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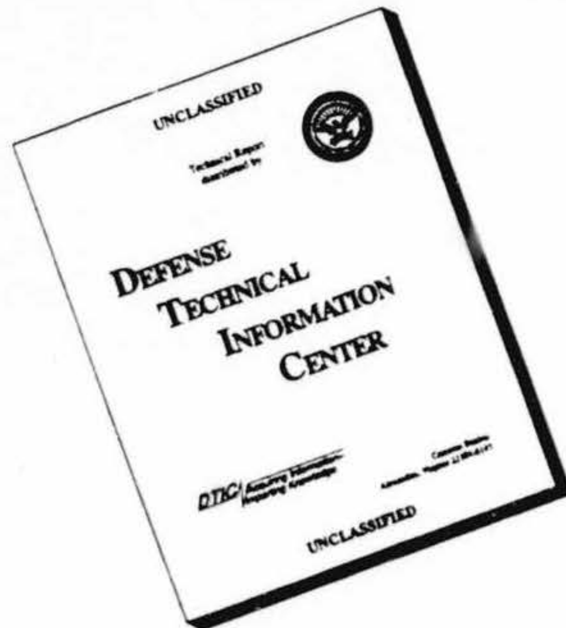

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INTERFERON INDUCERS AGAINST INFECTIOUS DISEASES

ANNUAL REPORT

JAKE BELLO, Ph.D., AND JUDITH O'MALLEY, Ph.D.

APRIL 1, 1988

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The purpose of this research is to develop interferon inducers which are safe effective antiviral agents. This involves the preparation of complexes of polyI·poly C with poly(L-lysine) and an anionic biopolymer. Effective inducer formulations have been prepared with the following anionic polymers: carboxymethylamylose, carboxymethyldextran, acetylcitryl-poly(lysine) and acetylcitryl-gelatin. The complex with carboxymethylamylose has also shown antiviral efficacy on tests at Fort Detrick.

Of the inducer formulations and their components so far tested for immunogenicity, all are non-immunogenic by the test used.

Absorption spectra and melting profiles (by absorbance or by fluorescence polarization of inducer complexes) have shown significant correlations with interferon induction.

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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).

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1. Problem Under Study.

The double-stranded synthetic polynucleotide poly I·poly C (i.e., poly-inosinic-poly-cyticylic acid, also to be called poly IC or IC) is an interferon (IFN) inducer. In primates it is not effective, 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. But CMC presents some difficulties: it is not excreted or metabolized, and it is suspected as a carcinogen (1).

The problem being addressed in this research is the preparation of effective, safe IFN inducers devoid of CMC. To this end our main efforts have been to develop anionic polymers to replace CMC. We are also seeking to replace CMC by modifying the PLL with engrafted polysaccharides.

2. Background.

Our expectation that replacements for CMC can be found is based on earlier work from this laboratory (2). We found that a complex of ICL, PLL and carboxymethyl dextran (CMdextran) could be prepared (called ICL-CM dextran), which was as effective an inducer in mice as ICLC, and of lower toxicity than ICLC. ICL-CM dextran was also as effective an IFN inducer in rhesus monkeys as is ICLC.

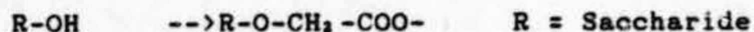
3. Rationale of the Research.

Since CMC has undesirable features, including non-excretion, non-metabolization, and possible carcinogenicity, we are seeking formulations without CMC. We are exploring two approaches to this goal. One is the replacement of CMC with other anionic biopolymers, selected on the basis of known or expected safety, and being excretable or metabolizable.

The CMC replacements were selected on the basis of a history of safe use as blood volume expanders, or being closely related to such. The second is the use of poly(L-lysine), PLL, covalently grafted to saccharide (without anionic groups). The PLL would bind to the poly IC, through its cationic groups, while the grafted saccharide would provide a solubilizing and hydrating effect. PLL-dextran grafts are being studied. Another reason for studying these graft polymers is that dextran is more readily cleared from the circulation of dogs than is CMdextran (3). We would 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 bearing terminal oligolysine.

A. Carboxymethyl Polysaccharides as CMC Replacements

The CMC replacements have been of two types: carboxymethyl polysaccharides other than CM cellulose and anionic derivatives of poly(L-lysine), PLL, and of gelatin. Carboxymethyl polysaccharides are anionic polymers in which the ionized carboxymethyl group has been introduced:


R-OH --> $R-O-CH_2-COO^-$ R = Saccharide

The substances investigated as CMC replacements are the following:

1. CMdextran. We have concentrated on relatively low molecular weight CMdextran. 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. We shall also examine higher molecular weights, as these appear to provide more readily soluble formulations (2).

