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T-cell stimulation and cytokine release induced by staphylococcal enterotoxin A (SEA) and the SEAD227A mutant

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SUMMARY

Previous work demonstrated that human cytotoxic T cells activated by superantigens can lyse major histocompatibility complex (MHC) class II-positive target cells as well as MHC class II-negative tumour cells coated with conjugates of monoclonal antibodies and superantigens. In order to decrease MHC class II affinity, and therefore unwanted binding of the superantigen staphylococcal enterotoxin A (SEA) to MHC class II molecules, a point mutation was introduced into the SEA gene. This mutation (SEAD227A) resulted in an approximately 3-log reduction of affinity to human leucocyte antigen (HLA)-DR, but cytotoxicity mediated by this mutant superantigen towards antibody-labelled tumour cells is as efficient as cytotoxicity mediated by the native superantigen. We therefore compared the T-cell activating potency of native and mutated SEA. Our data show that SEAD227A is 4- to 5-log less effective than native SEA when activation of resting T cells is assayed in terms of blast formation, expression of cell surface activation markers and cytokine release. Furthermore, presenting either SEA or SEAD227A to MHC class II-negative mononuclear cells by MHC class II-negative tumour cells did not result in significant blast formation of T cells, up-regulation of CD25 or cytokine release. This suggests that lysis of MHC class II-negative tumour cells is efficiently induced by monoclonal antibody targeted superantigen, while activation of resting T cells requires additional co-stimulatory signals.

INTRODUCTION

Enterotoxins produced by *Staphylococcus aureus* are potent mitogens for human T cells. These superantigens (SAg) activate a large proportion of T cells expressing an appropriate Vβ T-cell receptor (TCR) in a major histocompatibility complex (MHC) class II-dependent, but unrestricted fashion.[1]

Superantigens direct human cytotoxic T lymphocytes (CTL) to mediate strong cytotoxicity against MHC class II-positive target cells, which has been termed superantigen-dependent cell-mediated cytotoxicity (SDCC).[2,3] SDCC can be applied to MHC class II-negative tumour cells coated with a monoclonal antibody (mAb) conjugated to the superantigen staphylococcal enterotoxin A (SEA) *in vitro*[4–6] and *in vivo*.[7–9]

In order to reduce unwanted binding of SEA to MHC class II-positive cells, a mutant of SEA was constructed. To this purpose an alanine substitution of the aspartic acid residue at position 227 was introduced into the SEA gene resulting in the mutant SEAD227A. Previously, a major role for the aspartic acid at position 227 in MHC class II binding has been demonstrated by side-directed mutageneses.[10] Recent crystallography data of the toxin showed that the D227 residue is involved in binding a zinc ion.[11] The SAg SEA is suggested to bind to MHC class II molecules via the formation of a Zn^{2+} bridge. The mutant shows a 1000-fold reduced affinity to MHC class II but unaltered interaction with the TCR.[10] Using SEA-reactive T cells, lysis of antibody-coated tumour cells *in vitro* was comparable, independent of whether cytotoxicity was mediated by SEA or SEAD227A.[5,6]

We therefore investigated the T-cell stimulating effect of SEA as compared to SEAD227A in regard to the expression of different surface antigens (CD2, CD28, CD69, CD95, CD25, CD11a), the induction of T-cell blasts and the production of different inflammatory cytokines [interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour-necrosis factor-α (TNF-α)].

Our results show that the potency in T-cell activation by

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Abbreviations: CD, cluster of differentiation; CTL, cytotoxic T lymphocyte; IFN-γ interferon-γ; IL-2, interleukin-2; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; SAg, superantigen; SDCC, superantigen-dependent cell-mediated cytotoxicity; SEA/SEAD227A, staphylococcal enterotoxin A/staphylococcal enterotoxin A mutant D227A; TCR, T-cell receptor; TNF-α, tumour-necrosis factor-α.

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the mutant SEAD227A was severely reduced as compared to the wild-type superantigen. Furthermore, tumour cells coated with a conjugate of a tumour-specific mAb and SEA or SEAD227A, respectively, were not able to induce any substantial cytokine release in the absence of MHC class II-positive antigen-presenting cells, although both superantigens mediated equally high cytotoxicity *in vitro*. Earlier studies had suggested the importance of a second costimulatory signal, necessary for proliferation of resting T cells by the wild-type SEA.[12] The insufficiency to induce cytokine release and expression of cell surface activation markers by the wild-type and the mutant SEA presented on MHC class II-negative tumour cells supports and expands this findings. This implicates that distinct requirements for SEA induced cytotoxicity and activation of resting T cells exist.

