

THE TERMINATION OF IMMUNOLOGICAL UNRESPONSIVENESS
TO BOVINE SERUM ALBUMIN IN RABBITS

I. QUANTITATIVE AND QUALITATIVE RESPONSE TO
CROSS-REACTING ALBUMINS*

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Although immunological unresponsiveness to bovine serum albumin (BSA)¹ induced in neonatal rabbits is relatively stable, it can be terminated by injections of various heterologous albumins (1, 2) or by injections of altered BSA preparations (3-5). It has previously been reported that unresponsive rabbits produce lower levels of anti-BSA in response to these injections than do normal rabbits similarly immunized (1-5). These lower levels of antibody in the previously unresponsive rabbits could be explained by the partial inhibition of termination by small amounts of BSA present in the heterologous albumin preparations. Indeed, it has been shown that some commercial preparations of albumins are contaminated with BSA (6) and that the simultaneous injection of small amounts of BSA, along with the terminating antigen, suppressed the production of anti-BSA (7). It is also possible that the lower levels of antibody in unresponsive rabbits immunized with altered preparations of BSA may be due to partial prolongation of the unresponsive state by native determinants on the altered molecule, as has been indicated by the observations of Cinader et al. (8). The present investigation was designed to study the quantitative and quali-

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¹ Abbreviations used in this paper: BSA, bovine serum albumin; ESA, horse serum albumin; GPSA, guinea pig serum albumin; HGG, human gamma globulin; HSA, human serum albumin; I*, ¹²⁵I; PSA, pig serum albumin; N, nitrogen; SSA, sheep serum albumin; t₅₀, time in minutes for 50% of initially bound antigen to dissociate from antibody.

tative response of BSA-unresponsive rabbits free of significant levels of circulating BSA to injections of heterologous albumins.

Materials and Methods

Rabbits.—All rabbits used in these studies were of the New Zealand white strain.

Antigens.—Commercial preparations of serum albumins were obtained from the following sources: bovine (BSA), Armour Pharmaceutical Co., Chicago, Ill., Lot D71209; human (HSA), kindly supplied through the courtesy of the American Red Cross and prepared by E. R. Squibb & Sons, New York, N.Y., Lot 591R. Serum albumins from sheep (SSA), pig (PSA), horse (ESA), and guinea pig (GPSA) were prepared from whole serum by the method of Schwert (9). Albumins were trace-labeled with ^{131}I (I*) according to the method of McConahey and Dixon (10). Samples were counted in a gamma scintillation counter with a sodium iodide crystal.

Protein Nitrogen.—Protein nitrogen determinations were carried out by a modification of the micro-Kjeldahl technique (11) using a Technicon AutoAnalyzer (Technicon Corp., Ardsley, N.Y.).

Induction of Immunological Unresponsiveness to BSA.—Rabbits were made unresponsive to BSA by the injection of 100 mg during the first 24 hr of life followed by 400 mg in two equal doses before the 5th day of life. This procedure has proved sufficient to make all the rabbits thus treated unresponsive for as long as 6 months (2).

Termination of Unresponsiveness to BSA.—The unresponsive state to BSA was terminated by giving two courses of PSA, HSA, ESA, or GPSA spaced 2 wk apart. Each course consisted of four daily injections of 20 mg subcutaneously followed by 30 mg per kg of body weight intravenously on day 5. 7 days after the last injection all the rabbits were bled and the sera tested for antibody against BSA and five cross-reacting albumins.

Antibody Determinations.—Precipitating antibody was measured by the quantitative method of Talmage and Maurer (12) using I* antigen and the results are reported as μg antibody nitrogen (N) per 1.0 ml of serum. Tests for binding antibody were performed by the ammonium sulfate technique of Farr (13) employing 1.0 μg I* antigen N and increasing dilution of antisera, and the results are reported as μg I* antigen N bound to the globulin present in 1.0 ml of serum.

