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The Formation of Complexes between Polynucleotides, Carboxymethylcellulose and Polylysine Which Are Anti-viral in Mice without Inducing Interferon

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SUMMARY

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Poly(rI) and poly(rC) can each be wrapped with a colloidal complex of low viscosity carboxymethylcellulose and polylysine, without formation of precipitates, to form complexes containing 10 mg/ml polynucleotide material. From its resistance to ribonuclease treatment and inability to anneal to poly(rC) we conclude that the carboxymethylcellulose polylysine wrapped poly(rI) is really wrapped. Migration of wrapped poly(rI) and wrapped poly(rC) in agarose gel electrophoresis demonstrated that the negative charges of the polynucleotides were neutralized by wrapping. The double-stranded polynucleotide, poly(rI)·poly(rC), could also be wrapped by the same procedure but only to a final polynucleotide concentration of 2 mg/ml. The wrapping procedure should be effective for any polynucleotide. Mixtures of equal masses of wrapped poly(rI) and wrapped poly(rC) do not produce detectable serum interferon concentrations in mice when both polynucleotides are administered at 25 μg/mouse or less while wrapped poly(rI)·poly(rC) produces high concentrations of serum interferon. However, at equal polynucleotide doses the wrapped single-stranded polynucleotide preparations can produce the same degree of resistance to infection with encephalomyocarditis virus as wrapped poly(rI)·poly(rC).

INTRODUCTION

Certain regimens for treating mice with poly(rI) and poly(rC) or derivatives of poly(rC) have shown anti-viral activity against various picorna and toga-virus infections under conditions where the polynucleotides do not form double-stranded structures and interferon is not induced (1–3). Using poly(rI) and poly(rC) this situation was achieved by administering poly(rC) 4h after poly(rI) (1), but with poly(rI) and copolymers containing principally 5-hydroxycytidylate, the two polymers could be administered together since they do not anneal (3). These anti-viral effects are consequent on the polymeric nature of these materials since the nucleosides and nucleotides alone are without activity (4) and if the polymers are too short the activity is decreased or lost (5). Although it is clear from these results that degradation of poly(rI) and poly(rC) by nucleases in the animals is not rapid, it seemed possible that greater activity could be achieved by limiting nuclease attack of the polymers. A method for wrapping the double-stranded polynucleotide, poly(rI)·poly(rC), developed by Levy *et al.* (6), prevents its degradation by the high levels of double-strand specific nucleases which are to be found in the serum of primates. We

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have therefore adapted this wrapping procedure for use with single-stranded polynucleotides recognizing that they may also be degraded in primate blood and also that if poly(rI) and poly(rC) in the complex did not anneal to make the double-stranded polynucleotide they could be administered together without the attendant toxicity of double-stranded nucleic acids. We report here a physico-chemical analysis of wrapped polynucleotides and their ability to induce interferon and confer anti-viral protection to mice. The high viscosity carboxymethylcellulose used by Levy *et al.* (6) to wrap poly(rI)·poly(rC) was replaced by low viscosity material since this permitted the administration of polynucleotides at higher concentrations. We have therefore taken some care to check that the modified procedure is effective and we have also compared the anti-viral activity of wrapped single-stranded polynucleotides with that of wrapped poly(rI)·poly(rC).

Analytical data presented in this paper demonstrate by a number of criteria that single and double-stranded polynucleotides are indeed wrapped on slowly mixing the polynucleotides with a colloidal complex of carboxymethylcellulose and polylysine. The term 'wrapped' for such polynucleotide preparations is therefore justified, but its use is primarily for the sake of brevity when referring to colloidal complexes of polynucleotides, carboxymethylcellulose and polylysine.

MATERIALS AND METHODS

Chemicals. Sodium lauryl sulphate (specially pure) was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Poly-L-lysine was obtained from Sigma Chemical Co. as the hydrobromide (Type VI-A) and had a molecular weight of 3,400. Low viscosity carboxymethylcellulose (sodium salt having at 2%, 25–50 centipoises at 25°) was also obtained from Sigma Chemical Co.

T_1 ribonuclease (EC 3.1.4.8) was obtained from Sankyo Ltd., Japan. Micrococcal ribonuclease (EC 3.1.4.7) from *Staphylococcus* was obtained from MRE, Porton, U.K. and had an activity of 10^4 units/mg.

Preparation of wrapped polynucleotides. Poly(rI), poly(rC) and poly(rI)·poly(rC) were obtained from P-L Biochemicals, Milwaukee, Wisconsin. These polynucleotides all had $S_{20,w}$ values over 9.4 and molecular weights over 10^5.

