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Co-stimulation lowers the threshold for activation of naive T cells by bacterial superantigens

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Abstract

Staphylococcus enterotoxins bind class II MHC molecules on antigen presenting cells (APC) and stimulate T cells expressing appropriate V_β gene products. Although the role of non-TCR associated co-stimulatory receptors during antigen-specific T cell stimulation has been clearly established, the involvement of co-stimulatory activity in T cell activation by superantigens has been the matter of controversy. In this report, we examine the role of co-stimulation provided by selected APC populations in the response to bacterial exotoxins (staphylococcal enterotoxin A, staphylococcal enterotoxin B and toxic shock syndrome type 1). We demonstrate that the APC population able to activate naive T cells to IL-2 production is heterogeneous, comprising both adherent (presumably dendritic) and non-adherent (mostly B lymphocytes) cells. By stimulating naive T cells in the presence of graded doses of superantigens, we have observed that half-maximal IL-2 production was achieved at lower doses of superantigens in the presence of dendritic cells. Similarly, addition of antibodies to CD28 or B7.1-transfected cell lines increased the sensitivity of naive T cells to lower doses of superantigens. These observations indicate therefore that superantigens can be presented to naive T cells by APC displaying distinct levels of co-stimulatory activity, although with different efficacy. Thus, naive T cells are sensitive to CD28-mediated co-stimulation during superantigen-mediated responses but IL-2 production can be induced by high doses of superantigens in the presence of APC expressing weak co-stimulatory activity. These observations are compatible with the hypothesis that CD28-mediated signals participate in T cell activation by lowering T cell sensitivity to TCR ligands.

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 was introduced to describe this group of microbial antigens sharing several important features that distinguish them from conventional antigens (for review, see 1). Superantigens behave as bifunctional agents able to simultaneously interact with the variable V_β region of the TCR (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 superantigen 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. In fact, there is compelling evidence that the toxicity of superantigens *in vivo* is due to an exaggerated host T cell response as indicated by the fact that cyclosporin A is able to block the development of lethal shock (2). Moreover, SCID mice, which are naturally resistant to superantigen-induced toxicity, acquire superantigen sensitivity upon T cell repopulation (2). Thus, most of the biological effects of superantigens

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appear to be related to their ability to polyclonally activate subsets of T lymphocytes expressing selected V_β regions.

It has been recently demonstrated that activation of resting T cells initiated by engagement of the TCR with its physiological ligand antigen–MHC (signal 1) requires additional signals delivered by the antigen presenting cell (APC) (signal 2) (for review, see 3). Molecules belonging to the B7 family [B7.1/BB1 (4), B7.2 (5,6)] which interact with counter-receptors CD28 and/or CTLA-4 are thought to represent one of the most potent co-stimulatory molecules for T cell activation, and APC able to stimulate resting T cells, such as dendritic cells (DC) (7), have been shown to express high levels of CTLA-4-binding ligands (8). DC are known to induce potent T cell responses to superantigens in a CD28-dependent manner, suggesting thus a role for B7-related co-stimulatory molecules in the response to bacterial exotoxins (9–11). However, super-antigen-dependent proliferation can be induced by resting B cells (12), known to express low levels of co-stimulatory activity. In another study, CD28–B7-independent stimulation of naive T cells by superantigens has been reported (13). The conflicting results reported in the literature concerning the role of co-stimulation in the *in vitro* responses to super-antigens may be due to superantigen doses and origin, host species, and experimental conditions. Since pathogenicity of superantigens strongly correlates with their ability to activate naive T cells, the nature of the cells presenting superantigens *in vivo* may determine their toxicity *in vivo*.

In order to gain information on the role of APC and co-stimulation in the immune response to superantigens, we have performed a detailed analysis of the *in vitro* response of murine naive T cells to bacterial superantigens presented by distinct class II-expressing cell populations. Our data confirm that the ability to stimulate naive T cells *in vitro* is not restricted to a particular class II-positive cell population since both adherent and non-adherent cells can induce proliferation and IL-2 production in the presence of superantigens. Co-stimulatory activity (provided by purified DC, B7.1-transfected cells or antibodies to CD28) strongly enhances IL-2 production in response to superantigens *in vitro*. The comparative study of the *in vitro* response of murine T cells to several bacterial superantigens [staphylococcal enterotoxin A (SEA), staphylo-coccal enterotoxin B (SEB) and toxic shock syndrome type 1 (TSST-1)] known to display distinct affinities for murine MHC class II determinants also suggests that *in vitro* requirements for co-stimulatory activity are strongly dependent upon super-antigen concentration and affinity. Overall, these observations suggest that co-stimulation provided by molecules of the B7 family increases T cells responsiveness to superantigens by lowering the threshold of TCR-dependent activation.

