

# Leukocyte Common Antigen (CD45) Is Required for Immunoglobulin E-mediated Degranulation of Mast Cells

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## Summary

We demonstrate using primary mast cell cultures derived from wild-type and CD45-deficient mice that mast cell triggering through the high-affinity immunoglobulin E (IgE) receptor requires the cell surface tyrosine phosphatase CD45. Unlike wild-type cells, cross-linking of surface-bound IgE in mast cells deficient in CD45 does not induce degranulation. Degranulation in these mutant cells does occur after treatment with the calcium ionophore A23187 indicating that the degranulation machinery is intact in these cells. We also demonstrate that the tyrosine phosphatase inhibitors orthovanadate and pervanadate inhibit degranulation in wild-type mast cells, as does cross-linking of CD45 by anti-CD45 antibodies. Finally, we show that CD45-deficient mice are resistant to IgE-dependent systemic anaphylaxis. These results show that, like the T cell receptor and the antigen receptor on B cells, there is an absolute requirement for CD45 in signaling via the high affinity IgE receptor, expanding the number of receptors for which CD45 is an essential component.

Allergy is the most common human immune disorder, affecting as much as 20% of the Western population. The acute allergic response is mediated by mast cells and basophils. These cells express high affinity receptors for the IgE class of antibodies. Cross-linking of surface-bound IgE by Ag leads to activation of the cells and the release of granules containing inflammatory mediators such as histamine (1). FcεRI, the high affinity IgE receptor, is a four-chain receptor complex and a member of the multichain immune recognition receptor family (2). After cross-linking of this receptor complex, some of the earliest detectable events include tyrosine phosphorylation and activation of cytoplasmic tyrosine kinases (3–5), calcium flux (6, 7), and phosphatidyl inositol breakdown (8, 9). These events are shared in common with other members of the multichain immune recognition receptor family including the TCR and the Ag receptor on B cells.

Recent experiments have shown that the leukocyte common antigen (CD45), a cell surface protein with tyrosine phosphatase activity expressed on all nucleated hemopoietic cells, is an essential component of signaling through both the TCR (10–13) and Ag receptor (14). The similarities in some signaling properties amongst these different receptor systems prompted us to examine the possible role of CD45 in FcεRI-mediated signaling.

## Materials and Methods

**Cells and Cell Culture.** Supernatants from WEHI-3 cells were used as a source of IL-3. Recombinant murine steel factor (SLF)<sup>1</sup> was prepared using bacterial expression and purified by affinity purification (Berger, S. A., unpublished observation). Supernatants from cells expressing the appropriate vector (obtained from Dr. F. Melchers, Basel Institute for Immunology, Basel, Switzerland) were used as a source of murine IL-4. Bone marrow from CD45-exon 6  $-/-$  or  $+/+$  littermates were obtained from 6–8-wk-old mice and incubated in OPTI-MEM (GIBCO BRL, Gaithersburg, MD) plus 5% FCS, antibiotics, and 2-mercaptoethanol. Cells were split and refed every 5 d with fresh medium.

**Antibodies.** Anti-CD45 antibodies M1.89 (isotype IgG2b) and M1/9.3 were obtained from Dr. M. Julius (Wellesley Hospital Research Institute, Toronto, Canada) and conjugated with fluorescein or biotin. Antibody M5 (anti-MHC class II) was used as an isotype control for flow cytometry. Anti-DNP monoclonal IgE antibody SPE-7 was purchased from Sigma Chemical Co. (St. Louis, MO) and biotinylated. Streptavidin-PE was used to visualize biotinylated antibody.

**Degranulation Assay.** Cells were washed and incubated in Tyrode's buffer (10 mM HEPES buffer [pH 7.4], 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5.6 mM glucose, 0.1% BSA)

<sup>1</sup> Abbreviations used in this paper: HSA, human serum albumin; SLF, steel factor.

