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Role of 4-1BB Ligand in Costimulation of T Lymphocyte Growth and its Upregulation on M12 B Lymphomas by cAMP

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Summary

K46J B lymphomas express a T cell costimulatory activity that is not inhibited by CTLA-4Ig, anti-B7-1, anti-B7-2, anti-intercellular adhesion molecule 1 or antibodies to heat stable antigen. In this paper we report that this costimulatory activity is mediated at least in part by 4-1BB ligand, a member of the tumor necrosis factor (TNF) gene family that binds to 4-1BB, a T cell activation antigen with homology to the TNF/nerve growth factor receptor family. A fusion protein between 4-1BB and alkaline phosphatase (4-1BB-AP) blocks T cell activation by K46J lymphomas in both an antigen-specific system and with polyclonally (anti-CD3) activated T cells. 4-1BB-AP also blocks antigen presentation by normal spleen cells. When the antigen-presenting cells express B7 molecules as well as 4-1BB ligand, we find that B7 molecules and 4-1BB-AP both contribute to T cell activation. These data suggest that 4-1BB ligand plays an important role in costimulation of IL-2 production and proliferation by T cells. The B lymphoma M12 expresses low levels of 4-1BB-L but can be induced to express higher levels by treatment of the B cells with cAMP, which also induces B7-1 and B7-2 in these cells. Thus cAMP appears to coordinately induce several costimulatory molecules on B cells.

T lymphocyte activation requires two signals. Signal 1 is provided when an antigen/MHC complex binds to the TCR and signal 2 is provided by TCR-independent interactions (1). The binding of costimulatory molecules B7-1 or B7-2 to CD28 on the T cell is now widely recognized as providing an important costimulatory signal for inducing high level IL-2 production and proliferation of T cells (2, 3). However, other molecules have been reported to have costimulatory roles in T cell activation. For example, the heat stable antigen has been reported to have costimulatory activity for T cell activation that synergizes with B7 (4–6). Intercellular and vascular cell adhesion molecules 1 (ICAM-1 and VCAM-1)[1], the ligands, respectively, for LFA-1 and very late antigen 4 have also been shown to costimulate with anti-TCR in the costimulation of resting T cells (7). More recently, 4-1BB-ligand(L)-4-1BB interaction has been suggested to play a role in the costimulation of lymphocyte growth (8, 9).

4-1BB is a 55-kD protein expressed on activated CD4 and CD8 T cells (8). It is a member of the nerve growth factor (NGF)/TNF receptor gene family, characterized by the presence of cysteine-rich motifs in the extracellular domains (for reviews see references 10 and 11). 4-1BB mRNA is induced in T cells within hours of activation with anti-CD3 (12). However, surface expression of 4-1BB is not detected until 24–48 h after anti-CD3 treatment (8) and maximal expression appears to require costimulation or cytokine treatment of the T cells and takes several days (12a). Initially, it was reported that 4-1BB binds to extracellular matrix proteins (13). However, more recently a high affinity ligand for 4-1BB has been identified on activated macrophages (9) and mature B cells (14). 4-1BB-L shows homology to TNF, LT-α, LT-β, CD40L, and CD27L and thus appears to be a member of an emerging family of molecules that bind to members of the NGF/TNF receptor family (11). 4-1BB-L in CV-1 cells has been shown to costimulate with Con A in activation of thymocytes and with PHA in costimulation of splenic T cells (9). Moreover, antibody-mediated cross-linking of 4-1BB on anti-CD3-activated T cells profoundly enhances T cell proliferation (8).

In this report we have analyzed the ability of a 4-1BB-

[1] *Abbreviations used in this paper:* cAMP, cyclic AMP; 4-1BB-L, 4-1BB-ligand; ICAM-1, intercellular adhesion molecule 1.

alkaline phosphatase (AP) fusion protein to block T cell activation in the presence and absence of B7 molecules. These data provide further evidence for the importance of 4-1BB-L-4-1BB interaction in the costimulation of T lymphocyte activation.

Materials and Methods

Cell Lines, Antibodies and Reagents. The APC lines used in this study are described in Table 1. The BALB/c lymphomas M12 and K46J were originally described by Kim et al. (15). An Ia-negative variant of M12, M12.C3, transfected with truncated A^k (M12.C3.5C2,16) and the autoreactive A^k-restricted T hybrid C8.A3 (16) were obtained from Dr. Laurie Glimcher (Harvard Medical School, Boston, MA). K46J.73.35 (17), M12.C3.F6 (18), and M12.TDAK (19) transfectants have been described previously. Cells were maintained in RPMI-1640 containing 10% FCS (P.A. Biologicals, Sydney, Australia), 50 μM 2-ME, MEM nonessential amino acids (GIBCO BRL, Gaithersburg, MD), antibiotics, pyruvate, and glutamine as previously described (20).

