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A Study of the 'Termination' of Tolerance to BSA with DNP–BSA in Rabbits: Relative Affinities of the Antibodies for the Immunizing and the Paralysing Antigens

W. E. PAUL, G. W. SISKIND AND B. BENACERRAF

New York University School of Medicine, New York, N.Y.

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Summary. Rabbits rendered immunologically tolerant to BSA produced antibodies capable of precipitating BSA following immunization with DNP–BSA. Antigen binding studies using purified 'anti-BSA' antibodies produced by tolerant animals indicated that these antibodies bound DNP–BSA with far greater avidity than BSA. It was concluded that a true termination of tolerance had not occurred. The implications of the results with regard to induction of antibody formation and of tolerance are discussed.

INTRODUCTION

Rabbits immunologically tolerant to protein antigens such as bovine serum albumin (BSA) can be stimulated to synthesize antibodies capable of binding BSA by immunization with appropriate cross-reacting antigens (Weigle, 1961). This phenomenon which had been originally interpreted as a termination of tolerance is, nevertheless, restricted in its scope, since the antibodies produced which react with BSA are specific for configurations which are common to BSA and to the cross-reacting antigen which induced the immune response. Thus Weigle (1964) has demonstrated that after such 'anti-BSA' antisera have been precipitated at equivalence with the cross-reacting antigen used for immunization, they bind essentially no [^{131}I]BSA in the presence of a large excess of the cross-reacting antigen.

Similarly, an apparent termination of tolerance to BSA may also be observed following immunization of tolerant rabbits with hapten–BSA conjugates. The latter can also be considered as antigens cross-reacting with BSA (Weigle, 1962; Schecter, Bauminger, Sela, Nachtigal and Feldman, 1964; Nachtigal, Eschel-Zussman and Feldman, 1965).

If autoimmune reactions can be considered to result from a similar termination of tolerance to autologous components, this phenomenon constitutes an excellent model for the study of the pathogenesis of these diseases.

The possible mechanisms by which immunization with a cross-reacting antigen can bring about an apparent termination of the tolerant state will be investigated in this study. Two possibilities have been considered:

(1) Following immunization of a BSA-tolerant rabbit with a hapten–BSA conjugate, antibodies are formed which are directed against antigenic determinants to which the animal had been rendered unresponsive. This possibility thus represents a true termination of tolerance. Such anti-BSA antibodies would be expected to have an affinity for native

BSA equal to or greater than their affinity for the hapten–BSA conjugate which broke tolerance.

(2) Alternatively, the 'anti-BSA' antibodies formed are directed against new determinants present on the hapten–BSA conjugate to which the animal had never been unresponsive and indeed had never previously been exposed. These antigenic determinants represent alterations in the native BSA structure resulting presumably from coupling with hapten. These altered determinants are sufficiently similar to configurations of the native BSA that antibodies directed against them can also react with native BSA. According to the second alternative true termination of tolerance has not occurred. Such 'anti-BSA' antibodies would be expected to bind the hapten–BSA conjugate more strongly than they bind BSA.

The experiments reported here were undertaken in order to choose between the two possibilities discussed above. Rabbits which had been rendered tolerant to BSA as adults by a low dose method (Thorbecke and Benacerraf, 1967) were immunized with lightly conjugated 2,4-dinitrophenyl (DNP)–BSA. The antibodies capable of precipitating BSA which were produced by these animals were specifically purified, and their relative capacity to bind DNP–BSA and BSA was studied.