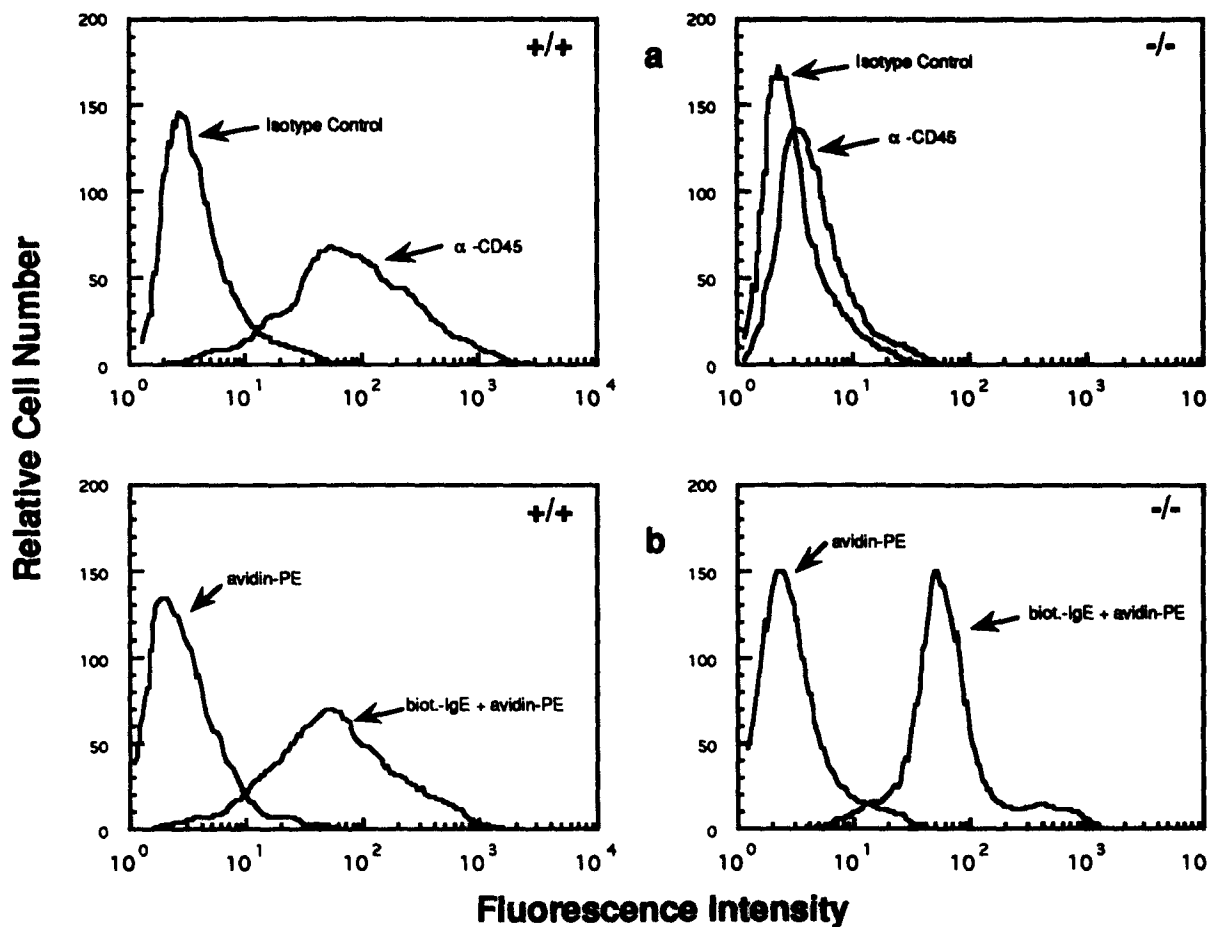
containing 10  $\mu\text{g/ml}$  anti-DNP IgE monoclonal antibody SPE-7 (Sigma Chemical Co.) for 1 h on ice. After removal of excess antibody, cells were exposed to varying amounts of the antigen DNP human serum albumin (HSA) (Sigma Chemical Co.) for 1 h at 37°C. Stock solutions of the calcium ionophore A23187 (Sigma Chemical Co.) were made up in DMSO and diluted in Tyrode's buffer. Cells were incubated in A23187 for 1 h at 37°C. Degree of degranulation was determined by measuring the release of  $\beta$ -hexosaminidase (15). Supernatant dilutions and cell pellets solubilized with Tyrode's + 0.5% Triton X-100 were incubated with the substrate *p*-Nitrophenyl-*N*-acetyl- $\beta$ -D-glucosamine (Sigma Chemical Co.) in 0.1 M NaCitrate (pH 4.5) for 40 min at 37°C. The reaction was terminated by the addition of 0.2 M glycine (pH 10.7). The release of the product 4-*p*-nitrophenol was followed by absorbance at 405 nm.

