

## CELLULAR INTERACTIONS IN IMMUNE REGULATION

### Hapten-specific Suppression by Non-T Cells and T Cell-mediated Reversal of Suppression\*

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Evidence has accumulated in recent years that the immune system has the capacity for self-regulation. This can be visualized as limiting the expression of antigen-specific B cells once an adequate immune response has ensued. The first clearly identified immunoregulatory activity involved serum antibody, which Uhr and co-workers (1, 2) showed could operate to control the normal immune response. The importance of suppressor T cells in immune regulation is demonstrated during normal immune responses (3, 4) as well as after tolerance induction (5). Another proposed mechanism for immunoregulation is an autoanti-idiotypic response in which there is specific recognition of the idiotypic determinants on the antigen-specific B and T cells (6-8).

The experiments presented here were carried out to characterize the capacity of lymphoid cells from immunized animals to regulate the response of B cells from naive animals to the immunizing hapten. Mice were immunized with trinitrophenylated (TNP)<sup>1</sup> bovine gamma globulin (BGG) in complete Freund's adjuvant (CFA), and their spleen cell population was analyzed at various times thereafter, both by mixed cell transfer and mixed cell culture experiments, for the presence of inhibitory activity, which affected the response to the TNP determinant. The inhibitory activity peaked 1 wk after immunization, specifically affected the response to the TNP determinant, affected the response to both T-dependent and T-independent antigens, was active in vivo and in vitro, and appeared to be mediated by a specific non-T cell and modulated by T cell activity. In addition, evidence is presented that shows that after antigen administration, T cells are stimulated that are capable of abrogating suppression of the response to a T-independent antigen.

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<sup>1</sup> *Abbreviations used in this paper:* BGG, bovine gamma globulin; C, complement; CFA, complete Freund's adjuvant; FCS, fetal calf serum; PAA, polyacrylamide beads; PFC, plaque-forming cell(s); RAMB, rabbit anti-mouse brain antiserum; RAMIg, rabbit anti-mouse immunoglobulin antiserum; SIg, surface immunoglobulin; SRBC, sheep erythrocytes; TNBSO<sub>3</sub>, 2,4,6-trinitrobenzene sulfonic acid, sodium salt; TNP, 2,4,6-trinitrophenyl group; TNP-F, trinitrophenylated-lysine-Ficoll; TNP-PAA, trinitrophenylated polyacrylamide beads.

### Materials and Methods

*Animals.* LAF<sub>1</sub> mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Adult cell transfer recipients and donors were 7–12 wk old. Spleen donors for in vitro experiments were 12–16 wk old.

*Antigens.* BGG was obtained as Fraction II from the Pentex division of Miles Laboratories Inc., Kankakee, Ill. TNP-BGG was prepared by the reaction of 2,4,6-trinitrobenzene sulfonic acid (TNBSO<sub>3</sub>; Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) in 0.28 M Na cacodylate, pH 7.2, for 1 h at room temperature, followed by extensive dialysis. The concentration of the conjugated protein was determined by dry weight analysis and its degree of derivitization was estimated spectrophotometrically from the absorbance at 348 nm ( $\epsilon$  for TNP-lysine = 15,400). The TNP-BGG preparation used in these experiments had 24 TNP groups per BGG molecule. Trinitrophenylated-lysine-Ficoll (TNP-F) was prepared by the reaction of  $\epsilon$ -TNP-L-lysine HCl (ICN Nutritional Biochemicals, Cleveland, Ohio) with Ficoll (400,000 mol wt; Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) activated by cyanogen bromide at pH 10.0 for 24 h, followed by extensive dialysis. Trinitrophenylated-polyacrylamide beads (TNP-PAA) were prepared according to the methods of Inman and Dintzis (9). The aminoethylated polyacrylamide was synthesized by the reaction of ethylenediamine (Sigma Chemical Co., St. Louis, Mo.) with Bio-gel-P30 (Bio-Rad Laboratories, Richmond, Calif.) for 30 min at 90°C. The reaction was terminated by the addition of an equal volume of crushed ice, and the activated gel was washed extensively with 0.1 M NaCl. 1 g of the aminoethylated material in 30 ml of 0.09 M sodium tetraborate, pH 9.3, was reacted with 3 mmol of TNBSO<sub>3</sub> at room temperature for 24 h. The product was extensively washed with 0.2 M NaCl-Tris base and then with 0.2 M NaCl.

