Tolerogenicity of Resting and Activated B Cells

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Summary

Antigen presentation by resting splenic B cells has been shown previously to induce T helper 1 cell (Th1) anergy. In contrast to expectations, it was found here that B cells treated with F(ab')$_2$ goat anti–mouse immunoglobulin (IgM) for 24 or 48 h also presented antigen (Ag) to Th1 cells in a manner that induced dramatic Ag-specific proliferative inactivation. The tolerogenicity of the anti-Ig–treated B cells was consistent with the observation that these B cells were only slightly more efficient than resting B cells in stimulating human gamma globulin (HGG)–induced proliferation of HGG-specific Th1 cells in primary cultures. The activated B cells were, however, more efficient than resting B cells in stimulating a primary mixed leukocyte reaction, and exhibited increased expression of major histocompatibility complex class II molecules, RL388 Ag and transferrin receptor. In addition, unlike resting B cells, which expressed little detectable B7, anti-Ig–treated B cells expressed high levels of B7. The functional capacity of the B7 expressed on the activated B cells was demonstrated by the fact that the Ag-presenting capacity of these B cells was inhibited by the addition to culture of CTLA4Ig, a soluble receptor for B7. It is unlikely that the tolerogenicity of the activated B cells was due to an inability of the Th1 cells to respond to B7 signals; the Th1 clones used in the experiments, unlike the Th2 clones tested, expressed CD28, the ligand for B7. In addition, anti-CD28 monoclonal antibody inhibited the induction of Th1 cell anergy when added to cultures of Th1 cells and Ag-pulsed fixed antigen-presenting cells. Taken together, the results indicate that B cells, even when activated, do not satisfy the costimulatory requirements of the Th1 cells used here, and therefore can present Ag in a tolerogenic fashion to Th1 cells. The costimulator deficiency of activated B cells may reflect an inadequacy in the level of B7 expressed or a lack of some other molecule.

Antigen-specific inactivation of Th1 cells can be induced in vitro by exposing the Th1 cells to Ag presented by APCs that lack costimulatory molecules. Tolerogenic APCs often consist of spleen cells that have been pulsed with Ag and then chemically fixed (1, 2). However, there is also evidence showing that nonfixed resting B cells are capable of presenting Ag to T cells in a tolerogenic fashion. It has been noted in several systems that lightly irradiated resting B cells are either ineffective or inferior APCs for the initiation of primary Th1 responses to mitogens or antigens (3–6). Using Th1 clones specific for human gamma globulin (HGG)[1], it was found that B cell inefficiency as APCs was associated with antigen-specific inactivation: Th1 cells exposed to HGG and lightly irradiated splenic B cells in primary cultures were unable to proliferate to HGG and immunogenic APCs in secondary cultures (7). In vivo, the role of B cells as tolerogenic APCs has been demonstrated in mice by using Fab fragments of rabbit anti–mouse IgD to induce tolerance specific for nonimmune rabbit Ig (8). Tolerance to rabbit Ig was apparently dependent on antigen presentation by B cells, since intravenous injection of soluble Fab nonimmune rabbit Ig, which is not targeted to B cells, resulted in a lower degree of tolerance, especially at lower doses. It has also been demonstrated that injection of a population of spleen cells consisting primarily of small resting B cells induced hyporesponsiveness to MHC alloantigen (9), and to the male-specific antigen H-Y in mice (10). Presumably, the tolerogenicity of resting B cells is due to the fact that although resting B cells can present Ag in conjunction with MHC class II molecules, they lack the costimulatory molecules required to fully activate T cells.

Although the identity of the costimulator molecule whose absence may be associated with B cell tolerogenicity is not known, a good candidate for such a molecule is the B7/BB-1 antigen. B7 is not expressed on resting B cells, but is expressed on dendritic cells (11) which are particularly potent accessory cells for T cells (12). The B7 molecule on APCs binds both CTLA-4, which is expressed on activated T cells (13), and CD28, which is expressed on >80% of resting peripheral CD4$^+$ T cells (14). Ligation of CD28 initiates a

signal transduction pathway which appears to be separate from that initiated by TCR ligation (for a review see reference 15), and costimulates proliferation and IL-2 production by normal T cells in conjunction with antigens or mitogens (16, 17). In terms of Th1 cells specifically, it has been shown that anti-CD28 mAb costimulates proliferation of Th1 cells to Ag-pulsed fixed spleen cells, and interferes with Th1 cell inactivation in these cultures (18). Thus, it appears that B7 ligation of CD28 on Th1 cells has the capacity to costimulate Th1 cell activation.

The potential for B cell–mediated ligation of CD28 on Th1 cells is enhanced in activated B cells through the upregulation of B7. The increased B7 expression on activated B cells (19–21) appears to correlate with increases in certain aspects of B cell Ag presentation. Activated B cells have been shown to be more efficient than resting B cells in promoting a MLR (22), and in costimulating Ag- or mitogen-induced T cell proliferation (23–26). Aside from B7, a number of other B lineage–associated and restricted Ags (e.g., CDw40, CD23, and CD25) are upregulated on activated B cells (27–29). Increased expression of MHC class II molecules is also observed on activated B cells (30). Because of the increased expression of B7 and other accessory molecules and activation Ags on stimulated as compared to resting B cells, it seemed likely that activated B cells would, unlike resting B cells, present Ag to Th1 cells in an immunogenic rather than a tolerogenic fashion. This study was conducted to test this point. In contrast to our expectations, activated B cells retained their ability to present Ag in a manner that led to Th1 inactivation.

Materials and Methods

Animals. 4–5-wk-old male A/J mice were purchased from the National Cancer Institute (Bethesda, MD). Experiments were performed by using 8–12-wk-old mice. Female Lewis rats were purchased from The Jackson Laboratory (Bar Harbor, ME).

Antigens and Antibodies. Soluble HGG was purified from Cohn fraction II of human plasma by DEAE-cellulose chromatography as previously described (31). Anti-Thy 1.2 mAb was purchased from New England Nuclear (Boston, MA). Hybridoma cells secreting 145-2C11 (hamster anti–mouse CD3ε) were provided by Dr. J. A. Bluestone (University of Chicago, Chicago, IL) and the mAb was purified as described (32). The sources and purification procedures for anti-RL388Ag mAb (RL388), anti–mouse transferrin receptor (TfR) mAb (R17.217), and polyclonal rat IgG were described previously (32). PE-conjugated anti-B220 mAb and fluorescein (Fl)-conjugated anti-Ia^k mAb (specificity-2) were purchased from PharMingen (San Diego, CA). The CTLA4Ig fusion protein and the purified human mouse chimeric mAb L6 protein were kindly provided by Dr. Peter Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). Hamster anti-CD28 (mAb 37.51, IgG2a) mAb was kindly provided by Dr. James Allison (University of California at Berkeley, Berkeley, CA). Biotinylated anti-CD28 mAb was purchased from Caltag Laboratories (San Francisco, CA). Fl-goat F(ab')$_2$ anti–human IgG (absorbed against mouse serum proteins) was purchased from Jackson Immuno-Research (West Grove, PA).

Th Clones and Lines. The HGG-specific CD4$^+$ Th1 clones were developed, maintained, and characterized as previously described (2). The Con A conditioned medium (CM) used in main-

taining the clones and as a source of IL-2 in the experiments described here, was prepared by collecting the supernatant from cultures of spleen cells from Lewis rats incubated at 10^6 cells/ml with 5 μg/ml Con A for 24 h.

