irradiation. Of the remaining six mice four produced ascites tumors which were tapped 16 days after the cells had been injected into the irradiated mice. The first collection of ascites fluids from these irradiated mice produced 7.8 ± 1.8 mL/mouse.

Table V-3. Development of ascites tumors in (RBF/Dn x BALB/c)F_1 mice.

Hybridoma cell line	% Mice developing tumors (N/T)	Total mL of ascites fluids/mouse	Time from inoculation with hybridoma to first collection (d)		
			Earliest	Latest	Ave ± SD
PC4.9A6	80 (4/5)	9.4 ± 5.2	26	32	29.0 ± 3.5
PC4.9D3	86 (6/7)	5.8 ± 3.0	26	36	33.5 ± 4.2
PC4.7C2	13 (1/8)	24.5	78	-	78
PC4.8D2	0 (0/8)	-	-	-	-

Table V-4. Titration of PC4.7C2 ascites fluids.

Sample	Dilution	IFA Result
PC4.7C2 (AF) (2/3/88) GBG	10^{-1}	4 no nonspecific Rx
PC4.7C2 (AF) (2/3/88) GBG	10^{-2}	4
PC4.7C2 (AF) (2/3/88) GBG	10^{-3}	4
PC4.7C2 (AF) (2/3/88) GBG	10^{-4}	4
PC4.7C2 (AF) (2/3/88) GBG	10^{-5}	2-3
PC4.7C2 (AF) (2/3/88) GBG	10^{-6}	0-1

Effect of total body irradiation on ascites production. All ten of the irradiated mice that received cell line PC4.7C2 developed ascites tumors that appeared 18 to 32 days (average 22 ± 5 d) after the cells were injected. Four of these ten irradiated mice also developed solid tumors, and in one case the solid tumor grew so rapidly that the mouse had to be killed prior to developing a sufficient volume of ascites fluids for harvest. From the nine ascites fluid-producing mice, the yields were 7.3 ± 4.9 mL/mouse. Some of the nonirradiated mice that received cell line PC4.7C2 also developed ascites fluid-producing tumors, but at a later time. Three of the six nonirradiated mice developed ascites tumors between 42 and 61 days postinoculation (average 50 ± 10 d p.i.). The three mice in this group that did not develop ascites fluid-producing tumors died between 13 and 20 days postinoculation after developing large solid tumors in the abdomen. Twelve of the thirteen irradiated mice that received cell line PC4.8D2 developed ascites fluid-producing tumors. Two previous experiments (Tables V-2 and V-3) failed to produce any PC4.8D2 ascites fluids. Only one of the four nonirradiated mice developed PC4.8D2 ascites tumors, and this was at 66 days after the cells were injected into the mice. The other three mice in the PC4.8D2 group did not develop any tumors during the 100 days that they were monitored. These data are summarized in Table V-5.

Production of a large pool of anti-Pichinde virus MAb PC4.9A6. The ascites production procedure described in this section was used to produce a large pool of ascites fluids arising from hybridoma cell line PC4.9A6. Of the 39 mice receiving these hybridoma cells, 37 developed ascites tumors. There was no indication of significant age- or sex-related biases in ascites production with the F_1 mice. The overall yield of ascites fluids was 349 mL for an average of just over 9 mL per mouse. A summary of the ascites production yields and recoveries is shown in Table V-6. MAb was purified from a 50-mL aliquot of this ascites pool. The final product was 21 mL of clear, colorless fluid containing IgG at a concentration of 8.4 mg/mL, for an overall yield of approximately 40%

Table V-5. Effect of irradiation on ascites production using RBF/Dn x FOX-NY hybridoma cell lines in (RBF/DnJ x BALB/c)F_1 mice.

Treatment[a] Prior to inoculation	Hybridoma cell line	Mice developing tumors/total (%)	Vol. of ascites fluids collected (mL/mouse)[b]	Time to ascites development (days)[c]		
				Earliest	Latest	Ave ± SD
Pristane + radiation	PC4.8D2	12/13[d] (92)	6.5 ± 3.4	27	67	40 ± 13
Pristane only	PC4.8D2	1/4[e] (25)	7	66		
Pristane + radiation	PC4.7C2	9/10[f] (90)	7.3 ± 4.9	18	32	22 ± 5
Pristane only	PC4.7C2	3/6[g] (50)	5.2 ± 2.5	42	61	50 ± 10

[a]All mice received 1.5 mL of pristane (i.p.), some received 350 rad of gamma radiation six days later. All then received 7 x 10^6 hybridoma cells (i.p) seven days after the injection of pristane.

[b]Mice that did not develop fluid-containing tumors were not included in this calculation.

[c]Time to ascites development was the time elapsed after inoculation with the hybridoma cells until the first ascites fluids were collected. The Earliest, Latest, and Average ± SD Times refer to the times of the initial ascites fluids collections from each mouse in the group.

[d]The lone mouse in this group not to develop ascites tumors survived over 100 days without developing tumors of any type.

[e]The three mice in this group not to develop ascites tumors survived over 100 days without developing tumors of any type.

[f]The lone mouse in this group not to develop ascites tumors died at 18 days postinoculation with a large solid tumor in the abdomen; no ascites fluids were present in the peritoneal cavity.

[g]The three mice in this group not to develop fluid-yielding ascites tumors died between 13 and 20 days postinoculation. All had developed large solid tumors in the abdomen; no ascites fluids were present in the peritoneal cavity.

Table V-6. Summary of large scale ascites fluid production in (RBF/DnJ x BALB.C)F_1 mice that were immunosuppressed by a combination of pristane injection and gamma irradiation followed by inoculation with hybridoma cell line PC4.9A6.

% Mice developing tumors (N/T)	Total mL of ascites fluids collected (vol/mouse)	Time from inoculation with hybridoma to first collection (d)		
		Earliest	Latest	Ave ± SD
95 (37/39)	349 (9.4 ± 4.4 mL/mouse)	8	12	9.8 ± 1.5

through the entire purification procedure. The antigen binding titers determined by indirect immunofluorescent antibody assay were $10^{5.5}$ for the crude ascites PC4.9A6, and $10^{5.5}$ for the purified pool MAb PC4.9A6.

D. Discussion

The FOX-NY cell line is a murine myeloma cell line that does not produce immunoglobulin heavy or light chains and thus is desirable as a fusion partner for hybridoma production. The FOX-NY cell line is deficient in the enzyme adenosine phosphoribosyltransferase (APRT), which forms the basis for the selection procedure described in the original reference (Taggart and Samloff, 1983) to the use of FOX-NY cells for hybridoma production. RBF/DnJ mice are characterized by a mutation resulting in a Robertsonian 8.12 translocation, which places portions of chromosomes 8 and 12 on a single 8.12 translocation chromosome. The gene coding for APRT is on chromosome 8 and the heavy chain immunoglobulin locus is on chromosome 12. Thus, in the RBF/DnJ mouse the enzyme marker sensitive to the selection procedure is genetically linked to the ability to produce immunoglobulin. Hybridoma cell lines derived from the fusion of FOX-NY myeloma cells to spleen cells from RBF/DnJ mice are stable antibody-producing hybridoma cell lines. However, in spite of the advantages of genetic stability, unless these

hybridoma cell lines can be readily propagated as ascites tumors their utility is seriously limited. When we attempted to produce ascites fluids from several RBF/DnJ x FOX-NY hybridoma cell lines, ascites production procedures that were suitable for conventional BALB/c-based hybridoma cell lines failed. In this section a series of experiments directed at efficient ascites production have been described.

The effect of pristane on ascites tumor formation. Mice primed by i.p. injection with pristane demonstrate an increased susceptibility to the development of spontaneous plasmacytomas. This phenomenon, apparently based upon suppression of normal immune surveillance, has been exploited for the propagation of hybridoma cells as ascites tumors. Freund and Blair (1982) demonstrated that pristane markedly depressed natural killer cell activity in BALB/c mice. It has been suggested that the BALB/c strain of mouse is particularly susceptible to plasmacytoma formation (Potter and Wax, 1981), and, because most murine hybridomas are derived from mice and cells of the BALB/c strain, the in vivo propagation of MAbs has usually been straight forward.

When BALB/c-derived hybridomas were seeded into BALB/c mice (Hoogenraad and Wraight, 1986), 0.1 mL of pristane was shown to be as effective as 0.5 mL of pristane. In contrast, we found that either 0.1 mL or 0.5 mL of pristane in the case of ascites tumor formation in BALB/c mice using hybridomas derived from RBF/Dn mice (FOX-NY x RBF/Dn hybridoma cell lines) yielded a very low percentage of responders. Employing a single FOX-NY x RBF/Dn hybridoma cell line, PC4.9A6, we found that an increased dosage of pristane resulted in an increased efficiency of ascites tumor formation in BALB/c mice. When 0.5 mL of pristane was used to prime BALB/c mice for ascites tumor formation with the PC4.9A6 hybridoma cell line, only one out of five mice responded, and the time between inoculation with the hybridoma cells and the formation of the tumor was long in comparison to mice receiving higher pristane dosages. All of the mice pretreated with either 1.0 or 2.0 mL of pristane produced ascites tumors. The

response in the 2.0-mL group was very uniform with an average time to first ascites fluid collection of 8 ± 1 days. The yield of ascites fluids was similar in the 1.0-mL (5.1 ± 1.9/mouse) and 2.0-mL (6.3 ± 3.5/mouse) groups of mice--both being much higher than the volumes obtained from the sporadic responders in the lower pristane volume groups.

FOX-NY x RBF/Dn hybridoma cell ascites formation in BALB/c mice primed by high pristane dosage. The results of pristane dose experiments using the PC4.9A6 hybridoma cell line in BALB/c mice indicated that this FOX-NY x RBF/Dn hybridoma cell could be grown as an ascites tumor in BALB/c mice if the mice were primed with 1-2 mL of pristane. However, we found that even with a high pristane dosage, ascites formation in this system was very dependent on the cell line. Of the three additional FOX-NY x RBF/Dn hybridoma cell lines examined only PC4.9D3 produced efficient ascites formation in the BALB/c mice, whereas 20% of the mice receiving the PC4.7C2 cell line developed ascites and none of the mice inoculated with PC4.8D2 developed ascites tumors. The ascites fluids produced in this experiment were of high titer, however the volumes were low. The mice were 12 weeks old when injected with pristane. Perhaps, younger mice would have been more suitable for ascites production. However, subsequent experiments with (RBF/Dn x BALB/c)F_1 mice demonstrated that mice of this age would produce ascites, and, if anything, the older mice produced larger volumes of ascites than did younger mice. The conclusion of this set of experiments was that even with large pristane doses, BALB/c mice do not develop FOX-NY x RBF/Dn hybridoma cell ascites tumors efficiently with some FOX-NY x RBF/Dn hybridoma cell lines.

Effect of using (RBF/Dn x BALB/c)F_1 mice. Although initial experiments utilizing increased dosages of pristane resulted in satisfactory production of MAb PC4.9A6 and PC4.9D3 and a small amount of MAb PC4.7C2, there was no success with MAb PC4.8D2. These four hybridoma cell lines carry cell surface antigens from both the RBF/Dn and BALB/c strains of mice. This may have accounted for the failure of some of

these hybridoma lines to grow as ascites tumors in either RBF/Dn or BALB/c strains of mice. In an experiment not described in this report, hybridoma cells of the line PC4.9A6 failed to grow as ascites in RBF/Dn mice. We next used (RBF/Dn x BALB/c)F1 mice as recipients. We determined that the use of (RBF/Dn x BALB/c)F_1 mice in place of BALB/c mice resulted in efficient ascites production with cell lines PC4.9A6 and PC4.9D3, the same as was observed with BALB/c mice. Although the time between when the PC4.9A6 and PC4.9D3 cells were put into the mice and the time of ascites appearance was longer (29-34 days as opposed to 8-13 days (see Tables V-1, V-3, and V-4), the volumes of ascites fluids were greater from the (RBF/Dn x BALB/c)F_1 mice (7.6 mL compared with 4.8 mL from the BALB/c mice). Ascites tumor formation with the PC4.7C2 cell line in both the (RBF/Dn x BALB/c)F_1 mice and BALB/c mice was delayed and sporadic. In BALB/c mice there were two out of ten mice that developed ascites tumors 45 and 46 days after inoculation with PC4.7C2 cells. In (RBF/Dn x BALB/c)F_1 mice one out of eight mice developed an ascites tumor, and, in this case, the tumor appeared 78 days after inoculation with PC4.7C2 cells. Neither the BALB/c nor the (RBF/Dn x BALB/c)F_1 mice developed ascites tumors following inoculation with PC4.8D2 cells.

That concluded the experiment with respect to the effect of (RBF/Dn x BALB/c)F_1 mice. However, in an attempt to get ascites from the surviving, nonproducing mice in the PC4.7C2 group, the mice were aggressively immunosuppressed with pristane and gamma irradiation. These mice were then inoculated with the corresponding hybridoma cell line that had been taken from the (RBF/Dn x BALB/c)F_1 mouse that had produced ascites fluids. This attempt was spawned by the observation that mice in an ongoing experiment were producing ascites, and the only difference was that those mice had been irradiated. Of the six mice in this group, four produced ascites tumors, which were drained 16 days after the cells had been injected into the irradiated mice. The first collection of ascites fluids from these irradiated mice produced 7.8 ± 1.8 mL/mouse. It appears that these mice will

produce 15-20 mL of ascites fluids per mouse. The results from this uncontrolled experiment indicate (1) that even relatively old (21-23 weeks old) mice can be induced to form ascites tumors, (2) that previous inoculation with a hybridoma cell line did not preclude the growth of that cell line as an ascites, as might be expected if the mice mounted an immune response to the surface antigens of the hybridoma cell line, and (3) that aggressive immunosuppression appears to be a key in growing the RBF/Dn x BALB/c hybridoma cell lines as ascites.

Effect of total body irradiation on ascites production. Previous experiments utilizing either (RBF/Dn x BALB/c)F_1 mice or BALB/c mice with increased dosages of pristane resulted in production of moderate amounts of MAbs PC4.9A6 and PC4.9D3 and a small amount of MAb PC4.7C2. No ascites was produced from the PC4.8D2 hybridoma cell line. That these four hybridoma cell lines carry different arrays of surface antigens from both the RBF/Dn and BALB/c strains of mice may account for the failure of some of these hybridoma lines to grow as ascites tumors in either RBF/Dn or BALB/c strains of mice. However, the failure of two out of four of these cell lines to grow as ascites in hybrid (RBF/Dn x BALB/c)F_1 mice that should be tolerant to surface antigens from the RBF/Dn and BALB/c strains was somewhat surprising. Since the efficiency of ascites production with cell line PC4.9A6 in BALB/c mice was directly related to pristane dosage, we thought that an even more aggressive immunosuppression might facilitate ascites production from the hitherto recalcitrant PC4.7C2 and PC4.8D2 hybridoma cell lines. The next step was to add total body irradiation to the immunosuppression protocol. These experiments, utilizing a combination of pristane priming and gamma irradiation, indicate that aggressively immunosuppressed (RBF/Dn x BALB/c)F_1 mice do develop ascites tumors at a high frequency even with the very refractory PC4.7C2 and PC4.8D2 hybridoma cell lines. Ten out of ten mice that were treated with pristane and then irradiated (350 rad) developed ascites tumors after receiving PC4.7C2 hybridoma cells. The ascites

tumor development with cell line PC4.8D2 has been slower than with PC4.7C2, but five out of 12 pristane-primed, irradiated mice developed ascites tumors after receiving PC4.8D2 hybridoma cells. With neither cell line have any of the nonirradiated mice developed ascites tumors. In the case of PC4.7C2 hybridoma cells ascites tumors have typically not appeared until 40 or more days post inoculation. The combination of pristane priming and gamma irradiation produced the best ascites yields to date. Pristane-primed, irradiated (RBF/DnJ x BALB/c)F_1 mice produced ascites from both the PC4.7C2 and PC4.8D2 hybridoma cells lines. This was the first time that the PC4.8D2 hybridoma cell line produced ascites.

E. Conclusions

1. By increasing the pristane dosage to 1.0-2.0 mL/mouse the efficiency of ascites tumor formation was dramatically increased. However, this procedure was not universally satisfactory for all RBF/DnJ x FOX-NY hybridoma cell lines. In these studies, two out of four cell lines failed to produce ascites even in recipient mice that had received 2.0 mL of pristane.

2. Using hybrid (RBF/Dn x BALB/c)F_1 mice as RBF/DnJ x FOX-NY hybridoma cell line recipients, even with increased pristane dosage levels, did not dramatically increase the ascites tumor yields over those obtained using BALB/c mice.

3. An aggressive immunosuppression regimen of pristane priming followed by total body irradiation (gamma, 350 rad) in hybrid (RBF/Dn x BALB/c)F_1 mice produced recipient mice suitable for the propagation of RBF/DnJ x FOX-NY hybridoma cell lines as ascites tumors.

TASK VI. IN VIVO TARGETING WITH ANTI-PICHINDE VIRUS MONOCLONAL ANTIBODIES.

A. Introduction

This report describes the biodistribution of anti-Pichinde virus MABS in infected and noninfected MHA hamsters. Fluorescein conjugates of MABs PC4.9A6 and PC4.9D3, which bind to antigens expressed on the surface of Pichinde virus-infected cells, were injected into hamsters, and the distribution of the labeled antibodies was examined by fluorescence microscopy of thin-sections prepared from spleen, liver, lung, heart, kidney, and brain of the hamsters.

B. Methods

Animals. Female MHA hamsters (Charles River Breeding Laboratories, Inc. Wilmington MA), 30 days old, were divided into infected and control groups. Those to be infected were inoculated subcutaneously (s.c.) in the groin with 10^4 LD_{50} units of the An4763 strain of Pichinde virus.

Monoclonal antibody conjugates. The MABS were conjugated to fluorescein isothiocyanate (FITC) by the procedure of Hebert et al. (1972) as described under Task III of this report. The FITC-labeled MABs had titers of approximately 1:6,000 by direct immunofluorescence assay on Pichinde virus infected Vero cells.

Treatment. Eight days after virus challenge, 0.5-mL doses of FITC-labeled MAbs were injected i.p. Three dosages were examined to avoid problems associated with too much or too little FITC-labeled MAb. The dosages were 250, 25, and 2.5 µg/animal with the MAb PC4.9A6 and 150, 15, and 1.5 µg/animal with the MAb PC4.9D3. The animals were killed by cervical dislocation 4 h after the antibody was administered. Tissues were immediately removed and frozen at -80°C in preparation for cryosectioning.

Virus levels in organs. The levels of Pichinde virus in 10% homogenates of the different organs were measured by an in vitro virus assay on Vero cell monolayers using the immunofluorescent cell count procedure as described under Task II of this report.

