CELLULAR IMMUNOLOGY 162, 315–320 (1995)

SHORT COMMUNICATION

Activation of Murine T Cells by Bacterial Superantigens Requires B7-Mediated Costimulation

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Received October 5, 1994; accepted January 24, 1995

***Staphylococcus* enterotoxins bind class II MHC molecules on antigen-presenting cells (APC) and stimulate T cells expressing appropriate Vβ gene products. Although the role of non-TcR-associated costimulatory receptors during antigen-specific T cell stimulation has been clearly established, the involvement of costimulatory activity in T cell activation by superantigens (SAgs) has been the matter of controversy. The aim of this study was to evaluate the role of the costimulatory–receptor ligand molecules CD28/B7 on bacterial SAg-mediated activation of naive murine T cells. We demonstrate in this report that a combination of monoclonal antibodies to murine B7.1 and B7.2 molecules inhibits the *in vitro* response of naive T cells to SAgs SEA, SEB, and TSST-1. The inhibition of T cell responses required simultaneous blocking of B7.1 and B7.2, suggesting that either B7.1 or B7.2 is sufficient to provide costimulatory signals to naive T cells in response to bacterial exotoxins. Inhibition of T cell activation by antibodies to B7-related molecules can be overcome by antibodies to CD28, a finding in agreement with the hypothesis that CD28-mediated signals participate in T cell activation by bacterial SAgs. These observations suggest that, as demonstrated for conventional antigen, T cell activation by SAgs requires the coordinated participation of TcR- and CD28-derived signals.**

INTRODUCTION

A growing list of proteins produced by bacteria, mycoplasma, and viruses has been shown to provoke a variety of pathological effects, including septic shock, in both human and mouse models. The term superantigen (SAgs) was introduced to describe this group of microbial antigens sharing several important features that distinguish them from conventional antigens (for review, see Ref. 1). SAgs behave as bifunctional agents able to simultaneously interact with the variable Vβ region of the T cell receptor (with little influence of the other TcR components) and with a conserved domain of the class II MHC molecule (independently of the polymorphic residues or the nature of the bound peptide). As a consequence, a given SAg is able to interact with a large fraction (1–30%) of the T cell repertoire, causing massive T cell activation and systemic release of inflammatory cytokines in the circulation. Numerous studies performed with conventional antigens have demonstrated that, in addition to TcR occupation, optimal T cell activation requires costimulatory signals delivered by the antigen-presenting cell (APC) (for review, see Ref. 2). APC-associated molecules of the B7 family (B7.1/BB1 (3), B7.2 (4, 5)) which interact with counter-receptors CD28 and/or CTLA4 are thought to represent an important signaling pathway in T cell costimulation. APC able to stimulate resting T cells, such as dendritic cells (6), have been shown to express high levels of B7.1 and B7.2 molecules (7, 8). Moreover, cell surface expression of B7.1 or B7.2 molecules enhances the ability of transfectants to provide costimulatory signals to naive T cells (9). A soluble CTLA4 fusion protein (an engineered molecule that binds with high affinity to B7 family molecules and therefore blocks its interaction with CD28) inhibits T cell activation during antigen-specific or allogeneic stimulation (10–12), further illustrating the importance of costimulation provided by B7 molecules in T cell responses.

While the role of CD28/B7 pathways has been well documented with conventional antigens, there have been conflicting reports on the requirement for CD28-mediated costimulation in the immune response to bacterial SAgs. Although numerous studies have indicated that signaling through CD28 enhances T cell responses to SAgs (8, 13–15), it is not clear to date whether CD28/

