

Postgraduate course

Histamine-releasing factors and IgE heterogeneity

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In my recent lecture to the Academy, I covered a wide range of topics. In this article, I will be more focused, directing attention to a family of HRFs that release mediators from human basophils by interacting with IgE. Of particular interest is that these factors interact with a subset of IgE molecules, thereby establishing a novel, functional heterogeneity of IgE. We have designated the IgE molecules that interact with HRF as IgE+ (by definition, the remaining IgE molecules are IgE-). IgE+ molecules occur only in atopic individuals, and it is our hypothesis that the clinical severity of the allergic response correlates with the acquisition of IgE+. We believe that the presence of IgE+ is related to the late-phase reaction and that HRF is responsible for the mediator release from human basophils that occurs at this time. The description of HRF and the subsequent appreciation of functional IgE heterogeneity could provide a partial answer to the question that has always bothered us as clinicians: why two patients with the same level of IgE antibody and the same degree of skin test sensitivity may differ so remarkably in their clinical response on exposure to antigen.

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Abbreviations used

HRF:	Histamine-releasing factor
IL-1,2,3,4:	Interleukin-1, 2, 3, and 4
D ₂ O:	Heavy water

It is rare in science for data presentation to be accomplished best in a chronologic fashion, that is, in the way it was acquired. This is because science progresses in bits and spurts with seemingly unrelated observations eventually becoming a part of the whole construct. I will try, however, to provide an almost chronologic version of this set of results, precisely because it indicates how the discovery of IgE heterogeneity was not anticipated but arose from the exigencies of the data. This work is obviously the product of a number of present and past Fellows and faculty members. I shall mention their contributions as I proceed.

Although Thueson et al.¹ had previously described an HRF from human lymphocytes, two unrelated lines of experimentation began this work in our laboratory. A by-product of the process of purifying human mast cells, a task that occupied us in the early 1980s, was alveolar macrophages.² It appeared wasteful to discard these cells, and therefore Liu et al.³ posed the question of whether macrophages communicated, controlled the function, of mast cells and/or basophils. After a number of false starts, it was determined that these macrophages, when they were cultured, produced a factor that could cause histamine release from human basophils and, at very much higher concentrations, could sometimes activate mast cells. When the characteristics of this macrophage HRF were examined, it was found that it induced release with the same kinetics, temperature, and calcium dependence as those observed with anti-IgE. Moreover, its relative inactivity with respect to human lung mast cells was

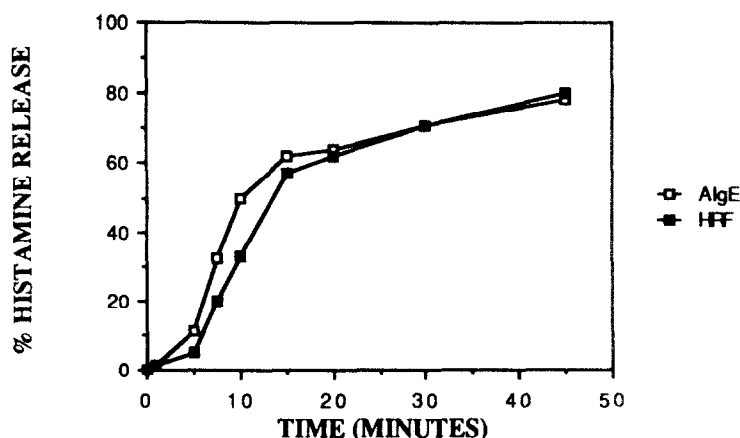


FIG. 1. Kinetics of basophil histamine release caused by anti-IgE and HRF. Similarity of the response to the two secretagogues led us to suspect that HRF interacted with IgE.

consistent with the fact that these cells are some 30 to 100 times less sensitive to anti-IgE than are basophils.⁴

The second line of experimentation that formed the basis for the work to be described in this article came from a deliberate attempt to understand the pathogenesis of the late-phase reaction. Nagy et al.⁵ had demonstrated after lower airway antigen challenge, as we had after upper airway antigen challenge,⁶ that the late-phase reaction was accompanied by the reappearance of mediators at the time of the increased clinical response. Our data indicated that the pattern of mediator release at this time resembled that of basophils more than that of mast cells, because prostaglandin D₂, a mast cell product not generated by basophils, was absent in the late-phase reaction. This was the first clue that basophils were responsible for mediator release during the late phase. We were relatively certain that the mediator release observed hours after challenge was not due to the persistence of antigen and therefore sought the secretagogue(s) responsible for these events. For this we turned to the skin blister technique previously described by Dunskey and Zweiman.⁷ In a series of experiments, Warner et al.⁸ demonstrated that an HRF could be found in blister fluid during the late-phase reaction, but not in control blisters or in either blister at early times after antigen exposure. In this tightly controlled system, we were able to demonstrate that the histamine releasing activity was not due to antigen persistence because appropriate IgG antibody did not block the reaction to HRF in antigen-sensitive individuals, and furthermore, the HRF in the late-phase blister fluid caused release from basophils derived from individuals who were not sensitive to the inciting antigen.

The response caused by this late-phase blister-derived HRF resembled anti-IgE in kinetics and in the other characteristics that define the IgE-related response.

After these observations in the skin, we attempted to document the appearance of a similar late-phase HRF in the upper airways challenge system developed by Naclerio et al.⁹ This was readily demonstrated, and to our surprise, MacDonald et al.¹⁰ found that control fluids, that is, nasal lavage obtained from individuals *before* antigen challenge, also had histamine releasing activity. Again, the characteristics of the induced release were similar to those of the factors described above, that is, it behaved very much like anti-IgE (Fig. 1). Thus, we turned to this unlikely source, nasal lavage of unchallenged individuals, as the best source of HRF with which to attempt to purify and characterize this molecule. The macrophage experimentation was not pursued because the release of HRF was highly variable, and we could find no way to increase production by activating the macrophages. It was discovered that individuals also varied in their production of nasal HRF, with some lavages yielding large amounts of releasing activity and others, negligible amounts. The presence of nasal HRF did not relate to the atopic state of the subject. Indeed, we have found that HRF production (as opposed to the ability to *respond* to these molecules) bears no relationship to clinical disease state.