20C9 antibody, specific for heat stable antigen (4) was provided as a culture supernatant by Dr. Yang Liu (New York University Medical Center, New York). The B7-1 specific hybridoma 1610A1 (21) was kindly provided by Dr. Hans Reiser (Dana Farber Cancer Institute, Boston, MA). The B7-2 specific hybridoma GL-1 (22), the CD8-specific, rat IgG2a-secreting hybridoma 53.72.6, the A^k-specific hybridoma 10-2.16, and the A^d-specific hybridoma MKD6 were obtained from the American Type Culture Collection (Rockville, MD). The ICAM-1–specific hybridoma YN1 (23) was kindly provided by Fumeo Takei (University of British Columbia, Vancouver, Canada) and the anti-CD3–secreting hybridoma 145-2C11 (24) was kindly provided by Dr. Jeff Bluestone (University of Chicago, Chicago, IL). Antibodies were purified using protein G–Sepharose and labeled with biotin according to Goding (25). The mAb 1AH2 is a subclone of the antibody 53A2 specific for 4-1BB described previously (8). The 4-1BB-AP fusion protein was prepared as previously described (14). The CTLA-4Ig fusion protein (26) was provided in purified form by Peter Linsley (Bristol-Myers Squibb, Seattle, WA). Human placental AP was purchased from Sigma Chemical Co. (St. Louis, MO) and anti-AP was obtained from Medix Biotech Inc. (Foster City, CA).

T Cell Isolation. 6–8-wk-old BALB/c female mice were obtained from Charles River Laboratory (St-Constant, Quebec, Canada). Spleen cell suspensions were isolated from 8–10-wk-old mice, treated with 0.15 mM NH_4Cl to lyse the RBC. MHC class II positive cells were depleted by treating the cell suspension with the A^d-specific antibody MKD6 plus low-tox baby rabbit complement (Cedarlane, Hornby, Ont., Canada). T cells were further purified by passage over T cell enrichment columns from R&D Systems Inc. (Minneapolis, MN) and were ~90% pure by anti-CD3 staining.

Antigen Presentation Assays. Either 10^5 C8.A3 T hybrids or 5×10^4 normal splenic T cells were mixed with 10^5 or 2×10^5 B lymphoma cells, respectively, and incubated overnight in the presence of various blocking antibodies. Supernatants were harvested and assayed for IL-2 content using the CTLL indicator line as described (27). For both the IL-2 and the proliferation assays, data are expressed as mean ± SD of triplicate cultures.

Primary MLR cultures were prepared from mouse spleen using 2×10^5 BALB/c responder and 8×10^5 C3H stimulator cells. Responder T cells were enriched by passage through Sephadex G-10 followed by Ab plus complement treatment of the eluted cells using anti-CD8 and anti-class II (MKD6) antibody. The stimulator population was depleted of T cells using anti-Thy 1.2 (HO13.4), anti-CD4 (RL172), and anti-CD8 mAb plus complement. Anti-Thy1 and anti-CD4 were provided by Michael Julius (University of Toronto). The remaining viable cells were irradiated (2,000 rads). IL-2 content of the supernatants was measured on day 4 and proliferation was measured on day 5, by harvesting cells that had been pulsed with [^{3}H]thymidine for the previous 8 h of culture.

Flow Cytometry. For flow cytometric analysis, cells were washed once in PBS and 10^6 cells in 200 μl of PBS, 1% FCS, 2 mM NaN_3 were incubated with irrelevant antibody to block Fc receptors before the addition of biotinylated antibodies. After incubation, samples were washed and incubated with 200 μl of a 1-μg/ml solution of PE-streptavidin (BioMeda, Foster City, CA), followed by further washing. Samples were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA). Samples were monitored for forward versus side scatter and FL2 (for PE staining). Alignment was checked using Immunocheck beads and Autocomp software (Becton Dickinson & Co.). Propidium iodide staining and forward and side scatter were used to gate on live cells. Data were analyzed and plotted using Lysis II software (Becton Dickinson & Co.). In some experiments, B lymphoma cells at a density of 5×10^5/ml were incubated overnight in complete medium containing 300 μM dibutyryl-cyclic (cAMP), as previously described (27).

Results

CTLA-4–independent Costimulation of T Cells by K46J B Lymphomas. Previous work from our laboratory at the University of Toronto and that of Laurie Glimcher has characterized the costimulatory requirements of a T cell hybrid, C8.A3. This T hybrid fails to respond to M12 B lymphomas expressing truncated A^k molecules (16) unless they have been stimulated with cAMP (27) to induce B7-1 and -2 expression (28–30) or unless they have been transfected with B7-1 cDNA (28). Here we show that in contrast to the results with M12 B lymphomas (16), the BALB/c B lymphoma, K46J, when transfected with genes encoding truncated A^k molecules, does not show the same antigen presentation defect (Table 1) and can stimulate C8.A3 T hybrids even in the presence of CTLA-4Ig (Fig. 1). The CTLA-4Ig was used at a concentration of 20 μg/ml, which is more than sufficient to give maximal inhibition of antigen presentation by M12 cells (29 and data not shown). These data suggest that K46J lymphomas express a costimulatory activity that does not involve CTLA-4 ligands.

