TERMINATION OF ACQUIRED IMMUNOLOGICAL TOLERANCE TO PROTEIN ANTIGENS FOLLOWING IMMUNIZATION WITH ALTERED PROTEIN ANTIGENS*, ‡

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It was previously shown that acquired tolerance to bovine serum albumin (BSA), induced by neonatal injection of rabbits with BSA, was terminated following the injection of certain serum albumins which cross-react with BSA (1). The ability of cross-reacting albumins to terminate the tolerant state was dependent on their serological relation to BSA. More distantly related albumins were more effective in terminating BSA tolerance than more closely related albumins. It was reasoned from these results that BSA tolerance may also be terminated by the injection of preparations of BSA that were structurally altered but still cross-reacted with native BSA. The present experiments were designed to study the immune response in tolerant rabbits to injections of enzymatically, chemically, and physically altered preparations of the same proteins and to study the effect of injections of these preparations on the tolerant state.

Materials and Methods

Antigens.—Both BSA (Lot V68802) and bovine gamma globulin (BGG) (Lot C-904) were obtained from Armour Pharmaceutical Company, Kankakee, Illinois. The proteins were trace-labeled with I^{131} (I*) by the method previously described (2). Protein-bound I* activity was determined in NaI crystal scintillation counters. The specific activity (counts/microgram N) of the I* proteins was determined so that the I* activity could be converted to micrograms of protein N.

Antisera.—Anti-BSA and anti-BGG were obtained from hyperimmunized albino rabbits prepared by a series of injections of either BSA or BGG given over a 2 to 4 month period, totaling 250 to 300 mg. The last injection was given 7 days before bleeding. The sera obtained from fifteen to twenty rabbits were pooled.

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Alteration of Antigens.—

(*a*) *BSA Complexed to Anti-BSA.*—BSA was added to hyperimmune rabbit anti-BSA at equivalence. The mixture was incubated at 37°C for 30 minutes and then overnight at 0–3°C. The precipitate which formed was washed twice with 0.15 M NaCl and resuspended in 0.15 M NaCl.

(*b*) *Pepsin-Degraded BSA.*—A 1 per cent BSA solution was partially degraded with pepsin[1] at pH 2.5 by the technique described by Kaminski and Tanner (3). The pepsin concentration was 0.001 mg/mg of protein and the period of incubation was 6 hours at 37°C. Enzymatic degradation was terminated by adjusting the pH to 7.2 with 0.5 M NaOH. Double diffusion in agar between pepsin-degraded BSA and anti-native BSA yielded three to four distinct precipitin bands (4).

(*c*) *Heat-Denatured BSA.*—An 0.5 per cent solution of BSA at pH 7.5 was placed in a boiling water bath for 1 hour (5). The heat-denatured BSA was purified by repeated precipitation at its isoelectric point.

(*d*) *Acetylated BSA.*—Acetylated BSA (acetyl-BSA) was prepared with acetic anhydride according to the procedure described by Olcott and Fraenkel-Conrat (6). The reaction was carried out for 1 hour, at which time about 85 per cent of the amino groups react (5).

(*e*) *Picrylated BSA.*—Picrylated BSA (picryl-BSA) was prepared with picryl-chloride according to the method described by Benacerraf and Gell (7). The number of picryl groups per molecule of BSA was 27.2 as determined by spectrophotometric analysis, using picryl-epsilon aminocaproic acid[2] as a standard.

(*f*) *Acetylated-Picrylated BSA.*—Acetylated-picrylated BSA (acetyl-picryl-BSA) was prepared by adding picryl-chloride in the manner previously described to acetyl-BSA. The number of picryl groups per molecule was 12.2.

(*g*) *Azoproteins.*—Azoproteins were prepared by the method described by Baker *et al.* (8). The diazonium derivatives of *p*-arsanilic and *p*-sulfanilic acids were prepared in HCl with $NaNO_2$ and coupled to the proteins at 0°C. The preparations were dialyzed in phosphate buffer, pH 7.5, ionic strength 0.1, for 12 to 17 days. Equal moles of the two acids were used. In the cases where both derivatives were coupled to the same protein preparations, equal molar amounts of each derivative were added so that the total molar quantities were the same as when only one of the derivatives was coupled to a protein. The azoproteins contained the following number of azo groups per molecule of protein: arsanil-BSA, 5.8; sulfanil-BSA, 6.9; arsanil-sulfanil-BSA, 6.5; arsanil-BGG, 14.0; sulfanil-BGG, 18.5; and arsanil-sulfanil-BGG, 16.9. The arsanil-BSA and the arsanil-sulfanil-BSA were also analyzed for arsenic (9). Arsanil-BSA contained 11.0 arsenic groups per molecule of protein and arsanil-sulfanil-BSA contained 3.8 arsenic groups per molecule of protein. Thus, as previously demonstrated by Gelewitz, Riedeman, and Klotz (10), azoproteins contain more haptenic groups than can be accounted for by the azo linkage.

