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Determination of Antibodies to Double-Stranded RNA by Enzyme-Linked Immunosorbent Assay (ELISA) (40909)

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Abstract. A sensitive enzyme-linked immunosorbent assay (ELISA) for antibodies to double-stranded RNA has been developed using polyinosinic acid·polycytidylic acid ($I_n \cdot C_n$) as antigen. Bound antibody was detected using alkaline phosphatase-conjugated goat anti-immunoglobulin and *p*-nitrophenylphosphate as substrate. The assay, as demonstrated for rabbit, NZB/NZW mouse, and grivet monkey sera, was far more sensitive and reproducible than quantitative complement fixation. $I_n \cdot C_n$ complexed with poly-L-lysine and adsorbed to polystyrene tubes resulted in optimal uptake of antibodies to double-stranded RNA. $I_n \cdot C_n$ without added poly-L-lysine or with other cationic substances adsorbed poorly, as measured by reduced antibody uptake. Specificity of the assay for antibodies to double-stranded RNA was demonstrated by reduction of antibody binding to antigen-coated tubes following adsorption of sera with double-stranded, but not single-stranded RNAs. Similar adsorption studies indicated that this method could be used also to assay for double-stranded RNA. Therefore, a simple ELISA can be used for quantitative determination of either antibodies to double-stranded RNA or double-stranded RNA antigen.

Double-stranded RNAs (dsRNA) have been the subject of considerable interest because of their diverse biological activities. They occur as replicative intermediates of certain viruses and as such act as inducers of interferon synthesis (1). They have been shown to alter immunological functions including macrophage-mediated resistance (2), as well as antibody and cell-mediated immune responses (3). In addition, the occurrence of spontaneous antibodies to dsRNA in NZB/NZW mice and in cases of human systemic lupus erythematosus has indicated that viruses and their replicative dsRNAs may be important in autoimmune disease development (4).

Double-stranded RNAs are potent interferon inducers in animals and man (5–7). As a result, dsRNA preparations are undergoing clinical evaluation as antiviral substances (7–9). Future evaluations should also include the potential for stimulation of antibodies, since antibodies neutralize the interferon-inducing capacity of dsRNA (10).

The present methods available for quantitation of antibodies to dsRNA are of two types—complement fixation and radioimmunoassay. The complement fixation assays are reliable, but suffer from interference by anticomplementary activity and are relatively insensitive. Radioimmunoassays are highly sensitive, but are more cumbersome, are dependent on more expensive equipment, and produce radioactive waste materials.

The need for simple, highly sensitive and specific methods for either quantitation of antibodies to dsRNA, or detection of nanogram amounts of dsRNA can be satisfied by an enzyme-linked immunosorbent assay (ELISA). The present report describes the development and use of such an assay.

Materials and methods. Antigens. Polyinosinic acid (I_n) and polycytidylic acid (C_n) were prepared at the Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey (11). Polynucleotide characteristics and complexing to form $I_n \cdot C_n$ were previously described by Field *et al.* (12). Polyadenylic acid (A_n) and polyuradylic acid (U_n) were purchased from Miles Laboratories, Elkhart, Indiana. MU9dsRNA, the replicative form dsRNA derived from MU9, an amber-coat protein mutant of MS2 bacteriophage, was previously described by Lago *et al.* (13).

Antisera. Rabbit sera to double-stranded

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RNA were prepared by repeated weekly intravenous injections of young adult New Zealand white rabbits with 1 mg $I_n \cdot C_n$. Serum reactivity was previously described by Field *et al.* (14). NZB/NZW mouse serum pools were obtained from untreated young (1 month) or old (12+ months) mice. NZB/NZW hybrid mice were obtained by mating NZB males and NZW females from colonies of inbred NZB and NZW mice maintained at the Merck, Sharp & Dohme Research Laboratories. Development of the autoimmune disease syndrome, including production of antibodies to $I_n \cdot C_n$ has been previously characterized (15). Grivet monkey (*Cercopithicus aethiops*) sera to double-stranded RNA were prepared by repeated weekly intravenous injections of young adult grivet monkeys with 1 mg $I_n \cdot C_n$ complexed with 0.5 mg methylated bovine serum albumin according to the methods of Plescia *et al.* (16).