We also tested the ability of K46J cells to costimulate with immobilized anti-CD3 antibody to drive IL-2 production by normal T cells isolated from BALB/c spleens. We found that K46J lymphomas can costimulate normal T cells and that this activity does not appear to be sensitive to inhibition with antibodies against ICAM-1 nor to antibodies to heat stable antigen added either alone or in combination with CTLA4Ig (data not shown). Thus the ability of K46J cells to deliver costimulatory signals is independent of B7 molecules, ICAM-1, and heat stable antigen.

K46J B Lymphomas Express an Anti-B7-2 (GL-1) Binding Molecule that Does Not Appear to be Involved in Costimulation. Although the costimulatory activity of K46J B lymphomas did not appear to be dependent on CTLA-4 ligands,

Table 1. *Effect of MHC II Truncation on Antigen-presenting Ability of M12 and K46J Lymphomas*

APC	A^k molecule expressed*	A^k specific fluorescence‡	IL-2 produced by C8.A3§	
			− cAMP	+ cAMP
M12.C3.F6	wt/wt	203	320	nt
M12.C3.5C2	−10/−12	43	<10	320
M12.TDAK	−10/−12	181	<10	320
K46J	none	0	<10	<10
K46J.73.35	−12/−12	57	160	160

* −10/−12 or −12/−12 indicates A^k molecules altered by site-directed mutagenesis to encode proteins lacking 10 or 12 amino acids from the COOH terminus of the β chain and 12 amino acids from the COOH terminus of the α chain. M12.TDAK and K46J express wild-type A^d and E^d molecules as well as the transfected A^k molecules, whereas M12.C3.F6 and M12.C3.5C2 were obtained by transfecting the Ia negative cell line M12.C3 and express only wild-type or truncated A^k.

‡ A^k surface expression was measured using FITC-10-2.16 antibody. Specific fluorescence refers to the signal obtained with FITC-10-2.16 minus that obtained with a negative control antibody.

§ C8.A3 are autoreactive, A^k-restricted T cell hybrids. IL-2 activity is expressed as the dilution of culture supernatant that gives 50% maximum thymidine incorporation by CTLL cells.

we analyzed the surface expression of these molecules on K46J B lymphomas. Surprisingly, we found that although K46J cells fail to bind CTLA-4Ig or anti-B7-1, they do stain with the B7-2–reactive antibody GL-1 but not with a control rat IgG2a antibody (data not shown). Despite the presence of a GL-1 (anti-B7-2) binding molecule on K46J cells, B7-2 does not appear to be responsible for the costimulatory activity of K46J B lymphomas in that GL-1 antibody does not inhibit antigen presentation by K46J B lymphomas (data not shown). Thus K46J cells express a molecule which binds B7-2–specific antibodies but does not appear to be involved in costimulation of T cells. Work is in progress to determine whether this molecule represents a mutated or posttranslationally modified form of B7-2 that lacks the CTLA-4 and CD28 binding site or whether it represents an unrelated molecule that expresses the GL-1 epitope.

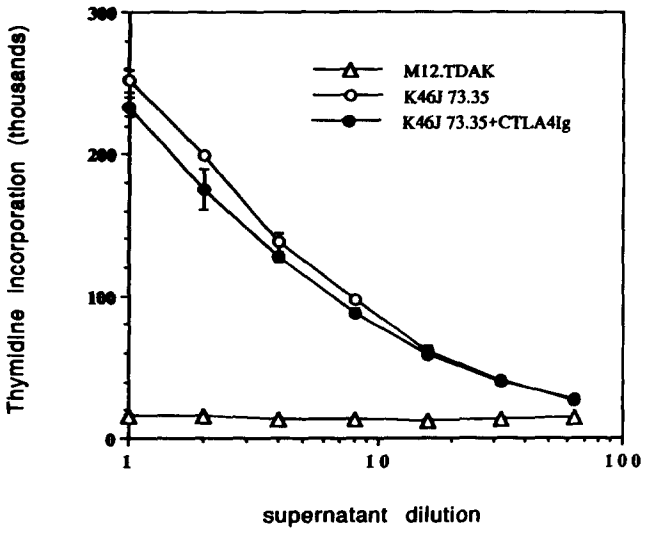

Figure 1. Antigen presentation by K46J B lymphomas is not blocked by CTLA-4Ig. 10^5 autoreactive, A^k-restricted C8.A3 T hybrids were cultured for 18 h with 10^5 K46J.73.75 or 10^5 M12.TDAK cells. Cell lines are described in Table 1. Untreated M12.TDAK are included as a negative control here, since they do not stimulate C8.A3 cells unless treated with cAMP to induce B7 molecules. Supernatants were harvested and assayed for the presence of IL-2 using CTLL cells. CTLA4Ig was added at a final concentration of 20 μg/ml.

