Antigenicity of Double-Stranded Ribonucleic Acids Including Poly I:C (36310)

A. KIRK FIELD, ALFRED A. TYTELL, GEORGE P. LAMPSON, AND MAURICE R. HILLEMAN

Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania 19486

The synthetic double-stranded ribonucleic acid, poly I:C (a complex of polyriboinosinic and polyribocytidylic acids, $rI_n:rC_n$), is a potent inducer of interferon and interference against viral infections *in vivo* and *in vitro* (1–3). The drug is of special interest in human medicine since it induces interferon in man and displays a remarkable lack of toxicity on intravenous administration. Furthermore, no human subject administered poly I:C intravenously has developed detectable antibodies reactive with poly I:C or denatured DNA (4).

It has been indicated in several reports that double-stranded RNA's may induce antibody in rabbits when given with adjuvants (5, 6). As part of the assessment for safety in man, however, it has been considered important to investigate more fully the antigenicity of double-stranded RNA's, to characterize the biological and physical properties of antibodies reactive with double-stranded RNA, and to study the effect of these antibodies on the biological effects associated with administration of double-stranded RNA's. The present report describes the findings in studies of polynucleotide immunology which are pertinent to the application of poly I:C in human and animal medicine.

Materials and Methods. Nucleic acids. Poly I and poly C were synthesized by Drs. E. Harris and C. H. Hoffman of the Merck Developmental Research Group, according to the method of Hoffman *et al.* (7). Poly A and poly U were purchased from Miles Laboratories, Elkhart, Indiana. Polynucleotide solutions were prepared as previously described (1). MU9-ds-RNA, the replicative form double-stranded RNA of bacteriophage MU9, was prepared from MU9 infected *E. coli* cultures by Drs. B. Lago and J. Birnbaum of the Department of Fermentation Research of Merck, Sharp & Dohme Research Laboratories. Cytoplasmic polyhedrosis virus RNA (CPV-ds-RNA) and rice dwarf virus RNA (RDV-ds-RNA) were the gifts of Dr. K. Miura, Nagoya University, Nagoya, Japan. Calf thymus DNA and yeast RNA were purchased from Worthington Biochemical Corp., Freehold, NJ.

Animal immunization. Young, adult, New Zealand albino rabbits, ICR mice, Hartley strain albino guinea pigs and grivet monkeys were given five weekly injections of antigen. A sixth dose was administered two weeks later and the animals were bled one week following the last dose. Some of the polynucleotides used to immunize were coupled with methylated bovine serum albumin (MBSA) according to methods described by Plescia *et al.* (5) and Nahon *et al.* (6). Pertinent details concerning the immunizations and antigen preparations are given in the text.

Serology. Quantitative complement fixation (CF) tests for antigen and antibody were performed according to Osler *et al.* (8) employing four units of antigen or rabbit antiserum and five 50% hemolytic units of guinea pig complement. The mouse and guinea pig sera were inactivated at 56° for 60 min, while the rabbit and grivet monkey sera were treated for 30 min. The rabbit and grivet monkey sera were adsorbed at 4° for 30 min with 5% sheep erythrocytes. Antibody against poly I:C was also assayed by radioimmunoprecipitation as described by Steinberg *et al.* (9). As shown in Table I, the CF test was as sensitive or more sensitive than

TABLE I. Comparison of Rabbit Antibody to Poly I:C Tested by Complement-Fixation Test and by Radioactive Antigen Precipitation Test.

Rabbit no.	50% Endpoint[a] obtained by	
	Complement-fixation	Radioimmuno-precipitation
1	115	80
2	320	320
3	320	80
4	320	120

[a] Numbers represent the highest dilution of serum which gave 50% fixation of complement or 50% precipitation of labeled antigen.

the radioimmunoprecipitation assay for detecting rabbit antibody against poly I:C.

Molecular species of antibody. IgM and IgG antibodies from 0.5 ml samples of rabbit sera were separated chromatographically on G-200 Sephadex. Collected fractions were treated as described above and tested for CF activity. *Interferon assays* of rabbit sera were performed as described earlier (1).

Hematology. Total and differential leukocyte counts were determined before and 3.5 and 6 hr after injection of poly I:C into normal and immunized rabbits, using blood samples taken from the medial artery of the rabbit ear. Changes in cell population were noted relative to the concentrations measured at the beginning of each experiment, prior to first injection of poly I:C.

