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CD45 Regulates Apoptosis Induced by Extracellular Adenosine Triphosphate and Cytotoxic T Lymphocytes

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Several lines of evidence implicate protein tyrosine phosphatases (PTP) in the regulation of apoptotic cell death. We have evaluated the role of CD45, the major PTP of hematopoietic cells, in apoptosis induced by extracellular ATP (ATP_e) and cytotoxic T lymphocytes (CTL). We observed that two $CD45^-$ clones obtained by mutagenesis of the Fas^- cell line L1210, exhibit a higher susceptibility to apoptosis induced by ATP_e, which was also evident in Ca^{2+}-free conditions, when compared to the parental cell line or $CD45^+$ variants. The $CD45^-$ cells were also more susceptible to death mediated by an alloreactive CTL clone. When the cytotoxic assay was performed in the presence of EGTA, a Ca^{2+} chelator, which prevents cytotoxic granule exocytosis and perforin polymerization on target cell membranes, only the $CD45^-$ target cells were killed by the CTL clone. These results suggest that a cytotoxic pathway other than the secretory or Fas-dependent pathways was responsible for the enhanced susceptibility of $CD45^-$ cells to death, and therefore provide further evidence for the role of ATP_e as a possible mediator of Ca^{2+}-independent target cell destruction by CTL.

Cytotoxic T lymphocytes (CTL) induce target cell destruction by causing lysis and/or nuclear DNA degradation (apoptosis). Two principal pathways are responsible for these events: a secretory pathway characterized by the Ca^{++}-dependent release of soluble lytic proteins (perforins and granzymes) from cytotoxic granules, and a non-secretory pathway that depends on the expression of ligands, e.g. tumor necrosis factor (TNFα) and Fas-L, on the surface of CTL, which in turn transduce a death signal by engaging their respective receptors expressed on the target cell surface (see review in 1). However, other mechanisms have also been shown to cause target cell death; in particular, it has been proposed that extracellular adenosine-triphosphate (ATP_e) represents an additional mediator of cytotoxicity (2-5). In fact, ATP_e is released from activated CTL (6) and, by interacting with cell surface purinoreceptors (7), induces cell death in a Ca^{++}-independent fashion through both colloido-osmotic lysis and apoptosis (2, 3, 8, 9). Recently, we demonstrated that protein tyrosine phosphatases (PTP) are involved in ATP_e-mediated apoptosis, through a pathway also involving protein tyrosine kinases (PTK) (10). It has been reported that CD45, the most important PTP in hematopoietic cells (11), negatively regulates apoptosis of immature B cells following IgM crosslinking (12), suppresses apoptosis of malignant T lymphoma cells (13), and is involved in the delivery of the apoptotic signal in immature thymocytes, as well as in T and B cells (14-18). In the present paper we

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The abbreviations used are: CTL, cytotoxic T lymphocytes; TNFα, tumor necrosis factor α; ATP_e, extracellular adenosine triphosphate; PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; 2-ME, 2-mercaptoethanol; FBS, foetal bovine serum; mAb, monoclonal antibody; WT, wild type.

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provide evidence, by means of $CD45^-$ cell mutants, that CD45 PTP is involved in the regulation of the apoptotic process induced by ATP_e and possibly by CTL.

MATERIALS AND METHODS

Cell cultures, antibodies, and flow cytometric analysis. The murine T cell leukaemia cell line L1210 (haplotype H-2^d) and its mutagenized derivatives (see below) were maintained in DMEM supplemented with 2 mM L-glutamine, 50 μM 2-mercaptoethanol (2-ME), 10 mM HEPES, 100 IU penicillin/ml, 100 μg streptomycin/ml, and 5% heat-inactivated foetal bovine serum (FBS, Irvine Scientific, Santa Ana, CA, USA). The alloreactive CTL clone G7 (H-2^b anti H-2^d) was isolated in our laboratory. The following antibodies were used in the flow cytometric analyses: anti-CD45 (M1/9.3.4, rat IgG_{2a}; obtained from the ATCC, Rockville, MD, USA), anti-H-2^d (mouse IgM; Cedarlane, Hornby, Canada); anti-ICAM-1 (YN1/1.7.4, rat IgG_{2a}; ATCC); anti-Fas (JO_2, hamster IgG; Pharmingen); R-phycoerythrin (R-PE)-conjugated anti-mouse and anti-rat, and FITC-conjugated anti-hamster (Jackson ImmunoResearch Laboratories, Inc, PA, USA). Cells were stained with the indicated primary antibody for 30′ in ice, washed, and then incubated with the conjugated secondary antibody, washed, and analyzed with a Coulter Epics Elite flow-cytometer (Coulter Corporation, Hialeah, FL, USA).