MATERIALS AND METHODS

Cell line

The neuroblastoma line IMR-32 was obtained from American Type Culture Collection (ATCC; Rockville, MD) and cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamate, 100 U/ml penicillin, 100 μg/ml streptomycin (Seromed; Berlin, Germany) and 10 μM sodium-pyruvate (Gibco; Gaithersburg, MD).

Purification of T cells

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by density gradient centrifugation (Lymphoprep®, Nycomed Pharma AS; Oslo, Norway) and incubated with anti-human leucocyte antigen (HLA)-DR antibody (Becton-Dickinson Immunocytometry Systems; San Jose, CA) in a concentration of 2×10^7 cells/ml. After washing once, the cell pellet was resuspended in 80 μl buffer, consisting of phosphate-buffered saline (PBS) supplemented with 5 mM ethylene diamine tetra-acetic acid (EDTA) and 0·5% bovine serum albumin (Gibco; Gaithersburg, MD) for each 10^7 cells. Subsequently, cells were incubated with 20 μl goat–anti-mouse IgG magnetic beads (Miltenyi Biotec; Bergisch Gladbach, Germany) for each 10^7 cells and incubated for 20 min at 4°. The cells were separated with a magnetic cell separator (MACS) on a separation column (Miltenyi Biotec) and a HLA-DR-negative and -positive fraction was collected. By staining with fluorescein isothiocyanate (FITC)-labelled goat–anti-mouse-IgG (Becton Dickinson), the negative fraction was shown to contain less than 2% HLA-DR-positive cells whereas in the positive fraction over 70% of the cells expressed HLA-DR. In freshly isolated PBMC approximately 30% of cells were HLA-DR positive.

SEA: production, mutation, fusion proteins

Recombinant SEA (rSEA) was expressed in *Escherichia coli* strain W3110 and purified as earlier described.[10] To reduce the MHC class II binding of SEA, an alanine substitution of the aspartic acid residue at position 227 was introduced in the SEA gene (SEAD227A).[10] Protein A–SEA and protein A–SEAD227A are recombinant fusion proteins produced by *E. coli* and include the genes encoding the IgG-binding domain of protein A[13] fused to the SEA and SEAD227A gene, respectively. Expression of the fusion proteins in *E. coli* is driven by a protein A promoter and secretion is directed by a protein A signal peptide. The fusion proteins were purified on an IgG affinity column.

Antibodies

The human/mouse chimeric anti-GD_2 mAb ch14.18 was developed by Gillies *et al.*[14] The chimeric construct consists of the variable region of the murine mAb 14.18[15] and the constant region of human IgG1,k. The antibody was produced and purified by Repligen (Needham Heights, MA) and was kindly provided by Dr Reisfeld, Scripps Clinic and Research Foundation, La Jolla, CA. Simultest® control antibody γ_1/γ_2, FITC-conjugated anti-CD3 and phycoerythrin (PE)-conjugated anti-CD2, anti-CD69 and anti-CD25 were all purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). FITC-conjugated anti-CD28, anti-Vβ22, anti-CD95 (Fas) and anti-CD11a (LFA-1) were purchased from Immunotech (Marseille, France).

Cytotoxicity assay

Cytotoxicity was measured by a standard 4-h ^{51}Cr-release-assay as described earlier.[5] Briefly, ^{51}Cr-labelled neuroblastoma target cells (IMR-32) were preincubated with the mAb ch14.18 (10 μg/ml) or PBS for 30 min at room temperature. Cells were washed and incubated with either protein A–SEA (5 μg/ml), protein A–SEAD227A (5 μg/ml) or PBS. Following the labelling procedure the target cells were incubated with SEA-reactive cytotoxic T cells (effectors) at various effector-

