REGULATION OF ANTIBODY FORMATION BY SERUM ANTIBODY

I. Removal of Specific Antibody by Means of Immunoadsorption*

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Injection of antibody into previously immunized animals can specifically suppress the expected antibody response (1–4). This finding led to the suggestion that serum antibody might act as a "feedback" type of mechanism. Experiments of this type, however, are not physiological in the sense that the serum antibody concentration of the immunized animal is raised to a higher level than normally present at that stage of immunization, and the passively administered antibody, usually obtained from well immunized animals, is probably of average higher binding affinity than the antibody in the recipient immunized animal. The question remains, therefore, as to whether the antibody molecules in the immunized animal are of sufficient concentration and binding affinity to regulate antibody formation in that animal.

One approach to answering this question is to remove antibody specifically from immunized animals and then to determine if the rate of antibody formation changes.

It is well recognized by immunologists, that hyperimmunized animals can be bled repeatedly and that serum antibody levels are maintained without further administration of antigen, suggesting an increased rate of synthesis. In addition, repeated plasmapheresis in humans has been shown to stimulate elevation of serum antibody levels to antigens injected many months previously (5, 6). Such experiments in which a large proportion of plasma is removed may induce profound *nonspecific* effects, for example, by reducing the concentration of immunoglobulin or other factors in the circulation. The critical point experimentally is to remove antibody of one specificity only and thus affect formation of antibody to that specificity only. Under such circumstances, interpretations can be made regarding the immunogenicity of the specific persisting antigen. This type of experiment can be accomplished by removing large samples of plasma from immunized animals repeatedly, exposing the plasma to antigen covalently bound to a particulate material, and returning the treated plasma without the immunoadsorbent to the animal.

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The present experiments describe such specific removal of a large portion of serum antibody from immunized rabbits and the effect of this removal upon antibody formation. The results suggest that serum antibody acts as a major regulatory mechanism for antibody formation to metabolizable antigens.

Materials and Methods

Antigens and Antibodies.—Bovine serum albumin (BSA)[1], 4× recrystallized was obtained from Nutritional Biochemicals Corp., Freehold, N.J. (Lot No. 1359). Bacteriophage T_2 (T_2) was prepared by the method of Adams (7). The BSA used gave a single line of precipitation on immunoelectrophoresis with rabbit anti-bovine serum. Human 7S gamma globulin (HGG) (Nutritional Biochemicals Corp.) gave a single line of precipitation on immunoelectrophoresis with rabbit anti-human serum.

Rabbit antibody to BSA contained 9.6 mg antibody protein/ml and gave a single line of precipitation on immunoelectrophoresis with bovine serum. Goat antibody to HGG contained 8.6 mg antibody protein/ml and rabbit antibody to ϕX had a neutralizing titer of K = 2700.

Immunization and Antibody Assays.—For passive immunization, albino rabbits, 3–4 kg, were injected intravenously with 16 mg of rabbit antibody to BSA and 0.2 ml of rabbit anti-ϕX (K = 2700). For active immunization, rabbits were injected intravenously with 10 mg/kg of BSA and 1–4 × 10^{10} T_2 in saline.

Serum anti-BSA titers were measured by the Farr technique (8) using 0.4 μg antigen N and an ABC precipitating line of 33%. Serum anti-T_2 titers were determined by the phage neutralization test (7).

Preparation of BAC-BSA.—Bromoacetyl cellulose (BAC) was prepared according to the method of Robbins et al. (9). In addition, two lots (Nos. 218 and 231) were purchased from Miles Laboratories, Inc., Elkhart, Ind.

^{125}I-BSA was prepared according to the method of Greenwood et al. (10). 96–99% of the counts of each preparation were precipitated by 5% trichloroacetic acid or by specific antibody.

In order to quantify the amount of BSA bound to BAC, 600 mg of BSA and 0.1 mg ^{125}I-BSA (10^6 counts/μg) were incorporated into BAC as previously described (11). The amount of radioactivity of all the washes, as well as the radioactivity remaining on the washed adsorbent, were measured so that the amount of BSA covalently bound to the BAC could be calculated by either method. Assuming that ^{125}I-BSA and BSA are equally bound to BAC during the coupling procedure, it was found that 54–65% of the BSA was covalently bound to BAC in the preparations used. The BAC-BSA was exhaustively washed with 0.15 M phosphate buffer pH 7.4 until the number of nondialyzable counts was at background level.