Rabbits were made tolerant to either BSA or BGG by the method previously described (11, 12). Newborn rabbits were given several subcutaneous injections totaling 500 mg of either BSA or BGG during the first 5 days after birth. Care was taken to insure an injection of at least 100 mg of protein during the first 24 hours following birth. When the rabbits reached maturity (at the 3 to 4 months of age) and prior to their use in the following experiments, they were injected intravenously with 20 mg of I*-labeled antigen and their failure to show an immune elimination of the labeled antigen within 2 weeks was used as an index of a tolerant state.

Injection of Native and Altered Proteins.—Both normal and tolerant rabbits were injected

[1] Worthington Biochemical Corporation, Freehold, New Jersey.

[2] Kindly supplied by Dr. Baruj Benacerraf.

with antigens either incorporated in Freund's adjuvant or precipitated by alum. Freund's adjuvant preparations were composed of nine parts bayol F,[3] one part arlacel C[4], and ten parts 0.15 M NaCl containing the antigen. Except where mentioned, rabbits were injected subcutaneously with 2 to 3 ml of adjuvant containing a total of 25 mg of protein and 0.5 mg of mycobacteria (*Mycobacterium tuberculosis var. hominis*). Injections of alum-precipitated antigens were given four times per week for 4 weeks (13) and the animals were bled 7 days after the last injection.

Elimination of I-BSA from the Circulation.*—After immunization with one of the altered proteins, the tolerant rabbits were bled for antibody and then immediately injected intravenously with 20 mg of an I*-labeled preparation of the protein used to induce tolerance. The sera were analyzed periodically for protein-bound I* activity. The immune elimination of I* activity was used as evidence for the loss of the tolerant state.

Antibody Analyses.—Precipitating antibody was measured by the quantitative immunochemical procedure described by Heidelberger and coworkers (13). In situations where the levels of precipitating antibody were low and could not be measured by this procedure, a quantitative technique (14) utilizing I* antigen, was employed. The ammonium sulfate technique of Farr (15) was used to detect either non-precipitating anti-BSA or small amounts of precipitating antibody. The ammonium sulfate technique was performed with either 1.0 or 0.1 μg I*-BSA N and increasing dilutions of antisera, and the results reported as the μg of I* -BSA N bound to the globulin in 1 ml of serum. Both non-precipitating anti-BGG and small amounts of precipitating anti-BGG were detected by the passive cutaneous anaphylaxis (PCA) test (16). The degree of cutaneous reaction was recorded as 0 to ++++. Antisera were diluted 1 to 5 and injected in an 0.1 ml volume. One ml of the antigen containing 500 mg protein per ml was injected in an 0.15 M NaCl solution containing 1 per cent Evans blue.

Inhibition Studies.—Rabbit serum containing 2.34 mg anti-BSA N was absorbed at equivalence with an altered BSA preparation. The remaining antibody to native BSA was determined in the presence of an excess of the same altered BSA preparation in an amount equal to 50 times the amount of BSA necessary to precipitate all of the anti-BSA at equivalence. The excess altered BSA was added to the antiserum at 0°C, and the mixture incubated for 30 minutes at 37°C and for 1 hour at 0–3°C prior to addition of native BSA.

RESULTS

Normal and BSA-Tolerant Rabbits Injected with BSA Complexed to Anti-BSA.—Four normal and six tolerant rabbits were injected with complete Freund's adjuvant, containing 3.75 mg of BSA complexed to anti-BSA at equivalence, once a week for 3 weeks and bled 14 days after the last injection. The sera of the four normal rabbits contained 86.6, 34.2, 72.2, and 86.6 μg anti-BSA N per ml, while none of the sera of the tolerant rabbits contained anti-BSA. Also, the tolerant rabbits failed to show an immune elimination of a subsequent injection of I*-BSA.