Methods

Mice

Female BALB/c mice, 6–8 weeks old, were purchased from Charles River Wiga (Sulzfeld, Germany).

Reagents

Hamster mAb 37.51 to murine CD28 and control mAb F531 (kindly provided by J. Allison, University of California, Berkeley, CA; 14) were used at 1/10,000 dilution of ascitic fluids. The rat mAb 1G10 to mouse B7.1 (15) was kindly provided by N. Nabavi (Hoffman-La Roche, Nutley, NJ). Concanavalin A (Con A) was obtained from Sigma (St Louis,MO). Purified SEA, SEB and TSST-1 were obtained from Toxin Technology (Sara-sota, FL). rIL-4 was produced in methotrexate-amplified Chinese hamster ovary cells and was a kind gift from Dr W. Fiers (State University, Gent, Belgium).

Cell lines

The I-E^d-restricted, myoglobin-specific T cell hybridoma 13-26-8–HG.1 (16) was derived by Dr A. Livingstone (Basel Institute of Immunology, Basel, Switzerland).

The B7.1 gene was introduced into the A20 B cell lymphoma cell line (available through the ATCC, Rockville, MD) by retroviral infection. Poly A^+ RNA was isolated from the BCL1 cell line (17) using the quickprep micro mRNA purification kit from Pharmacia (Uppsala, Sweden). First strand cDNA was made using an oligo(dT) primer and the superscript pre-amplification system from BRL (Gibco/BRL Life Technologies, Merelbeke, Belgium). Amplification was performed in a 50 µl volume containing 10 mmol/l Tris, pH 8.3, 50 mmol/l KCl, 2 mmol/l $MgCl_2$, 0.01% (w/v) gelatin, 30 pmol of each oligo-nucleotide primer, 0.2 mmol/l of each deoxynucleotide tri-phosphate and 1.25 U Taq polymerase (Cetus, Emmeryville, CA) on 1–2 µl of the first strand cDNA reaction material. The primers spanning the start and stop codons of the B7 sequence used were the sense primer, 5′-GCATAA-GCTTCTGAAGCTATGGCTTGCAATT-3′, and the antisense primer, 5′-GACCGTCTTCCTTTAGTTCTTCTCTCGAGGATC-3′. Each PCR cycle consisted of 94°C heat denaturation for 0.5 min and primer annealing at 55°C for 0.5 min followed by primer extention at 72°C for 1 min. Forty cycles were per-formed in a biomed thermocycler (Braun, Brussels, Belgium). The B7.1 polymerase chain reaction (PCR) product of 953 bp was blunted and cloned into the blunted *Eco*RI site of the retroviral vector pMXSVNeo-18 (kindly provided by Dr R. Ralston, Chiron, Emmeryville, CA) and sequenced using dideoxy chain termination sequencing procedures with [^{35}S]adATP and Sequenase (US Biochemicals, Cleveland, OH). Clones wich contained no PCR mistakes were used for transfection of the packaging cell line Y2, using a liposome transfection kit DOTAP (Boerhinger Mannheim, Bruxelles, Belgium). After 48 h the cells were transferred on culture medium containing 2 mg/ml geneticin G-418 sulfate (Gibco/BRL). Surviving clones were tested for B7 expression by staining with CTLA-4–Ig. High titer clones were selected by subsequent infection of 3T3-NIH fibroblasts. A20 cells were infected by co-culture on a Y2-B7 monolayer in the presence of 8 µg/ml polybrene during 48 h. The cells were gently removed from the monolayer and subcloned in culture medium containing 1 mg/ml geneticin G-418. Surviving clones were tested for B7 expression by FACS analysis.

Flow cytometry

Cells were analyzed by flow cytometry with a FACScan cytometer (Becton Dickinson, Mountain View, CA). N418; 14-4-4S (mouse anti I-$E^{k,d}$, available through the ATCC) and anti-CD3 [mAb 145-2C11 anti-CD3e (18)] were purified and modified in our laboratory according to standard procedure.

The FITC-coupled anti-mouse CD8, phycoerythrin (PE)-coupled anti-mouse κ and PE-coupled anti-mouse CD4 were purchased from Becton Dickinson (Oxnard, CA). In all cases, cells were gated according to size and scatter in order to eliminate dead cells and debris from analysis.

In vitro responses

Spleen cell suspensions were depleted of adherent cells by passage over Sephadex G10 (Pharmacia) columns as described (19). Splenic T cells were purified following G10 passage and removal of sIg^+ cells by Magnetic Cell Sorter (Miltenyi Biotec, Bergisch-Gladbach, Germany). The resulting population contained <1% Ig^+ or Ia^+ cells and comprised 70–80% of $CD3^+$ cells.