*Cell Culture.* Primary antibody responses were elicited in culture as described by Mishell and Dutton (10), using the T-dependent antigen, sheep erythrocytes (SRBC), and the T-independent antigen TNP-PAA. Spleens were teased in RPMI 1640, passed through sterile gauze to remove clumps, washed twice, and resuspended in culture medium. Cells were cultured at 10<sup>7</sup> or 5 × 10<sup>6</sup> cells per dish in modified minimal essential medium supplemented with 100 U penicillin/ml, 10  $\mu$ g streptomycin/ml, 2 mM glutamine, 5 × 10<sup>-5</sup> M 2-mercaptoethanol, and 10% fetal calf serum (FCS). Cultures were "fed" daily, as described by Mishell and Dutton (10), and were rocked at 7 oscillations per minute in a humidified atmosphere of 10% CO<sub>2</sub>, 7% O<sub>2</sub>, and 83% N<sub>2</sub>.

*Separation of Cells on Antibody-coated Dishes.* The basic technique used was that devised by Mage et al. (11) for selection of surface positive immunoglobulin (SIg<sup>+</sup>) cells from murine spleen cells. Purified rabbit anti-mouse Ig antibody (RAMIg) was prepared by affinity chromatography of hyperimmune rabbit anti-mouse Ig antiserum on a mouse Ig-sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) immunoabsorbent. After purification, the antibody was filtered and stored at 4°C as a 1 mg/ml solution in phosphate-buffered saline (PBS). To coat the plates (100-mm plastic dishes; 8-757; Fisher Scientific Co., Pittsburgh, Pa.) with Ig, 25  $\mu$ g of this solution was diluted in 10 ml for each plate and incubated overnight at 4°C. Plates were washed with PBS and then incubated for 1 h with 10 ml of PBS and 5% FCS. Plates were again washed, and 40 × 10<sup>6</sup> spleen cells in 10 ml PBS and 2% FCS was applied to each plate. After 1 h, the nonadherent cells were removed, the plates were washed, and the adherent, SIg<sup>+</sup> cells were flushed off the plates.

*Preparation of Supernate.* Mice were immunized by intraperitoneal injection of 500  $\mu$ g TNP-BGG in CFA. 1 wk later, spleens were removed and cell suspensions were prepared, as previously described. Cells were cultured for 24 h at 10<sup>7</sup> cells/ml in 60-mm tissue culture dishes (3002; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) at 3 ml per dish. After 24 h, cultures were harvested and centrifuged. The supernate was filtered through a 0.45- $\mu$ m pore size filter (Millipore Corp., Bedford, Mass.). This supernate is termed immune S/N. Control supernate was prepared in a similar manner from spleen cells of normal mice that had not been immunized.

*Cell Transfers.* In all experiments, LAF<sub>1</sub> adult mice were lethally irradiated before cell transfer by exposure to 800 rad from a gamma emitter. Spleens were teased in Hanks' balanced salt solution, passed through cotton gauze to remove clumps, washed twice with Hanks' balanced salt solution, and injected intravenously into irradiated hosts.

**Immunization.** Mice were immunized by the intraperitoneal injection of 500  $\mu\text{g}$  TNP-BGG emulsified in CFA containing 2 mg/ml *Mycobacterium butyricum* in a final volume of 0.2 ml. In some experiments, animals were immunized with 10  $\mu\text{g}$  TNP-F i.v. Cell transfer recipients were killed at various times after immunization, and their spleens were assayed for anti-TNP plaque-forming cells (PFC).

**PFC Assay.** Anti-TNP PFC were determined by the Dresser and Greaves (12) slide modification of the Jerne plaque assay (13). TNP-conjugated SRBC were prepared by the reaction of TNBSO<sub>3</sub> with washed SRBC, as described by Rittenberg and Pratt (14). Slides were incubated for 1 h at 37°C. Fresh-frozen guinea pig serum (absorbed with 50% packed SRBC) was added at a final dilution of 1:30 as a source of complement (C), and the slides were incubated for an additional 45 min. Detection of indirect anti-TNP plaques was effected by addition of rabbit anti-mouse Ig antiserum at a dilution that had been found to be optimal for plaque development.

**Antiserum Treatment.** In some experiments, the spleen cell population was depleted of T cells by treatment (at a concentration of  $4\text{--}5 \times 10^7$  cells/ml) with a 1:45 dilution of a rabbit anti-mouse brain antiserum (RAMB) and C. Agarose-absorbed guinea pig serum (1:12 dilution) was used as the C source. The C had been shown to be nontoxic for mouse cells. The cells were incubated with antiserum plus C for 30 min at 37°C and were then washed three times. The RAMB was prepared by the method of Golub (15) and was absorbed with agarose, mouse bone marrow cells, and mouse kidney powder. The absorbed antiserum killed >95% of mouse thymus cells, <5% of mouse bone marrow cells, and ~40% of mouse spleen cells. It prevented mouse spleen cells from producing an in vitro proliferative response to phytohemagglutinin.