MATERIAL AND METHODS

Crystallized BSA and bovine fibrinogen were purchased from Armour Pharmaceutical Company, Kankakee, Illinois, and rabbit γ-globulin was obtained from Mann Research Laboratories, New York. DNP_{10}–BSA with ten groups of DNP per molecule of BSA was prepared by the reaction at alkaline pH of BSA in 0·5 per cent Na_2CO_3 with 1-chloro-2,4-dinitrobenzene (Eastman Organic Chemicals, Rochester, New York) in *p*-dioxane solution at a molar ratio of 1 : 17. The reaction was allowed to proceed at room temperature for 2 hours. The product (DNP_{10}–BSA) was extensively dialysed against 0·005 M phosphate buffer, pH 7·6. Nitrogen content was determined by micro-Kjeldahl analysis and the degree of DNP substitution was estimated from the absorption at 360 mμ (Carsten and Eisen, 1953). DNP–bovine fibrinogen (DNP–BF) was prepared according to techniques described previously (Benacerraf and Levine, 1962). ε-DNP–L-lysine was purchased from Cyclo Chemical Corporation, Los Angeles, California.

BSA was labelled with ^{125}I and DNP_{10}–BSA with ^{131}I by the chloramine T method in the cold (0°) (Greenwood, Hunter and Glover, 1963; McConahey and Dixon, 1966). One or 2 ml of 0·05 M phosphate buffer, pH 7·6, solutions containing 1–2 mg/ml of these antigens were exposed to 2–5 mc of carrier free $Na^{131}I$ (E. R. Squibb & Sons, New York) or $Na^{125}I$ (New England Nuclear Corporation, Boston, Massachusetts). To the reaction mixture 0·1 mg of chloramine T in 0·1 ml of water was added rapidly with thorough mixing. After 1 minute 0·12 mg of $Na_2S_2O_3$ was added to stop the reaction. The products were then dialysed against several changes of 0·01 M phosphate, 0·15 M NaCl, pH 7·6 (PBS).

Induction of tolerance to BSA

Adult New Zealand white rabbits were rendered tolerant to BSA by a low dose method fully described by Thorbecke and Benacerraf (1967) in the accompanying publication. Approximately half of such animals failed to respond with a measurable haemagglutinin response to an intravenous immunization of 15 mg of alum-precipitated BSA followed

by a subsequent dose of soluble BSA 2 weeks later. Normal rabbits receiving such an immunization course formed high titres of anti-BSA haemagglutinins.

Immunization

Four tolerant and two normal New Zealand white rabbits were immunized by footpad injection with 10 mg of an emulsion of DNP_{10}–BSA in incomplete Freund's adjuvant. Two weeks later a total dose of 5 mg of DNP_{10}–BSA in 0·15 M NaCl was injected intradermally into four sites on the back. Four additional rabbits were immunized to BSA in a manner similar to that described for DNP_{10}–BSA. All rabbits were bled from the ear 1 week after the second immunization and then at weekly intervals until the termination of the experiment.

Precipitin analyses

All sera were tested for precipitating antibodies to DNP_{10}–BSA, DNP–BF and BSA by double diffusion in agar gel (Öuchterlony, 1962).

Quantitative precipitin analyses utilizing DNP_{10}–BSA, DNP–BF and BSA were performed as described by Eisen, Carsten and Belman (1954). Reaction mixtures were incubated at 37° for 1 hour and held at 4° for 2 days before analysis. Washed specific precipitates were dissolved in 0·02 M sodium lauryl sulphate and absorbancies at 278 and 360 mμ were determined. The extinction coefficient ($E_{1\,cm}^{1\%}$) for rabbit antibody at 278 mμ was taken as 13·5.

Antibody purification methods

Anti-BSA antibody was purified using the thiolated-BSA technique described by Singer, Fothergill and Shainoff (1960). Purified antibody obtained by this method was found to be 90 per cent precipitable by BSA at a concentration of 800 μg of antibody per ml. It should be noted that after precipitation of anti-DNP_{10}–BSA sera with BSA at equivalence, thiolated BSA precipitated no additional antibody.

Soluble complexes of BSA–anti-BSA for use in equilibrium dialysis studies were prepared from specific precipitates formed at equivalence. The precipitates were washed three times in cold PBS and dissolved in a twenty-fold excess of BSA.