Dissociation Rates.—Antibody avidity for BSA was measured by a modification of the Farr ammonium sulfate technique (4, 13, 14). The antigen-binding capacity of the serum was determined at the same antigen concentration and temperature used in the dissociation rate experiments (25°C, 2.0 μg antigen N per ml). 10 ml of each antibody solution was diluted in 10% normal rabbit serum to a concentration sufficient to bind 40% of the I*-antigen, and 10 ml of this diluted antiserum was mixed with 10 ml of I*-antigen at a concentration of 2.0 μg N per ml in 1% normal rabbit serum. The tubes were sealed and incubated overnight at 25°C to allow the antigen antibody interaction to come to equilibrium. The next day duplicate 1.0 ml samples were removed from each tube (for zero time controls) and added to 1.0 ml of ice-cold saturated ammonium sulfate. After 1 hr at 0°C the tubes were centrifuged and the precipitates washed with 50% saturated ammonium sulfate. A 1000-fold excess of cold antigen (in 0.5 ml) was then added to each tube at intervals of 1 min. At intervals of exactly 0.5, 1.0, 1.5, 3.0, and 6.0 hr after addition of the cold antigen, duplicate 1.0 ml samples were removed from each tube and added to 1.0 ml of ice-cold saturated ammonium sulfate and processed as were the zero time controls. Normal serum controls were included to correct for nonspecific precipitation. The per cent antigen I* bound at each time was calculated and plotted on semilogarithmic paper to determine the time (in minutes) for 50% of the initially bound antigen to dissociate from antibody (t_{50}).

RESULTS

Binding Antibody in Sera of Normal and BSA-Unresponsive Rabbits after Injections of Various Heterologous Albumins.—Rabbits were made unresponsive to BSA by injection of BSA at birth. At 12 wk of age, both the unresponsive rabbits and normal rabbits (of equivalent age and weight) were given two 1-wk courses of PSA, HSA, ESA, or GPSA totalling 340 mg. These animals were bled 7 days after the last injection of heterologous albumin and the antigen-binding capacities determined for BSA and five other albumins (cross-reactions with a hyperimmune anti-BSA serum ranging from 75% for SSA to 5% for GPSA). Both BSA unresponsive and normal rabbits produced high levels of binding antibody to the terminating antigen and lower levels reactive with the other albumins (Table I). There were no significant differences between normal and unresponsive rabbits in the amount of antibody that reacted with any of the albumins tested. This was true regardless which heterologous albumin was used to terminate the unresponsive state. 63 of 68 unresponsive rabbits (91.2%) and 68 of 78 normal rabbits (87.2%) produced antibody reactive with BSA. The antibody levels in the remaining 16 rabbits were low not only to the tolerated antigen, but to the terminating antigen and the other albumins as well.

Precipitating Antibody in Sera of Normal and BSA-Unresponsive Rabbits after Injections of Various Heterologous Albumins.—Normal and BSA unresponsive rabbits were given two 1-wk courses of immunization with various soluble serum albumins and bled 7 days after the last injection. The levels of precipitating antibody to BSA and five other albumins were measured using I* antigens. There were no significant differences in the amount of antibody precipitating with any of the albumins in sera of those normal and unresponsive rabbits which received either PSA or HSA (Table II). However, those normal rabbits immunized with GPSA produced greater quantities of precipitating antibody than did unresponsive rabbits similarly immunized.

Inhibition of the Termination of the Unresponsive State by the Simultaneous Injection of the Tolerated Antigen.—The inhibition of the termination of unresponsiveness by the simultaneous injection of BSA along with the terminating antigen was studied further. BSA unresponsive rabbits were given varying amounts of BSA simultaneously with HSA over two 1-wk courses of immunization. The antigen-binding capacities of the sera were determined for both BSA and HSA. Doses of 17.0 mg BSA or greater showed a marked inhibition of the termination of unresponsiveness. There was a decrease in the titer to BSA and to HSA with increasing amounts of BSA and only 2 of 14 rabbits receiving BSA showed an immune elimination of a subsequent injection of the tolerated antigen (Table III).

Dissociation Rates.—Grey (14) has shown that the association rates of antibody and antigen reactions were related to the net electrical charge of the antibody molecules, while the dissociation rates were independent of charge, and

TABLE I
Mean Antigen-Binding Capacities of Sera from Normal and BSA-Unresponsive Rabbits after Two Courses of Various Soluble Albumins*

