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Modulation of superantigen-induced T-cell deletion by antibody anti-Pgp-1 (CD44)

E. AYROLDI, L. CANNARILE & C. RICCARDI *Department of Clinical Medicine, Pathology and Pharmacology, Perugia University Medical School Perugia, Italy*

SUMMARY

We examined the effects of anti-Pgp-1 (CD44) antibody on the *in vitro* deletion of murine CD4 and CD8 single positive T cells induced by Staphylococcal enterotoxin B (SEB). Soluble anti-Pgp-1 antibody enhanced the apoptosis and decreased the proliferation of SEB-responding T cells. In contrast, cross-linked anti-Pgp-1 antibody provided costimulatory signals for the T-cell activation induced by anti-CD3 antibody. Hyaluronic acid (HA), a ligand of Pgp-1, did not affect proliferation and deletion induced by SEB, whereas it mimicked the effects of the cross-linked antibody in anti-CD3-driven proliferation. T-cell Pgp-1 surface expression after 48 hr incubation with SEB was unchanged as compared to unstimulated cells. However, when the memory T cells were established, some $V\beta 8^{+}$ (SEB-specific) T cells Pgp-1^{low} became Pgp-1^{high}, displaying a bimodal character. Moreover, the Pgp-1 increased expression correlated with an increase of Pgp-1 soluble form in the supernatant. These findings suggested that signals following the triggering of the Pgp-1 molecule are important in controlling T-cell survival.

INTRODUCTION

Immunological tolerance to self-antigens in the thymus can be acquired through either negative selection against autoreactive T cells or the induction of T-cell anergy. Negative selection is mediated by apoptosis.[1–4] The biological significance of these two processes in the thymus has been well defined. Bone marrow-derived cells cause deletion of thymocytes expressing the T-cell receptor (TCR) specific for self-antigen, whereas those expressing TCR specific for self-antigen expressed by the thymic epithelium no longer respond to further stimulation.[5–7]

In the periphery, contact with the antigen on the surface of specialized antigen-presenting cells (APC), leads to activation, anergy, or apoptosis, depending on a variety of coaccessory stimuli.[8–10] Recent data indicate that tolerance of the extra-thymic antigen is induced by elimination of mature T cells in peripheral lymphoid organs.[11–14]

Pgp-1 (CD44) is a heterogeneous family of molecules with important functions, among which regulation of cell–cell contact and cell matrix adhesion. It is expressed by different haematopoietic cell subpopulations. Pgp-1 is present on the surface of lymphocytes and modulates their homing, allowing their selective binding to specialized endothelium, the high endothelial venules (HEV), and to extracellular matrix.[15–22] It also plays a role in the biology and pathology of cell activation. In humans, anti-Pgp-1 antibodies increase CD2- or CD3-mediated peripheral T-cell activation.[22–25]

Recent evidence that negative selection is operative in mature peripheral T cells,[11–14] and that adhesion molecules play a role in controlling programmed cell death[26] prompted us to study the role of Pgp-1 in antigen-driven apoptosis and activation through investigation and comparison of its role in two T-cell activation models: first, a Staphylococcal enterotoxin B (SEB)-induced activation system, and second, an anti-CD3 activation system. Monitoring of the deletion and proliferation of anti-Pgp-1 treated activated T cells indicated that Pgp-1 is important in controlling T-cell activation and survival.

MATERIALS AND METHODS

Animals

C3H/HeN mice purchased from Charles River (Chalco, Milan, Italy) were used as donors of T cells at the age of 4 weeks.

Reagents

Hyaluronic acid (HA) was purchased from Sigma (St. Louis, MO).

Coculture of T cells and Dcek Hi 7 cells

Dcek Hi 7[27] cells (10^5/well, kindly provided by Dr L. D'Adamio, National Institutes of Health, Bethesda, MD) were suspended in Dulbecco's modified Eagle's minimal

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Abbreviations: APC, antigen-presenting cells; FITC, fluorescein isothiocyanate; HA, hyaluronic acid; SEB, Staphylococcal enterotoxin B; TCR, T-cell receptor.