Preparation of alkaline phosphatase-conjugated goat antisera. Goat IgG anti-rabbit, anti-mouse, and anti-monkey immunoglobulins were obtained from Cappel Laboratories, Inc., Downingtown, Pennsylvania. These included goat anti-rabbit immunoglobulins (IgG, IgA, IgM), goat anti-mouse IgG, and goat anti-monkey IgG. Conjugation of these antisera to alkaline phosphatase was done essentially as described by Engvall and Perlmann (17). In brief, one part by volume goat IgG (as furnished by the supplier) was mixed with six to eight parts pelleted alkaline phosphatase type VII (Cat. No. P-4502, Sigma Chemical Co., St. Louis, Mo.). After overnight dialysis at 4° against phosphate-buffered saline (PBS), glutaraldehyde (E. M. Grade, Polysciences, Inc., Warrington, Pa.) was added to a final concentration of 0.2%. The preparation was further dialyzed overnight against one change of PBS at 4° and finally against boric acid–sodium azide buffer, pH 7.85. Conjugated goat IgG preparations were stored at 4° in the dark and diluted just prior to use.

Antigen-coated tubes. $I_n \cdot C_n$ was combined in a 1.3:1 ratio (by weight) with poly-L-lysine (Sigma Chemical Co., mol wt $15-30 \times 10^3$) in PBS. One milliliter containing 1 μg $I_n \cdot C_n$ was adsorbed to each 10 × 75-mm polystyrene tube (Falcon Div. of Becton, Dickinson Co., Oxnard, Calif.) overnight at 37°, and subsequently washed three times with cold PBS containing 0.05% Tween 20. Antigen-coated tubes were stored dry at 4°.

Enzyme immunoassay. The following buffers were used in the ELISA:

buffer A—PBS containing 0.05% Tween 20 and 0.05% bovine serum albumin, fraction V (Reheis Chemical Co., Phoenix, Ariz.), pH 7.4;

buffer B—PBS containing 0.05% Tween 20, pH 7.4;

buffer C—0.05 M sodium carbonate containing 0.001 M $MgCl_2$, pH 9.8.

Test rabbit sera were diluted in buffer A. Antigen-coated tubes received 1 ml of diluted serum and were incubated at 37° for 30 min. Following three washes with buffer B, tubes received 1 ml enzyme-conjugated goat anti-rabbit immunoglobulins (IgG, IgM, IgA) freshly diluted in buffer A and were incubated at 37° for 30 min. The tubes were then washed twice with buffer B and finally washed with buffer C. The amount of alkaline phosphatase bound to each tube was determined by addition of 1 ml containing 1 mg *p*-nitrophenylphosphate freshly prepared in buffer C. Release of *p*-nitrophenylate by enzymatic hydrolysis was measured by optical density at 400 nm in a Gilford Stasar II spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Usual incubation was 30 min at 37°, after which enzymatic activity was stopped by addition of 1 ml of 1 *N* NaOH. Serum antibody titer was determined as the reciprocal dilution resulting in an optical density greater than the mean negative control value plus twice its standard deviation.

Quantitative complement fixation assay. Quantitative complement fixation (CF) assays for antigen and antibody were performed according to Osler *et al.* (18), employing 4 units of antigen or antiserum and 5 50% hemolytic units of guinea pig complement. All antisera tested were treated at 56° for 30 min. Rabbit and grivet monkey sera were adsorbed at 4° for 30 min with 5% sheep erythrocytes.

Results. Development of the enzyme-

linked immunosorbent assay. Rabbit antisera prepared by immunization with $I_n \cdot C_n$ were used to develop the ELISA. These sera were reactive with $I_n \cdot C_n$ and other dsRNA, but were unreactive with single-stranded RNA or double-stranded DNA, as determined by CF assay. CF titers for anti-dsRNA, as determined for 10 sera, ranged from 80 to 450, with a geometric mean of 214. Some sera tested were also reactive with polyinosinic acid (I_n), but this varied from animal to animal.