Preparation of Fixed APCs. Splenocytes in RPMI-1640 medium (M. A. Bioproducts, Walkersville, MD) supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 × 10^{-5} M 2-ME, and 5% FCS (M. A. Bioproducts) were incubated at 2 × 10^7/ml in medium alone, or with 1 mg/ml HGG for 3 h at 37°C. The spleen cells were then washed in RPMI, passed over a Ficoll-Hypaque (FH) gradient to remove the dead cells, and fixed by using a modification of a previously described method (2). Briefly, the spleen cells were incubated in 1 ml of 0.15% paraformaldehyde in HBSS for 1 min at 37°C. The reaction was stopped by adding 7 ml of cold 0.5% glycylglycine. The fixed APCs were washed three times in medium before use.

Primary Cultures for Inducing Th1 Cell Unresponsiveness. Th1 cells were harvested from culture 7–12 d after the last antigenic stimulation. They were passed over a FH gradient to remove old APCs, and incubated at 2.5 × 10^5 cells/ml in 0.2 ml medium consisting of 1 part Click's medium, 1 part supplemented RPMI-1640 medium, and 10% FCS. Also added to the primary cultures were untreated or activated B cells (5 × 10^6/ml) together with HGG. In some experiments, 5 × 10^6/ml HGG-pulsed fixed antigen presenting cells (FAPC) were added to the wells. The control cultures received B cells in the absence of HGG, or FAPC prepared in the absence of HGG. After incubation (48–72 h at 37°C), the cells in these primary cultures were harvested, washed, passed over a FH gradient to remove the dead B cells or fixed APCs, and the Th1 cells recultured in secondary cultures. In some experiments, the Th1 cells were rested for an additional 1–3 d before being recultured in secondary cultures.

Secondary Cultures for Monitoring Tolerized Th1 Cells. Th1 cells harvested from primary cultures were recultured at 5 × 10^4 cells/well with 5 × 10^5/well irradiated (3,000 rad of γ-radiation) naive spleen cells and HGG, or with 10% Con A CM in plates (model 3596; Costar Corp., Cambridge, MA) in a 0.2-ml final volume. Cultures were incubated for 36 h at 37°C. To measure DNA synthesis, cultures were pulsed for 2 or 4 h with [^{3}H]TdR, and incorporated radioactivity was determined by scintillation counting.

Immunofluorescent Staining and Flow Cytofluorometric Analysis. The formulation of staining medium and procedures for two-stage immunofluorescent (IF) staining were described previously (32). The mAb and polyclonal IgG were conjugated with Fl or PE as described previously, and were ultracentrifuged to eliminate aggregates (32). B cells from preincubation cultures were harvested at 36 h and viable B cells were isolated by FH density centrifugation and two-color IF stained for B220 versus membrane RL388 Ag, Ia^K, TfR, and B7. Non-B220$^+$ cells (T cells or macrophages) were excluded from analysis by gating on B220$^+$ cells. Staining B7 was a two-step procedure involving an initial treatment with CTLA4Ig (5 μg/ml) or human mouse chimeric mAb L6 (5 μg/ml), followed by treatment with Fl-labeled goat F(ab')$_2$ anti–human IgG in the presence of 5% normal mouse serum. Th cells or anti-CD3–activated spleen cells were one color–stained for B7 expression in a manner similar to that used for B cells. Th cells were also IF stained for CD28 using biotinylated anti-CD28 mAb and PE-streptavidin. The stained cells were examined by flow cytofluorometry using a FACS® IV flow cytometer (Becton Dickinson & Co., Mountain View, CA), and optical arrangements and machine settings for the FACS® IV were previously described in detail (32).

For two-color analyses using Fl reagents, Fl frequency distributions histograms obtained from PE-gated viable cells were generated as described (32).

Preparation of Splenic B Cells. Splenic B cells were isolated by double passage of T cell–depleted spleen cells over Sephadex G-10 (Pharmacia, Uppsala, Sweden) columns as described (7). The resulting spleen cell population was examined by cytofluorometric (FCF) analysis periodically and found to consist of >95% B220+ cells. The B cells were incubated at 5 × 10⁶/ml for 24 or 48 h in medium alone, or in the presence of F(ab')₂ goat anti–mouse IgM (Jackson ImmunoResearch; 10 μg/ml) ± 25 μg/ml LPS (*Escherichia coli* 0111:B4; Calbiochem-Novabiochem Corp., La Jolla, CA) or 4 ng/ml recombinant mouse IFN-γ (Genentech, San Francisco, CA). After preincubation, the B cells were irradiated at 950 rad or treated with 50 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, MO) for 45 min at 37°C, before use in primary cultures.

*Bioassays for Monitoring B Cell Ag Presentation.* To examine the ability of B cells to present HGG to the Th1 cells, B cells were added (5 × 10⁶/ml unless otherwise specified) to 0.2-ml cultures of FH-purified Th1 cells (2.5 × 10⁵/ml) in the absence or presence of HGG (500 μg/ml unless otherwise specified). Proliferation was determined by measuring the amount of [³H[TdR incorporated during a 4-h pulse at 36 h of culture. To examine the ability of B cells to stimulate a MLR, the A/J-derived B cells (1.25 × 10⁶/ml) were added to 0.2-ml cultures of C57BL/6 spleen cells (1.25 × 10⁶/ml). Proliferation was determined by measuring the amount of [³H]TdR incorporated during a 4-h pulse on day 4.

Results

Ag-presenting Capacity of Activated B Cells. This study to examine the tolerogenicity of activated B cells was begun by determining whether activated B cells were in fact more efficient than resting B cells in presenting HGG to HGG-specific Th1 cells. Resting splenic B cells were left untreated or treated with F(ab')₂ goat anti–mouse IgM alone or in combination with LPS or IFN-γ for 24 h. At this time, the B cells were tested for their ability to serve as APCs in two different assays, i.e., ability to present HGG to HGG-specific Th1 cells, and ability to present allo-specific Ag in a MLR. As can be seen in Fig. 1, the most effective APCs in terms of presenting HGG to the Th1 clones were untreated spleen cells. As shown previously (7), untreated resting B cells were

very inefficient in presenting HGG to the Th1 clones. At high Ag concentrations, the ability of B cells to present HGG to Th1 cells was enhanced slightly by treatment with anti-Ig or LPS. Combinations of anti-Ig and LPS or INF-γ were no more effective than anti-Ig alone in stimulating B cell presentation of HGG. In terms of stimulating a MLR, unseparated spleen cells were again the most efficient APCs. Resting B cells were incapable of stimulating a MLR. In contrast, anti-Ig–treated B cells did stimulate a MLR. Combinations of anti-Ig and LPS or IFN-γ were also tested for their effects on B cell Ag presentation in the MLR, and were found to be no more effective than anti-Ig alone. Taken together, these results illustrate that anti-Ig treatment enhances the ability of resting B cells to perform in a MLR, but has much less effect on the ability of B cells to present HGG to HGG-specific Th1 cells.