Expression of 4-1BB-L on B Lymphomas. As discussed above, 4-1BB, a member of the TNF/NGF receptor family, is an activation antigen on T cells that has been implicated in costimulation of T lymphocyte growth. Its ligand, 4-1BB-L, shows sequence homology to TNF and is found on activated macrophages, B cells, and B lymphomas. We examined K46J B lymphomas for the expression of 4-1BB-L using a soluble form of 4-1BB (14). Fig. 2 *A* shows that a fusion protein consisting of the extracellular domain of 4-1BB linked to AP (4-1BB-AP) stains K46J B lymphomas, whereas the AP itself does not. We therefore examined the T cell hybrid C8.A3 for expression of 4-1BB using mAb 1AH2. Resting C8.A3 cells did not express detectable 4-1BB (data not shown), however after overnight incubation with immobilized anti-CD3, 4-1BB surface expression was readily detected (Fig. 2 *B*).

It was of interest to determine whether 4-1BB ligand was also expressed on a B lymphoma that expressed B7 molecules. Fig. 2 *A* shows that M12 B lymphomas express low levels of 4-1BB-L constitutively but are induced to express much higher levels of 4-1BB-L upon overnight incubation in dibutyryl-cAMP. cAMP did not have an effect on 4-1BB-L expression on K46J cells, which already express high levels of the ligand (data not shown).

4-1BB-L Is Critically Involved in Costimulation by K46J B Lymphomas. Since activated C8.A3 cells express 4-1BB and K46J cells express its ligand, we tested the ability of the 4-1BB-AP fusion protein to inhibit IL-2 production by C8.A3 in response to K46J.73.75 cells. Fig. 3 *A* shows that the 4-1BB-AP fusion protein blocks IL-2 production by C8.A3 cells re-

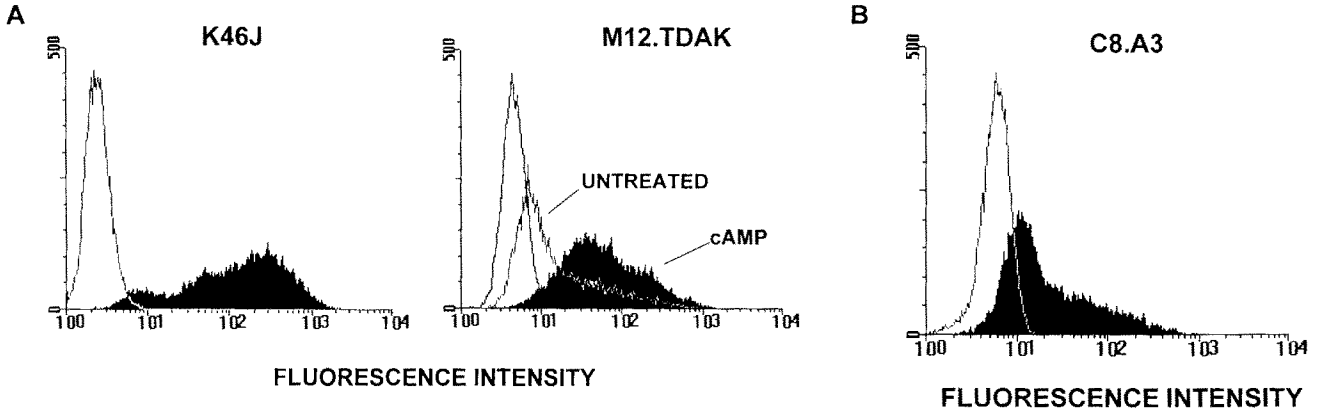

Figure 2. Expression of the 4-1BB-L and 4-1BB counter-receptors on (*A*) B cells and (*B*) T cells, respectively. (*A*) K46J B lymphoma cells were stained with either AP (*open histograms*) or 41BB-AP (*filled histograms*) followed by biotinylated anti-AP antibody. PE-streptavidin was used as the third step in the detection protocol. M12TDAK cells were untreated or treated with dibutyryl-cAMP as indicated on the figure and stained with 4-1BB-AP (*untreated, cAMP*) or AP alone (*unlabeled histogram*). (*B*) Filled histograms represent C8.A3 cells incubated overnight on plates coated with 10 μg/ml anti-CD3 antibody before flow cytometry analysis for 4-1BB expression, using biotinylated 1AH2 antibody. Open histograms represent staining of C8.A3 T cells with control biotinylated antibody. PE-streptavidin was used as the second step to detect biotinylated antibodies.

