Specificity of Enhanced Immunological Sensitization of Mice Following Injections of Antigens and Specific Antisera.* (27112)

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An enhanced immunological response to bovine serum albumin (BSA) was elicited in mice by simultaneous injection of BSA and rabbit antiserum (anti-BSA)(1). The optimal response was obtained when antigen and antiserum were injected in slight antigen excess(2). If the enhancing effect is derived from the antigen-antibody complex *per se* and limited to the specific antigen in complex, then specific enhancement may result from prolonged retention of antigen, increased molecular size due to complexing or preferential localization of complexed antigen by immunological competent cells. A nonspecific stimulation would be indicated if concomitantly injected unrelated antigen or nonspecific antibody elicited an enhanced antibody response.

This report is concerned with specificity of the enhanced antibody response. Specificity was determined by measuring antibody responses to BSA, rabbit gamma globulin (RGG) and fluid tetanus toxoid (FTT) when these antigens were administered in various combinations with specific rabbit antisera. Our findings show that the enhanced antibody response is specific and limited only to those antigens in complex.

Materials and methods. Animals. Eight-week-old male and female Swiss albino mice were used in these experiments. These mice were raised in our own colony and are specific pathogen-free.

Antigen. Crystalline BSA was obtained from Armour Laboratories. FTT and aluminum phosphate adsorbed tetanus toxoid were obtained from Lederle Laboratories. Fluid toxoid (33 ml) was concentrated by lyophilization, the dried toxoid was dissolved in 4.2 ml of 1% NaCl. It was dialyzed against 1% NaCl, the final concentration of the toxoid was 0.127%.

The RGG was fractionated from normal rabbit serum by repeated precipitation with ⅓ saturated ammonium sulfate at pH 7.8. BSA and RGG were labeled with I^{131} by the procedure previously described(2).

Antisera. Rabbits were immunized to BSA by a series of i.v. injections consisting of 10 mg of BSA 3 times a week for 6 weeks. One week later the rabbits were bled and sera pooled. The pooled anti-BSA contained 0.96 mg of antibody N/ml. When used the anti-BSA was diluted 1:3 with 1% NaCl. Rabbit tetanus antitoxin was obtained from rabbits immunized by subcutaneous injection of 0.5 ml of aluminum phosphate adsorbed tetanus toxoid. A series of 3 injections of toxoid was given at 2-week intervals and serum was obtained 2 weeks following last injection. The rabbit antitoxin, which contained 0.5 International Units (IU) of tetanus antitoxin per ml of pooled serum, was diluted 1:8 with 1% NaCl when used. Thus, in a 0.1 ml dose each animal received 0.0063 IU. All antigen preparations and antisera were filtered to remove possible bacterial contaminants. Subsequent bacteriological cultures demonstrated that these preparations were bacteria free.

Ammonium sulfate precipitation of antigen-antibody complexes. The mouse sera were titrated for anti-BSA by mixing with an equal volume of I^{131} BSA in decreasing concentrations, subsequently the I^{131} BSA-antibody complexes were precipitated by adjusting ammonium sulfate concentration to 37.5% saturation(3). The percentage of radioactivity (I^{131} BSA) precipitated by this procedure is proportional to the amount of antibody present(4). The exact procedure and composition of the reagents used has been described(2,4).

Titration of tetanus antitoxin. Pooled

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TABLE I. Titration of Mouse Sera for Anti-BSA and Tetanus Antitoxin. Mice injected with proteins indicated and bled on 14th day.

Group*	Inj. of antigen and antisera	Anti-BSA titration: mg of I^{131}-BSA added 0.114	0.04	0.013	0.0013 (% of I^{131}-BSA precipitated by ammonium sulfate)	Tetanus antitoxin inter. units 10^{-4}/ml	No. of MLD of toxin neutral./ml serum
1	FTT + anti-BSA + BSA	4.1	11.0	40.8	100.0	<4	<1.5
2	Anti-BSA + BSA	2.3	10.0	34.7	90.0	--	—
3	FTT + anti-BSA	2.8	15.6	51.2	88.0	<4	<1.5
4	FTT + BSA	.8	—	.9	2.9	<4	<1.5
5	BSA	.5	—	1.6	2.8	—	—
6	Normal mouse serum	.8	1.0	1.8	—	—	—
7	FTT	.8	—	.9	3.5	<4	<1.5
8	FTT + tetanus antitoxin	—	—	—	—	280	112
9	Tetanus antitoxin (only)	—	—	—	—	15	6

FTT = Fluid tetanus toxoid.
BSA = Bovine serum albumin.
* 15 mice per group.

sera were obtained from each group of mice and titrated as a single specimen with the Ehrlich method of titration. The antitoxin combining power of the tetanus toxin was determined by comparison with a standard obtained from the Nat. Inst. of Health. In these titrations, 1000 mouse MLD were neutralized by 0.025 International Unit of tetanus antitoxin. The titrations were read at the end of 4 days and minimal paralysis was used as the end point. Our procedures have been described previously(5).