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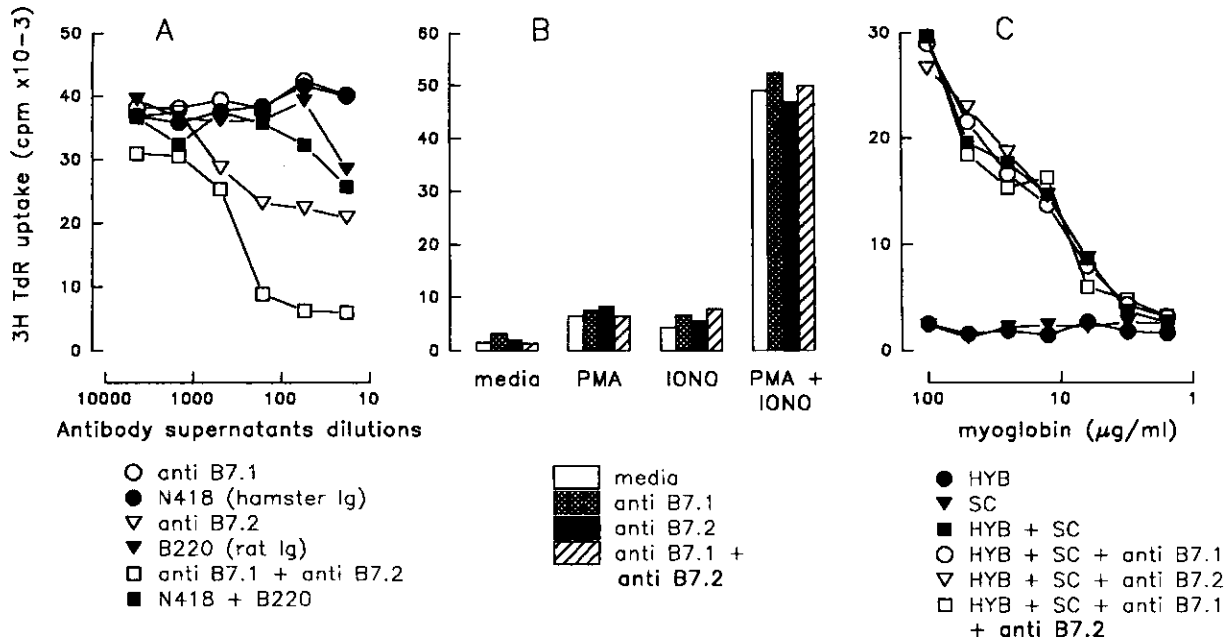

FIG. 1. Specificity of anti-B7.1 and anti-B7.2 mAbs-mediated inhibition of T cell responses *in vitro*. Spleen cells (5×10^5 cells/well, A and B) were stimulated *in vitro* in the presence of either anti-CD3 mAbs (1 μg/ml) or a combination of PMA (10 ng/ml) and ionomycin (60 ng/ml). The myoglobin-specific T cell hybridoma 13-26-8-HG.1 (5×10^5 cells/well) was stimulated by myoglobin in the presence of unirradiated, naive spleen cells (5×10^5 cells/well). Cultures were supplemented with graded doses of antibody-containing culture supernatants (A) or with a 1/200 final dilution of anti-B7.1- or anti-B7.2-containing supernatants. When indicated, a 1:1 mixture of antibodies, each present at a 1:400 dilution, was used. Culture supernatants were harvested following 24 hr of culture and assayed for IL-2 content by bioassay. Results are expressed as cpm of [^{3}H]TdR incorporation by the IL-2 dependent cell line CTL.L.

B7 interactions represent an obligatory step for SAg-mediated T cell activation. Recent studies indicate, for example, that CTLA-4Ig (16, 17) or anti-B7.1 antibody (18) has little or no effect on SAg-mediated naive T cell activation. In order to assess the role of costimulation in the immune response to SAgs, we have performed a detailed analysis of the *in vitro* response of murine naive T cells to bacterial SAgs using antibodies to B7.1 and B7.2 as blocking reagents. Our data demonstrate that T cell activation by SAgs *in vitro* is strictly dependent on costimulatory signals provided by either B7.1 or B7.2 molecules. Blocking of T cell responses to SAgs required the simultaneous presence of antibodies to B7.1 and B7.2, indicating that signals provided by B7.1 and B7.2 may be redundant for the *in vitro* response of murine T cells to bacterial exotoxins. These data show that, as demonstrated for conventional and alloantigens, T cell responses to bacterial SAgs require both TcR- and CD28-derived signals.