Binding assays

Antibody binding assays were carried out by a modification of the Farr technique (Farr, 1958). Five micrograms (100 μl) of purified antibody was incubated with 100 μl of a solution containing either 2, 4, 8 or 16 μg of antigen. Binding studies were carried out using [^{131}I]DNP_{10}–BSA, [^{125}I]BSA or a mixture of equal amounts of both radioiodinated antigens. When hapten inhibition of binding was to be studied, 100 μl of 3×10^{-3} M ε-DNP–L-lysine was added to appropriate tubes. All materials were dissolved in PBS containing a 1:10 dilution of normal rabbit serum. Siliconized tubes were used throughout.

The reaction mixtures were allowed to incubate at 4° overnight. An equal volume of cold 80 per cent saturated $(NH_4)_2SO_4$ was then added; tubes were held at 4° for 2 hours and then centrifuged. Two hundred microlitres of supernate was transferred to tubes for counting. Precipitates were washed three times with 0·5 ml portions of cold 40 per cent saturated $(NH_4)_2SO_4$ in 0·075 M NaCl, 0·005 M phosphate, pH 7·6, and then dissolved in 200 μl of saline.

Radioactivity was measured in a Nuclear-Chicago γ-ray spectrometer. With the window settings chosen, essentially no ^{125}I counts appeared in the ^{131}I window and 1·6 per cent of the ^{131}I counts appeared in the ^{125}I window. Appropriate corrections were made. Approximately 3 per cent of the [^{131}I]DNP_{10}–BSA and 1 per cent of the [^{125}I]BSA was non-specifically precipitated by 40 per cent cold saturated $(NH_4)_2SO_4$. In the presence of 100 μl of a 1:10 dilution of rabbit anti-BSA sera, more than 93 per cent of 1 μg of either antigen was precipitated by 40 per cent cold saturated $(NH_4)_2SO_4$.

Antigen binding was calculated from the radioactivity in the washed precipitates after correction for non-specifically precipitated radioactivity. A small error was introduced due to disassociation and solubilization of antigen during the washing procedure. This error was found to be sufficiently small to be safely ignored. Essentially identical results were obtained from measurements of the radioactivity remaining in the supernate. However, since the precision of results based upon precipitate analysis was found to be considerably better than that of results based upon the difference between total and supernatant radioactivity, the former method was used throughout.

Equilibrium dialyses (Eisen, 1964) were carried out on purified antibody preparations and on specific precipitates dissolved in BSA excess. Tritiated DNP–ε-aminocaproic acid ([^3H]DNP–EACA) with 156 mc/m-mole specific activity was prepared as previously described (Siskind, Paul and Benacerraf, 1966). When dissolved specific precipitates were used inside the dialysis bags, [^3H]DNP–EACA was dissolved in a solution containing a concentration of BSA and of rabbit γ-globulin equal to that inside the bag. Dialyses were carried out using washed Visking dialysis tubing. Bags were weighed before and after dialysis so that volume changes could be accurately determined. Tubes were rotated overnight at room temperature. Aliquots from inside and outside the bag were placed in 15 ml of Bray's solution (Bray, 1960) and radioactivity was measured in a Tricarb liquid scintillation counter (Packard Instrument Company, La Grange, Illinois).

RESULTS

PRECIPITIN ANALYSES

Three of the four rabbits tolerant to BSA produced significant amounts of antibody capable of precipitating BSA after immunization with DNP_{10}–BSA (Table 1). The

TABLE 1
QUANTITATIVE PRECIPITIN ANALYSES

Rabbit	Time after boost (weeks)	Immunizing antigen	Status when immunized	Antibody precipitated by: (mg/ml) BSA	DNP–BF	DNP_{10}–BSA	Antibody precipitable only by DNP_{10}–BSA* (% of total antibody)
1417	1	DNP_{10}–BSA	Tolerant	0·17	0·52	2·10	67
1420	1	DNP_{10}–BSA	Tolerant	0·27	0·45	1·01	29
1420	2	DNP_{10}–BSA	Tolerant	0·26	0·29	0·93	41
1421	1	DNP_{10}–BSA	Tolerant	0·13	0·03	0·61	74
A	1	DNP_{10}–BSA	Normal	0·96	0·33	2·38	46
B	1	DNP_{10}–BSA	Normal	2·26	0·33	3·90	34
1427	2	BSA	Normal	3·75			
1429	1	BSA	Normal	3·30			
1429	2	BSA	Normal	3·48			
1430	2	BSA	Normal	2·91			
1431	2	BSA	Normal	4·22			

