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REPLY TO
ATTENTION OF:

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MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-HDS/William Bush, Cameron Station, Bldg. 5, Alexandria, VA 22304-6145

SUBJECT: Request Change in Distribution Statement

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2. Point of contact for this request is Ms. Virginia Miller, DSN 343-7328.

CAREY O. LEVERETT
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SRI Project LSU-1956

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

Annual Report

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

April 1, 1989

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SRI International and Utah State University are developing 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 will be screened for antiviral activity both in vitro and in animal model systems.

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FOREWORD

For the protection of human subjects, the investigator(s) adhered to the policies of applicable Federal Law 45CFR56.

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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.

Research was conducted to: (1) synthesize modified analogs 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 <u>in vitro</u> and <u>in vivo</u> 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.

In the third year of this contract considerable progress was made to develop methodology for coupling ribavirin to a monoclonal antibody directed against an antigen expressed on the surface of Pichinde virus-infected cells.

The synthetic accomplishments were:

(1) Because direct conjugation of ribavirin to monoclonal antibody (MAb) PC4.9A6 only afforded a loading of four drugs per antibody, loading was enhanced using a polymeric carrier. Carboxymethyldextran was used as the carrier to which 5 ribavirin-5'-O-(6-aminohexyl)phosphate molecules were linked. This ribavirin-dextran polymer was linked to MAb PC4.9A6, giving a conjugate having 33 ribavirin molecules per antibody--a ninefold higher loading than the first approach. A variety of methods were investigated for separating the ribavirin-dextran-antibody conjugate from free antibody and ribavirin-dextran polymer. Methodology for the successful synthesis and separation of this conjugate is found in the final report for this project. This material was submitted for biological evaluation against Pichinde virus infection in Vero 76 cells.

(2) Methods were investigated for synthesizing a conjugate having higher loading. A pentadecamer of ribavirin-5'-O-phosphate was synthesized in 7% 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. 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 with an internalizing antibody.

The biological accomplishments were:

(1) The antiviral activity of the ribavirin derivatives that would be used for tethering to a MAb was determined. The phosphorylated ribavirin compounds displayed <u>in vitro</u> antiviral activity against Pichinde virus infection in Vero 76 cells. Unfortunately, the conjugate prepared from the adduct of ribavirin-5'-O-(6-aminohexyl)phosphate with carboxymethyldextran and MAb PC4.9A6 was inactive because the monoclonal antibody was not internalized by the cells. The ribavirin-5'-O-phosphate pentadecamer was also inactive because it too could not be taken up by the cells because of its size and polarity. Tethering of these compounds to a MAb that is internalized by the infected cell should provide an effective means of delivery.

(2) The effect of treatment with anti-Pichinde virus MAbs on the course of Pichinde virus infection in hamsters was determined to establish baseline values, which would be used to plan and interpret <u>in vivo</u> antibody-mediated drug delivery experiments. MABs were administered at the time of initial appearance of target antigen on

the surface of infected cells. The mortality data showed that none of four MAbs exerted a protective effect. These results indicated that at immunodrug conjugate doses of 2,000 µg of MAb per animal or less any significant decreases in mortality rates would be attributable to the immunodrug and not to any antiviral activity inherent in the MAb.

(3) An in vivo model that required less drug for evaluation was established. The current in vivo model for evaluating drugs against Pichinde virus used young adult (90 g) hamsters. A breeder colony of MHA hamsters was established. From this colony, weanling MHA hamsters (30 g) at 3 to 4 weeks of age were as sensitive to Pichinde virus challenge as were the young adult hamsters. Using weanlings in place of the adults will require smaller quantities of drug and immunodrug conjugate.

(4) The effectiveness of MAb PC4.9A6 for carrier-mediated drug delivery was evaluated using an immunotoxin repared from this MAb and the ribosomal-inactivating protein gelonin. Native gelonin does not enter cells on its own and, therefore, is not highly toxic, however if a mechanism for introducing it into cells is provided, it will inactive ribosomes in a catalytic manner. Immunotoxins enter target cells by receptor-mediated endocytosis. Therefore, a MAb that is internalized would transport this protein into the cell, where it would inhibit translation, causing cell death. Although a single gelonin molecule should be sufficient to kill a cell, the

PC4.9A6-gelonin immunotoxin failed to display antiviral activity against Pichinde virus in Vero 76 cells. Both the specific (activity against infected cells) and nonspecific (activity against uninfected cells) cytotoxicities observed for the PC4.9A6 immunotoxin were in the same range of 10-100 µg/ml. Because the PC4.9A6-gelonin immunotoxin failed to demonstrate an antiviral effect against Pichinde virus, the immunotoxin was evaluated for its ability to block translation in a cell-free protein synthesis system. The results of this experiment indicated that gelonin was present in the immunotoxin in a conjugated form that greatly reduced activity, but activity was increased 1,000-fold to that of the native protein upon release of gelonin from the MAb by reduction of the disulfide linkage--an event anticipated to occur within the target cell. These experiments indicate that although MAb PC4.9A6 binds to Pichinde virus-infected cells, it is not likely that it is internalized.

Below are listed summaries of each of the six subtasks in this project.

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

The ammonium salts of ribavirin-5'-O-(5-carboxypentyl)phosphate and ribavirin-5'-O-(6-aminohexyl)phosphate were synthesized and

characterized. Quantitate loading of the drug on the MAb, [3'-^{14}C]ribavirin-5'-O-hemisuccinate and [3'-^{14}C]ribavirin-5'-O-(5-carboxypentyl)phosphate were prepared from labeled ribavirin. The 5'-O-(6-aminohexyl)phosphate of the polyribonucleotide poly(ribavirin-3',5'-O-phosphate)$_{15}$-thymidine-5'-O-phosphate and the 5'-O-(6-aminohexyl)phosphate of the polynucleotide poly(thymidine-3',5'-O-phosphate)$_{10}$ were synthesized. Preliminary experiments to prepare conjugates of these compounds with MAbs were initiated.

II. In Vitro Antiviral Evaluation of Drugs Against Pichinde Virus

Several series of ribavirin derivatives were evaluated for antiviral activity against Pichinde virus using an assay based on detection of immunofluorescing foci of infected cells. One of the proposed approaches for achieving multiple loading of ribavirin in a targeted drug delivery system was to multiply link ribavirin to polymers using phosphate ester linkages. Several phosphorylated forms of ribavirin, corresponding to the ribonucleoside-5'-O-phosphates were synthesized at SRI and then evaluated at Utah State University for *in vitro* antiviral activity against Pichinde virus. Ribavirin, ribavirin-5'-O-monophosphate (RMP), ribavirin-5'-O-diphosphate (RDP), ribavirin-5'-O-triphosphate (RTP) and ribavirin-5'-O-(6-aminohexyl)phosphate (SRI 8699-28) were evaluated. The results indicated that the activity of the ribavirin derivatives is dependent on the initial state of phosphorylation. The relative

activities were: ribavirin > RMP = RDP > RTP. The carboxymethyl-dextran conjugate of ribavirin-5'-O-(6-aminohexyl)phosphate (SRI 8699-58) was prepared and its activity was compared with that of the unbound derivative and carboxymethyldextran (SRI-CD). The conjugate (SRI 8699-58) was 27-fold less active than ribavirin. Neither SRI 8699-58 nor SRI-CD displayed antiviral activity.

III. Production and Purification of a Large Pool Of Anti-Pichinde Virus Monoclonal Antibody PC4.9A6

A 350-ml pool of ascites fluids was produced in (RBF/DnJ x BALB/c)F_1 mice that were inoculated with hybridoma cell line PC4.9A6. The ascites fluids pool contained approximately 1,500 mg of anti-Pichinde virus MAb PC4.9A6. This represents the largest amount of anti-Pichinde virus MAb that we have produced to date. The yield of ascites fluids was 9.4 ± 4.4 ml/mouse and 95% of the inoculated mice produced ascites fluids. The average period between the inoculation with hybridoma cells and the first collection of ascites fluids was 9.8 ± 1.5 days. From a portion of this ascites fluids pool, MAb PC4.9A6 was purified by ammonium sulphate precipitation followed by affinity chromatography on protein A Sepharose columns. The overall efficiency of the purification procedure was 40%. The lyophilized, purified MAb PC4.9A6 was utilized for conjugation to ribavirin derivatives.

IV. Treatment of Pichinde Virus-Challenged MHA Hamsters with Anti-Pichinde Virus Monoclonal Antibodies. Effects on Various Measurable Parameters of Infection

The effect of treatment with anti-Pichinde virus MAbs on the course of Pichinde virus infections in MHA hamsters was examined to determine baseline values from which to plan and interpret future in vivo drug-targeting experiments. Antibodies were administered at the time of initial appearance of target antigen on the surface of infected cells. If the criterion for protection was based on mortality data, none of the four MAbs evaluated exerted a strong protective effect. However, there were survivors in three treatment groups. One or two animals (out of a total of seven per group) survived in the groups treated with 2 mg (the highest dosage tested) of MAb PC4.9A6, PC4.9D3, or PC4.8D2, indicating some protection. But the time-to-death values for all groups were within a single standard deviation of each other. None of the animals treated with the lower dosages of MAbs were protected. There was no apparent toxicity associated with MAb treatment. Data based on other parameters were not as clear-cut as survival data. For example, some liver and spleen scores in lethally infected animals were only slightly above normal. The same was true for some SGOT and SGPT values. Virus titers in the sera were variable, but in all cases there were measurable levels of virus in the sera from inoculated animals irregardless of treatment.

In the future, ribavirin-MAb immunodrug conjugates will be evaluated in Pichinde virus-infected hamsters. The results of the current study indicate that at immunodrug doses of 2,000 µg or less (based on MAb concentration) any significant increases in survival time or percent survivors would be attributable to the immunodrug and not the MAb.

V. Weanling Hamsters for In Vivo Antiviral Evaluations

The current in vivo model for evaluating antiviral drugs against Pichinde virus utilizes young-adult MHA hamsters weighing approximately 90 grams. If weanling MHA hamsters could be used in place of adults, less drug would be required for in vivo antiviral evaluations. We established a breeder colony of MHA hamsters from which weanlings at 3 to 4 weeks of age, weighing 30 grams, were obtained. These weanling MHA hamsters were found to be as sensitive to Pichinde virus challenge as were adult MHA hamsters. The symptoms and outcome (100% mortality rate) of Pichinde virus infection were the same in both adult and weanling MHA hamsters.

VI. Production and Characterization of an Immunotoxin Conjugate of Anti-Pichinde Virus Monoclonal Antibody PC4.9A6 Attached to Gelonin Through a Disulfide Linkage

An immunotoxin of MAb PC4.9A6 attached to gelonin through a disulfide linkage was prepared for evaluation against Pichinde

virus. Gelonin is a ribosome-inactivating glycoprotein extracted from the seeds of Gelonium multiflorium. Unlike other toxins such as abrin and ricin, gelonin does not have a B-chain with which to enter cells. Therefore, although gelonin is very inhibitory of protein synthesis in cell-free systems, it is not very cytotoxic in cell culture or in vivo unless provided with a mechanism for entering cells. MAb PC4.9A6 binds to a Pichinde virus antigen expressed on the surface of cells infected with Pichinde virus. The rationale for the construction and use of the MAb PC4.9A6-gelonin immunotoxin is that the PC4.9A6 will impart the cytospecificity and a mechanism for entry into Pichinde virus-infected cells. The immunotoxin may enter target cells by receptor-mediated endocytosis. The toxicity of the gelonin is then expressed after cleavage of the disulfide linkage inside the cell.

The MAb PC4.9A6-gelonin immunotoxin was evaluated for antiviral activity against Pichinde virus utilizing a focus-forming assay. In these studies, the possibility that monensin might enhance the activity of the MAb PC4.9A6-gelonin immunotoxin was examined. The MAb PC4.9A6-gelonin immunotoxin was evaluated alone and in the presence of 50 nM monensin. Under both sets of conditions the MAb PC4.9A6-gelonin immunotoxin failed to exhibit any antiviral activity over the concentration range 0.017-17 µg/ml. Since the MAb PC4.9A6-gelonin immunotoxin failed to demonstrate an antiviral effect against Pichinde virus, an effort was made to determine the cause for the lack of activity.

The immunotoxin was tested for its ability to block translation in a cell-free protein synthesis system. Experiments were conducted to determine whether the MAb PC4.9A6-gelonin conjugate contained gelonin in a conjugated form by comparing toxin activity before and after reduction with dithiothreitol. The effect of reduced and nonreduced MAb PC4.9A6-gelonin conjugate upon protein synthesis in a reticulocyte lysate system and the kinetics of the inhibition were examined. 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; 200- to 1,000-fold more of the nonreduced immunotoxin was required for the same degree of inhibition, indicating that gelonin was present in the immunotoxin in a conjugated form that greatly diminished activity. The activity increased up to 1,000-fold upon release of the gelonin from the conjugate by reduction of the disulfide linkage with dithiothreitol.

The cell-binding activity and specific cytotoxicity of the MAb PC4.9A6-gelonin immunotoxin were examined in vitro. The immunotoxin retained binding activity to Pichinde virus antigens on fixed cells. However, it was not clear whether the immunotoxin bound to Pichinde virus antigens on living cells. There was no detectable specific cytotoxicity associated with the immunotoxin with or without monensin present. The 50% inhibitory concentrations of immunotoxin ranged from 10 to 100 µg/ml under all conditions. These data give no indication of a specific targeting of cytotoxicity or of potent

nonspecific cytotoxicity. Possible reasons for the lack of specific cytotoxic activity were considered. The nonspecific cytotoxicity observed for the immunotoxin was 10-100 µg/ml immunotoxin or 1.6-16 µg/ml based on gelonin content.

STATEMENT OF PROBLEM UNDER STUDY

Under USAMRIID Contract No. DAMD17-86-C-6120, SRI International and Utah State University are developing methodology to assess the effectiveness of antiviral drug-monoclonal antibody conjugate therapy for the treatment of viral infections. Specifically, we are undertaking the preparation of monoclonal antibodies directed against viral antigens expressed on the surface of Pichinde virus, the conjugation of the antiviral drug ribavirin to these monoclonal antibodies, and the biological assessment of the antiviral activity of these conjugates compared with that of 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. The 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 annual progress report describes research accomplished in the third year of the contract (April 1, 1988 to March 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.

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

A. Introduction

Because of its high antiviral activity, ribavirin (Witkowski et al., 1972; Witkowski et al., 1973) was selected as the optimum drug to determine the potential of targeted drug delivery using MAbs. The 5'-position of the ribofuranose ring of ribavirin was selected for introduction of the tether functionality by which the drug would be linked to the MAb. The constraints on the covalent linkage of the tether group on ribavirin to the MAb were (1) sufficient stability to enable the conjugate to arrive intact at the surface of the virally infected cell, and (2) internalization of the drug

either (a) by chemical or enzymic hydrolysis of the conjugate at the cell surface to afford a high local concentration of the drug, which would then enter the cell by its usual active or passive transport process, or (b) by invagination of the conjugate by the cell followed by lysozomal cleavage of the conjugate to release the free drug within the cell. Carboxylic acid ester and phosphate groups were proposed for linking the 5'-position of ribavirin to MAbs, and various strategies were investigated for effecting the tethering. Two approaches were investigated for loading: (1) direct attachment of the functionalized drug to 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 access the biological activity of the MAb-drug conjugates, it is 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 alternative methods: (1) determination using radiolabeled ribavirin, (2) phosphorous analysis for the phosphate analogs, and (3) use of an ultraviolet chromophoric group attached to ribavirin.

B. Experimental Methods

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.

Ribavirin-5'-O-[5-Carbo-(4-nitro)phenoxypentyl]phosphate (4). To a stirred, room-temperature solution of 57.8 mg (0.132 mmol) of ribavirin-5'-O-(5-carboxypentyl)phosphate bisammonium salt (1) and 91.8 mg (0.66 mmol, 5 equiv.) of 4-nitrophenol (2) in 5 ml of water/dioxane (distilled from sodium) (1:1) was added 126.5 mg (0.66 mmol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDIC, 3). After stirring for 1 h, the reaction mixture was diluted with water (15 ml) and washed with chloroform (2 x 20 ml) and chloroform/acetic acid (20:5) (5 x 20 ml). The aqueous layer was concentrated to dryness to give 260 mg of a pale-yellow oil; TLC [chloroform-methanol-water (5:4:1)] R_f 0.31, 0.49 (4), 0.61, 0.98 (2) (UV); TLC R_f 0.24 (1), 0.49 (4) (by anisaldehyde-sulfuric acid reagent); ^1H NMR (D_2O) δ 0.9-1.25 (broad m, 6, 3 CH_2), 1.8 (broad t, J = 6 Hz, 2, CH_2CO_2), 3.35 (t, J = 6 Hz, 2 $POCH_2$), 3.65 (d, J = 5 Hz, 2, 5'-CH_2), 3.8-4.4 (m, 3, 2',3',4'-CH), 5.65 (d, J = 4 Hz, 1, 1'-CH), 6.55 (d, J = 9 Hz, 16% of 2, ArH o to NO_2 of 2), 6.92 (d, J = 9 Hz, 19% of 2, ArH of unidentified impurity), 7.16 (d, J = 9 Hz, 65% of 2, ArH o to NO_2 of 2), 7.75 (d, J = 9 Hz, 16% of 2, ArH o to OH of 2), 7.90 (d, J = 9 Hz, 19% of 2, ArH of unidentified impurity), 8.02 (d, J = 9 Hz, 65% of 2, ArH m to NO_2 of 2), 8.4 (broad s, 1, 5-ArH); UV (0.5 M NH_4HCO_3, pH 8) λ_{max} 404 nm

[A = 0.525 at 0.05-mg/ml concentration, indicating product contained 64.5 mg of 4 (87%)].

(8315-23)

[3'-^{14}C]-1-{5-[3-Carbo-(2-trimethylsilyl)ethoxypropionyl]-β-D-ribofuranosyl}-1,2,4-triazole-3-carboxamide (7). To a solution of 23.9 mg (0.098 mmol) of [3'-^{14}C]ribavirin (5) (specific activity 0.961 mCi/mmol) in 500 µl of anhydrous N-methylpyrrolidinone at room temperature under argon was added 27.8 mg (0.117 mmol) of 2-trimethylsilylethyl succinyl chloride (6). This reaction mixture was stirred for 15 h and then concentrated at reduced pressure. The residue was chromatographed (4 g of silica gel, methylene chloride/-methylene chloride-30% methanol gradient) to give 37.9 mg (87%) of 7 as a colorless oil, TLC (10% methanol/chloroform) R_f 0.22.

(8315-50)

[3'-^{14}C]Ribavirin-5'-O-hemisuccinate Ammonium Salt (9). To a solution of 37.9 mg (0.0853 mmol) of [3'-^{14}C]-1-{5-[3-carbo-(2-trimethylsilyl)ethoxypropionyl]-β-D-ribofuranosyl}-1,2,4-triazole-3-carboxamide (7) in 2 ml of anhydrous tetrahydrofuran was added 0.53 ml (0.53 mmol) of 1.0 M tetra(n-butyl)ammonium fluoride (8) in tetrahydrofuran. The reaction mixture was stirred at room temperature for 16 h. The solvent was removed at reduced pressure, and the residue was chromatographed (1 x 16-cm Pharmacia Sephadex DEAE A-25,

0.0-0.5 M ammonium bicarbonate gradient) to give 26.2 mg (87%) of 9 as a white solid (0.015 mCi, 16% radiochemical yield).

(8315-50)

[3-'^{14}C]Ribavirin-2',3'-O-acetonide (10). To a stirred suspension of 23.9 mg (0.098 mmol) of [3'-^{14}C]ribavirin (5), having specific activity of 0.961 mCi/mmol, in 160 µl of triethylorthoformate was added 60 µL of reagent-grade acetone. This solution was cooled in an ice bath under argon while 5 µl of ice-cold 70% perchloric acid was added. The yellow-orange reaction mixture was stirred at ice-bath temperature for 1 h and at room temperature for 3 h. The red solution was diluted with 5 ml of 5% sodium bicarbonate and extracted with ethyl acetate (seven 5-ml portions) until TLC (10% methanol/chloroform) indicated that no further product (R_f 0.25) was present. The combined extracts were dried (sodium sulfate) and concentrated to a pale-yellow oil, which was chromatographed (10 g of silica gel, chloroform/chloroform-20% methanol gradient) to give 21.7 mg (78%) of 10 as a white solid (0.065 mCi, radiochemical yield 69%).

(8315-45)

[3'-^{14}C]Ribavirin-2',3'-O-acetonide-5'-O-(2-cyanoethyl-5-carbomethoxypentyl)phosphate (12). To a stirred mixture of 21.7 mg (0.0763 mmol, 0.065 mCi) of [3'-^{14}C]ribavirin-2',3'-O-acetonide (10), which had been dried overnight at 0.005 mm Hg, and 5.7 ml (2.3 mmol) of 0.4 M tetrazole in acetonitrile (Applied Biosystems) under

argon was added dropwise 185 mg (0.534 mmol) of (5-carbomethoxypentyl)(2-cyanoethyl)(N,N-diisopropyl)phosphoramidite (11) in 0.2 ml of anhydrous acetonitrile. The mixture was stirred for 1 h at room temperature before 763 µl of 0.1 M iodine in tetrahydrofuran/2,6-lutidine/water (2:1:1) (Applied Biosystems) was added. Stirring was continued for 30 min. Next, 5 ml of 1 M sodium thiosulfite was added to reduce the unreacted iodine, and the aqueous layer was separated and extracted with methylene chloride (three 20-ml portions). The combined extracts were washed with 5% sodium bicarbonate (50 ml), dried (sodium sulfate), and concentrated to a pale-yellow oil, which was chromatographed (10 g of silica gel, methylene chloride/methylene chloride-5% methanol gradient) to give 36.3 mg (87%) of 12 as a colorless oil (0.045 mCi, radiochemical yield 69%), TLC (10% methanol/chloroform) R_f 0.50.

(8315-46)

[3'-^{14}C]Ribavirin-2',3'-O-acetonide-5'-O-(5-carboxypentyl)-phosphate Bisammonium Salt (13). A solution of 36.3 mg (0.0665 mmol, 0.045 mCi) of [3'-^{14}C]ribavirin-2',3'-O-acetonide-5'-O-(2-cyanoethyl-5-carbomethoxypentyl)phosphate (12) (specific activity 0.677 mCi/mmol) in 5 ml of concentrated ammonium hydroxide and 1 ml of pyridine was stirred at 55°C under argon overnight. Concentration at reduced pressure afforded 28.3 mg (90%) of 13 as a pale-yellow oil. This material was used in the next reaction without further purification.

