DEMONSTRATION OF AN ANTIBODY-MEDIATED TOLERANCE STATE AND ITS EFFECT ON ANTIBODY AFFINITY*

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Immunological tolerance may be operationally defined as a specific depression of the immune response resulting from prior exposure to antigen. The mechanisms and the cellular basis of tolerance induction vary with the experimental conditions under which tolerance is induced. Chiller et al. (1) have demonstrated that tolerance can result from specific inhibition of thymus-derived (T) lymphocytes or of both T lymphocytes and bone marrow-derived (B) lymphocytes. Pure B-cell tolerance has been described after the administration of a haptenic determinant conjugated to a nonimmunogenic carrier (2). In addition, recent work has suggested that some tolerance states may be mediated by the activity of suppressor T cells (3).

In the present paper, we will describe an experimental model for adult tolerance induction which appears to be mediated by the production of small amounts of high affinity antibody. It was found that a high degree of tolerance could be induced in adult mice by a single intravenous injection of antigen. The residual plaque-forming cells (PFC)¹ present in partially tolerant animals contained a larger subpopulation of cells producing very high affinity antibody than were present in normal mice immunized simultaneously. Cells from the tolerant animals transferred into lethally irradiated recipients yielded a response of greater magnitude and higher avidity than that produced by cells from normal mice transferred into irradiated animals. Tolerance was not abrogated by the

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¹ Abbreviations used in this paper: BGG, bovine gamma globulin; CFA, complete Freund’s adjuvant; DNFB, 1-fluoro-2,4-dinitrobenzene; EACA, ε-amino-N-caproic acid; HBSS, Hanks’ balanced salt solution; Ova, ovalbumin; PBS, phosphate-buffered saline; PFC, plaque-forming cells; RSA, rabbit serum albumin.

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injection of moderate numbers of normal spleen cells into tolerant mice. The tolerant state showed a carrier specificity similar to that of passive antibody-mediated immune suppression. These data are consistent with the hypothesis that this tolerant state is mediated by the production of small amounts of high affinity antibody in response to the tolerance-inducing injection of antigen. This high affinity antibody causes a suppression of a subsequent immune response to that antigen.

Materials And Methods

**Mice.** 5- to 7-wk old LAF, male mice (Jackson Laboratories, Bar Harbor, Maine) were used.

**Antigen and Hapten Preparation.** Dinitrophenyl (DNP) derivatives of bovine gamma globulin (BGG), rabbit serum albumin (RSA), and ovalbumin (Ova) were prepared by the reaction of 1-fluoro-2,4-dinitrobenzene (DNFB; Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) with the protein under alkaline conditions (4). The products were purified by extensive dialysis.

The concentrations of the derivatized proteins were calculated from their “dry weights” and the degree of hapten substitution was estimated from their absorbancy at 360 nm (ε for DNP-lysine = 17,400). The DNP derivatives of BGG, RSA, and Ova had an average of 50, 30, and 14 DNP groups/protein molecule, respectively.

Dinitrophenylated ε-amino-N-caproic acid (DNP-EACA) was prepared by the reaction of DNFB with EACA (Sigma Chemical Co., St. Louis, Mo.). Tritiated DNP-EACA was prepared by the reaction of [3H]DNFB (Amersham/Searle Corp., Arlington Heights, Ill.) and purified by thin-layer chromatography. These methods have been described in detail previously (5).

**Immunization.** Mice were immunized by the intraperitoneal injection of 0.4 mg DNP-BGG or DNP-RSA emulsified in an equal volume of complete Freund’s adjuvant (CFA; 1 mg/ml Mycobacterium butyricum). A total vol of 0.2 ml of emulsion was injected.

**Cell Transfer Studies.** Spleens were teased apart in Hanks’ balanced salt solution (HBSS; Grand Island Biological Co., Grand Island, N. Y.) and the suspension filtered through a thin layer of cotton gauze. The cells were washed once with HBSS and resuspended in HBSS for intravenous injection into lethally irradiated, syngeneic mice. The recipients, except as otherwise indicated, were irradiated with 800 R from a cesium source (Gammator-G, Radiation Machinery Corp., Parsippany, N. J.) 2-4 h before cell transfer. Animals were immunized by the intraperitoneal injection of antigen in CFA 24 h after cell transfer.

