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Sensitization of circulating basophils in guinea pig recipients of passive transfer of cutaneous basophil hypersensitivity (CBH) with immune serum: Antigen-specific histamine release in vitro

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An in vitro histamine release assay was used to test the hypothesis that passive sensitization of circulating basophils is associated with the activity of immune serum that transfers the ability to elicit cutaneous basophil hypersensitivity (CBH) reactions. Systemic i.v. transfer of several types of immune sera that mediate CBH also led to passive sensitization of circulating basophils for antigen-specific release of histamine in vitro. In addition, we found that immune serum passively sensitizes basophils in vitro. Thus immune sera had three activities that are probably interconnected: sera will 1) passively transfer CBH in vivo, 2) passively sensitize basophils in vivo, and 3) passively sensitize basophils in vitro. These results suggest that passive sensitization of circulating basophils by immune serum contributes to the mechanism by which antibodies transfer the ability to elicit CBH reactions.

The elicitation of cutaneous basophil hypersensitivity (CBH) reactions in guinea pigs has been shown to be dependent on thymic derived T lymphocytes and/or antibody-dependent mechanisms (1-4). We have previously shown that early after immunization the basophils in the peripheral circulation are not sensitized to the immunogen, as determined by an in vitro histamine release assay (5). However, by day 9 post-immunization, sensitized basophils are present in the circulation (5). These results suggest that the elicitation of early post-sensitization CBH is mediated by T lymphocytes alone, but that at later times, homocytotropic antibodies may also play a role. In addition, we have shown that by day 8 or 9, basophils that are arriving at CBH sites are sensitized to the immunizing antigen, which suggests that infiltrating basophils have a functional role at these delayed CBH reactions (6).

A number of different immunization procedures induce a serum activity that transfers delayed time course skin reactions of erythema and skin thickening and histologically shows an infiltrate composed of basophils, eosinophils, and mononuclear cells (2-4, 7). We showed previously that these serum-dependent CBH reactions are mediated by anaphylactic IgG1 (4) or IgE antibodies (8), and that host Fc receptors, such as those on cutaneous mast cells, are involved in these reactions (9, 10). Passive cutaneous anaphylaxis (PCA) reactions are also mediated by IgG1 or IgE antibodies and can be elicited after local passive transfer. In contrast, IgG1- or IgE antibody-mediated CBH reactions cannot be elicited after local passive transfer (9). They require systemic transfer. On the basis of these findings we suggest that although PCA reactions only require passive sensitization of cutaneous mast cells via Fc receptors, antibody-mediated CBH are more complex reactions and require passive sensitization that includes both skin mast cells and peripheral blood basophils.

In this report we show that the systemic transfer of immune sera not only leads to the ability to elicit CBH reactions in vivo, but also specifically sensitizes circulating basophils as determined by an in vitro histamine release assay. Transfer of antigen-specific antibody that mediates CBH also induces in vivo hapten-specific basophil sensitization. In addition, immune serum that transfers CBH in vivo and passively sensitizes basophils in vivo for in vitro release will also passively sensitize basophils in vitro. These results suggest that passive sensitization of circulating basophils by immune serum contributes to the mechanism by which antibodies transfer the ability to elicit CBH reactions.

Materials and Methods

Animals. In vivo passive sensitization was performed with outbred female Hartley albino guinea pigs that were obtained from Charles River Laboratories, Wilmington, MA, and were maintained by the division of Animal Care, Yale University School of Medicine. Female Hartley guinea pigs weighing from 200 to 300 g were used throughout the study. The in vitro passive sensitization data (see Figs. 3 and 4) were obtained by using female Hartley guinea pigs obtained from Bio Lab, St. Paul, MN.

Antigens and reagents. Oxazolone (Ox; 4-ethoxymethylene-2-phenoxy oxazolone), keyhole limpet hemocyanin (KLH), human serum albumin (HSA), and Ox coupled to KLH (Ox1100 KLH) and to HSA (Ox33 HSA) were obtained and prepared as described (2-4, 11). Picryl (Pic) ascars antigen (Pic-Asc) was prepared as described (12). Incomplete Freund's adjuvant (IFA) was obtained from Difco Laboratories, Detroit, MI. Phosphate-buffered saline (PBS) was 10 mM potassium phosphate-buffered 0.15 M NaCl, pH 7.4.

