ROLE OF IgD IN THE IMMUNE RESPONSE AND TOLERANCE

I. Anti-δ Pretreatment Facilitates Tolerance Induction in Adult B Cells In Vitro*

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Adult murine B cells, in contrast to neonatal B cells, are relatively resistant to in vitro tolerance induction (1–6). It has been suggested that the maturation of B cells from bearing only IgM receptors (μ-only cells) to those expressing both IgM and IgD receptors (μ + δ-cells) coincides with the development of resistance to in vitro tolerance induction (6–8). Early studies, however, suggested that the loss of tolerance susceptibility in the neonatal period occurred before IgD was detectable on most B cells (1). More recently, this question has been reexamined. Cambier et al. (8) reported that enzymatic digestion of IgD, for example, from the surface of murine B cells facilitated tolerance induction in adult T-dependent B cells. A more direct way of determining the role of IgD is by attempting to induce tolerance in vitro by pretreatment of adult B cells with an anti-δ serum before culture with tolerogen. In this paper, we report the results of the latter and other approaches. Our results suggest that μ + δ-cells do become susceptible to tolerance induction if the δ-receptor is blocked or modulated, and that mice suppressed for the δ-allotype remained susceptible to tolerance in vitro into adult life. We also suggest that adult μ-only cells are normally rendered tolerant in vitro but their unresponsiveness can be masked by the response of nontolerant μ + δ-cells.

Materials and Methods

Mice. C57BL/6 and its allotype-congenic partner strain (9), C57BL/6.Ig^e N14F4 (abbreviated "Ig^e"), were bred and maintained at the Walter and Eliza Hall Institute. Mice of either sex were used at various ages as described. Within an experiment, mice were of the same sex.

Antisera. "Anti-δ" serum was made in C57BL/6 mice against CBA/CaH WEHI spleen cells as described by Goding et al. (9). The antisera used contained antibodies against *H-2*^{k} and *Ia*^{k} antigens as well as to the IgD-like allotypic specificity (Ig 5-1). When tested against the C57BL/6.Ig^e spleen cells, only antibodies to the IgD allotype are detected (9). This serum is therefore

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referred to as "anti-δ." Anti-μ was a rabbit antiserum (no. 647) against mouse μ-chains, which had been previously absorbed with mouse IgG-Sepharose 4B and mouse thymocytes. The latter serum was the generous gift of Dr. James Goding, of this Institute.

Antigens. Human gamma globulin (HGG)[1] (Cohn fraction II, Commonwealth Serum Laboratories, Melbourne, Victoria, Australia) was conjugated with fluorescein isothiocyanate (BBL, Baltimore, Md.) at a hapten: protein ratio of 1:50 in pH 9.2 carbonate-bicarbonate buffer (10, 11). After 2 h at room temperature, the conjugated protein was separated from unreacted hapten by passage over a Biogel P-6 column (Bio-Rad Laboratories, Richmond, Calif.) and subsequent dialysis. The conjugates used had a final fluorescein: protein molar ratio of 6 (FLU_6HGG). For use as tolerogen, FLU_6HGG was deaggregated at 100,000 *g* for 60 min, passed through a 0.45 μm Millipore filter (Millipore Corp., Bedford, Mass.), and stored at 4°C for use within 2 wk. As immunogen, *Salmonella adelaide* polymerized flagellin SW1338 (POL) was similarly conjugated with FLU before dialysis and centrifugation at 26,000 rpm in a Spinco 50.1 rotor to pellet the FLU-POL. This antigen contained an average of 1 FLU group/38,000 mol wt monomeric flagellin subunit.

Basic Protocol

FIRST STAGE. Spleen cells from C57BL/6 and C57BL.Ig^e mice were cultured at 37°C in 5% CO_2 in microculture medium (MCM; reference 12) at a concentration of 5×10^6/2 ml MCM in Linbro FB-TC-24 trays (Linbro Chemical Co., New Haven, Conn.). The cultures included FLU_6HGG at 5 μg/ml (or no antigen) with or without 100 μl of a 1:10 dilution of anti-δ serum (or anti-μ; see Results). When antiserum was included, it was added 30 min before the addition of antigen.