Correspondence: E. Ayroldi, Institute of Pharmacology, University of Perugia, 06100 Perugia, Italy

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essential medium (DMEM), containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 2% L-glutamine in flat-bottomed 96-well plates (Costar, Cambridge, MA). Cells were allowed to sediment, adhere and form a confluent layer. Excess cells were then removed by repeated washing. SEB (Sigma) was added to some cultures 20 min before addition of T cells. Lymph nodes were ground between frosted glass slides to produce a cell suspension and passed twice through nylon columns, resulting in a 95% pure T-cell population, as established by CD4, CD8 expression. T cells were added to each well (2×10^5 cells/well) and incubated for 48 hr at 37°.

In some wells, [^{3}H]thymidine (1 μCi/well) was added 12 hr before harvesting and proliferation assayed as described elsewhere.[28]

Flow cytometry analysis

T cells were stained with Vβ-specific antibodies MR5-2 (mouse-anti-mouse Vβ8.1-8.2) or RR4-7 (rat-anti-mouse Vβ6) conjugated with fluorescein isothiocyanate (FITC, Pharmingen, San Diego, CA) and with anti-L3T4 (CD4) directly conjugated to R-phycoerythrin (PE) (Becton Dickinson, San Jose, CA), anti-mouse CD8α directly conjugated to Red 613 (Life Technologies, Gaithersburg, MD) and a 1:10 dilution of normal rat serum, in a single-step, triple staining.

A portion of T cells were stained with hamster IgG anti-mouse $\alpha\beta$ TCR (H57-597, Pharmingen) and anti-hamster-FITC antibody (Caltag Lab., San Francisco, CA) to assess possible down-regulation of TCR.

In selected experiments, a portion of T cells were stained with anti-Vβ8 or anti-Vβ6 FITC-conjugated and anti-interleukin-2 receptor (IL-2R), or anti-CD2, or anti-Pgp-1 conjugated with biotin (Pharmingen). A mixture of anti-CD4-PE and streptavidin-Reed 613 (Life Technologies) or CD8-Reed and streptavidin-PE, was used as second-step reagent.

Cells were then analysed on a FACScan flow cytometer gated to exclude non-viable cells, and the percentage of cells positive for a specific cell marker was calculated using Lysis II research software (Becton-Dickinson). Purified rat anti-mouse Pgp-1 (IM7), rat anti-mouse CD2 (RM2-5) and hamster anti-mouse CD3 (145-2C11) were purchased from Pharmingen and used at the concentrations indicated in the figures.

DNA labelling technique

Apoptosis was measured by flow cytometry according to a previously described procedure.[29] After culturing, the cells were centrifuged and the pellet gently resuspended in 1·5 ml hypotonic solution of propidium iodide (PI, 50 μg/ml in 0·1% sodium citrate plus 0·1% Triton-X-100, Sigma). The tubes were placed at 4° in the dark overnight. The PI-fluorescence of individual nuclei was measured by flow cytometry using standard FACScan equipment. The nuclei traversed the light beam of a 488 nm argon laser. A 560 nm dichroid mirror (DM 570) and a 600 nm band pass filter (band width 35 nm) were used to collect the red fluorescence due to PI staining of DNA, and the data were recorded on a logarithmic scale. All data were stored in a Hewlett Packard (HP 9000, model 310) computer. The percentage of apoptotic cell nuclei (sub-diploid DNA peak in the DNA fluorescence histogram) was calculated using specific FACScan research software (Lysis II).

Antibody cross-linking

Anti-mouse CD3-epsilon at various concentrations and/or anti-mouse Pgp-1 and/or anti-mouse CD2 were allowed to adhere in enzyme immunoassay (EIA)/radioimmunoassay (RIA) flat-bottomed, high-binding well plates (Costar) in 4° in 100 μl phosphate-buffered saline (PBS). After 18 hr, plates were washed, incubated at 37° for 2 hr with PBS supplemented with 10% FCS and washed again. T lymphocytes were then plated 2×10^5/well and incubated at 37° for 48 hr. HA coated plates were prepared by incubating HA and/or anti-CD3 at 37° for 8 hr, and then at 4° for 8 hr. In the experiments in which anti-Pgp-1 was used in soluble form, the antibody was added after incubation of anti-CD3 pretreated plates with 100 μl of FCS.