Terminating antigen	Status	No. of animals	Antigen tested					
			BSA	PSA	HSA	ESA	GPFA	SSA
PSA	Unresponsive	19	11.6 ± 1.8†	188.8 ± 23.0	3.3 ± 0.5	7.0 ± 1.2	9.4 ± 1.9	11.1 ± 1.8
	Normal	12	11.7 ± 1.9	249.1 ± 40.5	3.7 ± 0.8	9.2 ± 1.8	8.0 ± 2.2	10.1 ± 2.1
HSA	Unresponsive	26	0.9	0.2	0.6 < P < 0.7	0.3	0.6 < P < 0.7	0.7 < P < 0.8
	Normal	26	9.1 ± 1.8	8.3 ± 1.6	200.8 ± 29.3	8.9 ± 1.6	11.5 ± 2.1	4.7 ± 1.0
GPFA	Unresponsive	26	7.8 ± 1.2	6.9 ± 1.2	142.7 ± 14.6	7.6 ± 1.3	10.0 ± 2.0	5.9 ± 1.4
	Normal	9	0.5 < P < 0.6	0.4 < P < 0.5	0.05 < P < 0.1	0.5 < P < 0.6	0.6 < P < 0.7	0.4 < P < 0.5
ESA	Unresponsive	14	7.1 ± 1.3	32.6 ± 5.4	15.7 ± 3.8	10.8 ± 2.7	324.4 ± 58.0	17.6 ± 4.3
	Normal	15	8.7 ± 2.3	33.7 ± 5.8	14.2 ± 2.8	12.7 ± 3.7	289.3 ± 47.5	15.1 ± 3.6
SSA	Unresponsive	15	0.5 < P < 0.6	0.9	0.7 < P < 0.8	0.6 < P < 0.7	0.6 < P < 0.7	0.6 < P < 0.7
	Normal	26	6.6 ± 1.2	18.7 ± 2.6	15.1 ± 2.1	170.2 ± 16.6	11.9 ± 2.1	12.1 ± 1.0
			5.6 ± 1.0	14.4 ± 2.1	10.9 ± 1.8	139.4 ± 21.0	8.3 ± 1.5	10.1 ± 1.6
			0.5 < P < 0.6	0.2 < P < 0.3	0.1 < P < 0.2	0.2 < P < 0.3	0.1 < P < 0.2	0.4 < P < 0.5

* μg antigen N bound to the globulin (precipitated with 50% saturated $(\text{NH}_4)_2\text{SO}_4$) present in 1.0 ml of serum.

† Standard error of the mean.

TABLE II
Mean Precipitating Antibody from Normal and BSA-Unresponsive Rabbits after Two Courses of Various Soluble Albumins*

Terminating antigen	Status	No. of animals	Antigen tested					
			BSA	PSA	HSA	ESA	SSA	
PSA	Unresponsive	8	2.2 ± 0.8 ‡	268.6 ± 61.6	1.2 ± 0.6	2.4 ± 1.0	0.4 ± 0.4	1.4 ± 0.4
	Normal	12	3.4 ± 1.3	218.7 ± 32.0	0.2 ± 0.2	1.2 ± 0.4	0.2 ± 0.2	3.0 ± 1.3
HSA	Unresponsive	7	0.4 < P < 0.5	0.4 < P < 0.5	0.1 < P < 0.2	0.2 < P < 0.3	0.6 < P < 0.7	0.2 < P < 0.3
	Normal	12	5.2 ± 3.1	5.1 ± 4.3	255.4 ± 92.9	4.0 ± 1.7	0.6 ± 0.6	1.5 ± 1.0
GPSA	Unresponsive	9	8.2 ± 3.5	5.5 ± 1.8	278.5 ± 52.1	5.0 ± 1.0	1.9 ± 1.0	6.2 ± 2.9
	Normal	11	0.5 < P < 0.6	0.9	0.8 < P < 0.9	0.6 < P < 0.7	0.3	0.1 < P < 0.2
			0.2 ± 0.2	1.3 ± 0.5	0.7 ± 0.4	0.6 ± 0.4	267.7 ± 54.2	1.2 ± 0.6
			1.5 ± 0.6	8.8 ± 3.1	0.8 ± 0.4	2.3 ± 0.6	470.4 ± 66.2	3.9 ± 0.9
			0.05	0.02 < P < 0.05	0.8 < P < 0.9	0.02 < P < 0.05	0.02 < P < 0.05	0.02

* μ g antibody N per ml of serum.

‡ Standard error of the mean.

that therefore the dissociation rates were a more realistic measurement of avidity of antibody for antigen. Sera from normal and unresponsive rabbits obtained 7 days after immunization with heterologous albumins were assayed for their avidity for BSA by determining their dissociation rates with I*-BSA. Table IV shows that the antibody in sera of normal rabbits immunized with

TABLE III
Effect of Simultaneous Injection of BSA on the Termination of the BSA-Unresponsive State by HSA

BSA	Anti-BSA*	Immune elimination†
mg‡		
0	10.4	9/10
17	2.6	2/5
170	0.2	0/4
300	0	0/5

* μg antigen N bound by the globulin (precipitated with 50% saturated ammonium sulfate) present in 1.0 ml of serum.

† Fraction of rabbits showing on immune elimination of I*-BSA.

‡ Injected simultaneously with 340 mg HSA over two 1-wk courses of immunization.