Pre- and postimmunization rabbit sera were titrated for ELISA activity using polystyrene tubes coated with $I_n \cdot C_n$ complexed with poly-L-lysine. The postimmunization serum (CF titer 128) had a titer of 1600 by ELISA (Fig. 1). The preimmunization serum was inactive by ELISA at a 1:100 dilution, the highest concentration tested. The ELISA titers were not altered by heat treatment (56° for 30 min) of the rabbit sera.

Antigen ($I_n \cdot C_n$) binding to polystyrene tubes was evaluated by ELISA activity, as measured by OD_{400} using a 10^{-3} dilution of postimmunization rabbit serum (Table 1). Optimal ELISA activity was achieved using 1 μg $I_n \cdot C_n$/ml complexed with poly-L-lysine. $I_n \cdot C_n$ alone or in combination with other cationic materials, spermine or neamine, was ineffective in the assay.

The specificity of the ELISA for dsRNA was evaluated by reduction of antiserum binding to polystyrene-adsorbed $I_n \cdot C_n$ (Fig. 2). A 10^{-3} dilution of rabbit serum (ELISA titer 1600) was incubated overnight at 4° with various concentrations of test antigen prior to addition to $I_n \cdot C_n$ coated tubes. Inhibition was measured as the percentage reduction of OD_{400} resulting from antiserum preincubation with the test antigen, compared to similar preincubation in the absence of antigen. The dsRNAs ($I_n \cdot C_n$, $A_n \cdot U_n$ and MU9dsRNA) all blocked antibody binding significantly using 20 ng/ml. By contrast, I_n and C_n at 10-fold higher concentrations failed to block antibody binding. The failure of these single-stranded RNAs to inhibit ELISA activity was not the

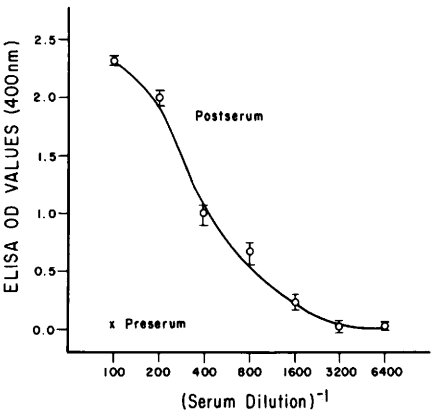

FIG. 1. Titration of rabbit antiserum to $I_n \cdot C_n$. Antiserum prepared from rabbits immunized with $I_n \cdot C_n$ was titrated for binding to polystyrene tubes coated with $I_n \cdot C_n$ complexed with poly-L-lysine. Antibody binding was measured by ELISA, as described under Materials and Methods. All values are presented as the mean ± SE.

TABLE I. ANTIGEN BINDING TO POLYSTYRENE TUBES

	Bound antibody activity (ELISA[b] OD at 400 nm) at the indicated antigen concentrations		
Antigen preparation[a]	10 μg/ml	1.0 μg/ml	0.1 μg/ml
$I_n \cdot C_n$ + poly-L-lysine	0.255 ± 0.032	0.500 ± 0.010	0.110 ± 0.008
$I_n \cdot C_n$	0.017 ± 0.005	0.011 ± 0.002	0.009 ± 0.003
$I_n \cdot C_n$ + spermine	0.031 ± 0.003	0.020 ± 0.011	0.009 ± 0.001
$I_n \cdot C_n$ + neamine	0.014 ± 0.003	0.055 ± 0.005	0.015 ± 0.002
poly-L-lysine	ND	0.022 ± 0.009	ND

[a] Antigens at the indicated concentrations were incubated overnight at 37° in polystyrene tubes followed by removal of unbound antigen by washing.

[b] Tubes containing antigen bound at the concentrations indicated were incubated with rabbit $I_n \cdot C_n$ antiserum at a 1:1000 dilution for 0.5 hr at 37°, washed free of unbound antibody, and further incubated with goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase. Enzyme activity was measured by increase in absorbancy at 400 nm upon incubation with *p*-nitrophenylphosphate. All values are presented as the mean ± SE.