Uptake of [^{3}H]thymidine was performed as previously described.[28]

Immunoprecipitation assay

Cells were lysed in 100 μl of ice-cold lysis buffer (50 mM Tris, pH 7·5, 0·3 M NaCl, 0·5% Triton, 2 mM EDTA, 0·4 M sodium orthovanadate, 2·5 mM leupeptin, 2·5 mM aprotinin) and homogenized. Lysates and supernatants were incubated at 4° for 1 hr with 100 μl of normal mouse serum, and then at 4° for 1 hr with protein A–Sepharose beads (Pharmacia, Uppsala, Sweden). Samples were microfuged at 13 ***g*** for 10 min at 4° to remove insoluble material. Supernatants were collected and incubated with 2 μg of Pgp-1 antibody (1 hr, 4°) and then protein A–Sepharose. After centrifugation, immunoprecipitates were washed three times with cold lysis buffer, boiled for 3 min and then analysed by electrophoresis in 10% SDS–PAGE gells followed by transfer to nitrocellulose (Bioblot-NC, Costar) for 5 hr at 250 mA at 4° in 25 mM Tris/glycine, pH 8·3, and 20% v/v methanol. Non-specific binding sites were blocked by immersing the membrane in 5% blocking reagent in Tris-buffered saline–Tween (TBS-T) for 1 hr at room temperature. The membranes were incubated with anti-Pgp-1 diluted 1:100 for 1 hr at room temperature. After washing with TBS-T buffer, membranes were probed for 1 hr at room temperature with HRP-labelled mouse anti-rat antibody, diluted 1:5000 (Amersham Life Science, Buckinghamshire, UK), then incubated with ECL Western blotting reagents (Amersham), and exposed to hyperfilm-ECL (Amersham) for 15 seconds.

Statistical analysis

Statistical comparisons were made with Student's *t*-test, $^*P < 0.01$.

RESULTS

Soluble anti-Pgp-1 monoclonal antibody (mAb), but not soluble anti-CD2 mAb increases SEB-induced deletion of T lymphocytes

To study the role of Pgp-1 in clonal deletion and proliferation of mature $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive T cells, we used an *in vitro* system consisting of the Superantigen Staphylococcus enterotoxin B (SEB), murine lymph node T cells and fibroblasts transfected with major histocompatibility complex (MHC) class II IE^k molecule (Dcek-Hi 7, 27) which allowed us to examine these phenomena in a cohort of purified lymph node T cells. SEB binds to the class II molecule on Dcek Hi 7 cells and mediates stimulation of mature T cells and clonal

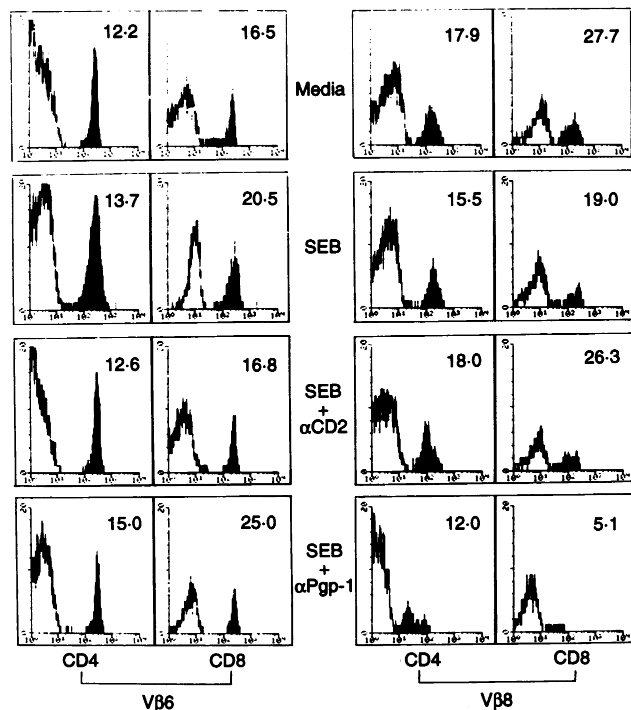

Figure 1. Effects of anti-Pgp-1 or anti-CD2 mAb on SEB-induced deletion of T lymphocytes. CD4 and CD8 single positive T lymphocytes (2×10^5/well) were coincubated with Dcek Hi 7 cells (10^5/well) in the presence or absence of SEB (10 μg/ml). In some wells, anti-Pgp-1 (10 μg/ml) or anti-CD2 (10 μg/ml) antibodies were added. After 48 hr, the cells were stained with anti-Vβ6 or anti-Vβ8, anti-CD4, anti-CD8 antibodies, and analysed with FACScan. Figure shows relative fluorescence intensity versus cell number. The number in the upper right corner represents the percentage of lymphocytes expressing the indicated Vβ gene.

