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# Murine B7 antigen provides an efficient costimulatory signal for activation of murine T lymphocytes via the T-cell receptor/CD3 complex

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**ABSTRACT** **We demonstrate that the murine B7 (mB7) protein is a potent costimulatory molecule for the activation of resting murine CD4$^+$ T cells through the T-cell receptor (TCR)/CD3 complex. Stable mB7-transfected Chinese hamster ovary cells, but not vector-transfected controls, synergize with anti-CD3 monoclonal antibody and Con A-induced T-cell activation, resulting ultimately in proliferation. mB7 exerted its effect by inducing production of interleukin 2 and expression of the interleukin 2 receptor. Thus, mB7 costimulates T-cell activation through the TCR/CD3 complex by positively modulating the normal pathway of T-cell expansion. In contrast to the pronounced effect of mB7 on the activation of T cells through the TCR/CD3 complex, the mB7-transfected CHO cell line costimulated T-cell activation via the glycosylphosphatidylinositol-anchored proteins Thy-1 and Ly-6A.2 only inefficiently. Finally, the combination of a calcium ionophore and mB7 is not sufficient to cause T-cell proliferation, while the combination of a calcium ionophore and phorbol 12-myristate 13-acetate (PMA) stimulates T cells efficiently. The signals that mB7 and PMA provide for murine T lymphocyte activation are therefore not interchangeable, although both costimulate activation through the TCR/CD3 complex.**

Stimulation of highly purified CD4$^+$ T lymphocytes with peptide–major histocompatibility complex (MHC) complexes, monoclonal antibodies (mAbs) against T-cell receptor (TCR)/CD3 complexes, lectins, or mAbs against the glycosyl-phosphatidylinositol (GPI)-anchored proteins Thy-1 and Ly-6A.2 does not lead to cell proliferation or lymphokine secretion. The above activation pathways require a second, costimulatory, signal for lymphokine gene expression and proliferation that is provided by an antigen-presenting cell (APC) or by pharmacological reagents such as phorbol 12-myristate 13-acetate (PMA) (1, 2).

The nature of the costimulatory signal appears to be dependent upon the subset of CD4$^+$ T cells that is activated. On the basis of their capacity to secrete specific lymphokines, two types of murine CD4$^+$ T-helper cells were defined originally. Th-1 clones synthesize interleukin (IL)-2, interferon $\gamma$, and lymphotoxin, whereas Th-2 clones secrete IL-4, IL-5, and IL-6 (3). In the case of Th-2 type CD4$^+$ T cells, this costimulatory signal appears to be IL-1 (4). In contrast, the nature of the costimulatory signal for Th-1-type CD4$^+$ T cells is unknown. Recent data in the human system suggest that B7, a 45- to 60-kDa cell surface glycoprotein present on activated B cells and on interferon-$\gamma$-stimulated monocytes/macrophages (5, 6), may be costimulatory for Th-1 CD4$^+$ T cells. Transfected cell lines that express human B7 or recombinant B7-immunoglobulin fusion protein can synergize with anti-CD3 or phytohemagglutinin-driven T-cell activation (7–9). Furthermore, binding of mAbs to CD28, a 44-kDa homodimeric T-cell glycoprotein that is a receptor for B7 (10), can augment the proliferation or lymphokine secretion of T cells stimulated with suboptimal doses of anti-CD3 mAbs (11, 12).

The gene encoding the murine homologue of B7 (mB7) has been recently cloned (13). The subject of the present report is the characterization of the function of mB7 in the activation of murine CD4$^+$ T lymphocytes. Our findings establish the costimulatory potential of mB7 in murine T-cell activation.

## MATERIALS AND METHODS

**Antibodies.** The following mAbs were used in this study: mAb M5/114 = anti-I-A$^{b,d,q}$, anti-I-E$^{d,k}$ (14); mAb 10-2.16 = anti-I-A$^k$ (15), mAb 116-13.1 = anti-Lyt-2.1 (16); mAb ADH4 = anti-Lyt-2.2 (17); mAb 145-2C11 = anti-CD3 (18); mAb G7 = anti-Thy-1 (19); mAb 8G12 = anti-TAP/Ly-6A.2 (20); mAb 7D4 = anti-IL-2 receptor (21); mAb 11B11 = anti-IL-4 (22); and mAb S4B6 = anti-IL-2 (23).