With a 15 to 30 kd preparation of HRF derived from pooled nasal lavages and partially purified by passage over Sephadex G-75, MacDonald et al. asked whether the release caused by this factor directly involved IgE. To this end we carried out cross-desensitization experiments between the nasal lavage HRF, the other HRFs, and anti-IgE. Treating baso-

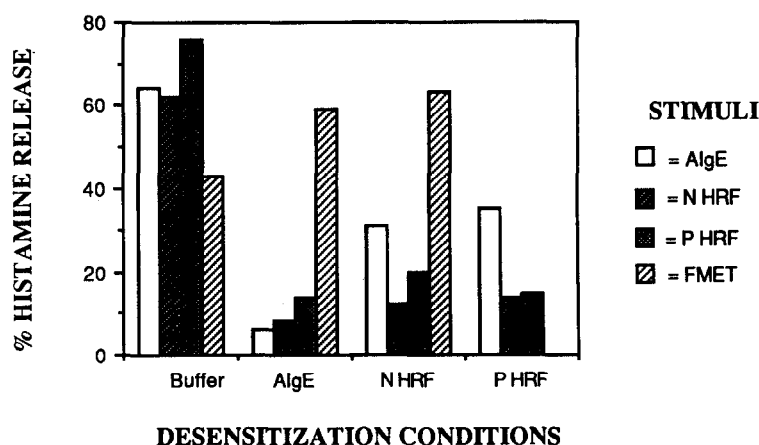


FIG. 2. Cross-desensitization experiments that indicate that the release caused by anti-IgE and HRF share common mechanisms, whereas the release caused by *N*-formyl-methionyl-leucyl-phenylalanine (FMET) does not. The experiment involves four conditions. In the first or control condition (*left side*), basophils are exposed to calcium-free buffer alone for 60 minutes, washed, and then challenged with anti-IgE, nasal lavage HRF (N HRF), a platelet-derived HRF (P HRF) (see text), or FMET. The histamine release observed here serves as the control for the rest of the experiment. In the second condition, basophils are exposed to anti-IgE, in calcium-free buffer for 1 hour (i.e., they are desensitized) and then washed and challenged as before. The response to rechallenge with anti-IgE falls, as expected, from about 65% to <10%. The point of the experiment, however, is that the response to the two HRFs is similarly decreased, whereas the response to the secretagogue that does not involve IgE (FMET) is not decreased but actually enhanced. In the last two parts of the experiment, the basophils are desensitized by exposure to the HRFs (rather than a-IgE), and cross desensitization is demonstrated; that is, the response to the homologous stimulus (i.e., N HRF) is decreased but that to the other HRF or to anti-IgE is similarly reduced. (There is no FMET challenge in this experiment after desensitization with P HRF.)

phils with anti-IgE in the absence of calcium obviates the response to anti-IgE or antigen when calcium is subsequently restored.¹¹ We found that anti-IgE desensitization also abrogated the basophil response to HRF. The converse was also true, that is, cells desensitized with any of the HRFs also had a decreased or absent response to anti-IgE as well as to autologous or nonautologous HRFs (Fig. 2). The response to formyl methionine peptide, which is not IgE mediated, was not affected by prior desensitization to HRF or anti-IgE.

In further pursuing the interaction of HRF with IgE, MacDonald et al.¹⁰ used a technique described by Pruzansky et al.¹² in which IgE is removed from the surface of basophils by brief exposure to lactic acid. This treatment removes about 90% of the cell-surface IgE; since the response to anti-IgE requires but a few IgE molecules, lactic acid stripping of cell-surface IgE decreases but does not completely block the response to anti-IgE. However, this treatment completely abolished the response to the several HRFs¹⁰ (Fig. 3).

At this point, our progress ceased. Although we could decrease the response to HRF by removing cell-surface IgE, Dr. Plaut³ could not, in general, restore it by resensitizing the cells with a convenient source

of IgE, that is, the PS myeloma kindly supplied by Dr. Ishizaka. This experiment was repeated many times, and although in some early experiments (perhaps with different batches of PS) some restoration could be observed, this could not be accomplished consistently. After spending several months attempting to define conditions to restore the response to HRF, we concluded that it could not be done with IgE from the PS myeloma. Drs. Sobotka and MacDonald¹⁰ then wondered whether other sources of IgE molecules would be more effective. This would indicate IgE heterogeneity, a rather heretical concept, since there is but one known gene for human IgE and, therefore, but one isotype. In spite of this theoretical difficulty, we attempted to restore IgE stripped cells with IgE containing sera obtained from individuals whose basophils responded to HRF. Once this experiment was attempted, it immediately became apparent that there were at least two types of IgE. Complete restoration of the response to HRF occurred when we used the IgE from HRF-responsive patients (IgE+), whereas the results with myeloma IgE (IgE-) were negative (Fig. 3). These investigators then carried out a series of experiments to demonstrate unequivocally that the restoring principle in the sera from HRF-responsive

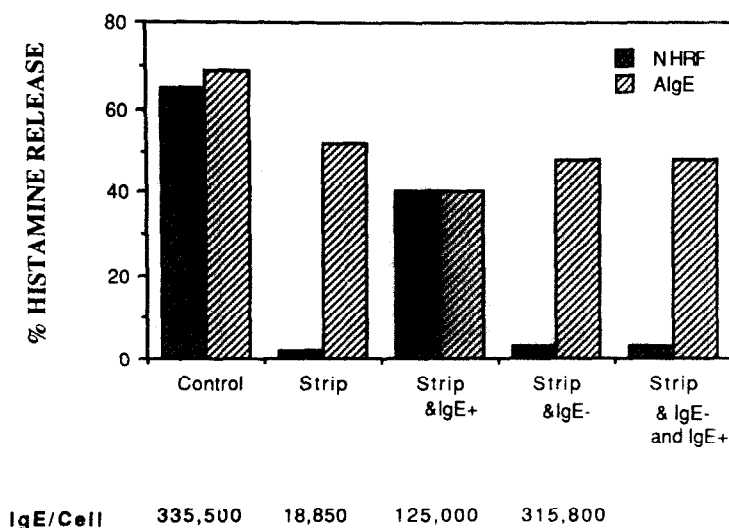


FIG. 3. An experiment that demonstrates that HRF causes basophil histamine release by interacting with one kind of IgE (IgE+). At the *left* (control) the untreated basophils have 335,000 IgE molecules on their surface and release 60% to 70% of their histamine after challenge with IgE or HRF. After treatment ("strip") with lactic acid, the number of IgE molecules on the basophils is reduced >90%. This decreases the response to a-IgE and eliminates the response to HRF. When the stripped basophils are sensitized with IgE+, the response to HRF is restored, but this does not occur with IgE-, even though higher concentrations were used for passive sensitization resulting in more IgE-molecules (316,000) on the basophil than when the IgE+ was used. Finally, this experiment demonstrates that, when stripped basophils are first sensitized with IgE-, their receptors are filled with these biologically inactive molecules and that IgE+ can no longer sensitize the cells for a response to HRF. Note that in the usual experiment, both IgE+ and IgE- completely restore the response to anti-IgE; this did not occur in the experiment illustrated.

individuals was indeed IgE. Thus, heating such sera at 56° C for 4 hours completely abolished the ability to resensitize the cells to HRF. In more definitive experimentation, passage of the sera over an anti-IgE immunosorbent column, with the removal of >90% of the serum IgE, also removed the activity.¹⁰ MacDonald et al.¹⁰ laboriously purified the IgE from an HRF-sensitive patient to >90% homogeneity and demonstrated that this highly purified IgE could also be used to sensitize basophils to HRF.

