

Ligation of CD45 and the isoforms CD45RA and CD45RB accelerates the rate of constitutive apoptosis in human eosinophils

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Background: Eosinophils are important effector cells in asthma pathogenesis, and an understanding of the mechanisms involved in eosinophil apoptosis induction might thus be relevant to the resolution of asthmatic inflammation.

Objective: Our aim was to determine the role of the common leukocyte antigen CD45 and the isoforms CD45RA, CD45RB, and CD45RO in human eosinophil apoptosis induction.

Methods: Immunostaining and flow cytometry were used to assess CD45 and CD45 isoform expression by eosinophils purified with use of density gradients and immunomagnetic negative selection. Apoptosis induction was measured by binding of fluorescein isothiocyanate-labeled annexin V to eosinophils cultured for 20 hours alone or with saturating quantities of mAb against CD45, CD45RA, CD45RB, CD45RO, CD9, CD11b, and isotype-matched controls in the presence or absence of GM-CSF.

Results: Freshly isolated eosinophils had high expression of CD45 and CD45RO, modest expression of CD45RB, and low expression of CD45RA. Eosinophils cultured alone for 20 hours were found to be approximately 20% to 25% apoptotic. Incubation with mAb against CD45, CD45RA, and CD45RB resulted in significant ($P < .005$) enhancement ($>100\%$) of their constitutive rate of apoptosis. Incubation with CD45RO, CD11b, CD9 mAb, or isotype controls had no significant effect on the rate of eosinophil constitutive apoptosis. The addition of GM-CSF inhibited eosinophil apoptosis but did not prevent CD45, CD45RA, or CD45RB mAb-dependent apoptosis induction. **Conclusion:** These data indicate that ligation of CD45, CD45RA, or CD45RB represents a novel pathway for the induction of apoptosis in human eosinophils. (*J Allergy Clin Immunol* 1999;104:1244-50.)

Key words: Eosinophils, apoptosis, CD45, allergy, asthma, cell surface molecules

Eosinophils play a protective role in host immunity to infections by parasitic worms and, detrimentally, are involved in the inflammation underlying the pathophysiologic mechanisms of asthma and other allergic dis-

Abbreviations used

FITC: Fluorescein isothiocyanate

PCD: Programmed cell death

PI: Propidium iodide

PTPase: Tyrosine protein phosphatase

eases.¹⁻³ Crucial steps in eosinophilic inflammation include augmented production of eosinophils in the bone marrow, their increased release into the circulation, and selective accumulation in the conducting airways.^{4,5} Apoptosis, or programmed cell death (PCD), is an important aspect of the resolution of inflammation because cells are phagocytosed whole or as discrete fragments bound by an intact membrane, thereby preventing exposure of adjacent tissues to their intracellular contents.⁶ Eosinophils die by apoptosis when cultured *in vitro*,⁷ being rapidly recognized and engulfed by macrophages that recognize apoptosis-associated changes in their plasma membrane.⁸ Withdrawal of viability-enhancing cytokines^{9,10}; treatment with glucocorticoids,¹¹ transforming growth factor- β ,¹² or IL-4¹³; mAb-dependent receptor ligation of CD69¹⁴; or the death receptor Fas (CD95)^{15,16} initiate eosinophil PCD. The clinical relevance of apoptosis in asthma therapy has been demonstrated in a study demonstrating that the clinical improvement after corticosteroid treatment of asthmatic patients was accompanied by the appearance in their induced sputum of apoptotic eosinophils, some of which had been engulfed by alveolar macrophages.¹⁷ Moreover, resolution of the late-phase reaction in antigen-induced cutaneous skin reactions in atopic subjects was associated with the ingestion of apoptotic eosinophils and neutrophils by macrophages.¹⁸ There is therefore growing interest in the mechanisms responsible for the induction and control of eosinophil apoptosis¹⁹ that might enable the development of more specific anti-inflammatory therapies for asthma.

