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CD45 molecule cross-linking inhibits natural killer cell-mediated lysis independently of lytic triggering

G. C. STARLING & D. N. J. HART *Department of Haematology, Christchurch Hospital, Christchurch, New Zealand*

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SUMMARY

The fact that certain CD45 [anti-leucocyte common antigen (LCA)] monoclonal antibodies (mAb) inhibit natural killer (NK) cell non-major histocompatibility complex (MHC)-restricted cytolysis led to the suggestion that these mAb block a 'trigger' for NK cell lytic activity. However, the discovery that the intracytoplasmic portion of the leucocyte common molecule has protein tyrosine phosphatase activity raises the possibility that the mAb initiate a direct inhibitory signal, independent of the triggering apparatus. To clarify this, we have tested the ability of CD45 antibodies to trigger NK cells and redirect cytotoxicity against mAb-producing hybridoma cells and autologous monocytes, an approach which has identified other cytotoxic trigger molecules. Peripheral blood NK cells failed to kill the CD45 antibody-producing hybridomas, although a CD3 antibody expressing hybridoma was susceptible to cytotoxic T-cell lysis. Furthermore, the CD45 mAb CMRF-12+26, 13.3 and HuLyM4 did not redirect lysis of autologous monocytes by NK cells, whereas the isotype-matched CD16 mAb did so. Bivalent CD45 antibody was necessary to block NK lysis of K562, as $F(ab')_2$ but not F(ab') fragments of CMRF-12+26 antibody inhibited killing. Capping of the LCA appeared to correlate with the ability of the CD45 mAb to block killing, suggesting that cross-linking of LCA molecular isoforms on the NK cell surface is required for CD45 mAb to inhibit non-MHC-restricted cytolysis.

INTRODUCTION

Natural killer (NK) cells are typically large granular lymphocytes which by definition lack a CD3/T-cell receptor complex (Hercend & Schmidt, 1988) and exhibit non-major histocompatibility complex (MHC)-restricted cytolytic activity. NK cells are found in greatest concentration in the blood and spleen, and lyse a range of cells, including cells infected with micro-organisms (especially viruses) and malignant cells (Herberman & Ortaldo, 1981; Fitzgerald & Lopez, 1986). The cell membrane antigens recognized by the CD2 (sheep red blood cell receptor), CD11b (receptor for the third component of complement), CD16 (FcRIII), CD56 (N-CAM) and CD57 (HNK-1) monoclonal antibodies (mAb) are expressed on the majority of NK cells. However, the molecular interactions whereby a NK cell recognizes its targets remains obscure, although some NK surface molecules (e.g. CD2 and CD16) can provide signals to activate the lytic machinery of NK cells (Siliciano *et al.*, 1985; Pantaleo *et al.*, 1988; Werfel *et al.*, 1989). Thus, the CD16 molecule, the low affinity Fc receptor for IgG, acts as both the recognition and signal molecule for antibody-dependent cellular cytotoxicity (Windebank *et al.*, 1988), as well as being involved in signalling for NK cell lymphokine production (Cassetella *et al.*, 1989). The leucocyte common antigen (LCA), or CD45 antigen, has also been proposed to act as a lytic trigger for NK cells (Targan & Newman, 1983), and a number of CD45 (anti-leucocyte common antigen) mAb: 13.1/13.3 (Newman, 1982), F2.5 (Sparrow & MacKenzie, 1983) and CMRF-12+26 (Starling *et al.*, 1987) have been reported to block the non-MHC-restricted lytic activity of NK cells.

The LCA family contains up to eight molecular isoforms, all derived from the same gene but having different molecular weights (180,000–220,000 MW) as a result of differential splicing of exons 4,5 and 6 of the LCA gene (Thomas, 1989). All species have a conserved intracellular domain, which has a repeated sequence with protein tyrosine phosphatase (PTPase) activity (Tonks *et al.*, 1988). The discovery of this enzymatic activity and the probability that the CD45 antigen interacts with other lymphocyte receptors raises the question as to whether the inhibition of non-MHC-restricted cytotoxicity by CD45 antibodies is due to the blockade of the NK triggering process or due to another mechanism. To study the possibility that the CD45 molecule might act as a lytic trigger on NK cells we tested the CD45 mAb 13.3, F2.5 (also known as HuLyM4) and CMRF-12+26 to see if, in addition to blocking NK lysis of sensitive

Abbreviations: CD, cluster of differentiation; LCA, leucocyte common antigen; mAb, monoclonal antibody; MHC, major histocompatibility complex; NK, natural killer; PTPase, protein tyrosine phosphatase.