Generation of $CD45^-$ L1210 cell lines. 60×10^6 L1210 cells were treated overnight with 4 mM ethyl methanesulfonate, then washed and cultured in DMEM-5% FBS for three days. After this period the cells were incubated with magnetic beads coated with monoclonal antibody (mAb) M1/9.3.4 and exposed to a magnetic field to remove the $CD45^+$ cells. After four rounds of negative selection, cells that lacked cell-surface expression of CD45 were subcloned by limiting dilution.

PTP activity. Protein phosphatase assays were performed using 1 μM angiotensin II, that had been phosphorylated by the protein tyrosine kinase, c-Fgr, in 6 mM imidazole/HCl, pH 7.2, 0.12 mg/ml bovine serum albumin, 0.12 mM EDTA, and 1.4 mM 2-ME. The reactions were started by addition of aliquots of cell membrane extracts (corresponding to 1-3 μg total protein), incubated at 30 °C for 10 min., and stopped by adding 10% (mass/vol.) trichloroacetic acid (17). The released [^{32}P]-phosphate was converted into its phosphomolybdic complex, extracted with isobutyl alcohol/toluene (1:1, by vol.) and quantified, all as described (19).

Northern blot analysis. mRNA (20 μg) was subjected to electrophoresis in a 0.8 % agarose-formaldehyde gel and transferred overnight to Hybond-N membrane (Amersham, Amersham, U.K.). The membrane was hybridized with a [^{32}P]-labelled DNA probe specific for CD45 (generously provided by J. Ashwell, Laboratory of Immune Cell Biology, National Cancer Institute, Bethesda, USA).

^{51}Cr and ^{125}IUdR short-term release assays. Indicated target cells (2×10^6) were labelled with 15 μCi of ^{125}IUdR (Amersham, Amersham U.K.) in DMEM-3% FBS for 14 hrs. After incubation, the cells were washed, pelleted, and labelled with 100 μCi ^{51}Cr ($Na_2{}^{51}CrO_4$, NEN Boston, MA, USA) for 1 hour at 37° C. Labelled target cells were mixed with ATP_e and CTLs at indicated concentrations and effector/target ratios, and processed as described (10).

RESULTS

To address the possible role of CD45 PTP in apoptotic target cell death mediated by ATP_e, L1210 cells were chemically mutagenized, and two $CD45^-$ mutants, hereafter referred to as clones 32 and 34, were selected by four rounds of cell sorting. Several clones which retained normal CD45 expression (clones 1, 2, 8, 36) were used as controls to verify that the procedure used to mutagenize and select the cells did not induce further alterations of the parental cell line. Fig. 1 presents flow cytometry analyses of clones 32, 34, 1, and parental L1210 cell (designated WT (wild type) in the figure) carried out to detect cell surface expression of CD45 and other molecules involved in cell-mediated cytotoxicity (MHC class I, ICAM-1, Fas). Analysis using a mAb against a CD45 epitope confirmed the lack of expression of CD45 on clones 32 and 34, while clone 1 maintained CD45 expression at a level comparable to that of the parental cell line, and thus was used as control in subsequent assays. The fact that similar levels of MHC class I and ICAM-1 molecules were detected on wild-type L1210 cells and mutagenized clones indicates that the mutation was specific for CD45. As reported by others, cell surface expression of the Fas molecule was undetectable on L1210 cells (20, 21), and, as expected, also remained absent from the mutagenized clones (Fig. 1). To determine the level of the defect in CD45 expression, RNA from wild-type L1210 cells and the mutagenized clones were subjected to Northern blot analysis using a CD45-specific cDNA probe. RNA from the fibroblast cell line L929, which does not express CD45, was included as a negative control. As shown in Fig. 2, Panel A, the CD45 mRNA was not detectable in $CD45^-$ L1210