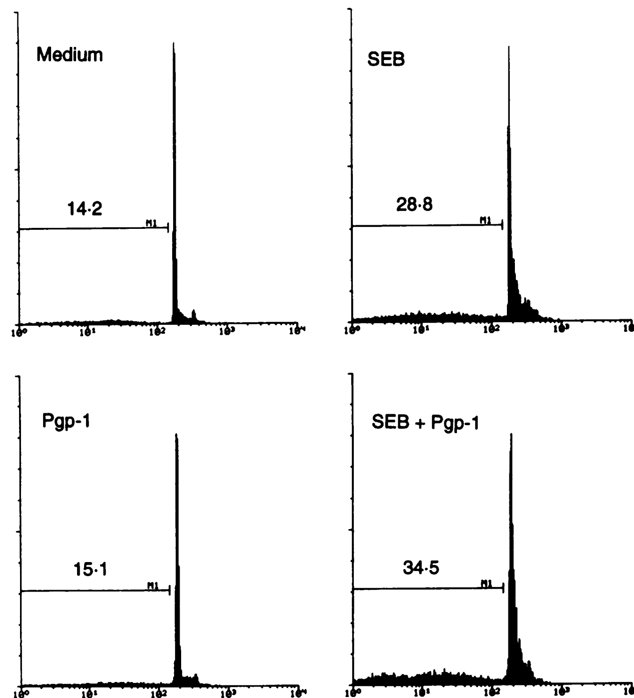

Figure 2. Flow cytometric analysis of PI-stained CD4$^+$ and CD8$^+$ nuclei after 48 hr coculture with Dcek Hi 7 cells with or without SEB (10 μg/ml) and/or Pgp-1 (10 μg/ml). The percentage of apoptotic nuclei (broad hypodiploid peak in $> 2 \times 10^2$ channels) is indicated in each histogram. PI fluorescence versus number of nuclei.

deletion of immature thymocytes bearing Vβ8.1, 8.2, 8.3. The model described has demonstrated that following SEB stimulation a fraction of Vβ8$^+$ mature T cells, either CD4$^+$ or CD8$^+$, are susceptible to deletion by apoptosis before or during the proliferative response.[13]

The effect of anti-Pgp-1 mAb in SEB-induced deletion was studied by adding the antibody to SEB-stimulated cells. Following 48 hr exposure to Dcek Hi 7 cells in the presence of SEB, with or without anti-Pgp-1 mAb, T cells were removed and the phenotypic analysis performed. As shown in a representative experiment (Fig. 1), the percentage of SEB-specific Vβ8$^+$ cells was significantly reduced in SEB-treated cells. Addition of anti-Pgp-1 mAb in SEB-treated T cells, further reduced the percentage of Vβ8$^+$ cells in both CD4$^+$CD8$^-$ and CD8$^+$CD4$^-$ subsets, with a significantly stronger effect on CD8$^+$ (12% CD4$^+$, 5·1% CD8$^+$ in anti-Pgp-1 + SEB group versus 15·5% CD4$^+$, 19% CD8$^+$ in SEB group, $P < 0{\cdot}01$). Anti-Pgp-1 alone did not affect deletion nor proliferation (not shown).

In parallel, a relative increase of the SEB non-specific Vβ6$^+$ cells occurred (Fig. 1). Antibodies against another adhesion molecule, CD2[30] were used as control. Addition of soluble anti-CD2 mAb to the SEB-treated group inhibited Vβ8$^+$ deletion (Fig. 1).

The decrease in the percentage of Vβ8$^+$ T cells could have been due to either down-regulation of TCR, or true apoptosis. An aliquot of the T cells used for evaluation of Vβ8 expression was therefore analysed to detect the presence of apoptotic cells by PI staining of DNA and subsequent FACScan analysis.[29] Figure 2 showed that anti-Pgp-1 significantly increased the apoptosis of SEB-treated T cells. Furthermore, lymphocytes were stained with antibody anti-$\alpha\beta$TCR, which reacted with 98% of the cells (not shown). These results suggested that the percentage decrease of Vβ8$^+$ T cells was due to apoptosis of SEB-specific cells.