SECOND STAGE. After 36–72 h the cells from each well were harvested and washed twice through undiluted fetal calf serum. After washing, the spleen cells were subsequently diluted in thymus "fillers" (12) and dispensed in 0.2-ml aliquots in Linbro IS-FB-96-TC culture trays. To eliminate the feedback effects which can occur when more than five antibody-forming cell precursors are cultured in the same well (12, 13), each well usually contained 5×10^4 cultured spleen cells, plus $1–2 \times 10^6$ thymocytes and 20 ng FLU-POL. Thymocytes from syngeneic C57BL/6 mice, Wistar, and DA rats were used with equal success as fillers (B. Pike and F. Melchers, personal communication). After 3 days at 37°C, each well was assayed vs. FLU-sheep erythrocytes (SRBC), sensitized with FLU-F(ab')$_2$ anti-SRBC (12), in Cunningham chambers (14). Routinely, 12–24 replicates were assayed.

Limit Dilution Microculture Analysis. In some experiments, cells were cultured at limit dilution as described by Pike and Nossal (12) and Stocker (13). Poisson statistical analysis of the number of responding and nonresponding wells was calculated as described by Lefkovits (15) applied to our culture system.

Anti-δ-Suppressed Mice. C57BL.Ig^e mice treated from birth with anti-δ serum ("suppressed" mice) or normal adult C57BL/6 serum (control mice) were prepared as described in detail by Layton et al.[2] These mice had virtually no detectable IgD$^+$ cells in spleen or lymph nodes at the time of sacrifice.

Results

Age Restrictions on Clonal Abortion. Previous studies in our laboratory (3) had established that neonatal, but not adult, spleen cells were susceptible to in vitro tolerance induction (i.e. "clonal abortion"; reference 4). Initially, we sought to reproduce this system using the FLU-hapten and C57BL/6.Ig^e mice to allow studies with anti-δ allotype serum (9) and to determine when neonatal cells "matured" to adultlike resistance to clonal abortion. Briefly, we found that Ig^e spleen cells from mice less than 7 days of age were consistently

[1] *Abbreviations used in this paper*: DNP, 2-4-dinitrophenyl; FLU, fluorescein; HGG, human gamma globulin; MCM, microculture medium; Pc, phosphorylcholine; PFC, plaque-forming cell; POL, polymerized flagellin; SRBC, sheep erythrocytes; TNP, trinitrophenyl.

[2] Layton, J. E., J. M. Teale, G. J. Johnson, D. W. Scott, and G. J. V. Nossal. 1977. The anti-δ-suppressed mouse. Manuscript in preparation.

