The discovery of T-cell (thymus derived) and B-cell (bone marrow derived) cooperation in the immune response (1–3) and the subsequent finding that either or both cells may be involved in immunologic tolerance (4, 5) has been critical to our understanding of both cellular immunology and tolerance. For example, split (6) or partial tolerance (7) is now understood as T-cell tolerance with B-cell immunity. Moreover, the T cell has been shown to play an increasing role in establishing and maintaining B-cell tolerance (8) to a number of antigens. Although experiments in vitro indicate that T cells are tolerant to self antigens (9) and it is known that either T or B cell need to be tolerant to a heterologous antigen for the intact animal to be unresponsive (10), it is not clear whether both cells are involved in tolerance to autologous antigen. To approach this question we have used one system of carrier-determined tolerance which closely resembles "self" tolerance (11, 12). Animals were made tolerant to a hapten conjugated to isologous gamma globulin and immunized with the identical hapten conjugated to heterologous carrier protein. Relatively pure populations of T and B cells from either normal or tolerant mouse spleen cells were obtained. The immune response of mixtures of either tolerant T and normal B cells, or conversely, normal T and tolerant B cells was examined in lethally irradiated isogeneic recipients. It was found that carrier-determined tolerance involves both T and B cells.

Materials and Methods

Animals. 3–8-week-old male BDF1 mice were purchased from Jackson Laboratories, Bar Harbor, Maine.

Preparation of Tolerogen. IgG1 (MOPC 245) was isolated from the serum of plasmacytoma-bearing mice by starch block electrophoresis. Twice recrystallized 2,4-dinitrobenzene sulfonic acid sodium salt (Eastman Kodak Co., Rochester, N.Y.) was bound to purified myeloma protein as previously described (11). The subscripts refer to the degree of substitution of the hapten carrier conjugate. The following preparations were used: DNP,n-γl and DNP,i-γl.

Preparation of Antigen. Keyhole limpet haemocyanin (KLH)1 was purchased from Pacific Bio

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1Abbreviations used in this paper: CFA, complete Freund’s adjuvant; FCS, fetal calf serum; HRBC, horse red blood cells; KLH, keyhole limpet haemocyanin; PBS, phosphate-buffered saline; PFC, plaque-forming cells.
Marine Supply Co., Venice, Calif. DNP, KLH was prepared as previously described (11).

Preparation of Cellular Immunoabsorbant. Mouse Fab was prepared from a pepsin digest of mouse Ig, emulsified in complete Freund's adjuvant, and used to immunize rabbits for the production of antimouse Fab. The resulting antisera were purified over Sepharose 4B mouse-Ig immunoabsorbant columns, concentrated, dialyzed, and stored at -20°C. The purified antibody was conjugated to cyanogen bromide-activated Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) by methods previously described (13). Approximately 0.3 mg of antibody was routinely bound/ml of activated Sephadex. 12-ml disposable syringes are fitted with polyethylene disks (Bel-Art Products, Pequannock, N. J.) and packed with 12-15 ml of the Sephadex conjugate. The columns were washed with Liebowitz-15 (Microbiological Associates, Bethesda, Md.) containing 5% fetal calf sera (FCS), 2.5 mM EDTA, 50 U/ml penicillin-streptomycin solution, incubated at 37°C for 60 min, and returned to 4°C for cell fractionation.

Cell Preparation and Cell Fractionation. 250-300 x 10⁶ mouse spleen cells from either normal or tolerant animals were teased into the above media without EDTA, aggregates sedimented, cells washed three times, and the resulting single cell suspension treated with iron carbonyl at 37°C for 30 min. The phagocytic cells were removed in a magnetic field. Cells were resuspended in EDTA containing media and placed on the 12-15-ml column in a concentration of approximately 30 x 10⁶ cells/ml. The cells were applied to the column at 4°C and collected by stepwise elution with 15-ml aliquots of EDTA-containing medium. To recover the adherent cells competitive absorption was carried out using medium with 10% autologous mouse sera as a source of gamma globulin (14). Usually 90-100% of the total cells were recovered. Cells viability, as determined by trypan blue exclusion test, before and after cells fractionation was 90-95%.

