IMMUNOLOGIC REACTIONS TO HAPTENS ON AUTOLOGOUS CARRIERS

I. Participation of Both Thymus-Derived and Bone Marrow-Derived Cells in the Secondary In Vitro Response*

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It is now well established that in mice the immune response to many antigens, including hapten-protein conjugates, requires the participation of two distinct classes of lymphocytes, one derived from the thymus (T cells)[1] and the second from the bone marrow (B cells) (1–3). Both cell types recognize antigens; T cells appear to be involved in cell-mediated immune reactions while the antibody response to a large number of antigens, including hapten-protein conjugates, involves the cooperation of both T and B cells. In the latter case, T cells have been shown to recognize the carrier while B cells manufacture antibody to the haptenic determinant (4). The ability of the cells to recognize antigens is explained by the "receptor hypothesis," which states that the lymphocytes have on their surface immunoglobulin-like molecules which are specific for antigens (5). The interaction of the membrane receptor with its specific antigen induces cell proliferation resulting in specific cellular and/or humoral immunity. There now exists a considerable amount of experimental evidence which supports the receptor hypothesis (6).

When lymphocytes from animals immunized with haptens coupled to foreign carriers are cultured in vitro, addition of the hapten-carrier complex or the carrier by itself induces specific cell proliferation which can be measured by the increased incorporation of tritiated thymidine ([^{3}H]T) into the DNA of dividing cells. This proliferation precedes the development of antibody-forming cells (B cells) and is thought to be due primarily to T cell stimulation (7). Less is known about the participation of T and B cells in responses to haptens on isologous carriers. We have previously shown that spleen cells from mice immunized with the hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) coupled to a nonimmunogenic isologous carrier, i.e. mouse gamma globulin (MGG), will respond in vitro specifically to that antigen

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[1] *Abbreviations used in this paper*: B cell, bone marrow-derived cell; BSA, bovine serum albumin; BSS, balanced salt solution; CFA, complete Freund's adjuvant; DNP, 2,4-dinitrophenyl; EACA, epsilon-aminocaproic acid; FDNB, *p*-fluorodinitrobenzene; [^{3}H]T, tritiated thymidine; MGG, mouse gamma globulin; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; OA, ovalbumin; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; T cell, thymus-derived cell; TCM, tissue culture medium.

(NIP-MGG) with increased DNA synthesis (8). The carrier alone does not stimulate the cells.

The present report describes experiments in which the participation of T and B cells in the total in vitro response was studied. Specific removal of T or B cells was accomplished by treating the lymphocytes with complement plus antiserum specific for surface antigens (theta), immunoglobulins (IgM), both heavy- and light-chain components (polyvalent antiserum), and by passage through anti-MGG-coated columns. The results demonstrate the following points: (a) the in vitro response is highly dependent on T cells; (*b*) a large portion of the in vitro response, as much as 40–50%, is contributed by B cells; (*c*) the responding B cells carry immunoglobulin determinants of IgM molecules; (*d*) responding T cells may have specificity directed to the hapten as well as to the hapten-protein complex.

Materials and Methods

Animals.—Adult inbred BALB/c mice were obtained from the American Medical Center, Denver, Colo.

Antisera.—AKR anti-C3H θ serum was prepared as previously described (9). Antiserum against mouse IgM was obtained from Meloy Laboratories, Inc., Falls Church, Va. The antisera were raised in goats to purified IgM (λ) obtained from mice bearing myeloma tumor MOPC 104E. During immunoelectrophoresis, the antiserum forms a single precipitin line corresponding to IgM when tested against whole mouse serum. Antisera against MGG were prepared by immunizing rabbits with heat-killed *Salmonella adelaide* bacteria coated with mouse anti-*Salmonella adelaide* antibodies. These antisera have antibodies directed against both heavy- and light-chain determinants of all mouse immunoglobulins. Before use, all sera were inactivated at 56°C for 30 min and absorbed with 1 ml of packed BALB/c erythrocytes (1 ml packed erythrocytes/5 ml antiserum). In addition, rabbit anti-MGG serum and goat anti-IgM serum were absorbed twice at 1/20 vol/vol with packed BALB/c thymocytes.

