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Immunology

Anergic self-reactive B cells present self antigen and respond normally to CD40-dependent T-cell signals but are defective in antigen-receptor-mediated functions

(transgenic/self tolerance/antigen processing/B-cell activation)

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ABSTRACT **B-cell tolerance to soluble protein self antigens such as hen egg lysozyme (HEL) is mediated by clonal anergy. Anergic B cells fail to mount antibody responses even in the presence of carrier-primed T cells, suggesting an inability to activate or respond to T helper cells. To investigate the nature of this defect, B cells from tolerant HEL/anti-HEL double-transgenic mice were incubated with a membrane preparation from activated T-cell clones expressing the CD40 ligand. These membranes, together with interleukin 4 and 5 deliver the downstream antigen-independent CD40-dependent B-cell-activating signals required for productive T–B collaboration. Anergic B cells responded to this stimulus by proliferating and secreting antibody at levels comparable to or better than control B cells. Furthermore, anergic B cells presented HEL acquired *in vivo* and could present the unrelated antigen, conalbumin, targeted for processing via surface IgD. In contrast, the low immunoglobulin receptor levels on anergic B cells were associated with reduced *de novo* presentation of HEL and a failure to upregulate costimulatory ligands for CD28. These defects in immunoglobulin-receptor-mediated functions could be overcome *in vivo*, suggesting a number of mechanisms for induction of autoantibody responses.**

Recent studies of B-cell activation by T-cell clones are consistent with a four-step model of T-cell–B-cell (T–B) collaboration (1–5). In the first step, B cells capture and internalize antigen via their cell surface immunoglobulin (Ig) receptor and process and then present peptide fragments in association with the major histocompatibility complex class II on the cell surface (6). In the second step, contact between the antigen-presenting B cell and a specifically primed T cell leads to T-cell activation via engagement of the T-cell receptor and a number of accessory molecules including CD28 (7). This interaction may be enhanced by the antigen-induced increase in a number of molecules on the B-cell surface including class II major histocompatibility complex, the ligands for CD28 (8, 9), and other adhesion molecules (10–12). Activation of T cells by B cells is accompanied by secretion of lymphokines and transient expression of new cell surface molecules, particularly the ligand for CD40 (2, 4). In the third step, B cells are induced to proliferate after contact with the activated T-cell surface (1). Interaction between CD40 and its ligand on the B-cell surface is essential for this cell-contact-mediated event (3). Finally, concomitant exposure of cell-contact-activated B cells to T-cell-derived lymphokines leads to their differentiation into antibody-secreting cells (1).

A double-transgenic (Dbl-Tg) model has been established to investigate the cellular basis of self tolerance in the B-cell repertoire. In this model, B cells expressing a transgene-encoded Ig receptor for the protein antigen hen egg lysozyme (HEL) were exposed throughout development to HEL in soluble form to render the B cells anergic (13). The anergic state was characterized by downregulation of IgM but not IgD antigen receptors and maturation arrest of the B cells in the splenic follicular mantle zone (14, 15). After removal from the tolerant environment, the self-reactive B cells failed to respond to T-cell help supplied by carrier-specific T cells, indicating that B-cell anergy is associated with a defect in the antigen-dependent interaction between B cells and primed T helper (Th) cells (16, 17). Theoretically this defect could be due to a failure by anergic B cells to activate or to respond to Th cells. Here we use the four-step model of T–B collaboration, mentioned previously, as the basis for defining the mechanism of B-cell anergy.

MATERIALS AND METHODS

Mice. Two lines of transgenic (Tg) mice on a C57BL6 (H-2^b) background were produced, one carrying the HEL gene under control of the mouse metallothionein-1 promoter (ML5) and the other expressing the rearranged heavy ($\mu + \delta$) and light chain genes encoding high-affinity anti-HEL IgM and IgD marked by the IgH^a allotype (MD4) (13). Dbl-Tg mice were created either by mating the two hemizygous lines or by reconstituting irradiated (950 rads; 1 rad = 0.01 Gy) ML5 Tg mice with 3×10^6 MD4 immunoglobulin-transgenic (Ig-Tg) bone marrow cells (13). For experiments involving the I-A^k-restricted HEL-specific hybridoma 3A9 (18), the Dbl-Tg mice and their Ig-Tg littermates were bred onto a CBA (H-2^k) background. Age-matched non-Tg mice were from the C57BL6, (C57BL6 × CBA)F_1, or B10BR strains. All mice were used between 8 and 20 weeks of age.