**Cell Cultures.** T cells were purified as previously described (24, 25). Briefly, splenocytes were depleted of erythrocytes by treatment with Tris/$NH_4Cl$. T cells were enriched by nylon wool fractionation (26). CD4$^+$ T cells from BALB/c or AKR mice were purified by treatment with a mixture of anti-MHC class II and anti-CD8 mAbs and rabbit complement (Cedarlane Laboratories, Hornby, ON, Canada) (24, 25). Chinese hamster ovary (CHO), CHO-mB7, and CHO-mB7.S8 cells were fixed for 15 min at room temperature with 1% paraformaldehyde in phosphate-buffered saline. Microcultures were set up in duplicates in 96-well plates as previously described (24, 25, 27). Lymphokine content was assayed on the HT-2 indicator cell line. The nature of the lymphokine produced in bulk cultures (IL-2 vs. IL-4) was determined as previously described (25) using the anti-IL-2 and anti-IL-4 mAb reagents listed above and provided by Abul Abbas (Harvard Medical School).

## RESULTS

**Construction of Stable mB7 Transfectants.** The *Xba–Eco*RI fragment (nucleotides 201–1716, ref. 13) of the mB7 cDNA was subcloned in the expression vector pCDNAI (Invitrogen, San Diego). Fifty micrograms of *Sfi* I-linearized mB7-pCDNAI was cotransfected with 5 $\mu$g of *Pvu* I-linearized pSV2-Neo-Sp65 (28) into CHO cells (7). A stable cell line, termed CHO-mB7, was obtained under G418 selection (400 $\mu$g/ml effective drug concentration). This cell line was subcloned. Both uncloned CHO-mB7 cells and the subclone CHO-mB7.S8 displayed high mB7 mRNA expression as

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Abbreviations: APC, antigen-presenting cell; GPI, glycosyl-phosphatidylinositol; IL, interleukin; mAb, monoclonal antibody; mB7, murine B7; MHC, major histocompatibility complex; PMA, phorbol 12-myristate 13-acetate; TCR, T-cell receptor.

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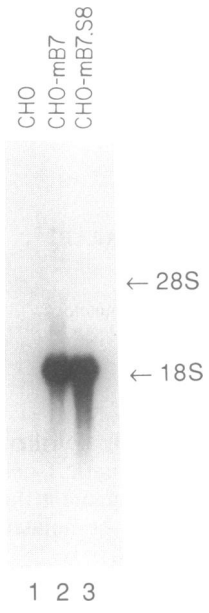


FIG. 1. Northern blot analysis of mB7-transfected CHO cells and controls. Total RNA, 20 μg, isolated from pSV2-Neo-Sp65-transfected CHO cells (lane 1), CHO-mB7 cells (lane 2), and CHO-mB7.S8 cells (lane 3), was size fractionated on an agarose/formaldehyde gel and transferred to nitrocellulose (29). The region was probed with $^{32}$P-labeled mB7 coding region cDNA (13). The mobilities of ribosomal RNAs are indicated.

assessed by Northern blot analysis (Fig. 1, lanes 2 and 3). mB7 expression was not observed in untransfected CHO cells or CHO cells that had been transfected with pSV2-Neo-Sp65 only (Fig. 1, lane 1, and unpublished data). In all functional experiments, we used CHO cells that were transfected with pSV2-Neo-Sp65 alone as controls.

