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Research Article

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Inhibition of Antibodies Binding Polyinosinic·Polycytidylic Acid in Human and Mouse Lupus Sera by Viral and Synthetic Ribonucleic Acids

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ABSTRACT The specificities of anti-RNA antibodies of diverse origin were studied by inhibition of the binding of radioative polyinosinic·polycytidylic acid. The antibodies were from human patients with systemic lupus erythematosus (SLE), older NZB/NZW F_1 mice who have SLE, and young NZB/NZW F_1 mice immunized with either synthetic or viral double-stranded (ds) RNA. The inhibitors were two viral ds and two synthetic ds RNAs, ribosomal RNA and transfer RNA. The human sera were more heterogeneous than the mouse lupus sera, and had greatest specificity for reovirus RNA. The mouse lupus sera were more homogeneous and, in general, were inhibited efficiently by all four ds RNAs. Sera from mice immunized with synthetic RNA reacted poorly with viral RNA, whereas sera from mice immunized with viral RNA reacted with all four ds RNAs and resembled the lupus sera. These results suggest a role for viruses in the induction of anti-RNA antibodies, and are compatible with the concept that virus infection as well as excessive antibody responses are involved in the pathogenesis of SLE.

INTRODUCTION

Antibodies to double-stranded (ds)[1] DNA and RNA occur with great frequency in patients with systemic lupus erythematosus (SLE) and rarely in patients with other connective tissue disorders (1–3). These antibodies are also present in New Zealand mice, particularly the NZB/NZW F_1 hybrid (B/W) which is an animal model for human SLE (1). Other antibodies reacting with single-stranded DNA and a variety of cellular components are found in lupus and in other connective tissue diseases.

It is possible to induce anti-RNA antibodies in normal strains of mice immunized with synthetic polyribonucleotides given in adjuvant but without protein carrier (4). Rabbits can be immunized with RNA complexed to protein carrier (5). Experimental immunization with native DNA has so far been unsuccessful, even when the DNA is complexed to protein.

This study attempts to explore the mechanism underlying the spontaneous formation of these anti-nucleic acid antibodies in SLE. Some clues to this problem can be sought in the antibody molecules themselves, since their specificity should reflect in mirror image the nature of the immunizing antigen.

We have studied this question using four different sets of anti-RNA antibodies. One set of antibodies came from patients with SLE and a second from older B/W mice with lupus. Both represent antibodies induced by naturally occurring immunogens during the course of disease. A third set of antibodies was prepared by immunizing young B/W mice with either polyinosinic·polycytidylic acid (rI·rC) or polyadenylic·polyuridylic acid (rA·rU). A fourth was prepared by immunizing young B/W mice with a viral ds RNA prepared from a fungal phage (mycophage). These latter two groups represent antibodies induced artificially by immunization.

The specificity of these four different sets of antibodies was studied by inhibiting the binding of radioactive RNA with various nucleic acids. Each of the four groups showed distinctive patterns of inhibition.

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[1] *Abbreviations used in this paper:* ds RNA and DNA, double-stranded RNA and DNA; rA·rU, polyadenylic·polyuridylic acid; rI·rC, polyinosinic·polycytidylic acid; SLE, systemic lupus erythematosus.

METHODS

Materials. Sera were obtained from human lupus patients and from NZB/NZW F_1 mice. The mice were bled by orbital sinus puncture. Sera were stored frozen at −20°C until studied. Heating of sera at 56°C for 30 min had no effect on RNA binding and this step was omitted.

Nonradioactive rI·rC and rA·rU, and ^{14}C-rI·rC were purchased from Miles Laboratories, Inc., Elkhart, Ind. ^{14}C-KB cell DNA was prepared as previously described (6). Ribosomal RNA was prepared by phenol extraction from rat liver (7). Rat liver, E. coli, and yeast transfer RNAs were given to us by Dr. Bernard Weinstein of Columbia University, New York. Reovirus ds RNA was a gift from Dr. George Acs of the Institute for Muscle Disease and mycophage ds RNA (from *Penicillium chrysogenum*) was a gift from Dr. K. W. Buck of the Imperial College of Science and Technology, London, England.

Immunization. 4–6-wk old female NZB/NZW F_1 mice were immunized intraperitoneally with three different ds RNAs. Equal volumes of rI·rC, rA·rU, or mycophage RNA (1 mg/ml) and complete Freund's adjuvant were emulsified and 0.3 ml of the mixture injected. The mice were bled 2 wk later for assay of anti-RNA antibodies.

Binding assay. The ammonium sulfate precipitation assay was used throughout. Serial dilutions of sera were incubated with 80 mμg of ^{14}C-rI·rC and the antigen–antibody complex precipitated by 35% ammonium sulfate as previously described (1). Dilutions of sera that gave 40–86% binding were then used for inhibition studies. This guaranteed that reactions were taking place on the linear part of the binding curve. For the human lupus sera, the mean binding was 62% (range 40–86); for the mouse lupus sera, the mean binding was 64% (range 41–86).