Percent degranulation = supernatant activity/(supernatant + pellet activities). Each determination is the average of three measurements.

**Preparation of Vanadate and PerVanadate.** Vanadate was prepared by dissolving sodium orthovanadate (Sigma Chemical Co.) to a final concentration of 10 mM in 0.2 M Hepes (pH 6). No color change was observed indicating the lack of significant decaVana-

date or Vanadyl ion in the preparation (16). PerVanadate was prepared by adding an equal concentration of hydrogen peroxide to the Vanadate solution. The solutions were used within 1 h of preparation.

**Systemic Anaphylaxis.** To observe systemic anaphylaxis, the assay described by Ando et al. (17) was used. Briefly, mice primed with a monoclonal IgE antibody are challenged 24 h later with Ag. Evan's blue dye is included in the challenge to visualize the fluid extravasation that accompanies the anaphylactic shock. This occurs within 15–20 min and is manifested by a general bluing of the extremities, particularly the front and hind feet, caused by leakage of the dye into the tissue. A titration of antibody from 20 to 0.01  $\mu\text{g}$  was done in  $+/+$  and  $-/-$  mice to optimize the discriminatory capacity of this assay. 0.1  $\mu\text{g}$  of SPE-7 was found to be an effective concentration, with dye extravasation occurring within 10–15 min. 8–12-wk-old CD45 $^{-/-}$ ,  $+/-$ , and  $+/+$  littermates (fourth backcross onto C57BL6 background) were injected intravenously with 0.2 ml PBS and 0.1% BSA containing 0.1  $\mu\text{g}$  IgE anti-DNP antibody SPE-7. 20–24 h later, these mice were injected intravenously with 0.2 ml PBS containing 0.5% Evan's blue dye with or without 1 mg DNP-HSA. After 20 min, the mice were killed and photographed.



**Figure 1.** Flow cytometric analysis of CD45 and IgE Receptor surface expression. (a)  $+/+$  or  $-/-$  mast cell cultures were stained FITC-labeled anti-CD45 antibody M1.89 (31) or with a similarly labeled isotype-matched control. Cell staining was analyzed with a FACScan<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA). (b)  $+/+$  or  $-/-$  mast cell cultures were incubated with biotinylated IgE monoclonal antibody SPE-7, followed by incubation with PE-coupled streptavidin. Control staining was with streptavidin-PE alone.

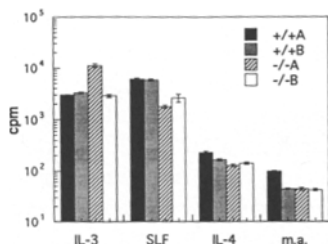
## Results and Discussion

Primary mast cell cultures were grown from bone marrow of  $+/+$  and  $CD45\text{-}exon6^{-/-}$  (hereafter referred to as  $-/-$ ) mice in the presence of IL-3. By 6 wk, >99% of the cells in the cultures were mast cells, as determined by staining with alcian blue or binding of IgE. We confirmed that CD45 was expressed on wild-type, but not  $-/-$  mast cells by flow cytometry. Fig. 1 *a* shows that  $-/-$  cells showed no significant staining with the anti-CD45 antibody M1.89, whereas  $+/+$  mast cells stained strongly. No significant differences were found in the kinetics of mast cell outgrowth or differentiation in the presence of IL-3 between  $+/+$  and  $-/-$  cells. We also found that the proliferative response of  $-/-$  mast cells to IL-3, IL-4, or SLF was similar to the  $+/+$  cultures (Fig. 2). Taken together, we conclude that the lack of CD45 does not prevent the emergence and differentiation of bone marrow-derived mast cells and that these cells appear to be normal in many respects.