Examination. Spleen, liver, lung, heart, kidney, and brain sections were mounted on slides. Sections were not fixed. The tissue sections were examined by epifluorescence microscopy. Infected tissues were always examined in comparison to those from noninfected animals. Because the livers from the animals that received labeled MAb PC4.9A6 at either 250 µg or 2.5 µg were the most intensely fluorescing of the sections, these were taken as a relative fluorescent intensity of 100%, and all other sections were scored by comparison against these sections.

C. Results

At the time of antibody injection the virus-inoculated hamsters showed obvious signs of infection, including bloody secretions from the eyes, and the anal and genital openings. One infected hamster died on the day of treatment prior to injection with FITC-MAb conjugate. Because we had started this experiment with an extra infected hamster, there were enough animals to include three in each group. Care was taken to compare all sections to livers from the animals that received FITC-MAb PC4.9A6 at either 250 µg or 2.5 µg, which were taken as the base-line readings and defined as 100%. The fluorescence in these sections was very striking compared with that in noninfected controls.

The readings for the middle concentration with either conjugate did not correlate with those observed with the highest or lowest concentrations. It was as if there were hardly any conjugate put into these animals. For that reason, only the data from the animals receiving the highest and lowest levels of labeled MAbs were used to produce the distribution graphs. The sections were observed and scored without the reader knowing which treatment groups were being read. The data are presented in Tables VI-1 to VI-6.

Table VI-1. Distribution of fluorescein-labeled anti-Pichinde virus MAbs in the livers of infected and noninfected hamsters.

Challenge	Treatment	Observations
PCV[a]-infected	PC4.9A6 at 250 µg	Many FCs[b] throughout liver; very intense fluorescence in epithelial layer. Intensity = 4+ or 100%.
PCV-infected	PC4.9A6 at 25 µg	Few FCs; most in epithelial layer. Intensity = 25%.
PCV-infected	PC4.9A6 at 2.5 µg	Many FCs throughout liver; very intense fluorescence in epithelial layer. Intensity = 4+ or 100%.
Noninfected	PC4.9A6 at 250 µg	No obvious FCs; only fluorescence is at margin in epithelial layer. Intensity = 7%.
Noninfected	PC4.9A6 at 25 µg	No FCs; no fluorescence at margin. Intensity = 0%.
Noninfected	PC4.9A6 at 2.5 µg	No FCs; slight fluorescence at margin in epithelial layer. Intensity = 3%.
PCV-infected	PC4.9D3 at 150 µg	Many FCs; intensity = 4+ around walls of vessels and 2+ in cells throughout liver. Intensity = 80%.
PCV-infected	PC4.9D3 at 15 µg	No FCs; no fluorescence at margin in epithelial layer. Intensity = 0%.
PCV-infected	PC4.9D3 at 1.5 µg	FCs throughout the liver; but less intense than with PC4.9A6. Intensity = 40%.
Noninfected	PC4.9D3 at 150 µg	No FCs; but slight fluorescence at margin in epithelial layer. Intensity = 6%.
Noninfected	PC4.9D3 at 15 µg	No FCs; no fluorescence at margin. Intensity = 0%.
Noninfected	PC4.9D3 at 1.5 µg	No FCs; no fluorescence at margin. Intensity = 0%.

[a]PCV = Pichinde virus.

[b]FCs = fluorescent cells.

Table VI-2. Distribution of fluorescein-labeled anti-Pichinde virus MAbs in the hearts of infected and noninfected hamsters.

Challenge	Treatment	Observations
PCV[a]-infected	PC4.9A6 at 250 µg	FCs[b] in rare areas of layer covering the heart and a few in the linings of ventricles. Intensity ≈ 15%.
PCV-infected	PC4.9A6 at 25 µg	No FCs or fluorescence.
PCV-infected	PC4.9A6 at 2.5 µg	A few FCs, no fluorescence. Intensity ≈ 2%.
Noninfected	PC4.9A6 at 250 µg	No FCs, slight diffuse fluorescence at margins. Intensity ≈ 2%.
Noninfected	PC4.9A6 at 25 µg	No FCs or fluorescence.
Noninfected	PC4.9A6 at 2.5 µg	No FCs or fluorescence.
PCV-infected	PC4.9D3 at 150 µg	Very few FCs, most of the fluorescence in cells covering heart. Intensity ≈ 10%.
PCV-infected	PC4.9D3 at 15 µg	No FCs or fluorescence.
PCV-infected	PC4.9D3 at 1.5 µg	No FCs or fluorescence.
Noninfected	PC4.9D3 at 150 µg	No FCs or fluorescence.
Noninfected	PC4.9D3 at 15 µg	No FCs or fluorescence.
Noninfected	PC4.9D3 at 1.5 µg	No FCs or fluorescence.

[a]PCV = Pichinde virus.

[b]FCs = fluorescent cells.

Table VI-3. Distribution of fluorescein-labeled anti-Pichinde virus monoclonal antibodies in the lungs of infected and noninfected hamsters.

Challenge	Treatment	Observations
PCV[a]-infected	PC4.9A6 at 250 µg	FCs[b] among epithelial cells covering the lung; quite patchy; some areas with no FCs others with many FCs. Solitary FCs in interior tissues of lungs. Intensity ≈ 30%.
PCV-infected	PC4.9A6 at 25 µg	No FCs or fluorescence.
PCV-infected	PC4.9A6 at 2.5 µg	Many FCs throughout interior of lungs. Intensity ≈ 20%.
Noninfected	PC4.9A6 at 25 µg	No FCs; very slight diffuse fluorescence (not FCs) in isolated areas along margins. Intensity ≈ 3%.
Noninfected	PC4.9A6 at 25 µg	No FCs or fluorescence.
Noninfected	PC4.9A6 at 2.5 µg	No FCs or fluorescence.
PCV-infected	PC4.9D3 at 150 µg	Many FCs throughout the interior of lung. Some patches of FCs in epithelial layer. Intensity ≈ 30%.
PCV-infected	PC4.9D3 at 15 µg	A few FCs in interior tissues. Intensity ≈ 4%.
PCV-infected	PC4.9D3 at 1.5 µg	No FCs or fluorescence.
Noninfected	PC4.9D3 at 150 µg	No FCs or fluorescence.
Noninfected	PC4.9D3 at 15 µg	No FCs or fluorescence.
Noninfected	PC4.9D3 at 1.5 µg	No FCs or fluorescence.

[a]PCV = Pichinde virus.

[b]FCs = fluorescent cells.

Table VI 4. Distribution of fluorescein-labeled anti-Pichinde virus monoclonal antibodies in the brains of infected and noninfected hamsters.

Challenge	Treatment	Observations
PCV[a]-infected	PC4.9A6 at 250 µg	FCs[b] and diffuse fluorescence in epithelial cells covering the brain and some on the linings of blood vessels; no FCs in the interior of the brain. Intensity ≈ 5%.
PCV-infected	PC4.9A6 at 25 µg	No FCs or fluorescence.
PCV-infected	PC4.9A6 at 2.5 µg	FCs in rare area of epithelial covering of brain; no FCs in interior tissues. Intensity ≈ 2%.
Noninfected	PC4.9A6 at 250 µg	No FCs or fluorescence.
Noninfected	PC4.9A6 at 25 µg	No FCs or fluorescence.
Noninfected	PC4.9A6 at 2.5 µg	No FCs or fluorescence.
PCV-infected	PC4.9D3 at 150 µg	A few FCs in epithelium covering brain. Intensity ≈ 5%.
PCV-infected	PC4.9D3 at 15 µg	No FCs or fluorescence.
PCV-infected	PC4.9D3 at 1.5 µg	A few FCs in epithelium covering brain.
Noninfected	PC4.9D3 at 150 µg	No FCs or fluorescence.
Noninfected	PC4.9D3 at 15 µg	No FCs or fluorescence.
Noninfected	PC4.9D3 at 1.5 µg	No FCs or fluorescence.

[a]PCV = Pichinde virus.

[b]FCs = fluorescent cells.

Table VI-5. Distribution of fluorescein-labeled anti-Pichinde virus monoclonal antibodies in the spleens of infected and noninfected hamsters.

Challenge	Treatment	Observations
PCV-infected	PC4.9A6 at 250 µg	Intense fluorescence in epithelium covering the spleen. Intensity ≈ 75% in that layer. Faint FCs[a] in interior tissue of spleen. Overall intensity ≈ 40%.
PCV-infected	PC4.9A6 at 25 µg	No FCs or fluorescence.
PCV-infected	PC4.9A6 at 2.5 µg	Many FCs; easiest to see in the epithelial cells covering the spleen, but many faint FCs in interior tissue of spleen. Intensity ≈ 5%inside and 40% in epithelial layer. Overall intensity ≈ 25%.
Noninfected	PC4.9A6 at 250 µg	Diffuse, relatively strong fluorescence over the exterior covering of spleen. Some intensely fluorescent debris-like clusters. Intensity ≈ 30%.
Noninfected	PC4.9A6 at 25 µg	About 10 solitary FCs, but not at margins. No bright infected areas. Intensity ≈ 5%.
Noninfected	PC4.9A6 at 2.5 µg	Some diffuse, weak fluorescence over the exterior covering of spleen. Intensity ≈ 3%.
PCV-infected	PC4.9D3 at 150 µg	Clearly defined FCs all along the epithelial covering of spleen. None in interior. Intensity ≈ 20%.
PCV-infected	PC4.9D3 at 15 µg	No FCs or fluorescence
PCV-infected	PC4.9D3 at 1.5 µg	A few areas of epithelial covering of spleen with bright FCs. Intensity ≈ 10%.
Noninfected	PC4.9D3 at 150 µg	Some isolated FCs and fluorescence at margins. Intensity ≈ 6%.
Noninfected	PC4.9D3 at 15 µg	No FCs or fluorescence.
Noninfected	PC4.9D3 at 1.5 µg	No FCs, but many fluorescent blobs near spleen sections. No fluorescence in spleen.

[a]PCV = Pichinde virus.

[b]FCs = fluorescent cells.

Table VI-6. Distribution of fluorescein-labeled anti-Pichinde virus monoclonal antibodies in the kidneys of infected and noninfected hamsters.

Challenge	Treatment	Observations
PCV[a]-infected	PC4.9A6 at 250 µg	Intense FCs[b] in epithelial covering of the kidney; some weak diffuse fluorescence in interior. Intensity ≈ 50%.
PCV-infected	PC4.9A6 at 25 µg	No FC or fluorescence.
PCV-infected	PC4.9A6 at 2.5 µg	Several areas of FCs in interior tissue of kidney; some FCs in epithelial covering of the kidney; but more inside. Intensity ≈ 40%.
Noninfected	PC4.9A6 at 250 µg	Diffuse, relatively strong fluorescence over epithelium of kidney, but no FCs. Intensity ≈ 20%.
Noninfected	PC4.9A6 at 25 µg	No FCs or fluorescence.
Noninfected	PC4.9A6 at 2.5 µg	No FCs or fluorescence.
PCV-infected	PC4.9D3 at 150 µg	Some FCs in interior tissue of kidney and in epithelial covering. Intensity ≈ 25%.
PCV-infected	PC4.9D3 at 15 µg	No FCs or fluorescence.
PCV-infected	PC4.9D3 at 1.5 µg	Strong FCs in interior of kidney; epithelium may have been lost in sectioning. Intensity ≈ 20%.
Noninfected	PC4.9D3 at 150 µg	Weak fluorescence at epithelium; no FCs. Intensity ≈ 2%.
Noninfected	PC4.9D3 at 15 µg	No FCs or fluorescence.
Noninfected	PC4.9D3 at 1.5 µg	Essentially no fluorescence, but a few isolated FCs. Intensity ≈ 2%.

[a]PCV = Pichinde virus.

[b]FCs = fluorescent cells.

The relative fluorescence intensities for sections from the different organs are shown in Figures VI-1 to VI-4. The levels of Pichinde virus in the different organs from infected hamsters was measured by an in vitro virus assay using immunofluorescing-cell counts. The results are shown in Table VI-7.

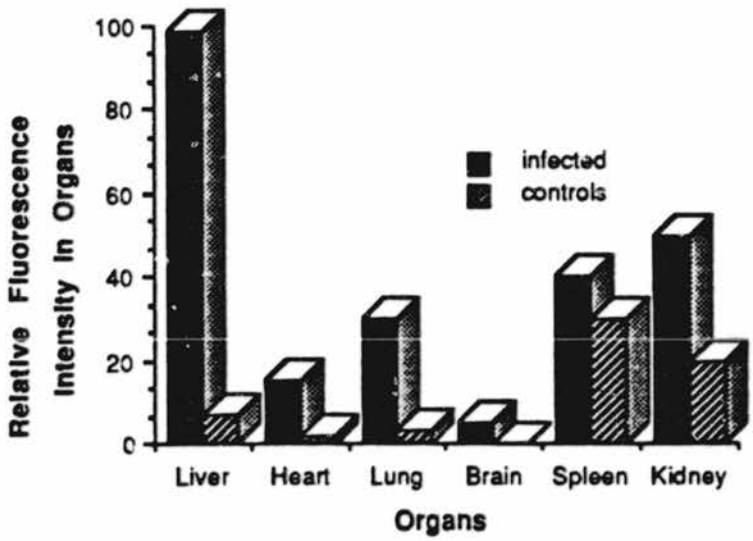


Figure VI-1. In vivo targeting with MAb PC4.9A6 at 250 µg per animal.

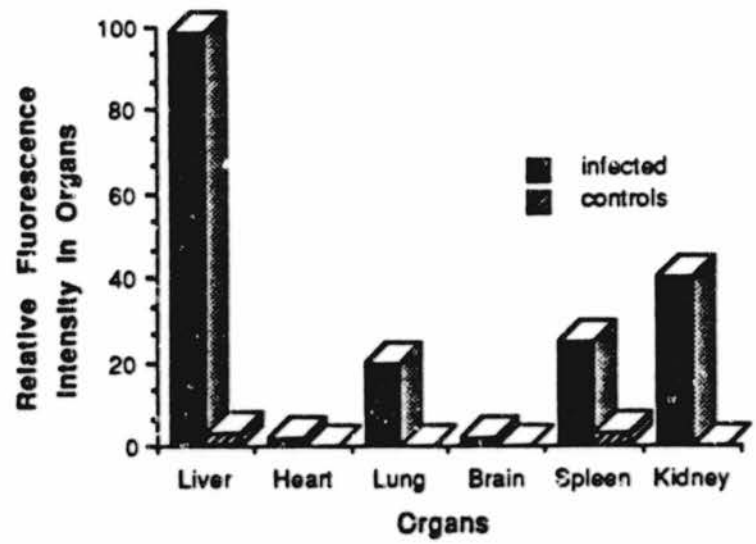


Figure VI-2. In vivo targeting with MAb PC4.9A6 at 2.5 µg per animal.

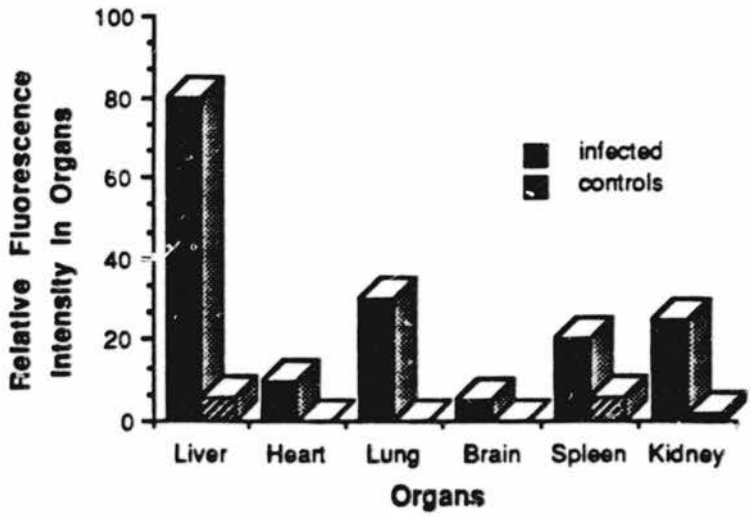


Figure VI-3. In vivo targeting with MAb PC4.9D3 at 150 µg per animal.

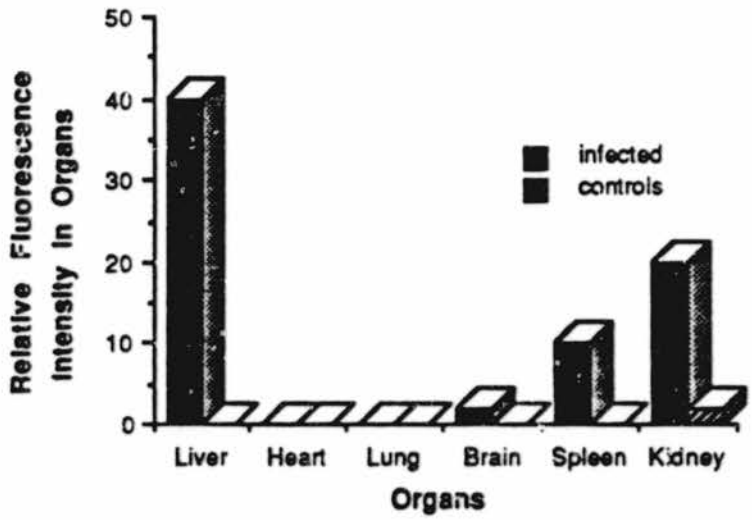


Figure VI-4. In vivo targeting with MAb PC4.9D3 at 1.5 µg per animal.

Table VI-7. Pichinde virus distribution in infected hamsters at 8 days postinoculation.

Organ	Virus Titer (IFCFU/g[a])
liver	3.1×10^8
brain	4.4×10^5
spleen	7×10^4
kidney	3×10^4
lung	$\leq 1 \times 10^4$
heart	$\leq 1 \times 10^4$

[a]The levels of Pichinde virus in 10% organ suspensions were measured by an in vitro assay on Vero cells. The data are reported as immunofluorescent cell forming units (IFCFU) per gram of organ tissue.

D. Discussion

There was a marked distribution of the labeled MAbs to the liver, kidney, spleen and lung tissues of the infected animals as compared to the noninfected animals. The difference was particularly dramatic for the livers. In infected and noninfected animals some of the fluorescence was diffuse and mainly in the epithelium covering the organs. The fact that this epithelial distribution was quite pronounced on brains from infected animals was interesting because there was a significant virus titer in the brain and because the MAbs would not be expected to cross the blood-brain barrier. The animals used in this study were killed by cervical dislocation. If a similar study were done again, it would be preferable to exsanguinate the animals and carefully rinse the organs before freezing them. These precautions would reduce the amount of blood in the tissues and might reduce the the background of labeled antibody. Some solitary isolated fluorescing cells were seen in the lungs and spleens in both infected and noninfected animals. These may have been macrophages that accumulated antibody as a result of being infected by Pichinde virus or by interacting with the Fc portions of the IgG_{2a} MAbs.

