IN VITRO TOLERANCE INDUCTION OF PRIMED, IgD-NEGATIVE MURINE SPLEEN CELLS*

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Induction of immunological unresponsiveness in lymphoid cells has been experimentally accomplished in a variety of ways both in vivo and in vitro (1–3). The ease of establishment of tolerance depends on several factors, including maturity and immunological status of the lymphoid cells. It is well established that neonatal lymphoid cells are more readily tolerized than are adult cells (1–7) and unprimed, virgin lymphoid cells are more susceptible to tolerance induction than primed cells (1, 8, 9). Nevertheless, under selected conditions, immunological tolerance using adult lymphoid cells, both primed (8, 9) and unprimed (4–7, 10–12), has been demonstrated. There is evidence that the restrictions to tolerizing adult spleen cells are related to the isotype of their surface immunoglobulin (Ig) receptors. The majority of adult, murine splenic B cells bear IgD as one of their surface Ig receptors, whereas neonatal splenic B cells predominantly bear IgM without IgD (13), which suggests that the presence of IgD interferes with tolerance induction. This is supported by the observation that removal or masking of surface IgD on adult spleen cells facilitates tolerance induction in vitro, with lesser amounts of tolerogen capable of inducing tolerance than would otherwise be required (14–16).

The difficulty in tolerizing primed spleen cells as compared with unprimed spleen cells has not been extensively investigated, but several studies have suggested that primed spleen cells can be made unresponsive. Mice primed with human gammaglobulin (HGG)[1] can be made tolerant to HGG by a series of injections of relatively large amounts of soluble HGG (8). On the other hand, HGG-primed B cells can be tolerized to HGG in vivo as effectively as nonprimed B cells when primed splenic T cells are removed before tolerance induction (9). Similarly, Katz et al. (17) have shown that hapten-primed B cells can be made unresponsive by exposure to haptenated conjugates that are unrelated to carriers used in priming, or by use of hapten-conjugated, nonimmunogenic carriers. Both conjugates avoid primed T cells. Thus, the problem of tolerizing primed spleen cells may be avoided if primed T cells are circumvented in some manner.

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[1] *Abbreviations used in this paper:* FCS, fetal calf serum; HGG, human gammaglobulin; KLH, keyhole limpet hemocyanin; OVA, ovalbumin; PBS, phosphate-buffered saline; PFC, plaque-forming cells; RBC, sheep erythrocytes; TGG, turkey gammaglobulin; TNP, trinitrophenyl.

The present report describes in vitro tolerance induction of spleen cells from mice primed with trinitrophenylated keyhole limpet hemocyanin (TNP-KLH), by methods similar to those used to tolerize nonprimed spleen cells in vitro. The data show that primed spleen cells can be made specifically unresponsive by addition of TNP conjugated to a carrier that is antigenically unrelated to the KLH used in priming, with unresponsiveness stable upon the removal of the conjugate by multiple washes, which indicates the induction of a tolerant state. Furthermore, examination of the surface isotype of the B cells susceptible to tolerance induction suggests that IgG^+, IgD^- B cells are the target cells for tolerance induction. The significance of a tolerance model using primed, potentially IgG-secreting spleen cells in the further examination of the role of IgD in tolerance induction is discussed.

Materials and Methods

Mice and Immunizations. A/J male mice from The Jackson Laboratory, Bar Harbor, Maine, were immunized intraperitoneally with 100–200 μg TNP-KLH in complete Freund's adjuvant at 9–10 wk of age (18).

TNP Conjugates. KLH was prepared as described and haptenated at 10 groups/100,000 mol wt (18). Turkey gammaglobulin (TGG) (19) and ovalbumin (OVA) (Miles Laboratories, Inc., Elhart, Ind.) were conjugated at a ratio of 10 and 20 TNP groups/molecule, respectively. The conjugates were sterilized by filtration (0.22 μm pore size) and stored at 4°C.

