Variations in the Contribution of Induced Interferon and Adjuvanticity to the Antiviral Effect of Different Polyinosinic Acid·Polycytidylic Acid Formulations in Mice Infected with Encephalomyocarditis Virus

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Treatment of mice with polyinosinic acid·polycytidylic acid [poly(I)·poly(C)] 6 h before infection and once daily on days 1, 2, 3, 4, 7, and 10 after infection with encephalomyocarditis virus was found to confer no additional protection as compared with a single treatment 6 h before infection. When complexed with a colloid formed between carboxymethylcellulose and polylysine, poly(I)·poly(C) conferred significant additional protection with the multiple treatment regimen compared with a single treatment 6 h before infection. The additional antiviral activity of multiple treatments could not be entirely attributed to interferon induction by the complexed form of poly(I)·poly(C), because free and complexed poly(I)·poly(C) both caused hyporesponsiveness to interferon induction after multiple treatments. However, mice protected against encephalomyocarditis virus infection by multiple treatments with the colloidal complex form of poly(I)·poly(C) showed a significant increase in resistance to reinfection, and this was attributable to adjuvant effects of the colloidal complex form of poly(I)·poly(C). The contribution of interferon induction and adjuvanticity of the poly(I)·poly(C) formulations varied with the times of treatment relative to infection, the dose of polynucleotide material, and the virus dose.

In vivo effects of double-stranded polyribonucleotides demonstrated in rodents do not occur in primates, probably as a result of high concentrations in primate serum of nucleases which rapidly degrade the polynucleotides (4). Complexes of polyinosinic acid·polycytidylic acid [poly(I)·poly(C)] with a colloid formed between carboxymethylcellulose and polylysine render the polynucleotide resistant to nuclease digestion (4, 9) and result in material which induces interferon in primates (4) and acts as an immunological adjuvant (3, 13). Analysis of the mode of action of an agent [poly(I)·poly(C)] known to inhibit virus replication and also to boost immune responses during infection when the concentration of viral antigens is varying, is likely to be complex, and the problem is made more difficult in the case of treatment of primates, in which situation the agent has to be formulated in a colloidal material. Use of the complexed form of poly(I)·poly(C) in treatments of rhesus monkeys infected with a virulent strain of yellow fever virus is accompanied by increased antibody titers to the virus (E. L. Stephen, W. L. Pannier, M. L. Sammons, S. Baron, H. B. Levy, and R. O. Spertzel, *Fed. Proc.*

34:960, 1975), whereas infections with hemorrhagic fever are accompanied by decreased antibody titers (5), so that the contributions of interferon induction and adjuvant effects may vary with different infections. However, the interferon-inducing and adjuvant activities of poly(I)·poly(C) occur in primates only when the polynucleotide is complexed with carboxymethylcellulose and polylysine, and it is not possible to determine, from primate studies, in what manner these activities are affected by formation of a colloidal complex. This is possible in mice in which single treatments with poly(I)·poly(C) and poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine both induce serum interferon to the same extent and also confer equal protection against virus infections (4, 9). The studies of Levy and co-workers (5, 7, 12; Stephen et al., *Fed. Proc.* 34:960, 1975) utilized multiple treatments of monkeys with complexed poly(I)·poly(C), which, if additionally protective by interferon induction compared with single treatments, could arise only if the usual hyporesponsiveness to interferon induction were overcome.

Thus it seemed important to determine, in mice, the relative roles of interferon induction and adjuvanticity in the protection by various

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treatment regimens with free and carboxymethylcellulose-polylysine-complexed poly(I)·poly(C). The results reported here demonstrate that multiple treatments postinfection with the colloidal complexed form of poly(I)·poly(C), unlike free poly(I)·poly(C), confer additional protection against encephalomyocarditis (EMC) virus infection of mice by adjuvant effects rather than additional interferon induction.

MATERIALS AND METHODS

Virus. EMC virus and Semliki Forest virus were grown and stored as previously described (11).

Mice. Female white mice (BK:W), 6 to 10 weeks old and weighing 18 to 22 g, were obtained from Bantin & Kingman Ltd., Hull, U.K. and maintained at 22°C with unlimited access to water and a maintenance diet of RHM PMD cubes obtained from Labsure, Poole, U.K. Except where stated, all treatments and infections were by the intraperitoneal route in 0.1 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (11).