Those instances where the antibodies were found in relatively high concentrations in organs of noninfected animals were limited to animals that received the highest dosages of antibody. In those animals receiving 250 µg of MAb PC4.9A6, the ratio of fluorescence intensity in the spleens and kidneys of the infected animals compared with the organs of noninfected animals was approximately 2:1, whereas the ratio in the livers was greater than 10:1. At the lower antibody dosages, the fluorescence intensity was always much greater (greater than 10:1) in organs from infected animals than in those from noninfected animals.

Stephen et al. (1980) studied the tissue tropism of Pichinde virus in MHA hamsters and reported virus titers of $10^{6.8}$ in both the plasma and spleen at 9 days p.i.; levels in other organs were not reported. Murphy et al. (1977) found that the major target organs for Pichinde virus infections in MHA hamsters were the liver, spleen, and kidney. They also observed viral antigen in brain sections from the infected animals. Jahrling et al. (1981) studied the tropism of Pichinde virus in strain-13 guinea pigs. They found infectious virus concentrations of 10^6 to 10^8 plaque-forming units (PFU)/mL in the liver, spleen, and lung; titers approaching 10^6 in kidney, and titers of 10^4 in brain. We, too, observed very high virus levels in the liver, but found lower concentrations in the spleen, lung and kidney. These lower virus levels (10^4-fold less) in the spleen and kidney correlate with the lower level of fluorescence in these organs after treatment with FITC-MAb conjugates. The virus levels that we observed in the brain were comparable with those reported by Jahrling et al. (1981).

E. Conclusions

1. The main target organ for Pichinde virus in the MHA hamster is the liver. Pichinde virus titers in the livers were a thousand-fold higher than titers observed in any other organs.

2. MAbs PC4.9A6 and PC4.9D3 after i.p. administration to Pichinde virus-infected hamsters concentrate in the livers of the infected animals. MAbs PC4.9A6 and PC4.9D3 reach at least 10-fold greater levels in the livers of infected animals than in livers of noninfected animals. These MAbs have potential for evaluating targeted delivery of antiviral drugs.

TASK VII. WEANLING HAMSTERS FOR IN VIVO ANTIVIRAL EVALUATIONS.

A. Introduction

Pichinde virus causes a fatal infection in adult MHA hamsters, and this model has been used for the in vivo evaluation of antiviral efficacy (Murphy et al. 1977, and Stephen et al. 1980). The Pichinde virus infection results in a severe hemorrhagic disease in which the principal target organs are the liver and spleen. The adult MHA hamsters weigh 70-100 grams, and for antiviral drugs that are available in very limited quantities, this relatively large animal requires more drug than would a smaller animal such as a mouse. Because there is not a satisfactory murine model for Pichinde virus infections, we chose to examine the use of weanling MHA hamsters. If weanling MHA hamsters could be used in place of adults, less drug would be required for in vivo antiviral evaluations.

B. Materials and Methods

Virus. The An4763 strain of Pichinde virus (obtained from Dr. Joseph D. Gangemi, School of Medicine, University of South Carolina, Columbia, SC) was used in these studies. The virus was twice plaque-purified in Vero cells and a large pool was prepared in these cells to be used as the challenge virus. The titer of this challenge virus pool as determined by the various virus titration procedures used in our laboratory is shown in Table VII-1.

Animals. Adult MHA hamsters were purchased from Charles River Laboratories. Weanling hamsters were obtained by breeding the MHA hamsters in our own animal care facility. MHA hamsters were weaned at 3 to 4 weeks of age, at which time they weighed approximately 30 grams.

Mode of infection. The hamsters were inoculated subcutaneously with 0.2 mL of virus diluted in Puck's balanced saline solution. The animals were examined daily and deaths as well as general appearance were recorded.

Table VII-1. Pichinde virus titers determined by immunofluorescence, plaque assay and in vivo assay in hamsters.

	Fluorescence		
Virus Pool	IFCFU[a]	Plaque PFU[b]	Hamster LD_{50}[c]
PCV[d] An4763 B (06/03/86)	1.6×10^5	4×10^5	5×10^5

[a]IFCFU, immunofluorescent cell-forming units/mL.

[b]PFU, plaque-forming units/mL.

[c]LD_{50}, number of lethal dose units/mL, determined in MHA hamsters.

[d]PCV, Pichinde virus.

C. Results

A titration of Pichinde virus pool PCV An4763 6-3-86 (B) was carried out in weanling and 8-week-old MHA hamsters. Half $\log_{10}$ dilutions were made of the virus over a range of 10^{-2} to 10^{-4}. The hamsters were inoculated subcutaneously in the groin with 0.2 mL of the appropriate virus dilution. The two groups of hamsters used in this titration weighed approximately 30 g and 90 g , respectively. There was a mixture of males and females in each group. All of the infected hamsters died regardless of age, weight, or sex within 14 days of infection. The average day of death in each group was just over 11 days p.i., as noted in Table VII-2.

Table VII-2. Titration of Pichinde virus in 3-4 week old and 8 week old MHA hamsters.

	Mortality Ratio		% Mortality	
Virus Dilution	3-4 week old	8 week old	3-4 week old	8 week old
10^{-2}	5/5	5/5	100	100
$10^{-2.5}$	5/5	5/5	100	100
10^{-3}	5/5	5/5	100	100
$10^{-3.5}$	5/5	5/5	100	100
10^{-4}	5/5	5/5	100	100

Average day of death for 3-4 week old hamsters: 11.16 ± 0.90

Average day of death for 8 week old hamsters: 11.44 ± 0.96

D. Discussion

Pichinde virus infections in MHA hamsters (Murphy et al., 1977, and Stephen et al., 1980) or in strain-13 guinea pigs (Jahrling et al., 1981) are used as models for arenavirus infections. The amount of drug available frequently limits the scope of in vivo antiviral evaluations. Adult guinea pigs weigh five times as much as adult hamsters, thus the hamster model is often chosen over the guinea pig model. A murine model might be even more desirable, but a satisfactory murine model for arenavirus infections is not available. This report compared weanling MHA hamsters to adult MHA hamsters as a model for arenavirus infection. Weanling MHA hamsters appear to be as sensitive to Pichinde virus challenge as are adult MHA hamsters. The symptoms (severe hemorrhagic disease) and outcome (100% mortality rate) of Pichinde virus infection were the same in both adult and weanling MHA hamsters. The use of weanling MHA hamsters will require threefold less compound for in vivo antiviral evaluations.

E. Conclusions

Weanling MHA hamsters, weighing 30 grams were as sensitive to Pichinde virus challenge as were young adult (90 gram) MHA hamsters. If the amount of drug is a limiting factor in future studies, weanling hamsters could be used in place of young adult hamsters.

TASK VIII. PREPARATION AND EVALUATION OF IMMUNOTOXINS AS THERAPIES FOR PICHINDE VIRUS INFECTIONS

A. Introduction

The basis for antibody-mediated delivery of antivirals is that infected and normal cells differ. Many viral infections are characterized by the expression of virus-specific antigens on the surface of infected cells. These may serve as target antigens. By using antibodies that bind to infected cells it may be possible to deliver drugs specifically to the infected cells and achieve a higher therapeutic index than is possible without the use of targeted delivery. Up to this time, our major efforts have been aimed at the antibody-mediated delivery of ribavirin to Pichinde virus infected cells. However, ribavirin must be present in relatively high concentrations to exert an antiviral effect so multiple loading technologies and compatible delivery systems are required for ribavirin so that antibody-mediated delivery of ribavirin will yield strong virus inhibitory activity. While the SRI efforts were focused on preparing such conjugates, USU researchers developed and studied several immunotoxins. In this case, rather than ribavirin attached to an antibody, an antiribosomal toxin is attached to the delivering antibody to shut off protein synthesis in virus-infected cells. Immunotoxins have been applied on an experimental basis for killing cancer cells (Arnon et al., 1985). A major difference between the virus-infected cells and cancer cells is that the target antigens are viral proteins embedded in the cytoplasmic membrane of the infected cell. These viral proteins are presumably on their way out of the cell and would not necessarily afford a route for internalization of immunotoxins.

The in vitro and in vivo evaluations of immunotoxins against Pichinde virus are described in this report. Immunotoxins consisting of anti-Pichinde virus antibodies attached to gelonin through a disulfide linkage were prepared. The heterobifunctional, cleavable cross-linker *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was used to

introduce dithiopyridyl groups into the immunoglobulin. Gelonin was modified with 2-iminothiolane. The thiolated gelonin was then reacted with the dithiopyridyl-substituted immunoglobulin. Using an exchange reaction, the gelonin and immunoglobulin were linked through a disulfide bond to produce the immunotoxin.

The coupling of gelonin to immunoglobulin by this method, which is similar to that described by Lambert et al. (1985), results in an immunotoxin with the structure:

$$\text{Immunoglobulin-NH·CO·CH}_2\text{·CH}_2\text{·S-S·gelonin}$$

Gelonin, a 30,000 Dalton, single-chain glycoprotein is obtained from the seeds of *Gelonium multiflorium* . Gelonin inactivates eucaryotic ribosomes in a catalytic manner, preventing the binding of elongation factor 2 and thus shutting down protein synthesis. Unlike other toxins such as abrin and ricin, gelonin does not have a B-chain (Falasca et al. 1982). Thus in the case of gelonin, there is no inherent mechanism for entering cells (Stirpe et al. 1980). Therefore, although gelonin is very inhibitory in cell-free protein synthesis systems, it is not very cytotoxic in cell culture or in vivo unless provided with a mechanism for entering cells. MAb PC4.9A6, which binds to the surface of cells infected with Pichinde virus, was used to impart cytospecificity to the immunotoxin. Immunotoxins enter cells by receptor-mediated endocytosis.

The PC4.9A6-gelonin immunotoxin produced in these studies was evaluated for its ability to block translation in a cell-free protein synthesis system and for antiviral activity against Pichinde virus in cell culture and in hamsters. As will be described, these studies indicated that the PC4.9A6-gelonin immunotoxin was not internalized and thus lacked significant antiviral activity. Subsequently, a series of immunotoxins were produced consisting of gelonin attached to either rabbit antibody to mouse immunoglobulins or of gelonin attached to recombinant protein G. These immunotoxins were used in an indirect approach to evaluate several monoclonal and polyclonal antisera for antibody-mediated

drug delivery. As a result of these latter studies, gelonin was linked to polyclonal antibodies specific for Pichinde virus. The polyclonal immunotoxin was very active against Pichinde virus. The experiments described in this report indicate that specific immunotoxins may be used to control viral infections.

B. Materials and Methods

Cells and media. African green monkey kidney cells (Vero) obtained from the American Type Culture Collection (ATCC, Rockville, MD) were cultured in minimum essential medium (MEM; GIBCO Laboratories, Grand Island, NY) containing 5% fetal FBS (HyClone Laboratories, Logan, UT). Cells growing as monolayers in cell-culture flasks were detached using trypsin and seeded into 24-well plates at 2×10^5 cells per well or onto 15-mm glass coverslips at the same density. For experiments utilizing 96-well plates, cells were seeded at 6×10^4 cells/well.

Virus. The An4763 strain of Pichinde virus (obtained from Dr. Joseph D. Gangemi, University of South Carolina School of Medicine, Columbia, SC) was used throughout these studies. Pichinde virus strain An4763 (stock 6/3/86-B) used in the in vivo experiments was produced in Vero cells and assayed in MHA hamsters. This particular stock of Pichinde virus had a titer of 1.6×10^6 LD_{50}/mL in MHA hamsters.

Hamsters. Female hamsters (strain MHA) 4 to 5 weeks old, weighing 40 to 50 g were obtained from Charles River Laboratories (Wilmington, MA).

Monoclonal antibodies. Derivation of the hybridoma cell lines that secrete anti-Pichinde virus monoclonal antibodies was described under Task III of this report. The IgG_{2a} concentrations of the ascites fluids pools used in these studies were between 10 and 15 mg/mL based on radial immunodiffusion assays utilizing murine IgG_{2a}-specific antisera.

Binding activity of monoclonal antibodies. The antigen-binding activity of the MAbs was assayed by indirect immunofluorescence. Antibody samples diluted (10-fold serial dilution series) in Dulbecco's phosphate-buffered saline (PBS) were added to Pichinde virus-infected acetone-fixed Vero cell cultures in 24-well plates. The antibodies on the infected cell cultures were incubated for 2 h at 37°C. The cell sheets were rinsed twice with PBS. The presence of anti-Pichinde virus antibody was detected by immunostaining with fluorescein-labeled goat antibody specific for mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN), 3 h at 37°C. Cell sheets were rinsed twice with distilled water and examined for fluorescing cells. The greatest dilution that still produced readily identifiable fluorescing cells was taken as the antigen-binding end-point titer of the antibody.

Immunofluorescence assay for determining Pichinde virus concentrations and extent of spread of Pichinde virus infections in vitro. The assay utilized fluorescein-labeled anti-Pichinde virus murine MAbs prepared in our laboratories (described under Task III of this report). The Pichinde virus assay was as described by Burns et al. (1988). Briefly, Vero cells in 24-well plates were inoculated with dilutions of the virus samples. At 20 h postinoculation (p.i.), the medium was removed and the infected cell cultures were allowed to dry thoroughly at room temperature. The cells were then fixed for 5 min in 80% acetone. For immunostaining, fluorescein-labeled antibody towards Pichinde virus was added to the fixed cells. Immunostained cells were detected by epifluorescence microscopy.

Buffers and reagents used in the conjugation of gelonin to monoclonal antibody. *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was dissolved in absolute ethanol to a concentration of 10 mM, prepared just before use. Stock solutions of 2-iminothiolane (0.1 M) were prepared just before use by dissolving 2-iminothiolane in 1.0 M triethanolamine-HCl buffer (1 M TEA buffer). The 1 M TEA

buffer was prepared by mixing equal volumes of 1 M triethanolamine-HCl and 1 M triethanolamine free base. The SPDP and 2-iminothiolane were obtained from Pierce Chemical Co., Rockford, IL. Recombinant protein G was purchased from Zymed Laboratories, Inc., South San Francisco, CA. Gelonin and other reagents were obtained from Sigma Chemical Co., St. Louis, MO. Phosphate saline buffer (PSB) was of the composition: 0.1 M Na_2HPO_4, 0.1 M NaCl, 1 mM EDTA, pH 7.5. Phosphate buffered saline (PBS) was of the composition: 0.01 M Na_2HPO_4, 0.15 M NaCl, pH 7.2. TEAE buffer was of the composition: 60 mM triethanolamine-HCl, 1 mM EDTA, pH 8.0. The TEAE buffer and the bis-tris/acetate buffer were flushed with argon by slowly bubbling argon through the buffers for 2 h;the argon-flushed buffers were stored in tightly sealed bottles under an argon atmosphere. Bis-tris/acetate buffer consisted of 5 mM bis-tris, 50 mM NaCl, 1 mM EDTA, pH 5.8. A stock solution of 20 mM iodoacetamide was prepared by dissolving iodoacetamide in sterile double-distilled water.

Columns used for separating conjugation reactants. Sephacryl S300 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) hydrated in PBS was used to prepare a column (1.5 x 70 cm) and equilibrated with PBS. Sephadex columns (1.5 x 35 cm) were prepared utilizing Sephadex G25-50 in PSB.

Immunoglobulin purification. The immunoglobulins from ascites fluids and sera were purified by three ammonium sulfate precipitations using an equal volume of saturated ammonium sulfate at each step. The precipitate was redissolved with distilled water and dialyzed exhaustively against PBS. The IgG concentration in the final product was determined by UV spectroscopy, assuming an extinction coefficient of 13.5 at 280 nm.

Preparation of immunotoxins. Introduction of 2-pyridyl disulfide residues. To 10 mg of purified immunoglobulin at a concentration of 5 mg/mL in PBS (done with both the rabbit anti-mouse IgG and the rabbit anti-Pichinde virus) or 5 mg of

recombinant protein G in PBS at 2.5 mg/mL was added 225 µg of SPDP (100 µL of a 10 mM solution) with rapid mixing. The reaction mixture was gently mixed (using rocker platform) at 37°C for 60 min. The excess SPDP and low-molecular-weight reaction products were removed from the reaction mixtures by gel chromatography on Sephadex G25 PD-10 columns equilibrated with PBS. A fresh column was used for each sample. The sample in a total volume of 2.5 mL was added and allowed to flow into the column, discarding the effluent derived during sample application. The column was eluted with PBS and the first 3.5 mL of eluant was collected. This fraction contained the derivatized sample with the low-molecular-weight products such as SPDP removed. The dithiopyridyl groups so introduced were stable for several days at pH 7.0 if held at 4°C.

Introduction of sulfhydryl groups into gelonin. Just prior to the conjugation reaction, gelonin (5 mg) was dissolved in 2 mL of argon-flushed TEAE buffer. The 2-iminothiolane stock solution was then prepared. To the 2 mL of gelonin was then added 50 µL of the 0.1 M 2-iminothiolane to make the reaction mixture 2.5 mM in 2-iminothiolane. The reactants were gently mixed under an argon atmosphere for 90 min at 4°C. The excess 2-iminothiolane was removed from the thiolated gelonin by gel chromatography on Sephadex G25 PD-10 columns equilibrated and eluted with argon-flushed 5 mM bis-tris/acetate buffer (pH 5.8). The thiolated gelonin was used immediately to minimize oxidation.

Reaction of thiol-containing gelonin and the 2-pyridyl disulfide-containing protein G or antibodies. To 10 mg (3.5 mL) of the 2-pyridyl dithio-derivatized antibodies or 5 mg (also in 3.5 mL) of the derivatized protein G was added 1.15 mL of the thiolated gelonin (approximately 1.5 mg of gelonin) in 5 mM bis-tris/acetate buffer (pH 5.8). The pH of the reaction mixture was measured and adjusted to pH 7.0 with TEAE buffer. The reaction mixture was then incubated at 4°C for 20 h. At the end of the conjugation reaction, the remaining free sulfhydryl groups were blocked by the addition

of 0.5 mL of 20 mM iodoacetamide followed by an additional h of incubation at 25°C. The conjugation reaction mixture was then centrifuged for 5 min to remove any precipitate.

Separation of immunotoxin conjugates from nonconjugated gelonin. The nonconjugated gelonin was removed from the reaction mixture by dialysis against PBS using Spectropore 50,0000 MWCO membranes (Spectrum Medical Industries, Inc., Los Angeles, CA). The optical densities of the various immunotoxin preparations were measured at 280 nm. Conjugates consisting of 1 gelonin molecule per IgG molecule would be approximately 16% gelonin by weight. From the OD_{280} of the immunotoxins (assuming absorbance coefficient of 1.0 at 1 mg/mL), the concentrations with respect to gelonin were calculated.