A colloidal complex was prepared by pumping a solution of the polylysine at 24 mg/ml into an equal volume of the carboxymethylcellulose at 80 mg/ml, both solutions being 0.85% or 0.17% NaCl, as specified in the text. These colloids containing 12 mg/ml polylysine and 40 mg/ml carboxymethylcellulose were used for wrapping single-stranded polynucleotides. A similar colloidal mixture prepared in the same manner but starting with 6 mg/ml polylysine and 20 mg/ml carboxymethylcellulose was used for wrapping poly(rI)·poly(rC). The colloidal complexes of polynucleotides with carboxymethylcellulose and polylysine were prepared at room temperature without the presence of buffers but 10 mM Hepes buffer, pH 7.5 was found to have no effect on preparation of the materials. The colloidal complexes were generally stored at 4° and were found to be stable for at least 24 hours.

Analysis of polynucleotides in agarose gels. Wrapped and unwrapped polynucleotides were analyzed in 0.8% (w/v) gels of agarose (Sigma, for use in electrophoresis). The agarose gels were made up in 20 mM Tris-HCl, 1 mM EDTA, 5 mM sodium acetate, pH 7.0 and then electrophoresed (anode in the lower tank) at 5 mA/gel for 20 minutes using the same buffer in both tanks. Gels were then scanned at 265 nm in a Joyce-Loebl UV-Scanner.

Interferon assay. Interferon determinations were carried out using a plaque reduction assay in L cells against EMC[1] as the challenge virus. Confluent monolayers of L cells in 35 mm wells of Linbro FB-6-TC multidishes (Flow Laboratories) were treated for 17 hr at 37° with serial tenfold dilutions of test serum samples. The cells were then infected with EMC virus and overlaid with Dulbecco's MEM containing 2% donor calf serum and 0.75% carboxymethylcellulose (Sigma, high viscosity). Plaques were counted two days later after

[1] The abbreviation used is: EMC, encephalomyocarditis.

staining with neutral red. The interferon titer was taken as the dilution which inhibited plaque formation by 50%. Parallel assays of Mouse Reference Interferon (Catalogue Number G002-904-511) obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, N.I.H., Bethesda, Maryland, were performed. In our system the international standard had a titer of 4000 U/ml compared with the quoted titer of 6485 U/ml. All the interferon titers in the text are the values determined in our assay multiplied by 1.62 to convert them to International Units. The highest concentrations of sera used were 1/10 dilutions so that the limit of our assay is taken to be 16.2 International Units.

Virus. EMC virus was grown and stored as previously described (4).

Mice. Female (BK:W) mice were obtained from Bantin and Kingman Ltd., Hull, U.K. All mice used were between 6 and 10 weeks old and weighed between 18 and 24 g. Mice were maintained at 22° with unlimited access to water and a diet of Labsure-RHM PMD nuts from Labsure, Poole, Dorset, U.K. All treatments were given by the intraperitoneal route and infections were initiated by intraperitoneal administration of virus except where indicated.

Statistical methods. To assess the prolongation of life achieved by various treatments of virus-infected mice, the survival time in hours (t) was obtained from records prepared twice daily and hence mean values of $10^2/t$ are estimated for each group of mice so as to include surviving mice, as described previously (3). Significant differences in survival times, including surviving mice, were calculated by the logrank method as described by Peto and Pike (7).

RESULTS

Preparation and characterization of carboxymethylcellulose-polylysine wrapped poly(rI)·poly(rC). Poly(rI)·poly(rC) at 4 mg/ml was pumped at about 20 ml/hr into an equal volume of a colloidal mixture containing 3 mg/ml polylysine and 10 mg/ml carboxymethylcellulose. No precipitate was formed and this material was suitable for intravenous injection. The absorption maximum of this wrapped poly(rI)·poly(rC) was 263 nm, 3 nm greater than that of unwrapped poly(rI)·poly(rC), and the ultraviolet absorption spectrum of the wrapped material was increased by 10% or less as observed by Levy *et al.* (6). Figure 1 shows the melting profile of this material diluted to 50 μg/ml compared with the melting profiles of poly(rI)·poly(rC) and the carboxymethylcellulose-polylysine separately. The Tm for the cooperative melting phase of poly(rI)·poly(rC) (66.5°) is increased by 10° in the complexed form.

