(2)

AD_____

INTERFERON INDUCERS AGAINST INFECTIOUS DISEASES

ANNUAL/FINAL REPORT

JAKE BELLO AND JUDITH O'MALLEY

June 15, 1991

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-87-C-7111

Roswell Park Memorial Institute
Buffalo, New York 14263

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 15, 1991	Annual/Final 23 Mar 87 - 22 May 91

4. TITLE AND SUBTITLE

Interferon Inducers Against Infectious Diseases

5. FUNDING NUMBERS

DAMD17-87-C-7111

61102A
3M161102BS12 AB
DA312543

6. AUTHOR(S)

Jake Bello and Judith O'Malley

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Roswell Park Memorial Institute
Buffalo, New York 14263

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Annual covers 14 June 1990 - 22 May 1991

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The double-stranded polyribonucleotide, poly IC(polyinosinic-polycytidylic acid) is an interferon inducer. In primates it is not an effective antiviral agent, presumably because circulating nucleases quickly degrade it. When complexed with cationic poly L-lysine (PLL) and anionic carboxymethylcellulose (CMC) the resultant complex, poly ICLC, is effective in humans. However, CMC appears not to be excreted or metabolized, and may be a carcinogen.

Our goal was to prepare effective, safe antiviral agents devoid of CMC, less toxic than poly ICLC, and non-immunogenic. To these ends, our efforts were to develop anionic polymers to replace CMC. We also replaced both PLL and CMC by modifying the PLL with engrafted polysaccharides.

Our research has resulted in the demonstration that effective IFN inducers can be formulated without using carboxymethylcellulose. Our new formulations have shown effective IFN induction and antiviral activity, some of them against two viruses, Rift Valley Fever and Punta Toro. Most of them are less toxic than poly ICLC. Of 7 types of formulations tested for immunogenicity, 6 were found to be non-immunogenic.

The most promising candidates for large-scale development as antivirals are IC-(PLL-Dextran), and ICL-β-cyclodextrin sulfate.

14. SUBJECT TERMS

Interferon inducers, antivirals, ds RNA, Rift Valley Fever virus, Punta Toro virus, Banzi virus

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements***.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	**PR**	- Project
G	- Grant	**TA**	- Task
PE	- Program Element	**WU**	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

- **DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."
- **DOE** - See authorities.
- **NASA** - See Handbook NHB 2200.2.
- **NTIS** - Leave blank.

Block 12b. Distribution Code.

- **DOD** - Leave blank.
- **DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
- **NASA** - Leave blank.
- **NTIS** - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

Foreword.

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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

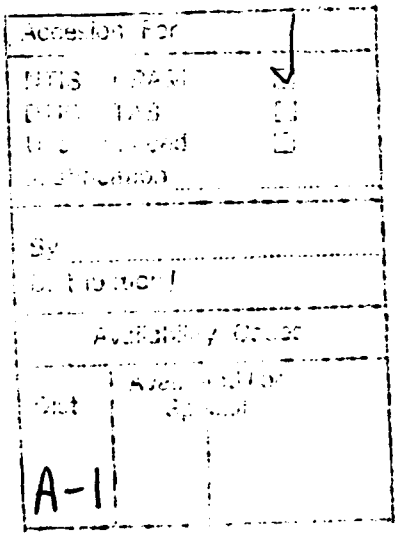


Table of Contents

Foreword....1

1. Problem Under Study....4

2. Background....4

3. Rationale of the Research....5

4. Experimental....8

5. Results....17

6. Conclusions....103

7. Bibliography....107

8. Glossary....109

9. Acknowledgements....110

Tables

1. IFN Induction in Mice by IC-(PLL-dextran)....
2. LD_{50} of IC-(PLL-Dextran)....
3. Time Course of IFN Induction by IC-(PLL-Dextran)....
in Cynomologous Monkey....
4. Melting Transitions of IC-(PLL-dextran)....
5. Protection of Mice against Rift Valley Fever Virus by IC-(PLL-dextran)....
6. Anti-Punta Toro Action of IC-(PLL-dextran)....
7. Induction of IFN in Mice by IC-(PLL-monosaccharides)....
8. ICL-ßCDSO$_4$; Compositions and IFN Titers....
9. LD_{50} Values for ICL-ßCDSO$_4$ in Mice....
10. Safety Testing of ICL-ßCDSO$_4$....
11. Anti-RVFV Action of ICL-ßCDSO$_4$....
12. IFN Induction in Mice by ICL-CM-ßCD....
13. LD_{50} Values of ICL-CMßCD....
14. Anti-RVFV Action of ICL-CMßCD....
15. Antiviral Action of ICL-CMßCD (Lot III-62) Against Punta Toro Virus in Mice....
16. IFN Induction by ICL-CMdextran....
17. IFN Induction by ICL-SO$_4$Gel....
18. Antiviral Action of ICL-SO$_4$ Gelatin Against Rift Valley Fever in Mice....
19. Action of ICL-SO$_4$Gel (III-198) Against Punta Toro Virus.
20. Toxicity of ICL-SO$_4$ Gelatin in Mice....
21. IFN Induction and LD_{50} by ICL-Gelatin in Mice....
22. Anti-RVFV Action of ICL-gel....
23. ICL-Gel: Action Against Punta Toro Virus in Mice....
24. IFN Induction in Mice by ICL-SuccGel and ICL-AcCitGel...

Tables, continued

25. IFN Induction by ICL-AcCitPLL in BALB/c Mice.....
26. IFN Induction by ICL-Hy.....
27. Melting Transitions of ICL-Amylose-SO_4.....
28. IFN Titers in Mice, Induced by ICL-Amylopectin-SO_4.....
29. IFN Induction by ICL-CMamylose.....
30. IFN Induction in Mice by ICL-CMamylose.....
31. Melting Temperatures of ICL-CMamylose.....
32. LD_{50} of ICL-CMamylose and Other Substances.....
33. Anti-Punta Toro Action of ICL-CMA (III-107).....
34. IFN Induction in Mice by ICL-C(open).....
35. Safety Testing in Mice.....
36. Safety Testing in Guinea Pigs.....
37. Polarization of Scattered Light.....
38. Substances Tested for Immunogenicity.....
39. Summary of LD_{50}Values.....
40. Summary of IFN Induction (as % of the Titer Induced by Poly-ICLC).....
41. Summary of Anti-RVFV Action in Mice.....

1. Problem Studied.

The double-stranded synthetic polynucleotide poly I.poly C (i.e., poly-inosinic acid-poly-cytidylic acid, also to be called poly IC or IC) is an interferon (IFN) inducer. In primates it is not effective as an antiviral agent, presumably because of circulating nucleases which quickly degrade it. When complexed with cationic poly L-lysine (PLL) and anionic carboxymethylcellulose, CMC, it is effective in humans[1]. But there are reservations about CMC. It appears not to be excreted or metabolized, and it is suspected as a carcinogen[2].

The problem addressed in this research was the preparation of effective, safe IFN inducers devoid of CMC. Since the IFN inducers are to be used as antiviral agents, it is not sufficient that they induce IFN, they must also show effective antiviral action. The formulations should be less toxic than ICLC, non-immunogenic, and metabolizable or excretable. To these ends our main efforts were to develop anionic polymers to replace CMC. We also replaced both PLL and CMC by modifying the PLL with engrafted polysaccharides.

2. Background.

Poly ICLC, Brief History.

Several interferon inducers have been shown to be of value as antiviral agents, as alternatives to exogenous IFN, or in conjunction with IFN or other agents. Most prominent is the double-stranded polyribonucleotide poly IC. To avoid hydrolysis of poly IC by serum nucleases, it can be protected by complexing with polylysine (PLL). This complex will be called poly ICL. Levy et al.[1] developed poly ICLC, which is poly IC complexed with PLL and carboxymethylcellulose (CMC). There are numerous reports on poly IC, and poly ICLC, which strongly support the further development of agents based on poly IC. Poly ICLC has shown favorable effects against viral infections such as yellow fever in monkeys, vaccinia virus in rabbits, simian hemorrhagic fever, encephalomyocarditis, and rabies in mice and monkeys[6]. Kende[7] reported that poly ICLC has prophylactic and therapeutic action against Rift Valley Fever virus (RVFV). The combination of poly ICLC and ribavirin showed therapeutic synergism against RVFV when administered 48 h after infection[7]. Imanishi[8] found that combined administration of IFN and poly ICLC to mice infected with herpes simplex (HSV) or ectromelia virus was more effective than either alone. Poly ICLC with HSV envelope antigen provided better protection against HSV than did the antigen alone[9]. Demyelinating encephalomyelitis responds to poly IC[10]. These agents may be effective against arboviruses and hepatitis, since IFN is effective against these infections[11-14]. Poly IC was shown to prevent varicella infection in leukemic children who had been exposed to infection[15]. IFN has been shown to circulate in some parasitic infections[16] and may be an aspect of the natural

host defense system in such infections. The use of IFN inducers may boost such defense. IFN shows a protective effect against malaria[17,18], trypanosomes[11] and some bacterial infections[16]. Lin et al.[19] showed that cells resistant to IFN retained sensitivity to poly IC, and suggested the combined use of IFN and poly IC.

Complexes based on poly IC are immune response modifiers[20,21], and radio-protective agents[22]. The spectrum of immunomodulating properties of poly IC has been reviewed by Johnson[23]. Poly ICLC augments macrophage and natural killer cell activity in mice[24,25]. ICLC increased secretion of colony stimulating factor by bone marrow cells, and increased bone marrow cells in the femur[25].

Other double-stranded RNA's such as poly(A)·poly(U), and the mismatched ds RNA poly(I)·poly(C_{12}U) known as Ampligen, have been shown to induce interferon-inducible genes[39].

A number of new candidate inducers have been developed in this work, which show a range of IFN-inducing ability, antiviral action, and reduced toxicity (compared with poly ICLC), which make them promising candidates for clinical testing.

3. Rationale of the Research:

Since CMC has undesirable features, including non-excretion, non-metabolization, and possible carcinogenicity, we sought formulations without CMC. We explored two approaches to this goal. One was the replacement of CMC with other anionic biopolymers, selected on the basis of known or expected safety, and being excretable or metabolizable. Most of the CMC replacements were selected on the basis of a history of safe use as blood volume expanders, or being closely related to such. These included gelatin, anionically-modified gelatin, carboxymethyl polysaccharides, sulfated polysaccharides, and anionic cyclodextrin. The second approach was the use of poly L-lysine (PLL) covalently grafted to a saccharide (without anionic groups). The PLL portion would bind to the poly IC, through its cationic groups, while the grafted saccharide would provide a solubilizing and hydrating effect. PLL-dextran and PLL-sugar grafts were studied. Another reason for studying these graft polymers is that dextran is readily cleared from the circulation of dogs[3]. We graft saccharide to only a fraction of the PLL residues. It may be expected that a graft polymer will be cleaved by trypsin-like enzymes and the fragments produced will be dextran chains bearing terminal oligolysine.

A. Chemical Studies: CMC Replacements.

1. Anionic Polysaccharides as CMC Replacements; Carboxymethylated and Sulfated Polysaccharides.

Carboxymethyl polysaccharides are anionic polymers in which the ionized carboxymethyl group has been introduced:

$$R\text{-}OH \quad \text{-->} R\text{-}O\text{-}CH_2\text{-}COO^- \qquad R = \text{Saccharide}$$

The carboxymethyl polysaccharides investigated as CMC replacements are the following:

a. CMdextran (CMD). We examined CMdextran of 10 kDa and 40 kDa molecular weight, and with average degrees of substitution (DS) of about 0.2 to 2 CM groups per glucose residue. The work of Chang et al.[3] showed that CMdextran is about 50-60% cleared from the circulation in dogs in about 1/2 hour, with no difference as to molecular weight of the CMdextran. The formulations of poly IC, PLL and CMD were effective IFN inducers in mice.

b. CMamylose (CMA). This was selected in expectation of low toxicity, and because of a report from China[4] (on its use as a blood volume expander) that it is non-immunogenic and is rapidly cleared from the body. In the work done so far, molecular weight has not been a concern.

c. CM-ß-cyclodextrin. ß-cyclodextrin is a small, cyclic glucose heptamer. It was carboxymethylated to introduce negative charges. It was thought that ß-cyclodextrin would have several advantages, which are described in detail below, related to excretion, purity and reproducibility inherent in a small molecule of definite size, rather than a large polymer of a range of molecular weights.

d. Sulfated poly- and oligosaccharides were also selected, because sulfate esters will bind differently, may be of low toxicity, and more metabolizable than carboxymethyl derivatives. The sulfated saccharides are:

a. Amylose sulfate.
b. Amylopectin sulfate.
c. ß-Cyclodextrin sulfate (for the same rationale as for CM-ß-cyclodextrin, as well as the potential advantages of sulfate esters).

ii. Anionically Modified Proteins and Polypeptides.

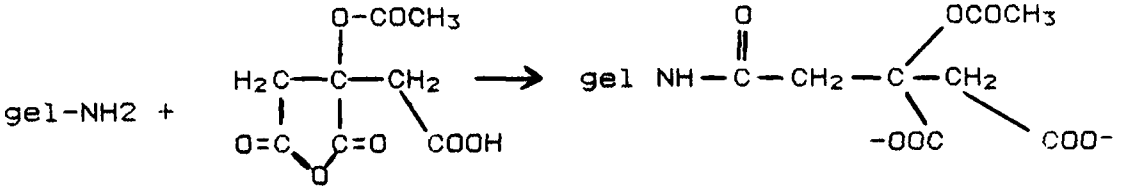
a. Gelatin.

We have investigated inducer formulations containing acetylcitryl gelatin, sulfated gelatin and unmodified gelatin. Gelatin is metabolizable and has been used as a plasma expander. Sulfated gelatin is prepared by reacting gelatin with sulfuric acid or chlorosulfonic acid. The numerous hydroxyl groups of gelatin (about 15 per 100 residues) react as follows:

$$R\text{-}OH \xrightarrow{} R\text{-}OSO_3H \xrightarrow[\text{neutralize}]{} R\text{-}OSO_3^-$$

The reactions are carried out with gelatin in cold sulfuric acid, or with gelatin dissolved in cold trifluoroacetic acid-sulfuric acid, or with chlorosulfonic acid on gelatin in H_2SO_4.

Acetylcitryl gelatin. This is a modified gelatin made by reaction of amino groups of gelatin with acetylcitric anhydride in water.



(and/or isomer with gelatin on the middle carboxyl)

The product is purified by dialysis, and is freeze-dried for storage. To our knowledge, this is the first use of this reagent for modifying a protein or polypeptide.

b. Anionically Modified Serum Albumin (HSA)

HSA was acylated with acetylcitric anhydride. This introduces a large amount of negative charge since HSA contains about 65 amino groups. It was difficult to prepare complexes from AcCit-HSA because of solubility difficulty with the modified albumin itself. As a side matter, it was found that acetylcitryl HSA was non-immunogenic in mice, although the parent HSA was strongly immmunogenic. This may be a way to prepare non-immunogenic proteins for therapeutic purposes.

c. Anionically Modified Poly(L-lysine):

AcCit-PLL. An acylation reaction with acetylcitric anhydride converts the positive ammonium groups of PLL to negative acetylcitryl groups. If AcCitPLL is metabolized, it will be converted to acetic and citric acids and to small oligol'sines or to lysine. If not metabolized, it may be excreted, if the molecular weight is not too large. Molecular weights of PLL from about 8 kDa to 55 kDa have been studied.

iii. PLL-Saccharide graft.

We grafted dextran to PLL. The reaction is a reductive alkylation of the amino groups of PLL with the terminal aldehyde groups of dextran. In the presence of sodium cyanoborohydride the intermediate Schiff base is reduced to a stable secondary amine:

$$RCHO + R'NH_2 \dashrightarrow R\text{-}CH{=}N\text{-}R' \xrightarrow{NaCNBH_3} RCH_2\text{-}NHR'$$

Formulations for biological testing are made from IC and the graft. No polyanion is used (although this would be another route to IFN inducers), and no unmodified PLL is incorporated.

4. Experimental

I. Chemical Studies.

A. Preparation of Inducer Formulations.

a. General Procedure

The procedure of Levy et al.[1] was used to prepare poly ICLC and also formulations in which a polyanionic replacement for CMC was used. This involves preparing a PLL-polyanion complex, then mixing this with poly IC. For IC-(PLL-dextran), and the analogous IC-(PLL-glucose) and IC-(PLL-ribose) the PLL-saccharide graft is mixed directly with poly IC. Some experimental formulations required modification in the ratios of components or the final concentration.

b. Sterilization of Components.

Poly IC was obtained sterile from the vendor (Pharmacia). Solutions of PLL·HB_r (Sigma), modified PLL's and modified gelatins were filtered through a 0.2 μ filter. The sterile components were mixed under a sterile hood and sealed in sterile serum vials.

It was found that standard butyl rubber septa emitted colored and turbid impurities into sterile saline. To eliminate this source of impurity, we boil the septa in water, which removes the light absorbing and turbid materials, or we use silicone septa which do not liberate impurities (at any rate impurities detectable by spectrophotometry.)

c. Quality of Saline Solutions.

Our inducer formulations have been made in sterile saline, using saline solutions packed for human use, injection or irrigation. These are packed in plastic. We examined sterile saline by light scattering and found that some lots gave markedly increased scattering at elevated temperature, increasing linearly up to 3-fold between room temperature and 90° C. Saline prepared in glass by us did not show this effect. The scattering effect of commercial saline probably arises from the plastic container, either from the polymer or from a plasticizer. The increased scattering at elevated temperature is suggestive of hydrophobic aggregation. As a result we routinely use saline made in glass.

B. Carboxymethylation of Polysaccharides.

The carboxymethylation was done by the procedure of Chang et al.[3], with modification. A typical experiment is carried out as follows: To a solution of 4 g of polysaccharide in 35 mL of NaOH solution (15 g of NaOH in 100 mL of water) at 60° C, is added 5.8 g of chloroacetic acid over 4 minutes. After 90 minutes the pH is brought to about 4.8 with glacial acetic acid, and the solution is dialyzed against water and freeze-dried. The dialysis procedure is used for CMamylose, but carboxymethyl dextran is precipitated with methanol, dissolved in water and reprecipitated.

C. Preparation of Carboxymethyl-ß-Cyclodextrin.

This reaction was carried out by a modification of the foregoing carboxymethylation procedure. To 2.2 g of ß-cyclodextrin in 5 mL water was added 2.34 g of sodium chloroacetate. NaOH (10 M) was added in small portions over 2 hr., until 2 mL had been added. After standing overnight at room temperature, the solution was heated at 50° for 4 hours. Methanol, 20 mL, was added to precipitate the CM-ß-cyclodextrin, followed by filtration and washing with 30 mL methanol, and vacuum-drying. The product was dissolved in 2 mL H_2O and passed through a column of Sephadex G-10, to separate it from NaCl and sodium glycolate (byproducts of the reaction). The fractions were tested for CM-cyclodextrin and cyclodextrin with iodine. These gave brown and purplish spots, respectively, on paper with iodine. NaCl-containing fractions were detected with $AgNO_3$. Some inducer formulations were prepared with unfractionated CM-ß-CD.

D. Analysis of Carboxymethyl Polysaccharides.

It is necessary to measure CM content of CM-polysaccharides, not so much to know the exact amount, as to be able to compare different batches; that is, reproducibility is the main consideration. The various methods described in the Hercules[8] manual for analysis of carboxymethyl cellulose are rather complex and time-consuming. The most convenient, the spectrophotometric uranyl procedure, is not applicable to all CM-polysaccharides, because of failure of some uranyl complexes to precipitate.

We have developed a simple, rapid, titrimetric procedure. The sample is dried in vacuo, weighed, and dissolved in a suitable volume of water. A portion is made 0.05 M in KCl, and is titrated with 0.1 M HCl to about pH 2.25, and a 0.05 M KCl blank is also titrated. A difference curve is drawn which gives the amount of HCl used to titrate the carboxyl groups. The difference curves show that the titration is complete at about pH 2.4. A control experiment is done with the parent polysaccharide, which was not carboxymethylated. (For cellulose itself, this cannot be done, because of insolubility).

Unmodified amylose presents a difficulty, in that it swells but does not dissolve. A modified titration was done: The amylose suspension was heated to 60° for 3 hrs. to swell the amylose; and after cooling the pH was lowered to 2.3 with a measured volume of 0.1 M HCl. The pH was measured over 45 minutes and again after 20 hours, to allow the penetration of H^+ into the swollen amylose. Almost no change was observed over 20 hours. Unmodified amylose was not expected to show any titratable groups, and this was verified.

E. Quality of Poly(L-lysine) Lots.

The poly(L-lysine), PLL, used must meet several criteria for suitability: absence of residual carbobenzyloxy (CBZ) protecting groups, absence of excessive light scattering, adequate molecular weight and, in general, having the characteristic conformational properties of PLL. Every batch of PLL is tested by spectrophotometry and circular dichroism. The substantial absence of residual carbobenzyloxy groups is demonstrated from the absorption spectrum in the benzene range, 280-250 nm, using a concentration of 5 or 10 mg/mL, which permits detection of one CBZ group per 1000 lysine residues. CBZ below about 2 per 1000 is considered satisfactory. The same spectrum also gives information on the presence of light scattering, which would be manifested as a rising slope at 350-300 nm. Light scattering would be evidence of aggregation or cross-linking. The spectrum also gives notice of the presence of other absorbing impurities.

We also measure the circular dichroism, CD, of PLL in 1 M $NaClO_4$ at 0°. When the molecular weight is sufficiently high, about 30,000, the CD spectrum will show 100% α-helix. Less than 100% helix indicates a substantial fraction of low molecular weight, or of other factors interfering with helix formation (e.g., chemical damage, presence of helix-breaking amino acids in the chain). PLL of molecular weight below 30,000 does not give 100% helix; and the lower the molecular weight the lower the helix content. Suitability of low molecular weight PLL is judged by conformity to calibration curves which have been amassed over some years.

F. Synthesis of PLL-Dextran and PLL-Sugar Grafts.

To 0.06 g of PLL-HBr in 5 mL H_2O was added 1 g of dextran and 0.063 g (1 mmole) $NaCNBH_3$, at room temperature. After two weeks of stirring, the reaction mixture was dialyzed against water and freeze-dried to yield 0.69 g of product. The crude material is purified on a 0.7 X 40 cm column of AG-50-X2 (protonated form). Unreacted dextran is eluted first with 200 mL H_2O, and the PLL-dextran graft is eluted with 400 mL 0.5 HCl, dialyzed against water and freeze-dried (yield 0.2 g). Then the product is put through Sephadex G-100, eluting with 0.2 M NaCl. Four peaks are obtained, representing unreacted PLL and three

products. The latter three are not well resolved, as expected for a series of species of varying degrees of substitution.

PLL-ribose and PLL-glucose grafts were prepared similarly using one-tenth as much sugar as dextran, but were not chromatographed.

The extent of grafting is calculated from elemental analyses for C, H and N on the graft and on the parent PLL, both calculated on the basis of the same counterion, chloride.

G. Acetylcitryl Gelatin, Acetylcitryl PLL, and Succinyl Gelatin.

To 0.4 g of PLL in 30 ml water maintained at pH 9, was added 1.9 g of acetylcitric anhydride during 2 hr. After an additional 20 minutes the solution was dialyzed against water and freeze-dried.

To 2.5 g of gelatin in 50 mL water maintained at pH 9 was added 3.76 g of acetylcitric anhydride. The remainder of the procedure was done as for PLL, above. Treatment with mixed-bed ion exchange resin to bring AcCitPLL and AcCitGel to their isoionic points, produced a pH of about 2.5 (originally 9.1 before reaction), indicating a high degree of substitution.

Succinyl gelatin was prepared from 5 g of gelatin with 2 g of succinic anhydride, in the same manner as for acetylcitryl gelatin.

H. Preparation of Sulfated Gelatin.

A gelatin solution (1%) is freeze-dried. The dry fibers are sulfated by one of the following procedures.

1) Gelatin (0.5 g) is dissolved in 9 ml of ice-cold concentrated H_2SO_4, stirred occasionally during 1 hour, and poured into 100 mL of ice-cold 4.7 M sodium acetate, pH 7. The gummy lump of precipitated SO_4gelatin is dissolved in H_2O (with gentle warming if needed) and dialyzed exhaustively against H_2O, and freeze-dried.

2) Gelatin (0.5 g) is dissolved in 10 mL ice-cold trifluoroacetic acid, 10 mL cold H_2SO_4 is added. The rest of the procedure is as above, except that 130 mL of 4.7 M sodium acetate is used.

3) Procedure[2], above, is carried out, except that the sulfation mixture is poured into ether chilled in Dry-Ice to precipitate the protein. The protein is filtered off on a fritted glass filter, washed with cold ether, cold acetone, dissolved in water, neutralized to about pH 6, dialyzed, and freeze-dried.

Freeze-dried material is dissolved in water and treated with mixed-bed ion-exchange resin (H^+ and OH^-). A pH of about 2.8 or less is indicative of a major degree of substitution.

Elemental analysis for sulfur is used to measure the extent of sulfation.

I. Synthesis of Sulfated Saccharides.

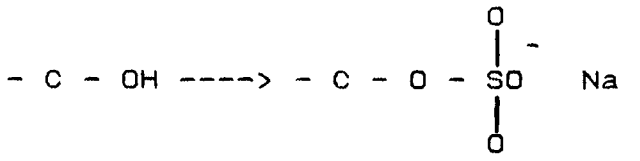
ß-cyclodextrin and amylose were sulfated with $SO_3 \cdot (CH_3)_3N$ in dimethylformamide, at temperatures and reactant ratios adjusted to obtain the desired degrees of sulfation.

For ß-CD, the reaction was carried out at 60-65° for 36 hrs, solvent was removed under vacuum, the syrup was extracted with methanol, and the methanol was cooled to 2°, resulting in the precipitation of the product. Product was purified by dissolving in water with NaOH (to pH 10), and the ß-$CDSO_4$ was precipitated with methanol, washed with methanol and tetrahydrofuran.

For amylose-SO_4 the reaction mixture was heated at 60-65° for 24 hr, then cooled to 5°, and the solvent was decanted. The residue was dissolved in H_2O with NaOH (pH 10), filtered and dialyzed against 2% $NaHCO_3$, then against H_2O, and freeze-dried. The degree of sulfation is proportional to the ratio of $SO_3 \cdot N(CH_3)_3$ to amylose. Amylopectin was sulfated similarly.

The degree of sulfation was determined from elemental analysis for sulfur. A secondary method was developed: the melting/decomposition temperature of sulfated amylose was found to be a monotonic function of the degree of sulfation.

The sulfation reaction results in the conversion of hydroxyl groups to anionic sulfate esters (sodium salts):



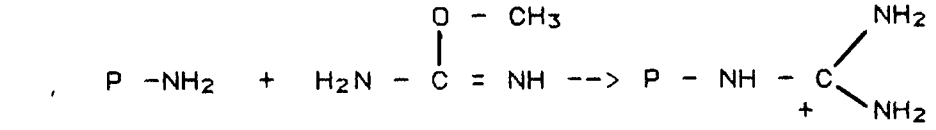
J. Methylated and Guanidinated PLL Derivatives.

a. Methylated PLL and methylated PLL-dextran grafts. Methylation was carried out on the polymer in H_2O at pH 9-10, room temperature, with dimethyl sulfate (10-fold excess), followed by neutralization, dialysis against 0.1 M NaCl, and then H_2O, and freeze-drying. Methylation was shown to be complete by ^1H nmr. The reaction is:

$$-NH_2 \quad + \quad (CH_3\text{-}O)_2SO_2 \quad \text{-->} \quad -N(CH_3)_3^+$$

b. Guanidinated PLL and PLL-dextran grafts. Guanidination was carried out on the polymer in H_2O at pH 9.5 with O-methyl-iso-urea·SO_4 (10-fold excess) followed by dialysis against 0.1 M NaCl, then against H_2O and freeze-drying. ^{13}C nmr showed the presence of guanidino groups.

The reaction is:



K. Ring-opening of CMC.

To 2 g of CMC in 0.1 M acetate buffer, pH 5, was added $NaIO_4$ (1 mole per two glucose residues or 1 mole per 10 glucose residues) at room temperature. After standing overnight in the dark, the pH was brought to 8, and a fourfold ratio of $NaBH_4$ was added (based on $NaIO_4$). After 4 hr., 4 mL of ethylene glycol was added to consume excess $NaBH_4$ and the solution was dialyzed against 0.1 M acetic acid, followed by extensive dialysis against water.

L. Fluorescent-labelled Polymers.

PLL and SO_4gelatin were labelled by reaction with dansyl chloride, followed by dialysis and freeze-drying. About 1-2 dansyl groups were introduced per 100 residues. CMC was labelled by first reacting CMC with $NaIO_4$ to oxidize one glucose residue in 80 to a dialdehyde, then reacting with fluorescein thiosemicarbazide, followed by dialysis and gel filtration on Sephadex G-75.

M. Melting Profiles.

Inducer complexes were diluted to contain 50 µg/mL of IC. Absorption spectra were recorded at room temperature from 325 nm to about 246 nm, using cells of 1 cm optical path and a Cary 219 or Uvikon 860 spectrophotometer, to ascertain that the spectra were normal and to reveal any significant degree of turbidity (manifested as absorbance at long wave length). A platinum resistance thermometer (PRT) was used, and X-Y recorder was operated off the spectrophotometer output and the PRT output). A Neslab circulating bath driven by a Neslab temperature programmer at 1° per minute was used.

Fluorescence melting profiles were carried out on complexes containing one component bearing a fluorescent label, dansyl on PLL or SO_4gelatin, and fluorescein on CMC (see below). Temperature was raised stepwise and fluorescence was measured at each step. Using vertically polarized illumination, the fluorescence intensity was measured with the emitted light passing through a vertically and then a horizontally oriented analyzer.

Light scattering profiles were measured in the same instrument, and usually on the same sample, as for fluorescence. The incident light was vertically oriented, and the scattered light intensity was read after passage through vertical and horizontal analyzers.

Fluorescence and scattered light measurements were made with an Aminco-Bowman spectrophotofluorometer fitted with a photon counter. For fluorescence, illumination was at 375 nm for dansyl groups and 450 nm for fluoresceinyl groups. Emitted light was measured at 550 nm, with a 550 nm interference filter to supplement the emission monochromator. Light scattering was done at 550 nm for both illumination and emission. Fluorescence intensities were read with the analyzer Polaroid vertical and horizontal, and the ratio of intensities was taken. (Strictly, this is not polarization or anisotropy; but it is suitable for our purposes).

N. Chromatography.

Gel filtration chromatography of inducer formulations was done on several types of column packings: Sephadex G200 and G25; Sephacryl S-400; Biogel A, 5 m; Biogel A, 15 m; Biogel A, 150 m; and Fractogel. In some cases fluorescent labels were incorporated into PLL or into the polyanion. Elution was followed by absorbance at 260 nm (for IC), and in some cases also by fluorescence and light scattering.

O. Digestion by Ribonuclease (RNase).

An inducer formulation is diluted to 50 µg/mL of poly IC in normal saline. Five µg of bovine pancreatic RNase is added, and the optical absorbance at 260° nm is measured with time, at 37°. The initial slope is measured for calculating the rate, and the total change in absorbance is measured, in order to estimate the extent of digestion.

II. Biological Studies

A. Interferon Induction in Mice.

The interferon inducers were evaluated for interferon production in BALB/c mice. Each inducer was compared to a standard poly ICLC preparation. Twenty gram mice were given a single i.v. injection of inducer containing 10 µg of poly IC. Blood was obtained by orbital bleeding 3 hours after injection. Usually there were 8 treated mice per group plus 2 mice that received placebo. Serum was assayed for interferon.

B. Interferon Assays.

Three-fold dilutions of serum were made in Minimal Essential Medium (MEM) containing 5% fetal bovine serum (FBS). The dilutions were done in 96-well microtiter plates, followed by addition of 20,000 murine L-cells/well. The cultures were incubated for 18-20 hours, the trays inverted to remove the medium, and vesicular stomatitis virus, at a multiplicity of infection of 0.15 plaque-forming units per cell, in MEM containing 2% FCS, was added to the cells. The cultures were incubated until virus controls showed marked cytopathic effects (24-48 hours). The medium was removed and antiviral activity determined by a standard colorimetric procedure which measures uptake of a vital dye, neutral red. Interferon titers are expressed in international reference units based upon standards received from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, NIH.

C. Antiviral Effect.

Formulations were submitted to Dr. Meir Kende, Fort Detrick, for testing against Rift Valley Fever virus and Banzi virus in mice, and to Dr. R. Sidwell, Utah State University for testing against Punta Toro virus in mice.

D. Toxicity Testing (LD_{50}).

LD_{50} was measured by injecting mice with doses of formulation containing 100, 200, 400, 600, 800 and sometimes 1000 µg of IC and observing the number surviving after 12 days.

E. Safety testing.

Selected inducers were evaluated for effect on weight in mice and guinea pigs according to the FDA Code of Federal Regulations on general safety.

Four Balb/c mice (16-20 g) and two Hartley guinea pigs (300-400 g) were injected intraperitoneally (i.p.) with 8 mg/kg of inducer in 0.5 ml or 5.0 ml, respectively. This dose was determined by results of experiments in which various doses of the standard inducer, Poly ICLC, were injected into mice and guinea pigs.

Results with the selected inducer are compared to those obtained with the Poly ICLC standard.

F. Testing for Immunogenicity. (This work was done by Dr. Oliver Roholt)

a. Immunization protocol.

Groups of 6-8 week old female BALB/c mice, 5-6 per group, were used. The mice in each group were injected subcutaneously

with one of the test antigens. The first injection consisted of 100 µg of the material in 0.1 or 0.2 mL of a 1:1 emulsion of Freund's complete adjuvant and the PBS solution of the material. Injections were with 1 mL disposable syringes, and although the emulsion is very viscous 25-guage needles were successfully used. After 3-week and 6-week periods a similar injection, but using Freund's incomplete adjuvant, was given.

b. Collection of serum.

About two weeks later the mice were bled by cutting off about 1/2 inch of the tail and letting the blood drip into a 1.5 ml plastic centrifuge tube. This method of bleeding consistently produces a better yield, is faster and easier than bleeding from the heart. Furthermore, the mice survive and can be again bled after a few days by removing 1/8-1/4" more of the tail; this can be done a number of times.

The tail-bleeding is facilitated and only really successful if the mice are warmed-up under an infra-red light for 4-5 minutes before bleeding. The whole group of mice is initially warmed-up and kept warm as individual mice are bled. Warming should be to a substantial degree and can be monitored by holding one's hand near the mice and by observing the activity of the mice. It is well to place a paper towel on the cage bottom (no sawdust) since the bare cage bottom may get uncomfortably hot. An individual mouse is removed from the cage by the tail and grasped firmly and gently in a crumpled paper towel with the tail held projecting from the towel by the thumb and forefinger. The tip of the tail (on a firm surface) is cut off with a razor blade and the drops of blood then permitted to fall into the centrifuge tube. With experience, about 15 good sized drops can be collected in less than a minute. The blood flow can be stopped by squeezing the tail for a half minute or so. A little bleeding may follow briefly when the mouse is returned to a clean cage but all of the mice have always survived.

Following overnight clotting of the blood, the tubes are centrifuged (Microfuge) for a good recovery of clear serum. As much of the serum as can be withdrawn (along with some red blood cells) using a disposable pipet with a control, not a rubber bulb, is transferred to a second 1 1/2 ml centrifuge tube and this is centrifuged. From this tube a good yield of clear, cell-free serum can be withdrawn from the very small red cell pellet, and stored in the freezer.

c. Testing sera for antibodies.