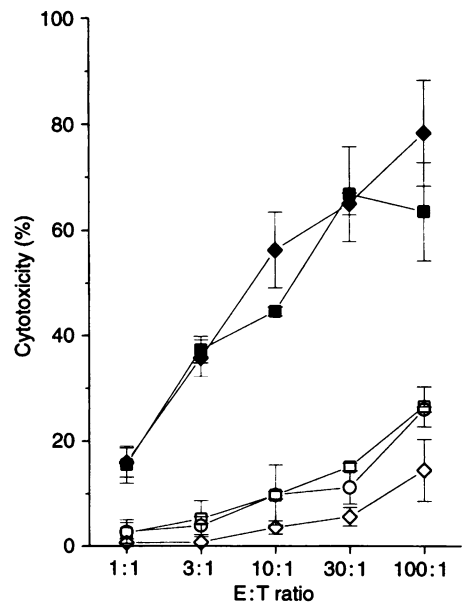

Figure 1. Antibody-dependent lysis of MHC class II-negative tumour cells mediated by SEA or SEAD227A: The ^{51}Cr-labelled neuroblastoma cells (IMR-32) were incubated with the anti-GD_2 antibody ch14.18 (10 μg/ml) or PBS and washed once. After adding the fusion proteins protein A–SEA, protein A–SEAD227A (5 μg/ml) or PBS, the target cells (5000 cells/well) were incubated with SEA-reactive T cells at different E:T-ratios. Cytotoxicity was assayed with a 4-hr ^{51}Cr-release assay and expressed as the mean ± SD. One out of two similar experiments is shown. Solid squares, ch14.18 + protein A–SEA; solid diamonds, ch14.18 + protein A-SEAD227A; open squares, protein A-SEA; open diamonds, protein A-SEAD227A; and open circles, ch14.18.

to-target (E:T) cell ratios. SEA-reactive T cells were established as described earlier.[5] After 4 h of incubation at 37° in 5% CO_2, released ^{51}Cr in 100 μl supernatant of each well was measured by a gamma-counter (Packard; Meriden, CT). Cytotoxicity is expressed as percentage specific cytotoxicity according to the formula:

$$\frac{\text{c.p.m. experimental release} - \text{c.p.m. spontaneous release}}{\text{c.p.m. total release} - \text{c.p.m. spontaneous release}} \times 100 = \text{Cytotoxicity (\%)}$$

Spontaneous release was determined in wells which contained only labelled target cells and total release was determined by lysing the tumour cells with 1 M HCl solution.

Stimulation assays

Freshly isolated mononuclear cells or HLA-DR-negative leucocytes were cultured in RPMI-1640 supplemented with 10% FCS, 2 mM glutamate, 100 U/ml penicillin, 100 μg/ml streptomycin (Seromed; Berlin, Germany) and 10 μM sodium-pyruvat (Gibco; Gaithersburg, MD). Three million cells/well were incubated in 24-well culture plates (Greiner, Germany) with either PBS, SEA, SEAD227A or 3×10^5 irradiated (30 Gy) IMR-32 neuroblastoma cells coated with ch14.18 (10 μg/ml) and protein A–SEA or protein A–SEAD227A (1 μg/ml). The cells of the HLA-DR-positive fraction after magnetic separation were irradiated (30 Gy) and supplemented as indicated. The cells were cultured for 48 h and then washed three times. After washing, the cells were again cultured for another 24 h without SEA to allow for remodulation of the TCR. After washing twice, aliquots (25 μl, $0{\cdot}5 \times 10^6$ cells) were incubated with the different mAbs for flow cytometry analysis.

Flow cytometry

Cells (2×10^7/ml, PBS/2% FCS) were incubated with either PE- or FITC-labelled antibodies for 30 minutes on ice. After washing, gates for forward (FSC) and sideward scatter (SSC) were set and 10 000 cells were acquired with a FACScan® flow