Correspondence: Dr D. N. J. Hart, Dept. of Haematology, Christchurch Hospital, Private Bag, Christchurch, New Zealand.

targets, they could redirect lysis against normally NK-insensitive targets, in the manner described for mAb to other conventional (e.g. CD2 and CD16) trigger molecules.

MATERIALS AND METHODS

Effector cells

Blood was extracted from normal volunteers, and spun over Ficoll–Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) to obtain peripheral blood mononuclear cells (PBMC). Further enrichment of effector cells was obtained by incubating PBMC overnight on plastic and passing the non-adherent cells over nylon-wool. Cells not sticking to the wool were centrifuged over a two-step isotonic Percoll gradient (Pharmacia Fine Chemicals), consisting of 30% and 45% Percoll in phosphate-buffered saline (PBS). Cells at the interface of the two layers were enriched for non-MHC-restricted cytotoxicity and large granular lymphocytes (LGL). These cells were further enriched for redirected killing and capping experiments by removing cells which bound to sheep red blood cells after 1 hr at 29° (Cosentino & Cathcart, 1987). The resulting cell population was commonly 70–80% $CD16^+$, 10–15% $CD3^+$.

Peripheral blood was also extracted from a patient with large granular lympho-proliferative disease (LGLD), whose cytotoxic lymphocytes had the surface phenotype $CD3^+$, $CD16^+$ and exhibited non-MHC-restricted cytotoxic activity against K562 (patient 5; Starling *et al.*, 1989). Cytotoxic T cells were derived from mixed lymphocyte culture of PBMC against the Epstein–Barr virus (EBV)-immortalized B-cell line, MANN, as described previously (Starling *et al.*, 1987).

Monoclonal antibodies used in this study

CMRF-12 and CMRF-26 (each CD45, IgG1) were produced in this laboratory, grown as ascitic fluid in BALB/c mice, and salt precipitated. F(ab′) and $F(ab')_2$ fragments were prepared as described previously (Prickett & Hart, 1990). HuLyM1 (CD2 non-activating epitope, IgG2b), HuLyM4 (CD45, IgG2a) and HuNK2 (IgG2a) were gifts from Professor I. F. C. McKenzie (University of Melbourne, Melbourne, Australia), and supplied as unpurified ascitic fluids. 13.3 (CD45, IgG1) was given to us by Dr W. Newman, Otsuka Pharmaceutical Co., Rockville, MD. Other CD16 mAb (My23 and VEP13) were available to us due to our participation in the 4th Leucocyte Differentiation Antigen Workshop (myeloid panel).

Target cells

Hybridoma cells producing the mAb CMRF-12, CMRF-26 (both produced in this laboratory from the NS-1 myeloma line) and OKT3, from the P3X63Ag8U1 myeloma line (American Type Culture Collection, Rockville, MD), were grown in culture in this laboratory and aliquots taken for use as targets. K562 (supplied by Dr H. Warren, Woden Valley Hospital, Canberra, Australia), was likewise grown in culture prior to radiolabelling. Autologous monocytes were obtained by overnight adherence to plastic.

^{51}Cr-release assay

A standard ^{51}Cr-release assay was used (Starling *et al.*, 1987). In brief, 2×10^6–5×10^6 target cells were incubated with 150 μCi of $Na_2{}^{51}CrO_4$ (Amersham International, Amersham, Bucks, U.K.) for 90 min at 37°. Prior to use of ^{51}Cr-labelled cells as targets, the cells were washed three times in PBS then 1×10^4 added to each microtitre well.

After 4 hr at 37°, plates were centrifuged and 100 μl (from a total volume of 200 μl) of supernatant were harvested. Radioactivity in the samples was determined in an LKB Wallac 1282 Compugamma (Turku, Finland). Percentage specific lysis (% SL) was calculated using the formula:

$$\% \text{ SL} = \frac{\text{test well} - \text{spontaneous release}}{\text{detergent release} - \text{spontaneous release}} \times 100,$$

where test well counts per minute (c.p.m.) were the mean of at least triplicate wells; spontaneous release was the mean of at least quadruplicate wells with medium and targets; and detergent release was the mean of at least quadruplicate wells containing 2% Tween 20 and target cells.