Ability of Activated B Cells to Present Ag in a Tolerogenic Fashion to Th1 Cells. Even though anti-Ig–treated B cells were not efficient stimulators of HGG-specific Th1 cell proliferation, it seemed likely that the ability of these B cells to stimulate a MLR was due to expression of costimulatory molecules that would decrease, to at least some degree, the tolerogenicity of the activated B cells. To test this possibility, resting splenic B cells were incubated for 24 h in medium alone, in the presence of anti-Ig alone, or in combination with LPS. The B cells were then lightly irradiated (950 rad) and incubated in primary cultures with Th1 cells in the presence or absence of HGG. After 3 d, the Th1 cells from the primary cultures were isolated and reincubated in secondary cultures stimulated with HGG and irradiated spleen cells as APCs, or with IL-2-containing Con A CM. As shown previously, Th1 cells from primary cultures containing HGG and resting B cells lost their ability to proliferate in Ag-stimulated secondary cultures (Fig. 2). In contrast to our expectation, B cells treated with anti-Ig in the presence or absence of LPS also induced a dramatic decrease in the Ag-specific proliferative capacity of Th1 cells. Th1 cells incubated in primary cultures containing either resting or activated B cells but no HGG proliferated normally in the HGG-stimulated secondary cultures. All groups of Th1 cells proliferated in a similar fashion
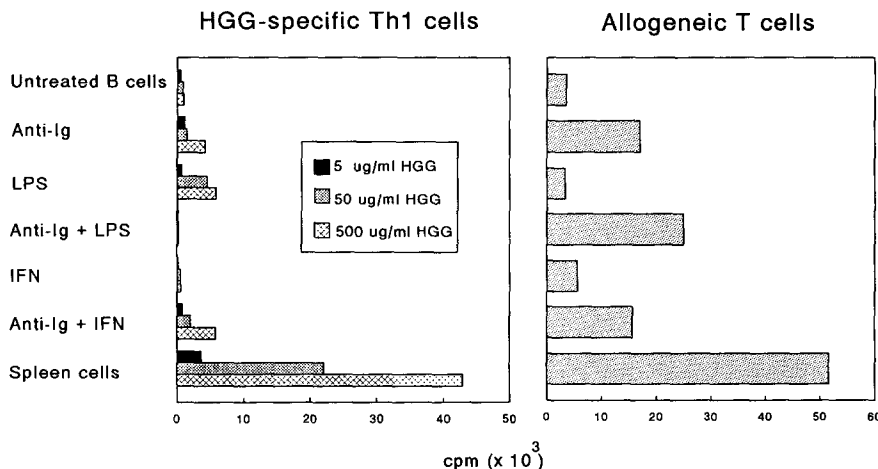
Figure 1. Stimulatory capacity of B cells treated with anti-Ig for 24 h. Splenic B cells treated with anti-Ig and/or LPS or IFN-γ for 24 h were compared with untreated splenic B cell and unseparated spleen cells for their ability to promote HGG-specific proliferation of HGG-specific Th1 clone JLB7, or for their ability to stimulate a primary MLR. In the absence of HGG none of the APC populations induced Th1 proliferation >1,000 cpm. Similar results were obtained with two other HGG-specific Th1 clones (12-11 and 4HE10).
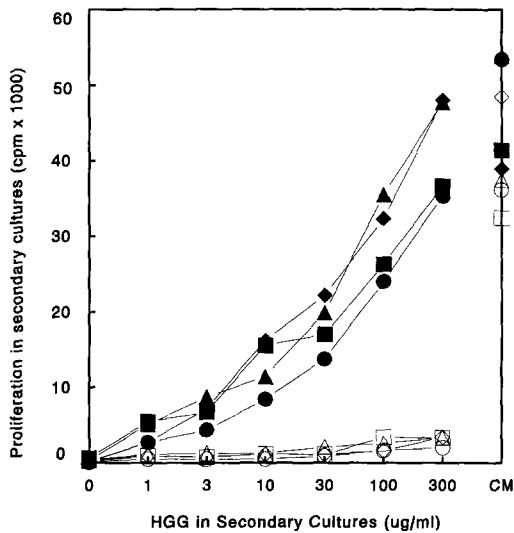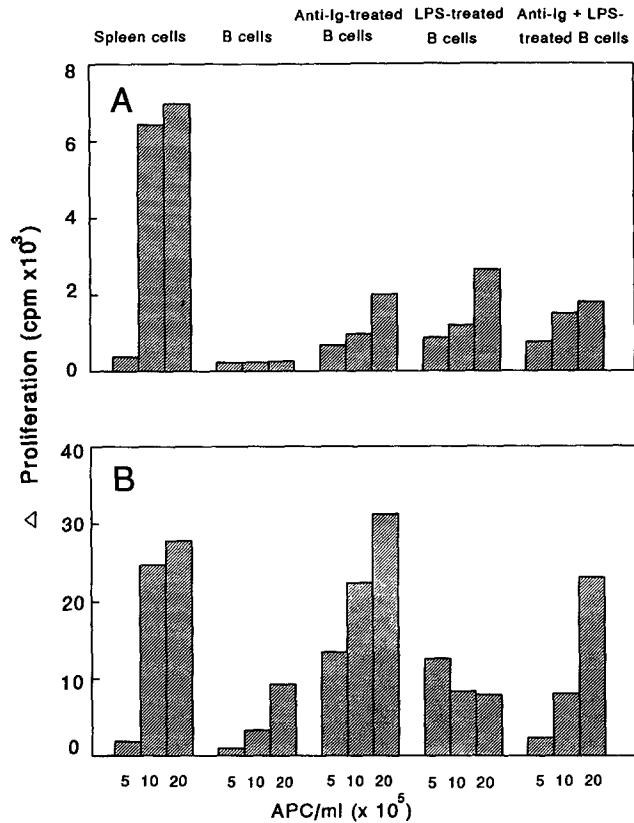
Figure 2. B cells treated with anti-Ig for 24 h still present Ag in a tolerogenic fashion to Th1 cells. Th1 cells (clone 12-11) were incubated in primary cultures with or without HGG together with B cells that were untreated, or that had been treated for 24 h with anti-Ig and/or LPS. After 3 d the Th1 were isolated from the primary cultures and reincubated in secondary cultures stimulated with HGG and APC. Additions to primary cultures: (■) B cells; (□) B cells + HGG; (●) anti-Ig–treated B cells; (○) anti-Ig–treated B cells + HGG; (▲) LPS-treated B cells; (△) LPS-treated B cells + HGG; (◆) anti-Ig and LPS-treated B cells; (◇) anti-Ig and LPS-treated B cells + HGG. Proliferation in the secondary cultures was measured. Very similar results were obtained in two other experiments using this as well as a second Th1 clone (4HE10).

Figure 3. Stimulatory capacity of B cells treated with anti-Ig for 48 h. Splenic B cells treated with anti-Ig in the presence or absence of LPS for 48 h were compared with untreated splenic B cells and unseparated spleen cells for their ability to (A) promote HGG-specific proliferation of the HGG-specific Th1 clone 12-11, or (B) stimulate a primary MLR.

in IL-2–containing secondary cultures. Based on these results, it appears that incubation of B cells with anti-Ig for 24 h does not inhibit the ability of the B cells to present HGG in a tolerogenic fashion to Th1 cells.

B cell tolerogenicity was dose dependent. A summary of five experiments revealed that in the presence of 500 μg/ml HGG, Th1 inactivation decreased from 78 to 66 to 49% when resting B cells were titrated from 2.5 × 10^6/ml to 5 × 10^5/ml to 10^5/ml, respectively, in primary cultures of Th1 cells. Experiments were also conducted in which Th1 cells were exposed in primary cultures to 500 μg/ml HGG and unseparated spleen cells in concentrations of 5 × 10^6/ml, 10^6/ml, or 2 × 10^5/ml. The Th1 cells were washed after 3 d, rested for 3 d, and reincubated in Ag-stimulated secondary cultures. The unseparated spleen cells were unable to inactivate Th1 cells by >16% at any concentration tested.