The ability of anti-CD2 or anti-Pgp-1 mAb to modulate

Table 1. Effect of HA on SEB-induced activation and proliferation

	CD4$^+$		CD8$^+$		
Groups	Vβ6$^+$	Vβ8$^+$	Vβ6$^+$	Vβ8$^+$	c.p.m.
Medium	13	27	15·8	38	576
SEB	14	22·9*	17·8	29*	1493
SEB + HA	13·5	22·1	17·2	28·5	1398

T lymphocytes (2×10^5/well) were cultured with Dcek Hi 7 cells (10^5/well) in the presence of SEB (10 μg/ml) or SEB + HA (10 μg/ml + 100 μg/ml). Three-colour analysis (anti-Vβ6 or anti-Vβ8 and anti-CD4 and anti-CD8 mAb) was performed after 48 hr.

Results are the average of three experiments, the standard errors (less than 10%), are omitted for clarity.

*$P < 0{\cdot}01$, line 2 versus line 1.

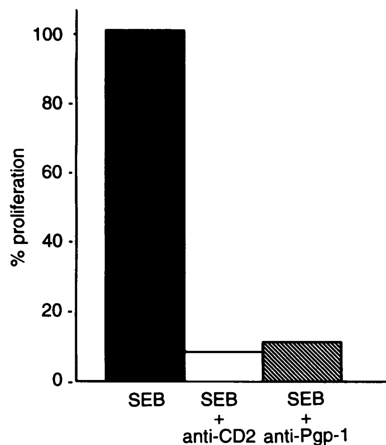

Figure 3. Effect of anti-Pgp-1 and anti-CD2 antibodies on SEB-induced T-cell proliferation. $CD4^+$ and $CD8^+$ T cells (2×10^5/well) were cocultured with Dcek Hi 7 cells (10^5/well) in the presence or absence of SEB (10 μg/ml). Anti-Pgp-1 (10 μg/ml) or anti-CD2 (10 μg/ml) were added to some wells. After 36 hr, cells were pulsed for 12 hr with 1 μCi/well [^{3}H]thymidine, as described in the Materials and Methods. The data are expressed as the percentage of SEB + anti-Pgp-1 or SEB + anti-CD2-treated cells relative to control (SEB alone, 100%). The results represent the average of three experiments. The standard errors were less than 10% of the mean.

SEB-induced activation was examined. As shown in a representative experiment in Fig. 3, both antibodies inhibited SEB-induced T-cell proliferation.

We then examined the effects of HA treatment on deletion and activation. When added to the SEB-treated group, soluble HA had no effect on deletion and proliferation, indicating that it could not mimic the effect of Pgp-1 mAb (Table 1).

Cross-linked Pgp-1 costimulates anti-CD3-triggered mature T cells

It was thus clear that soluble anti-Pgp-1 mAb, but not HA, increases SEB-driven deletion. We next looked to see whether cross-linked mAb activate T cells. Since the monolayer of APC in the SEB system prevents adherence of anti-Pgp-1 to the plastic, we used a model in which TCR was triggered by an anti-CD3 mAb to allow anti-Pgp-1 immobilization on the well bottom, resulting in cross-linking of the molecule on the cell surface.

Stimulation by cross-linked anti-Pgp-1 not only costimulated T cells triggered by suboptimal doses of anti-CD3, but also significantly increased T-cell proliferation induced by optimal concentrations of anti-CD3 antibodies. The effect on proliferation was dose-dependent (Fig. 4).

In this system, HA adhered to the plastic and mimicked the effects of anti-Pgp-1, suggesting that the conformational modification of Pgp-1 induced by the antibody cross-linking gives the same signal(s) as the interaction with its ligand.

A similar effect on T-cell proliferation was observed with cross-linked anti-CD2 antibody (Fig. 4). Figure 5 demonstrated that Pgp-1 only costimulated anti-CD3 triggered T cells when its antibody was cross-linked. Pre-treatment with FCS prevented its adherence and abolished its ability to costimulate.