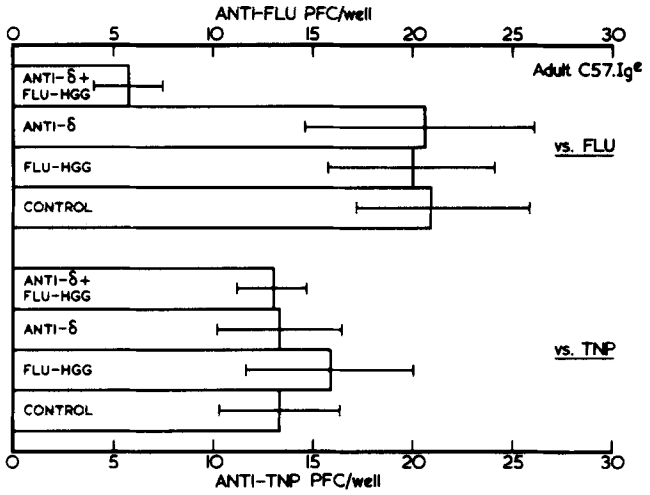

FIG. 1. Effect of anti-δ on tolerance induction. Spleen cells, from 4-mo-old C57.Ig^e mice, were cultured as described with no antigen, 5 μg/ml FLU_6HGG, anti-δ, or both FLU-HGG and anti-δ. After 36 h, the cells from each well were harvested, washed, and set up in secondary cultures at 4×10^4 cells with 1.5×10^6 C57BL/6 thymocytes per well (0.2 ml). All cultures were challenged with 100 ng/ml FLU-POL and TNP-POL as a specificity control. Only cultures exposed to FLU-HGG in the presence of anti-δ show tolerance. All groups responded equally to TNP.

rendered hyporesponsive in vitro during a 48- to 72-h incubation with 5 μg/ml FLU_6HGG (2×10^{-7} M hapten). Spleen cells from mice 2 wk of age or older, however, were generally resistant to this in vitro tolerance process (D. W. Scott, unpublished results).

Facilitating Effect of Anti-δ on Tolerance Induction. To determine whether the δ-receptor plays an important role in preventing tolerance induction in adult spleen cells, cells from 4-mo-old Ig^e mice were cultured with combinations of anti-δ and FLU-HGG as described in Materials and Methods. After 36–48 h, cells from each group were washed and then challenged with FLU-POL. When adult Ig^e spleen cells were *pre*cultured with 5 μg/ml FLU-HGG, or with anti-δ serum, no effect on subsequent responsiveness to FLU-POL was observed (Fig. 1). However, if both FLU-HGG and anti-δ were continually present during the first culture stage, then "hyporesponsiveness" of adult Ig^e spleen cells was induced (top bar). The effect is hapten-specific because a normal response to trinitrophenyl (TNP)-POL was observed (Fig. 1, bottom panel).

Strain Dependence. C57BL.Ig^e mice express the surface IgD allotype detected by our anti-δ serum; C57BL/6 mice do not (9, 16, 17). As shown in Fig. 2, if spleen cells from C57BL/6 mice are treated with FLU-HGG plus anti-δ, there is no effect (bottom panel); as in the experiment in Fig. 1, Ig^e spleen cells are rendered hyporesponsive by this protocol (top panel).

Lack of Effect of Anti-μ on Tolerance Induction. To insure that the facilitation of tolerance induction was a specific effect due to interaction with the δ-receptor, the effect of anti-μ was next tested. If anti-μ serum is substituted for anti-δ, no facilitation of tolerance induction is observed (Fig. 2, top bars) with Ig^e spleen cells. Similarly, anti-μ plus FLU-HGG pretreatment has no

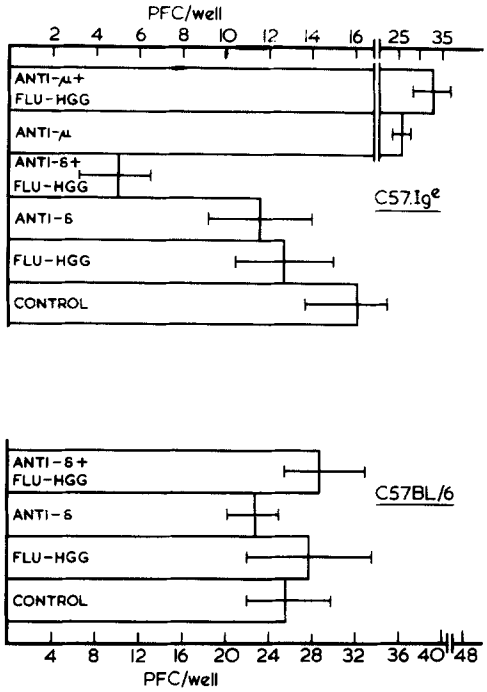

FIG. 2. Effect of anti-μ vs. anti-δ on tolerance induction in C57.Ig^e and C57BL/6 mice. Protocol as in Fig. 1 except that 1.4×10^6 Lewis rat thymus cells were used as fillers with cultured cells at 5×10^4/well.

adverse effects on C57BL/6 spleen cells (not shown in Fig. 2 for simplicity). In fact, a slight augmentation of responsiveness by anti-μ + FLU-HGG was observed in both strains.