Antigenicity and Tolerogenicity of B Cells Stimulated for 48 Rather than 24 h. It has been reported that although the Ag-presenting capacity of B cells is enhanced after 24 h in the presence of B cell stimulants, this enhanced capacity does not become resistant to chemical fixation or moderate doses of irradiation (3,000 rad) until after 48 h in the presence of the stimulants (32, 33). Therefore, it seemed possible that the unaltered tolerogenicity of B cells stimulated for 24 h with anti-Ig was due to the fact that some aspect of B cell Ag presentation only becomes evident after 48 h in culture. This possibility was rendered unlikely when it was found

that B cells treated with anti-Ig for 48 h were still inefficient stimulators of HGG-specific proliferation by Th1 cells even though they were now as efficient as unseparated spleen cells in stimulating a MLR (Fig. 3). The inability of anti-Ig to stimulate B cells capable of stimulating HGG-specific proliferation of Th1 cells was not enhanced if LPS was used in conjunction with anti-Ig to pretreat the B cells. In addition, experiments to test the tolerogenicity of B cells stimulated with anti-Ig for 48 h showed that these B cells did not lose their ability to present HGG in a tolerogenic fashion to Th1 cells (Fig. 4). The anti-Ig–treated B cells were tolerogenic whether they were lightly irradiated or mitomycin C treated before use as APCs. Thus, activation of B cells for 48 rather than 24 h did not alter the tolerogenicity of the B cells.

Expression of Accessory Molecules on Activated B Cells. Experiments were conducted to determine the extent to which anti-Ig treatment enhanced B cell expression of accessory molecules. Splenic B cells were incubated in medium alone, or in the presence of anti-Ig. After 36 h, the B cells were subjected to fluorescent staining and FCF analysis. The B220$^+$ B cells were examined for their expression of B7 molecules, as well as other Ags (e.g., MHC class II molecules, RL388, and TfR) which can be used as markers for B cell activation.
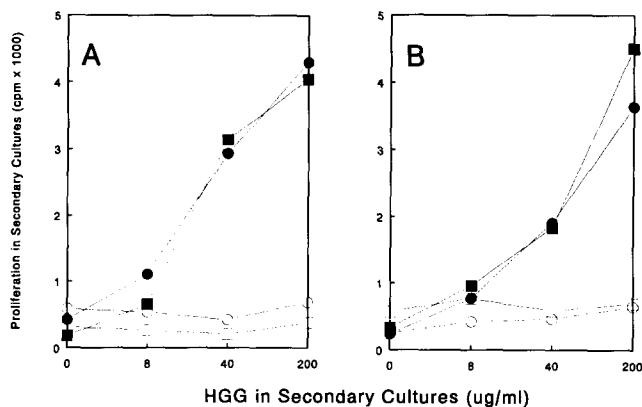
Figure 4. B cells treated with anti-Ig for 48 h still present Ag in a tolerogenic fashion to Th1 cells. Th1 cells (clone JLB7) were incubated in primary cultures with or without HGG together with B cells which were untreated or treated for 48 h with anti-Ig. The B cells were either irradiated at 950 rad (*A*), or treated with mitomycin C (*B*) before use in the primary cultures. Th1 cells isolated from the primary cultures were then reincubated in secondary cultures stimulated with HGG and splenic APC. Proliferation in the secondary cultures was measured. Th1 cells exposed in primary cultures to HGG in the absence of B cells were not inactivated. In experiment *B* Th1 cells exposed in primary cultures to HGG generated 2,261, 3,647, and 8,879 cpm when stimulated in secondary cultures with 8, 40, and 200 μg/ml HGG, respectively. Additions to primary cultures: (■) B cells; (□) B cells + HGG; (●) anti-Ig–treated B cells; (○) anti-Ig–treated B cells + HGG.

For example, hyperexpression of Ia molecules is used as a marker for early B cell activation (30), whereas upregulation of RL388 and TfR are observed during early and late G1 phase, respectively (32). Untreated resting B cells expressed moderate levels of MHC class II molecules, low levels of RL388 Ag and TfR, and no detectable B7 (Fig. 5). B cells treated with anti-Ig exhibited a large increase in their expression of Ia and RL388, and a definite shift in TfR expression. Most

1 = Resting B cells   2 = Anti-Ig-treated B cells
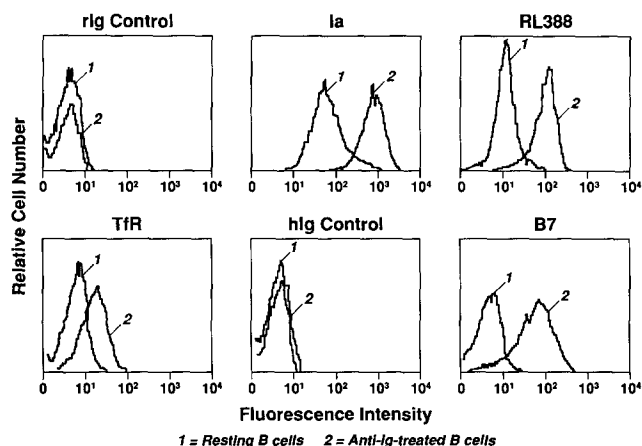
Figure 5. Expression of activation Ags on B cells stimulated with mitogens. Splenic B cells were cultured for 36 h in medium alone, or with anti-Ig. After incubation, the cells were two-color stained for B220 and either Ia, RL388, or TfR (with rat IgG control) or B7 (with human IgG control), and subjected to two-color FCF analysis. Shown are the Fl histograms of B200⁺ cells.
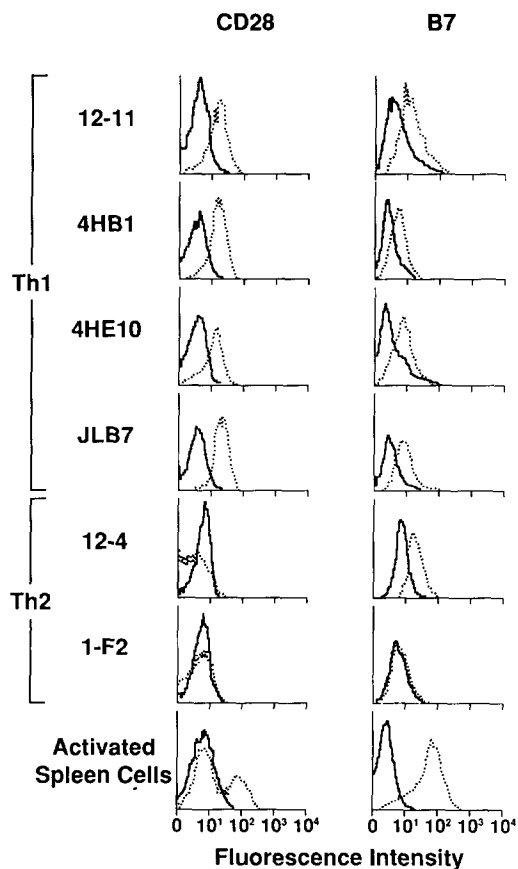
Figure 6. Expression of CD28 and B7 on HGG-specific Th cells. Unstimulated Th1 and Th2 cells or anti-CD3–treated spleen cells were stained for CD28 or B7, and subjected to FCF analysis. Shown are the Fl histograms of the positively stained cells (*dotted lines*) compared to their Ig controls (*solid lines*).
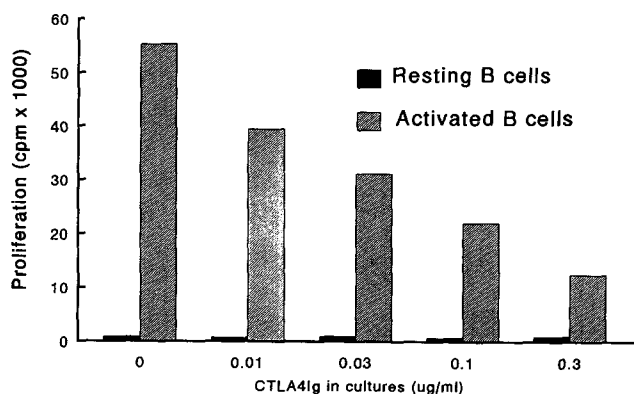
importantly, B7 expression on anti-Ig–treated B cells (mean fluorescence intensity [MFI], 77.1) was greatly enhanced as compared to resting B cells (MFI, 7.2). B7 expression was even higher (MFI, 243.8) on B cells treated with a combination of anti-Ig and LPS (data not shown). These results confirmed that treatment of B cells with anti-Ig in this system enhanced expression of several molecules, most notably B7, whose upregulation is associated with B cell activation.