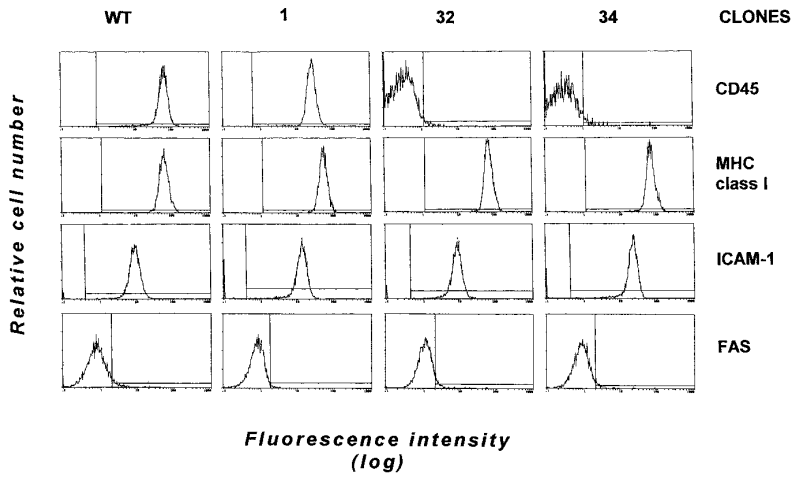

FIG. 1. Characterization of the L1210 WT and mutagenized clones. Wild-type L1210 cells and mutagenized clones were stained with M1/9.3.4 (anti-CD45), H-2^d (anti-MHC class I), YN1/1.7.4 (anti-ICAM-1), and JO_2 (anti-Fas) antibodies, and cell surface fluorescence was quantified by flow cytometry. The vertical line in each inset corresponds to the marker set at the upper limit of a sample of cells incubated with the conjugated secondary antibodies only, as a negative control for background fluorescence.

clones 32 and 34 or in L929 cells, but was clearly evident in wild-type L1210 cells and in $CD45^+$ clone 1. This observation indicated that the loss of CD45 from the cell surface of clones 32 and 34 reflected either a defect at the level of CD45 gene structure or rather than at the level of CD45 protein translation or processing. Since CD45 accounts for most of the total cellular PTPase activity in hematopoietic cells, we determined membrane-associated PTPase activity by measuring ^{32}P release from an angiotensin II substrate labelled with γ-^{32}-P-ATP. Results showed that PTPase activity was significantly reduced ($\approx$50%) in the $CD45^-$ clones compared to the $CD45^+$ clones, thus verifying the association between CD45 levels and PTPase activity (Fig. 2, Panel B).

To evaluate cell lysis and apoptosis induced by ATP_e, wild-type L1210 cells and mutagenized clones were labelled with ^{51}Cr for cell lysis assays and with $^{125}IUdR$ for DNA fragmentation assays. We observed that, while ATP_e-mediated cell lysis was nearly identical for all clones (Fig. 3, Panel A), DNA fragmentation was higher in $CD45^-$ than in $CD45^+$ clones (Fig. 3, Panel B). As ATP_e activity is known to be Ca^{++}-independent, additional experiments were performed in the presence of 4 mM EGTA and 3 mM $MgCl_2$ to chelate Ca^{++}. We observed that ATP_e maintained its ability to induce target DNA fragmentation under these conditions (albeit at a lower level compared to assays done in the absence of the chelator); again, fragmentation was more evident when $CD45^-$ target cells were used (Fig. 4).

We next evaluated the susceptibility of the $CD45^-$ target cells to CTL-mediated cytotoxicity. To this end, the clones were labelled with ^{51}Cr and $^{125}IUdR$ and used as specific target cells for an alloreactive CTL designated clone G7. As shown in Fig. 5 (Panel A) $^{125}IUdR$ release values obtained with $CD45^-$ clones were about twice the release values of $CD45^+$ clones. Comparable results were obtained in ^{51}Cr release assays (not shown). To study whether the higher susceptibility of $CD45^-$ variants to CTL-mediated killing was due to an increased binding to the CTL clone, we evaluated cell-cell binding by means of FACS analysis, and observed no difference in the number of cell-cell conjugates between $CD45^+$ and $CD45^-$ clones (not shown). To exclude the possibility that mediators released by CTL might account for the enhanced killing of $CD45^-$ target cells, experiments were performed in the presence