Chemicals. The double-stranded polyribonucleic acid complex, poly(I)·poly(C), was obtained from P-L Biochemicals, Milwaukee, Wis. A nuclease-resistant complex of poly(I)·poly(C) with a colloid formed between carboxymethylcellulose and polylysine was prepared as described previously (9). Doses of this material are given in terms of their polynucleotide content.

Interferon assay. Interferon concentrations in mouse serum samples were determined using an international standard, as previously described (10); the lowest concentration of interferon in the assays reported here was 10 IU. Each serum sample for assay was obtained by pooling serum from three mice per treatment group. Interferon determinations were carried out to assess whether repeated treatments with poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine hyporeactivated the interferon-inducing capacity of poly(I)·poly(C). The following experimental design was adopted. Mice were treated once daily for 4 days with 20 µg of the poly(I)·poly(C) formulations per mouse; then, after a further 2 days without treatments, the mice were challenged with 100 µg of the poly(I)·poly(C) formulations per mouse, and groups of three mice were sacrificed for serum 0, 2, 4, and 8 h after this challenge.

Statistical methods. The survival time of mice was obtained from records prepared twice daily for at least 25 days postinfection. A measure of the average survival time for a group of mice, which also takes account of surviving mice, was obtained by calculating the mean of the reciprocals of the survival times (t , in hours), taking the reciprocal of survivors to be zero, and multiplying this mean by 100 ($10^2/t$ values). These values are inversely proportional to survival time. For direct comparisons of survival times, the reciprocal of $10^2/t$ values may be used; these harmonic mean survival times are expressed in days. Significant differences in the survival of different groups of mice were tested for by calculating log rank χ^2 values which take account of surviving mice, as previously described (10, 11). One, two, and three asterisks after χ^2 values are used to indicate the 5, 1, and 0.1% significance levels,

respectively. For convenience of presentation, all deaths occurring in any one day are shown in mortality curves at one time only.

RESULTS

Comparison of single and multiple treatments for protection against EMC virus infection. Poly(I)·poly(C) and carboxymethylcellulose-polylysine-complexed poly(I)·poly(C) administered once before infection (−24 h or −6 h relative to infection) were found to confer equal protection against EMC virus infection over a range of virus doses from 1 to 200 50% lethal doses (LD_{50}). The data in Figure 1 show that doses of 10 or 30 µg of poly(I)·poly(C) per mouse, either alone or complexed with carboxymethylcellulose-polylysine, protected mice against infection with 100 LD_{50} of EMC virus when the polynucleotide material was given once at 6 h before infection, or at 6 h before infection and then on days 1, 2, 3, 4, 7, and 10 postinfection. All these treatments conferred significant protection compared with the infected control group ($P < 0.001$ in all cases). Treatment of mice with poly(I)·poly(C) on the 6 days post-

FIG. 1. Protective effect of (A) 10 µg and (B) 30 µg of poly(I)·poly(C) per mouse at 6 h before infection (Δ); poly(I)·poly(C) at 6 h before infection and on days 1, 2, 3, 4, 7, and 10 postinfection ($\square$); poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine at 6 h before infection ($\blacktriangle$); poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine at 6 h before infection and on days 1, 2, 3, 4, 7, and 10 postinfection ($\blacksquare$). Untreated control, infected only, 100 LD_{50} of EMC virus ($\bullet$). Number of mice per group, 40. See Table 1 for statistical analyses.

infection, in addition to 6 h before infection, was found to confer no additional protection over the single treatment given at 6 h before infection with polynucleotide doses of 10 or 30 μ g per mouse. In contrast, with poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine there was a significant increase in the protective effect with the treatments postinfection in addition to 6 h before infection (see Table 1 for results of statistical analyses). It should be noted that the significant difference between single and multiple treatments with the complexed form of poly(I)·poly(C) arises from an obvious increase in the protective effect of the multiple treatment regimen and a slight decrease in that of the single treatment compared with free poly(I)·poly(C), although this decrease was not significant (see Fig. 1 and Table 1). Similar results were obtained in all other experiments in which the free and complexed forms of poly(I)·poly(C) were compared in these treatment regimens.