## Results

**Suppression of the Response to TNP-BGG by Spleen Cells from Mice Immunized with TNP-BGG in CFA.** Mice were immunized with 500  $\mu\text{g}$  TNP-BGG in CFA. These will be referred to as immune mice. Lethally irradiated mice were reconstituted with normal spleen cells, with immune spleen cells (obtained 1 wk after immunization), or with a mixture of normal and immune spleen cells. Recipients were immunized 1 d after cell transfer with 500  $\mu\text{g}$  TNP-BGG in CFA. Animals were assayed for anti-TNP PFC at 2 or 3 wk after cell transfer. The results are presented in Table I. Mice reconstituted with immune spleen cells gave a secondary response, characterized by a greater number of indirect PFC, an increased ratio of indirect/direct PFC, and an earlier peaking of the response as compared with mice reconstituted with naive spleen cells (compare assays done at 2 and 3 wk, experiment 2, Table I). However, when mixtures of normal and immune cells were transferred, the PFC response was markedly more depressed than was expected. This was especially pronounced at 2 wk after cell transfer. It should be noted that in all cases the response of the recipient of a mixture of normal and immune cells was markedly lower than that of recipients of immune cells alone, and in some cases it was even lower than the response of animals receiving only naive cells. Generally, both direct and indirect PFC responses were depressed in animals reconstituted with a mixture of normal and immune cells.

It should be mentioned that the peak of the primary PFC response of normal LAF<sub>1</sub> mice to TNP-BGG in CFA has been previously shown (16) to occur 7–9 d after immunization. In the present studies, immune spleens were assayed for suppressor activity in mixed cell transfer experiments at 1, 2, and 3 wk after immunization. Suppressor activity was greatest at 1 wk after immunization (Table I), was decreased at 2 wk after immunization (Table II, experiment 1), and was not detectable at 3 wk after immunization (Table II, experiment 2).

**Suppression of the Response to TNP-F by Spleen Cells from Mice Immunized with TNP-BGG in CFA.**  $5 \times 10^7$  spleen cells from mice 1 wk after immunization with TNP-BGG in

TABLE I  
*Suppression of the Response to TNP-BGG by Spleen Cells from Mice That Had Been Immunized with TNP-BGG 1 wk Previously\**

Experiment	Cells transferred‡		Time of assay	Anti-TNP PFC/spleen	
	Normal	Immune		Direct	Indirect
1	+	0	2	6,520	16,500
	0	+		4,950	76,200
	+	+		1,920	18,480 ( $P < 0.001$ )
2	$10 \times 10^7$	0	2	24,600	53,500
	$5 \times 10^7$	0		16,970	32,850
	0	$5 \times 10^7$		6,650	72,520
	$5 \times 10^7$	$5 \times 10^7$	1,630	16,800 ( $P < 0.05$ )	
	0	$10 \times 10^7$	4,620	120,550	
	$5 \times 10^7$	$10 \times 10^7$	8,770	37,900 ( $P < 0.001$ )	
	$5 \times 10^7$	0	3	7,000	69,000
	0	$5 \times 10^7$		5,350	59,250
	$5 \times 10^7$	$5 \times 10^7$		11,175	54,750

\* LAF<sub>1</sub> mice were injected with 500  $\mu$ g TNP-BGG i.p. in CFA. 1 wk later, cells from these "immune" mice, from normal mice, or mixtures of cells from immune and normal mice were transferred to irradiated recipients. The recipients were immunized with 500  $\mu$ g TNP<sub>24</sub>-BGG in CFA 1 d after cell transfer and were assayed 2 or 3 wk later, as indicated under "time of assay." Data are presented as the mean of results obtained on groups of four mice. Significance of differences were evaluated by the use of the Student's *t* test. The indirect PFC responses of recipients of a mixture of normal and immune cells were compared with responses of recipients of normal cells alone.

‡ In experiment 1, the equivalent of one-half of a spleen of normal or immune mice was given per recipient. In experiment 2, the indicated numbers of cells were transferred.