MATERIALS AND METHODS

Mice. Female Balb/c and DBA/2 mice, 6–8 weeks old, were purchased from Charles River Wiga (Sulzfeld, Germany) and maintained in a pathogen-free environment in our own animal facility.

Reagents and antibodies. The following antibodies to murine determinants were used in this study: 37.51 (anti-CD28, hamster Ig) (19) and F531 (isotype-matched hamster Ig), 145-2C11 (anti-CD3ε, hamster Ig) (20), 16-10A1 (anti-B7.1, hamster Ig) (21), B220 (anti-CD45R, rat IgG2a), GL1 (anti-B7.2, rat IgG2a) (4), N418 (anti-CD11c, hamster Ig), and HBδ-6 (anti-IgD, rat IgG2a) (22). Concanavalin A, calcium ionophore A 23187, and phorbol myristate acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium ionophore was dissolved in DMSO at the concentration of 1 mg/ml. PMA was dissolved in anhydrous ethanol at a concentration of 10 μg/ml. Purified SEA, SEB, and TSST-1 were purchased from Toxin Technology, Inc. (Sarasota, FL). These reagents had no effect on IL-2 determination (not shown).

Cell lines. The I-E^d-restricted, myoglobin-specific T cell hybridoma 13-26-8-HG.1 (23) was derived by Dr. A. Livingstone (Basel Institute of Immunology, Basel, Switzerland).

In vitro responses. All *in vitro* immune responses were performed in serum-free medium containing RPMI 1640 (Seromed; Biochem KG, Berlin Germany) supplemented with 2% HY ultroser (Gibco BRL), penicillin, streptomycin, nonessential amino acids, sodium pyruvate, 2-ME, and L-glutamine (Flow ICN Biomedicals,