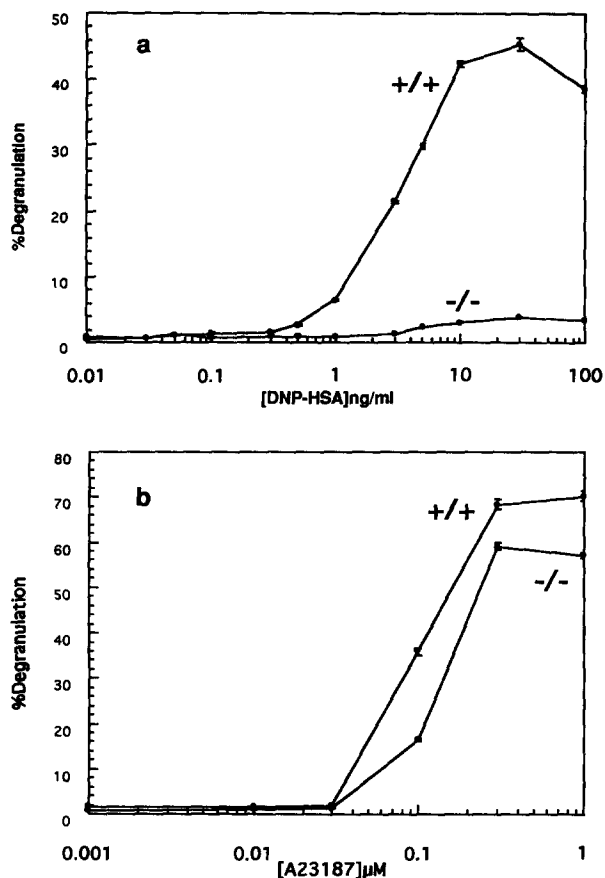
We tested for the ability to degranulate by first priming the cells with an IgE monoclonal anti-DNP, followed by incubation with DNP-HSA to induce receptor cross-linking. The degree of mast cell degranulation was determined by measuring the release of the granule enzyme  $\beta$ -hexosaminidase. As shown in Fig. 3 *a*,  $+/+$  mast cells were fully capable of degranulation in response to surface IgE cross-linking. In contrast, mast cells derived from  $-/-$  mice were incapable of significant degranulation.

We confirmed that this was not due to an absence of, or decrease in IgE receptor. Mast cells from  $+/+$  or  $-/-$  cultures were stained with biotinylated IgE and the cell surface expression was analyzed by flow cytometry. As shown in Fig. 1 *b* both  $-/-$  and  $+/+$  mast cell cultures stain strongly for IgE with a similar mean fluorescence intensity. The broader distribution of staining in the  $+/+$  cultures suggests somewhat greater heterogeneity of IgE receptor expression. However other cultures initiated at different times do not show such a broad distribution.

Calcium flux is an early event after Fc $\epsilon$ RI cross-linking (6, 7) and calcium ionophores are well known to be able to bypass the requirement for IgE receptor cross-linking in degranulation (18, 19). We used this property to confirm that



**Figure 2.**  $+/+$  and  $-/-$  mast cells respond equally to IL-3, IL-4, and SLF. Two separate cultures of  $+/+$  or  $-/-$  mast cells were established. At the time of the experiment, culture A was 9 wk old and culture B was 5 wk old. Cells were grown for 7 d in either a 1:50 dilution of medium from cells producing IL-3 or IL-4, or 100 ng/ml of SLF. Cells were pulsed with [<sup>3</sup>H]thymidine and incorporated counts were determined using scintillation counting. m.a. refers to cells cultured in medium alone.

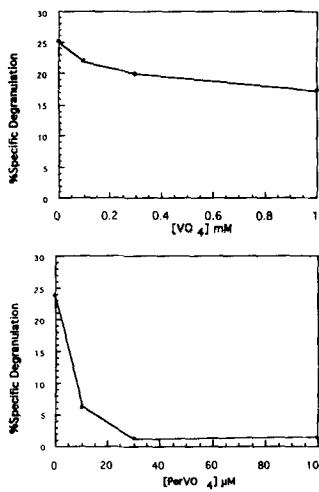


**Figure 3.** Degranulation in response to cross-linking antigen or calcium ionophore. (a) Degranulation by  $+/+$  or  $-/-$  mast cells after exposure to cross-linking Ag. (b) Degranulation by  $+/+$  or  $-/-$  mast cells after exposure to the calcium ionophore A23187.