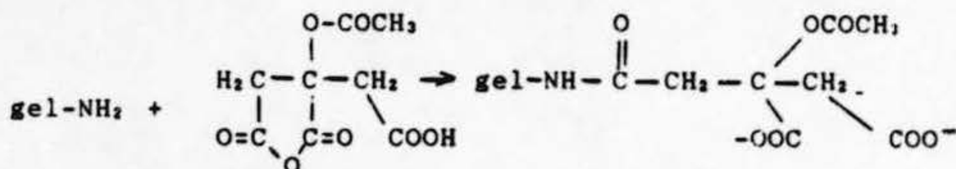
2. CMamylose. This was selected in expectation of low toxicity, and because of a report from China (4) (on its use as a blood volume expander) that it is non-immunogenic and is rapidly cleared from the body. In the work done so far, molecular weight has not been a concern. If results warrant it, we shall study the use of different molecular weights.

3. CM-Mannan. A carboxymethyl derivative of the starch mannan.

4. CM-Amylopectin. A carboxymethyl derivative of the starch amylopectin.

B. Anionically Modified Gelatin

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.

Gelatin and succinyl gelatin have a long history of use as blood volume expanders. AcCitgelatin is closely related to succinyl gelatin, but with twice as many carboxylate groups introduced per amino group acylated. Metabolism of AcCitgel should lead to smaller fragments which will be excreted or further metabolized to amino acids and to citric and acetic acids.

C. Anionically Modified Poly(L-lysine):

AcCit-PLL. An acylation reaction similar to that shown for the reaction with gelatin, 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 oligolysines or to lysine. If not metabolized, it may be excreted, if the molecular weight is not too large.

D. PLL-Saccharide graft.

We are grafting dextran, M_r 10,000, to PLL of M_r 6000.

4. Experimental

Preparation of Inducer Formulations.

a. General Procedure

ICLC was prepared according to Levy et al. (5) Experimental formulations were prepared in the same way, with modification, where necessary, in the ratios of components and/or the strength of saline or buffered saline.

b. Salt Gradient Dialysis

In some cases in which substantial insolubility occurred, the formulation was dialyzed against 2 M NaCl, and then against stepwise lower concentrations (2M->1.5->1.0->0.5->0.25->0.15) of NaCl until normal saline is reached, unless precipitation intervenes.

c. Sterilization of Components.

IC was obtained sterile from the vendor (Pharmacia). Solutions of PLL (Sigma), modified PLLs and modified gelatins were filtered through a 0.45 μ filter. The sterile components were mixed under a sterile hood and sealed in sterile serum vials.

Carboxymethylation of Polysaccharides.

The carboxymethylation is 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 dialyzed against water and freeze-dried.

In some experiments 200 mg of $NaBH_4$ was added before, or 20 minutes after, the chloroacetic acid was added. In some reactions the $NaBH_4$ was added 60 minutes after the chloroacetic acid. The reactions were neutralized and dialyzed.

The alternative purification procedure (following Chang et al.) was to bring the reaction mixture to pH 4.5, followed by precipitation with 100 mL methanol, decantation of methanol, redissolution in water followed by a second and a third precipitation, centrifugation, dissolution in water and freeze-drying.

Preparation of Carboxymethyl-β-Cyclodextrin.

To 2.2 g of β-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-β-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 dextrin with iodine. These gave brown and purplish spots, respectively, on paper with iodine. NaCl-containing fractions were detected with $AgNO_3$.

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 240 nm, using cells of 1 cm optical path and a Cary 219 spectrophotometer. Melting profiles were recorded at 246 nm with the Cary 219 Tm accessory and a Neslab circulating bath driven by a Neslab temperature programmer at 1° per minute.

Acetylcitryl Gelatin, Acetylcitryl PLL, and Succinyl Gelatin

Acetylcitric anhydride was made by the procedure of Klingemann (6), by reacting acetyl chloride and citric acid.

To 0.4 g of PLL in 30 ml water maintained at pH 9, was added 1.9 g of acetylcitric anhydride. After 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.

No analysis for degree of acylation was done, as a 5-16 fold excess of anhydride was used, and much experience among us and other protein chemists has shown that acylations usually go to completion. Treatment with mixed-bed ion exchange resin to bring AcCitPLL and AcCitGel to their isoionic points, produced a pH of about 2.5, indicating a high degree of substitution. Having worked out a titrimetric procedure for carboxyl groups in CM-polysaccharides, we shall apply it to the analysis of AcCitPLL and AcCitGel.

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

Spermine Grafting to Gelatin and PLL.

To 1 g of gelatin in 20 mL water was added 174 mg of spermine hydrochloride, and 1.1 g of dimethyl suberimidate was added over 10 minutes. The pH was maintained at about 9. After 5 minutes 5 mL of 5 M ammonium acetate was added. The solution was dialyzed against water and freeze-dried.

To 200 mg of PLL·HBr in 200 ml of $^1/_{10}$ PBS was added 340 mg spermine hydrochloride, and 40 mg of dimethyl suberimidate over 10 minutes. The pH was maintained at 9. After 3 minutes 2 mL of 2 M ammonium acetate was added, and the solution was dialyzed and freeze-dried.

Titration of carboxymethyl groups.

The sample is dried in vacuo over silica gel. A portion of about 50 mg is dissolved in 50 mL of water. For the titration of starting polysaccharide 100-150 mg is dissolved in 50 mL water. A volume of 15 mL is made 0.05 M in KCl with 2 M KCl, and titrated with 0.1 M HCl to about pH 2.25. A blank of the same volume of 0.05 M KCl is titrated, and a difference titration curve is drawn. Below pH 2.4 the difference curve is constant on the volume axis, indicating that the titration is complete.