Reticulocyte lysate system. A cell-free protein synthesis system was used to measure gelonin activity. Nuclease-treated rabbit reticulocyte lysate and L-[3,4,5-^{3}H] leucine, both obtained from New England Nuclear (Boston, MA), and untreated rabbit reticulocyte lysate, obtained from Promega (Madison, WI), were utilized. The general details of the reaction conditions were similar to those described by Pelham and Jackson (1976). Thin-layer chromatography (TLC) was used to separate the free and incorporated [^{3}H]leucine. Silica gel-impregnated glass-fiber sheets were obtained from Gelman Sciences Inc. (Ann Arbor, MI). Strips, 7 x 50 mm, cut from the sheets were developed in a TLC solvent prepared by mixing 15 mL of methanol, 5 mL of acetic acid and 30 mL of 15% (W/V) trichloroacetic acid (TCA).

Preparation of mRNA for cell-free translation. A few minutes before use, the messenger RNA was prepared from 30 µL of the untreated reticulocyte lysate to which was added 1 µL of 0.1 M EGTA to bind any Ca(II), which would reactivate the nuclease present in the nuclease-treated lysate. The resulting EGTA concentration was 6.25 mM. EGTA (0.05 M) was prepared by dissolving EGTA in sterile distilled water and adjusting to pH 7 with 1 N NaOH.

Effect of reduced and nonreduced immunotoxin upon cell-free protein synthesis. The assays were performed utilizing controls consisting of translation reaction mixtures with and without mRNA, both without immunotoxin. The PC4.9A6-gelonin immunotoxin was reduced with 100 mM dithiothreitol by adding 10 µL of 1 M dithiothreitol to 0.1 mL of the immunotoxin and incubating for 30 min at room temperature. Reduced and nonreduced immunotoxin were then diluted in PBS containing bovine serum albumin fraction V (BSA) at 0.1 mg/mL. By comparing the extent of translation with varying dilutions of reduced and nonreduced immunotoxin in the translation reaction mixture to the extent of incorporation in controls, the effect of immunotoxin was determined. After incorporation periods of 15, 30 and 60 min, aliquots of translation reaction mixtures were quenched by mixing with 1 M Tris, pH 10.7. The high pH also was used to deacylate [^{3}H]-leucyl- tRNA present in the mixture. The incorporated and free [^{3}H]-leucine were separated by thin-layer chromatography. The incorporated amino acids stayed at the origin while the free moved with the front. Developed strips were cut in half (horizontally) to allow determination of both free and incorporated counts on each strip. Halves were placed in separate vials and [^{3}H]-leucine was determined by liquid scintillation counting.

Cytotoxicity of MAb PC4.9A6-gelonin conjugate towards Pichinde virus-infected and noninfected Vero cells. Monolayer cultures of Vero cells in 96-well plates were inoculated with Pichinde virus at a MOI of 0.1; noninoculated plates were fed at this time with virus-free diluent (MEM containing 5% FBS). Two days p.i., the infected and noninfected cells were rinsed twice (to remove free virus, which might compete for immunotoxin). Varying concentrations (none, 50 nM, 500 nM and 5 µM) of monensin (ICN Biomedicals, Costa Mesa, CA) in 150 µL of MEM with 5% FBS were added; then 6 wells/dilution were treated with 50 µL of PC4.9A6-gelonin conjugate (neat through 10^{-6}) or diluent only; 72 h later, the cellular leucine pools were reduced by

removing media, rinsing twice with leucine-free medium and replacing with leucine-free MEM. After 30 min, the medium was removed from all wells. Each culture then received [^{3}H]leucine at approximately 0.25 µCi per well. The cultures were incubated at 37° C for 3 h (incorporation period). The medium was then carefully aspirated, taking care not to remove cells. Wells were carefully rinsed once with MEM (to lower nonspecific background counts). In earlier experiments, it was noticed that many cells remained attached or in clumps left in wells during the harvest. To avoid this problem, the cells were trypsinized by adding 0.1 mL of trypsin-EDTA and incubating for 30 min at 37°C. To deacylate leucyl-tRNA and to solubilize cells, 50 µL of 2 M NaOH was then added per well and allowed to stand for 10 min at room temperature. The base was then neutralized by addition of 0.1 mL of 20% TCA. The TCA precipitable [^{3}H]leucine was then harvested from the cells onto filter paper using a Skatron cell harvester (CH System 1208, Flow Laboratories, McLean, VA) utilizing distilled water for rinses.

Immunofluorescence assay for antiviral activity of antibody targeted antivirals. The following controls and MAb-gelonin conjugates were evaluated for antiviral activity against Pichinde virus: MAb PC4.9A6 alone, MAb Hy48 alone (a MAb also of the IgG_{2A} isotype but with no binding activity towards Pichinde virus or Vero cells), PC4.9A6-gelonin immunotoxin, Hy48-gelonin immunotoxin (negative control), and ribavirin (positive control). The conjugates were tested over a range of half-$\log_{10}$ dilutions with concentrations from 17 µg/mL down to 0.017 µg/mL. The same dilutions were also run in media containing 50 nM monensin.

For the antiviral evaluations, growth medium was removed from confluent monolayers of Vero cells growing in 96-well culture plates and 100 µL of Pichinde virus diluted in MEM with 2% fetal bovine serum was added. The virus inoculum was such that there were 5-10 immunofluorescent cell-forming units of Pichinde virus added to each well, resulting in an MOI of approximately 1:10,000. The inoculated cultures were incubated for

24 h at 37°C, at which time 100 µL of test compound diluted in MEM was added. The plates were sealed with plastic wrap and incubated at 37°C until the incubation was terminated at 96 h p.i. by pouring the medium from the cultures and letting the cell sheet air-dry prior to fixing with acetone. The fixed cells were immunostained to detect Pichinde virus-infected cells and the number and size of foci of fluorescing cells was determined. Each assay included toxicity controls, as well as virus and normal cell controls similar to a plaque reduction assay described previously (Huffman et al. 1973). Four wells were used for each compound dosage level, one for toxicity assessment (cells + sterile virus diluent + compound) and three wells for antiviral assessment (cells + virus + compound). Six wells in each panel were used for normal cell controls (cells + sterile virus diluent + drug diluent). Pichinde virus infection as detected by immunofluorescence was graded from 0 (normal cells) to 4 (virtually all cells fluorescing). The antiviral activity of a compound was determined in a manner analogous to measuring activity as a reduction in cytopathogenic effect, except reduction in immunofluorescent foci (IF) was the scoring parameter. The antiviral activity was expressed as the concentration of drug required to reduce the IF score by 50% (ED_{50}).

Binding of PC4.9A6-gelonin conjugate to Pichinde virus-infected Vero cells. Monolayer cultures of Vero cells in 24-well plates were inoculated with Pichinde virus at an MOI of 1:250. At 20 h p.i., the cultures were stained under the different conditions described below. To determine if the immunotoxin would bind to Pichinde virus antigens on viable cells, one of the culture plates was transferred directly to 4°C without fixing and dilutions of MAb PC4.9A6 or the PC4.9A6-gelonin immunotoxin were added to the culture medium. The plate was then incubated at 4°C for an additional 2 h. After the reaction with first antibody the plates were rinsed very gently two times with cold MEM. To detect either free MAb PC4.9A6 or immunotoxin, fluoroscein-labeled anti-mouse IgG was added and the cultures were incubated an additional 6 h at 4°C. Cultures

were then rinsed once with MEM and viewed by epifluorescence. To determine if the PC4.9A6-gelonin conjugate retained MAb PC4.9A6-binding affinity for fixed Pichinde virus antigens, cells in a parallel infected culture were fixed with 80% acetone prior to reaction at 4°C with MAb PC4.9A6 or MAb PC4.9A6-gelonin, with the remainder of the immunostaining procedure as described for the living cells.

Virus challenge in MHA hamsters. Hamsters were challenged with Pichinde virus by subcutaneous (s.c.) inoculation in the groin with 28 LD_{50} units of Pichinde virus.

In vivo passive protection studies. For the passive protection studies utilizing unmodified antibodies, the treatment with MAb was at 24 h after the animals were challenged with Pichinde virus. Ascites fluids were used neat or diluted in Puck's saline to yield the indicated IgG_{2a} concentrations in the 0.2 mL inocula, which was administered by intraperitoneal (i.p.) injection. Using a separate group of hamsters for each MAb, hamsters were treated with approximately 2,000 µg of MAb per treatment. Additionally, with MAb PC4.9A6, there were groups of animals treated with 200 µg and 20 µg. The toxicity controls received 0.4 mL of a 1:1:1:1 mixture of the four MAbs.

In vivo antiviral activity of PC4.9A6-gelonin conjugate towards Pichinde virus in MHA hamsters. After inoculation with one LD_{90} of Pichinde virus, hamsters were divided into six treatment groups of ten animals per group. The groups were as follows: (1) MAb PC4.9A6-treated (0.56 mg of MAb PC4.9A6 per kg per treatment); (2) gelonin-treated (0.1 mg/kg/treatment); (3) MAb PC4.9A6/gelonin mixture (0.56 mg/kg/treatment of MAb PC4.9A6 + 0.1 mg/kg/treatment of gelonin); (4) MAb PC4.9A6-gelonin immunotoxin (0.66 mg /kg/treatment); (5) ribavirin (20 mg/kg/treatment); and (6) saline. All treatments were a once a day intraperitoneal injection on days 3, 4, and 5 postinoculation except for the ribavirin treatment group which was treated twice a day (40 mg/kg/day) on days 1 through 14 postinoculation. The infected animals were housed in a room separate from the uninfected animals. The toxicity control group

consisted of five uninfected, 9A6-gelonin immunotoxin-treated (0.66 mg/kg/treatment) hamsters. The normal controls were five uninfected, untreated hamsters. The toxicity and normal controls were weighed individually immediately prior to the first treatment and then again prior to all subsequent immunotoxin treatments. After day 7 postinfection they were weighed on a weekly basis.

Scoring parameters and statistical evaluations for in vivo studies. Mortality rate and time to death were the main parameters used to measure the effect of the various treatments. Additionally, for the passive protection studies, at 8 days postinfection two animals from each group were killed, and liver, spleen, and blood were collected. Livers and spleens were examined visually and scored. Pichinde virus titers in the sera were determined. SGOT and SGPT levels in the sera were also determined. The mortality data were evaluated using chi-square analysis with Yates' correction.

In vitro antiviral assays based upon virus yield for evaluation of immunotoxins. For the evaluation of the indirect immunotoxins a different, more sensitive procedure was employed, which utilized 24-well plate cultures of Vero cells and used virus yield as the parameter indicating presence or absence of an antiviral effect. Vero cell cultures in 24-well plates were inoculated with Pichinde virus at a MOI of 1:10,000. Inocula were 0.25 mL/well, and all dilutions were in MEM. At 20 h postinfection, the cells were rinsed once with cold (4°C) MEM to remove free virus, then 250 µL of cold (4°C) MEM with varying concentrations of first antibody (rabbit anti-Pichinde virus) or rabbit anti-Pichinde virus-gelonin conjugate were added. The first incubation was for 1 h at 4°C, followed by 3 h at 25°C, and then the cell sheets were rinsed twice with cold (4°C) MEM. The indicated dilutions of the protein G-gelonin or anti-IgG-gelonin conjugates (4°C) were then added (0.5 mL/well), the second incubation was run for 30 min at 25°C and then, without removing the immunotoxin, the cultures were shifted to 37°C for 20 h before rinsing twice with MEM and adding maintenance medium to all wells. At four days

postinfection, the supernatant fluids were removed and saved for virus yield assays. Assays were immunofluorescent cell count assays for infectious Pichinde virus utilizing Vero cells.

C. Results

MAb PC4.9A6 displayed many desirable characteristics for a carrier antibody to be used in developing antibody-mediated drug delivery systems, i.e. a readily produced IgG class immunoglobulin, which binds to the surface of Pichinde virus-infected cells, little or no neutralizing or protective activity seen in vitro or in vivo, and yet specifically concentrating in the virus-infected tissues of Pichinde virus-infected hamsters. For these reasons, the initial studies utilized an immunotoxin produced by conjugating MAb PC4.9A6 to gelonin. The results from studies using PC4.9A6-gelonin immunotoxin are described first, followed by the results of subsequent studies utilizing other antibody-gelonin combinations.

Effect of PC4.9A6-gelonin immunotoxin upon protein synthesis in a reticulocyte lysate system. The first question addressed was whether the gelonin activity had survived the conjugation procedure. In order to determine if gelonin was present in a potentially active form in the immunotoxin, MAb PC4.9A6 attached to gelonin through a disulfide linkage (PC4.9A6-gelonin) was evaluated for its ability to block translation in a cell-free protein synthesis system. In the case of previously described gelonin immunotoxins, the toxicity of the gelonin was blocked while the gelonin was attached to the antibody but was expressed after reduction of the disulfide linkage with dithiothreitol (Lambert et al. 1985). The reduction cleaves the linkage between the gelonin and the antibody. The effect of reduced and nonreduced PC4.9A6-gelonin upon protein synthesis in a reticulocyte lysate system and the kinetics of the inhibition were examined. Varying dilutions of the immunotoxin were added at the initiation of the translation reaction

and samples were removed 15, 30 and 60 min into the translation reaction. The effect of reduced and nonreduced immunotoxin upon protein synthesis at 15 min into the reaction is shown in Figure VIII-1. The data reflect [^{3}H]leucine incorporation as a percentage of that of the control without immunotoxin. Similar results were obtained for 30- and 60-min incorporation times, the incorporated [^{3}H]leucine in 3 µL of the control reaction mixture (with mRNA but no immunotoxin) resulted in 150,000 to 300,000 cpm depending on the length of the incorporation reaction. The maximum incorporation was 60% of the added [^{3}H]leucine. The concentration of the immunotoxin in the undiluted starting material was 330 µg/mL; the concentration with respect to gelonin was 53 µg/mL. The concentration of reduced immunotoxin required to inhibit the incorporation of [^{3}H]leucine by 50% was taken as the end-point titer. Depending on the length of the incorporation reaction, the concentration of reduced immunotoxin required to inhibit the incorporation of [^{3}H]leucine by 50% was 3.3-8.7 x 10^{-4} µg/mL, while 200- to 1,000-fold more of the nonreduced immunotoxin was required for the same degree of inhibition. The results of these activity calculations are shown in Table VIII-1. Based on the gelonin content of the immunotoxin and the activity observed with native gelonin, greater than 75% of the original gelonin activity was displayed following reduction of the immunotoxin. Thus, the gelonin in the PC4.9A6-gelonin immunotoxin was present in a potentially active form. As expected, expression of the full toxic activity of the gelonin occurred only after cleavage of the disulfide linkage, a reaction likely to occur in the reducing environment of the lysosomal vesicles inside a target cell.

Cytotoxicity of PC4.9A6-gelonin immunotoxin towards Pichinde virus-infected and noninfected Vero cells. To determine whether this immunotoxin displayed specific cytotoxicity against Pichinde virus-infected cells, experiments were conducted to determine the effect of PC4.9A6-gelonin conjugate upon protein synthesis in Pichinde virus-infected and noninfected Vero cells. In these studies,

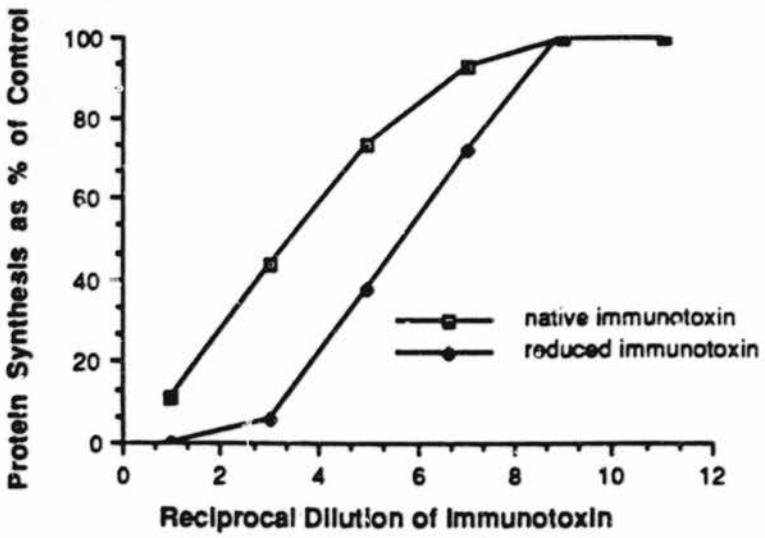


Figure VIII-1. Effect of native PC4.9A6-S-S-gelonin and reduced PC4.9A6-SH + HS-gelonin immunotoxins upon protein synthesis in a reticulocyte lysate cell-free translation system. The native immunotoxin was converted to the reduced form with dithiothreitol. Varying dilutions of the reduced or nonreduced immunotoxin were added at the initiation of the translation reaction. The reciprocal dilutions of immunotoxin are on a $\log_{10}$ scale. The data reflect [^{3}H]leucine incorporation after 15 min as a percentage of that of the control without immunotoxin. The incorporated [^{3}H]leucine in a 3-µL aliquot of the control reaction mixture with mRNA but no immunotoxin resulted in 150,000 cpm. The concentration of the immunotoxin in the undiluted starting material was 330 µg/mL; the concentration with respect to gelonin was 53 µg/mL. The concentration of reduced immunotoxin required to inhibit the incorporation of [^{3}H]leucine by 50% was 0.5 ng/mL, whereas 250-fold more of the nonreduced immunotoxin was required. Based on the gelonin content of the immunotoxin and the activity observed with native gelonin, less than 25% of the original gelonin activity was lost during the production of the immunotoxin. Thus, the gelonin in the PC4.9A6-gelonin immunotoxin was present in a potentially active form and expression of the full toxic activity of the gelonin occurred after cleavage of the disulfide linkage.

Table VIII-1. Gelonin activity in native and reduced PC4.9A6-gelonin immunotoxin.[a]

Assayed following incorporation period (min)	End-Point Titers[a] and 50% Inhibitory Concentrations[b]					
	Disulfide-linked Immunotoxin			Reduced Immunotoxin		
	Immunotoxin		Gelonin	Immunotoxin		Gelonin
	1/dilution	(μg/mL)	(μg/mL)	1/dilution	(μg/mL)	(μg/mL)
15	2.1×10^3	1.6×10^{-1}	2.5×10^{-2}	3.8×10^5	8.7×10^{-4}	1.4×10^{-4}
30	1.0×10^3	3.3×10^{-1}	5.3×10^{-2}	1.0×10^6	3.3×10^{-4}	5.3×10^{-5}
60	2.1×10^3	1.6×10^{-1}	2.5×10^{-2}	3.8×10^5	8.7×10^{-4}	1.4×10^{-4}

[a]The reciprocal of the dilution that diminished the incorporation of [^{3}H]leucine by 50% after the indicated incorporation periods was taken as the end-point titer of the immunotoxin.