Effect of Brief Pretreatment with Anti-δ on Tolerance Induction. We next examined whether anti-δ serum needs to be present throughout the first stage for tolerance to ensue. Spleen cells from 3-wk-old Ig^e mice were treated for 30 min at 0°C with anti-δ serum, then washed and cultured for 48 h with or without the tolerogen, FLU-HGG. The cells in each group were then washed and cultured at 10×10^4 well with rat thymus fillers. The data in Table I demonstrate that brief treatment with anti-δ was sufficient to render "resistant" cells susceptible to in vitro tolerance induction with FLU-HGG.

Ease of Tolerance Induction with Spleen Cells from δ-Suppressed Mice. Recently, Layton et al. (see above) have been able to suppress the expression of IgD-bearing lymphocytes in the lymphoid tissues of C57BL.Ig^e mice by treatment of these mice from birth with anti-δ allotype serum. These mice have virtually no detectable IgD-bearing cells by immunofluorescence, are slightly defective in B cell colony formation in agar, and have reduced numbers of precursors for the haptens FLU and 4-hydroxy-3-iodo-5-nitrophenyl (NIP) (see footnote 2 and below). Based on the results we have presented thus far, one might predict that spleen cells from these δ-suppressed mice would be highly susceptible to in vitro tolerance induction. As shown in Fig. 3 and Table

TABLE I
*Facilitation of Tolerance Induction by Pretreatment with Anti-δ Serum**

Group	Anti-δ	FLU-HGG	PFC/well
1	+	+	10.5 ± 3.5
2	+	−	43.8 ± 6.9
3	−	+	30.8 ± 6.8
4	−	−	30.3 ± 6.3

* Spleen cells from 18-day-old C57BL.Ig^e mice were incubated ± anti-δ for 30 min at 0°C, washed, and cultured 48 h at 37°C in MCM ± 5 μg/ml FLU_6HGG in Linbro trays. Cells from each group were then washed and diluted to 10^5/well in thymus fillers + FLU-POL and cultured for a final 3 days at 37°C. Mean plaque-forming cell (PFC) value of 12 wells.

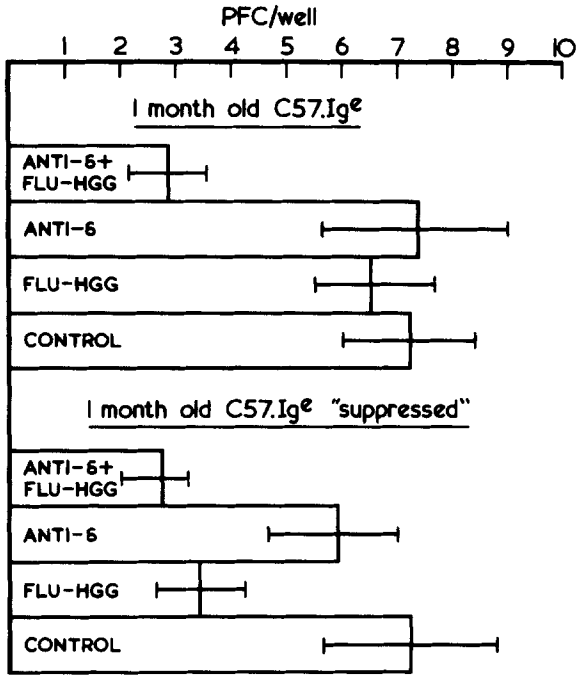

FIG. 3. Sensitivity to in vitro tolerance induction of spleen cells from anti-δ-suppressed mice (see fn. 2). Secondary cultures were at 10^5/well with 1.4×10^6 C57BL/6 mouse thymus fillers.