PLL-Dextran Graft.

To 0.06 g of PLL-HBr (M_r 6000) in 5 mL H_2O was added 1 g of dextran T-10 and 0.063 g (1 mmole) $NaCNBH_3$, at room temperature. After two weeks 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-500-X2 (protonated form). Unreacted dextran is eluted first with 200 mL H_2O, and the PLL-dextran graft is eluted with 400 mL 3 N NH_4OH, followed by evaporation to a small volume and freeze-drying (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.

Fluorescence Polarization.

Measurements of fluorescence were done with an Aminco-Bowman Spectrophotofluorometer, with excitation at 375 nm, and emission read at 550 nm. The emission side contained a 550 nm interference filter to supplement the two monochromators of the instrument.

Inducer formulations were made using PLL lightly modified (about one residue per 100) with fluorescent dansyl groups.

A sample of 0.1 mL in a 3 mm square cuvet, in a thermostatted cell holder, was heated stepwise. 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).

Biological Studies

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. There were 8 treated mice per group plus 2 mice that received placebo. Serum was assayed for interferon.

Interferon Assays

Three-fold dilutions of serum were made in Minimal Essential Medium (MEM) containing 10% 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.

Testing for Immunogenicity.

a. Immunization protocol

Ten 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. Two to three weeks later, the mice were etherized, the chest cavity opened to expose the heart and blood was drawn from the right auricle using a 25-gauge needle; occasionally, a good recovery required causing the heart to bleed into the chest cavity and the blood was drawn from the pool. A total of 0.5-0.7 mL was usually obtained and each bleeding placed in individual $1^1/_2$ mL plastic centrifuge tubes. The serum was removed following clotting and centrifugation and placed in individual tubes, and stored in the freezer.

b. 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. In the present case, careful examination of the slides after 24, 48 and 72 hours revealed no precipitin arcs in any case. 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.

i. Introductory Notes to the Results

Since ICLC and the new inducer formulation we are studying are composed of three components, there are many possible combinations using different proportions of the components. 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. Promising inducers are to be tested sufficiently to provide adequate statistics. The exploration of the possible combinations is limited by our biological testing capacity.

ii. IFN Induction vs. Antiviral Action.

Our biological test is for IFN induction in mice, which is measured by the effectiveness of the mouse serum in protecting human cells in culture against vesicular stomatitis virus. Direct protection of the mice is not measured. The interest of USMRDC is in direct protection. It is possible that a formulation with moderate to low IFN induction might be an effective antiviral agent. But IFN itself has antiviral action, and because further testing of all non-inducers would be burdensome, we concentrate on formulations shown to have at least moderate efficacy in IFN induction.

iii. Tables of IFN Induction.

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

In Tables 4-8, the composition of inducers is given in actual mg/mL, for the three components (in the order shown), and, where needed, normalized to 2 mg/mL of IC.

B. Melting Profiles.

Riley et al. (7) have noted that for ICLC to be an effective inducer, the IC component should manifest a melting temperature, Tm, substantially elevated above that of uncomplexed IC, which is about 60-63°, in normal saline. Tm is dependent on ionic strength; Riley et al. found a Tm of 53° in about $^{1}/_{10}$ the ionic strength we use. We have recently begun to measure melting profiles (i.e., absorbance (A) vs. temperature, as well as the absorption spectra on a routine basis. We find that an elevated Tm is required, in

agreement with Riley et al. Also, we find some unusual melting profiles (described under the individual inducer sections), and also preparations for which there is no melting profile at all, because the absorption spectra show that only the poly I component is present, not the poly C. Apparently the other components dissociate the IC and precipitate the poly C. The remaining poly I shows no Tm. These cases will be noted in their appropriate locations. The spectral and Tm data will enable us to avoid doing biological testing on such preparations. The melting temperature obtained is that of IC. In addition to the change in absorbance accompanying the melting, it also appears that in some cases a light scattering effect occurs at the melting temperatures. This is inferred from the fact that the absorbance change is sometimes twice as great (or more) than expected for IC melting.

C. Standard ICLC Formulations.

Every set of tests of experimental formulations must be accompanied by a test with a standard ICLC. At first we used lot #UI-84-101 prepared at the University of Iowa. As additional standards we prepared our own ICLC following the method of H. Levy et al. (5). Repeated comparisons of the Iowa lot with our own showed the former to give lower IFN titers than the latter at 3 hours after i.v. injection (Table 1). The average IFN titer for 6 tests of Iowa UI-84-101 was 246, and for 10 tests of in-house ICLC (two lots) was 1487, or six times as large. In Table I each horizontal line represents a separate test set, and entries for Iowa lot and in-house on the same line means that a direct comparison was made on the same day. The same lot of Iowa ICLC gives larger IFN titers in other laboratories but in different strains of mice.