(8315-47)

[3'-^{14}C]Ribavirin-5'-O-(5-carboxypentyl)phosphate Bisammonium Salt (14). A solution of 28.3 mg (0.06 mmol) of [3'-^{14}C]ribavirin-2',3'-O-acetonide-5'-O-(5-carboxypentyl)phosphate bisammonium salt (13) in 5 ml of 85% aqueous acetic acid was stirred for 15 h at 65-68°C. After concentration at reduced pressure, the reaction mixture was chromatographed (1 x 16-cm Pharmacia Sephadex DEAE A-25, 0.0-0.5 M ammonium bicarbonate gradient). Fractions 17-18, which contained product by TLC (chloroform/methanol/water, 5:4:1) R_f 0.22, were lyophilized to give 76.5 mg of a white solid containing 14 and salts (0.0183 mCi, radiochemical yield 41%); HPLC (10-μ Waters Radialpak 8 C_{18}, water, 1.7 mL/min, 220 nm) R_t 3.6 (0.3%), 4.3 (4.7%), 6.0 (0.5%), 7.8 (2.3%), and 51.8 min (92%).

6-(t-Butyloxycarbonylamino)-1-hexanol (17). A solution of di(t-butyl)dicarbonate (16) (4.8 g, 21 mmol) in 9.0 ml of dry tetrahydrofuran was added over a period of 10 min to a stirred and cooled (0-5°C) solution of 2.34 g (20 mmol) of 6-amino-1-hexanol (15) in 6.0 ml of dry tetrahydrofuran. The reaction mixture was stirred at this temperature for 30 min and at room temperature for 2 h. The clear solution that resulted was concentrated at reduced pressure to give a light-yellow oil; TLC (methylene chloride/acetone 9:1) R_f 0.17; ^{1}H NMR ($CDCl_3$) δ 1.20-1.90 (m, 8, $(CH_2)_4$), 1.40 (s, 9, $C(CH_3)_3$), 3.10 (q, J = 9 Hz, 2, NCH_2), 3.65 (q, J = 9 Hz, 2, OCH_2),

4.55 (broad s, 1, NH). This material was used directly in the next step.

(8699-25)

[6-(t-Butyloxycarbonylamino)hexyl](2-cyanoethyl)(N,N-diisopropyl)phosphoramidite (19). To a stirred solution of 4.35 g (20 mmol) of 6-(t-butyloxycarbonylamino)-1-hexanol (17) and 7.0 ml (40 mmol) of N,N-diisopropylethylamine in 50 ml of dry tetrahydrofuran was added over a period of 10 min by syringe 4.5 ml (20 mmol) of (N,N-diisopropylamino)(2-cyanoethoxy)chlorophosphine (18) at room temperature. The amine salt started to separate after a few minutes. After stirring for 1 h, the salt was removed by filtration and the filtrate was concentrated at reduced pressure. The colorless oil was dissolved in 300 ml of ethyl acetate and washed with cold 5% sodium bicarbonate (3 x 100 ml), dried (sodium sulfate), and concentrated at reduced pressure to give an oil, which was purified by chromatography (58 g of silica gel, ethyl acetate/hexane/triethylamine 25:70:5) to give 6.60 g (85%) of 19 as a colorless, viscous oil; TLC (ethyl acetate/hexane/triethylamine 25:70:5) R_f 0.41 (5% silver nitrate, 100°C to visualize); ^1H NMR ($CDCl_3$) ∂ 1.17 and 1.25 (d, J = 10 Hz, 12, $CH(CH_3)_2$), 1.30-1.80 (m, 10, $(CH_2)_4$, $CH(CH_3)_2$), 1.47 (s, 9, $C(CH_3)_3$), 2.65 (t, J = 9 Hz, 2, CH_2CN), 3.10 (q, J = 9 Hz, 2, NCH_2), 3.40-3.95 (m, 4, OCH_2), 4.50 (broad s, 1, NH); ^{31}P NMR ($CDCl_3$) 146.4 ppm.

(8699-26)

Ribavirin-2',3'-O-acetonide-5'-O-[(6-t-butyloxycarbonylamino-hexyl)(2-cyanoethyl)phosphate] (21). To a stirred solution of 284 mg (1.0 mmol) of ribavirin-2',3'-O-acetonide (20) and 700 mg (10 mmol) of resublimed (1H-tetrazole) in 25 ml of dry acetonitrile was added a solution of 1.00 g (2.59 mmol) of [6-(t-butyloxycarbonyl-amino)hexyl](2-cyanoethyl)(N,N-diisopropyl)phosphoramidite (19) in 10 ml of acetonitrile. The reaction mixture was stirred for 1 h before 4.3 ml of iodine in pyridine/tetrahydrofuran/water (Applied Biosystems) was added and stirring was continued for 30 min. The excess iodine was reduced by the addition of 26 ml of 1 M aqueous sodium thiosulfate and stirring for 10 min. The mixture was extracted with methylene chloride (4 x 100 ml) and the organic extract was washed with 5% aqueous sodium bicarbonate (2 x 100 ml), dried (sodium sulfate), and concentrated at reduced pressure to give a colorless viscous oil, which was purified by chromatography (60 g of silica gel, 1000 ml of 1% methanol/chloroform, followed by 1500 ml of 5% methanol/chloroform) to give 0.3 g (68% based on starting material consumed) of crude 21, containing 5% of acetonide and 20-30% of an unidentified tetrazole-containing product by ^{1}H NMR; TLC (10% methanol/methylene chloride) R_f 0.45; ^{1}H NMR ($CDCl_3$) δ 1.20-1.80, 1.42, 1.47, and 1.62 (m and 3 s, 23, $C(CH_3)_3$, $CH(CH_3)_2$, $(CH_2)_4$), 2.80 (m, 2, CH_2CN), 3.10 (q, J = 9 Hz, 2, CH_2N), 3.6-4.65 (m, 6, OCH_2), 4.85 (broad m, 1, 4'-H), 5.05 (m, 1, 3'-H), 5.32 (m, 1, 2'-H), 6.15 (s, 1, 1'-H), 6.55-6.90 (broad m, 1, NH), 7.50 (m, 2,

NH_2), 8.44 (s, 1, 5-ArH); ^{31}P NMR ($CDCl_3$) 0.0 (21) and 20.0 (impurity) ppm.

(8699-27,30)

Ribavirin-5'-O-(6-Aminohexyl)phosphate Ammonium Salt (22). A solution of 0.3 g (0.5 mmol) of ribavirin-2',3'-O-acetonide-5'-O-[(6-t-butyloxycarbonylaminohexyl)(2-cyanoethyl)phosphate] (21) in 5 ml of pyridine and 50 ml of ammonium hydroxide was stirred with heating at 55-60°C for 20 h. After concentration, the residue was dissolved in 30 ml of 85% acetic acid and stirred at 65-70°C for 20 h. Concentration afforded 0.3 g of crude 22 as a colorless, viscous oil: TLC (5% ammonium hydroxide/methanol) R_f 0.25 (22) and some higher R_f spots. Preparative TLC (2-mm, 20 x 20-cm plates, 5% ammonium hydroxide/methanol, methanol extraction) afforded 60 mg (27%) of 22 as a white solid; ^1H NMR (D_2O) δ 1.20, 1.45 (2 m, 8, $(CH_2)_4$), 2.77 (t, J = 8 Hz, 2, NCH_2), 3.66 (q, J = 7 Hz, 2, CH_2OP), 3.95 and 3.98 (2 t (1:2), J = 5 Hz, 1, 5'-H), 4.03 and 4.05 (2 dd (2:1), 1, J = 3 Hz, J = 2.5 Hz, 5'-H), 4.30 (m, 1, 4'-H), 4.50 (t, J = 5 Hz, 1, 3'-H), 4.68 (t, J = 4 Hz, 1, 2'-H), 6.0 (d, J = 4 Hz, 1, 1'-H), 8.73 (s, 1, 5-ArH); ^{31}P NMR 0.0 ppm.

(8699-28)

Carboxymethyldextran (25). A literature procedure (J. Pitha et al., 1980) was adapted. To a solution of 2.0 g (0.22 mmol) of T-9 dextran (Mw 9000) (23) in 2.0 ml of water was added 38 mL of 40% sodium hydroxide and 27.0 g (0.29 mol) of chloroacetic acid (24).

The suspension was stirred for 24 h, acidified to pH 3 with concentrated hydrochloric acid, and diluted with water (350 ml) until solution was achieved. The solution was ultrafiltered through a UM-2 membrane (14 diavolumes) before lyophilization to give 2.3 g of 25 as a fluffy white solid.

Carboxymethyl group-loading onto the dextran was determined by titration. A 0.30-g aliquot of the dry dextran was dissolved in 15 ml of water. The initial pH of this solution was 3.0 before dilution with 2.00 ml of 1.00 N sodium hydroxide, which brought the pH to 11.38. Back-titration with 0.200 N hydrochloric acid (2.95 ml) gave the equivalence point as pH 8.2, indicating 1.41 mmol of carboxylic acid residues/0.3 g or a loading of 58 carboxymethyl groups/mole dextran.

(8554-3)

Conjugate (26) of Ribavirin-5'-O-(6-Aminohexyl)phosphate with Carboxymethyldextran. The ammonium ion was first exchanged by diluting a solution of 30 mg (68 mol) of ribavirin-5'-O-(6-aminohexyl)phosphate ammonium salt (22) in 0.2 ml of water with 15 ml of pyridine. The solution was stirred for 30 min and then concentrated at high vacuum. This process was repeated four times, with 15-min stirring periods. The pyridium salt was obtained as 35 mg of a white solid and dried at high vacuum for 1 h.

The pyridinium salt was dissolved in 0.5 ml of water. To this solution was added 29.8 mg (2.48 mol) of carboxymethyldextran (25). After stirring at room temperature for 30 min, some polymer remained undissolved, so another 0.5 ml of water was added. The pH of this mixture was 4.2. It was adjusted to 5 by the addition of 0.5 N sodium hydroxide. Next,).191 g (1.0 mmol) of EDIC (3) was added. Since the pH of the mixture increased to 6.4, the pH was lowered to 5 by the addition of 1 N hydrochloric acid. The reaction mixture was stirred at room temperature for 17 h. Since TLC indicated the presence of 22, 100 mg (0.52 mmol) of EDIC was added and stirring was continued for 23 h. TLC indicated starting material. Another 100-mg (0.52 mmol) portion of EDIC in 0.5 ml of dioxane was added and stirring was continued for 24 h. Starting material still remained. The reaction mixture was concentrated at reduced pressure and dissolved in 1 ml of phosphate-buffered saline (PBS), pH 7.2, and purified on Sephadex G-75 (2.8 x 16 cm), with fractions of 6 ml being collected using PBS as the eluant and monitoring at 280 nm. The product 26 eluted in fractions 3 to 7. Recovered 6-aminohexyl ribavirin-5'-O-phosphate was obtained in fractions 8 to 11. The fractions containing the conjugate were combined and ultrafiltered for 20 diavolumes through a UM-2 membrane (1000 Mw cutoff) to remove salts. Lyophilization afforded 40 mg of a white solid.

Ribavirin loading was determined by measuring the amount of phosphate present (B. N. Ames, 1966). A 0.6-ml aliquot of a

solution of 0.53 mg of the conjugate in 4 ml of water was assayed. To a 13 x 100-mm test tube containing the sample was added 0.06 ml of 10% magnesium nitrate hexahydrate in 95% ethanol. This material was carefully ashed by a strong flame until the brown fumes disappeared. This procedure was done twice to ensure complete ashing. The tube was allowed to cool before 0.6 ml of 0.5 N HCl was added. The tube was capped by a marble and heated in a boiling water bath for 15 min to hydrolyze to phosphate any pyrophosphate that might have been formed during the ashing procedure. After cooling, to the tube was added 1.4 ml of a freshly prepared, ice-cold mixture made from 1 part of 10% ascorbic acid (prepared and kept refrigerated--one month stability) and 6 parts of 0.42% ammonium molybdate tetrahydrate in 1 N sulfuric acid (28.6 mL of concentrated sulfuric acid and 4.2 g of ammonium molybdate tetrahydrate diluted to 1000 ml with water). This mixture was incubated at 45°C for 20-25 min and the absorbance was measured at 820 nm against a blank to give a reading of 0.570. A standard curve was prepared using ribavirin-5'-O-phosphate as the organic phosphate source. From this standard the extinction coefficient of phosphate was calculated as 7.45×10^3. Loading was calculated as 9.6 moles of ribavirin per mole of dextran.

(8699-67)

3',5'-O-[1,1,3,3-(Tetraisopropyl)disiloxanyl]ribavirin (29).

A literature procedure was adapted (Gait, 1984). To a solution of 1.002 g (4.469 mmol) of ribavirin (27) in 17 ml of dimethylformamide

(dried over 4 Å molecular sieves) and 3 ml of pyridine (distilled from calcium hydride) was added dropwise over a period of 75 min a solution of 2.10 ml (4.47 mmol) of 1,3-dichloro-1,1,3,3-(tetraisopropyl)disiloxane (28) in 4 ml of dimethylformamide. The reaction mixture was stirred under argon at room temperature for 3 h and then neutralized with 7 ml of 2 M triethylammonium bicarbonate buffer, pH 7.5, prepared by bubbling carbon dioxide into a solution of 2 M triethylamine. The mixture was concentrated at reduced pressure to a white paste, which was diluted with 30 ml of methylene chloride and washed with 25 ml of 1 M aqueous sodium bicarbonate. The methylene chloride layer was washed with water (25 ml) and dried (8 g of magnesium sulfate). Concentration afforded a colorless oil, which was chromatographed (80 g of silica gel, 3 x 24-cm column, 1.5 L of 50-75% ethyl acetate/methylene chloride gradient). The product was eluted in fractions 3 to 5 (125 ml). Concentration afforded 1.14 g (55%) of a clear glass; TLC (50% ethyl acetate/-methylene chloride) R_f 0.29; IR ($CHCl_3$) 3500, 2940, 2860, 1700, 1580, 1460, 1230, 1030, 880 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.0 (s, 4, SiCH), 1.0 (s, 24, CH_3), 4.1 (m, 3, 4'-CH, 5'-CH_2), 4.5 (m, 2, 2',3'-CH), 5.3 (s, 1, 2'-OH), 5.9 (s, 1, 1'-CH), 6.1 and 7.0 (2 s, 2, NH_2), 8.4 (s, 1, 5-ArH).

(8460-1)

2'-O-,N-Bis(tetrahydropyranyl)-3',5'-O-[(1,1,3,3-tetraisopropyl)disiloxanyl]ribavirin (32). To a solution of 1.03 g (2.21 mmol) of 3',5'-O-[(1,1,3,3-tetraisopropyl)disiloxanyl]ribavirin (29)

in 10 ml of acetonitrile and 6 ml of dioxane (distilled from sodium) was added sequentially under argon 0.5 ml (7.1 mmol) of dihydropyran (30) (distilled from sodium) and 0.12 g (0.60 mmol) of anhydrous 2-mesitylsulfonic acid (31) (dried by vacuum). The reaction mixture was stirred at room temperature for 2.5 h [TLC (50% ethyl acetate/-methylene chloride) R_f 0.34, 0.65, 0.84, 0.91], neutralized with 1 ml of methanolic ammonia (saturated) and diluted with 40 ml of methylene chloride. This mixture was washed with 1 M aqueous sodium bicarbonate (20 ml) and water (20 ml), dried (magnesium sulfate), and concentrated to give a viscous, amber liquid, which was chromatographed (100 g of silica gel, 3 x 32-cm column, 2.5 L of 2.5-20% ethyl acetate/methylene chloride gradient). Fraction 16 (125 ml) afforded 1.06 g (67%) of 32 as a clear glass; TLC (10% ethyl acetate/methylene chloride) R_f 0.45, 0.49; IR ($CHCl_3$) 2940, 1630, 1440, 1220, 1060, 920, 890, 830 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.1 (broad s, 24, CH_3), 1.4-2.6 (m, 16, $(CH_2)_3$, SiCH), 3.5-4.7 (m, 11, 2',3',4'-CH, 5'-CH_2, OCHO, OCH_2), 5.10 (m, 1), 5.3 (m, 1), 5.9 (s, 1, 1'-CH), 7.45 and 8.45 (m, 13, ArH), 8.55 (s, 1, 5-ArH).

(8460-5)

2'-O-,N-Bis(tetrahydropyranyl)ribavirin (33). To a solution of 1.06 g (1.48 mmol) of 2'-O-,N-bis(tetrahydropyranyl)-3',5'-O-[(1,1,3,3-tetraisopropyl)disiloxanyl]ribavirin (32) in 20 ml of dioxane (distilled from sodium) was added 4.1 ml (1.63 mmol) of 1 M tetra(n-butyl)ammonium fluoride in tetrahydrofuran. After being stirred at room temperature for 1 h, the reaction mixture was

diluted with 0.6 ml (33 mmol) of water. The mixture was stirred for 5 min more and then triturated with petroleum ether (35-60°C). The solution was chromatographed (60 g of silica gel, 3 x 22-cm column, 0.5 L of 1-8% ethyl acetate/methylene chloride gradient). Fractions 8 to 11 (25 ml) afforded 0.39 g (81%) of 33 as a colorless glass; IR ($CHCl_3$) 3400, 2940, 1690, 1490, 980, 940, 850 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.8 (broad s, 12, $(CH_2)_3$), 3.5-5.0 (m, 11, 2',3',4'-CH, 5'-CH_2, OCHO, OCH_2), 5.4 (m, 1, OH), 6.2 (d, J = 6 Hz, 1, 1'-CH), 7.7 (2 s, 1, NH), 8.7 (s, 1, 5-ArH).

(8460-19)

Attempted Cleavage of 2'-O-,N-Bis(tetrahydropyranyl)ribavirin (33).

Method A. A solution of 5.2 mg of 2'-O-,N-bis(tetrahydropyranyl)ribavirin (33) in 1.0 ml of 0.01 M HCl, pH 2, and 1.0 ml of dioxane (distilled from sodium) was stirred at room temperature for 20 h [TLC (10% methanol/methylene chloride) R_f 0.66 and no 0.77 (33) or 0.28 (27)]. The reaction mixture was heated at 70°C for 3 days, at which time TLC showed no change.

(8460-46)

Method B. A solution of 3.7 mg of 2'-O-,N-bis(tetrahydropyranyl)ribavirin and 0.01 g (0.05 mmol) of anhydrous 2-mesitylsulfonic acid in 2 ml of methanol was stirred overnight under argon [TLC R_f 0.66 and no 0.77 (33) or 0.28 (27)]. The solution was then

heated at reflux for 2 days, but the R_f values of the reaction mixture remained unchanged.

(8460-46)

5'-O-(4,4'-Dimethoxytrityl)ribavirin (34). To a stirred suspension of 3.00 g (12.2 mmol) of ribavirin (27) in 40 ml of anhydrous pyridine (distilled from calcium hydride) was added, in four portions at intervals of 1 h, 4.83 g (14.1 mmol) of 4,4'-dimethoxytrityl chloride (DMTrCl, 34). The dark-orange reaction mixture was stirred for 18 h at, room temperature under argon and then concentrated at reduced pressure. The residue was diluted with dichloromethane (300 ml), washed with 5% aqueous sodium bicarbonate (200 ml), which was then back-extracted with dichloromethane (100 ml), washed with water (100 ml), and dried (sodium sulfate). Concentration afforded 10.7 g of a dark-yellow gum, which was chromatographed (200 g of silica gel, 4.5 x 46-cm column, 2.5 L of 0-10% methanol/dichloromethane gradient) to give in fractions 11 to 14 (500 ml) 5.09 g (77%) of 34 as a white solid; TLC (5% methanol/dichloromethane) R_f 0.16; IR (KBr) 3000-3500, 1700, 1620, 1520, 1180, 1030, 830 cm^{-1}. Anal. Calcd for $C_{29}H_{30}N_4O_7$: C, 63.72; H, 5.53; N, 10.25. Found: C, 63.69; H, 5.52; N, 10.17.

(8659-10)

2'-O-t-Butylsilyldimethyl-5'-O-(4,4'-dimethoxytrityl)ribavirin (37). Two different procedures were developed for the preparation of 37.

Method A (Dimethylformamide and Imidazole). The procedure of Ogilvie et al. (1978) was used. To a solution of 8.0 g (14.6 mmol) of 5'-O-(4,4'-dimethoxytrityl)ribavirin (35) in 60 ml of dimethylformamide (distilled from calcium hydride) was added in succession 1.60 g (23.5 mmol) of imidazole and 2.30 g (15.2 mmol) of t-butyldimethylsilyl chloride (36). The mixture was stirred at room temperature under argon for 20 h. The reaction mixture was quenched with 5% aqueous sodium bicarbonate (50 ml) and extracted with dichloromethane (3 x 50 ml). The extracts were washed with water (3 x 50 ml), dried (sodium sulfate), and concentrated at reduced pressure to give 13.7 g of a dark-yellow glass, which was chromatographed (200 g of silica gel, 4.5 x 42-cm column, 3.5 L of 0-100% ethyl acetate/dichloromethane gradient) to give three silylated products; TLC (50% ethyl acetate/dichloromethane) R_f 0.23 (37), 0.52 (38), and 0.77 (39):

2'-O-t-Butyldimethylsilyl-5'-O-dimethoxytritylribavirin (37): 0.3 g (3 %); TLC R_f 0.52; IR (KBr) 3000-3500, 2980, 2880, 1680, 1600, 1520, 1460, 1260, 1180, 1040 cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 0.02 (2 s, 6 H, $SiCH_3$), 0.81 (s, 9 H, $SiCCH_3$), 3.00 (m, 2 H, 5'-CH_2), 3.71 (s, 6 H, OCH_3), 4.05 (m, 4'-CH), 4.28 (m, 1, 3'-CH), 4.57 (m, 1, 2'-CH), 5.16 (m, 1 H, 3'-OH), 5.93 (m, 1 H, 1'-CH), 6.85 (m, 4 H, ArH),

7.22 (m, 7 H, ArH), 7.35 (m, 2 H, ArH), 7.67 and 7.68 (2 s, 2 H, NH_2), 8.89 (s, 1 H, 5-ArH); ^1H NMR (Me_2SO-d_6/D_2O) δ same spectrum except disappearance of 3'-OH at 5.16 ppm. Anal. Calcd for $C_{35}H_{44}N_4O_7Si$: C, 63.61; H, 6.71; N, 8.47. Found: C, 13.31; H, 7.05; N, 8.24.

3'-O-t-Butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)ribavirin (38): 1.29 g (13%); TLC R_f 0.16; IR (KBr) 3000-3500, 2980, 2880, 1700, 1620, 1580, 1520, 1460, 1260, 1180, 1040, 840 cm^{-1}; ^1H NMR (Me_2SO-d_6) 0.02 and 0.04 (2 s, 6 H, $SiCH_3$), 0.78 (s, 9 H, $SiCCH_3$), 3.00 (m, 2 H, 5'-CH_2), 3.70 (s, 6 H, OCH_3), 4.00 (m, 1 H, 4'-CH), 4.38 (m, 1 H, 3'-CH), 4.50 (m, 1 H, 2'-CH), 5.50 (m, 1 H, 2'-OH), 5.90 (m, 1 H, 1'-CH), 6.85 (m, 4 H, ArH), 7.20 (m, 7 H, ArH), 7.32 (m, 2 H, ArH), 7.63 and 7.64 (2 s, 2 H, NH_2), 8.90 (s, 1, 5-ArH); ^1H NMR (Me_2SO-d_6) δ same spectrum except disappearance of 2'-OH at 5.50 ppm. Anal. Calcd for $C_{35}H_{44}N_4O_7Si$: C, 63.61; H, 6.71; N, 8.47. Found: C, 63.35; H, 6.83; N, 8.17.