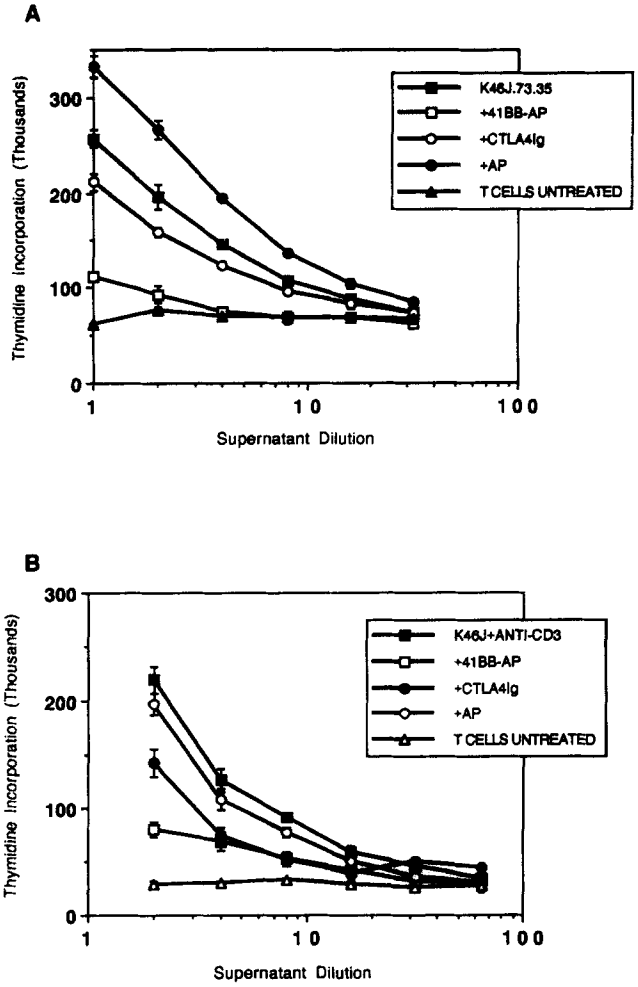

Figure 3. 4-1BB-AP blocks IL-2 production by C8.A3 T hybrids and by normal murine T cells. (*A*) C8.A3 T hybrids were incubated with A^k transfected K46J B lymphomas (*K46J.73.35*) and IL-2 production was assayed at 18 h using CTLL cells. (*B*) Normal murine T cells were co-cultured with K46J B cells and immobilized anti-CD3 and assayed for IL-2 production as described in Fig. 2. The 4-1BB-AP fusion protein and the AP were added at a final concentration of 10 μg/ml. CTLA4Ig was added at a final concentration of 20 μg/ml.

sponding to A^k-transfected K46J B lymphomas to near background levels whereas CTLA-4Ig has little or no effect. Fig. 3 *B* shows that IL-2 production by normal T cells to K46J plus anti-CD3 is also profoundly blocked by 4-1BB-AP fusion protein. In this and other experiments with normal T cells we observed some inhibition with CTLA4Ig also. Inasmuch as K46J does not express CTLA-4 ligands, this is likely due to failure to remove all the APC during T cell isolation.

Although AP itself did not block T cell activation by K46J cells, it is formally possible that the 4-1BB fusion protein was somehow inhibitory to the T cells by some means other than by blocking the interaction of 4-1BB with its ligand. To test this possibility, we looked at the response of C8.A3 T hybrids to immobilized anti-CD3. Although C8.A3 T hybrids require costimulation when responding to Ag/MHC II, they do respond to anti-CD3 alone, as in the case for most T cell hybrids. Fig. 4 shows that 4-1BB-AP does not affect IL-2 production by C8.A3 T cells responding to anti-CD3 immobilized on plastic.

To examine whether 4-1BB plays a role in T cell activation in the presence of B7 molecules, we tested the effect of 4-1BB-AP on T cell activation by M12 B lymphomas treated with cAMP. After overnight treatment with cAMP, M12 B lymphomas express limited amounts of B7-1 and high levels of B7-2 (data not shown). Fig. 5 shows that 4-1BB-AP profoundly blocks the activation of C8.A3 T cells by A^k-expressing M12 cells. Anti-B7-1 shows little or no effect in this experiment, presumably because high level B7-1 expression requires longer than the overnight APC activation carried