Normal and BSA-Tolerant Rabbits Injected with Heat-Denatured BSA.—Five normal and five BSA-tolerant rabbits were injected once a week for 3 weeks with Freund's adjuvant, containing 25 mg of heat-denatured BSA, and bled 14 days after the last injection. The sera of the five normal rabbits contained 367.0,

[3] Standard Oil Company, New Jersey.

[4] Atlas Powder Company, Wilmington, Delaware.

207.4, 237.0, 258.8, and 169.9 μg anti–heat-denatured BSA N and 170.1, 66.9, 88.0, 88.5, and 23.7 μg anti–native BSA. The five tolerant rabbits contained neither anti–heat-denatured BSA nor anti–native BSA and did not show an immune elimination of a subsequent injection of I*-BSA.

Normal and BSA-Tolerant Rabbits Injected with Pepsin-Degraded BSA.—Three normal and six tolerant rabbits were injected with an alum-precipitated preparation of pepsin-degraded BSA. Sera of the normal rabbits contained 405.8, 190.0, and 172.0 μg of antibody N per ml, which precipitated with pepsin-degraded BSA, and 281.8, 86.0, and 145.8 μg of antibody N per ml, which precipitated with native BSA. Sera from the tolerant rabbits, however, did not contain antibody which precipitated with either pepsin-degraded BSA or native BSA.

Normal and BSA-Tolerant Rabbits Injected with Acetyl-BSA.—Four normal and seven BSA-tolerant rabbits were injected with alum-precipitated acetyl-BSA. Sera of the normal rabbits contained 60.0, 198.5, 47.4, and 73.8 μg anti–acetyl-BSA N per ml and 3.1, 9.7, 4.4, and 30.4 μg anti–native BSA N per ml. The serum of only one of the tolerant rabbits contained anti–acetyl-BSA (81.0 μg N per ml) and none of the sera contained anti–native BSA. Six additional BSA-tolerant rabbits were injected once a week for 3 weeks with complete Freund's adjuvant, containing 25 mg of acetyl-BSA, and bled 14 days after the last injection. Their sera contained 0, 22.4, 0, 0, 8.6, and 0 μg anti–acetyl-BSA N per ml and no anti–native BSA. The tolerant rabbits injected with either alum-precipitated, acetyl-BSA or acetyl-BSA incorporated in Freund's adjuvant failed to show an immune elimination of a subsequent injection of I*-BSA.

Normal and BSA-Tolerant Rabbits Injected with Picryl-BSA.—Four normal and five BSA-tolerant rabbits were injected with an alum-precipitated preparation of picryl-BSA. Sera of the normal rabbits contained 87.8, 30.8, 35.6, and 33.6 μg anti–picryl-BSA N per ml and 9.7, 4.6, 3.4, and 4.8 μg anti-BSA N per ml. Sera of the five tolerant rabbits contained neither anti–picryl-BSA nor anti–native BSA. The tolerant rabbits also failed to show an immune elimination of a subsequent injection of I*-BSA. Also, two normal and five tolerant rabbits were injected once a week for 3 weeks with complete Freund's adjuvant containing 25 mg picryl-BSA and bled 14 days after the last injection. The sera of the two normal rabbits contained 579.5 and 385.0 μg anti–picryl-BSA N per ml and 161.0 and 131.0 μg anti–native BSA N per ml, while the sera of the five tolerant rabbits contained 0, 0, 0, 176.6, and 120.0 μg anti–picryl-BSA and no anti–native BSA. The tolerant rabbits also failed to show an immune elimination of a subsequent injection of I*-BSA.

BSA-Tolerant Rabbits Injected with Picryl-Acetyl-BSA.—Two of six BSA-tolerant rabbits injected once a week for 3 weeks with complete Freund's adjuvant containing 25 mg of acetyl-picryl-BSA lost their tolerant state (Table

I). These two rabbits contained small amounts of anti-BSA as indicated by the ammonium sulfate precipitation technique and showed an immune elimination of a subsequent injection of I*-BSA.

BSA-Tolerant Rabbits Injected with Azo-BSA.—Rabbits were injected once a week for 3 weeks with complete Freund's adjuvant, containing 25 mg of an azo-BSA, and bled 14 days after the last injection (Tables II, III, and IV). The sera were analyzed for antibody to both the native BSA and the azo groups. All sera containing anti–native BSA were absorbed with native BSA prior to analysis for anti–azo-BSA.