Because of the higher IFN titers obtained with our in-house ICLC 3 hours after injection, we have discontinued the use of the Iowa ICLC. Also, it appears logical to use ICLC made with the same lots of IC and PLL which we are using in our experimental inducer formulations. The 3 hour sampling time has been shown previously to be the peak time for elevated serum IFN after treatment with our ICLC. It is not within the scope of this project to measure the kinetics of IFN induction of each inducer that we test.

Table 1. Comparison of In-House ICLC with Iowa Lot U1-84-101

Quarterly Report Date	IFN Titer U/mL	
	Iowa	In-House
9/30/87	-	2585 (Lot BGI-43)
	-	933 (BGI-43)
	-	4170 (BGI-43)
	166	-
12/30/87	107	677 (BGI-43)
	242, 618[a]	939 (BGI-43)
	197	1748 (BGII-85)
	-	325 (BGII-85)
	-	1327 (BGII-85)
	148	672 (BGII-85)
	-	1316 (BGII-85)

[a] Two vials of same lot tested.

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, as noted in the report of December 30, 1987, because of failure of some uranyl complexes to precipitate.

We have now 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).

Carboxymethyl analyses for CM-polysaccharides used in this work are given in Table 2.

Samples 3 and 4, CMdextrans, were made from the same lot of T-10 dextran, but purified differently, #3 by dialysis and #4 by precipitation by methanol.

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 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 is not expected to show any titratable groups, and this is verified.

Table 2. Carboxyl Analysis of CM-Polysaccharides.

	Compound	Carboxyl groups per sugar residue
1	CMC, type 7H3SF (Hercules Co.)	0.50
2	CMamylose (Lot BGI-27)	0.50
3	CMdextran (Lot BGII-123, T-10)[a]	0.52
4	CMdextran (Lot BGII-124, T-10)[b]	0.40
5	CMmannan (Lot BGII-35)	0.01
6	Amylose (Control)	<0.02
7	Dextran (Control, T-10)	0
8	CMamylopectin	0.46

[a]Purified by dialysis.
[b]Purified by precipitation with methanol.

E. Suitability 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.

The substantial absence of residual CBZ groups is demonstrated from the absorption spectrum in the benzene range, 280-250 nm, using a concentration of 10 mg/mL, which permits detection of one CBZ group per 1000 lysine residues. CBZ below about 5 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 0.5 M $NaClO_4$. 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).

F. Summary of Results of Interferon Induction Experiments:

Forty seven preparations of known or potential interferon inducers, or of components of inducers, were tested for in-vivo interferon induction in BALB/c mice (Table 3). More than 500 serum samples were assayed for interferon. Certain preparations of ICL-AcCitPLL and ICL-CMamylose were equal to or superior to our standard ICLC's for induction. Certain preparations of other complexes, ICL-CMdextran and ICL-AcCitgel were highly effective as interferon inducers but were less active than the standard ICLC. Toxicity studies are currently underway to compare the therapeutic indices of the test compounds with the standard ICLC. Details of the results follow in separate sections describing the individual types of formulations.

Table 3

Complexes Tested for Interferon Induction

# of preparations	Complex	# IFN tests (8 mice each)
7	ICLC	25
6	ICL-AcCitPLL	7
1	ICL-SuccGel	1
9	ICL-CMdextran	9
6	ICL-CMamylose	10
6	ICL-AcCitGel	8
4	ICL-CMmannan	4
6	ICL-CMamylose	10
1	ICL-CMamylopectin	1
1	AcCitPLL	1
47		66

G. Inducers Containing Carboxymethyldextran.

In earlier work (2) on an NIH contract we had found complexes of ICL-dextran to be effective IFN inducers of low toxicity. Therefore, we have reinvestigated this type of inducer. The earlier work used dextran types T-10, T-40 and T-70 of three different molecular weights, M_r 10,000, 40,000 and 70,000, respectively. In our current work, we have been using T-10.

CMdextran was made by the procedure of Chang et al., (3) in which dextran is treated in NaOH with chloroacetic acid, and is isolated by precipitation with methanol, followed by centrifugation. We have also used a variant in which the reaction mixture is freed of reagents and byproducts by dialysis. The dialysis procedure was used to insure complete removal of byproduct NaCl and, in some cases, sodium borate. The sodium borate arose from $NaBH_4$ used to try to make purer CMdextran. When CMdextran is prepared, the reaction turns yellow and some yellow color may be carried over into the final product. Yellowing in carbohydrates is likely to arise from reactions of aldehyde groups. Also, aldehydes and their condensation products may possibly react with PLL or IC to generate covalent bonds. To prevent yellowing and destroy residual aldehyde, we carried out carboxymethylation in the presence of the reducing agent $NaBH_4$, or added $NaBH_4$ at the end of the reaction. $NaBH_4$ does, indeed, greatly reduce the extent of formation of yellow.

However, the ICL-CMdextran made with $NaBH_4$-treated CMdextran was essentially devoid of IFN inducer ability. The reason is not known. All further references to CMdextran will be to non-BH_4-treated material. (except for some work on fluorescence polarization.) It is of interest that $NaBH_4$ treatment in the preparation of CMamylose had no effect on IFN induction by ICL-CMamylose. Also, the carboxymethylation reaction mixture of amylose develops much less yellow color than does that of dextran.