**mB7 Costimulates T-Cell Proliferation Induced by Triggering of the TCR/CD3 Complex.** To test whether mB7 was capable of costimulating T-cell activation through the TCR/CD3 complex, we investigated initially whether mB7 was costimulatory for anti-CD3 mAb-driven T-cell proliferation. As expected, highly purified CD4$^+$ T cells did not respond to mitogenic concentrations of the anti-CD3 mAb 145-2C11 (Fig. 2*A*). In contrast, when purified CD4$^+$ T cells were incubated with anti-CD3 mAb in the presence of fixed CHO-mB7 cells, a dramatic increase in T-cell proliferation was observed. A representative experiment is shown in Fig. 2*A*. It should be noted that the effect of mB7 on T-cell activation was specific, as only a marginal increase in T-cell proliferation was induced by vector-transfected CHO cells (Fig. 2*A*). CHO-mB7.S8, a subclone of CHO-mB7, showed a costimulatory activity that was higher than that of uncloned CHO-mB7 cells (Fig. 2*A*). CHO-mB7.S8 cells were used in all subsequent experiments. As shown in Fig. 2*A*, CHO-mB7.S8 cells induced an at least 30-fold increase in T-cell proliferation compared with vector-transfected CHO controls. In >10 identical experiments this stimulation index ranged from approximately 10- to 50-fold. Titration experiments further demonstrated the potency of mB7 in providing the costimulatory signal: T cell proliferation was induced at T cell-to-CHO-mB7.S8 cell ratios of >100:1 (Fig. 2*B* and data not shown).

Activation of T cells by the lectin concanavalin A (Con A) is also thought to follow the TCR-mediated pathway of T-cell activation (1, 30, 31). As shown in Fig. 2*C*, mB7-transfected CHO cells were highly efficient at augmenting T-cell proliferation induced by this lectin. Vector-transfected CHO cells again served as negative control in the experiment.

**mB7 Does Not Effectively Costimulate T-Cell Proliferation Induced by mAbs Against the GPI-Anchored Proteins Thy-1 and Ly-6A.2.** We and others have previously shown that murine T cells can be activated with mAbs against GPI-linked proteins such as Thy-1 or Ly-6A.2 (19, 27, 32–34); however, the precise relationship of these pathways of T-cell activation to TCR-mediated stimulation is not understood (35–37). We therefore investigated whether CHO-mB7.S8 cells also supported alternative T-cell activation via the GPI-linked molecules. Two independent experiments, using purified T cells from two distinct strains of mice, are shown in Fig. 3. As shown in Fig. 3 *C*, *E*, and *F*, CHO-mB7.S8 cells do not efficiently costimulate anti-Thy-1- or anti-Ly-6A.2-induced T-cell proliferation. PMA can costimulate anti-Thy-1- and anti-Ly-6A.2-mediated activation in these experiments and thus serves as a positive control. In parallel cultures, mB7 induced efficient costimulation of anti-CD3 or Con A-mediated T-cell proliferation (Fig. 3 *A* and *B* and data not shown). These findings further support the specificity of the effects mediated by the mB7 antigen. It is also noteworthy that we have carried out these experiments with mouse strains of three independent MHC backgrounds: H-2$^d$, H-2$^k$, and H-2$^b$ (Fig. 3 and data not shown). Our results therefore extend to several haplotypes and provide evidence at the molecular level that T-cell activation via the GPI-anchored proteins is distinct from that via the TCR.

**mB7 Costimulates IL-2 Secretion and IL-2 Receptor Expression.** T-cell proliferation is dependent upon the synthesis of lymphokines that function as autocrine growth factors and on