The leukocyte common antigen CD45 is a member of a family of structurally related, membrane-spanning, glycoprotein tyrosine phosphatases (PTPase) and appears to be the dominant plasma membrane-located phosphatase found on the nucleated cells of the hemopoietic lineage.²⁰ CD45 is encoded by a single gene and is expressed as a protein with differently glycosylated multiple isoforms as a result of alternative splicing of primary messenger RNA transcripts.²¹ These isoforms include CD45RA, CD45RB,

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and CD45RO, which are expressed in characteristic cell type-specific patterns, leading to the hypothesis that differential CD45 isoform expression may influence the spectrum of specific associations with cell surface receptors involved in transmembrane signaling pathways.²² The protein tyrosine phosphatase activity of CD45 plays an important role in intracellular signal transduction in the immune system.^{23,24} Major unresolved issues include the physiologic function of the extracellular domain and the significance of the different CD45 isoforms. Although expression of CD45 and its isoforms by human eosinophils has been established,²⁵ there is a paucity of data on the function of this receptor in eosinophils. In this study we confirm the specific expression patterns of several CD45 isoforms and describe the acceleration of the constitutive rate of apoptosis in human peripheral blood eosinophils after mAb-dependent ligation of pan-CD45, CD45RA, or CD45RB but not CD45RO when cultured with and without GM-CSF.

MATERIAL AND METHODS

Antibodies

Details of the mAbs used in this study are as follows (clone identification in parentheses): CD11b (2LPM19C), CD45RO (UCHL1), IgG1 mouse negative control (Dak-G01), and IgG2a mouse negative control (Dak-G05) were all from Dako, High Wycombe, United Kingdom; CD16 (BL-LGL/1), CD45RB (BRA 11), and CD45 (BRA 55) were supplied by Sigma, Poole, United Kingdom; CD45RA (MEM55), CD45RA (F/8/11/13), CD45 (F/10/89/4), and CD45 were from Serotec, Oxford, United Kingdom; and CD45RA (MB1) and CD45RB (MT4) were from Immuno Quality Products, Gröningen, The Netherlands.

Eosinophil purification

Blood (80 mL) was obtained from healthy donors or individuals with mild allergic disease who were not taking any medication at the time of venesection and who gave informed consent (eosinophilia $\leq 0.5 \times 10^6$ eosinophils/mL). Eosinophils were purified by a slightly modified method that has been described in detail elsewhere.²⁶ Briefly, after dextran sedimentation to remove red blood cells, the leukocytes were centrifuged on isotonic Percoll cushions (1.129 g/mL, Pharmacia) at 400g for 20 minutes at 4°C. The granulocyte pellet was submitted to hypotonic lysis to remove any remaining erythrocytes, washed, and incubated with micromagnetic beads coated with anti-CD16 before passage through the magnetic-activated separation column (Miltenyi, Bisley, UK). With this method, eosinophils with a purity of at least 99% were obtained and 4% or less of the freshly isolated eosinophil preparations displayed positive staining with either annexin V–fluorescein isothiocyanate (FITC) or propidium iodide (PI) performed as described.²⁷

Flow cytometric analysis of eosinophil cell surface antigens

Freshly isolated eosinophils (2×10^5) were immunostained with the relevant antibodies or controls at saturating concentrations for 30 minutes on ice. After a wash and staining with FITC-conjugated antimouse Ig mAb, the cells were analysed on a flow cytometer (FACScan, Becton Dickinson). Flow cytometry was performed on eosinophils gated on the basis of their forward and side scatter profile with any cell debris excluded from analysis. Nonspecific fluorescence was controlled for by incubation with isotype-specific control antibodies.

Eosinophil culture

Freshly purified eosinophils were washed with RPMI 1640 (supplemented with 10% heat inactivated FCS, 100 IU/mL penicillin, 100 mg/mL streptomycin, and L-glutamine) and adjusted to a concentration of 2×10^6 /mL in the same medium. A total of 0.1 mL of cell suspension was incubated for 24 hours at 37°C with 5% carbon dioxide in a humidified atmosphere in 96-well flexible flat-bottomed plates (Becton Dickinson) with experimental antibodies or isotype-matched controls in the presence or absence of GM-CSF (R&D Systems, Oxon, UK) at a final concentration of 10^{-11} mol/L.