(8659-7,13)

2',3'-O-Bis(t-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-ribavirin (39): 2.60 g (27%); TLC R_f 0.77; IR (KBr) 3000-3500, 2960, 2880, 1700, 1640, 1520, 1460, 1270, 1190, 1050, 840 cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 0.01, 0.06, 0.09, and 0.20 (4 s, 12 H, $SiCH_3$), 0.75 and 0.78 (s, 18 H, $SiCCH_3$), 3.05 (dd, J = 4 Hz, 2 H, 5'-CH_2), 3.71 (s, 6 H, OCH_3), 4.00 (m, 1 H, 4'-CH), 4.20 (m, 1 H, 3'-CH), 4.65 (m, 1 H, 2'-CH), 5.90 (d, J = 5 Hz, 1 H, 1'-CH), 6.88 (m, 4 H, ArH), 7.28 (m,

7 H, ArH), 7.38 (m, 2, ArH), 7.60 and 7.68 (2 s, 2 H, NH_2); 8.95 (s, 1 H, 5-ArH); ^{1}H NMR (Me_2SO-d_6/D_2O) δ no exchangeable hydroxyl protons. Anal. Calcd for $C_{41}H_{58}N_4O_7Si_2$: C, 63.53; H, 7.54; N, 7.22. Found: C, 63.79; H, 7.34; N, 7.66.

Method B (Pyridine and Silver Nitrate). The procedure of Hakimelahi et al. (1981) was used. To a solution of 10.25 g (18.7 mmol) of 5'-O-(4,4'-dimethoxytrityl)ribavirin (35) in 20 ml of tetrahydrofuran (distilled from potassium benzophenone ketyl) and 7.0 ml (86 mmol) of anhydrous pyridine (distilled from calcium hydride) was added 3.81 (22.4 mmol) of silver nitrate. The reaction mixture was stirred for 30 min at ice-bath temperature before 3.66 g (24.3 mmol) of t-butyldimethylsilyl chloride (36) was added in one portion. A white emulsion formed instantly. The reaction mixture was slowly warmed to room temperature and stirred for 20 h under argon, while protected from light, and filtered into 5% aqueous sodium bicarbonate (75 ml). The filtrate was extracted with dichloromethane (3 x 75 ml). The extracts were washed with water (3 x 50 ml), dried (sodium sulfate) overnight, and concentrated at reduced pressure to give 15 g of a white foam, which was chromatographed twice to give 1.38 g (9%) of 2'-O,3'-O-bis(t-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)ribavirin (39), 3.99 g (32%) of 2'-O-t-butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)ribavirin (37), and 0.75 g (8%) of 3'-O-t-butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)ribavirin (38).

(8659-23)

Deprotection of 2'O-t-Butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)ribavirin (37). A reported procedure (Gait, 1984) was modified. A solution of 4.75 mg of 2'O-t-butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)ribvavirin (37) in 0.5 ml of 0.01 M HCl, pH 2, and 0.5 ml of dioxane (distilled from sodium) was stirred for 3.5 h at room temperature [TLC (5% methanol/methylene chloride) R_f 0.0, 0.12, 0.36, 0.86]. Stirring was continued for 3 days [TLC (25% methanol/methylene chloride) R_f 0.24 (ribavirin)].

(8460-46)

Deprotection of 3'-O-t-Butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)ribavirin (38). A solution of 8.00 mg of 3'-O-t-butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)ribavirin (38) in 0.5 ml of 0.01 M HCl, pH 2, and 0.5 ml of dioxane (distilled from sodium) was stirred for 3.5 h at room temperature [TLC (5% methanol/methylene chloride) R_f 0.0, 0.12, 0.36, 0.86]. Stirring was continued for 4 h more [TLC (25% methanol/methylene chloride) R_f 0.24 (ribavirin)].

(8460-46)

Desilylation of Mono- and Bissilylated 5'-O-(4,4'-Dimethoxytrityl)ribavirin. A solution of 7.43 g of a mixture of 3'-O-(t-butyldimethylsilyl)- and 2',3'-O-bis(di-t-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)ribavirin in 40 ml of 1 M tetra(n-butyl)-ammonium fluoride (40 mmol) in anhydrous tetrahydrofuran was stirred for 3 h at room temperature under argon. The progress of the

desilylation was monitored by TLC (10% methanol/methylene chloride). After disappearance of the starting material, the solution was concentrated and the residue was dissolved in 125 ml of methylene chloride. This solution was washed with water (2 x 20 ml), which was back-extracted with 80 ml of methylene chloride. The combined extract was dried (ammonium sulfate) and concentrated to give 8.2 g of a yellow solid, which was chromatographed on silica gel (3 x 29 cm, 0-10% methanol/methylene chloride gradient) to give 5.46 g (100% recovery) of 5'-O-(4,4'-dimethoxytrityl)ribavirin as a white solid.

(8659-38)

2'-O-t-Butyldimethylsilyl-5'-O-dimethoxytritylribavirin-3'-O-[(N,N-diisopropyl)(methyl)phosphoramidite] (43). A literature procedure was adapted (Usman et al., 1987). In a 50-ml round-bottomed flask equipped with a magnetic stirring bar and an argon bubbler were placed 3.9 ml (2.89 g, 22.4 mmol) of N,N-diisopropylethylamine (41), 1.40 (1.43 g, 7.26 mmol) of (N,N'-diisopropylamino)methylphosphoramidic chloride (2), 140 mg (1.14 mmol) of 4-dimethylaminopyridine (42), and 20 ml of tetrahydrofuran distilled from sodium/benzophenone. A solution of 3.7 g (5.59 mmol) of 2'-O-t-butyldimethylsilyl-5'-O-dimethoxytritylribavirin (37) in 25 ml of dry tetrahydrofuran was added dropwise using a pressure-equalizing dropping funnel over a period of 2 h. The cloudy solution was stirred at room temperature under argon for 20 h. The reaction mixture containing precipitated salts was diluted with ethyl acetate

(250 ml) and washed with brine (5 x 50 ml). The slightly yellow organic extracts were dried (sodium sulfate) and concentrated by rotary evaporation and on a vacuum pump to give 4.64 g of pale-yellow foam. This material was chromatographed (3 x 20-cm silica gel column, 600 ml of methylene chloride/hexane/triethylamine 55:35:10), to give after evaporation 3.27 g (71%) of 43 as a colorless foam; TLC (hexane/ethyl acetate/triethylamine 1:1:0.05) R_f 0.44; IR (KBr) 3450, 2650-2950, 1690, 1520, 1480, 1260, 1200, 1040, 840 cm^{-1}; ^1H NMR ($CDCl_3$) [pairs of diasteromeric signals (1:0.57) because of phosphorus] δ -0.08, -0.06, 0.04, and 0.06 (4 s, 6, $Si(CH_3)_2$), 0.82, 0.83, 0.98 and 1.00 (4 s, 9, $SiC(CH_3)_3$), 1.15 and 1.28 (m, 12, $N[CH(C\underline{H}_3)_2]_2$), 3.12 and 3.14 (m, 2, 5'-CH_2), 3.55 (m, 2, $N[CH(C\underline{H}_3)_2]_2$), 3.79 (3 s, 9, $ArOCH_3$, $POCH_3$), 4.28 and 4.30 (m, 1, 4'-H), 4.34, and 4.40 (m, 1, 3'-H), 4.92 and 4.98 (m, 1, 2'-H), 5.82 and 5.88 (dd, J = 15 Hz, J = 1 Hz, 1, 1'-H), 6.80 (m, 4, ArH), 7.24 (m, 7, ArH), 7.40 (m, 2, ArH), 7.52 (dd, J = 8 Hz, J = 1 Hz, 2, NH_2), 8.38 and 8.40 (d, J = 14 Hz, 5-ArH); ^{31}P NMR ($CDCl_3$) (internal standard 85% H_3PO_4) 147.21 and 148.61 ppm. Anal. Calcd for $C_{41}H_{65}N_5O_8P$: C, 61.36; H, 7.35; N, 8.51. Found: C, 61.31; H, 7.58; N, 8.38.

(8659-48)

5'-O-(6-Aminohexyl)phosphate of Poly(ribavirin-3',5'-O-phosphate)$_{15}$-thymidine-5'-O-phosphate Triethylammonium Salt (48).

1. Polymer Synthesis. A literature procedure (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 terminated by a protected 6-aminohexyl phosphate. The cycle modified for the synthesis of an RNA analog is given in Figure I-1. 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 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 5/0.45 M 1H-tetrazole	12
4	wait	480
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-1. Automated synthesis cycle.

The trityl protecting group was removed from the 5'-O-position of the nucleoside by treatment with 3 ml of 3% trichloroacetic acid in methylene chloride. A 0.1 M solution of 2'-O-t-butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)ribavirin-3'-O-[(N,N-diisopropyl)-(methyl)phosphoramidite] (43) in 0.18 ml of acetonitrile (0.162 g of 5/2.00 ml of acetonitrile) was activated by 0.18 ml (0.081 mmol) of 0.45 M 1H-tetrazole (9) 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 in 2,6-lutidine/-tetrahydrofuran 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 tetrahydrofuran/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 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-diisopropyl)phosphoramidite (46) 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 97%. Twelve 1-umole 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 (46) was removed from the columns and placed in four 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/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 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 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 3.0 ml of 1 M tetra(n-butyl)ammonium fluoride in tetrahydrofuran overnight to remove the 2'-O-silyl protecting groups. The reaction mixture was quenched with 3.0 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 acetate containing one drop of triethylamine and apportioned among twelve 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 50 L of 90% deionized formamide and the contents of each was apportioned among three PAGE wells (1.2 mm x 1.5-mm well, 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 one well 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 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 triethylammonium acetate, and extracted for 24 h at room temperature to elute the oligomer. This extraction process was repeated. 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 12 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 (30 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 triethylammonium acetate for purification by reverse-phase column chromatography on 24 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 triethylammonium 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 6.02 mg of 48 as a white solid.

5. Oligomer Characterization. This residue was dissolved in 10 ml of water for spectrophotometric analysis based on thymidine concentration. The absorbance at 260 nm was 0.779. Based on an extinction coefficient for thymidine of 8.8×10^4, the overall yield was 6.0 mg (7.4%). Based on the 97% 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 (Bio-Rad MA7Q anion exchange column, 20 mM Tris·HCl, pH 8.5, to 20 mM Tris·HCl, pH 8.5, 500 mM NaCl over 10 min at 1.0 ml/min, 260 nm) t_R 10.0 min (96%).

(8554-87)

Synthesis of the 5'-O-(6-Aminohexyl)phosphate of Poly-(thymidine-3',5'-O-phosphate)$_9$-thymidine-5'-O-phosphate Triethylammonium Salt.

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)$_9$-thymidine-5'-O-phosphate triethylammonium salt 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'-di ethoxytrityl)thymidine-3'-O-[(N,N-diisopropyl)-(methyl)phosphoramidite] in 0.15 ml of dry acetonitrile was activated with 0.15 ml of 0.45 M 1H-tetrazole in acetonitrile and then allowed to react with the deprotected 5'-hydroxyl group of thymidine attached via a 3'-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 10 thymidine units. The addition of the last unit in the sequence again required the reaction of the amino-linker phosphoramidite reagent [6-(N-4-methoxytritylamino)hexyl](2-cyanoethyl)(N,N-diisopropyl)phosphoramidite (46) 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 15 ml 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. To this residue was added 3 ml of 0.05 M triethylammonium acetate. Aliquots (0.50 ml) of this solution were added to each of six 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 triethylammonium 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 triethylammonium acetate to give a white solid, which was enriched in the desired oligomer.

4. Oligomer Characterization. The oligomer was dissolved in 90 ml of water. Its absorption at 260 nm was 0.656, indicating that the molarity of the thymidine units was 7.45×10^{-5} M. If no other UV absorbing material was present, the yield was 2.6 mg (21%).

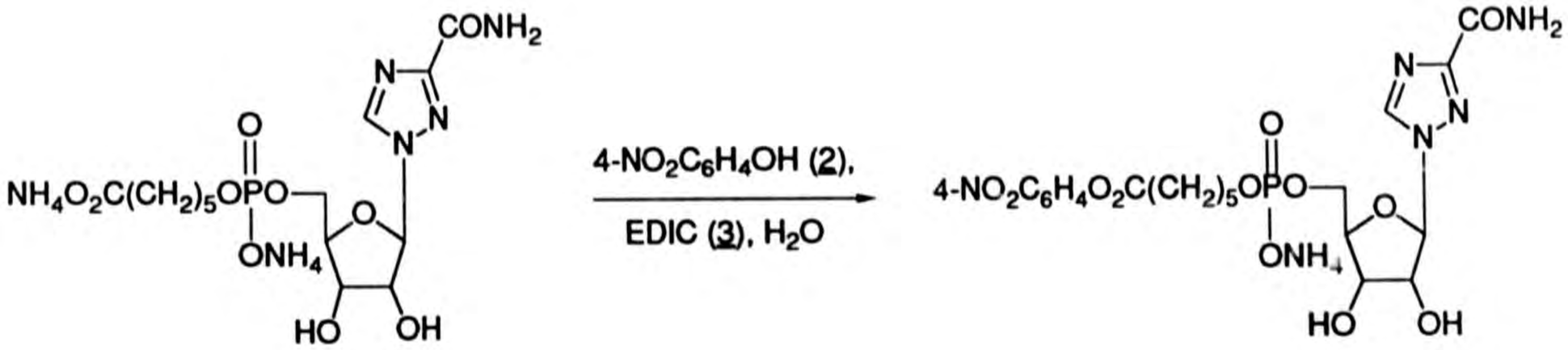
C. Results

Methods were investigated for coupling ribavirin-5'-O-(5-carboxypentyl)phosphate bisammonium salt (1, Scheme I-1) to MAbs. We had previously found that coupling could be achieved in modest yields using the corresponding derivative of 2',3'-dideoxycytidine, 6-aminohexyldextran, and EDIC as the coupling agent. Unfortunately, this methodology failed with 1. The 3'-hydroxyl group of the sugar ring of 1 may interfere in the reaction. We had previously demonstrated that the 4-nitrophenyl ester of ribavirin-5'-O-hemisuccinate could be prepared using dicyclohexylcarbodiimide and then coupled to a MAb. The 4-nitrophenyl group on the ester activates the carboxyl group to nucleophilic displacement and concomitantly releases the 4-nitrophenoxide anion, which is detected spectroscopically at 404 nm to determine indirectly the loading of ribavirin-5'-hemisuccinate onto the MAb. This methodology introduced about four ribavirins

onto the Mab as determined by 4-nitrophenoxide release. Because the linkage at the 5'-position of ribavirin is a carboxylate ester, which is labile, this conjugate was not deemed suitable for scale-up for in vivo studies and the more stable 5'-O-phosphate linkage was explored.

To prepare the 4-nitrophenyl ester 4, the bisammonium salt 1 was first converted to the bispyridinium salt to avoid formation of the carboxamide group (Scheme I-1). Coupling of the bispyridinium salt in pyridine using dicyclohexylcarbodiimide failed because of the low solubility of the salt in pyridine. However, the ester could be formed using EDIC (3) in water, in which the starting material was readily soluble. The extent of 4-nitrophenyl ester formation was determined from the ^{1}H NMR spectrum of the crude product. Two preliminary conjugations on MAb PC4.9A6 and albumin were attempted using 4-nitrophenyl ester 4. Unfortunately, only hydrolysis occurred in phosphate-buffered saline (PBS), pH 7.2, and no acylation of protein was detected. Perhaps the 4-nitrophenyl ester formed from the 6-substituted hexanoic acid was too lipophilic for nucleophilic attack by the ε-amino group of the protein lysines and, therefore, only attack by hydroxide could occur. Acylation in mixed solvent systems (water with dimethylformamide, tetrahydrofuran, or dioxane, which would 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.

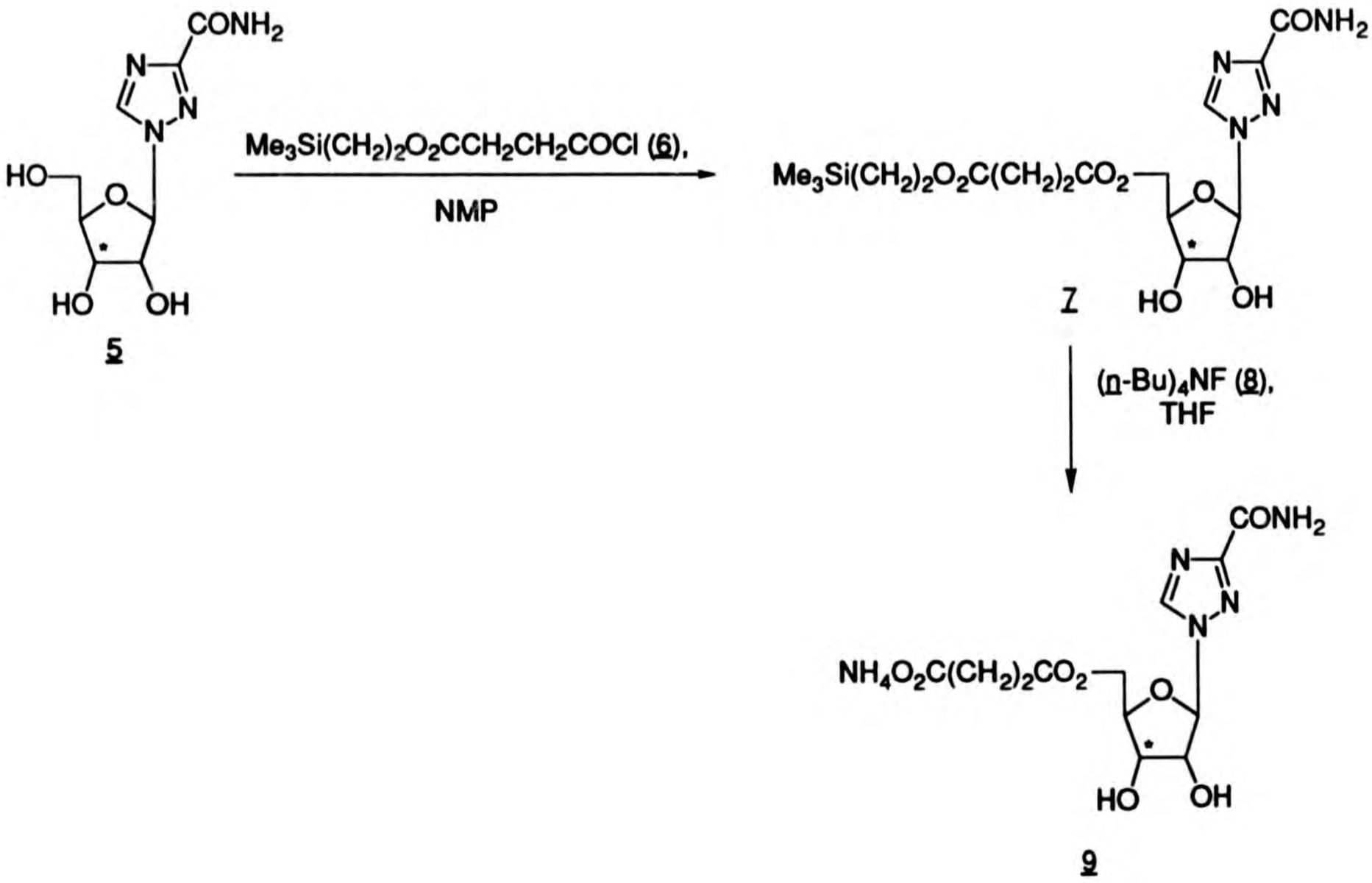


1 4

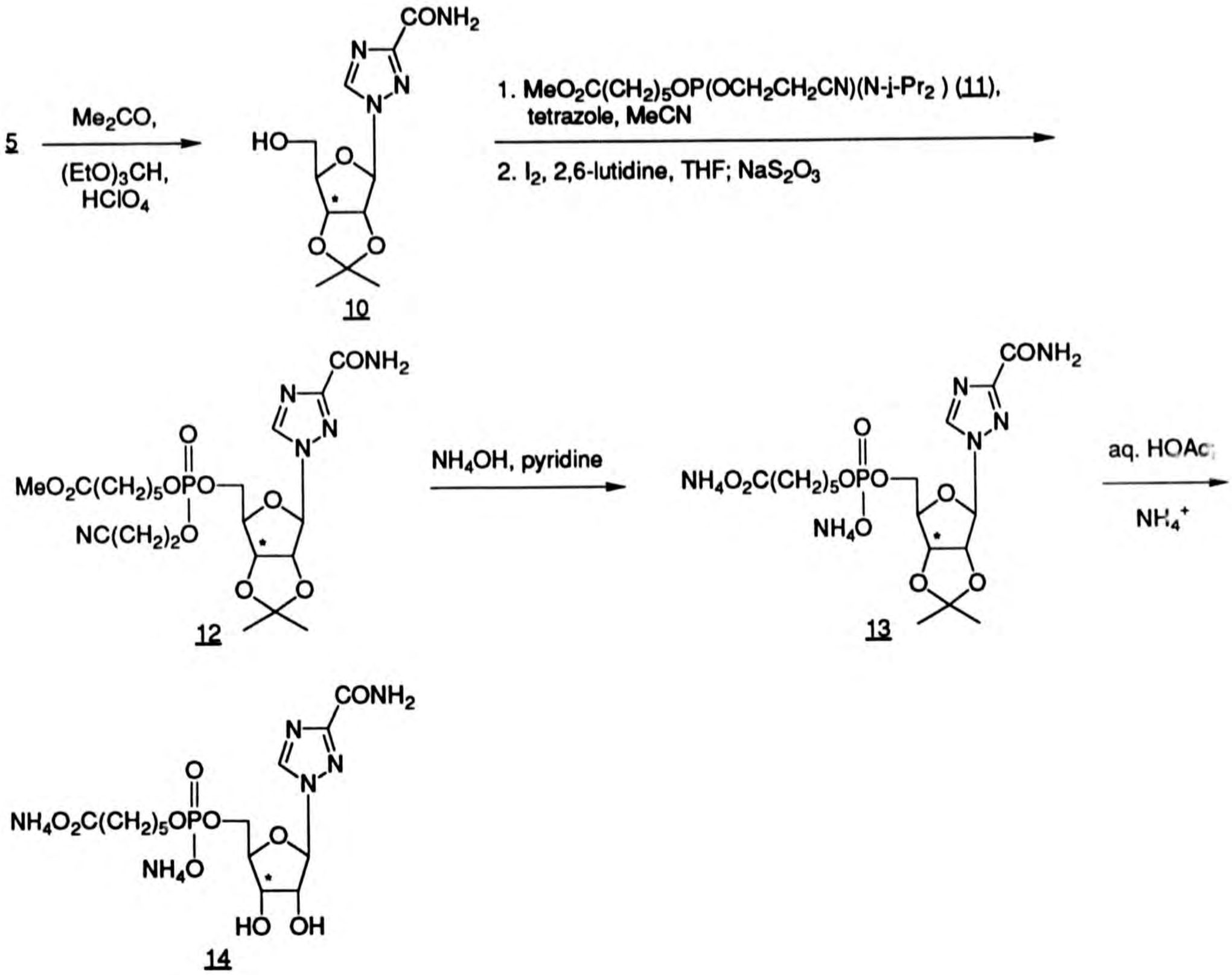
Scheme I-1. Synthesis of Ribavirin-5'-O-[5-Carbo-(4-nitrophenoxy)pentyl]phosphate (4)

Therefore, alternative methods of preparing conjugates were investigated. Because of the absence of a strong chromphore for ribavirin, we elected to use radiolabel as one method to determine loading. Therefore, [3'-^{14}C]ribavirin-5'-O-hemisuccinate bis-ammonium salt (9) and [3'-^{14}C]ribavirin-5'-O-(5-carboxypentyl)-phosphate bisammonium salt (14) were prepared by the routes outlined in Schemes I-2 and I-3, respectively. Although radioimmunoassay (loading of two ribavirins per antibody) and 4-nitrophenoxide release (loading of four ribavirins per antibody) indicated conjugate formation between MAb and 9, using the present sample of [^{14}C]9 and EDIC, no coupling with either MAb PC4.9A6 or ovalbumin was detected although the procedure of Austin et al. (1983) for the

preparation of the ribavirin-5'-O-hemisuccinate-ovalbumin conjugate was employed. In this present case, longer chromatographic times were employed rather than dialysis; perhaps hydrolysis occurred, releasing the free ribavirin, because no radioactivity was detected in the protein fractions.



