CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY 32, 142–150 (1984)

Histamine-Releasing Activity (HRA)

III. HRA Induces Human Basophil Histamine Release by Provoking Noncytotoxic Granule Exocytosis

ANN M. DVORAK, MICHAEL A. LETT-BROWN, DAVID O. THUESON, KATHRYN PYNE, PUTHALATH K. RAGHUPRASAD, STEPHEN J. GALLI, AND J. ANDREW GRANT

Departments of Pathology, Beth Israel Hospital and Harvard Medical School and the Charles A. Dana Research Institute, Beth Israel Hospital, Boston, Massachusetts 02215, and the Departments of Internal Medicine, Pharmacology and Toxicology, and Microbiology, University of Texas Medical Branch, Galveston, Texas 77550

Histamine-releasing activity (HRA) is an ~10,000–15,000 dalton, protease-sensitive factor that induces the rapid liberation of histamine from human basophils. Production of HRA by human peripheral blood mononuclear cells *in vitro* is augmented by concanavalin A or antigen, suggesting a mechanism whereby lymphocytes may regulate basophil mediator release *in vivo*. In order to determine whether HRA provokes conventional exocytosis of basophil granules or, alternatively, results in mediator release by some other mechanism such as vesicular transport or cytotoxicity, we investigated the ultrastructural features of human blood basophils purified over Percoll and exposed to HRA *in vitro*. HRA preparations induced a noncytotoxic pattern of basophil degranulation very similar to that previously observed in basophils triggered to release histamine in response to specific antigen, C5a, or mannitol. Thus, cytoplasmic granules were extruded singly hrough multiple separate points of fusion between perigranular membranes and the plasma membrane. Degranulating basophils exhibited plasma membrane activation but lacked a polarized configuration. By contrast, those basophils exposed to HRA that did not exhibit evidence of degranulation displayed a single elongated cellular process. The development of this polarized cellular configuration, similar in some respects to that of uropod-bearing motile guinea pig basophils, may have reflected chemokinetic or chemotactic effects of preparations containing HRA activity.

INTRODUCTION

Basophils have been identified in the inflammatory infiltrates accompanying a wide variety of delayed onset hypersensitivity reactions (1–6). During many of these processes, basophil cytoplasmic granules undergo a progressive and striking loss of dense content (7–9), changes likely to be accompanied by the liberation of histamine and other basophil-derived mediators. While the regulation of basophil mediator release in delayed reactions is probably complex (1–6), indirect evidence suggests that products of mononuclear cells are involved (1, 3–10). First, inflammatory processes containing partially degranulated basophils generally also include many mononuclear cells (1, 3–10). Second, mononuclear cells activated by specific antigen or other agents are known to elaborate several macromolecular factors with diverse biological effects, including basophil chemotaxis (11–13).

0090-1229/84 $1.50

We recently described a mononuclear cell product, histamine-releasing activity (HRA), that satisfies many of the requirements for a lymphokine capable of provoking basophil mediator release during delayed onset immunological reactions. HRA is an ~10,000–15,000 dalton, protease-sensitive activity elaborated by human peripheral blood mononuclear leukocytes that induces the rapid release of histamine from autologous or heterologous human basophils (14–16). The production of this factor by mononuclear cells *in vitro* is augmented by concanavalin A or by exposure to antigens that provoke cutaneous delayed hypersensitivity responses in the leukocyte donor (14). HRA can be distinguished by several criteria, including apparent molecular weight, from a variety of other mononuclear cell derived factors that provoke or augment basophil histamine release (15, 16), and antibodies that block histamine release induced by C5a or C3a have no effect on histamine release induced by HRA (15). We have shown that HRA and the previously described lymphokine, basophil chemotactic factor (BCF), have the same apparent molecular weight (13, 15) and that the two activities cannot be separated by ion-exchange chromatography (16). In addition, lymphokine activity purified by sequential Sephadex and CM-Sepharose C1-6B ion exchange chromatography showed one protein peak when analyzed by reverse phase high-performance liquid chromatography (Lett-Brown *et al.*, manuscript submitted). Although their precise chemical structures have not been defined, our results suggest that HRA and BCF activities may reside on very similar or even identical molecular species.