It was also found that an amount of immunoadsorbent containing 13 mg of BSA bound 4.1 mg of anti-BSA. 90% of the adsorbed anti-BSA was recovered after incubation of BAC-BSA with 0.1 N acetic acid, stirring at 37°C for 1 hr, and dialysis against 0.1 M sodium chloride–0.1 M phosphate buffer pH 7.4 overnight. In addition, it was found that the antibody-combining capacity of the BAC-BSA was fully regenerated by this procedure.

Cannulation of Rabbits.—Animals were anesthetized with nembutal and also received atropine 0.04 mg/kg and aqueous heparin 10 mg/kg intravenously. The femoral artery and vein were cannulated and a 35–40 cc sample of blood was withdrawn from the femoral artery.

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[1] *Abbreviations used in this paper:* BAC, bromacetyl cellulose; BSA, bovine serum albumin; HGG, human gamma globulin; T_2, bacteriophage T_2; TLCK, *p*-toluenesulfonyl lysine chloromethyl ketone.

The plasma was separated from the red cells by centrifugation at 6000 *g* for 5 min. The plasma was then incubated with portions of BAC-BSA for 15 min at 37°C, occasionally stirred, and then centrifuged at 105,000 *g* for 20 min to remove the immunoadsorbent. The adsorbed plasma and red cells were mixed and returned to the rabbit through the femoral vein. This entire maneuver, i.e., removal, adsorption, and replacement of blood, was repeated 4–6 times per animal over a period of 4–6 hr. The rabbits were given 1 ml of Wycillin-SM immediately after surgery and 4 days later. They were kept anti-coagulated with intramuscular injections of Coumadin for 1 wk after surgery.

Gel Filtration.—Sephadex G-200 was swelled in distilled water and equilibrated with 0.1 M phosphate buffer, pH 7.2. The outside and inside volumes were determined by using dextran blue 2000 and phenol red. BSA, rabbit 7S gamma globulin, and horse cytochrome *c* were used as markers.

Radioactive Counting.—Radioactive samples were counted in a New England Nuclear deep well counter to the 95% confidence level.

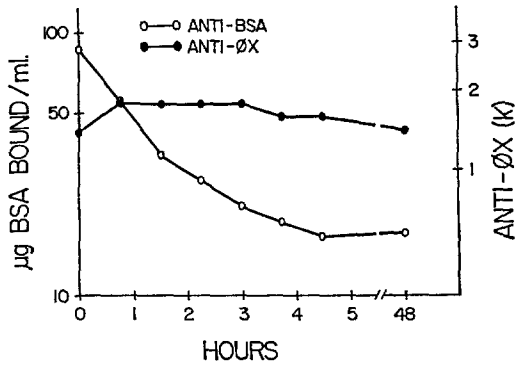

FIG. 1. Serum antibody levels to BSA and ϕX in a passively immunized rabbit in which anti-BSA was removed. Rabbit was injected intravenously with 16 mg of rabbit anti-BSA and 0.2 ml of rabbit anti-ϕX (K = 2700) 24 hr before removal of anti-BSA.

RESULTS

Antibody Removal from Passively Immunized Rabbits.—In order to determine whether serum antibody can be efficiently and specifically removed from immunized animals, 3 rabbits were injected intravenously with 16 mg of anti-BSA and with 0.2 ml of anti-ϕX (K of 2700). The following day, cannulation for removal of antibody was carried out. The rabbits were bled at frequent intervals during and after the procedure, and anti-BSA and anti-ϕX titers were measured.

Fig. 1 shows the antibody titers during the removal procedure in a representative animal. As can be seen, approximately 80% of serum antibody to BSA was removed after six bleedings and incubations with BAC–BSA during a 4.5 hr period without detectable effect on the serum antibody levels to ϕX. Serum anti-BSA titers 2 days after cannulation were equal to or lower than immediate postoperative levels. Similar results were obtained in two additional animals.

This experiment shows that the majority of antibody activity can be removed specifically in passively immunized animals.

Antibody Removal from Actively Immunized Rabbits.—The above technique was applied to actively immunized animals in order to determine the effect of removal of antibody to BSA on anti-BSA formation.