To determine whether or not the sera contained antibody, a precipitation-in-gel procedure was used. In this procedure, a commercially available apparatus (LKB) permits one to pour an approximately 1.3 mm thick layer of molten 1.5% agar solution in buffer onto labelled 1 inch X 3 inch microscope slides, after which a hexagonal array of 3 mm holes, 8 mm between holes, and

around a single central hole, is cut in the agar on each half of the slide.

The 5-6 sera obtained from each of the ten groups of mice were tested against one or more of the injected materials. Thus, 8-10 μL of each serum from the five individual mice injected with an antigen (say, carboxymethyl dextran) was placed in five individual wells of each of four of the hexagonal arrays on two labelled slides; the center well of each of the four arrays was then filled (8-10 μL) with one of the four solutions which the serum is to be tested against. Diffusion was allowed to proceed in a closed, moist chamber for 24 hours at room temperature; under these conditions, even the finest precipitin arcs ordinarily appear and little or no increase in the density of the arcs occurs with longer periods of time. Careful examination of the slides after 24, 48 and 72 hours revealed no precipitin arcs in any experimental case, but did show precipitin arcs (as expected) in the case of bovine serum albumin. Repetition of the procedure using an agar solution containing 0.5% polyethylene glycol which enhances the precipitin reaction in gel still did not result in any precipitin arcs.

5. Results.

A. Introductory Notes to the Results.

Each type of inducer candidate has three polymeric components, which means that many variations in composition are possible. In addition to the proportions of the components, other variables are molecular weight and degree of modification. In addition, reproducibility must be determined using a biological assay subject to variability in mouse response. The exploration of the possible combinations is limited by our biological testing capacity. The original mixing procedure of Levy has been used for the inducer formulations involving IC + PLL + polyanion.

Antiviral data were, mostly, obtained by Dr. Meir Kende. Some results were provided by Dr. Gan-Gemi, Medical School, University of South Carolina, and Dr. Sidwell, University of Utah. The antiviral data for each class of IFN inducer are summarized in the section for that inducer. In addition a summary of the overall antiviral results is given at the end of the report.

i. IFN Induction vs. Antiviral Action.

We test our experimental agents for their ability to induce IFN in mice. IFN induction is measured by the ability of mouse blood serum to protect cells in culture against vesicular stomatitis virus. Direct protection of mice against a virus is not measured here. Since there is no clear understanding yet of the optimum IFN blood titers, we do not reject agents which

induce modest IFN titers. Also, blood is taken three hours after injection of the inducer, which may not be near the time of peak IFN titer. As will be seen, modest IFN titers can accompany effective antiviral action. The latter is measured at Fort Detrick by Dr. Meir Kende, using Rift Valley Fever virus in mice and by Dr. R. Sidwell, at Utah State University using Punta Toro virus in mice. Dr. D. Gan-Gemi has measured the time course of IFN induction of one inducer in a monkey species.

ii. Tables of IFN Induction.

All inducer tests are done with a single dose containing 10 μg of IC per mouse. Each IFN titer for an experimental formulation shown is the average of the titers of 7-8 mice, and is compared with the average titer for 7-8 mice obtained with ICLC as the standard.

In the tables of IFN induction the composition of inducers is given in actual mg/mL, for each component (in the order shown). The solution to be tested is diluted with saline and a 200 L dose is injected i.v. In the course of the work we discovered that standard septa for serum bottles and standard sterile saline contain avoidable impurities (see Experimental). We make our own sterile saline and use silicone septa.

iii. Digestion by Ribonuclease.

It has been standard procedure, beginning with the pioneering work of Levy on ICLC, to measure the rate of digestion of poly C in the ICLC complex. A low rate of digestion (relative to the digestion of simple poly IC) has been taken as indicating that the poly IC is complexed. This is not necessarily the case. While a fast rate of digestion is usually indicative of uncomplexed poly IC, a slow rate is not necessarily indicative of the converse, because we have observed that some of the polyanions (i.e., CMC, CMamylose, amylose sulfate) are inhibitors of ribonuclease. Therefore, when a complex containing an RNase inhibitor is only slowly hydrolyzed by RNase, there is no way to tell if this is because of protection of poly IC within the complex or because of inhibition of the RNase. Therefore, we no longer routinely measure digestion by RNase, but do so only for formulations not containing an RNase inhibitor.

B. Summary of Results on Interferon Inducer-Antiviral Agents.

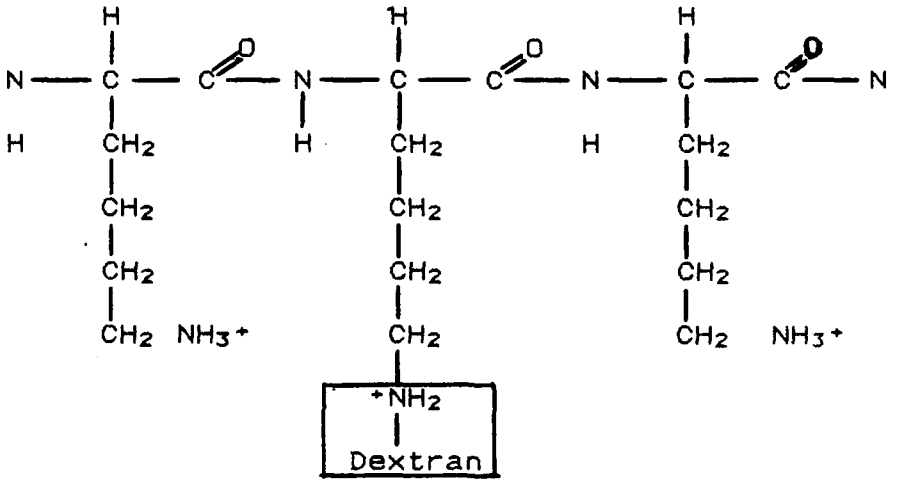
Results are presented below for IFN inducers based on poly IC, poly(L-lysine) and the replacement for carboxymethylcellulose (CMC). The results include IFN titers, antiviral action, LD_{50}, safety testing, and some physical data. The results are organized by the types of polymer being investigated as CMC replacements.

a. Poly(L-Lysine)-Dextran Grafts (IC-(PLL-dextran)).

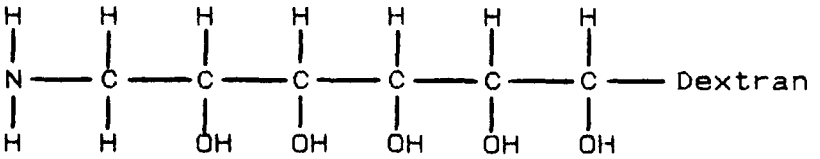
An approach that was studied is the replacement of both PLL and the anionic polymer by a PLL-polysaccharide graft. The rationale is that the positive charges would result in binding to the IC, with protection against serum nucleases, while the grafted polysaccharide would enhance solubility. (Since the graft polymer retains its positive charge, it could be used in place of PLL along with a polyanion. This more complex assembly has not been tried.)

The graft is made by the reductive alkylation method, in which aldehyde groups at the termini of the dextran chains condense with amino groups of PLL, in the presence of $NaCNBH_3$, to generate dextran-substituted amino groups. Reactions have been run with a large excess of dextran to PLL, since the alkylation reaction is not efficient. A number of grafts have been made, with PLLs and dextrans of different molecular weights. Both the dextranated and unmodified amino groups are protonated at pH 7. However, electrostatic binding to the IC would be expected to be stronger for unmodified lysine than for modified, because of steric constraints in the latter.

The PLL-dextran graft is prepared by the covalent attachment of dextran to poly(L-lysine), and contains the following structure:



The portion in the box has a PLL-dextran link as follows.



in which the terminal glucose residue of dextran is linked to the amino group of lysine.

Although IFN induction in mice (at our standard blood sampling time of 3 hours after injection) was typically about 10-30% of that induced by poly ICLC (Table 1), there was good antiviral action and lower toxicity than for poly ICLC (Table 2). Also, an initial pharmacokinetic study in a monkey showed that a *formulation of IC-(PLL-dextran) induced as high a titer of IFN as did poly ICLC, but with a different time-course (details below, Table 3).

Complexes of the graft with poly IC have no anionic polymer (except, of course, the poly IC). Only a fraction (see below) of the amino groups of PLL are engrafted with dextran. In principle this should permit digestion of the PLL backbone to liberate fragments small enough to be eliminated.

Work was done to study variations in several variables: 1) molecular weight of PLL; 2) molecular weight of dextran; 3) ratio of dextran to lysine; 4) ratio of graft polymer to poly IC.

i. Characterization of PLL-Dextran Graft Polymers.

Initially we characterized the PLL-dextran grafts by proton nuclear magnetic resonance (nmr). Approximate ratios of dextran to lysine were obtained, but it was not possible to obtain sufficient accuracy. We then turned to elemental analysis for carbon and nitrogen.

A graft (#BE661X), made from PLL of 62.5 kDa and T-10 (10 kDa) dextran was analyzed, along with PLL-HBr. The analyses for carbon and nitrogen of PLL·HBr were 92 and 90% of theory, indicating a probable 9% water in the sample. (It is difficult to remove all water from PLL·HBr, in vacuum over phosphoric anhydride, at room temperature. Therefore, analysis for hydrogen is not useful.) The graft polymer was in the form of the hydrochloride. The N analysis corresponds to a weight ratio of dextran to lysine of 12. This is in the range of 10-15 estimated from nmr. Another lot of graft polymer, BE132, prepared from PLL of 21.5 kDa and dextran of 10 kdal, was calculated to have a ratio of glucose residues to lysine residues equal to 17.

Because the average molecular weight of the dextran is 10,000, equivalent to 62 glucose residues per average dextran chain, there is about one dextran chain per 5 lysine residues in BE661X and one dextran chain per 3.65 lysine residues in BE132.

Table 1. IFN Induction in Mice by IC-(PLL-dextran)[a]

	Mol. wt. of PLL (kDa)	Mol. wt. of Dextran (kDa)	IFN titer	IFN titer of poly ICLC	IFN titer as % of poly ICLC titer
IV-18	6	10	86	799	11
IV-19	6	10	35	799	4
IV-20	6	70	84	799	11
III-135A[b]	21.5	10	45	357	8
III-190[b]	21.5	10	151	710	21
III-230[b]	21.5	10	173	1235	14
III-232[b]	21.5	10	77	1235	6
III-285	21.5	10	242	1667	15
III-297	21.5	10	76	810	9
III-298	21.5	10	118	810	15
V-17	21.5	10	174	1700	10
V-50	21.5	10	95	778	12
III-252	21.5	40	270	539	50
III-283	21.5	40	197	1667	12
V-100	21.5	40	84	778	11
III-253	21.5	70	204	539	38
III-284	21.5	70	253	1667	15
V-108	21.5	70	137	778	18
III-135B	38	10	78	357	13
V-120	50.5	10	74	778	10
III-286	62.5	10	351	1667	21
IV-131[b]	62.5	40	133	1097	12
IV-132[c]	62.5	40	430	1097	39
IV-224	62.5	40	157	866	18
IV-258[b]	62.5	40	134	1128	12
IV-259[c]	62.5	40	220	1128	20
V-144	62.5	40	159	306	52
IV-225	62.5	70	127	1039	12
V-146	62.5	70	303	306	100
IV-51	406	10	307	1183	26
IV-52	406	70	264	1183	22

[a] All lots made in the proportions: 2 mg poly IC per mL and 7.5 mg PLL-dextran per mL.

[b] PLL-dextran graft made with 2X the standard dextran to poly IC ratio.

[c] PLL-dextran graft made with 1/2 the standard dextran to poly IC ratio.

The weight ratio of dextran to PLL is 15:1 for BE661X (not including the chloride counterion of PLL). Since the weight ratio of PLL-dextran to poly IC in the IC-(PLL-dextran) inducer formulation is 7.5:1, the ratio (of residues) of PLL to poly IC in the inducer formulation is 0.5:1; or restating it as IC:PLL it is 2:1. This is the ratio for complete coverage of IC by engrafted PLL.

ii. Tryptic Digestion of PLL-Dextran Graft Polymer.

Part of the rationale for investigating the PLL-dextran graft polymer was the idea that the PLL backbone would be digestible by proteolytic enzymes, thereby degrading the polymer to pieces small enough to be excreted.

We have tested the digestion of a PLL-dextran graft by trypsin. The graft (BE661X), made from PLL of 62.5 kDa and dextran of 10 kDa, was digested by trypsin. The technique used to detect this was circular dichroism (CD), and the PLL-dextran graft was compared with PLL as a standard. Both PLL and PLL-dextran in 1 M $NaClO_4$ are in the α-helical conformation. The PLL and PLL-dextran graft were treated with trypsin overnight; $NaClO_4$ was added to 1 M, and the CD spectra were recorded at 1°C (to obtain maximum helix content, i.e., maximum sensitivity). In both cases the CD spectrum characteristic of the α-helix was replaced, after trypsin treatment, by the spectrum of unstructured, low molecular weight oligolysine. Thus, this PLL-dextran is digested by a common proteolytic enzyme.

iii. Molecular Weights.

We have examined the effects of varying the molecular weights of the PLL and dextran. We used PLL of molecular weights 6000, 21,500, 38,000, 50,500, 62,500 and 406,000, and dextrans of molecular weights of 10,000, 40,000 and 70,000, in various combinations. IFN inductions for these preparations are given in Table 1.

iv. Dextran-to-Lysine Ratio.

Most of the grafts were made with a constant input ratio of dextran to PLL, regardless of the molecular weights of the polymers. Experiments were also done with higher and lower ratios of dextran to PLL, namely twice and one-half the standard ratio (Table 1). These are lots IV-131 and IV-258 (2X) and lots IV-132 and IV-259 (1/2X). IV-132 induced more than three times as much IFN as did IV-131. Because IV-132 had all of its poly IC complexed, it appears that the complexed poly IC is the more active component than is free poly IC. The range of IFN induction for these four are similar to the general variation in IFN induction.

We have investigated the best proportions of IC and (PLL-dextran) in the complex. The complexes III-135A, III-135B and III-190 had weight ratios of 2:10 (i.e., 2 mg of IC and 10 mg of grafts per mL). Two batches of IC-(PLL-dextran) have been prepared, one of which, III-230, with a ratio of IC to (PLL-dextran) of 2:5, and the other, III-232, with a ratio 2:7.5. The IFN titers were 173 for III-230, and 77 for III-232, or 14 and 6%, respectively, of the ICLC standard (Table 5). These values are consistent with those found for III-135A which contained 1/2 the dextran of III-230 & 232, III-135B which contains PLL of MW 38,000, and III-190 which is the same composition of III-230 & 232. For III-230 (ratio 2:5) the melting profile showed two transitions, at 66° and 76°. The lower Tm may be a complex of only slightly greater stability than free IC. RNase hydrolysis of III-230 proceeds to 35% of the extent of free IC (corresponding to the ratio of the first transition ΔA to the total ΔA), but the rate of digestion by RNase is about one half of that expected if 35% of the IC is free. Either about 35% of the IC is weakly complexed, or IC double helix is only partially covered by PLL-dextran, and the intervening IC is somewhat stabilized by the adjacent covered segments. The 2:5 (III-230) complex has a second transition at 76°, or 8° lower than the single transition of the 2:10 (III-190). Thus, a higher ratio of (PLL-dextran) to IC raises Tm. This suggests a cooperative interaction in the latter.

At an IC to (PLL-dextran) ratio of 2:7.5, the low melting transition vanishes, and only the transition at high Tm is seen, at 83°. Thus, the stoichiometry has been narrowed to a ratio somewhere near 2:7.5.

Table 2. LD_{50}[a] of IC-(PLL-Dextran).

Compound Lot #	Molecular Weight of PLL, in kDa	Molecular Weight of Dextran, kDa	LD_{50}, mg/kg
Poly ICLC[b]	-	-	11[c]
IC-(PLL-dextran)			
IV-18	6	10	32
IV-19	6	10	29
IV-20	6	70	27
III-285	21.5	10	>40
III-190	21.5	10	25
III-230	21.5	10	35[d]
III-232	21.5	10	30
III-297	21.5	10	43
V-17	21.5	10	>40
III-283	21.5	40	40
V-100	21.5	40	33
III-284	21.5	70	44
V-108	21.5	70	>40
V-120	50	10	32
III-298	62.5	10	48
III-286	62.5	10	>40
IV-258	62.5	40	>50
IV-259	62.5	40	>32
IV-131	62.5	40	42
IV-132	62.5	40	31
V-144	62.5	40	30
V-146	62.5	70	>40
			average >35

[a] Dose required to kill 50% of mice.
[b] Standard ICLC.
[c] Average of 4 tests (13, 11, 10, 9 mg/kg).
[d] Average of 2 tests (33 and 37 mg/kg).

Although the IFN titers induced by most lots of IC-(PLL-dextran) are in the range of 10-30% of that of poly ICLC, they show effective antiviral activity. Our IFN titers are measured with blood drawn 3 h. after injection of the drug into mice. Pharmacokinetic studies would be needed to determine the times at which peak levels of each inducer occur. A start has been made. Dr. D. Gan-Gemi (School of Medicine, Univ. of South Carolina) has measured IFN induction by an IC-(PLL-dextran), lot III-252 in the squirrel monkey (Saimiri sciureus), with the following results (Table 3).

Table 3. Time Course of IFN Induction by IC-(PLL-Dextran) in Squirrel Monkey.

	IFN Titer		
	4 h.	12 h.	24 h.
Poly ICLC	22	98	870
IC-(PLL-dextran)	0	800	340

(This lot of IC-(PLL-dextran) gave an IFN titer of 270 units in mice at 3 h., 50% of that of the poly ICLC IFN titer).

In this primate, IC-(PLL-dextran) caused IFN to peak earlier than did poly ICLC, reaching essentially the same titer as did the latter, and still having at 24 h. 43% of its peak titer. This result shows that IC-(PLL-dextran) in one primate induces as high a titer as poly ICLC and does so earlier. This result emphasizes the need to obtain kinetic data in primates.

iii. Toxicity of IC-(PLL-Dextran).

The LD_{50} values for a number of lots of IC-(PLL-dextran) are given in Table 2. All lots of IC-(PLL-dextran), regardless of the molecular weights or proportions of components in the graft polymer, were less toxic than was poly ICLC. While sufficient data are not available for reliable statistics, there appears to be a trend toward the lowest molecular weight PLL (6 kDa) giving the lowest LD_{50} values. These materials also gave low IFN titers.

iv. Melting Profiles.

Melting profiles of the IC-(PLL-dextran) formulations show either one or two melting transitions. The first transition is either at 67-68° or at 70-75°, the second at 83-90°. The melting transition of poly IC alone is at about 64°. The transitions at 70° and above are surely for complexed poly IC. Those at 67-68° probably are also. There is no apparent correlation between Tm and IFN induction or LD_{50}.

For those cases with two Tm values, the second Tm usually had 65-85% of the total A of the whole transition. Some formulations had a Tm higher than that of free poly IC, in the range of 66-75°. The high end of this range probably represents complexed poly IC. The percentages of Δ A shown in Table 4 are rounded to the nearest 5%, as there is some overlap, usually, between the two transitions. The percentages for Δ A for Tm, represent the percentages of free poly IC. However, this must be taken with a caution, namely, that the total As for the transitions are not all the same. The variations probably represent differences in light scattering, arising from differences in the state of aggregation and molecular weight. Nor can the digestion by RNase be taken as a valid criterion of free poly IC, because of protective effects of PLL (or its derivatives) on adjacent uncovered poly IC. These considerations apply to all the Tm and RNase data.

Table 4. Melting Transitions of IC-(PLL-dextran).

Lot	Molecular Weight PLL (kDa)	Dextran (kDa)	Tm_1 °C	Δ Ac %	Tm_2 °C	Δ Ac %
IV-18	6	10	-		73	100
IV-19	6	40	-		75	100
IV-20	6	70	-		73	100
III-135A	21	10	-		85	100
III-190	21.5	10	-		87	100
III-230	21.5	10	66°	30	76	70
III-232	21.5	10	-		83	100
III-285	21.5	10	-		78	100
III-297	21.5	10	-		80	100
V-50	21.5	10	-		80	100
III-252	21.5	40	64°	20	83	80
III-283	21.5	40	63°	25	82	75
V-100	21.5	40	70	35	84	65
III-253	21.5	70	64°	25	83	75
III-284	21.5	70	63°	20	82	80
V-108	21.5	70	75	15	84	85
III-135B	38	10	-		85	100
III-286	62.5	10	-		83	100
III-298	62.5	10	-		85	100
IV-224	62.5	40	67°	35	90	65
IV-225	62.5	70	67°	40	87	60
IV-131[a]	62.5	40	67°	40	87	60
IV-258[a]	62.5	40	68°	50	85	50
IV-259[b]	62.5	40	-		87	100
IV-132[b]	62.5	40	-		88	100
V-144	62.5	40	67	25	88	75
V-146	62.5	70	68	30	88	70
IV-51	406	10	-		85	100
IV-52	406	70	73°	25	90	75
V-120	50	10	73	30	83	70

[a] PLL-dextran, with 2X the standard dextran-to-lysine ratio.
[b] PLL-dextran, with 1/2 the standard dextran-to-lysine ratio.
[c] % of Δ A of total transition.

v. Antiviral Action.

Thirteen lots of IC-(PLL-dextran) were tested against Rift Valley Fever virus (Table 5). The overall results were comparable to, or somewhat better than those with poly ICLC. The data show no correlation between anti-RVFV activity and molecular weights of the PLL and dextran components. Combined with the lower toxicity of IC-(PLL-dextran) these antiviral results show this class of formulation to be promising.

Table 5. Protection of Mice against Rift Valley Fever Virus by IC-(PLL-dextran).[a]

Preparation	Inducer dose µg/mouse	Survivors Experimental Day 22	Survivors Poly ICLC Standard Day 22
none	-	0	-
III-230	10	100	90
	2.5	100	100
III-232	10	90	90
	2.5	90	100
III-252	10	90	90
	2.5	50	100
III-253	10	100	90
	2.5	90	100
III-135A	2.5	100	87
III-135B	2.5	80	100
III-190	2.5	100	70
III-283	10	100	80
	2.5	100	50
III-284	10	90	80
	2.5	100	50
III-285	10	100	80
	2.5	100	50
III-286	10	100	80
	2.5	100	50
III-297	10	100	80
	2.5	100	50
III-298	10	100	80
	2.5	100	50
		(average 93%)	(average 78%)

[a] 10 mice per group except for lot III-135A (15 mice). 250 PFU of virus per mouse.

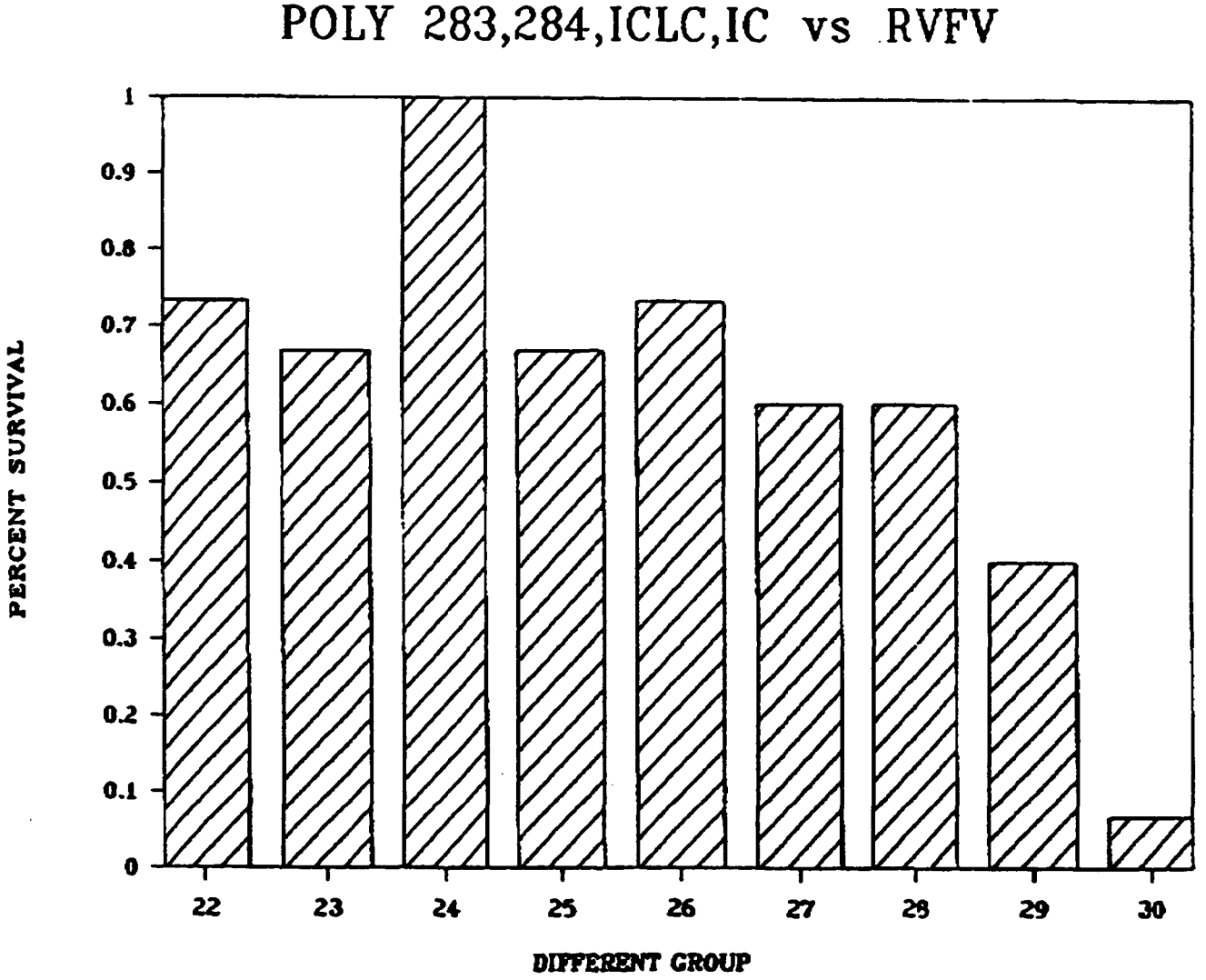


Fig. 1. Anti-RVFV action of IC-(PLL-Dextran) (lots III-283, III-284) at 21 days Post-Challenge.
Groups 22, 23: III-283, 20 µg/mouse and 5 µg per mouse.
Groups 24, 25: III-284, 20 and 5 µg/mouse.
Groups 26-29: Poly ICLC, 20, 5, 20 and 5 µg/mouse.
Group 30: RVFV control.

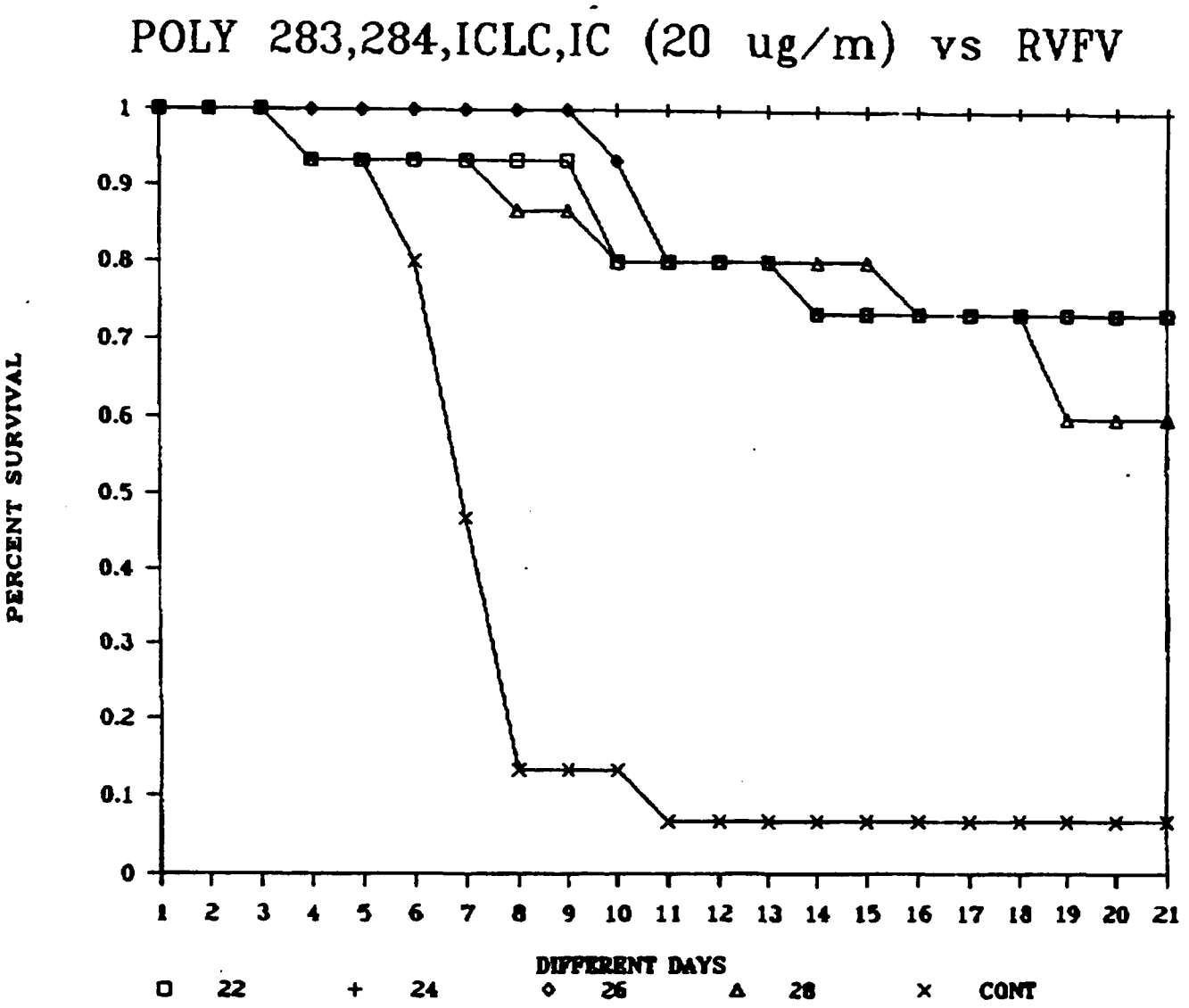


Fig. 2A. Anti-RVFV action of IC-(PLL-Dextran). Groups as in Fig. 1, at 20 µg/mouse.

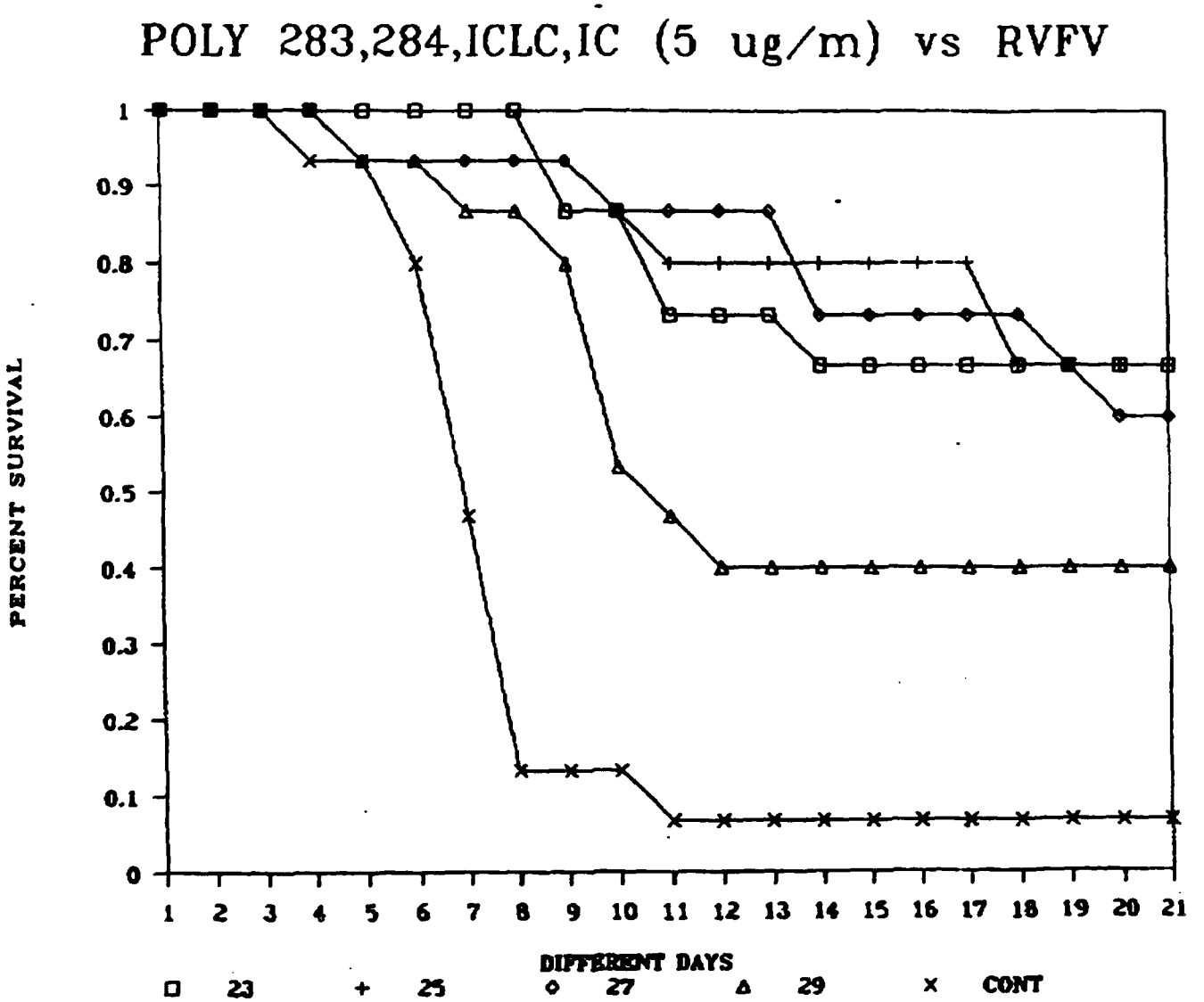


Fig. 28. Same as Fig. 2A, at 5 µg/mouse.