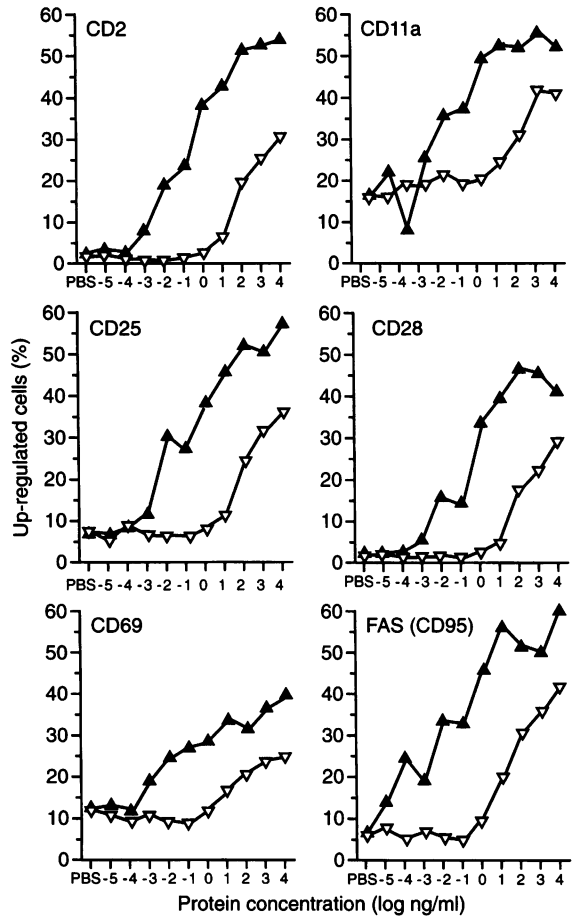

Figure 2. Expression of T-cell surface markers after stimulation with SEA or SEAD227A. Freshly isolated mononuclear cells were incubated with different concentrations of SEA or SEAD227A or PBS for 48 h. Antigen expression was examined after incubating cells with phycoerythrin- or fluorescein isothiocyanate-labelled antibodies. Cells were acquired by flow cytometry and only $CD3^+$ cells were evaluated for expression of the respective antigen with the exception of the IL-2-receptor expression where only $CD25^+$ cells were analysed. Analysis was performed using histograms plotting fluorescence of the appropriate antibody versus cell counts. Cells incubated with PBS served as control and regions were set to differentiate the fluorescence intensity of the stained control cells versus activated cells. Up-regulation was determined as the percentage of cells expressing higher fluorescence than control cells. The *x*-axis in the graph represents the protein concentration of SEA or SEAD227A added, the *y*-axis represents up-regulated cells as percentage of all cells. Solid triangles, SEA; and open triangles, SEAD227A.

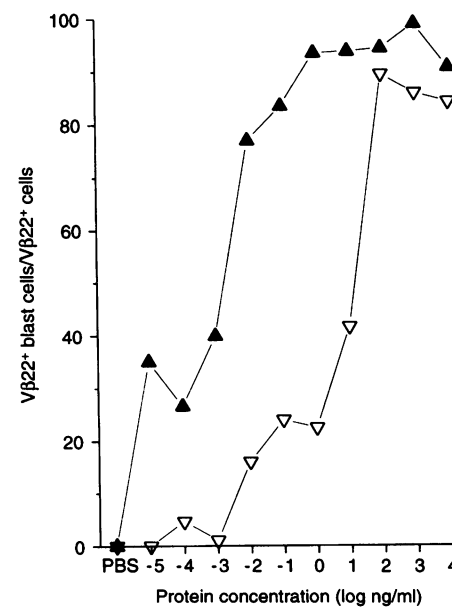

Figure 3. T-cell blast formation after incubation with SEA or SEAD227A. Freshly isolated PBMC were incubated with different concentrations of SEA or SEAD227A or PBS for 48 h, washed three times and cultured in superantigen free medium for another 24 h. The expression of Vβ22 was investigated by incubation with a fluorescein isothiocyanate-labelled monoclonal antibody as $V\beta22^+$ T cells are reactive to SEA. The term 'blast cells' refers to lymphocytes that exhibit a larger volume than resting control lymphocytes as determined by analysis of forward side scatter (FSC). $V\beta22^+$ blasts therefore reflect the portion of $V\beta22^+$ lymphocytes reacting with SEA. Shown is the number of $V\beta22^+$ blast cells in percentage of all $V\beta22^+$ cells (*y*-axis). One out of three similar experiments is shown: solid triangles, SEA; and open triangles, SEAD227A.

cytometer (Becton-Dickinson; Palo Alto, CA). Analysis of data was done with Lysis II® (Becton Dickinson) software.

Cytokine assays

Culture supernatants were collected 24, 48, 72 and 96 h after stimulation with SEA or SEAD227A and centrifuged once to remove particulate material. Interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour-necrosis-factor-α (TNF-α) were assayed using the appropriate enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN).

RESULTS

Antibody-dependent lysis of MHC class II-negative tumour cells mediated by SEA or SEAD227A

To compare mAb directed SDCC mediated by SEA and its mutant SEAD227A, SEA-reactive T cells were targeted to MHC class II-negative neuroblastoma cells (IMR-32) by the monoclonal anti-GD_2 antibody ch14.18 and the fusion proteins protein A–SEA and protein A–SEAD227A. The results shown in Fig. 1 demonstrate only marginal differences between the wild-type and its mutant in the E:T ratio-dependent lysis of the antigen-bearing tumour cells. Similar results were found for dose-dependent lysis (data not shown).