Cold target inhibition assay

The cold target inhibition assay is a variant of the ^{51}Cr-release assay. Various dilutions of 'hot' (^{51}Cr-labelled) and 'cold' (non-labelled) targets were added to microtitre wells, and mixed on a Cooke AM69 Microshaker (Dynatech AG, Bleichstrasse, Switzerland). Effectors were added at a fixed E:(hot)T ratio for each assay. The microtitre plates were then handled as for a normal ^{51}Cr-release assay.

Redirected killing against autologous monocytes

NK-enriched lymphocytes were incubated with individual mAb for 30 min at 4°, and non-binding antibody was washed off prior to the addition of the target monocytes to the microtitre wells (at 1×10^4 targets/well). This ensured that the monocyte Fc receptors were not blocked by mAb that was not bound to effector cells. Henceforth the redirected cytotoxicity assay was processed in the manner of the 4-hr ^{51}Cr-release assay.

Patching/capping of cell-surface antigens

To examine the ability of mAb to induce patching or capping of cell-surface antigens, cells were incubated with mAb as above but, instead of adding targets, $F(ab')_2$ fluorescein isothiocyanate-conjugated sheep anti-mouse (FITC-SAM) (Silenus Laboratories, Hawthorn, Australia) was added and incubated alongside the ^{51}Cr-release assay. After this incubation (4 hr), excess FITC-SAM was washed away and the cells fixed in 1% paraformaldehyde-1% BSA and stored at 4° until they were analysed. The cells were observed on a glass slide under a Leitz-Ortholux microscope (Ernst Leitz, Wetlar, FRG) using phase-contrast and fluorescence methods.

RESULTS

Blockade of the lysis of K562 by CMRF-12 + 26 does not involve the NK cell Fc receptor

In previous experiments (Starling *et al.*, 1987), we demonstrated that two mAb (CMRF-12 and CMRF-26) against pronase-sensitive epitopes of the CD45 molecule, when added together, were able to block up to 75% of PBMC-mediated non-MHC-restricted (NK) cytolytic activity, although each CD45 mAb alone only inhibited target cell lysis weakly. The NK cell Fc receptor may contribute indirectly to the effects of an antibody on NK function; for example the induction of non-MHC-restricted cytotoxicity by the CD2 mAb, 9.1, is dependent on the ability of the mAb to bind to both the CD2 antigen and to the Fc receptor (CD16) (Anasetti *et al.*, 1987). To examine whether or

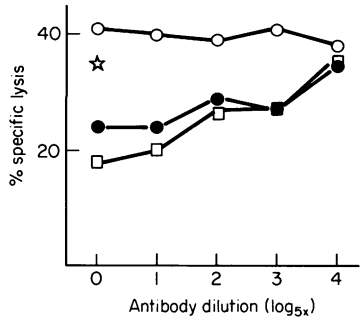

Figure 1. F(ab′)$_2$ but not F(ab′) fragments of CMRF-12+26 inhibit the lysis of K562. PBMC were incubated with F(ab′) (○), F(ab′)$_2$ (□) or whole immunoglobulin CMRF-12+26 (●) for 30 min prior to being washed and added to K562 targets. Lysis of targets incubated in the absence of mAb is indicated by the star. E:T ratio was 50:1. mAb concentration at dilution 0 was 500 μg/ml.

Table 1. CD45 mAb-producing hybridomas are not lysed by peripheral blood NK cells

^{51}Cr-hybridoma	Effector cell (E:T)	% SL
OKT3	MLC-CTL (25:1)	29
	CD3+LGLD (6:1)	28
	PBL (25:1)	8
CMRF-12	PBL (50:1)	4
CMRF-26	PBL (50:1)	0
CMRF-12+26	PBL (50:1)	5
CMRF-12+26	Percoll LGL (15:1)	2
NS-1	Percoll LGL (15:1)	0
K562	Percoll LGL (15:1)	60

mAb-producing hybridomas were labelled with ^{51}Cr and used as targets for the effector cells described above. A range of E:T ratios was tested, and the maximum % SL has been presented. This table is a summary of separate representative experiments.