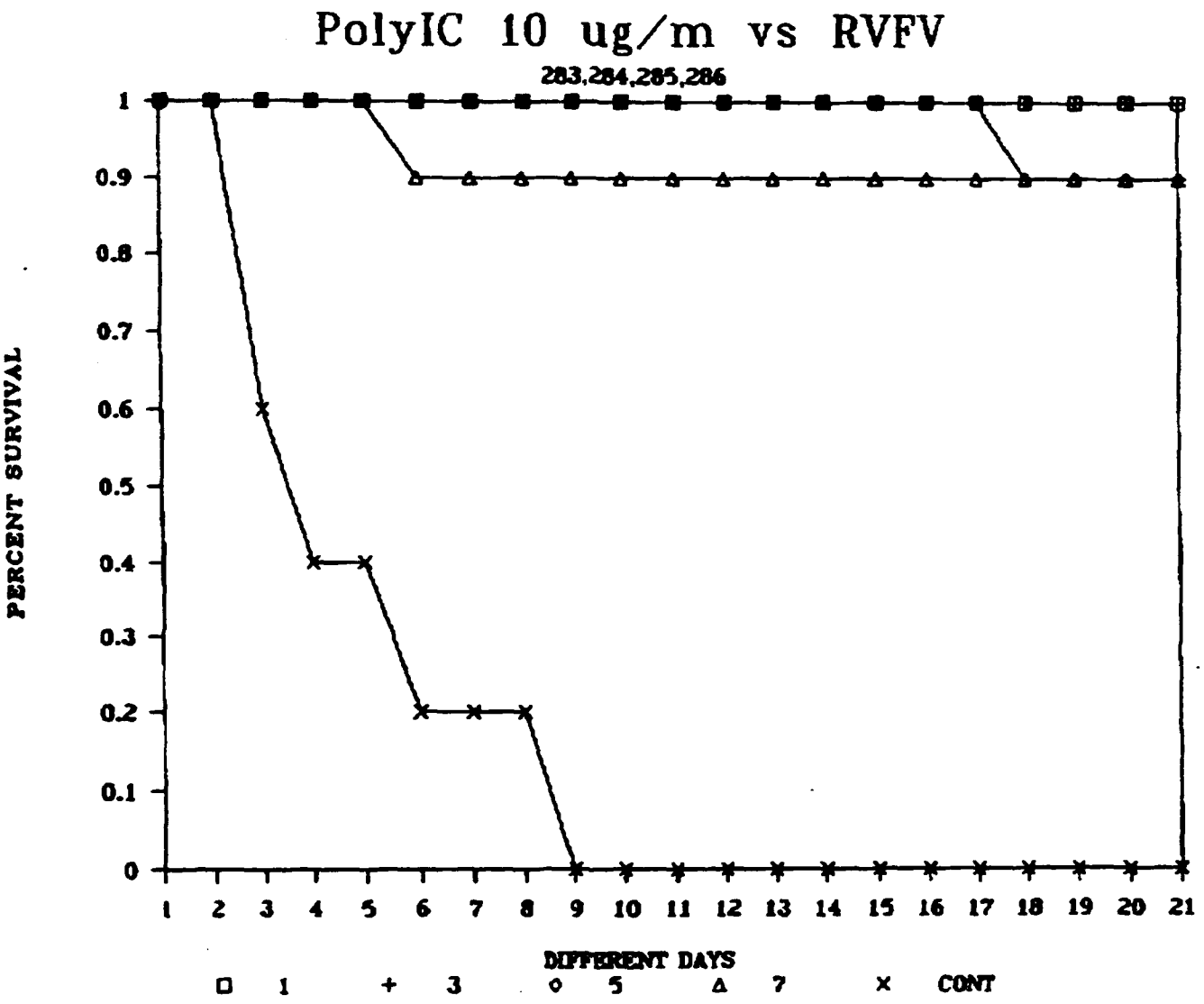


Fig. 3. IC-(PLL-Dextran), 10 µg/mouse.

Group 1, III-283; Group 3, III-284; Group 5, III-285; Group 7, III-286; X, control.

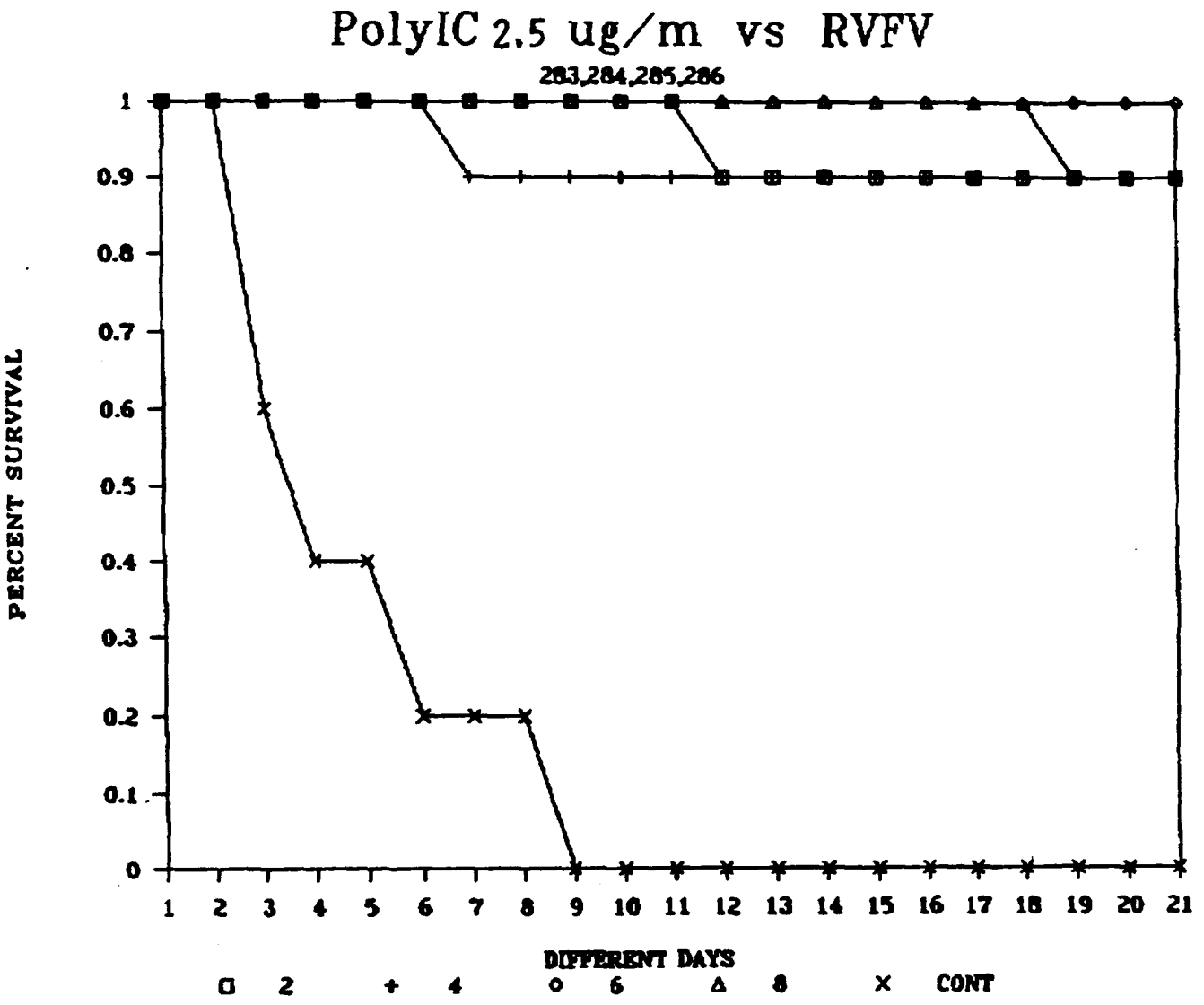


Fig. 4. Poly IC-(PLL-Dextran), 2.5 µg/mouse.

Group 2, III-283; Group 6, III-285; Group 4, III-284; Group 8, III-286; X, control.

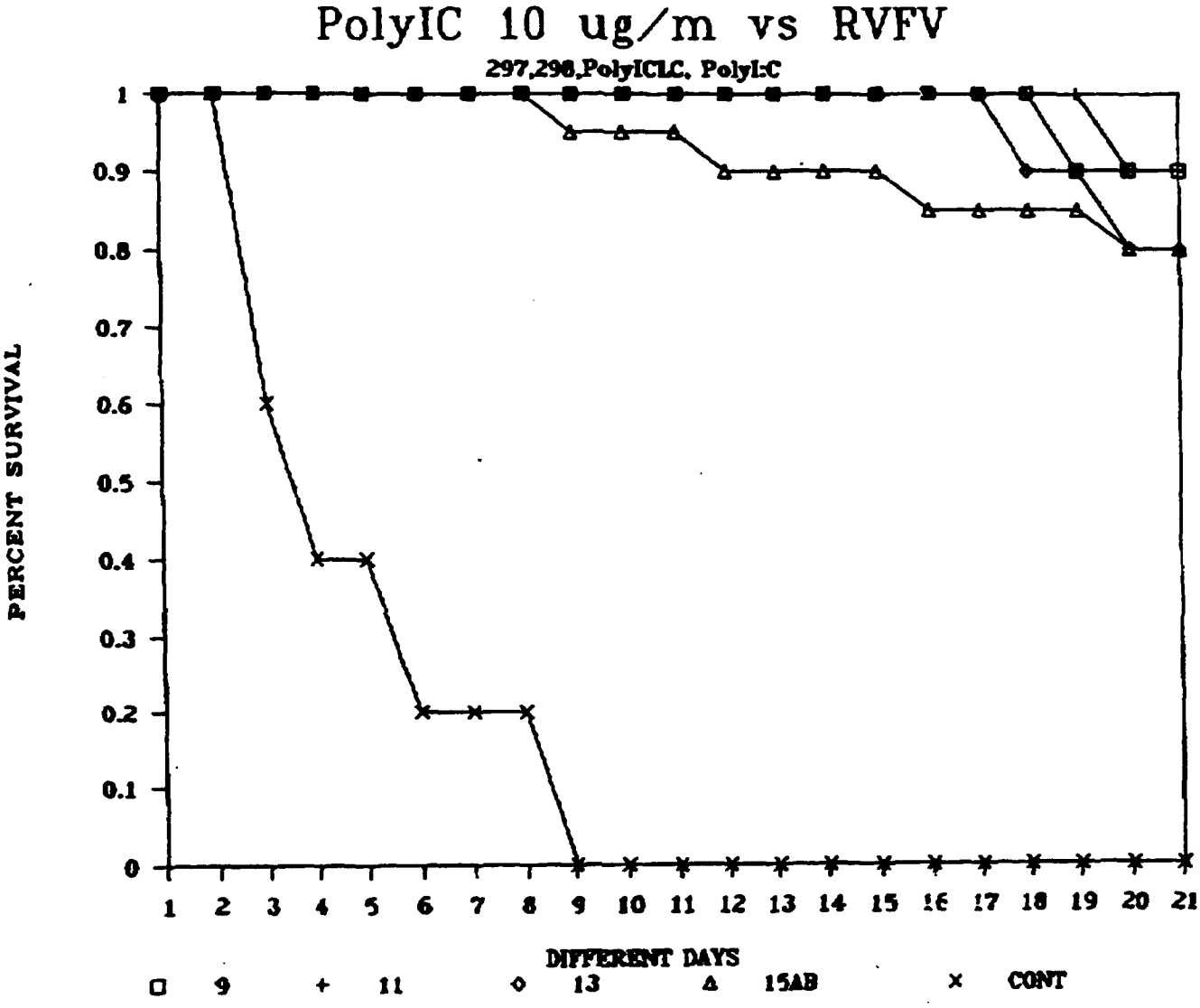


Fig. 5. IC-(PLL-Dextran), 10 µg/mouse.

Group 9, III-297; Group 11, III-298; Group 13, Poly ICLC; Group 15 A8, Poly IC; X, control.

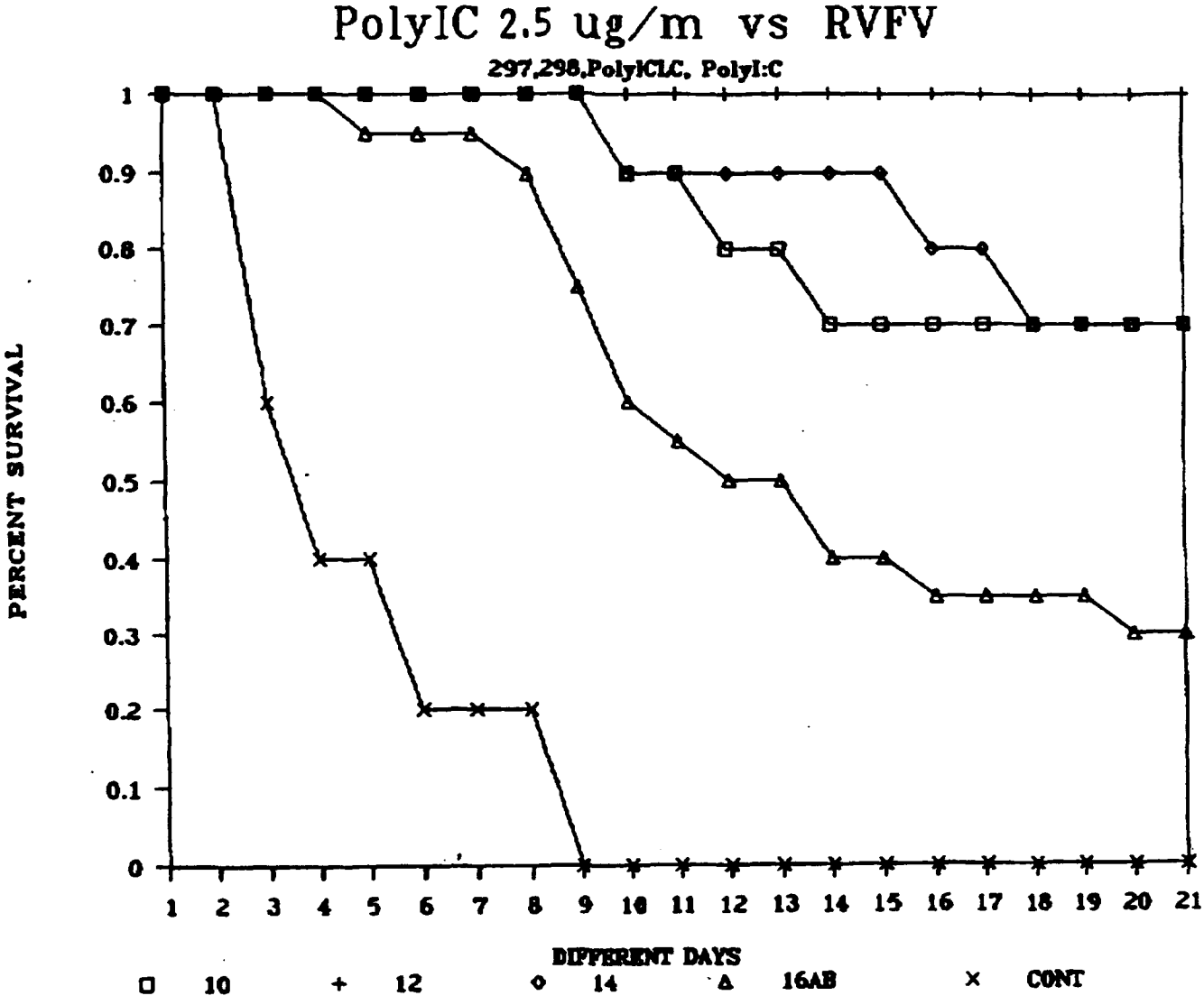


Fig. 6. Anti-RVFV action of IC-(PLL-Dextran).

Group 10, III-297 2.5 µg/mouse
Group 12, III-298 2.5 µg/mouse
Group 14, Poly ICLC 2.5 µg/mouse
Group 16AB, Poly IC 2.5 µg/mouse
X, Control

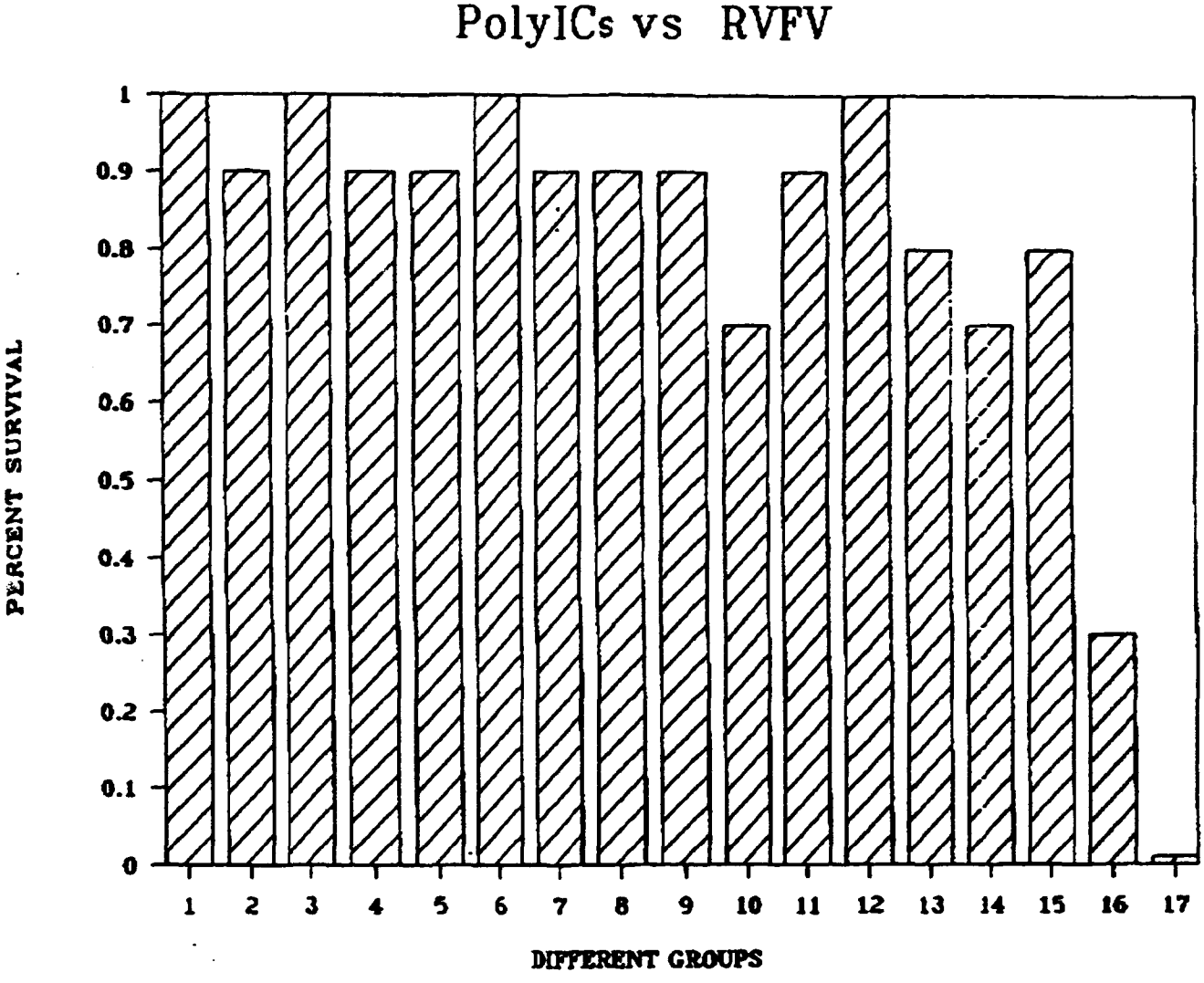


Fig. 7. Survival at 21 Days for Mice Treated with IC-(PLL-Dextran).

Groups in order from 1-12: III-283, III-283; III-284; III-284; III-285; III-285; III-286; III-286; III-297; III-297; III-298; III-298. Group 17 RVFV odd groups (except 17), 10 µg/mouse; even groups, 2.5 µg/mouse. Groups 13, 14, poly ICLC. Groups 15, 16, poly IC.

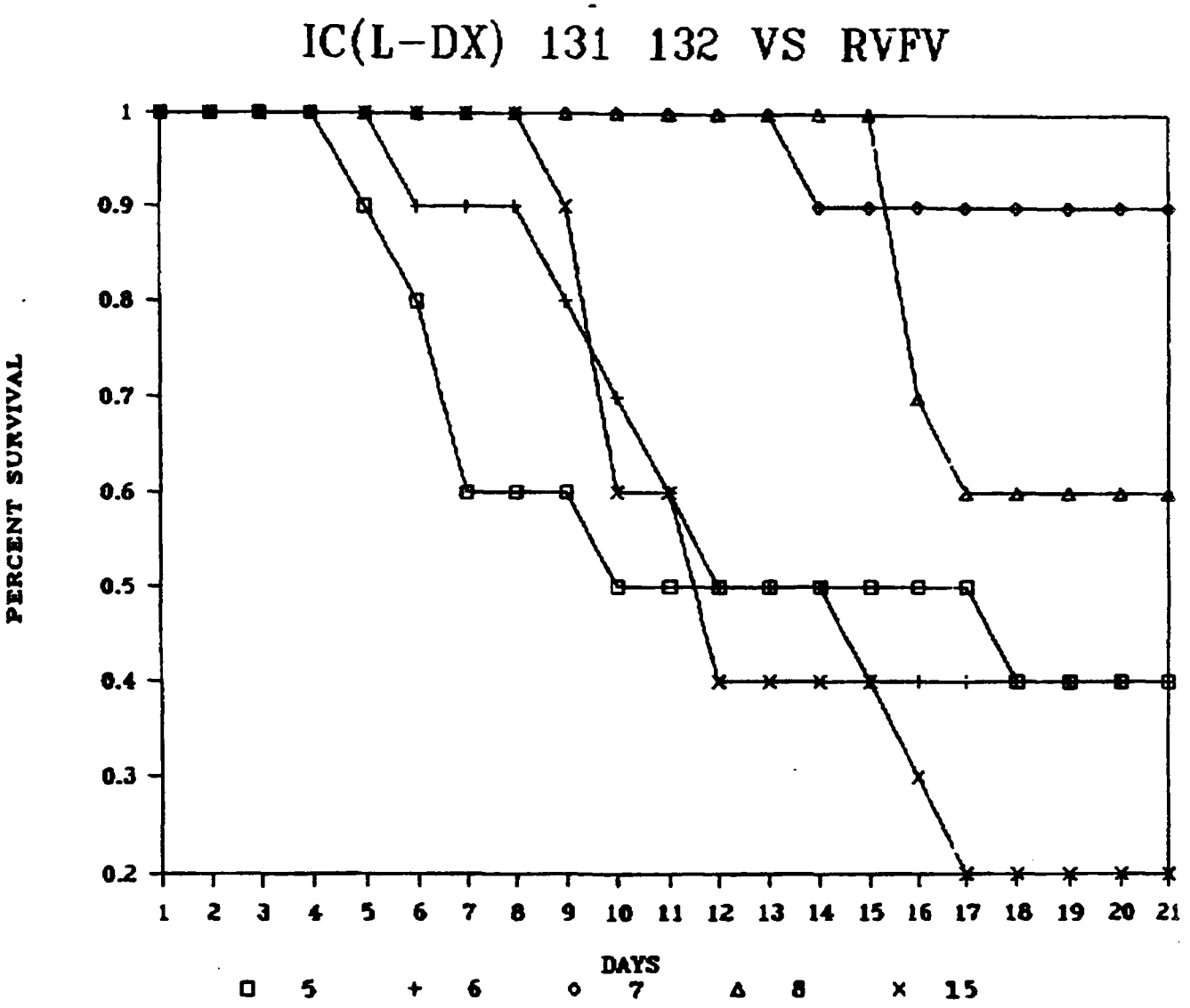


Fig. 8. Anti-RVFV action of IC-(PLL-Dextran), lots IV-131 and IV-132.

Groups 5, 6, lot IV-131 at 10 and 2.5 µg/mouse, IM.; Groups 7, 8, lot IV-132 at 10 and 2.5 µg/mouse, IP.

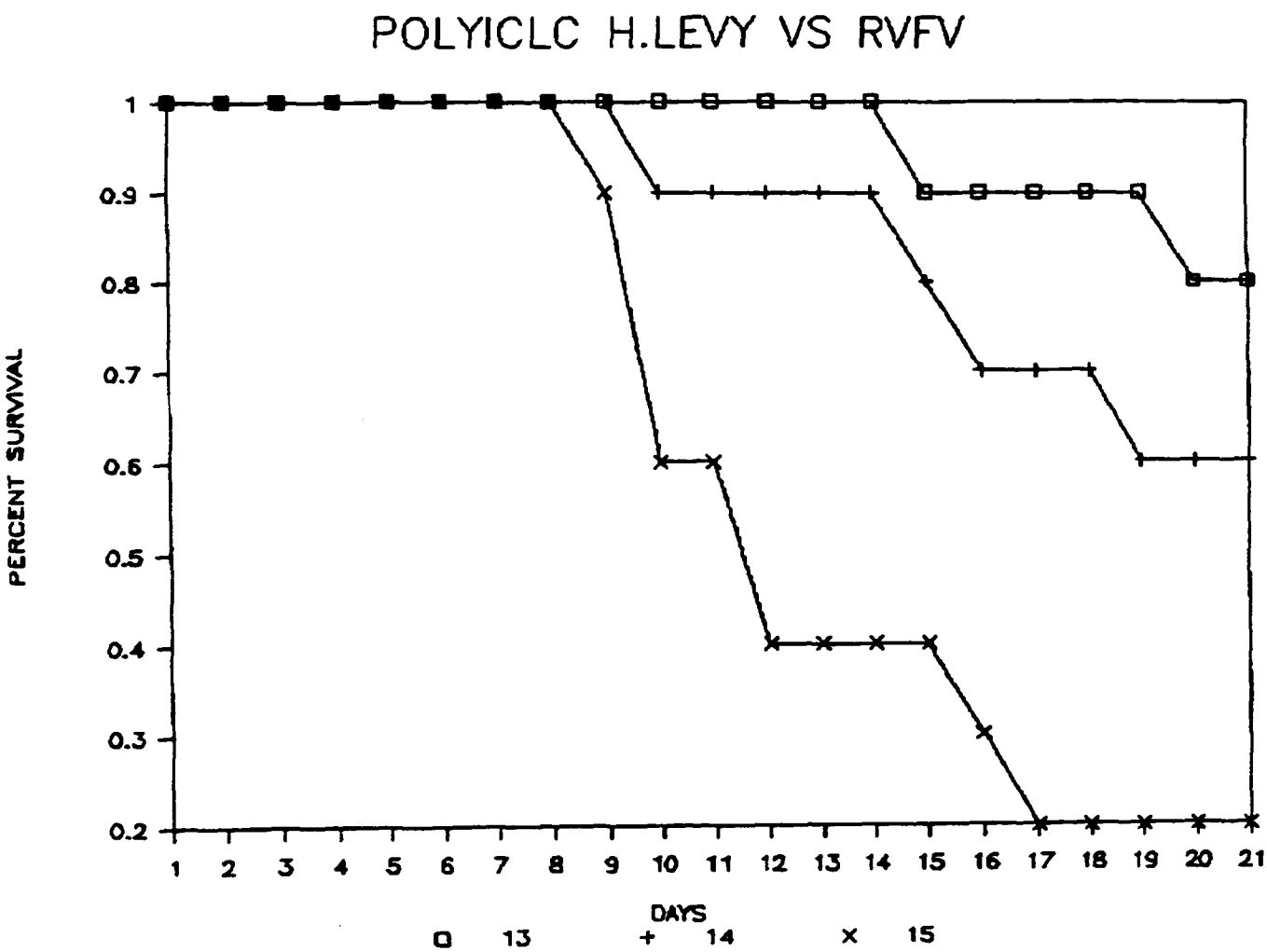


Fig. 9. Anti-RVFV action of poly ICLC, for comparison with Fig. 8.

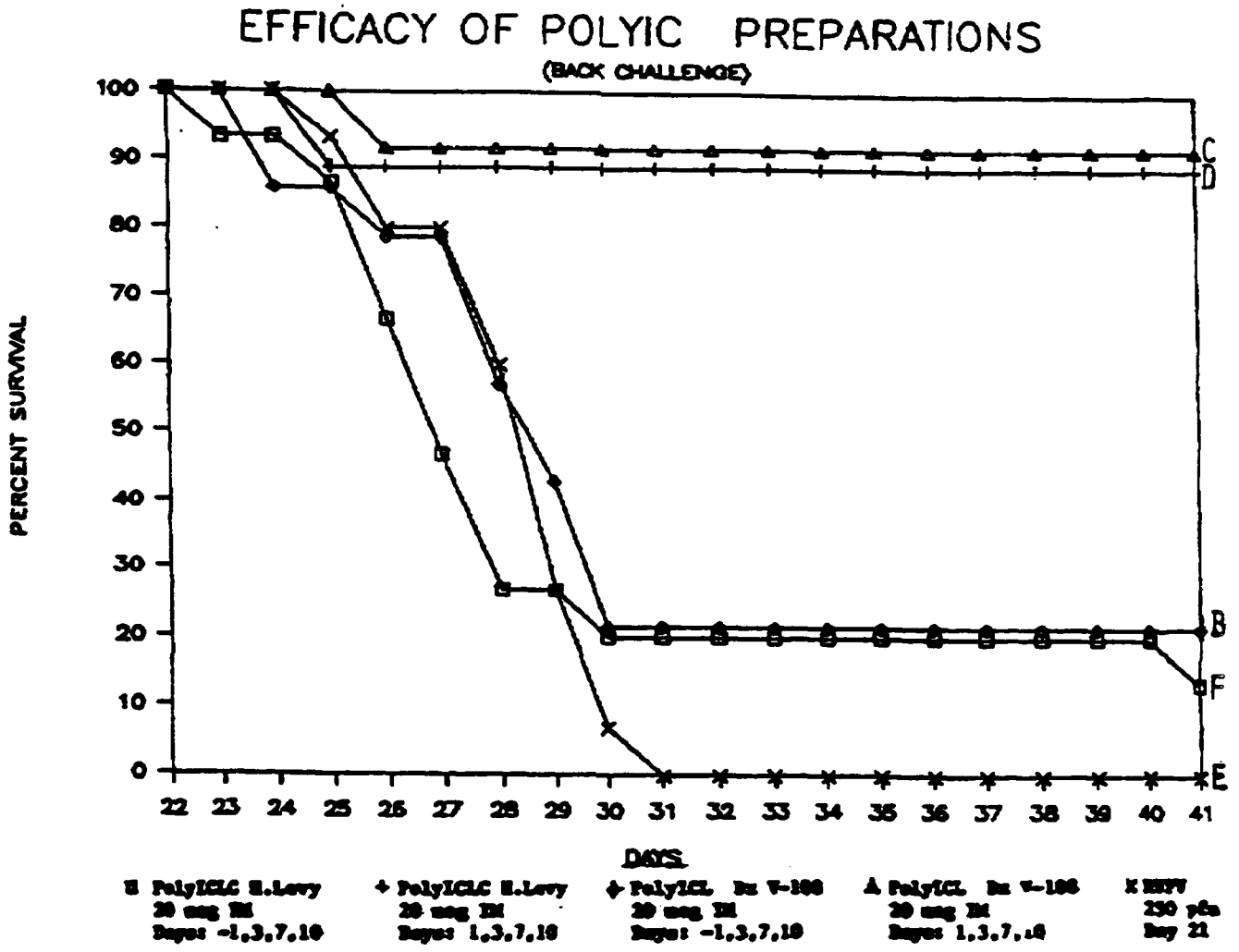


Fig. 10. Anti-RVFV action of IC-(PLL-Dextran) (Lot V-108).

A, Poly ICLC, 20 µg, IM, days -1, 3, 7, 10; B, V-108, 20 µg, IM, days -1, 3, 7, 10; C, V-108, 20 µg, IM, days 1, 3, 7, 10; D, Poly ICLC, 20 µg, IM, days 1, 3, 7, 10; E, RVFV control.

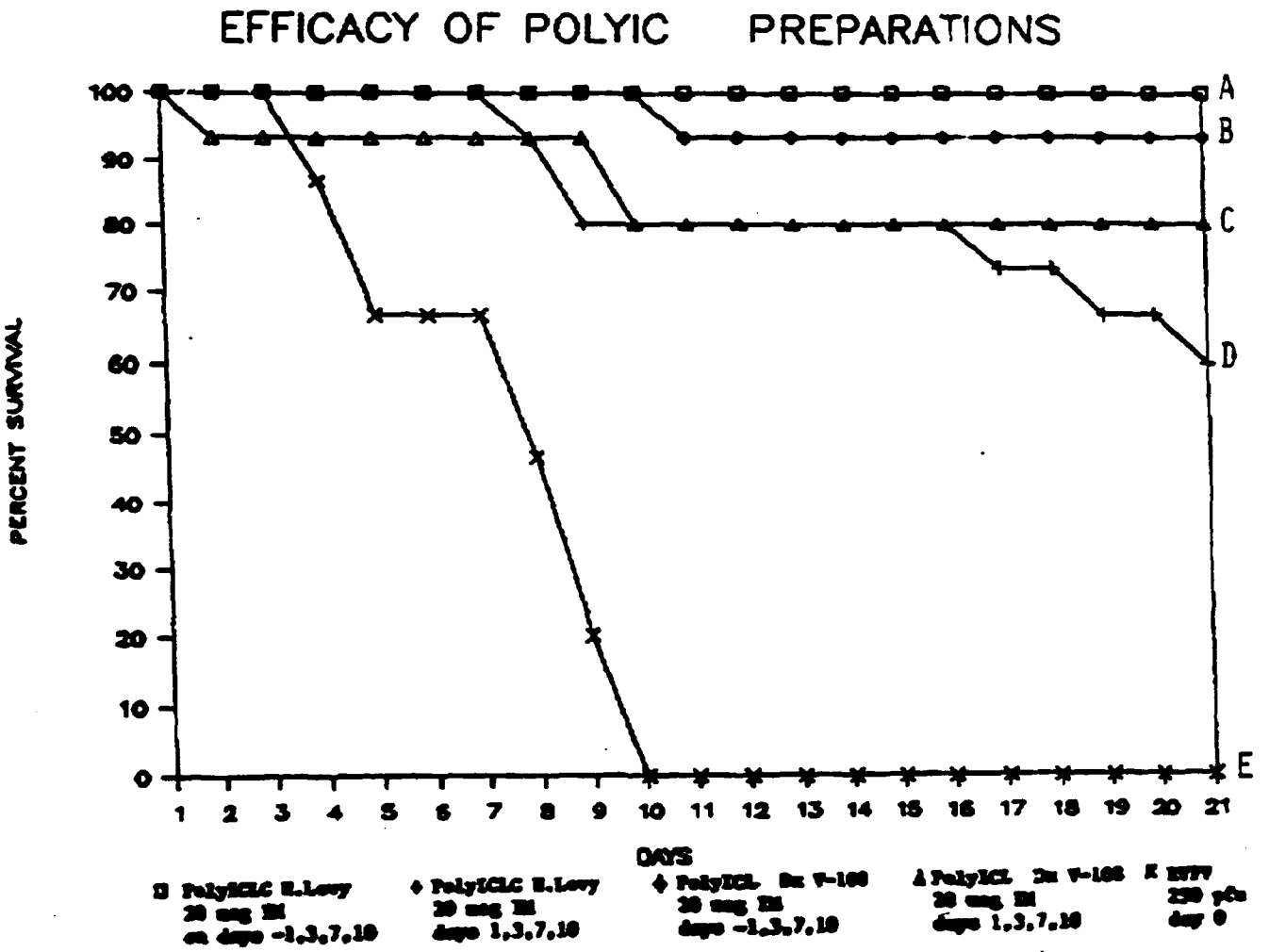


Fig. 11. Backchallenge. A, B, C, D, E as in Fig. 10.