* Antibody precipitable only by DNP_{10}–BSA = Antibody precipitated by DNP_{10}–BSA $-$ (Antibody precipitated by BSA $+$ Antibody precipitated by DNP–BF).

fourth tolerant rabbit (No. 1419) produced a small amount of such antibody (0·02 mg) at 3 weeks after the completion of the DNP_{10}–BSA immunization course; this animal's serum was not used in any further studies. Precipitation analysis of antisera from the three 'tolerant' rabbits (Nos. 1417, 1420 and 1421) and from two normal rabbits (A and B) immunized with DNP_{10}–BSA were performed with BSA, DNP–BF and DNP_{10}–BSA. Precipitin analyses on the sera of normal rabbits immunized with BSA (Table 1) were carried out with BSA alone. It may be seen that the rabbits tolerant to BSA at the time of DNP_{10}–BSA immunization produced far less antibody precipitable by BSA than did the normal animals immunized with DNP_{10}–BSA or with BSA. In addition, the fraction of antibody which could be precipitated by DNP_{10}–BSA but neither by BSA nor by DNP–BF was considerably higher in two of the three 'tolerant' rabbits than in the normals. On the other hand, the amount of anti-DNP antibody formed was of the same order of magnitude in the 'tolerant' and normal rabbits with the exception of one 'tolerant' rabbit (No. 1421) which had a rather low serum level of anti-DNP antibody.

BINDING OF IODINATED ALBUMINS

Antibodies capable of precipitating BSA were purified from the sera of tolerant and of normal rabbits immunized with DNP_{10}–BSA and of normal rabbits immunized with BSA.

Five micrograms of a pool of purified antibody from tolerant rabbits Nos. 1417 and 1421 bound 26 per cent of 2 μg of [^{131}I]DNP–BSA and 9 per cent of 2 μg of [^{125}I]BSA when exposed to these antigens independently. Five micrograms of antibody purified from the sera of normal rabbits immunized with DNP_{10}–BSA or from one normal rabbit immunized with BSA bound respectively essentially equal

TABLE 2

BINDING OF [^{131}I]DNP_{10}–BSA OR [^{125}I]BSA BY PURIFIED 'ANTI-BSA' ANTIBODIES

Rabbit	Time after boost (weeks)	Immunizing antigen	Status when immunized	Antigen bound* (per cent of 2 μg)	
				[^{131}I]DNP_{10}–BSA	[^{125}I]BSA
1417 and 1421	1	DNP_{10}–BSA	Tolerant	26	9
A	1	DNP_{10}–BSA	Normal	48	42
B	1	DNP_{10}–BSA	Normal	44	43
1429	1	BSA	Normal	46	46

* 5 μg of purified antibody was incubated with either 2 μg of [^{131}I]DNP_{10}–BSA or 2 μg of [^{125}I] BSA in a total volume of 200 μl.

amounts of [^{131}I]DNP_{10}–BSA and [^{125}I]BSA under the same conditions (Table 2). Thus the 'anti-BSA' antibody formed by BSA-tolerant rabbits appeared to bind DNP_{10}–BSA more strongly than BSA.