$-/-$  mast cells are capable of degranulation. As shown in Fig. 3 *b*, both  $+/+$  and  $-/-$  mast cells are capable of a similar degree of degranulation after incubation with the calcium ionophore A23187. This indicates that the degranulation machinery is intact in  $-/-$  cells and that the failure of  $-/-$  mast cells to degranulate in response to receptor cross-linking reflects the requirement of CD45 in Fc $\epsilon$ RI receptor signaling.

CD45 is highly expressed on hemopoietic cells and can represent upwards of 10% total cell surface protein (20). It is possible that the absence of CD45 expression throughout development results in secondary alterations in the  $-/-$  mast cell population which are responsible for the inability of  $-/-$  cells to degranulate in response to Fc $\epsilon$ RI receptor cross-linking. One prediction of this hypothesis is that short-term inhibitory treatments directed against CD45 in normal cells would have no effect on degranulation. To address this question, we treated  $+/+$  cells with both nonspecific and specific agents active against the CD45 tyrosine phosphatase.

In one series of experiments, we treated the cells with the tyrosine phosphatase inhibitors orthoVanadate or perVanadate. As shown in Fig. 4, both agents are capable of significant inhibition of specific degranulation. As reported in other



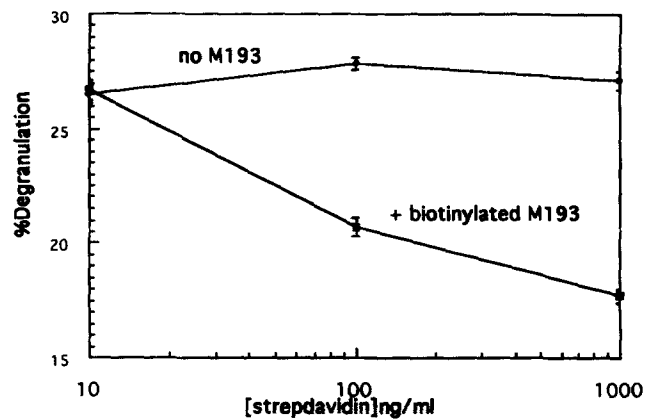
**Figure 4.** Effect of Vanadate or perVanadate on specific degranulation by +/+ mast cells. Cells were primed with anti-DNP antibody and then incubated for 1 h at 37°C with either Vanadate or perVanadate. 10 ng/ml of DNP-HSA was added to induce degranulation. After a further 1-h incubation at 37°C, degranulation was measured by the release of  $\beta$ -hexosaminidase. Specific degranulation was calculated by subtracting background degranulation (in the absence of added DNP-HSA) from degranulation in the presence of antigen.

systems (21, 22), perVanadate is much more potent than orthoVanadate, both in maximal amount of inhibition and concentration required.

Both orthoVanadate and perVanadate are relatively nonspecific phosphatase inhibitors. We therefore also tested for the ability of specific anti-CD45 antibodies to inhibit degranulation. Although the anti-CD45 antibody M1.93 failed to inhibit degranulation when incubated with mast cells (not shown), significant inhibition of degranulation could be achieved when cells were incubated with biotinylated M1.93 followed by aggregation with streptavidin (Fig. 5). Our interpretation of these results is that cross-linking of CD45 causes aggregation that inactivates the Fc $\epsilon$ R1-CD45 complex whereas incubation with the antibody alone does not disrupt this complex. This result is similar to those obtained from CD45 cross-linking studies on T cells (23).