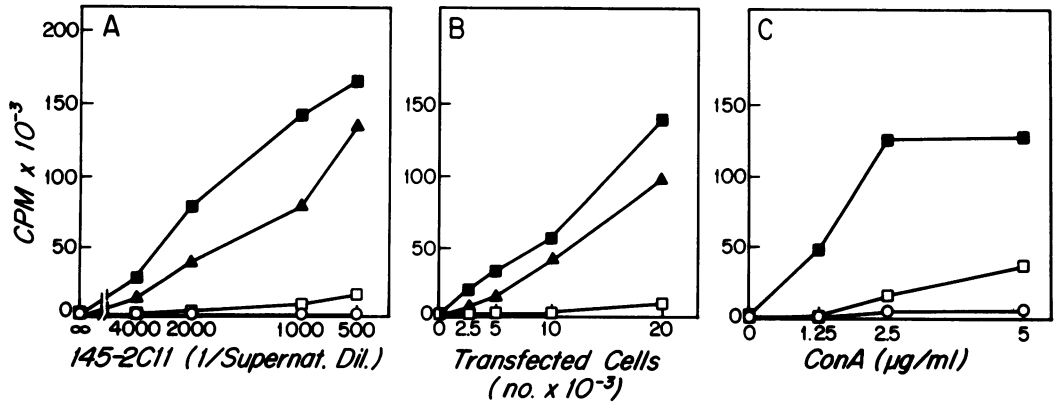


FIG. 2. mB7 induces T-cell proliferation in costimulation with anti-CD3 mAb or Con A. (*A* and *B*) Response to anti-CD3 mAb 145-2C11. (*C*) Response to Con A. Microcultures were set up as described (24) with 3 × 10$^5$ BALB/c CD4$^+$ T lymphocytes. All T-cell populations were depleted of detectable accessory cells, as demonstrated by the lack of an anti-CD3 or Con A response in the absence of a source of costimulatory activity (○). In the experiments represented in *A* and *C*, 2 × 10$^4$ paraformaldehyde-fixed CHO-mB7 cells (▲), CHO-mB7.S8 cells (■), or CHO cells (□) were added to the cultures. Indicated on the *x*-axis are concentrations of the stimulating agents [anti-CD3 mAb 145-2C11, given as reciprocal of supernatant dilution (*A*), or Con A (*C*)]. In the experiment represented in *B*, T cells were stimulated with a constant amount of anti-CD3 mAb 145-2C11 (1:1000 supernatant dilution). Indicated on the *x*-axis are concentrations of paraformaldehyde-fixed CHO-mB7 cells (▲), CHO-mB7.S8 cells (■), or CHO cells (□). All cultures were pulsed after 48 hr with 1 μCi (37 kBq) of [$^3$H]thymidine per well for the last 6 hr of the incubation period to assay T-cell proliferation.

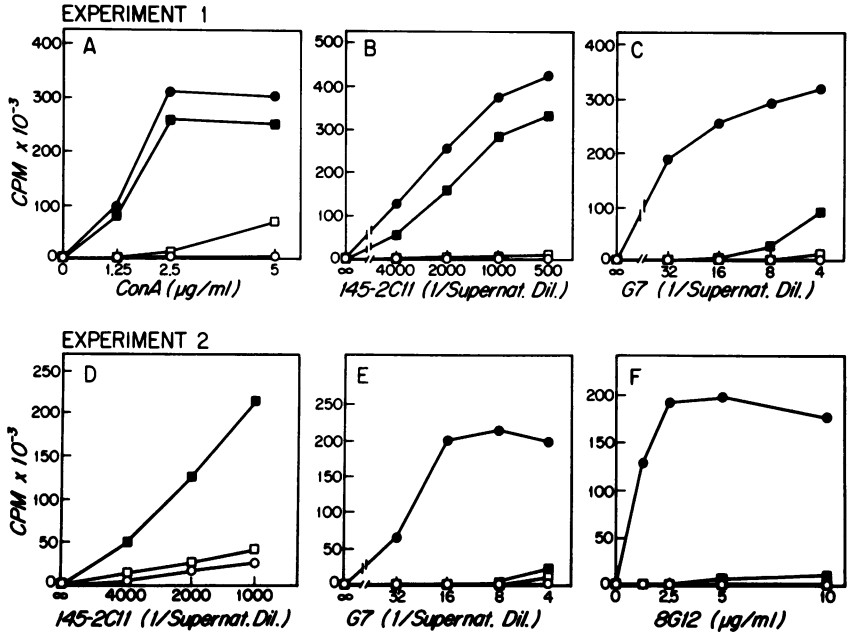


FIG. 3. Relative inefficiency of mB7 to induce T-cell proliferation in costimulation with anti-Thy-1 or anti-Ly-6A.2 mAbs. Microcultures were set up as described in the legend to Fig. 2 with 2 × $10^5$ BALB/c (experiment 1, *A–C*) or 2 × $10^5$ AKR (experiment 2, *D–F*) $CD4^+$ T cells. Medium (○), PMA at 10 ng/ml (●), or 2 × $10^4$ paraformaldehyde-fixed CHO-mB7.S8 cells (■) or CHO cells (□) were added to the cultures. Indicated on the *x*-axis are concentrations of the stimulating agents: Con A (*A*), anti-CD3 mAb 145-2C11 (*B*, *D*), anti-Thy-1 mAb G7 (*C*, *E*), or anti-Ly-6A.2 mAb 8G12 (*F*). After 48 hr, cultures were pulsed with 1 μCi of [$^3$H]thymidine per well for the last 6 hr of the incubation period.

