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Co-stimulation with B7 and targeted superantigen is required for MHC class II-independent T-cell proliferation but not cytotoxicity

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SUMMARY

The superantigen Staphylococcal enterotoxin A (SEA) conjugated to tumour-specific monoclonal antibodies (mAb) directs T cells to lyse tumour cells in the absence of major histocompatibility complex (MHC) class II. In contrast, the conjugate bound to MHC class II-negative tumour cells did not activate resting T cells to proliferate. The SEA–C215 mAb conjugate, when presented on the CA215 antigen-expressing Colo205 cells, required either signalling with CD28 mAb or CHO cells expressing the natural CD28 ligand, B7, to activate the T cells. The CD28/B7 co-stimulatory effect was further enhanced when the B7 and the tumour antigen were present on the same cell, decreasing the superantigen amount required for activation with a factor of 10^4. No influence of B7 was seen when the single CA215 or double CA215/B7 transfectants were used as targets for superantigen conjugate-dependent cytotoxicity. This suggests that the low affinity T-cell receptor (TcR) interaction of superantigen in the absence of MHC class II antigens is sufficient for induction of cytotoxicity but requires additional CD28/B7 signalling to result in proliferation of resting T cells.

INTRODUCTION

Bacterial superantigens (SA) bind to major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (APC) and activate T cells expressing certain T-cell receptor (TcR) Vβ sequences.[1] The activation leads to the production of large amounts of cytokines, resulting in T-cell proliferation and induction of cell-mediated cytotoxicity.[2–5] We have previously shown that conjugates of superantigens and tumour-specific monoclonal antibody (SA–mAb) can mediate T-cell-dependent killing of tumour cells independently of MHC class II molecule expression on the target cell.[6,7] This observation and other studies showing an increase of intracellular free Ca^{2+} after SA activation of T cells in a MHC class II-free system[8] indicate a direct SA–TcR interaction.

Participation of adhesion molecules is a crucial event in T-cell activation by antigens[9–11] as well as superantigens.[12–14] LFA-1/ICAM-1,[12,13] CD2/LFA-3[14] and CD28/B7[15] have been implicated in MHC class II-dependent superantigen activation of T cells. The CD28 molecules are expressed by 80% of all T cells[16] and its natural ligand B7 on activated B cells and monocytes.[17,18] Stimulation of CD28 leads to increased expression of interleukin-2 (IL-2)[16] by affecting both gene transcription[19] and mRNA stability.[20] Interaction of CD28 and B7 results in activation of a cyclosporin A-resistant protein kinase pathway.[21,22] CD28 activation has also been implied in the cytotoxicity of natural killer (NK) cells[23] and in the redirected anti-CD3-dependent cytotoxicity of tumour cells by small resting T cells.[24]

In the present study we have investigated the requirements for superantigen mAb activation of purified human resting MHC class II-negative T cells. The data presented show a crucial role for CD28 in the proliferation of resting T cells, whereas no requirement for CD28 co-signalling was seen in SA–mAb-targeted T-cell cytotoxicity. The results suggest a profound role for the CD28/B7 pathway in signal amplification when triggering resting T cells with a low TcR affinity.

Abbreviations: APC, antigen-presenting cell; mAb, monoclonal antibodies; MHC, major histocompatibility complex; SA, superantigen; SEA, Staphylococcal enterotoxin A; TcR, T-cell receptor; TIL, tumour infiltrating lymphocytes.

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MATERIALS AND METHODS

mAb and reagents

The B7/BB1 mAb was a generous gift from Dr E. Clark, University of Washington, Seattle, WA.[25] The CD28 mAb (9.3) was obtained from Bristol-Meyers, NY, the CD8 mAb (BMA081) from Behring, Marburg, Germany, and the CD2 mAb (T111) was a generous gift from Dr S. Schlossman, Boston, MA. The CD3 (OKT-3), the HNK-1 and the MHC class I (W6/32) mAb were obtained from the hybridoma cell line producing the mAb (American Type Culture Collection, Rockville, MD). The anti-SEA mAb (1A) was purchased from IGEN (Rockville, MD). FITC-conjugated CD3 (Leu4), PE-conju-

gated CD56 (Leu19) and PE-conjugated and unconjugated HLA-DR mAb were purchased from Becton Dickinson Laboratories (Mountain View, CA). FITC-labelled rabbit anti-mouse IgG was obtained from Dakopatts, Glostrup, Denmark. Recombinant Staphylococcal enterotoxin (SEA) was expressed in *Escherichia coli* and purified to homogeneity, as described elsewhere.[26] Recombinant IL-2 was obtained from Cetus Corp., Emeryville, CA ($1{\cdot}5 \times 10^6$ U/mg protein).