Pyrogenicity. Rectal temperatures in rabbits were measured by electronic temperature probe prior to and at hourly intervals for 6 hr after intravenous injection of poly I:C. The temperature index for each animal was the summation of temperature increments for all temperature determinations relative to the initial pretest temperature (10). Serum interferon determinations were performed using sera taken 6 hr after poly I:C injection.

Results. Antibody response to poly I:C in rabbits. Table II shows that nearly all rabbits developed antibody against poly I:C when given intravenously in repeated doses at 500 or 1,000 μg/animal. None of the animals responded to oral or nasal administration. Rabbits given poly I:C or MU9 double-stranded RNA coupled with MBSA responded with far greater antibody titer against the homologous antigen. Less than half the rabbits developed antibody, and in very low titer, when immunized with denatured DNA that was coupled with MBSA.

Failure of other animal species to develop antibody against poly I:C. Groups of 37 and 91 ICR mice were given weekly intravenous injections of poly I:C or poly I:C plus MBSA at a dose of 10,000 μg/kg. Similarly, 50 ICR mice were given weekly intranasal doses of poly I:C. Groups of 4 or 5 guinea pigs were given weekly doses at 4,000 μg/kg of poly I:C intranasally or intravenously, and groups of 5 monkeys were given weekly doses at 250–500 μg/kg of poly I:C in-

TABLE II. Antibody Response of Rabbits to Injection of Nucleic Acids.

Immunization procedure			CF antibody response	
Antigen	Dose (μg/animal)	Route	No. rabbits positive/no. tested	Geometric mean serum titer
Poly I:C	500	Intravenous	5/7	12
	1000	Intravenous	26/29	63
	1000	Oral or nasal	0/14	0
Poly I:C + MBSA	500	Intravenous	4/4	510
	1000	Intravenous	6/6	350
MU9-ds-RNA + MBSA	500	Intravenous	5/5	200
Denatured[a] DNA + MBSA	500	Intravenous	4/9	4.6

[a] Calf thymus DNA was denatured by heating to 120–125° for 15 min followed by quenching in an ethanol–dry ice bath.

TABLE III. Interferon Induction by Complexed Polyribonucleotides in Immunized and Non-Immunized Rabbits.

Immunization	Homologous geometric mean CF antibody titer at time of interferon induction	Serum interferon titer 2 hr after injection with homologous polynucleotide		
		0 μg	2.5 μg	10 μg
Poly I:C	320	0	4.8	33
Control	0	0	190	540
MU9-ds-RNA	110	0	0	3.2
Control	0	0	4.5	160

tranasally or intravenously. None of the sera taken one week following the sixth injection showed detectable CF antibody against poly I:C or denatured DNA. By contrast, all of 10 rabbits given 200 μg/kg of poly I:C developed CF antibody against poly I:C with a geometric mean CF titer of 1:170. In additional experiments, guinea pigs injected with 1,000 μg/kg dose of denatured calf thymus DNA or the same amount of DNA combined with an equal amount of poly I:C also failed to develop CF antibodies against either polynucleotide.

Altered biological responses in rabbits immunized against poly I:C. Interferon. Groups of rabbits were given repeated intravenous doses of poly I:C or MU9-ds-RNA coupled with MBSA to induce high levels of CF antibody against the polynucleotides. Rabbits given phosphate buffered saline solution in similar volumes and regimen served as controls. The animals from each treatment regimen were divided into three groups and given 0, 2.5, or 10 μg of homologous polynucleotide intravenously. Serum samples were taken 2 hr later and titrated for interferon content (Table III). The presence of high level circulating antibody greatly reduced but did not eliminate the interferon response to injection of the homologous polynucleotide. The suppression of interferon response in the immunized animals was probably due to the presence of specific antibody since the interferon-inducing capacity of poly I:C could be neutralized by exposure to anti-poly I:C antibody *in vitro*. Groups of four rabbits were injected with 2 μg poly I:C that had been treated for 2 hr with immune serum, purified anti-poly I:C antibody, normal serum or phosphate buffered saline (PBS). All three anti-poly I:C preparations caused a marked (10-fold or greater) reduction of interferon-inducing capacity of the poly I:C compared with the poly I:C controls as measured in the sera of rabbits 2 hr after injection (Table IV).

Hematology. Groups of 6 rabbits that were previously immunized with repeated doses of poly I:C (mean CF titer 1:230) or with PBS (controls) were given 100 μg of poly I:C intravenously and observed for hematological changes (Fig. 1). The poly I:C caused marked lymphocytopenia in both the poly I:C immunized and control animals observed

TABLE IV. Neutralization of Interferon-Inducing Capacity of Poly I:C by Antibody Against Poly I:C.