To demonstrate that we were dealing with an IgE receptor, we stripped the IgE from basophils with lactic acid and then filled the open receptors with IgE-, that is, with myeloma IgE. Under these circumstances, the IgE+ could no longer sensitize basophils, and they failed to respond to HRF. MacDonald et al.¹⁰ also prepared an immunosorbent column, using the purified IgE+ and demonstrated that HRF could be absorbed to this column and later eluted with glycine buffers. This experiment provided definitive evidence of an interaction between HRF and IgE+, since columns made with IgE- were without ability to bind HRF.

A new source of HRF was discovered by Orchard et al.¹³ pursuing an observation made some years ago

by Knauer et al.,¹⁴ that platelet products had the ability to enhance IgE-mediated release. They found that under appropriate conditions, human platelets could also generate a factor (probably the same enhancing factor, but at higher concentrations) that could itself cause basophil histamine release. This factor had the same characteristics of the other HRFs in being able to stimulate release only when cells were supplied with IgE+. We subsequently found that two cell lines, U937 and RPM18866, spontaneously produced molecules with the characteristics of HRF.¹⁵

A major goal of this work has been to purify and characterize the various HRFs, and in this we have been less than completely successful. Efforts with HRF derived from nasal lavages, platelets, and the U937 cell line indicated that most of the activity eluted from sizing columns with approximate molecular weights of 15 to 30 kd, although at times activity in a higher molecular weight fraction was observed. Further attempts at purification have been frustrating in that functional activity can be obtained in preparations without detectable protein. We have recently entered into a collaboration using scaled-up production methods and hope that purification and characterization will be forthcoming. It should be noted at this point that,

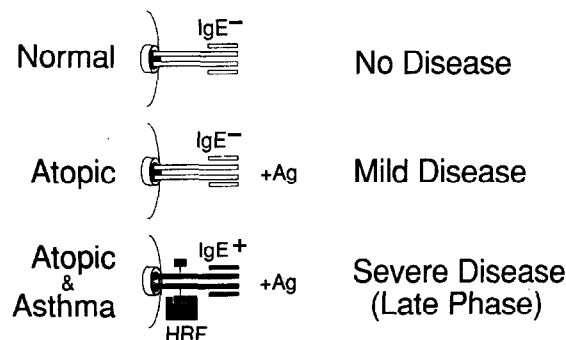


FIG. 4. A cartoon of the possible clinical significance of IgE heterogeneity (see text).

whereas all the HRF molecules have similar molecular weights, all cross-desensitize to one another and to anti-IgE, and all require an interaction with IgE+ to cause histamine release, they are not identical. In experiments carried out with a panel of basophil donors tested with platelet, nasal lavage, and U937 HRFs, there was a differential response among the donors, indicating that there must be some differences between the HRF molecules. Thus, we currently think of these molecules as constituting a family with similar characteristics but differing in structure in a fashion yet to be ascertained.

As mentioned earlier, HRF acts as a releasing agent only with the basophils of atopic individuals. Another thread in this research was derived from an early observation that D₂O is a selective secretagogue for the basophils of atopic individuals, particularly those with atopic asthma.¹⁶ Since this D₂O-induced release demonstrated the characteristics of IgE-mediated release, we questioned whether sensitivity to D₂O was also dependent on IgE+. This question was answered in the affirmative. Removal of responder basophil cell-surface IgE rendered the cells unresponsive to D₂O. This response could be restored by resensitization with IgE+ but not with IgE-. Cells from donors originally unresponsive to D₂O could be rendered sensitive by passive sensitization with IgE+. This represents a second secretagogue that demonstrates the functional heterogeneity of IgE. This experiment also mitigates our concern that HRF is a ubiquitous antigen and that IgE+ is an antibody to that antigen.

Based on our inability to purify HRF, we had every reason to be concerned that it was a molecule that had already been described. Haak-Frendscho et al.¹⁸ and Subramanian et al.¹⁹ had reported that IL-1 could cause histamine release. Since this and other interleukins have about the same molecular weight as HRF, we examined whether these recombinant interleukins (kindly supplied by Steve Gillis) caused basophil his-

tamine release. We could not confirm that IL-1 caused release, although Massey et al.²⁰ found that it did enhance the effect of other IgE-mediated secretagogues. Neither recombinant IL-2 or IL-4 caused release, nor did they enhance IgE-mediated release. To our surprise, however, MacDonald et al.²¹ found that IL-3, a known promoter of mast cell and basophil growth, did induce histamine release. It was clear, however, that IL-3 differed from HRF in several respects. First, the kinetics of release were much slower and quite distinct from those associated with HRF or anti-IgE. Furthermore, IL-3 did not cross-desensitize with either HRF or anti-IgE. It did not, in fact, desensitize to itself. In this characteristic and with respect to kinetics, it behaved very much like the calcium ionophore A23187. Given this information, it was surprising to discover that the response to IL-3 was also IgE dependent. Again, removal of cell-surface IgE abolished the response to IL-3, which could only be restored with IgE+. Highly purified IgE+ restored the response and saturation of receptors with IgE- before sensitization with IgE+ obviated the response. Thus, IL-3 constitutes a third secretagogue that demonstrates functional IgE heterogeneity. We do not know that IL-3 binds to IgE+; we only know that this molecule must be on the cell surface for it to cause release. Its ionophore-like characteristics suggest the possibility that IL-3 is interacting with the putative IgE-dependent calcium binding/transporting protein.

This discussion thus far has stressed the interaction of HRF with basophils rather than mast cells. In an effort to determine whether this interaction was clinically relevant, we started from the previously mentioned observation that the pattern of mediator release (i.e., the absence of prostaglandin D₂) in the late-phase reaction suggested basophil involvement. In further experiments exploring this question, Bascom et al.²² studied cell traffic during the late-phase reaction in the upper airways and confirmed that there is an influx of basophils, along with eosinophils and neutrophils. Basophils increase approximately eight-fold, and thus are present and able to respond to HRF released from whatever in vivo source. Other investigators have noted an increase in basophils in chronic allergic rhinitis and in chronic dermal reactions to antigen.^{23, 24} It is our hypothesis that mediator release in late-phase reactions or in the subacute and chronic allergic response results from the interaction of HRF with basophils drawn to the site by chemotactic factors. Our studies with systemic steroids reinforce our hypothesis regarding the importance of basophils in late-phase reactions. Patients treated with prednisone for 48 hours before nasal antigen challenge failed to

have the late-phase response that was observed with placebo treatment. Steroid therapy also decreased the eosinophil and basophil infiltration without significantly decreasing the number of neutrophils that enter the upper airways.²⁵ That an HRF-basophil interaction is relevant in the late-phase reaction is further suggested by the finding that the clinical severity of the late-phase reaction in the nose correlates significantly with the response of basophils of those patients to nasal lavage HRF.