T-cell stimulating potency of SEA versus SEAD227A

In order to investigate the T-cell stimulating effect of SEA and its mutant SEAD227A, we assayed the expression of the cell surface antigens CD2, CD11a, CD28, CD69, CD95, and CD25 on $CD3^+$ T cells after incubating mononuclear cells with different concentrations of SEA or SEAD227A for 48 h (Fig. 2). In addition, as Vβ22-positive T cells are activated by SEA, the proportion of $V\beta22^+$ blast cells in relation to all $V\beta22^+$ T cells was analysed (Fig. 3). The results demonstrate, that the T-cell stimulating capacity of SEAD227A is reduced approximately 4–5-log in terms of enhanced cell surface antigen expression and blast formation when compared to SEA. In regard to MHC class II binding, the affinity of the mutant SEAD227A was below the detection limit as calculated from Scatchard plots,[10] while MHC class II-dependent SDCC and PBM proliferation suggested an approximately 3-log reduction of MHC class II affinity.[5,6,10]

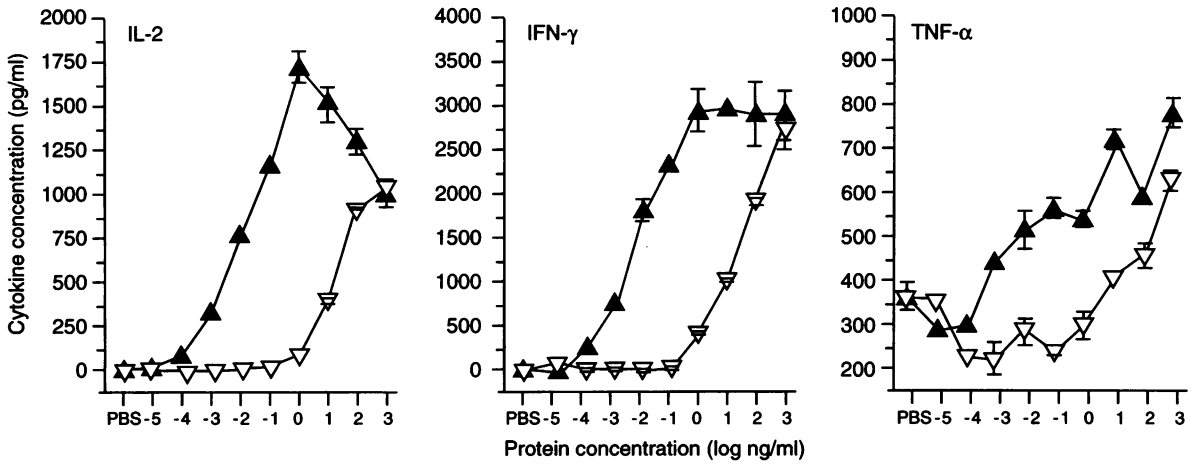

Figure 4. Dose-dependent cytokine release after stimulation with SEA or SEAD227A. Freshly isolated PBMC were incubated with different concentrations of SEA or SEAD227A or PBS for 48 h and the supernatant was collected and centrifuged to remove all particulate material. IL-2, IFN-γ and TNF-α concentrations (pg/ml) were assayed using the appropriate ELISA kits. Solid triangles, SEA; and open triangles, SEAD227A.

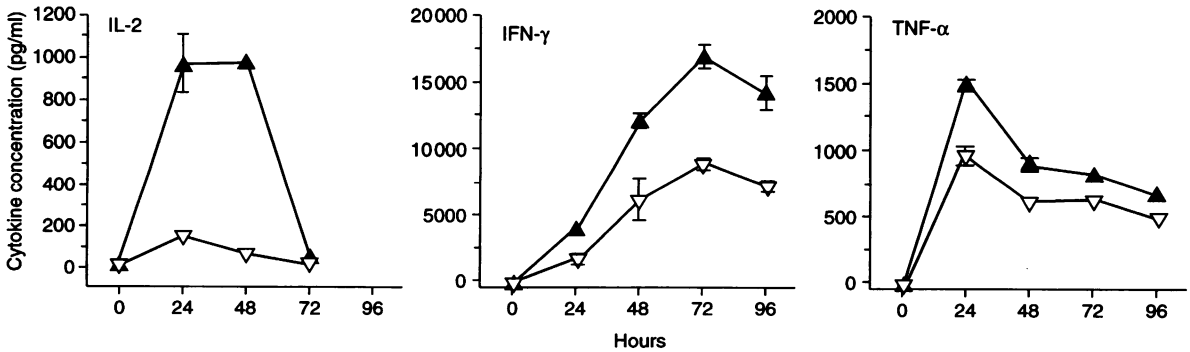

Figure 5. Time-dependent cytokine release after stimulation with SEA or SEAD227A. PBMC were incubated with 10 ng/ml SEA or SEAD227A for 24, 48, 72 and 96 h and the supernatants were collected. Cytokine concentrations (pg/ml) were measured using an appropriate ELISA kit. Solid triangles, SEA; and open triangles, SEAD227A.