TABLE I

Production of Antibody in BSA-Tolerant Rabbits Injected‡ with Picryl-Acetyl-BSA

Rabbit No.	Antibody to native BSA		Antibody N precipitating with		Immune elimination of I*-BSA
	Precipitating N	Binding§	Picryl-BSA	Acetyl-BSA	
	μg		μg	μg	
1	0	2.4	42.7	24.4	+
2	0	0	0	0	–
3	0	2.0	29.7	19.6	+
4	0	0	20.4	12.0	–
5	0	0	18.6	14.8	–
6	0	0	0	0	–

‡ Injected once a week for 3 weeks with 25 mg of picryl-acetyl-BSA in complete Freund's adjuvant and bled 14 days after the last injection.

§ The μg I*-BSA N bound by the globulin fraction (precipitated with 50 per cent $(NH_4)_2SO_4$) of 1 ml of serum.

The sera of three normal rabbits injected with arsanil-BSA contained some antibody precipitating with both arsanil-BSA and native BSA (Table II). Sera of the tolerant rabbits did not contain antibody to native BSA and these rabbits did not show immune elimination of a subsequent injection of I*-BSA. The sera of three of the four tolerant rabbits did contain small amounts of antibody to the arsanil-BSA as detected by the PCA test, but precipitation tests were negative.

Sera of the three normal rabbits injected with sulfanil-BSA contained moderate to high levels of antibody that precipitated with both native and sulfanil-BSA (Table III). The sera of three tolerant rabbits injected with sulfanil-BSA contained antibody which precipitated with sulfanil-BSA but no antibody which precipitated with native BSA. Their sera did contain a small amount of anti–native BSA as detected by the ammonium sulfate technique. In addition, the three tolerant rabbits showed an immune elimination of a subsequent injection of I*-BSA.

The sera of three normal rabbits injected with arsanil-sulfanil-BSA contained moderate to large amounts of antibody which precipitated with both native and arsanil-sulfanil-BSA (Table IV). The sera of nine tolerant rabbits injected with arsanil-sulfanil-BSA contained moderate to large amounts of antibody

TABLE II

Production of Antibody in Normal and BSA-Tolerant Rabbits Injected‡ with Arsanil-BSA in Freund's Adjuvant

Rabbit No.	Antibody to native BSA		Antibody to arsanil-BSA§		Immune elimination of I*-BSA
	Precipitating N	Binding‖	Precipitating N	PCA¶	
	μg		μg		
Normal 1	33.0		17.4		
2	22.2		52.2		
3	3.2		64.5		
Tolerant 4	0	0	0	+++	−
5	0	0	0	++++	−
6	0	0	0	0	−
7	0	0	0	+++	−

‡ Injected once a week for 3 weeks with 25 mg of arsanil-BSA in complete Freund's adjuvant and bled 14 days after the last injection.

§ Absorbed with native BSA prior to determinations.

‖ The microgram I*-BSA N bound by the globulin fraction (precipitated with 50 per cent $(NH_4)_2SO_4$) of 1 ml of serum.

¶ Passive cutaneous anaphylaxis.

TABLE III

Production of Antibody in Both Normal and BSA-Tolerant Rabbits Injected‡ with Sulfanil-BSA in Freund's Adjuvant

Rabbit No.	Antibody to native BSA		Antibody N precipitating with sulfanil-BSA‖	Immune elimination of I*-BSA
	Precipitating N	Binding§		
	μg		μg	
Normal 1	312.0		158.0	
2	336.0		149.0	
3	652.8		307.4	
Tolerant 4	0	3.3	226.4	+
5	0	2.5	146.2	+
6	0	4.2	64.8	+

‡ Injected once a week for 3 weeks with 25 mg arsanil-BSA in complete Freund's adjuvant and bled 14 days after the last injection.

§ The microgram I*-BSA N bound by the globulin fraction (precipitated with 50 per cent $(NH_4)_2SO_4$) of 1 ml of serum.

‖ Absorbed with native BSA prior to determinations.

which precipitated with arsanil-sulfanil-BSA and small to large amounts of antibody which precipitated with native BSA. All of the tolerant rabbits showed an immune elimination of a subsequent injection of I*-BSA.