not the blockade of lysis of K562 by CMRF-12+26 was dependent on the NK cell Fc receptor, F(ab′)$_2$ portions of the CMRF-12 and CMRF-26 mAb were produced. These bivalent F(ab′)$_2$ preparations inhibited NK lysis of K562 to a similar extent as the IgG CMRF-12+26 (Fig. 1), indicating that the CD45 mAb binding to the LCA was sufficient to inhibit lysis and implying that the CD16 molecule was not involved. Monovalent F(ab′) fragments of CMRF-12+26 did not inhibit the lysis of K562, which was evidence that cross-linking of CD45 molecules was necessary to obtain the inhibition of lysis. Both the F(ab′) and F(ab′)$_2$ fragments bound to effectors over the course of the assay to a similar extent, as determined by radioimmune binding assay (not shown); therefore failure of F(ab′) to block was not due to differing rates of dissociation of antibody fragment.

Peripheral blood lymphocytes do not lyse CD45 hybridomas

Hybridoma cells producing mAb against various cytotoxic trigger molecules are targets for lymphocytes bearing the trigger

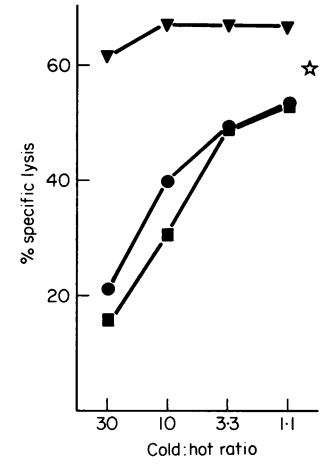

Figure 2. CMRF-12+26 hybridomas inhibit the lysis of ^{51}Cr-K562 by PBMC. Lysis of K562 in the absence of inhibitors is indicated by the star. The E:(hot) T ratio was 50:1. Inhibitors used were cold K562 (■), cold CMRF-12+26 (●), and cold OKTd (▼).

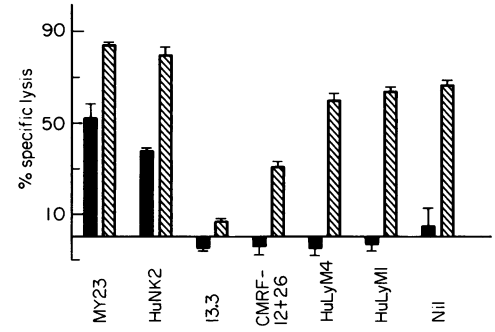

Figure 3. CD16 but not CD45 mAb redirect the lysis of NK cells against autologous monocytes. Lymphocytes enriched for NK cells were incubated with antibody for 30 min at 4° and then added to ^{51}Cr-labelled autologous monocytes (solid bars) or K562 (hatched bars) at an E:T ratio of 33:1. % specific lysis ± 1 SD is illustrated.

molecule (Itoh, Balch & Platsoucas, 1987; Ferrini *et al.*, 1987; Lanier, Ruitenberg & Phillips, 1989). Hybridomas producing CD3 (OKT3) and CD45 (CMRF-12 and CMRF-26) mAb were labelled with ^{51}Cr and used as targets in a Cr-release assay. Both the cytotoxic T lymphocytes derived from secondary MLR stimulation and the CD3$^+$ cells from a patient with LGLD lysed the CD3-producing hybridoma. No PBL-mediated NK lysis of the two CD45 hybridomas was detected when tested either individually or as mixed targets, nor did Percoll-enriched LGL kill the CMRF-12+26 hybridomas, although they were active against K562 (Table 1). NK cells were capable of binding to the CD45 hybridomas, as both of the unlabelled hybridoma lines inhibited the lysis of ^{51}Cr-labelled K562. Cold OKT3 hybridoma cells did not inhibit NK-mediated K562 lysis (Fig. 2), consistent with the fact that the the vast majority of non-MHC-restricted cytotoxicity is elicited by CD3$^-$ NK cells.

Redirected killing of autologous monocytes

Cytotoxic lymphocytes can be redirected to kill autologous monocytes by the specific binding of mAb to the cytotoxic cell

Table 2. Induction of capping on NK cells by mAb

Antibody	Cell-bound fluorescence
CD16	
My23	Monopolar capping*
HuNK2	Monopolar capping
CD45	
13.3	Bipolar† and monopolar capping
HuLyM4	Weak caping/patch‡/ring§
CMRF-12+26	Ring fluorescence
CD2	
HuLyM1	Patching/capping

Representative experiment using the same preparation of NK-enriched cells as used for the experiment shown by Fig. 3.