Serum donors. Groups of three to six guinea pigs were immunized by four different protocols to produce serum that transfers the ability to elicit CBH reactions: 1) Two hundred micrograms of KLH in 0.1 ml PBS intradermally (7); 4) After intradermal skin testing with 200 µg KLH ox day 7, animals were bled on day 9. 2) One hundred micrograms of Ox-KLH or KLH emulsified in IFA. This was distributed subcutaneously among the four footpads in 0.1-ml volumes (3, 4). Animals were skin tested on day 7 by ear painting with 4% Ox in olive oil and were bled 24 or 48 hr later. 3) Ox in IFA. Ox was dissolved in heated IFA that was then emulsified with plain PBS. Then 100 µg Ox was distributed subcutaneously among the four footpads in 0.1-ml volumes (3, 4). Cutaneous challenge and subsequent bleeding were as in protocol 2 above. 4) Pic-Asc in alum: Guinea pigs received monthly ip injections of 1 µg Pic-Asc conjugate in alum (12). The serum had a PCA titer of 1:1600 when a 4-hr sensitization period was used, and a PCA titer of 1:50 when a 7-day sensitization period was used. These findings indicated a preponderance of IgG1 vs IgE anaphylactic antibody.

Serum transfers. After immunization and skin testing, serum donors were anesthetized with ether and exsanguinated by severing the vessels of the neck or via cardiac puncture by using 20-gauge, 1.5-in. needles and plastic...
Buffers. HEPES-buffered saline contained 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 137 mM NaCl and 5 mM KCl and was adjusted with NaOH. HG consisted of HEPES-buffered saline plus 0.5 mg/ml gelatin and 1 mg/ml dextrose. HGCM was HG containing 2 mM CaCl₂ and 1 mM MgCl₂.

In vitro release of histamine from blood basophils. This assay has been described previously (13). Blood was harvested by using plastic syringes containing sufficient ethylenediaminetetraacetic acid (EDTA) to provide a final concentration of at least 10 mM. Ten milliliters of EDTA-containing blood were added to 5 ml of 3% gelatin in 0.9% NaCl at 37°C. After mixing, tubes were incubated for 45 min in a 37°C water bath. The leukocyte-rich plasma was withdrawn and centrifuged (30 min at 150 x G). The pellet cells were resuspended in HG containing 4 mM EDTA. Residual erythrocytes were lysed by hypotonic shock. Cells were washed in HG and resuspended in the desired volume of HGCM at a concentration of 1 x 10⁶ cells/ml.

Antigens were diluted in HGCM, and different concentrations were added in 0.3-ml aliquots to test tubes and equilibrated for 10 min at a 37°C water bath. Three-tenths milliliter of leukocyte suspension at 37°C was then placed in each tube. Six percent percolitic acid was added to three samples (controls) to obtain the total histamine content per tube. Tubes containing cells and buffer alone were used to measure the spontaneous release. In these series of experiments the spontaneous release for serum recipients was 22 ± 15 (mean ± SD) for 54 animals. This value is higher than the spontaneous release we have found for donor animals (5, 13, and herein), which is 5 to 10%. These differences may reflect a nonspecific effect of serum transfer on background release from recipient guinea pig basophils. Concanavalin A (Con A; Sigma, St. Louis, MO) at a final concentration of 2.5 µg/ml was used to induce histamine release as a positive control (13). After 60 min, the reaction was stopped by centrifugation (800 x G, 10 min) at 4°C. The supernatants were assayed for histamine content. All experimental samples were run in duplicate.

Assay of histamine. Histamine was measured by an automated fluorometric technique (14). The percentage of histamine released was calculated with the formula:

\[
\text{% Release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Complete} - \text{spontaneous release}} \times 100
\]

In vitro passive sensitization. For these experiments, EDTA-containing blood from three animals was pooled, then allowed to sediment as described previously, and after washing of the cell pellet, the cells were finally resuspended in HGCM at a concentration of 2 x 10⁹ cells/ml in HG containing 4 mM EDTA. An equal volume of immune serum was then added. The cell suspension was incubated at 37°C in a shaking water bath. Aliquots were removed at the initiation of incubation and after 1, 2, 4, and 8 hr. Each aliquot of cell suspension was diluted by a 10-fold excess of HG buffer at 4°C. The suspension was centrifuged (1000 rpm, 15 min), and the cells were suspended in the desired volume of HGCM at a concentration of 1 to 5 x 10⁶ cells/ml.

The histamine release assay was carried out as described above.

Statistics. One-way analysis of variance and a studentized range test were used to compute the significance of differences between groups. Results are expressed as the mean ± SD.

RESULTS

In vivo passive transfer of anti-KLH immune serum induces specific sensitization of circulating basophils as detected by histamine release in vitro. As we reported previously, after intradermal immunization of guinea pigs with KLH, basophils became sensitized by day 9, releasing more than 50% of their histamine in vitro in the presence of the immunizing antigen (5, 13). The sensitization is specific, because the basophils do not release histamine in the presence of ovalbumin (OVA). These animals served as donors of immune serum that was transferred systemically in 2-ml volumes, and resulted in specific sensitization of basophils in the recipients. Figure 1 shows two of 11 successful transfer experiments. As illustrated, the degree of sensitization varied from experiment to experiment in both the donors and the recipients. In the case of experiment A in Figure 1, the donor basophils released 75% of their histamine and recipient basophils released 50% of their histamine in the presence of 100 µg/ml KLH, whereas in experiment B, only 28% release occurred with donor basophils and 16% release with recipient basophils.