Since mast cells are the primary mediators of the acute allergic response *in vivo*, our *in vitro* results would predict that CD45-deficient mice are resistant to IgE-mediated anaphylaxis. To test this hypothesis, CD45<sup>-/-</sup>, CD45<sup>+/-</sup>, and CD45<sup>+/+</sup> mice were injected with anti-DNP IgE antibody, followed by challenge with antigen 20–24 h later. Evan's blue dye was included in the antigenic challenge to visualize the fluid extravasation that occurs during anaphylaxis. As shown in Fig. 6, 20 min after the antigenic challenge, CD45<sup>+/+</sup> and CD45<sup>+/-</sup> mice showed a marked blue coloring in the feet (and other extremities), whereas CD45<sup>-/-</sup> mice showed no dye extravasation above background levels. These results indicate that CD45<sup>-/-</sup> mice are resistant to IgE-mediated anaphylaxis.

In T cells, the primary role of CD45 is the dephosphorylation of the tyrosine kinases p56<sup>lck</sup> (24, 25) and p59<sup>lyn</sup> (26). This activates these kinases, leading to increased tyrosine phosphorylation of substrates. Similarly, in B cells, CD45 may be responsible for activating src-like tyrosine kinases as well (14, 27). Studies have shown that the tyrosine kinases lyn, yes, and ptk72 are activated in mast cell or basophil cell lines (3–5). It remains to be determined if CD45 is responsible for activating these kinases in primary mast cell cultures.