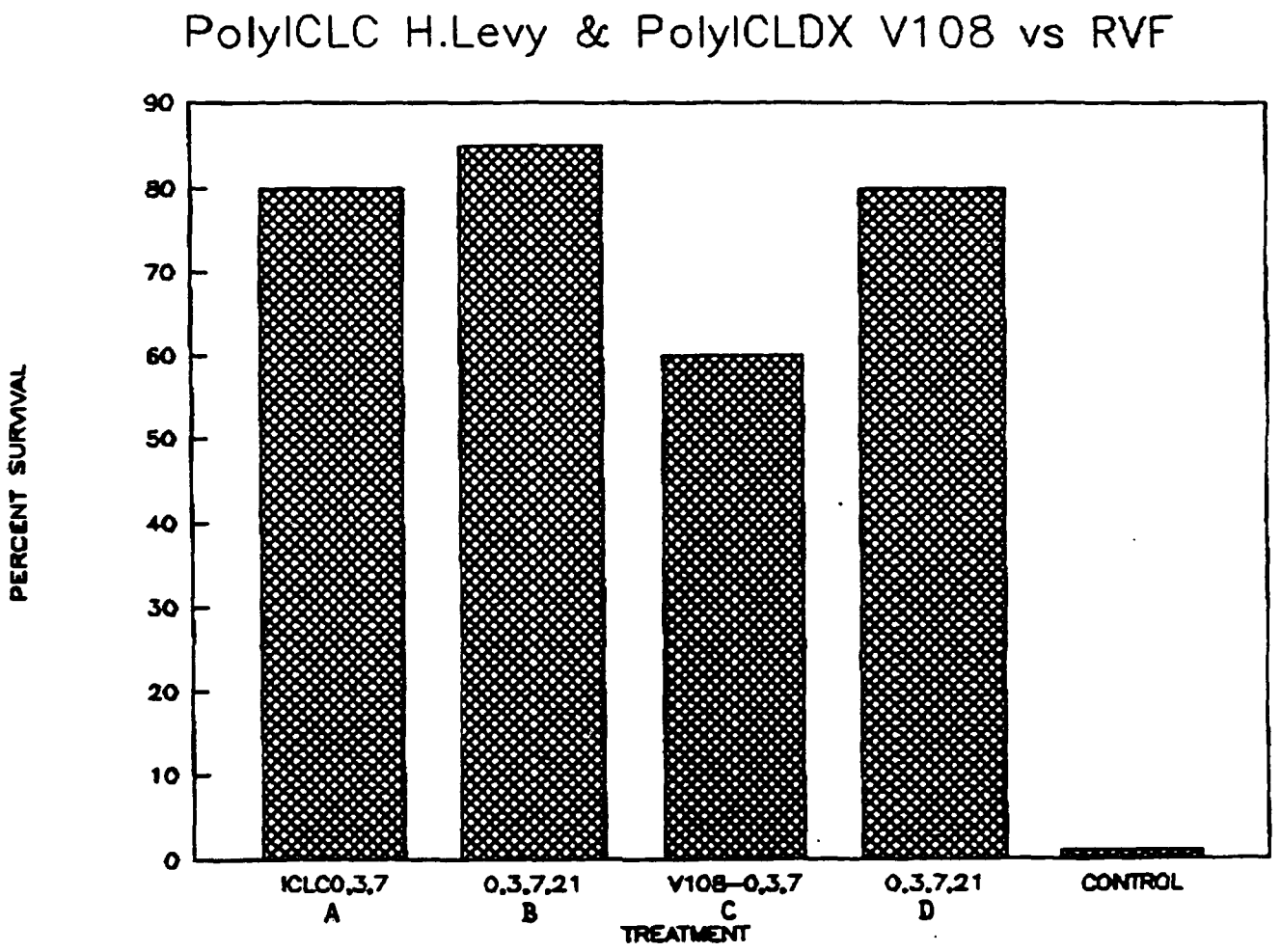


Fig. 12. Anti-RVFV action of IC-(PLL-Dextran) (Lot V-108) and Poly ICLC.

From left to right: A) 20 µg, IP, days 0, 3 and 7; B) 20 µg on days 0, 3, 7 and 2 µg on day 21; C) 20 µg on days 0, 3, 7; D) 20 µg on days 0, 3, 7 and 2 µg on day 21.

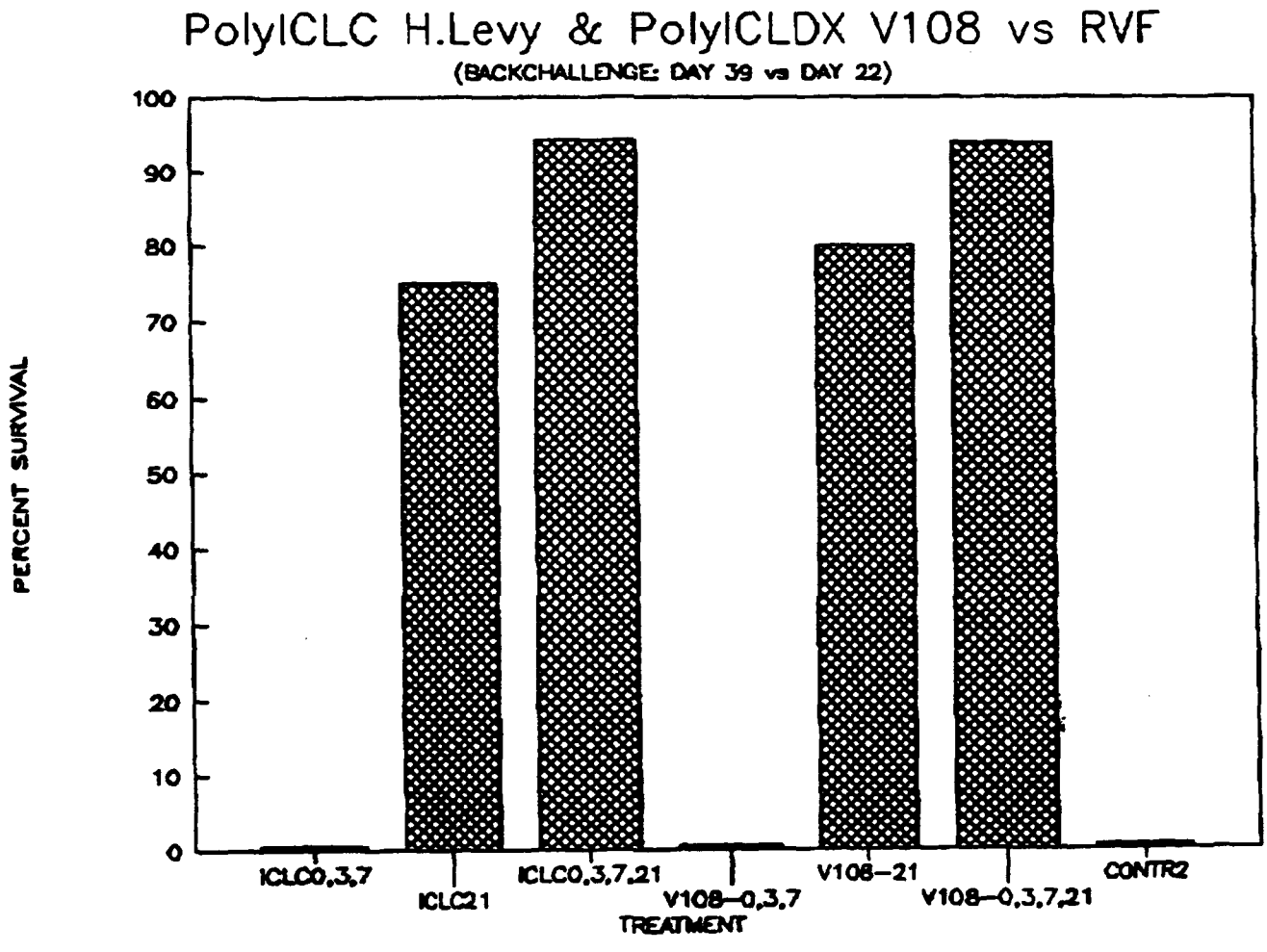


Fig. 13. Backchallenge with RVFV on Day 22, with IC-(PLL-Dextran) (lot V-108) or Poly ICLC given on day 22.

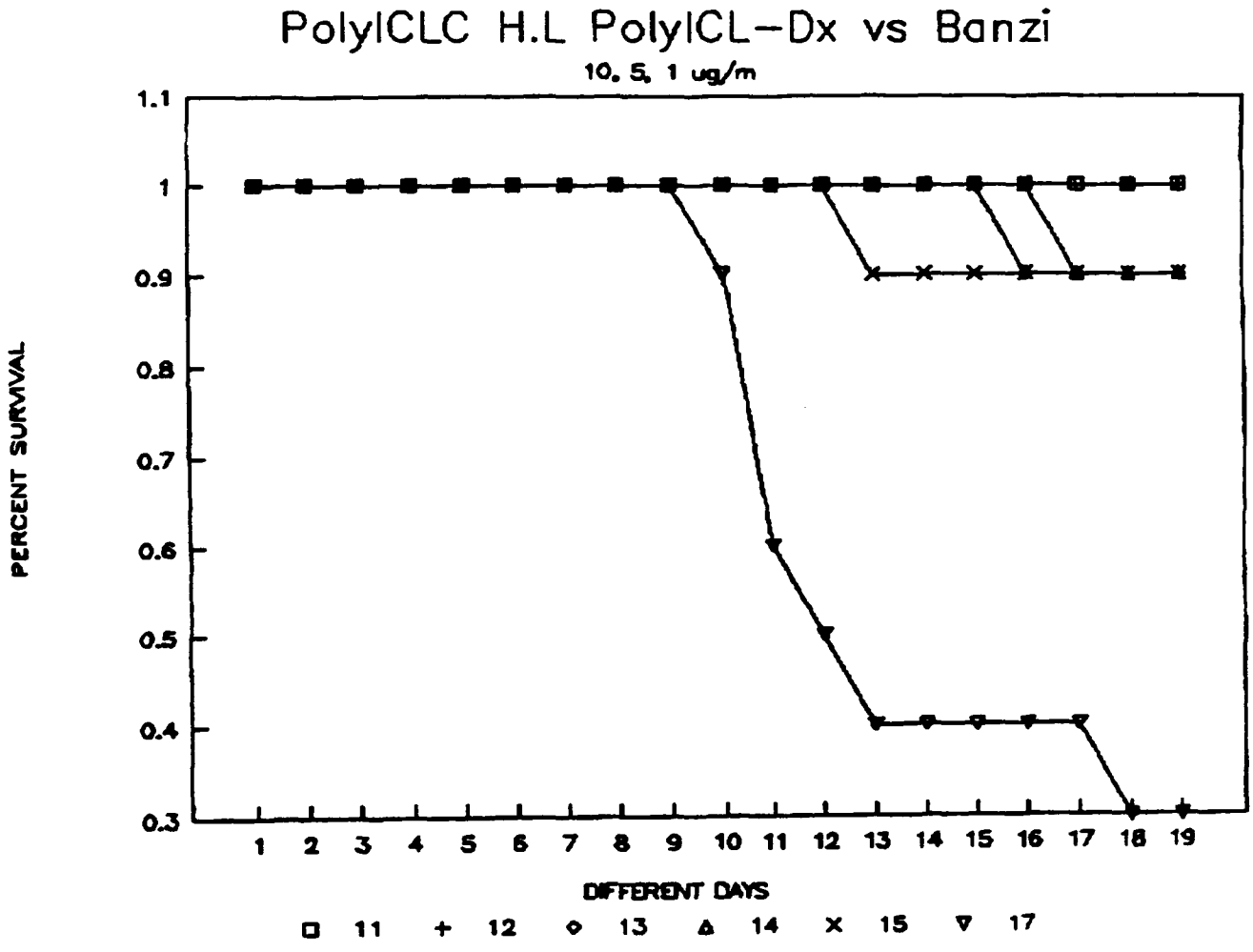


Fig. 14. IC-(PLL-Dextran) and Poly ICLC vs Banzi Virus.

Groups 11, 12, 13, Poly ICLC at 10, 5, 1 µg/mouse, respectively. Groups 14, 15, IC-(PLL-Dextran, Lot IV-52, at 10 and 5 µg/mouse, respectively, Group 17, virus control.

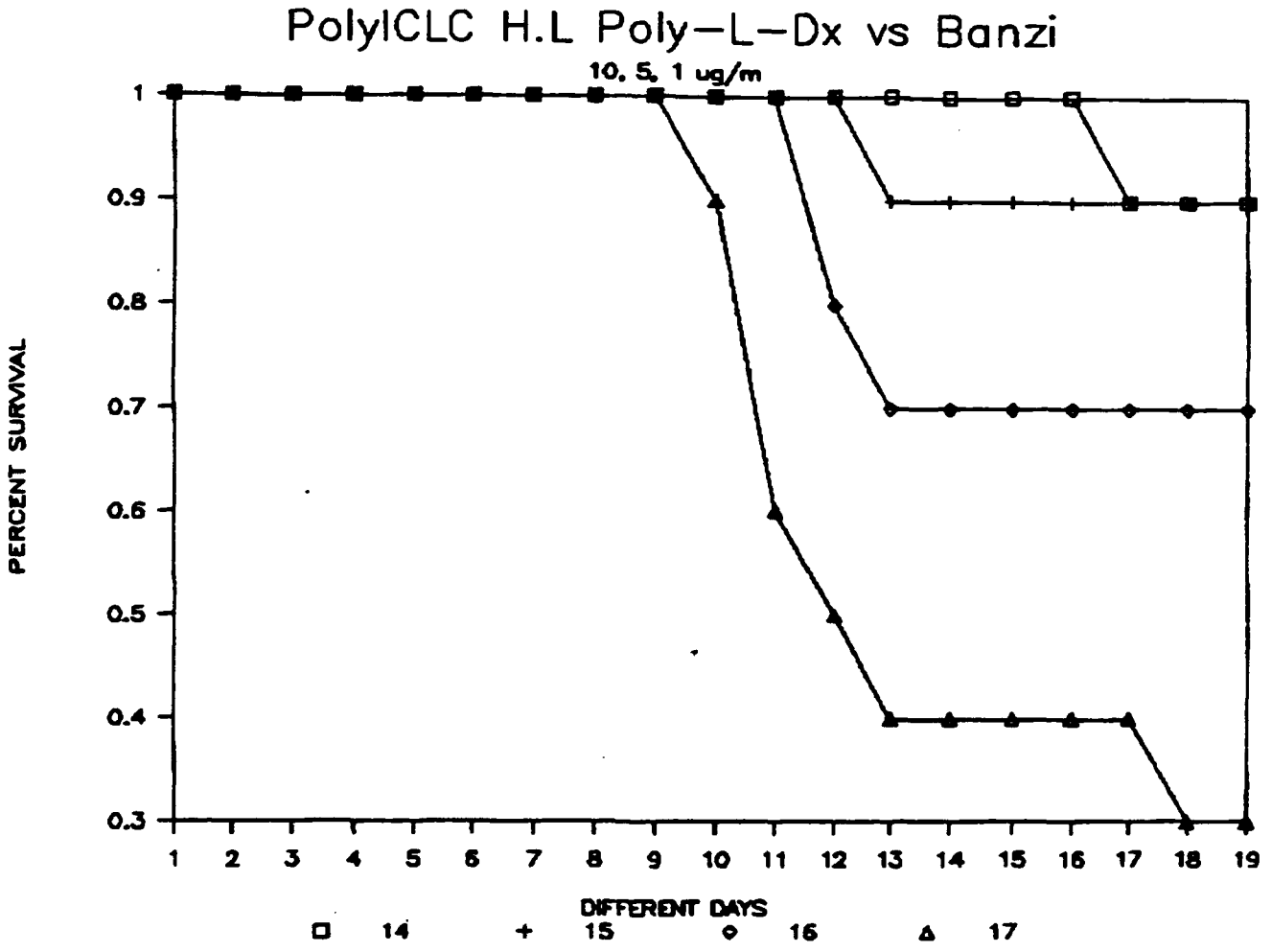


Fig. 15. Action of IC-(PLL-Dextran) against Banzi Virus. Lot #IV-52.

Group 14, 10 µg/mouse; group 15, 5 µg/mouse; Group 16, 1 µg/mouse, Group 17, virus control.

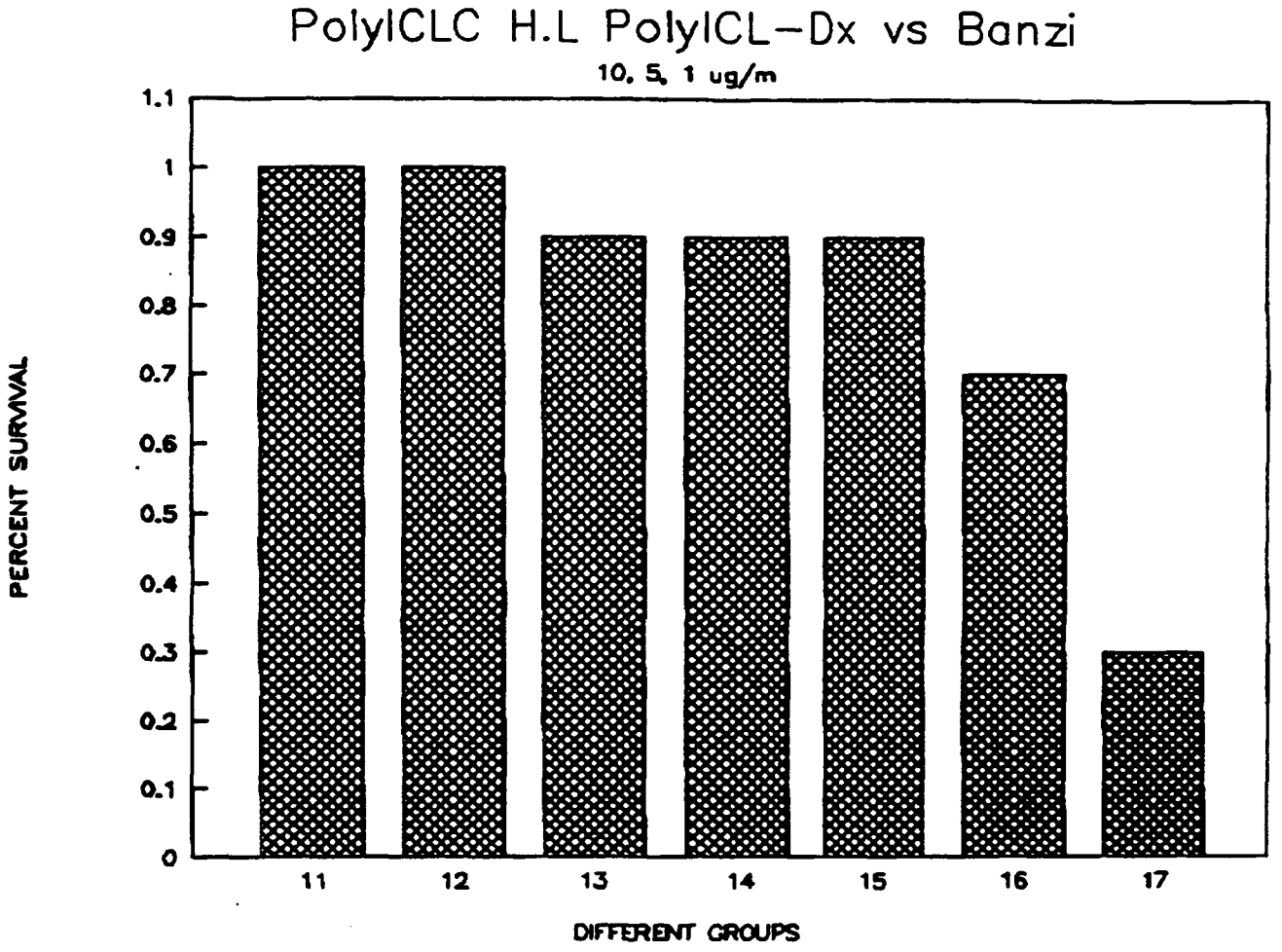


Fig. 16. Anti-Banzi Action of IC-(PLL-Dextran) and Poly ICLC at 19 Days. Groups as in Figs. 14 and 15.

Dr. Kende's results for the anti-viral efficacy of several lots of IC-(PLL-dextran) are given in Figs. 1-16.

Two lots of IC(PLL-Dextran) were tested against Punta Toro virus in mice (by Dr. R. Sidwell, Utah State University), with the results shown in Table 6. The results were compared with two tests of poly ICLC, and show lot III-190 to be as good as the average of the two tests on poly ICLC, except at the three lowest doses.

Table 6. Anti-Punta Toro Action of IC-(PLL-dextran).

Inducer dose mg/kg	Survivors			
	Lot	Lot	poly ICLC[a]	
	III-232	III-190	I	II
1.0	9/10	10/10	-	-
0.5	9/10	10/10	10/10	-
0.25	9/10	10/10	-	-
0.125	9/10	10/10	10/10	9/10
0.1	6/10	10/10	10/10	9/10
0.032	1/10	7/10	9/10	3/10
0.01	1/10	1/10	9/10	3/10
0.0032	0/10	0/10	3/9	1/10

[a] Blanks, not determined.

Some tests were also done against Banzi Virus (by Dr. Kende). The results (Figs. 14-16) show that lot IV-52 was somewhat less effective than poly ICLC, especially at the lowest dose, 1 µg/mouse. At 5 and 10 µg/mouse there was little, if any, difference.

b. PLL-Monosaccharide Grafts.

The PLL-dextran grafts described above contain long polysaccharide chains. We decided to examine the possibility of using short chains, beginning with the monosaccharides ribose and glucose, and the disaccharide lactose. The basic concept is the same as for PLL-dextran, but the use of a small saccharide, such as glucose, in place of a large polysaccharide (dextran) might have advantages. A small sugar is a very reproducible, crystalline, standard substance of very high purity, free of endotoxins, etc. We considered it possible that the use of a

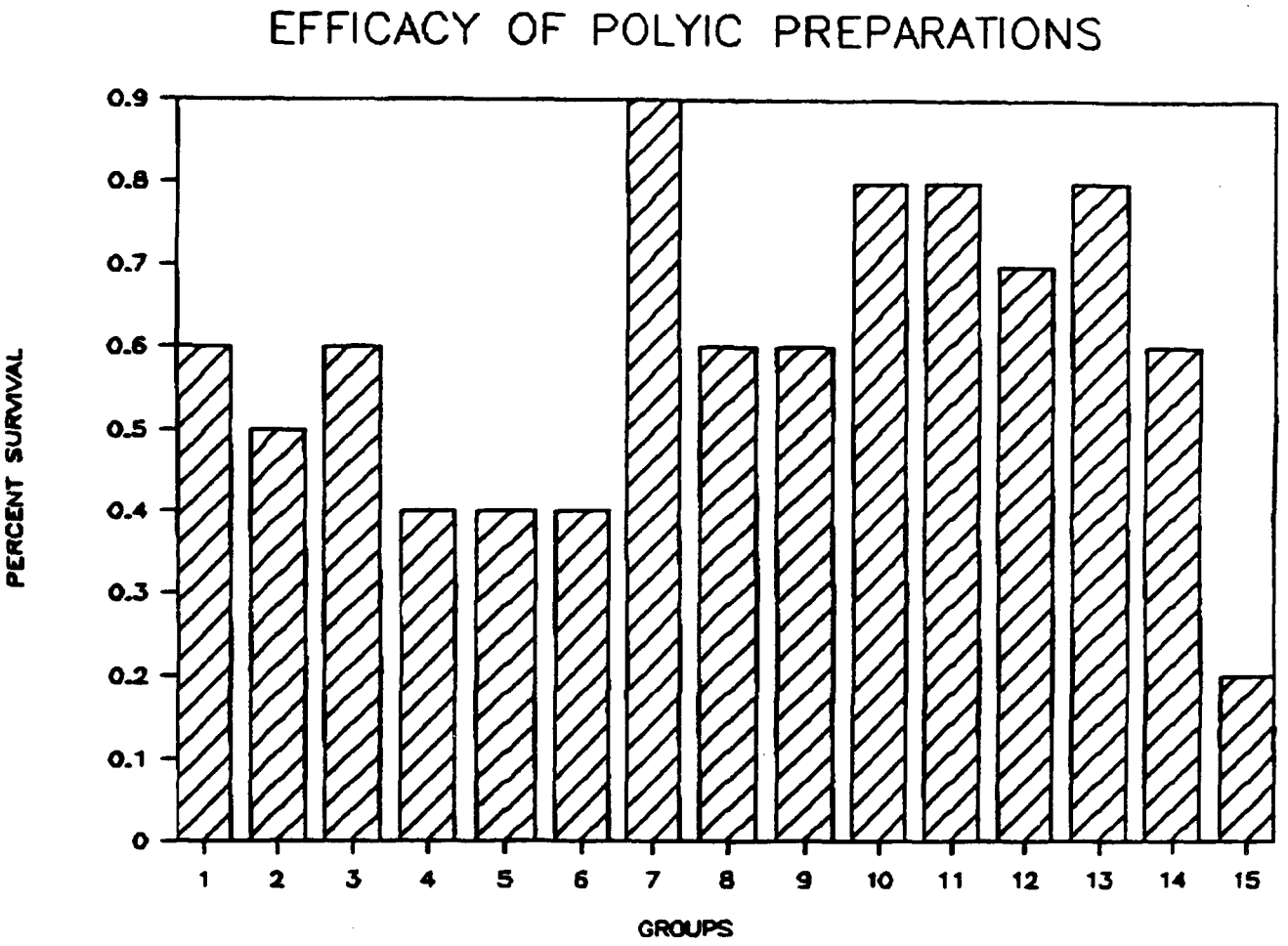


Fig. 17. Survival of Mice at 21 days, after RVFV challenge and treatment with IC-(PLL-CM-ßCD), IC-(PLL-Dextran) with IC-(PLL-glucose), or IC-(PLL-cyclo-dextrin sulfate).

Groups 1,2, ICL-CMßCD, 10 and 2.5 µg/mouse, respectively, lot IV-119; Groups 3-4, IC-(PLL-Dextran), lot IV-131, at 10 and 2.5 µg/mouse, IP; Groups 5-6, ditto, IM; Groups 7,8, IC-(PLL-Dextran) lot IV-132, at 10 and 2.5 µg/mouse, IP; Groups 9,10, ICL-ßCD-SO_4, lots IV-190, IV-191, 5 µg/mouse, IP; Groups 11,12, IC-(PLL-Glucose) lot IV-175, 10 and 2.5 µg/mouse IP; Groups 13,14, Poly ICLC at 10 and 2.5 µg/mouse, IP, Group 15, control, RVFV alone.

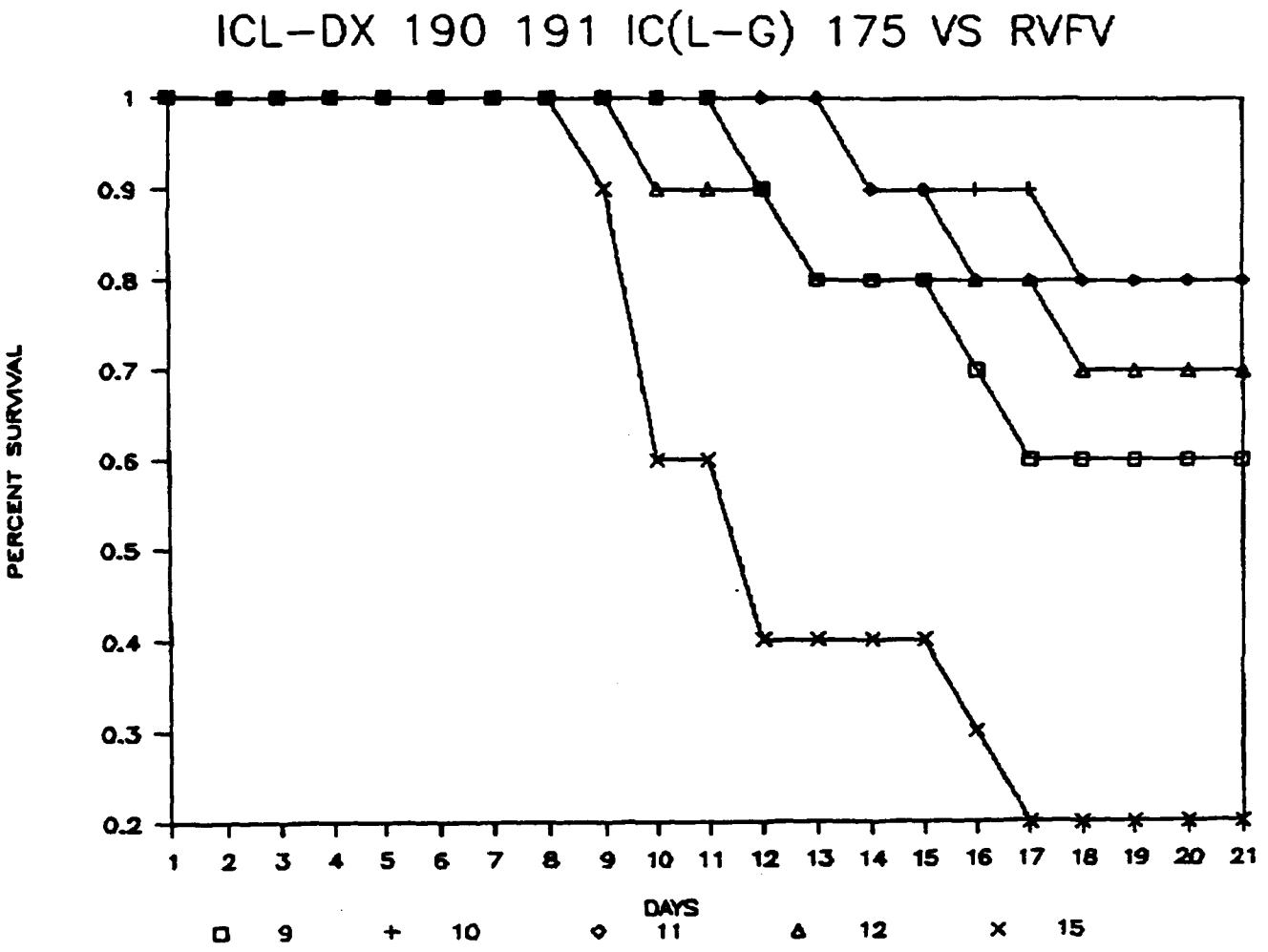


Fig. 18. Antiviral-action (RVFV) of IC-(PLL-glucose) (lot IV-175) and ICL-βCDSO$_4$ (lots IV-190, IV-191) Groups as in Fig. 17.

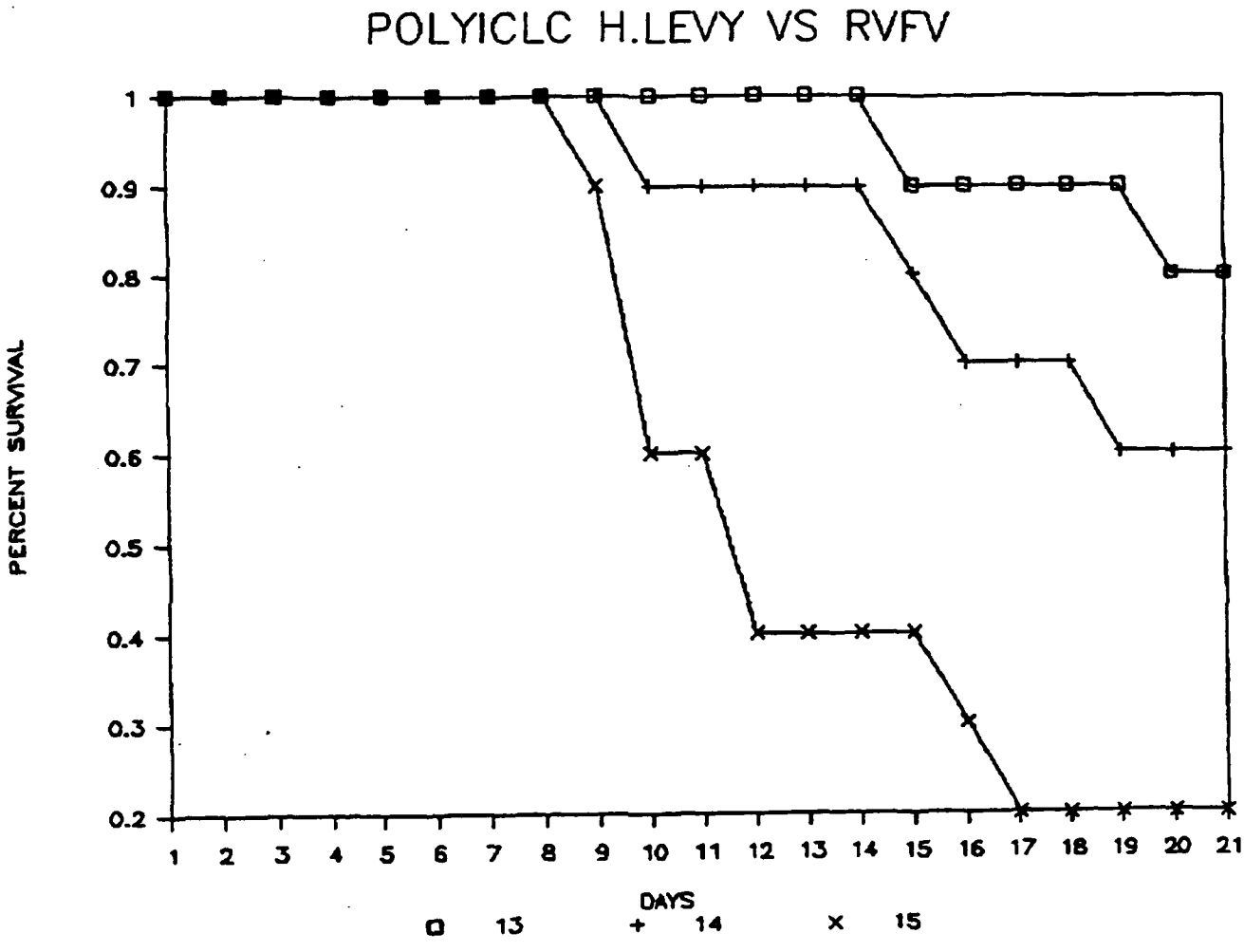
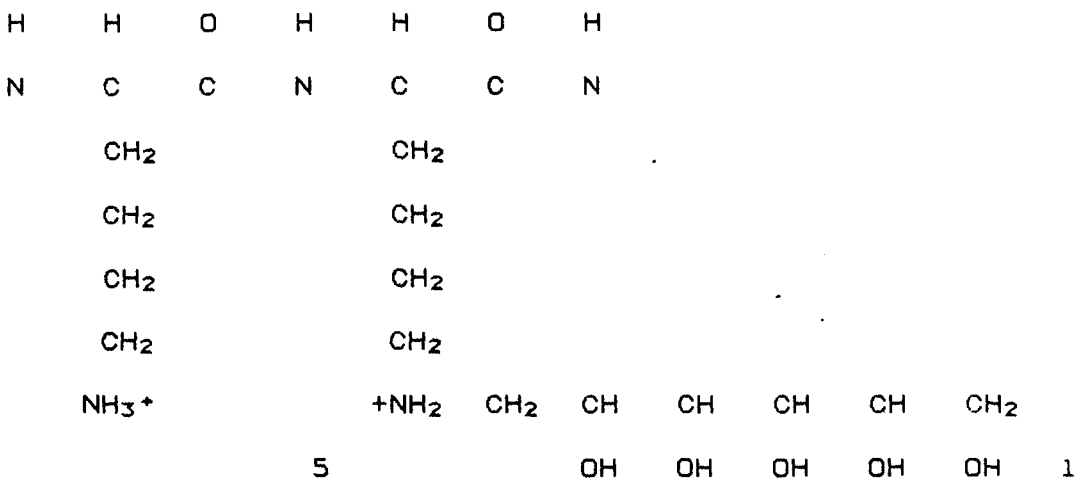


Fig. 19. Anti-RVFV of Poly ICLC, as reference for Figs. 17 and 18. Group 13, 10 µg/mouse; group 14, 2.5 µg; group 15, control, RVFV.

small saccharide might facilitate metabolism or excretion. The sugars were grafted to PLL by the same chemistry as for PLL-dextran, i.e., reaction of PLL, sugar and sodium cyanoborohydride.