Scheme I-2. Synthesis of [3'-^{14}C]Ribavirin-5'-O-hemisuccinate Bisammonium Salt (9)

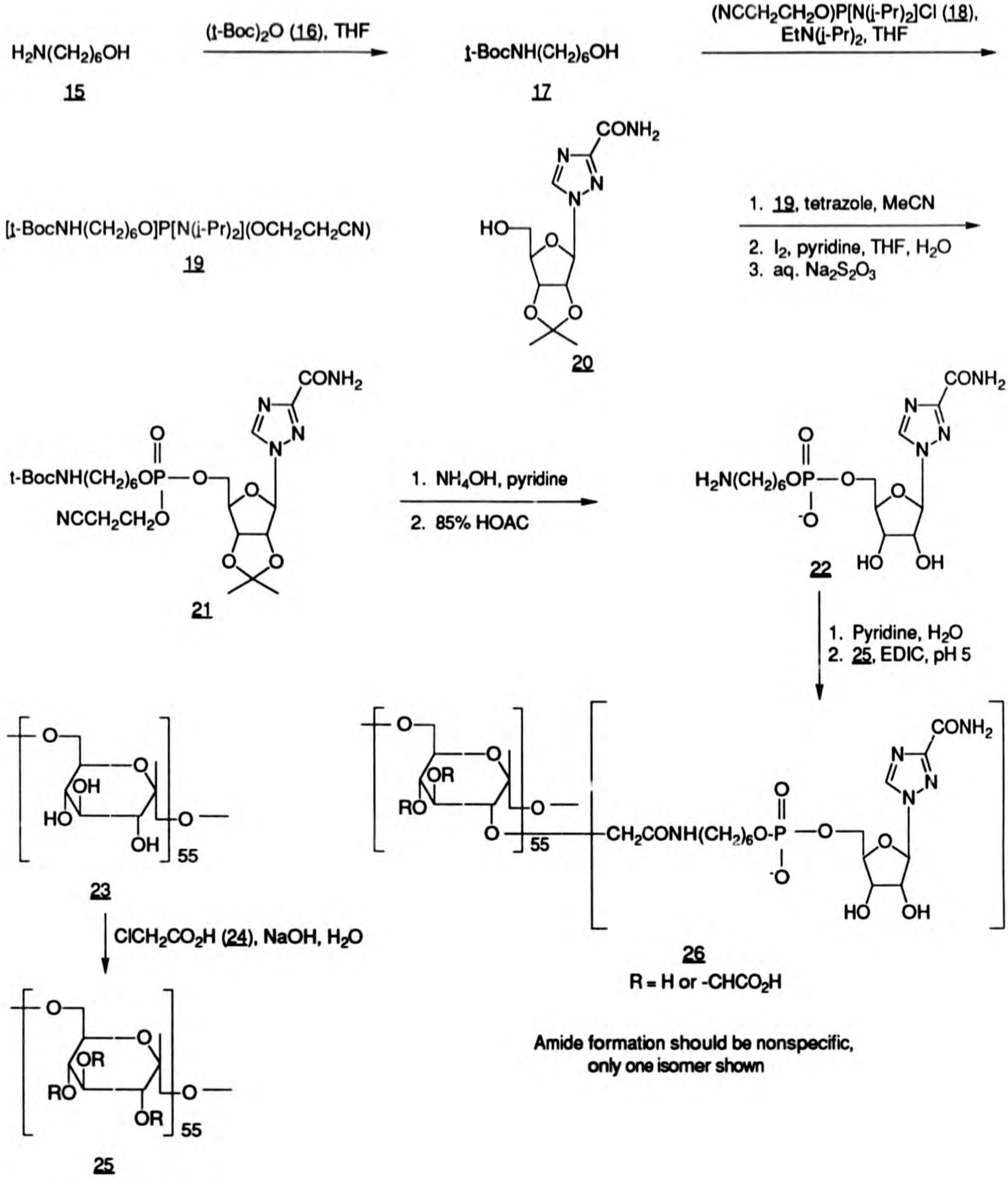


Scheme I-3. Synthesis of [3'-^{14}C]Ribavirin-5'-O-(5-carboxypentyl)phosphate Bisammonium Salt (14)

Because concurrent work on preparing conjugates using the 5'-O-(5-carboxypentylphosphate) of ribavirin was not promising, studies using [^{14}C]14 to accurately measure loading were postponed and research was directed toward the 5'-O-(6-aminohexylphosphate) derivative (22), instead. To achieve higher loadings, several molecules of the ribavirin derivative were first to be linked onto a polymer, which would then be linked to the MAb. The polymer selected was carboxymethyldextran (Mw 12,200), which was prepared by

alkylation of dextran (Mw 9,000) with chloroacetic acid in the presence of potassium hydroxide. Titration indicated that 58 carboxymethyl groups (one per glucose residue) were loaded onto the dextran. The carboxyl groups would be used to form amide bonds with the amino groups of the tether on ribavirin and the MAb. Ribavirin-5'-O-(6-aminohexyl)phosphate ammonium salt (22) was first prepared and then linked to carboxymethyl dextran (25) by the routes outlined in Scheme I-4. The starting material for this synthesis was ribavirin-2',3'-O-acetonide (20), which was prepared by treatment of ribavirin with acetone in triethyl orthoformate in the presence of perchloric acid. The conversion of 20 to 22 using (2-cyanoethyl)-(N,N-diisopropylamino)[6-(N-trifluoroacetylaminohexyl)]-phosphoramidite was described in Annual Report No. 2 (M. I. Dawson et al., 1988). To increase the stability of the phosphoramidite to chromatographic purification on silica gel, the synthesis outlined in Scheme I-4 was modified to use the t-butyloxycarbonyl (t-Boc) group rather than the trifluoroacetyl group for protecting the primary amine. After removal of the 2-cyanoethyl protecting group by treatment with ammonium hydroxide/pyridine and the acetonide and t-Boc protecting groups by treatment with 85% acetic acid, the product (22) was obtained by preparative thin-layer chromatography on silica gel using 5% ammonium hydroxide in methanol as the eluant.

To ensure that the carboxyl groups on the carboxymethyldextran were not converted to primary amide groups during the coupling of the 6-aminohexylphosphate derivative, the ammonium counter ions were



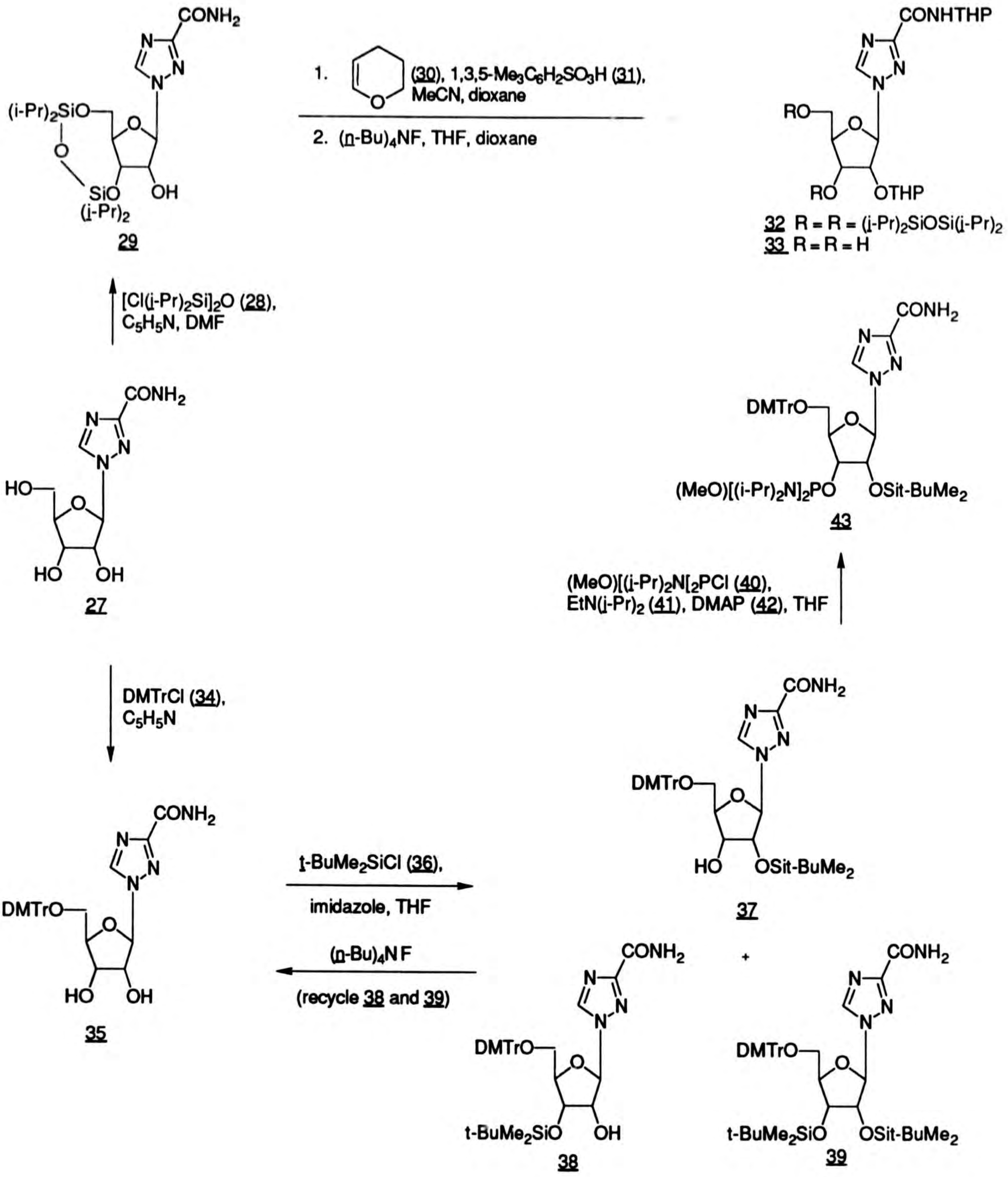
Scheme I-4. Synthesis of Conjugate 26 of Ribavirin-5'-O-(6-aminohexyl)phosphate with Carboxymethyldextran

replaced with pyridinium ions. Coupling was effected with EDIC. A loading of ten molecules of the derivative onto the functionalized dextran was established using a colorimetric assay for inorganic phosphate (Ames, 1966). Work was initiated to tether the functionalized dextran to the MAb. Thus far, conjugate yields have been low using EDIC as the coupling reagent, and purification of the conjugate from unreacted dextran has proved problematic. Carboxymethyldextran behaves as an extended rigid-rod polymer, showing a far larger hydrodynamic volume than a globular protein of similar molecular weight would display. In fact, this modified polymer is excluded in the void volume on chromatography on Sephadex G-150. Purification of the conjugate from the dextran by size-exclusion has not been successful. Ion exchange chromatographic separation has also been ineffective. We have recently found a report of separation of the two using ammonium sulfate precipitation (Schechter et al., 1987), which we are now pursuing.

The synthesis of the 5'-O-(6-aminohexyl)phosphate of poly(ribavirin-3',5'-O-phosphate)$_{15}$-thymidine-5'-O-phosphate triethylammonium salt (48) (Scheme I-6) has been achieved. This oligomer was designed to give multiple loadings of ribavirin onto the MAb. As with the monomeric version, phosphatases were expected to cleave the oligomer, releasing ribavirin. The terminal aminohexyl tether group of 48 provides the tether functionality for linkage directly to the MAb or to a polymeric carrier. The terminal thymidine group was used to initiate the synthesis on a solid support and serve as a

chromophore for detection and quantitation. The first synthetic target in the synthesis of 48 was a ribavirin phosphoramidate reagent, which necessitated the protection of the 3'-hydroxyl group of ribavirin. Tetrahydropyranyl was the first protective group investigated. The 3'- and 5'-hydroxyl groups of ribavirin were first protected as the 1,1,3,3-(tetraisopropyl)disiloxanyl cyclic ether, permitting selective tetrahydropyranylation at the 2'-hydroxyl (Scheme I-5). Unfortunately, under the conditions for formation of the tetrahydropyranyl ether, the amide also reacted, giving the bistetrahydropyranyl derivative 32. Cleavage of the amide protecting group under conditions that would remove the 2'-O-tetrahydropyranyl ether failed.

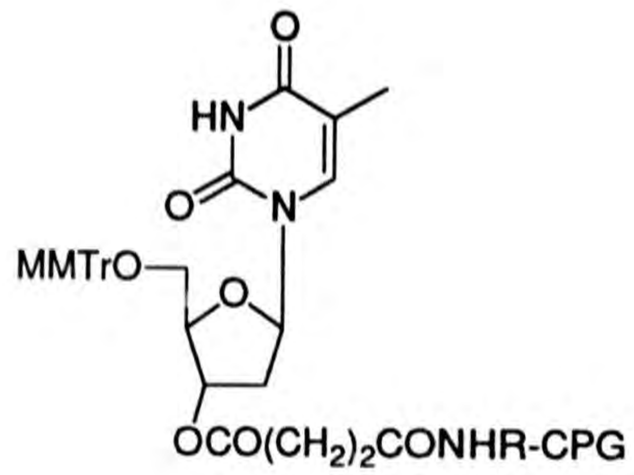
Therefore, the t-butyldimethylsilyl protecting group was investigated next. The 5'-hydroxyl of rivavirin was protected as the 4,4'-dimethoxytrityl ether to give 35, which was allowed to react with t-butyldimethylsilyl chloride (36) in the presence of imidazole (Ogilvie et al., 1978; Ogilvie et al., 1979; Hakimelahi et al., 1981). Like the natural nucleosides, the aromatic ring system of ribavirin permited selective protection of the less hindered 2'-hydroxyl group. A mixture of the 2'- and 3'-O-t-butyldimethylsilyl ethers (37 and 38, respectively) and the 2',3'-O-bis-t-butyldimethylsilyl ether 39 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 to regenerate 35. The position of the



Scheme I-5. Synthesis of a Ribavirin Phosphoramidate Reagent 43

t-butyldimethylsilyl protecting group was established by ^1H NMR spectroscopy in Me_2SO-d_6 and Me_2SO-d_6/D_2O. The signals for the 3'-proton of 37 and the 2'-proton of 38 collapsed after addition of D_2O because of exchange of the hydroxyl protons. Olgilvie and coworkers (1979) also determined th sition of silylation on the ribofuranose ring by comparision of the ^1H NMR spectra with the ^{13}C NMR spectra. Silylation of a hydroxyl group led to a downfield shift of the proton to which the silyl group was attached. For example, in the present case, silylation of 35 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 43 by an in situ generation of the phosphatidylating reagent using (N,N-diisopropylamino)methylphosphoramidic chloride (40), N,N-diisopropylamine (41), and a catalytic amount of 4-dimethylaminopyridine (DMAP, 42) in tetrahydrofuran. After workup and chromatography, a 71% yield of 43 was obtained.

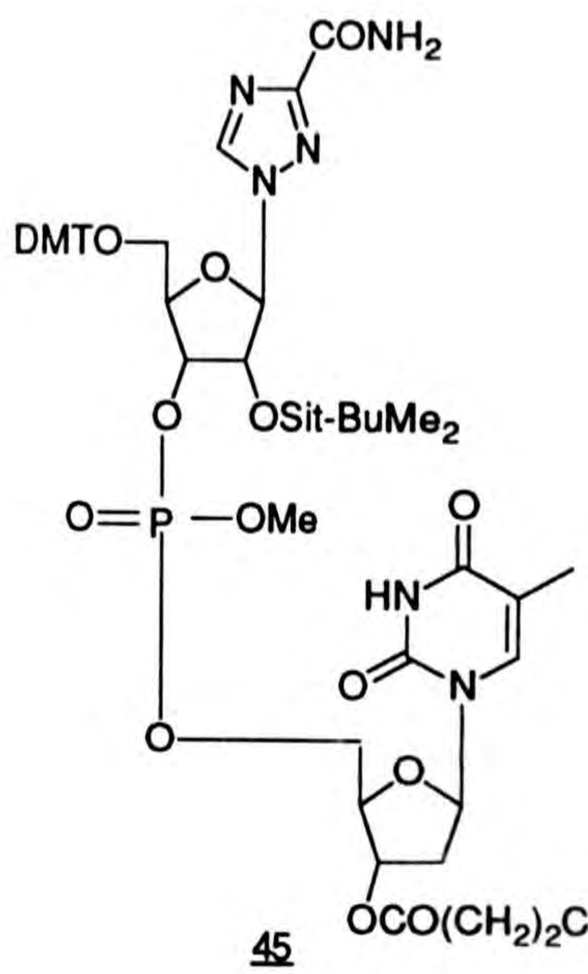
Phosphoramidite 43 was used to prepare the 5'-O-(6-aminohexyl)phosphate of poly(ribavirin-3',5'-O-phosphate)$_{15}$-thymidine-5'-O-phosphate (48) by the sequence outlined in Scheme I-6. This oligoribonucleotide was prepared on a solid support using an Applied BioSystems DNA Synthesizer, with modifications of the program to account for the decreased reactivity of the phosphoramidite because of the steric hindrance caused by the t-butyldimethylsilyl ether protecting group of the 2'-hydroxyl function. The procedure



44

1. Cl_3CCO_2H, CH_2Cl_2
2. MeCN wash
3. 43, tetrazole, MeCN

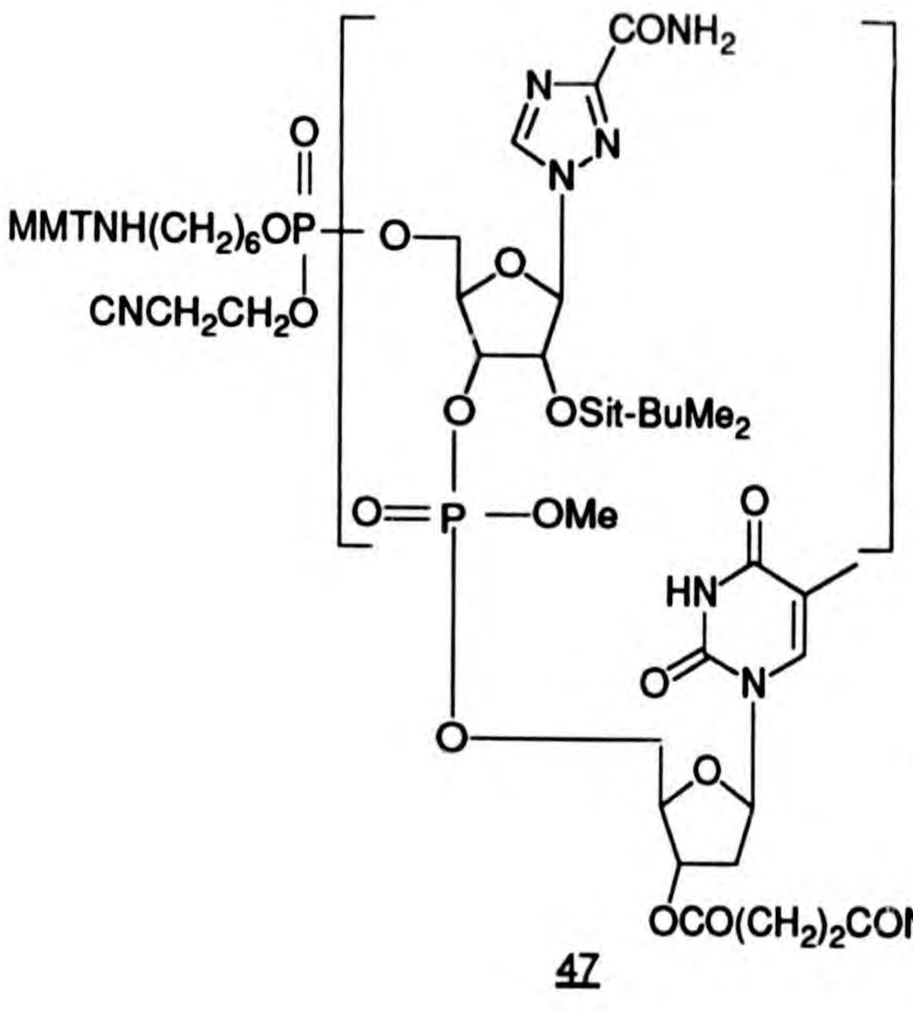
4. Wait
5. Ac_2O, DMAP, THF
6. I_2, pyridine, THF, H_2O



45

1. Steps 1-7 repeated 14 times
2. Cl_3CCO_2H, CH_2Cl_2
3. MeCN wash
4. $[MMTNH(CH_2)_6O]P[N(i\text{-}Pr)_2](OCH_2CH_2CN)$ (46), tetrazole, MeCN