**PFC Assay.** PFC were assayed by the method of Kennedy and Axelrad (6) using sheep red blood cells (SRBC) coated with DNP-Ova by a chronic chloride technique (7). The washed DNP-Ova-coupled SRBC were adsorbed as monolayers to poly-L-lysine (Miles Laboratories, Inc., Kankakee, Ill.) coated plastic petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Spleens from experimental animals were teased apart, the cells washed once and resuspended in HBSS containing 0.1 mg Na heparin/ml (Sigma Chemical Co.). Guinea pig complement absorbed with SRBC, was added at a final concentration of 10% (vol/vol) and the cell suspension placed onto the DNP-Ova-coupled SRBC monolayer and incubated at 37°C for 1 h. Plaques were counted using a dissecting microscope. Indirect PFC were developed by adding rabbit antismouse gamma globulin (final dilution of 1:400) to the cell suspension. This concentration of developing antiserum inhibited approximately 75% of direct PFC and resulted in maximum development of indirect PFC.

**Determination of PFC Avidity.** The avidity of anti-DNP PFC was determined by inhibition of plaque formation with DNP-EACA (8). Plaque formation by cells producing high avidity antibody is inhibited by low concentrations of hapten whereas low avidity antibody-forming cells require a high hapten concentration for inhibition. Thus, inhibition of plaque formation at a series of hapten concentrations permits calculation of the distribution of avidities of the PFC. Nine hapten concentrations, ranging from 1 x 10^-9 to 1 x 10^-4 M, in one-half log increments, were used.

**Measurement of Antibody Concentration and Affinity.** Anti-DNP antibody concentration was measured in mouse antiserum by a modification of the Klinman and Taylor immunoadsorbent
method (9). The affinity of the antibody for \[^{[H]}\text{DNP-EACA}\] was assayed by the Farr technique (10). The details of how these procedures are performed in our laboratory have been previously reported (5, 11, 12).

**Preparation of Anti-DNP-BGG Antibody.** LAF, mice were injected intraperitoneally with 500 \(\mu\)g of DNP-BGG in CFA and boosted 6 wk later by the intraperitoneal injection of 500 \(\mu\)g DNP-BGG in phosphate-buffered saline (PBS: 0.15 M NaCl, 0.01 M potassium phosphate buffer, pH 7.4). Animals were bled 7 days later.

## Results

**Induction, Duration, and Specificity of Tolerance.** Mice were injected intravenously with varying amounts of DNP-BGG in PBS or with PBS alone. 5 days later, all animals received 0.4 mg DNP-BGG in CFA, intraperitoneally. The mice were sacrificed at various times thereafter, and their spleens assayed for the number and avidity of anti-DNP PFC. 8 wk after immunization some animals were boosted by the intraperitoneal injection of 0.4 mg DNP-BGG in PBS and their spleens assayed for anti-DNP PFC 1 wk later.

The intravenous injection of DNP-BGG before immunization with DNP-BGG in CFA resulted in a marked reduction in the number of anti-DNP PFC (Table I).

### Table I

**Induction of Tolerance to DNP-BGG in Adult Mice**

| Tolerance-inducing dose of DNP-BGG (mg) | Time between tolerance induction and antigen challenge (days) | Time between antigen challenge and sacrifice (days) | Indirect anti-DNP PFC (PFC/spleen \(X10^{-2}\)) |
|----------------------------------------|------------------------------------------------------------|--------------------------------------------------|---------------------------------------------|
| 0.0                                    | 5                                                          | 7                                                | 147.1                                       |
| 0.02                                   | 5                                                          | 7                                                | 46.9                                        |
| 0.5                                    | 5                                                          | 7                                                | 26.7                                        |
| 5.0                                    | 5                                                          | 7                                                | 4.1                                         |
| 0.0                                    | 5                                                          | 14                                               | 37.8                                        |
| 0.02                                   | 5                                                          | 14                                               | 18.8                                        |
| 0.5                                    | 5                                                          | 14                                               | 7.8                                         |
| 5.0                                    | 5                                                          | 14                                               | 2.6                                         |
| 0.0                                    | 5                                                          | 28                                               | 17.6                                        |
| 0.02                                   | 5                                                          | 28                                               | 8.5                                         |
| 0.5                                    | 5                                                          | 28                                               | 3.3                                         |
| 5.0                                    | 5                                                          | 28                                               | 0.9                                         |
| 0.0                                    | 14                                                         | 7                                                | 18.7/168.7†                                  |
| 0.5                                    | 14                                                         | 7                                                | 7.5/149.7‡                                   |
| 5.0                                    | 14                                                         | 7                                                | 4.4/46.1                                    |

* Mice were injected intravenously with the indicated dose of DNP-BGG and were challenged with 0.4 mg DNP-BGG in CFA intraperitoneally, when indicated. Results are expressed as the geometric means of groups of 5–7 mice.