Reagents. Conalbumin and HEL were purchased from Sigma. The peptide containing HEL residues 46–61 [HEL-(46–61)] was synthesized by P. Peake (Centenary Institute). Recombinant interleukin (IL) 4 and IL-5 were kindly provided by R. Kastelein and R. Coffman (DNAX) and used at 100 units/ml or 1 ng/ml, respectively. In some experiments supernatant from concanavalin A-stimulated D10G4.1 cells (19) served as the source of cytokines, a 1:100 dilution being equivalent to IL-4 at 142 units/ml (HT-2 bioassay) (20) and IL-5 at 31 units/ml (BCL1 bioassay) (21). Affinity-purified goat anti-mouse IgM was purchased from Cappell.

Purified Cell Populations. B cells were prepared from single-cell suspensions of spleen cells (three mice per group) by depleting erythrocytes and T cells as described (1).

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Abbreviations: CFA, complete Freund's adjuvant; Tg, transgenic; Ig, immunoglobulin; Dbl-Tg, double Tg; HEL, hen egg lysozyme; IgD–Con, conalbumin–Fab anti-IgD^a; Ig-Tg, immunoglobulin Tg; Th, T helper; LPS, lipopolysaccharide.

HEL-primed T cells depleted of B cells with anti-mouse-Ig-conjugated Dynabeads (Dynal, Oslo) were obtained from inguinal lymph nodes of mice primed with HEL in complete Freund's adjuvant (CFA) (100 μg) as described (13).

Membrane Preparation and Fusion Proteins. Plasma membranes containing the CD40 ligand were prepared as described (1, 22) from activated HDK-1 Th1 (20) or D10 Th2 (19) T-cell clones. CD40-Ig fusion protein was prepared as outlined (23). Transfected cell lines secreting fusion proteins of murine CD40 ligand–murine CD8 (24) and CTLA-4–human IgG1 Fc (CTLA-4–Ig) (25) were kindly provided by P. Lane (Basel Institute).

Flow Cytometric Analysis and Sorting. Binding of CD40 ligand–CD8 and CTLA-4–Ig to B cells was detected with anti-CD8 biotin and biotinylated mouse anti-human IgG $F(ab')_2$ (Jackson ImmunoResearch), respectively, followed by streptavidin-phycoerythrin. To detect IgD^a and IgM^a expression, fluorescein isothiocyanate conjugated anti-IgD(AMS 9.1) and anti-IgM (RS3.1) coupled to allophycocyanin (15) were included with the fusion protein. Fluorescence intensity was determined by dual laser flow cytometry on a FACStar Plus (Becton Dickinson). For cell sorting, a single-cell deposition unit was used to place 25,000 selected cells directly into wells of a microtiter tray. Dbl-Tg spleen cells sorted for HEL binding were stained with HyHEL5-biotin, which labels HEL bound to the Tg receptor (15) followed by streptavidin-phycoerythrin.

Proliferation. Purified B cells (2×10^4 cells per well) were cultured for 48 h in flat-bottom microtiter plates (Nunc) with various concentrations of Th membranes in 100 μl of B-cell medium (1). HEL (20 ng/ml), IL-4 (100 units/ml), and IL-5 (1 ng/ml) were added to half of the cultures. During the last 4 h, the wells were pulse-labeled with [^{3}H]thymidine (1 μCi per well; 1 Ci = 37 GBq; Amersham). For cultures with lipopolysaccharide (LPS from *Escherichia coli* 0111:B4, Difco), 5×10^4 B cells per well were used.

***In Vitro* Antibody Responses.** Purified B cells were cultured for 8 days in flat-bottom microtiter plates with various concentrations of Th membranes in 200 μl of B-cell medium containing D10 supernatant with or without addition of HEL. Supernatants were removed daily from triplicate cultures between days 3 and 8 and assayed for cumulative antibody production. Specific antibody was measured by ELISA for IgM^a (transgene allotype) as described for anti-HEL IgM^a (13) except that the wells were coated with the monoclonal antibody RS3-1 at 2.5 μg/ml. Total antibody was assayed by ELISA in which wells were coated with rabbit anti-mouse Ig (Dakopatts) at 5 μg/ml, binding being detected with alkaline phosphatase-conjugated goat anti-mouse Ig (Sigma) in phosphate-buffered saline containing 0.1% bovine serum albumin and 5% (vol/vol) rabbit serum.