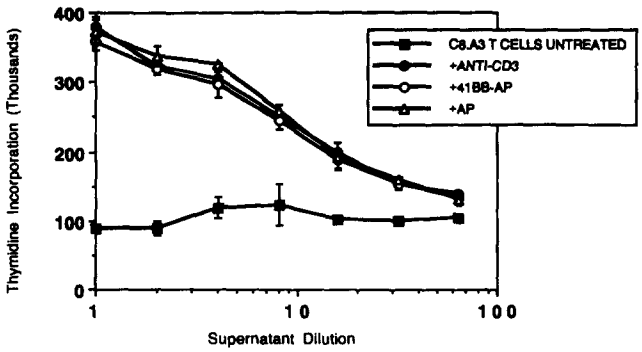

Figure 4. Soluble 4-1BB does not inhibit anti-CD3 activated C8.A3 T hybrids. 10^5 C8.A3 T cells were incubated overnight on plates coated with 10 μg/ml anti-CD3. IL-2 production was assayed using CTLL cells. The 41BB-AP fusion protein and the AP were added at the beginning of the incubation at a final concentration of 10 μg/ml.

out here (28). Anti-B7-2 gave inhibition comparable to that observed with 4-1BB-AP alone. In combination, anti-B7-1, anti-B7-2, and 4-1BB-AP completely inhibited IL-2 production by C8.A3 in response to cAMP-treated M12 cells. Thus B7 molecules and 4-1BB-L each contribute independently to T cell activation when expressed on the same APC.

Role of 4-1BB in Antigen Presentation by Splenic APC. 4-1BB-L has been identified on activated macrophages as well as on B cells. To determine whether 4-1BB-L plays a role in T cell activation by splenic APC as well as by K46J B lymphomas we tested the ability of 4-1BB-AP to block T cell responses in a BALB/c anti-C3H MLR. Fig. 6 shows that both 4-1BB-AP and CTLA-4Ig added individually profoundly block proliferation and IL-2 production by the alloreactive T cells. The combination of the two completely abrogated the response. These data suggest that both 4-1BB-L and B7 molecules play a role in costimulation by splenic APC.

Discussion

In this report we have shown that a fusion protein between 4-1BB and AP profoundly blocks T cell activation both by B lymphomas and by unfractionated spleen cells. The effect of the 4-1BB-AP fusion protein appears to be on APC–T cell interaction, since 4-1BB-AP does not block T cell activation by immobilized anti-CD3. These data suggest that the fusion protein is blocking antigen presentation by blocking the interaction between 4-1BB and its ligand on the APC. 4-1BB-L, a member of the TNF gene family, recently has been identified as a high affinity ligand for 4-1BB on activated B cells and macrophages (9). However, the present experiments do not rule out that alternate low affinity ligands for 4-1BB, namely extracellular matrix components, also play a role (13).

We find that 4-1BB plays a role in costimulation of T cell activation both in the presence of B7 molecules and in their apparent absence. M12 cells, when treated overnight with dibutyryl-cAMP, express both B7-2 and 4-1BB-L as well as low levels of B7-1. Under these conditions of antigen presentation we find that CTLA-4Ig and 4-1BB-AP each inhibit substantially. In contrast to the effects of CTLA4Ig alone (29) the combination of anti-B7-1, anti-B7-2, and 4-1BB-AP completely blocks antigen presentation by cAMP-treated M12 cells, suggesting that these molecules account for the majority of the costimulatory ligands on cAMP-treated M12 cells. When spleen cells are used as stimulators in an MLR, we find that CTLA-4Ig and 4-1BB-AP each inhibit almost completely on their own. This suggests that high level IL-2 production may require multiple costimulatory signals, perhaps acting sequentially. Removal of either one of these signals is sufficient to abrogate the major part of the response.

On K46J B lymphomas which lack functional B7 molecules, 4-1BB-L appears to be capable of costimulating C8.A3 T cells in the absence of B7 molecules. Thus 4-1BB–4-1BB ligand interaction in the absence of B7, can costimulate IL-2 production. However, we do not know whether other molecules on K46J are required or whether 4-1BB-L alone is sufficient for costimulation. This question will require analysis of the function of 4-1BB-L in the absence of other APC molecules. Goodwin et al. (9) showed costimulation of splenic T cells with PHA plus 4-1BB-L transfected CV-1 cells. However, the fact that they used a lectin in these studies may have