† Direct PFC/indirect PFC.
The degree of depression (tolerance) was related to the amount of antigen injected. The difference between tolerant and nontolerant groups was greatest at 1 wk after immunization and decreased thereafter as a consequence of a more rapid decrease in the number of PFC in normal, as compared with tolerant animals.

Tolerance induced with 0.5 mg DNP-BGG was of relatively short duration (Table I). By 2 wk after the intravenous injection of antigen the animals upon immunization with DNP-BGG in CFA responded indistinguishably from controls. In contrast, animals treated with 5.0 mg DNP-BGG were still tolerant. Specificity of the tolerant state was demonstrated by the observation (Table II)

| Tolerance induction | Anti-SRBC PFC/spleen $\times 10^{-3}$ |
|---------------------|--------------------------------------|
|                     | Direct PFC                           | Indirect PFC                        |
| -                   | 52.3                                 | 178.3                                |
| +                   | 41.1                                 | 148.9                                |

* Animals were injected intravenously with 5.0 mg DNP-BGG or with PBS and were challenged 5 days later with $5 \times 10^8$ SRBC intravenously. Spleens were assayed for anti-SRBC PFC 1 wk later. Results are expressed as the geometric means of groups of 7 mice.

that the intravenous injection of DNP-BGG had no effect on the immune response to SRBC.

Effect of Tolerance on Avidity of PFC and Affinity of Serum Antibody. The distribution of avidities of PFC in normal and in tolerant animals is presented in Fig. 1. Normal animals initially had PFC of predominantly low avidity and showed a progressive increase in avidity with time after immunization. In contrast, early after immunization animals made tolerant by the intravenous injection of 0.02 or 0.5 mg DNP-BGG had populations of higher avidity PFC than did normal animals. This could be seen at 1 and at 2 wk after immunization with antigen in CFA. At 4 wk after intraperitoneal immunization all animals had PFC of equally high avidity. On the other hand, animals rendered tolerant by the intravenous injection of 5.0 mg DNP-BGG had only low avidity anti-DNP PFC throughout the period of observation.

Antibody concentration and affinity were measured in the serum of a small number of tolerant and normal animals (Table III). As was observed at the PFC level, animals made tolerant with 0.5 mg of antigen had higher affinity serum antibody than did normal animals early after immunization. Animals tolerized with 5.0 mg of antigen had very low affinity serum antibody throughout the period of observation. Thus, measurements on serum antibody confirm the results described above on the avidity of antibody formed by PFC.

The high avidity of the residual PFC and serum antibody in animals rendered tolerant by a low dose of soluble antigen is very different from previous studies in which tolerance has been associated with decreased antibody affinity (13-18). This difference in results suggested that the tolerance being studied in the present model is due to a distinctly different mechanism. Passive antibody is
FIG. 1. Each histogram illustrates the distribution of indirect anti-DNP PFC with respect to avidity for DNP-EACA in the spleen of an individual mouse. The abscissa represents the log of the inverse of the hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. Avidity increases to the right. Counting from the top down: rows 1 and 2 contain data on normal mice; rows 3 and 4 on mice tolerized with 0.02 mg DNP-BGG; rows 5 and 6 mice tolerized with 0.5 mg DNP-BGG; and rows 7 and 8 mice tolerized with 5.0 mg DNP-BGG. Animals were immunized with DNP-BGG in CFA 5 days after the tolerizing injection of antigen and sacrificed at the time indicated below each column of histograms. Animals in the right most column were boosted with DNP-BGG in PBS 8 wk after primary immunization.
known to cause a specific suppression in antibody synthesis with an increase in the average affinity of the residual antibody (16, 19, 20). It was, therefore, hypothesized that, in the present model, tolerance induced by relatively low doses of antigen was the result of the synthesis of small amounts of high affinity antibody in response to the intravenous injection of antigen. This high affinity antibody suppresses the immune response to antigen in CFA in a fashion analogous to that of passively administered antibody.