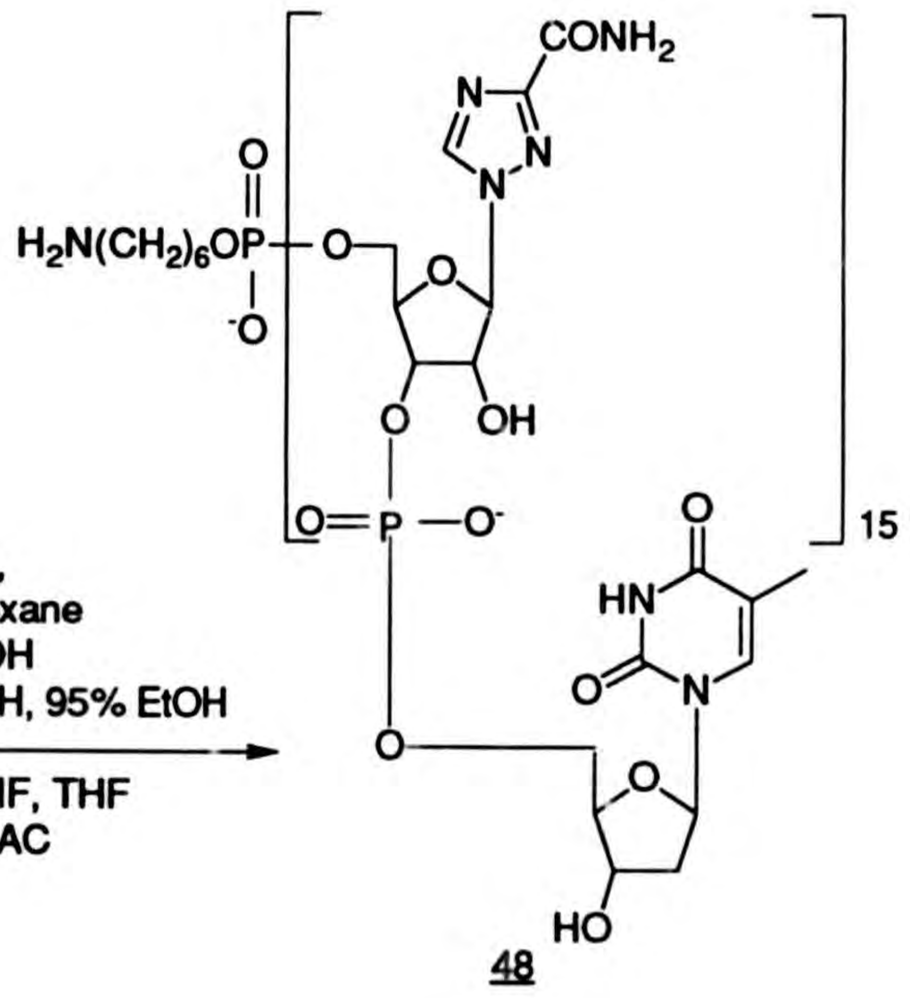
5. Wait
6. Ac_2O, DMAP, THF
7. I_2, pyridine, THF, H_2O



47

1. HSC_6H_5, Et_3N, dioxane
2. 95% EtOH
3. c. NH_4OH, 95% EtOH

4. $(n\text{-}Bu)_4NF$, THF
5. 80% HOAC



48

Scheme I-6. Synthesis of 5'-O-(6-Aminohexyl)phosphate of Poly(ribavirin-3',5'-O-phosphate)$_{15}$-thymidine-5'-O-phosphate Triethylammonium Salt (48)

followed that developed by Olgilvie and co-workers for the synthesis of poly(ribonucleotides) (Usman et al., 1987). The synthesis was conducted on controlled pore glass (CPG) to which thymidine had been attached through a 3'-O-hemisuccinate group. The 5'-O-monomethoxytrityl protecting group on thymidine was first removed by treatment with trichloroacetic acid to permit formation of a phosphite ester linkage between the 5'-O-position of the thymidine and the 3'-O-position of the ribavirin phosphoramidite 43. Unreacted hydroxyl groups on thymidine were blocked to further reaction by acetylation in the presence of DMAP before the 3',5'-O-phosphite ester linkage was oxidized to the phosphate by iodine/pyridine. Trichloroacetic acid was then used to remove the dimethoxytrityl group from the 5-position of ribavirin, which now resided in the 2-position of the nascent polymer chain. This process was repeated 14 times so that 15 ribavirin phosphate units were incorporated after thymidine. The oligoribonucleotide was terminated by reaction with [6-(4-monomethoxytrityl)aminohexyl](methoxy)-N,N'-(diisopropyl)aminophosphoramidite (46) to introduce amino group functionality on the spacer arm. The methyl group was cleaved from the phosphate groups of the polymer by treatment with thiophenol-triethylamine before the polymer was cleaved from its support by treatment with ammonium hydroxide. Next, the t-butyldimethylsilyl groups were removed by treatment with tetra(n-butyl)ammonium fluoride. The oligoribonucleotide was purified by electrophoresis on a 20% acrylamide gel before the trityl groups were removed by treatment with 80% acetic acid. During this reporting period, this synthesis was accomplished

on a 6-mg scale starting with 12 1-μmole thymidine-containing CPG columns. Since this time, we have repeated the synthesis to obtain 24 mg of oligomer, which should be sufficient for the conjugation work.

A probe reaction was conducted to establish the linking conditions for attachment of the oligomer to carboxymethyldextran. A loading ratio of 10 molecules of the 5'-O-(6-aminohexyl)phosphate of poly(thymidine-3',5'-O-phosphate)$_{10}$ onto dextran as determined by ultraviolet analysis at 260 nm was achieved using EDIC as the coupling agent.

D. Conclusions

In the period covered by this third annual report, an adduct of ribavirin-5'-O-(6-aminohexyl)phosphate with carboxymethyldextran having a loading of five molecules of ribavirin per dextran was linked to MAb PC4.9A6 to give an immunodrug conjugate having a loading of 33 molecules of ribavirin per antibody. This material was submitted for biological evaluation for activity against Pichinde virus infection in Vero 76 cells. In addition a penta-decamer of ribavirin-5'-O-phosphate was synthesized to enhance loading efficacy. This ribavirin polymer had a 6-aminohexyl group at the 5'-O-phosphate ester terminus for tethering to a polymeric carrier and a thymidine at the 3'-hydroxyl group terminus in order to determine 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 considered to be too large and polar for uptake by these cells. Conjugation of the polymer to an internalizing monoclonal antibody should provide a method for entry into the cells.

II. In Vitro Antiviral Screening of Drugs Against Pichinde Virus.

A. Introduction

The *in vitro* antiviral activity of several ribavirin derivatives was measured against Pichinde virus using an antiviral assay based on detection of immunofluorescing foci of infected cells. One of the proposed approaches for achieving multiple loading of ribavirin in a targeted drug-delivery system is using a polymer carrier to which ribavirin is linked as a phosphate ester. Several phosphorylated forms of ribavirin corresponding to ribonucleoside-5'-O-phosphates were synthesized at SRI and then evaluated at Utah State University for *in vitro* antiviral activity against Pichinde virus. Ribavirin-5'-O-monophosphate (RMP), ribavirin-5'-O-diphosphate (RDP), ribavirin-5'-O-triphosphate (RTP), and ribavirin-5'-O-(6-aminohexyl)phosphate (SRI 8699-28) were evaluated for antiviral activity against Pichinde virus in Vero 76 cells. The assay was performed using an immunofluorescent cell count assay to detect Pichinde virus-infected Vero cells in 96-well culture plates. The presence or absence of fluorescing cells was easy to score; the assay was read after 24 h incubation of virus, drug, and cells. The ribavirin-dextran conjugate [6-(ribavirin-5'-O-phosphate)pentylcarboxamidomethyl](carboxymethyl)dextran (SRI 8699-58) and carboxymethyldextran (SRI-CD) were also evaluated.

B. Materials and Methods

Cells. The Vero 76 line of African green monkey kidney cells (American Type Culture Collection, 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 6×10^4 cells/well in 96-well polystyrene cell culture plates.

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

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 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 toward 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 adsorbent 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 dose of compound, 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.

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 dessicant, at room temperature. Once placed into solution, all compounds were stored at 4°C.

C. Results

The immunofluorescence assay was used to measure the antiviral activity of ribavirin and several phosphorylated ribavirin derivatives against Pichinde virus. Data from these experiments are shown in Table II-1. The second set of evaluations dealt with the ribavirin-dextran conjugate. The antiviral activity of SRI 8699-28, 8699-58, and SRI-CD against Pichinde virus is shown in Table II-2. The data in Table II-2 are presented on the basis of molarity in terms of ribavirin content; therefore Table II-3 shows the corresponding data for the antiviral evaluation of ribavirin.

D. Discussion

The use of conjugation or multiple-loading strategies employing polyribavirin derivatives linked through phosphate ester bonds could result in various phosphorylated forms of ribavirin within the target cell. The following compounds were evaluated against Pichinde virus in Vero cells: ribavirin, RMP, RDP, RTP, and ribavirin-5'-O-(6-aminohexyl)phosphate. The results indicated that the activity of the ribavirin derivatives is dependent on the state

of phosphorylation of the compound, (ribavirin > RMP = RDP > RTP). The activity ranking is most likely because of 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.

Ribavirin-5'-O-(6-aminohexyl)phosphate bisammonium salt retained antiviral activity but the activity was 27-fold less than that of ribavirin. Neither the carboxymethyldextran-ribavirin conjugate (SRI 8699-58) nor carboxymethyldextran (SRI-CD) displayed antiviral activity in the cell culture system. The lack of activity may be caused either by the inability of the conjugate to be cleaved to release ribavirin in the culture medium or by the inability of the conjugate to be taken up by the cells and then cleaved.

Table II-1. Results of immunofluorescence assay for antiviral activity against Pichinde virus. Percent of virus controls in cell cultures receiving the indicated compounds at varying concentrations.

Conc. (µg/ml)	Percent of virus controls (number of fluorescing cells)				
	Ribavirin	RMP	RDP	RTP	SRI 8699-28
1000	0	0	0	0	0
320	0	0	15.1 (7)	36.6 (16)	32.7 (14)
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)[1]	84.6 (39)	65.9 (31)	94.9 (41)	80.9 (39)
3.2	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}[2]:	5.1	27.4	41	211.6	238.5
ED_{50} (µM)[2]:	20.1	79.4	80.5	351	542
MTD:[3]	>1000	1000	>1000	>1000	>1000
TI:[4]	>196	36.4	>24.4	>4.51	>4.2

[1]Average number of fluorescing cells indicated in parentheses.

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

[3]MTD: Maximum Tolerated Dose, greatest concentration of compound that still did not produce cytotoxicity.

[4]TI: Therapeutic Index, MTD/ED_{50}.

Table II-2. Results of immunofluorescent cell count assay for antiviral activity against Pichinde virus. Immunofluorescing cells were counted in virus controls and in cultures treated with the indicated concentrations of the various compounds. Data listed as percent of the virus controls.

Concentration[1] (mM)	Percent of virus controls (number of fluorescing cells)		
	SRI 8699-58	SRI 8699-28	SRI-CD
2.25	94 (27.3)[2]	0	92 (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): compound SRI 8699-28 has a molecular weight of 440. 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 x 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/ED50.

Table II-3. Results of immunofluorescence cell count assay for antiviral activity of ribavirin against Pichinde virus. Immunofluoresoing cells were counted in virus controls and in cultures treated with the indicated concentrations of ribavirin. Data listed as percent of the virus controls.

Ribavirin (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/ED50.

III. Production and Purification of a Large Pool of Anti-Pichinde Virus Monoclonal Antibody PC4.9A6

A. Introduction

With this project there is a continuing requirement for purified MAb PC4.9A6 to be used for conjugation to the ribavirin derivatives. The most efficient way to produce large quantities of a MAb is to grow the corresponding hybridoma cell line in mice as ascites tumors and then isolate the antibodies from the ascites fluids. Our previous studies (Section VIII in Annual Report No. 2 for this project, Dawson et al., 1988) have indicated that optimal ascites production from hybridoma line PC4.9A6, derived from the fusion of FOX-NY cells with the spleen cells of an RBF/Dn mouse, is achieved in gamma-irradiated, pristane-primed (RBF/Dn x BALB/c)F1 mice. This section describes the production of a large pool of ascites fluids arising from the growth of hybridoma cell line PC4.9A6 in mice using these recently developed techniques, and the subsequent purification of anti-Pichinde virus MAb PC4.9A6 by ammonium sulfate precipitation followed by protein A affinity chromatography.

B. Materials and Methods

Hybridomas. Hybridoma cell line PC4.9A6 was derived from the fusion of FOX-NY myeloma cells with the spleen cells from an RBF/Dn

mouse that had been hyperimmunized with Pichinde virus. The PC4.9A6 cell line had been cloned twice and was in the exponential growth phase when injected into recipient mice. The viability of the cells was determined by trypan blue exclusion. At the time of inoculation over 95% of the hybridoma cells were viable. The PC4.9A6 cell line secretes an immunoglobulin (IgG) of the IgG_{2A} subclass. MAb PC4.9A6 binds to a viral antigen expressed on the surface of cells infected with Pichinde virus.

Mice. Hybrid mice were bred in the Utah State University Laboratory Animal Research Center using RBF/DnJ males from Jackson Laboratories and BALB/c females from Simonsen Laboratories. After being weaned, 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 to 21 weeks old (18.6 ± 2.2) when inoculated with the PC4.9A6 hybridoma cells.

Cells. PC4.9A6 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 one hour.

Pristane Priming, Irradiation, and Hybridoma Cell Inoculation. 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. 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 once every one to two days after the tumors were readily apparent, usually within three days of the initial appearance of the tumor. Ascites fluid was collected by inserting an 18-gauge needle (no syringe) into the swollen abdomen and allowing the fluid 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.

Determination of IgG_{2a} concentrations. The concentration of immunoglobulin 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 (ICN Biomedicals Inc., Costa Mesa, CA). The procedures were as described in the supplier's product enclosure.

Precipitation with Ammonium Sulphate. A 50-ml aliquot of the pool of PC4.9A6 ascites fluids was the starting material for the purification of the MAb. Immunoglobulins were precipitated from the

ascites fluids by dropwise addition of an equal volume of saturated ammonium sulphate. The suspension was allowed to stand several hours at 4°C before the precipitate was collected by centrifugation at 500 x g for 15 min. The precipitated immunoglobulins were dissolved in sufficient Dulbecco's phosphate-buffered saline (PBS)--0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, and 0.14 M NaCl, pH 7.2--to yield a solution having the original sample volume. The immunoglobulins were precipitated again by addition of an equal volume of saturated ammonium sulphate. The precipitated immunoglobulins were collected as before by centrifugation. The precipitation was performed once more. The semipurified immunoglobulins were dissolved in PBS and then dialyzed against PBS. The dialysis fluids were changed four times at intervals of a few hours or more.

Affinity Chromatography Using Protein A Sepharose. The ammonium sulphate-purified fraction from the ascites fluids was further purified by affinity chromatography on protein A using the Bio-Rad Affi-Gel Protein A MAPS II Kit (BIO-RAD 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 will result in an optical density of 1.35 at 280 nm). The IgG_{2a} concentration was determined by RID. The final buffer exchange was into PBS without Ca or Mg (0.01 M Na_2HPO_4, 0.15 M NaCl, pH 7.2).

Lyophilization of Purified Monoclonal Antibody. The purified MAb was aliquoted to vaccine septum vials, frozen in a -90°C freezer, and then lyophilized using a model FDX-1-84-VP lyophilizer (FTS Systems, Inc., Stone Ridge, NY). Lyophilization to dryness was accomplished in 4 h. After lyophilization, the vials were sealed and stored at -20°C.

Antigen-Binding Activity of Monoclonal Antibodies. The antigen-binding activity was determined by an indirect immunofluorescent antibody assay. 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 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.

C. Results

There was no indication of significant age- or sex-related biases in ascites production in these 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 III-1. The 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% 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 of MAb PC4.9A6; the titration data are shown in Table III-2. The purification yields are summarized in Table III-3.

D. Conclusions and Discussion

The purified pool of MAb PC4.9A6 had a very high protein concentration, approximately 840 µg/ml, and a very high concentration of active antibody against Pichinde virus. The yield calculations (Table III-3) indicated that there was no significant loss of antigen-binding activity through the purification procedure. The high protein concentration of this purified MAb PC4.9A6 should facilitate subsequent ribavirin conjugation reactions. The yield of MAb PC4.9A6 in this purification was approximately 40%. Since the

IgG_{2a} content does not necessarily indicate active antibody, the antigen-binding activity of the purified MAb, measured by indirect immunofluorescence, was determined. The residual 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 affect the antigen-binding activity of MAb PC4.9A6. The purified MAb PC4.9A6 analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions migrated as two sharp bands corresponding to the heavy and light immunoglobulin chains. The purity appeared to be in excess of 95%.

Table III-1. Summary of ascites fluid production in (RBF/DnJ x BALB/c)F1 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/total mice (%)	Total vol. of ascites fluids collected (ml)	Time from inoculation with hybridoma to first collection (d)		
		Earliest	Latest	Avg ± SD
37/39 (95%)	349 (9.4 ± 4.4 ml/mouse)	8	12	9.8 ± 1.5

Table III-2. Titration of starting ascites fluids and purified MAb PC4.9A6 by indirect immunofluorescent antibody assay on Pichinde virus-infected Vero cells.

Sample	Dilution	Reading (0 to 4+)
Positive control Fluorescein-labeled MAb 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 MAb PC4.9A6	10^{-3}	4+
Purified MAb PC4.9A6	$10^{-3.5}$	4+
Purified MAb PC4.9A6	10^{-4}	3+
Purified MAb PC4.9A6	$10^{-4.5}$	3+
Purified MAb PC4.9A6	10^{-5}	3+
Purified MAb PC4.9A6	$10^{-5.5}$	1+
Purified MAb PC4.9A6	10^{-6}	-
Purified MAb PC4.9A6	$10^{-6.5}$	+/-
Purified MAb PC4.9A6	10^{-7}	+/-
Purified MAb PC4.9A6	$10^{-7.5}$	+/-
Purified MAb PC4.9A6	10^{-8}	-

Table III-3. Purification of MAb PC4.9A6. Yields and recoveries.

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

[1]Protein (mg/ml) determined by optical density measured at 280 nm.

[2]IgG_{2a} (mg/ml) determined by radial immunodiffusion.

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

IV. Treatment of Pichinde Virus-Challenged MHA Hamsters with Anti-Pichinde Virus Monoclonal Antibodies. Effects on Various Measurable Parameters of Infection.

A. Introduction

Prior to evaluating immunodrug conjugates in the hamster Pichinde virus model, it was necessary to determine whether treatment with the MAbs alone would affect the course of the Pichinde virus infection. In passive protection studies, treatment usually has consisted of a single i.p. injection of the antibody, either just prior to virus challenge (for protection studies) or a few days after challenge, at which time the infection is firmly established (for curative or recovery studies). In rodents, such studies generally use MAbs in ascites fluids at a total dose of immunoglobulin of 500 µg or less per animal (Brandriss et al., 1986). We have derived several MAbs that bind to antigens expressed on the surface of Pichinde virus-infected cells in vitro and in vivo. These antibodies lack in vitro virus-neutralizing activity and appear to have potential as carriers for MAb-mediated drug-targeting studies. The objective of this study was to determine the effect of the anti-Pichinde virus MAbs on the course of Pichinde virus infections in MHA hamsters, thereby setting a baseline from which to plan and interpret future in vivo drug-targeting experiments.

B. Materials and Methods

Monoclonal Antibodies. The derivation of the hybridoma cell lines that secrete anti-Pichinde virus MAbs has been described in a previous report (Section IV of Annual Report No. 2, Dawson et al., 1988). The studies described in this report used MAbs PC4.7C2, PC4.8D2, PC4.9D3, and PC4.9A6 of the IgG_{2a} subclass that bind to Pichinde virus antigens expressed on the surface of Pichinde virus-infected cells. None had in vitro Pichinde virus-neutralizing activity at dilutions in excess of 1:20. Anti-Pichinde virus MAbs PC4.9A6 and PC4.7C2 recognize the same epitope, whereas PC4.9D3 and PC4.8D2 recognize distinct, nonoverlapping epitopes. Thus, a total of three epitopes are recognized. The IgG_{2a} concentrations of the ascites fluids pools used in these studies were between 10 to 15 mg/ml based on RID measurements utilizing murine IgG_{2a}-specific antisera.

Virus. Pichinde virus strain CoAn 4763 [stock dated 06/03/86(B)] was produced in Vero cells and titered in MHA hamsters. In MHA hamsters this particular stock of Pichinde virus had a titer of 1.6×10^6 LD_{50}/ml.

Animals. Female hamsters, strain MHA, were obtained from Charles River Laboratories (Wilmington, MA). The hamsters were 4 to 5 weeks old and weighed 50 g when challenged with Pichinde virus.

Virus Challenge. Hamsters were challenged with Pichinde virus by subcutaneous inoculation in the groin with 28 LD_{50} units of Pichinde virus (0.2 ml of a 10^{-4} dilution of the pool containing 28 LD_{50} units of Pichinde virus).

Monoclonal Antibody Treatment. The MAb treatment occurred 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 were administered by i.p. injection.

Amount of Monoclonal Antibody Used. With a separate group of hamsters for each MAb, hamsters were treated with approximately 2,000 µg of MAb per treatment (0.2 ml of undiluted ascites). In addition, groups of animals were treated with 200 µg (1:10 dilution) and 20 µg (1:100 dilution) of MAb PC4.9A6. The toxicity controls received 0.4 ml of a 1:1:1:1 mixture of the four MAbs.

Binding Activity of Monoclonal Antibodies. The antigen-binding activity of the MAbs was assayed by an indirect immunofluorescence assay. Antibody samples diluted (10-fold serial dilutions) in Dulbecco's 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 and incubating for 3 h. Cell sheets were rinsed twice with water and examined for fluorescing cells. The greatest dilution that still yielded readily identifiable fluorescing cells was taken as the antigen-binding end-point titer of the antibody.

Types of Observations. Mortality rate and time to death were the main parameters used to measure the effect of treatment. In addition, at 8 days postinjection (p.i.), two animals from each group were killed and liver, spleen, and blood were collected. Livers and spleens were examined visually and given a score on a scale of 0 to 4 as described in the footnotes to Table IV-2. Pichinde-virus titers in the sera were determined. SGOT and SGPT levels in the sera were also determined.

C. Results

The ascites fluids generated from hybridoma cell lines PC4.9A6, PC4.9D3, PC4.8D2, and PC4.7C2 had antigen-binding titers of $10^{5.5}$ to $10^{6.5}$ by indirect immunofluorescence assay (IFA) on Pichinde virus-infected Vero cells. These fluids were used directly or with dilution in PBS to yield the IgG concentrations indicated for treatments.

The first deaths occurred at 8 days post-virus challenge. The mortality data and time to death for all groups are shown in Table IV-1. The parameters measured from assays of the organs and sera from the animals sacrificed at 8 days p.i. are shown in Table IV-2.

D. Discussion and Conclusions

Pichinde virus-infected cells presenting suitable cell-surface target antigens do not begin to appear until 16 to 24 h post-challenge. The treatment schedule used in these studies was chosen to duplicate conditions that would be used in antibody-mediated drug-targeting studies--i.e., antibody was administered at the time of initial appearance of target antigen on the surface of infected cells. Although an earlier treatment would probably be more effective for a drug that would be taken up nonselectively, treatment commencing with the appearance of viral target antigens on the cell surface would be more suitable for antibody-mediated drug-targeting. The goal in this and future studies is to use a very simple treatment schedule that will optimize the advantages of an antibody-targeted drug over the nontargeted drug. These initial studies examined the effects of anti-Pichinde virus MAbs on Pichinde virus infections in MHA hamsters.