Adoptive Transfer. Approximately 5×10^5 purified splenic B cells and 1×10^7 HEL-primed T cells from F_1 donors were injected intravenously into 750-rad-irradiated (C57BL6 × CBA)F_1 recipients and challenged with 100 μg of HEL in CFA intraperitoneally. Serum levels of specific anti-HEL IgM^a antibody were measured by ELISA 10 days later (13).

Antigen Presentation. The 3A9 T hybridoma cells, which secrete IL-2 and IL-3 on recognition of HEL-(46–61) peptide were provided by A. Gautam (John Curtin School of Medical Research), who originally obtained them from Paul Allen (Washington University School of Medicine, St. Louis) (18). T-cell-depleted spleen cells from Tg or non-Tg (C57BL6 × CBA)F_1 mice were incubated for 24 h with 1×10^5 3A9 cells in 200 μl of RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum (CSL), 50 μM 2-mercaptoethanol, and 2 mM glutamine. The cultures included various concentrations of HEL or the HEL-(46–61) peptide. Activation of the T hybridoma was determined by measuring IL-3 production in endpoint units using the IL-3-dependent cell line, 32Dcl (26).

For targeting conalbumin processing through surface IgD^a, a Fab fragment of AMS9.1 (27) was chemically coupled to conalbumin at a molar ratio of 1:1 as described (28) to form IgD–Con. Presentation of conalbumin was detected by incubating B cells (10^5 cells per well) with an equivalent number of D10 T-hybridoma cells under the same conditions as for 3A9 except that IL-4 served as the readout. IL-4 (in units/ml) was measured by ELISA using 11B11 (29) at 5 μg/ml as the coat and biotinylated BVD6/24G2 monoclonal antibody (hybridoma generously provided by J. Abrams, DNAX) at 0.6 μg/ml for detection.

RESULTS

Th Membrane Induces a Proliferative Response in Anergic B Cells from Dbl-Tg Mice. Initially, the mechanism of B-cell anergy was examined by determining whether a plasma membrane preparation from activated T-cell clones containing the CD40 ligand could reverse the anergic state. These Th membranes can bypass the first two antigen-dependent steps in the four-step model of T–B collaboration described above by delivering the cell-contact-mediated antigen-independent CD40-dependent activation signals responsible for inducing proliferation of B cells followed by their differentiation into antibody-secreting cells on addition of the Th2 lymphokines IL-4 and IL-5 (1, 3).

Th membrane induced strong proliferative responses in B cells purified from the spleens of non-Tg, Ig-Tg, and Dbl-Tg littermates in the presence or absence of HEL (Fig. 1 *a–c*). In the standard 48-h culture, the highest response was obtained from Dbl-Tg B cells and was accompanied by upregulation of surface IgM (data not shown). The greater response of these cells was due to the fact that proliferation by these B cells peaked earlier than it did for the other two cell types (data not shown), presumably reflecting prior exposure to HEL *in vivo*.