The in vitro release was specific, because OVA caused no release (Fig. 1). We have shown previously that basophils from animals immunized with OVA will release histamine in vitro in the presence of OVA at concentrations as low as 1 µg/ml (5). In the current experiments, release of histamine was obtained from basophils of donors and recipients with KLH concentrations as low as 1, 0.1, or even 0.01 µg/ml (Figs. 1 and 2). Histologic examination showed that the intradermal test sites in both donors and recipients had a pronounced infiltrate of basophils, as reported previously (7).

Passive transfer of CBH with immune serum has been shown previously to be mediated by anaphylactic IgG1 (4) or IgE antibodies (8). In order to test for which immunoglobulin isotype might be involved, immune sera from animals immunized with KLH emulsified with IFA was heated in a 56°C water bath for 4 hr. Figure 2 shows the results of passive transfer of untreated and heat-inactivated immune sera. Animals were passively sensitized with 2, 1, or 0.5 ml of sera. At all doses, there was no significant difference either in the in vitro histamine release from the recipient basophils or in the in vivo diameter of erythema at the skin test sites. Because guinea pig IgE is destroyed by heating at 56°C for 1 hr (15), these results suggest that the serum antibody isotype mediating both CBH and basophil sensitization is IgG1.

In vitro passive sensitization of basophils for histamine release with anti-KLH immune serum. Figures 3 and 4 show two of seven successful experiments in which different pools of immune serum (from animals immunized with KLH intradermally) were used to passively sensitize basophils in vitro. In both experiments, significant antigen-dependent histamine release occurred after preincubation of the cells for 30 min in the presence of immune serum. Also, Figure 3 shows that during the first 2 hr of preincubation, there was no change in the total cell-associated histamine, which suggests that during this period of time the basophils remained viable. However, in three of seven experiments, signif-

Figure 1. Comparison of basophil sensitization in donor animals (immunized with KLH) and recipients of sera from KLH-immune animals. Two experiments (A and B) are shown. Each group consisted of two guinea pigs and results are expressed as mean ± S.D. Total cell-associated histamine was 75 ± 17 ng/ml (mean ± SD for four donors) and 9 ± 1.6 ng/ml (mean ± S.E.M for four recipients). Donor values were higher than the induced basophils (see Reference 5).
caused by factors in the immune serum, such as activated histamine could be due to either loss of viability of a pool of immune serum. Washed cells were incubated in vitro with buffer, 100 μg/ml OVA or 100 μg/ml KLH. *p < 0.01 vs spontaneous release in buffer or release in OVA; **p < 0.05 vs total histamine present at time 0.

Our results show that immune sera can passively sensitize basophils in vitro, the optimum preincubation period being 30 to 60 min. Furthermore, the sensitization is specific, because KLH immune sera will only sensitize basophils to release in the presence of KLH and not another antigen (e.g., OVA, Fig. 4). We have also noted in a number of experiments that as the time of preincubation is increased, the amount of histamine released by specific antigen decreases, which suggests that a deactivation phenomenon may be occurring. Preincubation of cells in the presence of either normal guinea pig serum or OVA immune serum did not lead to in vitro release of histamine in the presence of KLH (data not shown).

In vivo passive transfer of hapten-specific immune serum induces hapten-specific sensitization of circulating basophils. It was shown previously that under certain conditions CBH is hapten specific and that this reaction could be transferred passively with immune serum. Table 1 confirms these results and furthermore shows that the basophils of the recipient guinea pigs are specifically sensitized to the relevant hapten. Passive transfer of sera from animals immunized with either Ox-KLH or Ox alone emulsified in IFA resulted in sensitized basophils that released histamine in vitro in the presence of Ox bound to another carrier, HSA. However, basophils from recipients of sera

![Image](http://www.jimmunol.org/Downloadedfrom.png)
from Pic-Asc- or KLH-immunized animals did not release histamine in vitro in the presence of Ox-HSA.

The reverse experiment to show specific sensitization to Pic-Asc was attempted but was not successful because the in vitro challenge antigen Pic-HSA caused nonspecific histamine release from leukocytes of unimmunized animals.

Depending on the immunization protocol employed, the passive transfer of hapten-specific CBH and recipient basophil sensitization were dependent on the volume of sera transferred. With the Ox-KLH + IFA immune sera, a reduction in the volume transferred from 2 ml to 0.7 ml reduced both the percent of change in ear thickness and the percent of release of histamine in vitro. However, with the Ox + IFA immune sera, little difference was seen in either ear thickness change or percent of histamine release (Table I). The potency of Ox + IFA immune sera in the ability to transfer CBH was previously noted (3).

