Small B Cells as Antigen-presenting Cells in the Induction of Tolerance to Soluble Protein Antigens

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Summary

We have investigated the ability of resting B cells, acting as antigen-presenting cells, to induce tolerance to soluble protein antigens in mice, using an antigen targeted specifically to B cells. We inject mice intravenously with ultracentrifuged Fab fragments of rabbit anti-mouse immunoglobulin D (IgD) (Fab anti-δ). Treatment with Fab anti-δ results in profound tolerance to challenge with 100 μg Fab nonimmune rabbit Ig (Fab NRG), precipitated in alum, as measured by antibody production. Tolerance to rabbit Fab is antigen specific, since the treated mice make normal antibody responses to a control antigen, chicken Ig. Tolerance is dependent on antigen presentation by B cells, since intravenous injection of soluble Fab NRG, which is not targeted to B cells, results in a much lower frequency and degree of tolerance, especially at lower doses. T cell help in this system is affected, since T cells from Fab anti-δ-treated mice fail to provide help for an adoptive primary antibody response to Fab NRG when transferred together with normal B cells into severe combined immunodeficient (SCID) mice. The antigen-specific B cell compartment is also affected during tolerance induction, since B cells from treated animals make less antibody than normal B cells when transferred into SCID mice with normal T cells. Although the mechanism of nonresponsiveness in the helper T cell compartment remains to be determined, we think it is likely that the precursors of helper T cells are inactivated or deleted by encountering antigen presented by small, resting B cells, which lack accessory signals necessary to induce helper T cell proliferation and differentiation to effector function. Our experiments suggest a role for small B cells as antigen-specific, tolerizing antigen-presenting cells in acquired tolerance to foreign protein antigens and in self-tolerance to soluble self-proteins.

Effective antigen presentation results from the interaction of a T cell receptor with self-MHC plus a specific foreign peptide. During T/B cell collaboration in the antibody response, additional signals are required for this interaction to be productive from both the T and B cell perspectives. These additional signals include adhesion and/or signaling by cell surface molecules and cytokines, some of which remain to be identified. A two-signal hypothesis was first put forth by Bretscher and Cohn (1) for B cells and by Lafferty et al. (2) for T cells. A more specific model has been proposed by Schwartz and colleagues (3, 4) who have shown that fixed APCs or signals that engage the TCR alone deliver an abortive signal to type 1 Th cell lines, which results in anergy. This abortive signal produces a rise in intracellular Ca²⁺ and an increase in the expression of IL-2 receptors, but no secretion of IL-2, and the cells remain unresponsive to complete activation signals for weeks (4).

B cells get help from T cells in the antibody response by acting as antigen-specific APCs, and they can drive T cell proliferation in vitro and in vivo (5, 6, and reviewed in reference 7). On the other hand, resting B cells have been found to be ineffective (8-11) or inferior (12-14) APCs for primary responses to alloantigens and foreign antigens. Overall, the evidence indicates that unmanipulated, small, resting B cells are poor APCs for primary T cell responses, although they can provide the necessary interactions to get help from an already activated T cell.

In this paper, we propose that presentation of antigen by a small, resting B cell to a small resting T cell is tolerogenic and results in loss of T cell activity. This model is supported by evidence from Ryan et al. (15) that in vivo injection of small (accessory cell-depleted) spleen cells from allogeneic donors into normal mice produced hyporesponsiveness in MLR which lasted at least 13 d posttransfer. More recent evidence of B cell involvement in tolerance induction, to the bm12 mutation in the I-A<sup>d</sup> MHC class II molecule, comes from Hori et al. (16), who have shown that injection of (bm12 × B6)<sub>F<sub>1</sub></sub> accessory cell-depleted spleen cells into B6 mice results in prolonged survival of bm12 skin grafts and abolishes the MLR against bm12 cells. The predominant MHC class II-bearing cell in this population is a small B cell.