APC were obtained following complement-mediated lysis (normal rabbit serum produced in our laboratory according to standard procedure) of anti-Thy-1.2 (clone HO-13-4A, available through the ATCC) labeled spleen cells or G10 passed spleen cells. The resulting population contained <1% $CD3^+$ cells. Splenic DC were purified as previously described (20) and contained >95% of $N418^+$ [anti-CD11c mAb (21)] cells and no detectable levels of $CD3^+$ or sIg^+ cells (<1%). Peritoneal macrophages were purified from exudate cells obtained following extensive washing of the peritoneal cavity with 0.34 M sucrose solution at 4°C. After overnight culture in FCS-containing media, non-adherent cells were discarded and adherent cells were collected by scraping with a plastic rubber policeman. The resulting population contained at least 90% of macrophages, as assessed by morphology and specific staining using Mac-1 mAb (ATCC).

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

An adequate number of responder and stimulator cells (see Results for cells numbers) was stimulated by lectins, mAbs or superantigens in a total volume of 0.2 ml in 96-well U-bottom plates. Cultures were maintained at 37°C in a humidified incubator (7% CO_2). Supernatants were collected after 18–24 h of culture, frozen and assayed for IL-2 content using a standard bioassay using a subclone of the CTLL cell line insensitive to murine IL-4 (21). Proliferation of this cell line induced by supernatant from stimulated T cell cultures is completely inhibitable by the anti-murine IL-2 mAb S4B6. Standard curves were generated using human rIL-2 and results are expressed as U/ml or c.p.m.. IFN-γ was quantitated by two-site ELISA using mAb F1 and Db-1, kindly provided by Dr Billiau (KUL, Leuven, Belgium) and P. H. Van Der Meide (TNO Health Research, Rijswijk, The Netherlands) respectively.

Results

Role of co-stimulation in the in vitro immune response of splenic T cells to SEB

The present study was undertaken in order to investigate the role of co-stimulation provided by accessory cells for the *in vitro* response of unprimed T cells to bacterial superantigens. It has been previously established that removal of Sephadex G10-adherent cells from the splenic APC population abrogated the ability of these cells to stimulate an *in vitro* primary alloreactive response (23). Since percentages of B (sIg^+) and T ($Thy\text{-}1^+$) cells are not appreciably altered by passage over G10 columns (19 and our own observations), it is assumed that APC able to initiate primary *in vitro* mixed lymphocyte reaction mostly comprise G10-adherent cells such as macrophages and/or DC. This observation suggested to us that class II-bearing B cells, although able to process and present antigen (24), were unable to provide the co-stimulatory signals required for the activation of resting T cells. In order to test this hypothesis, we included in our analysis a myoglobin-specific, $V_\beta 8$-bearing T cell hybridoma, as numerous studies have suggested that T cell hybrids responded optimally to TCR ligation in the absence of co-stimulatory signals provided by APC (25 and this study). We therefore assumed that the capacity of selected APC populations to generate the adequate TCR ligand could be monitored by their ability to stimulate a T cell hybridoma.

As a first approach, we compared the accessory cell function of unfractionated or G10-passed purified splenic APC (obtained following complement-mediated lysis of Thy-1-bearing spleen cells). Purified splenic T cells were stimulated by SEB (0.5 μg/ml) in the presence of selected APC populations and IL-2 production was assayed following overnight culture. Figure 1 indicates that unfractionated splenic

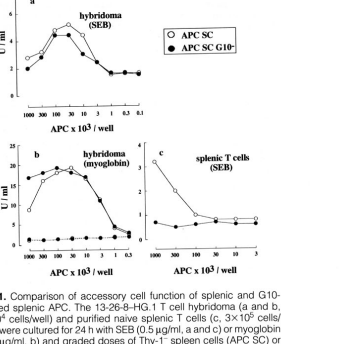

Fig. 1. Comparison of accessory cell function of splenic and G10-passed splenic APC. The 13-2E-8-HG.1 T cell hybridoma (a and b, 3×10^4 cells/well) and purified naive splenic T cells (c, 3×10^5 cells/well) were cultured for 24 h with SEB (0.5 μg/ml, a and c) or myoglobin (100 μg/ml, b) and graded doses of $Thy\text{-}1^-$ spleen cells (APC SC) or $Thy\text{-}1^-$, G10-passed spleen cells (APC SC G10$^-$) as indicated. Culture supernatants were assayed for IL-2 content by bioassay and results are expressed as U/ml.