the cell surface expression of the respective growth factor receptors. It was therefore of interest to study whether mB7 could costimulate both lymphokine secretion and upregulation of lymphokine receptors. We initially analyzed whether mB7 could costimulate lymphokine secretion after activation of T cells with anti-CD3 mAb or Con A. As expected, incubation with these reagents alone did not result in the secretion of lymphokines, as demonstrated by the lack of support for proliferation of the IL-2/IL-4-dependent HT-2 indicator cell line. In the presence of fixed CHO-mB7.S8 cells but not upon incubation with control CHO cells, growth factor activity was detected in culture supernatants in a dose-dependent manner (Fig. 4*A* and data not shown). In contrast, mB7 induced only marginal costimulation of anti-Thy-1 or anti-Ly-6A.2-induced lymphokine production. PMA again served as positive control in this experiment, providing a source of costimulatory activity for both anti-Thy-1- and anti-Ly-6A.2-mediated T-cell activation (Fig. 4 *B* and *C*).

$CD4^+$ T lymphocytes synthesize IL-2, IL-4, or a combination of both. The former two populations have been termed Th-1 and Th-2 cells, respectively (1). Previous studies in humans have demonstrated that triggering of CD28 by mAb or by B7 results in the production of IL-2 but not of IL-4. We therefore decided to determine the nature of the lymphokine produced by T cells in our system. For this purpose, T cells were stimulated with anti-CD3 mAb (Fig. 5*A*) or Con A (Fig. 5*B*) in the presence of either CHO-mB7.S8 or CHO cells. Supernatants were harvested after 24 hr and assayed for lymphokine content in the HT-2 assay. Addition of anti-IL-2 mAb to the assay medium virtually eliminated proliferation of the indicator cell line. In contrast, an anti-IL-4 mAb did not affect the growth of HT-2 cells (Fig. 5). Thus IL-2 is the major lymphokine synthesized by bulk $CD4^+$ T cells after costimulation with mB7 and either anti-CD3 mAb or Con A.

Normally, T-cell activation leads to cell surface expression of the IL-2 receptor. We therefore investigated whether mB7 could stimulate the cell surface expression of this growth factor receptor. Incubation of purified $CD4^+$ T cells with Con A in the presence of CHO-mB7.S8 cells, but not with CHO-mB7.S8 cells alone, leads to cell surface expression of the IL-2 receptor. Incubation of T cells with vector-transfected CHO cells, with or without Con A, did not result in IL-2 receptor surface expression (Fig. 6). Thus, the effect of mB7 is specific.

**T-Cell Activation by mB7 in the Presence of PMA but Not in the Presence of Calcium Ionophore.** T cells can be triggered to