[b]The 50% inhibitory concentrations were calculated from the end-point dilution titers, based on an immunotoxin concentration of 330 μg/mL in the undiluted starting material and a concentration with respect to gelonin of 53 μg/mL.

the possibility that the ionophore monensin might enhance the activity of the immunotoxin was examined. Monensin has been shown in previous studies to potentiate the cytotoxicity of ricin-containing immunotoxins (Casellas et al.,1985). At 48 h postinoculation, the immunotoxin was added for the cytotoxicity studies. At the same time, cells in parallel control plates were immunostained for Pichinde virus antigens to determine what percentage of the cells in the inoculated cultures were infected. About 25% of the cells were heavily decorated with fluorescing antibody; these were definitely displaying Pichinde virus antigens. Perhaps most or even all the remaining cells were also infected, but the intensity of staining was much lower than that on the 25% that were brightly fluorescing. The cytotoxicities of the different combinations of PC4.9A6-gelonin and monensin were studied by measuring the incorporation of [^{3}H]leucine by Vero cell cultures 72 h after

treatment with immunotoxin. The 50% inhibitory concentrations of immunotoxin were 21 µg/mL and 125 µg/mL towards infected and control cultures respectively whether without monensin or with 50 nM monensin (Figures VIII-2 and VIII-3). When monensin was present at 500 nM or 5 µM (Figures VIII-4 and VIII-5) there was no difference between the 50% inhibitory concentrations (ID_{50}) of immunotoxin toward Pichinde virus-infected and normal cells. In all instances these ID_{50}s were approximately 100 µg/mL. Thus, in the absence of monensin or with monensin at the lowest concentration (50 nM) there was a slight specific toxicity towards Pichinde virus-infected cells. The possibility that only 25% of the cells were displaying Pichinde virus antigens confuses the interpretation of these experiments. Because subsequent attempts to obtain Pichinde virus-infected Vero cell cultures in which 80% or more of the cells displayed Pichinde virus antigens on the cell surface failed, these specific cytotoxicity studies were abandoned in favor of the approach described below.

In vitro antiviral testing of PC4.9A6-gelonin immunotoxin against Pichinde virus. The experiments we had conducted demonstrated that the PC4.9A6-gelonin immunotoxin displayed potent activity in a reticulocyte lysate system, but whether or not the immunotoxin possessed antiviral activity towards Pichinde virus had not been determined. The antiviral activity was evaluated by observing for the inhibition of fluorescing foci-formation in an immunofluorescence assay for Pichinde virus-infected cells. Vero cell monolayers were inoculated with Pichinde virus at a MOI of 1:10,000. The immunotoxin and controls were added 24 h later. Four days postinfection, the monolayers were fixed, stained, and examined by immunofluorescence for foci of Pichinde virus-infected cells. In untreated, control cultures, large fluorescing foci (several hundred cells) were observed while cultures that had been treated with powerful antivirals exhibited only single fluorescing cells and occasional small (2-8 cells) fluorescing foci. Using this assay, the PC4.9A6-gelonin immunotoxin was evaluated alone and in the presence of 50

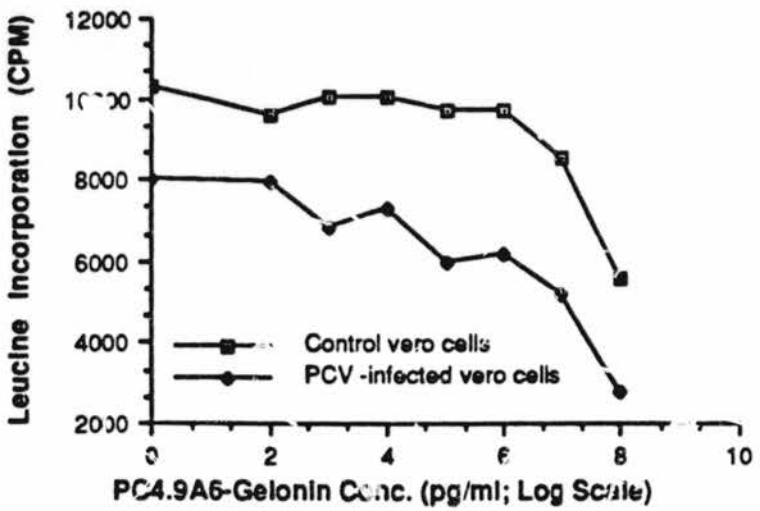


Figure VIII-2. Effect of immunotoxin PC4.9A6-gelonin upon protein synthesis in Pichinde virus-infected and noninfected Vero cells in the absence of monensin. Monolayer cultures of Vero cells in 96-well plates were inoculated with Pichinde virus at a MOI of 1:10; noninoculated plates were fed at this time with virus-free diluent. Forty-eight h postinoculation, cells were rinsed twice and treated with 200 µL of varying concentrations of PC4.9A6-gelonin immunotoxin using 6 wells/dilution. Seventy-two h later, cells were rinsed twice with leucine-free medium, then incubated 30 min in leucine-free medium. [^{3}H]Leucine at 0.25 µCi per well was added and the cultures were incubated at 37°C for 3 h. Culture medium was then removed, the leucyl-tRNA in the cells was deacylated and the amount of [^{3}H]leucine incorporated into protein was determined.

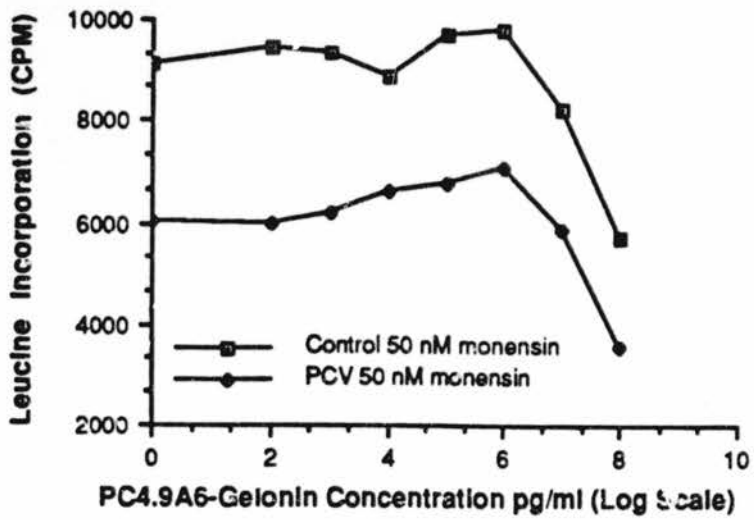


Figure VIII-3. Effect of immunotoxin PC4.9A6-gelonin upon protein synthesis in Pichinde virus-infected and noninfected Vero cells in the presence of 50 nM monensin. Monolayer cultures of Vero cells in 96-well plates were inoculated with Pichinde virus at a MOI of 1:10; noninoculated plates were fed at this time with virus-free diluent. Forty-eight h postinoculation, cells were rinsed twice and treated with 200 µL of varying concentrations of PC4.9A6-gelonin immunotoxin in medium containing 50 nM monensin using 6 wells/dilution. Seventy-two h later, cells were rinsed twice with leucine-free medium, then incubated 30 min in leucine-free medium . [^{3}H]Leucine at 0.25 µCi per well was added and the cultures were incubated at 37°C for 3 h. Culture medium was then removed, the leucyl-tRNA in the cells was deacylated and the amount of [^{3}H]leucine incorporated into protein was determined.

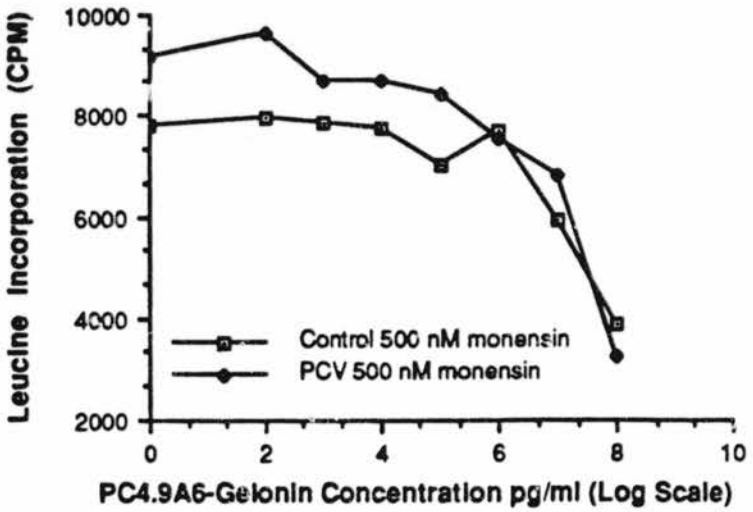


Figure VIII-4. Effect of immunotoxin PC4.9A6-gelonin upon protein synthesis in Pichinde virus-infected and noninfected Vero cells in the presence of 500 nM monensin. Monolayer cultures of Vero cells in 96-well plates were inoculated with Pichinde virus at a MOI of 1:10; noninoculated plates were fed at this time with virus-free diluent. Forty-eight h postinoculation, cells were rinsed twice and treated with 200 µL of varying concentrations of PC4.9A6-gelonin immunotoxin in medium containing 500 nM monensin using 6 wells/dilution. Seventy-two h later, cells were rinsed twice with leucine-free medium, then incubated 30 min in leucine-free medium. [^{3}H]Leucine at 0.25 µCi per well was added and the cultures were incubated at 37°C for 3 h. Culture medium was then removed, the leucyl-tRNA in the cells was deacylated and the amount of [^{3}H]leucine incorporated into protein was determined.

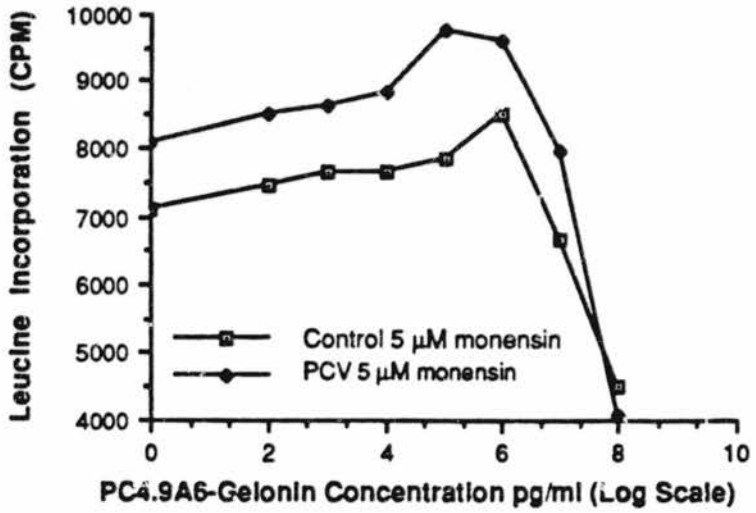


Figure VIII-5. Effect of immunotoxin PC4.9A6-gelonin upon protein synthesis in Pichinde virus-infected and noninfected Vero cells in the presence of 5 µM monensin. Monolayer cultures of Vero cells in 96-well plates were inoculated with Pichinde virus at a MOI of 1:10; noninoculated plates were fed at this time with virus-free diluent. Forty-eight h postinoculation, cells were rinsed twice and treated with 200 µL of varying concentrations of PC4.9A6-gelonin immunotoxin in medium containing 5 µM monensin using 6 wells/dilution. Seventy-two h later, cells were rinsed twice with leucine-free medium, then incubated 30 min in leucine-free medium . [^{3}H]Leucine at 0.25 µCi per well was added and the cultures were incubated at 37°C for 3 h. Culture medium was then removed, the leucyl-tRNA in the cells was deacylated and the amount of [^{3}H]leucine incorporated into protein was determined.

nM monensin. Casellas et al. (1985) reported that the in vitro activity of immunotoxins composed of the purified A-chain of ricin covalently attached to a MAb directed at an antigen present on mouse leukemia cells was enhanced 7- to 10-fold in the presence of monensin. With or without monensin, MAb PC4.9A6-gelonin immunotoxin failed to exhibit any antiviral activity over the concentration range 0.017-17 µg/mL (Table VIII-2). The positive control in this assay was ribavirin. When ribavirin was added to the infected cells at 24 h postinfection, there was a dose-dependent inhibition of immunofluorescent foci formation. At the highest noncytotoxic level of ribavirin, 320 µg/mL, there were no fluorescent foci formed by 96 h postinfection, and only occasional solitary fluorescing cells were observed (a score of 0, on a scale of 0-4). As the concentration of ribavirin was reduced, the size and number of fluorescent foci increased. The concentration that reduced the immunofluorescing foci score by 50% (ED_{50}) for ribavirin was 24 µg/mL; the maximum tolerated dose (MTD) was 1,000 µg/mL; and the therapeutic index (MTD/ ED_{50}) was 42.

Ability of PC4.9A6-gelonin to bind to Pichinde virus infected cells. Although the PC4.9A6-gelonin immunotoxin exhibited the ability to block protein synthesis in a cell-free system, the antiviral assays indicated no antiviral activity. Therefore, the ability of PC4.9A6-gelonin immunotoxin to bind to Pichinde virus-infected Vero cells was examined. In Pichinde virus-infected cultures, which were stained by indirect immunofluorescence (first antibody was either immunotoxin or free PC4.9A6, and second antibody was fluorescein labeled goat anti-mouse), strongly fluorescing cells were observed. The immunotoxin PC4.9A6-gelonin at 10^{-2} dilution gave strong staining of Pichinde virus-infected cells and was detectable to a dilution of 10^{-3}. The MAb PC4.9A6 solution, which contained 10 times as much IgG as did the immunotoxin solution, was detectable to a dilution of 10^{-4} under these staining conditions. Thus the immunotoxin retained antigen-binding activity.

Table VIII-2. Antiviral activity of ribavirin and PC4.9A6-gelonin immunotoxin against Pichinde virus.[a]

Ribavirin conc (μg/mL)	Immunotoxin conc (μg/mL)	Extent of Pichinde virus infection[b] when treated with Ribavirin	MAb PC4.9A6-gelonin	MAb PC4.9A6-gelonin + 50 nM monensin
1000	17	CT[c]	4	4
320	5.4	0	4	4
100	1.7	0	4	4
32	0.54	1	4	4
10	0.17	4	4	4
3.2	0.054	4	4	4
1.0	0.017	4	4	4
0	0	4	4	4

[a]Test material at the indicated concentration was added 24 h after inoculation of Vero cells with Pichinde virus at MOI of 1:10,000.

[b]Extent of infection was measured four days postinfection by immunofluorescence, graded on a scale of 0 (occasional, solitary fluorescing cells) to 4 (confluent fluorescing foci).

[c]CT, Cytotoxicity was observed at ribavirin concentration of 1,000 μg/mL.

In vivo activity of PC4.9A6-gelonin immunotoxin in MHA hamsters. Although the PC4.9A6-gelonin immunotoxin failed to display in vitro antiviral activity, there remained the possibility that different populations of target cells and different mechanisms for internalization of the immunotoxin in vivo might result in a greater in vivo effect than predicted by the in vitro results. MHA hamsters were inoculated with one LD_{90} of Pichinde virus and then treated by intraperitoneal injection on days 3, 4 and 5 post-infection, except as noted for ribavirin treated positive controls. The data are summarized in Table VIII-3. Treatment with MAb alone, gelonin alone, a nonconjugated mixture of the two, or the PC4.9A6-gelonin immunotoxin produced no significant increases in survivors or in mean survival time. The only hamsters to show significant resistance to Pichinde virus infection were those in the ribavirin treatment group. The immunotoxin toxicity controls all survived. Thus, the PC4.9A6-gelonin immunotoxin, although very toxic against protein synthesis in a reticulocyte lysate system, failed to display antiviral activity in either an in vitro or in vivo system.

Effect of direct and indirect immunotoxins (using polyclonal antisera) on the extent of Pichinde virus infection of Vero cells. The PC4.9A6-gelonin immunotoxin displayed only weak antiviral activity, thus three additional immunotoxins were produced. Two were indirect immunotoxins consisting of gelonin attached to either rabbit antibody to mouse immunoglobulins or of gelonin attached to recombinant protein G (a protein similar to protein A which binds to the Fc region of immunoglobulins). The third was gelonin conjugated to IgG isolated from the serum of rabbits hyperimmunized towards Pichinde virus, referred to as hyperimmune rabbit serum-immunotoxin (HRS-gelonin). All three were produced utilizing the disulfide linkage technique as used for the conjugation of MAb PC4.9A6 to gelonin. Utilizing the indirect immunotoxins allowed the evaluation of MAbs and polyclonal antisera for antibody-mediated delivery of therapeutics in several virus systems. The initial evaluation of these

Table VIII-3. Effect of PC4.9A6-gelonin immunotoxin on Pichinde virus infection in MHA hamsters.

Treatment[a]	Start of Treatment	Dose (mg/kg/day)	Survivors/Total	Mean survival time (days)
Saline	72 h post	0	4/20	12.3
MAb PC4.9A6 only	72 h post	0.56	0/10	10.5
Gelonin only	72 h post	0.10	4/10	13.0
MAb PC4.9A6/Gelonin[b]	72 h post	0.56/0.10	1/10	13.8
MAb PC4.9A6-Gelonin[c]	72 h post	0.66	0/10[d]	10.7
Ribavirin[e]	24 h post	40	9/10[f]	20

[a]Treatment: by intraperitoneal injection once daily on days 3, 4 and 5 postinfection unless otherwise noted.

[b]A noncovalently linked mixture of MAb PC4.9A6 and gelonin.

[c]A covalently linked conjugate of MAb PC4.9A6 and gelonin.

[d]The toxicity controls receiving MAb PC4.9A6-gelonin at 0.66 mg/kg/day survived. Although they did lose weight after the first day of treatment, they began to gain weight at the same rate as nontreated controls after the third day of treatment.

[e]Ribavirin twice daily by intraperitoneal injection beginning 24 h postinfection through day 14.

[f]$P < 0.005$.

immunotoxins was to determine their effect on Pichinde virus yield in Vero cell culture. Pichinde virus infected cell cultures (MOI of 1:10,000, approximately 20 infected foci per well) were treated with the immunotoxins or antibody/immunotoxin combinations at 20 h postinfection and then the infections were allowed to proceed. At four days postinfection, the culture fluids were removed for virus assays. The untreated control cultures yielded Pichinde virus titers of 2×10^4 fluorescent cell-forming units per mL. Both indirect and direct immunotoxins were evaluated against Pichinde virus in Vero cells. The virus yields in terms of percent of control are summarized in Figure VIII-6. Only the hyperimmune rabbit serum in combination with the indirect immunotoxin or the hyperimmune rabbit serum conjugated directly to gelonin produced a strong antiviral effect, virus yield reductions of 96% and 80% respectively, whereas MAb PC4.9A6 in combination with the indirect immunotoxin reduced the yield to $70 \pm 24\%$ of that of the untreated controls. Other treatment combinations failed to reduce virus yields.