* Fluorescence concentrated at one pole of the cell.

† Fluorescence concentrated at two poles.

‡ Fluorescence in patches randomly distributed on cell membrane.

§ Fluorescence in an evenly distributed ring over membrane surface.

trigger molecule [via its F(ab′) portions] and to the monocyte Fc receptor via the Ig Fc portion (Itoh *et al.*, 1988). Itoh *et al.* (1988) showed that CTL could kill monocytes if redirected by CD3 mAb (against the T-cell signal transduction molecular complex), and our preliminary results confirmed that IgG1 and IgG2a CD3 mAb could redirect killing of monocytes by PBL (not shown). The IgG1 (My23) and IgG2a (HuNK2) CD16 mAb were able to redirect the cytotoxic activity of NK cells against monocytes. However, the isotype-matched CD45 mAb, 13.3, CMRF-12+26 (all IgG1) and HuLyM4 (IgG2a) did not redirect the NK cells to lyse the autologous monocytes (Fig. 3), despite the fact that the anti-CD45 mAb inhibited the lysis of K562 in parallel experiments, as described previously, for each antibody. Failure to redirect killing was independent of the mAb concentration (not shown), as a wide range of CD45 mAb concentrations was tested in these experiments, and none produced killing of the monocytes greater than in the no-mAb control. HuLyM1 also failed to redirect lysis, as was expected for an antibody against a non-activating CD2 epitope. The percentage specific lysis values less than zero depicted in Fig. 3 probably result from effector cell uptake of spontaneously released ^{51}Cr from the monocytes.

Capping of CD45 antigens on the NK surface

The requirement of bivalency of the CD45 mAb to inhibit the lysis of K562 was evidence that CD45 cross-linking was required for the inhibitory activity. We therefore tested the effects of the CD2, CD16 and CD45 mAb on the distribution of their target antigens in the cell membrane. Effector cells were labelled with mAb at identical concentrations to those used in the redirected killing assay, and after washing were removed from the assay and FITC-SAM added to the cells instead of incubation with the monocytes. These were incubated for the period of the ^{51}Cr-release assay, and washed and fixed for examination by ultraviolet microscopy for cell-bound fluorescence. These results are summarized in Table 2. The CD16 mAb induced the strongest capping of its antigen on the NK cells, whereas the CD45 mAb CMRF-12+26 and HuLyM4 induced little visible cell-surface movement of their antigens. The 13.3 mAb did induce the LCA to form both bipolar (capping at both ends of the cell) and monopolar (capping focused at one pole of the cell) caps.

DISCUSSION

The ability of CD45 antibodies to inhibit the non-MHC-restricted cytotoxic activity of NK cells prompted us to investigate the role of the leucocyte common molecule in NK cell-mediated cytolysis. The inability of CD45 mAb to redirect the lytic machinery of NK cells against CD45 mAb-producing hybridomas and autologous monocytes suggests that the LCA is not a trigger molecule in the classical sense of the word. Biochemical data which demonstrate that CD45 mAb do not induce the turnover of cell membrane phospholipids (Seaman *et al.*, 1987) or the induction of Ca^{2+} flux (Anasetti *et al.*, 1987) support this conclusion, as the triggering of the effector cell for lysis appears to involve both events (Anasetti *et al.*, 1987; Seaman *et al.*, 1987; Windebank *et al.*, 1988). If the CD45 antibodies are not blocking a stimulatory pathway, then an alternative explanation is that they induce an inhibitory pathway in NK cells. This possibility would fit with the fact that CD45 mAb can inhibit a wide range of cellular functions, including the activation of T and B cells as well as non-MHC-restricted cytotoxicity (reviewed by Thomas, 1989). The recent discovery that the LCA mediates protein tyrosine phosphatase (PTPase) activity fits well with this concept and it is conceivable that a number of receptors are under the regulatory control of the LCA PTPase. For example, the CD3ζ chain has recently been proposed as being a site for the action of PTPase activity in T cells (Kiener & Mittler, 1989), and CD45 PTPase activity can activate the CD4-associated molecule, $p56^{lck}$ (Mustelin, Coggeshall & Altman, 1989). NK cells have recently been shown to possess the CD3ζ molecule (Anderson *et al.*, 1989), which is found in association with the CD16 antigen (Lanier, Yu & Phillips, 1989). The interaction of CD3ζ, or other signal-transducing molecules regulated by phosphorylation, with the putative NK cell receptor appears to be another potential site of action of the LCA PTPase.