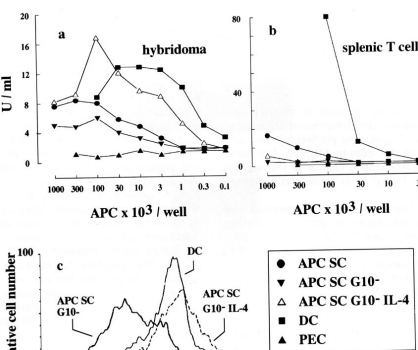

Fig. 2. (a and b) Comparaison of accessory cell function of selected APC populations. Responder T cells (see legend to Fig. 1 for details) were stimulated with SEB (0.5 μg/ml) and selected APC populations including Thy-1$^-$ spleen cells (APC SC), Thy-1$^-$, G10-passed spleen cells (APC SC G10$^-$), purified splenic DC and purified PEC. All APC populations were cultured 18 h in complete media (or media containing rIL-4 when indicated). After 24 h, culture supernatants were assayed for IL-2 content by bioassay and results are expressed as U/ml. (c) I-E cell surface expression on selected APC populations. The indicated APC populations were stained with FITC-labeled anti-I-E^{k,d} mAb (clone 14.4.45) and analyzed using a FACScan. All stainings were performed in the presence of saturating doses of 24G2, a rat mAb to mouse FcRII/III, in order to inhibit FcR binding of staining reagents.

APC efficiently presented SEB to both splenic T cells and T cell hybridoma. No IL-2 production was observed when T cells or SEB were omitted from the culture (data not shown). Removal of Sephadex-adherent cells from the splenic APC population did not significantly modify the ability of these cells to present SEB or antigen (myoglobin) to a T cell hybridoma (Fig. 1a and b). In marked contrast, the G10-passed APC fraction was unable to induce IL-2 production from splenic T cells in the presence of 0.5 μg/ml of SEB. In the light of the two-signals theory of T cell activation, these data suggest that G10-passed APC were able to process antigen and generate an appropriate TCR ligand since they correctly presented SEB and antigen to a T cell hybridoma, but failed to deliver the required co-stimulatory signal(s) to naive T cells in this culture conditions. Note than nominal antigen represent a better stimulus for a murine T cell hybridoma than SEB (compare Fig. 1a and b), an observation that probably relates to the finding that SEB binds with low affinity to murine class II molecules (26). Accordingly, a recent reports demonstrates that bacterial superantigens display a higher affinity for human class II proteins than for murine Ia antigens and that murine T cell hybrids are optimally stimulated by superantigens presented by human APC.

In order to identify the antigen-cell population(s) able to restore the response of naive T cells to SEB, splenic T cells were stimulated in the presence of purified splenic DC known to represent APC for naive T cells (7,27) or peritoneal exudate cells (PEC). T cells were also stimulated in the presence of unfractionated and G10-passed APC, obtained as previously described. Of note, the purification procedure of DC requires an overnight culture step during which co-stimulatory activity appears to be up-regulated (T. Sornasse and M. Moser, in preparation). All APC populations were therefore cultured overnight in complete media or, when indicated, in media supplemented with recombinant murine IL-4. By comparing the response of naive T cells and of a T cell hybridoma, we were able to reveal the ability of selected APC populations to generate a TCR ligand (signal 1) and to deliver the adequate co-stimulatory signal(s) (signal 2) to naive T cells. The results presented in Fig. 2 can be summarized as follows. The ability of APC to deliver signal 1 strongly correlated with the cell surface expression of the I-E molecule, known to bind and

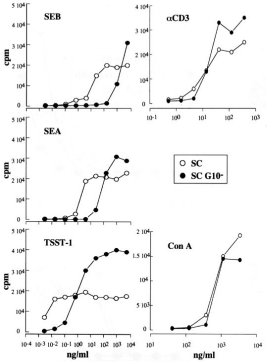

Fig. 3. Role of G10 adherent APC populations in naive T cell responsiveness to bacterial superantigens, anti-CD3 mAbs and Con A. Unfractionated (SC) or G10-passed (SC G10$^-$) responder spleen cells (10^6 cells/well) were cultured for 24 h in the presence of graded doses of bacterial superantigens or T cell mitogens as indicated. Culture supernatants were assayed for IL-2 content by bioassay. Results are expressed as c.p.m. of [^{3}H]thymidine incorporation by the IL-2-dependent cell line CTLL.