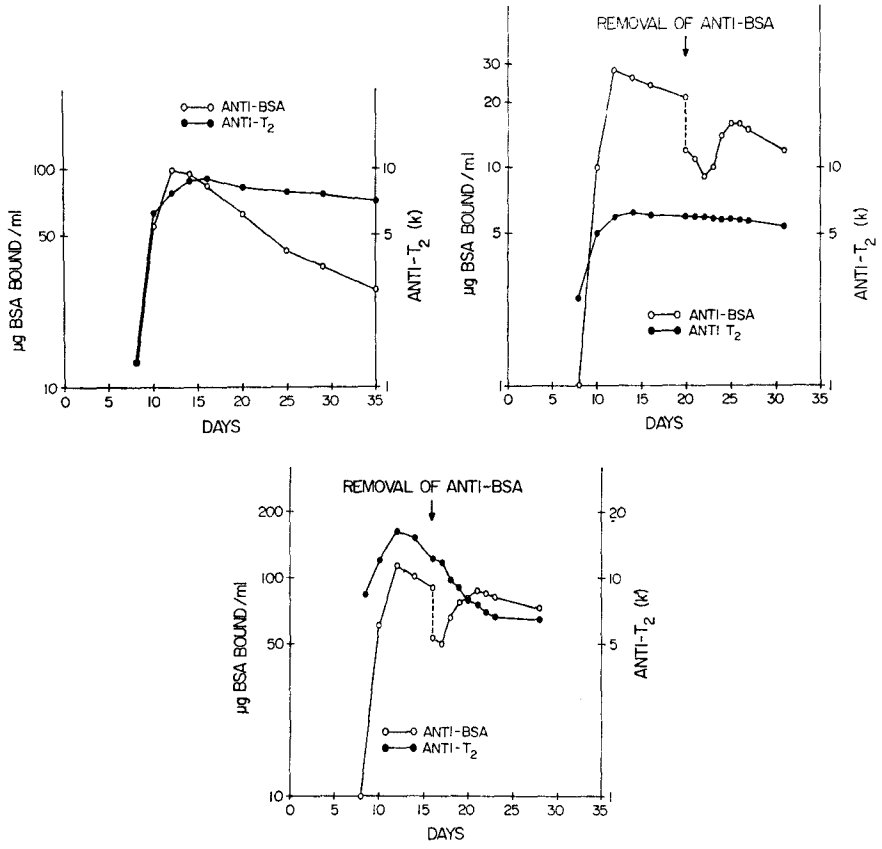

FIG. 2. Serum antibody levels to BSA and T_2 in actively immunized rabbits in which anti-BSA was removed. Rabbits were injected intravenously with 10 mg/kg of BSA and 2×10^{10} T_2 in saline. A: Average serum antibody levels in seven control immunized animals. B and C: two representative animals in which anti-BSA was removed.

For this purpose, six rabbits were injected intravenously with 10 mg/kg of BSA and $1\text{–}4 \times 10^{10}$ T_2 phage in saline. The anti-BSA levels in the sera of such animals reached a peak titer within 2 wk after antigen injection. The animals were cannulated on days 16–20 when serum anti-BSA levels were falling, indicating that rate of synthesis had slowed. Antibody titers to BSA and T_2 were measured at frequent intervals before and after cannulation.

Fig. 2 shows the average serum antibody levels to BSA and T_2 in seven control immunized animals and antibody levels in two representative animals in which antibody to BSA was removed. As can be seen in 2a, both anti-BSA and anti-T_2 peak titers were reached by 2 wk. Thereafter, anti-BSA levels declined steadily, and anti-T_2 remained at a plateau. In the cannulated animals, between 38% and 43% of the antibody to BSA was removed by the experimental procedure (2b, c). Anti-BSA levels continued to fall for 1–2 days after cannulation. At this point anti-BSA levels began to rise and reached peak levels usually 5 days later. Anti-T_2 levels in the cannulated animals were unaffected by the adsorption procedure and were similar to those seen in the control immunized animals.