Antigen.—MGG was prepared and purified as previously described (8). Bovine serum albumin (BSA, fraction V) was purchased from Pentex Biochemical, Kankakee, Ill. The haptens used were NIP and 2,4-dinitrophenyl group (DNP). NIP-protein coupling was carried out according to the method of Brownstone et al. (10) and yielded a ratio of 8–10 NIP/molecule of NIP-MGG. DNP-BSA coupling was accomplished by the method of Little and Eisen (11) and yielded an approximate ratio of 4–5 DNP/molecule of DNP-BSA.

Immunization.—BALB/c mice were immunized intraperitoneally with 400 μg NIP-MGG in complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich.).

Skin Painting.—BALB/c mice were sensitized to *p*-fluorodinitrobenzene (FDNB) according to the method of Taylor and Iverson (12). The mice were painted once a week for 4 or 5 wk and sacrificed for preparation of lymph node cell suspensions within 7 days of the last painting.

Cell Preparation and Culture.—Single-cell suspensions of spleen cells from NIP-MGG (CFA)-immunized mice and lymph nodes (popliteal, inguinal, axillary, and cervical) from FDNB-sensitized mice were prepared and cultured as previously described (8). Briefly, 4×10^6 spleen or lymph node cells were cultured with and without antigen in disposable glass culture tubes (16 × 100 mm) with Morton closures in 1 ml RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) containing 5% heat-inactivated rat serum, 100 U penicillin G, and 100 μg streptomycin/ml (complete tissue culture medium, TCM). On the 3rd day of culture at 37°C in a humidified CO_2 incubator (5% CO_2 and 95% air) each tube

was pulsed for 5 h with 1 μCi of [^{3}H]T (6 Ci/mmol, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.). DNA synthesis was determined by precipitating TCA-insoluble material on glass fiber filters (H. Reeve Angel & Co., Inc., Clifton, N. J.) and by counting in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Activation of Thymus Cells by NIP-MGG.—100 $\times$ 10^6 BALB/c thymus cells were injected intravenously on two successive days (200 $\times$ 10^6 total cells) into BALB/c recipients which had received 750 R ^{60}Co irradiation. 4–6 h after the second intravenous injection, each mouse received 400 μg of NIP-MGG (CFA) or CFA only intraperitoneally. 8 days later, the mice were sacrificed and the spleen cells cultured.

Treatment of Cell Cultures with Antisera.—Spleen or lymph node cells were suspended in RPMI-1640 medium without serum at 5 $\times$ 10^6 cells/ml. 0.8 ml (4 $\times$ 10^6 cells) was added to each culture tube together with 0.1 ml of dilutions of antisera or normal sera and 0.1 ml of fresh guinea pig serum (diluted 1:3) previously absorbed with agarose (13). After 45 min incubation at 37°C, 5 ml of cold Earle's balanced salt solution (BSS) was added to each tube, the tubes centrifuged for 5 min, and the supernatant discarded. The cell pellets were then gently resuspended in 1 ml of TCM and cultured for 3 days with and without antigen or phytohemagglutinin (PHA) (Difco).

In Vitro Blocking of Hapten-Specific Stimulation.—In blocking experiments, 4 $\times$ 10^6 spleen cells suspended in TCM were incubated for 3 h at 37°C with different concentrations of NIP-epsilon-aminocaproic acid (NIP-EACA) prepared according to the method of Brownstone et al. (10). At the end of 3 h, antigen or PHA was added, the NIP-EACA was not removed, and the cells were cultured for 3 days as described.