Determination of eosinophil apoptosis by flow cytometry

Analysis of eosinophil apoptosis and viability was performed with a combination of annexin V–FITC and PI. We have demonstrated that assessment of human eosinophil apoptosis with this technique is more accurate and sensitive compared with a number of other techniques, including detection of chromatin fragmentation in an electrophoretic gel (DNA laddering) and morphologic assessment.²⁷ Furthermore, annexin V–FITC provides a rapid and reproducible measure of apoptosis in eosinophils, allowing differentiation between early apoptotic cells, late apoptotic cells undergoing secondary necrosis, and necrotic eosinophils. Eosinophils cultured in the presence of experimental mAb and the presence or absence of GM-CSF were washed in cold PBS and resuspended in binding buffer (HEPES-buffered PBS supplemented with 2.5 mmol/L calcium chloride) before the addition of FITC-conjugated annexin V (Bender MedSystems, Loughborough, UK) for 10 minutes at room temperature. Cells were washed and resuspended in binding buffer and PI was added to a final concentration of 1 mg/mL; cells were immediately analyzed on a flow cytometer as described above. All eosinophil apoptosis data presented in this study reflect a combination of early (annexin V⁺ and PI⁻) and late (annexin V⁺ and PI⁺) apoptotic eosinophils.

Statistics

Data were analyzed with either Kruskal-Wallis ANOVA or the Mann-Whitney *U* test as appropriate, with a *P* value of less than .05 considered statistically significant. All data are presented as the mean ($\pm$ SEM).

RESULTS

Ligation of CD45 and the CD45RA and CD45RB isoforms accelerates eosinophil constitutive apoptosis

A representative experiment showing simultaneous measurement of apoptosis and secondary necrosis 20 hours after purified eosinophils were ligated with mAb against CD45 and the isoforms RA, RB, and RO together with appropriate controls is shown in Fig 1. The dot plots have a typical sigmoid shape: as eosinophils ligated with CD45, CD45RA, or CD45RB become apoptotic, they bind annexin V–FITC. Although apoptotic, over time these cells exhibit secondary necrosis as their membrane integrity is lost and they fail to exclude PI. The cells are in the late apoptotic stage, that is, they are both annexin V and PI positive. Fig 2 summarizes the results from 6 separate experiments analyzing the effect on eosinophil apoptosis induction after ligation with pan CD45 mAb and CD45RA, CD45RB, or CD45RO mAb. In general, the pan CD45 and CD45RA and CD45RB