Turning now to Table 4 and the IFN induction observed for ICL-CMdextran, we note that all of the preparations listed were made with dialyzed CMdextran, with the sole exception of BGII-233, which was prepared by methanol precipitation. Only one (BGII-11a) of the five ICL-CMdextrans prepared with dialyzed CMdextran gave substantial IFN induction. The next highest was BGII-182, prepared with the same proportions (but twice the total concentration) of components as BGII-11a.

BGII-233, made with precipitated CMdextran, was an effective inducer, with 1232 U/mL, or 45% of a higher-than-usual standard. BGII-191, BGII-208 and BGII-233, all made with the same ratios of components, differ markedly in IFN titers induced in mice. The first two were made with dialyzed CMdextran and BGII-233 with precipitated CMdextran. However, we cannot yet conclude that precipitation is superior to dialysis since BGII-11a (also made with dialyzed CMdextran) was a fairly effective inducer. The $NaBH_4$-treated samples were also dialyzed; it is not apparent if the low titers for these arose from $NaBH_4$ or from dialysis. Further work is planned in order to decide between dialysis and precipitation (or a combination of the two), and to settle on the best proportion of components. Also, this work has been done with CMdextran containing 0.5 CM group per glucose residue. Other degrees of substitution and other molecular weights of dextran will be studied.

We have, in the latter part of the year, begun to record the absorption spectra and melting profiles of inducer formulations, with a view to correlating biological and physical properties, and to predicting the former from the latter.

BGII-191 and BGII-208, which gave essentially no IFN induction, showed no melting of the IC, and the spectrum of BGII-208 showed the presence of poly I only, no poly C. The latter is thought to have precipitated, probably as a complex with PLL and CMdextran. (The spectrum of BGII-191 was not measured, but is presumed to be similar). BGII-184a which gave almost no IFN, showed mostly a 63° Tm (melting temperature) and a small melting transition at 78°. Most of the IC was probably not complexed. Only BGII-182 and BGII-233 gave Tm in the range expected, 77 and 78°, respectively, but with a difference. The melting profile of BGII-182 was normal, with a ΔA of about 40% of the original absorbance (A), and a flat profile above Tm. BGII-233 gave a ΔA greater than 100% of A, and above Tm the absorbance decreased sharply to about its original value. This is a remarkable observation, and may involve light scattering in addition to, or instead of, normal polynucleotide melting. This is to be studied further.

ICLC itself gives a normal melting profile and a Tm of 81°.

Table 4. IFN Induction by ICL-CMdextran.

Lot Number	Composition IC, PLL, CMdextran mg/mL	IFN Titers Exptl. Formulation	 ICLC Standard	 % of ICLC Standard
BGII-11b*	2, 1.5, 5	67	2585	2
BGII-29a**	0.5, 1.5, 5 (2, 6, 20)	46	933	5
BGII-29c*	0.5, 1.5, 2.5 (2, 6, 10)	40	933	4
BGII-11a	1, 0.75, 2.5 (2, 1.5, 5)	903	2585	35
BGII-184a***	2, 0.75, 5	26	575/736	4
BGII-182	2, 1.5, 5	295	1641	18
BGII-191	0.5, 1.5, 5 (2, 6, 20)	18	1641	1
BGII-208	0.5, 1.5, 5 (2, 6, 20)	6	1826	0
BGII-233	0.5, 1.5, 5 (2, 6, 20)	1232	2609	45

* $NaBH_4$ treatment before adding chloroacetic acid.
** $NaBH_4$ treatment after adding chloroacetic acid.
*** Tm values of 63° (80% of the ΔA) and 78° (20% of the ΔA).

H. Inducers Containing Carboxymethylamylose (CMamylose).

Six formulations were made of ICL-CMamylose, three with the components in the concentrations of 2, 1.5 and 5 mg/mL for IC, PLL and CMamylose, respectively, and three with the concentrations of 2, 0.75 and 2.5 mg/mL (Table 5). The former group gave IFN titers averaging 43% of the standard ICLCs, the latter 70%. 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.

Because, on general principles, it is usually desired to use the minimum amount of any agent, it appears tnat th lower ratios of PLL and CMamylose to IC are preferable. This conclusion must be further tested by comparison of toxicities of the two formulations.

Lot BGII-166 was submitted to Dr. Meir Kende at Fort Detrick for testing against Rift Valley Fever virus. The result indicated protection similar to that of the ICLC he used.

Tentatively, ICL-CMamylose appears to be a promising candidate, subject to reproducibility and toxicity considerations.

CMamylose, by itself showed no IFN induction, so that the observed induction arose from the IC component.

BGII-166 gave a melting profile with a high ΔA, about 100%, and a Tm of 81.5°. BGII-127 and BGII-143 gave normal IC-type absorption spectra, but melting profiles have not been run.