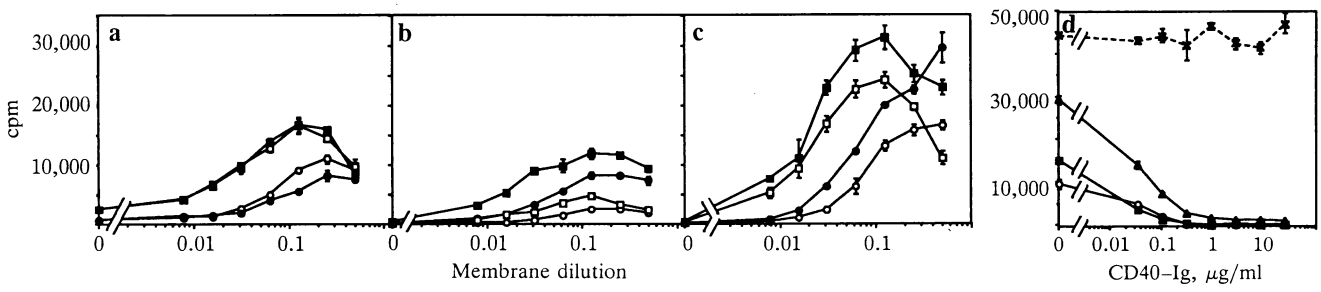

FIG. 1. Proliferation of splenic B cells from non-Tg (*a*), Ig-Tg (*b*), and Dbl-Tg (*c*) mice in response to stimulation with various concentrations of Th membranes. (*a–c*) Responses of 2×10^4 B cells per well cultured with Th membrane (HDK-1) alone (open symbols) or membrane plus HEL (solid symbols) in the presence (squares) or absence (circles) of recombinant IL-4 and IL-5. (*d*) Inhibitory effect of CD40–Ig fusion protein on the proliferative response to Th membrane of B cells from non-Tg (triangles), Ig-Tg (circles), and Dbl-Tg (squares) mice. Non-Tg B cells (5×10^4 cells) cultured with LPS (×) served as the specificity control since LPS stimulation is not CD40-dependent.

Th Membrane Induces Antibody Production by Anergic B Cells from Dbl-Tg Mice. The effect of Th membranes on total antibody (Ig) and IgM^a secretion by anergic B cells was tested by adding lymphokines to cultures containing 1×10^4 or 2×10^4 purified B cells in the presence or absence of HEL. At both cell numbers, Dbl-Tg B cells secreted similar or higher amounts of total Ig (data not shown) and IgM^a compared to Ig-Tg B cells (Fig. 2 *a* and *b*). The presence of HEL in the cultures at 20 ng/ml increased the yield of antibody from Ig-Tg B cells but had little influence on Dbl-Tg B cells.

Soluble CD40 Specifically Inhibits the Stimulatory Effects of Th Membrane. The level of CD40 on the surface of anergic B cells from Dbl-Tg mice was compared with those on Ig-Tg and non-Tg B cells by flow cytometric analysis using a CD40 ligand–CD8 fusion protein. The results indicated that anergy was not associated with a decrease in CD40 expression (data not shown).

To confirm that the interaction between the CD40 receptor and ligand pair was important for Th membrane activation of anergic B cells, a soluble CD40–Ig fusion protein was added to cultures of Tg B cells stimulated with Th membranes and lymphokines. Low concentrations of CD40–Ig (0.1–1 μg/ml) resulted in almost complete inhibition of both proliferation and antibody production (Figs. 1*d* and 2*c*). Inhibition was specific since CD40–Ig did not influence the responses of non-Tg B cells to LPS (Figs. 1*d* and 2*d*). Thus these results established that anergic B cells can respond to Th cell signals delivered by the CD40 pathway and confirmed the reversibility of B-cell anergy (30).

Anergic B Cells from Dbl-Tg Mice Constitutively Present Self Antigen and Process Antigen *de Novo*. Antigen processing and presentation was examined by culturing B cells from Tg or non-Tg mice on a (CBA × C57BL6)F_1 background with the HEL-specific hybridoma 3A9 using IL-3 release as the indicator of antigen recognition. Activation of this hybridoma did not require, nor was it enhanced by costimulation through CD28 (data not shown), thereby allowing processing to be tested independently of costimulatory activity. As expected, exposure of Ig-Tg B cells to HEL resulted in secretion of significant amounts of IL-3 by 3A9, although the level differed from one experiment to the next (Fig. 3 *a* vs. *b*). In contrast Dbl-Tg B cells consistently stimulated 3A9 in the absence of exogenous antigen.

Constitutively presented self antigen could have been acquired and processed from the serum of Dbl-Tg mice or from transgene-encoded HEL expressed within the B cells from these mice. To distinguish between these possibilities, irradiated HEL Tg mice were reconstituted with Ig-Tg bone marrow to create a chimera resembling Dbl-Tg mice. B cells from these chimeras, despite lacking the HEL transgene, could still present HEL constitutively (Fig. 3*c*). Moreover, when they were sorted into HEL-binding and non-HEL-binding populations, only the former population of B cells stimulated 3A9 (Fig. 3*c*). Thus the B cells utilized their antigen receptors to process and present self antigen *in vivo*.