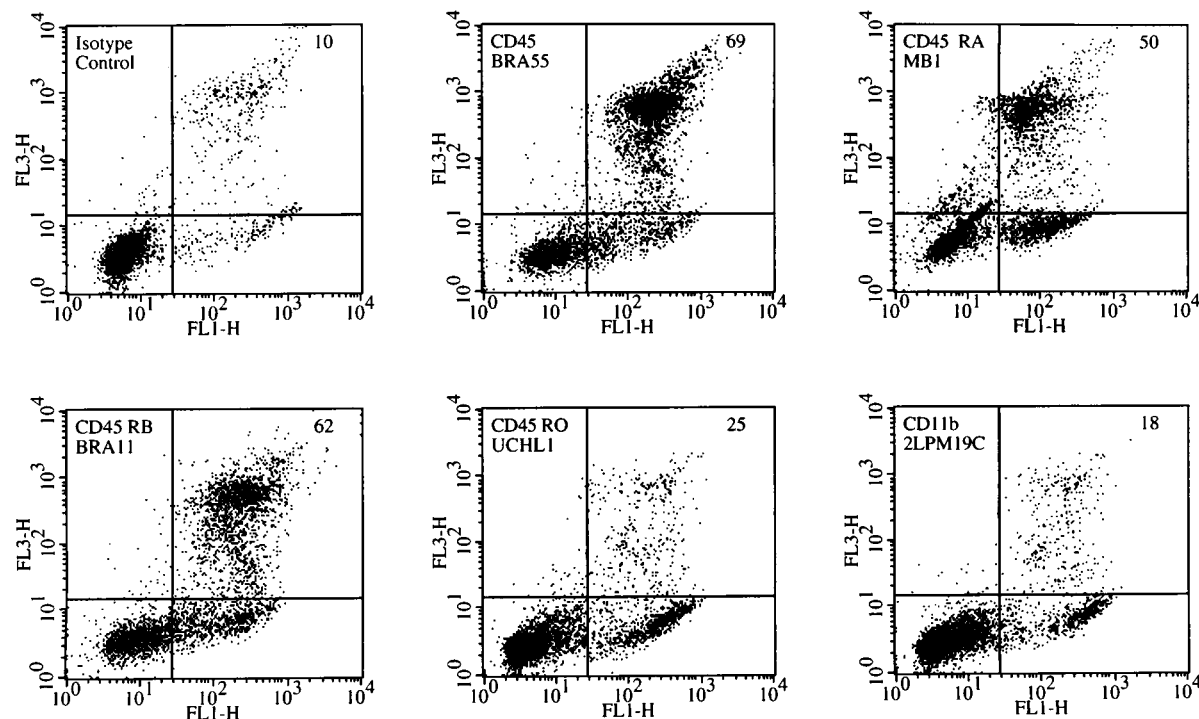


FIG 1. Representative experiment showing flow cytometric analysis of eosinophil binding of annexin V-FITC (FL1-H) and uptake of PI (FL3-H) after incubation for 20 hours with mAb against pan-CD45, CD45RA, CD45RB and CD45RO, CD11b, or an isotype control. Figures in top right hand corner represent total number of early apoptotic eosinophils (annexin V-FITC⁺ and PI⁻) and late apoptotic eosinophils (annexin V-FITC⁺ and PI⁺). Fewer than 4% of freshly isolated eosinophils were annexin V-FITC or PI positive.

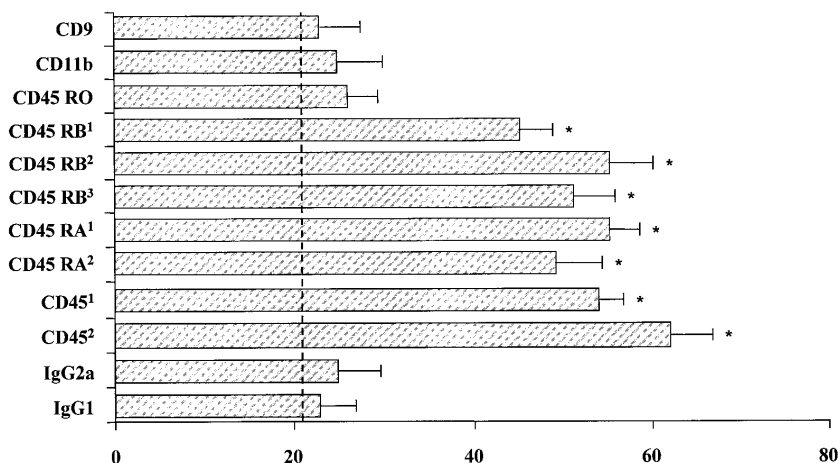


FIG 2. Effect of culture of eosinophils for 20 hours with panel of mAbs against pan CD45 (F-10-89-4¹ and Bra 55²), CD45RA (F-8-11-13¹ and MB1²), CD45RB (MT-4¹, MEM55², and Bra 11³), and CD45RO (UCHL1) on rate of their constitutive apoptosis compared with CD9 and CD11b and isotype controls. Dotted line, Constitutive apoptosis of untreated eosinophils. Each bar represents mean ($\pm$ SEM) of 6 experiments (asterisk, $P < .01$).

mAb more than doubled the rate of constitutive eosinophil apoptosis relative to isotype control, irrespective of the clone used. Significant apoptosis was observed after mAb-dependent ligation with pan CD45 (BRA 55, $P = .01$, and F-10-89-4, $P = .004$), CD45 RA (F-8-11-13, $P = .004$, and MB1, $P = .001$), and CD45 RB (MEM 55, $P = 0.005$; BRA 11, $P = .004$; and MT4, $P =$