CFA,  $5 \times 10^7$  normal spleen cells, or mixtures of  $5 \times 10^7$  normal and  $5 \times 10^7$  immune spleen cells were transferred into irradiated recipients that had received 800 rad 24 h previously. The recipients were challenged with 10  $\mu$ g TNP-F i.v. on the day of cell transfer, and their splenic anti-TNP PFC were assayed 7 d later. The results are presented in Table III. In each experiment, mice reconstituted with TNP-BGG immune spleen cells gave a markedly lower response to the T-independent antigen TNP-F than did animals reconstituted with naive spleen cells. Mice reconstituted with mixtures of normal and immune spleen cells gave anti-TNP responses to TNP-F that were depressed by 38–67% as compared with those of mice reconstituted with only normal spleen cells. In experiment 3, immune spleen cells were treated with RAMB plus C before transfer. This treatment did not affect the response of the immune cells. The response of mice reconstituted with normal plus RAMB-treated immune spleen cells was still suppressed (Table III).

To rule out the possibility that the peak response of the immune population was missed by assaying only at day 7, a transfer was performed in which groups were assayed daily from 3–7 d after antigen injection. There was no difference in the magnitude of the PFC response of mice reconstituted with immune spleen cells from 4–7 d after immunization. On day 3 the response was considerably lower. The

TABLE II  
*Suppression of the Response to TNP-BGG by Spleen Cells from Mice That  
 Had Been Immunized with TNP-BGG 2 or 3 wk Previously\**

Experiment	Cells transferred		Time of assay <i>wk</i>	Anti-TNP PFC/spleen	
	Normal	Immune		Direct	Indirect
1‡	+	0	2	14,633	37,400
	0	+		16,225	126,750
	+	+		7,675	100,350
	+	0	3	8,825	28,800
	0	+		4,900	60,900
	+	+		2,525	46,350
2§	+	0	2	12,200	34,300
	0	+		4,350	85,400
	+	+		15,320	87,300

\* LAF<sub>1</sub> mice were injected with 500 µg TNP<sub>24</sub>-BGG i.p. in CFA. After 2 wk (experiment 1) or 3 wk (experiment 2), 5 × 10<sup>7</sup> spleen cells from these "immune" mice, 5 × 10<sup>7</sup> spleen cells from normal mice, or mixtures of spleen cells from both immune and normal mice (5 × 10<sup>7</sup> each) were transferred to irradiated recipients. The recipients were immunized with 500 µg TNP<sub>24</sub>-BGG in CFA 1 d after cell transfer and were assayed 2 or 3 wk later, as indicated under "time of assay." Data are presented as the mean of results obtained on groups of four–five mice.

‡ Transfer 2 wk postimmunization.

§ Transfer 3 wk postimmunization.

response of mice reconstituted with cells from normal donors peaked at 6–7 d after immunization, and suppression was most pronounced when assayed at this time (data not shown). Essentially, no indirect plaques were detected in the response to TNP-F under the conditions of these experiments.

To be certain that the suppressive activity was not the result of exposure to CFA, donors were immunized with TNP-BGG in CFA or CFA alone before transfer (experiment 2, Table III). The response by mice reconstituted with spleen cells from animals preimmunized with CFA alone is equivalent to the response of mice reconstituted with normal spleen cells. Mixing cells from mice preimmunized with CFA with cells from naive mice does not result in suppression of the response as compared with that of mice reconstituted only with cells from normal donors.

*Suppression of the In Vitro Anti-TNP PFC Response by Immune Spleen Cells.* Spleen cells from normal mice or from mice immunized 1 wk previously with TNP-BGG in CFA were challenged in vitro with SRBC or with the T-independent antigen TNP-PAA. A representative experiment is presented in Table IV. Spleen cells from immune mice produce far fewer PFC in response to TNP-PAA than do spleen cells from normal mice. Although the anti-SRBC response of immune spleen cells is somewhat lower than that of normal spleen cells in this experiment, the response is still within the usual range of these cultures (Table V). When 5 × 10<sup>6</sup> spleen cells from immune mice were mixed with 5 × 10<sup>6</sup> cells from normal mice, the anti-TNP-PAA response was suppressed 91%. The suppressive activity was TNP specific in that there was no suppression of the anti-SRBC response (Tables IV and V). Even 1 × 10<sup>6</sup> spleen cells

TABLE III  
*Suppression of the Response to TNP-F by Spleen Cells from Mice That Had  
 Been Immunized with TNP-BGG\**

Experi- ment	Cells transferred		Direct anti-TNP PFC‡  <i>PFC/spleen</i>
	Normal	Immune	
1	+	0	47,300
	0	+	4,400
	+	+	29,000 (39%) $P < 0.02$
2	+	0	45,800
	0	+	19,500
	+	+	15,000 (67%) $P < 0.001$
	0	CFA	48,000 N.S.
	+	CFA	56,000 (-22%) NS
3	+	0	59,900
	0	+	13,400
	+	+	34,100 (40%) $P < 0.05$
	0	RAMB	15,900
	+	RAMB	30,200

\* LAF<sub>1</sub> mice were injected with 500  $\mu$ g TNP-BGG i.p. in CFA or with CFA alone. 1 wk later,  $5 \times 10^7$  spleen cells from these mice were transferred alone or together with  $5 \times 10^7$  normal spleen cells into recipients that had been given 800 rad 24 h previously. Mice were challenged with 10  $\mu$ g TNP-F i.v. at the time of cell transfer, and were assayed for anti-TNP PFC 7 d later. Data are presented as the mean of results with groups of four mice.