What other molecular interactions could be involved in the inhibition of non-MHC-restricted cytolysis by CD45 mAb? Here we have shown that cross-linking of CD45 molecules is necessary, as F(ab′) fragments of the antibody did not inhibit the lysis of K562. Newman, Fast & Rose (1983) have likewise demonstrated the need for bivalency in the blockade of lysis by 13.1 mAb. Although a simple steric effect cannot be discounted as yet, cross-linking may be necessary to bring the CD45 molecules into contact with other important cell-surface molecules, rendering them susceptible to down-regulation via dephosphorylation by PTPase activity. Conversely, cross-linking of CD45 molecules may stop them associating with other cell-activating molecules similar to CD4 (not itself expressed on NK cells), thereby preventing CD45 PTPase-mediated dephosphorylation and activation of such a hypothetical molecule. Further evidence that cross-linking rather than steric effects are the important features of the interactions comes from Ledbetter

et al.'s. (1988) observations that CD45 molecules can markedly alter cellular function when cross-linked to relevant T- and B-cell receptors.

Another mechanism by which the CD45 antigen may influence NK cell function is via a role in the organization of the cytoskeleton, because it is associated with the cytoskeletal protein, fodrin (Suchard & Bourguignon, 1987). Fodrin interacts with actin in the cytoskeleton, and the ability of actin to orientate itself in the cell is dependent on the phosphorylation state of fodrin (Wang, Kong & Wang, 1988). The initial steps of the NK cell–target interaction involve the orientation of the NK cell cytoplasm towards the target cell (reviewed by Carpen & Saksela, 1988), and cross-linking of CD45 mAb may affect the ability of the NK cell to organize its cytoskeleton towards the target. Little is known about the distribution of the CD45 molecule on the NK cell-surface during the lysis of target cell, and it would be interesting to follow CD45 movement on the cell membrane in NK-cell mediated cytolysis using F(ab′) fragments of CD45 antibody (which do not block killing) in association with immunogold labelling and transmission electron microscopy. The antibody 13.3, which can block non-MHC-restricted cytolysis at very low mAb concentrations (Newman *et al.*, 1983), compared to CMRF-12+26 and HuLyM4 (not shown), induced more cell membrane capping than the other CD45 mAb, raising the possibility that this mAb is best at inhibiting non-MHC-restricted cytolysis because it is the most effective at interfering with normal cytoskeletal changes in the NK cell prior to target cell lysis.

Other workers have suggested that CD45 is involved in the adhesion between NK cells and their targets (Sparrow & MacKenzie, 1983; Gilbert, Zaroukian & Esselman, 1988). Using the single cell assay, Sparrow & MacKenzie (1983) showed that HuLyM4 inhibited conjugate formation, whereas 13.3 did not inhibit the binding stages of cytolysis. The functional differences between the two mAb, which have been confirmed in our laboratory (unpublished data) may result from epitope differences in the two CD45 mAb. If a ligand–receptor interaction was involved in the function of the LCA epitopes recognized by CMRF-12+26 and 13.3, then F(ab′) fragments of mAb could be expected to block this interaction (and therefore block killing). That the F(ab′) fragments of 13.3 and CMRF-12+26 did not inhibit lysis is evidence that the epitopes recognized by these mAb are not involved in adhesion, although other epitopes could be. There is limited evidence to suggest that the CD45 molecule, as defined by CMRF-12 and CMRF-26 mAb, is involved in the adhesion of T cells to dendritic cells (Prickett & Hart, 1990). A number of molecules, including CD2, LFA-1, CD4 and CD8, have both adhesive and activating roles and antibodies to these molecules may likewise have profoundly different effects on cellular function. Thus, variable effects may be seen according to whether a CD45 antibody blocks LCA extracellular portion interactions with the natural ligands, cross-links the LCA effectively in the membrane or acts more like a natural ligand in initiating PTPase activity. The variation in the external portion of the different CD45 antigens is an indication that the LCA could bind to a variety of ligands selected for by the differing external portions of each individual CD45 isoform. The restricted expression of these isoforms on some cellular populations suggests that this might allow for selective regulation of different cell types. Obviously the identification of ligands for the LCA isoforms would be a major step forward in the understanding of the immunobiology of the CD45 antigen.

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