Table I shows the results in tabular form for the two rabbits illustrated in the

TABLE I

Serum Antibody Titers in Rabbits Immunized with BSA and Bacteriophage T_2 in Which Anti-BSA Has Been Removed

Rabbit No.*	Serum antibody titers					
	Precannulation‡		Postremoval		Peak of rebound	
	Anti-BSA§	Anti-T_2‖	Anti-BSA	Anti-T_2	Anti-BSA	Anti-T_2
1-25	120	16.4	48	13.5	75	11.2
1-26	86	12.4	54	12.0	74	7.6
1-28	20	7.4	14	8.2	17	6.2
1-31	97	37	36	36	65	38
1-32	42	35	16	35	28	35
1-33	21	6.1	12	6.2	16	5.8

* Rabbits were injected with 10 mg/kg BSA and 1–4 × 10^{10} T_2 in saline intravenously.

‡ 16–20 days after immunization, antibody to BSA was removed.

§ μg BSA bound/ml.

‖ Neutralizing titer (K).

preceding Fig. and four additional animals. As can be seen, in all animals there was a significant removal of serum antibody to BSA followed by a pronounced rise in serum antibody levels above postoperative levels reaching peak values usually 5 days after cannulation. Antibody levels to T_2 were not affected.

Antibody adsorbed by the BAC-BSA was eluted with acetic acid and dialyzed as previously described. Some of the material precipitated during dialysis. The amount of anti-BSA in the supernatant, as determined by the Farr technique, accounted for 30–73% of the fall in serum antibody titer during the removal procedure in the six animals (a plasma volume of 30 ml/kg was assumed for each rabbit). In contrast, three of six eluates did not show detectable antibody to T_2; three had trace amounts (K's less than 0.8). These results are consistent, therefore, with the specificity of removal of antibody to BSA.

We interpret the subsequent rise of anti-BSA levels in the experimental animals as caused by an increase in the rate of antibody formation to BSA. We ex-

clude a change in the rate of catabolism of immunoglobulin in such animals, a priori, an unlikely possibility, because anti-T_2 levels were similar in the control and cannulated animals. Redistribution of anti-BSA between extra- and intravascular compartments cannot account for this considerable elevation in titer because no such change in titers was seen in the passively immunized animals and, furthermore, in four of the six actively immunized cannulated animals, there was a continued fall in serum antibody levels for 24–48 hr before the rise in serum antibody level began.

Release of BSA from BAC-BSA.—The next and obvious question to arise was whether this stimulation of antibody formation was caused by antigen released from the immunoadsorbent into the circulation of the animals. In the

TABLE II

Radioactivity in Plasma Pretreated with BAC-BSA

Rabbit No.	Radioactivity in adsorbed plasma			Maximal BSA present‡
	Postdialysis*	Coprecipitated by BSA-anti-BSA	Coprecipitated by HGG-anti-HGG	
	cpm	cpm	cpm	μg
1-25	2890	680	—§	2.7
1-26	2278	612	—	3.2
1-28	3795	799	—	4.4
1-31	1564	646	646	3.3
1-32	1700	680	646	3.7
1-33	1632	663	680	3.5

* Adsorbed plasma samples were dialyzed against 0.1 M sodium chloride–0.1 M phosphate buffer, pH 7.4, overnight. The total radioactivity returned to each animal is shown.

‡ Calculated from cpm coprecipitated by BSA–anti-BSA.

§ —, not done.

preceding experiments, the BSA had been iodinated with ^{125}I before binding to the BAC. Portions of the treated plasma samples which had been returned to the immunized rabbits were saved and tested for the presence of antigen released from the immunoadsorbent.

Table II shows the maximal amount of radioactive material returned to each animal and its specific coprecipitability. Table III shows two representative experiments in which the adsorbed plasma was treated with either 100 μg of BSA or HGG, and an excess of specific antiserum. As can be seen from the two tables, extremely minute amounts of radioactivity were released from the immunoadsorbent. If all the radioactivity that coprecipitated with BSA–anti-BSA represented BSA (10 cpm above background/ml plasma), the maximal amount that could have been administered to each animal ranged from 2.7 to 4.4 μg. However, an equal amount of radioactivity was coprecipitated with an immunologically, unrelated antigen-antibody precipitate. The BSA–anti-BSA

precipitate, under the same conditions as above, coprecipitated 97% of ^{125}I–BSA, whereas HGG–anti-HGG coprecipitated only 4%. Thus, no evidence was obtained that this released material is antigen.