Analysis of Surface Characteristics. Unfractionated, nonretained, and serum-eluted cell populations were studied with respect to surface Ig properties. The presence of surface Ig was detected by using a direct fluoresceinated antibody technique. To approximately 2 x 10⁶ cells was added 0.1 ml of fluoresceinated rabbit antimouse Fab and the reaction mixture incubated at 4°C for 30 min. The cells were then washed three times with cold phosphate-buffered saline (PBS) containing 10% FCS, suspended in a glycerol-PBS buffer, and viewed with a Zeiss Universal fluorescence microscope (Carl Zeiss, Inc., New York) with an Osram 100 watt ultraviolet light source and with phase contrast. Routinely 200 viable cells were counted on the phase contrast and percentage staining with fluoresceinated rabbit gamma globulin was used. In these experiments, fluoresceinated normal rabbit IgG stained less than 2% of the cells.

Hemolytic Plaque Assay. Compound J-(N-2,4-dinitrophenyl alanyl glycylglycine Boc hydrazide (purchased from Regis Chemical Co., Morton Grove, Ill.) was covalently bound to sheep red blood cells (SRBC, Colorado Serum Co., Denver, Colo.) as described by Inman et al. (15). These target cells were used in the direct hemolytic plaque assay as previously described (11). In some instances, horse red blood cells (HRBC, Colorado Serum Co.) were used in the Jerne hemolytic plaque assay.

Statistical Analysis. Statistical analysis was done according to the Student's t test. For the PFC/spleen, the geometric mean of each group was calculated.

Results

Transfer of Tolerance with Spleen Cells. Preliminary experiments were done to determine whether spleen cells obtained from tolerant animals could transfer tolerance to lethally irradiated recipient mice. Two treatment schedules were used for tolerance induction. 6-wk-old BDF, mice were injected with a single i.v. dose (0.2 mg or 1 mg) of DNP, KLH; 3-wk-old BDF, mice were treated weekly with 0.2 mg of DNP, KLH i.v. for 4 wk. 1 wk after the last injection of tolerogen spleen cell suspension from tolerant animals were used to repopulate lethally irradiated (300 rad) recipient animals. Normal spleen cells from age-matched controls were also used. Immediately after reconstitution, they were immunized i.p. with 1 mg of DNP-KLH in complete Freund's adjuvant (CFA). The number of antibody-forming cells to DNP was determined 8 days later. As seen in Table I, the ability to transfer tolerance with spleen cells of animals

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Transfer of Tolerance to DNP by Spleen Cells from Animals Made Tolerant for 1 Wk

| Donor treatment | Spleen cells transferred | No. irradiated BDF recipient mice | DNP-PFC/S | (±SE) |
|-----------------|--------------------------|-----------------------------------|-----------|-------|
| None            | $40 \times 10^6$         | 6                                 | 49,101    | (8,044) |
| 0.2 mg DNP$_{15\gamma 1}$ | $40 \times 10^6$      | 6                                 | 42,192    | (4,824) |
| None            | $40 \times 10^6$         | 6                                 | 44,404    | (7,613) |
| 1 mg DNP$_{15\gamma 1}$  | $40 \times 10^6$      | 6                                 | 8,392     | (1,419) |
| None            | $40 \times 10^6$         | 6                                 | 47,504    | (4,034) |
| 0.2 mg DNP$_{15\gamma 1} 4\times$ | $40 \times 10^6$ | 6                                 | 2,177     | (1,038) |

Table I

Transfer of Tolerance to DNP by Spleen Cells from Animals Made Tolerant for 1 Wk

| Donor treatment | Spleen cells transferred | No. irradiated BDF recipient mice | DNP-PFC/S | (±SE) |
|-----------------|--------------------------|-----------------------------------|-----------|-------|
| None            | $40 \times 10^6$         | 6                                 | 49,101    | (8,044) |
| 0.2 mg DNP$_{15\gamma 1}$ | $40 \times 10^6$      | 6                                 | 42,192    | (4,824) |
| None            | $40 \times 10^6$         | 6                                 | 44,404    | (7,613) |
| 1 mg DNP$_{15\gamma 1}$  | $40 \times 10^6$      | 6                                 | 8,392     | (1,419) |
| None            | $40 \times 10^6$         | 6                                 | 47,504    | (4,034) |
| 0.2 mg DNP$_{15\gamma 1} 4\times$ | $40 \times 10^6$ | 6                                 | 2,177     | (1,038) |

given a single injection of tolerogen 1 wk previously was dose dependent. Although animals injected i.v. with 0.2 mg of DNP$_{15\gamma 1}$ are themselves tolerant, their spleen cells fail to transfer tolerance to irradiated hosts. In contrast, spleen cells from donor animals injected with 1 mg of DNP$_{15\gamma 1}$ exhibit a significantly (P > 0.001) lower number of DNP antibody-forming cells than normal spleen cells after adoptive transfer to lethally irradiated recipients. Similarly, the spleen cells of animals treated weekly with 0.2 mg of DNP$_{15\gamma 1}$ for 1 mo remained tolerant in irradiated recipient animals.