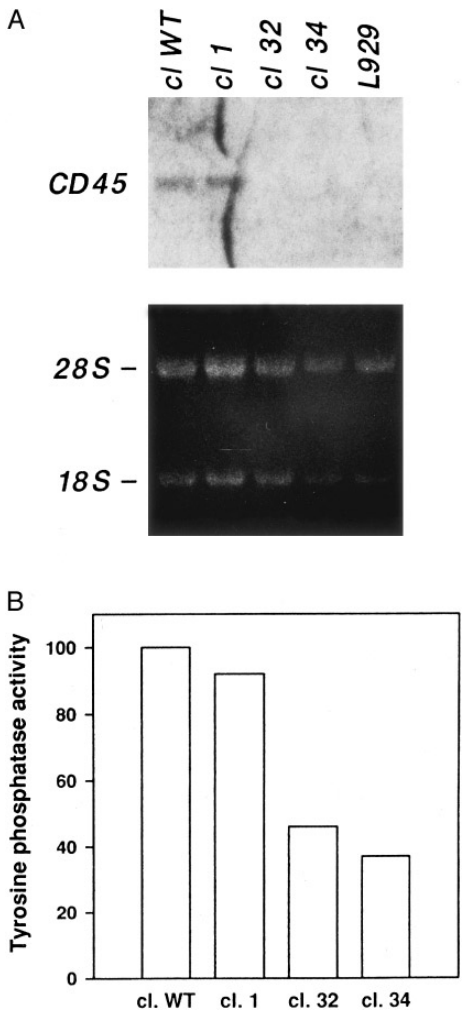

FIG. 2. CD45$^-$ L1210 variants lack transcripts for CD45 and have a reduced PTP activity. Panel A: total RNA (20μg/lane) extracted from wild-type L1210 cells, mutagenized clones, and from L929 cells (negative control) was separated by electrophoresis, transferred to a Hybond-N membrane, and incubated with a [^{32}P]-labelled CD45 cDNA probe. Shown beneath the blot is the ethidium bromide-stained membrane. Panel B: the membrane-associated PTP activity of wild-type L1210 cells and mutagenized L1210 clones was assayed in vitro by measuring the release of [^{32}P] from angiotensin II previously phosphorylated using the PTK c-Fgr. The indicated values represent the percentage of the PTP activity of wild-type L1210 cells, considered as 100%.

of 4 mM EGTA and 3 mM $MgCl_2$ in order to inhibit granule exocytosis and perforin assembly on the target cell membrane. As shown in Fig. 5 (Panel B), DNA fragmentation in CD45$^+$ clones was no longer detected in the presence of this chelator, while DNA fragmentation of CD45$^-$ clones was only reduced. These results indicated that a cytotoxic pathway other than the secretory pathway was responsible for the enhanced susceptibility of CD45$^-$ target cells to death.

DISCUSSION

Evidence is accumulating ATP_e acts as a lytic mediator possibly involved in the non secretory Ca^{++}-independent pathway of cell-mediated cytotoxicity (2, 3). In fact, this nucleotide is

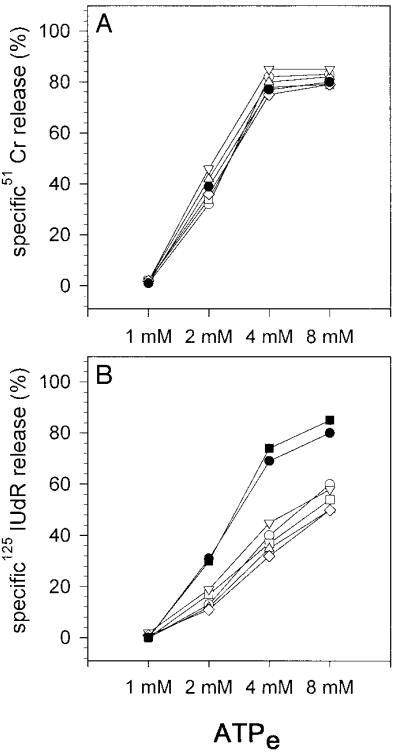

FIG. 3. CD45$^-$ L1210 clones exhibit a higher susceptibility to apoptosis induced by ATP_e. Target cells, prelabelled with ^{51}Cr and ^{125}IUdR, were incubated with different concentrations of ATP_e for 4 hours. Plates were then centrifuged and supernatant was harvested to quantify released ^{51}Cr (Panel A) and ^{125}IUdR (Panel B). The CD45$^+$ and CD45$^-$ cell lines are represented by white and black symbols, respectively. Target cells are wild-type L1210 cells, ○ ; clone 1, □ ; clone 2, △ ; clone 8, ▽ ; clone 36, ◇ ; clone 32, ● ; clone 34, ■. Spontaneous ^{51}Cr and ^{125}IUdR release never exceeded 15%. Comparable results were obtained in three separate experiments.