Treatment of poly I:C[a] (2 μg/dose)		Geometric mean rabbit serum interferon titer at 2 hr (4 rabbits/group)
Substance	Anti-poly I:C antibody titer	
Buffered saline	—	190
Normal rabbit serum	0	320
Immune rabbit serum	230	13
Poly I:C IgM antibody	95	10
Poly I:C IgG antibody	320	10
None	—	0[b]

[a] Poly I:C incubated for 2 hr at 4° with indicated substance before intravenous injection into rabbits.

[b] Normal rabbit serum.

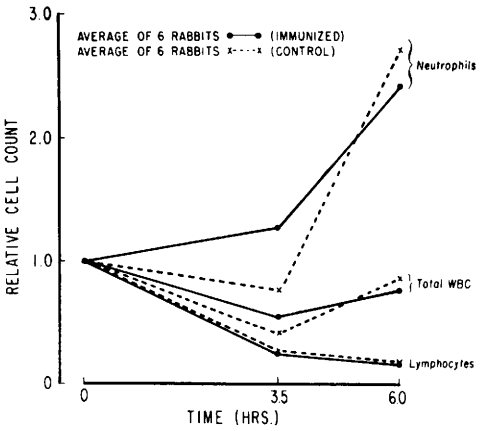

FIG. 1. Influence of previous poly I:C immunization of rabbits on leukocytic response to 100 μg of poly I:C given intravenously.

at 3.5 and 6 hr after injection (Fig. 1). This change was accompanied by a 20% decrease in neutrophils in the controls and 25% increase in the test animals at 3.5 hr; by 6 hr both groups of animals displayed marked neutrophilia.

Pyrogenic response. The fever response curves in the same rabbits employed in the hematology study (Fig. 1) are shown in Fig. 2. There was only slightly less fever in the immunized rabbits on poly I:C injection compared with the controls even though the interferon response was markedly suppressed in the previously vaccinated animals with high serum antibody titer against poly I:C.

Characterization of poly I:C antisera prepared in rabbits. Characteristics of antibody. The sera from rabbits (see Table II) immunized with poly I:C or with poly I:C coupled with MBSA were fractionated and tested for content of IgM or IgG antibodies. Table V shows that the antibody response to poly I:C was primarily of the IgM type while that prepared from animals immunized with poly I:C + MBSA was predominantly IgG. Repeated courses of injection with poly I:C resulted in a shift in the antibody response from IgM to IgG and was accompanied also by the development of antibody against poly I, which was not detectable after the first course of immunization.

Specificity of antisera. Individual sera

TABLE V. IgM and IgG Antibody Responses in Rabbits Immunized with Poly I:C or Poly I:C Coupled with Methylated Bovine Serum Albumin.

Antigen	μg/Animal	Anti-poly I:C CF antibody titer of fractions[a] IgM	IgG
Poly I:C	1000	39	5
Poly I:C + MBSA	500	100	380

[a] Geometric mean titer of fractions separated by chromatography on G-200 Sephadex.

from 10 rabbits immunized with poly I:C were tested for capacity to react with homologous and heterologous nucleic acids. The sera were each used at four H_{50} units when tested for complement fixation with 0.1 μg poly I:C. To conserve space, the data are not given in tabular form. Nine sera were capable of detecting less than 1 μg double-stranded RNA (poly I:C, poly A:U, MU9-ds-RNA). One serum sample did not react with MU9-ds-RNA. Six sera were also capable of detecting as little as 0.02 μg poly I or greater. The remaining four sera were capable of detecting 5 μg/ml poly I or more. None of the 10 sera were capable of detecting poly C, yeast RNA or calf thymus DNA at 10 μg/ml or less.

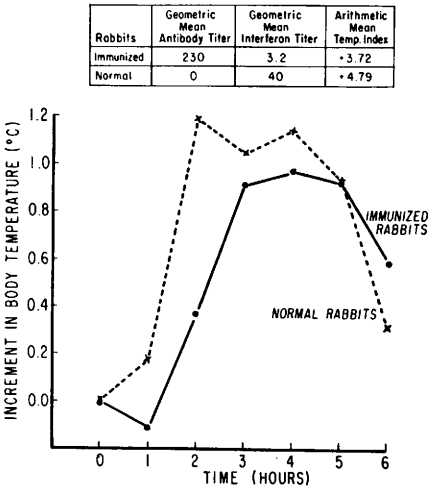

FIG. 2. Influence of previous poly I:C immunization of rabbits on the febrile response to 2 μg poly I:C given intravenously.