Generation and Assay of Antibody-producing Cells. Spleen cells from mice primed with TNP-KLH and boosted intraperitoneally with 20 μg soluble TNP-KLH were cultured 14–22 d later in microtiter plates in minimum essential medium containing 7.5% fetal calf serum (FCS), 2-mercaptoethanol (5×10^{-5} M), and nutrient and antibiotic additions (18). TNP-KLH was added at a final concentration of 0.005 μg/ml. Indirect (IgG) plaque-forming cells (PFC) to TNP were measured against TNP-coupled burro erythrocytes 4–5 d later (18). Indirect PFC were developed using a sheep anti-mouse Ig serum that prevented the development of IgM PFC by > 75%. Only indirect PFC were measured in this study because the IgM response was routinely 20% or less than that of the IgG response. Results are expressed as the mean with standard deviation (SD) of duplicate cultures; PFC were normalized per 10^7 input spleen cells.

Induction of Unresponsiveness. Unresponsiveness to TNP-KLH was induced in spleen cells from mice primed and boosted to TNP-KLH either by adding soluble TNP conjugates simultaneously with the addition of TNP-KLH to the spleen cell cultures, or by preincubating the spleen cells with the conjugates, followed by extensive washing before addition of TNP-KLH, carried out as follows. Spleen cells were cultured at 4×10^6/ml per culture well of a 24-hole plate (3008, Falcon Labware, Div. of Becton, Dickinson and Co., Oxnard, Calif.) for 1 h at 37°C, at which time TNP-TGG was added. After 20 h culture at 37°C, the cells were washed three times with phosphate-buffered saline (PBS) (18) containing 5% FCS. To minimize the transfer of TNP-TGG by macrophages and also to aid in recovery of cells from the plastic after 20 h incubation, the number of plastic-adherent cells was reduced by adsorbing 30×10^6 spleen cells at 37°C in 5 ml medium per 100-mm Diam culture dish (Falcon Labware). After 30 min, nonadherent cells were collected by vigorous pipetting. This process depleted nonspecific esterase-staining cells by ~50% and had no inhibitory effect on the response to TNP-KLH. The adherence procedure was not necessary for induction of unresponsiveness, but was used for the technical purposes outlined above.

Removal of Various Ig-bearing Cells. IgM-, IgD-, and IgG_1-bearing B cells were depleted by physical adherence to glutaraldehyde-fixed, tanned sheep erythrocytes (RBC) coupled with antisera specific for the respective heavy chain determinants and removal of RBC-lymphoid cell aggregates, or rosettes, on a Ficoll-Isopaque gradient (18). Preparation and specificity of the IgM reagent has been described (18). The anti-IgD rosetting reagent used was an immunoadsorbent purified rabbit anti-mouse IgD antiserum (13), which was generously supplied by Dr. Ellen Vitetta, University of Texas Southwestern Medical School, Dallas, Tex. Goat anti-mouse IgG_1 was supplied by Dr. Hans Spiegelberg, Scripps Clinic and Research Foundation, La Jolla, Calif. The antiserum was immunoadsorbent purified, with specificity

restricted to the heavy chain. The capacity of anti-IgM- and anti-IgD-coupled RBC reagents to deplete their respective B cell subpopulations was assessed using fluoresceinated anti-IgM and anti-IgD [$F(ab)_2$ preparations]. Approximately 50% of the spleen cells were IgM^+ and 55% were IgD^+ as assessed by fluorescence microscopy. After depletion with anti-IgM or anti-IgD coupled RBC, respective B cell subpopulations were reduced to < 1%. All Ig-bearing cells were depleted with RBC coupled with a multispecific goat anti-mouse Fab antiserum (18).

Depletion of Ly 2.2 Cells. Spleen cells at 50×10^6/ml were incubated at 4°C for 30 min with anti-Lyt 2.2 serum obtained from Dr. F. W. Shen, Memorial Sloan-Kettering Cancer Center, New York. The cells were then washed twice with PBS/5% FCS and incubated at 37°C for 30 min in minimum essential medium containing 0.1% sodium azide and rabbit:guinea pig serum (1:2) as a source of complement. The complement had been adsorbed previously on mouse tissues to avoid toxicity.