Expression of CD28 and B7 on HGG-specific Th Clones. The fact that activated B cells expressing high levels of B7 retained the ability to present Ag in a tolerogenic fashion to Th1 cells could be explained if Th1 cells lacked CD28 and were therefore resistant to B7-mediated signaling. This possibility was examined and rejected after IF staining and FCF analysis of the Th1 cells. Moderate to high levels of CD28 were expressed on all of the HGG-specific Th1 clones examined (Fig. 6). In contrast, the Th2 clones examined expressed no detectable CD28. CD28 expression was not enhanced when the Th2 cells were stimulated for 36 h with anti-CD3 mAb (data not shown). Since B7 has been shown to be expressed on human T cell clones (34), the expression of B7 on the HGG-specific murine Th clones was also examined. The levels of
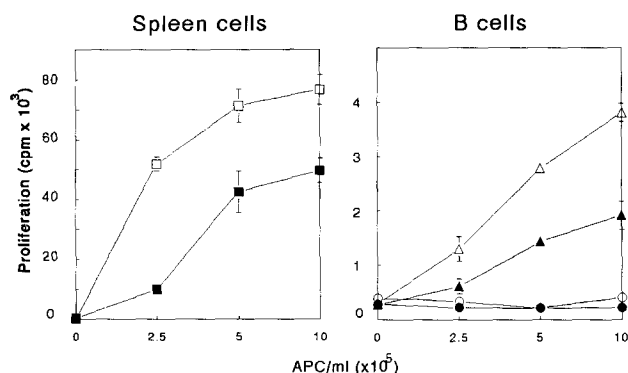
B7 expressed by the different clones varied from moderate to low, and was not restricted to a particular Th cell subset. High levels of B7 were detected on spleen cells treated with anti-CD3 mAb. Untreated spleen cells expressed very low levels of B7 (data not shown).

Confirming Roles for B7 and CD28 in this System. Even though CD28 was expressed on the HGG-specific Th1 cells, and B7 was expressed on the activated B cells used in these experiments, it seemed possible that for some reason in this system B7 and CD28 did not mediate the same effects observed in other systems. Accordingly, experiments were conducted to test the functional roles of B7 and CD28 in our system. This included an examination of whether the increased B7 expression observed on B cells treated with mitogens was associated with the increased capacity of the B cells to serve as APCs. The involvement of B7 in stimulating a MLR has been demonstrated by others (16, 35) who showed that blocking B7 interaction with CD28 by the use of B7 mAb or CTLA4Ig inhibited the MLR. In the experiment described here, CTLA4Ig was titrated into cultures containing resting or anti-Ig–treated splenic B cells from A/J mice (H-2ᵃ) as stimulator cells together with C57Bl/6 (H-2ᵇ) splenic responder cells. As described in Fig. 3, anti-Ig–treated B cells, unlike resting B cells, were efficient stimulators of a MLR (Fig. 7). Addition of CTLA4Ig blocked, in a dose-dependent fashion, the ability of activated B cells to stimulate the MLR. The effect of CTLA4Ig on HGG-induced proliferation of the Th1 cells was also examined. CTLA4Ig inhibited by 50% the modest ability of anti-Ig–treated B cells to promote HGG-specific proliferation (Fig. 8). The APC capacity of unseparated spleen cells was also inhibited by CTLA4Ig. These results suggest that the activated B cells used in these experiments express functionally active B7 molecules, and that the HGG-specific Th1 cells are responsive to B7-mediated signaling.

As confirmation that the HGG-specific Th1 cells were responsive to B7–CD28 interactions, anti-CD28 mAb was tested for its ability to interfere with the ability of HGG-pulsed fixed spleen cells to inactivate HGG-specific Th1 cells. In three separate experiments, Th1 cells were incubated in

Spleen cells   B cells

Figure 8. Ability of CTLA4Ig to inhibit HGG-specific Ag presentation by activated B cells. APCs preincubated for 1 h in the presence (*solid symbols*) or absence (*open symbols*) of CTLA4Ig (10 μg/ml) were titrated to cultures of Th1 cells (clone 12-11) and HGG. The APCs consisted of either unseparated spleen cells (*squares*), or resting (*circles*) or anti-Ig–treated (*triangles*) B cells.

the presence or absence of anti-CD28 mAb together with HGG presented by either paraformaldehyde-fixed unseparated spleen cells (HGG-FAPC), resting B cells, or anti-Ig–treated B cells. Control cultures of the Th cells received B cells in the absence of HGG, or fixed spleen cells prepared in the absence of HGG (FAPC). The Th1 cells were subsequently isolated from the primary cultures and reincubated in Ag-stimulated secondary cultures. As described previously, Th1 cells exposed in primary cultures to HGG-FAPC, unlike Th1 cells exposed to only FAPC, lost their ability to proliferate
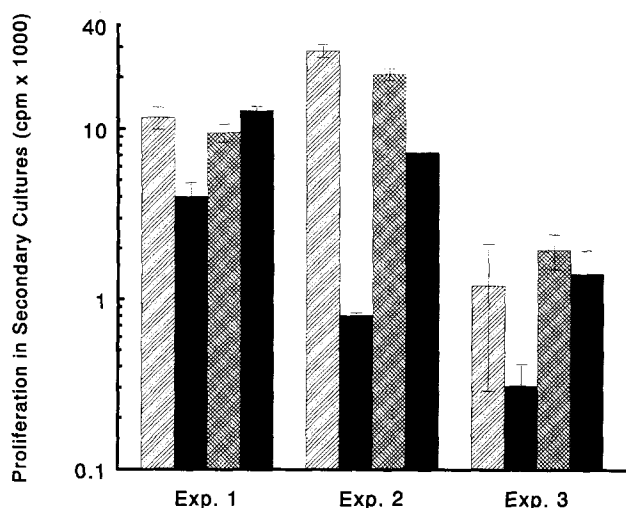

Figure 7. Ability of CTLA4Ig to block MLR stimulated by activated B cells. CTLA4Ig was titrated to cultures containing C57BL/6 spleen cells as responder cells, and stimulator cells consisting of untreated B cells or B cells treated for 48 h with anti-Ig Ab.