Ears of animals that received serum from donors immunized with Ox-KLH + IFA or Ox + IFA were challenged by contact painting with 4% Ox in olive oil. The ears were removed for histologic examination 24 hr later and showed the expected infiltrate of basophils, as reported previously (2–4).

**DISCUSSION**

Previous studies have demonstrated that CBH can be transferred passively with immune serum employing several immunization protocols (2–4, 7, 16, 17). Haynes et al. (4) reported that low affinity IgG1 antibodies can mediate the transfers, and Graziano and Askenase (10) showed that host Fc receptors are involved in these antibody-mediated CBH reactions. Basophils infiltrating these delayed hypersensitivity reactions are sensitized to the immunizing antigen and can be triggered with soluble antigen to undergo anaphylactic degranulation, with the immediate release of vasoactive mediators, such as histamine (6, 9). This phenomenon has been called "cutaneous basophil anaphylaxis," and has been shown recently to be dependent on passive sensitization of basophils arriving at CBH reactions by IgG1 antibodies acquired in the circulation (18).

In this report we have extended these findings in two ways. First, we have shown that after passive transfer of immune serum, the circulating basophils become sensitized and will release over 50% of their histamine content upon challenge in vitro with specific antigen (Fig. 1). Both IgG1 and IgE have been shown to passively transfer CBH. However, Figure 2 shows that heat-treated sera (at 56°C for 4 hr) were as efficient as untreated sera at transferring both CBH and basophil sensitization. Also, IgE responses to protein antigens only occur in Hartley guinea pigs treated with cyclophosphamide (12). These findings suggest that IgG1, and not IgE, mediated both the transfer of CBH and systemic basophil sensitization in the current study. Second, we have shown that immune serum can passively sensitize guinea pig basophils in vitro (Figs. 3 and 4). Although the sera were used at a relative concentration of 50%, the degree of basophil sensitization as judged by in vitro histamine release was low compared with the in vivo transfers (cf. Fig. 3, <15% release, and Fig. 4, <25% release, with Fig. 1, >50% release). When the sera were used in vitro at lower concentrations, basophil sensitization was less effective (data not shown). These results suggest that unknown in vivo effects such as cofactors may be involved in the binding of IgG1 to Fc receptors on basophils, or that these receptors become unavailable when basophils are processed in vitro. It is remarkable that putative guinea pig IgG1 antibody can passively sensitize basophils in a manner that resists washing. Basophil-sensitizing IgG antibodies have not been demonstrated convincingly in other species. In rats and mice, some IgG antibody isotypes attach to mast cells with low affinity, and thus are easily washed off.

Although most results were achieved with sera from animals immune to a protein antigen (KLH), we also showed that hapten-specific immune sera will transfer both hapten-specific CBH and hapten-specific basophil sensitization (Table I). When sera from animals immunized with Ox-KLH or with reactive Ox alone were transferred systemically to recipients, 24-hr ear skin tests showed a significant increase in mean thickness, ranging from 10 to 20%, compared with 1 to 4% in controls (Table I). This is in accordance with previous studies in which it was shown that a 10% increase in ear thickness is statistically significant (2–4). In addition, basophils isolated from the recipients released histamine in vitro with Ox-HSA (Table I). No histamine release occurred in the presence of Ox-HSA with basophils isolated from unimmunized animals (data not shown) or from animals passively sensitized with immune sera to KLH or to Pic-Asc (Table I).

Our data support the hypothesis that sensitization of circulating basophils may be a prerequisite for the elicitation of antibody-mediated CBH reactions. According to this hypothesis, mast cell sensitization is also required in antibody-mediated CBH. Upon cutaneous challenge with antigen, these sensitized mast cells release chemotactic factors. Among cells that are recruited to the local site are sensitized basophils that upon interaction with residual skin test antigen can release mediators (6, 19). We propose that among these mediators, not only are there present the previously characterized eosinophil chemotactic factors (19, 20), but also chemotactic factors for basophils and perhaps lymphocytes. That the latter concept may be true is supported by the finding that rat mast cells release chemotactic factors for lymphocytes (21). Release of these mediators would cause a further accumulation of leukocytes at the reaction site, some of which would be passively sensitized with antibody and thus able to be activated by remaining antigen to release additional mediators. Our results do not exclude the possibility that passively transferred antibody may also sensitize monocytes, eosinophils, or lymphocytes and that these cells may also contribute to elicitation of antibody-mediated CBH. Sensitization of lymphocytes could lead to the release of basophil chemotactic factors (22, 23) and the basophil-activating lymphokine, histamine-releasing activity (24, 25). These lymphokines may also lead to an increased infiltrate of basophils and cause their degranulation and release of mediators at the CBH site.

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