Starting in the early sixties, a series of investigators showed
that injection of soluble protein antigens (particularly when ultracentrifuged to remove aggregates) produced tolerance to a challenge with a more immunogenic form of the same antigen (usually in CFA or IFA). These antigens include BSA (17), human gamma globulin (HGG)1 (18), bovine gamma globulin (19), and flagellin (20). Injection of low doses of these proteins, in either a single or multiple dose(s) over several weeks, produced tolerance in normal animals that lasted up to several weeks. This type of low zone, soluble protein antigen tolerance has been shown to involve the inactivation of Th cell function (21, 22). The APC that mediates tolerance induction has not been defined. Using low doses of deaggregated antigen, the most likely APCs are the relatively rare antigen-specific B cells, which have antigen-specific Ig molecules on their surface. These B cells could concentrate and process the antigen, but would not be activated by binding antigen since deaggregated antigen would not crosslink B cell membrane Ig. As outlined above, the interaction of an antigen-specific resting T cell with an antigen-specific resting B cell may be ineffective due to a lack of costimulatory signal or signals, which are already present or unnecessary in previously activated cells.

Our laboratory has previously shown that small B cells can process and present Fab fragments of rabbit anti-mouse Ig very efficiently to our rabbit Ig-specific Th cell lines (23). Fab anti-δ alone does not activate B cells in vitro; they show no increases in MHC class II expression, size, or DNA synthesis (23). Since we know that small, resting B cells can process this antigen while remaining in a resting state (24), we felt that this would be a good system for looking at antigen presentation by small B cells to naive T cells. The Fab anti-δ is ultracentrifuged and injected intravenously into normal mice that are challenged intraperitoneally 7 d later with Fab NRG precipitated in alum. Confirming our preliminary experiments reported earlier (25), these treated mice are profoundly tolerant to rabbit Fab compared with untreated control mice. Comparable treatment of mice with an Fab rabbit Ig molecule not targeted to B cells, or with a cross-linking F(ab')2 anti-δ, is much less effective. Since the treated animals are tolerant in the Th cell compartment, the most probable interpretation of these results is that Fab anti-δ is presented on B cells and induces tolerance in T cells specific for rabbit Fab.

**Materials and Methods**

**Mice.** BALB/c × DBA/2 mice (CD2)F1 and BALB/c mice were from the National Cancer Institute (Frederick, MD). Female, age-matched mice were used in each experiment and the mice were 8–20 wk old at the start of each experiment. Mice with SCID (CB.17 SCID mice) (26) and CB.17 mice were bred at the University of Massachusetts Medical Center animal facility and were from stock kindly provided by Donald Mosier (Medical Biology Institute, La Jolla, CA). The mice were injected and bled from their lateral tail veins.

1. **Abbreviations used in this paper:** anti-ars, rabbit anti-azophenyl arsonate; HGG, human gamma globulin; NRG, nonimmune rabbit Ig.

**Antigens and Antibodies.** Rabbit anti–mouse IgD was prepared in rabbits as previously described (27). The antibody was purified from rabbit sera by affinity chromatography on IgD columns, removing anti-IgG and anti-IgM crossreactivity first. Immunoprecipitation of δ chains but not μ chains from 125I-labeled B cells was done to check specificity (23). Fab fragments were prepared by incubating intact antibody with papain coupled to agarose (Sigma Chemical Co., St. Louis, MO) and then separated from Fab fragments by passage over protein A-Sepharose columns. F(ab')2 fragments were prepared by pepsin digestion. Rabbit anti–azophenyl arsonate (anti-ars) antibody was prepared in rabbits and purified by affinity chromatography. Nonimmune rabbit Ig (NRG), prepared from Cohn fractions II and III, was purchased from Sigma Chemical Co. Absorbed Fab NRG was prepared by incubating Fab NRG (at 1 mg/ml) with whole mouse spleen cells (one spleen/1 ml of Fab NRG) for 1 h at 4°C. Chicken Ig was purchased from Calbiochem-Behring Corp. (La Jolla, CA). BSA was purchased from United States Biochemical Corp. (Cleveland, OH). Rabbit anti-mouse H+L chain or isotype-specific horseradish peroxidase–coupled antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). CFA was purchased from Gibco Laboratories (Grand Island, NY). Killed Bordetella pertussis was purchased from the Biological Laboratories at the State Laboratory Institute (Jamaica Plain, MA). Endotoxin levels in antigens were measured by Pyrotell Limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). All antigens used had <15 ng endotoxin/mouse at the maximum injected dose.