In preparation for testing the protection of wrapped polynucleotides from nuclease attack, several attempts were made to degrade poly(rI)·poly(rC) with various nucleases. Only micrococcal ribonuclease was

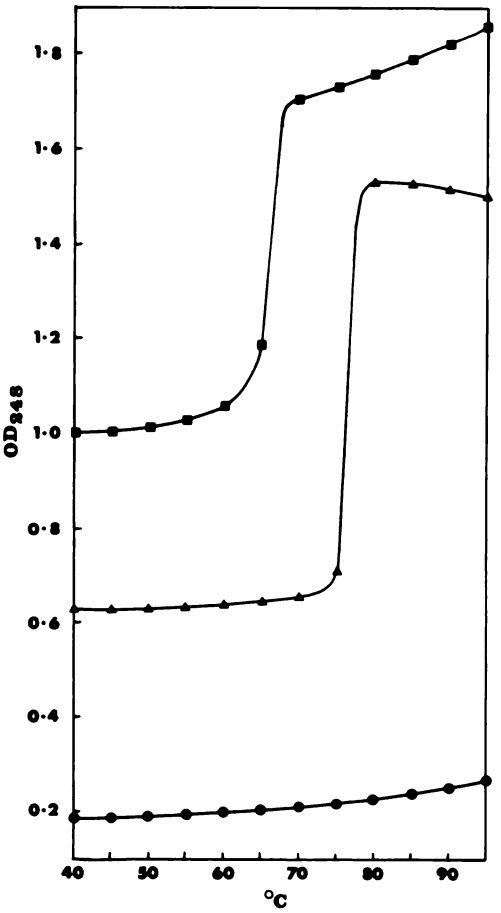

FIG. 1. *Melting profiles of poly(rI)·poly(rC)* (■——■); *wrapped poly(rI)·poly(rC)* (▲——▲) *and the wrapping agent alone* (●——●)

found to degrade poly(rI)·poly(rC), in contrast to the report of Levy *et al.* (6) who claimed that pancreatic ribonuclease was also effective. In control experiments we found that addition of carboxymethylcellulose-polylysine to poly(rI)·poly(rC) at 50 μg/ml just prior to the addition of enzyme could completely inhibit the degradation of the polynucleotide, suggesting inactivation of the enzyme by the colloid or perhaps very rapid complexing of the polynucleotide. We therefore examined the ability of nuclease to degrade poly(rI)·poly(rC) in the free state and when mixed with wrapped poly(rI)·poly(rC). The results, in Fig. 2, show that with 750 units/ml of the nuclease, free poly(rI)·poly(rC) is completely degraded within 2 hours since there is an increase in optical density of 100%. The change in optical density of the carboxymethylcellulose-polylysine wrapped poly(rI)·poly(rC) is insignificant over a period of 5 hours. However, a mixture containing both the free and the wrapped poly(rI)·poly(rC) at the same final concentrations showed a change in optical density over 5 hours that could be entirely accounted for by degradation of all the free poly(rI)·poly(rC). From the initial slopes we estimate that the rate of degradation of poly(rI)·poly(rC) is inhibited by 62% by the wrapped poly(rI)·poly(rC). That the changes in optical density occurring in the presence of micrococcal nuclease represent degradation of the polynucleotides and that the absence of optical density changes for wrapped poly(rI)·poly(rC) represent no degradation, was confirmed by looking for nucleotide monophosphates by thin layer chromatography of the incubation products after 5 hours.

Preparation and characterization of carboxymethylcellulose-polylysine wrapped poly(rI) and poly(rC). A homogeneous material without any precipitate was obtained on pumping poly(rC) at 20 mg/ml in 0.85% NaCl into an equal volume of a colloidal complex containing 40 mg/ml carboxymethylcellulose and 12 mg/ml polylysine. This material remained stable for several hours at 4° but did not redissolve after lyophilization. The use of these higher concentrations was not possible with poly(rI) since a precipitated material was formed even when the pumping rate was reduced to 5 ml/hr and at NaCl concentrations of 0 to 1.5%. However, a satisfactory complex was formed when poly(rI) at 4 mg/ml was

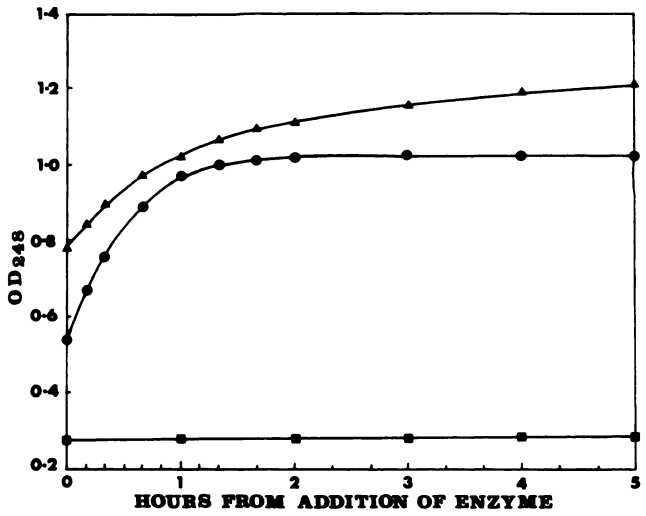

FIG. 2. *The effect on optical density at 248 nm of 750 units/ml micrococcal nuclease added to poly(rI)·poly(rC), wrapped poly(rI)·poly(rC) and a mixture of these two*