Analysis of the extent of grafting of glucose to PLL was attempted by nmr analysis, but the complexity of the spectrum and overlapping of peaks did not lend itself to simple analysis. We have obtained an elemental analysis. Analysis for nitrogen gave 12.05% compared with 16.97% (theory) for PLL-HCl. This corresponds to an average of 1 glucose residue for 6 lysine residues. (Carbon analysis is not useful, because the glucose residue and the lysine·HCl residue have identical percentages of carbon.)

The PLL-glucose graft polymer is expected to have the structure



with the modified lysine-glucose residues interspersed, presumably at random, in the proportions shown.

Formulations of poly IC with the PLL-ribose, PLL-lactose and PLL-glucose grafts were made. The IC-(PLL-ribose) gave a single melting transition at 78°; IC-(PLL-glucose) gave two transitions, at 66° and 77° (35 and 65%, respectively, of the total A). The 77° and 78° Tms demonstrate that a complex of poly IC with the PLL-saccharide graft exists.

Dr. Kende's results with IC-(PLL-glucose) at 10 and 2.5 µg/mouse gave 80 and 70% survival in mice challenged with Rift Valley Fever virus, compared with 80 and 60% for poly ICLC. Thus, this formulation has substantial antiviral activity despite apparently modest IFN inducing power (Table 7).

Table 7. Induction of IFN in Mice by IC-(PLL-monosaccharides).

Lot	IFN titer	IFN titer of Poly ICLC	% of Poly ICLC titer
IV-161 IC-(PLL-Ribose)	52	1020	5
IV-175 IC-(PLL-Glucose)	156	1020	15
V-76 IC-(PLL-Lactose)	15	1956	1

Dr. Kende's results with RVFV are shown graphically in Figs. 17, 18 (groups 11, 12), and are comparable to poly ICLC (groups 13, 14).

Because the IFN titers were obtained at a standard time of 3 h after injection, pharmacokinetics would be needed in order to see the true extent of IFN induction. The complexes of poly IC with PLL-ribose and PLL-glucose were made at a graft-to-PLL ratio of 2:1; other ratios (and other degrees of grafting) should be studied.

The data obtained suggest that IC-(PLL-glucose) may be as good as IC-(PLL-dextran).

c. <u>β-Cyclodextrin Sulfate (βCDSO$_4$)</u>.

i. Rationale.

Carboxymethylcellulose is a polymer of high molecular weight, as are carboxymethyl dextran, the anionic amyloses and gelatin. The use of anionic cyclodextrins, small saccharides, appeared attractive for the following reasons:

1. Cyclodextrins have been described in many pharmaceutical preparations;
2. They are expected to be non-immunogenic;
3. They are small enough to be excreted, if not metabolized;
4. As small, organic compounds of definite size and structure they should be amenable to purification by common techniques of organic chemistry;
5. They should be obtainable free of microorganisms, pyrogens, etc.;
6. It may be possible to obtain more reproducible IFN inducer formulations.

7. There will be no variation of molecular weight and molecular weight distribution from ba ch to batch, because cyclodextrins are molecules of defined size and structure.

Cyclodextrins are cyclic oligomers of glucose, and contain 6, 7, 8 or 9 glucose residues. The 7-mer, ß-cyclodextrin has been used in this initial work, because of its low cost and high solubility in water.

Sulfated cyclodextrin was prepared by the action of sulfur trioxide-trimethylamine in dimethylformamide, and was obtained as the sodium salt (details in Experimental Section). A series of experiments with varied temperatures and ratios of reactants led to procedures for the synthesis of ßCDSO$_4$ with different degrees of substitution (DS). DS was determined from analysis for sulfur.

ii. IFN Induction.

Formulations were made with ßCDSO$_4$ containing 11 SO$_4$ groups per ß-CD; one pair of lots (IV-153 and IV-190) contained twice as much ßCD-SO$_4$ as the other pair (IV-152 and IV-191). The compositions (in mg/mL of components) and IFN titers in mice are shown in Table 8.

Table 8. ICL-ßCDSO$_4$; Compositions and IFN Titers.

Lot	IC[a]	PLL[a]	ß-CD-SO$_4$[a]
IV-153	2	1.5	2.5
IV-191	2	1.5	2.5
IV-152	2	1.5	5.0
IV-190	2	1.5	5.0

	IFN titer	IFN titer of Poly ICLC	% of Poly ICLC
IV-153	2166	1020	212
IV-191	1481	733	202
IV-152	1000	1020	98
IV-190	755	733	103

[a] In mg/mL.

Thus, one pair of formulations is as effective as IFN inducers as is poly ICLC, and the other pair is twice as effective.

The reproducibility is good, but more tests would be needed. In principle one expects better reproducibility with a small molecule like ßCDSO$_4$ than with high polymeric anions. The other inducers are made from three high polymers, which interact electrostatically (chiefly, but, no doubt, with other contributions), and may lock together in a (partially) random manner; the nature of the ternary complex may vary with slight variations in mixing procedures, molecular weights and molecular weight distributions. If one component at least is a small, fairly rigid molecule, one element of randomness is eliminated, and a more reproducible product may result. Additional experiments would be required.

iii. Toxicity and Safety Testing.

LD_{50} of two lots of ICL-ßCDSO$_4$ are shown in Table 9. ßCD-SO$_4$ itself showed no toxicity at the highest dose tested, 250 mg/kg; LD_{50} must be substantially greater.

Table 9. LD_{50} Values for ICL-ßCDSO$_4$ in Mice:

Inducer	LD_{50} mg/kg
Poly ICLC	11
ICL-ßCDSO$_4$, IV-190	36
ILC-ßCDSO$_4$, IV-191	24

The two lots of ICL-ßCDSO$_4$ are less toxic than is poly IC, by factors of 2 and 3.5. Since IV-191 induces twice the titer of poly ICLC, its ratio of effectiveness to toxicity is correspondingly better.

These two lots of ICL-ßCDSO$_4$ were safety-tested and gave somewhat smaller losses of weight in mice than did poly ICLC (Table 10). The weight losses and toxicity were in the same order, IV-190 being better than IV-191 in both respects. In guinea pigs these resulted in weight gains comparable to the saline controls, instead of the weight losses shown with poly ICLC.

Table 10. Safety Testing of ICL-ßCDSO$_4$.

Lot #	Weight Change at 48 Hr.	
	Mice	Guinea Pigs
IV-190	-11.8	+3.8
IV-191	-15.5	+3.7
ICLC, IV-241	-19.1	-4.5
Saline	+ 3.2	+3.9

Antiviral Action of ICL-ßCDSO$_4$.

IV-190 and IV-191 were as effective against RVFV as poly ICLC (Table 11). The anti-viral action is graphically shown in Figs. 17 and 18 (groups 9 and 10), with poly ICLC as reference in Fig. 19.

Table 11. Anti-RVFV Action of ICL-ßCDSO$_4$.

Lot #	Inducer dose µg/mouse	Survivors	
		ICL-ßCDSO$_4$	poly ICLC
IV-190	2.5	60%	60%
IV-191	2.5	80%	60%

iv. Melting Profiles.

ICL-CDSO$_4$, lots IV-153 and IV-191 have melting transitions, Tm, at 83°. IV-152 and IV-190 have two Tm's, at 67° and 83° in roughly equal proportions. Digestion of IV-152 by ribonuclease proceded at 60% of the rate for poly IC or for poly IC in the presence of ßCDSO$_4$. (ßCDSO$_4$ does not inhibit the action of RNase.) Thus, roughly half of the poly IC of IV-152 and IV-190 is uncomplexed. Since uncomplexed poly IC induces little IFN (about 10% of the titer induced by ICLC), the high titers shown above for IV-152 and IV-190 presumably arise from that fraction of poly IC which is complexed, which means that the IFN from the latter would be equivalent (per unit weight) to the titer induced by IV-153 and IV-191, which have their poly IC completely complexed.

The presence of uncomplexed poly IC in IV-152 and IV-190, which contain twice the amount of ßCDSO$_4$ as in IV-153 and IV-191, indicates that the interaction of PLL with ß-CDSO$_4$ is fairly strong, resulting in the excess ß-CDSO$_4$ pulling PLL out of its complex with poly IC. Attempts to prevent this by making formulations with a ßCDSO$_4$ having about half the number of SO$_4$ groups per ß-CD gave only insoluble complexes.

The 67° Tm is about 4° higher than that of poly IC alone. Yet the Rnase action is indicative of bare poly IC. A tentative explanation is that a bare poly IC segment between two covered poly IC segments will be somewhat stabilized by the very stable covered segments. Yet this stabilized bare segment must be as digestable by RNase as is simple poly IC. This can be explained on the basis of RNase causing local unwinding of the stabilized bare poly IC and initial cleavage. After the first cleavage, the further unwinding would proceed easily, leading to rapid digestion.

v. Summary.

On the basis of high IFN titers, antiviral action and reduced toxicity, ICL-ß-CDSO$_4$ appears to be a promising candidate.

d. Carboxymethyl-ß-cyclodextrin, CMßCD.

A second approach to the use of anionic cyclodextrin is via carboxymethyl-ß-cyclodextrin, CMßCD. CMßCDs of several degrees of substitution were synthesized, after working out the conditions of temperature, time, proportions of reagents and methods of isolation and purification.

The first lots (III-62, III-254, IV-119, of Table 12) of CMßCD had been fractionated by column chromatography. We decided to see if unfractionated CMßCD would be suitable. This would greatly reduce the time and cost of making CMßCD. The formulation of a soluble ICL-CMßCD with unfractionated CMßCD required a series of experiments at several ratios of components. The lot (V-162) eventually prepared had the ratio of components shown in Table 12, and the highest IFN titer of all of these formulations. We were not able to measure LD$_{50}$, because of lack of resources as the contract approached termination.

i. IFN Induction.

Four formulations of poly ICL-CM-CD were prepared. The induction of IFN by these agents in mice is shown in Table 12.

Table 12. IFN Induction[a] in Mice by ICL-CM-ßCD.

Lot #	Composition poly IC	PLL	CM-ß-CD[a]	IFN titer	% of poly ICLC titer
III-62	2	1.5	5	443	55
III-254	2	1.5	5	773	143
IV-119	2	0.75	2.5	343	31
V-162	2	0.75	5	1170	382

[a] Degree of substitution is 4.2 carboxymethyl groups per ß-CD for each lot.

IV-119 contained about one-half of its poly IC uncomplexed (two Tm's, at 63 and 83°), while for III-62 and III-254 all of the poly IC was complexed (Tm = 83° for III-62 and 85° for III-254).

Lot III-62 in its first trial in mice induced an IFN titer of 123 units or 9% of that of the ICLC standard. On cold storage for several months III-62 showed an increase in IFN titer to 443 units, or 55% of the ICLC standard. The melting profile also changed from two transitions at 83 and 87° to a single transition at 78.5°. It appears that ICL-CM-ß-cdextrin improved on aging. If so, we would try to accelerate the aging process, perhaps by storage at room temperature or above.

When the three polymers are joined into a complex the initially formed complex may not be near its thermodynamically most stable structure, and there may be a gradual process of rearrangement leading to the most stable arrangement. This may be slow, and might be speeded by annealing at a temperature below the melting transition. In advance, one cannot know if this would result in superior performance, but it might result in better reproducibility. Annealing may be applicable to any of the inducers we have studied.

Lot V-162 was tested against RVFV in mice (Dr. Kende). At 20 µg/mouse, there was 100% survival, and at 5 µg, 70%.

ii. LD_{50}.

LD_{50} of ICL-CMßCD, lot III-254 was 14 mg/kg, slightly higher than that of poly ICLC (Table 13). Lot IV-119 had LD_{50} >40 mg/kg; this lot contained half of the usual amount of PLL and CMßCD. The CM-ß-cyclodextrin had an average of 0.6 carboxymethyl groups per glucose residue, or 4.2 per ß-CD molecule. There are other degrees of carboxymethylation to consider, as well as α- and γ-cyclodextrins (i.e., with 6 and 8 glucose residues, respectively).

Table 13. LD_{50} Values of ICL-CMßCD.

III-254	14 mg/kg
IV-119	>40 mg/kg
poly ICLC	11 mg/kg

iii. Antiviral Activity.

The action of these lots of ICL-CMßCD was tested against Rift Valley Fever Virus (III-254, IV-119) and Punta Toro virus (III-62), with the results given in Tables 14 and 15. These were as effective against these viruses as was poly ICLC. III-62 was at least as good, overall, as ICLC in all of the following parameters: safety testing, % of survival, mean survival time, liver damage, enzyme assays and viral titers.

Table 14. Anti-RVFV Action of ICL-CMßCD.

Lot	Inducer dose µg/mouse	Rift Valley Fever Virus Survivors	
		ICL-CMßCD	Poly ICLC
III-254	10	9/10	9/10
	2.5	9/10	10/10
IV-119	10	6/10	5/10
	2.5	5/10	3/10

The anti-RVFV results of Dr. Kende on lot IV-119 are shown graphically in Figs. 17 and 20 (groups 1 and 2).

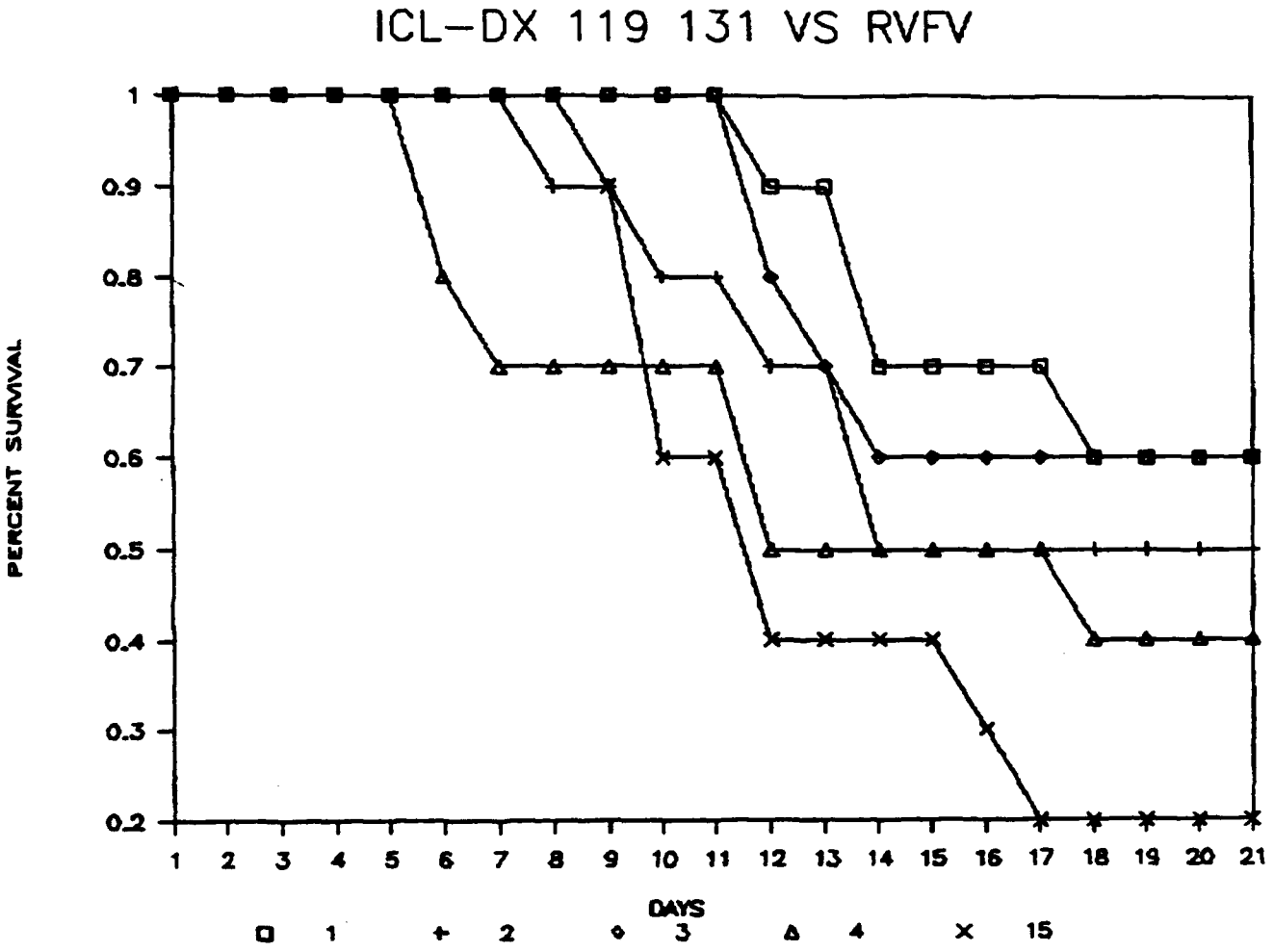


Fig. 20. Anti-RVFV action of ICL-carboxymethyl-ß-cyclodextrin (ICL-CMßCD)-(lot IV-119), compared with IC-PLL-Dextran) (lot IV-131).

Groups 1,2, ICL-CMßCD, 2.5 µg/mouse, respectively. Groups 3,4, IC-(PLL-dextran), 10 and 2.5 µg/mouse.

Table 15. Antiviral Action of ICL-CMßCD (Lot III-62) Against Punta Toro Virus in Mice[a,b]

Inducer dose mg/kg mouse[c]	Survivors	MLS[c]	ML VT[d] Log10	MS VT[e] Log10
III-62				
1	10/10	0.1	0.0	0.0
0.25	9/9	0.1	0.0	0.0
0.1	10/10	0.8	1.6	1.0
0.032	10/10	0.9	2.6	2.8
0.01	5/5	1.5	2.2	2.0
0.0032	3/10	2.3	2.3	3.5
Poly ICLC				
0.1	10/10	1.6	1.5	1.8
0.032	9/10	2.0	3.3	2.9
0.01	9/10	2.1	1.5	0.9
0.0032	3/9	2.7	3.4	3.2

[a] Data from R.W. Sidwell, Utah State U.
[b] Every other day IP Treatment.
[c] Mean Liver Score, a measure of liver damage.
[d] Mean Liver Virus Titer.
[e] Mean Serum Virus Titer.

e. Formulations with CMdextran.

During the first year of the contract we prepared and tested a series of ICL-CMdextran formulations, using a variety of proportions of components. The CMdextran was made from dextran of 10 kDa molecular weight, and several procedures were studied for the preparation and purification of CMdextran. Some formulations gave good IFN induction, but some did not.

We developed a standardized procedure for isolation and purification of CMdextran. This procedure (Experimental section) had given the best results. We extended the range of CM-substitution (DS = degree of substitution) from 0.2 to 2 CM groups per glucose residue. (Earlier we had used a DS of 0.5.) We also used CMdextran of 40 kDa molecular weight. In the new series all formulations, except one, induced substantial titers of IFN, Table 16 shows results for a series of ICL-CMdextran formulations with 10 kDa CMdextrans. The titers for all but batch II-278 were good to high, and even II-278, the lowest, gave a titer of 245 units.

Two batches were tested for toxicity; they were more toxic than was ICLC. Toxicity was tested by a method differing from the usual LD_{50} procedure. Mice were injected with a dose of inducer containing 150 g poly IC on each of four consecutive days. The percentage surviving was compared with the result of similar treatment with poly ICLC. Lots II-238 and III-45A resulted in 0% survival compared with 51% for poly ICLC. LD_{50} was not done, as ICL-CMdextran research was discontinued.

When CMdextran of 40 kDa molecular weight was used, we also obtained high titers with DS of 0.46 and 1.1. One of these, III-59 with DS = 1.1 was much less toxic than ICLC, with 87% survival compared with 0% for poly ICLC (by the method of the previous paragraph). III-59 was tested for anti-RVFV effect and gave no protection (Table 16a), despite its high IFN titer.

Table 16. IFN Induction by ICL-CMdextran.

Lot Number	Composition IC, PLL, CMdextran	IFN Titers		
		Exptl. Formulation	ICLC Standard	% of ICLC Standard
		10 kDa[a]		
II-238 DS = 0.4	0.5/1.5/5	1302	643	202
II-266 DS = 0.22	0.5/0.33/1.25	669	1450	46
II-278 DS = 0.22	0.25/0.75/1.25	245	1450	17
III-45A[c] DS = 2.0	2/1.5/5	1020	1669	61
III-45B DS = 2.0	0.5/0.38/1.25	496 1247	808 1669	61 75
III-90 DS = 2.0	0.5/0.38/1.25	618	808	76
		40 kDa[b]		
III-59 DS = 0.46	0.5/0.38/1.25	830 479	1280 808	65 59
III-79 DS = 1.1	2/1.5/5	994 856	1280 808	78 106

[a] CMdextran made from 10 kDa dextran.
[b] CMdextran made from 40 kDa dextran.

f. ICL-SO_4Gelatin.

Gelatin and sulfated gelatin have been investigated as replacements for CMC. Gelatin would be metabolized, and probably also sulfated gelatin. Gelatin, unmodified or modified in several ways, has a long history of use as a plasma volume expander. As a replacement for CMC, gelatin, per se, would appear to be an unlikely candidate since its net charge at pH 7 is slightly positive or only slightly negative (depending on whether the gelatin was extracted from tissue by acid or alkali). To increase the negative charge, we tried sulfation of the hydroxyl groups of gelatin, of which there are about fifteen per hundred residues:

$$R\text{-}OH + H_2SO_4 \longrightarrow R\text{-}OSO_3\text{-}Na^+$$

$$\text{or } ROH + ClSO_3H \longrightarrow R\text{-}OSO_3\text{-}Na^+$$

i. Analysis of Sulfated Gelatin.

The extent of sulfation of gelatin had previously been qualitatively estimated by determining its isoionic point, obtained by treatment with excess mixed-bed ion-exchange resin and measuring the pH. The greater the number of sulfate groups the lower the pH. The difficulty with this procedure is the poor electrode response in salt-free solution.

We now have obtained elemental sulfur analysis for two batches of SO_4Gel, 34-208 (with neutralization of the sulfuric acid by aqueous sodium acetate) and 35-6 (with removal of the sulfuric acid by ether). The former had 3.56% S and the latter 3.75%. These correspond to about one sulfate (as the sodium salt) per 8 peptide residues, or 88% of maximum (allowing for 10% water which is tightly held by gelatin).

ii. IFN Induction.

Formulations of IC, PLL and SO_4Gel were prepared and tested in mice for IFN induction (Table 17). The earliest preparations, III-31A, III-31B and III-43 gave 47-72% of the IFN titers of ICLC standards. III-111 gave a substantial titer (427 units), but no ICLC standard was run. III-31A, III-31B, and III-111 were of lower toxicity than ICLC.

A series of formulations (III-106, III-155, III-164 and III-175B) gave very little IFN. All of these showed two melting transitions, one of which corresponded to uncomplexed IC and accounted for 50-85% of the total IC. The reasons for these failures are not known.

New batches of SO_4Gel were synthesized and incorporated into three inducer formulations III-198, III-199 and III-200. They were all effective inducers; the variation in titers between two tests of III-198 and of III-199 is not unusual as can be seen by the results with ICLC. III-198 and 199 had high melting

temperatures and were resistant to hydrolysis by ribonuclease, both being hydrolyzed at 9% of the rate for IC.

While ICL-SO4Gel is a promising IFN inducer, of lower toxicity than ICLC, two batches (III-31A and III-31B) were found to be immunogenic when administered under conditions designed to promote immunogenicity, i.e., at a very high dose of 100 µg/mouse (or 2 mg/kg).

Table 17. IFN Induction by ICL-SO_4Gel.

Lot #	Composition IC:PLL:X	IFN Titer	IFN titer of ICLC Std.	% of ICLC Std.
	ICL-SO_4Gel			
III-31A	2/0.375/5	952	2046	47
III-31B	2/0.375/5	1345	2046	66
III-43	2/0.75/6	1203	1669	72
III-111	2/0.75/5	427	-	-
III-155	2/0.375/5	38	357	11
III-164	2/0.75/5	14	357	4
III-106	2/0.375/6	37	756	5
III-175B	2/0.375/2.5	23	351	7
III-198	2/0.75/6	1263	394	321
III-199	2/0.75/6	344	394	87
III-200	2/0.75/6	122	394	31
IV-261	2/0.75/6	399	1128	35
V-81	2/0.375/6	21	1956	1

[a] 172% if one mouse with very high titer is deleted.

The SO_4gel prepared by the ether-precipitation procedure was incorporated into an ICL-SO_4Gel formulation, V-81, which gave a low IFN titer in mice, 21 units. No difference was observed when the IFN titer was measured 3 or 6 hours after administration to the mice. Lot V-81 gave an LD_{50} of >40 mg/kg. The new procedure for making sulfated gelatin appears to be not as good as the old (on the basis of IFN titer), despite the similarities in extent of sulfation. V-81 gave a melting profile with two transition temperatures, 63° and 83°. The latter accounted for 67% of the total transition. Therefore, most of the poly IC was complexed. The reason for the low IFN is not apparent.

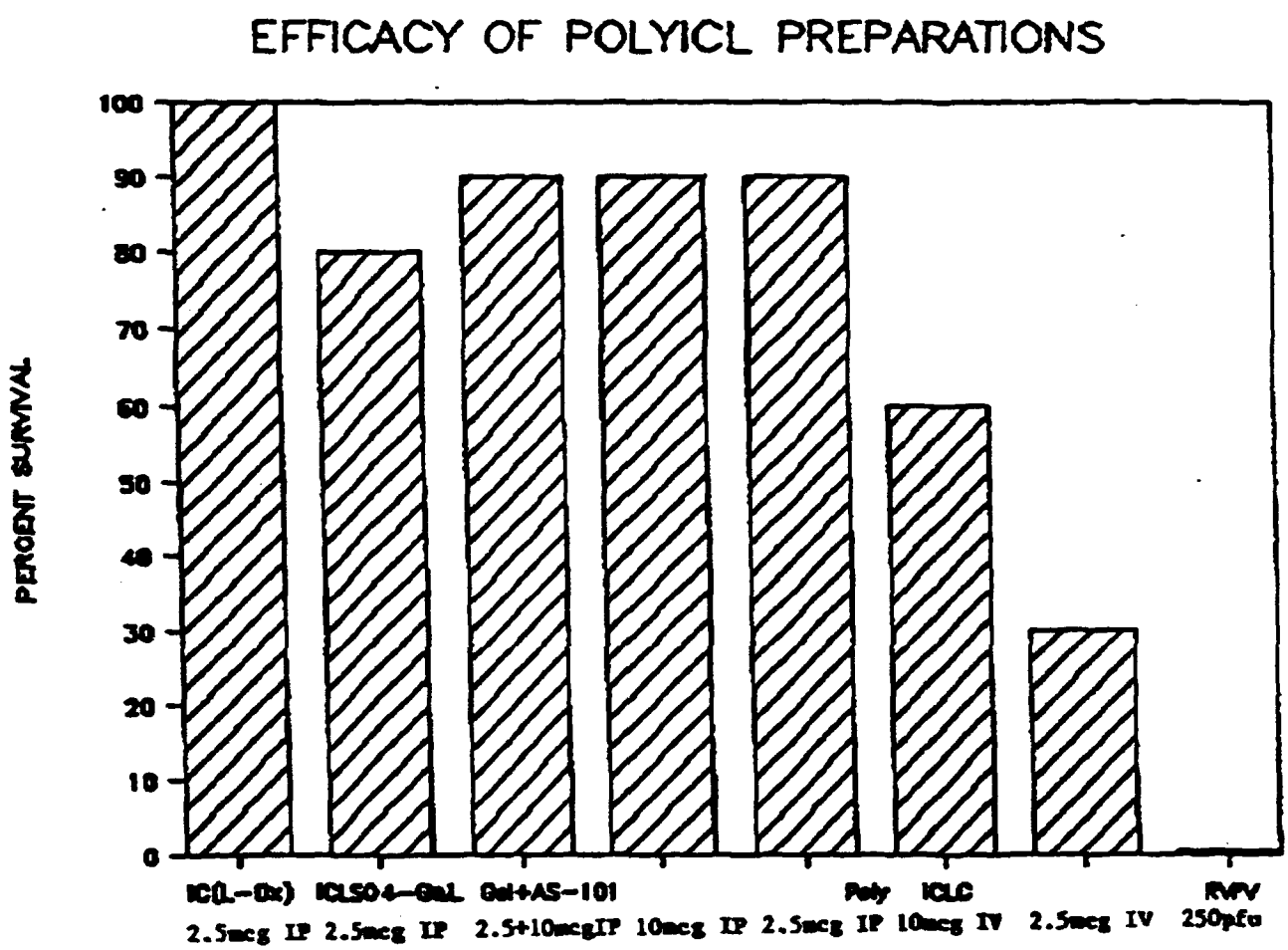


Fig. 21. Anti-RVFV action of ICL-SO_4 Gelatin (lot III-198) and ICL-Gelatin (lot III-210), compared with Poly ICLC and a lot (III-190) of IC-(PLL-Dextran).

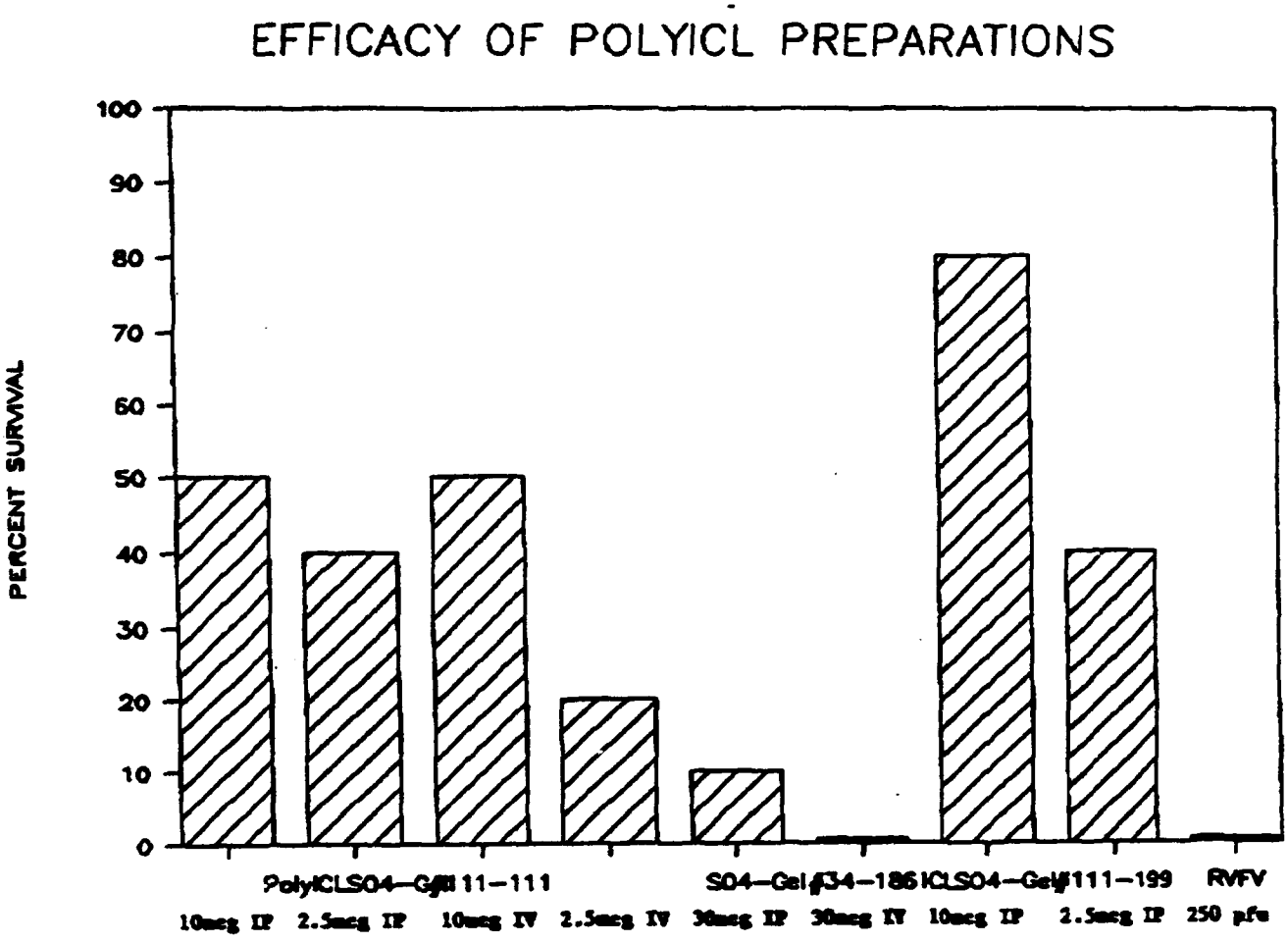


Fig. 22. Anti-RVFV action of ICL-SO_4Gelatin (lots III-111, III-199). SO_4-Gel (34-186) is control with SO_4-gelatin alone without poly IC.

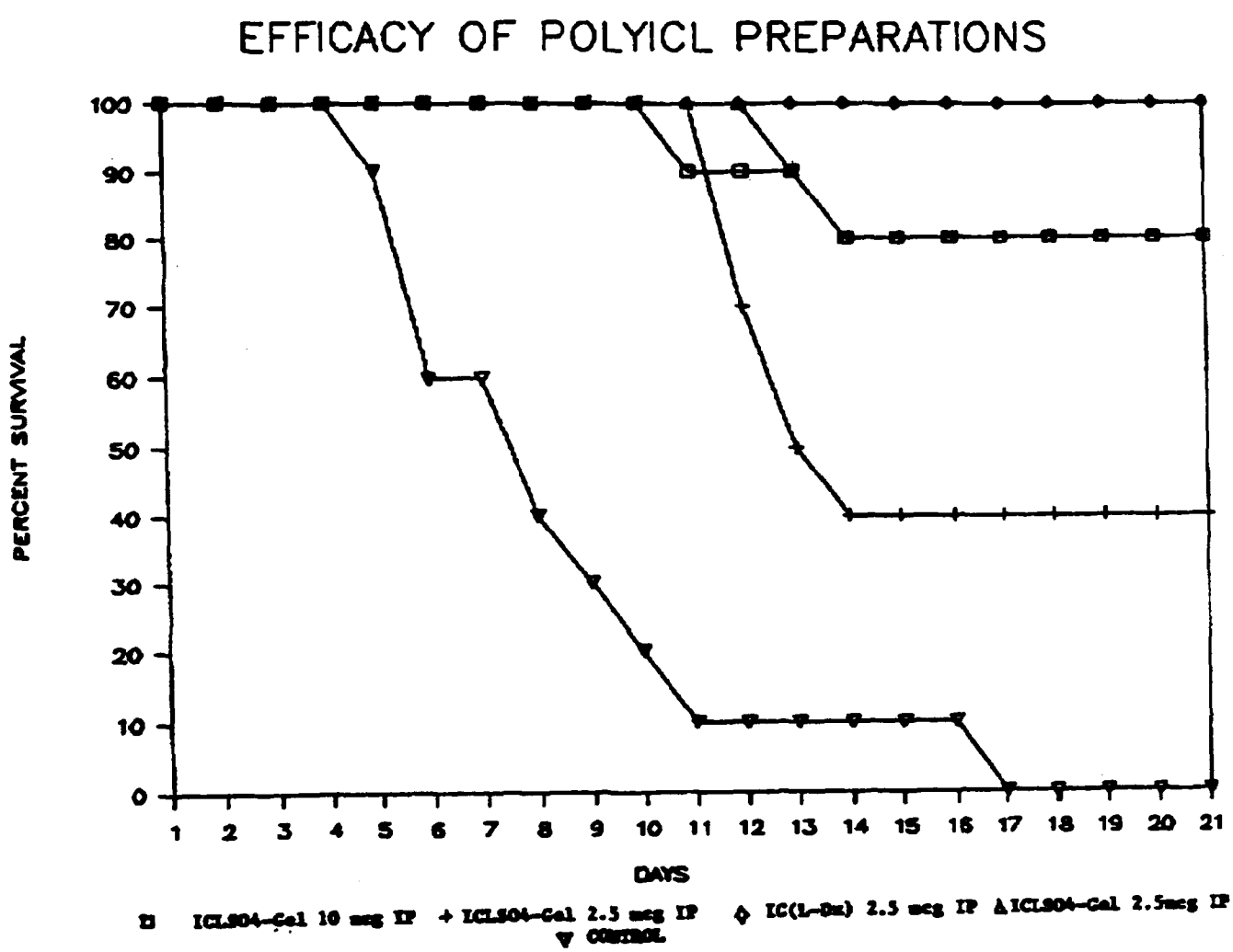


Fig. 23. Anti-RVFV action of ICL-SO_4 compared with IC-(PLL-Dextran). Lot numbers from left to right at bottom of Figure are: III-199,. III-199, III-190, III-198.