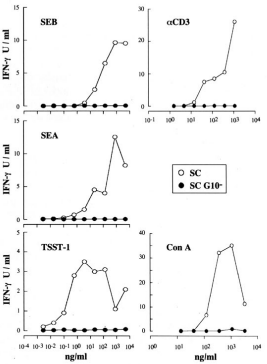

Fig. 4. G10-adherent spleen cells are strictly required for IFN-γ production in response to all doses of T cell mitogens tested. For experimental conditions, see legend to Fig. 3. Supernatants collected after 24 h of culture were assayed for IFN-γ content by ELISA and the results expressed as U/ml.

present SEB. DC expressed high levels of class II molecules and were indeed very potent stimulators of the T cell hybrid; accordingly, up-regulation of I-E cell surface-associated molecules by IL-4 on B cells resulted in ~10-fold increase in the ability of these cells to stimulate the T cell line. In contrast, PEC, which expressed low levels of I-E molecules in the absence of external stimuli, were unable to present SEB in culture to the T cell hybridoma.

Among cells able to present SEB to the T cell hybridoma, only purified DC (Fig. 2b, closed squares) and unfractionated spleen APC (Fig. 2b, closed circles) were found to significantly induce IL-2 production from naive T cells. In particular, B cells cultured in the presence of IL-4 were only weak stimulators for naive T cells in spite of high levels of cell surface I-E expression (Fig. 2c). Data presented in Fig. 2 suggest therefore that although DC and IL-4-treated B cells express similar levels of cell-surface associated I-E molecules and are able to optimally stimulate a T cell hybridoma, they display distinct co-stimulatory activity for naive T cells.

Response of unfractionated and G10-passed responding cell populations to graded doses of superantigens and T cell mitogens

In order to establish whether activation of naive T cells was strictly dependent on the co-stimulatory activity provided by adherent accessory cells, G10-passed spleen cells were stimulated by increasing doses of superantigens and T cell mitogens. Dose–response curves obtained with different superantigens and mitogens are shown in Fig. 3. G10-passed spleen cells (containing both Thy-1$^+$ and I-E$^+$ cells) were able to amount an *in vitro* T cell response to superantigens but required higher doses of superantigens for detectable IL-2 production. In contrast, removal of adherent cells from the responding population did not significantly affect their response to Con A or anti-CD3 mAbs, indicating thus that the different threshold of activation observed with superantigens was not due to a generalized hyporeactivity of the responding T cell population present in the G10-passed fraction.

As previously reported by others (28), we have observed that although TSST-1 only reacts with a small subpopulation of naive T cells, it induces high levels of IL-2 production *in vitro* (see Fig. 3). Note also that IL-2 production in the G10-passed population induced by saturating doses of superantigen generally exceeds the response of the untreated population. Although further studies (including precursor frequency analysis and sorting experiments using antibodies to CD45) are needed to elucidate this observation, flow cytometry analysis suggest that the G10 population is enriched for small resting T cells, a cell population that has been shown to be hyperresponsive to superantigens *in vitro* (29).

In contrast to what was observed for IL-2 production,

depletion of adherent cells from the responding population completely abrogated the IFN-γ response induced by all mitogens tested (Fig. 4). IFN-γ secretion was fully restored by addition of unfractionated splenic APC or purified DC to a G10-passed, responder cell population (data not shown). This observation suggests a functional heterogeneity among splenic APC. IL-2 production can be induced by both adherent and non-adherent APC, while IFN-γ production is only observed in the presence of adherent spleen cells.

Anti-CD28 mAbs, DC and B7.1-bearing B cell lines restore IL-2 production of naive T cells to low doses of superantigens

The previous observation suggested that co-stimulatory activity provided by adherent spleen cells modulated the *in vitro* IL-2 production of naive T cells by lowering the threshold of activation to superantigens. Since antibodies to CD28 have been shown to provide a co-stimulatory stimulus to naive T cells (14), we tested whether they were able to restore the sensitivity of G10-passed responding cells to control levels. Figure 5 shows that addition of anti-CD28 mAbs up-regulated the response of G10-passed spleen cells to superantigens by lowering the threshold of activation and increasing the total amount of IL-2 produced at plateau levels. Following a similar rationale, we wished to test whether the addition of purified DC was also able to modify the *in vitro* response of naive T cells to superantigens. Figure 6 shows that addition of DC cells to G10-passed spleen cells augmented their *in vitro* response to superantigens by both lowering the threshold of activation and increasing the plateau level of IL-2 production. As shown before, a similar result was obtained by supplementing the cultures with antibodies to anti-CD28. In order to test the ability of one of the recently identified CD28 ligands to provide a co-stimulatory stimulus to G10-passed responder cells, we tested the ability of a B7.1-tranfected cell line to present SEB to G10-passed responding cell populations. A stable cell line expressing high levels of the murine B7.1 molecule (A20-B7.1) was produced following transfection of the corresponding gene. As shown in Fig. 7, the parental and the transfected cell line displayed equivalent amounts of cell surface I-E molecules while expressing different levels of the B7.1 molecule. In agreement with data presented in the previous sections, both cell lines were equally effective in stimulating the $V_{\beta}8$-bearing T cell hybridoma in the presence of SEB or myoglobin (Fig. 8a and b), thus confirming that this hybridoma is insensitive to B7.1-mediated co-stimulation. In contrast, the B7.1-transfected cell line proved to be more effective in presenting superantigens to G10-passed cell populations (Fig. 8c and e). Note that this experiment was performed using lower cell densities (3×10^5 responder cells/well), in order to favor antigen presentation by the A20 cell line. B7.1-mediated co-stimulation affected the *in vitro* immune response by increasing the sensitivity of unprimed T cells to lower doses of SEB and SEA. Of note, the *in vitro* response of T cells to anti-CD3 antibodies and lectins was also sensitive to B7.1-mediated co-stimulation, although to a lesser extent.