In contrast to our progress in understanding the physicochemical characteristics of HRA, and the factors controlling its production by mononuclear cells *in vitro*, the mechanism by which HRA induces basophil histamine release has been difficult to define. Previous ultrastructural analysis has demonstrated that basophil histamine release induced by specific antigen (17), the complement fragment C5a (18), or mannitol (19) reflects the ability of these agents to stimulate exocytosis (20); the direct fusion of membranes surrounding individual cytoplasmic granules with the plasma membrane. However, basophils may also be able to release mediators by a different mechanism, involving the transport of small quanta of granule-associated material from the granules to the plasma membrane within cytoplasmic vesicles (1, 3–10, 21). Because such apparent vesicular transport, or "piecemeal degranulation," has been observed in basophils participating in immunological reactions of delayed-onset, we have suggested that this pattern of mediator release may be regulated by lymphokines (1, 3–10, 21). Although HRA represents a prime candidate for such a lymphokine, our previous work was performed with populations containing only ~1% basophils and did not evaluate the nature of HRA-induced mediator release at the ultrastructural level. Indeed, because basophils represented such a minor fraction of these populations, even our tentative conclusion that HRA-induced histamine release reflected a noncytotoxic effect was based on indirect evidence (15). The recent development of a Percoll method for rapidly isolating viable peripheral blood leukocytes highly enriched for basophils (22) has now permitted direct ultrastructural analysis of the effects of HRA on human basophils. In the experiments reported here, we show that preparations containing HRA can induce basophil histamine release by

provoking conventional exocytosis of cytoplasmic granules, in the absence of basophil cytotoxicity.

MATERIALS AND METHODS

Preparation of leukocytes. Forty-five to 100 ml of peripheral venous blood from either of two normal volunteers was anticoagulated with 10 m*M* EDTA. A cell population enriched for basophils (12–39%) was then obtained by centrifugation on a Percoll layer as previously described (22). Briefly, 10 ml volumes of anticoagulated blood were carefully layered on 10 ml Percoll (density = 1.076) in 50 ml plastic tubes with conical bottoms. The tubes were centrifuged at 600*g* for 10 min at room temperature. Following removal of the plasma layer and mononuclear cell layer at the interface, the Percoll layer was removed separately. The Percoll layer was diluted with saline (137 m*M* NaCl, 5 m*M* KCl) buffered at pH 7.4 with 10 m*M* HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Sigma, St. Louis, Mo.) containing 0.3 mg/ml human serum albumin (fraction V, Sigma). The diluted Percoll layer was centrifuged and the cell pellet washed in HEPES buffered saline. Cells were resuspended (10×10^6/ml) in HEPES-buffered saline containing albumin, calcium (2 m*M*), and magnesium (1 m*M*).

Preparation of HRA. HRA was prepared in previously described (14–16) using human peripheral blood mononuclear cells stimulated with streptokinase–streptodornase (SKSD, Varidase, Lederle, Pearl River, N.Y.). These leukocyte preparations contain few, if any, neutrophils (usually <1%). Partial purification of HRA from supernatants was accomplished by Sephadex G-75 chromatography followed by CM-Sepharose C1-6B chromatography as previously described (16). The active fractions contained both HRA and the previously reported lymphokine, basophil chemotactic factor (BCF, 13). Data derived from molecular sieve and ion exchange chromatography suggest that HRA and BCF activities reside on similar or identical molecular species (Lett-Brown *et al.*, manuscript submitted), and the term HRA will be used to designate preparations containing both activities.

Release of basophil histamine. Aliquots (0.1 ml) of the basophil-enriched leukocyte suspension at 37°C were added to test tubes (Falcon No 2052) containing an equal amount of 6% perchloric acid (to obtain the total histamine content), buffer (to measure spontaneous histamine release), or HRA. The reactions were stopped at 3 min (Donor A) or 10 min (Donor B) by the addition of 1.8 ml buffer containing 10 m*M* EDTA at 4°C and were centrifuged (800*g*, 10 min) immediately at 4°C. The supernatants were assayed for histamine by the automated fluorometric technique (23).