To detect antibodies to DNA, 50 mμg of ^{14}C-KB cell DNA was used and precipitation was achieved with 50% ammonium sulfate (1).

Inhibition assay. The appropriate dilution of serum was then incubated with or without one of the RNA inhibitors in borate buffer (pH 8.0) for 30 min at 37°C. In some experiments a 4-fold excess of inhibitor was used and the results were expressed as per cent inhibition. In the majority of experiments, four different concentrations of inhibitor (varying from 30 to 6000 mμg) were used. 80 mμg of ^{14}C-rI·rC was added and a second 30 min incubation at 37°C followed. After overnight incubation at 4°C, an equal volume (0.1 ml) of ammonium sulfate was added to bring the final concentration to 35%. The tubes were kept at 4°C for 1 hr before separation of precipitate and supernatant fractions by centrifugation at 11,500 *g* for 7.5 mins. Each fraction was placed in Bray's solution and radioactivity determined in a Nuclear Chicago liquid scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill.) The per cent of bound antigen was determined for each point and from that the per cent of inhibition of binding for each concentration of inhibitor was calculated. The per cent inhibition was plotted against the $\log_{10}$ of the inhibitor concentration and that concentration giving 50% inhibition was calculated. This concentration was called the I_{50} for the serum under study.

Absorption. A human lupus serum was divided into four 1 ml portions. A slight excess of antigen (either calf thymus DNA, rI·rC, or rA·rU) was added to each of three samples while the fourth was carried through the procedure as a control. After incubation at 37°C for 1 hr and at 4°C for 3 days, the precipitates were removed by centrifugation at 11,500 *g*. Each supernatant was again absorbed with the same antigen for 4 days at 4°C. The supernatants recovered after a second centrifugation were assayed for ^{14}C-rI·rC binding.

RESULTS

Antibodies to ds RNA and to ds DNA are predominantly different molecular populations. Six human lupus sera containing both antibody activities were studied in a differential inhibition experiment. The binding of radioactive DNA by antibody was inhibited 82% by prior incubation with a 4-fold excess of nonradioactive DNA, but only 15% by prior incubation with rI·rC. Conversely, the binding of radioactive rI·rC was inhibited 57% by prior incubation with rI·rC and only 6% by DNA. The reduction of rI·rC binding would have been even greater if viral ds RNA was used as the competitive inhibitor (*vide infra*).

One serum was absorbed with either rI·rC or DNA in an attempt to remove the antibodies to ds RNA. Ab-

TABLE I

Comparative Ability of Different RNAs to Inhibit ^{14}C-rI·rC Binding by Human Lupus Sera

	Per cent inhibition by:							
					Ribosomal RNA	Transfer RNA	Transfer RNA	Transfer RNA
Serum	Reo	Phage	rI.rC	rA.rU	Rat liver	Rat liver	*E. coli*	Yeast
LN	100	100	86	81	42	32	32	25
IP	100	81	16	36	17	4	10	0
HR	100	62	77	36	54	21	37	23
BB	100	57	52	15	21	0	0	0
BD	91	51	21	42	7	0	0	0
AB	85	67	45	62	18	2	11	2
ML	74	62	23	93	35	3	17	2
GG	68	31	84	15	11	13	8	3
BA	47	59	25	30	19	3	0	8
Mean	85	63	47	45	24	8	12	7

TABLE II

Inhibition of Mouse Lupus Sera Binding ^{14}C-rI·rC

Serum	Binding by uninhibited serum	I_{50} (50% inhibitor concentration, mμg)			
		Reo	Phage	rI·rC	rA·rU
	%				
168	72	30	160	210	60
59	41	30	170	200	N.S.
172	71	95	220	200	180
92	43	120	N.S.	640	500
58	67	170	230	250	230
187	57	150	210	900	*
96	42	150	N.S.	150	30
93	55	150	N.S.	500	*
178	60	150	*	30	22
118	66	200	420	550	30
100	84	200	N.S.	730	10
197	84	200	560	1200	1200
159	86	220	480	1300	540
64	49	300	300	170	300
31	66	950	*	250	350
188	85	950	*	1200	950
Median I_{50}		150	360	375	300
% of serum giving I_{50}		100	75	100	87

* 50% inhibition was not achieved at a 75-fold excess of inhibitor.
N.S. = not studied.

sorption with the synthetic ds RNA reduced the binding of ^{14}C-rI·rC 70%, whereas absorption with DNA resulted in only a 16% reduction in ^{14}C rI·rC binding.