**Immune Response to Intravenous Antigen.** If tolerance in the present system is mediated by the production of small amounts of high affinity antibody in response to the intravenous tolerizing injection of antigen, then one would expect to detect an increase in PFC after the intravenous injection. As indicated in

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**Table III**

*Effect of Tolerance Induction on Serum Antibody Affinity*

| Time after immunization | Normal mice | Tolerance (dose 0.5 mg) | Tolerance (dose 5.0 mg) |
|-------------------------|-------------|------------------------|------------------------|
|                         | Antibody concentration | Affinity $\Delta F_{10%}$ | Antibody concentration | Affinity $\Delta F_{10%}$ | Antibody concentration | Affinity $\Delta F_{10%}$ |
|                         | mg/ml | kcal/mol | mg/ml | kcal/mol | mg/ml | kcal/mol |
| days                    |       |         |       |         |       |         |
| 7                       | 0.16  | 8.93    | 0.14  | 9.08    | 0.05  | NM†     |
|                         | 0.11  | 9.30    | 0.06  | 9.78    | 0.05  | NM      |
|                         | 0.20  | 8.84    | 0.10  | 9.73    | 0.03  | NM      |
|                         | 0.23  | 8.83    | -     | -       | 0.04  | NM      |
| Average                 | 0.18  | 8.98    | 0.10  | 9.55    | 0.04  | -       |
| 14                      | 0.52  | 9.58    | -     | -       | 0.26  | 8.93    |
|                         | 1.17  | 9.17    | -     | -       | 0.15  | 8.57    |
|                         | 0.95  | 9.27    | -     | -       | -     | -       |
| Average                 | 0.88  | 9.34    |       |         | 0.21  | 8.75    |
| 28                      | 0.46  | 9.67    | 0.07  | 10.43   | -     | -       |
|                         | 0.74  | 10.11   | 0.06  | 10.37   | -     | -       |
|                         | 1.82  | 9.59    | 0.09  | 9.85    | -     | -       |
|                         | 1.51  | 9.46    | 0.08  | 10.15   | -     | -       |
|                         | 0.52  | 9.91    | 0.08  | 10.12   | -     | -       |
| Average                 | 1.01  | 9.75    | 0.08  | 10.18   |       |         |
| 56                      | 1.10  | 10.14   | -     | -       | 0.05  | 10.75   |
|                         | 1.31  | 11.32   | -     | -       | 0.25  | 8.82    |
|                         | 1.86  | 9.85    | -     | -       | 0.11  | 9.39    |
|                         | 2.52  | 10.32   | -     | -       | 0.16  | 9.62    |
|                         | 1.53  | 10.30   | -     | -       | 0.20  | 8.01    |
|                         | 0.72  | 10.37   | -     | -       |       |         |
| Average                 | 1.51  | 10.38   | 0.15  | 9.32    |       |         |

* Mice rendered tolerant by the intravenous injection of 0.5 or 5.0 mg DNP-BGG and immunized 5 days later with 0.4 mg DNP-BGG in CFA. Animals were bled at the times indicated. Antibody concentration was determined by a solid-phase immunoadsorbent technique and affinity assayed by the Farr technique using DNP-EACA as ligand.

† NM, not measurable.
Table IV, the injection of either 0.5 or 5.0 mg DNP-BGG elicited a significant PFC response.

The distribution of avidities of PFC elicited by the intravenous injection of antigen is shown in Fig. 2. A relatively high avidity population of PFC is already present at 5 days after antigen injection. The avidity of the PFC present 5 days after intravenous antigen is roughly comparable to the avidity of PFC 2 wk after immunization with DNP-BGG in CFA (compare Fig. 1 and 2). Thus, the intravenous injection of DNP-BGG stimulates a small but significant immune response involving the selective proliferation of high avidity antibody-forming cells.

Transfer of Normal Cells into Tolerant Animals. If tolerance results from the production of high affinity antibody in response to the tolerance-inducing

Table IV

| Antigen dose (mg) | Anti-DNP PFC/spleen $\times 10^{-2}$ | Direct PFC | Indirect PFC |
|------------------|-----------------------------------|------------|--------------|
| 0.0              | 0.2                               | 0.1        |
| 0.5              | 2.3                               | 8.3        |
| 5.0              | 0.3                               | 4.6        |

* Spleens were assayed for anti-DNP PFC 5 days after the intravenous injection of the indicated dose of DNP-BGG. Results are shown as the geometric means of groups of 5–7 mice.