HRF is remarkably selective in causing release only from atopic individuals. Thus, MacDonald et al.²⁶ found that the basophils of 32 of 64 patients with ragweed-induced allergic rhinitis responded to nasal lavage HRF, whereas none of 22 skin test-negative individuals had a response. Fisher et al.²⁷ have carried out similar studies with platelet-derived HRF. No normal individuals had basophils that responded to this factor, whereas approximately half of individuals with atopic rhinitis and close to 70% of individuals with atopic asthma were responsive. Interestingly, none of seven individuals with intrinsic asthma responded to the platelet HRF, suggesting that HRF can effectively discriminate between intrinsic and extrinsic asthma. Fisher et al.²⁷ went on to study the production of HRF by these subsets of patients and found that all platelets released similar quantities of HRF. Thus, as noted earlier, it is not the production of, but the ability to *respond* to HRF that differentiates atopic from non-atopic individuals. Another interesting observation made by Weiss et al.²⁸ is that, whereas the basophils of patients with atopic rhinitis or atopic asthma respond to platelet HRF, when autologous platelet HRF is injected back into the skin, only patients with atopic asthma respond with an immediate wheal-and-flare reaction.

With these clinical studies in mind and knowing that the response to HRF is conditioned on a patient having IgE+, the significance of IgE heterogeneity becomes clear. We can classify individuals into three types (Fig. 4): (1) those who fail to have IgE antibody to an antigen and are thus not allergic, (2) those who have IgE- antibodies to an allergen and have positive skin tests and clinical symptoms, but these are relatively mild, and (3) those individuals with more severe atopy, particularly atopic asthma, who have IgE+ antibodies to antigen and, by virtue of this antibody, have more severe disease. Thus, we suggest that the type of IgE antibody a patient has influences the clinical severity of his disease. These observations may, within a short period of time, be of clinical use. Thus, it should be relatively easy to determine which patients have atopic asthma and which have intrinsic asthma with an assay for IgE+. Moreover, it should

soon be possible to quantitate what percentage of IgE antibody to a particular antigen is of the IgE+ variety. It may well be that clinicians will wish to carry out immunotherapy with only those antigens to which patients have a substantial majority of IgE+ antibody. It may even be that part of the clinical efficacy of immunotherapy is related to the conversion of IgE+ to IgE-.

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T cell localization in rabbit models of acute and chronic experimental hypersensitivity pneumonitis

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Cell-mediated hypersensitivity has been increasingly implicated in immunologic diseases of the lung, including hypersensitivity pneumonitis (HSP) (extrinsic allergic alveolitis). We used a T cell-specific monoclonal antibody (L11/135) to localize T cells in the parenchyma and bronchus-associated lymphoid tissue of ethanol-fixed, paraffin-embedded lung sections in rabbit models of experimental HSP to define further their possible role in pathogenesis. T cells appeared within 4 hours in early lesions of rabbit models of acute HSP and heavily infiltrated alveolitis lesions at 8 and 24 hours after aerosol challenge. T cells were also present in lesions of rabbit models with chronic alveolitis and occurred peripherally in granulomas. Variable aggregate and follicular forms of bronchus-associated lymphoid tissue rich in T cells occurred in both experimental and control animals. Our findings document early and continuing presence of T cells in lesions in rabbit models of experimental HSP. (J ALLERGY CLIN IMMUNOL 1988;81:821-8.)

Effector mechanisms responsible for immunologic lung injury are incompletely understood.¹ HSP is an immunologic lung disease of known cause secondary to inhalation of a variety of antigens, primarily organic dusts. Early notions of pathogenesis incriminated precipitating antibody and the formation of local antigen-antibody complexes. More recent evidence implicates cell-mediated hypersensitivity.²

We developed rabbit models of HSP to help clarify mechanisms involved in immunologic inflammation of the lung. Preimmunized animals develop acute HSP with initial inhalation of antigen, but disease wanes with long-term inhalational antigen challenges to yield "desensitized" animals.³ If MDP, the minimal adjuvant-active component of mycobacterial cell walls, is added to the aerosol for repeated challenges, chronic granulomatous interstitial pneumonitis results.⁴ A series of experiments has suggested the im-

Abbreviations used

BALT:	Bronchus-associated lymphoid tissue
HSP:	Hypersensitivity pneumonitis
MDP:	Muramyl dipeptide
OA:	Ovalbumin
FCA:	Freund's complete adjuvant
PBS:	Phosphate-buffered saline
GALT:	Gut-associated lymphoid tissue

portance of cellular mechanisms in the pathogenesis of experimental HSP.^{2,5,6} The kinetics and identity of mononuclear cell infiltrations have not been reported, however, nor have T cell composition and localization in BALT been reported in these models.

The present study localized thymus-derived lymphocytes (T cells) in the lungs of rabbits of acute and chronic experimental HSP, with a rabbit T cell-specific monoclonal antibody and immunohistopathology. We found early influx of T cells in rabbit models with acute HSP and the localization of T cells in alveolitis lesions in models of both acute and chronic HSP. In contrast, relatively few T cells were present throughout lung parenchyma of desensitized animals and other control animals. BALT, rich in T cells, was prominent

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TABLE I. Histopathology and T cell localization in BALT in rabbit models of HSP

Rabbit groups	Alveolitis	BALT		
		Airways	BALT	% Follicular
Acute (8)				
4 hr (3)	0.5	3.7 ± 1.2	1.5 ± 0.7	62 ± 31
8 hr (2)	1.5	3.0 ± 0.0	2.5 ± 0.7	54 ± 30
24 hr (3)	2.5	2.6 ± 0.9	2.4 ± 0.3	63 ± 13
Chronic (4)	3.0	5.4 ± 2.1	1.4 ± 0.1	32 ± 12
Desensitized (4)	0.5	5.8 ± 3.1	1.9 ± 0.3	42 ± 10
Immunized-saline challenged (3)	0.5	2.3 ± 0.8	1.7 ± 0.6	38 ± 33
Normal (3)	0	1.6 ± 0.1	1.5 ± 0.3	54 ± 14
ANOVA <i>p</i> value for BALT		0.05	0.05	0.36

Numbers in parentheses denote numbers of rabbits in each group; alveolitis was graded on a scale of 0 to 3.0 (no alveolitis, mild, moderate, and marked); Airways = number of airways containing BALT per lung cross-section examined ($\bar{x} \pm SD$); BALT = number of BALT forms per airway with BALT ($\bar{x} \pm SD$); % follicular = percentage of BALT forms that were follicular; others were aggregate forms ($\bar{x} \pm SD$); ANOVA = Analysis of variance; on Tukey's follow-up, no statistically significant differences among the groups were found.

in both experimental and control rabbits, containing aggregate and follicular forms in all animals.