Cytokine release after stimulation with SEA or SEAD227A

The release of the cytokines interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour-necrosis factor-α (TNF-α) by superantigen-stimulated PBMC was measured after incubation with different concentrations of SEA or SEAD227A for 48 h (Fig. 4). Again, production of all three cytokines was reduced in the same range as blast formation and expression of cell surface molecules. A time kinetic revealed no difference between SEA and SEAD227A (Fig. 5), although the amount of cytokines induced by SEAD227A was markedly reduced. IL-2 and TNF-α production peaked after an incubation time of 24 hours and rapidly decreased thereafter, whereas the production of IFN-γ peaked after 72 h and declined more slowly.

T-cell stimulation by superantigens presented on MHC II-negative tumour cells

Stimulation of T cells by SAg is not MHC-restricted, but dependent on MHC class II expression.[1] Therefore, removal of MHC class II-positive cells from the PBMC preparation resulted in only marginal activation of T cells by SEA (Fig. 6). Activation could partially be restored by supplementing autologous HLA-DR-positive cells. However, superantigen immobilized on MHC II$^-$,[5] B7- and ICAM-1-negative (data not shown) tumour cells did not restore activation of T cells in terms of blast formation or CD25 expression suggesting that polyvalent presentation on a cell surface is not sufficient to induce T-cell proliferation.

Cytokine release by SEA or SEAD227A presented on the tumour surface

In accordance with the results described above, there was only marginal cytokine release seen in the HLA-DR-negative cell fraction when either SEA, SEAD227A or MHC class II-negative neuroblastoma cells coated with mAb and SEA or SEAD227A were added (Fig. 7). Again, addition of irradiated HLA-DR-positive cells together with SEA or SEAD227A resulted in restored cytokine release.

DISCUSSION

We demonstrated that the T-cell stimulating activity of the mutated superantigen staphylococcus enterotoxin D227A is reduced by several log compared to the wild-type. In terms of blast formation, up-regulation of cell surface activation markers and cytokine release, we found the mutant to be 4- to 5-log less effective than native SEA. Furthermore, after removal of MHC class II-positive cells, presenting native or mutant SEA by MHC class II-negative tumour cells did not result in significant T-cell activation. This suggests that MHC class II-positive antigen-presenting cells are required for SAg-induced activation but not for cytotoxicity.

Superantigen-activated T cells can be targeted by conjugates of superantigens and monoclonal antibodies to lyse MHC class II-negative tumour cells.[4–6] By introducing a point mutation into the SEA gene, MHC class II affinity of SEA was markedly reduced. This could be explained by the resolution of the crystal structure combined with site-directed mutagenesis which demonstrated that a zinc ion is co-ordinated in the SEA molecule by participation of the H187, H225 and