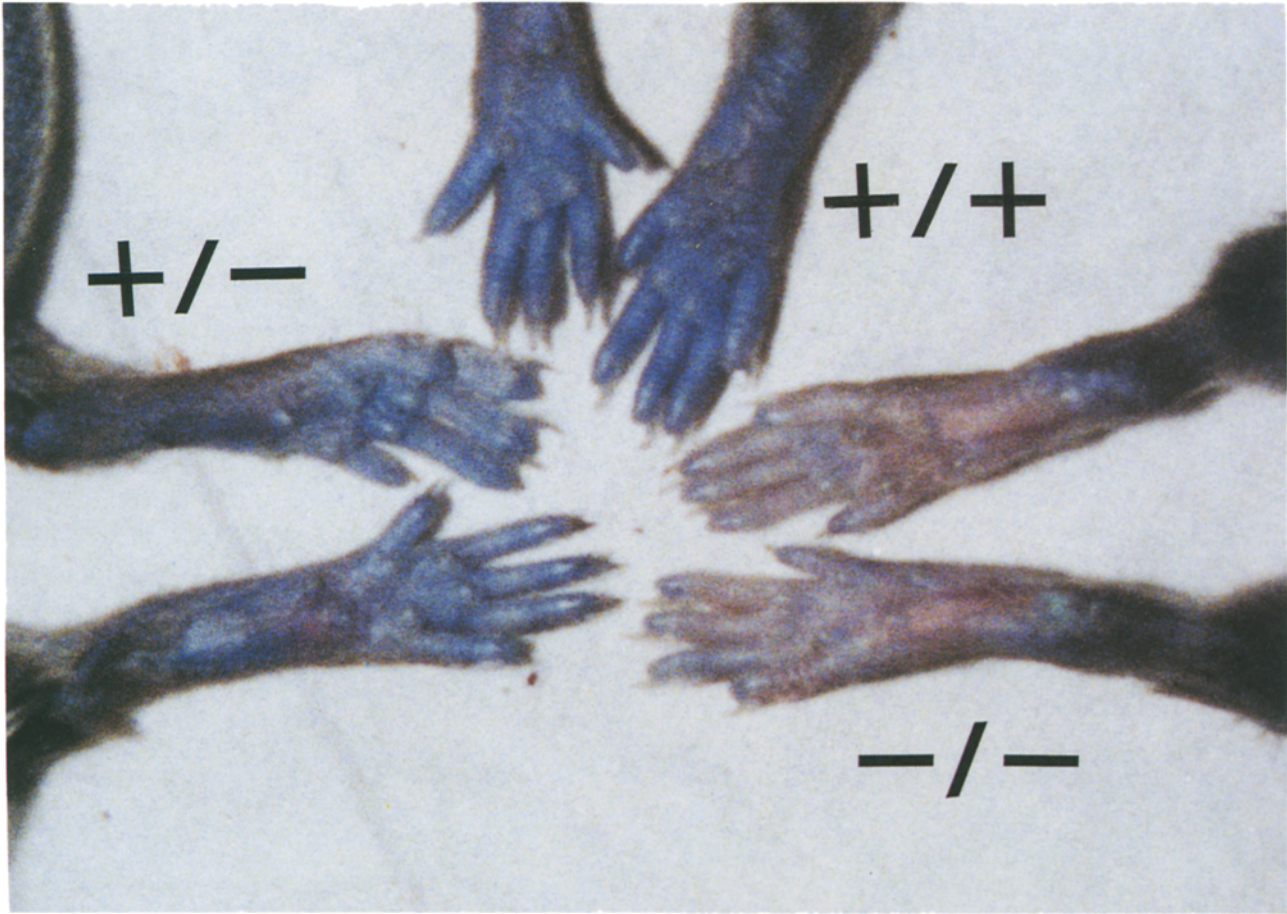


**Figure 5.** Anti-CD45 antibody M1.93 inhibits degranulation when cross-linked by streptavidin. +/+ cells were incubated with IgE antibody and biotinylated anti-CD45 antibody M1.93 on ice for 1 h. Excess antibody was washed off and cells were then incubated with streptavidin for 1 h at 37°C. 10 ng/ml of DNP-HSA was added to induce degranulation and the incubation was continued for another hour at 37°C. Percent degranulation was measured by the release of  $\beta$ -hexosaminidase. Using the Student's *t* test distribution, the probability that the percent degranulation is not different in the presence of cross-linking agent (*p* value) is 0.0003 and 0.0007 for 100 ng/ml and 1,000 ng/ml, respectively.

Mast cells from CD45<sup>-/-</sup> mice are fully capable of responding to signals from the growth factor SLF and the cytokines IL-3 and IL-4. In addition, a previous study (13) found that colony formation by bone marrow-derived hemopoietic cells was normal in response to IL-3. In contrast, Broxmeyer et al. (28) reported that antisense oligodeoxynucleotides to CD45, or cross-linking anti-CD45 antibodies could inhibit colony formation by human bone marrow cells in response to GM-CSF, IL-3, and SLF. These results may reflect different requirements or involvement of CD45 in human bone marrow progenitors.

Hook et al. (29) found that extensive incubation of human basophils with anti-CD45 antibodies was required for inhibition of degranulation. We find that incubation of mast cells for a short time with antibody alone is ineffective in inhibiting degranulation, but that significant inhibition can be obtained if the cell surface CD45 is cross-linked. It is possible that the longer incubation times used by Hook et al. (29) are required for significant inhibition. Alternatively, inhibition by anti-CD45 antibodies may be epitope specific. We are currently testing other known anti-CD45 antibodies to determine their ability to inhibit mast cell degranulation.

It is generally accepted that mast cells and basophils are the primary mediators of the acute allergic response. Recently Dombrowicz et al. (30), using mice genetically deleted for the  $\alpha$ -chain of the Fc $\epsilon$ R1 receptor, were able to show that these mice were resistant to anaphylaxis. These results confirm that it is signaling through the high affinity IgE receptor that is required for the acute allergic response. Our observation that CD45-deficient mice are also resistant to anaphylaxis provides additional evidence for the involvement of CD45



**Figure 6.** CD45-deficient mice are resistant to IgE-mediated systemic anaphylaxis. +/+, +/-, and -/- mice were injected intravenously with 0.2 ml PBS containing 0.1% BSA and 0.1  $\mu$ g IgE anti-DNP monoclonal SPE-7. 20 h later, these mice were injected intravenously with 0.2 ml PBS containing 1 mg DNP-HSA and 0.5% Evan's blue dye. 20 min after the Ag challenge, the mice were killed and photographed. The photograph shows the hind feet of +/+, +/-, and -/- animals. Control mice injected with Evan's blue dye only showed no dye extravasation.

in signaling through the Fc $\epsilon$ R1 pathway and confirms the importance of this signaling pathway in the anaphylactic response.

The IgE receptor system is the third example of a multichain immune recognition receptor requiring CD45 for ac-

tivation. Other members of this family include the high and low affinity IgG receptors, which display significant homology to the Fc $\epsilon$ RI receptor. It is therefore possible that all members of this family require CD45 as an essential component of the signaling complex.

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