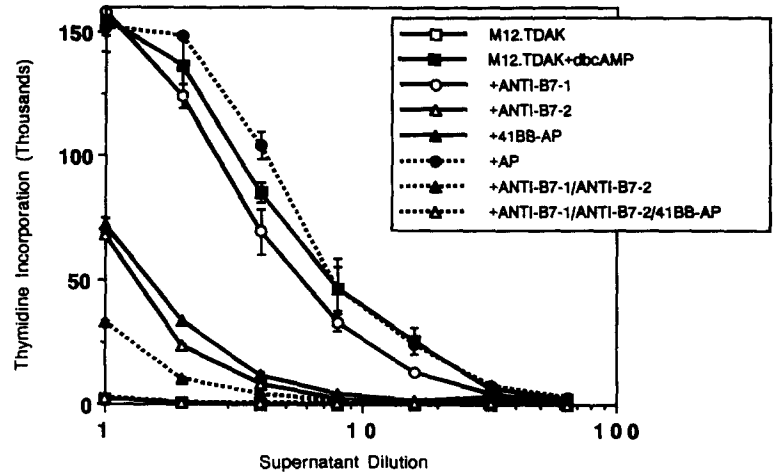

Figure 5. 4-1BB-AP and anti-B7 antibodies have additive effects on the ability of M12 B cells to costimulate T cells. C8.A3 T cells were incubated with M12.TDAK B lymphomas and IL-2 production was assayed using CTLL cells as described in Materials and Methods. Anti-B7-1 (1610A1) and anti-B7-2 (GL-1) antibodies were added to cultures at a final concentration of 20 μg/ml. The 4-1BB-AP fusion protein and the AP were added at a final concentration of 10 μg/ml.

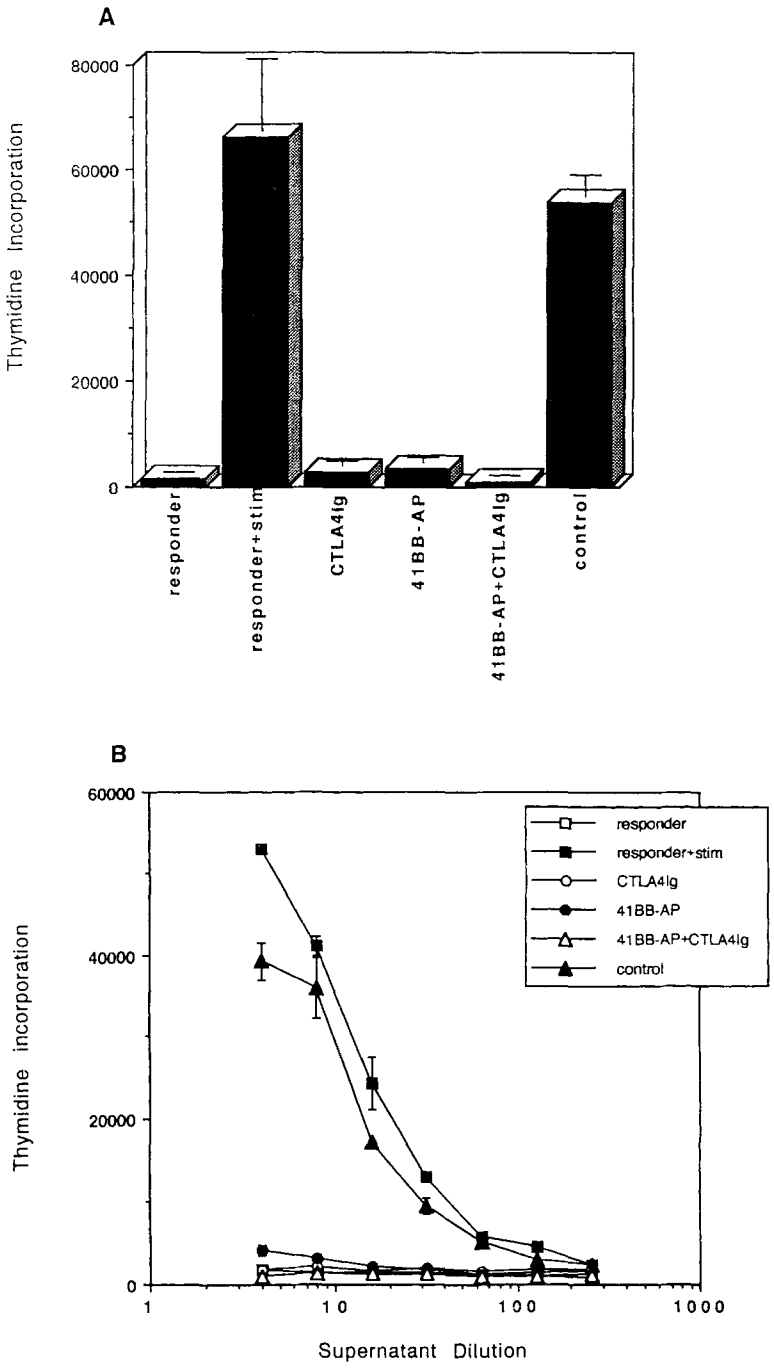

Figure 6. 4-1BB-AP blocks proliferation and IL-2 production in a 5-d BALB/c anti-C3H MLR. (*A*) Proliferation of T cells, measured at 5 d. (*B*) IL-2 content of the supernatant at 4 d was measured using CTLL cells. CTLA-4Ig and 4-1BB-AP were each added at 10 μg/ml. Control refers to the addition of 10 μg/ml human IgG1. In other experiments, AP was used as a control with a similar lack of effect.

resulted in cross-linking of several T cell surface proteins. On the other hand, Pollok et al. (8) have shown that the 53A2 mAb, specific for 4-1BB, can costimulate with anti-CD3 to activate splenic T cells, again suggesting that 4-1BB–4-1BB-L interaction can function independently of B7 molecules to costimulate lymphocyte growth.