The C215 mAb reacting with human colon cancer[27] recognizes a glycoprotein of approximately 35,000–37,000 MW under reduced conditions (CA215[28]). The SEA–C215 mAb conjugates were produced by coupling SEA to the C215 mAb as described in ref. 6. The conjugates contained an average of 1 SEA molecule per mAb.

mAb were bound to polystyrene beads coupled with goat anti-mouse IgG (Dynabeads M-450, Dynal A/S, Norway) according to the manufacturers' recommendation. Approximately 10% of the added mAb bound to the beads, as determined with ^{125}I-labelled mAb. In the experiments using $0{\cdot}1 \times 10^6$ beads, the mAb concentration was 2 ng/ml.

Transfected cell lines

Chinese hamster ovary (CHO) K1 cells were transfected with human cDNA encoding the B7 gene (a generous gift from Dr B. Dupont, Sloan Kettering Institute, NY) or the CA215 gene (generously provided by Dr P. Lind, Kabi-Pharmacia Bioscience Center, Stockholm, Sweden). The B7 coding region cDNA was expressed under the control of a human cytomegalovirus immediate early promoter in the vector pEE6 hCMV (a gift from Dr C. Bebbington, Celltech, Slough, U.K.) and the CA215 coding region cDNA under the control of a SV40 immediate early promoter in the neomycin-resistant vector pKGE839 (P. Lind, Kabi-Pharmacia Bioscience Center). These constructs were transfected by electroporation to CHO of CHO–B7 cells to create CHO–B7, CHO–CA215 and CHO–CA215–B7 double transfectants. The B7 transfectants were selected for expression 48 hr after electroporation by FACS sorting. Positive cells were repeatedly sorted and finally cloned after approximately 4 weeks in culture. The CA215 transfectants were selected in medium containing 1 mg/ml G418 (geneticin; Gibco BRL, Paisley, U.K.), followed by FACS sorting. The transfectants were routinely analysed for B7 and CA215 expression by FACS.

Cell separation and culture

PBMC from healthy donors were isolated by density centrifugation over Ficoll–Hypaque. T cells were enriched by separation over gelatin columns,[29] followed by depletion by panning with HNK1 and HLA-DR mAb. Cells obtained by this procedure were more than 98% CD3 positive. All tests on purified T cells were performed with $0{\cdot}1 \times 10^6$ cells/well in 200-μl volumes, using flat-bottomed 96-well plates (Nunc, Roskilde, Denmark). Colo205 cells, which express the CA215 antigen,[6] low LFA-3 amounts[30] but no MHC class II antigens,[6] B7 antigens (data not shown) or ICAM-1,[30] were obtained from the American Type Culture Collection. Mitomycin C (Sigma Chemical Co., St Louis, MO)-treated Colo205 or transfected CHO cells were preincubated with SEA–C215 mAb conjugates as described,[6] and added to the T cells. DNA synthesis was studied after exposure of cultures to [^{3}H]thymidine ([^{3}H]TdR) as described earlier.[31]

All the cell cultures were done in RPMI-1640 medium supplemented with 2% 200 mM L-glutamine, 1% HEPES, 2·8% (v/v), 7·5% $NaHCO_3$, 20 μg/ml gentamicin (Gibco BRL) and 10% fetal bovine serum. All cell lines were free of mycoplasma infection.

Cytotoxicity assay

The cytotoxicity was measured in a standard 4-hr ^{51}Cr-release assay employing human T-cell lines obtained by repeated stimulation with SEA-coated B-cell lymphoma cells[32] as effector cells. The percentage cytotoxicity was computed according to the formula: percentage cytotoxicity = 100 × [experimental release (c.p.m.) − spontaneous release (c.p.m.)/maximum release (c.p.m.) − spontaneous release (c.p.m.)].

Flow cytometry

Analyses and sorting were performed according to standard settings in a FACStar Plus Flow Cytometer (Becton Dickinson) equipped with one argon laser and one helium/neon laser.