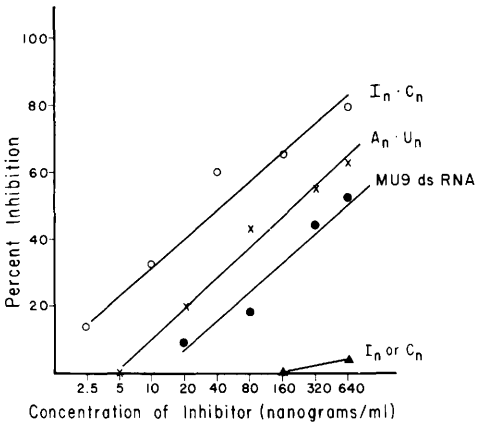

FIG. 2. Blocking of antibody binding to $I_n \cdot C_n$. Polynucleotides at the indicated concentrations were incubated overnight at 4° with a 10^{-3} dilution of the positive rabbit serum titrated in Fig. 1. Residual free antibody was determined by ELISA as described under Materials and Methods.

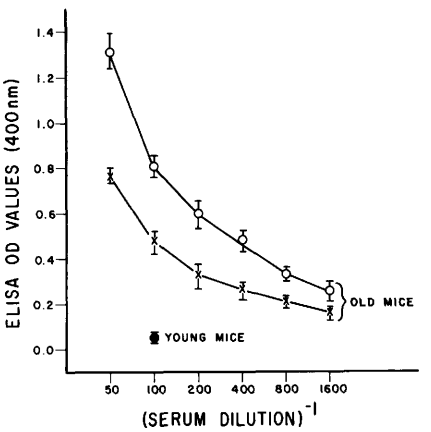

FIG. 3. Titration of NZB/NZW serum binding to $I_n \cdot C_n$. Pooled sera from 12+-month-old unimmunized NZB/NZW mice were titrated for antibody binding to $I_n \cdot C_n$ complexed with poly-L-lysine. Antibody binding was measured by ELISA, as described under Materials and Methods. Serum binding was for 30 min at 37° (X) or overnight at 4° (○). All values are presented as the mean ± SE.

result of their relative sensitivity to degradation by serum ribonuclease. No hydrolysis of either I_n or C_n was detected, as measured by hyperchromicity at 260 nm, following overnight incubation of the rabbit antiserum (10^{-3} dilution) with homopolynucleotide solutions (10 μg/ml).

Application of the enzyme-linked immunosorbent assay for antibodies in other animal species; NZB/NZW mice. NZB/NZW mice spontaneously develop antibodies to nucleic acids, including dsRNA (4, 19). The incidence of mice with detectable antibodies to dsRNA increases with age, and is greater in females than in males (15). Serum pools from young mice (1 month) and old mice (12+ months) were compared for antibodies to dsRNA. The procedure employed was similar to that developed for rabbits, with one exception. Antigen-coated ($I_n \cdot C_n$ plus poly-L-lysine) tubes were incubated with serum dilutions overnight at 4°, rather than at 37° for 30 min. The altered incubation resulted in a fourfold increase in antibody titer and in reproducible results. Whereas pooled sera from young mice were unreactive, pooled 12+-month serum had high titers (1600) of antibody to dsRNA (Fig. 3). The specificity for dsRNA was also demonstrated by antibody blocking (Fig. 4). Double-stranded RNAs ($I_n \cdot C_n$, $A_n \cdot U_n$ and MU9dsRNA) blocked antibody binding to $I_n \cdot C_n$ coated tubes whereas I_n or C_n alone did not. CF assay determinations for similar samples resulted in titers never exceeding 40, and were often obscured by anticomplementary activity.