II, this is, indeed, the case. In Fig. 3 (top), we reiterate the basic observation that anti-δ plus FLU-HGG causes hyporesponsiveness in 1-mo-old littermate controls. The lower panel shows that a degree of tolerance is observed with suppressed spleen cells by *antigen alone*. In the experiment recorded in Table II, not only are spleen cells from suppressed mice easily tolerized in vitro by antigen alone, but also the precursor frequency to FLU (determined by limit dilution analysis; references 12, 13, 15) is dramatically reduced.

TABLE II
*Susceptibility of Adult Spleen Cells from δ-Suppressed Mice to In Vitro Tolerance Induction**

Group	FLU-HGG	Anti-FLU PFC/well	(Precursor frequency)$^{-1}$
Suppressed‡	+	2.2 ± 0.9	104,821
Suppressed‡	−	16.6 ± 4.8	36,995
Control	+	20.6 ± 5.4	18,229
Control	−	27.5 ± 3.7	17,013

* Spleen cells from both groups were incubated with 5 μg/ml of FLU_6HGG or no antigen for 36 h at 37°C in Linbro trays at 5 × 10^6/2 ml MCM. They were then harvested, washed, and challenged in secondary microcultures (with 1.5 × 10^6 DA rat thymus fillers per well) with FLU-POL. For PFC determinations, cells were recultured at 10^5/well; for precursor analysis, cells were diluted to 10^4/well.

‡ C57BL.Ig^e mice, treated with anti-δ allotype serum from birth, are referred to as "suppressed." At 1 mo of age, these mice had virtually no detectable IgD$^+$ B cells (see fn. 2). Control mice were littermates of the same age, but were injected with normal C57BL serum.

Discussion

The results presented in this paper argue in favor of the hypothesis that surface IgD receptors are among the factors controlling the susceptibility of murine B cells to tolerance induction. When IgD is blocked or modulated off the membrane with anti-δ allotype serum, adult B cells are no longer resistant to our in vitro tolerance regimen. That is, they exhibit phenotypically the same degree of sensitivity to tolerogenesis as do spleen cells from neonatal mice.

We have shown that this tolerance facilitation process is antigen-(hapten) specific, strain-specific (occurs only in the appropriate strain expressing the relevant Ig5-1 allotype), and isotype-specific (occurs with anti-δ but not anti-μ). The latter point is an important one because an anti-μ reagent failed to promote tolerance induction under conditions of time and temperature identical to those used for anti-δ. It is noteworthy that anti-Ig reagents have been used previously to block the induction of tolerance in vitro (18). Our results are consistent with the view that interaction of tolerogen with IgM receptors can provide a negative (tolerogenic) signal, whereas the presence of available IgD receptors on the cell surface prevents this from occurring. Thus, IgD may be a receptor, which in some, as yet unclear, manner prevents an encounter between cell and antigen being tolerogenic. In other words, IgD can function as a "failsafe" receptor.

Very recently, Cambier et al. (8) presented evidence consistent with our results in a system employing papain to cleave membrane IgD. These workers took advantage of the finding (19, 20) that IgD seems quite sensitive to proteolytic degradation, whereas other cell surface molecules are relatively resistant to proteolysis. Cambier et al. found that adult B cells, after papain treatment, became susceptible to in vitro tolerogenesis in the presence of $TNP_{17}HGG$ (8). The extent of tolerance susceptibility seemed to correlate with the relative paucity of IgD on the surface; furthermore, if the cells were

allowed to regenerate (in the absence of tolerogen) the molecules cleaved by papain, they again became resistant to tolerance induction. Unfortunately, as stated by Cambier et al. (8), papain may remove other molecules which interfere with tolerance induction (and which may be resynthesized at the same rate as surface IgD). In fact, only approximately 10% of the macromolecules released by papain treatment can be identified as IgD (R. M. E. Parkhouse, personal communication). Hence, our demonstration that anti-δ pretreatment of adult B cells will facilitate tolerance induction provides more direct evidence for the role of IgD as a failsafe receptor.