In order to study more critically the relative binding avidity of these antibodies for DNP_{10}–BSA and for BSA, 5 μg of antibody was incubated with a mixture of equal amounts of [^{131}I]DNP_{10}–BSA and [^{125}I]BSA (Table 3). Rabbits which were tolerant to BSA at the time of immunization with DNP_{10}–BSA produced an 'anti-BSA' antibody which bound little or no BSA when in competition with DNP_{10}–BSA. On the other hand,

normal rabbits immunized with DNP_{10}–BSA produced an 'anti-BSA' antibody which bound BSA only slightly less well than it bound DNP_{10}–BSA in a competitive situation. Finally, normal rabbits immunized with BSA produced an anti-BSA antibody which bound BSA to a somewhat greater degree than it did DNP_{10}–BSA. Identical results were obtained with total antigen amounts of 2, 4, 8 and 16 μg although only the data for the 2 μg tubes are presented. Thus tolerant rabbits do not seem to produce any antibody which binds BSA as well as or better than DNP_{10}–BSA.

TABLE 3

COMPETITIVE BINDING OF [^{131}I]DNP_{10}–BSA AND [^{125}I]BSA BY PURIFIED 'ANTI-BSA' ANTIBODIES

Rabbit	Time after boost (weeks)	Immunizing antigen	Status when immunized	Antigen bound*	
				[^{131}I]DNP_{10}–BSA (% of 1 μg)	[^{125}I]BSA (% of 1 μg)
1417 and 1421	1	DNP_{10}–BSA	Tolerant	41	1·5
1420	2	DNP_{10}–BSA	Tolerant	26	4·6
1421	2	DNP_{10}–BSA	Tolerant	23	< 0·5
A	1	DNP_{10}–BSA	Normal	57	40
B	1	DNP_{10}–BSA	Normal	49	31
1429	1	BSA	Normal	39	60
1430	1	BSA	Normal	43	61
1431	1	BSA	Normal	50	63

* 5 μg of purified antibody was incubated with a mixture of 1 μg of [^{125}I]BSA and 1 μg of [^{131}I]DNP_{10}–BSA in a total volume of 200 μl.

HAPTEN INHIBITION AND HAPTEN BINDING STUDIES

Since the 'anti-BSA' antibodies formed by BSA-tolerant rabbits immunized with DNP_{10}–BSA bind DNP_{10}–BSA with much greater avidity than they do BSA, the possibility exists that the combining sites of these antibodies are partially complementary to the DNP group itself. This possibility was explored by studies of hapten binding and of hapten inhibition of antigen binding.

Attempts were made to demonstrate inhibition of binding of the radio-iodinated albumins by ε-DNP–L-lysine. The hapten was present either in the original incubation mixture or in the $(NH_4)_2SO_4$ washing solution at a final concentration of 10^{-3} M. In repeated experiments ε-DNP–L-lysine had no measurable effect on the binding characteristics of the purified 'anti-BSA' antibodies.

In a typical experiment 5 μg of a pool of 'anti-BSA' antibody purified from the sera of tolerant rabbits (Nos. 1417 and 1421) was incubated with a mixture of 2 μg of [^{131}I] DNP_{10}–BSA and 2 μg of [^{125}I]BSA in the presence or absence of 10^{-3} M ε-DNP–L-lysine. In both cases, no measurable [^{125}I]BSA was bound; in the presence of ε-DNP–L-lysine, 97 per cent as much [^{131}I]DNP_{10}–BSA was bound as was bound in its absence. Thus the combining site of 'anti-BSA' antibodies formed by BSA-tolerant rabbits immunized with DNP_{10}–BSA does not appear to be significantly fitted to the DNP group. This finding was confirmed by equilibrium dialysis experiments.

Equilibrium dialyses of purified 'anti-BSA' antibodies from tolerant rabbits immunized with DNP_{10}–BSA and from normal rabbits immunized with BSA were performed. The results are presented in Table 4. No significant binding of [^{3}H]DNP–EACA was observed. As a final attempt to demonstrate DNP binding by 'anti-BSA' antibodies produced by

tolerant rabbits, equilibrium dialysis studies were carried out on immune complexes of antibody and BSA, formed at equivalence and dissolved in a twenty-fold BSA excess. Equilibrium dialyses were performed using [^{3}H]DNP–EACA in a solution of BSA. Similar measurements were made with anti-BSA antibody produced by BSA immunization of normal rabbits (Table 5). In no instance could any significant binding of [^{3}H]