Antigen degradation. Degradation of I^{131}-labeled proteins was followed by whole body measurement of residual radioactivity(2,6,7). This was measured in a crystal scintillator with a well sufficiently large to contain a mouse. NaI (1%) was added to the drinking water to reduce I^{131} uptake by the thyroid.

Experiments and results. To determine the specificity of the enhanced response a series of antigen-antisera combinations was tested. The mice were injected i.v. with one, 2, or 3 of the following: 0.1 ml of FTT; 0.1 ml of antitoxin serum; 0.1 ml of anti-BSA serum; 0.15 mg of BSA, as shown in Table I. The ratios of BSA/anti-BSA and toxoid/antitoxin were in slight antigen excess. All mice were bled 14 days later and the pooled sera titrated as a single sample with respect to anti-BSA, anti-RGG, and tetanus antitoxin. Experimental conditions and the order of injections are indicated in Table I. Twenty minutes prior to the above injections, the mice were treated with an antihistaminic agent (Thephorin) to prevent fatal anaphylaxis(8). A dose of 0.5 mg of Thephorin was given by intraperitoneal injection.

The sera were tested for anti-BSA by addition of I^{131}-BSA and subsequent precipitation of antigen-antibody complexes with ammonium sulfate. The percentage of radioactivity (I^{131}-BSA) precipitated by the sera from mice injected with BSA/anti-BSA (groups 1 and 2) and anti-BSA only (group 3) (Table I) is significantly greater than that precipitated with normal mouse serum (group 6). These data demonstrate an enhanced antibody response in group 1 injected with FTT, anti-BSA and BSA in antigen excess and in group 2 injected with anti-BSA and BSA in antigen excess. In contrast, the mice injected with FTT and BSA (group 4) and BSA only (group 5) had little if any antibody to BSA in that the values obtained are comparable to those obtained with normal mouse serum (group 6) and serum from mice injected with FTT only (group 7). It should be emphasized that a significant amount of antibody to BSA was found in the serum of mice injected with FTT and anti-BSA (group 3). Since anti-BSA was passively administered to these animals (group 3) it was necessary to determine if the anti-BSA found in groups 1, 2, and 3 fourteen days later was of mouse origin or rabbit origin. The anti-BSA in groups 1 and 2 was of mouse origin and

TABLE II. % of I^{131}-RGG* Degraded in Normal Mice Injected with the Test Serum Indicated.

Serum from groups indicated in Table I	% I^{131}-RGG degraded in 16 hr
Group 1 (FTT + anti-BSA + BSA)	88; 86
" 2 (anti-BSA + BSA)	94; 92
" 3 (FTT + anti-BSA)	24; 21; 20; 20
" 4 (FTT + BSA)	24; 24; 21; 19
Control (I^{131}-RGG only)	22; 21; 18; 16

* All animals inj. with I^{131}-labeled rabbit gamma globulin (0.0013 mg) by i.v. route.

that in group 3 was of rabbit origin, as shown in a succeeding experiment.

Tetanus antitoxin was not detectable in the sera of mice injected with FTT only (group 7) or in mice given FTT in combination with BSA, anti-BSA, and BSA with anti-BSA (groups 4, 3, and 1). Thus, simultaneous antigenic stimulation with BSA and the anti-BSA complex in slight antigen excess did not elicit an enhanced antibody response to tetanus toxoid. An enhanced antitoxin response was obtained in the animals (group 8) injected with the FTT-antitoxin complex in slight antigen excess. An 18-fold increase in antitoxin titer was found in the serum from these animals in comparison with serum from animals injected with antitoxin only (group 9). These data demonstrate an enhanced antibody response elicited by the antigen-antibody complex; moreover, the enhancement effect is limited to the specific antigen in the complex.