Grivet monkey. The ELISA for grivet monkey sera was performed essentially as described for the rabbit serum assay. Monkey antisera were produced by repeated injections of young adult grivet monkeys with $I_n \cdot C_n$ complexed with methylated

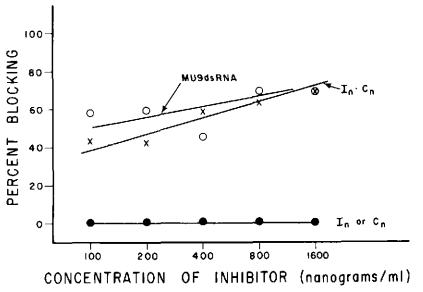

FIG. 4. Blocking of NZB/NZW antibody binding to $I_n \cdot C_n$. Experimental conditions were as described for Fig. 2 using NZB/NZW pooled sera from 12+-month-old mice. Residual-free antibody was determined by ELISA using antibody incubation overnight at 4°.

bovine serum albumin, since injection with $I_n \cdot C_n$ alone was not effective in stimulating antibody production. Significant ELISA antibody activity to $I_n \cdot C_n$ was detected in serum diluted 1:12,600 (Fig. 5), and activity at 1:50,000 has been obtained for sera from hyperimmune animals. These same serum samples were either negative by CF assay or contained activity often obscured by anticomplementary activity.

Discussion. The data described in the present report demonstrate that an enzyme-linked immunosorbent assay specific for antibodies to dsRNA has been developed for the sera from rabbit, mouse, and grivet monkeys. The assay is dependent on providing surface-coated antigen in the form of $I_n \cdot C_n$ complexed with poly-L-lysine and fixed to polystyrene tubes. As demonstrated by inhibition experiments, the assay is specific for antibodies to dsRNA, and is useful in detecting nanogram quantities of dsRNA.

As indicated by our data and also by the work of Payne and Kalmakoff (20), the specificity of activity for dsRNA, but not for single-stranded RNA (I_n or C_n) was not due to the relative ribonuclease sensitivity of single-stranded RNA. Under the conditions of our assays, using high dilutions of antiserum to dsRNA, little or no hydrolysis of RNA was apparent. The assay, as demonstrated using rabbit antiserum at high dilution (10^{-3}) detected less than 10 ng dsRNA/ml, without interference by single-stranded RNA. This observation is in marked contrast to the demonstrated cross-reactivity of this same serum for I_n when the serum was used at a 30-fold higher concentration necessary for the complement fixation assay. The increased sensitivity for antibody to dsRNA provided by the ELISA procedure has apparently permitted dsRNA detection using the antiserum diluted beyond the extinction of I_n antibody activity.

Detection of IgG antibodies to dsRNA in NZB/NZW sera was strongly influenced by incubation conditions. Overnight incubation at 4° resulted in a fourfold increase in antibody titer and consistent reproducibility not obtained by the 30-min incubation at 37° used for the assays of rabbit or grivet monkey sera. While this observation is of practical importance in development of the assay of NZB/NZW sera, the reason for this increase of antibody titer is not apparent.

The present ELISA, adapted for evaluation of grivet monkey sera, provides the sensitivity required for monitoring antibodies to dsRNA which might arise during repeated interferon inductions with $I_n \cdot C_n$. As such, the assay is now in use as a rapid screen of sera at low dilutions without nonspecific interference, as was encountered by anticomplementary activity in the CF test. With increased use of $I_n \cdot C_n$ as an anti-viral or anti-tumor agent (7–9) in human clinical trials, awareness of the potential for stimulation of antibody may be very important. A technique with the specificity, sensitivity, and simplicity of the enzyme-linked immunosorbent assay should be useful in evaluating an immune response.

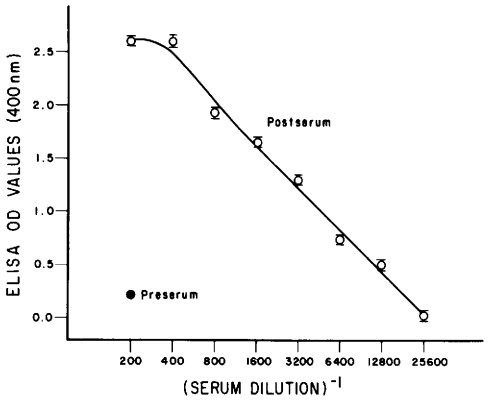

FIG. 5. Titration of grivet monkey serum binding to $I_n \cdot C_n$. Antiserum prepared from a grivet monkey immunized by $I_n \cdot C_n$ complexed with methylated bovine serum albumin was titrated as described in Fig. 1. All values are presented as the mean ± SE.

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