The C8.A3 T hybrids used here, although costimulatory molecule dependent when stimulated using Ag/MHC, are costimulatory molecule independent when stimulated with anti-CD3. Therefore, it was of interest to determine whether 4-1BB-L–4-1BB interaction plays a role in normal T cell activation. We found that K46J B lymphomas can costimulate with anti-CD3 to activate normal splenic T cells to produce IL-2 and induce T cell proliferation. This costimulatory activity of K46J was blocked by 4-1BB-AP, suggesting the importance of 4-1BB–4-1BB-L interaction in normal T cell activation. It is difficult however to conclude from these data that 4-1BB-L alone is costimulatory because we often observed a small amount of inhibition with CTLA-4Ig in these experiments. This inhibition with CTLA-4Ig is presumably

due to residual APC in the T cell preparations, since K46J cells do not stain with CTLA-4Ig.

It is perhaps surprising that 4-1BB-AP blocks T cell activation by M12 B lymphomas which also express B7 molecules, because B7-transfected CHO cells have been shown to be sufficient to provide costimulation for T cells (30–33). In those studies the levels of B7 used were higher than those found on activated B cells and furthermore, the T cells were stimulated with anti-CD3. It is likely that under normal conditions when the physiological ligand for TCR (Ag/MHC) is used that several costimulatory molecules will contribute to T cell activation.

On C8.A3 T cells, 4-1BB is detectable on the cell surface after overnight incubation with anti-CD3 (Fig. 2 *B*), consistent with our observation that 4-1BB–4-1BB-L plays a role in antigen presentation within the first 24 h of activation of C8.A3 T cells. However, for normal splenic T cells maximal surface expression of 4-1BB requires costimulation or exposure to cytokines and takes several days (12a). Therefore, one must ask how 4-1BB-AP can block T cell activation before the surface expression of 4-1BB is detectable. Previous work (12) has established that 4-1BB mRNA is detectable within a few hours of stimulation with anti-CD3. It is possible that low levels of 4-1BB are present on the cell surface within hours of contact of the T cells with APC and that these levels are sufficient to initiate T cell activation. These initial signals would then result in further amplification of 4-1BB expression on the T cell. Alternatively, other molecules on K46J cells may contribute to the costimulatory signals which then allow induction of more 4-1BB on the T cell and further amplification of the costimulatory signals.

Cross-linking of a number of surface receptors on T cells has been shown to augment T cell activation. However, many of these interactions function to enhance signals induced via the TCR, i.e., "signal 1". What distinguishes the CD28–B7 interaction is that CD28 engagement results in the induction of a distinct signal transduction pathway, hence the widely accepted view that CD28 is a major recipient of "signal 2" (2, 3). 4-1BB has been shown to associate with $p56^{lck}$ (34), but at this time little else is known about its signal transducing properties. It remains to be seen whether signals emanating from 4-1BB intersect those signal transducing pathways activated by TCR and/or CD28.

4-1BB-L and 4-1BB are members of an emerging family of cytokine-like, cytokine receptor–like molecules, including TNF, TNFR; CD40L, CD40; and CD70, CD27 (9, 35). It is of interest, that CD27 on T cells interacting with CD70 on human B cells shares many of the features of the 4-1BB–4-1BB-L system (36, 37). CD27 is present at low levels on mature T cells and upregulated by TCR signaling. Its ligand, CD70 is absent on resting B cells but upregulated by CD40 signaling. CD70 has also been shown to be costimulatory for T cells and to have additive effects with B7, however CD70 has more of an effect on TNF-α production than on IL-2 and IL-4 production by human T cells (36). It is tempting to speculate that various combinations of B7, 4-1BB-L, CD27L, and perhaps other costimulatory molecules, might influence the phenotype of T cell response one obtains.

We have shown that in M12 cells, both B7 molecules and 4-1BB-L are upregulated by cAMP treatment. Thus several costimulatory molecules on APC can be induced by activation. During antigen presentation, it is likely that both 4-1BB and 4-1BB-L are upregulated reciprocally allowing an amplification of the initial signals in much the same way as B7 and CD28 molecules and CD27 and CD70 are upregulated during T cell activation. This back and forth interaction between the two cells might serve to reinforce initial signaling events that take place upon the engagement of just a few TCR and a few costimulatory receptors by their ligands, rendering the T cell exquisitely sensitive to antigenic challenge.

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