TABLE IV
Mean Dissociation Rates of Antibody from Normal and BSA-Unresponsive Rabbits after Two Courses of Various Soluble Albumins*

Terminating antigen	Status	No. of animals	t_{50} *
PSA	Unresponsive	7	6.3 ± 1.0
	Normal	11	11.2 ± 1.0
	<i>P</i>		0.01
HSA	Unresponsive	10	20.4 ± 5.0
	Normal	14	42.9 ± 3.6
	<i>P</i>		0.01
ESA	Unresponsive	14	74.2 ± 8.1
	Normal	9	45.4 ± 5.6
	<i>P</i>		0.02

* Time in minutes for 50% of initially bound I*BSA to dissociate from antibody in the presence of a 1000-fold excess of unlabeled BSA.

PSA or HSA was more avid than antibody in sera of unresponsive rabbits similarly treated. However, if ESA was used to terminate unresponsiveness, the antibody in sera from the previously unresponsive rabbits was more avid than that of antibody from sera of normal rabbits.

DISCUSSION

The data presented above indicate that the establishment of the unresponsive state to BSA in neonatal rabbits does not result in death of precursor cells or

loss of specific receptors on these cells, suggesting that the unresponsive rabbit has a cellular potential to produce antibody to BSA. Normal rabbits and rabbits made unresponsive by neonatal injection of BSA made similar amounts of both binding and precipitating antibody to BSA after immunization with aqueous preparations of BSA, HSA, GPSA, or ESA. The avidity of the antibody produced was dependent on the antigen used to terminate the unresponsive state and not on whether the antibody came from normal or unresponsive rabbits. Thus after injection of normal and unresponsive rabbits with these heterologous albumins, no quantitative or qualitative difference in the anti-BSA produced could be detected, suggesting that the unresponsive rabbit had the potential to produce a normal response to the BSA-related determinants on cross-reacting albumins.

It has been shown that each of the albumins used shares several determinants with BSA (15), and since there were few significant differences between tolerant and normal rabbits in the amount of antibody reacting with any of the albumins, the specificity of the antibody in sera from previously unresponsive rabbits may be the same as that in sera from normal rabbits similarly immunized. Previous reports (1-5), however, all showed lower levels of antibody in unresponsive animals after termination of the unresponsive state with either hapten-substituted antigens or with certain cross-reacting antigens. The lower levels in those unresponsive animals immunized with hapten-substituted antigens could be explained by prolongation of the unresponsive state by native determinants on the substituted molecule very similar or identical to those on the tolerated antigen. Cinader et al. (8) have shown that the injection of certain hapten-substituted HSA preparations into HSA-unresponsive rabbits did prolong the duration of the HSA-unresponsive state and that the ability to prolong this unresponsive state depended on the degree of substitution. Whether complete or partial inhibition was obtained depended on the number and specificity of the native determinants remaining. These findings are not unexpected since it has been shown that simultaneous injection of native BSA along with the terminating antigen can suppress the production of anti-BSA in response to injections of heterologous albumins. Indeed, very small amounts of BSA can reduce the level of anti-BSA to 7.5% of that formed in control unresponsive rabbits, and completely abolish the capacity of these animals to respond to a subsequent injection of BSA. The injection of 20 mg BSA 2 wk before, or 3.0 mg BSA 1 day before the start of injections of the terminating antigen has no effect on the production of anti-BSA. This would indicate that a certain level of BSA must be maintained for inhibition to occur and that a direct competition for cellular receptors between shared determinants on BSA and the terminating antigen is most likely responsible for this inhibition. In addition, it appears that the lower levels of anti-BSA reported in other studies (1, 2) in which the unresponsive state was terminated with natural cross-reacting antigens could be

explained by a contamination of the terminating antigen preparations with the tolerated antigen (6). In these studies, the amount of contaminating BSA may not have been sufficient to completely inhibit the termination but may have been sufficient to reduce the amount of anti-BSA produced. In the normal animal, contaminating BSA would have had no inhibitory effect.

The avidity for BSA of the antibody produced in unresponsive and normal rabbits seems to depend on the heterologous albumin used to terminate the unresponsive state. Those unresponsive rabbits immunized with either PSA or HSA produced antibody with a lower avidity for BSA than did normal rabbits similarly treated. However, if ESA was used as the terminating antigen, the avidity of antibody from BSA unresponsive rabbits was greater than that of antibody from normal rabbits. This could be explained if one assumes that there is a spectrum of cells producing antibody toward a given determinant and that the receptors on these cells vary in their avidity for that determinant. It is possible that in this spectrum some cells would not be able to detect the structural differences (if any) between the determinants on BSA and those on the terminating antigen. Interaction between the terminating antigen and those receptors in the unresponsive animal would then have the same effect as interaction between these receptors and BSA (the tolerated antigen), i.e., an inhibition of antibody formation. When the terminating antigen was PSA or HSA, the cells thus affected would be those cells producing high avidity antibody, whereas when ESA was the terminating antigen those cells affected would be the low avidity producers. The number of cells affected by any of these antigens would necessarily be few since quantitative differences are not seen between these unresponsive and normal animals.