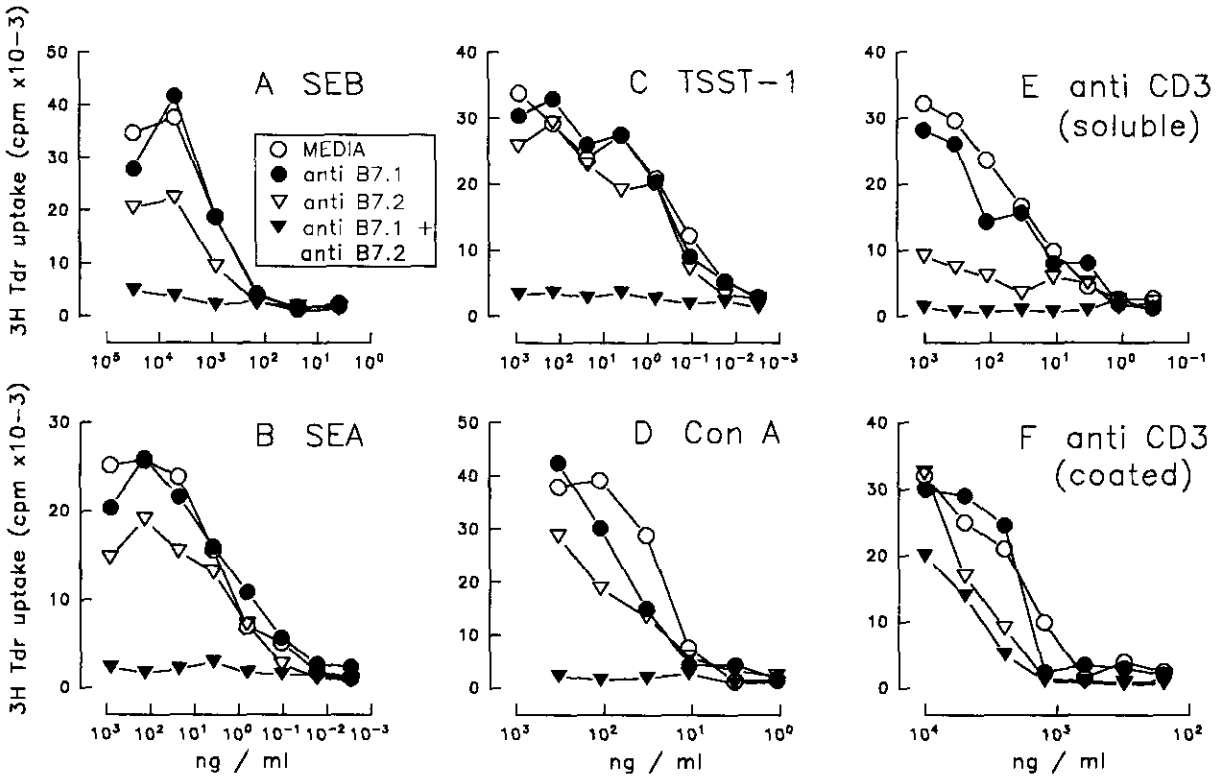

FIG. 2. Role of B7.1 and B7.2 molecules in T cell activation to bacterial superantigens, anti-CD3 mAbs, and concanavalin A. Spleen cells (5×10^5 cells/well) were cultured for 24 hr in the presence of graded doses of bacterial SAgs or T cell mitogens as indicated. mAbs to B7.1 and B7.2 were added as described in the legend of Fig. 1. Results are expressed as cpm of [^{3}H]TdR incorporation by the IL-2-dependent cell line CTL.L.

Bucks, UK). An adequate number of spleen cells (see results for cell numbers) was stimulated by lectins, mAbs, or SAgs in a total volume of 0.2 ml in 96-well U-bottom plates. Splenic $CD4^+$ cells were purified by depleting adherent cells over Sephadex G-10 (Pharmacia, Uppsala, Sweden) and following removal of sIg^+ and $CD8^+$ cells by a magnetic cell sorter (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturers' recommendations. The resulting population contained <1% Ig^+ and <1% $CD8^+$ cells and consisted of between 75 and 80% $CD4^+$ cells. APC were obtained following complement-mediated lysis of anti-Thy-1.2 (clone HO-13.4, available through the ATCC)-labeled spleen cells and consisted of <1% $CD3^+$ cells. Cultures were maintained at 37°C in a humidified incubator (7% CO_2). Supernatants were collected after 18–24 hr of culture, frozen, and assayed for IL-2 content by a bioassay using a subclone of the CTL.L cell line insensitive to murine IL-4 (24).

RESULTS

Inhibition of T Cell Activation by Antibodies to B7.1 and B7.2

In order to assess the specificity of anti-B7-mediated inhibition, naive spleen cells were incubated with graded doses of monoclonal antibodies (mAbs) to B7.1 (clone A16-10A1) and B7.2 (clone GL1) and stimulated *in vitro* by soluble anti-CD3ε mAbs. Isotype-matched hamster mAbs to CD11c (clone N418) and rat mAbs to CD45R (clone B220) were used as controls. As shown in Fig. 1A, mAbs to B7.2 markedly inhibited CD3-mediated T cell responses, while mAbs to B7.1 had no effect (see also Fig. 2F). A combination of both mAbs strongly inhibited IL-2 production induced by mAbs to the TcR complex, whereas all isotype-matched controls remained ineffective. In order to further verify the specificity of the anti-B7-mediated inhibition, the blocking properties of these mAbs on costimulatory-independent responses were assayed. As shown in Fig. 1, neither the IL-2 response induced by pharmacological agents (PMA + ionomycine) (Fig. 1B) nor the antigen-induced IL-2 production by a CD28-negative T cell hybridoma (Fig. 1C) was significantly inhibited by a fixed dose (1/200 final dilution of culture supernatant) of mAbs to B7.1 and B7.2 molecules.