RESULTS

CD28 mAb co-stimulates SEA–mAb-dependent T-cell proliferation

Highly purified resting T cells failed to proliferate when triggered by SEA–mAb immobilized on beads. When bead-bound CD28 mAb were used as co-stimulator for SEA–C215 mAb conjugate or CD3 mAb activation (the latter used in a suboptimal stimulatory dose), a strong proliferation was induced (Fig. 1). Also, CD2 mAb showed some co-stimulatory activity with both SEA–C215 mAb conjugate and CD3 mAb, while CD8 mAb did not. It was clearly advantageous for the co-stimulatory signal to be present on the same bead as the superantigen (data not shown). SEA–C215 mAb bound to the

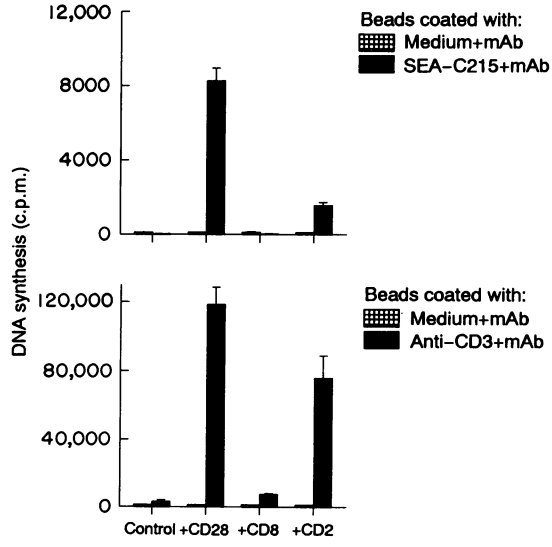

Figure 1. T-cell activation by beads coupled SEA–C215 mAb conjugate and mAb. Beads were incubated with medium, CD28, CD8 or CD2 mAb, SEA–C215 mAb conjugate, CD3 mAb or an equal mixture of mAb and SEA–C215 mAb conjugate or CD3 mAb or as control with medium alone, and tested for activation of T cells at a T-cell/bead ratio of 1:1. The values presented are the mean ± SD of triplicates.

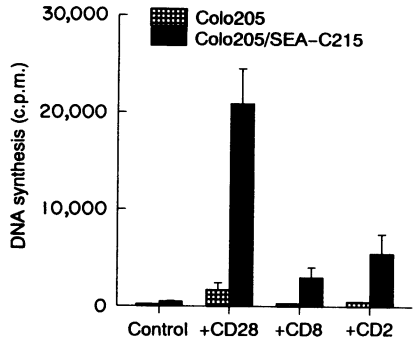

Figure 2. CD28 requirement of tumour-cell-bound SEA–C215 mAb conjugate activation of T cells. Colo205 cells were precoated with SEA–C215 mAb conjugate (1 μg/ml) or medium and mixed with control beads, CD28, CD8 or CD2 mAb beads at a T-cell/tumour cell/bead ratio of 1:1:0·1. The values presented are the mean ± SD of triplicates.

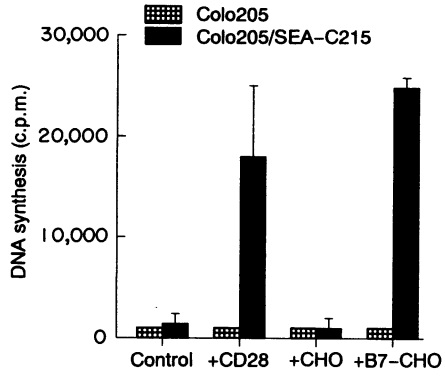

Figure 3. T-cell activation by tumour cell-bound SEA–C215 mAb conjugates and B7-expressing CHO cells. Colo205 cells, precoated or not with SEA–C215 mAb conjugates (1 μg/ml) or medium were mixed with CD28 mAb or medium-precoated beads, CHO cells or B7-transfected CHO cells and tested for T-cell activation, at a T-cell/tumour cell/bead or CHO cell ratio of 1:1:0·1. The values presented are the mean ± SD of triplicates.

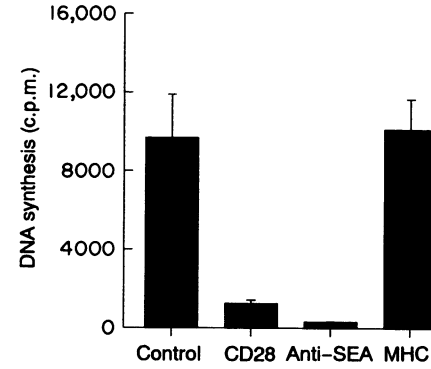

Figure 4. Inhibition of T-cell activation by tumour cell-bound SEA–C215 mAb conjugate and B7-CHO cells. Colo205 cells preincubated with 1 μg/ml SEA–C215 mAb conjugate were mixed with B7-transfected CHO cells and medium (control) or 1 μg/ml CD28, SEA or MHC class I mAb and tested for T-cell activation. The T-cell tumour cell/B7–CHO cell ratio was 1:1:0·1. The values presented are the mean ± SD of triplicates.