.001). There were no significant differences observed in apoptosis induction between the different CD45 clones used in this study. In contrast, levels of apoptosis induction of 20% to 25% were seen with isotype controls or with mAb against CD9 or CD11b, which was comparable to the constitutive rate of apoptosis seen in untreated cultured eosinophils at 20 hours. Interestingly, no signif-

TABLE I. Expression of CD45 and CD45 isoforms by freshly isolated human eosinophils

	Clone	Percent positive ($\pm$ SEM)	Mean fluorescence ($\pm$ SEM)
Nil	—	1.26 (0.12)	6.92 (0.86)
IgG1	Dak-G01	2.09 (0.36)	7.85 (1.00)
IgG2a	Dak-G05	1.91 (0.30)	7.69 (0.88)
CD45	Bra-55	98.88 (0.42)	201.99 (40.65)
CD45	F-10-89-4	99.37 (0.16)	251.53 (30.77)
CD45RA	F-8-11-13	30.18 (10.42)	14.58 (2.24)
CD45RA	MB1	8.80 (4.97)	9.3 (0.93)
CD45RB	MEM55	15.45 (6.76)	10.33 (0.90)
CD45RB	Bra-11	67.27 (6.86)	21.56 (3.21)
CD45RB	MT4	81.47 (7.19)	23.17 (2.67)
CD45RO	UCHL-1	99.22 (0.19)	204.47 (32.35)
CD11b	2LPM19C	99.31 (0.20)	193.2 (21.13)
CD9	P1/33/2	99.13 (0.20)	479.61 (92.78)

icant apoptosis was observed after ligation with the CD45RO isotype mAb despite high and consistent expression of this isoform by eosinophils. No significant differences in the degree of CD45 mAb-dependent apoptosis induction were observed in eosinophils isolated from healthy or atopic donors (data not shown).

The differential expression of CD45 isoforms

We also determined differential eosinophil expression of CD45 compared with the isoforms CD45RA, CD45RB, and CD45RO by flow cytometric analysis of the binding of an extensive panel of specific mAb. Table I shows the mean expression levels of these surface antigens in terms of specific mean fluorescence from 10 independent preparations of freshly isolated eosinophils. Although we observed a degree of variation between the various clones for CD45 and the isoforms CD45RA and CD45RB, in general expression was found to be reproducible with high and comparable levels of CD45 and CD45RO, modest to weak expression of CD45RB, and relatively low levels of CD45RA expression by eosinophils.

Ligation of CD45 and isoforms: dose-response and time course

A series of experiments was subsequently performed to assess the impact on eosinophil apoptosis of both the mAb concentration and duration of CD45 ligation. Fig 3, A, shows the effect of saturating and suboptimal concentrations of representative CD45, CD45RA, and CD45RB mAb compared with the effect of isotype mAb. We demonstrated a statistically significant dose-dependent increase in apoptosis with increasing mAb concentration, which was maximal at the saturating concentration (10–20 μ g/mL) for each mAb. Freshly isolated eosinophils were also cultured for 0, 5, 12, and 24 hours in the presence of CD45 mAb before assessment of apoptosis induction by annexin V–FITC staining. A representative time course experiment is shown in Fig 3, B. After 24 hours of receptor ligation a clear-cut difference was seen between eosinophils incubated with CD45, CD45RA, or CD45RB compared with cells ligated with control or CD45RO mAb. By 48 hours the degree of CD45 mAb-

induced apoptosis approached that of the constitutive rate of eosinophil apoptosis and could not be distinguished at 72 hours (data not shown).

Effect of GM-CSF on CD45-dependent apoptosis induction

The cytokine GM-CSF has been shown to prevent eosinophil apoptosis for up to 2 weeks in culture.^{9,10} Therefore we investigated whether the addition of GM-CSF to eosinophils would prevent the apoptosis-promoting effects of CD45 and CD45 isoform mAb-dependent receptor ligation. Fig 4 shows that addition of GM-CSF at a concentration previously demonstrated to inhibit eosinophil apoptosis (10^{–11} mol/L) only marginally and nonsignificantly diminished CD45, CD45RA, and CD45RB receptor-mediated apoptosis induction. In contrast, significant ($P < .05$) inhibition of eosinophil apoptosis by addition of GM-CSF was observed in the presence of isotype controls, CD45RO, and CD11b. The use of increased concentrations of GM-CSF (10^{–8}, 10^{–9}, and 10^{–10} mol/L) did not inhibit CD45, CD45RA, and CD45RB receptor-mediated acceleration of constitutive eosinophil apoptosis (data not shown).