A Combination of Anti-B7.1 and Anti-B7.2 Inhibits Bacterial Superantigen-Induced IL-2 Response

In order to study the role of CD28/B7 interactions in response to bacterial SAgs, naive spleen cells were stimulated *in vitro* by graded doses of SEA, SEB, and TSST-

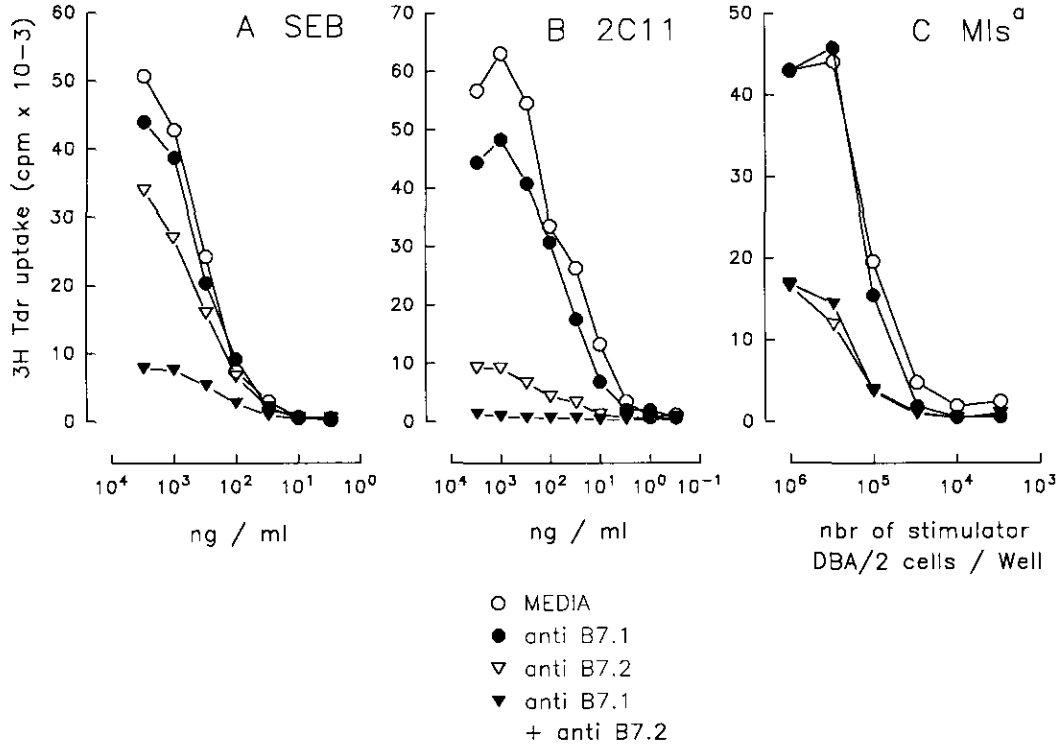

FIG. 3. Role of B7.1 and B7.2 molecules in T cell activation to SEB, anti-CD3 mAbs, and Mls^a. CD4 Balb/c T cells (3×10^5 cells/well) were stimulated for 24 hr as follows: A, SEB and syngeneic APCs (3×10^5 cells/well); B, anti-CD3 MAbs and syngeneic APCs (3×10^5 cells/well); and C, splenic APC from DBA/2 mouse previously injected with anti IgD MAbs (300 μg in PBS) as described (22). MAbs to B7.1 and B7.2 were added as described in the legend of Fig. 1. Results are expressed as cpm of [^{3}H]TdR incorporation by the IL-2-dependent cell line CTL.L.