**Tolerance Induction.** Molecules used as tolerogens were centrifuged at 150,000 g in an airfuge (Beckman Instruments, Inc., Palo Alto, CA) for 60 min, and the top 60% of the solution was used. Tolerogens were injected in a volume of 0.2 ml in PBS intravenously into the lateral tail vein of the mice. After 7 d, the animals were challenged intraperitoneally with 100 μg of Fab NRG precipitated in alum. Most animals also received 100 μg chicken Ig precipitated in alum at a separate site intraperitoneally. The animals were bled from the tail veins at weekly intervals, and the serum was tested for anti-rabbit Fab antibodies.

**ELISA.** Antibody titers were measured by a sandwich ELISA in which serial dilutions of mouse sera starting at 1:40 were incubated with antigen (Fab[NRG] or chicken Ig) bound to flexible polyvinylchloride plates (Becton Dickinson Labware, Oxnard, CA) and blocked with 1% BSA in PBS. The plates were washed with PBS + 0.05% Tween 20 (poloxylethylene-sorbitan mono-laureate) (Sigma Chemical Co.) and incubated with rabbit anti–mouse IgG H+L chain or isotype antibody coupled to horseradish peroxidase. The substrate and color developer were 3,3',5,5'-tetramethyl-benzidine (ICN Biocoligicals, Lisle, IL) with H2O2 added. The reactions were stopped by the addition of 2 M H2SO4. Plates were read at 405 vs. 530 nm on an ELISA platereader (Dynatech Laboratories, Inc., Alexandria, VA), and antibody concentrations determined by comparing OD with a mouse anti-rabbit Fab standard that had been purified by affinity chromatography. Analysis of antibody titers was made by logistic analysis (Immunosoft, Dynatech Laboratories, Inc.), and those dilutions in the most accurate range of the plate reader were averaged to provide micromgram/milliliter equivalents.

**Adoptive Transfer.** BALB/c mice were injected intravenously with 100 μg Fab anti-δ. On day 5, spleen and lymph node cells were harvested. The spleen cells were depleted of T cells as previously described (23) by treatment with an anti-T cell cocktail (anti-Thy-1, anti-CD4, and anti-CD8) followed by a mouse anti–rat κ antibody and thenagarose-absorbed guinea pig complement (tolerant B cells). The lymph node cells were depleted of B cells by
panning on rabbit anti-mouse Ig-coated plates by the method of Wysocki and Sato (28) (tolerant T cells). These cells, along with T and B cells collected and separated from untreated control BALB/c mice (normal T and normal B), were transferred intravenously into SCID mice. 2 d later, the reconstituted SCID mice were challenged intraperitoneally with 100 μg Fab NRG + 20 μg chicken Ig, each precipitated in alum. All SCID mice were shown to have <5 μg/ml Ig (by ELISA) before transfer. T cells were <1% slg+ and B cells were <1% Thy-1+ when stained with biotinylated anti-mouse Ig or anti-Thy-1 plus FITC-avidin.

Results

Antiδ Injected into Mice Induces Tolerance to Rabbit Globulin. Our model system involves injection of 100 μg of ultracentrifuged Fab rabbit anti-δ intravenously into normal CD2F1 mice followed by challenge with Fab NRG precipitated in alum 7 d later. These animals become profoundly tolerant to rabbit Fab as measured by antibody production in an ELISA. Fig. 1 shows the results of one such experiment. These mice were followed weekly until day 64 postchallenge, when three of the six tolerant mice still made <5% of the mean control anti-rabbit Fab response. The difference between the control and tolerant mice is generally greatest at day 21 or 28 postchallenge. At this point the control mice approach their peak response and the tolerant mice remain unresponsive. In all mice to date, 35 of 50 mice made <1% of the mean control anti-rabbit Ig response at day 21 postchallenge. Only two of the remaining mice made responses >10% (15 and 20%) (data not shown).

Tolerance generated by this method is antigen specific as shown by the response to an unrelated antigen given at the same time as the rabbit Fab challenge. As shown in Fig. 2, these animals all made normal responses to challenge with chicken Ig, also precipitated in alum, given at a different site.