Constitutive presentation of HEL to 3A9 by B cells was studied in a series of four experiments and proved to be 3–20 times less efficient than presentation of exogenous HEL by Ig-Tg B cells at concentrations (10^{-7}–10^{-2} μg/ml) that required processing via the antigen-specific receptor (Fig. 3 *a* and *b*). In one of these experiments (Fig. 3*a*), the small difference in 3A9 stimulation could be explained entirely on the basis of the lower numbers of HEL-binding B cells present in F_1 Dbl-Tg spleen. Thus only 25% of T-cell-depleted spleen cells from this source bound HEL compared to 90% from Ig-Tg spleen (data not shown). In another experiment (Fig. 3*b*), the difference was much greater (20-fold) and indicated that on a per cell basis, constitutive presentation was lower than Ig-mediated presentation by nontolerant B cells. Under these conditions, addition of exogenous HEL failed to enhance stimulation of 3A9 by the Dbl-Tg B cells until concentrations sufficient to bypass the specific Ig receptor were attained (10–100 μg/ml) (31, 32).

These observations suggested impairment of Ig-receptor-dependent antigen presentation as a result of either the 10- to 30-fold reduction in IgM levels on anergic B cells (33) or impairment of Ig-receptor-mediated processing *per se*. To distinguish between these two possibilities, the ability of Ig-Tg and Dbl-Tg B cells to present conalbumin chemically coupled to anti-IgD^a (IgD–Con) to the specific T-cell clone D10 was tested. This reagent effectively targeted conalbumin presentation through IgD^{a+} (CBA) and not IgD^{b+} (B10.BR) B cells, whereas B cells from both strains presented high concentrations of soluble conalbumin equally effectively (Fig. 3*d* and data not shown). Sorted HEL-binding B cells from Dbl-Tg mice presented IgD–Con to D10 at ≈50% the level of sorted Ig-Tg B cells. Thus, there was no major processing defect associated with surface IgD on anergic B cells, implying that the low levels of surface IgM were primarily responsible for reduced *de novo* antigen processing by anergic B cells.

Antigen-Receptor-Induced Expression of Costimulatory Ligands Is Reduced on Anergic B Cells from Dbl-Tg Mice. An important receptor for costimulation of primed T cells to deliver help to B cells is CD28. Two known ligands for CD28, B7 and GL1, are poorly expressed on resting B cells but can be induced to high levels after antigen-receptor crosslinking (8, 12). To examine the ability of B cells to express these ligands, a CTLA-4–Ig fusion protein was used (25). CTLA-4 is homologous to CD28 and has a high affinity for B7 and GL1 (8, 9, 34). After stimulation with anti-IgM, non-Tg, and Ig-Tg B cells expressed high levels of CTLA-4 ligand (Fig. 4 *a* and *c*). The constitutive level on Dbl-Tg B cells, which express