Six tolerant rabbits were injected with alum-precipitated arsanil-sulfanil-BSA. The serum from only one of these rabbits contained antibody which pre-

TABLE IV

Production of Antibody in Normal and BSA-Tolerant Rabbits Injected‡ with Arsanil-Sulfanil-BSA in Freund's Adjuvant

Rabbit No.	Antibody to native BSA		Precipitating antibody N to arsanil-sulfanil-BSA‖	Immune elimination of I*-BSA
	Precipitating N	Binding§		
	μg		μg	
Normal 1	556.8		208.2	
2	321.6		371.2	
3	360.0		140.4	
Tolerant 4	—	—	—	+
5	302.4	85.8	206.9	+
6	46.8	21.1	111.3	+
7	22.8	19.2	186.8	+
8	146.4	23.8	313.4	+
9	42.0	15.8	413.6	+
10	15.0	11.6	111.0	+
11	—	—	—	+
12	331.2	115.5	242.5	+
13	32.2	17.5	235.2	+
14	153.6	26.4	345.3	+

‡ Injected once a week for 3 weeks with 25 mg of arsanil-sulfanil-BSA in complete Freund's adjuvant and bled 14 days after the last injection.

§ The microgram I*-BSA N bound by the globulin fraction (precipitated with 50 per cent $(NH_4)_2SO_4$) of 1 ml of serum.

‖ Absorbed with native BSA prior to determinations.

cipitated with native BSA (Table V). Also, this serum was the only serum that contained antibody which precipitated with arsanil-sulfanil-BSA. However, the sera of the other five tolerant rabbits contained small amounts of antibody to arsanil-sulfanil-BSA as shown by PCA reaction and all six rabbits showed an immune elimination of a subsequent injection of I*-BSA.

BGG-Tolerant Rabbits Injected with Azo-BGG.—All the tolerant rabbits received one injection of complete Freund's adjuvant, containing 25 mg of an azo-BGG, and were bled 28 days later. There was no significant difference among the results obtained with arsanil-BGG, sulfanil-BGG, and arsanil-sulfanil-BGG injected rabbits (Table VI). The sera of all rabbits except one contained antibody which precipitated with the azo-BGG used for injection.

TABLE V

Production of Antibody in BSA-Tolerant Rabbits Injected‡ with Arsanil-Sulfanil-BSA Precipitated with Alum

Rabbit No.	Antibody to native BSA		Antibody to azo groups		Immune elimination of I*-BSA
	Precipitating N	Binding§	Precipitating N	PCA‖	
	μg		μg		
1	0	0	0	+	+
2	0	0	0	+	+
3	0	0	0	+++	+
4	0	0	0	++	+
5	16.3	3.6	35.5	++++	+
6	0	0	0	+	+

‡ Injected four times per week for 4 weeks with alum-precipitated, arsanil-sulfanil-BSA.

§ The microgram I*-BSA N bound by the globulin fraction (precipitated with 50 per cent $(NH_4)_2SO_4$) of 1 ml of serum.

‖ Passive cutaneous anaphylaxis.

TABLE VI

Production of Antibody in BGG-Tolerant Rabbits Injected‡ with Azo-BGG in Freund's Adjuvant

Azo-BGG injected	Rabbit No.	Antibody to native BGG		Antibody (N) to azo-BGG	Immune elimination
		Precipitating N	PCA§		
		μg		μg	
Arsanil	1	0	+	117.4	+
	2	0	++	137.6	+
	3	0	−	32.4	+
Sulfanil	4	0	±	29.6	+
	5	5.2	+++	22.4	+
	6	0	+	173.6	+
Arsanil-sulfanil	7	0	+	7.4	+
	8	0	+	0	+
	9	0	+	24.6	+

‡ One injection of 25 mg protein and bled 28 days later.

§ Passive cutaneous anaphylaxis.

Only one rabbit (injected with sulfanil-BGG) produced precipitating antibody to the native BGG. Most of the rabbits produced antibody to the native BGG as detected by the PCA test and all rabbits showed an immune elimination of a subsequent injection of I*-BGG. Five BGG-tolerant rabbits injected in a similar manner with native BGG failed either to produce circulating anti-BGG or to show an immune elimination of a subsequent injection of I*-BGG.

Serological Relationship between Altered and Anti-Native Proteins.—The serological relationship between the altered BSA preparations and anti-BSA was examined by both direct precipitation and inhibition (Table VII). Pooled hyperimmune rabbit serum containing 2.34 mg antibody N per ml was used to study cross-reactions between altered BSA and anti-native BSA. With heat-denatured, acetyl-, and picryl-BSA preparations, the cross-reactions were greater when carried out with concentrated antiserum than with antiserum diluted 1 to 15. Homologous and heterologous reactions were carried out in

TABLE VII

Cross-Reactions between Altered BSA Preparations and Anti-Native BSA‡

Antigen	Cross-reaction		
	By direct precipitation		By inhibition§
	Antiserum diluted (1-15)	Antiserum conc. (0.5 ml)	
	per cent	*per cent*	*per cent*
Heat-denatured BSA	30	48	100
Acetyl-BSA	52	80	100
Picryl-BSA	78	100	100
Arsanil-BSA		75	100
Sulfanil-BSA		80	100
Arsanil-sulfanil-BSA		84	100

‡ 2.34 mg antibody N per ml of pooled hyperimmune serum.