Figure 9. Ability of anti-CD28 mAb to block HGG-specific inactivation of Th1 cells. HGG-specific Th1 cells (clone 12-11) were incubated in primary cultures containing fixed spleen cells prepared in the absence of HGG (FAPC) or the presence of HGG (HGG-FAPC) (*Exp. 1*); resting B cells ± HGG (*Exp. 2*); or anti-Ig–treated B cells ± HGG (*Exp. 3*). Anti-CD28 mAb was also added to some primary cultures. Additions to primary cultures: (▨) APC; (■) APC + HGG; (▩) APC + anti-CD28; (▩) APC + HGG + anti-CD28. Th1 cells isolated from the primary cultures were subsequently reincubated in secondary cultures stimulated with HGG and APC. Proliferation was measured in the secondary cultures.

in HGG-stimulated secondary cultures (Fig. 9). The Ag-specific inactivation induced by HGG-FAPC was prevented if anti-CD28 mAb was also present in the primary cultures. Similarly, anti-CD28 mAb inhibited Th1 cell anergy induced by resting or anti-Ig–treated B cells. Taken together, these results show that at least some of the functional effects of CD28 and B7 described in other systems can be demonstrated in the present system.

Discussion

It seemed likely that the previously documented ability of resting B cells to present Ag to Th1 cells in a tolerogenic rather than stimulatory fashion (7) was due to a lack of B cell costimulatory molecules, notably B7. However, in contrast to expectations, treatments that were shown to enhance certain aspects of B cell Ag presentation, as well as B cell expression of accessory molecules including B7, were found here to have no effect on B cell tolerogenicity. Similar to untreated resting B cells, anti-Ig–activated B cells presented Ag to Th1 cells in a manner that induced Ag-specific proliferative inactivation. There is evidence to suggest that a similar phenomenon can also occur in vivo. Fuchs and Matzinger (10) showed that LPS-treated B cells from male mice were similar to resting B cells in their capacity to induce H-Y–specific inactivation when injected into female mice. This inactivation, which was demonstrated as a decrease in cytotoxic T cell responses and prolonged skin graft survival, was not induced if dendritic cells rather than B cells were injected. In potential contrast to our findings, it was shown in another study (8) that although divalent F(ab′)2 fragments of rabbit anti–mouse IgD did induce unresponsiveness to nonimmune rabbit Ig in mice, the F(ab′)2 fragments were less effective than monovalent fragments in inducing this inactivation. The authors suggested that the F(ab′)2 fragments of anti–mouse Ig activated the B cells by crosslinking surface Ig, and thereby reduced the tolerogenicity of resting B cells. However, the authors could not rule out the perhaps more likely possibility that the divalent fragments formed complexes with circulating IgD that were then captured and presented by professional APCs rather than B cells. In other studies, it was reported that even resting B cells possessed more costimulatory capacity for Th1 cells than described here (33), and could interfere with Th1 cell tolerance induction when added to cultures of Th1 cells in the presence of peptide and fixed spleen cells (36). The apparent disparity between these findings and our and others' results showing that resting B cells are poor costimulators for Th1 cells (3, 7), could be explained if certain Th1 clones have fewer requirements for costimulatory activity. Along these lines, it was shown subsequently that the Th1 clone A.E7, which was featured in both studies describing efficient B cell APC activity for Th1 cells, differed from other Th1 clones tested in that the A.E7 cells could bypass costimulator requirements and proliferate to immobilized anti-CD3 in the absence of accessory cells (37). Therefore, the results obtained using the A.E7 clone may not reflect the responses of other Th1 clones, or naive T cells, with more stringent costimulator requirements. Taken together, the

reported results of others are not inconsistent with our finding that resting B cells can present Ag in a tolerogenic fashion to Th1 cells, and that activation of the B cells does not inhibit this B cell activity.

The ability of anti-Ig–treated B cells to present Ag in a tolerogenic fashion to Th1 cells in the present system cannot be attributed to the fact that for some reason these B cells were not activated. Although B cell treatment with anti-Ig did not reverse B cell tolerogenicity, it did enhance certain aspects of B cell Ag presentation. Unlike resting B cells, B cells incubated with anti-Ig mAb for 24 or 48 h, efficiently stimulated a MLR, and were somewhat more effective in stimulating HGG-induced proliferation of HGG-specific Th1 cells. The activation state of the stimulated, but still tolerogenic B cells was further demonstrated by the enhanced expression of MHC class II molecules, RL388 and TfR, on these B cells. In addition, unlike untreated splenic B cells which exhibited undetectable levels of B7, splenic B cells treated with anti-Ig expressed high levels of B7. The functional relevance of the B7 expressed on the activated B cells used here was demonstrated by showing that the ability of the mitogen-activated B cells to stimulate a MLR or promote Ag-specific Th1 proliferation was inhibited by the addition of CTLA4Ig. CTLA4Ig, a soluble fusion protein incorporating the extracellular domain of CTLA-4 with the CH2 and CH3 region of human Ig/Cγ1 blocks B7 activity (35). Whereas it has not been reported that CTLA4Ig binds to FcRs on murine APCs, the possibility exists that CTLA4Ig blocks HGG-specific Ag presentation by inhibiting FcR-mediated uptake of HGG by APCs rather than by blocking B7 expression on APCs. Such a mechanism is, however, unlikely, and would not account for CTLA4Ig-mediated inhibition of a MLR. Thus, the results suggest that the levels of B7 expressed on the anti-Ig–stimulated B cells used in these experiments were functionally effective. It should be mentioned that even though anti-Ig treatment of B cells can stimulate expression of accessory molecules and DNA synthesis by these cells (32, 38), it can also make the B cells refractory to further stimulation (39). The anti-Ig–treated B cells used here were not tested for their subsequent proliferative capacity. However, even if the B cells were ultimately unresponsive, the results show that B cells that express B7, and are at least partially activated, can tolerize Th1 cells.

The tolerogenicity of activated B cells cannot be attributed to the fact that the Th1 cells used in this study were incapable of CD28-mediated signaling. All the Th1 cells used in the experiments were found to express CD28. The association of CD28 expression with Th1 clones fits in with the previous observation that mitogen-stimulated human CD28+ T cells make only those lymphokines produced by Th1 cells (40). The functional competence of the CD28 expressed by the Th1 cells in this study was demonstrated when anti-CD28 mAb was shown to inhibit tolerance induction in HGG-specific Th1 cells exposed to Ag-pulsed fixed APCs or Ag-presenting B cells. This finding is similar to that of Harding et al. (18) who showed that anti-CD28 mAb inhibited T cell inactivation when added to cultures of Th1 cells specific for λ repressor protein and Ag-pulsed fixed APCs.

Taken together, the results presented here suggest that the Th1 clones used in this study can be activated through CD28.

Although the HGG-specific Th1 cells appear to have functionally active receptors for B7, upregulation of B7 expression on anti-Ig–treated B cells was not sufficient to alter the tolerogenicity of these B cells for Th1 cells. This observation was somewhat surprising in view of the reported correlation between B7 expression and APC costimulatory activity. The ability of B cell tumor lines to stimulate a MLR has been shown to correlate with expression of B7 (41), and B7-transfected CHO cells, unlike untranslated cells, can costimulate a MLR, as well as mitogen-induced and Ag-specific T cell proliferation (42). In addition, blocking B7-mediated signaling appears to inhibit APC immunogenicity and enhance APC tolerogenicity. For example, it has been shown that CTLA4Ig treatment induced long-term tolerance to human pancreatic islet cells in mice (43), and caused T cell hyporesponsiveness to alloantigens in a human mixed leukocyte culture (44).