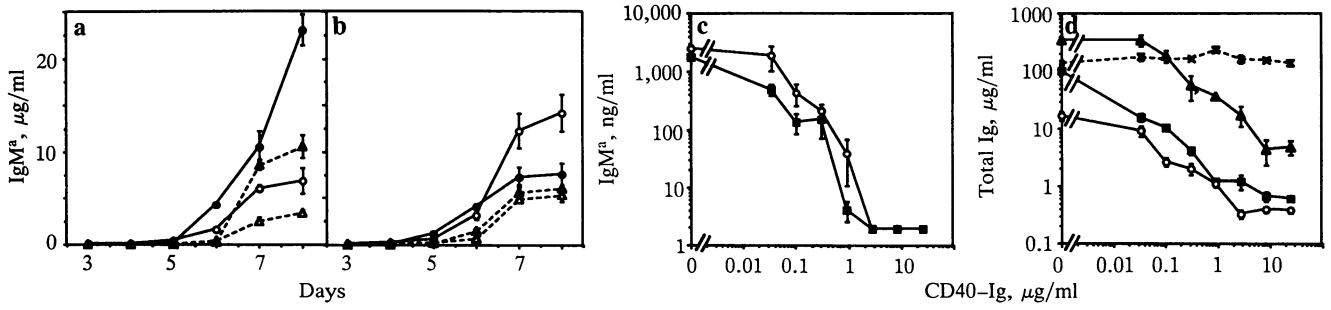

FIG. 2. Cumulative transgene-specific IgM^a antibody production over 8 days by splenic B cells from Ig-Tg (*a*) and Dbl-Tg (*b*) mice in response to stimulation with Th membrane. B cells at 10^4 cells per well (triangles) or 2×10^4 cells per well (circles) were cultured in the presence of Th membrane and lymphokines without HEL (open symbols) or with HEL (solid symbols). Unstimulated B cells from non-Tg, Ig-Tg, and Dbl-Tg cultures secreted total Ig at 25, 90, and 85 ng/ml, respectively, and IgM^a at <8 ng/ml. The inhibitory effect of CD40-Ig on IgM^a (*c*) and total Ig (*d*) responses to Th membrane and lymphokines by 2×10^4 B cells per well from non-Tg (triangles), Ig-Tg (circles), and Dbl-Tg (squares) mice is shown. Non-Tg B cells (5×10^4 cells per well) cultured with LPS (×) served as the specificity control.

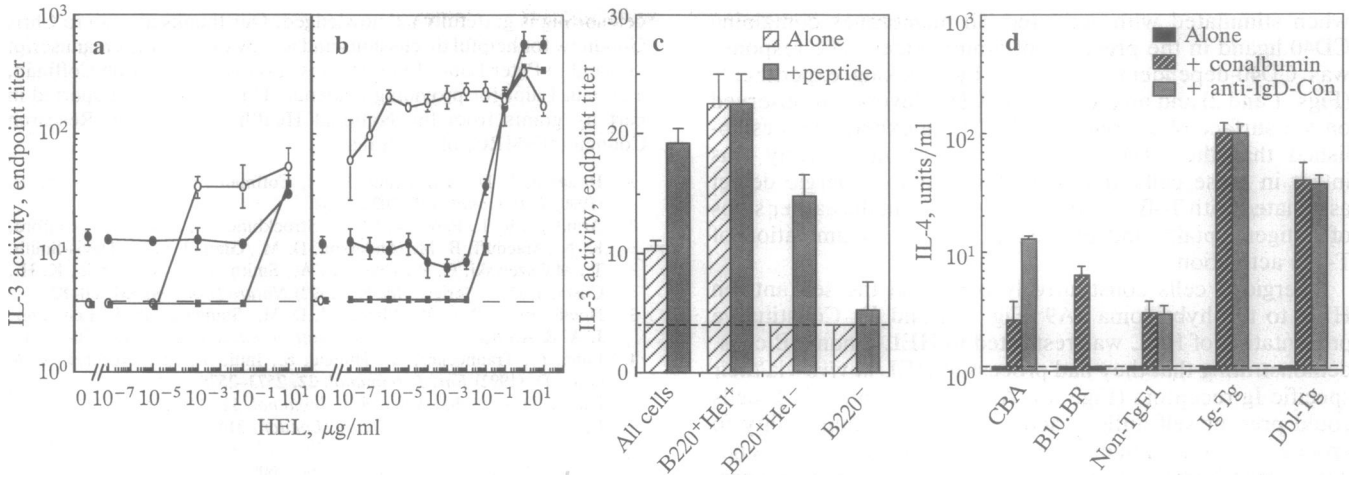

FIG. 3. Anergic B cells present HEL constitutively and can process antigen via IgD. (*a* and *b*) IL-3 released from 1×10^5 3A9 cells after incubation with various concentrations of HEL in the presence of 5×10^4 T-cell-depleted B cells from non-Tg (solid squares), Ig-Tg (open circles), or Dbl-Tg (solid circles) mice. (*c*) IL-3 released from 3A9 cells incubated with cells sorted from the spleen of three Ig-Tg into HEL Tg bone marrow chimeras. Cells were sorted into four groups either without discrimination (all cells) or according to staining with B220 and HyHEL5. A total of 25,000 cells per well was incubated alone or with HEL-(46–61) peptide at 25 μg/ml and 3A9. (*d*) Presentation of conalbumin to D10. Cells from the strains indicated were incubated with 1×10^5 resting D10 cells for 24 h alone, with conalbumin at 100 μg/ml, or with IgD-Con at 2.5 μg/ml. T-cell-depleted spleen cells from CBA, B10.BR, and non-Tg (C57BL6 × CBA)F_1 cells were added at 1×10^5 cells per culture. Ig-Tg and Dbl-Tg cells were sorted directly at 25,000 cells per well. Ig-Tg cells were sorted as $B220^+$ (98% purity) and Dbl-Tg cells were sorted as HEL^+ (98% purity). All data points show the mean and SEM of triplicate cultures.