Results

Effect of the Presence of TNP-TGG on the Secondary In Vitro Antibody Response to TNP-KLH. Spleen cells from mice primed and boosted with TNP-KLH were cultured with TNP-KLH and various amounts of soluble TNP-TGG to determine the effect of TNP coupled to an unrelated carrier on the generation of PFC to TNP over a 4-d culture interval. The first experiment in Table I shows that TNP-TGG was inhibitory, with 0.1 μg/ml and 1 μg/ml causing 40 and 77% suppression of the PFC response, respectively. In addition, suppression was dependent upon the hapten, TNP, as shown by the failure of nonhaptenated TGG to be suppressive (experiment 2, Table I).

Effect of TNP-TGG on the Kinetic Response to TNP-KLH. Antibody-producing cells in the previous experiments were measured on day 4, a day previously determined as optimal for the IgG response to TNP-KLH in vitro. To determine whether the addition of TNP-TGG might be inducing a shift in antibody synthesis, the antibody response with and without TNP-TGG present was measured on various days of culture. Fig. 1 shows that the response to TNP-KLH without TNP-TGG rose sharply between days 3 and 4, peaked on days 4 and 5, and began to fall by day 6. Cultures containing TNP-TGG were markedly suppressed whether PFC were measured on day 4, 5, or 6. Thus, suppression by TNP-TGG was not due to a shift in antibody synthesis, but rather to a suppression of the generation of antibody-producing cells.

TABLE I

*Effect of TNP-TGG on the Secondary In Vitro Antibody Response to TNP-KLH**

Experiment	Addition	PFC ± SD	Percent suppression
	μg/ml		
1	None	27,346 ± 5,729	—
	TNP-TGG (0.01)	27,599 ± 716	0
	TNP-TGG (0.1)	16,548 ± 1,790	40
	TNP-TGG (1)	6,330 ± 3,581	77
2	None	107,760 ± 31,220	—
	TNP-TGG (1)	680 ± 509	100
	TGG (1)	112,000 ± 27,627	0

* Spleen cells from mice primed and boosted with TNP-KLH were incubated with TNP-KLH antigen and soluble TNP-TGG or TGG. After 4 d of culture, IgG PFC to TNP were measured. Background PFC were <500 in all cases.

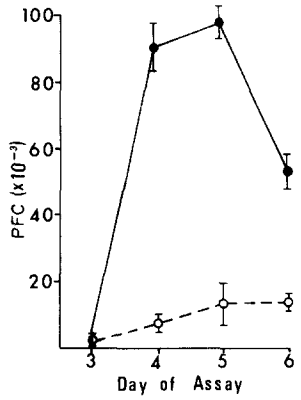

FIG. 1. Kinetics of the secondary response of TNP-KLH-primed and boosted spleen cells with and without TNP conjugate. Spleen cells with and without TNP-TGG (1 μg/ml) added simultaneously with TNP-KLH were cultured for various time periods and IgG PFC to TNP were measured. Background PFC were <140 on all d. ●, without TNP-TGG; ○, with TNP-TGG.

TABLE II

Determination of the Effect of Time of Addition of TNP Conjugate to TNP-KLH Primed and Boosted Spleen Cell Cultures *

Experiment	TNP Conjugate	Time of addition	PFC ± SD	Percent suppression
		hr		
1‡	−	—	23,940 ± 8,910	—
	+	0	6,854 ± 213	71
	+	20	26,059 ± 1,280	0
2§	−	—	12,148 ± 1,499	—
	+	0	<72	100
	+	1	968 ± 862	92
	+	4	12,900 ± 0	0

* Spleen cells were incubated with TNP-KLH antigen and TNP conjugates added either at 0 time or at various times thereafter at a concentration of 1 μg/ml. After 4 d of culture, IgG PFC to TNP were measured. Background PFC were <500 in all cases.

‡ TNP-TGG conjugate.

§ TNP-OVA conjugate.

Effect of the Time of Addition of TNP Conjugate. TNP conjugate was added either simultaneously with the addition of TNP-KLH or at various times thereafter to determine the phase of the antibody response inhibited by the conjugate. The first experiment in Table II shows that TNP-TGG added at culture initiation with TNP-KLH resulted in 71% suppression. However, if TNP-TGG was added 20 h later, the response was not suppressed. To determine more precisely when TNP conjugate had to be added to be suppressive, TNP conjugate was added at 0, 1, and 4 h after TNP-KLH. The second experiment in Table II shows that TNP-OVA added at 0 and 1 h was highly suppressive, whereas addition 4 h after TNP-KLH was not suppressive. TNP-OVA conjugate suppressed responses in a manner similar to TNP-TGG, although TNP-TGG was used in most experiments reported here.