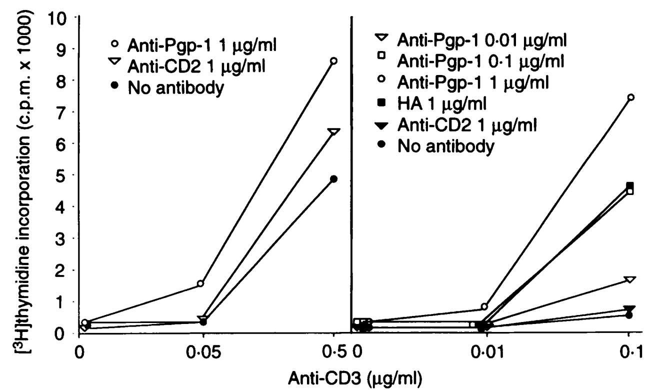

Figure 4. Effects of anti-Pgp-1, HA and anti-CD2 on anti-CD3-induced proliferation. Different doses of anti-CD3, and/or anti-Pgp-1, and/or HA, and/or anti-CD2, were allowed to adhere in 96-well flat-bottomed plates as described above. T lymphocytes (2×10^5/well) were cultured for 48 hr on coated plates. The wells were then pulsed for the last 12 hr with 1 μCi of [^{3}H]thymidine. The results represent the average of three experiments (each in triplicate culture). The standard errors were routinely less than 10% of the mean.

Modulation of Pgp-1 surface expression: shedding of the molecule

Anti-CD3 mAb rapidly up-regulates Pgp-1 expression,[31] and anti-Pgp-1 mAb causes rapid Pgp-1 shedding from human lymphocytes.[32] The effect of SEB on this shedding is unknown.

Pgp-1 expression on SEB-treated cells was therefore investigated. As shown in Fig. 6, Pgp-1 expression was unchanged by 48 hr exposure to SEB. However, IL-2 receptor expression was selectively evident on $V\beta8^+$ cells after 48 hr, demonstrating that T cells were activated and deleted (Fig. 1) at the same time. After 96 hr, 38·8% $CD4^+$ and 31% $CD8^+$ Pgp-1^{low} cells became Pgp-1^{high} (Fig. 6).

The expression of CD2 (control) was also modulated. Part of the cells expressed CD2 at high intensity after 96 hr (Figure 6).

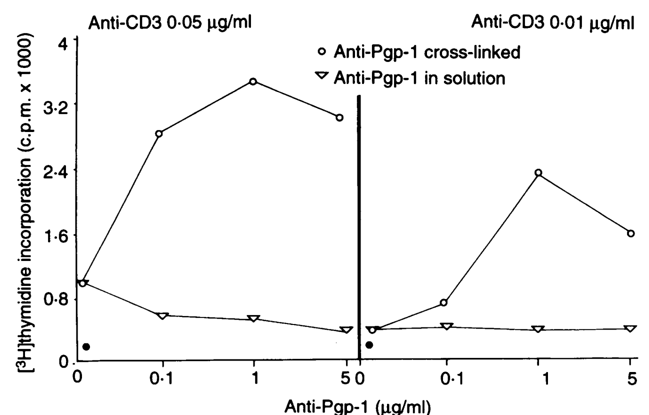

Figure 5. Anti-Pgp-1 cross-linked, but not in solution, costimulates anti-CD3-triggered lymphocytes. Anti-CD3- and/or anti-Pgp-1-coated plates were prepared as described. Some anti-CD3-coated plates were incubated with 100 μl of FCS, and Pgp-1 antibody was added together with lymphocytes (2×10^5/well). Uptake of thymidine was performed as described in the Materials and Methods. Standard errors were less than 10% of the mean of three experiments.