Electron microscopy. In parallel experiments, reactions were carried out as above and stopped by the addition of dilute Karnovsky's fixative directly to the cell suspension (24). The cells were fixed for 1 hr at room temperature and washed in cold 0.1 *M* sodium cacodylate buffer, pH 7.4. Cells were then resuspended in 1 ml of Hanks' solution containing 0.5 mg of cationized ferritin (Miles Laboratories, Inc., ref. 25), an electron dense tracer that binds to negatively charged membranes exposed to the exterior, and placed on a rotary shaker at low speed for 30 min at room temperature. Cells were then washed again in 0.1 *M* sodium

cacodylate buffer, transferred to microtubes and centrifuged at 1500*g* for 1 min. The cell pellet was rapidly mixed with warm 2% agar and centrifuged again. The agar pellet was then postfixed for 2 hr at 4°C in 2% aqueous osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 *M* sodium phosphate buffer pH 6.0 (17, 18, 26) at room temperature for 2 hr prior to dehydration in a graded series of alcohols, infiltration, and embedding in a propylene oxide–Epon sequence. Sections were cut with a diamond knife, placed on copper grids, and stained with lead citrate. All basophils encountered in one randomly selected block from each sample (total basophils = 76) were photographed using a Philips 400T electron microscope and 8 × 10 in. prints were prepared. All basophils were analyzed for viability, cell shape, evidence of degranulation, and number of cytoplasmic granules.

RESULTS

Control Basophils. Basophils purified over Percoll appeared well preserved and viable. Nearly all such cells were round in configuration, with irregular, short surface processes, and contained a full complement of cytoplasmic granules uniformly filled with dense particulate material (Fig. 1A). Basophils incubated in buffer for 3 or 10 min maintained their round shape and intact cytoplasmic granules and released little histamine (3 and 2%, respectively).

Basophils exposed to HRA. HRA preparations induced the rapid, noncytotoxic exocytosis of basophil granules (Table 1, Figs. 1B, 2, 3A), a reaction that was observed in 14 of the 29 basophils studied in experiment A and 2 of the 12 examined in experiment B (total in the two experiments: 16/41 = 39%). In each

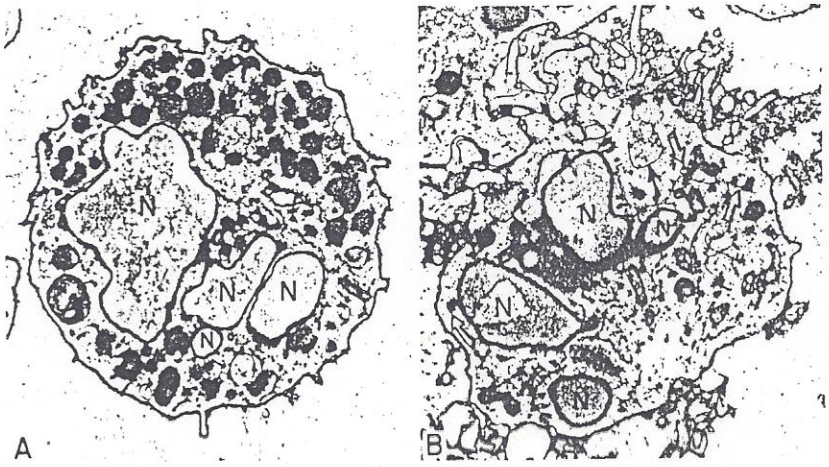

FIG. 1. (A) Control basophil from donor A incubated in buffer for 3 min at 37°C. The cell has a round shape and numerous intact particle-filled cytoplasmic granules. The extracellular tracer cationized ferritin is confined to the cell surface, which exhibits irregular short processes. (B) HRA-stimulated basophil from the same donor. After 3 min at 37°C, the cell exhibits evidence of degranulation. Granule extrusion is indicated by solid arrows. Note that the electron dense tracer, cationized ferritin, is attached to the opening of two granule-containing sacs. Although surface processes are markedly elongated and irregular in areas of granule release, the overall cell configuration appears round. N, Nucleus; open arrows, glycogen. (A) 14,000×; (B) 15,000×.