Characterization and optimization of the antiviral activity of gelonin-polyclonal anti-Pichinde virus immunotoxin. Because the greatest antiviral activity was associated with the polyclonal sera, subsequent evaluations utilized the hyperimmune rabbit serum. Although the indirect immunotoxin approach, wherein gelonin was linked to protein G produced a greater antiviral effect in the initial studies, the direct immunotoxin, consisting of hyperimmune IgG linked to gelonin (HRS-gelonin), was chosen for the subsequent evaluations and optimization. Reasons for this were because (1) the direct system is simpler, (2) there is only one reagent to vary rather than doing checkerboard titrations with two reagents, and (3) there are fewer incubations and rinses. The characterization studies were designed to answer three questions: (1) What is the time course for the expression of antiviral activity? (2) At what time posttreatment should the antiviral effect be measured? and (3) What is the relationship between immunotoxin concentration, antiviral activity, and nonspecific cytotoxicity?

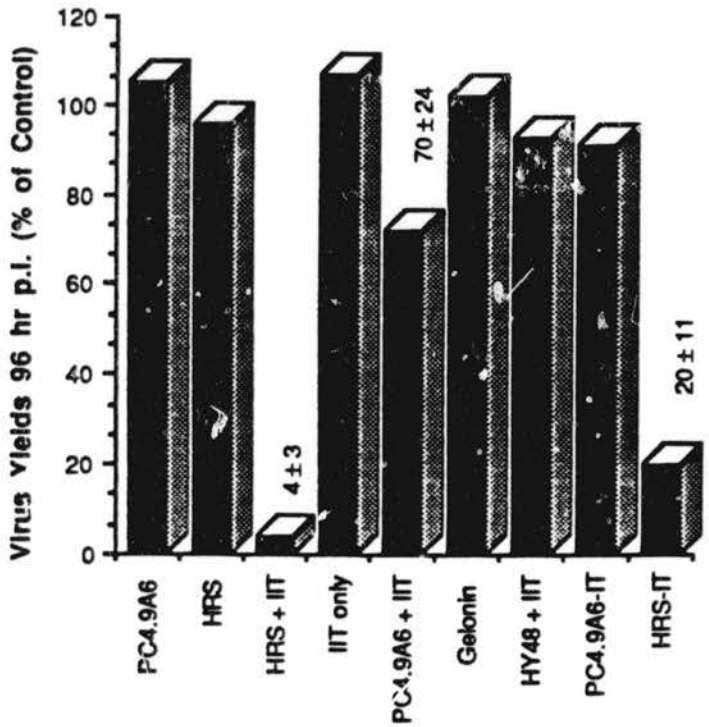


Figure VIII-6. Effect of immunotoxins on Pichinde virus yield. Vero cell cultures in 24-well plates were inoculated with Pichinde virus at a MOI of 1:10,000 (20 foci-forming units per well). Cultures were treated at 20 h postinfection with: PC4.9A6 (a nonneutralizing MAb that reacts with Pichinde virus antigens expressed on the surface of infected cells), HRS (hyperimmune rabbit serum, actually IgG isolated from the serum of rabbits hyperimmunized towards Pichinde virus), HRS + IIT (HRS followed by an indirect immunotoxin consisting of gelonin linked to protein G), IIT only (indirect immunotoxin consisting of gelonin linked to protein G); MAb PC4.9A6 + IIT (MAb PC4.9A6 followed by indirect immunotoxin); gelonin, the toxin used to produce the immunotoxins (in its native form, without a means for binding to cells, gelonin is relatively nontoxic); MAb HY48 + IIT (HY48 is an isotype-matched MAb that has no affinity for Pichinde virus or Vero cells); PC4.9A6-IT (an immunotoxin consisting of PC4.9A6 conjugated to gelonin through a disulfide linkage); and HRS-IT (an immunotoxin consisting of rabbit polyclonal anti-Pichinde virus IgG conjugated to gelonin through a disulfide linkage). Cultures were harvested at 96 h postinfection and assayed for Pichinde virus by an immunofluorescent cell count procedure. The control cultures produced 2×10^4 fluorescent cell-forming units of Pichinde virus per mL.

The effect of the HRS-gelonin immunotoxin upon Pichinde virus yield as a function of time postinoculation is shown in Figure VIII-7. Immunotoxin, HRS-gelonin, was added at 20 h postinfection, cultures were harvested and Pichinde virus titers determined each day for 5 days. The initial MOI (1:10,000) was very low (20 focus forming units/well), so virus levels increased for several days before reaching a plateau of 10^6 per mL. The rise in virus yields from the immunotoxin-treated cultures lagged behind those of the control cultures by one day. These experiments utilized a single treatment at 20 h post-infection and, under these conditions, the greatest difference between virus yields from treated and nontreated cultures was at one day posttreatment. However, with the low MOI used, there was too much variation in virus yields at 48 h postinfection, so 72 h postinfection was selected as the optimum time for measuring virus yields in subsequent studies. The effect of immunotoxin concentration upon the antiviral activity, measuring virus yields at 72 h postinfection is shown in Figure VIII-8. The ED_{50} was 16 nM (the molecular weight of the immunotoxin consisting of 1 molecule of IgG linked to 1 molecule of gelonin is 190,000). A toxicity experiment utilizing inhibition of protein synthesis in noninfected cells was run in parallel. And those data are also shown in Figure VIII-8. There was no apparent toxicity over the concentration range evaluated. From these data it follows that the therapeutic index for the HRS-gelonin immunotoxin is greater than 50 compared with a therapeutic index of 32 for ribavirin in a similar in vitro system. The antiviral activities of the HRS-gelonin immunotoxin, ribavirin, and controls (all against Pichinde virus) are summarized in Table VIII-4.

D. Discussion

Examples of active immunotoxins have been reported although none dealt with targeting virus-infected cells. A related approach that did not use antibody, but rather utilized a *Pseudomonas* exotoxin-CD4 recombinant protein has been shown to mediate in-

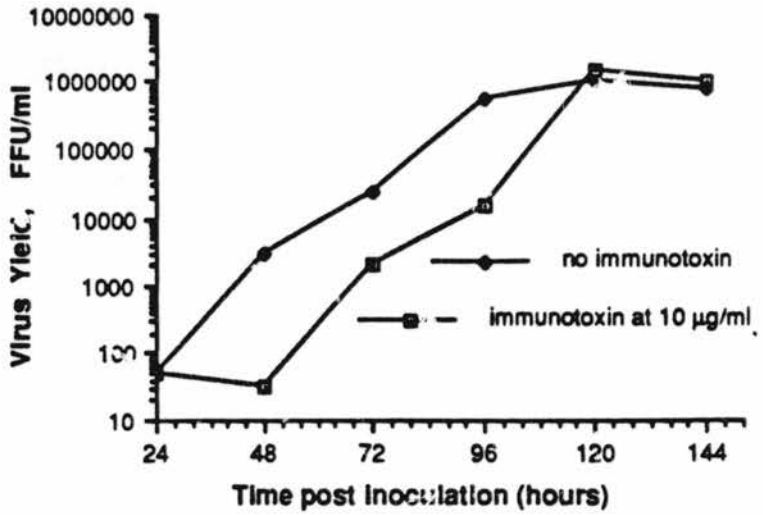


Figure VIII-7. Time course for the expression of antiviral activity associated with HRS-gelonin immunotoxin against Pichinde virus. Virus titers are on a logarithmic scale, thus the differences in virus yields between the nontreated cultures and the treated cultures (Vero cells in 24-well plates) are on the order of 20- to 100-fold. Immunotoxin (10 µg/mL, equivalent to 50 nM) was added at 20 h postinfection, cultures were harvested and Pichinde virus titers determined each day for 5 days. The initial MOI was 1:10,000 (20 focus-forming units/well), and virus levels increased for several days before reaching a plateau of 10^6 per mL. These experiments utilized only a single treatment at 20 h postinoculation.

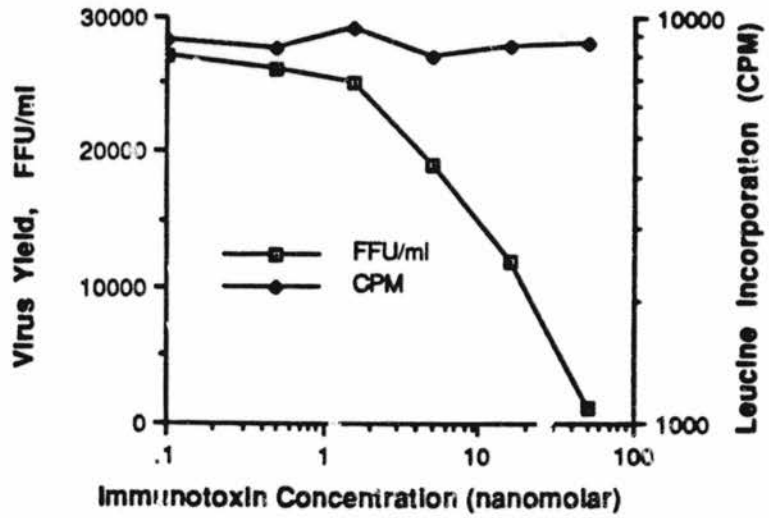


Figure VIII-8. Effect of immunotoxin concentration upon Pichinde virus yields from infected cells and upon protein synthesis in noninfected Vero cells. Vero cell cultures in 24-well plates were inoculated with Pichinde virus at a MOI of 1:10,000, which produced 20 foci of infected cells per well by 20 h postinfection. Immunotoxin, molecular weight 190,000, was added at 20 h postinoculation. Pichinde virus yields were determined at 72 h post inoculation. A toxicity experiment utilizing inhibition of leucine incorporation in noninfected cells was run in parallel. There was no apparent toxicity over the concentration range evaluated.

Table VIII-4. Comparison of antiviral activity[a] of immunotoxins and controls against Pichinde virus in Vero cells.

	Effective Dose (ED_{50}), µg/mL	Maximum Tolerated Dose (MTD), µg/mL	Therapeutic Index (MTD/ED_{50})
Ribavirin	10 [40 µM]	320 [1.3 mM]	32
HRS[b]	>350	350	<1
HRS-gelonin[c]	3.2 [0.016 µM]	>160 [>0.8 µM]	>50
HY48-gelonin[d]	>160	>160	---

[a]Single treatment by adding test material to medium at 20 h postinfection. Toxicity read daily; antiviral activity read at 96 h postinfection.

[b]HRS (hyperimmune rabbit serum), IgG isolated from the serum of rabbits that had been hyperimmunized with Pichinde virus.

[c]HRS-gelonin, an immunotoxin consisting of rabbit polyclonal anti-Pichinde virus IgG conjugated to gelonin through a disulfide linkage.

[d]HY48-gelonin, a negative control immunotoxin consisting of an isotype-matched MAb (HY48) with no affinity for Pichinde virus or Vero cells conjugated to gelonin through a disulfide linkage.

vitro killing of HIV-infected cells (Chaudhary et al. 1988). Thorpe et al. (1981) produced conjugates consisting of gelonin attached through a disulfide linkage to anti-Thy 1.1 MAb. These immunotoxins were cytotoxic in vitro and in vivo to lymphocytes bearing the Thy 1.1 set of surface antigens. Stirpe et al. (1980) reported that, at a concentration of 100 µg/mL, gelonin only slightly inhibited protein synthesis in intact HeLa cells. However, when gelonin was attached to concanavalin A, the complex inhibited HeLa cell protein synthesis 50% at a gelonin concentration of 0.2 µg/mL.

In the studies described in this report, several immunotoxins were developed and evaluated against Pichinde virus. Gelonin, an anti-ribosomal toxin with no inherent means for entering cells, was attached to the delivering antibodies, the objective being to shut off protein synthesis in virus-infected cells. Immunotoxins have been used for killing cancer cells and selected populations of lymphocytes. However, a major difference between the previous applications and the studies described here is that the target antigens, viral proteins embedded in the cytoplasmic membrane of the infected cell, are presumably on their way out of the cell. Gelonin immunotoxins must be internalized by the target cell before they can exert their toxic effect. If MAbs are to be used for delivering gelonin, large numbers of candidate MAbs may need to be screened before antibodies are found that bind to suitably internalized antigen-MAb complexes. Our initial studies utilized an immunotoxin produced by conjugating MAb PC4.9A6 to gelonin. MAb PC4.9A6 displays many desirable characteristics for evaluating antibody-mediated drug delivery systems. It is an IgG class immunoglobulin, which is readily produced via cultivation of the PC4.9A6 hybridoma cell line as an ascites tumor in mice. Purification of MAb PC4.9A6 is uncomplicated, and the ability to bind to infected cells is retained. Although MAb PC4.9A6 binds to the surface of Pichinde virus-infected cells, no neutralizing or protective activity is displayed in vitro or in vivo. This latter quality is desirable because any protective effect associated with a

PC4.9A6 immunotoxin would be attributable to an immunotoxin effect and not an inherent protective activity associated with the antibody.

The immunotoxin of MAb PC4.9A6 attached to gelonin through a disulfide linkage was produced, and experiments were conducted to determine if the MAb PC4.9A6-gelonin conjugate contained active gelonin. This was done by measuring the ability of MAb PC4.9A6-gelonin to block translation in a cell-free protein synthesis system, comparing toxin activity before and after reduction with dithiothreitol. The gelonin was present in MAb PC4.9A6-gelonin in a conjugated configuration that resulted in a greatly diminished activity; however, the activity increased 200 to 1,000-fold upon release of the gelonin from the conjugate by reduction with dithiothreitol. Utilizing a similar cell-free protein synthesis system, Lambert et al. (1985) reported that gelonin at 7.4×10^{-4} µg/mL completely shut down protein synthesis within 15 min. In the experiments described in this report, the concentration of gelonin in the reduced immunotoxin, which was required to inhibit the incorporation of [^{3}H]leucine by 50% was 0.5-1.4 $\times 10^{-4}$ µg/mL. Thus MAb PC4.9A6-gelonin retained the potential toxicity of the gelonin.

In the subsequent cytotoxicity studies, the possibility that monensin might enhance the activity of the PC4.9A6-gelonin immunotoxin was examined. Monensin has been shown in previous studies by others to potentiate the cytotoxicity of ricin-containing immunotoxins. Casellas et al. (1985) reported that the in vitro activity of immunotoxins composed of the purified A-chain of ricin covalently attached to a MAb directed at an antigen present on mouse leukemia cells was greatly enhanced (7- to 10-fold) by the carboxylic ionophore monensin. They presented data indicating that the rate of cell-killing may account for the relative ineffectiveness of immunotoxins in vitro. When the rate of cell-killing was compared for ricin and immunotoxins, the time for ricin to kill 90% of the cells was on the order of 2 h as compared to 4-60 h for immunotoxins. The kinetics were found to be very dependent on the MAb used to produce the immunoconjugate. For

instance, immunotoxins composed of MAbs of the IgM class took much longer to kill 90% of the target cells than did immunotoxins composed of MAbs of the IgG class. The data suggested that immunotoxins with rapid in vitro cell-killing kinetics worked well in vivo. The authors speculated that the immunotoxins were degraded within lysosomes. Treatment of cells with monensin disturbed the acidification of the lysosomes and greatly increased both the kinetics of cell-killing and the activity of the immunotoxins.

Experiments were conducted to determine if the PC4.9A6-gelonin immunotoxin exhibited specific cytotoxicity towards Pichinde virus-infected Vero cells. There was only a slight specific cytotoxicity associated with the PC4.9A6-gelonin immunotoxin either without monensin or with monensin at 50 nM. The 50% inhibitory concentrations of immunotoxin ranged from 20-125 μg/mL under all conditions. These studies gave no indication of a strong, specific targeting of cytotoxicity nor of any potentiating activity on the part of monensin. In previously reported studies, Lambert et al. (1985) examined the cytotoxicity of gelonin conjugates directed at human lymphocytes by measuring the [^{3}H]thymidine incorporation as an index of cytotoxicity. They found that the ID_{50} for one of their gelonin conjugates on the appropriate target cells was 30 pM (a 30 pM solution of gelonin would contain gelonin at 1×10^{-3} μg/mL) as compared with an ID_{50} of 0.4 μM (10 μg/mL) for native gelonin. This latter observation agreed with the cytotoxicity we have observed for the PC4.9A6-gelonin immunotoxin (20 to 125 μg/mL immunotoxin or 3.2 - 20 μg/mL based on gelonin content).

The possibility that only 25% of the cells were displaying Pichinde virus antigens confuses the interpretation of the specific cytotoxicity experiments described above. Indeed, there may have been a strong specific cytotoxicity that was masked by the undisturbed protein synthesis taking place in cells not displaying Pichinde virus antigens. Subsequent attempts to obtain Pichinde virus-infected Vero cell cultures wherein most of the cells displayed Pichinde virus antigens on the cell surface failed, so these specific

cytotoxicity studies were abandoned in favor of an approach based on measuring the inhibition of Pichinde virus infection in cell culture.

PC4.9A6-gelonin immunotoxin displayed potent activity in a reticulocyte lysate system, but specific cytotoxicity towards Pichinde virus-infected cells had not been demonstrated. To determine if the PC4.9A6-gelonin immunotoxin possessed antiviral activity towards Pichinde virus, the immunotoxin was evaluated by observing for the inhibition of fluorescing foci-formation in an immunofluorescence assay for Pichinde virus-infected cells. This antiviral assay was not dependent on infection of a large percentage of the cells, rather a very low MOI (1:10,000) was used. Infected Vero cells were treated 24 h postinoculation at which time Pichinde virus antigens were expressed on the surface of infected cells, but prior to extensive spread of the infection. The positive control in these studies was ribavirin. Four days postinfection, the cells were examined for Pichinde virus-infected cells. In untreated control cultures large fluorescing foci (several hundred cells) were observed. Using this assay, with or without monensin the PC4.9A6-gelonin immunotoxin failed to exhibit any inhibition of foci formation. Ribavirin caused a dose-dependent inhibition of immunofluorescent foci formation. At the highest noncytotoxic level of ribavirin, 320 µg/mL, there were no fluorescent foci formed by 96 h post-infection and only occasional solitary fluorescing cells were observed (a score of 0, on a scale of 0-4). Although the PC4.9A6-gelonin immunotoxin exhibited the ability to block protein synthesis in a cell free system, the antiviral assays indicated no antiviral activity.

It is possible that the PC4.9A6-gelonin immunotoxin did not enter the target cells. This could have been due to a loss of ability to bind to infected cells. For that reason, experiments described in this report were designed to determine if the PC4.9A6-gelonin immunotoxin exhibited specific binding to Pichinde virus infected-cells. It was shown that the immunotoxin retained binding activity to Pichinde virus antigens. Another potential problem was that the immunotoxin may have bound to the cells but was not internalized.

Because the inclusion of monensin did not increase the specific cytotoxicity of the PC4.9A6-gelonin immunotoxin, additional antibodies, which might bind to viral antigens that were more readily internalized, were utilized in subsequent experiments. But before abandoning MAb PC4.9A6-gelonin, an in vivo antiviral study was conducted.