§ Anti-BSA serum was absorbed at equivalence with an altered BSA preparation and the remaining anti-BSA determined in the presence of an excess of the same altered preparation in a concentration fifty times that necessary to precipitate all of the anti-BSA at equivalence.

both concentrated and diluted antiserum. With concentrated antiserum, only picryl-BSA gave a 100 per cent cross-reaction by direct precipitation, but all of the altered BSA preparations gave a 100 per cent cross-reaction when inhibition tests were employed.

Pooled hyperimmune rabbit serum containing 1.01 mg antibody N per ml was used to study the cross-reactions between azo-BGG preparations and anti-native BGG. As determined by direct precipitation, arsanil-BGG, sulfanil-BGG, and arsanil-sulfanil-BGG cross-reacted with rabbit anti-BGG 76, 80, and 83 per cent respectively.

DISCUSSION

Mature rabbits made tolerant to BSA by neonatal injections of BSA lost their tolerant state following the injection of certain altered BSA preparations. BSA which had diazonium derivatives of both arsanilic and sulfanilic acids

coupled to the same molecule was more effective than any other altered preparations employed (Table VIII). Tolerance was terminated in all of the rabbits injected with Freund's adjuvant containing arsanil-sulfanil-BSA and precipitating antibody was produced to both the native BSA and the azo groups. Arsanil-sulfanil-BSA was also effective in terminating tolerance even when it was precipitated by alum. However, picryl-acetyl-BSA, which terminated the tolerant state in a portion of the animals when incorporated into complete Freund's adjuvant, had no effect when precipitated by alum. BSA tolerance

TABLE VIII

Antibody Response to Altered BSA in BSA-Tolerant Rabbits‡

Altered BSA-Injected	No. of rabbits	Anti-native BSA		Immune elimination of I*-BSA	Anti-Altered BSA§
		Binding	Precipitating		
Heat-denatured	5	–	–	–	–
Acetyl	7	–	–	–	+(2)
Picryl	5	–	–	–	+(2)
Acetyl-picryl	6	+(2)	–	+(2)	+(4)
Arsanil	4	–	–	–	–
Sulfanil	3	+(3)	–	+(3)	+(3)
Arsanil-sulfanil	9	+(9)	+(9)	+(9)	+(9)
BSA-(native)	7	–	–	–	

‡ Injected once a week for 3 weeks with 25 mg of antigen in complete Freund's adjuvant and bled 14 days after the last injection.

§ Precipitating antibody.

Figures in parenthesis show the total number of rabbits which gave positive reactions.

was also terminated following injections of Freund's adjuvant containing sulfanil-BSA, but only small amounts of binding antibody were produced to the native BSA. The injection of Freund's adjuvant containing native BSA, BSA which had been complexed to anti-BSA, or BSA which had been heat-denatured, acetylated, picrylated, or coupled to a diazonium derivative of arsanilic acid failed to terminate the tolerant state. Also, only an occasional animal produced precipitating antibody to these altered proteins and then usually in small amounts.

Other workers have studied the antibody response to chemically altered antigens in rabbits made tolerant to native antigens and observed either little or no effect on the tolerant state. In all of these studies the altered antigens were injected in a soluble form rather than incorporated into adjuvant and only a single hapten was coupled to an antigen. Denhardt and Owen (17) injected soluble sulfanil-BSA into BSA-tolerant rabbits and failed to observe an antibody response. Similarly, Boyden and Sorkin (18) failed to observe an antibody

response in HSA-tolerant rabbits injected with soluble sulfanil-HSA. On the other hand, Cinader and coworkers (19, 20) observed that the injection of soluble HSA coupled to the diazonium derivative of *p*-aminosulphonic acid (DHSA), into rabbits tolerant to HSA resulted in the production of hemagglutinating anti-DHSA in three of eight rabbits. However, only one of the three rabbits was shown to give an immune elimination of a subsequent injection of I*-HSA. These data are in sharp contrast to the present data showing the production of small to large amounts of precipitating anti-BSA by BSA-tolerant rabbits injected with arsanil-sulfanil-BSA incorporated into complete Freund's adjuvant.