B Cells Must Be Targeted for Tolerance to Occur. To show that the tolerance was due to presentation by B cells, we used a rabbit Ig molecule that was not targeted to B cells. For this purpose we used either Fab NRG or Fab rabbit anti-ars.
of 50 μg/mouse, the two treatments were almost equivalent, but at lower doses Fab anti-δ was a clearly superior tolerogen. Treatment with 0.5 μg Fab anti-δ was more effective than treatment with 5 μg/mouse of absorbed Fab NRG. By targeting the rabbit Fab fragment to the majority of B cells, we have made a much more efficient tolerogen. These data support our hypothesis that small B cells are tolerance-inducing APCs.

Divalent F(ab′)2 Anti-δ Does Not Induce Tolerance. Since activated B cells have been shown to initiate some T cell responses in vitro (8), activated B cells may no longer be tolerogenic as APCs. Finkelman et al. (29) have shown that intravenous injection of whole goat anti-mouse δ induces polyclonal B cell activation followed by the appearance of large numbers of surface IgG-positive cells and T cell-dependent IgG secretion. In addition, Golub and Weigle (30) showed that injection of LPS around the time of the tolerogen (HGG) blocked the induction of tolerance. We tested the effects of B cell activation in our system by injecting 100 μg of ultracentrifuged F(ab′)2 fragments of rabbit anti-δ into mice followed by challenge as above. This divalent antigen can crosslink the surface IgD molecules, and, as shown in Fig. 3, the F(ab′)2 anti-δ did not induce tolerance. We were not able in this system to rule out effects caused by aggregation of the small amount of serum IgD (31), but the simplest interpretation of these results is that the B cells must remain in a resting state to induce tolerance.

Adjuvants. Although most of our tolerance induction has been tested with alum-precipitated challenge, we wanted to know if mice were still tolerant after challenge with other adjuvants since different adjuvants may stimulate different subsets of T cells (32, 33). We treated animals with Fab anti-δ and then challenged them with 100 μg Fab NRG either precipitated in alum, or precipitated in alum plus 2 × 10^9 B. pertussis (both injected intraperitoneally), or emulsified in CFA and injected at the base of the tail. As shown in Fig. 5 a, the alum-challenged animals with or without B. pertussis remain tolerant. CFA partially breaks tolerance, since anti-δ-treated mice challenged with CFA make 18% of the mean control response (1,156 μg/ml) at day 35 postchallenge.

Since murine Th cell subsets have been defined in vitro that induce switching to different Ig isotypes (34), we were interested to see if there was a different spectrum of isotypes in the residual antibody response in the tolerant mice versus the controls. Individual isotype responses, from the sera shown in Fig. 5 a, were measured by ELISA using horseradish peroxidase-coupled goat anti-mouse isotype antibodies.
juvants, the predominant isotype in all groups (whether control normal T cells only or normal B cells only followed by chal-

anti-chicken Ig (data not shown). SCID mice that received then tested for anti-chicken Ig antibodies at days 7 and 14 partment.

there is also some effect on the antigen-specific B cell com-
tolerant B cells with normal T cells, which indicates that mice. There is also a decrease in the responses generated using higher anti-rabbit Fab titers, which were 2% of the control experiment but not shown were three mice that received 2 x 10^7 normal mice. The SCID recipient mice were challenged and bled

T Cells and B Cells Transferred

1. Each group shown contained five SCID mice that received 8 x 10^7 pooled spleen B cells and 2 x 10^7 pooled lymph node T cells from either tolerant or normal mice. Mice were bled weekly and anti-rabbit Fab levels measured by ELISA. Symbols, in the last two groups only, represent the anti-rabbit Fab responses of the individual mice. Also included in this experiment but not shown were three mice that received 2 x 10^7 normal T cells alone or 8 x 10^7 normal B cells alone. Neither of these groups made an anti-rabbit Fab response.