It is interesting to note that Cambier et al. (6, 8) found that adult T-independent (T-I) B cells are hypersusceptible to tolerance induction in vitro as opposed to T-dependent (TD) B cells (1, 6), and that papain treatment has no effect on the threshold dose of tolerance required for T-I B cells. Our system uses haptenated POL, normally considered to be a T-I antigen, as the challenge immunogen. Superficially, this would seem to be at odds with the Cambier results mentioned above. However, it is useful to recall that our secondary culture system includes excess thymus filler cells, which could provide some T cell "help," after appropriate in vitro activition by the carrier POL. It is noteworthy that this filler function is quite radiosensitive (B. Pike, personal communication).

Moreover, although haptenated POL can behave as a T-I antigen in stimulating bulk cultures of spleen cells from nu/nu mice to form antihapten PFC, we have consistently found that the number of PFC generated in such bulk cultures is considerably less than that from cultures of spleen cells from the nu/+ littermates. This difference is not seen when each type of suspension is cultured at limit dilution with thymus filler cells. These findings could indicate a thymus-dependent component to the antihapten-POL response as measured at limit dilution, a conclusion supported by the finding of some IgG production by days 5 and 6 of the in vitro response.[3] Thus, although we do not observe in vitro tolerogenesis of adult spleen cells by the low concentrations of antigen used by Cambier et al. (6, 8), adult cultures in fact serving as the negative control for all our work on clonal abortion (3, 4), the differences in the readout system are sufficient to ensure that, at present, no conflict of data exists. A further important difference in experimental design is that our tolerogens have always been oligovalent, carrying three to six haptens per molecule of carrier, whereas Cambier et al. used $TNP_{17}HGG$ (2, 6, 8). In our experience, heavily haptenated proteins tend to aggregate spontaneously. Their effects in tissue culture might then mimic those of agents such as antigen-antibody complexes (21) or hapten-gelatin (22), which not only blockade adult B cells but actually prevent fully differentiated antibody-forming cells from making a plaque, a phenomenon we termed "effector cell blockade" (23). It remains to be determined what the relationships among all these different forms of B-cell suppression really are (cf. reference 27).

Data obtained with spleen cells from mice suppressed for the δ-allotype (see

[3] Nossal, G. J. V., and B. Pike. 1977. Further evidence for the clonal selection theory of B lymphocyte stimulation. Manuscript submitted for publication.

footnote 2), by prolonged treatment with anti-δ serum from birth, are consistent with our hypothesis that IgD acts in some way as a failsafe receptor against tolerance. Interestingly, some spleen cells from suppressed mice can reexpress IgD after 24- to 48-h culture in vitro (see fn. 2). These results, as well as the anti-δ pretreatment experiments, indicate that IgD-bearing cells are indeed susceptible to in vitro tolerance induction if exposed to tolerogen before their δ-receptor is resynthesized. We are presently investigating whether spleen cells from δ-suppressed mice lose their susceptibility to tolerogenesis after 24–48 h in vitro.

We have also noted that the frequency of anti-FLU precursors in suppressed spleen cells exposed to FLU-HGG (i.e. tolerized) is greatly reduced by this process (Table II). A similar tolerance susceptibility, in terms of clonable precursors, was observed with spleen cells from male CBA/N mice (N. Klinman, personal communication), which seem to have a paucity of surface IgD receptors, among other defects. Thus, we are inducing the adult counterpart of "clonal abortion" (3, 4) by manipulation of the δ-receptor in vivo or in vitro.