Transfer of Tolerance with T and B Cells

Donor animals tolerant for 4 wk. Spleen cells of animals tolerant for 1 mo were initially used to study the cellular basis of tolerance. A week after the last tolerogen injection, the spleen cell suspensions from these tolerant animals were fractionated on the cellular immunoabsorbant column to obtain pure samples of T (nonimmunoglobulin-bearing cells) and B (immunoglobulin-bearing) cells to be referred to as tolerant T and tolerant B cells. Similarly, spleen cell suspensions of untreated 7-wk-old BDF$_1$ mice were fractionated on another cellular absorbant column to obtain normal T and B cells. A mixture of tolerant T and normal B cells, or conversely, normal T cells and tolerant B cells was used to repopulate lethally irradiated (960 rad) isogenic 8-wk-old BDF$_1$ mice. The total number of cells used to restore one irradiated host was $20 \times 10^6$ and the T/B ratio 1/1 with the exception of one instance where $40 \times 10^6$ cells were transferred with a T/B ratio of 3/1. Immediately after cell transfer, the recipient animals were immunized with 1 mg of DNP-KLH given i.p. in CFA. 8 days later, the direct hemolytic plaque assay was done. The results (Table II) show that as expected, the whole spleen cells population readily transferred tolerance.

Also, tolerance can be readily transferred with a mixture of tolerant B cells and normal T cells in a ratio of 1/1 corresponding to that found in the whole spleen. On the other hand, when the ratio of tolerant T cells and normal B cells was 1/1, no diminution of the immune response was observed. However, at a 3/1 ratio it was diminished. This suggests that a T/B ratio in excess of the one found in the spleen is required to demonstrate T-cell tolerance.

This question was examined in the next series of experiments in which the
hapten specificity of T-cell tolerance was sought. Lethally irradiated BDF, mice were repopulated with T cells from 4-wk tolerant mice and normal B cells. 25 × 10^6 cells were transferred, and the T/B ratio was 4/1. As a control, mice were repopulated with normal T and B cells in the same ratio. Animals of both groups were immunized with 1 mg of DNP-KLH given i.p. in CFA and 0.2 ml of 20% of HRBC. 8 days later, the direct hemolytic plaque assay to both antigens was done. The results show (Table III) that animals repopulated with tolerant T cells and normal B cells in a ratio of 4/1 are tolerant as compared to controls repopulated with the same ratio of normal T and normal B cells. Perhaps of greater importance, T cell tolerance was hapten specific. It is not clear why animals repopulated with fractionated populations of T and B cells have a lower response to HRBC than animals repopulated with the whole spleen cell population in which the transfer of tolerance was also hapten specific.

### Table II

**Donor Animals Tolerant for 4 Wk**

| No. irradiated BDF, recipient mice | T/B ratio | Restoring cells | DNP-PFC/S (±SE) | P |
|------------------------------------|-----------|----------------|----------------|---|
| 6                                  | 1/1       | 20 × 10^6 N. Spl.* | 19,159 (1,638) | <0.001 |
| 5                                  | 1/1       | 10 × 10^6 N. T | 3,319 (515) | <0.001 |
| 6                                  | 3/1       | 30 × 10^6 Tol. T | 10,559 (1,553) | <0.025 |

* N. T., normal T cell; Tol. T, tolerant T cell; Tol. B, tolerant B cell; N. B, normal B cell; N. Spl., normal spleen; Tol. Spl., tolerant spleen.

### Table III

**Donor Animals Tolerant for 4 Wk**

| No. irradiated BDF, recipient mice | T/B ratio | Restoring cells | DNP-PFC/S (±SE) | P | HRBC-PFC/S (±SE) |
|------------------------------------|-----------|----------------|----------------|---|-----------------|
| 6                                  | 1/1       | 20 × 10^6 N. Spl.* | 10,175 (1,638) | <0.005 | 18,285 (3,465) |
| 5                                  | 1/1       | 10 × 10^6 N. T | 11,408 (4,418) | <0.005 | 4,699 (4,521) |
| 5                                  | 3/1       | 20 × 10^6 Tol. T | 1,328 (520) | <0.005 | 3,189 (1,157) |

* N. Spl., normal spleen cells; Tol. Spl., tolerant spleen cells; N. T, normal T cell; N. B, normal B cell; Tol. T, tolerant T cell.
To determine whether tolerance was also hapten specific at the B cell level, a similar experiment was done in which animals were repopulated with normal T cell and tolerant B cells in a 1/1 ratio. Table IV shows that while the number of anti-DNP PFC was depressed, the response to HRBC was the same as in the controls. Thus, carrier-determined tolerance is hapten specific for both T and B cells.