●——●, 50 μg/ml poly(rI)·poly(rC); ■——■, 25 μg/ml wrapped poly(rI)·poly(rC); ▲——▲, 50 μg/ml poly(rI)·poly(rC) plus 25 μg/ml wrapped poly(rI)·poly(rC). Enzyme was added at time zero and the samples incubated at 37° in 0.85% NaCl, 10 mM Hepes, 0.5 mM $CaCl_2$.

pumped into the colloidal complex containing 3 mg/ml polylysine and 10 mg/ml carboxymethylcellulose in 0.17% NaCl. This complex did not precipitate and it could be lyophilized and redissolved at 5 times the original concentration also without precipitating. Thus preparations containing up to 10 mg/ml poly(rI), 6 mg/ml polylysine and 20 mg/ml carboxymethylcellulose could be obtained. Mixtures containing equal weights of wrapped poly(rI) and wrapped poly(rC) both at 5 mg/ml were prepared by adding freshly prepared wrapped poly(rC) to lyophilized preparations of wrapped poly(rI).

A direct check of the wrapping procedures was carried out by examining the migration of the materials in 0.8% agarose gels. In these gels polynucleotides do not seem to be sieved by size since polynucleotides of a wide range of sizes (tRNA and ribosomal RNA) were found to migrate in the same position as bromo-phenol blue. Therefore a change in migration rate of a polynucleotide mixed with other materials will reflect principally a change in charge rather than a change in size of the materials. The results, in Fig. 3, show that the migration rate of both poly(rI) and poly(rC) is greatly retarded when the polynucleotides have been complexed with carboxymethylcellulose-polylysine. This retardation is not due to blocking of the gel by the carboxymethylcellulose-polylysine since electrophoresis of mixtures of poly(rI) and wrapped poly(rI) gives rise to two bands, one in the position of free poly(rI) and the other in the position of the complex. The complexes between carboxymethylcellulose-polylysine and poly(rI) or poly(rC) were not maintained when 0.2% sodium lauryl sulphate was included in the buffers. We conclude that a complex is formed between poly(rI) or poly(rC) when mixed with carboxymethylcellulose-polylysine and that the negative charges of the polynucleotides are then largely neutralized, probably by the amino residues of polylysine.

A further check on the formation of a complex between carboxymethylcellulose-polylysine and poly(rI) was carried out by testing the availability of the poly(rI) for annealing to poly(rC) and degradation by T_1 RNase. Figure 4a shows that the hypochromic effect due to the annealing of poly(rC) and poly(rI) is maximal at 250 nm when compared with the sum of the absorption spectra of poly(rC) and poly(rI) determined separately. In contrast (Fig. 4b) the absorption spectrum of a mixture of poly(rC) and carboxymethylcellulose-polylysine wrapped poly(rI) does not differ

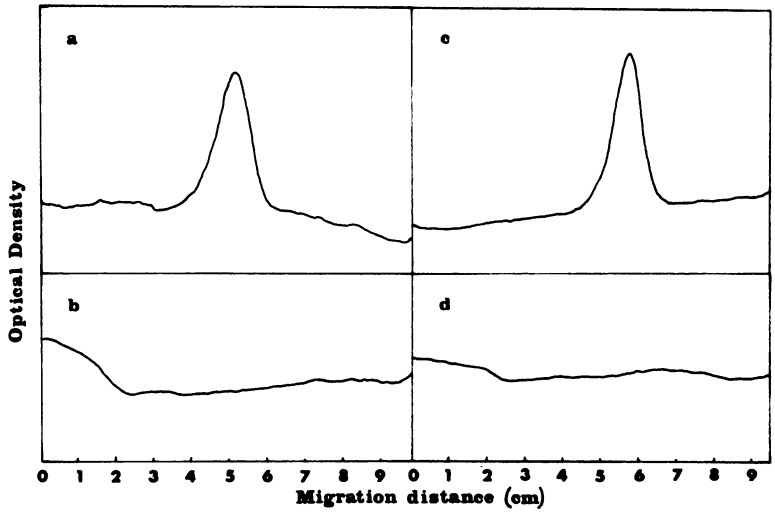

FIG. 3. *Ultraviolet scans at 265 nm of 0.8% agarose gels run for 20 minutes at 5 mA/gel containing: (a) poly(rI), (b) wrapped poly(rI), (c) poly(rC), (d) wrapped poly(rC)*

Each gel contained 200 μg polynucleotide.