How do these results relate to the relatively late appearance of IgD in B-lymphocyte ontogeny (16)? That is, resistance to tolerance may appear even though the majority of B cells are in fact μ-only cells (1, 3). Firstly, our results and those of Klinman and his colleagues (1) argue against the notion that IgM acts *solely* as a tolerogenic receptor, as originally postulated (7). Most probably, some μ-only cells appearing early in ontogeny can discriminate between tolerogenic and immunogenic signals and are stimulatable. If this were not true, we would not be able to engender a "positive control" response in neonatal spleen cultures in which IgD-bearing cells are rare or absent. Furthermore, spleen cells from mice 2 wk of age are quite resistant to tolerogenesis by FLU hapten conjugates although about half of their splenic B cells are still μ-only. It is worth pointing out that measurement of the percentage of IgM-vs. IgD-bearing cells at a given time reflects the total B-cell repertoire of specificities, which Klinman and co-workers (24, 25) have shown to appear in a programmed fashion. That is, 2-4-dinitrophenyl (DNP) precursors appear before FLU precursors, which antedate phosphorylcholine(Pc)-specific precursors. Moreover, acquisition of resistance to tolerance induction follows the same sequential pattern of diversification. Hence, it is possible that DNP precursors become IgD positive at an earlier time than Pc-specific B cells. Thus, it one were to isolate FLU precursors from 7-day-old mice (which are susceptible to tolerance induction), one might predict that these FLU-specific cells would be mostly IgM only cells. FLU precursors from 2- to 3-wk-old mice resistant to tolerogenesis would be predicted to be IgM-IgD doubles (μ + δ-cells) although on a total B-cell basis, most cells would still be IgM only (μ-only cells). Hence the dynamic maturation is a programmed sequence which is clonotype-specific as well as isotype-related. We are currently testing this hypothesis with cells isolated off FLU-gelatin dishes (22).

An alternative, but not mutually exclusive hypothesis, is that the μ-only cells in the spleens of older mice are T-I B cells, as previously suggested (6–8). These cells might normally be rendered unresponsive in vitro but their tolerant state would not be detectable in our thymus filler readout system. Formal

proof of the susceptibility of μ-only cells to tolerogenesis is currently under investigation with antihapten affinity columns (26)[4] to separate μ-only from μ + δ-cells.

Finally, it is interesting to consider that cells which have differentiated to express a new surface isotype (IgD) and are also resistant to tolerance induction may revert back to a "less differentiated" behavior by the simple removal or blockade of their IgD receptor. Thus the μ + δ-cell behaves phenotypically like a neonatal μ-only B cell under these circumstances. This suggests that the appearance of IgD simply represents the acquisition of an additional level of control, which is a reversible event. How the δ-receptor acts as a failsafe receptor and the nature of the negative signals which the μ-receptor can provide are totally unknown. The model presented in this paper, using anti-δ and allotype-congenic mice, should provide some answers to these questions.

Summary

Adult spleen cells from C57BL.Ig^e mice, which generally are resistant to in vitro tolerance induction in the B-cell compartment, became hyporesponsive (tolerant) when cultured with antigen in the presence of an anti-allotype serum. Both antigen and anti-δ had to be present for this effect, which was hapten-specific and did not occur in C57BL/6 mice, which lack the Ig5-1 allotype of the δ-chain detected in this system. Preculture with anti-μ serum plus antigen, in contrast, did not cause tolerance induction in adult spleen B cells of either strain. These results suggest that the surface IgD may act as a failsafe receptor to prevent tolerance induction in adult B cells. Tolerance studies with spleen cells from mice with markedly reduced numbers of IgD^{+ve} cells, because of a regimen of repeated injections of anti-δ serum beginning at birth (δ-suppressed mice), confirmed the importance of membrane IgD in preventing tolerance, because such δ-suppressed mice were hypersusceptible to tolerance by antigen alone. Inasmuch as immature B cells lack IgD on their surface, these studies suggest that acquisition of IgD is an important maturational step in the ability of murine B cells to discriminate tolerogenic and immunogenic signals.

Note Added in Proof: Recently, Vitetta et al., have also found that a heterologous anti-δ, but not an anti-μ, will facilitate tolerance induction in adult T-D B cells in vitro (*J. Exp. Med.* 146: 1804). Their results confirm our basic findings.

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