Table 5. IFN Induction by ICL-CMamylose

Lot Number	Composition IC, PLL, CMamylose mg/mL	IFN Titer Experimental Formulation	ICLC Standard	% of ICLC Standard
BGII-5	2, 1.5, 5	1061	2585	41
BGII-9	2, 1.5, 5	1053	2585	41
BGI-127	2, 1.5, 5	394	672	59
BGI-127	2, 1.5, 5	658	1316	50
BGI-127	2, 1.5, 5	263	1038	25
	Average	686		43
BGII-143	2, 0.75, 2.5	908	1316	69
BGII-143	2, 0.75, 2.5	448	1035	43
BGII-166	2, 0.75, 2.5	1052	575	183
BGII-193	2, 0.75, 2.5	678	1826	37
BGII-193	2, 0.75, 2.5	533	2609	20
	Average	724		70

I. Inducers Containing Carboxymethylmannan and Carboxymethylamylopectin.

Mannan was carboxymethylated in the same manner as dextran and amylose. However, titration of the supposed CMmannan showed essentially no carboxyl groups. This explains why on mixing PLL with "CMmannan" no precipitate formed, contrary to the cases of PLL-CMC, PLL-CMdextran and PLL-CMamylose. In the latter cases precipitation must arise from interactions between the oppositely charged polymers, but does not arise with PLL plus "CMmannan" since the latter has no negative charges. The reason for the resistance of mannan to carboxymethylation is not known.

Two mixtures of IC with PLL and "CMmannan" gave small IFN titers, 248 and 290 U/mL. These titers probably arose from ICL, since the uncharged "CMmannan" is not likely to have entered the complex. A comparison with standard ICLC was done with the Iowa ICLC, which gave 166 U/mL. Since the Iowa ICLC give very low titers in our strain of mouse, it cannot be assumed that the ICl-CMmannan titers were high.

A complex of IC, PLL and CMamylopectin did not induce IFN. These types of complexes have been put aside for now.

J. Inducers Containing Acetylcitryl PLL.

Two preparations of AcCitPLL were made with PLL of M_r 55,000 and 14,000. First, ICL-AcCitPLL(55) complexes were prepared and tested (Table 6). The number in parenthesis after ICL-AcCitPLL indicates the molecular weight in thousands of the PLL from which AcCitPLL was prepared. Complexes with the 55,000 AcCitPLL were effective IFN inducers when the IC, PLL and AcCitPLL were combined in the ratio 2:6:10 by weight (BGII-29b; BGI-119). When less PLL and AcCitPLL were used relative to IC very little or no IFN induction was obtained (BGII-43b, BGII-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, M_r 14,000. Two ICL-AcCitPLL(14) complexes were prepared and tested. BGII-120 made with IC:PLL:AcCitPLL(14) in the ratio 2:6:10 gave a moderate IFN induction of 32% of the standard, while BGII-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 BGII-142 are attractive, but there is a difficulty. BGII-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 7 were obtained with the supernate after allowing the precipitate to settle. Solubility problems were also encountered with the M_r 55,000 AcCitPLL complexes, but of much less severity; small amounts of precipitate were present, which dissolved on gently warming. We shall try to overcome the solubility problem.

We shall examine inducer formulations made with AcCitPLL of still lower molecular weight, on the (untested) hypothesis that lower molecular weight AcCitPLL will be more readily eliminated.

The melting profile of BGII-142 was similar to that of the ICL-CMdextran BGII-233 (see above). Tm was 75°, ΔA was about 100% (or twice the expected), and above Tm the absorbance decreased to its original value.

BGII-195, ICL-AcCitPLL(55), 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. Since effective inducers can be formulated with AcCitPLL, we plan to try to eliminate the problem of partial solubility, and to study the toxicity of these formulations.

Table 6. IFN Induction by ILC-AcCitPLL in BALB/c Mice[a]

Lot Number	Composition[b] IC, PLL, AcCitPLL	Mol. Wt.[a]	IFN, U/mL Exptl. Formul.	ICLC Standard	IFN % of Standard
BGI-43b	2, 1.5, 5	55,000	125	1173	11
BGI-29b	0.5, 1.5, 2.5 (2, 6, 10)	55,000	891	933	95
BGII-119	0.5, 1.5, 2.5 (2, 6, 10)	55,000	1145	1327	86
BGII-195	2, 1.5, 1.75	55,000	0	1826	0
BGII-120	0.5, 1.5, 2.5 (2, 6, 10)	14,000	429	1327	32
BGII-142	2, 1.5, 5	14,000	1684	1316	128
BGII-142	2, 1.5, 5	14,000	2840	1035	274

[a]molecular weight of PLL-HBr from which AcCitPLL was prepared.

K. Acetylcitryl 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, BGI-43b in Table 8 induced a small 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.

Formulations with two compositions of components were prepared and tested (Table 7). The best lot was BGI-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. Perhaps there was a low mouse response in this test.

The ICL-AcCitGel formulations have presented difficulty in solubility. BGI-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. BGI-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, and most recent formulation, was BGII-234, with a titer of 17% of standard. But this may have been induced by uncomplexed IC. BGII-234 gave a normal melting profile and a Tm of 63.5°, indicative of uncomplexed IC.