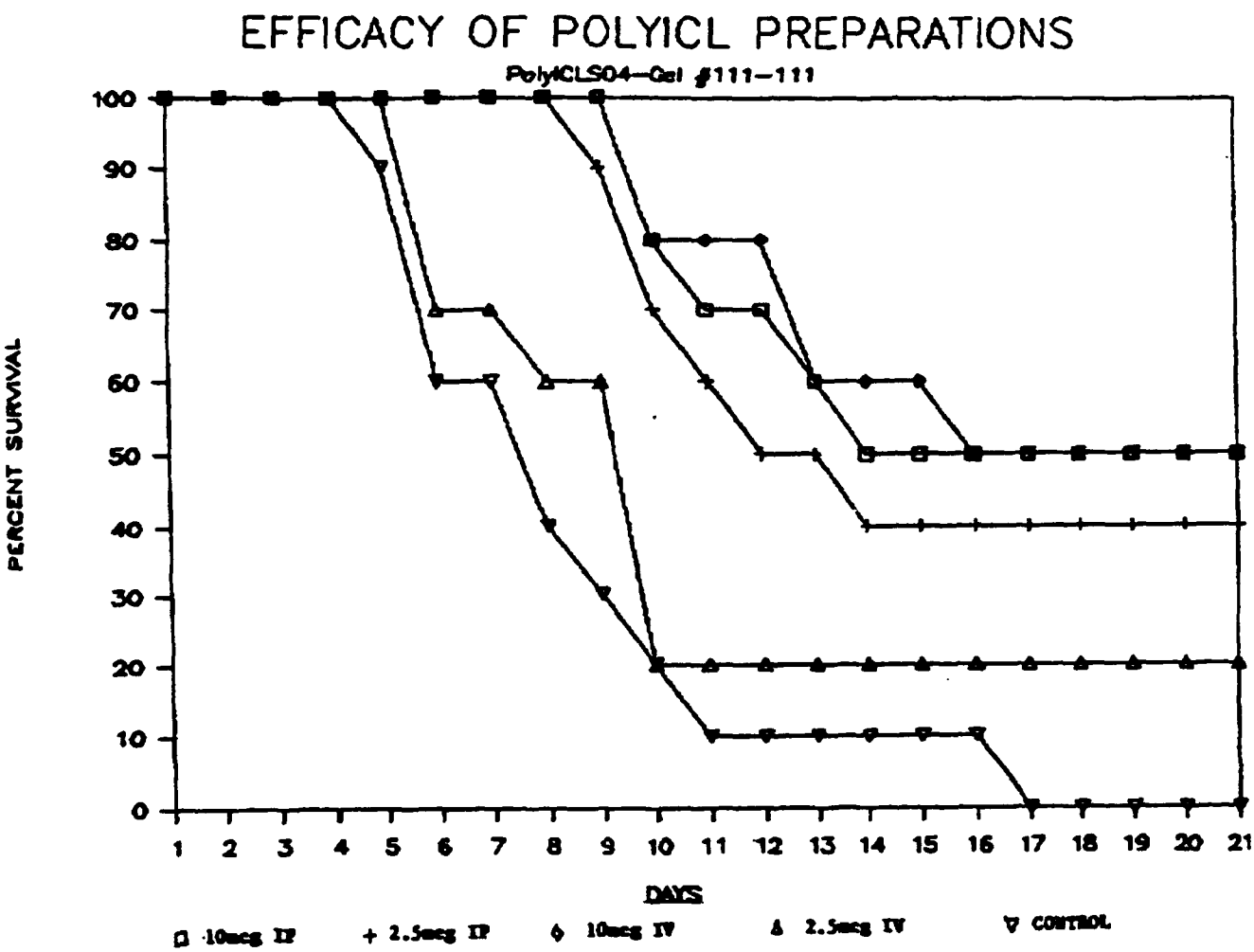


Fig. 24. Anti-RVFV action of ICL-SO_4Gel (lot III-111).

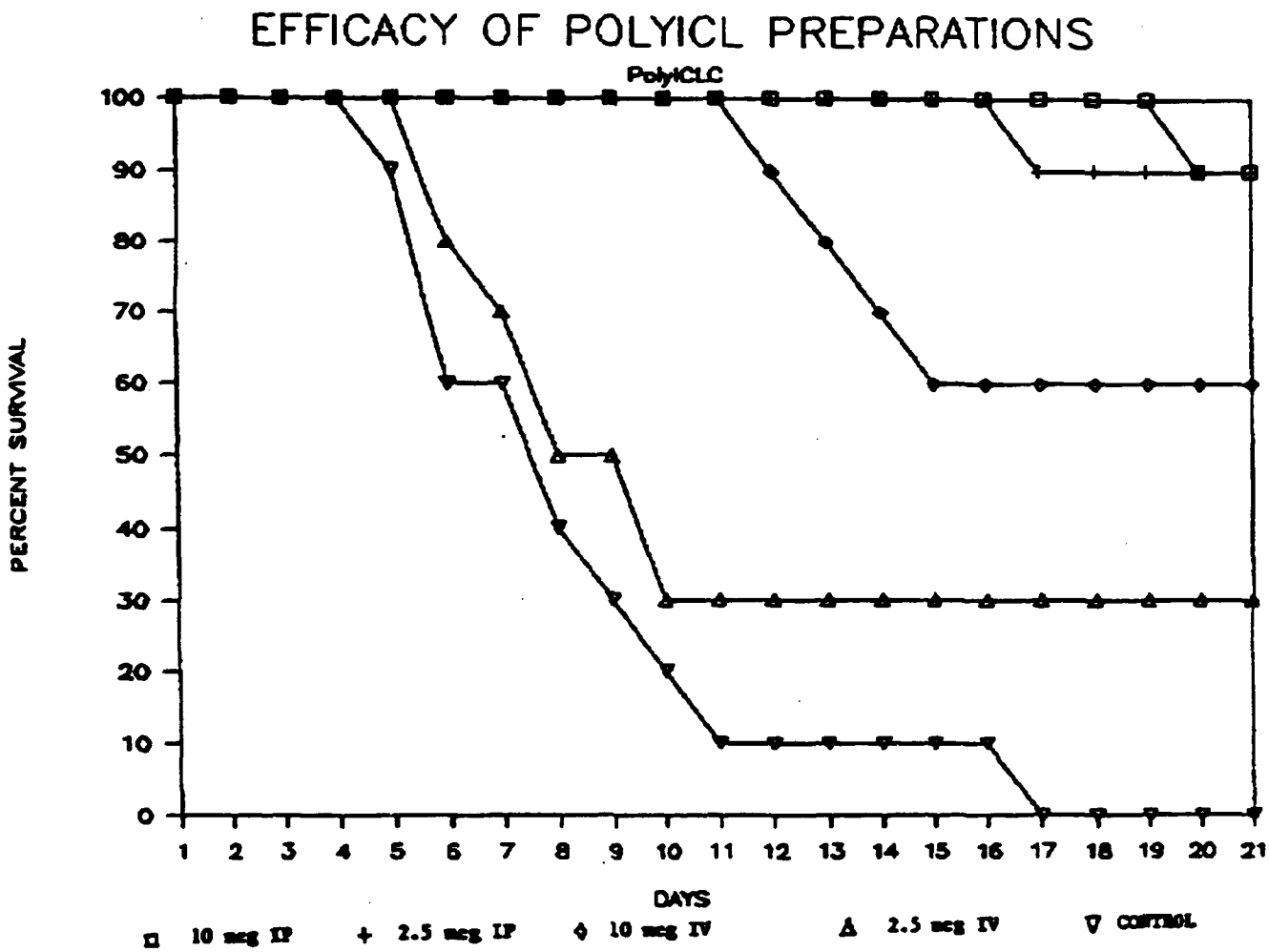


Fig. 25. Anti-RVFV action of Poly ICLC, for comparison with Figs. 23 and 24.

iii. Antiviral Action.

Several lots of ICL-SO_4 gelatin were tested against Rift Valley Fever virus (by Dr. Kende) with the results of Table 18. These were moderately effective against RVFV.

Table 18. Antiviral Action of ICL-SO_4 Gelatin Against Rift Valley Fever in Mice[a]

Lot No.	Dose µg		Survivors Exptl. Agent	ICLC
III-106	2.5	IV	5/10	9/10
III-111	10	IP	5/10	10/10
III-111	2.5	IP	4/10	9/10
III-111	10	IV	5/10	6/10
III-111	2.5	IV	2/10	3/10
III-198	2.5	IP	8/10	9/10
III-198	10	IP	6/10	-
III-199	10	IP	8/10	10/10

[a] Mice challenged with 250 pfu of virus, followed by drug at 0, 3 and 7, or 0, 2, 4 and 6 days. In some cases a second viral challenge was done at day 22 with 500 pfu, as noted. The surviving fraction is for day 19-21, except where noted after a second challenge.

Graphic display of anti-RVFV action of ICL-SO_4Gel is shown in Figs. 21-25. Figs. 22 and 26 also show a control with SO_4-gelatin, without poly IC. Some antiviral action may inhere in this material.

Lot III-198 was submitted for testing against Punta Toro virus (Dr. Sidwell, Utah State Univ.), with the survival results comparable to those obtained with Poly ICLC (Table 19). Other assays (enzymes and viral titers) for III-198 were similar to the averages for two trials of poly ICLC.

Table 19. Action of ICL-SO_4Gel (III-198) Against Punta Toro Virus.

Inducer dose mg/kg	Survivors		
	ICL-SO_4Gel	Poly ICLC Trial 1	Trial 2
1	10/10	10/10	-
0.25	10/10	10/10	-
0.1	9/10	10/10	9/10
0.032	7/10	9/10	1/10
0.01	6/10	9/10	3/10

iv. LD_{50} and Safety Testing.

Toxicities of ICL-SO_4 gelatin lots are shown in Table 20. All lots tested were less toxic than was ICLC. With this type of formulation the data suggest that the higher the IFN titer, the lower the LD_{50}. Nevertheless, ICL-SO_4 gelatin has the advantage over ICLC that all components are metabolizable.

Table 20. Toxicity of ICL-SO_4 Gelatin in Mice.

Lot #	LD_{50} mg/Kg
ICLC	10
III-106	25
III-111	25
III-186	21
IV-261	>40

Safety testing of ICL-SO_4Gel (IV-261) in mice resulted in a weight loss half as large as with ICLC (9.6% vs. 19.1%), but in guinea pigs IV-261 caused a larger weight loss than did ICLC. (Tables 35 and 36).

Overall ICL-SO_4gel is an effective inducer, an effective antiviral agent, and is of lower toxicity than poly ICLC. In addition SO_4gel should be metabolized (to be tested by enzymic digestion).

v. Immunogenicity of ICL-SO_4Gel.

Two lots of ICL-SO_4gel (III-31A, III-31B) were antigenic in mice, but lot III-199 was not antigenic. None of the components of III-31A and III-31B was antigenic, nor did sera raised against these cross-react with SO_4gel. The earlier work was done with 100 µg of contained poly IC; the new work on III-199 was done with 10 µg of poly IC per mouse. Antigenicity of ICL-SO_4gel should be reexamined.

vi. Tryptic Digestion of Sulfated Gelatin.

An important aspect of the research is that the inducer be excretable or metabolizable. Gelatin is digested, being a protein. We have carried out tryptic digestion of sulfated gelatin and of gelatin. The results of digestion were followed by circular dichroism, observing the behavior of the 220 nm band at 0°, which is characteristic of the formation of the helical, collogen-related structure, which requires that the gelatin be of high molecular weight. Action of trypsin on both leads to the same result, namely, the disappearance of most of the 220 nm band, showing that the molecular weight had been much reduced. Thus, sulfated gelatin is digested by trypsin, an important step toward complete digestion or excretion of small peptides.

g. ICL-Gelatin.

As a control for ICL-SO_4 gelatin we prepared a formulation with unmodified gelatin. It was not expected that gelatin would complex well with PLL, since at pH 7 the gelatin would have a small net positive charge, nor was it expected to complex with IC, since the small net positive charge on gelatin was not expected to compete with the large charge and high charge density of PLL. However, a complex of all three components was formed, as shown by the fact that a soluble complex was formed, whereas without gelatin the ICL precipitates. The ICL-gelatin was an effective inducer. Three lots were prepared and tested, and induced high titers of IFN in mice (Table 21).

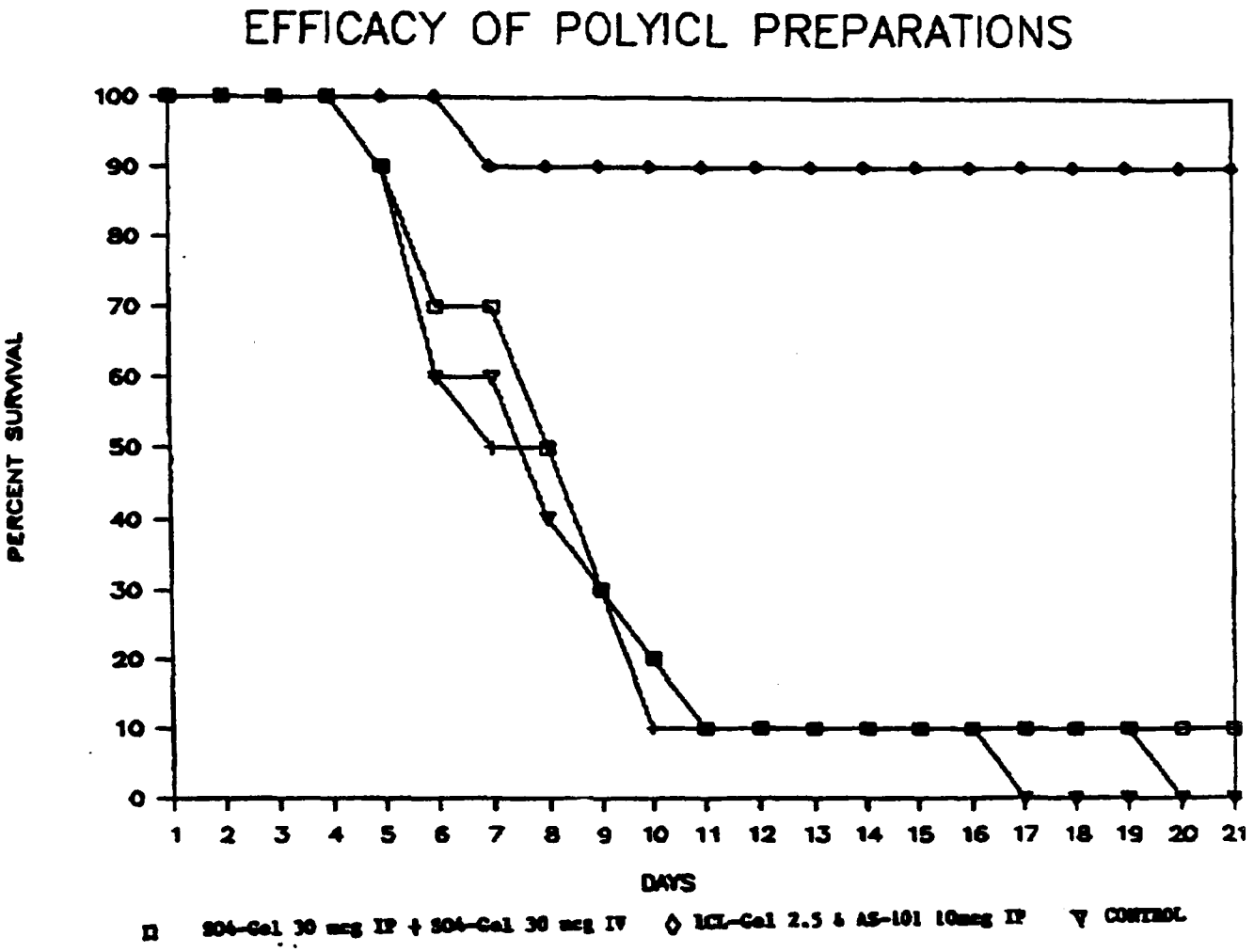


Fig. 26. Anti-RVFV action of ICL-gelatin (lot III-210). SO_4-gel is a SO_4-gelatin alone, for reference to Fig. 22, showing a possible slight action of SO_4-gelatin.

Table 21. IFN Induction and LD_{50} by ICL-Gelatin in Mice.

	IFN Induction			LD_{50}
	Experimental	ICLC Standard	% of ICLC	
ICL-Gel (IP = 5)[a] III-276 2:1.5:5[b]	856	1535	56	21 mg/kg
ICL-Gel (IP = 9)[a] III-210 2:0.75:6[b]	340	670	51	-
ICL-Gel[c] (IP = 9)[a] IV-243 2:0.75:6[b]	989	1039	95	11 mg/kg
IC-Gel (IP = 9)[a] III-222 2:0:6[c]	<6	670	<1	-

[a] Isoelectric point of the gelatin.
[b] Composition in mg/mL of IC, PLL and gelatin.
[c] Control IC-gel, without PLL.

ICL-gel is a moderately effective protector against RVFV Table 22) at 10 µg per mouse, better at 2.5 µg; but these should be repeated. This is a promising complex in the light of the extensive experience with gelatin as a safe plasma volume expander. Gelatin is usually immunogenic; the gelatin we are using is claimed to be non-immunogenic, and was found to be so by our screening procedure. ICL-gel had a high Tm (see below), and was hydrolyzed by ribonuclease at 5% of the rate of uncomplexed IC. A control of IC-gel without PLL (III-222) was not an inducer, and was not a complex, as shown by the Tm of 63°, the normal Tm of uncomplexed IC.

Table 22. Anti-RVFV Action of ICL-gel (Lot III-210).

Inducer dose, µg/mouse	Survivors	
	Lot III-210	Poly ICLC
10	6/10	9/10
2.5	9/10	9/10

Graphic results with ICL-gel are shown in Figs. 21 and 26.

Lot III-210 was tested against Punta Toro virus, Table 23 (data of Dr. Sidwell). III-210 was about as effective as the average of two trials for poly ICLC. Other data in these trials

(enzyme assays and viral titers) were comparable to the averages for the two poly ICLC trials.

Table 23. ICL-Gel: Action Against Punta Toro Virus in Mice[a]

Dose mg/kg	Survivors		
	Lot II-210	Poly ICLC Trial 1	Trial 2
1.0	10/10		
0.25	10/10		
0.1	9/10	10/10	9/10
0.032	9/10	9/10	1/10
0.01	9/10	9/10	3/10

[a] Data of Sidwell. Lot III-210.

Lot, IV-243 had an LD_{50} of 11 mg/kg, the same as for poly ICLC. (III-210 was not tested for toxicity.) The potential advantage of ICL-gel is that gelatin is metabolizable.

ICL-Gel (III-210 and III-213) had been made with a gelatin of isoelectric point (IP) of pH 9, an acid-process gelatin. At physiological pH this will have a slightly positive net charge. We prepared ICL-gel with a base-process gelatin, with isoelectric point of pH 5, which has a negative net charge at physiologic pH. This lot, III-276, gave an IFN titer of 856 units or 56% of that of poly ICLC, a value comparable to those of ICL-Gel formulations made with acid process gelatin (which runs from about 50 to 95%). III-276 had an LD_{50} of 21 mg/kg, twice as high as for III-243 and poly ICLC. Thus, both major types of gelatin give effective inducers. A choice between them would depend on other properties, such as toxicity, immunogenicity, antiviral action, etc.

Although the two types of gelatin have different net charges, they contain both positive and negative charges but in different proportions. This may result in differences in the organization of the complexes with poly IC and PLL, which could affect the rate of digestion of the poly IC and the kinetics of IFN induction. Twice as much poly(L-lysine) is required to complex the poly IC when IP 5 gelatin was used as when IP 9 gelatin was used. The net negative charge on IP 5 gelatin (resulting from a greater number of negative carboxylate groups) would probably bind cationic poly(L-lysine) more strongly than does IP 9 gelatin (net positive charge), thereby competing more effectively against poly IC for binding to poly(L-lysine) and requiring more poly(L-lysine) to form the ternary complex.

Melting profiles were done on III-276, III-210 and IV-243. III-276 gave two melting steps, 63° and 79.5°, in the proportion of 40% to 60%, respectively. The 63° Tm is probably that of free poly IC. III-276 was less toxic than poly ICLC (Table 21). III-210 and IV-243 had Tm values of 80° and 82°, respectively; all poly IC is complexed.

Overall, ICL-gelatin is an effective inducer, and an effective antiviral agent. LD_{50} should be resolved as to the source of the difference between III-243 and III-276. The advantage of ICL-gel is that all components are metabolizable.

Storage Behavior of ICL-Gelatin.

ICL-gel on storage at 4°C sets to a white, opaque gel. On warming to room temperature, a clear solution is regenerated, and the ICL-gel shows a Tm of 79.5°, near to the original Tm of 80°. A possible explanation of the above observations is the following: Because of its low charge density gelatin binds rather weakly to ICL. At low temperature, gelatin-gelatin interactions (i.e., the interactions which lead to gelation) become stronger, shifting the equilibrium, as shown, leading to gelation of the gelatin and precipitation of the ICL.

Warm	<---->	Cold
ICL-gel	<---->	ICL (ppt) + gelatin (gelled)

There is a difference between the melting profiles before and after cold storage. Before, the absorbance decreases above Tm; after cold gelation and reliquefaction, the melting profile is normal with a flat plateau above Tm. Aside from the obvious appearance and disappearance of the opaque gel, there is another difference, namely, the order of mixing the components. The original ICL-gel is prepared by mixing PLL and gelatin, after which IC is added. When the opaque gel is thawed, the mixing procedure is that the ICL precipitate interacts with gelatin, i.e., the mixing procedure is essentially reversed. The original mixing procedure is based on Levy's procedure for making ICLC. We have chosen, so far, to use the same procedure for the inducer formulations involving IC + PLL + polyanion, to avoid doubling all of the formulations and their biological tests. However, it may be desirable to try the reverse procedure, since this may result in different particle structures and biological properties.

h. Acetylcitryl (AcCitGel) and Succinyl Gelatins.

The introduction of carboxylates into gelatin is simple and straightforward. Reaction in water at pH 9 with an anhydride acylates the amino groups. We have done one experiment with succinylated gelatin, which converts a positively charged group

to one with one negative charge, or a change of -2 in net charge. The ICL-SuccGel, I-43b in Table 23 induced a low IFN titer.

Since there are only about 3-4 amino groups per 100 amino acid residues in gelatin, the density of negative charges introduced by succinylation is not large (although they are in addition to the 8-12 carboxylate residues per 100 amino acid residues naturally present in gelatin). In order to increase the number of negative charges we turned to acylation with acetylcitric anhydride, which introduces two negative charges per amino, or a change of -3 in net charge. Treatment of AcCitgel with chymotrypsin resulted in cleavage to smaller fragments, detected by gel filtration through Sephadex G-50. Thus AcCitgelatin should be metabolizable.

Formulations with several compositions of components were prepared and tested (Table 24). The best lot was I-43a which gave a titer of 3123 U/mL or 75% of that of a very high titer for standard ICLC. When this was retested after 10 weeks storage at 4° the titer was 116; but the ICLC standard gave 325, a very low value.

The ICL-AcCitGel formulations have presented difficulty in solubility. I-43a was obtained in solution by stepwise gradient dialysis from 2 M NaCl down to normal saline. However, attempts to repeat this have not been successful, as precipitation occurred. I-43a may have been kinetically stable for the first mouse assay, but not thermodynamically stable. The next best lot, BGII-39 gave 859 U/mL, 21% of standard. The standard was unusually high and BGII-39 may be of value.

The next best was II-234, with a titer of 17% of standard. But this may have been induced by uncomplexed IC. II-234 gave a normal melting profile and a Tm of 63.5°, indicative of uncomplexed IC. Because of difficulties with solubility and reproducibility, this line of research was terminated.

Table 24. IFN Induction in Mice by ICL-SuccGel and ICL-AcCitGel.

Lot Number and type of Gelatin	Composition IC, PLL, Gel mg/mL	IFN Titer Exptl. Formulations	IFN Titer ICLC Standard	IFN Titer % of ICLC Standard
SuccGel				
I-43b	2, 1.5, 5	158	1173	13
AcCitGel				
I-43a*	2, 1.5, 5	3123**	4170	75
II-34	0.5, 1.5, 2.5	24	4170	1
II-39	0.5, 1.5, 2.5	859***	4170	21
II-39a	2, 1.5, 2.5	38	325	12
II-121	2, 1.5, 2.5	324	1327	24
II-234#	2, 1.5, 5	433	2609	17

* Gradient dialyzed from 2 M NaCl.
** Retested after 10 weeks at 0°; 116 units compared with 325 for ICLC standard.
***Retested after 10 weeks at 0°; no IFN.
Melting profile showed that IC was not complexed.

i. Inducers Containing Acetylcitryl PLL.

Two preparations of AcCitPLL were made with PLL of two molecular weights, 55 kDa and 14 kDa. First, ICL-AcCitPLL(55) complexes were prepared and tested (Table 25). The number in parenthesis after ICL-AcCitPLL indicates the molecular weight in thousands of the PLL from which AcCitPLL was prepared. Complexes with the 55 kDa AcCitPLL were effective IFN inducers when the IC, PLL and AcCitPLL were combined in the ratio 2:6:10 by weight (II-29b; I-119). When less PLL and AcCitPLL were used relative to IC very little or no IFN induction was obtained (II-43b, II-195). Since both the PLL and AcCitPLL were reduced, we do not know which of these components may be crucial, or if the higher content of both is needed for IFN induction.

We then made AcCitPLL with a PLL of lower molecular weight, 14 kDa. Two ICL-AcCitPLL^{14} complexes were prepared and tested. II-120 made with IC:PLL:AcCitPLL^{14} in the ratio 2:6:10 gave a moderate IFN induction of 32% of the standard, while II-142 with the components ratios of 2:1.5:5 gave 128 and 274% of standard in two mouse tests. In the case of the M_r 14,000 AcCitPLL, less PLL and AcCitPLL gave better results.

The results for II-142 are attractive, but there is a difficulty. II-142 is not completely soluble in normal saline. It was prepared in 2 M NaCl, and on 40-fold dilution with normal saline for injection, some precipitate appears. The IFN data in Table 25 were obtained with the supernate after allowing the

precipitate to settle. Solubility problems were also encountered with the 55kDa AcCitPLL complexes, but of much less severity; small amounts of precipitate were present, which dissolved on gently warming.

II-195, ICL-AcCitPLL, which gave no IFN titer, showed no Tm, only a very broad shallow increase in Tm, and a spectrum indicating a preponderance of poly I and relatively little poly C; apparently, with the ratio of components used, IC is dissociated, and the poly C component is precipitated, leaving in solution only the inactive poly I.

Table 25. IFN Induction by ICL-AcCitPLL in BALB/c Mice

Lot Number	Composition[a] IC, PLL, AcCitPLL	IFN, U/mL Exptl. Formul.	ICLC Standard	IFN % of Standard
I-43b	2, 1.5, 5	125	1173	11
I-29b	0.5, 1.5, 2.5	891	933	95
II-119	0.5, 1.5, 2.5	1145	1327	86
II-195	2, 1.5, 1.75	0	1826	0
II-120	0.5, 1.5, 2.5	429	1327	32
II-142	2, 1.5, 5	1684	1316	128
II-142	2, 1.5, 5	2840	1035	274

[a] In mg/mL of the components in the order IC, PLL, AcCitPLL.

j. Hyaluronic Acid Complex (ILC-Hy).

Hyaluronic acid is a natural mammalian polysaccharide with negative charges. It is used for medical purposes, e.g., to replace the vitreous in ophthalmic surgery. Two formulations were made with poly IC, PLL and hyaluronic acid (ICL-Hy), lots V-167 and V-178. Lot V-167 was formulated with high molecular weight hyaluronic acid, as received without sterilization. Because of its very high viscosity it could not be sterilized by filtration. Lot V-178 was sterilized by autoclaving the hyaluronic acid, which reduced its viscosity (and presumably its molecular weight).

Table 26. IFN Induction by ICL-Hy.

Lot #	Titer	Titer as % of Titer Induced by ICLC	LD_{50}
V-167	461	39%	-
V-178	658	56%	26 mg/Kg[a]

[a] Compared with 11 mg/Kg for ICLC.

This type of formulation gives a substantial IFN titer (Table 26), and is less toxic than ICLC. Lot V-178 gave an LD_{50} of 26 mg/Kg, or 2.6 times as high as ICLC. The melting profile gave a Tm of 83°, demonstrating that poly IC is complexed. All of its components are digestible, and the hyaluronic acid is a substance already in clinical use. Additional work should be done to see if formulations with higher LD_{50} values can be prepared. Against RVFV in mice ICL-Hy was as effectivve as poly ICLC; 100% survival at 20 µg/mouse and 95% at 5 µg/mouse.

k. Amylose Sulfate ($AmSO_4$) and Amylopectin Sulfate ($AmpSO_4$).

We have studied sulfated amylose and amylopectin. Good results had been obtained with carboxymethyl amylose (CMA). The rationale was that sulfatases may hydrolyze the sulfate esters, restoring the amylose, which could then be metabolized. We have prepared $AmSO_4$ of several degrees of sulfation by reaction of amylose with sulfur trioxide-trimethylamine complex in dimethylformamide. Reaction conditions (time, temperature and ratios of reactants) to obtain different degrees of substitution were worked out.

Formulations were made with poly IC, PLL and $AmSO_4$ at DS = 0.4, 0.7 and 1.4 (sulfate per glucose residue), and melting profiles were obtained (Table 27).

Table 27. Melting Transitions of ICL-Amylose-SO_4.

Lot No.	Composition, IC:PLL:Amylose-SO_4 (by weight)	DS of Amylose-SO_4	Tm, °C
IV-212	2:0.75:2.5	0.4	84
IV-211	2:0.75:2.5	0.7	68
IV-210	2:0.75:2.5	1.4	64
IV-212A	2:1.5:5	0.4	84.5
IV-211A	2:1.5:5	0.4	64

The Tm data show that complexes were formed with amylose-SO_4 of DS 0.4 at both proportions of PLL and amylose-SO_4, but no complex was observed at the higher DS values. (The Tm of 68° for IV-211, being 4° higher than for poly IC alone, may indicate a complex. This has not been established, because ribonuclease digestion cannot be used, because $AmSO_4$ is an inhibitor of RNase.) Apparently at high DS, the negative charge on amylose-SO_4 pulls PLL out of interaction with poly IC, leaving bare poly IC.

The first ICL-$AmSO_4$ (IV-212) to be tested in vivo induced in mice an IFN titer of 183 units or 18% of that of poly ICLC. This is in the same range as the titers induced by IC-(PLL-dextran). The time course of IFN induction should be done, in order to see if this might be an effective inducer. Also, lower degrees of substitution may be useful.

ICL-Amylopectin-SO_4.

Amylopectin sulfate has been incorporated into an inducer, ICL-Amylopectin-SO_4 (lots V-129, V-177) which gave high IFN titers (Table 28). The very high percentage for V-129 is the result of a rather low titer for a new lot of ICLC. Compared with the average titer for other lots of ICLC, the titer obtained with V-129 would be about 130%. LD_{50} was 25 mg/kg, compared with 11 mg/kg average for ICLC. This combination of properties makes this an attractive candidate.

Table 28. IFN Titers in Mice, Induced by ICL-Amylopectin-SO_4.

Experimental Inducer, Lot #	IFN Titer	Poly ICLC IFN Titer	% of Titer Induced by Poly ICLC
V-129	1330	306	435%
V-177	995	1181[a]	84%

[a] Average of two lots (V-171, V-183); 1227 and 1134, respectively.

Lots V-129 and V-177 gave melting profiles with single transitions, at 79 and 81°, respectively, demonstrating the presence of complexed poly IC.

ICL-amylopectin-SO_4 was as effective against RVFV in mice as was poly ICLC: 100% survival at 20 µg/mouse, and 90% at 4 µg/mouse.

1. ICL-Carboxymethyl Amylose (ICL-CMA).

(Carboxymethylamylose (CMA) is an analog of CMC). But it has been tested as a blood volume expander, with good results[4]. Formulations first were made of ICL-CMamylose with the components in the concentrations of 2, 1.5 and 5 mg/mL for IC, PLL and CMamylose, respectively, or with the concentrations of 2, 0.75 and 2.5 mg/mL (Table 29). The former group gave IFN titers averaging 43% of the standard ICLCs, the latter 70%.

Table 29. IFN Induction by ICL-CMamylose.

Part a.

Lot Number	D.S.	Composition IC,PLL,CMamylose mg/mL	IFN Titer Experimental Formulation	ICLC Standard	% of ICLC Standard
II-5[a]	0.5	2, 1.5, 5	1061	2585	41
II-9	"	2, 1.5, 5	1053	2585	41
I-127	"	2, 1.5, 5	394	672	59
I-127	"	2, 1.5, 5	658	1316	50
I-127	"	2, 1.5, 5	263	1038	25
II-143	"	2, 0.75, 2.5	908	1316	69
II-143	"	2, 0.75, 2.5	448	1035	43
II-166	"	2, 0.75, 2.5	1052	575	183
II-193	"	2, 0.75, 2.5	678	1826	37
II-193	"	2, 0.75, 2.5	533	2609	20

Part b.

III-107	"	2, 0.75, 2.5	787	756	104
II-166	"	2, 0.75, 5	764	643	119
III-167A	0.22	2, 0.75, 2.5	256	351	73
III-167B	0.43	2, 0.75, 5	123	351	35

[a] Lots II-5 through II-193 were made with CMamylose with DS = 0.5.

However, the latter average may have been distorted by one preparation with a titer 183% of the standard (an unusually low standard). If we average the absolute titers the two sets have 686 and 724, respectively; i.e., there is no difference.

Subsequent work was done with the composition 2/0.75/02.5 and 2/0.75/5, with the results in Table 29b. Lot III-107 was retested after one year and gave 684 units, or 64% of the titer with ICLC (1039 units).