In spite of the lack of impressive in vitro activity, the possibility remained that the PC4.9A6-gelonin immunotoxin might demonstrate in vivo activity. Because Pichinde virus infects macrophages and other cells of the reticuloendothelial system, in vitro evaluations utilizing Vero cells may or may not faithfully predict in vivo activity of immunotoxins. For that reason, the PC4.9A6-gelonin immunotoxin was evaluated in Pichinde virus-infected MHA hamsters. However, there was no therapeutic effect associated with the PC4.9A6-gelonin immunotoxin in Pichinde virus-infected hamsters. There was a slight toxicity associated with the treatment as indicated by weight loss in treated noninfected control animals, indicating that the maximum tolerated dose was probably approached in these studies. There are many possible explanations for the lack of antiviral activity. For instance, the PC4.9A6-gelonin immunotoxin might not be internalized even if it has bound to target cells. If the immunotoxin is internalized the gelonin might not reach the ribosomes in an active form. It is also possible that the hamster mounted an immune response to the immunotoxin. However, this latter possibility did not seem plausible, considering that the animals died as a result of the infection less than two weeks after treatment.

Because neither the in vitro nor in vivo experiments utilizing MAb PC4.9A6-gelonin produced evidence for specific targeting of cytotoxicity, cell-culture experiments using both the indirect and direct protocols with polyclonal antisera towards Pichinde virus were initiated. One of the reasons the MAb PC4.9A6-gelonin immunotoxin may not have shown strong antiviral activity is because it may not have been directed against a Pichinde virus antigen that is readily internalized following binding by immunotoxin. It was thought

that the use of the indirect approach might allow identification of antibodies suitable for the targeted delivery approach and also validate the general premise of using antibodies for the targeted delivery of antivirals. The use of polyclonal antisera was intended to provide binding to a large number of Pichinde virus antigens on the infected cells and thus increase the chances for binding to a readily internalized antigen.

When polyclonal antisera were used for both direct and indirect immunotoxin approaches, the virus yield data showed a strong antiviral effect with the direct immunotoxin, with an even stronger effect observed with the indirect immunotoxin. Both the indirect and direct protocols utilized the same anti-Pichinde virus polyclonal antisera. Explanations for this difference may be in the particular batches of immunotoxins utilized or there is perhaps something about the conjugation directly to the rabbit anti-Pichinde virus caused some loss of antibody activity. Another explanation could be that there was an amplification associated with the sandwiching of the first antibody and the indirect immunotoxin, resulting in a greater number of immunotoxin molecules being bound per viral antigen in the case of the indirect procedure. Yet another possibility is that the indirect system leads to an enhanced internalization of the immunotoxin; such a situation could be the result of increased capping efficiency as the result of cross-linking of antigens expressed on the cell surface. The success with the indirect immunotoxin approach suggests that protein G-gelonin indirect immunotoxins could be used to screen for MAbs suitable as targeted delivery vehicles.

The experiments utilizing polyclonal antibodies for the targeted delivery of antivirals demonstrated very strong antiviral effects against Pichinde virus. A single treatment with HRS-gelonin at 20 h postinfection reduced the virus yield by over 1,000-fold with no detectable nonspecific cytotoxicity. It is important to note that the IgG from the hyperimmune rabbit serum displayed little or no Pichinde virus-neutralizing activity (SN_{50} of less than 1:10) in the absence of complement, and that the controls shown in

Table VIII-4 and in Figure VIII-6 show that the antiviral activity is not inherent in the hyperimmune rabbit serum. Thus, the antiviral activity was associated with the immunotoxin and could not be attributed to neutralization.

The experiments to determine the time course for the expression of immunotoxin activity indicated that multiple treatments with immunotoxin might produce even more dramatic reductions in virus yields. The virus yields from the immunotoxin treated cultures lagged behind those of the control cultures by one day. It appeared that the treatment at 20 h postinfection halted viral replication in those cells that had been infected for one day, perhaps killing these cells; however, by 20 h postinfection the infection had spread to adjacent cells too, and although infected, these cells would not yet display viral antigens on their surface and would thus escape detection by the immunotoxin. We have yet to conduct experiments wherein infected cultures are treated repeatedly with immunotoxins. With repeated treatments the virus levels might be held very low.

Ribavirin, the drug of choice for treating arenavirus infections (Huggins et al. 1984), was used as the positive control in the studies we have described. The summarizing data in Table VIII-4 compare the in vitro activities of HRS-gelonin and ribavirin. The ED_{50}, MTD, and therapeutic index for HRS-gelonin are each more favorable than the corresponding parameters for ribavirin. On a weight/volume basis, the ED_{50} for HRS-gelonin was a third of that for ribavirin (3.2 µg/mL compared to 10 µg/mL). However, comparison of the ED_{50} values expressed in terms of molar concentrations (0.016 µM compared to 40 µM) indicates that one molecule of the immunotoxin mediated an antiviral effect equivalent to that of 2,500 molecules of ribavirin. One of the major advantages envisioned for antibody-mediated delivery of antiviral substances is a reduction in nonspecific cytotoxicity (side-effects). The data in Table VIII-4 indicate that this potential advantage may be realized. We have yet to achieve a maximum tolerated dosage with the HRS-gelonin immunotoxin. It can be anticipated that immunotoxins produced with

suitable MAbs will be even more specific than the HRS-gelonin utilized in these studies. Thus, although much characterization and optimization remain to be completed, the anti-Pichinde virus immunotoxins described in this report appear promising for use in developing antibody-mediated delivery of antiviral substances.

E. Conclusions

1. The PC4.9A6-gelonin immunotoxin produced and described in this report retained the antigen-binding activity of MAb PC4.9A6 and the anti-ribosomal toxicity of gelonin, but lacked specific cytotoxicity towards Pichinde virus infected cells. Apparently the MAb PC4.9A6-gelonin immunotoxin was not internalized by target cells.

2. Indirect immunotoxins consisting of protein G linked to gelonin through a disulfide bond are potent immunotoxins, which can be used to screen for virus specific antibodies to be used for antibody-mediated targeted delivery of antivirals.

3. The experiments utilizing polyclonal antibodies for the targeted delivery of antivirals demonstrated a very strong antiviral effect against Pichinde virus. A single treatment with HRS-gelonin at 20 h postinfection reduced the virus yield by over 1,000-fold with no detectable nonspecific cytotoxicity. The experiments to determine the time course for the expression of immunotoxin activity indicated that multiple treatments with immunotoxin might produce even more dramatic reductions in virus yields.

4. The ED_{50}, MTD, and therapeutic index for HRS-gelonin immunotoxin were each more favorable than the corresponding parameters for ribavirin. The ED_{50} for HRS-gelonin was 0.016 μM compared to 40 μM for ribavirin. One molecule of the HRS-gelonin immunotoxin mediated an antiviral effect equivalent to that of 2,500 molecules of ribavirin. Thus, the anti-Pichinde virus immunotoxins described in this report appear promising for use in developing antibody-mediated delivery of antiviral substances.

CONCLUSIONS

In the period covered by this final report, ten ribavirin derivatives were prepared. Two of these derivatives were used to synthesize immunoconjugates with MAb PC4.9A6. First, a conjugate of ribavirin-5'-*O*-hemisuccinate and MAb PC4.9A6 was prepared. A loading of four ribavirins per MAb was achieved. This loading was deemed insufficient for biological activity because of the high ED_{50} value for ribavirin; therefore, investigations with polymeric carriers were pursued because they would afford higher loadings. In addition, because a carboxylate ester linkage at the 5'-*O*-position of ribavirin was found to be too labile for extended stability in the plasma and delivery to the target cells, 5'-*O*-phosphate ester linkages were investigated. An adduct of ribavirin-5'-*O*-(6-aminohexyl)-phosphate with (carboxymethyl)dextran having a loading of 9.6 molecules of ribavirin per dextran was linked to MAb PC4.9A6 to give the second immunodrug conjugate having a loading of 33 molecules of ribavirin per antibody. This immunoconjugate had no antiviral activity against Pichinde virus infection in Vero 76 cells. The lack of activity is understandable because the MAb was not internalized within the infected cell to deliver the conjugate to the necessary enzymes to effect cleavage of the conjugate to release an active form of the drug.

To achieve higher loading levels of ribavirin, a pentadecamer of ribavirin-5'-*O*-phosphate was designed and synthesized based on oligoribonucleotide chemistry and structure. This polymer has a 6-aminohexyl group at the 5'-*O*-phosphate ester terminus for tethering to a polymeric carrier and a thymidine at the 3'-*O*-phosphate ester terminus for determination of drug loading and to facilitate purification. The polymer was inactive against Pichinde virus infection in Vero 76 cells. This inactivity was expected because the polymer was probably too polar for absorption by these cells. Conjugation of the polymer

to an internalizing MAb should provide a method for entry into these cells. Methodology was developed for tethering the polymer to a carrier protein that would bind to a MAb.

LITERATURE CITED

Allen, L. B., Boswell, K. H., Khwaja, T. A., Meyer, R. B., Jr., Sidwell, R. W., and Witkowski, J. T. Synthesis and antiviral activity of some phosphates of the broad-spectrum antiviral nucleoside, 1-β-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin). J. Med. Chem. **21**, 742-746, 1978.

Ames. B. N. Assay of inorganic phosphate, total phosphate and phosphatases. **In**: Methods of Enzymology, Vol. VIII. Complex Carbohydrates. Neufeld, E. F. and Ginsburg, V., Eds. Academic Press, New York, 1966, pp. 116-117.

Arnon, R., and Sela, M. In vitro and in vivo efficacy of conjugates of daunomycin with anti-tumor antibodies. Immunol. Rev. **62**, 5-27, 1982.

Arnon, R., Schechter, B., and Wilchek, M. Monoclonal antibodies for immunotargeting of drugs in cancer therapy. **In**: Monoclonal Antibodies and Cancer Therapy, R. A. Reisfeld and S. Sell, eds., Alan R. Liss, Inc., New York, NY, 1985, pp. 243-256.

Auperin, D. D., Galinski, M., and Bishop, D.H.L. The sequences of the N protein gene and intergenic region of the S RNA of Pichinde arenavirus. Virology **134**, 209-219, 1984.

Austin, R. K., Trefts, P. E., Hintz, M., Connor, J. D., and Kagnoff, M. F. Sensitive radioimmunoassay for the broad-spectrum antiviral agent ribavirin. Antimicrob. Agents Chemother. **24**, 696-701, 1983.

Bjorn, M. J., Groetsema, G., and Scalapino, L. Antibody-Pseudomonas exotoxin A conjugates cytotoxic to human breast cancer cells in vitro. Cancer Res. **46**, 3262-3267, 1986.

Buchmeier, M. J., Gee, S. R., and Rawls, W. E. Antigens of Pichinde virus I. Relationship of soluble antigens derived from infected BHK-21 cells to the structural components of the virion. J. Virol. **22**, 175-186, 1977.

Buchmeier, M. J., Lewicki, H. A., Tomori, O., and Oldstone, M.B.A. Monoclonal antibodies to lymphocytic ch ngitis and Pichinde viruses: Generation, characterization, and cross-rea ith other arenaviruses. Virology **113**, 73-85, 1981.

Buchmeier, M. J., and Oldstone, M.B.A. Protein staructure of lymphocytic choriomeningitis virus: Evidence for a cell-associated precursor of the virion glycopeptides. Virology **99**, 111-120, 1979.

Buchmeier, M. J., and Parekh, B. S. Protein structure and expression among arenaviruses. Current Topics in Microbiol. and Immunol. **133**, 41-57, 1987.

Burns, N. J., Barnett, B. B., Huffman, J. H., Dawson, M. I., Sidwell, R. W., DeClerq, E., and Kende, M. A newly developed immunofluorescent assay for determining the Pichinde virus-inhibitory effects of selected nucleoside analogues. Antiviral Res. **10**, 89-98, 1988.

Carlsson, J., Drevin, H., and Axen, R. Protein thiolation and reversible protein-protein conjugation. Biochem. J. **173**, 723-737, 1978.

Casals, J. Arenaviruses. **In**: Viral Infections of Humans. A. S. Evans (Ed.), Plenum Medical Book Company, New York, 1982, pp. 127-150.

Casellas, P., Blythman, H. E., Bourrie, B.J.P., Canat, X., Carriere, D., Laurent, G., and Jansen, F. K. Potentiation of cytotoxicity induced by immunotoxins. **In**: Monoclonal Antibodies and Cancer Therapy. R. A. Reisfeld and S. Sell, eds., Alan R. Liss, Inc., New York, NY, 1985, pp. 263-274.

Chaudhary, V. K., Mizukami, T., Fuerst, T. R., FitzGerald, D. J., Moss, B., Pastan, I., and Berger, A. Selective killing of HIV-infected cells by recombinant human CD4-Pseudomonas exotoxin hydrid molecule. Nature **335**, 369-372, 1988.

Corey, E. J. and Venkateswarlu, A. Protection of hydroxyl groups as tert-butyldimethylsilyl derivatives. J. Am. Chem. Soc. **94**, 6190-6191, 1972.

Dawson, M. I., Abrams, H., Satyam, A., Torkelson, S., Ross, B., Shiba, A., Reeve, M., George, D., Gaston, I., McGrath, M., and Lifson, J. Effect of derivatized nucleotide antiviral drugs on HIV-1 relication in cells of T-lymphoblastoid and monocyte/macrophage lineage in vitro. Proc. N. Y. Acad. Sci. (in press).

Dawson, M. I., Sidwell, R. W., and Barnett, B. B. Development of Methods for Carrier-Mediated Targeted Delivery of Antiviral Compounds Using Monoclonal Antibodies (USAMRIID Contract No. DAMD17-86-C-6120). Annual Report No. 1, April 1987, SRI International, Menlo Park, CA.

Dawson, M. I., Sidwell, R. W., and Barnett, B. B. Development of Methods for Carrier-Mediated Targeted Delivery of Antiviral Compounds Using Monoclonal Antibodies (USAMRIID Contract No. DAMD17-86-C-6120). Annual Report No. 2, April 1988, SRI International, Menlo Park, CA.

Dawson, M. I., Sidwell, R. W., and Barnett, B. B. Development of Methods for Carrier-Mediated Targeted Delivery of Antiviral Compounds Using Monoclonal Antibodies

(USAMRIID Contract No. DAMD17-86-C-6120). Annual Report No. 3, April 1989. SRI International, Menlo Park, CA.

Deakin, H., Org. M. G., and Stocken, L. A. 'Glucose 6-phosphate-dehydrogenase' activity and thiol content of thymus nuclei from control and x-irradiated rats. Biochem. J. **89**, 296-304, 1963.

Dillman, R. O., Shawler, D. L., Johnson, D. E., Meyer, D. L., Koziol, J. A., and Frincke, J. M. Preclinical trials with combinations and conjugates of T101 monoclonal antibody and doxorubicin. Cancer Res. **46**, 4886-4891, 1986.

Embleton, M. J., Byers, V. S., Lee, H. M., Scannon, P., Blackhall, N. W., and Baldwin, R. W. Sensitivity and selectivity of ricin toxin A chain-monoclonal antibody 791T/36 conjugates against human tumor cell lines. Cancer Res. **46**, 5524-5528, 1986.

Falasca, A., Gasperi-Campani, A., Abbondanza, A., Barbieri, L., and Stirpe, F. Properties of the ribosome-inactivating proteins gelonin. *Momordica charantia* inhibitor and dianthins. Biochem. J. **207**, 505-509, 1982.

Forrester, J. A., McIntosh, D. P., Cumber, A. J., Parnell, G. D., and Ross, W.C.J. Delivery of ricin and abrin-A chains to human carcinoma cells in culture following covalent linkage to monoclonal antibody LICR-LOND-Fib75. Cancer Drug Deliv. **1**, 283-292, 1984.

Freund, Y. R., and Blair, P. B. Depression of natural killer activity and mitogen responsiveness in mice treated with pristane. J. Immunol. **129**, 2826-2830, 1982.

Fuertes, M., Witkowski, J. T., Streeter, D. G., and Robins, R. K. Synthesis and enzymatic activity of 1,2,4-triazole-3-carboxamide 6'-deoxyhomoribonucleoside-6'-phosphonic acid and related compounds. J. Med. Chem. **17**, 642-645, 1974.

Gilliland, D. G., Steplewski, Z., Collier, R. J., Mitchell, K. F., Chang, T. H., and Koprowski, H. Antibody-directed cytotoxic agents: Use of monoclonal antibody to direct the action of toxin A chains to colorectal carcinoma cells. Proc. Natl. Acad. Sci. USA **77**, 4539-4543, 1980.

Habeeb, A. F. Determination of free amino groups in proteins by trinitrobenzene sulfonic acid. Anal. Biochem. **14**, 328-336, 1966.

Hakimelahi, G. G., Proba, Z. A., and Ogilvie, K. K. New catalysts and procedures for the dimethoxytritylation and selective silylation of ribonucleosides. Can. J. Chem. **60**, 1106-1113, 1981.

Hampton, A. and Magrath, D. I. Synthesis of an isopropylidene derivative of an alkali-labile nucleoside: 2',3'-O-Isopropylidene-9-ß-D-ribofuranosylpurine. J. Am. Chem. Soc. **79**, 3250-3251, 1957.

Hampton, A. Nucleosides. II. A new procedure for the conversion of ribonucleotides to 2',3'-O-isopropylidene derivatives. J. Am. Chem. Soc. **83**, 3640-3645, 1961.

Harnish, D. G., Leung, W. C., and Rawls, W. E. Characterization of polypeptides immunoprecipitable from Pichinde virus-infected BHK-21 cells. J. Virol. **38**, 840-848, 1981.

Hebert, G. A., Pitman, B., McKinney, R. M., and Cherry, W. B. **In**: The Preparation and Characterization of Fluorescent Antibody Reagents. United States Public Health Service, Atlanta, GA, 1972, pp. 1-41.

Hoogenraad, N. J., and Wraight, C. J. The effect of pristane on ascites tumor formation and monoclonal antibody production. **In**: Methods in Enzymology, Vol. 121. J. L. Langone and H. Van Vunakis, eds., Academic Press, Orlando, FL, 1986, pp. 375-381.

Horsfall, F. L., Jr. General principles and historical aspects. **In**: Viral and Rickettsial Infections of Man. F. L. Horsfall, Jr., and I. Tamm (Eds.), Lippincott, Philadelphia, 1965, p. 1.

Huffman, J. H., Sidwell, R. W., Khare, G. P., Witkowski, J. T., Allen, L. B., and Robins, R. K. In vitro effect of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) on deoxyribonucleic acid and ribonucleic acid viruses. Antimicrob. Ag. and Chemother. 3, 235-241, 1973.