DISCUSSION

In the current study we have made the novel observation that mAb-dependent ligation of the common leukocyte antigen CD45 and the isoforms CD45RA and CD45RB, but not CD45RO, significantly accelerated the rate of constitutive apoptosis of human eosinophils irrespective of which mAb clone was used. Furthermore, we also fully characterized eosinophil expression of CD45RA, CD45RB, and CD45RO. CD45RO was the most predominant isoform of CD45 expressed by eosinophils followed by modest expression of CD45RB and very low but consistent levels of CD45RA, data that in most respects are in agreement with Matsumoto et al.²⁵ However, these workers found no eosinophil expression of CD45RA. The reasons for this discrepancy are not clear but may be related to differences in sources of CD45RA mAb because, in the current study, both CD45RA mAbs revealed low but consistent expres-

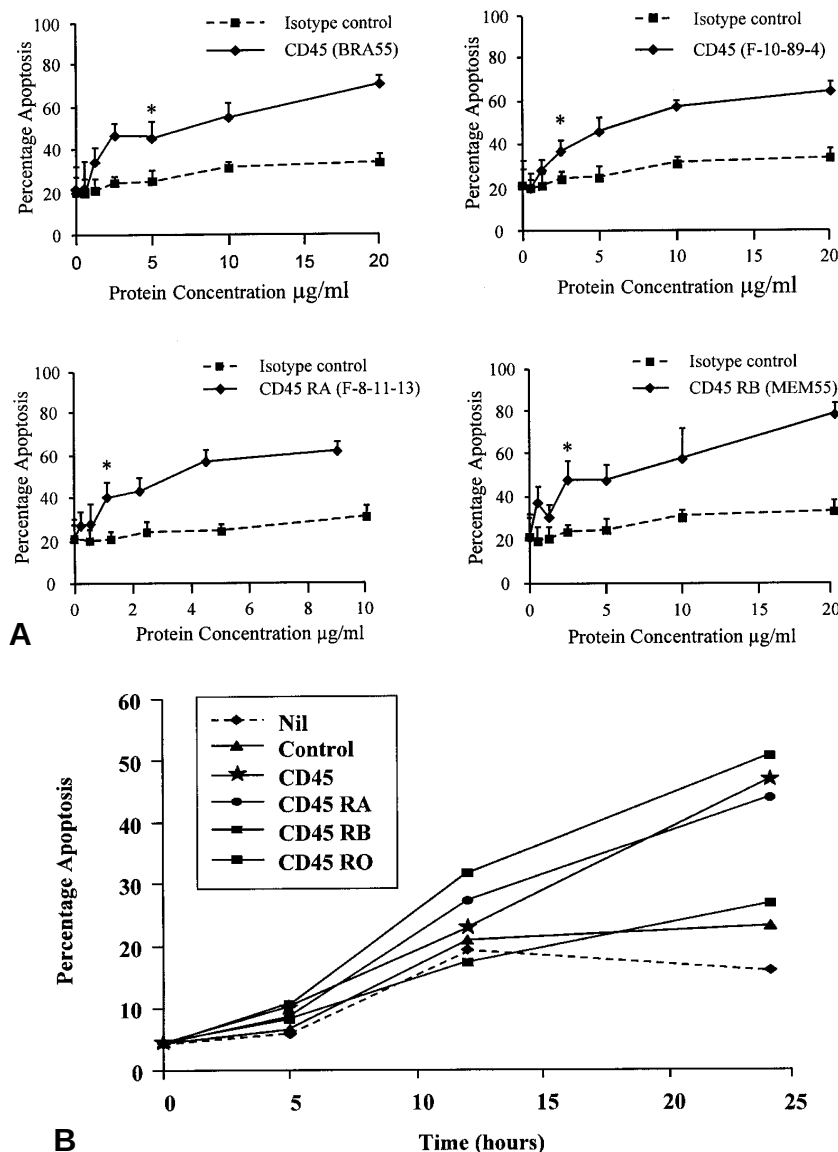


FIG 3. A, Dose-response of effect of increasing concentrations of representative pan CD45, CD45RA, CD45RB, and CD45RO mAb on constitutive rate of eosinophil apoptosis compared with isotype control after 20 hours of incubation. Asterisk, Significant effect ($P < .05$) of relevant mAb at that point and at all higher concentrations. Each point represents mean ($\pm$ SEM) of 5 experiments. **B**, Time course of effect of mAb-dependent ligation of CD45, CD45RA, CD45RB, and CD45RO on constitutive rate of eosinophil apoptosis compared with isotype control and untreated eosinophils. This is representative example of 5 independent experiments that gave near identical results.