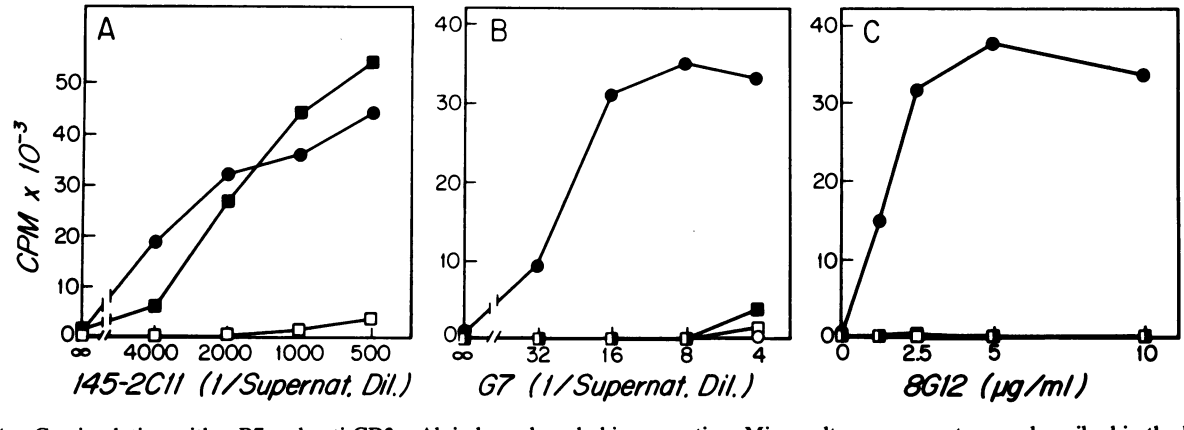


FIG. 4. Costimulation with mB7 and anti-CD3 mAb induces lymphokine secretion. Microcultures were set up as described in the legend to Fig. 2 with 2 × $10^5$ purified AKR T lymphocytes. Then 2 × $10^4$ paraformaldehyde-fixed CHO-mB7.S8 cells (■), 2 × $10^4$ paraformaldehyde-fixed CHO cells (□), PMA at 10 ng/ml (●), or medium (○) was added to the cultures. Anti-CD3 mAb 145-2C11 (*A*), anti-Thy-1 mAb G7 (*B*), and anti-Ly-6A.2 mAb 8G12 (*C*) were added at the indicated concentrations. After 20–24 hr, aliquots of the culture supernatants were harvested, irradiated (8000 rads; 1 rad = 0.01 Gy), and analyzed for lymphokine content in the HT-2 bioassay. Indicated on the *y*-axis is the incorporation of [$^3$H]thymidine by the IL-2/IL-4-responsive HT-2 indicator cell line.

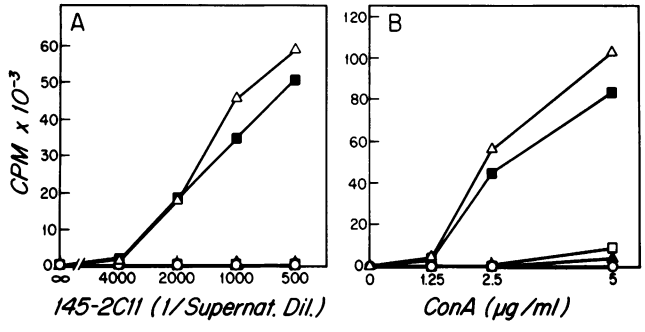


FIG. 5. Costimulation of T cells with mB7 and either anti-CD3 mAb or Con A induces IL-2 secretion. Microcultures were set up as described in the legend to Fig. 3 with $2 \times 10^5$ purified AKR T lymphocytes. Medium (○), $2 \times 10^4$ fixed CHO-mB7.S8 cells (■), or $2 \times 10^4$ fixed CHO cells (□) were added to the cultures. T cells were stimulated with anti-CD3 mAb 145-2C11 (*A*) or Con A (*B*). After 20–24 hr, aliquots of the culture supernatants were harvested, irradiated (8000 rads), and analyzed for lymphokine content in the HT-2 assay. A fine determination (IL-2 vs. IL-4) was performed by adding anti-IL-4 mAb (△) or anti-IL-2 mAb (▲) to the HT-2 culture medium where indicated. The sensitivity of the HT-2 indicator line to IL-4 and the efficiency and specificity of the anti-IL-4 and anti-IL-2 mAb reagents were confirmed in IL-2 and IL-4 titration experiments run in parallel (data not shown).

proliferate, at least in bulk cultures, by a combination of pharmacological reagents, such as a calcium ionophore and PMA (38). Because of the ability of mB7 to substitute for PMA as a source of costimulatory activity in anti-CD3 mAb- or Con A-mediated T-cell activation, we examined whether mB7 could synergize with the calcium ionophore A23187 to trigger T-cell proliferation. As shown in Fig. 7*B*, this is not the case. In contrast, PMA and A23187 can synergize. Thus PMA serves as a positive control in this experiment. In agreement with previous findings (13), some T-cell proliferation was, however, induced by a combination of CHO-mB7.S8 cells and PMA (Fig. 7*A*), although this stimulatory effect was of a much lesser magnitude than that observed in