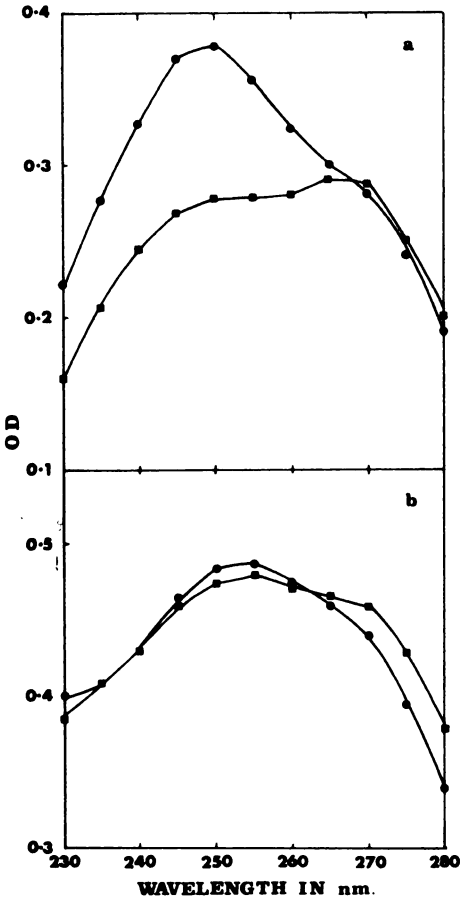

FIG. 4. *(a) The mean absorption spectrum of poly(rC) and poly(rI) determined separately (●——●) and of poly(rC) mixed with poly(rI) (■——■); (b) the mean absorption spectrum of poly(rC) and wrapped poly(rI) determined separately (●——●) and of poly(rC) mixed with wrapped poly(rI) (■——■)*

Each polynucleotide was at a concentration of 25 μg/ml.

markedly from the sum of the spectra of the two components determined separately. These observations indicate that the wrapped poly(rI) is not capable of hydrogen bonding to poly(rC).

Figure 5 shows the effect of T_1 ribonuclease on 25 μg/ml poly(rI) either alone, wrapped with the complex, or rapidly mixed with the colloidal carboxymethylcellulose-polylysine immediately before treatment with enzyme. The change in absorption at 248 nm shows that free poly(rI) is entirely degraded within 20 minutes while the wrapped poly(rI) shows only one tenth of the maximal change in absorption even after 160 minutes. Addition of carboxymethylcellulose-polylysine just before the enzyme causes a decrease in the rate of degradation of the poly(rI), but after 160 minutes the change in optical density corresponds to complete degradation of the poly(rI). From the initial rates of degradation we estimate that the activity of T_1 ribonuclease was inhibited by 66% by the complex. These results indicate that about 10% of the poly(rI) in a wrapped complex may be exposed and is degraded rapidly but the remainder is not available for degradation by the ribonuclease (Fig. 5). That the changes in optical density occurring in the presence of T_1 RNase represent degradation of poly(rI) and that the small change in optical density of the wrapped poly(rI) represents only minor degradation was confirmed by looking for inosine monophosphate by thin layer chromatography of the incubation products after 3 hours.

We have examined the possibility of dissociating the wrapped complexes of poly(rI) and poly(rC) thus allowing the formation of poly(rI)·poly(rC) when the two complexes are mixed. This dissociation is most likely to occur at elevated temperatures. Upon heating a mixture of the two complexes there was an initial *decrease* in absorbance up to 60° and thereafter the curve was the same as that obtained with wrapped poly(rI)·poly(rC). On cooling to 30° the absorbance fell below that of the starting material. These observations indicate that the wrapped single-stranded polynucleotides dissociate from the wrapping agent during heating and anneal to form poly(rI)·poly(rC) within the complex. Thus wrapped poly(rI)·poly(rC) would appear to be a more stable configuration for the materials in these mixtures. On the other hand dissociation and formation of poly(rI)·poly(rC) did not occur when the wrapped single-stranded polynucleotides were kept below 40° for up to 24 hours.

Determination of serum interferon and anti-viral activity after treatment of mice with various wrapped polynucleotides. Wrapped poly(rI)·poly(rC) (100 μg/mouse i.p.) induced high titers of serum interferon

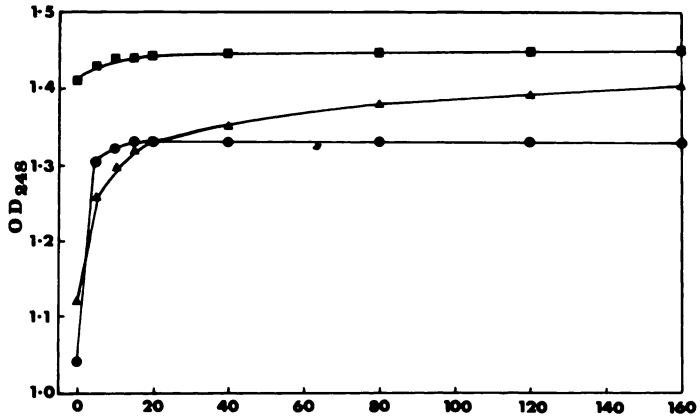

FIG. 5. *The effect on optical density at 248 nm of 10 μg/ml T_1 ribonuclease added to poly(rI)* (●——●), *wrapped poly(rI)* (■——■), *and poly(rI) with carboxymethylcellulose-polylysine added just before the enzyme* (▲——▲)