Huggins, J. W., Jahrling, P. B., Kende, M., and Canonico, P. G. Efficacy of ribavirin against virulent RNA virus infections. **In**: Clinical Application of Ribavirin, R. A. Smith, ed., Academic Press, New York, NY, 1984, pp. 49-63.

Hurwitz, E. Attempts at site directed experimental chemotherapy with antibody drug conjugates. In: Optimization of Drug Delivery. H. Bundgard, A. Bagger Hansen, and H. Kofod (Eds.), Munksgaard, Copenhagen, 1982, p. 255.

Hurwitz, E., Arnon, R., Sahar, E., and Danon, Y. A conjugate of adriamycin and monoclonal antibodies to Thy-1 antigen inhibits human neuroblastoma cells in vitro. Ann. N.Y. Acad. Sci. **417**, 125-136, 1983.

Hurwitz, E., Kashi, R., Arnon, R., Wilchek, M., and Sela, M. The covalent linking of two nucleoside analogues to antibodies. J. Med. Chem. **28**, 137-140, 1985.

Jahrling, P. B., Hesse, R. A., Rhoderick, J. B., Elwell, M. A., and Moe, J. B. Pathogenesis of a Pichinde virus strain adapted to produce lethal infections in guinea pigs. Infect. and Immuno. **32**, 872-880, 1981.

Johnson, K. M., Halstead, S. B., and Cohen, S. N. Hemorrhagic fevers of Southeast Asia and South America: A comparative appraisal. Prog. Med. Virol. **9**, 105-158, 1967.

Just, G. and Grozinger, K. A selective, mild cleavage of trichloroethyl esters, carbamates, and carbonates to carboxylic acids, amines, and phenols using zinc/tetrahydrofuran, pH 4.2-4.7 buffer. Synthesis, 457-458, 1976.

Kato, Y., Umemoto, N., Kayama, Y., Fukushima, H., Takeda, Y., Hara, T., and Tsukada, Y. A novel method of conjugation of daunomycin with antibody with a poly-L-glutamic acid derivative as intermediate drug carrier. An anti-α-fetoprotein antibody-daunomycin conjugate. J. Med. Chem. **27**, 1602-1607, 1984.

Kreishman, G. P., Witkowski, J. T., Robins, R. K., and Schweizer, M. P. The use of proton and carbon-13 nuclear magnetic resonance for assignment of the glycosylation site in 3- and 5-substituted 1-ß-D-ribofuranosyl-1,2,4-triazoles. J. Am. Chem. Soc. **94**, 5894-5896, 1972.

Kulkarni, P. N., Blair, A. H., and Ghose, T. I. Covalent binding of methotrexate to immunoglobulins and the effect of antibody-linked drug on tumor growth in vivo. Cancer Res. **41**, 2700-2706, 1981.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680-685, 1970.

Lambert, J. M., Senter, P. D., Yau-Young, A., Blättler, W. A., and Goldmacher, V. S. Purified immunotoxins that are reactive with human lymphoid cells. Monoclonal antibodies conjugated to the ribosome-inactivating proteins gelonin and the pokeweed antiviral proteins. J. Biol. Chem. **260**, 12035-12041, 1985.

Lowry, O. H., Rosenbrough, J. J., Farr, A. L., and Randall, R. S. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265-275, 1951.

Meyers, A. I., Roland, D. M., Comins, D. L., Henning, R., Fleming, M. P., and Shimizu, K. Progress toward the total synthesis of maytansinoids. Synthesis of (±)-4,5-deoxymaysine (N-methylmaysenine). J. Am. Chem. Soc. **101**, 4732-4734, 1979.

Murphy, F. A., Buchmeier, M. J., and Rawls, W. E. The reticuloendothelium as the target in a virus infection. Pichinde virus pathogenesis in two strains of hamsters. Lab. Invest. **37**, 502-515, 1977.

Ogilvie, K. K., Beaucage, S. L., Schifman, A. L., Theriault, N. Y., and Sadana, K. L. The synthesis of oligoribonucleotides. II. The use of silyl protecting groups in nucleoside and nucleotide chemistry. VII. Can. J. Chem. **56**, 2768-2780, 1978.

Ogilvie, K. K., Schifman, A. L., and Penney, C. L. The synthesis of oligoribonucleotides. III. the use of silyl protecting greoups in nucleoside and nucleotide chemistry. VIII. Can. J. Chem. **57**, 2230-2238, 1979.

Okabayashi, T., Mihara, S., Repke, D. B., and Moffatt, J. D. A radioimmunoassay for 1-ß-D-arabinofuranosylcytosine. Cancer Res., **37**, 619-624, 1977.

Onishi, H., and Nagai, T. Preparation of dextran T70-methotrexate conjugate and dextran T70-mycophenolic acid conjugate, and in vitro effect of dextran T70-methotrexate on dihydrofolate reductase. Chem. Pharm. Bull. Tokyo **34**, 2561-2567, 1986.

Parekh, B. S., and Buchmeier, M. J. Proteins of lymphocytic choriomeningitis virus: antigenic topography of the viral glycoproteins. Virology **153**:168-178, 1986.

Pelham, R. B., and Jackson, R. J. An efficient mRNA-dependent translation systaem from reticulocyte lysates. Eur. J. Biochem. **67**, 247-256, 1976.

Pimm, M. V., Jones, J. A., Price, M. R., Middle, J. G., Embleton, M. J., and Baldwin, R. W. Tumour localization of monoclonal antibody against rat mammary carcinoma and suppression of tumour growth with adriamycin-antibody conjugates. Cancer Immunol. Immunother. **12**, 125-134, 1982.

Potter, M., and Wax, J. S. Genetics of susceptibility to pristane-induced plasmacytomas in BALB/cAn: reduced susceptibility in BALB/cJ with a brief description of pristane-induced arthritis. J. Immunol. **127**, 1591, 1981.

Schecter, B., Pauzner, R., Arnon, R., Haimovich, J., and Wilchek, M. Selective cytotoxicity against tumor cells by cisplatin complexed to antitumor antibodies via carboxymethyl dextran. Cancer Immunol. Immunother. **25**, 225-230, 1987.

Seeger, R. C., Danon, Y. L., Rayner, S. A., and Hoover, F. Definition of a Thy-1 determinant on human neuroblastoma, glioma, sarcoma and teratoma cells with a monoclonal antibody. J. Immunol. **128**, 983-989, 1982.

Smith, J. F., and Pifat, D. Y. Morphogenesis of sandfly fever viruses (Bunyaviridae family). Virology **121**, 61-81, 1982.

S ephen, E. L., Jones, D. E., Peters, C. J., Eddy, G. A., Loizeaux, P. S., and Jahrling, P. B. Ribavirin treatment of toga-, arena-, and bunyavirus infections in subhuman primates and other laboratory animal species. **In**: Ribavirin: A Broad Spectrum Antiviral Agent, R. A. Smith and W. Kirkpatrick, eds., Academic Press, New York, NY, 1980, pp. 169-183.

Stirpe, F., Olsnes, S., and Pihl, A. Gelonin, a new inhibitor of protein synthesis, nontoxic to intact cells. J. Biol. Chem. **255**, 6947-6953, 1980.

Streeter, D. G., Witkowski, J. T., Khare, G. P., Sidwell, R. W., Bauer, R. J., Robins, R. K., and Simon, L. N. Mechanism of action of 1-ß-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent. Proc. Natl. Acad. Sci. USA **70**, 1174-1178, 1973.

Taggart, R. T., and Samloff, I. M. Stable antibody-producing murine hybridomas. Science **219**, 1228-1230, 1983.

Thorpe, P. E., Brown, A.N.F., Ross, W.C.J., Cumber, A. J., Detre, S. I., Edwards, D. C., Davies, A.J.S., and Stirpe, F. Cytotoxicity acquired by conjugation of an anti-thy 1.1 monoclonal antibody and the ribosome-inactivating protein, gelonin. Eur. J. Biochem. **116**, 447-454, 1981.

Tsukada, Y., Bischof, W.K.-D., Hibi, N., Hirai, H., Hurwitz, E., and Sela, M. Effect of a conjugate of daunomycin and antibodies to rat α-fetoprotein on the growth of α-fetoprotein-producing tumor cells. Proc. Natl. Acad. Sci. USA **79**, 621-625, 1982.

Tsukada, Y., Hurwitz, E., Kashi, R., Sela, M., Hibi, N., Hara, A., and Hirai, H. Effect of a conjugate of daunomycin and purified polyclonal or monoclonal antibodies to rat

α-fetoprotein on the growth of α-fetoprotein producing tumor cells. Ann. N.Y. Acad. Sci., **417**, 262-269, 1983.

Usman, N., Ogilvie, K. K., M.-Y. Jiang, and Cedergren, R. J. Automated chemical synthesis of long oligoribonucleotides using 2'-O-silylated ribonucleotide 3'-O-phosphoramidites on a controlled-pore glass support: synthesis of a 43-nucleotide sequence similar to the 3'-half molecule of an Escherichia coli formylmethionine tRNA. J. Am. Chem. Soc. **109**, 7845-7854, 1987.

Vezza, A. C., Gard, G. P., Compans, R. W., and Bishop, D.H.L. Structural components of the arenavirus Pichinde. J. Virol. **23**, 776-786, 1977.

Wissner A. and Grudsinskas, C. V. Reaction of tert-butyldimethylsilyl esters with oxalyl chloride-dimethylformamide: Preparation of carboxylic acid chlorides under neutral conditions. J. Org. chem. **43**, 3972-3974, 1978.

Witkowski, J. T., Robins, R. K., Sidwell, R. W., and Simon, L. N. Design, synthesis, and broad spectrum antiviral activity of 1-ß-D-riboruranosyl-1,2,4-triazole-3-carboxamide and related nucleosides. J. Med. Chem. **15**, 1150-1154, 1972.

Witkowski, J. T., Robins, R. K., Khare, G. P., and Sidwell, R. W. Synthesis and antiviral activity of 1,2,4-triazole-3-thiocarboxamide and 1,2,4-triazole-3-carboxamide ribonucleosides. J. Med. Chem. **16**, 935-937. 1973.

Youle, R. J. and Neville, D. M., Jr. Anti-Thy 1.2 monoclonal antibody linked to ricin is a potent cell-type-specific toxin. Proc. Natl. Acad. Sci. USA **77**, 5483-5486, 1980.

APPENDIX A

The hybridization procedure that was used to produce the hybridoma cell lines described in this report was as follows:

1. Female RBF/DnJ mice were immunized against Pichinde virus at six months, eight weeks, and six weeks prior to fusion. Each injection was intramuscular with a 50/50 mixture of Pichinde virus-infected suckling mouse brain in Freund's adjuvant. A booster was administered five days prior to the fusion by direct injection into the tail vein with 0.2 mL of Pichinde virus antigen in saline.

2. The spleen donor mouse was anesthesized with ether and exsanguinated. Blood was collected from the subclavean artery. The serum was used as a positive control in antibody assays.

3. The animal was saturated with 70% ethanol. The skin was peeled from the entire peritoneal and chest area, and pinned back out of the way. The animal was again saturated with 70% ethanol.

4. The spleen was aseptically removed and placed in serum-free RPMI-1640 medium (RPMI-1640-SF) in a petri dish. Excess fat and connective tissue was removed from the spleen. The spleen was transfered to a #60 mesh, hat-shaped stainless-steel screen inverted over a 15-mL plastic centrifuge tube in an ice bath. The spleen was kept moist by constant dropwise addition of Tris-NH_4Cl, which also served to lyse erythrocytes.

5. The spleen was minced with sterile scissors. The spleen was dissociated into a cell suspension by forcing it through the stainless-steel strainer, using the sterile plunger from a 3.0-mL disposable syringe and rinsing with Tris-NH_4Cl. This process was

continued until only connective tissue remained. The total volume of cell suspension was 10 ml.

6. Cells were collected by centrifugation at 400 x *g* for 5 min.

7. The supernatant fluids were decanted and the cells resuspended in 12 mL of RPMI-1640-SF, with 0.01 M HEPES. Large chunks were allowed to settle for 2 min and the cell suspension was transfered to a duplicate tube.

8. Cells were collected by centrifugation at 400 x *g* for 5 min.

9. The above spleen cells and the myeloma cells were washed three times in RPMI-1640-SF, with 0.01 M HEPES.

10. Spleen cells and myeloma cells were resuspended in RPMI-1640-SF, with no HEPES. HEPES was avoided during all steps while cells were permeablized with DMSO. Cells were counted. Results in the PC4 fusion were: spleen cells, 10 mL at 6.3 x 10^6/mL, with 82% viable; FOX-NY cells, 10 mL at 1.3 [illegible] mL, with 93% viable.

11. Spleen cells and myeloma cells were combined in a 15-mL conical polypropylene tube at a ratio of five spleen cells to one myeloma cell and sedimented at 400 x *g* for 5 min.

12. The supernatant fluids were drained from the tube; the last drops were removed with a sterile swab. Then, the cells were spread up the walls of the tube by swizzling.

13. To the cell slurry was added 1 mL of fusion mixture (50% PEG and 5% DMSO) and warmed to 37°C to get effecient fusion over 60 sec with gentle swizzling between fingers. The slurry was not vortexed or pipetted to disrupt cells. The slurry was incubated at 37°C for 90 sec. Clumps were visible at this stage. These clumps were allowed to remain intact as cells were fusing at this time.

14. The fusion mixture was diluted by dropwise addition of RPMI-1640-SF, no HEPES, and mixed by gently swizzling tube between fingers with no rough agitation. Diluent was added as follows: 1.0 mL over 30 sec, 3.0 mL over 30 sec, and finally 10.0 mL over 60 sec.

15. The suspension was allowed to stand at room temperature for 5 min before cells were collected by centrifugation at 400 x *g* for 5 min. Supernatant fluids were removed, and cells were resuspended in 15 mL of RPMI-1640-SF no HEPES. Cells were counted. The result from PC4 fusion was: 15 mL at 4.4 x 10^6/mL with 70% viable.

16. Cells were collected by centrifugation at 400 x *g* for 5 min, and resuspended in 240 mL, so there were approximately 2 x 10^5 viable cells/mL in RPMI-1640 with 15% FBS, and adenine and thymidine, but without aminopterin. (The FBS was used without heat inactivation).

17. The fusion mixture was distributed at 1 mL/well to 24-well plates that had been seeded with macrophages six days earlier. The following controls were included: FOX-NY cells only, spleen cells only, and a nonfused mixture of FOX-NY cells and spleen cells. Wells of all three controls were maintained in RPMI with and without aminopterin. The media are referred to as AAT media meaning RPMI-1640 with aminopterin, adenine, and thymidine; and AT media which is same as AAT except without aminopterin. The FOX-NY cells quickly died in the wells with aminopterin. In wells without aminopterin, they overgrew within seven days to the point where most appeared dead. The spleen cell and nonfused mixture control supernatants were very useful as controls in the IFA-screening procedures as an indication of the level of antibody contributed by residual spleen cells.

18. The fusion cell suspension was incubated 24 h. Then 1 mL of AAT medium containing aminopterin (2X) was added to each well. From this point on, the cells were

protected from light, and the handling of the plates were minimized. On days 7, 9, 11, 14, 17, 21, and 24, the cells were fed by removing 1 mL of medium, which was replaced with 1 mL of AAT medium.

19. Supernatants from the fusion plates were assayed several times, beginning when colonies of a few hundred cells became visible.

20. Those hybridomas that secreted antibody towards Pichinde virus were cloned. For cloning, one well of a 24-well plate was cloned into all 96-wells of a 96-well cell culture plate.

21. RPMI-1640 containing adenine and thymidine, but no aminopterin, was used for the first cloning. One half of the plate was seeded with a cell suspension diluted sufficiently to yield 1 cell/well (in 0.3 mL). The remaining wells were seeded with an average of 0.5 cells/well (in 0.3 mL).

22. On day 4 or 5, all wells were examined using low power (40X). The lid over those wells with one clone was circled, and an X was placed over those wells with more than a single clone. Only those wells with a single clone were expanded.

23. When colonies covered 20-50% of the floor of the well, 0.1 mL of the supernatant medium was removed and assayed for antibody activity. The removed medium was replaced with fresh RPMI containing AT.

24. Selected clones were expanded using RPMI containing AT. The ratio for expanding was one well from the 96-well plate to one well of a 24-well plate at 2 mL/well.

25. When colonies covered half of the floor of the well, they were expanded again to three wells of a 24-well plate using RPMI-1640 containing AT as above. Supernatant fluids were reassayed for antibody to confirm positives.

26. When clones were 50% confluent, they were expanded by placing all three wells into one 25-cm^2 flask containing 10 mL of RPMI containing AT. Again when cell

densities reached 0.5-1.0 x 10^6 cells/mL, clones were transfered to a 75-cm^2 flask with 20 mL of RPMI.

27. When cell densities reached 0.5-1.0 x 10^6 cells/mL. The cultures were expanded as needed using RPMI-1640 without AT.

Procedure for the isolation of peritoneal macrophages

1. BALB/c mice, 8-30 weeks old, were sacrificed by cervical dislocation. One mouse at a time was processed through step 5. Macrophages were pooled at that step.

2. The mouse was disinfected by dropping it into a beaker of 70% (w/v) ethanol in water. The mouse was pinned to a clean dissection board.

3. Using sterile forceps and scissors, the abdominal skin was opened and pulled back to completely expose the peritoneal wall. Skin flaps were secured with pins.

4. Using a second pair of sterile forceps, the abdominal muscle layer was lifted and a 5-mL aliquot of sterile, cold RPMI-1640 (without serum) was injected using a 5-cc syringe and a 20-gauge needle. After gentle massage of the abdomen for 10 sec, the solution was withdrawn by keeping the bevel of the needle down and lifting the peritoneal membrane to form a "tent".

5. The fluids containing the macrophages were placed into a sterile centrifuge tube in an ice bath. The intestine was not punctured. NOTE: The withdrawn suspension was clear. If it was noticeably cloudy, the solution and the mouse was discarded.

6. Step 4 was repeated, and the macrophage suspensions were pooled and centrifuged at 500 x *g* for 5 min. NOTE: A pellet was not readily visible.

7. The supernatant fluids were decanted and the cell pellet resuspended in a small amount (approximately 2 mL/mouse) of serum-free medium. Using trypan blue, the

macrophages were counted. The red blood cells or lymphocytes were not included. The macrophages were diluted to the desired cell number.

8. One mouse typically yielded about 1 x10^6 macrophages with 2.5 x 10^4/mL used in 24-well plates or 5 x 10^3 macrophages/0.2 mL in 96-well plates.

9. The macrophages were resuspended in HAT or other selective media and placed into the culture plates before the fusion products (hybrids). This was done 24 h before the addition of fusion products so that macrophages wells could be checked for contamination.

10. Plates of macrophages were used on days 1-6. Plates were useable after longer periods if media was changed.

11. The macrophages attached and cleared the culture of debris in 6-10 days.

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