Preparation of Rabbit Anti-MGG-Coated Column.—The method used was essentially that of Golstein et al. (14). Briefly, polymetaacrylic plastic particles with an average diameter of 250 μm (Degalan V26, Degussa Wolfgang AG., Hanau am Main, W. Germany) were washed several times with sterile distilled water followed by two washes with sterile phosphate-buffered saline (PBS), pH 7.4. The beads were then mixed with ammonium sulfate-precipitated pooled MGG (2 mg/ml in PBS) and incubated at 45°C for 1 h. After overnight incubation at 4°C, the beads were poured into each of two 30-ml syringes and washed with 100 ml of sterile saline. Each syringe was then filled with either normal rabbit serum or rabbit anti-MGG serum diluted 1:5 in saline and incubated at 4°C for 2 h. Each column was then washed with 70 ml of Earle's BSS containing penicillin and streptomycin and 30 ml of RPMI-1640 medium with antibiotics. After this washing, 2–3 ml (400 $\times$ 10^6–600 $\times$ 10^6 cells) of the spleen cell suspension were added to each column and eluted with RPMI-1640 medium at a rate of 2–3 ml/min. Approximately 21% of the cells were recovered from the normal rabbit serum-coated column and 14% from the anti-MGG-coated column. The eluted cells were then washed once and cultured as described.

RESULTS

We have previously demonstrated that mice immunized with NIP-MGG (CFA) produce NIP-specific plaque-forming cells and the spleen cells from these mice respond to the antigen in vitro with increased DNA synthesis (8). To investigate the participation of T and B cells in the total in vitro response to the hapten-protein conjugate, spleen cells from immunized mice were pretreated in vitro in ways to selectively remove T or B cells. After treatment, the cells were washed and cultured for 3 days with or without antigen. The incorporation of [^{3}H]T into the DNA of the antigen-stimulated cells was used to measure the response. As a monitor for T cell function in the treated cell sus-

pensions, separate cultures were stimulated with PHA. Previous studies have shown that the lymphocytes which respond to PHA in vitro are thymus derived (15).

Inhibition of the In Vitro Response of Spleen Cells to NIP-MGG by Anti-θ, Anti-IgM, or Anti-MGG Sera.—Table I shows the results obtained from treating spleen cell cultures from immunized mice with three separate antisera

TABLE I

Inhibition of In Vitro NIP-MGG and PHA Responses of Primed Mouse Spleen Cells by Pretreatment with Anti-θ, Anti-IgM, or Polyvalent Anti-MGG Antiserum and Complement

Treatment of cells in vitro	Final serum dilution	Stimulant added			Percent inhibition of response*	
		None	NIP-MGG (500 μg/ml)	PHA	NIP-MGG	PHA
		cpm (range)	*cpm (range)*	*cpm (range)*		
NMS‡ + C§	(1:20)	851 (564-1,019)	8,151 (3,988-18,753)	18,894 (13,257-26,921)		
Anti-θ + C	(1:20)	578 (325-969)	2,228 (1,358-3,233)	3,686 (783-9,872)	77.4	82.8
NGS‖ + C	(1:40)	1,562 (1,070-2,439)	9,940 (5,269-17,477)	25,035 (9,997-47,190)		
Anti-IgM + C	(1:40)	772 (636-934)	5,263 (3,036-8,513)	27,265 (15,093-44,274)	46.4	0
NRS¶ + C	(1:40)	723 (521-1,038)	6,886 (2,544-13,299)	ND**		
Anti-MGG‡‡ + C	(1:40)	1,146 (1,038-1,231)	4,287 (1,946-7,280)	ND	49.0	

BALB/c mice were immunized with 400 μg NIP-MGG (CFA) i.p. 2-4 wk later, spleen cell suspensions were prepared, treated with different antisera plus complement (C), and cultured for 3 days with or without NIP-MGG or PHA. Results are expressed as the mean of four separate experiments together with experimental ranges.

* Calculated as described in Results.

‡ Normal AKR mouse serum.

§ Fresh guinea pig serum, final dilution 1:30.

‖ Normal goat serum.

¶ Normal rabbit serum.