1 in the presence of a fixed dose of either anti-B7.1 or anti-B7.2 mAbs (1/200 dilution of culture supernatant) or a combination of the two mAbs (at 1/400 dilution each). Anti-B7.2 mAbs, but not anti-B7.1 mAbs, weakly inhibited SAgs-induced *in vitro* response (see Figs. 2A and 2B). However, a combination of both anti-B7.1 and anti-B7.2 mAbs was required in order to significantly inhibit the IL-2 production in response to all bacterial exotoxins tested. Similarly, a combination of anti-B7.1 and anti-B7.2 mAbs completely inhibited the response of naive T cells to soluble anti-CD3 or concanavalin A (Figs. 2D and 2E). Note that the response of naive T cells to plastic-bound anti-CD3 is weakly affected by mAbs to B7, suggesting that these reagents only inhibit APC-mediated T cell activation (Fig. 2F). Of interest, anti-B7.2 mAbs significantly inhibited the response of naive spleen cells to soluble anti-CD3, while they only moderately affect the SAgs or the lectin-induced response (Fig. 2). Similarly, Hathcock *et al.* have recently demonstrated that the anti-B7.2 mAbs are sufficient to block the T cell response to a retroviral superantigen (25). In order to further confirm that the *in vitro* response to bacterial superantigens requires the coordinate blocking of both B7.1 and B7.2 molecules, the following experiment was performed. CD4$^+$ Balb/c spleen cells (H-2d, Mls^{a-}) were stimulated in culture by SEB or soluble anti-CD3 in the presence of autologous APCs or by graded numbers of DBA/2 (H-2d, Mls^{a+}) APCs. The inhibitory properties of anti-B7 mAbs on bacterial SAgs, anti-CD3 antibodies, and retroviral SAgs *in vitro* T cell responses are shown in Fig. 3. In agreement with previous findings, anti-B7.2 mAbs significantly inhibited anti-CD3- and Mls^a-specific responses, while a combination of anti-B7.1 and anti-B7.2 was required in order to affect the *in vitro* response to SEB.

Reversal of Anti-B7-Mediated Inhibition of in Vitro Responses by Antibodies to CD28

In order to further confirm the role of CD28-mediated signals in response to bacterial SAgs, we attempted to overcome the inhibitory effect of anti-B7 mAbs by stimulating naive T cells in the presence of mAbs to CD28. As shown in Fig. 4, addition of anti-CD28 mAbs completely restores the response of naive T cells to TSST-1 or to soluble anti-CD3 in the presence of blocking concentrations of anti-B7 mAbs.

DISCUSSION

The observations reported in this study indicate that a combination of mAbs to B7.1 and B7.2 inhibits the *in vitro* response of naive T cells to bacterial exotoxins. Anti-B7 mAbs appear to inhibit T cell responses by dis-

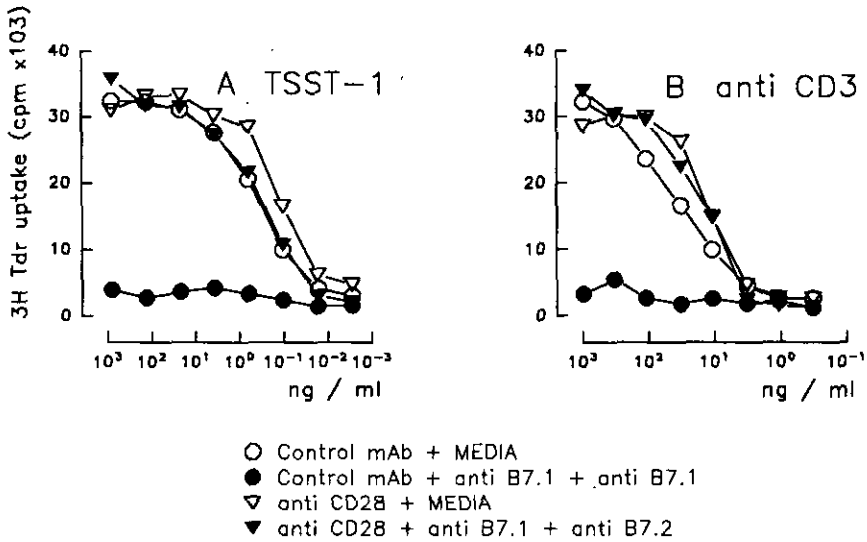

FIG. 4. Reversal of anti-B7.1- and anti-B7.2-mediated inhibition by mAbs to CD28. Spleen cells (5×10^5 cells/well) were stimulated by graded doses of agonists (TSST-1 or anti-CD3 mAbs) in the presence of mAbs to CD28 (clone 37.51) or control mAbs (clone F531). Both mAbs are used at 1/10000 dilution of ascites. mAbs to B7.1 and B7.2 are used as described in the legend of Figure 1. Results are expressed as cpm of [^{3}H]TdR incorporation by the IL-2-dependent cell line CTL.L.