**Donor Animals Tolerant for 1 Wk.** The next series of experiments were designed to determine whether both T and B cells were tolerant in animals tolerant for 1 wk. Spleen cells of animals injected with a single injection of 1 mg of DNP_{125} i.v. 1 wk before were fractionated to obtain populations of T and B cells from tolerant animals (tolerant T and tolerant B cells). Similarly, T- and B-cell suspensions were obtained from 7-wk-old BDF1 mice. As before, mixtures of tolerant T cells and normal B cells, or conversely, normal T cells and tolerant B cells were used to repopulate lethally irradiated mice. Each recipient animal received $25 \times 10^6$ cells. The T/B ratio was 4/1. Immediately after cell transfer, recipient animals were immunized with 1 mg of DNP-KLH given i.p. in CFA. 8 days later, the direct hemolytic plaque assay was done. The results of three separate experiments were summarized in Table V. Tolerance is transferred with the whole spleen population and similarly at a 4/1 ratio with a mixture of tolerant T cells and normal B cells. On the other hand, $20 \times 10^6$ normal T cell

| No. irradiated BDF1, recipient mice | T/B ratio | Restoring cells | DNP-PFC/S (±SE) | HRBC-PFC/S (±SE) |
|-------------------------------------|-----------|----------------|-----------------|-----------------|
| 4                                   | 20 x 10^6 N. Spl.* | 15,960 (2,841) | 7,459 (2,058) |
| 3                                   | 20 x 10^6 Tol. Spl. | 1,773 (1,358) | 7,195 (2,282) |
| 5                                   | 1/1       | 10 x 10^6 N. T 10 x 10^6 Tol. B | 4,407 (948) | 8,195 (951) |

* See footnotes Table II and III.

**Table V**

| Donor Animals Tolerant for 1 Wk |
|---------------------------------|
| No. irradiated BDF1, recipient mice | T/B ratio | Restoring cells | DNP-PFC/S (±SE) | HRBC-PFC/S (±SE) | P |
|-------------------------------------|-----------|----------------|-----------------|-----------------|---|
| 13                                  | 25 x 10^6 N. Spl.* | 15,063 (2,292) |                |                |   |
| 10                                  | 25 x 10^6 Tol. Spl. | 6,952 (1,560) |                |                | <0.01 |
| 13                                  | 4/1       | 20 x 10^6 Tol. T 5 x 10^6 N. B | 6,956 (1,781) |                | <0.025 |
| 15                                  | 4/1       | 20 x 10^6 N. T 5 x 10^6 Tol. B | 17,114 (2,636) |                | >0.5 |

* See footnotes Table II and III.
and $5 \times 10^6$ tolerant B cell fails to transfer tolerance. These results suggest that tolerance was only observed in the T-cell population. To resolve this question, the experiment was repeated but the ratio of tolerant B cells and normal T cells was 1/1, corresponding to the one found in the spleen. Under these conditions, tolerance could be shown in the B-cell population as well (Table VI). Why it was not demonstrated with $5 \times 10^6$-tolerant B cells in the previous experiments is not clear. Again, as shown above (Table V) at a 4/1 ratio, T cells also transfer tolerance.

Discussion

The data described above indicates that hapten-specific carrier-determined tolerance affects the T- and B-cell populations. The observation that both cell types are involved, has several implications: First, it confirms that both T cells and B cells are the target of tolerance induction to a protein antigen (4), as well as to a hapten linked to isologous protein (16). Secondly, it shows that hapten-specific carrier-determined tolerance is not necessarily only an example of B-cell tolerance. Lastly, it raises questions concerning the cellular recognition by T cells of a hapten bound to a "self" carrier and how tolerant T cells influence normal B cells.