** Not done.

‡‡ Polyvalent antiserum.

plus complement. The results are expressed as the mean of four separate experiments together with the experimental ranges. The percent inhibition of the response was calculated according to the following equation:

$$100 - \frac{[(\text{Antiserum-treated}) \text{ cpm stimulated} - \text{cpm unstimulated}]}{[(\text{Normal serum-treated}) \text{ cpm stimulated} - \text{cpm unstimulated}]} \times 100.$$

Treatment of the spleen cells with anti-θ complement resulted in a 77% inhibition of the NIP-MGG response. The same treatment of replicate cultures inhibited the PHA response by 83% showing that a very large portion of the T cells were killed by the antiserum.

The existence of immunoglobulin-like molecules on the surface of antibody-

forming cell precursors (B cells) is now a well established fact. Both IgG and IgM and the heavy chains of these molecules have been demonstrated on the surface of these lymphocytes (6, 16–19). To determine the participation of B cells in the in vitro response to NIP-MGG, spleen cells were pretreated with purified antimouse IgM antiserum plus complement (Table I). The anti-IgM treatment resulted in a 46% inhibition of the NIP-MGG response. Absorption of the antiserum with purified IgM completely removed its inhibitory activity. Of more significance was the finding that the same anti-IgM treatment had no effect on the PHA response of the spleen cells. We have interpreted these results as strong evidence that the anti-IgM was killing only B cells, some of which were responding to the NIP-MGG in vitro. Similar results were obtained by treating the spleen cells with polyvalent anti-MGG serum. This treatment reduced the NIP-MGG response by 49%. Although the cell type, i.e. B cell or T cell, that is being affected by the polyvalent anti-MGG cannot be determined from these results, the similarity between the inhibition produced by the anti-MGG and that produced by pretreatment with anti-IgM suggests that the effect is primarily at the B cell level.

Inhibition by Anti-θ or Anti-IgM Serum of the Response of Lymphocytes from Mice Skin Painted with FDNB.—Painting the skin of mice with highly reactive compounds such as FDNB has been shown to be an effective way of inducing delayed hypersensitivity, primarily a T cell-mediated immune reaction. Mice sensitized in this way form little if any antibody specific for DNP which shows it is a very poor method for inducing humoral antibody, i.e., activating B cells. We have tested, by hemagglutination (20), the sera from 12 skin-painted mice used in these studies for the presence of anti-DNP antibodies, but none was found. Based on these findings, FDNB sensitization provided us with an additional hapten-specific in vitro response, mediated primarily if not totally by T cells, on which we could test the specificity of the different antisera with respect to the cell populations that were being killed. Lymph nodes from skin-painted mice were removed and single-cell suspensions cultured as previously described. DNP-BSA was found to be the best stimulating antigen in vitro while BSA by itself did not stimulate the lymph node cells.

Table II shows the results obtained from treating the lymph node cells with anti-θ or anti-IgM serum plus complement. The results are presented as in Table I and represent the mean of three to four experiments.

Treatment of the lymph node cells with anti-θ inhibited the DNP-BSA response by 66%. These results agree favorably with those obtained by treating spleen cells from NIP-MGG-immunized mice with anti-θ serum. More important in this series of experiments was the finding that treatment of the lymph node cells with anti-IgM had no inhibitory effect on the DNP-BSA response. These results together with the PHA stimulation shown in Table I are even stronger evidence that the anti-IgM serum is not killing T cells and that the inhibition of the NIP-MGG response by anti-IgM treatment is due to elimination of B cells.

TABLE II

Effect of Pretreatment by Anti-θ or Anti-IgM Antiserum Plus Complement on the In Vitro DNP-BSA Response of Lymph Node Cells from Mice Sensitized to p-Flurodinitrobenzene

Treatment of cells in vitro	Stimulated added		Percent inhibition of response*
	None	DNP-BSA (100 μg/ml)	
	cpm (range)	*cpm (range)*	
NMS‡ + C§	380 (315–444)	1,087 (1,032–1,142)	
Anti-θ + C	241 (128–355)	480 (307–653)	66.2
NGS‖ + C	551 (368–879)	2,318 (1,060–3,957)	
Anti-IgM + C	551 (342–812)	2,484 (994–3,896)	0

BALB/c mice were skin-painted once a week for 4–5 wk. Within 7 days of the last painting lymph node cell suspensions were prepared, treated with different antisera plus complement, and cultured for 3 days with or without DNP-BSA. The results are expressed as the mean of three to four experiments together with experimental ranges.