‡ Numbers in parentheses indicate the percentage of suppression of the PFC response as compared with the response of mice reconstituted with normal spleen cells alone. Significance of differences were evaluated by use of the Student's *t* test.

from immune mice caused marked suppression of the response of  $5 \times 10^6$  normal spleen cells (Table IV).

Experiments were carried out to characterize the nature of the suppressive activity (Table V). Spleen cells from either the immune or normal mice, or both, were treated with RAMB antiserum and C. All controls were treated with C alone. The anti-TNP-PAA responses of mixtures of normal and immune spleen were suppressed 70–90%, calculated on the basis of simultaneous cultures of the individual cell populations comprising the mixtures. RAMB antiserum and C treatment of the immune spleen cell population did not alter the suppression. When the normal spleen cell population was treated with RAMB antiserum plus C and mixed with immune spleen cells, the degree of suppression was significantly decreased, although it was not eliminated. However, when RAMB plus C-treated immune cells were mixed with RAMB plus C-treated normal cells, the anti-TNP response was markedly suppressed.

Comparable results were obtained when anti-Thy-1 was used instead of RAMB antiserum. For example, in some experiments the immune spleen cells were treated with a monoclonal anti-Thy-1.2 and C instead of the RAMB. The cells remaining after treatment were possibly enriched for suppressive activity (Table IV). Certainly, suppression was not eliminated by anti-Thy-1.2 treatment of the immune cell population.

TABLE IV  
*Suppression of the In Vitro Anti-TNP PFC Response to TNP-PAA by Spleen Cells from TNP-BGG-immunized Mice\**

Experiment	Cells cultured		Direct anti-SRBC PFC	Direct anti-TNP PFC‡
	Normal	Immune		
1	$5 \times 10^6$	0	675	9,080
	$1 \times 10^7$	0	1,485	16,760
	0	$1 \times 10^6$	0	0
	0	$2 \times 10^6$	105	0
	0	$5 \times 10^6$	305	320
	$5 \times 10^6$	$1 \times 10^6$	835	2,850 (69%)
	$5 \times 10^6$	$2 \times 10^6$	720	1,795 (80%)
	$5 \times 10^6$	$5 \times 10^6$	880	850 (91%)
2	$5 \times 10^6$	0	544	14,916
	0	$5 \times 10^6$	250	1,254
	$5 \times 10^6$	$5 \times 10^6$	587	1,122 (92%)
	$5 \times 10^6$	$2.5 \times 10^6$	ND	4,774 (68%)
	$5 \times 10^6$	$1.25 \times 10^6$	ND	6,534 (56%)
	$5 \times 10^6$	$2.5 \times 10^6$ (anti-Thy-1.2)§	592	2,178 (85%)

\* Spleen cells from 3- 4-mo-old normal or TNP-BGG-immune (1 wk after antigen in CFA) mice were cultured for 4 d with TNP-PAA or SRBC. Values represent direct anti-TNP or anti-SRBC plaque-forming cells per culture. Data from one experiment that is representative of three similar experiments are presented.

‡ Numbers in parentheses indicate the percentage of suppression from the expected response of mixed cultures. The expected response was calculated from the results of simultaneous cultures of the individual cell populations comprising the mixtures.

§ In experiment 2, immune spleen cells ( $5 \times 10^7$ /ml) were treated with monoclonal anti-Thy-1.2 (final concentration 1:100,000) and rabbit C for 45 min at 37°C and washed.  $2.5 \times 10^6$  treated cells were added to the normal spleen cells where indicated.

|| Not done.