20- to 30-fold less surface IgM (33), was comparable to that on the other B cells but increased only slightly after stimulation (Fig. 4*e*). When Tg B cells were exposed to HEL at 100 ng/ml, a similar trend was observed, enhanced expression of CTLA-4–Ig ligand binding being restricted to Ig-Tg B cells (Fig. 4 *b*, *d*, and *f*).

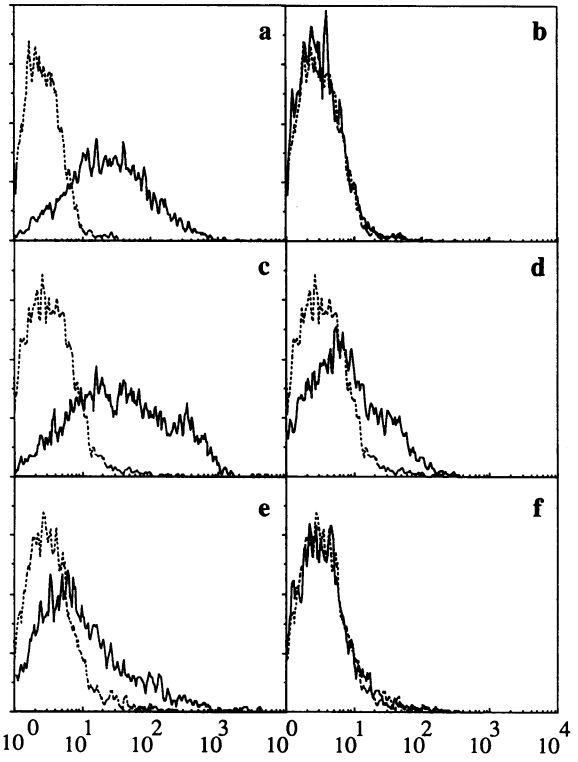

FIG. 4. Expression of CTLA-4 ligand on non-Tg and Tg B cells. CTLA-4 ligand levels were determined before and after Ig-receptor engagement. Approximately 1×10^6 B cells from the spleen of non-Tg (*a* and *b*), Ig-Tg (*c* and *d*), and Dbl-Tg (*e* and *f*) mice were incubated for 30 h either alone (unstimulated, dashed line) or with anti-IgM at 20 μg/ml (solid line) (*a*, *c*, and *e*) or HEL at 100 ng/ml (solid line) (*b*, *d*, and *f*). Cells recovered after culture were triple labeled for IgD^a, IgM^a, and CTLA-4–Ig and examined by dual laser flow cytometry. Profiles show CTLA-4–Ig labeling of live non-Tg B cells vs. gated IgH^a allotype surface Ig-expressing B cells from Ig-Tg and Dbl-Tg mice. Control unstained profiles were identical to unstimulated cells. The ordinate shows cell numbers measured; the abscissa shows fluorescence intensity.

Anergic B Cells Can Be Induced to Secrete Antibody *in Vivo*. The evidence described above suggested that anergic B cells might be stimulated *in vivo* under conditions where help is provided by HEL-specific T cells and where nonspecific inflammatory signals might bypass an Ig-receptor-mediated block in costimulation. To test this, 5×10^5 B cells from Dbl-Tg mice, 10^7 HEL-primed T cells, and 100 μg of HEL in CFA were transferred into irradiated recipients. As shown in Table 1, Dbl-Tg B cells could indeed be stimulated under these conditions to mount a substantial anti-HEL IgM^a response of comparable magnitude to that of Ig-Tg B cells.

DISCUSSION

Anergy (35) in the Tg model used here is an intrinsic property of B cells that prevents them from responding to T-cell help once they have been removed from the tolerant environment (16). Here we have examined the nature of this defect within the framework of the four-step model of T–B collaboration as described (1–5).