If the criteria for protection were based on mortality data, none of the four MAbs exerted a strong protective effect. However, there were survivors in three treatment groups. One or two animals

out of a total of seven per group survived in the groups treated with 2 mg of MAb PC4.9A6, PC4.9D3, or PC4.8D2, indicating there was some protection. However, the time to death values for all groups were within a single standard deviation of each other. None of the animals treated with the lower levels of MAb PC4.9A6 were protected. There was no apparent toxicity associated with the MAb treatments.

Interpretations based on the other parameters were not as clearly defined as those based on survival data. For example, some liver and spleen scores in lethally infected animals were only slightly above normal. The same was true for some SGOT and SGPT values. Virus titers in the sera were variable, but if more animals had been sampled, these data may have been predictive. In all cases there were measurable levels of virus in the sera whether the animals had been treated or not. Since increased SGOT and SGPT levels in the serum are indicative of liver cell damage, we used this parameter as an additional indicator of the severity of Pichinde virus infections. Elevated SGOT and SGPT levels did coincide with Pichinde virus infections; however, it was not apparent whether the severity of infection could be deduced from the SGOT and SGPT levels. Likewise, virus titers in sera did not appear to have a direct correlation with the severity of infection. In all these instances the ultimate indicator of the severity of infection was the mortality data.

Stephen et al. (1980) examined the course of Pichinde virus infection in MHA hamsters. Virus was detectable in the blood by 3 days p.i. and peaked at 10^7 PFU/ml on day 6. Deaths in the untreated animals began at 8 days p.i., and over half had died by 11-days p.i. These results are similar to what we have noted in both this and previous studies with the Pichinde virus-MHA hamster model. The biological half-life of murine MAb in rats was estimated at 5.2 days by Worrell et al. (1986). Thus, it appears reasonable to use a single treatment with MAb in Pichinde virus infections of MHA hamsters where the disease runs a relatively rapid course.

There are many examples in cancer immunotargeting where the therapeutic index of a drug has been improved by conjugation to tumor-specific antibodies. For example, the conjugate of adriamycin and mouse MAb to surface differentiation antigen Thy 1.1 had a strong chemotherapeutic effect on lung metastasis in mice inoculated with neuroblastoma N115 cells (Arnon et al., 1985). Treatment was a single injection of immunodrug or vehicle five days after the injection of tumor cells. Free drug (300 µg/mouse) resulted in a mean survival time of 35 days, compared to 45 days for nontreated control mice. Free adriamycin (375 µg/mouse) was toxic, with half of the mice dying by day 12, whereas adriamycin-anti-Thy 1.1 immunoconjugates administered (650 µg of adriamycin/mouse) were not toxic and more than 50% of the tumor-bearing mice survived for over 90 days.

In the future ribavirin-MAb immunodrug conjugates will be evaluated in Pichinde virus-infected hamsters. The results of this current study indicate that at immunodrug doses of 2,000 µg or less (based on MAb concentration), any significant increases in survival time or percent survivors will be attributable to the immunodrug and not to neutralization by the MAb.

Table IV-1. Protection by MAb against lethal challenge with Pichinde virus.[1]

Cage No.	Treatment[2]	Survivors/total[3]	Time to Death (days) (mean ± s.d.)
1-3	No virus All 4 MAbs (1 mg each) (toxicity controls)	10/10	-
4-6	Diluent (virus controls)	0/7	10.9 ± 1.7
7-9	MAb PC4.7C2 (2 mg)	0/7	10.9 ± 1.7
10-12	MAb PC4.8D2 (2 mg)	1/7	13.0 ± 3.0
13-15	MAb PC4.9D3 (2 mg)	2/7	12.4 ± 1.8
16-18	MAb PC4.9A6 (2 mg)	1/7	11.5 ± 1.1
19-21	MAb PC4.9A6 (0.2 mg)	0/7	10.9 ± 1.1
22-24	MAb PC4.9A6 (0.02 mg)	0/7	11.4 ± 0.8

[1]Female MHA hamsters, 4 to 5 weeks old, were challenged s.c. in the hind flank with 28 LD_{50} units of Pichinde virus strain An4763.

[2]Treatment by i.p. injection at 24 h postinfection with the indicated amount of MAb.

[3]Animals were observed for 21 days following virus challenge.

Table IV-2. Treatment of Pichinde virus-challenged MHA hamsters[1] with MAbs. Effect upon measurable parameters of infection at 8 days postchallenge.

Treatment[2]	Animal[3] I.D. no.	Liver scores[4]	Spleen scores[5]	Pichinde virus titers[6] in sera	SGOT[7]	SGPT[7]
No virus, All four MAbs	1-1	0	0	0	73	72
MAbs (1 mg each)	1-2	0	0	0	84	59
(Toxicity Controls)						
Diluent	4-1	2	2	3×10^6	405	610
(virus controls)	4-2	3	3	2×10^7	5,000	6,950
MAb PC4.7C2	7-1	0	1	2×10^7	62	60
(2 mg)	7-2	3.5	3	$\geq 3 \times 10^7$	2,900	2,200
MAb PC4.8D2	10-1	1	1.5	3×10^4	90	110
(2 mg)	10-2	1.5	2	1×10^5	125	132
MAb PC4.9D3	13-1	1	1.5	$\geq 3 \times 10^7$	108	43
(2 mg)	13-2	1.5	0	2×10^7	1,225	1,400
MAb PC4.9A6	16-1	1	1.5	3×10^6	170	175
(2 mg)	16-2	2	1.5	6×10^4	445	610
MAb PC4.9A6	19-1	1.5	1	$\geq 3 \times 10^7$	410	450
(0.2 mg)	19-2	2	1	$\geq 3 \times 10^7$	525	705
MAb PC4.9A6	22-1	1	1	$\geq 3 \times 10^7$	2,100	2,100
(0.02 mg)	22-2	1.5	1	$\geq 3 \times 10^7$	1,290	900

[1]Female MHA hamsters, 4 to 5 weeks old, were challenged s.c. in the nind flank with 28 LD_{50} units of Pichinde virus strain An4763.

[2]Treatment was via i.p. injection at 24 h p.i. with the indicated amount of MAb.

[3]Two animals from each treatment group were sacrificed at 8 days p.i., and the various observations were made. The number is the identification number for each of the sacrificed animals.

[4]Liver scores were determined by visual examination, at necropsy, of livers from infected and noninfected animals, using a scale of 0 to 4. A normal liver, appearing deep red in color and demonstrating a soft, resilient consistency, was scored 0. Livers from moribund animals were discolored with an increasingly tan appearance and had lost much of their resiliency, with 4 being the most extreme discoloration and consistency.

[5]Spleen scores were determined as for livers, using a scale of 0 to 4, except that splenomegaly was a prime parameter. Spleens from moribund animals were as much as 3 to 4 times larger than spleens from normal controls.

[6]Pichinde virus titers determined by immunofluorescence, expressed in fluorescent cell forming units (FCFU) per ml of serum.

[7]SGOT and SGPT concentrations in sera expressed in Sigma/Fraenkel units.

V. Weanling Hamsters for In Vivo Antiviral Evaluations

A. Introduction

Pichinde virus causes a fatal infection in adult MHA hamsters. This model has been used for the in vivo evaluation of antiviral efficacy (Murphy et al., 1977; Stephen et al., 1980). The infection results in a severe hemorrhagic disease in which the principal target organs are the liver and spleen. We have used this animal model for the past two years. However, for testing antiviral drugs that are available in very limited quantities, the relatively large adult MHA hamster (70-100 g) is an unsatisfactory model. Since there is not currently 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. We established a breeder colony of MHA hamsters from which weanlings at 3 to 5 weeks of age, weighing approximately 30 grams, were obtained.

B. Materials and Methods

Virus. The An4763 strain of Pichinde virus from Dr. Joseph D. Gangemi, University of South Carolina School of Medicine, Columbia, SC, was used in these studies. The virus was twice plaque-purified in Vero cells. 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 various virus titration procedures is shown in Table V-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 g.

Mode of Infection. The hamsters were inoculated subcutaneously (s.c.) 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.

C. Results

A titration of Pichinde virus pool PCV An4763 [dated 06/03/86 (B)] was carried out in weanling (30 g weight) and 8-week-old MHA hamsters (90 g weight). Half $\log_{10}$ dilutions were made of the virus over a range of 10^{-2} to 10^{-4}. The male and female hamsters were inoculated s.c. in the groin with 0.2 ml of the appropriate virus dilution. In this titration all of the infected hamsters died within 14 days of infection regardless of age, weight, or sex. The average day of death in each group is noted in Tables V-2 and V-3.

D. Discussion and Conclusions

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 mean that threefold less compound will be required for the initial *in vivo* antiviral testing.

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

Pichinde virus pool	Fluorescence (IFCFU)[1]	Plaque (PFU)[2]	Hamster LD_{50}[3]
An4763 [06/03/86 (B)]	1.6×10^6	4×10^5	5×10^5

[1]IFCFU: immunofluorescent cell-forming units/ml.

[2]PFU: plaque-forming units/ml.

[3]LD_{50}: number of lethal dose dose units/ml, determined in MHA hamsters.

Table V-2. Titration of Pichinde virus in 3- to 4-week-old MHA hamsters.

Virus dilution	Mortality ratio	Mortality (%)
10^{-2}	5/5	100
$10^{-2.5}$	5/5	100
10^{-3}	5/5	100
$10^{-3.5}$	5/5	100
10^{-4}	5/5	100

Average day of death: 11.16 ± 0.90.

Table V-3. Titration of Pichinde virus in 8-week-old MHA hamsters.

Virus dilution	Mortality ratio (deaths/total)	Mortality (%)
10^{-2}	5/5	100
$10^{-2.5}$	5/5	100
10^{-3}	5/5	100
$10^{-3.5}$	5/5	100
10^{-4}	5/5	100

Average day of death: 11.44 ± 0.96.

VI. Production and Characterization of an Immunotoxin Conjugate Consisting of Anti-Pichinde Virus Monoclonal Antibody PC4.9A6 Attached to Gelonin Through a Disulfide Linkage

A. Introduction

An immunotoxin consisting of MAb PC4.9A6 attached to gelonin through a disulfide linkage was prepared for evaluation against Pichinde virus. The heterobifunctional reagent N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) was used to introduce dithiopyridyl groups into the immunoglobulin. Gelonin was modified with 2-imino-thiolane. The sulfhydryl groups introduced into gelonin were then reacted with the dithiopyridyl-substituted immunoglobulin to produce the immunotoxin. The coupling of gelonin to immunoglobulin by this method, which is similar to that described by Lambert et al. (1985), is reported to result in active immunotoxins with the structure:

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

Gelonin is a 30,000-dalton, single-chain glycoprotein obtained from the seeds of Gelonium multiflorum (Stirpe et al., 1980; Falasca et al., 1982). Unlike related toxins such as abrin, ricin, and diphtheria toxin, gelonin does not have a B chain that facilitates entry of the other toxins into cells. Gelonin enzymatically inactivates the 60S ribosome subunit. It is very active in cell-free protein synthesis systems; however, it is not very cytotoxic in

cell culture or in vivo unless provided with a mechanism for entering cells. The objective in this study was to use MAb PC4.9A6, which binds to the surface of cells infected with Pichinde virus, to impart cytospecificity to the immunotoxin. Immunotoxins enter cells by receptor-mediated endocytosis.

The MAb PC4.9A6-gelonin immunotoxin produced in these studies was evaluated for antiviral activity against Pichinde virus utilizing a focus-forming assay that we developed for this purpose. The possibility that the ionophore monensin might enhance the activity of the MAb PC4.9A6-gelonin immunotoxin was examined. In previous studies monensin has been shown to potentiate the cytotoxicity of ricin-containing immunotoxins (Casellas et al., 1985). The immunotoxin consisting of MAb PC4.9A6 attached to gelonin through a disulfide linkage was evaluated for its ability to block translation in a cell-free protein synthesis system. In the case of the previously described gelonin-immunotoxins, the toxicity of the gelonin is expressed after cleavage of the disulfide linkage with dithiothreitol (Lambert et al., 1985). The effect of varying dilutions of reduced and nonreduced MAb PC4.9A6-gelonin conjugate upon protein synthesis in a reticulocyte lysate system and the kinetics of the inhibition were examined. Experiments to determine whether the MAb PC4.9A6-gelonin immunotoxin exhibited specific binding to Pichinde virus-infected cells and to determine the effect of the MAb PC4.9A6-gelonin conjugate on protein synthesis in

Pichinde virus- infected and noninfected Vero cells were also conducted and are described in this section.

B. Materials and Methods

Buffers and Reagents Used in the Conjugation of Gelonin to Monoclonal Antibody. SPDP (Pierce Chemical Co., Rockford, IL), dissolved in absolute ethanol to a concentration of 10 mM, was prepared just before use. Iminothiolane (Pierce); gelonin (Sigma Chemical Co. St. Louis, MO); Sephacryl S300 (Pharmacia); Sephadex G25-50 (Sigma); dithiothreitol (Sigma); and miscellaneous biochemicals (Sigma) were used. Buffer compositions were:

- phosphate-saline buffer (PSB): 0.1 M Na_2HPO_4, 0.1 M NaCl, and 1 mM EDTA, pH 7.5;

- PBS: 0.01 M Na_2HPO_4, and 0.15 M NaCl, pH 7.2;

- TEAE buffer: 60 mM triethanolamine-HCl and 1 mM EDTA, pH 8.0;

- Bis-tris/acetate buffer: 5 mM bis-tris, 50 mM NaCl, 1 mM EDTA, pH 5.8; and

- 1 M TEA buffer: equal volumes of 1 M triethanolamine-HCl and 1 M triethanolamine-base.

The TEAE and the bis-tris/acetate buffers were flushed slowly with bubbling argon for 2 h. The argon-flushed buffers were stored in tightly sealed bottles under an argon atmosphere. Stock solutions of 2-iminothiolane (0.1 M) were prepared just before use by dissolving 2-iminothiolane in 1 M TEA buffer. A stock solution of 20 mM iodoacetamide was prepared by dissolving iodoacetamide (Sigma) in sterile, doubly distilled water.

Columns Used for Separating Conjugation Reactants. Sephacryl S300 was swollen for several days in PBS and brought to room temperature just before pouring the column (1.5 x 70 cm). The column was equilibrated with PBS at room temperature. Sephadex columns (1.5 x 35 cm) were prepared using Sephadex G25-50. The resin was swollen in PSB and held in a double boiler for 10 min to degas.

Immunoglobulin Purification. MAb PC4.9A6 used for these studies was purified by affinity chromatography from an ascites fluids pool. The IgG concentration in the purified MAb PC4.9A6 was determined by UV spectroscopy, assuming an extinction coefficient at 280 nm of 13.5 for IgG; the concentration was 11.1 mg/ml. An indirect immunofluorescence assay utilizing fluorescein-labeled goat antibody to mouse IgG as second antibody and Pichinde virus-infected Vero cells as targets was used to determine that the anti-Pichinde virus antibody titer was $10^{-5.5}$ in the purified MAb preparation.

Conjugation of Gelonin to Monoclonal Antibody PC4.9A6. The MAb was modified for conjugation by the introduction of 2-pyridyldisulfide residues. This was accomplished by adding to 33.3 mg of MAb PC4.9A6 at a concentration of 11.1 mg/ml in PBS 300 µg of the SPDP (133 µl of a 10 mM solution) with rapid mixing. The reaction mixture was gently mixed using a rocker platform at 37°C for 60 min. A slight precipitate present at the end of the incubation was removed by centrifugation. The excess SPDP and low-molecular-weight reaction products were removed from the reaction mixture by gel chromatography on Sephadex G25 equilibrated with PSB. The effluent fractions from the column were monitored at 280 nm. The fractions containing the majority of the IgG were pooled, yielding 25 mg of modified IgG in a total volume of 5 ml.

Just prior to the conjugation reaction, sulfhydryl groups were introduced into gelonin. Gelonin (6 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 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 in a sealed tube for 90 min at 4°C. The excess 2-iminothiolane was removed from the thiolated gelonin by gel chromatography on Sephadex G25 equilibrated with argon-flushed 5 mM bis-tris/acetate buffer. The fractions were monitored at 280 nm. The peak fractions contained 4.2 mg of thiolated gelonin at 0.84 mg/ml in 5 ml bis-tris, pH 5.8, calculated on the basis of a extinction coefficient at 280

nm of 6.7 for gelonin. The thiolated gelonin was used immediately to minimize oxidation.

To 25 mg (5.0 ml) of the 2-pyridyldithio-derivatized MAb PC4.9A6 in PBS was added 4.2 mg of thiolated gelonin in 5 ml of argon-flushed 5 mM bis-tris/acetate buffer, pH 5.8. The pH of the reaction mixture was adjusted to pH 7.0 with TEAE buffer. The reaction mixture was then incubated at 4°C for 20 h. A precipitate was noted soon after the reactants were combined, and after 20 h there appeared to be even more precipitate. At the end of the conjugation reaction, the remaining free sulfhydryl groups were blocked by the addition of 1 ml of 20 mM iodoacetamide followed by an additional hour of incubation at 37°C. It was thought that raising the temperature might also reduce some of the precipitate, but it did not dissolve. The reaction mixture was then centrifuged for 5 min, yielding a large pellet of white precipitate. The supernatant fluids were removed and found to have an OD_{280} of 2.52 and total volume of 11 ml, corresponding to approximately 25 mg of conjugate or 21 mg of IgG if the extinction coefficient of IgG was used. The supernatant fluids were concentrated by ultrafiltration using a Centriprep-30 (Amicon, Danvers, MA). The resulting 4.0 ml of solution had an OD_{280} of 2.9, indicating approximately 11.6 mg of protein. Apparently there was a 65% loss of protein during this concentration step. The concentrated reaction mixture was fractionated on a Sephacryl S300 column using PSB, collecting fractions of 1 ml/tube.

Using PSB at 37°C, an attempt was made to resuspend the precipitate that was formed during the conjugation reaction. The resulting supernatant fluids had an optical density of 0.08 at 280 nm and a total volume of 12 ml. Thus, the precipitate was not very soluble in PSB or distilled water.

Cells. The Vero 76 line of African green monkey kidney cells obtained from the American Type Culture Collection (Rockville, MD) was used in these studies. Cells were passaged in polystyrene disposable cell-culture flasks using MEM (GIBCO Laboratories, Grand Island, NY) containing 5% fetal bovine serum (HyClone Laboratories, Logan, UT). For antiviral assays, cells were seeded at 6×10^4 cells/well in 96-well polystyrene cell culture plates.

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

Immunofluorescence Assay for Antibody-Targeted Antiviral

Compounds. The following controls and MAb-gelonin conjugates were tested in a 4-day antiviral test wherein the compounds were added at 24-h postinfection: MAb PC4.9A6 only, MAb Hy48 only (an irrelevant MAb also of the IgG_{2A} isotype), MAb PC4.9A6-gelonin immunotoxin, MAb 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 media 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 to 10 immuno-fluorescent cell-forming units of Pichinde virus added to each well, resulting in a multiplicity of infection (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 media 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).

The 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 as the reduction in immunofluorescent foci (IF) as the scoring parameter in a manner analogous to measuring activity as a reduction in cytopathogenic effect. The antiviral activity was expressed as the concentration of drug required to reduce the IF value by 50% (ED_{50}).

Reticulocyte Lysate System. To measure gelonin activity, a cell-free protein synthesis system was established. The system utilized nuclease-treated rabbit reticulocyte lysate and L-[3,4,5-^{3}H]leucine (New England Nuclear, Boston, MA), with untreated rabbit reticulocyte lysate (Promega, Madison, WI) serving as the mRNA source. 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 on silica gel-impregnated glass fiber sheets (Gelman Sciences, Inc., Ann Arbor, Michigan) cut into 7 x 50-mm strips and developed in 15 ml of methanol, 5 ml of acetic acid, and 30 ml of 15% (W/V) trichloroacetic acid (TCA). Tris buffer (1 M Tris, pH 10.7) was prepared by dissolving Trizma base (Sigma) in distilled water.

Preparation of mRNA for Cell-free Translation Experiment. A few minutes before the assay, the "mRNA" 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, which would otherwise reactivate the nuclease present in the 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.

Preparation of Reduced and Nonreduced Immunotoxin for Cell-free Translation Experiment. The MAb 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 (BSA) at 0.1 mg/ml.

Effect of Reduced and Nonreduced Immunotoxin on Protein Synthesis. Controls consisted of translation reaction mixtures with and without mRNA, and both without immunotoxin. By comparing the extent of translation with varying dilutions of reduced and nonreduced immunotoxin to the extent of incorporation in controls, the effect of immunotoxin was determined.

After incorporation periods of 15, 30, and 60 min, aliquots of the translation reaction mixtures were quenched by mixing with 1 M Tris, pH 10.7. The high pH also was intended to deacylate [^{3}H]-leucyl-tRNA present in the mixture. The incorporated and free

[^{3}H]leucine were separated by TCL. The incorporated amino acids stayed at the origin while the free moved with the front. Developed strips were cut in half--crosswise 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.

Binding of Monoclonal Antibody 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 a MOI of 1:250. At 20 h p.i. the cultures were stained under different conditions as described below. To determine whether 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 MAb PC4.9A6-gelonin immunotoxin were added to the culture media. The plate was then incubated at 4°C for an additional 20 h. After the reaction with the first antibody the plates were very gently rinsed twice 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 whether the MAb PC4.9A6-gelcnin conjugate retained the 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

the MAb PC4.9A6-gelonin, with the remainder of the immunostaining procedure being as described for the living cells.

Cytotoxicity of MAb PC4.9A6-Gelonin Conjugate toward Pichinde Virus-Infected and Noninfected Vero Cells with and without Monensin. A 500 μM stock solution of monensin (ICN Biochemicals, Cleveland, OH) was prepared in absolute ethanol. Working solutions were prepared from the stock solution by further dilution in cell culture medium. Monolayer cultures of Vero cells in 96-well plates were inoculated with Pichinde virus at an MOI of 0.1; noninoculated plates were fed at this time with virus-free diluent (MEM with 5% FBS). Two days p.i., the infected and noninfected cells were rinsed twice to remove free virus, which might compete for the immunotoxin. Varying concentrations of monensin (none, 50 nM, 500 nM, and 5 μM) in 150 μl of MEM with 5% FBS were added. Then six wells/dilution were treated with 50 μl of MAb PC4.9A6-gelonin conjugate (neat through 10^{-6}) or diluent only. Seventy-two hours later, the cellular leucine pools were reduced by removing media, rinsing twice with leucine-free medium, and replacing with MEM without leucine. 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 from wells, 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 the cells, 50 µl of 2 M NaOH was then added per well and the contents were allowed to stand for 10 min at room temperature. The base was then neutralized by the addition of 0.1 ml of 20% TCA. Cells were harvested with a Skatron cell harvester (CH System 1208, Flow Laboratories, McLean, VA) using distilled water for rinsing.