sion of this receptor. In general, there are few data available on the function of eosinophil CD45. Ligation of eosinophil CD45, CD45RO, or CD45RB with specific mAb for 24 hours enhanced expression of CD11b.²⁵ Another study demonstrated enhanced respiratory burst activity in eosinophils stimulated with TNF- α or GM-CSF after mAb-dependent cross-linking of CD45.²⁸ The latter study used very different conditions from the current study: after incubation of eosinophils with CD45, cross-linking of the receptor was a requirement for any measurable change in respiratory burst activity. Taken together with our findings, these data suggest that, in

keeping with many receptors, CD45 has more than one function that, depending on the activation status of the eosinophil, can regulate mediator release or induce apoptosis. This is analogous to eosinophil interaction with the proteins of the extracellular matrix; depending on the experimental conditions, this can either modulate or enhance eosinophil degranulation or induce prolonged survival.²⁹

Apoptosis induction in eosinophils ligated with CD45 mAb appeared to be a specific effect because addition of isotype-specific controls and anti-CD9 or anti-CD11b mAb had no significant effect at any time point despite

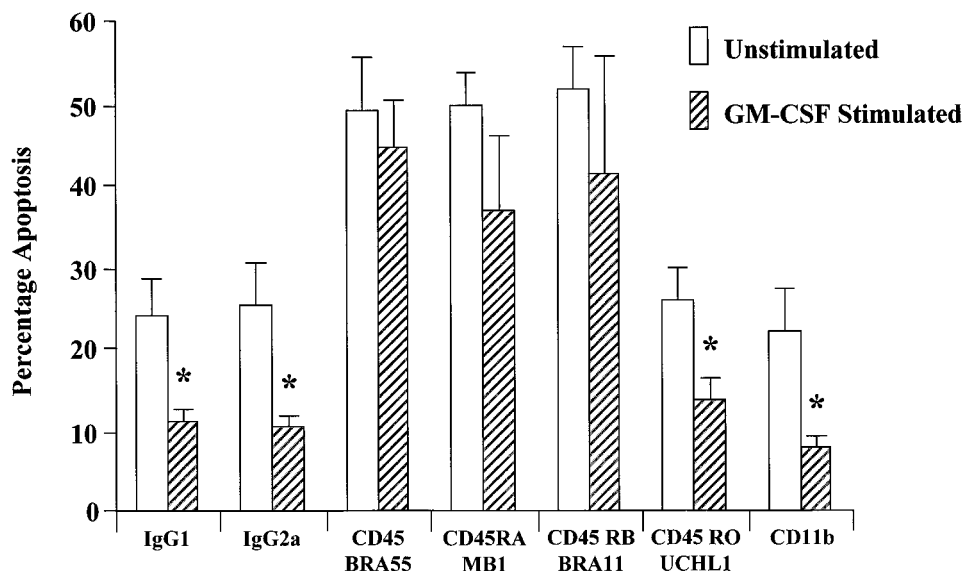


FIG 4. Effect of presence or absence of GM-CSF (10^{-11} mol/L) on apoptosis induction in eosinophils ligated with representative CD45RA, CD45RB, and CD45RO mAbs compared with mAb to CD11b and isotype controls and cultured for 20 hours. Asterisk, Significant ($P < .05$) inhibition of apoptosis by GM-CSF was seen with isotype controls, CD45RO, and CD11b. Each bar represents mean ($\pm$ SEM) of 6 experiments.