* Calculated as described in Results.

‡ Normal AKR mouse serum.

§ Fresh guinea pig serum, final dilution 1:30.

‖ Normal goat serum.

Inhibition of the In Vitro NIP-MGG Response by Filtration of Immune Spleen Cells through a Column Coated with Anti-MGG Antiserum.—Golstein et al. have recently reported that passage of immune spleen cells through a column coated with anti-MGG antiserum results in the specific and almost complete retention of B cells on the column (14). T cells, on the other hand, pass through the column freely. As another means of investigating the participation of hapten-specific B cells in the in vitro NIP-MGG response, immune spleen cells were passed through anti-MGG-coated columns. After passage, the cells were washed and cultured with and without antigen or PHA. The filtered cells were also tested for NIP-specific plaque-forming cells (21) and rosette-forming cells (22) to determine the extent of B cell depletion.

The combined results of three separate experiments are shown in Table III. Passage of the spleen cells through the anti-MGG columns resulted in a 48% inhibition of the NIP-MGG response. These same cells had an average of 78% fewer NIP-specific plaque-forming cells and 86% fewer NIP-specific rosette-forming cells (data not shown), which demonstrates that the column filtration caused extensive B cell depletion. On the other hand, the ability of the filtered cells to respond to PHA was unimpaired, demonstrating that the column filtration had not selectively depleted the spleen cell suspension of T cells. These results give strong support to our previous conclusion that the inhibition of the

TABLE III

Immune Spleen Cell Responses to NIP-MGG and PHA after Passage of the Cells through Bead Columns Coated with Rabbit Polyvalent Anti-MGG Serum or Normal Rabbit Serum

Column coated with	Stimulant added			Percent inhibition of response*	
	None	NIP-MGG (500 μg/ml)	PHA	NIP-MGG	PHA
	cpm	*cpm*	*cpm*		
NRS‡	863 (353–1,505)	10,867 (4,788–17,105)	70,594 (49,345–86,266)		
Anti-MGG§	566 (460–752)	5,707 (3,958–7,492)	71,559 (61,767–78,952)	47.5	0

BALB/c mice were immunized with 400 μg NIP-MGG (CFA) i.p. 2-4 wk later, spleen cell suspensions were prepared and passed through bead columns coated with either polyvalent anti-MGG serum or NRS. The cells which passed through the column were cultured for 3 days with NIP-MGG or PHA. The results are expressed as the mean of three separate experiments together with experimental ranges.

* Calculated as described in Results.

‡ Normal rabbit serum.

§ Polyvalent antiserum.

NIP-MGG response after anti-IgM and anti-MGG treatment was the result of B cell inactivation.

Activation of Thymus Cells by NIP-MGG.—In a previous report, we demonstrated that nearly 50% of the in vitro response of primed spleen cells to NIP-MGG could be blocked by NIP-EACA and that the inhibition was hapten specific (8). In experimental systems utilizing haptens coupled to heterologous protein carriers where both the hapten-protein complex and the carrier alone can stimulate cells in vitro, such blocking would generally be thought to occur via B cells since B cells have hapten specificity whereas T cells have carrier specificity. However, with NIP-MGG, the carrier is an isologous protein and cannot stimulate the spleen cells by itself. Therefore, using NIP-MGG, it was of interest to determine what part the hapten was playing in stimulating T cells to proliferate in vitro. To achieve this with a "relatively pure" T cell population, thymus cells were activated by NIP-MGG (see Materials and Methods), and the effect of NIP-EACA on the antigen-induced proliferative response was determined. Representative results of such experiments are shown in Table IV.