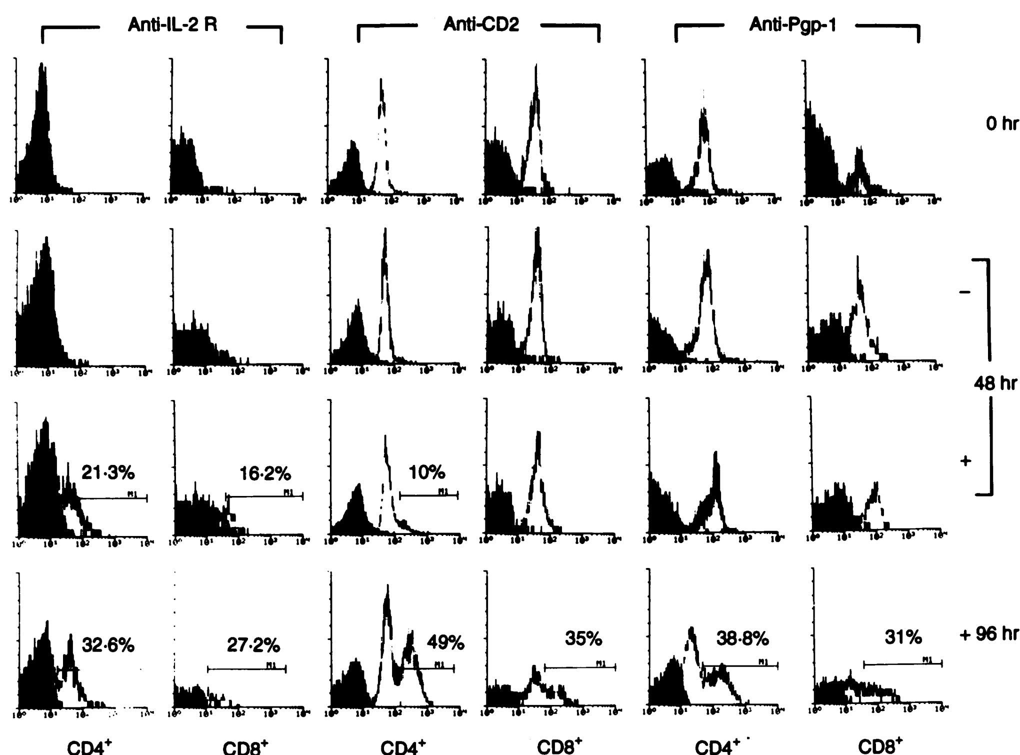

Figure 6. Surface expression of IL-2R, CD2, Pgp-1, on SEB-stimulated $V\beta 8^+$ T lymphocytes. Mature T cells, CD4 and CD8 single positive were cocultured with Dcek Hi 7 cells in the presence or absence of SEB. After 48 or 96 hr, T cells were removed and three-colour analysis was performed as described.

To investigate whether SEB induces shedding, we immunoprecipitated Pgp-1 from supernatants and lysates of cells treated for 96 hr with APC in the presence or absence of SEB. A soluble Pgp-1 form was isolated from the culture supernatant of SEB-treated lymphocytes. A small amount of shed Pgp-1 was detected in the supernatant of the control (Fig. 7). The molecular mass of the soluble Pgp-1 was slightly lower than that of membrane Pgp-1. These data suggest that shedding is an important regulatory mechanism of surface Pgp-1 expression in superantigen-induced T cell activation.

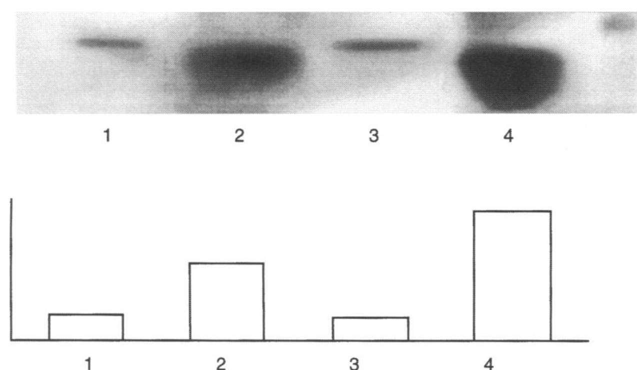

Figure 7. Effect of SEB on shedding of Pgp-1 molecule. T lymphocytes were either untreated or treated with SEB (10 μg/ml) for 96 hr. Cell lysates and supernatants were immunoprecipitated with anti-Pgp-1 and analysed by SDS-PAGE gel. 1, control; 2, control supernatant; 3, SEB; 4, SEB nupernatant.