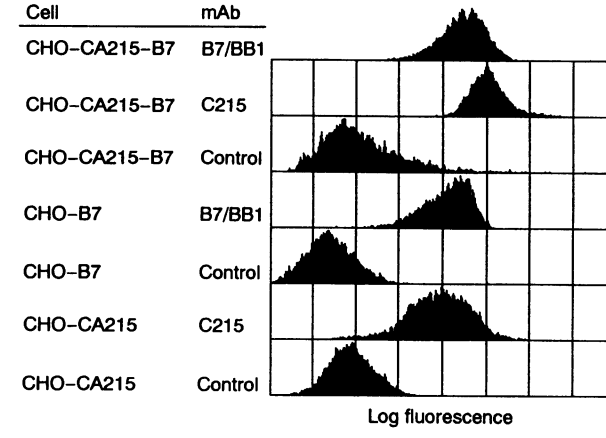

Figure 5. Flow cytometric analysis of CHO–CA215, CHO–B7 and CHO–CA215–B7 cells. Cells were stained with FITC-labelled C215 mAb or B7/BB1 mAb with PBS as control and FITC-labelled rabbit anti-mouse IgG.

CA215-positive B7-negative Colo205 tumour cell line has been demonstrated to trigger cytotoxic T cells.[6] In contrast, resting T cells failed to respond to SEA–C215 mAb bound to the colon carcinoma cells (Fig. 2). The ability of CD28 mAb to act as a co-stimulator with SEA–C215 mAb was also seen when triggering T cells with SEA–C215 mAb-coated Colo205 cells (Fig. 2).

B7 transfectants co-stimulate tumour cell-bound SEA–mAb-dependent T-cell proliferation

To test if B7, the natural ligand for CD28, acts as a co-signal in superantigen–mAb conjugate-induced T-cell activation, B7 cDNA were transfected into CHO cells. The transfectants were mixed with SEA–C215 mAb conjugate bound to Colo205 cells and tested for T-cell activation (Fig. 3). The CHO–B7 cells were potent co-stimulators of the superantigen–mAb conjugate activation of T cells. The strong activation obtained with SEA–C215 mAb conjugate with B7-expressing transfectants was inhibited by addition of mAb to CD28 and SEA but not by a control mAb to human MHC class I (Fig. 4), suggesting the involvement of CD28 on the T cells. Whether the effect of the CD28 mAb was due to negative signalling to the T cells or specific blocking of B7 binding remains to be evaluated. To analyse whether the activation of T cells is influenced by the presence of B7 and SEA on separate or the same cells, CHO–B7 and CHO cells were transfected with CA215 cDNA. The resulting transfectants expressed similar amounts of the CA215 molecules (Fig. 5). When the cells were used as presenters of superantigen C215–mAb conjugates to T cells it was clear that the B7/CA215 double transfectant was the optimal inducer of superantigen-dependent T-cell activation, requiring $10^4\times$ less SEA–C215 mAb conjugate than when the B7 signal was on different CHO cells (Fig. 6).

B7 transfectants as targets for SEA–mAb-dependent cytotoxicity

To investigate the involvement of the CD28/B7 pathway in the T-cell cytotoxicity, we tested the sensitivity of CHO–CA215 and

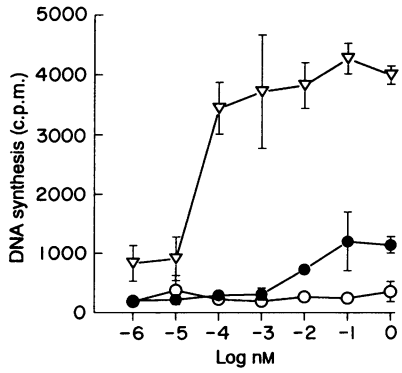

Figure 6. SEA–C215 mAb conjugate-dependent T-cell activation by cells expressing the C215 and B7 molecules on the same or separate cells. CHO–CA215 or CHO–CA215–B7 transfectants were mixed with CHO or CHO–B7 cells and tested for T-cell activation in the presence of SEA–C215 mAb conjugate at various concentrations, at a T-cell/CHO cell/CHO ratio of 1:0·01:0·01. (○) CHO–CA215+CHO cells; (●) CHO–CA215+CHO–B7 cells; (▽) CHO–CA215–B7+CHO cells. The values presented are the mean ± SD of triplicates.