Effect of Preincubation of Spleen Cells with TNP-TGG. The fact that suppression by

TNP conjugates could only be induced early in the antibody response suggested that inhibition entailed more than simple competition between TNP-KLH antigen and TNP conjugate for hapten-specific B cells, interfering with specific T-B cell cooperation. Rather, the data suggested that exposure of primed spleen cells early in the immune response to the hapten-conjugate resulted in their inactivation, and the presence of soluble TNP conjugate was no longer required for suppression. To determine whether this was the case, spleen cells were preincubated with TNP-TGG and then washed extensively before culture with TNP-KLH and compared with conjugate preincubated cells that had not been washed free of soluble conjugate before addition of TNP-KLH. Table III shows that culture of the primed spleen cells for 20 h with 0.1 μg/ml TNP-TGG before the addition of TNP-KLH suppressed the PFC response by 90% (group C), whereas preincubation with 10-fold lesser amount, 0.01 μg/ml, was not suppressive (group B). Preincubation with 0.1 μg/ml TNP-TGG, followed by extensive washing before reculture and addition of TNP-KLH, resulted in 83% suppression (group E), as effective as when TNP-TGG was continually present (group C). PFC were measured 5 d after TNP-KLH addition, rather than the usual 4 d, to give the suppressed cells more opportunity to respond. Because the continual presence of 0.01 μg/ml TNP-TGG was not suppressive (group B), it is unlikely that suppression by preincubation with 0.1 μg/ml followed by extensive washing was due to transfer of suppressible amounts of soluble TNP-TGG. Furthermore, the second experiment in the table shows that preincubation of spleen cells in the cold, followed by washing, did not result in suppression (group G), whereas the continual presence of TNP-TGG was suppressive (group H). Thus, failure to suppress by preincubating cells at 4°C followed by washing showed that the procedure itself was sufficient to remove soluble TNP-TGG and, moreover, suggested that the process of induction of

TABLE III

In Vitro Induction of Unresponsiveness to TNP-KLH by Preincubation of TNP-KLH Primed and Boosted Spleen Cells with TNP Conjugate

Experiment	Group	Treatment of spleen cells before addition of TNP-KLH	PFC ± SD*	Percent suppression
1	A	Culture 20 h	38,760 ± 1,075	—
	B	Culture 20 h with TNP-TGG‡	40,800 ± 5,280	0
	C	Culture 20 h with TNP-TGG§	3,927 ± 1,612	90
	D	Culture 20 h, wash, reculture	16,467 ± 1,791	—
	E	Culture 20 h with TNP-TGG§, wash, reculture	2,755 ± 134	83‖
2	F	None	26,592 ± 5,363	—
	G	4°C, 60 min with TNP-TGG¶, wash	31,667 ± 1,791	0
	H	None, TNP-TGG§ present throughout culture	2,470 ± 986	91**

* IgG PFC to TNP were measured 5 d after addition of TNP-KLH.
‡ 0.01 μg/ml.
§ 0.1 μg/ml.
‖ Suppression was calculated relative to group D.
¶ 1 μg/ml.
** Suppression was calculated relative to group F.

unresponsiveness with TNP-TGG involved a relatively rapid, energy-dependent, direct inactivation of the primed spleen cells, which prevented their subsequent response to TNP-KLH. Thus, tolerance, for at least a short period, appeared to be induced by preincubation with the conjugate at 37°C.

Determination of Whether Suppressor Cells are Involved in Tolerance Induction. Spleen cells from mice primed and boosted with TNP-KLH were depleted of suppressor T cells with anti-Ly 2.2 serum and complement before culture to determine their possible role in tolerance induction. Table IV shows that depletion of the suppressor T cell population did not abrogate the capacity of TNP-TGG to inhibit the response to TNP-KLH.