All samples were incubated at 30°C with poly(rI) at a concentration of 50 μg/ml. The time zero (enzyme addition) points have been adjusted for the effect of dilution and optical density of the added materials.

around 4 hours after administration to mice (Table 1). The amounts of interferon produced at the various times were the same as those induced by free poly(rI)·poly(rC) (data not shown). If the polynucleotides were mixed after wrapping, an equivalent amount (50 μg/mouse each i.p., CP-I_{50} plus CP-C_{50}, Table 1) gave a maximum interferon titer only 1.5% of that observed with wrapped poly(rI)·poly(rC). A fourfold increase in the doses of wrapped poly(rI) and wrapped poly(rC) in the mixture slightly increased the maximum interferon titer up to 2.5% of that observed with wrapped poly(rI)·poly(rC) (Table 1). Neither the wrapping agent alone at the highest dose used in the mixtures, nor wrapped polynucleotides administered separately, caused detectable interferon induction (Table 1).

To minimize the possibility of inducing interferon with mixtures of wrapped poly(rI) and poly(rC) these two complexes were each administered at polynucleotide doses of 25 μg/mouse and their anti-viral activity compared with that of wrapped poly(rI)·poly(rC) at a polynucleotide dose of 50 μg/mouse. The results, in Fig. 6, show that these two treatments confer essentially similar protection against infection. For convenience of presentation all deaths occurring within any one day are shown in Fig. 6 at one time only. Logrank χ^2 analyses showed no differences between the two wrapped polynucleotide treatments, which were both highly protective ($p < 0.001$) compared with the infected control, and treatment with the wrapping material alone was not protective. Analysis of serum from mice treated with a mixture of wrapped poly(rI) and poly(rC) at these and lower doses showed no detectable interferon. In this experiment wrapped poly(rC) alone at 25 μg/mouse of polynucleotide showed no anti-viral activity and wrapped poly(rI) at this dose conferred only slight protection. At higher doses these materials became more anti-viral but this activity was not significantly altered by wrapping. Essentially similar results were obtained when the treatments were given at 6 hours, rather than 24 hours, before infection. We conclude that mixtures of the wrapped single-stranded polynucleotides confer essentially the same degree of protection on a weight basis as wrapped poly(rI)·poly(rC), and that with the former treatment no circulating interferon can be detected. Moreover, we conclude that combinations of wrapped poly(rI) and wrapped poly(rC) show greater anti-viral activity than either material alone.

To check whether the anti-viral activity of the wrapped single-stranded polynucleotide preparations was due entirely or in

TABLE 1

Interferon titrations of serum samples taken at various times after treating mice intraperitoneally with carboxymethylcellulose-polylysine wrapped polynucleotides

Pooled serum from 5 mice used at each assay time.

Treatment of mice	Times after treatments at which serum samples were taken				
	0h	2h	4h	8h	24h
Saline	<16.2	<16.2	<16.2	<16.2	<16.2
CP-I:C_{100}[a]	<16.2	6480	>6480	3240	1620
CP-I_{50} + CP-C_{50}[b]	<16.2	16.2	16.2	97.2	<16.2
CP-I_{200} + CP-C_{200}[b]	<16.2	81.0	81.0	162.0	32.4
CP-I_{200}[b]	<16.2	<16.2	<16.2	<16.2	<16.2
CP-C_{200}[b]	<16.2	<16.2	<16.2	16.2	<16.2
CP alone[c]	<16.2	<16.2	<16.2	<16.2	<16.2

[a] CP-I:C_{100} = wrapped poly(rI)·poly(rC) at 100 μg/mouse polynucleotide administered in 0.1 ml.

[b] CP-I and CP-C = wrapped poly(rI) or wrapped poly(rC), the dose of polynucleotide material (μg/mouse) being indicated by the suffix, in each case the material being administered in 0.1 ml/mouse.

[c] CP = 0.1 ml/mouse of the wrapping agent containing 10 mg/ml carboxymethylcellulose and 3 mg/ml polylysine.