The previous experiment demonstrated enhanced antibody responses to BSA and FTT when these antigens were injected with specific antisera. Since heterologous antisera were used, it was of interest to also determine if an enhanced response was elicited to rabbit gamma globulin. This was tested by the capacity of passively immunized mice to degrade I^{131}-labeled RGG at an accelerated rate(8). As shown in Table II, normal mice were passively immunized with serum from groups 1, 2, 3 and 4 as indicated in Table I. The test dose of 0.0013 mg of I^{131}-RGG was injected immediately by intravenous route. The amount of I^{131}-RGG degraded in 16 hours was determined by wholebody measurement of residual radioactivity. The data (Table II) show that sera from mice previously injected with antigen and specific antiserum (groups 1 and 2) passively immunized normal mice to RGG since these mice degraded 86 to 94% of the I^{131}-RGG within 16 hours. Sera from mice previously injected with FTT and anti-BSA (group 3) and antigen only (group 4) failed to passively immunize normal mice since these animals degraded only 19 to 24% of the I^{131}-RGG in 16 hours. Similar values were obtained in control animals injected with I^{131}-RGG only. The data demonstrate an enhanced response to RGG when antigen is injected with specific rabbit antiserum.

Discussion. These experiments demonstrate an enhanced antibody response to FTT, BSA and RGG when mice were injected with these antigens and specific antisera. Specificity of the enhanced response was shown with respect to the antigen-antiserum system used in that the enhancing effect was limited to the antigens in complex. When mice were injected with a combination of anti-BSA plus BSA in slight antigen excess plus FTT, these animals gave an enhanced response to BSA only; antibody to FTT could not be detected. A single injection of BSA or FTT only, and a combination of BSA and FTT failed to elicit a detectable antibody response. An enhanced antitoxin response was obtained when FTT was injected with rabbit tetanus antitoxin. The response to RGG was also enhanced in mice injected with antigen and RGG prepared from anti-BSA rabbit antiserum. Evidence for this was obtained when normal mice were passively immunized with serum from mice previously injected with FTT plus anti-BSA plus BSA (group 1) and anti-BSA plus BSA (group 2). These animals as well as the mice in group 3 previously immunized with FTT plus anti-BSA degraded I^{131}-RGG at an accelerated rate. Since anti-BSA survived in mice passively immunized with serum from mice previously injected with FTT plus anti-BSA (group 3), the possibility arose that the enhanced response to BSA observed in groups 1 and 2 may represent surviving anti-BSA of rabbit origin rather than newly formed anti-BSA of mouse origin. Mouse anti-RGG was added

to the sera of groups 1, 2 and 3 to test for neutralization of antibody to BSA. Antibody to BSA was neutralized only in serum from mice injected with FTT plus anti-BSA (group 3) demonstrating that the observed anti-BSA in group 3 was of rabbit origin while the anti-BSA demonstrated in groups 1 and 2 was of mouse origin.

Summary. An enhanced antibody response to bovine serum albumin and fluid tetanus toxoid was found in mice when these antigens were injected in slight antigen excess with specific heterologous antisera. The specificity of the enhanced response was limited to specific antigens in complex. An enhanced response was also demonstrated to rabbit gamma globulin by testing the capacity of passively immunized mice to degrade I^{131}-labeled RGG at an accelerated rate.

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Use of an Antiserum Against Cerebrospinal Fluid in Demonstration of Trace Proteins in Biological Fluids.‡ (27113)

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The proteins of serum(1) and cerebrospinal fluid(2,3,4) have been extensively studied by immunoelectrophoresis with the aid of antisera prepared against human serum. Since protein concentration in cerebrospinal fluid (CSF) is much lower than in serum, it is necessary to concentrate CSF 50-to-100-fold before comparable immunoelectrophoresis diagrams can be obtained. Recently, antisera against such concentrated CSF have been prepared and examined by Chevance(5), Clausen(6) and in this laboratory. Such antisera reveal, upon immunoelectrophoresis of CSF, precipitation lines not seen in the diagrams obtained with human serum.

The present studies were undertaken in an attempt to determine whether the proteins responsible for these lines are 1) specific for CSF or 2) present in such small quantities in biological fluids that concentration of such fluids is necessary before these proteins can be demonstrated by immunoelectrophoresis. In contrast to the conclusion of Clausen(6), the results of immunoelectrophoretic and Ouchterlony plate studies performed with antisera to CSF after "absorption" with plasma or serum, using concentrated CSF, ascitic fluid, pleural fluid, urine, and plasma fractions obtained by starch block electrophoresis as the antigens, indicate that the proteins are not specific for CSF but can be found in small quantities in serum and other biological fluids.

Materials and methods. Preparation of antisera. Cerebrospinal fluid was obtained from patients admitted to the Neurological and Psychiatric Services of Bellevue Hospital, New York City. All grossly normal fluids were pooled and concentrated to a protein content of approximately 35 mg per ml by means of vacuum dialysis (Membranfiltergesellschaft, Göttingen, Germany). They were then mixed with equal parts of complete Freund's adjuvant and injected into the footpads of white New Zealand rabbits (2 ml of

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