Discussion

We have shown here that targeting a monovalent soluble protein antigen to B cells in vivo increases the efficiency of induction of tolerance in the Th cell compartment. This result contrasts with early experiments showing that cell-associated antigen is exceptionally immunogenic (35) and more recent experiments showing that T cell determinants on an allotypic IgG molecule are at least 100 times more immunogenic in vivo when the IgG molecule is directed against class II MHC alleles on APC (36). In experiments superficially resembling ours, Finkelman et al. (29) obtained massive, T cell-dependent polyclonal B cell activation and Ig secretion instead of tolerance when they injected goat anti-δ antibodies into mice. In our experiments, instead of intact anti-δ antibody, we used monovalent, ultracentrifuged Fab fragments. We have shown that Fab anti-δ does not activate small B cells through their antigen receptors but can be efficiently presented to T cell lines in vitro (23, 24). Resting B cells have been shown to be defective APCs for primary responses to foreign antigens (10, 11). We propose that the result of defective antigen presentation in vivo by resting B cells to unprimed T cells is tolerance in the T cell compartment. This hypothesis offers an attractive explanation for the phenomenon of low-zone acquired tolerance to soluble proteins (17), and suggests a role for self-reactive B cells in maintaining tolerance to soluble self-proteins.

Tolerance to soluble protein antigens is usually described as a loss of reactivity to a normally immunogenic protein induced by administration of that protein by an alternate route, dose, or form of administration. One consequence of these methods of administration is that the antigen may be processed by an alternate or inappropriate APC. In the case of low doses of soluble protein antigens, the tolerizing APC may be the small antigen-specific B cell. Although these cells are present at low frequency (between one in 10^4 or 10^5 for protein antigens) (37), they may be the only cells with the ability to pick up enough of a low-dose, soluble antigen to affect the T cell response. If no other APC can gather enough antigen, these few small B cells could present it over time to the antigen-specific T cells. Resting B cells may also act as tolerizing APC in high-zone tolerance to soluble proteins, because at high antigen concentrations, all the B cells can take up protein antigens nonspecifically (23), and B cells far outnumber other constitutively class II-positive cells.

Fab anti-δ was much more effective at tolerance induction than Fab fragments of rabbit IgG (Fab NRG), particularly at lower concentrations of tolerogen (Fig. 4). We think this is because antigen-specific naïve T cells are much more likely to encounter antigen presented by a resting B cell in the anti-δ-treated animals: Fab anti-δ can be presented by every IgD-positive B cell, while Fab NRG is presented effectively only
by rare antigen-specific B cells. In our experiments, single injections of ultracentrifuged Fab NRG produced variable effects on the antibody response to subsequent challenge with Fab NRG in adjuvant, ranging from no effect (25) to tolerance (Fig. 4). We have not yet identified the source of this variability, which does not correlate with the low levels of endotoxin in our tolerogen preparations. It may be a consequence of the condition of the mice or of improving technique in intravenous injection, since Fab NRG has become increasingly tolerogenic over the course of these experiments. In classical low-zone tolerance, repeated injections of tolerogen were required (17), although single injections of whole nonspecific IgG have been very effective (18, 19). Whole IgG may be a more effective tolerogen than Fab fragments because of its longer half-life in vivo, 5–6 d vs. ≈4 h (38, 39).

Tolerance generated in this system is not permanent. Over the times tested most mice made an antibody response to the alum depot of rabbit Fab. Since the original tolerogen is present for such a short time and mice are not challenged for an additional 6 d, it is possible that newly emerging immune cells can reconstitute the anti-rabbit Fab response. Mice treated with Fab anti-δ have been challenged as early as 48 h after treatment (data not shown), and there is no significant difference in the decay of tolerance between mice challenged at 2 d posttreatment or 7 d posttreatment. Alternatively, a very small number of peripheral pre-Th cells that escape tolerance induction during the anti-δ treatment may expand over the weeks after challenge.