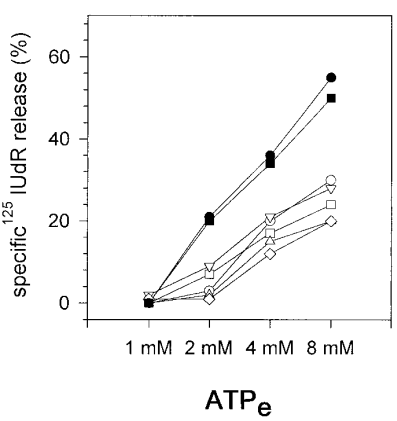

FIG. 4. CD45$^-$ L1210 clones exhibit a higher susceptibility to apoptosis induced by ATP_e in the presence of EGTA/$MgCl_2$. Target cells, prelabelled with ^{125}IUdR, were incubated with different concentrations of ATP_e in the presence of 4 mM EGTA and 3 mM $MgCl_2$ for 4 hrs and harvested to quantify released ^{125}IUdR. Spontaneous ^{125}IUdR release never exceeded 15%. Comparable results were obtained in three separate experiments.

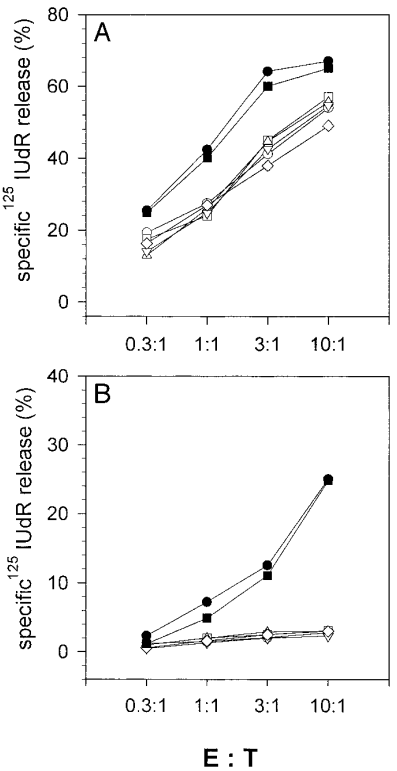

FIG. 5. CD45$^-$ L1210 clones are more susceptible to cell death mediated by CTL compared to CD45$^+$ L1210 clones. Target cells, prelabelled with ^{125}IUdR, were incubated with anti-H-2^d CTL at different E:T cell ratios in the absence (Panel A) or presence of 4 mM EGTA and 3 mM $MgCl_2$ (Panel B). Target cells are wild-type L1210 cells, ○ ; clone 1, □ ; clone 2, △ ; clone 8, ▽ ; clone 36, ◇ ; clone 32, ● ; clone 34, ■ . Spontaneous ^{125}IUdR never exceeded 15%. Comparable results were obtained in three separate experiments.

secreted by CTL after treatment with activating ligands, and is able to induce both lysis and DNA fragmentation in many cell types, even in the presence of EGTA (2). The results presented here indicate that CD45, the principal PTP of lymphocytes, exerts a negative control on cellular DNA degradation induced by ATP_e. This is demonstrated by the finding that two CD45$^-$ mutant clones from the L1210 tumor cell line were more susceptible to DNA degradation induced by ATP_e than wild-type L1210 cells or clones that expressed the CD45 molecule (Fig. 3). Results of additional experiments showed that the K^+-ionophore valinomycin induced comparable degree of DNA fragmentation in CD45$^+$ and CD45$^-$ clones, thus suggesting that the high susceptibility of cells lacking CD45 to ATP_e concerns the early stages of the pathway that leads to apoptotic cell death (data not shown).