Fig. 2. Each histogram illustrates the distribution of indirect anti-DNP PFC with respect to avidity for DNP-EACA in the spleen of an individual mouse 5 days after the intravenous injection of 0.5 mg DNP-BGG. The abscissa represents the log of the inverse of the hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. Avidity increases to the right.
injection of antigen then one would expect that the injection of normal immunocompetent cells into tolerant animals should not alter their unresponsive state. 3 days after the tolerizing injection of DNP-BGG, mice were irradiated with 360 R, were given normal spleen cells intravenously, and were immunized 1 day later with DNP-BGG in CFA. As indicated in Table V, injection of normal spleen cells into tolerant animals did not alter their response to antigen. That is, the responsiveness of normal spleen cells to DNP-BGG appeared to be blocked in the tolerant animals. There was perhaps a slight tendency to overcome tolerance induced with 5.0 mg of antigen when a high dose of spleen cells was used. However, this effect was of small magnitude and only seen in the direct PFC response. Thus, tolerance could not be terminated by injection of normal spleen cells into tolerant mice. Furthermore, the tolerant state was not terminated by the transfer of normal peritoneal exudate cells into tolerant animals (Table V).

Transfer of Spleen Cells from Tolerant Animals into Normal Recipients. If tolerance were due to the production of high affinity antibody one might predict that cells from tolerant animals would transfer a high affinity, secondary type response to lethally irradiated syngeneic mice. Cells from DNP-BGG tolerant mice (transferred 5 days after tolerance induction) were capable of reconstituting

| Exp. | Tolerance-inducing dose of DNP-BGG | Number of cells transferred (× 10⁸) | Anti-DNP PFC/spleen × 10⁻³ |
|------|-----------------------------------|-----------------------------------|---------------------------|
|      |                                   | Direct PFC                        | Indirect PFC              |
| 1    | 0.0                               | 5.2                               | 27.0                      |
|      | 0.5                               | 1.2                               | 12.5                      |
|      | 5.0                               | 0.6                               | 1.4                       |
| 2    | 0.0                               | 7.0                               | 41.5                      |
|      | 0.0                               | None                             | 2.3                       |
|      | 0.5                               | 1.8                               | 6.4                       |
|      | 5.0                               | 4.2                               | 4.2                       |
| 3    | 0.0                               | 5.8                               | 14.3                      |
|      | 0.5                               | 2.2                               | 7.1                       |
|      | 5.0                               | 1.2                               | 2.8                       |
| 4    | 0.0                               | 7.6                               | 98.5                      |
|      | 0.0                               | 7.5                               | 61.5                      |
|      | 5.0                               | 2.1                               | 7.0                       |
|      | 5.0                               | 1.5                               | 4.3                       |

* Mice were injected with the indicated dose of DNP-BGG intravenously. 4 days later they received 360 R irradiation and the indicated number of normal, syngeneic, spleen, or peritoneal cells. 1 day later all animals were intraperitoneally immunized with 0.4 mg DNP-BGG in CFA. In exprs. 1 and 2, spleens were assayed 10 days after antigen challenge. In expr. 3 spleens were assayed 21 days after antigen challenge. Results are expressed as the geometric means of groups of 5-7 mice.

† Peritoneal cells were obtained by washing the peritoneal cavity of normal mice with HBSS.
the anti-DNP PFC response in such irradiated recipients (Table VI). In fact, the responses of animals reconstituted with cells from tolerant donors were three- to sevenfold greater than the responses of animals receiving equal numbers of spleen cells from normal donors.

If a 2-wk delay is imposed between tolerance induction and cell transfer, spleens from mice tolerized with 0.5 mg DNP-BGG still showed an augmented immune response as compared with normal spleen cells. In contrast, recipients of spleens from mice injected with 5.0 mg DNP-BGG gave a PFC response identical in magnitude to that of recipients of normal spleen cells.

**Table VI**

*Transfer of Spleen Cells from DNP-BGG Tolerant Mice into Lethally Irradiated Syngeneic Mice*

| Exp. | Tolerance-inducing dose of DNP-BGG | Time before transfer | Anti-DNP PFC/spleen $\times 10^{-3}$ |
|------|-----------------------------------|----------------------|-------------------------------------|
|      | mg  | days | Direct PFC | Indirect PFC |
| 1    | 0.0 | 5    | 1.9 | 6.2 |
|      | 0.5 | 5    | 3.1 | 21.7 |
|      | 5.0 | 5    | 2.7 | 18.1 |
| 2    | 0.0 | 5    | 1.7 | 3.9 |
|      | 0.5 | 5    | 3.1 | 28.8 |
|      | 5.0 | 5    | 4.5 | 21.6 |
| 3    | 0.0 | 14   | 0.2 | 0.4 |
|      | 0.5 | 14   | 2.5 | 21.6 |
|      | 5.0 | 14   | 0.2 | 0.5 |

* Animals were injected intravenously with the dose of DNP-BGG indicated. 5 or 14 days later $2 \times 10^7$ spleen cells from these mice were injected into 800 R irradiated, syngeneic recipients. All recipients were immunized with 0.4 mg DNP-BGG in CFA, intraperitoneally, 1 day after cell transfer. Spleens were assayed for anti-DNP PFC 10 days after immunization. The results are expressed as the geometric means of groups of 5–7 animals.