TABLE V  
*Effect of RAMB Plus C Treatment of the Suppressive Activity of Immune Spleen Cells\**

Cells cultured	Direct anti-SRBC PFC			Direct anti-TNP PFC‡				
	Normal	Immune	Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
			<i>PFC/culture</i>			<i>PFC/culture</i>		
+	0		630	390	980	7,830	7,350	8,075
0	+		1,050	1,314	770	680	450	2,365
0	RAMB-treated		0	20	0	390	200	820
+	+		1,338	1,760	1,838	885 (90%)	640 (92%)	3,115 (70%)
+	RAMB-treated		903	390	890	585 (93%)	180 (96%)	1,460 (84%)
RAMB-treated	0		ND§	0	0	ND	5,930	6,800
RAMB-treated	+		ND	3,060	2,330	ND	4,595 (28%)	5,315 (42%)
RAMB-treated	RAMB-treated		ND	ND	0	ND	820 (87%)	1,190 (84%)

\*  $5 \times 10^6$  or  $10 \times 10^6$  viable spleen cells were cultured for 4 d with SRBC or with TNP-PAA. Mixtures were composed of  $5 \times 10^6$  viable spleen cells of each of the indicated cell types. Normal spleen cells were obtained from 3- 4-mo-old mice. Immune spleen cells were obtained from mice immunized with TNP-BGG in CFA 1 wk before killing. To deplete spleens of T cells, the cell suspensions were incubated at  $4-5 \times 10^7$  cells/ml with RAMB antiserum (1:45 final concentration) plus agarose-adsorbed guinea pig C (1:12) at 37°C for 30 min. The cells were then washed three times, and the cells remaining after treatment of  $5 \times 10^6$  cells were added to each dish. Controls were treated with agarose-adsorbed guinea pig C (1:12).

‡ Numbers in parentheses indicate the percentage of suppression from the expected response of mixed cultures. The expected response was calculated from the results of simultaneous cultures of the individual cell populations comprising the mixtures.

§ Not done.

Because it has been reported (17) that in certain systems, specific suppressor T cells may be resistant to anti-theta antiserum and C treatment, other purification steps were taken to analyze the nature of the suppressive activity. SIg<sup>+</sup> cells were positively selected from the immune spleen cell population by panning on plastic dishes coated with RAMIg (Table VI). It was found that the SIg<sup>+</sup> cells were enriched for suppressive activity compared with unseparated immune spleen cells. The RAMIg adherent spleen cells were then treated with anti-Ly-1,2 and C to eliminate any remaining T cells. These cells still suppressed the normal response (Table VI).

*Suppression of the In Vitro Anti-TNP PFC Response by Supernate from Immune Spleen Cells.* The following experiment was done to test whether a soluble suppressive substance was generated by the immune spleen cells. 7 d after immunization with 500 µg TNP-BGG in CFA, spleen cells were put into culture without antigen. After 24 h, the cultures were centrifuged and the supernate passed through a Millipore membrane. This immune supernate was compared with the effect of supernates obtained simultaneously from cultures of spleen cells from unimmunized cells. At a concentration of 50% (~4 × 10<sup>6</sup> cell equivalents) the immune S/N suppressed the anti-TNP response of normal spleen cells (control response) by ~50% (Table VII). Control S/N had either no effect or an enhancing effect on the control response.

### Discussion

The results presented here demonstrate the development of specific suppressive activity during the course of an immune response to TNP-BGG. This activity was effective in depressing the in vivo response to TNP on both T-dependent and T-independent carriers. When TNP-BGG immune spleen cells were transferred to an irradiated mouse and challenged with TNP-BGG, they produced a characteristic secondary response. Suppression was only evident when mixtures of normal and immune spleen cells were challenged, suggesting that induction of suppressive activity in the normal spleen cells might be taking place. However, when mice reconstituted with TNP-BGG immune spleen cells were challenged with TNP-F, the magnitude of

TABLE VI  
*Evidence That the Cells Responsible for the Suppression Activity Are Not T Lymphocytes\**

Group	Cells added/ culture	Direct anti-	Percentage of
		TNP PFC	suppression
		<i>PFC/culture</i>	<i>%</i>
Control response	—	9,868	—
Immune spleen cells	1 × 10 <sup>6</sup>	4,208	57
	5 × 10 <sup>6</sup>	1,085	89
RAMIg-adherent immune spleen cells	1 × 10 <sup>6</sup>	2,431	75
	5 × 10 <sup>6</sup>	1,002	90
RAMIg-adherent, anti-Ly-1,2 plus C-treated immune spleen cells	1 × 10 <sup>6</sup>	1,250	87
	5 × 10 <sup>6</sup>	552	94

\* SIg<sup>+</sup> cells from the immune spleen cell population were purified by adherence to RAMIg-coated plastic dishes. Immune spleen cells, SIg<sup>+</sup>-purified immune spleen cells, or anti-Ly-1,2 antiserum plus C-treated SIg<sup>+</sup>-purified immune spleen cells were added in the numbers indicated to 5 × 10<sup>6</sup> normal spleen cells. The effect of the added immune cells on the direct anti-TNP PFC response to TNP-PAA by normal spleen cells was determined after 4 d of culture with antigen. The data are presented as PFC/culture and percentage of suppression from the control response by normal spleen cells.