high and consistent expression of the latter 2 receptors. The negative findings with isotype controls and CD9- and CD11b-specific mAbs also rules out any apoptosis induction by the very low levels of azide added as a preservative to the commercial mAb used in these studies. CD45 ligation has been shown to induce the apoptosis of lymphocytes,³⁰ CD4⁺ and CD8⁺ thymocytes,³¹ and T and B lymphocytes.³² This is the first description of accelerated eosinophil apoptosis after mAb-dependent ligation of pan-CD45, and we further demonstrate a potent apoptosis-inducing effect by the CD45 isoforms CD45RA and CD45RB. Interestingly, ligation of CD45RO antibody caused little or no apoptosis despite being highly expressed by eosinophils, whereas, in contrast, ligation of the weakly expressed CD45RA was a potent inducer of eosinophil apoptosis. This was an unexpected observation because mAb-dependent ligation of CD45RO would be expected to activate the same PTPase pathways that follow mAb-dependent CD45 ligation in other leukocytes. To our knowledge, the only clone currently available for CD45RO is that used in this study (UCHL-1) and its failure to induce eosinophil apoptosis might be a consequence of its inability to initiate full activity of its ligand after binding. Thus the possibility cannot be excluded that another CD45RO mAb might have induced eosinophil apoptosis if it were available. Evidence for this notion is provided by the observation that specific ligation of CD45RO with its physiologic ligand, the lectin galectin-1, mediated apoptosis in activated T cells.³³ The ability of galectin-1 to induce apoptosis in eosinophils is currently under investigation.

Our data demonstrate that the presence of GM-CSF was unable to significantly inhibit the onset or magnitude of apoptosis when the cells were ligated with mAb to

pan-CD45 or any of the isoforms of CD45. These findings are in agreement with those of a previous study¹⁵ that demonstrated that IL-5 did not prevent mAb-dependent Fas-mediated apoptosis in eosinophils. These observations suggest that mAb-dependent ligation of both Fas and CD45 or CD45 isoforms initiates apoptosis-signaling pathways independent of IL-5 or GM-CSF. The eosinophil viability-promoting effects of GM-CSF are associated with tyrosine phosphorylation of proteins of 150, 74, and 40 kd. It has also been reported that inhibition of PTPase activity increases tyrosine phosphorylation in human eosinophils.³⁴ Therefore it is feasible to assume that activation of CD45 through mAb ligation is sufficient to stimulate PTPase activity, thereby attenuating the action of GM-CSF. An alternative explanation is that, although the inhibition of eosinophil apoptosis by cytokines such as GM-CSF appears to be regulated by early events in signal transduction, ligation of CD45 might be upstream of these events, resulting in an effect on tyrosine phosphorylation that leads to the signals responsible for apoptosis. Other intracellular proteins might be responsible for the development of CD45- and CD45 isoform-dependent eosinophil apoptosis. For example, the IL-1 β converting enzyme like proteases or caspases have been implicated as universal apoptosis effectors associated with the downstream events of programmed cell death.³⁵ Other factors important in the control of apoptosis in many cellular systems include the Bcl-2 family of proteins.³⁶ Bcl-2 and Bcl-x_L inhibit cell death, whereas other members such as Bax and Bcl-x_s promote apoptosis. There are several reports demonstrating constitutive expression of Bcl-2^{37,38} or Bax and Bcl-x³⁹ by human eosinophils, whereas a decrease in Bcl-x_L messenger RNA and protein levels was found to be asso-

ciated with eosinophil apoptosis.⁴⁰ The relative contribution of those intracellular proteins important in eosinophil apoptosis induction after mAb-dependent CD45 ligation is currently under investigation.

In conclusion, our results demonstrate that mAb-dependent ligation of the pan-leukocyte receptor CD45 and the isoforms CD45RA and CD45RB markedly accelerates the constitutive rate of apoptosis in human eosinophils. These findings contribute to our understanding of the mechanisms responsible for induction of apoptosis in human eosinophils, which may aid the development of more targeted anti-inflammatory therapy for asthma.

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