TABLE VI. Cross Reactivity of Rabbit Polynucleotide Antisera with Polynucleotide Antigens.

Antigen tested		Minimum antigen detected (μg/ml)[a] using antisera prepared against			
Kind	Structure	Poly I:C	Poly I:C + MBSA	Poly I + MBSA	Denatured calf thymus DNA + MBSA
Poly I:C	ds[b]-RNA	0.02–0.04	0.02–0.08	0.04	>10
Poly I	ss-RNA	>5	0.008–0.02	0.02	0.08
Poly C	ss-RNA	>10	>10	>10	>10
Poly A:U	ds-RNA	0.04–0.08	0.04–0.1	>10	>10
Poly A	ss-RNA	>10	>10	>10	0.16
Poly U	ss-RNA	>10	>10	>10	>10
MU9-ds-RNA	ds-RNA	0.04–0.08	0.08–0.4	>10	>10
Denatured MU9-ds-RNA	ss-RNA	5	2–5	ND[c]	ND
CPV-ds-RNA	ds-RNA	ND	0.06	>10	ND
Denatured CPV-ds-RNA	ss-RNA	ND	0.3	ND	ND
RDV-ds-RNA	ds-RNA	ND	0.08	>10	ND
Yeast RNA	ss-RNA	>10	>10	>10	5.0
Calf thymus DNA	ds-DNA	>10	>10	>10	1.25
Denatured calf thymus DNA	ss-DNA	>10	>10	>10	0.02

[a] Antigen titrations were carried out against $4H_{50}$ units of antiserum, as determined by reaction with the homologous antigen.

[b] ss = Single strand; ds = Double strand.

[c] ND = Not done.

In further studies, rabbit antisera prepared by immunizing with four different nucleic acid preparations were tested with 14 different antigens (Table VI). Antisera prepared against poly I:C plus MBSA or poly I:C were capable of detecting submicrogram quantities of double-stranded RNA (poly I:C, poly A:U, MU9-ds-RNA, CPV-ds-RNA, RDV-ds-RNA). The dependence of reactivity on the helical structure was evidenced by the greater than 10-fold reduction upon denaturation of double-stranded MU9 and CPV RNA's. Whereas the antiserum prepared against poly I:C plus MBSA was strongly reactive with poly I, only poor reactivity was observed for antiserum prepared against poly I:C. No reactivity with any other single-stranded RNA, native or denatured DNA was observed. This was further emphasized by the lack of reactivity with yeast sRNA, liver sRNA, tobacco mosaic virus RNA, poly G, poly X, hypoxanthine, inosine, $(I_p)_2I$, cytidylic acid or poly dAT (not shown in Table VI).

Antiserum prepared from rabbits immunized with poly I plus MBSA was reactive with poly I and poly I:C, but not with any other single-stranded or double-stranded RNA or with native or denatured DNA.

Antiserum prepared from rabbits immunized with denatured calf thymus DNA plus MBSA could detect 0.1 μg or less of denatured DNA, poly I and poly A, 1.25 μg native DNA/ml, and 5 μg yeast RNA/ml. However, this antiserum was unreactive with double-stranded RNA's (poly I:C, poly A:U, MU9-ds-RNA), and the polypyrimidines tested (poly C, poly U). These data indicate that little or no cross reactivity existed between rabbit antibodies to double-stranded

RNA's and antibodies to DNA.

Discussion. The present studies give precise definition to the antigenicity of poly I:C and to the antisera prepared against double-stranded polynucleotides. Antigen–antibody reactions could be measured either by the complement-fixation test or by the radioimmunoprecipitation assay. The CF test was either as sensitive or more sensitive than the precipitation assay and was thus employed routinely.

The findings showed distinct differences in the capacity of various animals to respond immunologically to intravenous injection of poly I:C. Poly I:C was antigenic in New Zealand albino rabbits. Talal and Steinberg previously reported the antigenicity of poly I:C in New Zealand black and white mice (11). Failure of antigenic response was noted in the present studies in ICR mice, guinea pigs, and grivet monkeys. Talal and Steinberg (11) reported lack of response in C_3H/He, C_{57}Bl/6 and BALB/c mice and Field *et al.* (4) recorded lack of antibody stimulation in human subjects. None of the rabbits in the present studies produced circulating anti-poly I:C antibodies in response to oral or nasal administration of poly I:C.