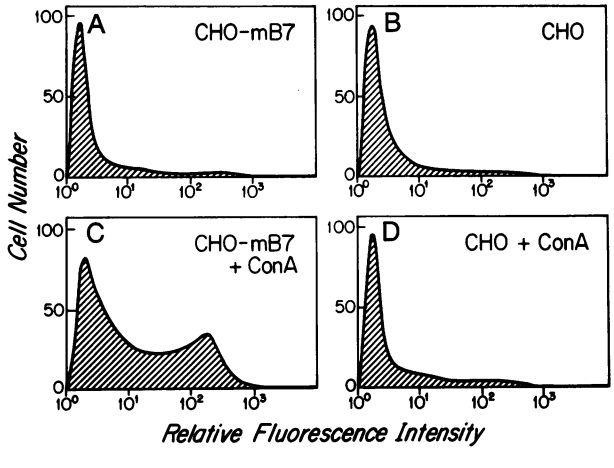


FIG. 6. Costimulation with mB7 induces IL-2 receptor expression. Purified BALB/c CD4$^+$ T cells ($10^7$) were incubated in 10 ml of culture medium in 15-ml conical tubes in the presence of $4 \times 10^6$ paraformaldehyde-fixed CHO-mB7.S8 cells (*A* and *C*, labeled in the panels as CHO-mB7 cells) or CHO cells (*B* and *D*). Where indicated (*C* and *D*), Con A was added to the culture medium at a final concentration of 2.5 μg/ml. Cells were analyzed after 24 hr with the anti-IL-2 receptor mAb 7D4 and a second-step FITC-goat anti-rat immunoglobulin antibody (Kierkegaard and Perry Laboratories, Gaithersburg, MD). Staining with second-step reagent only was performed as a negative control (data not shown). Samples were analyzed on a FACScan (Becton Dickinson). Five thousand cells were analyzed per sample.

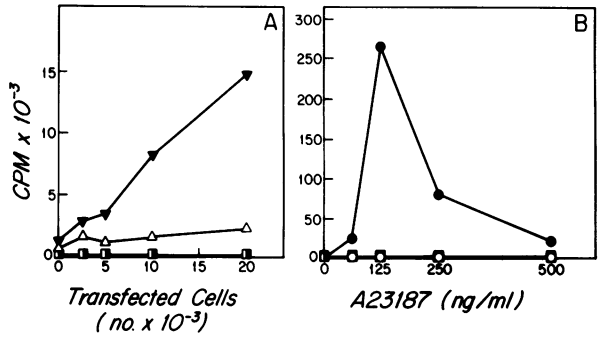


FIG. 7. Costimulation of T cells with mB7 and PMA but not with mB7 and calcium ionophore induces cell proliferation. (*A*) Costimulation of T cells with CHO-mB7.S8 cells and PMA. Purified BALB/c CD4$^+$ T lymphocytes ($2 \times 10^5$) were stimulated with paraformaldehyde-fixed CHO-mB7.S8 cells (▼, ■) or CHO cells (□, △). PMA was added at 10 ng/ml where indicated (▼, △). Indicated on the *x*-axis is the number of transfected cells ($2.5 \times 10^3$–$2 \times 10^4$) added to each culture. (*B*) Coculture of T cells with CHO-mB7 cells and A23187. In this experiment, $2 \times 10^4$ paraformaldehyde-fixed CHO-mB7.S8 cells (■), $2 \times 10^4$ paraformaldehyde-fixed CHO cells (□), PMA at 10 ng/ml (●), or medium alone (○) was added to $2 \times 10^5$ purified BALB/c CD4$^+$ T lymphocytes. Indicated on the *x*-axis are concentrations of the calcium ionophore A23187 (Calbiochem, La Jolla, CA). After 48 hr, cultures in *A* and *B* were pulsed with 1 μCi of [$^3$H]thymidine per well for the last 6 hr of the incubation period to assay for T-cell proliferation.

the human system (7). It is also noteworthy that the combination of CHO-mB7.S8 cells and PMA is much less stimulatory than the combinations of either anti-CD3 mAb + CHO-mB7.S8 cells or anti-CD3 mAb + PMA (compare Fig. 7*A* with Fig. 2 or 3).