TABLE 4

LACK OF DNP–EACA BINDING BY PURIFIED 'ANTI–BSA' ANTIBODIES*

Rabbit	Time after boost (weeks)	Immunizing antigen	Status when immunized	DNP–EACA† 'bound' (mM × 10^{+7})
—	—	—	—	+0·0126‡
1420	1	DNP_{10}–BSA	Tolerant	+0·0072
1429	2	BSA	Normal	+0·0088
1429 + anti-DNP–BGG§	2	BSA	Normal	+0·0136

* 100 μg of purified antibody (13×10^{-7} mM of combining sites) in 1·0 ml of PBS was placed inside of dialysis bag. $0{\cdot}9 \times 10^{-7}$ mM of [^{3}H]DNP–EACA in 0·9 ml of PBS was placed outside of dialysis bag.

† DNP–EACA 'bound' = (conc. DNP–EACA inside bag − conc. DNP–EACA outside bag) volume inside bag.

‡ No antibody present; 1·0 ml of PBS placed inside bag.

§ In this case, rabbit anti-DNP bovine γ-globulin serum was added to anti-BSA serum prior to purification of antibodies (see text).

TABLE 5

LACK OF DNP–EACA BINDING BY SOLUBLE IMMUNE COMPLEXES OF 'ANTI-BSA' AND BSA*

Rabbit	Time after boost (weeks)	Immunizing antigen	Status when immunized	DNP–EACA† 'bound' (mM × 10^{+7})
—	—	—	—	0·0029‡
1420	1	DNP_{10}–BSA	Tolerant	−0·0062
1429	2	BSA	Normal	−0·0179
1429 + anti-DNP–BGG§	2	BSA	Normal	+0·0052

* 0·7 ml of soluble immune complexes containing 342 μg of 'anti-BSA' (46×10^{-7} mM of antibody combining sites) and 1·09 mg of BSA were placed inside of dialysis bags. 0·7 ml of a solution containing $0{\cdot}7 \times 10^{-7}$ mM of [^{3}H]DNP–EACA, 342 μg of rabbit γ-globulin and 1·09 mg of BSA were placed outside of dialysis bag.

† DNP–EACA 'bound' = (conc. DNP–EACA inside bag − conc. DNP–EACA outside bag) volume inside bag.

‡ No antibody present; 342 μg of rabbit γ-globulin and 1·09 mg of BSA were placed within dialysis bag.

§ In this case, rabbit anti-DNP bovine γ-globulin serum was added to anti-BSA serum prior to formation of specific precipitates (see text).

DNP–EACA be detected. As control experiments for both isolation procedures employed (Tables 4 and 5), to some sera of normal rabbits immunized with BSA, rabbit anti-DNP bovine γ-globulin serum was added prior to precipitation so that the ratio of anti-DNP to anti-BSA antibody was the same as that present in the sera of tolerant rabbits immunized with DNP_{10}–BSA. In no instance could any significant binding of [^{3}H]DNP–EACA

be detected, indicating the lack of contamination of the anti-BSA antibodies with antibodies of other specificities.

DISCUSSION

Rabbits immunized with DNP–BSA while tolerant to BSA produced antibodies capable of precipitating BSA. In competitive binding experiments such antibodies bound DNP–BSA preferentially to BSA. Indeed, in the presence of an equal amount of DNP–BSA they bound little or no BSA. Thus these 'anti-BSA' antibodies exhibited a far greater avidity for DNP–BSA than for BSA. Contrasting with the behaviour of tolerant rabbits, normal rabbits immunized with DNP–BSA produced 'anti-BSA' antibodies which bound DNP–BSA only slightly better than BSA. Control experiments demonstrated that contamination of purified 'anti-BSA' antibodies with anti-DNP antibodies cannot account for the binding characteristics of the 'anti-BSA' antibody produced by tolerant rabbits. Furthermore, it is clear from the hapten binding studies that the purified anti-BSA antibodies were not contaminated with any significant amount of anti-DNP antibody.