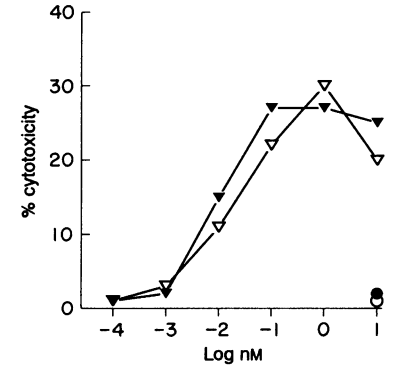

Figure 7. SEA–C215 mAb conjugate-dependent cytotoxicity against CHO transfectants. ^{51}Cr-labelled CHO, CHO–B7, CHO–CA215 and CHO–CA215–B7 transfectants were mixed with SEA–C215 mAb conjugate at various concentrations and tested for cytotoxicity employing a T-cell line as effector. (○) CHO cells; (●) CHO–B7 cells; (▽) CHO–CA215 cells; (▼) CHO–CA215–B7. The T-cell/tumour cell ratio was 30:1. One representative experiment out of three is shown.

CHO–B7/CA215 to SEA–C215 mAb targeted cytotoxic T lymphocyte (CTL). The CHO–CA215 cells were strongly lysed at picomolar amounts of SEA–C215 mAb (Fig. 7). No influence of B7 molecules on the cytotoxicity was seen. Further, T-cell lines, sorted into $CD8^+CD28^+$ and $CD8^+CD28^-$ cells and used in the cytotoxicity assay, were found to be equally efficient in killing the transfectants (data not shown). This strengthens the conclusion that the CD28/B7 system was not involved in the killing by the T-cell lines.

DISCUSSION

We have previously shown that SEA coupled to mAb against human colon carcinomas efficiently directs T-cell lines and tumour infiltrating lymphocytes (TIL) to kill tumour cells in a MHC class II-independent manner.[6,7] In contrast, resting human purified T cells fail to respond to SEA–mAb bound to Colo205 cells. Earlier, Fleischer *et al.*[33] reported that CD8 and CD2 stimulation induced proliferation in SA-activated resting human T cells. The influence of CD8 and CD2 triggering seems to be of marginal importance compared to the strong proliferation induced by CD28 co-stimulation. In this study we show that CD28/B7 interaction greatly enhances the SA–mAb activation of purified human resting MHC class II-negative T cells. The CD2/LFA-3 pathway has recently been implied as a strong co-factor in superantigen activation of T cells,[12-14] but in conjunction with MHC class II expression. However, the low level of proliferation induced by CD2 mAb and LFA-3 transfectants (data not shown) suggests that this pathway is of minor importance in superantigen MHC class II-independent activation of T cells. Green *et al.*[34] showed recently that T-cell activation with soluble superantigen in an APC-free system, could be obtained by co-stimulatory CD28 mAb. We extended this finding by demonstrating the impact of the natural CD28 ligand, B7, in SEA–C215 mAb-mediated activation of resting human T cells. B7 also interacts with the CTLA-4 molecules, which show close similarities at the protein level with CD28, but is predominantly expressed on activated T cells.[35] Co-stimulation through CTLA-4 might occur during the late phase of the culture since CD28 mAb were not able to completely inhibit the superantigen/B7 activation of T cells upon prolonged culture (>4 days, data not shown). Indeed, recently, it has been demonstrated that CD28 and CTLA-4 may act synergistically.[36] The advantage of presenting the superantigen signal on the same cells as B7 was evident. Similarly, Liu and Janeway[37] showed an increase of CD4 cell proliferation triggered by TcR mAb and co-stimuli presented by the same cell.

In contrast to resting T cells, human cytotoxic T-cell lines lysed the target cells irrespective of the B7 expression. The CD28/B7 interaction has been shown to enhance the killing of tumour cells by NK cell lines[22] and CD3-redirected resting T cells but not of T-cell clones,[23] which supports our observations.

The results suggest that the low affinity interaction between superantigen and the T-cell receptor, which strongly triggers cytotoxicity by T-cell lines, is insufficient to induce proliferation in resting T cells or the T-cell lines (data not shown) unless co-activation through CD28/B7 occurs. This indicates that the requirement for B7/CD28 interaction is dictated by the effector function studied rather than the activation state of the T cells. Rapid tyrosine phosphorylation is seen when triggering of T cells through TcR[38] or CD28[22] occurs. It remains to be investigated whether synergistic effects are seen in this signal transduction pathway during a low affinity TcR interaction concomitantly with CD28 activation.

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