Although the studies described above argue convincingly that blocking B7-mediated signaling inhibits the costimulatory activity of B7-expressing APCs, and enables these APCs to present Ag in a tolerogenic fashion to CD4$^+$ T cells, the results of our study suggest that the reverse may not necessarily be true. It appears that increasing the B7 expression on tolerogenic APCs does not necessarily enable these APCs to present Ag in an immunogenic fashion to CD4$^+$ T cells. Similar to our findings using activated B cells, Freedman et al., (45) found that IFN-γ treatment did not enhance the costimulatory capacity of the monocytes, even though it significantly enhanced B7 expression on monocytes. The authors proposed that the contrast between the costimulatory activity of B7-expressing monocytes and the costimulatory capacity of B7-transfected cells was linked to a quantitative difference in B7 expression. It has been reported that CD28 can induce two different levels of costimulation, one that is mediated by a pathway independent of the TCR and requires minimal CD28 receptor oligomerization, and one that requires a high degree of receptor crosslinking and delivers a more potent primary signal to the T cells (for review see reference 15). If B7 is present on the APCs in sufficient quantities (e.g., on B7-transfected APCs) to induce extensive CD28 crosslinking, this costimulatory molecule may be all that is required for Th1 cell activation. On the other hand, it appears that under more physiological conditions, the level of B7 expression on activated B cells is not sufficient to fully costimulate Ag-specific proliferation of the Ag-specific Th1

cells. Presentation of Ag to Ag-specific Th1 cells may have more stringent requirements for costimulatory signals than other bioassays such as a primary MLR. Primary MLRs are often, as is the case in this study, performed in the presence of responder adherent cells which may provide accessory cell signals that amplify or even costimulate the signals provided by the alloantigen-bearing B cells. In addition, both Th1 and Th2 CD4$^+$ T cells, which have been shown to have different costimulator requirements (3), may participate in the MLR responder population.

The results from this study can be explained if Th1 cell activation requires a certain cumulative level of costimulatory signals in addition to TCR ligation. This level of costimulatory activity may not be attainable on B cells. Two possible exceptions must be mentioned. First, Th1 cells which for some reason have less stringent costimulator requirements than the Th1 cells used here, may be stimulated by B cell APCs. Second, it is possible that unlike the activating regimes used in this study, certain B cell stimulatory treatments (e.g., interaction with Ag-activated Th2 cells) may induce more potent B cell costimulators, and turn B cells into efficient APCs for Th1 cells. Bearing in mind these two possible caveats, it is possible to propose a model in which physiological levels of B7 on APCs are required but not sufficient for Ag-induced Th1 cell activation. This would mean that blocking B7 would inhibit Th1 cell activation and thus lead to tolerance induction, but that expression of B7 at physiological levels, in the absence of other costimulatory signal(s), would not lead to complete Th1 cell activation. Th1 cell activation may require an additional, non-B7 costimulator signal, such as has been implicated in Th1 stimulation by brain microvessel smooth muscle cells/pericytes (46).

The role of B7 in stimulating Th2 activation is unclear. The Th2 cells tested in this study did not express CD28, eliminating this means of B7-mediated signaling. However, it is possible that these Th2 cells have lost CD28 expression as a result of long-term tissue culture. Alternatively, these Th2 cells may express the other ligand for B7, CTLA4, and are therefore still susceptible to B7-mediated signaling. It is also possible that Th2 cells, which can be activated to produce IL-4 by TCR crosslinking alone (47), may not require signaling from B7 and/or other putative costimulatory molecules. A lack of costimulatory requirements by Th2 cells would explain why resting B cells, which can crosslink the TCR, but appear to lack costimulator molecules, are efficient APCs for Ag-induced Th2 cell but not Th1 cell proliferation (3, 7).

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Note added in proof: It has recently been shown that activated B cells express CTLA-4 counter-receptors which are distinct from the molecule termed B7 (48, 49). Since these CTLA-4 counter-receptors are apparently expressed by 48 h after B cell activation, a time at which we show that activated B cells are tolerogenic, it seems unlikely that expression of any of the family of B7 molecules is sufficient to interfere with B cell tolerogenicity in our system.

References

1. Jenkins, M.K., and R.H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* 165:302.
2. Gilbert, K.M., K.D. Hoang, and W.O. Weigle. 1990. Th1 and Th2 clones differ in their response to a tolerogenic signal. *J. Immunol.* 144:2063.
3. Gajewski, T.F., M. Pinnas, T. Wong, and F.W. Fitch. 1991. Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen-presenting cell populations. *J. Immunol.* 146:1750.
4. Baumhuter, S., C. Bron, and G.J. Corradin. 1985. Different antigen-presenting cells differ in their capacity to induce lymphokine production and proliferation of an apo-cytochrome c-specific T cell clone. *J. Immunol.* 135:989.
5. Taira, S., T. Kato, K. Yamamoto, T. Inoue, and H. Nariuchi. 1993. Differential requirement for humoral factors for IL-2R expression of murine T cell subsets, Th1, Th2, and CD8Th clones. *Cell. Immunol.* 147:41.
6. Kakiuchi, T., J. Mizuguchi, and H. Nariuchi. 1988. Molecular analysis of the dissociation between IL-2 production and proliferation in a response of a T cell clone to the antigen presented by B cells. *J. Immunol.* 141:3278.
7. Gilbert, K.M., and W.O. Weigle. 1992. B cell presentation of a tolerogenic signal to Th clones. *Cell Immunol.* 139:58.
8. Eynon, E.E., and D.C. Parker. 1992. Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. *J. Exp. Med.* 175:131.
9. Hori, S., S. Sato, S. Kitagawa, T. Azuma, S. Kukudo, T. Hamaoka, and H. Fujiwara. 1989. Tolerance induction of allo class II H-2 antigen-reactive L3T4+ helper T cells and prolonged survival of the corresponding class H-2 disparate skin graft. *J. Immunol.* 143:1447.
10. Fuchs, E.J., and P. Matzinger. 1992. B cells turn off virgin but not memory T cells. *Science (Wash. DC).* 258:1156.
11. Larsen, C.P., S.C. Ritchie, T.C. Pearson, P.S. Linsley, and R.P. Lowry. 1992. Functional expression of the costimulatory molecule, B7/BB1, on murine dendritic cell populations. *J. Exp. Med.* 176:1215.
12. Langhoff, E., and R.M. Steinman. 1989. Clonal expansion of human T lymphocytes initiated by dendritic cells. *J. Exp. Med.* 169:315.
13. Brunet, J.F., F. Denizot, M.F. Luciani, M. Roux-Dosseto, M. Suzan, M.G. Matte, and P. Golstein. 1987. A new member of the immunoglobulin super family, CTLA-4. *Nature (Lond.).* 328:267.
14. Linsley, P.S., E.A. Clark, and J.A. Ledbetter. 1990. The T cell antigen CD28 mediates adhesion with B cells by interacting with the activation antigen, B7/BB-1. *Proc. Natl. Acad. Sci. USA.* 87:5031.
15. June, C.H., J.A. Ledbetter, P.S. Linsley, and C.B. Thompson. 1990. Role of the CD28 receptor in T-cell activation. *Immunol. Today.* 11:211.
16. Azuma, M., M. Cayabyab, D. Buck, J.H. Phillips, and L.L. Lanier. 1992. CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. *J. Exp. Med.* 175:353.
17. Linsley, P.S., W. Brady, L.S. Grosmaire, A. Aruffo, N.K. Damle, and J.A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.* 173:721.
18. Harding, F.A., J.G. McArthur, J.A. Gross, D.H. Raultet, and J.P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature (Lond.).* 356:607.
19. Freeman, G.J., A.S. Freedman, J.M. Segil, G. Lee, J.F. Whitman, and L.M. Nadler. 1989. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J. Immunol.* 143:2714.
20. Qin, S., S. Cobbold, R.J. Benjamin, and H. Waldmann. 1989. Induction of classical transplantation tolerance in the adult. *J. Exp. Med.* 169:779.
21. Yokochi, T., R.D. Holly, and E.A. Clark. 1982. B lymphoblast antigen (BB-1) expressed on Epstein-Barr Virus–activated B cell blasts, B lymphoblastoid cell lines, and Burkitt's lymphomas. *J. Immunol.* 128:823.
22. Hawrylowicz, C.M., and E.R. Unanue. 1988. Regulation of antigen-presentation-I IFN-gamma induces antigen-presenting properties on B cells. *J. Immunol.* 141:4083.
23. Gosselin, E.J., H.-P. Tony, and D.C. Parker. 1988. Characterization of antigen processing and presentation by resting B lymphocytes. *J. Immunol.* 140:1408.
24. Casten, L.A., E.K. Lakey, M.L. Jelachich, E. Margoliash, and S.K. Pierce. 1985. Anti-immunoglobulin augments the B-cell-presentation function independently of internalization of receptor-antigen. *Proc. Natl. Acad. Sci. USA.* 82:5890.
25. Kakiuchi, T., R.W. Chesnut, and H.M. Grey. 1983. B cells as antigen-presenting cells: the requirement for B cell activation. *J. Immunol.* 131:109.
26. Krieger, J.I., S.F. Grammer, H.M. Grey, and R.W. Chesnut. 1985. Antigen presentation by splenic B cells: resting B cells are ineffective, whereas activated B cells are effective accessory cells for T cell responses. *J. Immunol.* 135:2937.
27. Ledbetter, J.A., G. Shu, M. Gallagher, and E.A. Clark. 1987. Augmentation of normal and malignant B cell proliferation by monoclonal antibody to the B cell-specific antigen BP50 (CDW40). *J. Immunol.* 138:788.
28. Thorley-Lawson, D.A., L.M. Nadler, A.K. Bhan, and R.T. Schooley. 1985. Blast-2 (EBVCS) an early cell surface marker of human B cell activation is super induced by Epstein-Barr Virus. *J. Immunol.* 134:3007.
29. Waldmann, T.A., C.K. Goldman, R.J. Robb, J.M. Depp, W.J. Leonard, S.O. Sharrow, K.F. Bongiovanni, S.J. Korsmeyer, and W.C. Greene. 1984. Expression of interleukin 2 receptors on activated human B cells. *J. Exp. Med.* 160:1450.
30. Monroe, J.G., and J.C. Cambier. 1983. Level of mIa expression