Further work with AcCitgel is planned. The main problem is to find conditions for obtaining stable, soluble complexes. In particular, we wish to try to obtain preparations comparable to BGI-43.

Table 7. IFN Induction by ICL-SuccGel and ICL-AcCitGel.

Lot Number and type of Gelatin	Composition IC, PLL, Gel mg/mL	IFN Titer Exptl. Formulations	ICLC Standard	% of ICLC Standard
SuccGel				
BGI-43b	2, 1.5, 5	158	1173	13
AcCitGel				
BGI-43a*	(2. 1.5, 5)	3123**	4170	75
BGII-34	0.5, 1.5, 2.5 (2, 6, 20)	24	4170	1
BGII-39	0.5, 1.5, 2.5 (2, 6, 10)	859***	4170	21
BGII-39a	2, 1.5, 2.5	38	325	12
BGII-121	2, 1.5, 2.5	324	1327	24
BGII-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.

L. Fluorescence Polarization.

Some exploratory work was done on the use of fluorescence polarization to study the inducer complexes. Our goal is to see if this property might be useful as a predictor of biological activity.

Our experiments so far 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. The change in mobility will change the extent of polarization of the emitted light.

Some melting profiles were done. 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 made with CMdextran treated with $NaBH_4$ (see CMdextran section) 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 $NaBH_4$ 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. This question will be examined further.

These preliminary results show promise for the study of complexes. We hope to be able to use fluorescence to study the complexes at room temperature to predict biological efficacy.

M. Poly(lysine)-Dextran Grafts.

An approach being studied is the replacement of 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.

We have been investigating the preparation and purification of a graft between PLL (M_r 6000) and T-10 dextran (M_r 10,000). 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.

We have worked out the separation of unreacted dextran from the product by ion-exchange chromatography, and the separation of unmodified PLL from PLL-dextran by gel filtration. A clear separation of unreacted PLL from PLL-dextran was realized. The latter appears as several overlapping peaks, as expected, since there must be a series of species with different numbers of dextran chains per PLL chain. Preliminary estimates indicate that several dextran chains (3-6, very tentatively) are grafted to an average PLL chain of about 28 lysine residues. It is desired to have a substantial fraction of the lysine amino groups unmodified for effective binding to IC, and to permit enzymic digestion in vivo to generate fragments small enough to be excreted.

N. Immunology.

It is desirable that an inducer formulation be non-immunogenic, so that repeated doses do not result in the production of high levels of circulating antibodies.

A survey has been made of most of the polymer components of the formulations as to immunogenicity in BALB/c mice. In the initial work we wished to see if any inducer is a strong immunogen. Future work will be directed toward more sensitive methods to be applied to the most promising inducer candidates.

Tests of the antigenicity of inducer components have been done by the gel-precipitation method. This method is not as sensitive as the ELISA method, but the latter requires more exploratory research to establish that the specific antibodies will be adsorbed to the wall of the assay vessel.

In the method used, the substance to be tested was injected into BALB/c mice with complete Freund's adjuvant. Additional injections were made after 3 and 6 weeks, using Freund's incomplete adjuvant. After 2-3 weeks blood was obtained, clotted, and centrifuged, and the clear plasma tested. Each potential antigen was injected into 5 mice, and serum from each mouse was tested against the antigen.

The substances tested were:

- A CMdextran (no $NaBH_4$ treatment)
- B CMdextran ($NaBH_4$-treated)
- C CMamylose
- D PLL
- E Succinylated gelatin
- F ICL-CMdextran
- G AcCitPLL
- H ICL-CMamylose
- I AcCitgelatin
- J ICL-AcCitgelatin

Serum A was tested against B, D and F
Serum B was tested against A and B
Serum C was tested against C
Serum D was tested against A-J (except B)
Serum E was tested against E
Serum F was tested against A, D and F
Serum G was tested against D and G
Serum H was tested against A, C and H
Serum I was tested against A, I and J
Serum J was tested against A, I and J.

All were negative.

Although each of the ten compounds listed above was injected into five mice and no antibodies were detected in the serum of any of the mice, the results must be considered preliminary. It is likely that in some cases a level of antibody too low to be detected was present. The injection protocol used is relatively standard and ordinarily leads to the induction of antibodies even with proteins which appear to be only weakly immunogenic, or not immunogenic, when injected without adjuvant; the immunogenicity may also differ depending on the experimental species used.

The assay procedure used here was selected on the basis of its simplicity and fair degree of sensitivity. Assays employing radioactivity, enzymes or fluorescence are more sensitive but are correspondingly more involved. It appears that some such method must be adapted to the present problem.

A substantial portion of each of the sera collected in the present work is frozen away and should be examined for antibody by a more sensitive method. Also, the immunogenicity of these and other compounds involved in this project should be tested in other species. The more sensitive and involved procedures will be reserved for inducers of the greatest promise.

Miscellaneous:

Under the heading we include several projects which have not progressed to the extent of having provided data suitable for discussion.