The immunogenicity of the plasma released material was investigated. BAC-BSA was incubated with fresh normal plasma, and release of radioactivity equivalent to that in the above experiments was achieved. This amount of plasma was then injected into each of four BSA-primed rabbits. In this experiment, the injection of plasma was performed 4–8 days later than the time of cannulation in the earlier experiments in order to allow serum antibody levels to fall further and, therefore, to more closely approximate those observed in the immunized animals after removal of antibody. Additional BSA-primed rabbits

TABLE III

Immunoprecipitation of Radioactive Plasma

Plasma No.	Antigen and antibody*	Radioactivity of adsorbed plasma			
		After addition of antigen and antibody		After addition of BSA and anti-BSA to supernatant*	
		Precipitate	Supernatant	Precipitate	Supernatant
		cpm	cpm	cpm	cpm
1-31	BSA and anti-BSA	9	11	—§	—
	HGG and anti-HGG	10	10	1	8
1-32	BSA and anti-BSA	10	12	—	—
	HGG and anti-HGG	9	10	1	9

* 100 μg of antigen and 0.5 ml of specific antiserum were incubated with 1 ml of adsorbed plasma at 37°C for 1 hr and 4°C for 18–24 hr. The precipitates were washed twice with cold saline.

§ —, not done.

were injected with an amount of BAC–BSA equivalent to the plasma released radioactivity or with BSA.

Fig. 3 illustrates the responses of individual animals. As can be seen, there was no detectable response of primed animals to treated plasma, the immunoadsorbent itself, or BSA in amounts less than, or equal to, 20 μg. Indeed, with 100 μg of BSA only one of two animals showed a booster response.

It should be emphasized that an optimal control is an animal similarly immunized but without the usual concentration *and quality* of serum antibody. Thus, it can be argued that immunized, antibody-depleted animals have antibody of very low average binding affinity and are, therefore, much more susceptible to exogenous antigen than conventionally immunized animals.

Regardless, these experiments provide no evidence that immunogenic material released into plasma by the immunoadsorbent accounts for the antibody response after antibody removal.