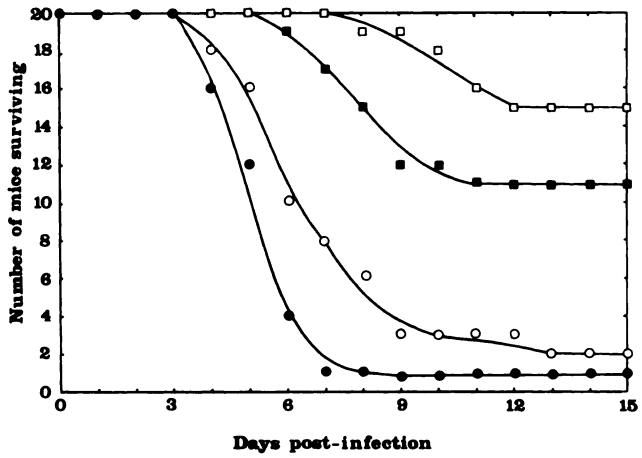

FIG. 6. *The protective effect of intraperitoneally-administered wrapped polynucleotides against intraperitoneal infection of mice with 80 × LD_{50} EMC virus*

(●——●), infected control; (□——□), 50 μg/mouse wrapped poly(rI)·poly(rC) (■——■), 25 μg/mouse wrapped poly(rI) and 25 μg/mouse wrapped poly(rC); (○——○), the wrapping agent alone.

part to some local effect in the peritoneal cavity which, for example, merely limits transport of virus from this site, we examined the anti-viral effect of wrapped polynucleotides against infections initiated by intraperitoneal or intravenous injection of the virus. The results, in Table 2, show that highly significant protection occurs with the mixture of wrapped poly(rI) and wrapped poly(rC) given intraperitoneally even against intravenous infection. However, with this mixture the degree of protection against intravenous infection is significantly less than that conferred by wrapped poly(rI)·poly(rC) while no such difference occurs between these treatments in the case of intraperitoneal infections (Table 2). The wrapped poly(rI) also confers significantly greater protection against intraperitoneal infection than intravenous infection. It is therefore likely that a small component of the anti-viral effect of the wrapped single-stranded polynucleotide preparations arises by some local effect when the materials and virus are both administered into the peritoneal cavity.

TABLE 2

Comparison of the protective effects of poly(rI)·poly(rC), poly(rI) and poly(rC) wrapped with carboxymethylcellulose-polylysine against infection of mice with encephalomyocarditis virus by the intraperitoneal (i.p.) or intravenous (i.v.) routes

Number of mice per group = 20. All treatments given i.p. and mice injected with a virus dose of 100 × LD_{50}.

Route of infection	Treatment 6h before infection	$10^2/t$	χ^2_{inf} [c]	p	χ^2_{diff} [d]	p
i.p.	CP-I:C[a]	0.14	27.4	<0.001	1.4	NS
	CP-I + CP-C[b]	0.25	24.8	<0.001		
	CP-I	0.40	12.7	<0.001		
	CP-C	0.71	0.9	NS		
	none	0.76	—			
i.v.	CP-I:C	0.17	32.2	<0.001	6.01	<0.05
	CP-I + CP-C	0.40	15.2	<0.001		
	CP-I	0.65	5.4	<0.05		
	CP-C	0.80	0.1	NS		
	none	0.81	—			

[a] CP-I:C = wrapped poly(rI).poly(rC) at 50 μg/mouse of polynucleotide.

[b] CP-I and CP-C = wrapped poly(rI) and wrapped poly(rC) at 25 μg/mouse of polynucleotide.

[c] χ^2_{inf} = logrank comparisons with the infected only (no treatment) groups.

[d] χ^2_{diff} = logrank comparisons between bracketed groups.

DISCUSSION

The results reported here demonstrate that colloidal complexes of carboxymethylcellulose and polylysine can effectively wrap single-stranded polynucleotides in that they become resistant to nuclease digestion, unavailable for hydrogen bonding, and that the negative charges of the polynucleotides are neutralized. The observation that mixtures of wrapped poly(rI) and wrapped poly(rC) do not induce significant amounts of interferon also indicates that wrapping of these single-stranded polynucleotides precludes formation of a double-stranded polynucleotide. The fact that sodium lauryl sulphate breaks down the complexes indicates that no covalent bonds are generated during formation of the complexes and that the bonds maintaining the complexes are probably ionic. It is unlikely that hydrogen bonding to the nucleic acid bases plays a significant role in forming the complexes since poly(rI)·poly(rC) can be wrapped as effectively as the single-stranded polynucleotides. Since both single- and double-stranded polynucleotides can be effectively wrapped by the procedures described here, the method should be effective for any polynucleotide.

Determination of the interferon-inducing capacity of wrapped single-stranded polynucleotides, particularly wrapped poly(rI), would seem important in view of the greater importance attributed to poly(rI) for interferon induction by poly(rI)·poly(rC) (9, 10). Moreover, certain conformations of poly(rI), but not that from P-L Biochemicals, can induce interferon (11) and an interferon-inducing conformation may be formed on wrapping poly(rI). Our results argue against this and the notion that poly(rC) simply serves to limit nuclease digestion of poly(rI) since our wrapped poly(rI) is nuclease resistant but does not induce interferon (see Table 1).