An alternative procedure used was the mixing of tolerized and nontolerized spleen cells to determine whether the tolerized cells inhibited the antibody response of the latter. Spleen cells from primed and boosted mice were tolerized by preincubation with TNP-TGG for 20 h, as described previously in Table III, washed, and then added to primed and boosted spleen cells that had not been incubated with TNP-TGG. Table V shows that preincubation of the spleen cells with TNP-TGG tolerized the spleen cells (i.e., reduced the response to TNP-KLH from 48,703 to 11,702 PFC), but mixing tolerized and untreated spleen cells did not suppress the capacity of the latter to respond to TNP-KLH. Thus, tolerization probably results from a direct inactivation of specific B cells occurring within a 20-h interval of incubation with tolerogen, with suppressor cells not involved in tolerance induction.

TABLE IV

*Failure of Removal of Suppressor T Cells to Abrogate Suppression with TNP-TGG Conjugate**

Treatment	TNP-TGG	PFC ± SD	Percent suppression
None	−	118,613 ± 1,478	—
None	+	14,820 ± 1,347	88
Anti-Ly 2.2 + C	−	119,040 ± 7,680	—
Anti-Ly 2.2 + C	+	16,000 ± 3,730	87

* Spleen cells from mice primed and boosted with TNP-KLH were depleted of suppressor T cells by treatment with anti-Ly 2.2 + C before culture. TNP-TGG (0.1 μg/ml) was added at culture initiation with TNP-KLH. IgG PFC to TNP were measured after 4 d.

TABLE V

*Effect of Tolerized Spleen Cells on the Response of Nontolerized Spleen Cells**

Spleen cell preparation	Cell number/culture	PFC ± SD
	$\times 10^{-5}$	
Untreated	5	48,703 ± 894
Tolerized	5	11,702 ± 1,341
Untreated + tolerized	2.5 + 2.5	41,829 ± 3,444

* Spleen cells from mice primed and boosted with TNP-KLH were cultured for 20 h with and without TNP-TGG at 0.3 μg/ml, washed, and then recultured with TNP-KLH separately or mixed together. IgG PFC to TNP were measured 4 d later. The theoretical PFC value if no suppression was exhibited is 30,202 (i.e., half the PFC response of the untreated cells alone + half the PFC response of the tolerized cells alone).

Assessment of the Role of IgD-bearing Cells in the Secondary Response to TNP-KLH. Because the surface isotype of spleen cells has been implicated in tolerance induction, and the presence of surface IgD makes cells more difficult to tolerize, it was investigated whether the relative ease in tolerizing primed spleen cells observed here correlated with a lack of surface IgD on the TNP-primed, specific splenic B cells. As stated previously (see Materials and Methods), the antibody response to TNP-KLH that is generated by spleen cells from mice primed and boosted with TNP-KLH is predominantly an amplified, indirect antibody response with a relatively insignificant IgM component. The latter is >75% suppressed by the amplifier antiserum used in the plaquing procedure. Thus, to determine whether the precursors of this predominantly IgG response to TNP bore surface IgD, the spleen cells were depleted of surface IgD^+ cells and the subsequent antibody response was measured. Spleen cells were depleted of IgD-bearing cells by physical adherence to RBC coated with heavy chain specific rabbit anti-mouse IgD serum and removing the lymphoid-RBC conjugates or rosettes on a density gradient. The depleted cells were then cultured with TNP-KLH, and antibody generation was measured 4 d later. Table VI shows that depletion of 99% of the IgD^+ spleen cells, as assessed by fluorescence microscopy before and after depletion (see Materials and Methods), did not reduce the response to TNP. This failure to deplete potential antibody-producing cells occurs in spite of the fact that the majority of the splenic B cells were removed (>90% as defined by elimination of bacterial endotoxin-induced mitogenesis and the large number (~60%) of B cells forming rosettes with the reagent).