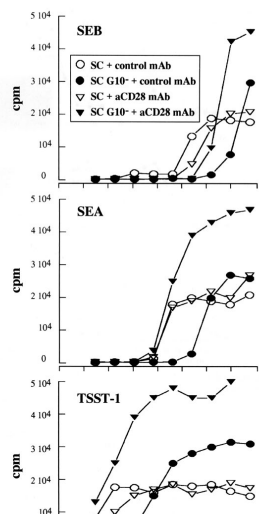

Fig. 5. Co-stimulatory effect of mAbs to CD28. Unfractionated (SC) or G10-passed (SC G10⁻) spleen cells were stimulated by graded doses of bacterial superantigens and antibodies to CD28 (clone 37.51) or control antibody (clone F531). Both antibodies were used at 1:10,000 dilution of ascites. After 24 h, culture supernatants were assayed for IL-2 content by bioassay. Results are expressed as c.p.m. of [^{3}H]thymidine incorporation by the IL-2-dependent cell line CTLL.

Discussion

An important feature of superantigens is the fact that they do not undergo processing but directly engage MHC class II molecules on the APC and subsequently bind to V_{β} segments of the TCR (for review, see 1). As superantigens do not require APC capture and internalization, they can potentially bind to all class II$^+$ cells *in vivo*, in contrast to classical antigens which appear to be preferentially captured and presented to T lymphocytes by APC of the DC lineage (30). The focus of the experiments reported herein was to compare the ability of selected APC populations to stimulate naive T cells in the presence of bacterial exotoxins.

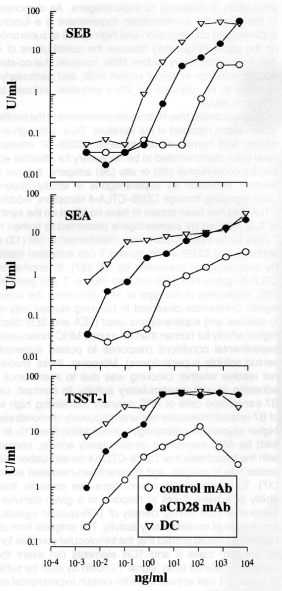

Fig. 6. Co-stimulatory effect of purified DC and antibody to CD28. G10-passed (SC G10') spleen cells (10^6 cells/well) were stimulated by graded doses of bacterial superantigens in the presence of antibodies to CD28 (clone 37.51) or isogenic control antibody (clone F531) (1:10,000 dilution of ascites) or purified syngeneic DC (10^4 cells/well). After 24 h, culture supernatants were assayed for IL-2 content by bioassay and results are expressed as U/ml.

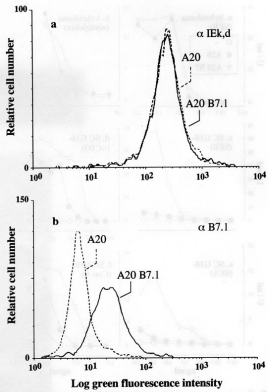

Fig. 7. FACS analysis of wild-type A20 and B7.1-transfected A20 cell lines. Cell surface antigen expression was assessed by incubating cells with FITC-labeled antibodies to I-$E^{k,d}$ (a) or B7.1 (b). All stainings were performed in the presence of saturating doses of 24G2, a rat mAb to mouse FcRII/III, in order to inhibit FcR binding of staining reagents.