rupting an obligatory interaction between CD28/CTLA4 and B7.1/B7.2. This conclusion is supported by the finding that anti-B7.1 and anti-B7.2 mAbs do not inhibit APC-independent T cell activation induced by pharmacological agents bypassing the early steps of signal transduction nor the response of a CD28-negative T cell hybridoma. Moreover, the combination of anti-B7 mAbs failed to completely abrogate the response of naive spleen cells to plastic-bound anti-CD3 mAbs. Taken together, these observations suggest that mAbs to B7 molecules do not affect the viability nor the ability of splenic APCs to generate a TcR ligand, but inhibit their ability to provide a costimulatory signal to naive T cells. Finally, anti-CD28 mAbs could overcome the inhibitory effect of anti-B7.1 and anti-B7.2 immunoglobulins, further suggesting that anti-B7 mAbs inhibit T cell responses by preventing the interaction of B7.1 and B7.2 molecules with their appropriate ligands. B7.1 and B7.2 molecules appear to provide redundant costimulatory signals to naive T cells in response to SAgs, as suggested by the finding that only a combination of mAbs to both B7.1 and B7.2 is inhibitory in this model. In contrast, and as previously reported by others (25), the response of naive T cells to soluble anti-CD3 or to Mls^a is markedly inhibited by mAbs to B7.2 alone. Because B7.1 and B7.2 transfectants have been found equally effective in providing T cell costimulation *in vitro* (7, 8), these results suggest that the sensitivity of a particular *in vitro* T cell response to anti-B7-mediated inhibition will be dictated by the phenotype of the APC. In particular, it has been clearly demonstrated that soluble anti-CD3 mAbs require the presence of FcR$^+$ APCs in order to efficiently aggregate the TcR complex. As recently shown, FcR$^+$ naive B cells express mainly the B7.2 protein (25), while dendritic cells (known to weakly express FcR) bear both B7.1 and B7.2 cell surface-associated molecules (7). The observations reported here are therefore compatible with the hypothesis that T cell responses to mitogens presented predominantly by B cells (anti-CD3 or Mls determinants) are sensitive to anti-B7.2 inhibition with poor synergistic blocking activity of antibodies to B7.1 and B7.2. In contrast, responses to bacterial SAgs, lectins (this work), alloantigens, and conventional antigens (26) which can all be presented by cells bearing either B7.1 or B7.2 molecules are only inhibited by a combination of both B7 mAbs.

Finally, *in vivo* administration of bacterial SAgs leads to polyclonal T cell activation and lymphokine production (including IL-2), followed by anergy and reduction in the number T cells bearing specific Vβ TcR segments (for review, see Ref. 1). Thus, as the *in vitro* studies described here indicate a strict requirement for B7-mediated costimulation in response to bacterial exotoxins, we postulate that *in vivo* production of T-cell-derived lymphokines observed 2 hr following SEB administration (27) results from SAg presentation by APC constitutively expressing B7.1 and/or B7.2 molecules. Further experiments are needed in order to confirm the contribution of the CD28/B7 pathway in the *in vivo* activation of T cells by bacterial exotoxins and before an anti-B7-based therapy in the treatment of toxic shock syndrome involving SAgs can be envisaged.

ACKNOWLEDGMENTS

The authors thank G. Dewasme, M. Swanepoel, and F. Tielemans for expert technical assistance, J. Allison, A. Livingstone, H. Reiser,

H. Bazin, and R. J. Hodes for providing reagents, and S. Barcy and J. Ismaili for helpful discussion. This work presents research results of the Belgian Program in Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming. The scientific responsibility is assumed by its authors. E.M. and T.D.S. are supported by a grant from the IRSIA. M.M. and O.L. are research associates from the National Funds of Scientific Research (Belgium).

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