on mitogen-stimulated murine B lymphocytes is dependent on position in cell cycle. *J. Immunol.* 130:626.

31. Parks, D.E., M.V. Doyle, and W.O. Weigle. 1978. Induction and mode of action of suppressor cells generated against human gamma globulin. I. An immunologic unresponsive state devoid of demonstrable suppressor cells. *J. Exp. Med.* 148:625.

32. Ernst, D.N., D.N. McQuitty, W.O. Weigle, and M.V. Hobbs. 1988. Expression of membrane activation antigens on murine B lymphocytes stimulated with lipopolysaccharide. *Cell Immunol.* 114:161.

33. Weaver, C.T., C.M. Hawrylowicz, and E.R. Unanue. 1988. T helper cell subsets require the expression of distinct costimulatory signals by antigen-presenting cells. *Proc. Natl. Acad. Sci. USA.* 85:8181.

34. Azuma, M.A., H. Yssel, J.H. Phillips, H. Spits, and L.L. Lanier. 1993. Functional expression of B7/BB1 on activated T lymphocytes. *J. Exp. Med.* 177:845.

35. Linsley, P.S., W. Brady, M. Urnes, L.S. Grosmaire, N.K. Damle, and J.A. Ledbetter. 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* 174:561.

36. Ashwell, J.D., M.K. Jenkins, and R.H. Schwartz. 1988. Effect of gamma radiation on resting B lymphocytes II. Functional characterization of the antigen-presentation defect. *J. Immunol.* 141:2536.

37. Williams, I.R., and E.R. Unanue. 1990. Costimulatory requirements of murine Th1 clones. *J. Immunol.* 145:85.

38. Klaus, G.G.B., C.M. Hawrylowicz, and C.J. Carter. 1985. Activation and proliferation signals in mouse B cells. *Immunology.* 55:411.

39. Warner, G.L., and D.W. Scott. 1991. A polyclonal model for B cell tolerance. *J. Immunol.* 146:2185.

40. Thompson, C.B., T. Lindsten, J.A. Ledbetter, S.L. Kunkel, H.A. Young, S.G. Emerson, J.M. Leiden, and C.H. June. 1989. CD28 activation pathways regulates the production of multiple T-cell–derived lymphokines/cytokines. *Proc. Natl. Acad. Sci. USA.* 86:1333.

41. Jenkins, M.K., P.S. Taylor, S.D. Norton, and K.B. Urdahl. 1991.

CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol.* 147:2461,

42. Galvin, F., G.J. Freeman, Z. Razi-Wolf, W. Hall, B. Benacerraf, L. Nadler, and H. Reiser. 1992. Murine B7 antigen provides a sufficient costimulatory signal for antigen-specific and MHC-restricted T cell activation. *J. Immunol.* 149:3802.

43. Lenschow, D.J., Y. Zeng, J.R. Thistlethwaite, A. Montag, W. Brady, M.G. Gibson, P.S. Linsley, and J.A. Bluestone. 1992. Long-term survival of zenogeneic pancreatic islet grafts induced by CTLA4Ig. *Science (Wash. DC).* 257:789.

44. Tan, P., C. Anasetti, J.A. Hansen, J. Melrose, M. Brunvand, J. Bradshaw, J.A. Ledbetter, and P.S. Linsley. 1993. Induction of alloantigen-specific hyporesponsivness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. *J. Exp. Med.* 177:165.

45. Freedman, A.S., G.J. Freeman, K. Rhynhart, and L.M. Nadler. 1991. Selective induction of B7/BB-1 on interferon-gamma stimulated monocytes: a potential mechanism for amplification of T cell activation through the CD28 pathway. *Cell. Immunol.* 137:429.

46. Fabry, Z., M. Sandor, T.F. Gajewski, J.A. Herlein, M.M. Waldschmidt, R.G. Lynch, and M.N. Hart. 1993. Differential activation of Th1 and Th2 CD4$^+$ cells by murine brain microvessel endothelial cells and smooth muscle/pericytes. *J. Immunol.* 151:38.

47. Munoz, E., A. Zubiaga, D. Olson, and B.T. Huber. 1989. Control of lymphokine expression in T helper 2 cells. *Proc. Natl. Acad. Sci. USA.* 86:9461.

48. Freeman, G.J., J.G. Gribben, V.A. Boussiotis, J.W. Ng, V.A. Restivo, Jr., L.A. Lombard, G.S. Gray, and L.M. Nadler. 1993. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. *Science (Wash. DC).* 262:909.

49. Hathcock, K.S., G. Laszlo, H.B. Dickler, J. Bradshaw, P. Linsley, and R.J. Hodes. 1993. Identification of an alternative CTLA-4 ligand costimulatory for T cell activation. *Science (Wash. DC).* 262:905.