MATERIAL AND METHODS

These studies followed the guidelines given in the United States Public Health Services/National Institutes of Health guide for the Care and Use of Laboratory Animals and were approved by the University of Iowa Animal Care and Use Committee.

Animals

Female New Zealand white rabbits weighing 2 to 3 kg were purchased from a local supplier. They received food and water ad libitum.

Reagents

OA, crystallized five times, was purchased from Miles Laboratories (Elkhart, Ind.). MDP was synthesized in the laboratory of Dr. Charles Barfknecht, University of Iowa College of Pharmacy.⁷ A mouse monoclonal antibody (L11/135) of IgG isotype directed against rabbit T cells⁸ was obtained from American Type Culture Collection (Rockville, Md.). Control mouse monoclonal antibodies of IgG isotype included antihuman leukocyte antigen (gift of Dr. Zuhair Ballas) and anti-Ia (gift of Dr. K. Knight). L11/135 has been demonstrated to identify all rabbit peripheral T cells.⁸ Biotinylated antimouse IgG antibody and avidin-biotin-peroxidase complexes were contained in Vectastain ABC Kits (Vector Laboratories, Burlingame, Calif.). Chromogen substrate (0.1% diaminobenzidine tetrachloride) was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Preparation of rabbit models of HSP

Experimental rabbits of acute and chronic HSP and desensitized rabbit models were prepared as previously described.^{3,4,9} Briefly, experimental rabbits were sensitized

with 1 mg of OA emulsified in FCA in 0.2 ml total volume distributed among eight toe pads of the four feet. Inhalational challenges began 2 to 3 weeks later and were administered in plexiglass chambers. Chronic challenges with ultrasonically nebulized aerosol consisted of three weekly challenges for 4 to 6 weeks; for each challenge, groups of three or four rabbits received, during about 45 minutes, a total chamber dose of 10 ml of saline containing 100 mg of OA with 1 mg of MDP for chronic HSP, or without MDP for desensitization. The animals were sacrificed 48 hours after the last challenge.

Acute challenge consisted of a single challenge as above; animals were sacrificed 4, 8, and 24 hours later.

Control animals included a group of animals sensitized with OA in FCA and challenged chronically with saline, and a group of normal rabbits.

Histopathology

Animals were overdosed with intravenous pentobarbital. The chest was opened, and 50 ml of blood was obtained from the heart. The lungs were perfused with 6% dextran (Gentran 75; Travenol Laboratories, Deerfield, Ill.) via a catheter in the pulmonary artery with egress from an amputated left atrial appendage. The trachea was clamped, and lungs were removed in toto. Fixation involved intratracheal instillation of cold ethanol with subsequent processing of whole lung cross-sections according to the method of Saint-Marie.¹⁰ Paraffin sections were subsequently processed and stained with hematoxylin and eosin or subjected to the avidin-biotin-immunoperoxidase technique described below. Representative rabbits were used for examination of Peyer's patches and appendix, fixed and processed identically to lung.

Full translobar sections of lung were examined for alveolitis and T cell localization in parenchyma, BALT, airways, and vessels from all rabbits in each group. Representative areas were selected for illustration.

To determine whether areas of parenchyma uninvolved



FIG. 1A, Acute experimental hypersensitivity pneumonitis. Immunoperoxidase stain with monoclonal L11/135 anti-T cell antiserum as primary antibody. Lightly counterstained with hematoxylin. Four hours after aerosolized antigen illustrating region distal to a terminal bronchiole with early cell collections predominantly positive for T cell antigen. (Original magnification $\times 150$.)

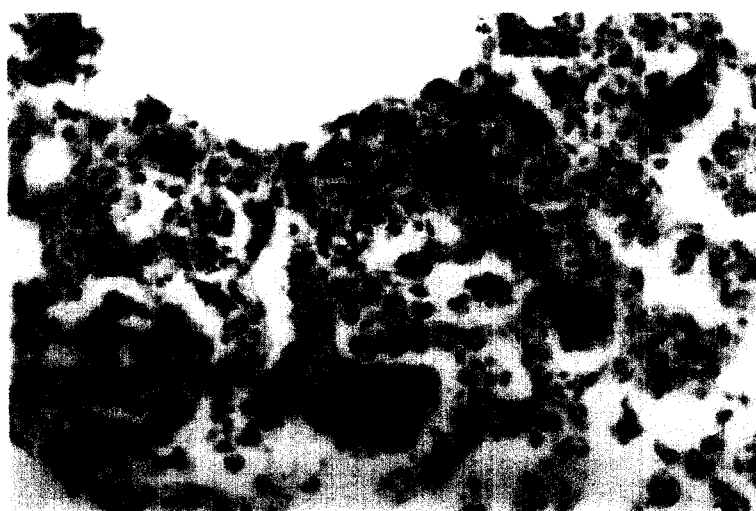


Fig. 1B, Color photomicrograph of acute alveolitis 24 hours after aerosolized antigen. Heavy infiltration with predominantly mononuclear cells staining brown for T cell antigen. (Original magnification $\times 240$.)

with alveolitis contained different numbers of T cells among the different groups of animals, the number of T cells within 100 alveolar septa and spaces for each of three animals in each group was counted. Uninvolved areas were insufficient for this purpose in animals with 24-hour acute HSP and in animals with chronic HSP. No attempt was made to count the numbers of T cells within the alveolitis lesions themselves.

A semiquantitative estimate of BALT was achieved in each group by counting numbers of airways containing BALT in multiple sections, and the number of BALT forms (whether aggregate or follicular) in each airway containing BALT (Table I).

Immunoperoxidase technique

The method used is a slight modification of that described by Hsu et al.¹¹ Paraffin sections were placed for 30 minutes on a slide warmer set at 56° C, deparaffinized with xylene, and hydrated in a graded alcohol series. Sections were then incubated with 1% H₂O₂/methanol for 30 minutes to quench endogenous peroxidase activity, rinsed in distilled water and PBS, followed by 15-minute incubation in 1% normal horse serum to help suppress nonspecific background staining. Excess was removed by shaking, followed by incubation for 60 minutes with L11/135 anti-T cell antibodies (at 1:160 dilution), normal mouse serum (at 1:100 and 1:1000), or control monoclonal antibodies (at 1:160).



FIG. 2. Chronic experimental hypersensitivity pneumonitis, stained as described in Fig. 1. Color photomicrograph demonstrating brown staining T cells within thickened interstitium, within alveoli, and surrounding a granulomatous lesion in an area of chronic alveolitis. (Original magnification $\times 240$.)