The binding of ^{14}C-rI·rC by nine human lupus sera was studied by competitive inhibition using a 4-fold excess of two synthetic ds RNAs (rI·rC and rA·rU) and six naturally occurring RNAs. Two were viral (reovirus and mycophage), one was ribosomal, and three were transfer RNAs of diverse origins. The ds viral RNAs showed the most complete inhibition (Table I). In general, the synthetic ds RNAs were the second most efficient inhibitors, although three sera (HR, BB, ML) were inhibited as well by mammalian ribosomal RNA as by one of the synthetic RNAs. Ribosomal RNA is single-stranded whereas double-stranded regions occur in the structure of transfer RNA. Simple nucleotides, polyribonucleotides, nucleosides, or free bases failed to inhibit.

The comparative inhibition by viral and synthetic ds RNA was explored further in a study involving mouse as well as human antibodies to ds RNA. The NZB/NZW F_1 mice spontaneously develop SLE and produce antibodies that bind ^{14}C-rI·rC during the course of their disease. 16 sera from such mice spontaneously making antibodies to RNA were compared with 18 human lupus sera. Four inhibitors (reovirus and phage ds RNA, rI·rC, and rA·rU) were studied at several dilutions to permit a determination of the amount of inhibitor RNA that would result in a 50% reduction of ^{14}C-rI·rC binding (called the I_{50}). The data are presented in Tables II and III. The per cent binding by uninhibited serum is included for comparison. In general, the mouse antibodies were well inhibited by all four ds RNAs at the concentrations used. An I_{50} could be determined for virtually each inhibitor and each serum. The human antibodies were much more heterogeneous and more difficult to inhibit. The median I_{50} was higher than for the mouse sera, ranging from 750 to > 6000 mμg. Reovirus RNA was the best inhibitor, and an I_{50} could be determined for 72% of the sera. However, even at a great excess of inhibitor, 45% or more of the sera were not inhibited 50% by the three other ds RNAs. Only three human sera (LN, AB, DD) resembled the mice in that all four inhibitors competed efficiently to prevent binding of ^{14}C-rI·rC.

TABLE III

Inhibition of Human Lupus Sera Binding ^{14}C-rI·rC

Serum	Binding by uninhibited serum	I_{50} (50% inhibitor concentration, mμg)			
		Reo	Phage	rI·rC	rA·rU
	%				
LN	46	170	550	400	150
AB	80	220	1200	340	280
IP	42	225	750	*	*
HR	74	240	3500	560	*
BD	80	500	3700	*	*
BB	83	600	800	3000	*
BA	86	600	2500	*	*
DD	40	700	1000	600	30
DB	44	700	*	*	*
ML	56	800	*	*	700
BK	78	900	1400	*	5000
GG	70	3000	*	1050	*
MO	68	4500	*	210	*
PR	40	*	*	600	3000
AS	50	*	*	1100	*
MW	82	*	*	6000	3000
AC	41	*	*	*	*
AF	52	*	*	*	*
Median I_{50}		750	>3700	4500	>6000
% of serum giving I_{50}		72	50	55	39

* 50% inhibition was not achieved at a 75-fold excess of inhibitor.

One serum (MO), which was inhibited by rI·rC but not by rA·rU, was exhaustively absorbed with either rI·rC or rA·rU. Absorption with the former reduced binding by 70%, whereas absorption with rA·rU did not reduce binding at all. This experiment indicates agreement between the inhibition and absorption methods for determining antibody specificity.

4–6-wk old NZB/NZW F_1 mice are clinically free of disease and do not have serum antibodies to ds RNA. We immunized such mice with rI·rC, rA·rU, or viral ds RNA in Freund's adjuvant. Most mice so treated produced antibodies which bound ^{14}C-rI·rC and could therefore be studied in a competitive inhibition assay. The cumulative data are presented in Table IV. Mice immunized with phage RNA (group 1) produced antibodies that were inhibited well by both naturally occurring and synthetic ds RNAs. An I_{50} could be determined for most sera. In this respect they had properties that seemed intermediate between those of antibodies appearing in mice and humans afflicted with lupus (groups 4 and 5). By contrast, most mice immunized with either rI·rC (group 2) or rA·rU (group 3) produced antibodies that were inhibited efficiently only by synthetic ds RNA. For example, the mean inhibition by reovirus RNA was 20% for the 20 such sera failing to give an I_{50}, i.e., 10 sera from group 2 and 10 sera from group 3 in Table IV. In spite of the genetically homogeneous background of the mice and the immunologic cross-reactivity of ds RNAs, the specificities of antibodies produced in response to viral ds RNA are remarkably different from those induced by synthetic ds RNA.