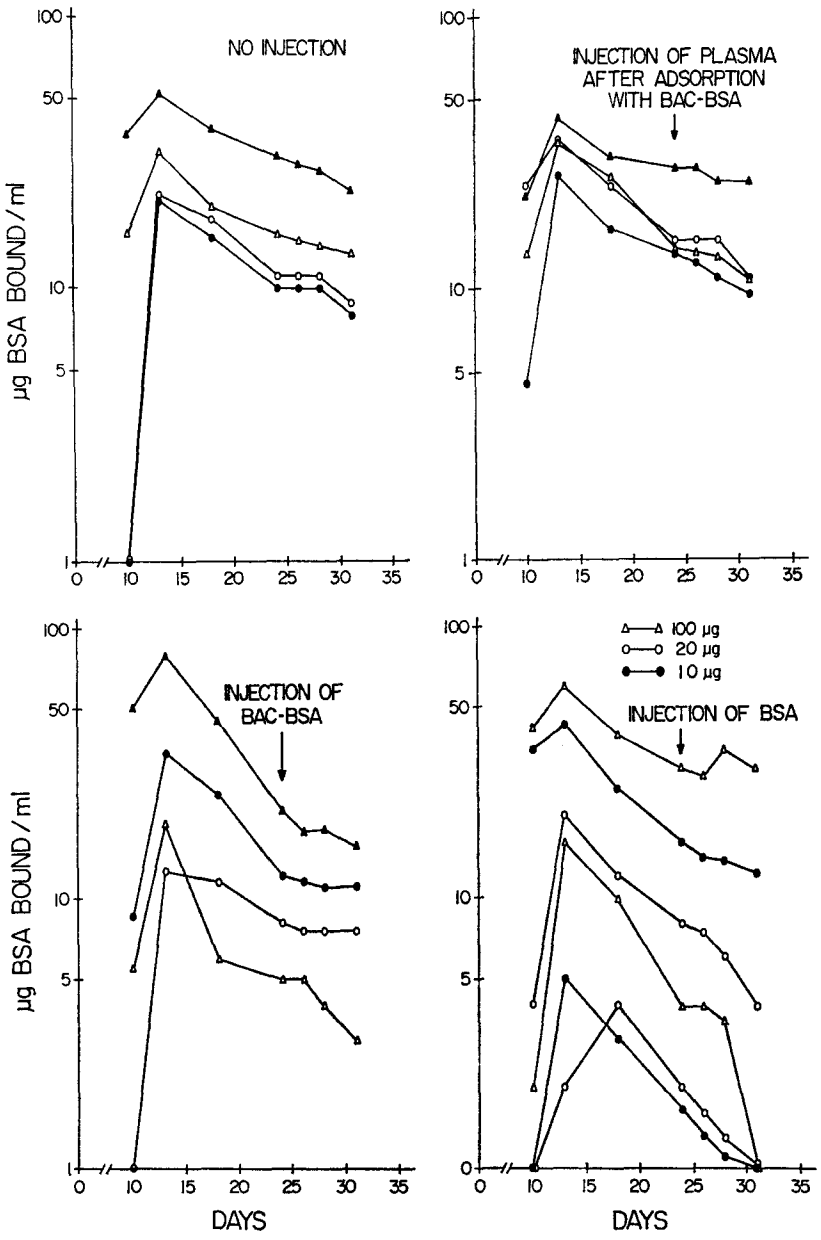

FIG. 3. Immunogenicity of plasma adsorbed with BAC-BSA or BSA in primed rabbits. Serum antibody levels to BSA of individual animals are shown. Animals received 30 mg BSA intravenously and except for controls were challenged 24 days later with the indicated material.

The physico-chemical characteristics of the radioactive material released into adsorbed plasma was investigated by gel filtration on Sephadex G-200. Unlabeled BSA, rabbit 7S gamma globulin, and horse cytochrome *c* were added as markers. As can be seen in Fig. 4, there is little or no radioactivity associated with the BSA peak.

Additional experiments revealed that the release of trace amounts of radioactivity from BAC-BSA into fresh plasma occurred within 1 min, was not increased by incubation for an additional hr, was independent of temperature

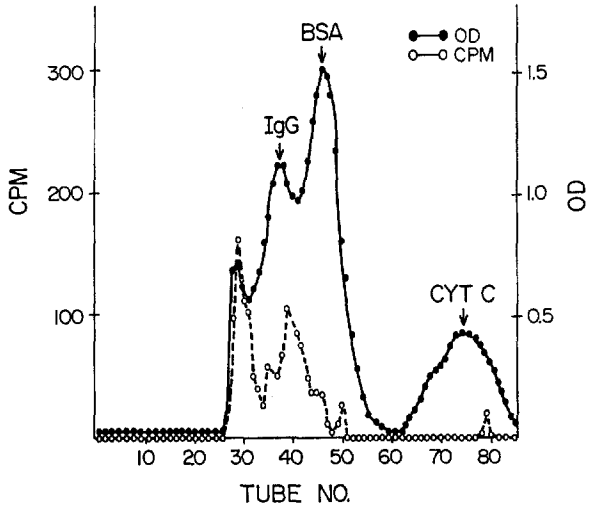

FIG. 4. Gel filtration on Sephadex G-200 of plasma previously adsorbed with ^{125}I-BSA-BAC. BSA, rabbit 7S gamma globulin and horse cytochrome c were used as markers. Eluting solvent was 0.1 M phosphate buffer pH 7.2.

(0°–37°C), and was not diminished by repeated exposure to plasma. It occurred in plasma previously heated to 80°C for 3 min and was not inhibited by TLCK, 2 mg/ml (*p*-toluenesulfonyl lysine chloromethyl ketone), a potent inhibitor of proteolytic enzymes (12). The released radioactivity was not extractable by chloroform. Nondialyzable counts were not released into physiological salt solutions, but were released into 60% sucrose.

These studies, thus, have not elucidated the nature of the radioactive material or the mechanism of its release. They suggest, however, that the release is not mediated by an enzymatic process, and that the radioactivity is not associated with lipids. The physico-chemical data are consistent with the possibility that the released radioactivity is BSA bound to fragments of cellulose or aggregated BSA, although no support for this was obtained from immunoprecipitation or immunization experiments.

DISCUSSION

A major problem in the evaluation of antibody-induced suppression has been to determine whether or not this mechanism operates during the normal immune response.