Anergic B cells were fully responsive to downstream T-cell-derived helper signals. Both Ig-Tg and Dbl-Tg B cells proliferated and secreted similar amounts of Tg antibody

Table 1. Stimulation of Dbl-Tg B cells *in vivo*

B cells	HEL-primed T cells	Serum levels of anti-HEL IgM^a, ng/ml
None	−	48 ± 14
	+	70 ± 1
Non-Tg	−	60 ± 11
	+	135 ± 113
Ig-Tg	−	59 ± 43
	+	40,500 ± 5002
Dbl-Tg	−	78 ± 3.6
	+	22,917 ± 2108

Irradiated recipients were given 5×10^5 B cells, 1×10^7 HEL-primed T cells, as indicated, and HEL at 100 μg in CFA. Serum levels of anti-HEL IgM^a are the mean ± SEM.

when stimulated with activated Th membranes containing CD40 ligand in the presence of lymphokines. The response was CD40-dependent, as shown by blocking experiments (Figs. 1 and 2) and no reduction in CD40 levels was observed on the surface of anergic B cells. These experiments established that the CD40-dependent activation pathway was intact in these cells and suggested that the anergic defect associated with T–B collaboration lies within the earlier steps of antigen uptake and processing and/or costimulation of T-cell activation.

Anergic B cells constitutively presented the self antigen HEL to the hybridoma 3A9 (Fig. 3 *a* and *b*). Constitutive presentation of HEL was restricted to HEL-binding B cells, demonstrating that they had processed HEL *in vivo* via their specific Ig receptors (Fig. 3*c*). Although the anergic B cells could present self antigen acquired *in vivo*, their ability to process the same antigen *de novo* was reduced. This was not due to inactivation of processing machinery since an unrelated antigen, conalbumin, targeted through surface IgD was efficiently presented (Fig. 3*d*). Rather the decrease in *de novo* HEL processing appeared to result from the 4- to 10-fold reduction in HEL-binding capacity associated with downregulation of surface IgM (13). Consequently, anergic B cells would be expected to process a carrier coupled to HEL less efficiently than Ig-Tg B cells (16), a difference that would be magnified even further as a result of prior Ig-receptor occupancy by HEL acquired *in vivo* (33). Nevertheless, an alternative explanation for B-cell anergy is required since recovery of surface IgM levels on Dbl-Tg B cells after adoptive transfer is not accompanied by a return in their ability to respond to carrier-specific T-cell help (30). The most likely mechanism for rendering B cells unresponsive to T-cell help is a failure in upregulation of CD28 ligands after crosslinking of their antigen receptors with HEL (Fig. 4). Similar conclusions have been reached by Cooke *et al.* (36) who showed in the same Tg model that antigenic stimulation enhances provision of T-cell help directed to allogeneic class II major histocompatibility complex on B cells. Whether impaired costimulation by anergic antigen-presenting B cells might induce T-cell anergy (37, 38) in this situation remains to be determined.

Despite their failure to upregulate CD28 ligands, anergic B cells could respond efficiently to T-cell help *in vivo* if primed T cells were directed to HEL and the antigen was administered in CFA (Table 1). This protocol presumably worked since anergic B cells were not required to process a new carrier and the inflammatory stimulus associated with CFA may have enhanced nonspecifically the costimulator levels on the B-cell surface.

The demonstration that anergic B cells are responsive to T-cell help both *in vitro* and *in vivo* not only confirmed the previous report of reversibility of B-cell anergy (30) but also suggested at least two mechanisms by which self-reactive anergic B cells can be stimulated to secrete autoantibody. In the first, high levels of a self epitope associated with foreign carrier epitopes could compete with self antigen on anergic B cells and be presented to carrier-primed T cells (39). A concomitant inflammatory stimulus or microbial infection would help bypass the block in costimulation. Alternatively, T-cell activation during chronic antigen stimulation could result in expression of B-cell stimulatory ligands such as the CD40 ligand as well as the elaboration of lymphokines. Bystander exposure of anergic B cells to these T-cell-derived signals would then lead to a breakdown in self tolerance accompanied by autoantibody production.

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