A. Preparation of Modified Gelatin and PLL of Increased Positive Charge.

The elimination of CMC can, in principle, be carried further from the ternary complexes such as ICLC or the others described in this report, to binary complexes of IC with a polycation. Indeed ICL has been studied, but has not been successful because of severe, adverse reactions (H. Levy, personal communications). The difficulty may lie in the PLL. A possible way to avoid this problem, may be use other cationic polymers.

To this end we reacted gelatin with spermine and the bifunctional reagent dimethyl suberimidate to graft spermine onto the amino groups of gelatin and PLL, following the general procedure of Wang and Moore(9). These products have not yet been characterized or tried in an inducer formulation.

B. Carboxymethyl β-Cyclodextrin.

The polysaccharides from which the carboxymethyl derivatives are made, are not accurately characterized entities. They are heterodisperse as to molecular weight, may vary in branching and purity.

The use of a small saccharide of well-defined structure appears attractive. We have begun to study the carboxymethyl derivatives of β-cyclodextrin, a cyclic oligosaccharide consisting of seven glucose units. Cyclodextrins of smaller and larger ring size are known.

The potential advantages of CM-β-cyclodextrin are:

1) Cyclodextrins have been described in many pharmaceutical preparations, and appear to be essentially non-toxic;

2) CM-Cyclodextrins are expected to be non-immunogenic;

3) CM-Cyclodextrins are expected to be very rapidly cleared;

4) As small organic compounds of definite size and structure they should be amenable to purification by common techniques, free of microorganisms, pyrogens etc.

We have carboxymethylated β-cyclodextrin, and are in the process of purifying it by the use of organic solvents and chromatography.

C. Preparation for Testing for Nuclease Resistance.

In his various publications (e.g., Levy et al. (5)) Hilton Levy reported that bovine pancreatic RNase hydrolyzes IC. However, Stebbing and Dawson (10) reported that RNase does not hydrolyze IC, although experimental details were not given. We have verified Levy's finding that RNase does hydrolyze IC at pH 7 in 0.15 M NaCl.

6. Conclusions

The results show that effective interferon inducers can be formulated without CMC. At least four CMC replacements, CMdextran, CMamylose, acetylcitryl PLL and acetylcitryl gelatin, have been combined with IC and PLL to give formulations which induce, in mice, from moderate to superior titers of interferon.

One of these, CMamylose, gave an inducer as effective against Rift Valley fever as is ICLC.

Most of the formulation types and components are non-immunogenic by the test used so far. If they are immunogens, they must be weak immunogens.

We have also seen that melting profiles and absorption spectra are correlated, at least partially. While we cannot yet always relate these physical properties to efficacy of IFN induction, we can predict that formulations which gave abnormal spectra and/or melting profiles will fail, and can now avoid needless biological tests. The fluorescence work, although preliminary, also gives promise of a correlation between physical and biological properties. The combination of the two types of optical probes may be useful.

Among the tasks before us are to:

a) test reproducibility and stability of formulations;

b) solve solubility problems;

c) find the optimum proportions of components;

d) test toxicity;

e) test pyrogenicity;

f) test immunogenicity with more sensitive methods;

g) screen new formulations embodying PLL-dextran grafts and carboxymethyl-cyclodextrin;

h) screen other polymers described in the original proposal (e.g., sulfated gelatin);

i) test formulations for resistance to nucleases, and for correlation of physical properties with biological properties.

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8. Glossary.

IFN:	Interferon
IC:	PolyI·PolyC, Poly IC
PLL:	Poly(L-lysine)
ICL:	Complex of IC with PLL
ICLC:	Complex of IC with PLL and CMC
CM:	Carboxymethyl
CMC	Carboxymethylcellulose
AcCitPLL	Acetylcitryl derivative of PLL
AcCitGel:	Acetylcitryl derivative of gelatin
Tm:	Melting, or transition, temperature
CMamylose:	Carboxymethyl amylose
ICL-CMamylose:	Complex of IC, PLL and CMamylose
CMdextran:	Carboxymethyl dextran
ICL-CMdextran:	Complex of IC, PLL and CMdextran
ICL-AcCitPLL:	Complex of IC, PLL and AcCitPLL
ICL-AcCitGel:	Complex of IC, PLL and AcCitGel
CMmannan:	Carboxymethylmannan
CMamylopectin:	Carboxymethylamylopectin
U:	Units of interferon
CBZ:	Carbobenzyloxy
A:	Absorbance (spectrophotometer)
Mr:	Molecular weight
RNase:	Ribonuclease

SUPPLEMENTARY

INFORMATION

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

REPLY TO ATTENTION OF
SGRD-RMI-S (70-1y)

ERRATA

0 5 AUG 1994

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

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research, Development, Acquisition and Logistics Command (USAMRDALC)(Provisional), has reexamined the need for the limited distribution statement on technical reports for Contract No. DAMD17-87-C-7111. Request the limited distribution statement for AD No. ADB122935 [redacted] be changed to "Approved for public release; distribution unlimited," and a copy of these reports be released to the National Technical Information Service.

2. Point of contact for this request is Mrs. Judy Pawlus, DSN 343-7322.

ERRATA - AD-B122935

for CAREY O. LEVERETT
LTC, MS
Deputy Chief of Staff for
Information Management

151.20/14