C. Results

The conjugation of gelonin to MAb affords a mixture that contains free gelonin (Mw 30,000 daltons), free antibody (160,000), the immunotoxin conjugate (190,000) and very minor fractions of immunotoxin at 220,000 and 250,000 daltons resulting from two or three gelonins attached to a single antibody. As Figure VI-1 illustrates, the gelonin was readily separated from the higher-molecular-weight fractions, but the free antibody and immunotoxin eluted as a single peak. Alternative column matrices based on affinity chromatography are under development and are described in the Discussion section below. According to a the report by Lambert et al. (1985), the reaction conditions of the synthesis should have resulted in a conjugate consisting of 1 gelonin molecule per IgG molecule. Such an immunotoxin would be approximately 16% gelonin by weight. The OD_{280} of the immunotoxin was 0.33, indicating a protein concentration of 330 µg/ml. The concentration with respect to

gelonin was approximately 53 µg/ml. It is realized that some free antibody and some immunotoxin containing more than a single gelonin molecule per IgG will be present in the "immunotoxin." The immunotoxin was recovered in a volume of 13 ml--thus the final pool contained 4.3 mg of immunotoxin.

Monensin was included in some of these studies as a possible enhancer of immunotoxin activity in cell culture. Monensin alone was initially examined for cytotoxicity and antiviral activity against Pichinde virus in Vero cells. The results are shown in Table VI-1. At high concentrations, monensin exhibited an antiviral effect against Pichinde virus. However, at 50 nM monensin--the concentration used for immunotoxin-potentiating studies--there was no detectable antiviral activity.

In the assays for evaluating antibody-targeted antivirals, the infections were terminated 96 h p.i., at which time most of the cells in the nontreated, virus control wells were infected, as indicated by immunofluorescence in over 90% of the cells; individual foci of fluorescing cells were no longer distinguishable because most foci were overlapping. Using this assay, the MAb PC4.9A6-gelonin immunotoxin was evaluated alone and in the presence of 50 nM monensin. Under both sets of conditions the MAb PC4.9A6-gelonin immunotoxin failed to exhibit any antiviral activity over the concentration range of 0.017 to 17 µg/ml (Table VI-2). The positive control in this assay was ribavirin. When ribavirin was added to

the infected cells at 20 h p.i., dose-dependent inhibition of immunofluorescent foci formation occurred. At the highest noncytotoxic level of ribavirin (320 µg/ml), no fluorescent foci were formed by 96 h p.i.; only occasional solitary fluorescing cells were observed. This result was scored as 0-1. As the concentration of ribavirin was reduced, the size and number of fluorescent foci increased. The fifty percent effective dose (ED_{50}) for ribavirin was 24 µg/ml, the maximum tolerated dose was 1,000 µg/ml, and the therapeutic index was 42.

The gelonin activity in various dilutions of the native and reduced immunotoxin MAb PC4.9A6-gelonin was examined by measuring the inhibitory activity in a reticulocyte lysate cell-free translation system. The immunotoxin dilutions were added to the translation system at the initiation of the translation reaction. The effect of reduced and nonreduced immunotoxin on protein synthesis is shown in Figures VI-2, VI-3, and VI-4. The data indicate [^{3}H]leucine incorporation as a percentage of that of the control without immunotoxin. The incorporated [^{3}H]leucine in 3 µl of the control reaction mixture with mRNA but no immunotoxin had 150,000 to 300,000 cpm, depending on the length of the incorporation reaction. The maximum incorporation of tritiated leucine in this experiment was 60% of the added [^{3}H]leucine.

The concentration of the immunotoxin in the undiluted starting material was 330 µg/ml and the concentration of conjugated gelonin

was 53 µg/ml. The reciprocal of the immunotoxin dilution that diminished the incorporation of [^{3}H]leucine by 50% was taken as the end-point titer. Those titers were determined by interpolation from Figures VI-2, VI-3, and VI-4. 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; 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 VI-3.

Although the immunotoxin exhibited the ability to block protein synthesis in a cell-free system, the antiviral assays indicated no antiviral activity. Therefore, binding of MAb PC4.9A6-gelonin immunotoxin to Pichinde virus-infected Vero cells was examined. In the Pichinde virus-inoculated cultures, which were acetone-fixed and stained by indirect immunofluorescence, strongly fluorescing cells were observed. The immunotoxin MAb 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, which contained 10 times as much protein as did the immunotoxin, was detectable to a dilution of 10^{-4} under these staining conditions.

The cytotoxicity of the MAb PC4.9A6-gelonin conjugate toward Pichinde virus-infected and noninfected Vero cells was then examined. At the time that the immunotoxin was added for the cytotoxicity studies, cells in a parallel control plate were fixed

and immunostained for Pichinde-virus antigen to determine what percentage of cells in the inoculated cultures were infected. About 25% of the cells were heavily decorated with fluorescing antibody, indicating 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 immunotoxin MAb 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 averages of the raw counts from six wells are shown in Table VI-4. The data in Figures VI-5 and VI-6 show that the 50% inhibitory concentrations of immunotoxin were 21 µg/ml and 125 µg/ml toward infected and control cultures, respectively, regardless of whether 50 nM was present. When monensin was present at 500 nM or 5 µM (Figures VI-7 and VI-8), there was no difference between the 50% inhibitory concentrations of immunotoxin toward Pichinde virus-infected or normal cells, in all instances it being approximately 100 µg/ml.

D. Discussion and Conclusions

During the synthesis of the MAb PC4 9A6-gelonin immunotoxin, a large volume of precipitate formed during the reaction of the thiol-containing gelonin and the 2-pyridyldisulfide-containing MAb PC4.9A6. The precipitate may have been aggregates of excessively cross-linked gelonin and MAb. The formation of multiply

cross-linked product might be minimized by lowering the number of thiol groups introduced into the gelonin and/or lowering the number of pyridyldisulfide groups introduced onto the MAb. For future conjugations, the extent of the derivatization of both the gelonin and the MAb will be measured. Lowering the concentrations of both the derivatized gelonin and the MAb in the conjugation reaction mixture would also lower the proportion of multiply cross-linked products.

The interpretation of the various immunotoxin characterization studies is complicated by the fact that the Sephacryl chromatography did not cleanly separate the immunotoxin from the free antibody. An effort is now under way to develop a method for attaining a clean separation of immunotoxin from free antibody. Antisera toward gelonin is currently being produced in rabbits. The resulting antibodies will be used to produce affinity chromatography columns, which will be be used to separate immunotoxin from free MAb. The rabbit antisera toward gelonin will also be used to produce fluorescein-labeled anti-gelonin antibody that will be used to detect binding of immunotoxin to Pichinde virus-infected cells. Rabbit antisera toward murine IgG_{2a} MAb is also being produced and will be used to produce affinity columns for the purification of immunotoxins.

The MAb PC4.9A6-gelonin immunotoxin produced in these studies was evaluated for antiviral activity against Pichinde virus

utilizing a focus-forming assay that we developed for this purpose. Vero cell monolayers were inoculated with Pichinde virus at a very low MOI (1:10,000) and the immunotoxin and appropriate controls were added 24 h p.i. Four days p.i., the monolayers were fixed, stained, and examined for the presence of immunofluorescing foci of Pichinde virus-infected cells. In the untreated control cultures large fluorescing foci of several hundred cells were present, whereas cultures that had been treated with ribavirin exhibited only single fluorescing cells and occasional small fluorescing foci of two to eight cells.

Although no antiviral activity was observed with the MAb PC4.9A6-gelonin immunotoxin, previous reports by others, dealing with specific populations of lymphocytes or malignant cells with specific cell-surface markers, suggest that the immunotoxin approach may be feasible for killing virus-infected cells. Lambert et al. (1985) examined the cytotoxicity of MAb-gelonin conjugates against human lymphoid cells by measuring [^{3}H]thymidine incorporation as an index of cytotoxicity. They found that the ID_{50} for one MAb-gelonin conjugate on the appropriate target cells was 30 pM (a 30 pM solution of gelonin would contain gelonin at 1×10^{-3} µg/ml) compared with an ID_{50} of 0.4 µM (10 µg/ml) for native gelonin. The conjugates were not toxic to cells lacking the relevant cell-surface antigen. The development of the maximum toxic effect required 2 to 3 days' exposure. At 24 h a 100-fold higher concentration of conjugate was required to achieve the same amount of toxicity as was

observed in a 48-h exposure. There was not an appreciable difference between the 48-h and 72-h exposures with respect to cytotoxicity, indicating that it should be possible to produce Pichinde virus antigen-specific immunotoxins. Because the MAb PCV4.9A6-gelonin immunotoxin failed to demonstrate an antiviral effect against Pichinde virus, an effort was mounted to evaluate the characteristics of the MAb PCV4.9A6-gelonin immunotoxin to determine the cause of the problem.

The possibility that monensin might enhance the activity of the MAb PC4.9A6-gelonin immunotoxin was examined. Monensin has been shown in previous studies to potentiate the cytotoxicity of ricin-containing immunotoxins. Casellas et al. (1985) reported that the in vitro activity of immunotoxins of the purified A chain of ricin covalently linked to a MAb directed at an antigen present on mouse leukemia cells was greatly enhanced (7- to 10-fold) by the carboxylic acid-containing 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 compared to 4 to 60 h for immunotoxins. The kinetics was 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.

In this current study, some of the toxicity associated with monensin (Table VI-1) may have been due to the presence of ethanol, the solvent used in preparing the 500 mM stock solution of monensin. The ethanol concentration was approximately 5%, 1.6%, and 0.5% in the cultures treated with 25 mM, 7.9 mM, and 2.5 mM monensin, respectively. However, the data in Table VI-1 indicate that monensin may have a significant antiviral effect against Pichinde virus. Monensin at 50 nM, the level previously used by others (Casellas et al., 1985) to potentiate the activity of ricin immunotoxins, had no inherent antiviral activity nor did it cause the MAb PC4.9A6-gelonin immunotoxin to exhibit detectable antiviral activity.

The portions of this study utilizing the inhibition of cell-free protein synthesis were intended to determine whether the immunotoxin contained active gelonin. Lambert et al. (1985) reported that gelonin at 7.4×10^{-4} µg/ml shut down protein synthesis completely within 15 min. In the experiments described in this report, the concentration of gelonin in the reduced immunotoxin that was required to inhibit the incorporation of [^{3}H]leucine by 50% was

0.5 to 1.4 x 10^{-4} µg/ml. The conjugated gelonin in the MAb PC4.9A6-gelonin had greatly diminished activity; however, the activity increased 200- to 1,000-fold on release of the gelonin from the conjugate by reduction with dithiothreitol.

Experiments described in this section were designed to determine if the MAb PC4.9A6-gelonin immunotoxin exhibited specific binding to Pichinde virus-infected cells and to determine the effect of MAb PC4.9A6-gelonin conjugate on protein synthesis in Pichinde virus-infected and noninfected Vero cells. The immunotoxin retained binding activity to Pichinde-virus antigens on fixed cells. At this time it still is not clear if the MAb PCV4.9A6-gelonin immunotoxin binds to Pichinde-virus antigens on viable cells. The cell-binding data did not conclusively show that the immunotoxin binds to Pichinde virus-infected cells. The problem with the cell-binding experiments was that a portion of the immunotoxin preparation could have contained free MAb because the Sephacryl chromatography removed free gelonin from the immunotoxin but did not cleanly separate the immunotoxin from free antibody. Therefore, the binding that was observed could have been by free antibody present in the immunotoxin preparation. Antisera toward gelonin and murine monoclonal IgG_{2a} antibodies are currently being produced in rabbits. The resulting antisera will be used to produce affinity-chromatography columns that will be be used to separate immunotoxin from free MAb.

There was only a slight specific cytotoxicity associated with the MAb PC4.9A6-gelonin immunotoxin either without monensin or with monensin at 50 nM. The 50% inhibitory concentrations of immunotoxin ranged from 20 to 125 µg/ml under all conditions. The data gave no indication of a strong, specific targeting of cytotoxicity, of potent nonspecific cytotoxicity, or of any potentiating activity by monensin. The cytotoxicity observed for the MAb PC4.9A6-gelonin immunotoxin was 3.2 to 20 µg/ml based on gelonin content.

It is possible that the immunotoxin was not entering cells because of the lack of significant binding to infected cells, or because the free MAb PC4.9A6 antibody competed more effectively for the limited number of cell surface-binding sites. Another potential problem is that the immunotoxin may bind to the cells but not be internalized. 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 MAb-gelonin conjugates on the appropriate target cells was 30 pM (a 30 pM solution would contain 1×10^{-3} µg/ml of gelonin) compared with 0.4 µM (10 µg/ml) for native gelonin. This observation agreed with the cytotoxicity we observed for the MAb PC4.9A6-gelonin immunotoxin (20 to 125 µg/ml of immunotoxin or 3.2 to 20 µg/ml based on gelonin content).

Other examples of active gelonin immunotoxins have been reported although none involved targeting virus-infected cells. Thorpe et al. (1981) produced conjugates of gelonin attached through a disulfide linkage to anti-Thy 1.1 MAb. The 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 gelonin at a concentration of 100 µg/ml only slightly inhibited protein synthesis in intact HeLa cells; however, attachment to concanavalin A inhibited protein synthesis 50% at a gelonin concentration of 0.2 µg/ml.

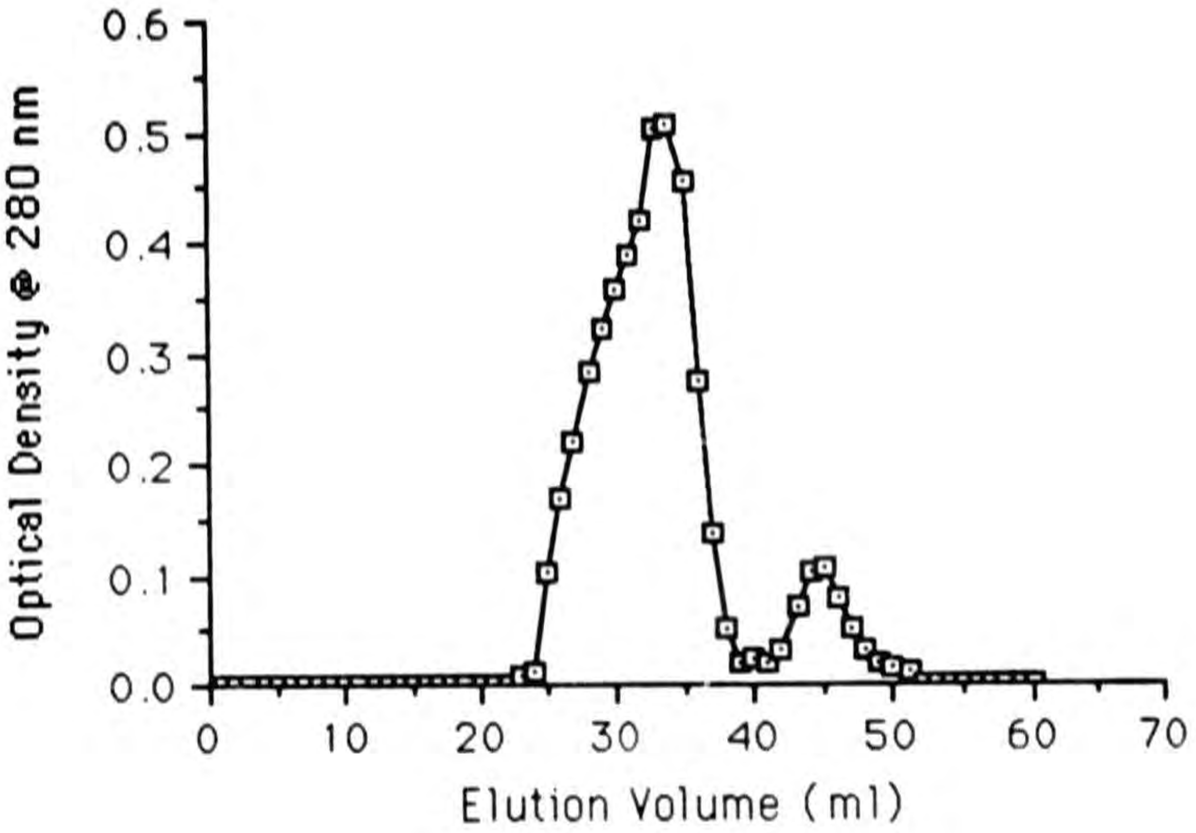


Figure VI-1. Elution profile of a MAb PC4.9A6-gelonin conjugation reaction mixture fractionation on a Sephacryl S300 column.

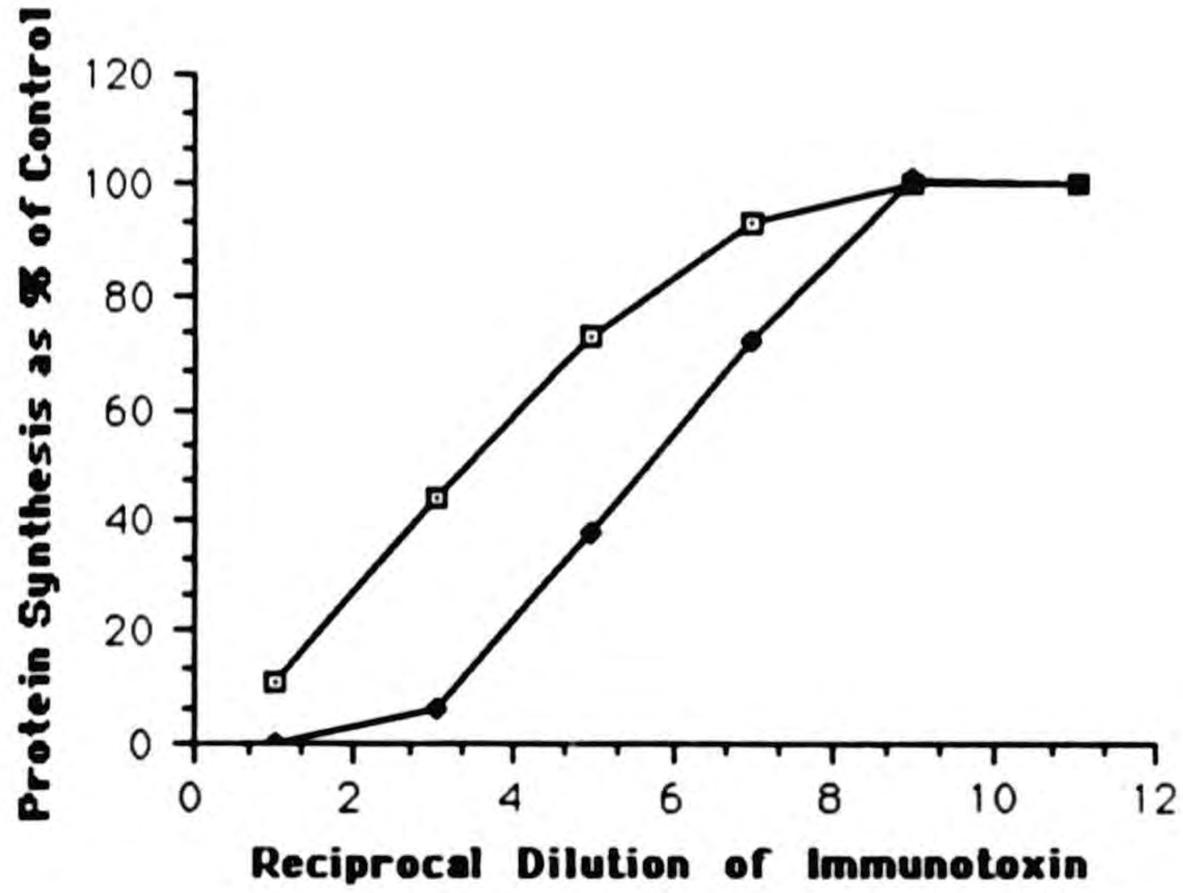


Figure VI-2. Effect of native (□) and reduced immunotoxin MAb PC4.9A6-gelonin (♦) on [^{3}H]leucine incorporation when added to a reticulocyte lysate cell-free translation system. These data indicate extent of incorporation after a 15-min reaction time. The reciprocal dilutions of immunotoxin are on a $\log_{10}$ scale.

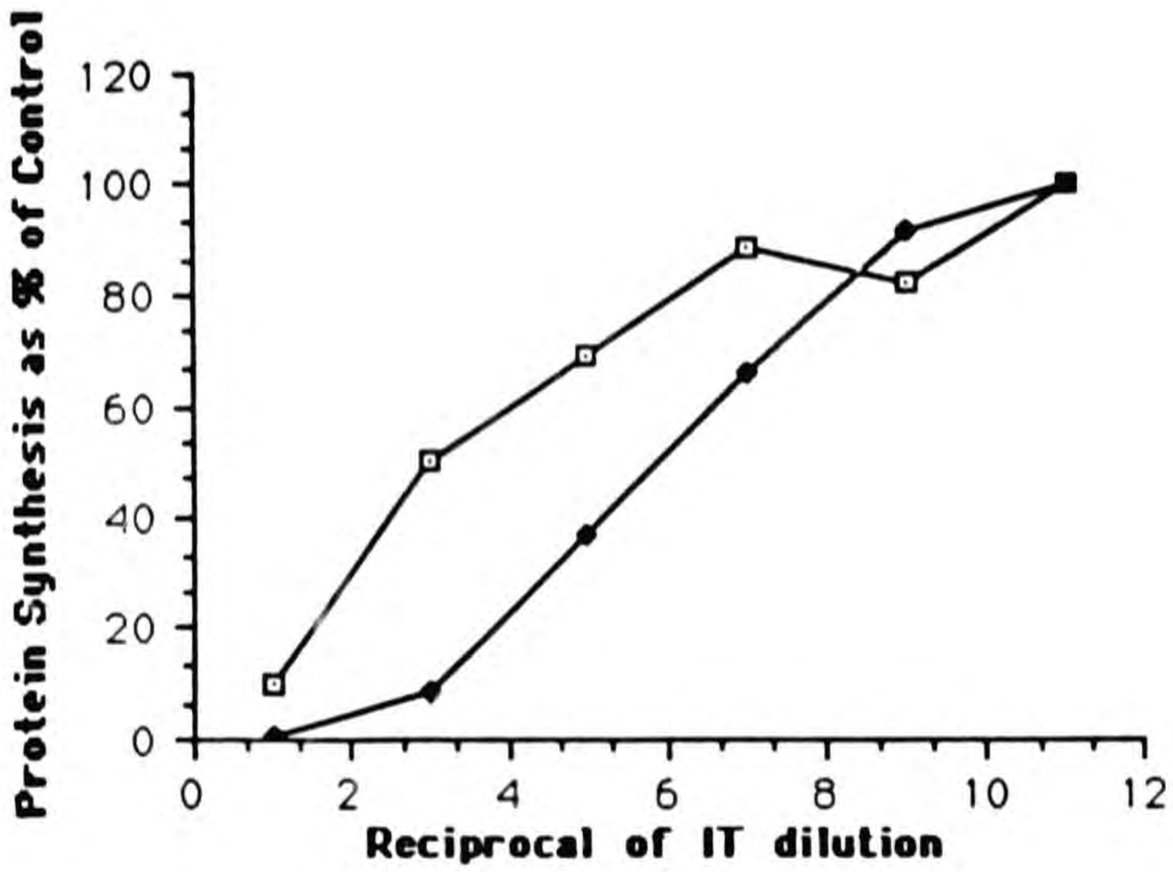


Figure VI-3. Effect of native (□) and reduced immunotoxin MAb PC4.9A6-gelonin (♦) on [^{3}H]leucine incorporation when added to a reticulocyte lysate cell-free translation system. These data indicate extent of incorporation after a 30-min reaction time. The reciprocal dilutions of immunotoxin are on a $\log_{10}$ scale.