Our results support the role of ATP_e as a mediator of Ca^{++}-independent target cell destruction. In fact, when mutant clones and wild-type L1210 cells were used as targets of the specific alloreactive CTL clone G7, the CD45$^-$ clones showed a higher susceptibility to DNA fragmentation than the mutagenized CD45$^+$ clones or wild-type L1210 cells (Fig. 5, Panel A). The higher susceptibility of CD45$^-$ clones was evident even in the presence of EGTA/$MgCl_2$, added to the cytotoxic assay in order to prevent cytotoxic granule release (Fig. 5, Panel B). In fact, under these experimental conditions target cells expressing the CD45 molecule were not killed, while the mutagenized CD45$^-$ clones maintained their susceptibility to CTL-mediated activities. It should be noted that wild-type L1210 cells and mutagenized clones did not express

Fas, as assayed by cytofluorimetric analysis (Fig. 1); moreover, they did not undergo DNA destruction following incubation with anti-Fas mAb (data not shown). We also evaluated the possible role of TNFα, which is considered a further mediator of Ca^{++}-independent apoptosis, although this cytokine requires several hours in order to exert its cytotoxic effect (22, 23). We observed by means of paraformaldehyde-fixed CTL and soluble TNF-α that $CD45^+$ and $CD45^-$ cells were not killed in a 4-hour assay, even when EGTA was added to the medium (not shown). Therefore, the enhanced $CD45^-$ target cell susceptibility to CTL cannot be ascribed to the secretory and Fas-dependent pathways. Instead, we hypothesize that under our experimental conditions, target cell destruction might be due to ATP_e released by CTL. In Ca^{++}-free conditions we observed a difference in the susceptibility of $CD45^-$ and $CD45^+$ target cells to ATP_e and CTL-mediated death; in fact, in the presence of EGTA, $CD45^+$ target cell destruction by CTL was no longer detected (Fig. 5, Panel B), while ATP_e-induced destruction was only partially reduced (Fig. 4). This discrepancy can be explained by the fact that CTL release low levels of ATP_e and therefore the cytotoxic activity of this mediator is detectable only when $CD45^-$ cells, which are highly susceptible to its toxic activity, are employed as targets.

An unresolved question remains whether ATP_e exerts lytic effects by interacting with signal transducing purinoreceptors, and the cascade of intracellular events leading to ATP_e-dependent apoptosis remains to be determined. On the basis of their pharmacological properties and, more recently, on characterization of their genes, ATP_e receptors fall into two classes of P2 purinoreceptors termed P2y and P2x (7). Interestingly, one member of the P2x family can operate both as an ion channel that is permeable only to small cations, and as a nonselective pore, leading to osmotic cell death (24). Although the association between P2x receptors and ATP_e-induced apoptosis has not been conclusively demonstrated, this possibility is supported by the observation that these receptors share sequence similarity with RP-2, a protein expressed in thymocytes undergoing apoptosis (25). Because the ATP_e receptor does not seem to have intrinsic kinase activity, one possibility is that CD45 might dephosphorylate PTK coupled with purinoreceptors or, alternatively, various proteins phosphorylated by PTK. If this is the case, the loss of the phosphatase activity of CD45 would induce a constitutive hyperphosphorylation state in the cell, which ultimately would be responsible for an increased susceptibility to the apoptotic process. Preliminary experiments to verify this possibility indicated a constitutively higher level of protein phosphorylation in $CD45^-$ mutant cells compared to $CD45^+$ cells (unpublished results).

Accumulating evidence indicates that CD45 PTP is a positive as well as a negative regulator of different cellular processes. However, it is not clear how CD45 can regulate apoptotic cell death. In fact, while our results and previous studies (12, 13) indicate that CD45 negatively regulates apoptosis, there is also some indications for its positive role (14, 17). Very recently, Klaus et al. (15) showed that mAb engagement of CD45 on human T and B lymphocytes induces apoptotic cell death, without inducing DNA fragmentation, and that co-ligating CD45 and CD95 (i.e., Fas) either enhances or inhibits CD45-induced cell death, depending on the dose of the mAbs used. In contrast, it has also been reported that in $CD45^-$ mutant cells, CD95-mediated apoptosis is independent from CD45 (26). The fact that L1210 cells lack Fas expression presents us with the opportunity to study the role of this molecule by stably transfecting $CD45^-$ clones with the Fas gene; preliminary experiments suggest that apoptosis induced by anti-Fas mAb is not regulated by CD45 molecule (unpublished results).

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