Spleen cells from irradiated BALB/c mice receiving thymus cells and CFA only did not respond in vitro to either NIP-MGG or MGG (group A). However, the spleens from these mice did contain functional T cells as shown by their ability to respond to PHA. In contrast to this, spleen cells from mice receiving thymus cells and NIP-MGG (CFA) responded very well to NIP-MGG in vitro although they were unable to respond to MGG by itself (group B). The importance of the hapten for T cell stimulation was demonstrated by the blocking effect of NIP-EACA. The response to NIP-MGG was inhibited in a

TABLE IV

In Vitro Response of T Cells Activated by NIP-MGG; Requirement for and Response to the Hapten NIP

Experiment	Group	Thymus cell recipients injected with	Treatment in vitro	Stimulant added	cpm ± SE	% Inhibition*
			μg/ml	μg/ml		
1	A	CFA‡	None	None	1,308 ± 25	
		CFA	None	NIP-MGG (500)	1,187 ± 60	
		CFA	None	MGG (500)	1,161 ± 31	
		CFA	None	PHA	10,914 ± 389	
	B	NIP-MGG (CFA)	None	None	1,100 ± 247	
		NIP-MGG (CFA)	None	NIP-MGG (500)	5,327 ± 363	
		NIP-MGG (CFA)	None	MGG (500)	1,135 ± 303	
		NIP-MGG (CFA)	NIP-EACA (50)	NIP-MGG (500)	4,629 ± 1,019	16.5
		NIP-MGG (CFA)	NIP-EACA (100)	NIP-MGG (500)	3,579 ± 111	41.4
2		NIP-MGG (CFA)	None	None	754 ± 18	
		NIP-MGG (CFA)	NMS§ + C	NIP-MGG (500)	3,568 ± 253	
		NIP-MGG (CFA)	Anti-θ + C	NIP-MGG (500)	663 ± 118	100

Lethally irradiated BALB/c mice received 200 × 10^6 syngeneic thymus cells i.v. together with 400 μg NIP-MGG (CFA) or CFA only i.p. 8 days later spleen cell suspensions were prepared and cultured for 3 days with or without antigen or PHA. Blocking of the response was accomplished by incubating the cultures for 3 h with NIP-EACA before NIP-MGG was added for the remainder of the 3 days. Results are expressed as the mean of triplicate cultures ± standard error.

* Calculated as described in Results.

‡ Complete Freund's adjuvant.

§ Normal AKR mouse serum.

dose-dependent way by the NIP-EACA with a maximum inhibition of 41%. Finally, to insure that the response we were detecting was in fact T cell, activated spleen cells were treated with anti-θ serum plus complement. As shown in experiment 2, Table IV, such treatment completely abolished the response to NIP-MGG.

DISCUSSION

The present report investigates the participation of both T and B cells in the in vitro response of spleen cells from mice immunized with the hapten NIP coupled to a nonimmunogenic isologous gamma globulin carrier (MGG). Previously, the use of defined haptens coupled to heterologous carriers has yielded valuable information regarding the interaction of antigen and cells (3). The production of antihapten antibodies depends on cooperation between T and B cells. Furthermore, the in vivo experiments of Mitchison (4) together with the recent in vitro experiment of Cheers et al. (23) clearly demonstrate that in the cooperative system T cells recognize the foreign carrier and B cells recognize the haptenic group and manufacture antihapten antibodies.

The use of haptens coupled to nonimmunogenic isologous carriers for induction of specific immunity or tolerance is relatively new and consequently less is known about cell interactions and about how the molecule is recognized as foreign. The present results clearly demonstrate that both T and B cells are involved in the in vitro secondary response to NIP-MGG.