When the original lot of amylose was largely exhausted, a new preparation of CMamylose was made, using a new batch of amylose. This gave ICL-CMamylose formulations of low IFN inducing ability (III-246, III-248, and III-255, Table 30). That this was not a defect in our technique of formulation was shown by making a new batch of ICL-CMamylose equivalent to III-107 (reported earlier) from some remaining original CMamylose; this batch, III-258, gave 247% as much IFN as did standard ICLC. A third batch of amylose was carboxymethylated. The ICL-CMamylose formulations from this gave good IFN titers, 989 units, and 534 units, or 64% and 35% of standard ICLC for two formulations differing in the PLL content.

There was still a difference from ICL-CMamylose batches made with the original first batch of amylose, namely, that twice as much PLL was needed with batch-3 CM-amylose to complex all of the IC as with batch-1. That is, III-278 had two Tm values, 63° and 81°, while III-281 had only an 81° Tm (Table 31).

The difficulty with the second batch of amylose suggests that one cannot depend on amylose batches to be reproducible. At present we do not know what properties of amylose are critical. We must also bear in mind that the low IFN titers of formulations from the second batch of amylose may have been only a change in kinetics, resulting from a subtle variation in the amylose (molecular weight, molecular weight distribution, etc).

Comparison of III-278 and III-281 is instructive. III-278 had one-half as much PLL as did III-281. III-278 had two Tms, at 62° and 81° (in the ratio of 40:60 for ΔA), and III-281 had only one Tm, at 81° (Table 31). III-281 had nearly twice the IFN titer of III-278. In this case we can think that the 62° Tm of III-278 is that of IC alone, since it vanished with more PLL. Also, since III-281 had almost twice as much of the 81° complex and twice the IFN titer, it appears that the IFN titer may be proportional to the concentration of complexed IC.

Table 30. IFN Induction in Mice by ICL-CMamylose.

Lot and Composition[a]	Amylose Lot	IFN Induction Exptl. Formulation	IFN Induction ICLC Standard	IFN Induction % of ICLC Standard
III-246 2:0.75:2.5	2	46	799	6
III-248 2:0.75:2.5	2	5	799	1
III-255 2:1.5:2.5	2	127	1535	8
III-258[b] 2:0.75:2.5	1	1329	539	247
III-278 2:0.75:2.5	3	534	1535	35
III-281 2:1.5:2.5	3	989	1535	64

[a] Composition in mg/mL of the components in the order: IC, PLL, CMamylose.
[b] Equivalent to III-107 (Annual Report, April 1, 1989, Table 3).

Table 31. Melting Temperatures of ICL-CMamylose.

Lot and Composition[a]	Tm 1 °C	% of Total Δ A	Tm 2 °C	% of Total Δ A
III-246 2:0.75:2.5	61	55	80	45
III-248 2:0.75:2.5	63	25	80	75
III-255 2:1.5:2.5	62	80	82	20
III-258 2:0.75:2.5	-	-	79	100
III-278 2:0.75:2.5	62	40	81	60
III-281 2:1.5:2.5	-	-	81	100

[a] Composition in mg/mL of the components in the order: IC, PLL, CMamylose.

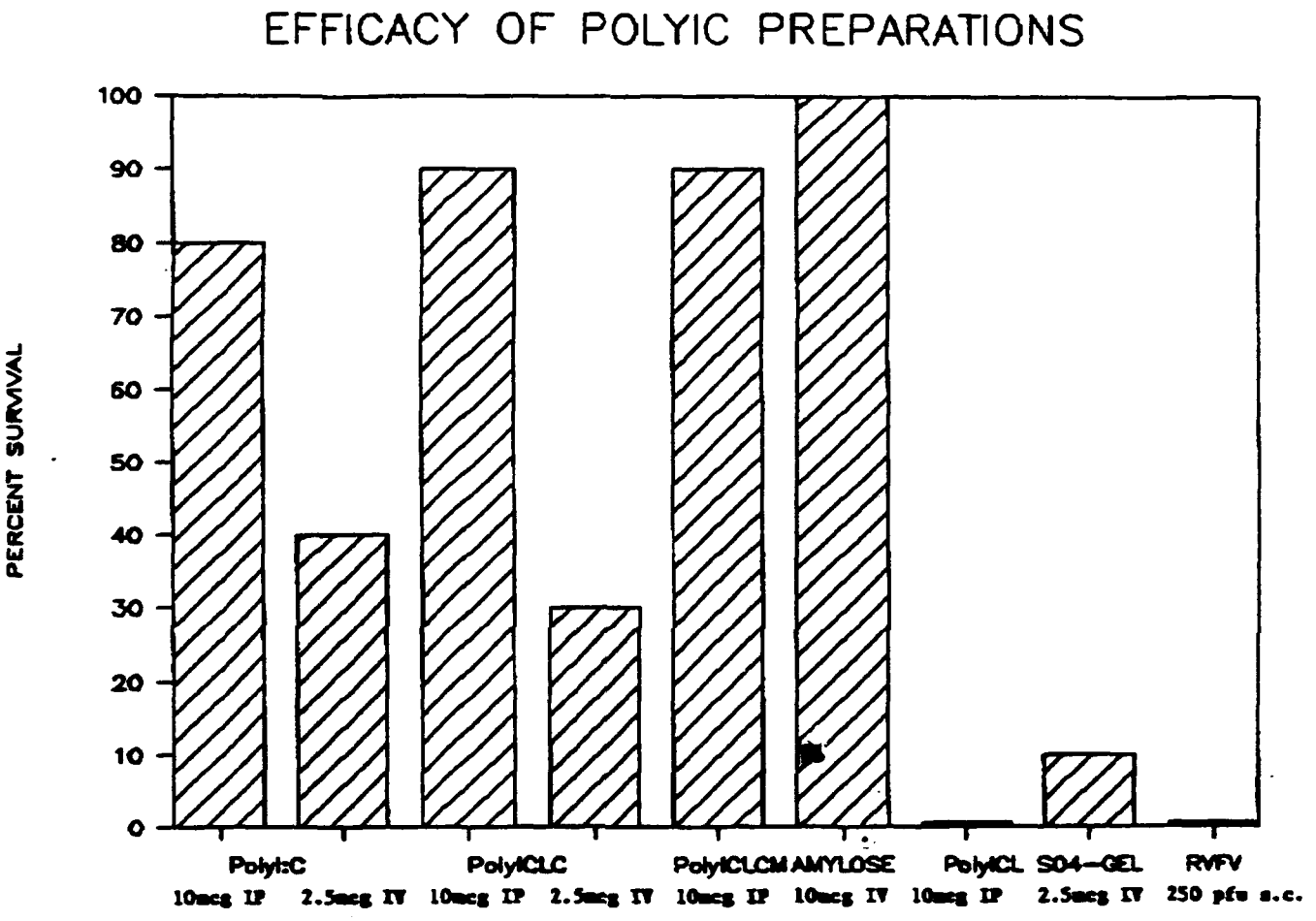


Fig. 27. Anti-RVFV action of ICL-CMamylose (lot III-107).

Table 32. LD_{50} of ICL-CMamylose and Other Substances.

Substance	LD_{50}
Poly ICLC Standard	11 mg/Kg[a]
ICL-CMA III-281	25 mg/Kg
Poly IC	33 mg/Kg[b]
PLL	>30 mg/Kg
CMC	>75 mg/Kg

[a] Average of four measurements: 13, 11, 11, and 9 mg/Kg.
[b] Average of three measurements: 26, 37 and 35 mg/Kg.

ICL-CMamylose complex III-281 gave an IFN titer of 64% of that of the ICLC standard (989 units vs. 1535 units, Table 30). LD_{50} of this preparation and those for the poly IC and CMC batches we are using are shown in Table 32.

Thus, ICL-CMA III-281 has more than twice the LD_{50} of ICLC. It is notable that poly IC alone is less toxic than is ICLC or ICL-CMA. Also, CMC is less toxic. However, the combination of ingredients tends to be more toxic, especially for poly ICLC.

Antiviral Action of ICL-CMamylose.

Lot III-107 was tested by Dr. Kende against RVFV. The results for III-107 are shown in Fig. 27. III-107 was as effective as poly ICLC. Against Punta Toro virus III-107 was effective at doses of 10 and 2.5 mg/kg, but not at 0.1 mg/kg (Table 33). (There were no data between 2.5 and 0.1 mg/kg.)

Table 33. Anti-Punta Toro Action of ICL-CMA (III-107).

Inducer dose mg/kg	Survivors	
	III-107	poly ICLC
10	9/10	10/10
2.5	10/10	10/10
0.1	1/10	19/20

m. Methylated and Guanidinated PLL and PLL-Dextran Graft Polymers.

Is PLL the optimum cationic polypeptide? The PLL used in formulations with poly IC and polyanions and in making graft polymers with dextran, has a certain degree of binding strength toward poly IC and toward CMC or a replacement polyanion. This binding strength might not be optimum for our purpose. Therefore, we investigated chemically modified PLLs which would have weaker or stronger binding to poly IC or to a polyanion.

Methylation of the amino groups of PLL weakens binding to polynucleotides, as measured by the ability of salt to dissociate the cationic polypeptide from polynucleotide[5]. Guanidinated amino groups strengthen binding. Therefore we methylated PLL to poly(N^{ϵ},N^{ϵ},N^{ϵ}-trimethyl-L-lysine) (PTMLL)[5], and we methylated and guanidinated a PLL-dextran graft. In place of the control guanidinated PLL we used poly(L-arginine), PLA.

i. Methylated Poly(L-lysine).

A mixture of poly IC, PTMLL and CMC showed a melting transition similar to that of poly IC. Apparently the poly IC was free, not bound to PTMLL. The formulation did not induce any significant titer of IFN, and gave, in fact, values of about 6 units/mL, essentially nil. The binding of PTMLL to poly IC may be weakened more than its binding to CMC, so that CMC outcompetes the poly IC. In the absence of CMC, PTMLL does bind to polynucleotides including poly IC, but more weakly than does PLL[5].

ii. Poly(L-arginine).

With poly IC, poly(L-arginine) (PLA) and CMC an insoluble complex formed, when these components were mixed in the same ratio as for poly ICLC. With the proportion of PLA reduced by one-half (poly IC and CMC held at the standard concentration), a clear solution (lot IV-111) was obtained, but the melting transition was low, 67°, and no significant titer of IFN was induced.

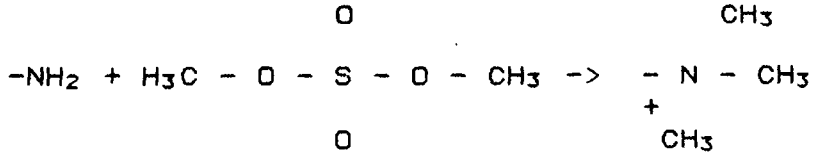
The 67° Tm for poly IC-PLA-CMC, compared with 63-64° for poly IC suggests that a complex may have been formed, and this is supported by the formation of a precipitate in the case where more PLA was used. The nearly zero IFN titer for IV-III, compared with about 100 for free poly IC, may mean that IC, PLA and CMC are bound in a complex of low activity, or the pharmacokinetics are different.

iii. Modified Graft Polymers.

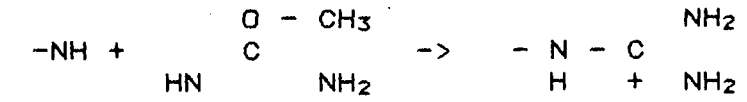
Formulations of poly IC with methylated and guanidinated PLL-dextran graft polymers had melting transitions of 63-64°, the same as for poly IC alone. Apparently, no complex was formed. The result for guanidinated PLL-dextran is unexpected as strengthened binding is expected.

These results suggest that ammonium groups of PLL and of PLL-dextran have near-optimum binding properties in poly ICLC. It is possible that binding might be fine-tuned by partial chemical modification of PLL, or by the use of copolymers of lysine with other amino acids. With anionic replacements for CMC, PLL might not be optimum.

Methylation was carried out by treating PLL or PLL-dextran with dimethyl sulfate in water at pH 9-10, followed by thorough dialysis. The reaction is:



The methylated amine is a quaternary ammonium ion, positively charged at all pH values. Guanidination was carried out with O-methyl-isourea sulfate in water at pH 9-10:



The guanidium group is positively charged.

n. Single-Stranded RNA in Formulations.

We have studied the use of single-stranded polyribonucleotides as polyanionic replacements for CMC. Some are antiviral agents in themselves, which may provide improved antiviral activity.

i. The rationale in each case was ease of digestion or excretion, and biological compatibility. The polynucleotides were:

Poly(guanylate), PG Poly(inosinate), PI

Poly(uridylate), PU Poly(cytidylate), PC

Two formulations with PG, using different ratios of PLL and PG to poly IC, gave 19 and 13% of IFN titer of poly ICLC. The poly IC is probably not complexed, as the Tm's were about 65°.

A formulation of poly IC, PLL and PU (composition 2:1.5:5) gave 14% of the IFN titer of poly ICLC. The melting transition of ICL-PU was 68°, 3-4° higher than that of poly IC.

Poly I complexed with IC and PLL was insoluble. Poly C gave a soluble complex, designated ICL-Cyt (where Cyt stands for poly C, to avoid confusion with the use of C for CMC). One complex of composition 2:1.5:5, for IC:PLL:Cyt, gave 271 units of IFN, or

36% of that of the ICLC standard. This is a substantial titer, and indicates that further work should be done.

o. Miscellaneous Formulations.

i. A formulation of poly IC, PLL and Poly(galacturonic acid) gave 4% of the IFN titer of poly ICLC. The melting profile showed two Tm values, 83° and 94°; both are for complexed poly IC.

ii. We also replaced PLL by a random copolymer of lysine and alanine. This "dilutes" the positive charges on PLL and increases hydrophobicity. The copolymer had the composition 67% Lysine 33% alanine, or a 2:1 ratio, and is designated L_2A. A formulation (IV-78) containing IC, L_2A and CMC in the proportions 1:1.5:2 by weight induced in mice an IFN titer of 130 units, or 14% of that of poly ICLC. Thus, replacing one-third of the lysine residues by alanine has reduced the IFN titer (or changed the time course of its induction). Tm for IV-78 was 75°, demonstrating the existence of a complex.

iii. Heat-annealed poly ICLC.

Elsewhere in this report we have raised the question of annealing of an inducer. We have tested this idea by heating poly ICLC above Tm, then cooling during one hour to room temperature. This would allow the three polymeric components to interact more slowly than by direct mixing.

The result was a total loss of IFN induction and antiviral action against Punta Toro virus. Heating above Tm is too drastic. We hypothesize that the separated strands of poly I and poly C may have interacted with PLL and CMC before reforming the double helix of poly IC. More gentle annealing below Tm (i.e., below the temperature at which the poly IC double helix is dissociated) may be better.

iv. Ring-Opened CM-Polysaccharides.

A polysaccharide chain is like a string of fairly rigid beads on a string, i.e. the cyclic sugar residues linked through the ether linkages. Rotation may occur around C-O bonds of the ether linkages, but not around the ring bonds (except for the limited conformational flexibility permitted to a ring). If greater flexibility were permitted perhaps improved properties would result. We have begun to study this possibility, beginning with CMC.

CMC glucose rings were opened by reaction with $NaIO_4$, followed by reduction of the newly-formed aldehyde groups to alcohols with $NaBH_4$. The resulting polymer is chemically very similar to the original polysaccharide, but has greater flexibility. Two levels of $NaIO_4$ treatment were done, 1 per 10 glucose residues, and 1 per 2 residues. These were formulated

into inducer preparations with different ratios of PLL, and are designated ICL-C (open).

The 1/2 type was formulated in the proportions of 2:1.5:5 (III-228Ia), 2:0.75:5 (III-228Ib) and 2:0.375:5 (III-228Ic), for the components in the order IC:PLL:CMC(open). The first of these was not adequately soluble. The other two gave clear solutions. III-228Ib gave a Tm of 79.5, while III-228Ic gave a three-step melting profile with Tm's of: 57° (30% of total Δ A), 67° (10% of total Δ A) and 79.5° (60% total of Δ A). The lowest Tm for III-228Ic is below Tm for uncomplexed IC, and appears to represent IC which is destabilized. Therefore, it must be complexed, but in a manner not heretofore seen. Sample III-228Ib induced an IFN titer of 666 units or 54% of the standard (Table 34).

With the 1/10-ring opened CMC, complexes of 2:1.5:5 III-228IIa, 2:0.75:5 III-228IIb, and 2:0.375:5 III-228IIc were soluble. Melting profiles of these gave Tm values of 75.4° for III-228IIa, 80° for III-228IIb, and two transitions, at 63° and 80.5° for III-228IIc. For the last the ratio of A of the 63° transition to A of the 80.5° transition was 30% to 70%, and the low Tm transition of 63° was similar to that of IC alone. The IFN titers (Table 34) of III-228IIa and III-228IIb were 430 units and 1021 units, or 35% and 83% of the standard ICLC.

Table 34. IFN Induction in Mice by ICL-C(open)

Batch #	Composition IC:PLL:X [X = CMC or CMC (open)]	IFN Titers Mean Titer	IFN Titers % of ICLC-STD.
ICLC III-201 (Standard)	2:1.5:5	1235	(100)
ICL-C(open)[a] III-228Ib	2:0.75:5	666	54
ICL-C(open)[b] III-228IIa	2:1.5:5	430	35
ICL-C(open)[b] III-228IIb	2:0.75:5	1021	83

[a] CMC reacted with $NaIO_4$ to open 1/2 of the glucose rings.
[b] CMC reacted with $NaIO_4$ to open 1/10 of the glucose rings.

v. IC-Chitosan.

Chitosan is a positively charged polysaccharide. It was thought that it might replace PLL, without requiring a third

polymeric component. Complexes were made with poly IC, which were insoluble.

p. Safety testing.

Several types of formulations were tested for safety in mice and guinea pigs: ICL-SO_4Gel, ICL-ßCDSO_4, ICL-CMA, IC-(PLL-dextran) and poly ICLC, with the results shown in Tables 35 and 36. The results are discussed in the sections on individual IFN inducers. Overall, all of our experimental formulations resulted in smaller weight losses in mice than did poly ICLC. In guinea pigs, ICL-SO_4gel, one lot of IC-(PLL-dextran) and a lot of ICL-CMA caused larger losses than did poly ICLC at 48 hours, but all recovered and showed weight gains as large or larger than did controls which received only saline. Two lots of ICL-ßCDSO_4 and one of IC-(PLL-dextran) resulted in weight gains at 48 hours.

Table 35. Safety Testing in Mice[a]

Compound	Wt. loss or gain, % at 48 hr
Saline	+3.2%
ICLC (IV-241)	-19.1%
ICLSO_4Gel (IV-261)	-9.6%
ICL-ßCDSO_4 (IV-190)	-11.8%
ICL-ßCDSO_4 (IV-191)	-15.5%
ICLCMamylose (III-107)	-15.0%
IC(PLL-Dextran) (IV-258)	-9.1%
IC(PLL-Dextran) (IV-259)	-8.5%

[a] 4 mice per group. Dose: 8 mg/kg (160 µg/mouse), as poly IC.

Table 36. Safety Testing in Guinea Pigs[a]

Compound	Wt. loss or gain, % at 48 hr
Saline	+3.9%
ICLC (IV-241)	-4.5%
ICLSO_4Gel (IV-261)	-13.1%
ICL-ßCDSO_4 (IV-190)	+3.8%
ICL-ßCDSO_4 (IV-191)	+3.7%
ICLCMamylose (III-107)	-8.9%
IC(PLL-Dextran) (IV-258)	-9.0%
IC(PLL-Dextran) (IV-259)	+3.6%

[a] Two guinea pigs per group. Dose, 8 mg/kg, as poly IC.

q. Induction Kinetics.

A number of inducer formulations were tested in the laboratory of Dr. Sidwell, at Utah, and compared with two lots of ICLC, one of ours and one from Dr. Hilton Levy. IFN induction was tested at 2, 6, 12 and 24 hr. after injection into mice. The formulations tested were:

1. ICL-CMamylose, lot III-107
2. ICL-CMdextran, lot III-59
3. ICL-CMßCD, lot III-62
4. ICL-gelatin, lot III-210
5. ICL-SO_4gelatin, lot III-198
6. IC-(PLL-dextran), lot III-190
7. IC-(PLL-dextran), lot III-232

These were tested at 0.1 mg/kg, compared with our standard dose of 0.5 mg/kg.

All of the above gave IFN values at least as high as our lot of ICLC. Inducers 1, 3, 4, 5 and 6 gave peak values as high or higher than the average value of Levy's ICLC over the period of

2-12 hr. No. 7 gave somewhat less IFN. All but #2 gave good titers from 2-12 hrs (2-24 hr for #'s 1, 3, 4).

Sidwell's data for IC-(PLL-dextran) are important in that they show good IFN induction, early induction, and prolonged induction in C57BL/6 mice.

r. Immunogenicity Testing.

We tested individual components and formulations composed of these components.

A. Individual components.
The following were tested:

CM-Dextran (no $NaBH_4$ treatment) (2 lots)
CM-Dextran (no BH_4 treated)
CM-Amylose (2 lots)
PLL (5 lots)
Succ-gelatin
AcCitgelatin
AcCit-PLL
SO_4Gelatin (2 lots)
PLL-Dextran (5 lots)
Methylated PLL
Oxidized-CMC
Gelatin
Poly IC
CMC

None of these resulted in the generation of antibodies against themselves. None of these resulted in the generation of antibodies against IFN-inducer formulations of which they were a component.

B. IFN-Inducer Formulations.
The following formulations were tested for immunogenicity:

ICL-CMdextran
ICL-CMamylose (2 lots)
ICL-AcCitgelatin
ICL-AcCitPLL
ICL-SO_4Gel (3 lots)
IC-(PLL-dextran) (3 lots)
ICL-CM-ß-CD
ICLC
ICL-gelatin

None of these was immunogenic when serum was tested against it or against the CMC replacement used in the fomulation, except two lots of ICL-SO_4 gelatin. These lots of ICL-SO_4 gelatin generated antibodies against themselves, but not against SO_4gelatin. One lot of ICL-SO_4gelatin (III-199) was not antigenic.

s. Molecular Seive Chromatography of Inducer Formulations.

A question concerning all of the inducer formulations is whether or not some of the polyanion (CMC, etc.) is not bound, i.e., is some of the polyanion free? Also, is any of the PLL present as a binary complex with the third component, i.e., without IC, and does more than one type of ternary complex form? To answer these questions we turned to molecular sieve chromatography, to see if all components move together, or if some move separately from the ternary complex. It is emphasized that these experiments are introductory, to see if the method is suitable, to learn the difficulties, and to try to find the best procedures.

In order to follow the elution of the components conveniently it was necessary to label the PLL and polyanion with fluorescent labels. The IC can be monitored by its absorbance. The PLL was lightly labelled with fluorescent dansyl groups, as was sulfated gelatin. CMC was labelled with fluorescein thiosemicarbazide, after oxidation of about 1% of glucose residues with sodium periodate.

The ternary complexes were passed through Biogel A5m, exclusion limit 5×10^6 daltons, and the fractions were measured for absorbance at 260 nm (for IC), fluorescence (excitation at 340 for dansyl, 440 for fluorescein; emission at 550 for both), and for light scattering at 550 nm. The last is sensitive to aggregates.

Fig. 28 shows the elution of IC alone, which emerges at the void volume. This result shows that some future work should be done with a gel of greater exclusion limit. The emergence of IC at the void volume does not mean that its molecular weight is 5×10^6 or more. The elongated double helix results in behavior similar to a larger, more compact polymer.

Fig. 29 shows chromatography of ICL* (L* indicates fluorescent dansyl-labelled poly(lysine). Most of the L* emerges with the IC. The nature of the small fluorescent peak at fraction 8 is not yet established. Light scattering shows a peak corresponding to the A_{260} and dansyl fluorescence, as well as a second peak at fraction 8. Since poly(lysine) is a very weak scatterer, the meaning of this peak is not known. It does not contain IC. For solubility reasons the ratio of L* to IC is lower than in the complexes to follow, and no uncomplexed L* is expected. This must be further studied.

Fig. 30 shows chromatography of ICL*C. A_{260}, dansyl fluorescence and light scattering all peak at fraction 4. Since this experiment tells us nothing about the CMC, we also made ICLC* (CMC labelled with fluorescein), and chromatographed it, with the results of Fig. 31. The IC (A_{260}) appears essentially in one peak (fraction 6). A very small peak is at fraction 3; this has not yet been identified. Also, we do not yet know why

the main peak emerges at fraction 6 instead of 3. A second large fluorescent peak appears at fractions 8-9, showing that the CMC is not all bound in the complex. It will be necessary to rechromatograph fraction 6 in order to see if it emerges as a single peak, or if some ICLC* dissociates to give, again, the fraction 8-9 peak. If, on rechromatography of fraction 6, a single peak is obtained, this would mean that ICLC uses excess CMC, and that it may be possible to use less of it, with a reduction in toxicity.

However, if rechromatography of the fraction 6 peak should show a second peak at fraction 8-9, this would indicate that ICLC dissociates when diluted on passage through the column. This may be important, since it would be expected that inducer complexes dissociate in-vivo, and such dissociation may be required in order to expose IC for cellular interaction. The rate and extent of dissociation may determine the kinetics of IFN appearance, the magnitude of the titer, and duration of IFN induction, the duration of antiviral effects, and toxicity.

We also chromatographed ICL-SO_4Gel* (dansyl-label on sulfated gelatin), with the results seen in Fig. 32. IC peaks at fraction 4 (A_{260}), as do fluorescence and light scattering. Excess fluorescence emerges, as a shoulder, at fractions 6-7. Thus, some excess SO_4Gel may be present. The fraction 4 peak should be rechromatographed for the reasons given above in connection with ICLC.

Chromatography of ICL*CMDextran (dansyl fluorophore on poly-lysine) is shown in Fig. 33. Two peaks were observed, fractions 3 and 7-8, both of which show the presence of IC and of L*, and both of which show light scattering, which probably indicates the presence of all three components. Labelled CMDextran would be needed to verify this. The fact that light scattering of fraction 3 is smaller than that of fraction 7-8 cannot yet be explained, since light scattering is a complex phenomenon affected by the size, shape, refractive index, anisotropy and hydration of the particles, and is dependent on the angle of observation. All of our scattering experiments were done at an observation angle of 90°.

The polarization of fluorescence and of scattering was measured for the two peaks, and with both of these methods the polarization was greater for fractions 7-8 than for 4. For fluorescence the polarizations were 1.0 and 4.2 for the first and second peaks, and for scattering the polarizations were 13 and 22, indicative of structural differences between the two fractions. It would be interesting to see if each of the two peaks rechromatograph as a single peak, and if they differ in biological properties, chemical constitution and physical properties.

Returning to the question of dissociation of complexes, we have chromatographed ICLC and ICL-SO_4gel at different times after dilution, from a few minutes to 7 days. Chromatograms differ with the appearance of late-eluting (smaller size?) fractions, indicating that these complexes undergo dissociation. These experiments are quite preliminary; more extensive work is required. One problem to be examined is the possibility that the column packing may interact preferentially with one or more component of the complex, thereby producing a dissociation artefact. Nevertheless, chromatography of formulations may be useful to permit the preparations of formulations without excess components (which may reduce toxicity) or to isolate complexes of superior properties.

In the Figures to follow, light scattering and fluorescence intensities are drawn to arbitrary scales.

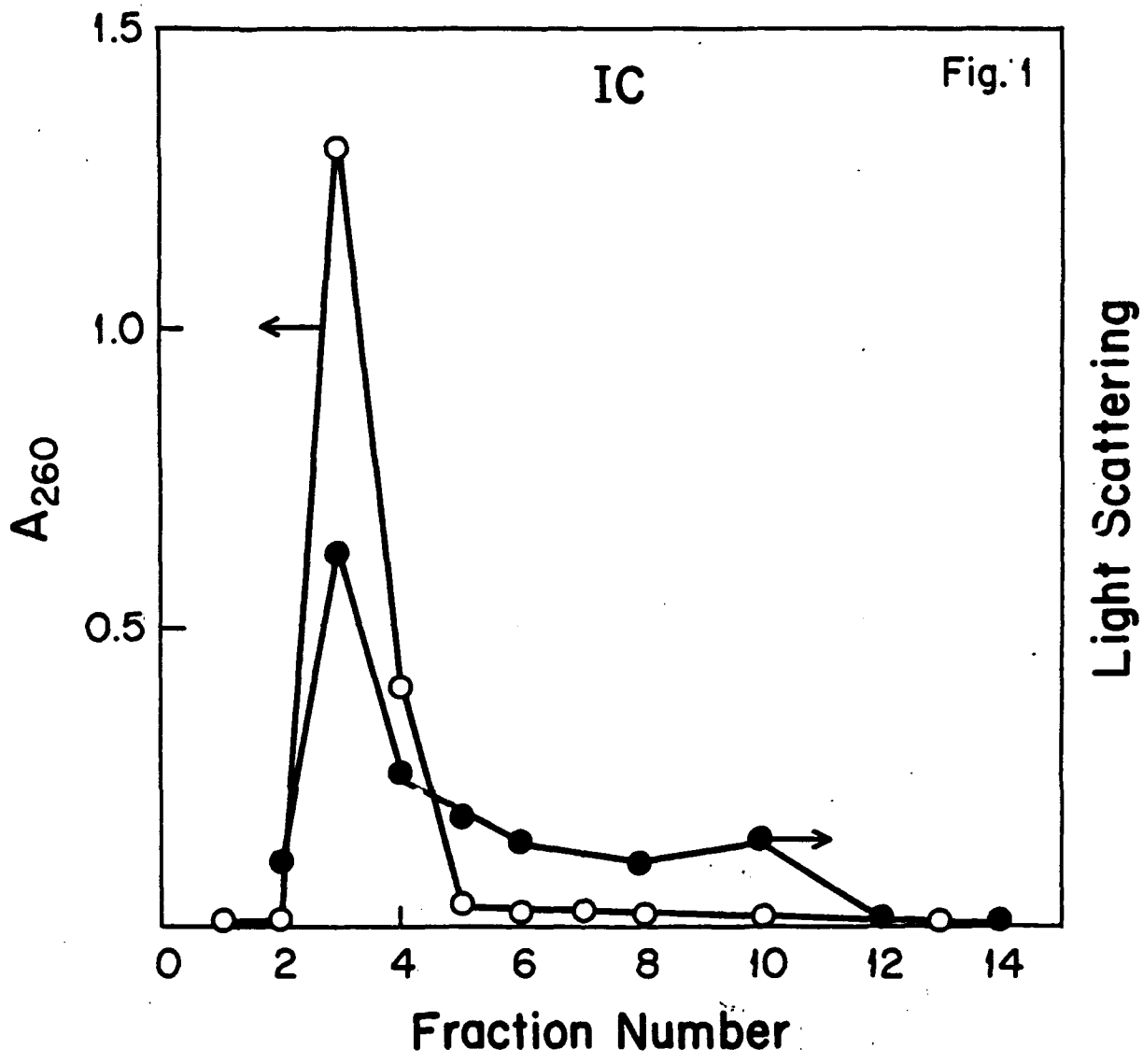


Figure 28. Molecular sieve chromatography of poly I·poly C on Biogel A5m, elution with standard saline.

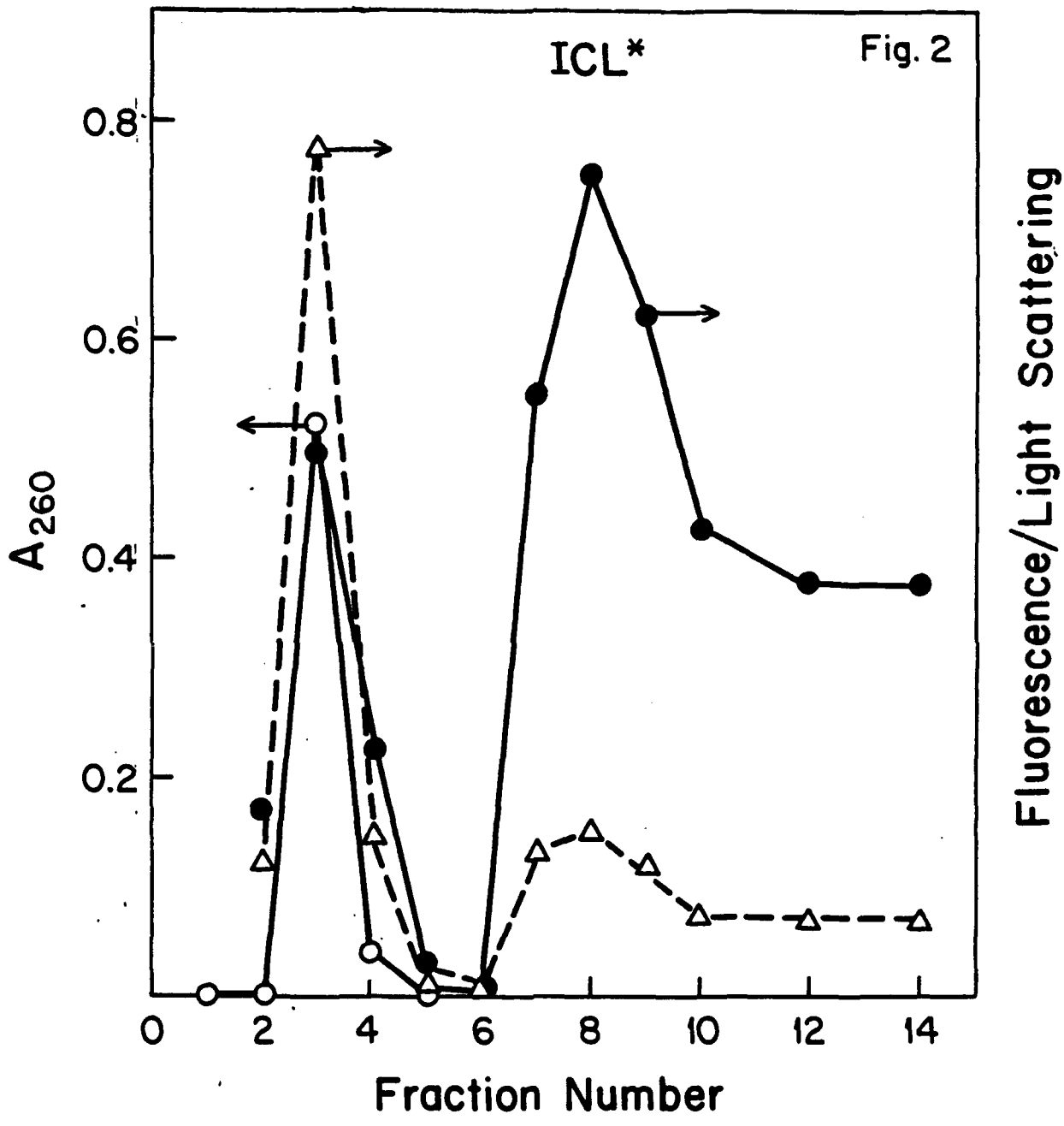


Figure 29. Molecular sieve chromatography of polyI polyC poly(L-lysine) with fluorescent label on poly(L-lysine); method as for Figure 28.