TABLE 1

HRA-INDUCED DEGRANULATION OR STIMULATION OF CELLULAR POLARIZATION IN HUMAN BASOPHILS[a]

		Percentage Histamine release	Round basophils		Degranulated basophils		Basophils with elongated process	
			% total (No.)	Granules/cell	% total (No.)	Granules/cell	% total (No.)	Granules/cell
A	Control, 0 min	NA[b]	100 (12)	19 ± 1.9	0	NA	0	NA
	Control, 3 min	3 ± 0	100 (14)	22.4 ± 2.4	0	NA	0	NA
	HRA, 3 min	32 ± 1[c]	0	NA	48 (14)	2.5 ± 1.6[d]	52 (15)	23.2 ± 3.0
B	Control, 0 min	NA	100 (5)	16.0 ± 2.5	0	NA	0	NA
	Control, 10 min	2 ± 0	100 (4)	17.2 ± 2.5	0	NA	0	NA
	HRA, 10 min	31 ± 2[c]	0	NA	17 (2)	0.5[e]	83 (10)	16.7 ± 3.4

[a] Seventy-six basophils from two donors (A and B) were photographed in the electron microscope. Basophils were classified as "degranulated," "round," or as having a single elongated process, and the number of granules present in each cell was determined (see text). Results are expressed as percentages, M ± SE, or M (granules/cell, HRA, 10 min).

[b] NA, Not applicable.

[c] $P < 0.001$ (Student's t test) when compared to corresponding control histamine release.

[d] $P < 0.001$ (Student's t test) when compared to any group of nondegranulated basophils (control, 0 min; control, 3 min; or HRA, 3 min). The differences among groups of nondegranulated basophils are not significant ($P > 0.05$).

[e] $P < 0.002$ vs any group of nondegranulated basophils. The other differences are not significant.

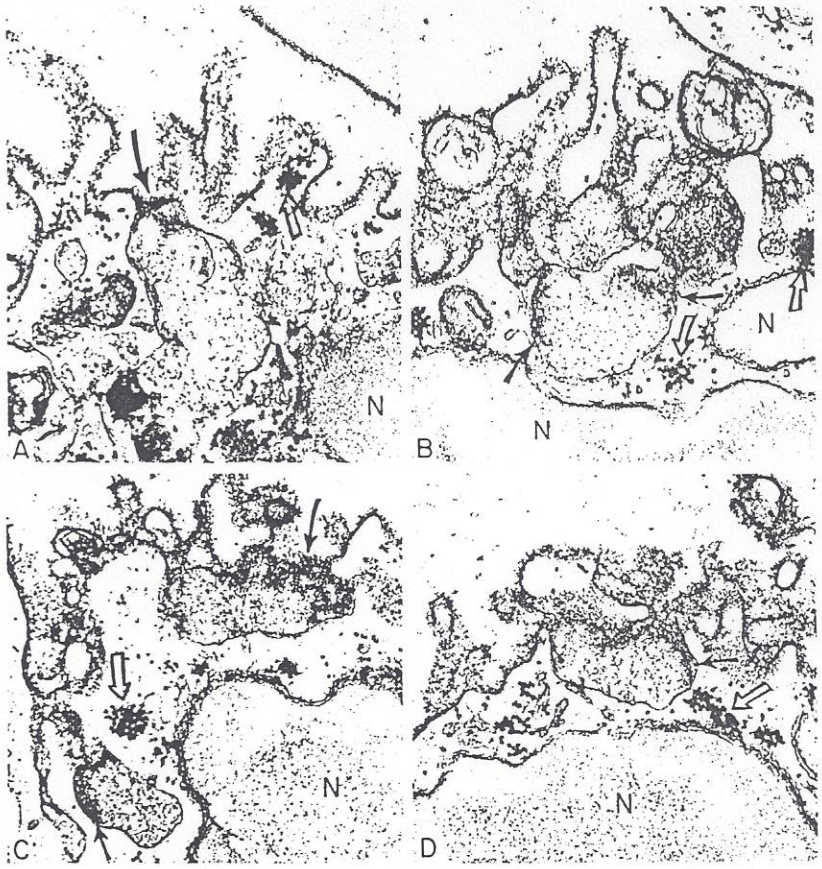

FIG. 2. Higher magnification micrographs of three separate basophils exposed to HRA for 3 min at 37°C (A, B are of separate regions of the same cell) showing granules whose particulate content is in continuity with the exterior. Granule content at the surface of narrow (A,B) or wider openings in the plasma membrane (C–D) is coated with the tracer cationized ferritin (arrows), which also stains the cell membrane. Open arrows, glycogen; N, nucleus. (A and B) 43,000×; (C and D) 41,000×.

experiment, exposure of basophils to HRA preparations resulted in a ~30% release of cell-associated histamine (Table 1). Basophils undergoing degranulation displayed prominent irregular surface processes, many of which partially enclosed membrane-free, extruded granules. As in basophil degranulation induced by antigen, most of the cytoplasmic granules were extruded to the exterior singly through multiple separate openings in the plasma membrane. Some of these areas of fusion between perigranular and plasma membranes were quite narrow at the time of cell fixation, but were readily demonstrated because they permitted entry of the electron dense tracer cationized ferritin (Fig. 2). Basophils undergoing anaphylactic degranulation formed aggregates with each other, other leukocytes in the preparation, and platelets, findings similar to those reported by Pruzansky *et al.* (27).