Others have shown that a hapten linked to T-independent antigens induces B-cell tolerance (17–20) even in nude mice which are presumably without T cell (21). However, it is not known when T cells were present whether they were also tolerant. Our results clearly show that a hapten-specific tolerant T cell prevents a normal B cell from forming antihapten antibody. Why more tolerant T cells are needed than tolerant B cells to demonstrate tolerance is unclear. In our system, DNP isologous gamma globulin can be considered analogous to a T-independent antigen since we presume that no carrier cell exists for "self" IgG. However, the possibility that T cells recognize self antigen cannot be ruled out, and, in fact, the data show that DNP isologous IgG is recognized by T cells. Moreover, in a system analogous to our model, it was shown that mice made tolerant to the hapten NIP conjugated to mouse gamma globulin (NIP-MGG) do not respond to NIP-MGG in vitro (22). The authors conclude that tolerance

| Donor Animals Tolerant for 1 Wk |
|-------------------------------|
| No. irradiated BDF, recipient mice | T/B ratio | Restoring cells | DNP-PFC/S | ($\pm$SE) | $P$ |
|-------------------------------|-----------|----------------|-----------|----------|-----|
| 5                             | 5         | $25 \times 10^6$ N. Spl. | 26,028    | (3,594)  |     |
| 5                             | 4/1       | $20 \times 10^6$ Tol. T  | 4,739     | (822)    | 0.001|
|                               | 5/1       | $5 \times 10^6$ N. B    |           |          |     |
| 5                             | 1/1       | $10 \times 10^6$ N. T   | 3,069     | (1,617)  | 0.001|
|                               | 10 x $10^6$ Tol. B |               |           |          |     |

* See footnotes Tables II and III.
induced by NIP-MGG involves not only B cells but also T cells, again implying that a hapten autologous carrier is recognized by T cells and that such cells can be rendered tolerant. It is worth mentioning that the ability to render the T cell tolerant in carrier-determined tolerance might be important for the therapeutic application of this experimental model. For example, in allograft transplantation and in prevention of autoimmune disease (23) T-cell tolerance might be critical.

It is conceivable that T-independent antigens are only independent as far as the helper T cell, but T-dependent as far as suppressor T cells are concerned. Perhaps, T-independent antigens are T-dependent tolerogen. If this is the case, the predominant 19S antibody response provoked by T-independent antigen would represent an example of partial tolerance since it is known that T cells are required to make IgG antibody, and T cells are more susceptible to tolerance induction than B cells.

Finally, one critical question that cannot be answered by these results is how T and B cells are made tolerant. While B-cell tolerance can be readily explained, for example, by receptor blockade, (24) how tolerant T cells turn off normal B cells is unknown. At least two possibilities that are not mutually exclusive can be offered. We are dealing with either a specific tolerant T cell and/or a hapten-specific suppressor T cell. There is no evidence to support the latter. The former is consistent with our recent finding that the receptor of the antigen-binding cell is specifically blocked by the tolerogen (24). Thus, receptor blockade on T cell by DNP-isologous IgG would prevent the cooperative effect of DNP-KLH. The recent observation that DNP-mouse gamma globulin raises hapten-specific helper T cell is consistent with this interpretation. (25) Alternatively, after a T-cell receptor blockade, a T-cell product (which might be the tolerogen itself) might suppress the B cell. The view of a T-cell product to suppress B cells is consistent with requirement of a relatively large number of tolerant T cells turning off normal B cells upon transfer to irradiated hosts. An attractive possibility would be that a suppressor T cell is a T cell with its receptor occupied by the tolerogen. Further studies are in progress to determine the cellular origin of the tolerant cell. In any event, our results clearly show that B cell antibody production failed in the presence of a hapten-specific tolerant T cell. Whether this results from active suppression or lack of help cannot be determined from the above data.

Summary

BDF, mice were made tolerant by a single i.v. injection of 1 mg of DNP-γ l or by weekly i.v. injections of 0.2 mg of DNP-γ l given for a month. In both instances, spleen cells of tolerant animals were fractionated to obtain pure populations of T cells (nonimmunoglobulin-bearing cells), referred to as tolerant T cells, and B cells (immunoglobulin-bearing cells) referred to as tolerant B cells. The control cells were similarly fractionated to obtain normal T and B cells. Mixtures of tolerant T cells and normal B cells, or conversely, normal T cells and tolerant B cells were used to repopulate lethally irradiated recipients. These recipients were then immunized with dinitrophenyl-keyhole limpet haemocyanin and in certain instances with other antigen horse red blood cells. The
immune response to both antigens was measured using the direct hemolytic plaque assay.

It was found that both T and B cells were tolerant and that tolerance was hapten specific at both T- and B-cell levels. While B-cell tolerance was demonstrated at a 1/1 T/B ratio, a 4/1 T/B ratio was necessary to show T-cell tolerance. Thus, the hapten-specific carrier-determined tolerance involves not only B cells but also T cells. The implication of this finding for the cellular mechanism of tolerance in an experimental model closely related to self tolerance is discussed.

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