From comparisons of the binding characteristics of 'anti-BSA' antibodies synthesized by tolerant and normal animals, it is apparent that the production of those antibodies best adapted to the antigenic determinants of BSA remained inhibited in the tolerant animals. Thus, as discussed in detail below, from an immunochemical point of view tolerance to BSA was maintained even after immunization with DNP–BSA. Similar results were obtained by Linscott and Weigle (1965). The critical interpretation of their findings, however, is complicated by their use of whole antisera containing antibodies directed solely at determinants specific for the hapten–protein conjugate, rather than of purified antibodies of defined specificities.

One may consider that the stimulation of immunocompetent cells to proliferate and produce antibody or, alternatively, to be rendered tolerant is dependent upon interaction of antigen (either 'processed' or native) with preformed 'antibody' present on or in these cells. This selection theory presumes the existence of precommitted immune cells, present in the animal prior to contact with antigen. The nature of the 'antibody' associated with such a cell can be specified at present only in so far as its antigen binding site must be identical to that present in the antibody molecules to be produced by that cell (or its progeny) subsequent to antigenic stimulation. Based upon such considerations it would be predicted that cells bearing 'antibody' of low affinity for a particular antigen would not be either stimulated or inhibited (rendered tolerant) unless very high doses of that antigen were used. With lower antigen concentrations insufficient antigen would be bound by such 'low affinity cells' to trigger subsequent steps in the immune process. Moreover, these antibodies and the cells associated with them capable of binding one antigen with low affinity might well bind a related (cross-reacting) antigen with significantly higher affinity. This accounts for the response of tolerant animals to cross-reacting antigens.

In the process of induction of antibody synthesis cells bearing such cross-reacting 'antibody' would not be stimulated by the antigens for which they have low affinity. Although with normal doses of antigen these cells are not stimulated, it is reasonable from thermodynamic considerations that in the presence of very high concentrations of antigen such cells might be triggered, and thus the range of specificities (heterogeneity) of the antibody produced would be increased. Since the differences in affinities involved are probably orders of magnitude, the antigen concentrations required to stimulate such

cells must be very high indeed. Precisely the same line of reasoning would be expected to hold for the induction of tolerance. That is, cells capable of producing low affinity antibody should require much higher concentrations of antigen to render them non-responsive. The data reported here indicate that the production of antibody capable of binding BSA by BSA-tolerant rabbits upon immunization with DNP–BSA involves synthesis of antibody by cells not involved in the normal response to BSA. The cells capable of producing high affinity anti-BSA antibodies best adapted to the antigenic determinants of the molecule remained unresponsive to the DNP–BSA challenge. Thus on a cellular level no true termination of tolerance has occurred.

From the theoretical considerations above, it would be predicted that if tolerance had been induced with significantly higher concentrations of BSA, cells bearing lower affinity antibody (cross-reacting antibody in the experimental situation discussed here) would also have been rendered tolerant, and that under these conditions immunization with DNP–BSA would result in the production of little or no antibody capable of binding BSA. That is, the normal immune response to DNP–BSA would be further restricted in that BSA cross-reacting antibodies would not be produced. A review of the literature bearing upon immunization of tolerant animals with cross-reacting antigens provides experimental support for this prediction. Thus in the experiments reported here, tolerance was induced in adult rabbits with very low doses of BSA (approximately 9 mg) and immunization with DNP–BSA produced significant amounts of precipitable 'anti-BSA' antibodies in all animals and little impairment of the anti-DNP–BSA response was noted. The low dose tolerance method used in these experiments was, therefore, particularly suited for the production of a sufficient amount of antibodies cross-reacting with the tolerance-inducing antigen to allow their isolation and study. Similarly, Nachtigal *et al.* (1965) found that rabbits rendered tolerant to human serum albumin (HSA) with relatively low doses of antigen (adult animals given 200 mg of antigen over a 10-week period) produced amounts of 'anti-HSA' antibody capable of causing precipitation in gel after immunization with *p*-azophenyl sulphonyl–HSA. Furthermore, Schecter *et al.* (1964) utilizing rabbits made tolerant as newborns by a rather low dose of HSA (80 mg within the 1st week of life) observed the production of precipitating amounts of 'anti-HSA' antibody upon immunization with poly-L-tyrosyl–HSA.