Purified DC were found much more efficient on a per cell basis than resting or IL-4-treated B cells in stimulating naive T cells to produce IL-2 in the presence of SEB, in spite of similar I-E cell surface expression and comparable activating properties when presenting superantigens to a T cell hybrid (see experiments performed with IL-4-treated B cells, Fig. 2). This result indicates that, in contrast to what has been previously suggested by Nisanian *et al.* (31), high efficiency of DC in presenting SEB to naive T cells relates to the presence of co-stimulatory activity rather than to the higher amounts of MHC–superantigen complexes that they present. Removal of adherent cells from the responding population did not completely abrogate IL-2 response to superantigens, but rather reduced sensitivity of naive T cells to limiting doses of superantigens. Reduced IL-2 secretion was not observed in response to anti-CD3 mAbs or lectins, suggesting that G10 passage did not significantly affect T cell responsiveness to TCR cross-linking. G10-passed spleen cells failed to produce detectable levels of IFN-γ in response to superantigens, anti-CD3 mAbs and lectin, confirming previous work from Radbruch *et al.* (32), who showed that adherent cells were required for superantigens-induced IFN-γ secretion. IFN-γ production was restored by addition of T cell depleted spleen cells or purified DC to the G10-passed responding population, demonstrating that lack of IFN-γ response was solely attributable to an APC defect (data not shown). Note that the complete absence of IFN-γ production in this culture demonstrates that residual IL-2 response induced by superantigens cannot be attributed to adherent cells contaminating the G10-passed responding population. The nature of co-stimulatory activity provided by adherent spleen cells for IL-2 production is not entirely clear as it may encompass soluble secreted molecules as well as membrane associated factors. Reconstitution

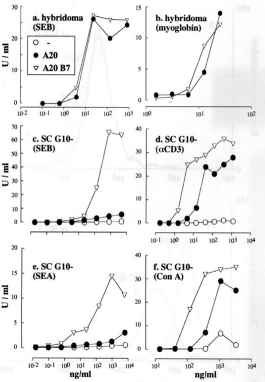

Fig. 8. Co-stimulatory activity of the A20-B7.1-transfected cell line. The 13-26-8–HG.1 T cell hybridoma (a and b, 3×10^4 cells/well) or G10-passed (SC G10$^-$) spleen cells (c, d, 3×10^5 cells/well) were stimulated by graded doses of superantigens or T cell mitogens in the presence of A20 or B7.1-transfected A20 cell line (10^5 cells/well). After 24 h, culture supernatants were assayed for IL-2 content by bioassay and results are expressed as U/ml.

experiments using antibodies to CD28, B7.1-transfected cell lines or DC expressing B7.1 and B7.2 molecules (see 33 and our own observations) indicated that co-stimulatory activity provided by the CD28–CTLA-4 receptors increased T cell responsiveness to limiting doses of superantigen and resulted in sustained levels of IL-2 production at saturing doses of superantigen. Increased plateau levels of IL-2 production are in agreement with observations indicating that signals transmitted through CD28 cause a marked rise in cytokine mRNA stability (34). Of note, these experiments were not designed in order to identify the nature of co-stimulatory factors provided by adherent cells, but rather to confirm that APC products distinct from the MHC–superantigen complex can decrease the superantigen threshold of naive T cells for IL-2 production.

This study clearly demonstrates that the APC population able to activate naive T cells in response to superantigens is heterogeneous. Both adherent (comprising DC and macrophages) and non-adherent APC (mostly B cells) have been shown to induce naive T cells to IL-2 production, with different efficacy. Although co-stimulation provided by adherent spleen cells (in particular DC) increases T cell reactivity to superantigens, it may not represent an obligatory signal for IL-2 production in response to superantigens. As demonstrated in this work, the co-stimulation requirement is a function of superantigen concentration and high doses of superantigens (in the μg/ml range) may obscure the contributions of APC-derived co-stimulatory factors. Note, however, that co-stimulation provided by adherent spleen cells, and particularly DC, appears to be required for IFN-γ secretion induced by all mitogens tested.