## DISCUSSION

T lymphocytes require at least two stimuli that together lead ultimately to complete cell activation (1). In the case of a T helper cell response, both signals are provided by an APC. The first signal, imparted through the TCR, is a complex of peptide and MHC class II protein. The second signal for T-cell activation is also provided by the APC. The role of the APC in providing the second signal has been established through the use of lectins such as Con A and through the use of anti-TCR antibodies. Purified T cells respond to these stimuli only in the presence of certain MHC class II$^+$ and MHC class II$^-$ accessory cells (24, 39). Thus a second signal, distinct from the MHC proteins, is required for T-cell activation.

In the present report we establish that mB7 is a potent costimulatory molecule for the activation of resting murine CD4$^+$ T cells through the TCR/CD3 complex. Stable mB7-transfected CHO cells, but not control transfectants, synergize with anti-CD3- and Con A-induced T-cell activation, resulting ultimately in proliferation. Thus the functional effects of the mB7 antigen on murine T lymphocyte activation are specific.

CD4$^+$ T lymphocytes synthesize IL-2 (Th-1), IL-4 (Th-2), or a combination of both (3). In our study we found, using bulk populations of CD4$^+$ T cells, that mB7 exerted its effect by inducing IL-2 production and IL-2 receptor expression. Thus, mB7 costimulates T-cell activation through the TCR/CD3 complex by positively modulating the normal pathway of T-cell expansion of the major T-cell subset. Because of the precursor frequency ratio of Th-1 and Th-2 cells in normal mice (40, 41), our experiments cannot rule out the possibility that the mB7 antigen also has an effect on IL-4-secreting T cells. Analyzing the effect of mB7 on a panel of IL-2- or IL-4-secreting T-cell clones should allow us to resolve this question.

Our results raise a number of interesting issues. First, in contrast to the pronounced effect of mB7 on activation of T cells through the TCR/CD3 complex, mB7-transfected CHO cells costimulated T-cell activation via the GPI-anchored proteins Thy-1 and Ly-6A.2 only inefficiently when the pharmacological reagent PMA was used as a standard source of costimulatory activity. Our data point to a molecular difference between the TCR-mediated pathway of T-cell activation and that via the GPI-anchored proteins. Our findings also raise the issue whether there exists a costimulatory molecule for the activation via the GPI-anchored proteins that is expressed on normal APCs and distinct from mB7.

Second, the combination of a calcium ionophore and mB7 is not sufficient to cause T-cell proliferation. In contrast, the combination of a calcium ionophore and PMA stimulates T cells efficiently. The signals that mB7 and PMA provide for T-lymphocyte activation are therefore not interchangeable, although both costimulate activation through the TCR/CD3 complex. It is in fact clear from our data that PMA and mB7 can synergize with one another to cause T-cell proliferation, although an effect of greater magnitude is observed in T-cell cultures that have been stimulated with anti-CD3 mAb + PMA, Con A + PMA, anti-CD3 + mB7, and Con A + mB7. This result is also consistent with data obtained with anti-CD28 mAbs. Taken together, our data strongly suggest that the function of mB7 cannot be merely the activation of protein kinase C or the modulation of the intracellular calcium concentrations. They also point to a distinct signaling pathway in T cells triggered by binding of the mB7 antigen to the T cell surface.

On human T cells there are at least two receptors for B7. The first is the CD28 antigen (10). Binding of mAbs to CD28 can augment the proliferation or lymphokine secretion of T cells stimulated with suboptimal doses of anti-CD3 mAbs (11, 12). The second is the CTLA-4 molecule (42). This protein, structurally related to CD28, was identified by differential screening of a murine cytolytic T-cell cDNA library, and CTLA-4 transcripts were found in cytotoxic T cells (43). Preliminary data, however, suggest that the molecule is also expressed by $CD4^+$ T cells (G.J.F. and David Lombard, unpublished data). Blocking experiments with anti-CD28 and anti-CTLA-4 antibodies will therefore be required to clarify through which structure the mB7 molecule exerts its effect on $CD4^+$ T cells.

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