L11/135 and control monoclonals were of IgG isotype. After washings in PBS, sections were incubated in turn with biotinylated horse antimouse IgG secondary antibody for 30 minutes and an avidin-biotin-peroxidase complex for 45 minutes (both supplied in Vectastain ABC kits), and with chromogen substrate (0.1% diaminobenzidine tetrahydrochloride) for 5 to 10 minutes. After each incubation, washings were carried out with PBS and, finally, with distilled water. Sections were counterstained lightly with Gill-2 hematoxylin (Lerner Laboratories, New Haven, Conn.) for 1 to 2 minutes, dehydrated with alcohols, dipped in xylene, and mounted with Permount (Fisher Scientific Co., Fair Lawn, N.J.).

Evaluation of results

To compare responses of more than two groups of animals, one-way analysis of variance was performed for determination of overall differences. When a significant overall difference was found, pair-wise differences between individual groups were evaluated with Tukey's studentized range test.¹² These analyses used an SAS program at the University of Iowa Weeg Computer Center.¹³

RESULTS

Histopathology (Table I)

Lesions produced in the various groups were similar to those described previously for these established models.^{3, 4, 9} The lungs of normal rabbits and control groups that were immunized and challenged with saline were clean except for minimal scattered small areas (involving one or two alveoli) in occasional animals of increased tightly packed nonspecific interstitial cellularity. Rabbits receiving toe pad injections of FCA developed a few scattered adjuvant granulomata in lung parenchyma, often peripheral in distri-

bution, and easily distinguished from acute or chronic alveolitis produced by inhaled antigen.⁹ Progressively increasing numbers and sizes of areas of alveolitis, characterized by thickened septa infiltrated with mononuclear and a few polymorphonuclear cells, and mild to moderate alveolar filling with similar cells, were found at 4, 8, and 24 hours in animals with acute HSP. These areas were typically located in areas distal to terminal bronchioles. Rabbits in the group with chronic HSP had widespread interstitial infiltrates and alveolar filling with primarily mononuclear and scattered giant cells. Small to moderate-sized granulomata were present in interstitial locations. Desensitized rabbits were characterized by minimal scattered areas of mild alveolitis without granulomata. All rabbits examined from each group had both aggregate and follicular forms of BALF. Multiple sections (up to 12) from normal animals revealed generally smaller BALF than from immunized and aerosol-challenged animals, but no apparent qualitative differences were observed.

T cell localization in parenchyma

In general, parenchymal T cells were concentrated in alveolitis lesions typically located distal to terminal airways. In areas without alveolitis, T cell numbers were not increased, ranging from means of 0.52 to 0.64 T cells per alveolus in the various groups.

T cell influxes into lung parenchyma after acute inhalational challenge with antigen (Fig. 1). Sensitized animals receiving aerosolized antigen and examined 4, 8, and 24 hours later demonstrated consistent, scattered, localized foci of T cells by 4 hours (Fig. 1, A), increased numbers at 8 hours, and large

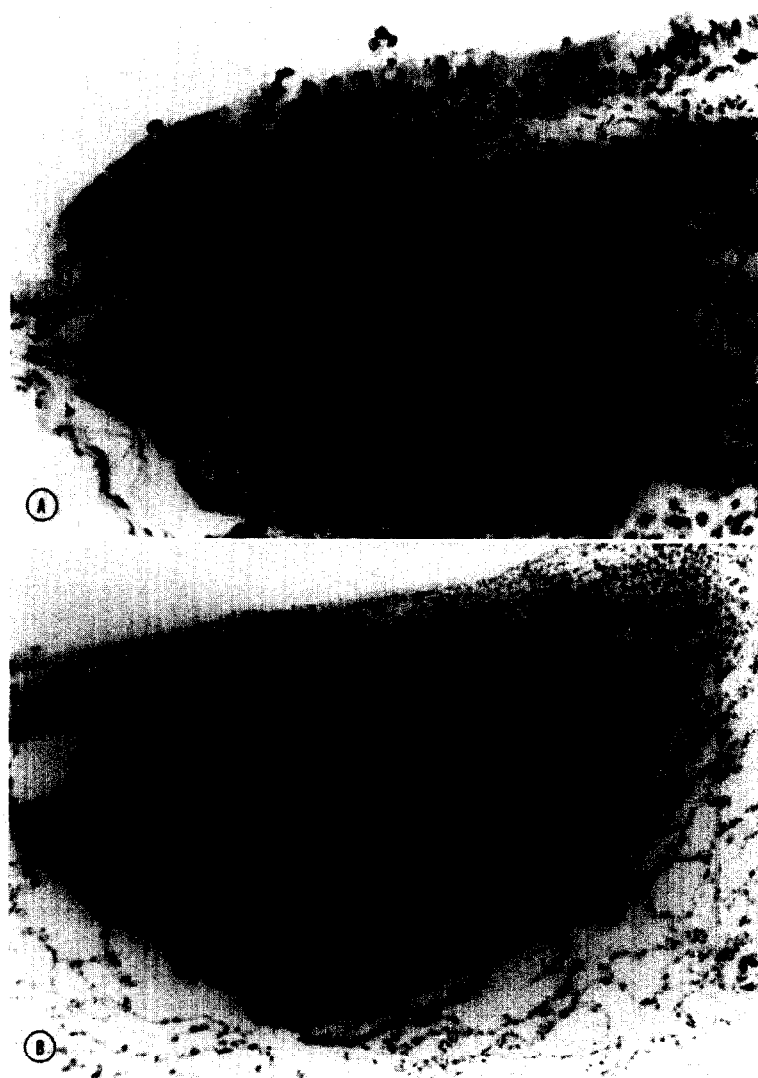


FIG. 3. Examples of aggregate and follicular BALT with localization of T cell antigen prepared by the immunohistochemical techniques described in legend to **Fig. 1**. **A**, Aggregate BALT illustrating darkly stained T cells scattered throughout and infiltrating the overlying specialized lymphoepithelium. (Original magnification $\times 240$.) **B**, Follicular BALT with collar of darkly staining T cells and pale central follicles with cells predominantly negative for T cell antigen. T cells are also infiltrating the overlying specialized lymphoepithelium. (Original magnification $\times 100$.)

numbers of T cells in areas of alveolitis at 24 hours (Fig. 1, B).

T cell localization after chronic aerosol challenges (Fig. 2). Sensitized animals, receiving aerosol challenges three times a week with OA and MDP for 6 weeks, developed chronic alveolitis, and T cells were prominent in alveolitis lesions (Fig. 2). T cells were localized more peripherally than centrally in granulomatous areas. Alveolar walls without apparent infiltrates demonstrated sparse T cells similar to those in control rabbits.