Our results on the use of low rather than high viscosity carboxymethylcellulose in the preparation of wrapped polynucleotides show clear advantages when compared with the results reported by Levy *et al* (6). In particular, no precipitate is formed on adding the polylysine and the colloidal complex can be made at least four times more concentrated. This increase in concentration of the wrapping agent enabled us to prepare wrapped polynucleotides at higher concentrations than those used by Levy *et al.* (6) and avoidance of precipitation greatly shortened the time needed for preparation. These features should greatly facilitate studies on the activity of the preparations against virus infections of primates. Levy *et al.* (6) observed that the toxicity of their

wrapped poly(rI)·poly(rC) was greater by the intraperitoneal than the intravenous route and that intraperitoneal toxicity was associated with the formation of gelatinous masses in the peritoneal cavity. The preparations we have used have not given these problems, suggesting further advantages in the use of low rather than high viscosity carboxymethylcellulose.

Although agreeing with the main conclusions of Levy *et al.* (6), that wrapped poly(rI)·poly(rC) induces interferon in mice as effectively as the free polynucleotide, our results differ from theirs in matters of detail. We were unable to degrade free poly(rI)·poly(rC) with pancreatic RNase and resistance to this enzyme under the conditions used is considered diagnostic for double-stranded polynucleotides (8). The data of Levy *et al.* (6) showing degradation by this enzyme are therefore puzzling and we feel that their data demonstrating protection from this degradation needs further examination since we have evidence that nucleases are inhibited directly by the colloidal complex of carboxymethylcellulose and polylysine, a mechanism that is unrelated to the wrapping process. Nevertheless our data show that complexes of the type we have produced, and therefore presumably those described by Levy *et al.* (6), are genuinely protected from attack by nucleases which do degrade poly(rI)·poly(rC) under conditions where the nuclease remains active. Features of the carboxymethylcellulose-polylysine complex important for effective wrapping of polynucleotides have not been extensively explored but we find that a decrease in the molecular weight of the polylysine to 2000 results in material which does not effectively wrap polynucleotides.

Our results confirm that wrapped poly(rI)·poly(rC) is as good an interferon inducer in mice as the unwrapped polynucleotide, as previously found by Levy *et al.* (6). The anti-viral activities of poly(rI) or poly(rC) administered separately to mice are also not significantly increased by wrapping implying that, *in vivo*, nuclease digestion is not a major factor limiting their anti-viral activity. Presumably the polynucleotides do not remain long in the circulation and are taken up into compartments low in nuclease activity. Mixtures of wrapped poly(rI) and wrapped poly(rC) appear to be as anti-viral as wrapped poly(rI)·poly(rC) when the two treatments involve equal masses of polynucleotide materials. This degree of anti-viral activity for the mixture of wrapped single-stranded polynucleotides is slightly higher than anticipated from similar treatment regimens which also do not cause interferon induction, such as sequential treatments with poly(rI) and poly(rC) or mixtures of poly(rI) and copolymers containing 5-hydroxycytidylate (1, 3). The slightly lower activity of these mixtures compared with wrapped poly(rI)·poly(rC) against intravenous infection indicates an additional local effect when treatment and infection are both by the intraperitoneal route.

The serum interferon titrations associated with treatment with wrapped poly(rI) and wrapped poly(rC) would seem to be too low to account for the observed anti-viral activities, particularly at total polynucleotide doses of 50 μg/mouse or less when no interferon can be detected. It should be noted that the interferon-inducing capacity of mixtures of wrapped poly(rI) and wrapped poly(rC) was tested at doses four times greater than wrapped poly(rI)·poly(rC) (Table 1). The low levels of serum interferon detected with mixtures of the higher concentrations of the wrapped single-stranded polynucleotides could possibly arise by formation *in vivo* of small amounts of poly(rI)·poly(rC), for example as a result of incomplete wrapping of the polynucleotides. The mechanism of action of these wrapped single-stranded polynucleotide treatments is obscure but may be due to direct inhibition of virus replication, in the manner proposed for unwrapped polynucleotides (1, 2, 4). It is possible that the wrapped single-stranded polynucleotides are anti-viral by mechanisms different from those concerned with the unwrapped polynucleotides and this possibility is presently being investigated. It may be argued that tissue rather than serum interferon titrations are more relevant to protection against infection and that wrapped single-stranded polynucleotides induce tissue in-

terferon. However, other similar unwrapped polynucleotide treatments are protective with evidence against the notion that interferon is involved (1, 2, 3).

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