The only experiment to our knowledge which indicates that this mechanism is a physiological one is the demonstration by Britton and Möller (13), that injection of *Escherichia coli* lipopolysaccharide into mice results in a cyclical variation in serum antibody levels and the number of antibody-forming cells in the spleen. They suggest that the metabolism of serum antibody and the lack of metabolism of the antigen are responsible for the cyclical nature of this type of immune response. Their experiment shows, therefore, that serum antibody is a major regulatory mechanism for nonmetabolizable antigens.

We interpret the present experiments as indicating that serum antibody also acts as a regulatory mechanism in the conventional antibody response to metabolizable antigens. Thus, in all six actively immunized rabbits studied after removal of a large portion of serum antibody, there was a pronounced rise in serum antibody levels beginning 1–2 days later, and reaching peak titers usually at 5 days.

The obvious possibility, that the secondary response was caused by injection of antigen released from the immunoadsorbent, did not receive support from several lines of evidence: (*a*) A minute proportion of ^{125}I–BSA-BAC was released into plasma, less than one part in 7×10^5. (*b*) Immunoprecipitation studies revealed that about half of the minute number of nondialyzable counts (approximately 20 cpm above background per ml of adsorbed plasma) was coprecipitated with either BSA or HGG and their specific antibodies. (*c*) The same amount of material released into plasma by BAC–BSA or an equivalent amount of immunoadsorbent was not immunogenic for primed animals with serum antibody titers approximating those of the actively immunized animals after antibody removal. Indeed, 100 μg of "free" BSA was required to stimulate a response in one of two primed animals. (*d*) The plasma-released radioactivity did not elute with BSA on gel filtration through Sephadex G-200.

We therefore interpret the increased rate of antibody formation in these experiments as resulting from persisting antigen which is rendered more immunogenic after the removal of serum antibody. The mechanism by which this change in immunogenicity occurs is not known. The specificity of suppression indicates that serum antibody interacts with persisting immunogen. There is a potential for chemical reaction, therefore, between persisting immunogen, circulating antibody, and antibody receptors on preantibody secreting cells; this reaction may not be in equilibrium. Regarding the immunized rabbits in the present experiments, after injection of approximately 30 mg of BSA in saline intravenously 3 wk previously, only minute amounts of undegraded, immunogenic BSA are presumed to remain (14–16). The immunized animals

after removal of antibody, however, still had considerable antibody approximately 100 μg antibody protein per ml. Thus, serum antibody was in considerable excess. The question arises, therefore, as to how persistent immunogen can stimulate cells despite the continuous presence of a large excess of antibody. We have considered at least three possibilities, none of which is mutually exclusive to explain this stimulation: (*a*) The average binding affinity of the postcannulated serum antibody may have been markedly decreased by a preferential adsorption of antibody of high binding affinity by the immunoadsorbent, whereas the antibody receptors on the preantibody-forming cells continue to have a high average binding affinity. (*b*) There may be a gradient between concentration of antibody in the serum and the immunogenic site within the lymphoid tissue. (*c*) Stimulation of preantibody secreting cells to proliferate and differentiate may be irreversible, thus favoring this pathway. We have no information at the present time to exclude any of these possibilities.

Regardless of mechanism, the present studies indicate that serum antibody plays a significant role in controlling the rate of antibody formation to a metabolizable antigen.

SUMMARY

Rabbits were injected intravenously with bovine serum albumin (BSA) and bacteriophage T_2 (T_2). 2–3 wk later, anti-BSA was removed from such animals by a procedure which involved exposure of removed plasma to an immunoadsorbent (^{125}I-BSA bound to bromoacetyl cellulose) and return of the adsorbed plasma to the animal. This resulted in removal of the majority of antibody activity to BSA without affecting antibody levels to T_2. 1–2 days later, anti-BSA levels began to rise, and reached peak levels usually 5 days after the removal of antibody. Antibody levels to T_2 did not change. No evidence was obtained that BSA was released from the immunoadsorbent into the circulation of the rabbits. Thus, only trace amounts of radioactivity were released into the plasma; most of the radioactivity was equally coprecipitable with BSA or human gamma globulin and their specific antibodies; the released material was not demonstrated to be immunogenic in primed rabbits; and the released material did not elute with BSA on gel filtration. The results are interpreted as evidence that serum antibody acts as a regulatory mechanism for antibody formation during the conventional antibody response to a metabolizable antigen.

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