This hypothesis may help understand some of the conflicting observations reported in the literature. Thus, although in both murine and human model systems, CD28–B7 interactions have been demonstrated to be necessary for effective activation by conventional (35) or allo (36) antigens, it is not clear whether activation by superantigens is strictly dependent upon signaling through CD28–CTLA-4 receptors. Addition of CTLA-4 Ig has been shown to have no effect on the synthesis of IL-2 induced by superantigens presented to human naive T cells by an Epstein–Barr virus-transformed B cell (13) while antibodies to CD28 inhibit human T cell activation mediated by superantigens presented by DC (37). In another study, CTLA-4–Ig-blocked SEB-induced murine T cell proliferation (38), regardless of the type of APC delivering the activation signal. Differences observed in blocking studies may relate to species and superantigens used (SEA and SEB display a higher affinity for human than for murine MHC molecules) and experimental conditions (response to pulsed superantigen versus soluble superantigens). Moreover, these studies do not resolve whether blocking was due to interference with adhesion or with co-stimulatory activity. In contrast, use of B7-transfected cells or APC naturally expressing high levels of B7-related molecules has unambiguously demonstrated an higher efficacy of superantigen presentation (from 10- to 100-fold) by APC expressing co-stimulatory activity, compatible with the hypothesis that CD28–CTLA-4 co-stimulation plays a similar role in antigen- and superantigen-mediated activation (37). To explain these observations, we consider that the ability of a naive T cell to respond to a given stimulus is a function of TCR affinity, density of TCR-specific ligands and expression of co-stimulatory activity. The simplest form of this hypothesis would predict that the trimolecular complex formed by antigen, class II and TCR elements (an event that is commonly refered to as 'signal 1') could by itself be sufficient to initialize T cell activation under certain experimental conditions. Anti-TCR antibodies presumably display a higher affinity for TCR than natural antigen–MHC ligands and can be used at high local concentrations. As a consequence, naive T cells can be stimulated in the absence of class II-bearing cells by plastic-bound anti-CD3 mAbs when cultured at high cell densities (39). Although total absence of APC is difficult to prove, these studies suggest that activation of naive T cells by antibodies to the TCR complex may occur independently of CD28-derived signals. Bacterial exotoxins presumably bind to an unoccupied site on the MHC molecule and recent studies in a human model (37) have demonstrated that 100% of MHC class II$^+$ molecules occupancy is attained at 50 nM dose of SEA (corresponding to a working concentration of 1–5 μg/ml). Sufficient TCR cross-linking might therefore be induced at high superantigens doses, rendering co-stimulation provided by adherent cells dispensable. This observation does not negate the possibility that other co-stimulatory

molecules expressed by most lymphoid cells (such as CD11a–CD18) play a role in B cell-mediated stimulation of naive T cell in the presence of high concentrations of superantigens. In marked contrast, stimulation of T cells by peptide antigens is limited by the presence of naturally-processed peptide which binds to MHC molecules on APCs. It has been estimated that even when added at saturating doses, only 0.1–5% of total MHC molecules are occupied by a given antigenic peptide (40). Thus, under physiological situations, antigen-stimulation of T cells appears strictly dependent on co-stimulatory factors provided by APC, as 'signal 1' provided by antigen–MHC complexes is presumably too weak to induce activation. Accordingly, G10-passed APC do not stimulate alloreactive T cells (23 and our own observations) but stimulate naive T cells to produce IL-2 in the presence of adequate concentrations of superantigens. Of interest, a recent study performed by C. Melief and colleagues demonstrated that DC and the antigen processing defective mutant line RMA-S were the only APC able to induce primary cytotoxic T lymphocyte responses (41). RMA-S cells display a reduced cell surface expression of MHC class I molecules which can partly be restored by addition of MHC class I binding peptides. This results in elevated expression of MHC class I molecules preferentially loaded with one particular peptide, presumably because no competition with naturally processed, self peptides occurred in this mutant cell line. Of relevance, the non-mutated, parental RMA cell line was unable to induce primary *in vitro* responses suggesting that increased expression of antigen–MHC complexes confered to the mutant cell line the ability to stimulate naive T cells. Taken together, these observations suggest that co-stimulatory molecules play a predominant role in T cell activation when T cells are stimulated by low avidity TCR ligands.

One conclusion that clearly follows from this study is that superantigens can be presented to naive T cells by cells expressing different co-stimulatory activities. By binding with high affinity to an unoccupied site on the MHC class II molecule, bacterial exotoxins bypass physiological requirements for antigen capture and processing, and are able to generate high avidity ligands able to efficiently cross-link TCR even when presented by cells bearing weak co-stimulatory activity. This is particularly relevant as superantigens have been shown to both polyclonally activate naive T cell *in vivo* (causing both intravascular and extravascular inflammation) and induce anergy and/or clonal elimination (42). Understanding the pathophysiological effects of superantigens will therefore require the identification of the cells presenting superantigens to naive T cell *in vivo*, as numerous studies (for review, see 3) have suggested that functional consequences of TCR occupancy (memory versus anergy) is determined by co-stimulatory signals provided by APC.

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Abbreviations

APC	antigen presenting cell
Con A	concanavalin A
DC	dendritic cell
PE	phycoerythrin
PEC	peritoneal exudate cell
PCR	polymerase chain reaction
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B
TSST-1	toxic shock syndrome type 1

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