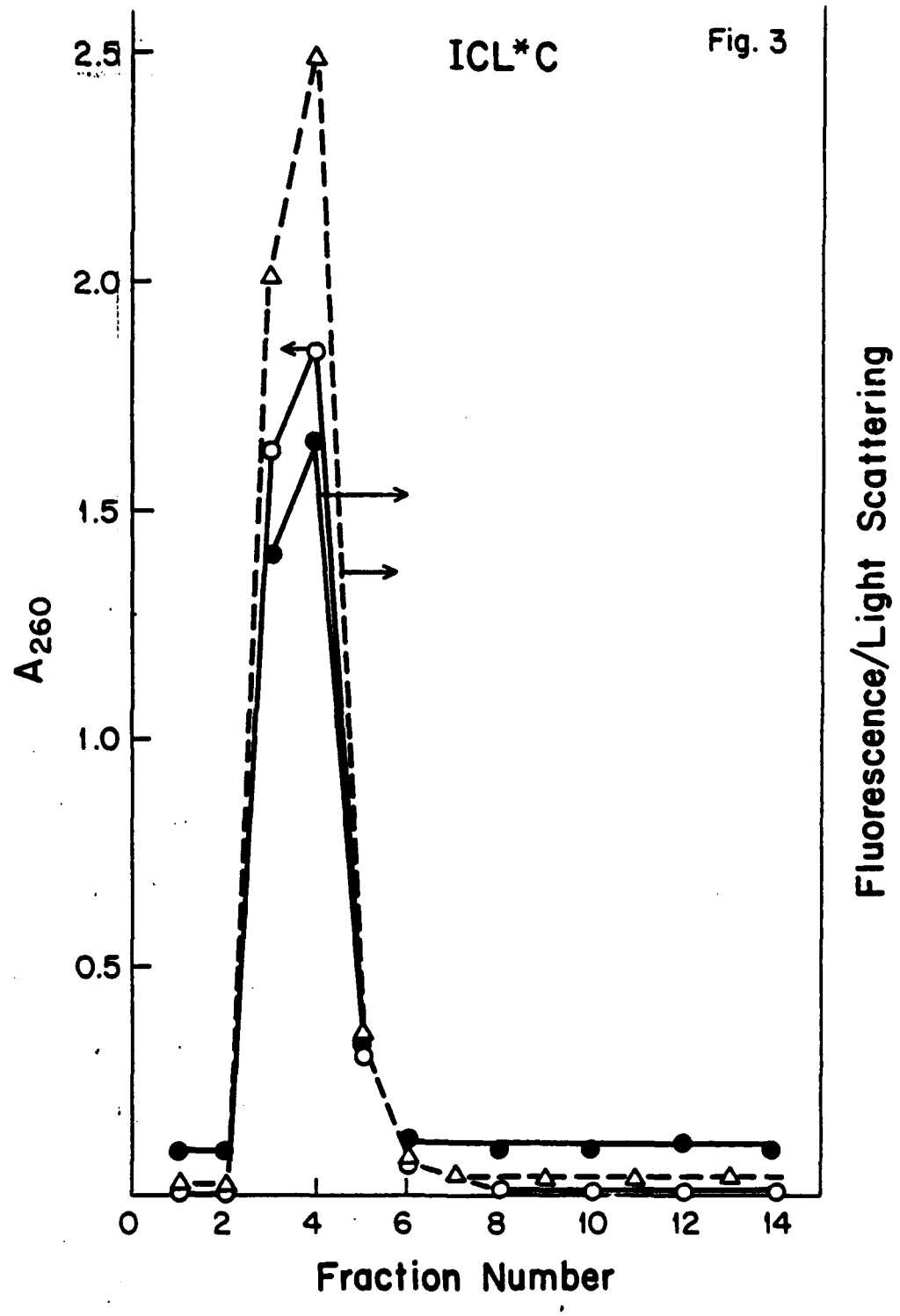


Figure 30. Molecular sieve chromatography of ICLC with fluorescent label on poly(L-lysine); method as for Figure 28.

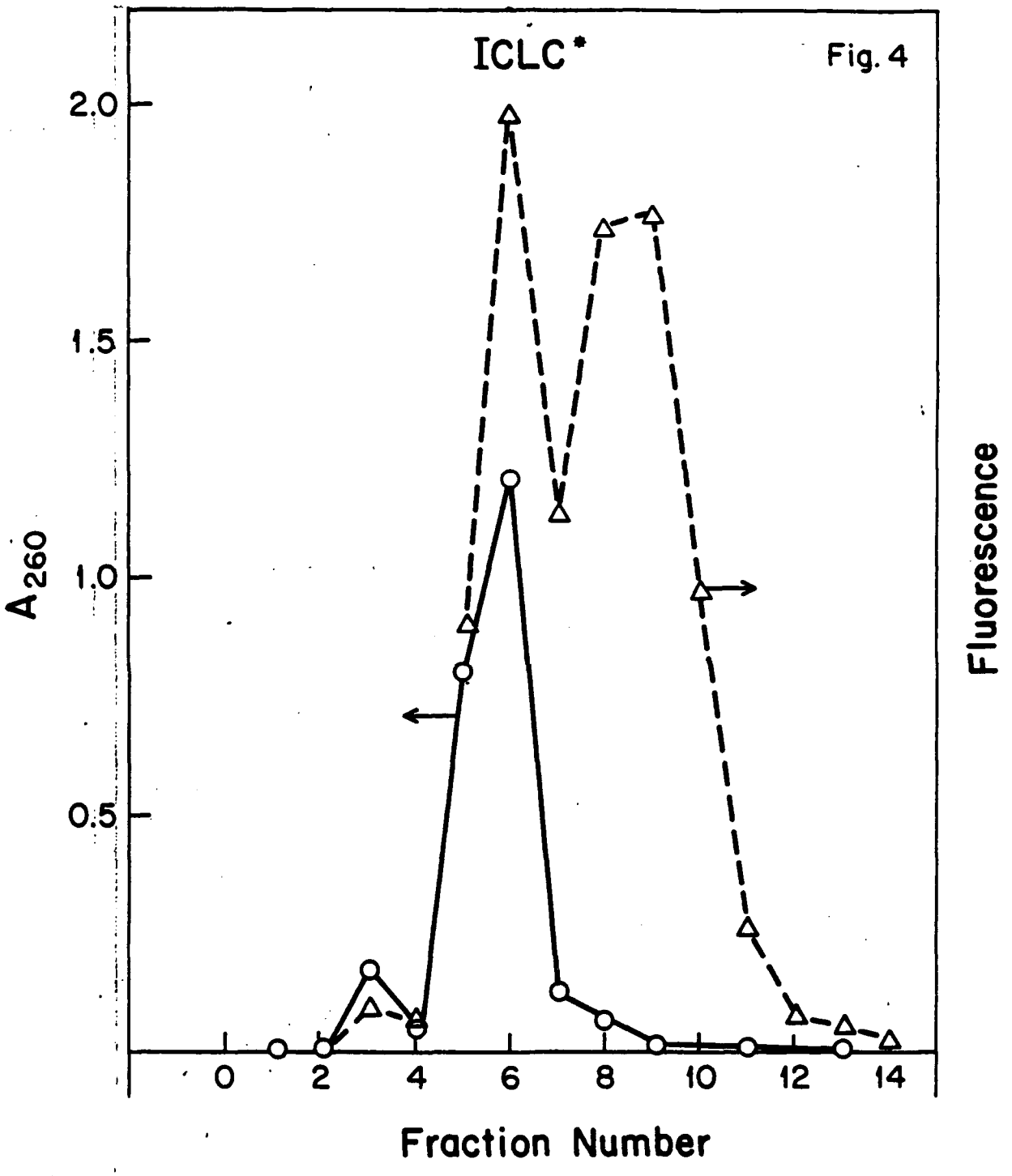


Figure 31. Molecular sieve chromatography of ICLC with fluorescent label on CMC; method as for Figure 28.

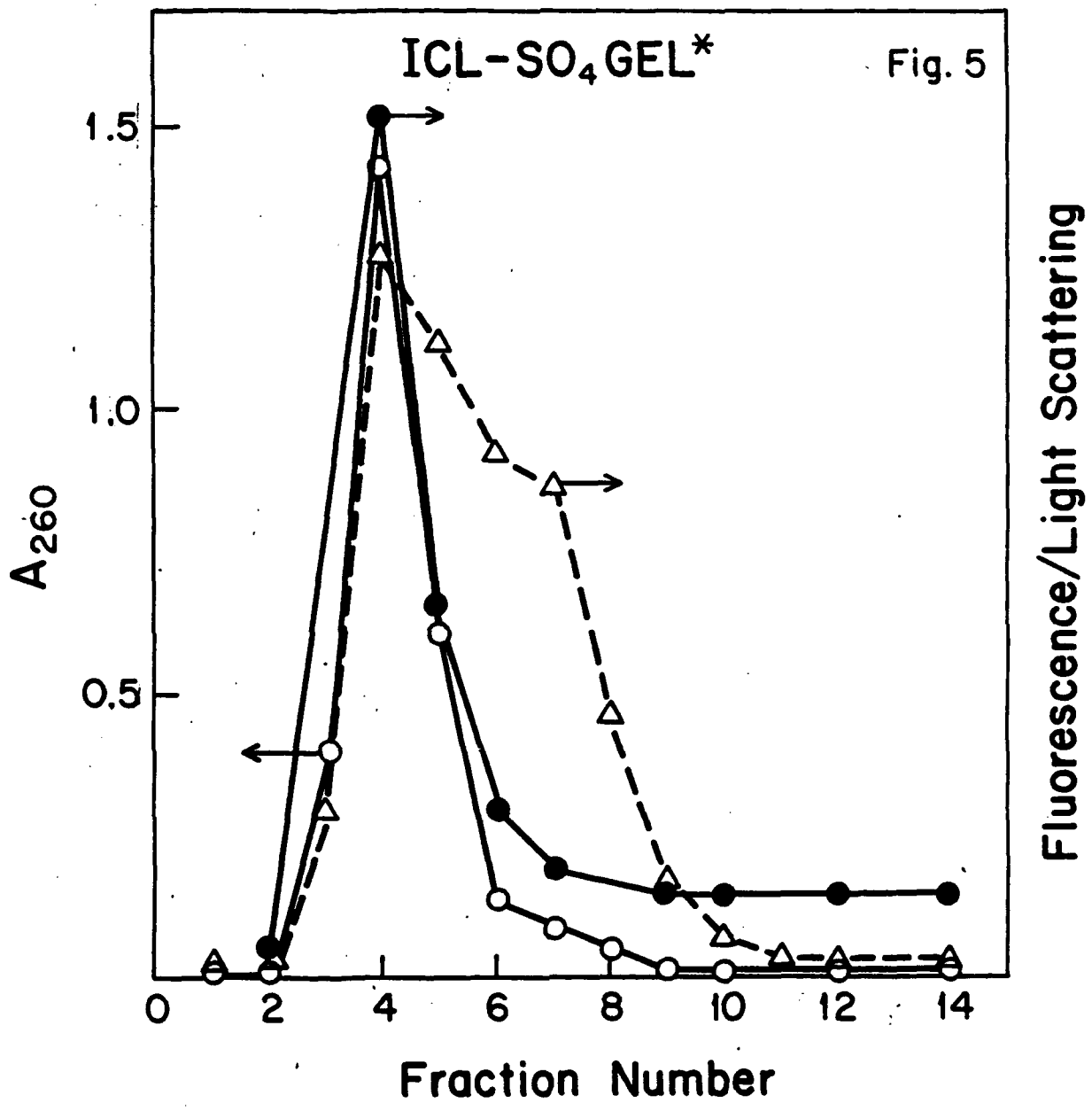


Figure 32. Molecular sieve chromatography of ICL-SO_4Gel with fluorescent label on gelatin; method as for Figure 28.

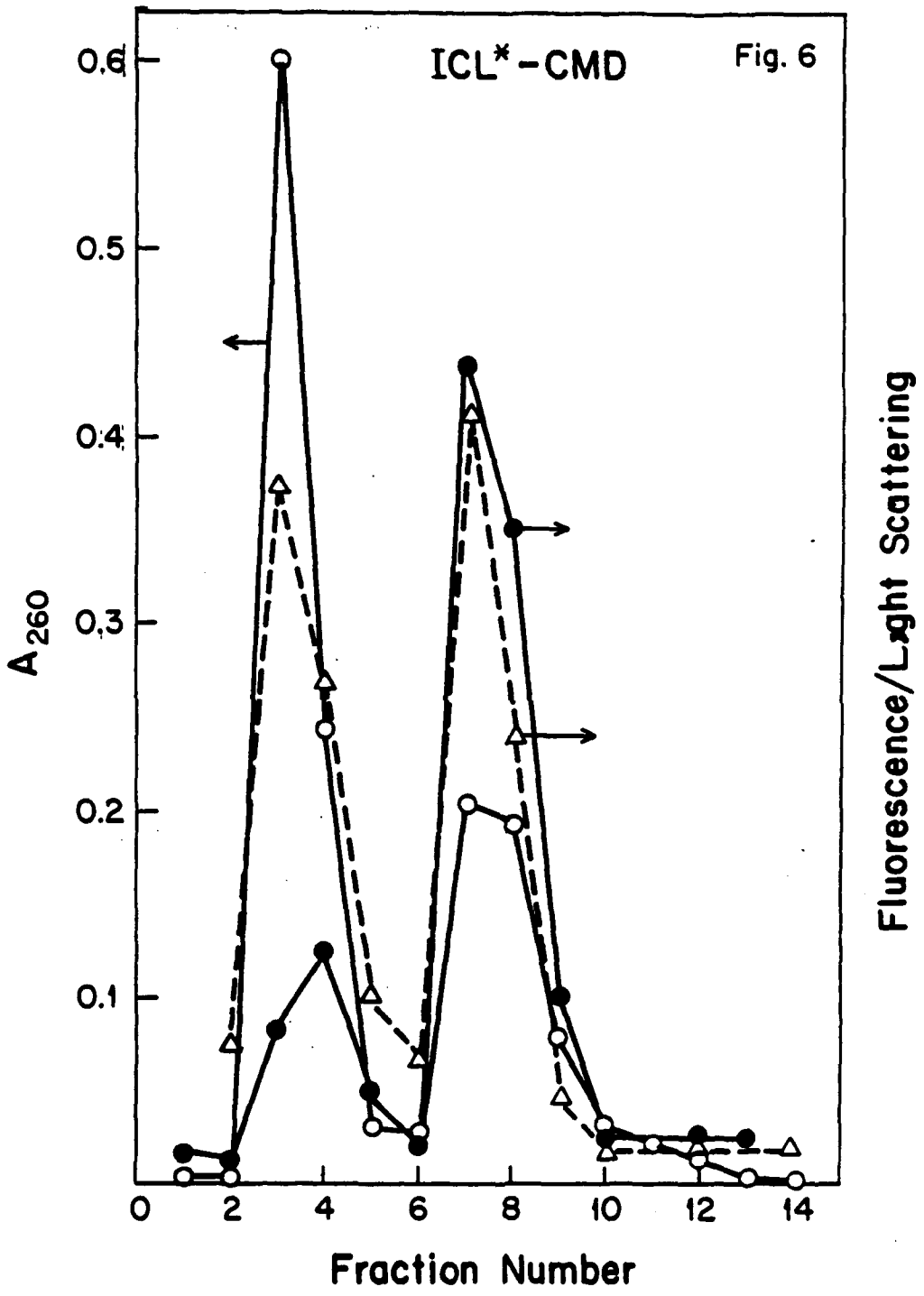


Figure 33. Molecular sieve chromatography of ICL-CMD with fluorescent label on poly(L-lysine).

u. Melting Profiles of Complexes by Light Scattering and Fluorescence.

The inducer formulations which were chromatographed (see above) were also subjected to melting transitions, monitored by absorbance at 260 nm (for IC), and, where appropriate, by fluorescence (for dansyl-PLL, dansyl-SO_4Gel and fluoresceinyl-CMC) and by light scattering. Both intensity and polarization were measured for fluorescence and scattering.

In all cases the intensity of light scattering increased on melting. But the polarization of scattered light decreased for ICLC and ICL-CMamylose (Table 37). Two batches of ICL-CMdextran were measured. One (III-238) which was a good IFN inducer, showed a decrease in polarization, and one (III-191), essentially a non-inducer, showed an increase in polarization of scattering. Thus, on the basis of a few experiments, and only one with a poor inducer, we tentatively suggest the possibility of a correlation between IFN titer and polarization of light scattering. Also, at room temperature the good inducers have polarizations of 20 or more, while the poor inducer has a value of 9. It may be possible to use light scattering to learn about particle characteristics (size, shape, hydration, etc.) associated with IFN induction. As noted earlier, light scattering is a complex phenomenon, and the change in scattering intensity may arise from several causes, one of which is a change in the degree of anisotropy, which can change the relative scattering in the forward direction vs 90°. A scattering polarization decrease may indicate a greater degree of anistropy.

Furthermore, when the complex is prepared with dansyl-PLL, the melting transition is accompanied by an increase in fluorescence intensity and an increase in fluorescence polarization, indicative of a tighter binding of the PLL. This was unexpected. When the complex contained dansyl-SO_4Gel or fluoresceinyl-CMC, the magnitude of fluorescence increase was small, compared with dansyl-PLL. This indicated a relatively smaller structural change for these components; these may be on the outside of the complex, relatively less rigidly bound on the complex than is PLL, or presumably, IC.

Our experiments have been on melting profiles of inducer complexes. For these experiments complexes were made using PLL covalently modified with fluorescent dansyl groups (about one per hundred lysine residues). The sample is illuminated with polarized light and the emitted fluorescent light is measured after passing through a polarizer oriented first vertically and then horizontally. The ratio of the two readings is calculated and is called the polarization. (This is not strictly correct, but is adequate to our purpose.)

When the IC melts, the structure of the complex must change. This is expected to change the mobility of the PLL, and therefore, the mobility of the fluorescent dansyl group attached to it. The change in mobility will change the extent of polarization of the emitted light.

Some melting profiles were done by fluorescence polarization. An ICL-AcCitgel (ratios of components IC:PLL:AcCitGel = 2:6:12) gave a Tm of 71°, with a sharp decrease in polarization, indicative of a loosening of structure. This lot was not tested for biological activity.

A useful result was obtained for two ICL-CMdextrans. One (II-191) made with CMdextran treated with $NaBH_4$ gave a Tm of 53°, an indication that the IC was free and not complexed. This probably explains the absence of IFN induction by such preparations. An ICL-CMdextran made with CMdextran <u>not</u> treated with $NaBH_4$ (II-238) gave a Tm above 81°, but with a gradual premelting change between 60 and 80°. Since what we observe is the fluorescence of the dansyl group on the PLL, not the IC directly, it is not certain that the Tm of 53° reflects the melting of the IC. However, 53° is the Tm of IC alone at the ionic strength used (a lower ionic strength than in the cases of the 63° Tm reported elsewhere in the report), and melting of IC would alter the physical state of the dansyl-PLL.

These preliminary results show promise for the study of complexes.

Table 37. Polarization of Scattered Light.

Complex	Polarization 25°C	Above Tm
ICLC III-95	22	4
ICLC III-94	26	12
ICL-CMamylose III-97	27	7
ICL-CMdextran II-238	23	9 (1300 units of IFN)
ICL-CMdextran II-191	9	13 (18 units of IFN)

11. Immunogenicity Studies.

The investigation of the immunogenicity in mice of compounds and complexes under study in this project, as well as some control substances, was continued. The substances tested are listed in Table 38.

Table 38. Substances Tested for Immunogenicity.

Carboxymethyl dextran
Carboxymethyl dextran treated with $NaBH_4$
Carboxymethyl amylose
Poly-L-lysine (5 lots, molecular weights 12,000 to 52,000)
Succinyl gelatin
Poly IC·poly-L-lysine-carboxymethyl dextran
Acetyl-citryl-poly-L-lysine
Poly IC·poly-L-lysine·carboxymethyl amylose
Acetyl-citryl-gelatin
Poly IC·poly-L-lysine·acetyl-citryl-gelatin
IC (Pharmacia Lot #292947)
ICLC
CMC
Bovine Plasma Albumin
SO_4Gel #1
SO_4Gel #2
Gelatin
Ac·Cit·Gel (BG I-3)
CM-Amylose (BG II-127; DS = 0.50)
CM-Dextran (BGII-256; T-10, DS = 0.22)
ICL-CM-Amylose (BGII-193)
ICL-CM-Dextran (BGII-238)
ICL-CM-Dextran (BGII-266)
ICL-SO_4-Gel (BGIII-31A)
ICL-SO_4-Gel (BGIII-31B)
ICL-SO_4-Gel (BGIII-199)
ICL-Ac-Cit-PLL (14k, BGIII-22)
CM-Dextran (d.s. = 2.02)
ICL-CMDextran (BGIII-45A; CMDextran is sample EE, above)

None of the IFN-inducer formulations were immunogenic (except) two lots of ICL-SO_4gel), or cross-reacted with any of their components. The two lots of ICL-SO_4gel were immunogenic when the dose was 100 µg/mouse, a very high dose. Lot III-199 of ICL-SO_4gel at 10 µg/mouse was not immunogenic but did not cross-react with SO_4gel.

Of the substances tested, two different preparations of ICL-SO4Gel showed weak to strong immunogenicity among the five mice in each of the two test groups. The five strongest antisera of the ten obtained against the ICL-SO_4Gel were tested against gelatin and sulfated gelatin or complexes. None of these cross reacted. Further work would be necessary to determine what structure(s) on the ICL-SO_4Gel are responsible for the immunogenicity. The immunogenicity may arise from the SO_4Gel component in a different conformation not present in free SO_4Gel, or from some combination of groups in two or all three components.

None of the remaining substances showed any immunogenicity; each serum was tested against the injected material and in most cases against one or more related substances.

Bovine plasma albumin, used as a positive immunization and test control, was, of course, strongly immunogenic.

These tests were done under conditions designed to enhance a positive response, in order to increase the probability of detecting such a response if the test substance is antigenic. Thus, 100 µg of substance (i.e., 100 µg of the IC component) is injected with Freund's adjuvant.

6. LD_{50}.

These values are summarized in Table 39.

7. Conclusions.

Our research has resulted in the demonstration that effective IFN inducers can be formulated without using carboxymethylcellulose. Our new formulations have shown effective IFN induction and antiviral activity, some of them against two viruses, Rift Valley Fever and Punta Toro. Most of them are less toxic than is poly ICLC.

An important finding was that an IC-(PLL-dextran) which was a moderate inducer of IFN in mice, under our standard protocol (blood tested for IFN three hours after injection) was as effective an inducer as poly ICLC in a monkey, and gave a peak titer earlier than did poly ICLC.

Many formulations were tested for antigenicity; none was found to be antigenic by the precipitation-in-gel method, except two lots of ICL-SO_4gel. A more recent lot of ICL-SO_4gel was not antigenic.

Table 39. Summary of LD_{50} Values.

Inducer		LD_{50} (mg/kg)		
Poly ICLC		11	(average of 5 lots)[a]	
IC-PLL-Dext	(See Table 2 for individual LD_{50}'s)			
	6 + 10[b]	31	(average of 2 lots)	
	6 + 70	27	(1 lot)	
	22 + 10	>35	(average of 5 lots)	
	63 + 10	>44	(average of 2 lots)	
	63 + 40	37	(average of 2 lots)	
			overall average:	>35
ICL-ßCDSO$_4$	IV-190	36	average 30	
	IV-191	24		
ICL-CMßCD	III-254	14		
	IV-119	>40		
ICL-SO$_4$Gel	IV-261	>40		
	III-106	21	average 28	
	III-111	25		
	III-186	25		
ICLGel	IV-243	11		
ICL-CMA	III-281	25	average 25	
	III-107	25		

[a] Individual LD_{50}'s: 9,10,11,11,10.
[b] The two numbers refer to the molecular weights of the PLL and Dextran, respectively, in thousands. The LD_{50} values of the 12 lots are displayed in Table 2.

The effective interferon inducers are:

IC-(PLL-dextran)
ICL-CMamylose
ICL-amylose sulfate
ICL-amylopectin sulfate
ICL-CM-ß-cyclodextrin
ICL-ß-cyclodextrin sulfate
ICL-CMdextran
ICL-gelatin
ICL-SO_4gelatin
ICL-Hyaluronic acid
ICL-oxidized CMC

ICL-ßCDSO_4 (2 lots tested) had an LD_{50} 2.7 times as high as poly ICLC (Table 27), good IFN induction (Table 28) and good antiviral action (Table 29). The ß-CDSO_4 itself was non-toxic).

ICL-SO_4 gelatin (ICL-SO_4gel) had an average LD_{50} 2.5 times that of poly ICLC (Table 27), good IFN induction (Table 28), but was somewhat less effective than poly ICLC against RVFV. However, against Punta Toro virus it was at least as effective as poly ICLC (Table 16). The probability that SO_4 gelatin is metabolized to amino acids is an attractive feature of this inducer.

ICL-CMA (ICL-CMamylose) had an LD_{50} of 2.3 times that of poly ICLC (Table 27), and was a good IFN inducer (Table 28) and antiviral agent (Table 29). CMamylose has been reported to be safe at doses more than 1000 times greater than ours (as a blood volume expander, ref. 4). The chief drawback is the unsuitability of some batches of amylose; but this can be checked, and good batches can be used.

The inducer types effective against viruses were:

Inducer Type	Rift Valley Fever	Punta Toro
IC-(PLL-dextran)	+	+
ICL-CMamylose	+	+
ICL-gelatin	+	+
ICL-SO_4gelatin	+	+
ICL-CM-ß-cyclodextrin	+	+
ICL-CMdextran	-	+
ICL-(PLL-glucose)	+	NT*

*Not tested.

The LD_{50} values of the several classes of IFN inducers are summarized in Table 39. The most extensively studied was IC-(PLL-dextran), 12 lots of which were tested and gave an average LD_{50} greater than 35 mg/kg, or more than three times the LD_{50} of poly ICLC. Although its average IFN titer (at our standard time of 3 hours after injection into mice) was

17% of that of poly ICLC (Table 28), IC-(PLL-dextran) was an effective anti-viral agent (Table 29). Either 17% is adequate, or a higher titer is achieved at some time after the 3-hour test. The initial monkey result (Table 3) gave a high titer for a batch of IC-(PLL-dextran).

Overall, this is a promising antiviral candidate.

Table 40. Summary of IFN Induction (as % of the Titer Induced by Poly-ICLC).

IC-(PLL-Dextran)	17%	(ave. of 24 lots)
IC-PLL-glucose)	15%	(one lot)
ICL-ßCDSO$_4$	154%	(ave. of 4 lots)
ICL-CMßCD	76%	(ave. of 3 lots)
ICL-SO$_4$gelatin	63%	(ave. of 12 lots)
ICL-gelatin	67%	(ave. of 3 lots)
ICL-CMamylose	115%	(ave. of 3 lots)[a]

[a] Does not include 3 lots made with Amylose Lot #2.

Table 41. Summary of Anti-RVFV Action in Mice.

	% Surviving Mice	
	Experimental agent	Poly ICLC
IC-(PLL-Dextran)	93	78
ICL-ßCDSO$_4$	70	60
ICL-ßCMCD	73	68
ICL-gelSO$_4$	70	90
ICL-gel	75	90
ICL-CMamylose[a]	80	80

Addenda

A. References

1. Levy, H.B., Baer, G., Baron, S., Buckler, C.E., Gibbs, C.J., Iadarola, M.J., London, W.T., and Rice, J. (1975) J. Infect. Dis. 132, 434-439.
2. U.S. Environmental Protection Agency, Office of Toxic Substances, March 1976.
3. "An Assessment of the Potential Use of Anionic Dextrans as a Plasma Substitute", Chang, L.S., Crawford, M.P. and West, M.D. (1980) J. Biomed. Eng. 2, 41-44.

4.a. Calvert, P. (1979) Nature 280, 108.

b. Plasma Substitute Joint Research Group (1978) Acta Chimica Sinica, 36, 49-76.

5. Granados, E.N. and Bello, J. (1980) Biochemistry 19, 3227-3223.
6. Baer, G.M., Shaddock, J.H., Moore, S.A., Yager, P.A., Baron, S., and Levy, H.B. (1977) J. Infect. Dis. 136, 286-291.
7. Kende, M. (1985) J. Biol. Response Modifiers 4, 503-511.
8. Imanishi, J., Matsubara, M., Won, C.-J., Nomura, H., and Kishida, T. (1981) Antimicrobial Agents and Chemotherapy 19, 922-924.
9. Hilfenhaus, J., Christ, H., Kohler, R., Moser, H., Kirchner, H., and Levy, H.B. (1981) Med. Microbiol. Immunol. 669, 25-235.
10. Salazar, A.M., Engel, W.K., and Levy, H.B. (1981) Arc. Neurol. 38, 382-383.
11. Friedman, R.M. In: Interferons, a Primer, pp. 118-124, ed. R.M. Friedman, Academic Press, New York, 1981.
12. Friedman, R.M., In: Interferons, a Primer, pp. 26-45, ed. R.M. Friedman, Academic Press, New York, 1981.
13. Greenberg, H.B., Pollard, R.B., Lutwick, L.J., Gregory, P.B., Robinson, W.S., and Merigan, T.C. (1976) New Engl. J. Med. 295, 517-522.
14. Dolen, J.G., Carter, W.A., Horoszewicz, J.S., Vladutiu, A.O., Leibowitz, A.I., and Nolan, J.P. (1979) Am. J. Med. 67, 127-131.
15. Freeman, A.I., Bogger-Goren, S., Brecher, M.L., and O'Malley, J. (1981) J. Interferon Res. 1, 457-462.
16. Baron, S., Howie, V., Langford, M., Macdonald, E.M., Stanton, G.J., Reitmeyer, J., and Weingent, D.A. (1982) Texas Reports on Biology and Medicine 41, 150-157.
17. Jahiel, R.I., Vilcek, J., Nussenzweig, R., and Vanderberg, J. (1968) Science 161, 802-804.
18. Schultz, W.W., Huang, K.-Y., and Gordon, F.B. (1968) Nature 220, 709-710.
19. Lin, S.L., Greene, J.J., and T'so, P.O.P. (1982) Nature 297. 417-419.
20. Harrington, D.G., Crabbs, C.L., Hilmas, D.E., Brown, J.R., Higbee, G.A., Cole, F.E., and Levy, H.B. (1979) Infection and Immunity 24, 160-166.

21. Levy, H.B., Lvovsky, E., Riley, F., Harrington, D., Anderson, A., Moe, J., Hilfenhaus, J., and Stephen, E. (1980) Ann. N.Y. Acad. Sci. 33-41.
22. Lvovsky, E., Baze, G.B., Hilmas, D.E., and Levy, H.B. (1977) Texas Reports Biol. Med. 35, 388-393.
23. Johnson, A.G. (1985) J. Biol. Response Modifiers 4, 481-483.
24. Wiltrout, R.H., Salup, P.R., Twilley, T.A., and Talmadge, J.E. (1985) J. Biol. Response Modifiers 4, 512-517.
25. Chirigos, M.A., Schlik, E., Ruffmann, R., Budzynski, W., Sinibaldi, P., and Gruys, E. (1985) J. Biol. Response Modifiers 4, 621-627.
26. Torrence, P.F. How Interferon Works. In Biological Response Modifiers. New Approaches to Disease Intervention. ed. P.F. Torrence, Academic Press, Orlando, 1985, pp. 77-105.
27. Johnston, M.I., and Torrence, P.F. In Interferon: Mechanisms of Production and Action. ed. R. Friedman, Elsevier, Amsterdam, 1984, pp. 189-298.
28. Nilsen, T.W., Maroney, P.A., and Baglioni, C. J. Virol. 42, 1039-1045, 1982.
29. Wallach, D., Fellous, M., and Revel, M. Nature 299, 833-836, 1982.
30. Hovanessian, A.G., Meurs, E., Aujean, O., Vaquero, C., Stefano, S., and Falcoff, E. Virology 104, 195-204, 1980.
31. Borden, E.C., Holland, J.F., Dao, T.L., Gutterman, J.U. Weiner, L., Chang, Y.C., and Patel, J. The American Cancer Society Phase II trial. Ann. Int. Med. 97, 1-6, 1982.
32. Borden, E.C., Hawkins, M.J., Sielaff, K.M., Storer, B.M., Schiesel, J.D., and Smalley, R.V.J. Interferon Res. 8, 357-366, 1988.
33. Paulnock, D.M., Havlin, K.A., Storer, B.M., Spear, G.T., Sieloff, K.M., and Borden, E.C. J. Interferon Res. 9, 457-473, 1989.
34. Shanghai Institute of Organic Chemistry (1978) Carboxymethylamylose as a New Plasma Substitute, Hua Hsueh Pao, 36, 49-76.
35. Elin, R.M., and Wolff, S.M. (1973) J. Infect. Dis. 128, 349.
36. Granados, E.N., Dawidzik, J., O'Malley, J., McGarry, M., and Bello, J. J. Interferon Res. 4, 155-160, 1984.
37. Kemeny, D.M., and Challacombe, S.J. "ELISA and Other Solid Phase Immunoassays" Wiley, N.Y. (1988)
38. Heu, S.M., Raine, L., Fanger, H. (1980) Am. J. Clin. Pathol. 75, 734-738.
39. Memet, S., Besancon, F., Bourgeade, M-F., Thang, M.N. J. Interferon Res. 11, 131-141, 1991.

8. Glossary.

A:	Absorbance (spectrophotometer)
$AmSO_4$:	Sulfated amylose
CM:	Carboxymethyl
CMC:	Carboxymethylcellulose
CMßCD:	Carboxymethyl-ß-cyclodextrin
CMamylose:	Carboxymethyl amylose
CMA:	Carboxymethyl amylose
$ßCDSO_4$:	ß-Cyclodextrin sulfate
CMdextran:	Carboxymethyl dextran
Gel:	Gelatin
HYL:	Hyaluronic acid
IC:	Poly-IC
ICL:	Complex of IC with PLL
ICLC:	Complex of IC with PLL and CMC
ICL-CMamylose:	Complex of IC, PLL and CMamylose
ICL-CMA:	Complex of IC, PLL and CMamylose
ICL-CMdextran:	Complex of IC, PLL and CMdextran
IC-(PLL-Dextran):	Complex of IC and PLL-Dextran
IC-(PLL-Dextran):	Complex of IC and PLL-glucose
IC-(PLL-Ribose):	Complex of IC and PLL-Ribose
IFN:	Interferon
kDa:	Molecular weight in thousands
PLL:	Poly(L-lysine)
PLL-Dextran:	Graft polymer of PLL and dextran
PLL-Glucose:	Graft polymer of PLL and glucose
Poly IC; IC:	PolyI·PolyC
PTMLL	Poly(Trimethyl-L-lysine)
RNase:	Ribonuclease
RVFV	Rift Valley Fever virus
SO_4Gel	Sulfated gelatin
Tm:	Melting, or transition, temperature

9. Acknowledgements.

The work on the synthesis of CM-ß-cyclodextrin and PLL-dextran grafts was carried out by Dr. Kazuo Ueda. The synthesis of sulfates of ß-cyclodextrin, amylose and amylopectin were done by Dr. Shoufang Zhang. Preparation of inducer complexes, ribonuclease digestions, melting profiles and the synthesis of various modified polymers were done by Ms. Barbara Grossmayer. Biological work (IFN induction, LD_{50}, safety testing) was done by Ms. Jean Dawidzik. Synthesis of methylated and guanidinated polymers were done by Dr. Herbert Iijima. NMR spectra, instrument maintenance, design and construction were done by Mr. George Potienko. Typing of reports and record keeping were done by Mrs. Christine Pajek. Immunology was done by Dr. Oliver Roholt.