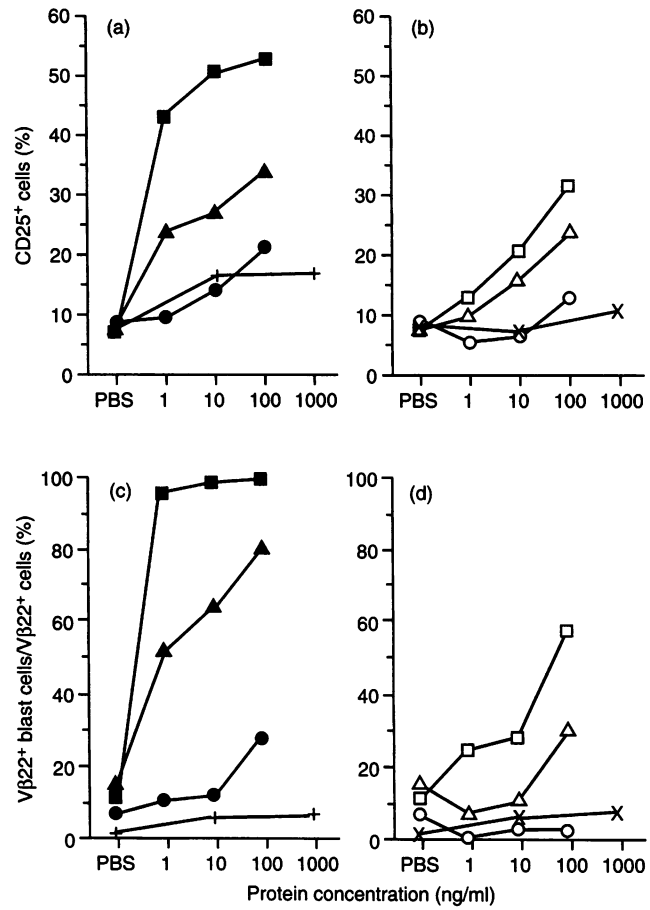

Figure 6. T-cell stimulation by SEA or SEAD227A presented on tumour cell surface. Freshly isolated PBMC, separated HLA-DR-negative lymphocytes or HLA-DR-negative cells supplemented with 10% autologous HLA-DR-positive cells (irradiated with 30 Gy) were incubated with different doses of either SEA (a, c) or SEAD227A (b, d). For presentation of the SAg on tumour surface, irradiated (30 Gy) IMR-32 neuroblastoma cells were coated with ch14.18 (10 μg/ml) and protein A–SEA (a, c) or protein A–SEAD227A (b, d) and added to HLA-DR-negative lymphocytes at a ratio of 1:10. The cells were incubated for 48 h in the presence of different doses of the superantigen, washed three times and cultured in superantigen free medium for another 24 h. CD25 (a, b) expression was analysed by using a PE-labelled antibody. Vβ22 expression (c, d) was investigated by incubation with a fluorescein isothiocyanate-labelled monoclonal antibody. For details, see legends to Fig. 1 and 2. One out of three similar experiments is shown. PBMC incubated with SEA, solid squares; or SEAD227A, open squares; HLA-DR-negative cells incubated with SEA, solid circles; or SEAD227A, open circles; HLA-DR-negative cells +10% autologous HLA-DR-positive cells incubated with SEA, solid triangles; or SEAD227A, open triangles; and HLA-DR-negative cells +10% IMR-32 +ch14.18 incubated with protein A–SEA, plus; or protein A-SEAD227A, crosses.

D227 amino acids.[10,11] The SEAD227A mutation interfered with zinc ion co-ordination, resulting in reduced MHC class II binding. The residual MHC class II binding in this mutant is mediated by a low-affinity N-terminal site.[10] The D227A amino acid substitution did not alter the gross conformation of SEA as determined by analysis with circular dichroism or reactivity with anti-SEA antibodies.[10]

With regard to the MHC class II-dependent T-cell activat-

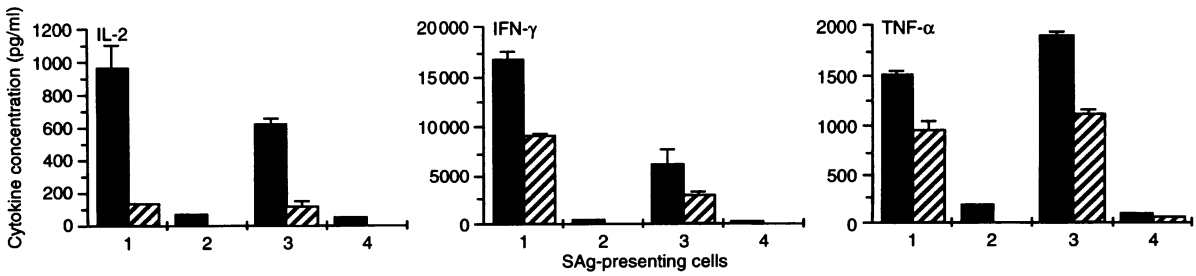

Figure 7. Cytokine release by SEA or SEAD227A presented on the tumour surface. Freshly isolated PBMC (1), HLA-DR-negative lymphocytes (2), HLA-DR-negative lymphocytes supplemented with 10% irradiated autologous HLA-DR-positive cells (3) were incubated with 10 ng/ml SEA or SEAD227A for 24 h (IL-2, TNF-α) or 72 h (IFN-γ) and the supernatants were collected. Fraction 4 comprises purified HLA-DR-negative lymphocytes supplemented with 10% irradiated IMR-32 cells coated with ch14.18 (10 μg/ml) and protein A–SEA or protein A–SEAD227A (1 μg/ml). Cytokine concentrations were assayed using the appropriate ELISA kit. One out of two similar experiments is shown. Solid bars, SEA; hatched bars, SEAD227A. SAg-presenting cells: 1, PBMC; 2, HLA-DR-negative cells; 3, HLA-DR-negative cells + 10% autologous HLA-DR-positive cells; and 4, HLA-DR-negative cells + 10% (IMR-32 + ch14.18).