TABLE VII  
*Suppression of the In Vitro Anti-TNP PFC Response to TNP-PAA by Supernate  
 from Cultured Immune Spleen Cells\**

Group	Direct anti-TNP PFC	
	Experiment 1	Experiment 2
Control response	7,580	987
10% Immune supernate	6,050 (19%)	ND‡
25% Immune supernate	5,300 (30%)	ND
50% Immune supernate	3,780 (51%)	555 (44%)
10% Control supernate	8,140 (-7%)	ND
25% Control supernate	7,620	ND
50% Control supernate	7,800 (-2%)	1,300 (-32%)

\* Spleen cells from 3-mo-old normal mice were cultured for 4 d with TNP-PAA (control response). Where indicated, Millipore-filtered supernate from 24-h cultures of immune or nonimmune (control) spleen cells was added to the normal spleen cell cultures. Immune spleen cells were obtained 7 d after injection of 500  $\mu$ g TNP-BGG i.p. in CFA. In experiment 1,  $10 \times 10^6$  cells were cultured in dishes, as described in Materials and Methods. In experiment 2, cells were cultured in microtiter plates (Falcon 3040) at  $1 \times 10^6$  cells per well. Data in experiment 1 are given as PFC per culture and in experiment 2 as PFC per well ( $10^6$  cells). Numbers in parentheses indicate the percentage of suppression of the control response.

‡ Not done.

their anti-TNP PFC response was markedly depressed when compared with mice reconstituted with normal spleen cells. Suppression of the challenge with the T-independent antigen was readily demonstrated by mixed cell transfer experiments. The difference between these two sets of experiments lies in the challenge antigen. In the first group, it is probable that sufficient carrier (BGG)-specific help was present to overcome the effects of the suppressive activity, and a secondary response was obtained. When a T-independent carrier (Ficoll) was used for challenge, the carrier-specific help was not operative, and the response of both the immune cells alone and the mixture of immune and normal cells was suppressed. Comparable results were also obtained in vitro studies.

We believe that the cell responsible for suppression is a B cell because (a) immune cells treated with RAMB and C suppressed both the in vivo anti-TNP-F response and the in vitro TNP-PAA response, and (b) SIg plus cells obtained from the immune population by elution from plastic dishes coated with RAMIg were enriched for suppressive activity, and further treatment of these cells with anti-Ly-1 and anti-Ly-2, and C did not affect their suppressive activity. Because this population is relatively depleted of macrophages and the suppression observed was antigen specific, it is unlikely that macrophages are responsible for the suppression. However, we have not formally ruled out a role of the macrophages that could possibly passively carry the suppressive activity. Although the initial experiments were performed using an RAMB antiserum, treatment of immune spleen with a monoclonal anti-theta or positive selection on anti-Ig-coated plates followed by treatment with anti-Ly-1,2 and C also failed to eliminate the suppressive activity, which confirmed that it was not a T cell.

The mechanism of suppression has not been characterized. Two possibilities exist. One is classical antibody feedback, that is, the production of anti-TNP antibody by the B cells, which could bind antigen and block its interaction with specific cells. The

other possibility is anti-idiotypic antibody induced by the receptors of the immune B cells. The anti-idiotypic antibody might bind the receptors of the assay B cells and inhibit antibody secretion. Precedents exist in the literature for both mechanisms (1, 2, 18). A soluble specific suppressor factor was detected in the supernate from immune spleen cells cultured in the absence of antigen. However, the suppressor factor was not further characterized. It is unlikely that the immune spleen contains a highly suppressive antigen fraction because such "processed" antigen would usually be regarded as immunogenic. It would have to exert differential effects on normal and immune cells because treatment of normal spleen cells with RAMB decreased suppression when immune T cells were present. Furthermore, the activity of the soluble suppressor factor was removed by incubation with antigen.

Recently, Zubler et al. (19) have demonstrated that surface Ig-bearing, Ly-1<sup>-</sup>2<sup>-</sup>3<sup>-</sup>, B lymphocytes, after incubation in vitro with SRBC, could exert potent specific suppressive effects on primary in vitro responses. There are many similarities between their system and the one studied here. However, it must be stressed that we have shown that this regulatory mechanism operates in vivo as well as in vitro. That this mechanism can operate in vivo emphasizes its possible importance in the normal regulation of the immune response.