In contrast to animals with chronic HSP, T cells were much less prominent in lungs of rabbits receiving

only OA (without MDP) three times a week for 4 to 6 weeks (the desensitized group). A few areas of mild alveolitis were scattered widely in the lungs of these animals, and clusters of T cells were present in these areas. Alveolar walls without disease demonstrated sparse T cells similar to those in control rabbits.

Control groups. T cells were scattered sparsely throughout the alveolar septa (51 ± 15 cells per 100 alveolar septa and spaces in the normal group). Occasional nests of T cells were associated with mild nonspecific cellular collections, and T cells also surrounded adjuvant granulomata in the sensitized/saline challenged control animals.

T cell localization in BALT (Fig. 3)

No statistically significant differences among the groups were found in respect to number of airways containing BALT per lung cross-section examined, number of BALT forms per airway with BALT, or percent follicular versus aggregate forms of BALT, as indicated in Table I, although there appeared to be a trend toward more airways with BALT in animals with chronic HSP and desensitized animals.

In aggregate forms of BALT, T cells were scattered throughout the lymphoid tissue and infiltrated the lymphoepithelium (Fig. 3, A), whereas in follicular forms, a peripheral demarcated parafollicular T cell zone surrounded the unstained follicle (Fig. 3, B). In fortuitous cuts, T cells were observed to heavily infiltrate overlying specialized epithelium. No difference in architecture was found between experimental and control groups.

T cell localization in GALT

Because BALT and GALT have been compared and to some extent are considered analogous structures, we examined GALT (Peyer's patches and appendix) for localization of T cells. Heavy T cell staining was localized between follicles toward the luminal side in both appendix and Peyer's patches and in the corona area overlying the follicle. Results were consistent with previously reported T cell localization^{14, 15} and help to confirm the specificity of the L11/135 anti-T cell antibody for purposes of the experiments reported here. No apparent differences were found between experimental and control animals.

DISCUSSION

These studies revealed an influx within 4 hours of T cells into areas of developing alveolitis in an established rabbit model of acute HSP. T cells were also a prominent infiltrative constituent of chronic experimental HSP. The localization of T cells in early, acute HSP corresponded to foci of alveolitis, and, in general, T cells appeared concentrated in areas of alveolitis in both acute and chronic lesions. Chronic granulomatous lesions centrally contained very few T cells, but these were dominant cells in the periphery of granulomata.

Our findings are consistent with the hypothesis that the pathogenesis of experimental HSP requires participation of T effector cells.^{2, 16, 17} We have previously reported systemic and local antigen-specific lymphocyte responses in these models as measured by *in vitro* incorporation of ³H-thymidine¹⁸ and demonstrated that such responses were primarily T cell dependent.¹⁹ We have also found increased numbers of bronchoalveolar lavage T cells as quantitated by flow cytometry in

animals with acute and chronic HSP compared to control animals.²⁰ The present study documents localization of T cells in the lesions themselves. Dissection of T cell subsets is not yet possible in the rabbit. To what extent suppressor T cells are involved is uncertain, but early influx of T cells by 4 hours into acute lesions, and localization of T cells in areas of alveolitis in acute and chronic disease, favor the conclusion that these T cells are effector cells rather than suppressors. In addition, there was no evidence that desensitized rabbits had unusual numbers or locations of pulmonary T cells that might be responsible for suppressing disease.

The L11/135 monoclonal antibody used in these studies is specific for a differentiation antigen present on all rabbit T cells and does not react with B cells, monocytes/macrophages, or granulocytes.⁸ The antigen was well preserved in ethanol-fixed tissue sections, and we have also found excellent staining in formalin-fixed, routinely prepared paraffin sections with this antibody and the avidin-biotin-immunoperoxidase technique.²¹ T cell antigen was also easily identified in BALT and GALT, described here, and in lymph nodes, spleen, and thymus (not presented).

BALT has been defined by Bienenstock²² to exist in aggregate follicular and solitary follicular forms and to be covered at some point by specialized lymphoepithelium that separates lymphoid tissue from airways. A proliferative response, as measured by DNA synthesis, has been demonstrated in BALT lymphocytes after local antigenic stimulation,²³ a finding consistent with our impression, not confirmed statistically, that BALT was somewhat more prominent in rabbits chronically challenged with aerosolized antigen than in normal, unchallenged animals.

Some controversy exists concerning the presence of typical parafollicular T cell-dependent areas in BALT.²² Racz et al.²⁴ postulated a T cell-dependent area of the BALT after finding interdigitating reticular cells in the parafollicular area. Sell et al.¹⁵ depicted a thymus-dependent area in a schematic drawing of BALT and reported that most BALT cells were positive with use of a polyclonal anti-T cell antibody.¹⁵ Our findings with monoclonal L11/135 antibodies are consistent with the concept of a well-demarcated T cell-dependent area in follicular forms of BALT. T cells were also observed heavily infiltrating the specialized lymphoepithelium. These morphologic features of BALT are not particularly reminiscent of GALT in which the thymus-dependent area is wedged between apical portions of follicles and underlies mucosa uninvaded by T cells.

Early notions on the pathogenesis of HSP empha-

sized precipitating antibodies, immune complexes, and a localized Arthus reaction involving the lung.²⁵ Tenuous evidence exists for immune complexes in pathogenesis of pulmonary disease, reviewed elsewhere,²⁶ and current information favors the primary importance of cell-mediated (delayed) hypersensitivity in HSP.⁴ Human studies have used bronchoalveolar lavage to study cell populations in HSP. Increased numbers of lymphocytes attributable in general to T8+ cells after recent exposure have been reported by several investigators.²⁷⁻²⁹ Other investigators, however, have observed no phenotypic aberrations of T cell subsets but have found evidence of increased T cell proliferation, activation, and function.^{30,31} Recent evidence has been presented that T-lymphocytes also predominate within alveolar infiltrates of human interstitial pneumonitis studied by use of immunohistochemical techniques.³² In our rabbit models of HSP, we have evaluated carrier requirements for acute HSP and found that lesions were dependent on carrier (i.e., cellular responses), not on antihapten, antibodies.⁵ The disease was abrogated by cyclosporine.⁶ The requirement for FCA or MDP in immunization^{4,33} and MDP in the antigen-containing aerosol for chronic HSP⁴ are additional points favoring the necessary participation of cellular mechanisms in experimental HSP. The present article provides direct documentation of T cell involvement in both acute and chronic HSP in these rabbit models. Further study is needed, however, to define the specific effector T cell subsets and molecular mechanisms responsible for the development of inflammation in hypersensitivity lung diseases.