Thus in studies where tolerance was induced using comparatively small amounts of antigen, immunization with cross-reacting antigens produced significant amounts of antibody capable of binding the tolerated antigen. In contrast, in a number of studies in which tolerance was induced with larger doses of antigen, immunization with cross-reacting antigen either failed to induce formation of antibody reacting with the tolerated antigen or induced formation of very small amounts of such antibody.

For example, Weigle (1961, 1962, 1964; Linscott and Weigle, 1965) using rabbits rendered tolerant with 500 mg of BSA administered during the first 5 days of life found that many cross-reacting antigens were ineffective or only minimally effective in stimulating the formation of 'anti-BSA' antibodies. Even with the most effective cross-reacting antigens, precipitating amounts of 'anti-BSA' antibodies were rarely observed. Similarly, Humphrey (1964) was unable to 'terminate' tolerance induced in neonatal rabbits with high doses of albumins by immunization with cross-reacting albumins.

Restriction in the immunological response of tolerant animals to cross-reacting antigens has been observed by a number of workers who utilized high doses of antigen to induce tolerance (Cinader and Dubert, 1955; Weigle, 1961). Thus induction of tolerance with

large doses of antigen appears to suppress a greater range of antibody-producing cells than is the case when tolerance is induced with lower doses of antigen.

Finally, one must consider the possibility that the appearance of antibody capable of binding a tolerated antigen may subsequently accelerate a true termination of tolerance. The disposition of the tolerated antigen within and its excretion from the body is undoubtedly changed by its combination with antibody. Thus, newly arising populations of immunocompetent cells might not have been exposed to the tolerizing antigen and would be expected to respond to subsequent injections of the formerly tolerated antigen with the production of antibodies. This latter phenomenon appears to have been observed by both Weigle (1961, 1962, 1964) and Nachtigal *et al.* (1965). Such a mechanism is not likely to be of major importance in the true termination of natural tolerance because of the continued production and presence of the tolerizing material.

Although immunization with cross-reacting antigens does not offer a model for the true termination of natural tolerance, it may nevertheless be of importance in understanding the production of 'autoantibodies' in a variety of human disorders. These experimental models indeed demonstrate that an important distinction must be made between the capacity of an antibody simply to bind, even though weakly, with structures to which it has some complementarity and the more exacting immunological specificity which controls its synthesis at the cellular level. Thus autologous constituents against which tolerance has been established can, nevertheless, be expected to bind to some extent antibodies which are induced by cross-reacting antigens. This mechanism for 'autoantibody' formation would probably be operative only in cases in which the bodily constituent under normal conditions reaches the immuno competent cells in low concentration. One would expect also that such 'autoantibodies' would be produced for periods of time not much greater than the duration of persistence of the cross-reacting antigen in the body. Thus, the production of chronic 'autoimmune' disease due to continued synthesis of cross-reactive antibody would depend upon the persistence of or repeated exposure to the cross-reacting antigen.

In view of the possible importance of such antibodies, it is of interest to consider the extent of alteration of a tolerated antigen required to render it immunogenic. In this experiment no significant binding of DNP–EACA by 'anti-BSA' antibodies produced by tolerant rabbits could be demonstrated and no inhibition of 'anti-BSA' binding of DNP–BSA could be effected by ε-DNP–L-lysine. Thus, the cross-reactive antibody has little or no specificity for the DNP group and must be directed mainly at perturbations produced in the BSA molecule resulting from the presence of DNP groups or due to the coupling procedure. This would suggest that very subtle alterations of the fine structure are sufficient for the production of cross-reactive antigens capable of stimulating the production of antibody reactive with tolerated antigens.

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