Antibody prepared against poly I:C either alone or coupled with MBSA reacted only with double-stranded RNA's and not with single-stranded RNA's or with DNA. The one exception was the variable reactivity with poly I. The only serological difference observed between use of poly I:C and poly I:C plus MBSA as an immunogen given intravenously was the relatively poor reactivity of anti-poly I:C sera with poly I. Nahon *et al.* (6) have also reported that antisera prepared from rabbits immunized with poly I:C plus MBSA was reactive with poly I alone and not reactive with poly C, poly A, poly U or a variety of DNA's. However, in contrast to our results, they did find reaction with a variety of single-stranded RNA's. Yet Schwartz and Stollar (12), using antisera prepared from rabbits injected with poly A:U plus MBSA, and Schur and Monroe (13), using antisera prepared from rabbits injected with poly I:C plus MBSA observed the same lack of reactivity with DNA or single-stranded RNA, and recognized the specificity of reaction with double-stranded RNA's. In our studies denaturation of double-stranded RNA's (MU9-ds-RNA and CPV-ds-RNA) rendered them less reactive with poly I:C antiserum and demonstrated the importance of retaining the double-helical structure.

Antiserum prepared against poly I reacted with poly I and with poly I:C but not with other single-stranded or double-stranded RNA's which did not contain the poly I strand. Antibody against denatured calf thymus DNA reacted strongly with homologous antigen, poly I, poly A, and poorly with yeast RNA and native DNA.

The antibody produced in rabbits against poly I:C was predominantly of the IgM class but this was shifted to IgG on prolonged repeated administration. Complexing of poly I:C with MBSA resulted in predominantly IgG antibody with higher titer.

Prior immunization of rabbits with poly I:C or MU9-ds-RNA rendered them far less responsive to induction of interferon. This was likely due to circulating antibody since such suppression of effect was obtained when poly I:C was neutralized with whole poly I:C antiserum or with separated IgM or IgG antibodies against the polynucleotide. Similar suppression of interferon response to poly I:C was also reported by Steinberg *et al.* (14) in NZB/NZW mice immunized with poly I:C. Suppression of interferon stimulation was marked but was never total, as indicated by low level response in the rabbits. Unlike the interferon induction, circulating antibody had little or no effect on the alteration of circulating leukocyte population produced by poly I:C or on the pyrogenic reaction to poly I:C given intravenously, suggesting that both these responses are not directly related to the interferon inducing response to poly I:C (15).

The findings in the present study are of importance in providing orientation for clinical trials of induction of interferon and resistance to viral infections by poly I:C. Antibody against double-stranded RNA's may be implicated in patients with systemic lupus, rheumatoid arthritis and Sjogrens syndrome (13), and one would not wish to stim-

ulate such antibody in human subjects. New Zealand albino rabbits and New Zealand mice appeared immunologically responsive to poly I:C, but only when the drug is given parenterally. Humans have not responded immunologically even when large amounts were given in repeated doses by the intravenous route. The data give added assurance of safety for intranasal use of poly I:C in man.

Summary. Rabbits repeatedly injected intravenously with poly I:C developed antibodies reactive with poly I:C. However, oral or nasal administration of poly I:C to rabbits failed to stimulate detectable circulating antibodies. Intravenous injection of ICR mice, guinea pigs, and grivet monkeys also failed to stimulate a detectable circulating antibody. Combination of poly I:C with methylated bovine serum albumin enhanced the antigenicity of poly I:C in rabbits. Antibodies produced in response to poly I:C were primarily the IgM type, whereas those produced in response to poly I:C plus methylated bovine serum albumin were mainly the IgG type. Hyperimmunization with poly I:C resulted in shift to predominance of IgG antibodies.

Rabbits with high antibody titers to poly I:C showed reduced serum interferon production in response to induction with poly I:C. However, blood leukocyte alterations and febrile responses to poly I:C in immunized and normal rabbits were essentially similar. Both the IgM and IgG antibodies to poly I:C were capable of neutralizing the capacity of poly I:C to induce interferon in unimmunized rabbits.

Antisera reactive with poly I:C were similarly reactive with other double-stranded RNA's, were variably reactive with poly I, but were unreactive with other single-stranded RNA's or DNA.

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