The addition of foreign determinants on BSA probably was more responsible for its ability to terminate tolerance than the destruction of native determinants. Although most of the altered BSA preparations used in the above experiments only precipitated a portion of preparations of anti–native BSA, all of the altered BSA preparations were able to completely inhibit the precipitation of anti–native BSA by native BSA. Thus, the altered BSA preparations must still contain either all or most of the determinants present on native BSA.

The degree of cross-reaction between altered BSA and anti–native BSA as revealed by direct precipitation and inhibition, suggests that caution should be taken in interpretation of data obtained by studying cross-reactions by only direct precipitation. Based on direct precipitation the cross-reactions between altered BSA preparations and anti–native BSA ranged from 30 to 100 per cent and depended on the concentration of antiserum. The per cent of cross-reacreactions was higher with concentrated serum than with diluted serum. A similar effect of dilution was observed by Kabat and Schor (21), where more antibody was precipitated by homologous antigen in concentrated antisera than in diluted serum when the values obtained with diluted sera were converted to antibody per ml of concentrated sera. Moreover, the results obtained in the present study by direct precipitation were not meaningful, since all of the altered BSA preparations showed a 100 per cent cross-reaction with anti-BSA when inhibition studies were employed. Schlamowitz (22) showed that acetylation of dog intestinal phosphatase destroyed its ability to precipitate with rabbit antibody, but it still could form soluble complexes with the antibody. Similarly, Nisonoff and Pressman (23) found that acetylation of rabbit anti-BSA destroyed its ability to precipitate with BSA, but that the acetylated antibody still combined with BSA to form soluble complexes. They attributed this result to electrostatic repulsion effects among the antibody molecules.

The ability of injections of a heterologous serum albumin to terminate the tolerant state of BSA-tolerant rabbits appeared to depend on the serological relationship between that albumin and BSA (1). On the other hand, there was no evidence that the ability of injections of an altered BSA preparation to terminate BSA tolerance was dependent on the serological relationship between

the altered BSA and native BSA. However, such a dependence might exist which could not be shown by the serological techniques employed.

A difference in the sites on the protein molecule to which the haptens are coupled may explain why arsanil-sulfanil-BSA is more effective in terminating BSA tolerance than picryl-acetyl-BSA. Picryl and acetyl groups are coupled mainly to the epsilon amino groups of lysine, which are believed not to be involved in antigenicity, while at least a portion of the diazonium derivatives are coupled to tyrosine, which is thought to contribute to the antigenicity of proteins.

The results summarized in Table VIII indicate that injection of BSA molecules containing two different haptens in combination was more effective in terminating the tolerant state than the injection of BSA molecules containing only one of the two haptens. However, quite different results were obtained when azo-BGG preparations were injected into BGG-tolerant rabbits. Injection of BGG preparations coupled to a single diazonium derivative was as effective in terminating BGG tolerance as injection of BGG coupled to two different derivatives. The discrepancy between these two systems may be explained by the antigenic heterogenicity of gamma globulin (24, 25). Certain determinants may be present on such a small proportion of the native BGG molecules that their concentration is too low for the rabbit to develop tolerance to them. That tolerance need not be established to every determinant on a protein to render that protein non-antigenic was shown by the failure of sheep serum albumin to elicit an immune response in BSA-tolerant rabbits (1). Thus, the addition of a single hapten to BGG may be sufficient to permit some BGG molecules to be recognized as foreign in the BGG-tolerant rabbit. Whether the presence of two or more different, foreign determinants on a substance is necessary to permit it to be recognized as foreign will have to await further experimentation employing altered proteins in which the exact nature of the alteration is known. However, it appears reasonable to assume that the area of the surface of a protein molecule, responsible for its being recognized as foreign, includes more than one single determinant.

The termination of tolerance by injection of related antigens can be explained if it is accepted that two steps are involved in an antibody response. The first step, recognition, would be selective in that only genetically appropriate cells could respond and this recognition apparently involves multiple determinant groups on the antigen. The second step, antibody production, would involve the synthesis of antibody to individual determinant groups on the molecule once it has been recognized as foreign. Cells or subcellular units capable of recognizing BSA as foreign would be absent in the BSA tolerant rabbit, but cells or subcellular units capable of recognizing certain altered BSA molecules would be present. Once an altered or cross-reacting protein is recognized as foreign, it then (and only then) may be given access to the antibody-producing sites where

antibody may be made to both foreign and previously tolerated determinants. Altered or cross-reacting substances that are not recognized as foreign may be unable to confront the antibody-producing sites and thus antibody could not be made to either tolerated or foreign determinants.