The increase in the protective effect of poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine that occurred with additional treatments postinfection was found to be quite general and occurred with treatments ranging from 5 to 100 μ g of the polynucleotide per mouse and virus doses from 1 to 240 LD₅₀. This additional protective effect of the poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine was not due to a local effect at the site of administration, as occurs with polycarboxylates (1), since treatments by the intravenous route had the same effect against intraperitoneal infections (data not shown). The known pharmacological properties of the double-stranded polynucleotide complex, namely, interferon induction and adjuvanticity, were therefore examined as a basis for explaining the antiviral effects of

the materials in various treatment regimens.

Interferon-inducing capacity of the poly(I)·poly(C) formulations. Although previous studies have also shown no significant increase in the antiviral activity or interferon-inducing capacity in mice of single treatments with poly(I)·poly(C) when complexed with carboxymethylcellulose-polylysine (4, 9), it is possible that multiple treatments are more antiviral in mice by virtue of interferon induction because hyporesponsiveness to interferon induction does not occur with the complexed form of poly(I)·poly(C). This possibility was examined by determining the concentration of serum interferon over 8 h after administering 100 μ g of poly(I)·poly(C) per mouse to mice that had been previously treated on 4 consecutive days with poly(I)·poly(C) or poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine. The results in Table 2 show that the pretreatment regimen with poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine or free poly(I)·poly(C) both resulted in marked reduction in serum interferon after a challenge dose of 100 μ g of poly(I)·poly(C) per mouse, compared with interferon in mice pretreated with either saline or the carboxymethylcellulose-polylysine alone. The hyporesponsiveness to interferon induction was also observed after a challenge dose of 100 μ g of poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine per mouse. As expected, there was no detectable serum interferon in mice challenged with saline and pretreated with complexed or free poly(I)·poly(C) (see Table 2). Thus the increase in the protective effect of poly(I)·poly(C) when complexed with carboxymethylcellulose-polylysine would not seem to be due to absence of hyporesponsiveness to treatment with the complexed form of poly(I)·poly(C), and the colloidal complex alone does not cause any interferon induction or affect interferon induction by the free or complexed formulation of poly(I)·poly(C).

Resistance to reinfection of mice treated with the different poly(I)·poly(C) formulations. In situations where antiviral antibodies are stimulated to levels sufficient to confer protection against a lethal infection, it is to be expected that surviving animals would be resistant to reinfection with the same virus. We found that mice surviving EMC virus infections of around 10 LD₅₀ by virtue of treatment, 6 h before infection, with 10 μ g of poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine per mouse were significantly protected against reinfection, whereas mice surviving such infections by virtue of treatment with 10 μ g of free poly(I)·poly(C) per mouse were susceptible to reinfection. The same doses of these materials

TABLE 1. Comparison of the antiviral effect in EMC-infected mice of single and multiple treatments of poly(I)·poly(C) and poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine^a

Treatments	Treatment		χ^2_{diff}
	Single: -6 h only	Multiple: -6 h and days 1, 2, 3, 4, 7, 10	
I:C ₁₀	0.32	0.26	0.41
I:C ₃₀	0.21	0.20	0.09
CP-I:C ₁₀	0.37	0.16	8.73 ^b
CP-I:C ₃₀	0.29	0.13	9.85 ^b

^a Values of $10^2/t$ with log rank χ^2 comparisons. Number of mice per group, 40. Value of $10^2/t$ for infected control = 0.77. I:C, Poly(I)·poly(C); CP, carboxymethylcellulose-polylysine. Subscripts: 10, 10- μ g dose per mouse; 30, 30 μ g per mouse.

^b 1% significance level.

both conferred significant resistance to reinfection when the initial virus dose was greater than 50 LD₅₀ of EMC virus. Thus the equal protective effects conferred by treatments at 6 h before infection with 100 LD₅₀ of EMC virus, using the free and complexed forms of poly(I)·poly(C) (Table 1), would seem to be coincidental: although the materials induced essentially equal

amounts of interferon, with the former material protection would seem to be due to interferon, whereas with the latter material protection would seem to be due to a combination of interferon induction and adjuvant effects.