TABLE IV

Inhibition of Antibodies to RNA Induced by Immunization or Appearing Spontaneously in Lupus

Group	Immunized with	Number of sera giving I_{50}* / Number of sera studied			
		Reo	Phage	rI·rC	rA·rU
1	Phage	10/13	6/10	9/13	11/13
2	rI·rC	2/12	1/8	12/12	10/12
3	rA·rU	3/13	1/7	11/13	12/13
4	Human lupus	13/18	9/18	10/18	7/18
5	Mouse lupus	16/16	9/12	16/16	13/15

* I_{50} could be determined at a 75-fold excess of inhibitor.

DISCUSSION

50% or more of patients with SLE and B/W mice have antibodies to ds DNA or RNA as detected by the ammonium sulfate precipitation method. The present study was designed to investigate the specificity of the anti-RNA antibodies and indirectly the nature of the immunogenic RNA.

These antibodies are inhibited relatively poorly by ribosomal and transfer RNA, suggesting that these mammalian RNAs may not be the naturally occurring immunogens. The anti-RNA antibodies react better with two viral and two synthetic ds RNAs, although individual differences exist. The mouse sera are more homogeneous than the human, suggesting a similarity of antibody specificity. This can reflect either a uniform genetic background, a common immunologic stimulus, or both.

The New Zealand mice are highly inbred and therefore of a more similar genetic composition than the outbred human lupus population. Therefore, it was necessary to show that the specificity of anti-RNA antibodies produced by New Zealand mice was not simply dependent upon genetic factors but could be influenced by the nature of the immunizing RNA. This was shown by immunization of young New Zealand mice with either synthetic or viral RNAs. The specificity of these antisera were strikingly dissimilar. Antisera induced by synthetic RNA reacted very poorly with viral ds RNA. Antisera induced by viral RNA resembled in specificity the antibodies that occur spontaneously in human and mouse lupus.

The immunologic stimulus for the spontaneous antibodies might be a viral ds RNA or a ds RNA induced in mammalian cells as a consequence of viral infection (8). New Zealand mice harbor the RNA-containing murine leukemia virus (9). Some RNA viruses (such as reovirus and mycophage) have ds RNA in their virions. Others are single stranded but produce a ds replicative form during their reproductive cycle. Still others (including the murine leukemia viruses) replicate by an unusual mechanism in which DNA copies of the RNA are synthesized by an RNA-dependent DNA polymerase (10, 11). These various nucleic acid complexes might become immunogenic, particularly in a host that is easily immunized by an antigen of this nature. We have previously shown that B/W mice are immunologically hyperresponsive and make antibody to rI·rC given without adjuvant (1, 4).

If the relative homogeneity of the mouse lupus sera is taken to reflect a common immunologic stimulus, what implications can be drawn from the greater heterogeneity of the human lupus sera? Do the differences in antibody specificity reflect different immunologic stimuli, possibly different viral RNAs that are immunogenic in different individuals? Lupus patients have high titers of anti-viral serum antibodies (12), suggesting that they may be immunologically hyperreactive to viral antigens. Their kidneys contain endothelial cytoplasmic inclusions (13, 14) suggestive of virus infection. In the present report, the

lupus sera were most reactive with reovirus ds RNA. These findings, taken together, are compatible with the concept that virus infection as well as excessive antibody responses to nucleic acids are implicated in the pathogenesis of SLE. They suggest but by no means prove that viral RNA may induce the formation of anti-nucleic acid antibodies.

Previous studies with human lupus anti-RNA antibodies showed precipitation with viral as well as synthetic ds RNA but not with ribosomal or transfer RNA (2). Stollar found that rabbit anti-RNA antibodies and human lupus anti-DNA antibodies cross-reacted with RNA : DNA hybrids, although antibodies raised against the hybrids did not react with ds RNA or DNA (15). Another study showed that rabbit antibodies to rA·rU precipitated with some DNAs (5).

The I_{50} for any serum and inhibitor is a complex function dependent upon the binding characteristics of the antibody for rI·rC and the molecular configuration of the inhibitor RNA. In preliminary experiments we observed in the direct binding assay that antigen-binding curves, i.e. per cent of ^{14}C-rI·rC bound vs. $\log_5$ of antiserum dilution, varied in slope. A relatively steep curve correlated with greater strength of binding and a low I_{50} in subsequent inhibition studies. A serum giving a steep curve was inhibited even when the nonradioactive RNA was added after the ^{14}C-rI·rC. A relatively flat curve correlated with weaker binding and such sera were poorly inhibited giving a high I_{50}. Such sera were not inhibited when the nonradioactive RNA was added after the ^{14}C-rI·rC. A serum with high capacity for rI·rC but a flat binding curve might produce the occasionally noted paradox of ^{14}C-rI·rC binding with poor inhibition by rI·rC.

The efficient inhibition by reovirus RNA is all the more remarkable because sera were selected on the basis of rI·rC binding. Despite the selection against reovirus RNA, the latter was a very good inhibitor. An even greater specificity for virus RNA might be demonstrated when radioactive viral RNA becomes available.

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