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Enhanced bronchoalveolar lavage mast cell histamine releasability in allergic dogs with and without airway hyperresponsiveness

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To determine if mast cells from the airway lumen of Basenji-greyhound (BG) dogs differ functionally from mast cells of control dogs, we compared spontaneous release and A 23187-induced and C5-induced histamine release from bronchoalveolar lavage (BAL) fluid of 14 BG and five allergic and five nonallergic control dogs. No dog received antigen, agonist, or therapeutic aerosols for 4 weeks before BAL. The fluid recovered was centrifuged, and the number of mast cells was quantitated. Aliquots containing equal numbers of mast cells were incubated with A 23187 or C5 for 30 minutes, and histamine release was measured by an automated fluorometric method. Spontaneous release, A 23187-induced release, C5-induced release, and total histamine content per mast cell were calculated. The total amount of histamine per mast cell was not significantly different in BGs and allergic and nonallergic control dogs. Mast cells obtained by BAL released histamine to A 23187 and C5 in a dose-related manner. Spontaneous histamine release and A 23187-induced histamine release was significantly greater in BGs and allergic control dogs compared to nonallergic control dogs. C5-induced histamine release was significantly greater in BGs than in allergic as well as nonallergic control dogs. These data suggest that BAL mast cell histamine releasability must be defined with respect to each stimulus and that mast cells obtained from BAL from control dogs differ with respect to histamine releasability from mast cells of allergic dogs with and without airway hyperresponsiveness. This study suggests that mast cells obtained from BAL can be used to study mast cell function in the control and the allergic state. (J ALLERGY CLIN IMMUNOL 1988;81:829-35.)

BAL is a relatively safe procedure that can be performed repeatedly in subjects with pertinent and well-characterized disease states. Mast cells have been identified in the BAL fluid from animals¹⁻⁴ and humans,⁵⁻⁸ suggesting that these cells are present in the airway lumen. These cells are easier to study than cells in tissue because they are already dispersed. It is not necessary to subject them to the mechanical trauma and long incubations with proteolytic enzymes required with resected lung specimens.

Mediator release in the airway lumen or from the airway mucosa is likely important in the pathogenesis of asthma.⁹ In attempts to understand the pathophysiology of asthma, considerable interest has focused on

Abbreviations used

BAL: Bronchoalveolar lavage
BG: Basenji greyhound
FMLP: *N*-formyl-methionyl-leucyl-phenylalanine

the airway mast cell, since distinct differences exist between mast cells and basophils and between mast cells of different tissues within the same species.

The BG dog model of asthma is extremely well characterized with respect to atopy^{10, 11} and persistent airway hyperresponsiveness.^{11, 12} This model has 5% to 9% mast cells in BAL. These cells appear morphologically similar to mast cells of control animals.⁴ Functionally, however, these cells may differ from those of a control population.

We therefore attempted to answer whether mast cells obtained from BAL in a group of BG dogs functionally differed, with respect to histamine releasability, from cells obtained in allergic and nonallergic control dogs.

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TABLE I. Summary of study subjects

	No. of dogs	Hyperresponsive to		Atopic dogs†
		Histamine*	Methacholine*	
BG	14	yes	yes	yes
Basenjis	5	no	no	yes
Greyhounds	5	no	no	no

*Data from reference 14.

†Data from reference 10.

TABLE II. Percent of mast cells in BAL fluid and histamine content per metachromatic cell in three dog groups (mean + SEM)

	N	Total histamine (pg/metachromatic cell)	Metachromatic cells (% of total cells)
BG	14	1.1 ± 0.08	5.3 ± 0.24*
Basenjis	5	1.0 ± 0.08	2.2 ± 0.18*
Greyhounds	5	1.2 ± 0.08	1.1 ± 0.12*

*All three groups were significantly different from each other ($p < 0.02$ to $p < 0.001$).

METHODS

Study subjects

The study followed the published "Guiding Principles in the Care and Use of Animals," approved by the Council of the American Physiological Society.

Studies were performed in 14 BG dogs, five purebred Basenjis (allergic control dogs) and five greyhounds (non-allergic control dogs) (Table I). Dogs were of both sexes and ranged in age from 2 to 5 years. None of these animals had intestinal helminthiasis or microfilaria. The BG dogs and Basenjis, but not the greyhounds, displayed signs of atopy.¹⁰ An atopic dog was defined as one demonstrating at least three of the four features of atopic dermatitis and immediate skin test reactivity to commonly occurring allergens.¹³ The BG dogs used in this study were hyperresponsive to aerosols of histamine and methacholine compared to either the purebred Basenjis or the greyhounds.¹⁴

No dog received antigen, agonist, or therapeutic aerosol challenges for at least 4 weeks before BAL. Preliminary studies demonstrated that histamine releasability to A 23187 and C5 were similar after 4 weeks to that of dogs that had never received an aerosol challenge.

BAL

Dogs were anesthetized with thiopental (15 mg/kg) intravenously, intubated with an endotracheal tube, and mechanically ventilated throughout the procedure. A flexible fiberoptic bronchoscope (American Cystoscope Makers, Inc., Pelham Manor, N.Y.) with a 6-mm external diameter, introduced through the tube, was wedged into a segmental bronchus of the right middle lobe. A loading volume of 100 ml of sterile saline warmed to 37° C, to which was added antibiotic-antimycotic mixture (Gibco, Grand Island, N.Y.)

(with an ending concentration of 1 U/ml of penicillin, 1 µg/ml of streptomycin, and 0.002 µg/ml of amphotericin [Fungizone; E. R. Squibb, Princeton, N.J.]), and 2 U/ml of sodium heparin was introduced into the lobe through the bronchoscope and aspirated immediately into a collection trap by use of 50 to 100 mm Hg suction. Fifty-ml increments were then introduced and immediately aspirated until a total of 400 ml of fluid had been used. Consistently, 75% to 85% of the fluid introduced was recovered. BAL was performed in two dogs on each day, and cells from a BG and a control dog were processed simultaneously on most days.

Processing of fluid

The lavage fluids recovered from each dog were pooled and filtered through surgical gauze to remove any visible mucus. The remaining fluid was centrifuged at 400 g for 10 minutes at 4° C in 50-ml plastic centrifuge tubes (Corning Glass Works, Corning, N.Y.) in the swinging bucket rotor of a Beckman TJ-6 refrigerated centrifuge (Beckman Instruments, Palo Alto, Calif.). The supernatant was discarded, and cells were resuspended in 0.9% NaCl and washed. They were then centrifuged at 400 g for 10 minutes at 4° C. The resulting cellular pellet was resuspended in 1 ml of Gey's balanced salt solution (Gibco) and enumerated with a Coulter counter (Coulter Electronics; Hialeah, Fla.). An aliquot was taken for quantitation of metachromatic cells.

Quantitation of metachromatic cells

Cell counts were adjusted to 2 to 4×10^6 /ml, spread on slides in a cytocentrifuge (Shandon-Elliot, Sewickley, Pa.), fixed with Mota's solution (1% lead acetate, 47.5% ethanol, and 0.5% acetic acid) for 5 minutes, and washed with dis-