The distribution of avidities of PFC in irradiated mice reconstituted with spleen cells from tolerant or normal animals and immunized with DNP-BGG is illustrated in Fig. 3. It is clear that the recipients of cells from tolerant animals produce subpopulations of high avidity PFC which are not present in the recipients of normal spleen cells.

Thus, the spleen cells from donors made tolerant with 0.5 mg DNP-BGG behave as if they had been primed to give a secondary response upon transfer into irradiated recipients. The response of animals reconstituted with cells from tolerant donors is greater in magnitude and affinity than that of animals reconstituted with normal spleen cells. The data are thus consistent with the concept that the tolerance induced with 0.5 mg DNP-BGG is mediated by the production of small amounts of high affinity antibody. When mice are made tolerant with a larger dose of antigen (5 mg) a primed population of cells is initially present. However, by 2 wk after tolerance induction with 5 mg
Fig. 3. Each histogram illustrates the distribution of indirect anti-DNP PFC with respect to avidity for DNP-EACA in the spleen of an individual lethally irradiated mouse reconstituted with spleen cells from either normal mice or from mice made tolerant by the intravenous injection of 0.5 or 5.0 mg DNP-BGG 5 days before use as cell donors. The abscissa represents the log of the inverse of the hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. Avidity increases to the right. The mice were immunized with DNP-BGG in CFA 1 day after cell transfer and were sacrificed for assay 10 days after immunization.

DNP-BGG the spleen cells behave like cells from normal donors, suggesting that additional factors are involved when tolerance is induced with larger doses of antigen.

Carrier Specificity of Antibody-Mediated Suppression and of Tolerance. If the tolerance described here results from the production of small amounts of high affinity antibody, then passive antibody should duplicate both the effect on affinity and the carrier specificity characteristics of this tolerance state. As illustrated in Fig. 4, a low dose of passive antibody reproduces the effect on avidity which is seen in the tolerant mice. That is, both antibody-mediated suppression and the tolerance state studied here result in an increase in high avidity PFC.

Serum antibody is known to exhibit significant carrier specificity (21). The carrier specificity of antibody-mediated immune suppression was therefore
FIG. 4. Each histogram illustrates the distribution of indirect anti-DNP PFC with respect to avidity for DNP-EACA in the spleen of an individual mouse. The abscissa represents the log of the inverse of the hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. Avidity increases to the right. The top row received normal mouse serum (NMS) and the bottom row received mouse anti-DNP-BGG antiserum 1 day before immunization with 0.1 mg DNP-BGG in CFA. Assays were carried out 7 days after immunization.

studied. Mice were given various doses of either mouse anti-DNP-BGG antibody or normal mouse serum intraperitoneally. 1 day later all animals were immunized intraperitoneally with either DNP-BGG or DNP-RSA in CFA and their anti-DNP PFC responses were assayed 1 wk after immunization. Injection of 0.05 ml of mouse anti-DNP-BGG antiserum markedly suppressed the immune response to DNP-BGG but caused little suppression of the PFC response to DNP-RSA (Table VII). The carrier specificity of tolerance was then examined. Mice made tolerant by the intravenous injection of DNP-BGG were immunized 5 days later with either DNP-BGG or DNP-RSA in CFA. DNP-BGG tolerant animals showed a much greater depression in their response to DNP-BGG than in their response to DNP-RSA (Table VIII). This was true for both the direct and indirect PFC response. Thus, the tolerant state which we have been studying and antibody-mediated immune suppression both exhibit a similar marked degree of carrier specificity.