Clearly any adjuvant effect of the poly(I)·poly(C) preparations will be dependent on the amount of viral antigen appearing during infection, and this may be limited by the virus dose and the extent to which interferon induction suppresses virus replication. On this basis one would expect that the lower the amount of interferon induced by the treatment, the greater the possibility that viral antigens may reach a concentration sufficient for an adjuvant effect to confer protection and hence resistance to reinfection. We therefore examined the resistance to reinfection conferred by various doses of poly(I)·poly(C). Poly(I)·poly(C) conferred dose-dependent protection against EMC virus infection that was greater for a virus dose of 10 LD₅₀ than 100 LD₅₀ (see Fig. 2A). However, on reinfection with 100 LD₅₀ of EMC virus 21 days after the first infection, there was a difference in the response depending on the initial virus dose and the dose of poly(I)·poly(C). In all groups, mice protected against an initial dose of 10 LD₅₀ of EMC virus were not significantly resistant to reinfection, regardless of the poly(I)·poly(C) dose. In contrast, mice protected against an initial dose of 100 LD₅₀ of EMC virus were significantly resistant to reinfection; maximum resistance was conferred by a poly(I)·poly(C) dose of

TABLE 2. Serum interferon concentrations in mice after challenge with poly(I)·poly(C) or poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine^a

Challenge at time 0	Pretreatment	Interferon titers at time after challenge:			
		0 h	2 h	4 h	8 h
I:C	Saline	<10	4,250	5,135	2,870
	I:C	<10	50	85	25
	CP-I:C	<10	50	380	160
	CP	<10	4,045	5,000	2,900
CP-I:C	Saline	<10	2,690	4,900	3,160
	I:C	<10	0	145	140
	CP-I:C	<10	30	420	340
	CP	<10	4,400	7,920	4,580
Saline	I:C	<10	<10	<10	<10
	CP-IC	<10	<10	<10	<10

^a Challenge dose = 100 µg per mouse. Mice were pretreated once daily for 4 days with 20 µg of poly(I)·poly(C) per mouse in the free (I:C) or complexed (CP-I:C) form.

FIG. 2. Protective effect of various doses of poly(I)·poly(C) against primary infections of 100 LD₅₀ of EMC virus (●) and 10 LD₅₀ of EMC virus (■). (A) Harmonic mean survival time (HMS), in days, of mice treated with poly(I)·poly(C); (B) HMS after reinfecting the surviving mice 21 days after the primary infection. Poly(I)·poly(C) was administered intraperitoneally 6 h before the primary infection, and the reinfection virus dose was 100 LD₅₀.

10 μ g per mouse (Fig. 2B). Presumably, at up to 10 μ g per mouse the adjuvant effect of poly(I)·poly(C) progressively increases, but at higher doses (30 and 100 μ g per mouse), the interferon induced suppresses virus replication to such an extent that the adjuvant effect decreases.

Treatments postinfection with both the formulations of poly(I)·poly(C) resulted in surviving mice which showed significant resistance to reinfection at all virus doses examined (10 to 240 LD₅₀). The data in Table 3 show that mice surviving an infection of 12 LD₅₀ as a result of 10 μ g of poly(I)·poly(C) per mouse 6 h before infection were susceptible to reinfection (χ^2_{inf} , not significant), whereas mice surviving as a result of the colloidal complex form of poly(I)·poly(C) 6 h before the primary infection were resistant. However, multiple treatments of mice, 6 h before infection and on 6 days after infection, conferred significant resistance to reinfection with poly(I)·poly(C) treatments as well as with treatments with the colloidal complex form of poly(I)·poly(C) (see Table 3). Again, resistance to reinfection is explicable on the basis of a stimulation of antibodies to EMC virus by adjuvant effects of the treatments. It is to be expected that mice treated with either of the formulations of poly(I)·poly(C) at 6 h before infection and postinfection should be partially protected by interferon induction, which will suppress production of viral antigen, but in mice also treated postinfection, adjuvant effects of the treatments should be increased. Resistance to reinfection appears to be specific because mice surviving EMC virus infection by virtue of treatment with the colloidal complex of poly(I)·poly(C) were unaffected with respect to their susceptibility to Semliki Forest virus.

TABLE 3. Susceptibility to reinfection of mice^a

Treatment	Treatment			
	Single: -6 h only		Multiple: -6 h and days 1, 2, 3, 4, 7, 10	
	10 ² /t	χ^2_{inf} ^b	10 ² /t	χ^2_{inf} ^b
I:C	0.52	0.59	0.05	13.56***
CP-I:C	0.07	13.76***	0.00	14.60***

^a Susceptibility to reinfection of mice surviving a primary infection of 12 LD₅₀ of EMC virus as a result of treatments of 10 μ g of poly(I)·poly(C) (I:C) per mouse or 10 μ g of poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine (CP-I:C) per mouse.