The participation of T cells was demonstrated in two ways. First, treatment with anti-θ serum resulted in a 77% inhibition of the secondary in vitro response to NIP-MGG. That this inhibition was due to removal of T cells was shown by the 83% inhibition by anti-θ of the PHA response of replicate cultures. Furthermore, the activation experiments clearly demonstrated that a relatively pure population of adoptively transferred and stimulated thymus cells could be specifically restimulated by NIP-MGG in vitro and that this response was sensitive to anti-θ treatment. However, the demonstration that removing T cells almost completely abrogates the response does not necessarily mean that only T cells are responding. In hapten-protein stimulation, as is the case with many other antigens, antigen-induced proliferation of B cells requires the helper effect of T cells; i.e., B cells cannot be made to proliferate by interaction with antigen alone. Thus, killing of antigen-specific T cells would not only eliminate the bulk of the T cell response, but would also indirectly by removal of helper cells, inhibit hapten-specific B cell proliferation. The inhibition of the NIP-MGG response by anti-θ treatment therefore shows that the total in vitro response depends on T cells, but it does not necessarily mean that all of the cells responding to the antigen are themselves T cells.

The participation of B cells in the secondary response was also investigated. There now exists substantial evidence to support the receptor hypothesis demonstrating the existence of immunoglobulin determinants on the surface of lymphocytes. Determinants of both μ and γ-type have been clearly demonstrated on the surface of B cells by immunoflurorescence, autoradiography, and cytotoxicity using anti-μ or anti-γ serum plus complement (16–19, 24). In this report, we tested the active participation of B cells in the secondary in vitro response to NIP-MGG by stimulating primed cells before and after procedures designed to remove B cells selectively. These procedures were (*a*) passage of spleen cells through a column coated with anti-MGG, a technique which has been previously shown to specifically remove B cells (14), and (*b*) treatment of primed spleen cells with anti-IgM or anti-MGG serum plus complement. Anti-MGG column treatment of NIP-MGG-primed spleen cells caused a significant reduction in the number of NIP-specific plaque-forming cells and NIP-specific rosette-forming cells, both of which are functions of B cells. Furthermore, the in vitro proliferative response to NIP-MGG of the passed cells was inhibited 47% while their ability to respond to PHA was not affected. Treatment of primed spleen cells with anti-IgM or anti-MGG serum plus complement also caused a significant inhibition of the in vitro NIP-MGG response (46 and 49% respectively), but had no effect on the PHA response of the same cells. These results, showing that procedures which selectively deplete populations of B cells entail a 40–50% reduction in the secondary response to NIP-MGG, clearly indicate that B cells contribute to the response.

In such experiments, it is crucial to establish the specificity of reagents designed to selectively eliminate one cell or another. That only B and not T cells

were eliminated by our anti-IgM antiserum was shown in two ways. First, PHA responses were not diminished after anti-IgM treatment. Secondly, anti-IgM treatment of lymph node cells from FDNB-sensitized mice had no effect on the ability of these cells to respond to DNP-BSA in vitro, a response which seems to be mediated totally by T cells. Some investigators, however, have reported the existence of μ-chains on "immune" T cells (25). Our results do not substantiate these findings. This discrepancy is characteristic of the current controversy about the nature of T cell receptors (26). With regard to this controversy, it should be noted that in our investigation of T cell participation, the only anti-T cell reagent we used was anti-θ serum; there seems to be little doubt regarding its specificity for T cells (27).

The results reported in this paper also have implications for tolerance experiments. The induction of hapten-specific tolerance using DNP coupled to an isologous carrier has been recently reported by Golan and Borel (28). In our first report (8), we also demonstrated that hapten-specific tolerance to NIP could be induced in mice challenged with NIP-MGG (CFA) by pretreatment with soluble NIP-MGG. With respect to current immunological views, this hapten-specific tolerance results from B cell inactivation. However, spleen cells from those tolerant mice were also unable to respond to NIP-MGG in vitro. The results presented in this paper clearly show that T cells respond to NIP-MGG in vitro. Therefore, it is clear that pretreatment of mice with soluble NIP-MGG results not only in B cell tolerance to NIP but also in T cell tolerance to NIP-MGG.