The termination of acquired tolerance is not readily explained by the clonal selection theory of antibody production. This theory suggests that acquired tolerance results from the destruction or inhibition of cells which are destined to make antibody to a specific antigen (26). The ability of the injection of altered or cross-reacting antigens to cause a termination of acquired tolerance suggests that there are cells in the tolerant rabbit that are capable of producing antibody to the tolerated antigen if properly stimulated. That such cells can be stimulated has been demonstrated with the fluorescent antibody technique (27). However, the present results can be explained by a selection theory (possibly clonal) where selection would take place at the recognition level rather than at the antibody production level. Thus, any potential antibody-producing cell would be capable of making antibody to any antigen (even self), if that antigen were first recognized as foreign. To insure a total tolerance, contact with the antigen in early life would have to destroy only the cells or subcellular units responsible for recognition. By injecting related antigens which are recognized as foreign, both related and unrelated determinants may be permitted to confront the antibody-producing sites of the previously tolerant animals where antibody may be made to both the unrelated and the related determinants. The component that limits the antibody response may be the recognition unit rather than the antibody-producing site. The recognition phase may involve either preparation of the antigen to permit it to enter the antibody-producing site or transfer of information to the antibody-producing site. The recognition units might be contained in a cell type different from the cells involved in antibody synthesis, in a cell type the same as the cells involved in antibody synthesis but possessing a different function, or in the same cells that are involved in antibody production. The above postulation is partially based on the assumption that a single cell is potentially capable of making antibody to many different antigenic determinants. However, all cells would not be expected to respond in the same manner to a given determinant. The antibody-producing cells may vary widely in the quality and quantity of antibody they produce to the same antigenic determinant, thus accounting for the heterogeneity of antibody.

The present results can be discussed in relation to autoimmune diseases if it is accepted that the tolerance induced in neonatal rabbits to heterologous proteins is similar to the tolerance of normal rabbits to their own body constituents. Thus, BSA, which is heterologous to the normal rabbit, may be considered to be autologous to the BSA-tolerant rabbit. The termination of tolerance to BSA then may involve mechanisms similar to those involved in autoimmunity. If these assumptions are correct, the termination of acquired tolerance may be

viewed as an experimental model of autoimmunity. Accordingly, an individual could either contact a substance related to a constituent of the body, or as a result of trauma, inflammation, etc., a constituent of the body could become altered. In either case, an autoimmune response might result with accompanying autoantibodies.

SUMMARY

Acquired tolerance to BSA in rabbits was terminated following the injection of certain preparations of altered BSA. Injections of Freund's adjuvant containing BSA complexed to anti-BSA, heat-denatured BSA, acetyl-BSA, picryl-BSA, or arsanil-BSA failed to terminate the tolerant state. Except in an occasional tolerant rabbit, injections of these preparations failed to cause the production of precipitating antibody to the altered preparation. Similar results were obtained following injections of alum-precipitated preparations of pepsin-degraded BSA, acetyl-BSA, and picryl-BSA. Injections of Freund's adjuvant containing sulfanil-BSA terminated the tolerant state, but only small amounts of non-precipitating anti-BSA were produced. Injections of Freund's adjuvant containing picryl-acetyl-BSA terminated the tolerant state in two of six rabbits, but again, only small amounts of non-precipitating anti-BSA were produced. Injections of an alum-precipitated preparation of picryl-acetyl-BSA failed to terminate the tolerant state. On the other hand, injections of Freund's adjuvant containing arsanil-sulfanil-BSA terminated the tolerant state in eleven of eleven rabbits and caused the production of precipitating anti-BSA in all nine of the rabbits tested. The tolerant state was terminated also in six of six rabbits injected with an alum-precipitated preparation of arsanil-sulfanil-BSA. Only one of these rabbits produced precipitating anti-BSA. In addition, the injection of BGG-tolerant rabbits with arsanil-BGG, sulfanil-BGG, or arsanil-sulfanil-BGG terminated the tolerant state. These results were discussed in relation to both the clonal selection theory of antibody production and autoimmunity.

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