Characterization of the requirements for demonstration of suppressive activity in vitro suggests that its occurrence may be dependent upon complex interactions among different cell populations. Removal of T cells from the immune spleen by incubation with RAMB antiserum and C did not decrease suppression. In contrast, removing T cells from the normal spleen cell population markedly reduced the suppressor activity, suggesting that an interaction between immune spleen cells and normal T cells is involved in generating suppressor activity. However, when T cells were eliminated from both immune and normal spleen cell preparations, the mixture generated marked suppressor activity. This clearly indicates that although T cells may be involved in regulating the degree of suppression, they are not necessary for the generation or manifestation of suppressor activity in this system.

The role of the T cell in this system is interesting. It appears that the T cells in the normal spleen cell population augment the suppression (i.e., their depletion decreases suppression), whereas T cells in the immune spleen cell population decrease the suppression (i.e., their deletion, when T cells have also been deleted from the normal spleen cell population, increases suppression). Thus, the T cell population in the immune spleen acts as an antagonist of suppression. We would suggest the term "abrosuppressor" to refer to this T cell-mediated antagonism of suppression. The abrosuppressor does not appear to be a traditional helper T cell because it prevents the suppression of the response to a highly T-independent antigen (TNP-PAA). The term "contrasuppressor" has recently been proposed (20) to describe a nonspecific activity generated upon culture of normal neonatal mouse spleen cells, which reversed the nonspecific suppressor activity generated upon culture of adult spleen cells. It was suggested that the contrasuppressor acted by rendering the helper T cell less responsive to suppression. The contrasuppressor appears to be distinct from what we are referring to as an abrosuppressor in that the latter activity is specific rather than nonspecific, and because it affects the responses to a T-independent antigen, it does not appear to act via an effect on helper T cells.

The nature of the interaction between immune and normal spleen cell populations,

which leads to the development of specific suppressor activity, is not obvious. It is possible that T cells in the normal spleen cell population interact with the antigen-specific immune spleen cells to generate idiotype-specific suppressor T cells (21). It is known that Ig-associated structures on activated B cells can trigger Ly-1<sup>+</sup>2<sup>-</sup> inducer cells to stimulate Ly-2<sup>+</sup> T suppressor activity (22). Mechanisms such as these could explain how the removal of T cells from the normal spleen cell population can decrease suppression.

It seems likely from much of the current literature in immunology that suppression is dominant in the naive state of the immune system. That is, the immune system normally exists in a suppressed, stable, steady state. In part, the stability of this suppressed state appears to be the result of a network of interactions involving idiotypes and anti-idiotypes as suggested by Jerne, and in part this suppressed stable state appears to result from a constant level of suppressor T cell activity. On the basis of this line of reasoning, we would propose the hypothesis that the initiation of an immune response involves temporarily abrogating this chronic state of suppression. The results reported here suggest that specific abrosuppressor cells can be stimulated by antigen and can act to block suppressor cell activity. It is suggested that the stimulation of specific abrosuppressor activity may be essential for the induction of an immune response to take place; in its absence suppression would remain dominant and no immune response would occur.

### Summary

The ability of lymphoid cells from immunized animals to regulate the response of naive B cells to the immunizing hapten was studied. Mice were immunized with trinitrophenylated (TNP) bovine gamma globulin (BGG) in complete Freund's adjuvant, and their spleen cells were examined *in vivo* and *in vitro* for the presence of specific inhibitory activity. This activity was found to peak 1 wk after immunization, was active against TNP on both T-dependent (BGG) and T-independent (Ficoll and polyacrylamide beads) carriers, and was demonstrable both by mixed cell transfers and mixed cell culture experiments. In *in vitro* studies, it was shown that the inhibition of the response to TNP-polyacrylamide beads by immune spleen cells was mediated by a non-T cell, possibly a B cell, because the suppressor activity was enriched in a purified B cell preparation. A role for macrophages was not formally ruled out. A specific suppressor factor was produced *in vitro* by immune spleen cells cultured in the absence of antigen. The suppressor activity was modulated by T cells because elimination of T cells from the normal spleen cell population decreased suppression; elimination of T cells from the immune spleen cell population did not effect suppression, but elimination of T cells from both the normal and immune spleen cell populations allowed the expression of marked specific suppression. Thus, T cells present in the normal spleen cell population augment the degree of suppression, whereas T cells present in the immune spleen cell population decrease the degree of suppression; that is, T cells present in the immune spleen cell population had the ability to specifically abrogate suppression ("abrosuppression") in a T-independent immune response. It is proposed that the response to a T-independent antigen is regulated by specific suppressor activity generated by a non-T cell and augmented by the interaction of this cell with a T cell. The suppressor activity can be blocked by a specific abrosuppressor T cell. It is suggested that, because suppressor activity appears

dominant in the naive state of the immune system, the induction of specific abro-suppressor activity may be essential if an immune response is to take place.

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