TABLE VI

*Role of Cells Bearing IgD Receptors in the Secondary In Vitro Antibody Response to TNP-KLH**

Experiment	Treatment before culture	PFC ± SD
1	None	100,040 ± 14,086
	Depleted of IgD^+ cells‡	166,400 ± 18,102
2	None	59,533 ± 12,538
	Depleted of IgM^+ cells§	88,667 ± 14,329
3	None	62,574 ± 2,508
	Depleted of IgM^+ and IgD^+ cells	118,623 ± 4,049
4	None	41,769 ± 1,755
	Depleted of Ig^+ cells	4,491 ± 2,773
5	None	17,010 ± 2,673
	Depleted of IgG_1^+ cells	2,121 ± 964

* Primed cells were depleted of spleen cells bearing various surface Ig isotypes by physical adherence to RBC coupled with specific antisera and removing the lymphoid cells with adherent RBC on a Ficoll-Isopaque gradient. When both anti-IgM and anti-IgD depletions were done (experiment 3), the IgM^+ cells were depleted first, followed by depletion of IgD^+ cells. The IgG PFC response to TNP was measured after 4 d of culture with TNP-KLH.

‡ This procedure reduces the proliferative response to bacterial endotoxin by >90%. Also, ~60% of the spleen cells form rosettes with the anti-IgD coupled RBC and IgD-bearing cells are reduced 99% as assessed by immunofluorescence.

§ This procedure eliminates the secondary IgM response to TNP by >99% (18). Also, ~55% of the spleen cells form rosettes with the anti-IgM coupled RBC, and IgM-bearing cells are reduced 99% as assessed by immunofluorescence.

To determine the isotype of the precursor cells of the antibody response, depletion experiments were done with other class-specific antisera. Experiments 2 and 3 in Table VI show that removal of IgM^+ cells, or both IgD^+ and IgM^+ cells, did not reduce the response to TNP. Depletion of IgM-bearing cells has been shown previously to prevent total generation of the secondary IgM response to TNP by these primed and boosted spleen cells (18). Depletion of IgM^+ or IgD^+ cells or both resulted in increased PFC responses, which probably reflects enrichment of TNP-specific B cells, as would be expected upon depletion of the bulk of splenic B cells by the rosetting procedure. Although the antibody response was not derived from IgD or IgM precursor cells, it was derived from Ig-bearing cells, as shown by depletion with a multispecific anti-mouse Ig reagent that reduced the subsequent PFC response from 41,769 to 4,491 (experiment 4, Table VI). Furthermore, the Ig-bearing cells were predominantly of the IgG class, as shown by the capacity of anti-IgG_1 subclass-specific antisera-coupled RBC to deplete subsequent antibody-producing cells by 88% (experiment 5, Table VI).

It should be noted that the variation in the magnitude of the antibody response to TNP-KLH which can be observed in the five experiments shown in Table VI and throughtout this study is typical of the response of spleen cells from mice primed and boosted with TNP-KLH. The reasons for this variation are not known, but mouse to mouse variation, the length of time after priming, and/or less than optimal in vitro eliciting antigen concentration may be contributing factors. To investigate the last possibility, the effect of depleting IgD^+ or IgM^+ cells was measured using spleen cells cultured with a range of concentrations of TNP-KLH to ensure that IgD^+ or IgM^+ cells were not involved at essentially all the eliciting antigen concentrations. It was found that at no concentration of TNP-KLH, suboptimal or supraoptimal, did depletion of IgD^+ or IgM^+ cells reduce the antibody response (data not shown).

The surface Ig isotype of the spleen cells involved in the antibody response to TNP-KLH was also assessed by blocking with isotype-specific antisera added directly to the spleen cell cultures. The antisera used were the same immunoadsorbent purified antisera to IgM, IgD, and IgG that were used in the depletion experiments described in Table VI. Table VII shows that only anti-IgG_1 blocked the antibody response, and anti-IgM and anti-IgD had no inhibitory effect. Thus, both the negative selection of isotype-specific B cells (Table VI) and the blocking with specific antisera (Table VII)

TABLE VII

Inhibition of the Indirect, Secondary Antibody Response to TNP-KLH with Anti-IgG, but Not Anti-IgD or Anti-IgM

Antiserum added at culture initiation*	Percent inhibition of indirect PFC
Anti-IgD	0
Anti-IgM	0
Anti-IgG_1	90

* Immunoadsorbent purified antisera to IgD, IgM, and IgG_1 were added at 10 μg/ml to cultures of spleen cells from mice primed and boosted with TNP-KLH. After 4 d, the indirect antibody response to TNP was measured. Inhibition of the response by antisera was measured relative to the control PFC response of 15,624 ± 713 PFC.

indicate that the B cells that are responsible for the antibody response and are thus susceptible to tolerance induction are IgD^-, IgM^-, and IgG^+ B cells.