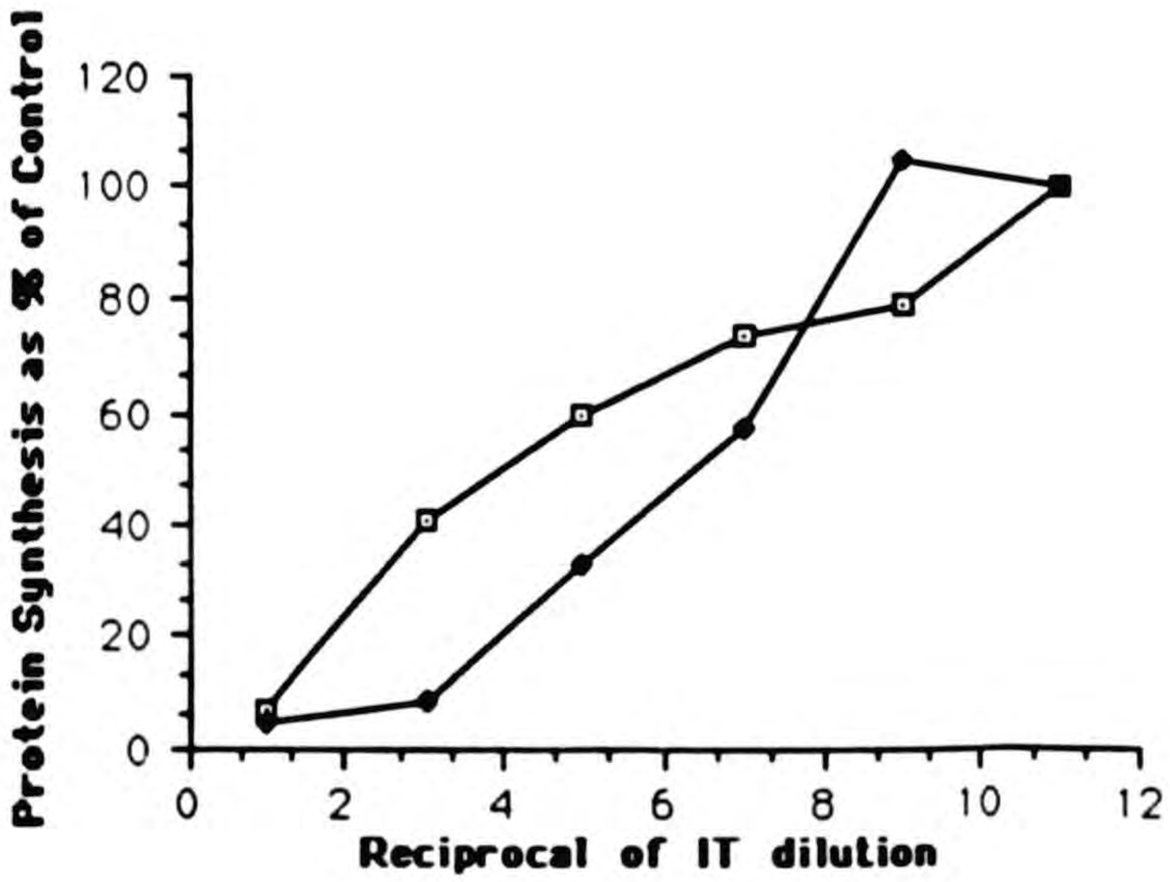


Figure VI-4. Effect of native (□) and reduced immunotoxin MAb PC4.9A6-gelonin (♦) on [^{3}H]leucine incorporation when added to a reticulocyte lysate cell-free translation system. These data indicate extent of incorporation after a 60-min reaction time. The reciprocal dilutions of immunotoxin are on a $\log_{10}$ scale.

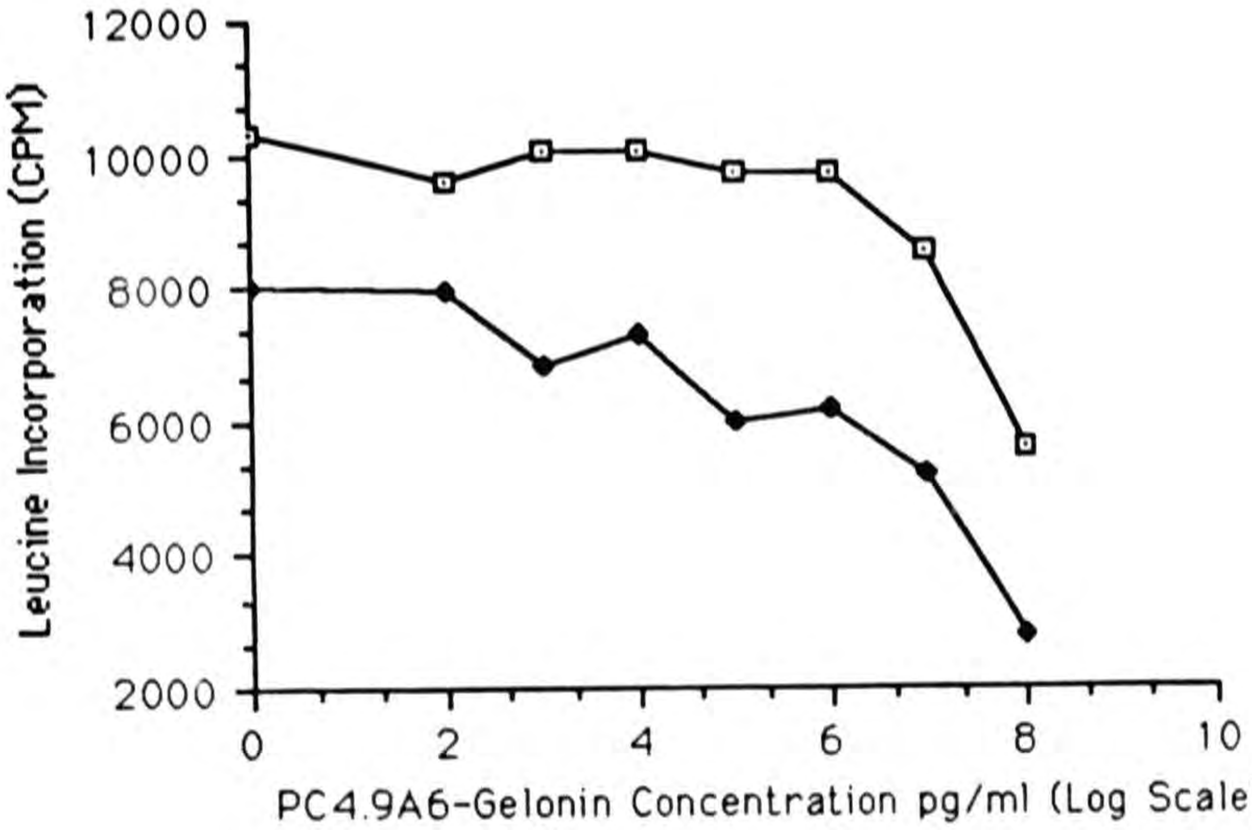


Figure VI-5. Effect of immunotoxin MAb PC4.9A6-gelonin on protein synthesis in Pichinde virus-infected (♦) and control (□) Vero cells in the absence of monensin.

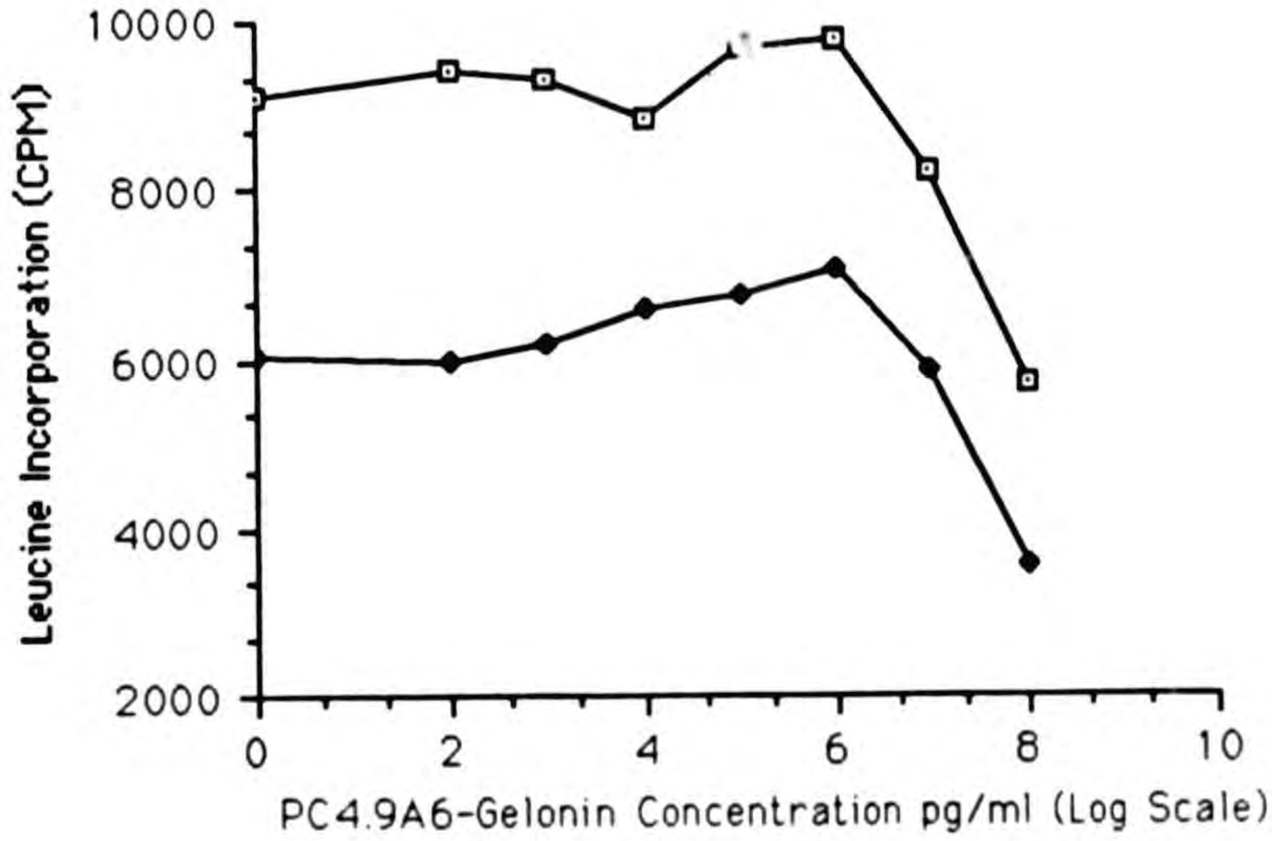


Figure VI-6. Effect of immunotoxin MAb PC4.9A6-gelonin on protein synthesis in Pichinde virus-infected (♦) and control (□) Vero cells in the presence of 50 nM monensin.

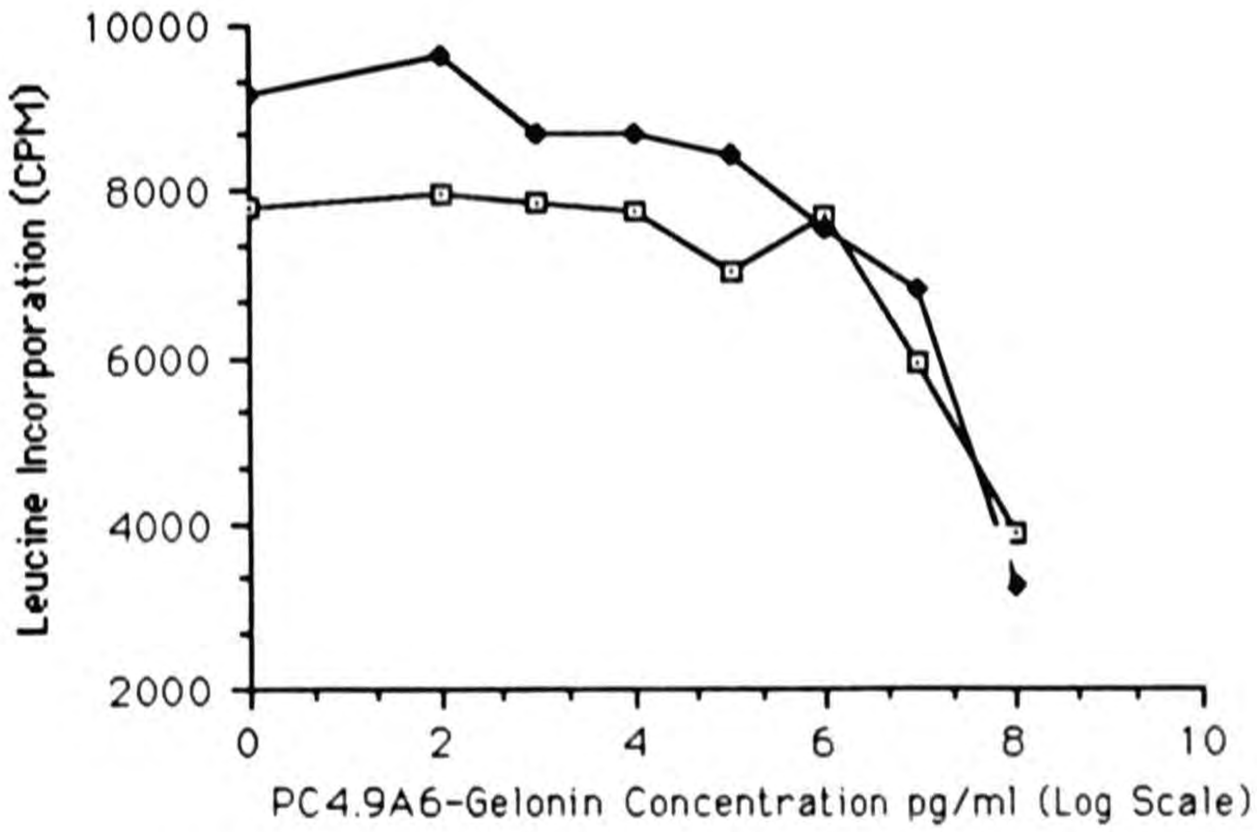


Figure VI-7. Effect of immunotoxin MAb PC4.9A6-gelonin on protein synthesis in Pichinde virus-infected (♦) and control (□) Vero cells in the presence of 500 nM monensin.

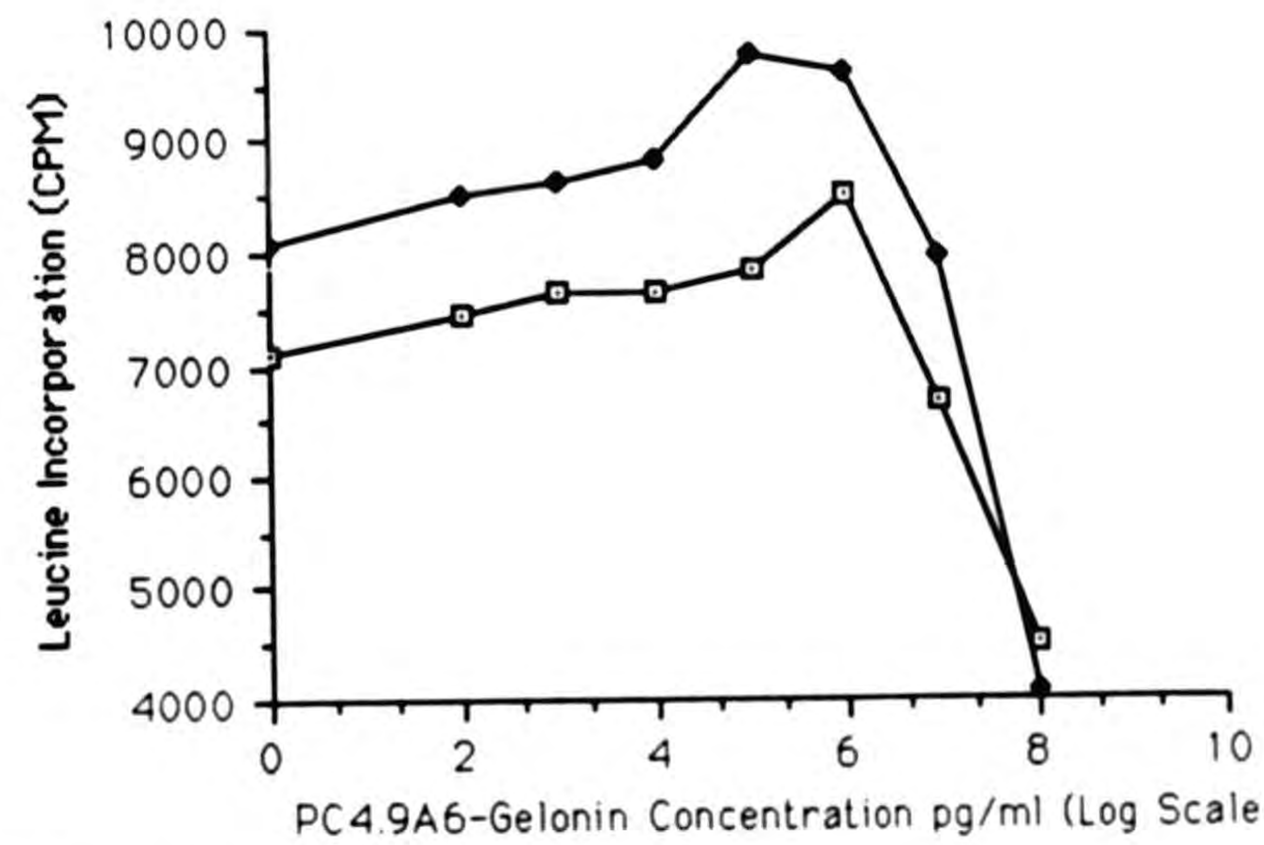


Figure VI-8. Effect of immunotoxin MAb PC4.9A6-gelonin on protein synthesis in Pichinde virus-infected (♦) and control (□) Vero cells in the presence of 5 μM monensin.

Table VI-1. Anti-Pichinde virus activity of monensin in 24-h immunofluorescent cell assay. Percent of virus control values and average number of fluorescing cells in cell cultures receiving monensin at various concentrations.

Monensin Concentration (mg/ml)	(molarity)	Number of fluorescing cells	Percent of virus controls
17.3	25 mM	toxic	toxic
5.5	7.9 mM	1	3.1
1.73	2.5 mM	10	24.1
0.55	790 µM	39	90.3
0.173	250 µM	48	112.8
0.055	79 µM	42	98.8
0.0173	25 µM	>43	>100.0

ED_{50}: 1.3 mg/ml.

MTD: 5.48 mg/ml.

Therapeutic Index: 4.21.

Table VI-2. Antiviral activity of MAb PC4.9A6-gelonin immunotoxin against Pichinde virus.[1]

Ribavirin conc (µg/ml)	Immunotoxin conc (µg/ml)	Extent of Pichinde virus infection[2] when treated with: Ribavirin	MAb PC4.9A6-gelonin	MAb PC4.9A6-gelonin + 50 nM monensin
1000	17	CT[3]	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

[1]Determined by Immunofluorescing foci assay. Test material at the indicated concentrations was added 24 h after inoculation of Vero cells with Pichinde virus at an MOI of 1:10,000.

[2]Measured by immunofluorescence, graded on a scale of 0 (normal cells) to 4 (confluent fluorescing foci).

[3]CT: Cytotoxicity observed at 1,000 µg/ml of ribavirin.

Table VI-3. Gelonin activity in native and reduced immunotoxin MAb PC4.9A6-gelonin. 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.

Assayed following incorporation period (min)	End-Point Titers and IC_{50}[1]					
	Disulfide-linked Immunotoxin			Reduced Immunotoxin		
	1/dilution	Immunotoxin[1] (μg/ml)	Gelonin (μg/ml)	1/dilution (μg/ml)	Immunotoxin (μg/ml)	Gelonin (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}

[1] IC_{50}: 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 of 53 μg/ml with respect to gelonin.

Table VI-4. Effect of monensin and immunotoxin MAb PC4.9A6-gelonin on incorporation of [^{3}H]leucine by Pichinde virus-infected and control Vero cell cultures 72 h after treatment with immunotoxin.

	[^{3}H]Leucine incorporated (cpm) in control and Pichinde virus-infected Vero cells at varying concentrations of immunotoxin and monensin in medium							
	Monensin (nM)							
Immuno-toxin (pg/ml)	0		50		500		5000	
	Control	Virus	Control	Virus	Control	Virus	Control	Virus
none	10271	7989	9125	6008	7801	9167	7087	8071
102	9560	7971	9440	5988	7972	9631	7433	8501
103	10054	6825	9317	6187	7849	8716	7628	8638
104	10040	7281	8829	6624	7766	8714	7647	8830
105	9689	5982	9703	6758	7004	8410	7834	9761
106	9734	6199	9789	7087	7692	7529	8494	9598
107	8558	5223	8238	5879	5935	6811	6686	7942
108	5612	2760	5692	3545	3866	3243	4529	4068

CONCLUSIONS

The results of the third year of this project indicate that:

(1) Polymeric versions of ribavirin can be synthesized.

(2) An immunodrug conjugate having a loading of 33 ribavirins per MAb can be synthesized.

(3) Multiple loading of ribavirin onto virus-specific antibodies using either a polymeric carrier or a polymeric version of ribavirin appears to be a promising approach for carrier-mediated targeted delivery. In the future, both the dextran multiply loaded by ribavirin and the poly(ribavirin-5'-O-phosphate) should be targeted by attachment to virus-specific internalizing antibodies.

(4) Four anti-Pichinde virus MAbs that bind to the surface of Pichinde virus-infected cells and concentrate in the virus-infected target tissues of infected hamsters had no protective effect against the virus in infected hamsters. Lack of protective activity is desirable for antibodies developed for evaluating carrier-mediated drug delivery because any significant increases in survival time or percent survivors would be attributable to the antibody-drug

conjugate and not to any antiviral activity inherent in the antibody.

(5) Weanling MHA hamsters, weighing 30 g, were as sensitive to Pichinde virus challenge as were young adult (90 g) MHA hamsters. If the amount of drug is a limiting factor in future studies, weanling hamsters that can be reared in the Utah State University animal care facility could be used in place of young adults.

(6) An immunotoxin consisting of anti-Pichinde virus MAb PC4.9A6 attached to gelonin failed to display antiviral activity against Pichinde virus in Vero 76 cells. However, this immunotoxin blocked translation in a cell-free protein synthesis system. The PC4.9A6-gelonin immunotoxin probably did not enter the Pichinde virus-infected cells. Therefore, other carrier antibodies should be examined using the immunotoxin approach before further drug conjugations experiments proceed.

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