DISCUSSION

Engagement of the CD3/TCR complex by antigen associated with MHC molecules triggers the biochemical events responsible for T-cell activation.[8] However, antigen-specific T-cell proliferation requires costimulatory signals delivered by interaction of T-cell surface receptors with their putative ligands on the APC surface.[9] The molecular basis of costimulation involves at least four pairs of receptor–ligand: B7/CD28,[33] CD2/LFA-3,[34] LFA-1/ICAM-1,[35] and CD44/HA.[24] In the absence of costimuli, triggering of TCR induces anergy.[9] Functional anergy was demonstrated in an *in vivo* model, using SEB as antigen, and followed by apoptosis of a large part of the responding T cells.[12] While clonal anergy of the mature T cells persists for at least 3 months after tolerization, and follows an initial activation phase, another model of peripheral tolerance has recently been described. Activation by SEB of peripheral T cells *in vivo* and *in vitro* results in depletion of a large part of $V\beta 8^+$ antigen-specific T cells.[13] This depletion is due to programmed cell death, which precedes or occurs at the same time as proliferation.[13] This model was used to investigate the role of Pgp-1 in SEB-driven deletion, since adhesion molecules may play a substantial role in the control of cell survival. Epithelial and endometrial cells, for example, undergo apoptosis when they lose contact with their underlying matrix, indicating that integrin signals control the apoptotic response.[26] Furthermore, Pgp-1 activation rescues $CD4^+$ $CD3^+$ T-cell hybridomas from programmed DEX- or CD3-induced cell death.[36]

This paper provides the evidence that Pgp-1 may control

SEB-specific apoptosis. Soluble anti-Pgp-1 antibody added to the SEB and APC system significantly increased the percentage of $V\beta8^+$ cells deleted (Fig. 1) as a result of apoptosis (Fig. 2). The fact that the same antibody, cross-linked to the plastic, but not in solution, costimulated CD3-treated lymphocytes (Fig. 5), while in a system with SEB and APC, where cross-linking to the plastic was prevented by adherent APC, apoptosis was increased and proliferation decreased, suggested that soluble mAb blocked rather than triggered a signal. Cross-linking of Pgp-1, in fact, is required to trigger the costimulatory signal, and monovalent Fab fragments are inactive.[37] Adhered HA mimicked the Pgp-1 mAb biological effects by costimulating CD3-primed lymphocytes (Fig. 4), whereas its soluble form did not affect SEB-induced proliferation or deletion (Table 1). Soluble exogenous HA, which can compete with cell-bound endogenous HA for binding to Pgp-1, should not (theoretically) exert any effect. Another simple interpretation of our finding is that Pgp-1 on T cells recognizes and directly binds to other molecules (HA is only one of the ligands of Pgp-1) important for cell survival. This binding was prevented by the antibody. Experiments are in progress to test this interpretation. The hypothesis that the soluble antibody blocks a costimulatory signal is further supported by the similar behaviour of the anti-CD2 antibody: its cross-linked form costimulated anti-CD3-driven proliferation (Fig. 4), whereas the soluble form inhibited proliferation and deletion induced by SEB (Figs. 1, 3), since it antagonizes CD2/LFA-3 binding[30] and prevents the coaccessary function of APC.

The different effects triggered by the same anti-Pgp-1 antibody may depend on the system used. Pgp-1 (clone IM7), in fact, inhibits the proliferation of normal human peripheral blood lymphocytes stimulated by anti-CD3, but acts in synergy with anti-CD2.[38]

We do not know how addition of Pgp-1 antibody to the SEB system augmented apoptosis. Pgp-1 triggering provides coaccessory stimuli to SEB-driven activation.[39] SEB-induced deletion and activation requires not only TCR engagement, but also signal(s) delivered by APC, whose chemical inactivation inhibits both phenomena.[40] Therefore, it may be speculated that while Pgp-1 antibody blocks one of the 'second signals'[9] necessary for activation, the concomitant presence of 'apoptotic signal(s)' induces an imbalance between the pathways of activation and deletion and results in increased cell death.

A number of functionally important surface molecules e.g. the tumour necrosis factor receptor,[41] are regulated by shedding, in response to external stimuli. Pgp-1 is down-regulated on granulocytes by phorbol 12-myristate 13-acetate or ionomycin, and on lymphocytes and granulocytes by anti-Pgp-1 mAb.[31] Shedding rather then internalization is responsible for both modulations.

Peripheral T cells responded to SEB by shedding Pgp-1 (Fig. 7). The presence in serum of soluble Pgp-1[31] with a molecular weight corresponding to that of Pgp-1 shed from lymphocytes *in vitro* suggests *in vivo*, that Pgp-1 is shed perhaps for self-control of the immune response. Release of the soluble form, by competing with the natural ligand of Pgp-1, could modulate the triggering effect of Pgp-1, and therefore activation and apoptosis.

Our results demonstrate that Pgp-1 is involved in controlling SEB-induced T-cell death. The *in vivo* significance of these observations remains to be studied.

ACKNOWLEDGMENTS

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