Discussion

The above results demonstrate that primed spleen cells can be made unresponsive in vitro by using a tolerization protocol that involves preincubation with tolerogen followed by extensive washing and is similar to protocols used to tolerize nonprimed spleen cells (4–7). Tolerance was probably facilitated by the circumvention of primed T cells by using, as tolerogen, a haptenated-carrier complex not used in priming. Other research has shown that tolerance induction in nonprimed B cells cannot be established if T cells primed to the tolerogen are present (3, 4, 9). Excessive amounts of tolerogen were not required, 0.1 μg TNP-TGG/ml being adequate, which is significantly less than that used in most nonprimed tolerance models with adult spleen cells (4–7). The small amount of tolerogen required for unresponsiveness may be due to the fact that primed B cells were used instead of nonprimed cells. Primed cells usually have higher avidity receptors for antigen and lesser amounts of surface Ig (20), factors that may make it easier to saturate their surface receptors with tolerogen to initiate the tolerance process. It is well established that tolerance is more easily achieved with high than low avidity antibody precursor cells (21). Furthermore, the surface isotype of the B cells being tolerized was shown to be IgG only, without detectable IgM. Because IgM often has much lower avidity for antigen than IgG, a cell bearing only surface IgG could be expected on the whole to have a higher avidity for antigen than cells bearing IgM. Thus, the lack of surface IgM on these primed B cells could aid tolerization.

In addition to the possible high avidity of the B cells for tolerogen, the ease of tolerance induction may also be directed by the fact that the B lymphocytes being tolerized did not bear detectable surface IgD, a surface isotype shown to interfere with tolerance induction. Neonatal spleen cells, for example, which for the most part lack IgD receptors (13), or adult spleen cells stripped of surface IgD (14–16), are tolerized by amounts of highly haptenated TNP-conjugates in the 0.1–1 μg/ml concentration range, similar to the concentration used here to tolerize adult primed B cells. However, others (22) have reported that adult cells in general are less tolerizable than neonatal spleen cells whether they bear IgD or not.

Some of the controversy over the role of IgD in B cell tolerance may be a consequence of subtle differences in tolerance protocols, or perhaps, of different methods of defining IgD-positive or -negative B cells. The method used here, depletion of IgD-bearing B cells by rosetting with anti-IgD-coated RBC, appeared to be highly sensitive, because > 60% of the spleen cells were removed, and mitogenesis by the B cell activator, endotoxin, was essentially eliminated, which indicates the depletion of the majority of the splenic B cells. The reagent may be very sensitive in the detection of IgD-bearing cells, because the antiserum to mouse IgD was elicited in a heterologous species (13). Such antiserum may recognize a greater number of antigenic determinants and be of higher titer than that generated within appropriate strains of mice (23).

An advantage of the present model of tolerance induction using primed spleen cells is that the target cell subpopulation is an IgD-negative, mature B cell. It has been difficult to study the role of IgD in tolerization of unprimed, mature, adult B cells,

because the response to antigen is due predominantly to IgD-bearing cells (13, 22). To study the tolerizability of IgD^- B cells, the small number of IgD^- B cells have to be enriched with the cell sorter (22) or IgD^+ cells must be stripped of IgD (14–16). In contrast, the entire response of the primed and boosted spleen cells examined in our study was derived from IgD^- B cells. This is not surprising in view of what is known about the maturation sequence of B cells defined in terms of their surface Ig receptors. In general, neonatal cells bear only IgM, whereas adult cells bear both IgM and IgD, or IgD only (13). After priming, the cells retain IgD, but lose IgM and gain IgG receptors. As the cells mature in response to antigen, IgD is eventually lost, with the remaining cells bearing only IgG (20). We anticipate future studies using primed spleen cells obtained at shorter intervals after priming than in the present work, to follow tolerizability of primed B cells as they mature from IgD positive to IgD negative.