Discussion

The data presented in this paper demonstrate that tolerance to a haptenic determinant can be induced in adult mice by a single intravenous injection of antigen. Tolerance produced by the intravenous administration of 0.5 mg DNP-BGG appeared to result from the production of small amounts of high
affinity anti-DNP-BGG antibody. This hypothesis is supported by several observations. (a) Early after immunization tolerant animals have higher affinity serum antibody and a population of higher avidity PFC than are present in normal animals. It is known (16, 19, 20) that antibody-mediated immune suppression is associated with an increase in affinity of the residual antibody. (b) Animals only intravenously injected with DNP-BGG produced a significant anti-DNP PFC response of high avidity. Thus, as has been shown previously (22), intravenous antigen is capable of causing a selective proliferation of high avidity

**Table VII**

Carrier Specificity of Immune Suppression After Passively Administered Anti-DNP-BGG Antibody

| Anti-DNP antibody treatment | Antigen | Direct anti-DNP PFC | Indirect anti-DNP PFC |
|----------------------------|---------|---------------------|----------------------|
|                            |         | PFC/spleen × 10⁻³   | Depression            |
|                            |         |                     |                      |
| -                          | DNP-BGG | 7.0                 | -                    |
| +                          | DNP-BGG | 0.1                 | 98                   |
| -                          | DNP-RSA | 1.5                 | -                    |
| +                          | DNP-RSA | 0.8                 | 47                   |

*Animals were injected with 0.05 ml of mouse anti-DNP-BGG antiserum or with an equal volume of normal mouse serum. 1 day later they were immunized intraperitoneally with 0.1 mg of either DNP-BGG or DNP-RSA in CFA. Spleens were assayed 1 wk after immunization and the results are expressed as the geometric means of groups of 5-7 mice.

**Table VIII**

Carrier Specificity of Tolerance to DNP-BGG

| Tolerance-inducing dose of DNA-BGG | Antigen | Direct anti-DNP PFC | Indirect anti-DNP PFC |
|-----------------------------------|---------|---------------------|----------------------|
| mg                                |         | PFC/spleen × 10⁻³   | Depression            |
|                                  |         |                     |                      |
| 0.0                               | DNP-BGG | 10.3                | -                    |
| 0.0                               | DNP-RSA | 3.9                 | -                    |
| 0.5                               | DNP-BGG | 1.1                 | 89                   |
| 0.5                               | DNP-RSA | 3.3                 | 15                   |
| 5.0                               | DNP-BGG | 1.4                 | 86                   |
| 5.0                               | DNP-RSA | 3.2                 | 18                   |

*Mice were injected intravenously with the indicated dose of DNP-BGG. 5 days later the animals were immunized intraperitoneally with 0.4 mg of either DNP-BGG or DNP-RSA in CFA. 1 wk after immunization spleens were assayed for anti-DNP PFC and the results are expressed as the geometric means of groups of 5-7 mice.
anti-DNP-producing cells. (c) Spleen cells from normal donors injected into sublethally irradiated, tolerant mice did not respond to antigen suggesting that their function was suppressed in the tolerant animals. (d) Spleen cells from tolerant mice injected into lethally irradiated, syngeneic recipients produced a PFC response of greater magnitude and higher avidity than that produced by spleen cells from normal animals. The data suggest that the tolerizing injection of antigen produces a selective proliferation of high affinity antibody-producing cells so that upon cell transfer a high avidity secondary response is observed. (e) The marked carrier specificity of the tolerant state was shown to be identical to the carrier specificity of antibody-mediated immune suppression.

The ability of antibody to suppress immune responsiveness (23) and the effect of this immune suppression on antibody affinity (16, 19, 20) have been well documented. It appears that the major mechanism by which circulating antibody depresses the immune response is, in effect, by competing with immunocompetent cells for available antigen. This results in a preferential failure of stimulation of low affinity antibody-forming cells.

Carrier specificity is generally regarded as characteristic of T-lymphocyte function. It was therefore initially surprising to find that the tolerant state described here had a high degree of carrier specificity. However, it must be remembered that serum antibody itself has marked carrier specificity. Thus it has been shown (11) that antihapten antibody binds the original immunizing hapten-protein conjugate with higher avidity than it binds the hapten alone or the hapten on a different carrier. Since high affinity passive antibody is far more effective in causing suppression than is low affinity antibody (24), and since serum antibody has marked carrier specificity, one would predict that antibody-mediated suppression should exhibit carrier specificity. This prediction was confirmed in the present study. Thus, the carrier specificity of the tolerance state which we have been studying is clearly consistent with the hypothesis that this tolerance is mediated by the suppressive effects of circulating high affinity antibody.