ing properties, the fusion of the SEA molecule to protein A had only marginal influence when compared to the corresponding unfused protein. Targeting of SEAD227A to the cell surface by a protein A–SEAD227A fusion protein demonstrated that the mutant displayed a similar ability as the corresponding wild-type SEA molecule to trigger the T-cell receptor on cytotoxic T cells.[5,6,10] Thus, the MHC class II binding site in the C-terminal of the molecule is perturbed but a functional TCR epitope is retained. However, presentation of SEA on MHC class II-negative tumour cells, sufficient to induce tumour cell lysis, did not result in T-cell proliferation,[12] blast formation and consecutive cytokine release.

Furthermore, lysis of MHC class II-positive cells is reduced approximately 1000-fold when comparing SEA and its mutant,[5] whereas stimulation of T cells required 4- to 5-log higher concentrations of SEAD227A as compared to the wild-type. This result suggests that MHC class II-dependent stimulation of resting T cells by SAg is more affected by the mutation than the cytotoxic effect of effector CTL and support the proposed different mechanism of cytotoxicity versus events associated with activation of T-cell proliferation[12] and cytokine production. Alternatively, this might reflect different requirements for resting and activated T cells.

Expression of cell surface markers on target cells is important for activation of T cells by superantigens. Different studies have predicted that in the absence of antigen-presenting cells and adhesion molecules on the tumour cell surface, neither activation of T cells nor cytokine release can be achieved.[12,16,17] For example, T-cell proliferation could be induced by SEA presented on B7 transfected cells irrespective of the MHC class II expression,[12] suggesting an important role of the CD28/B7 pathway in SAg-induced T-cell proliferation. Moreover, the interaction of CD11a/CD18 and its receptor intracellular adhesion molecule-1 (ICAM-1) on SAg-presenting cells seems to offer an additional pathway.[18] The neuroblastoma cell line IMR-32 used in our study expressed neither B7 nor ICAM-1. Therefore, SEA or SEAD227A was presented on the surface of MHC class II-negative tumour cells in the absence of proper co-stimulatory signals. This did not result in T-cell activation and cytokine release. Consistent with this observation, adhesion molecules were not necessary for superantigen-induced cytokine gene expression in T cells, but were necessary for secretion.[19] Additionally, studies demonstrated that cross-linking of two MHC class II molecules by one single SEA molecule could induce cytokine gene expression in monocytic cells.[20]

Cytokines produced by superantigen-stimulated T cells have been shown to suppress the growth of human colon carcinoma cells[21] and injection of superantigens into tumour-bearing mice resulted in inhibition of tumour growth, probably mediated by cytokines.[22] SDCC therefore is likely to be enhanced by SEA-induced cytokines of tumour-infiltrating T cells. Indeed tumour-infiltrating lymphocytes activated by antibody-targeted superantigens have been shown to produce tumoricidal cytokines.[7,23] The possible effect of superantigen-induced cytokines against target-antigen-negative bystander tumour cells not sensitive to antibody-mediated SDCC has therefore to be addressed. Thus, in an *in vivo* setting, most likely higher doses of the mutant SEA as compared to native SEA would be required to achieve a similar cytotoxic effect. The presence of $B7^+$/ICAM-1^+ monocytes in the tumour area may be crucial to obtain proper co-stimulation of T cells with SAg presented on tumour cells. Indeed B7 co-stimulates T cells when presented in trans-position on a cell distinct from the antigen-presenting cell, providing a proper antigen–MHC complex for TCR interaction.

Altogether, antibody-directed SDCC either mediated by SEA or SEAD227A towards MHC class II-negative tumour cells is very effective using preactivated T-cell lines *in vitro*, but stimulation of T cells with ensuing release of cytokines is markedly reduced by the mutation. In a conceivable clinical setting, one has to consider not only the probably reduced side-effects of the mutant, but also the possibly reduced anti-tumour effect. However, tumour cells expressing adhesion molecules like B7 or ICAM-1, naturally, such as lymphoma and leukemic cells or after transfection could be excellent target cells for mutant SEA-based tumour therapy.

Moreover, the recruitment of $B7^+$/ICAM-1^+ macrophages and dendritic cells to the tumour area may critically affect the outcome of SAg-based tumour therapy. Combined administration of cytokines, such as granulocyte–macrophage colony-stimulating factor and SEA-mutants may be used to increase the presence of such cells in the tumour area.

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