The type of unresponsiveness or tolerance being studied here is presumed to be a form of receptor binding leading either to deletion or abortion of specific B cells. Active suppression by T cells appears not to be involved, as removal of suppressor T cells had no effect on unresponsiveness elicited by the continuous presence of TNP-TGG, and spleen cells that were tolerized by preincubation with TNP-TGG did not suppress nontolerized cells when mixed together. Evidence suggests that continuous incubation with TNP conjugate induced tolerance similar to that induced by the preincubation, tolerance protocol. The stringent time requirement for addition of conjugate relative to TNP-KLH to elicit suppression, i.e., within 4 h, strongly suggests that the conjugate did not compete passively with TNP-KLH for hapten-reactive B cells, interferring with T-B cell collaboration over the course of the antibody response. Competition by TNP conjugate presumably would have disrupted T-B cell cooperation at times later in the 4–5-d antibody response than the first 4 h. Thus, TNP conjugate appears to have inactivated B cells before their triggering by antigen whether the primed spleen cells were cultured continually with conjugate or were preincubated with conjugate and washed. Moreover, inactivation was effected through an active process, because preincubation with TNP-TGG at 4°C was not inhibitory, with 37°C required to induce tolerance. Thus, as a working hypothesis it is postulated that tolerance of primed B cells with TNP conjugate involves saturation of surface IgG receptors, followed by an energy-dependent inactivation of the B cell. Whether the B cell is deleted or aborted, through an inability to accept T cell help, for example, is open to investigation.

In contrast to the present study, others (24) have shown suppression of TNP-KLH-primed and boosted spleen cells with TNP-TGG when the conjugate was present continually, but not when the spleen cells were preincubated and washed free of TNP-TGG. However, the amount of TNP-TGG used relative to the eliciting antigen concentration was 100 times that in our study, so that a passive competition between conjugate and antigen might have occurred. Furthermore, the failure to elicit tolerance by preincubation may have been because the particular spleen cells used were relatively insusceptible to tolerization. The primed spleen cells were from animals boosted 2–3 mo earlier, in contrast to the 2–3 w in the present study, so that the IgG response was significantly less than reported here and had a large IgM component.

Finally, if the lack of surface IgD on the responding B cells examined here did facilitate tolerance induction, one might ask why secondary, IgG-bearing B cells

should lose or not bear surface IgD, making them vulnerable to tolerization to potentially harmful antigens. Perhaps the explanation is that the IgG response, extremely dependent on primed T cells, is safeguarded against tolerance induction by primed T cells; it is known that it is difficult to tolerize B cells with primed T cells present (3, 4, 8, 9). Thus, only in situations where primed T cells are absent, as in unprimed animals, would the presence of IgD on B cells be necessary to avoid unwanted tolerance induction. The use of haptenated-conjugates unrelated to the carrier used in priming to circumvent primed T cells, as done in this study, is an experimental stituation presumably not encountered routinely in nature. However, use of primed B cells and such conjugates may be a convenient and useful means to further probe how B cells are activated or inactivated by antigen.

Summary

The above observations demonstrated induction of immunological tolerance in vitro in primed IgD^-, IgG^+ B cells. In these studies, addition of trinitrophenylated (TNP) turkey gammaglobulin (TGG) or TNP ovalbumin conjugates suppressed the secondary in vitro response in mice primed with TNP keyhole limpet hemocyanin (TNP-KLH). Suppression was not a reflection of a shift in kinetics of the antibody response, was not dependent on suppressor T cells, and could only be elicited when conjugate was added within 4 h of addition of TNP-KLH. Moreover, preincubation of the primed spleen cells with TNP-TGG for 20 h at 37°C, followed by extensive washing, was as effective in inhibiting the response to TNP-KLH as when TNP-TGG was present throughout the 5 d of culture, reflecting induction of a tolerant state. Amounts of conjugate in the concentration range that have been shown by others to tolerize immature or neonatal B cells or mature B cells that have been stripped of surface IgD were sufficient to induce tolerance. The target cells being tolerized did not bear IgD, as determined by B cell depletion and blocking procedures with anti-IgD. Whether the lack of surface IgD on the primed cells contributed to the relative ease of tolerance induction was not established by these studies, but the advantages of using primed B cells to examine further the role of surface IgD in tolerance susceptibility was discussed.

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