Previous studies on the effect of tolerance on antibody affinity have suggested that B-lymphocyte tolerance mainly effects those cells which preferentially capture antigen, that is, high affinity antibody-producing cells. Consequently, B-cell tolerance has been found to be associated with a marked depression in antibody affinity (13, 15-18). The tolerant state described in the current work is thus distinctive in that it is associated with an increase in antibody affinity. This effect on affinity is consistent with the hypothesis that this form of tolerance is mediated by high affinity circulating antibody formed in response to the tolerizing injection of antigen.

It should be noted that the antigen preparation used for tolerance induction is a highly substituted protein which has a tendency to aggregate and partially precipitate from solution in normal saline at neutral pH. The antigen was not ultracentrifuged or otherwise treated so as to remove aggregated protein before its use in tolerance induction. Thus, the antigen preparation is one which would generally be regarded as highly immunogenic. The preparation of antigen used in inducing the type of tolerance described here is, therefore, quite different from antigen preparations freed of aggregated or denatured material which have been previously shown to readily induce tolerance (25, 26).
The tolerance described in the present paper thus appears to involve a stimulation of high affinity antibody synthesis and results in an expanded population of specific memory cells. It should be noted that upon subsequent antigen injection high affinity memory cells, which arose in response to the initial tolerizing injection of antigen, would be preferentially stimulated. High affinity antibody would be promptly formed and would tend to suppress further antibody synthesis. Crowle and Hu (27) have previously described what also appears to be a state of antibody-mediated tolerance.

Tolerance induced by the intravenous injection of a 10-fold higher dose (5.0 mg) of DNP-BGG, while sharing some similarities with the tolerant state induced with lower doses of antigen, is different in several important respects. The degree of tolerance was more marked and of longer duration after a 5.0-mg dose of antigen than after lower doses. The avidities of PFC in animals tolerant to a 5.0-mg dose of antigen were low and failed to increase with time. When spleen cells from animals made tolerant with 5.0 mg DNP-BGG were transferred to irradiated recipients 2 wk after the intravenous injection of antigen, they failed to respond like spleen cells from mice tolerant to a 0.5-mg dose of antigen, but responded instead like spleen cells from normal donors. Tolerance after 5.0 mg of antigen must therefore involve at least two separate mechanisms. The first mechanism is probably the production of anti-DNP-BGG antibody. This conclusion is supported by the observations that there is a PFC response to the injection of 5.0 mg DNP-BGG and that normal spleen cells do not restore responsiveness to tolerant animals. The second mechanism appears to be a selective loss of high affinity anti-DNP antibody-producing cells. This hypothesis is supported by: (a) the failure of PFC in animals tolerant to 5.0 mg doses of antigen to increase in avidity with time after immunization; (b) the longer persistence of tolerance in these animals; (c) the absence of a primed population of PFC in spleens of animals tolerant to 5.0 mg doses of antigen 2 wk after tolerance induction. This initial production of antibody followed by a loss of high affinity antibody-producing cells which occurs after injection of 5.0 mg of antigen could result from an “exhaustive terminal differentiation” of antibody-producing cells. Cells with high affinity antigen receptors would naturally be preferentially affected by such a process. The loss of such populations of cell as a result of “terminal differentiation” would leave the animals with a small population of cells producing low affinity antibody. Such a mechanism for tolerance has been postulated by Sterz (28) and by Sterz and Trnka (29) and seen to require the injection of a large dose of antigen.

Summary

We have described a model of immunological tolerance induced, in adult mice, by a single injection of a moderate dose of a hapten-protein conjugate. The data suggest that the mechanism of this tolerance state is the production of small amounts of high affinity antibody in response to the tolerance-inducing antigen injection. This antibody acts to inhibit the response to a subsequent challenge with antigen in complete Freund’s adjuvant by a mechanism comparable to that
of passive antibody-mediated immune suppression. It was shown that a small but high avidity PFC response occurs after the tolerizing injection of antigen. The small number of PFC produced by partially tolerant mice are of high avidity and their serum antibody is of high affinity. Tolerance was not terminated by transfer of normal syngeneic spleen or peritoneal cells into tolerant animals. Spleen cells from tolerant mice, when transferred into lethally irradiated, syngeneic animals, produced a PFC response which is greater in magnitude and of higher avidity than that produced by spleen cells from normal donors. The tolerance state had a significant degree of carrier specificity which was shown to be comparable to the carrier specificity of antibody-mediated immune suppression. Thus, evidence was presented to show that one mechanism of tolerance in adult animals is the suppressive effect of small amounts of high affinity antibody formed in response to the tolerizing injection of antigen.

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