^b χ^2_{inf} = Log rank comparisons between the treated groups and an infected control group of mice for which the 10²/t value was 0.61. The reinfection virus dose was 100 LD₅₀ given 21 days after the primary infection. ***, 0.1% significance level.

DISCUSSION

The interferon-inducing capacity of poly(I)·poly(C) in mice is not significantly altered by complexing the polynucleotide with carboxymethylcellulose-polylysine (4, 9), and from the data presented here (Table 2), hyporesponsiveness to repeated treatments occurs with the colloidal complexed material in mice, as it does in primates (8). Significant interferon induction and adjuvanticity in primates only occurs with poly(I)·poly(C) when it is complexed with carboxymethylcellulose-polylysine. However, antiviral effects in primates observed after multiple treatments with carboxymethylcellulose-polylysine-complexed poly(I)·poly(C) may be partly or in some cases entirely attributable to adjuvant effects, depending on the virus. The present studies show that the adjuvant effects of poly(I)·poly(C) in mice were increased when the polynucleotide was complexed with carboxymethylcellulose-polylysine, and that above 10 μ g of polynucleotide per mouse there was an inverse relationship between the dose of material and its adjuvant effects with treatments at 6 h before infection. With treatments before infection, interferon induction appears to be the major factor causing antiviral effects, as it is with low virus doses (around 1 LD₁₀₀ = 12 LD₅₀), when adjuvant effects must be less pronounced due to lower viral antigen concentrations. The relatively lower adjuvant effects in treatment regimens including a preinfection treatment may also be due in part to inherent lower adjuvant effects at this time, since this has been observed with other double-stranded polynucleotides administered before nonviral antigens (2, 6).

Whereas resistance to reinfection is here attributed to an adjuvant effect, this term is used broadly because antibody titers to EMC virus were not determined. Nevertheless, the effect would appear to be specific since we observed resistance to reinfection with EMC virus but not Semliki Forest virus. Moreover, protection against a primary and resistance to reinfection were not restricted to infections by the same route as the treatments, as occurs with some nonspecific stimulants (1).

Although Levy and co-workers have generally attributed the antiviral effects of the colloidal poly(I)·poly(C) complex in primates to interferon induction (5, 7, 12; Stephen et al., Fed. Proc. 34:960, 1975), the use of daily treatments postinfection, in the same regimen as that utilized in the present mouse studies, indicates that adjuvant effects may be of considerable importance. This conclusion is supported by the observation of hyporeactivation that occurs in primates (8) and the present studies with mice

which show that although the interferon-inducing capacity of poly(I)·poly(C) is not significantly affected by complexing it with carboxymethylcellulose-polylysine, its adjuvant effects are increased. What determines the relative contribution of the interferon-inducing or adjuvant effects of poly(I)·poly(C) complexed with carboxymethylcellulose-polylysine to antiviral effects against different virus infections of primates remains unresolved since the protection studies in mice reported here involved only one virus. However, the immunogenicity and rate of replication of a virus are likely to be important factors since a minimum virus concentration must be achieved for an adjuvant effect to contribute to protection and for pathological lesions to develop.

The methods for preparing carboxymethylcellulose-polylysine-complexed poly(I)·poly(C) described by Levy et al. (4) and Stebbing and Dawson (9) are slightly different. However, there is no reason to suspect that the pharmacological properties of the two materials, in primates, are different. Both materials induce essentially the same concentrations of interferon in primates, and the multiple treatment regimen, using our material, protects rhesus and squirrel monkeys against infection with yellow fever virus (our unpublished data). Moreover, our primate studies indicated variations in adjuvant effects because protection of rhesus monkeys was associated with increased antibody titers whereas protection of squirrel monkeys was associated with absence of detectable antibodies to yellow fever virus. Nevertheless, it is possible that the effects in mice of carboxymethylcellulose-polylysine-complexed poly(I)·poly(C), described here, do not occur in primates with the slightly different material described by Levy et al. (5, 7, 12; Stephen et al., Fed. Proc. 34:960, 1975).

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