The presence of a normal complement of antibody-producing cells to BSA in rabbits rendered unresponsive to BSA by neonatal injections can most readily be explained if the immune response to BSA in rabbits involves the interaction of two cell types, both containing receptor sites for antigen, and if unresponsiveness lies in a cell other than the precursor cell. That two cells are involved in the induction of antibody formation has been shown by several recent experiments. Synergistic effects have been shown between thymus and bone marrow cells in the induction of an antibody response to red blood cells (16, 17) and to human gamma globulin (HGG) (18) and that the bone marrow-derived cells are the precursor cells (17). It has also been shown that mice thymectomized after the induction of unresponsiveness escape from unresponsiveness much later than nonthymectomized controls (19); that animals injected directly into the thymus with antigen showed a greater degree of unresponsiveness than did animals in which the antigen was injected into the spleen or lymph nodes (20); and that specific cellular receptors may exist on both the thymus and the bone marrow-derived cells (21). The specificity of these receptors of the two cells interacting with the antigen would necessarily be different, since there does not seem to be

a repetition of determinants on the surface of monomeric albumins (22 and footnote 2). The bone marrow cell could then carry receptors of the same specificity as the antibody it produces and the specificity of the thymus cell receptor may be toward determinants on the terminating antigen unrelated to those on BSA. In view of the carrier antibody theories, it appears that, in a normal rabbit, BSA may be presented to the antibody-forming cell by specific receptors on a thymus-derived cell reacting with determinants of HSA unrelated to those on BSA. If this is the case, the BSA-unresponsive rabbit, even though it seems to possess a normal complement of antibody-forming cells, would be unable to make an immune response to BSA, since the thymus-derived cells would be missing. The presence of a defect at only the thymus-derived cell is not in agreement with observations in mice, where the defect of unresponsiveness to human γ -globulin was shown to be at both the thymus and bone marrow-derived cells (21). However, in the latter experiments the mice were rendered unresponsive as adults, whereas in the present experiments unresponsiveness was induced by neonatal injections.

Other explanations for the termination of unresponsiveness have been given. It has been proposed that the difference between unresponsive and normal animals is the avidity of cellular receptors for the determinants on the tolerated antigen and similar determinants on the terminating antigen (5, 23) with the avidity being much greater for the terminating antigen. If this is the case, then the number of cells bearing specific receptors with a great enough avidity for the tolerated antigen have to be too few to result in a detectable antibody response, whereas the number of cells responding to the cross-reacting antigen have to be much greater. However, the role of avidity suggested above appears unlikely, since the injection of relatively small amounts of the BSA simultaneously with large amounts of the terminating antigen results in a marked inhibition of the termination of unresponsiveness. Bretscher and Cohn (24) have suggested that carrier antibody (either free or cell-bound) may be necessary for antibody formation and that the carrier antibody for the tolerated antigen is missing in unresponsive animals. This carrier antibody would necessarily be of a special class, since passive anti-BSA or soluble BSA-anti-BSA complexes have no effect on the ability of a BSA-unresponsive rabbit to respond to BSA (2). A carrier-like material may still be the missing factor, but would have different properties than suggested by Bretscher and Cohn.

SUMMARY

Rabbits made unresponsive to BSA at birth were given two courses of immunization with various cross-reacting albumins at 3 months of age. Normal

² Benjamin, D. C., and W. O. Weigle. The structural basis for the cross-reactivity of serum albumins. Manuscript in preparation.

control rabbits, of equivalent age and weight, were similarly immunized. Sera obtained 7 days after the last injection were assayed for binding and precipitating antibody to six albumins and for their avidity for BSA. No significant differences were found between unresponsive and normal rabbits in the amount of antibody reacting with any of the six albumins used. This was the case regardless which albumin was used to terminate the unresponsive state. Avidity differences were seen and seemed to depend on the antigen used and not on the immunological status of the animal. The simultaneous injection of small amounts of BSA inhibited the termination of unresponsiveness. These results were discussed in the light of the more recent theories of the termination of unresponsiveness and of antibody formation.

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