Finally, the results from the thymus cell activation experiments reported here deserve some special comment because, we believe, they suggest the existence of hapten-specific T cells. Mitchison (29), Iverson (30), and Taylor and Iverson (12) have all reported the existence of hapten-specific helper cells using FDNB-sensitized mice. However, the interpretation of these results as clearly showing helper cell activity mediated via the haptenic DNP group has recently become unclear by the demonstration of Janeway that a similar effect can be produced by transfusion of serum containing anti-DNP antibodies (31). In our system, thymus cells could be specifically activated by NIP-MGG and the cells responded very well in vitro to the hapten-carrier complex but not to the carrier alone. Furthermore, 41% of the response could be blocked by the addition of 100 μg NIP-EACA. The inability of the carrier (MGG) alone to stimulate the cells demonstrates the absolute necessity of the hapten for antigen recognition by the T cells. (In a similar system using NIP-ovalbumin [OA]-activated thymus cells, OA by itself stimulates the cells nearly as well as does NIP-OA, demonstrating that the hapten is not required for this in vitro response to a heterologous carrier.) We believe that with NIP-MGG the antigen recognition involves cell receptors directed in part to the hapten and in part to the carrier. Blocking of the response would therefore be the result of steric hindrance produced by NIP-EACA binding at this receptor site. However, the fact that

60% of the response is not blocked by NIP-EACA allows for an alternative explanation.

It is possible that the activation of T cells to NIP-MGG results in the expansion of two separate clones of cells. One clone clearly has specificity for NIP-MGG while a second clone may have specificity for NIP only. The receptors on the cells specific for NIP-MGG would be expected to have relatively higher affinity for the complex and lower affinity for NIP alone. The hapten-specific cells would have "receptors" with higher affinity for the hapten alone and lower affinity for the complex. Addition of NIP-MGG would result in stimulation of both cell clones. However, NIP-EACA would bind preferentially to the cells with NIP specificity, thus preventing them from responding to NIP-MGG.

Definitive results supporting this latter interpretation are not yet available. However, it seems clear that profound differences may exist with respect to T cell recognition of haptens depending primarily on whether the carrier is an isologous or heterologous protein. We believe that the use of haptens coupled to isologous carriers opens new areas and approaches for the study of both immunity and tolerance directed to a defined antigenic group.

SUMMARY

Both thymus-derived (T) and bone marrow-derived (B) lymphocytes participate in the response to a hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP), coupled to a nonimmunogenic isologous carrier, mouse gamma globulin (MGG). Spleen cells from mice immunized with NIP-MGG show increased DNA synthesis in vitro when cultured with NIP-MGG. The participation of and requirement for T cells in the response was demonstrated by treating the spleen cells with anti-θ serum. This treatment resulted in a 77% inhibition of the antigen response. Furthermore, adoptively transferred normal thymus cells could be specifically "activated" by NIP-MGG in vivo and they responded secondarily to the antigen in vitro.

The active participation of B cells in the secondary response was demonstrated by passing the immune spleen cells through a column coated with polyvalent anti-MGG serum. Column filtration reduced the number of NIP-specific plaque-forming cells and NIP-specific rosette-forming cells (both functions of B cells) and produced a 47% inhibition of the NIP-MGG response. The ability of the cells to respond to phytohemagglutinin (PHA) was not affected by column filtration showing that T cells were not being selectively removed. The participation of B cells in the in vitro NIP-MGG response was also shown by treatment of the spleen cells with antiserum specific for MGG and MGG determinants. B cells were removed by treatment with anti-IgM or polyvalent anti-MGG serum plus complement, resulting in a respective 46 and 49% inhibition of the response to NIP-MGG. (Treatment with anti-IgM serum had no effect on T cells.)

The contribution of the hapten NIP to stimulation of T cells was investi-

gated using NIP-MGG-activated thymus cells. These activated T cells responded in vitro very well to the NIP-MGG complex but not to the MGG carrier alone demonstrating the requirement of the hapten for T cell stimulation. The response was also partially inhibited (41%) by incubating the activated cells with NIP coupled to a single amino acid (epsilon-aminocaproic acid) before addition of NIP-MGG. These results demonstrated that T cells recognize the hapten NIP when it is coupled to the isologous carrier MGG.

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