We looked at T cell function in these anti-δ-treated mice in two ways. First, in classical carrier-specific help for an anitihapten response, we found diminished help for an anti-DNP antibody response when mice were challenged with DNP-NRG [data not shown]. The second look at T cell function was in the adoptive transfer system. Here we reconstituted SCID mice with combinations of T and B cells from tolerant and control mice and then challenged these mice with Fab NRG and looked at the anti-rabbit Fab antibody response. These mice had a clear defect in rabbit Fab–specific T cell help, since the mice receiving tolerant T plus normal B cells made very reduced amounts of anti-rabbit Fab. The adoptive transfer into SCID mice showed that the rabbit Fab–specific B cells had also been affected by the anti-δ treatment. The response of mice receiving tolerant T plus tolerant B cells was even smaller than that of mice receiving tolerant T plus normal B cells, and B cells from tolerant mice plus normal T cells transferred into SCID mice made approximately one-third of the response of mice that received normal B plus normal T cells. This response was delayed and did not reach the control response by the termination of the experiment (day 35). This delayed response may be due to recovery of B cell function, or the clonal expansion of a smaller number of responsive B cells. All mice receiving both T and B cells, with and without anti-δ treatment, made equivalent responses to chicken Ig. This indicates that B cell function has not been generally affected by anti-δ. For the rabbit Fab–specific B cells, the interaction with Fab anti-δ is a cross-linking interaction, since the membrane IgM and IgD on the antigen-specific B cells can recognize the rabbit Fab fragments bound to membrane IgD on the same cell or adjacent B cells. Therefore, the antigen-specific B cells could get a signal through their antigen receptors in the absence of T cell help. Goodnow et al. (40) have shown using antibody transgenic mice that transfer of mature antigen-specific B cells into antigen-bearing, T cell–tolerant mice results in B cell anergy. Also, B cell tolerance may require the involvement of slgM, which can occur only in the rabbit Fab–specific B cell population. Alternatively, it is possible that the transferred B cells, from anti-δ–treated mice, retained some cell-associated or processed antigen, and induced tolerance in T cells after transfer into SCID recipients.

Tolerance in the T cell compartment has been shown to occur by at least three different mechanisms (3). Clonal deletion and anergy have both been shown to account for tolerance in the thymus (41, 42) and in the periphery (43, 44). In addition, antigen-specific suppression by T cells has been described in peripheral tolerance to BSA (45) and to high doses of fowl gamma globulin (46). Recently, it has been proposed that Th1 and Th2 CD4+ T cell subsets may negatively regulate one another in vivo, accounting for earlier observations that immune responses tend towards either delayed hypersensitivity or antibody formation (34). We are currently investigating the mechanism of the T cell defect with mixing experiments to look for active suppression. We are also looking for any set of T cells from tolerant animals able to proliferate in response to antigen in vitro, since Whiteley et al. (47) found a proliferating population of T cells in tolerant animals that was unable to help antibody production. To date, we have been unable to demonstrate suppressor T cells in our mixing experiments, and find that in vitro proliferative responses of primed lymph node cells from anti-δ–treated mice are reduced or absent. Our IgG subclass profiles provide no evidence of activity of a different T cell subset in tolerant mice.

The most straightforward mechanisms for the induction of tolerance by restig B cells would be anergy or deletion of naive antigen-specific T cells as a result of antigen presentation without appropriate costimulation. Resting B cells lack costimulatory activity (8, 48). Activation of B cells induces costimulatory activity (8, 49), including the B7 molecule, which engages CD28 on the T cell (50, 51). This may explain why F(αb')2 anti-δ is a less effective tolerogen (Fig. 3), and whole IgG anti-δ results in a polyclonal antibody response (29).

It may be possible to induce tolerance to polypeptide drugs or antibodies used as therapeutic agents with this technique by coupling the drug to the Fab portion of an anti-δ molecule, or in the case of mAbs, by injecting a species-specific Fab anti-δ intravenously before the start of the therapy. As we have proposed (52), small B cells, acting as tolerizing APCs, may maintain peripheral tolerance to their own Ig isotypes and idiotypes as well as to self-proteins not expressed or present at very low levels in the thymus. B cells capable of presenting low concentrations of self proteins must be autoreactive. Autoreactive B cells may persist in the periphery either as anergic cells at high self-antigen concentrations (40)
or as functional cells at low self antigen concentrations (53). Both kinds of cells may play a role in maintenance of peripheral T cell tolerance. When autoreactive B cells are activated by crossreacting foreign antigens and acquire costimulatory activity, they may be able to present self-antigens very efficiently to T cells, and break self-tolerance in the T cell compartment (54).

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