Some basophils exposed to HRA did not degranulate. These basophils contained approximately the same number of granules per cell as control basophils incubated in buffer (Table 1) but differed from control cells by displaying a single, thin, elongated surface process (Fig. 3B). Nondegranulated basophils in preparations exposed to HRA, like control basophils, did not form aggregates with other cells or platelets.

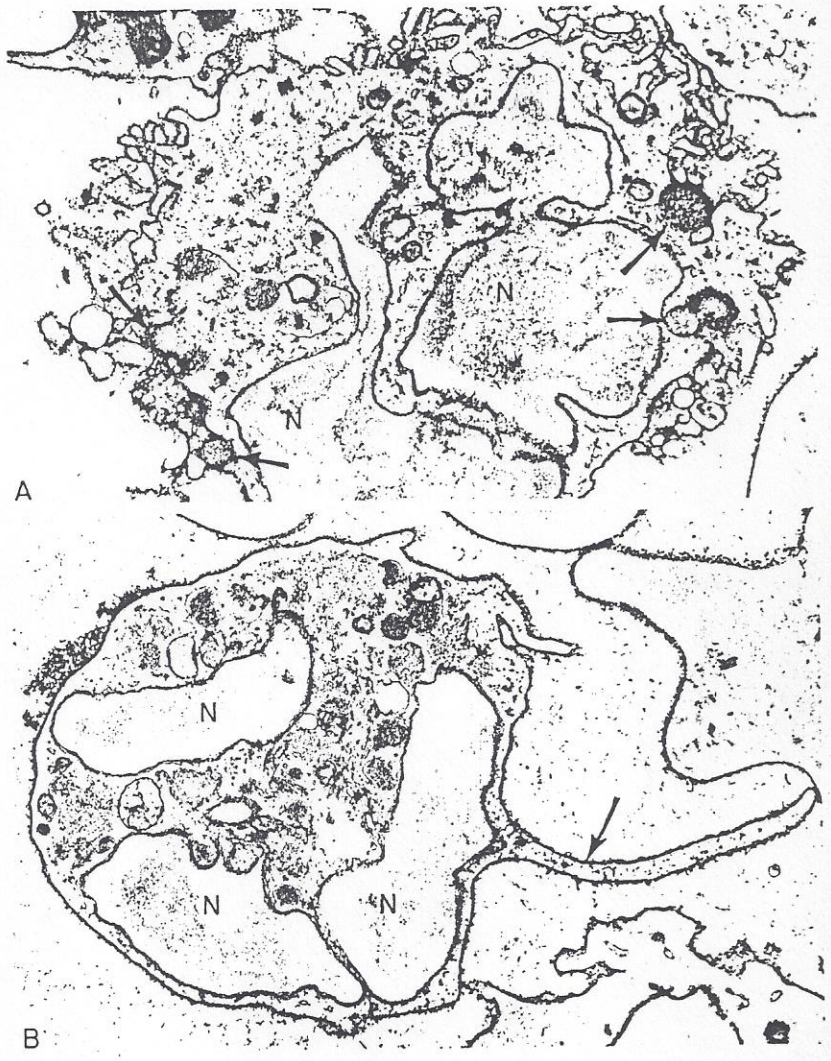

FIG. 3. Basophils from Donor A were incubated with HRA for 3 min at 37°C. All of the basophils in this preparation either exhibited degranulation (A) or displayed elongate surface processes (B). No basophils exhibiting both features were seen. The basophil undergoing degranulation (A) has numerous irregular surface processes. Many cytoplasmic granules are being extruded through multiple separate plasma membrane openings (arrows). The basophil in B displays one very elongated surface process (arrow), but no evidence of degranulation. N, Nucleus. (A) 20,500×; (b) 21,500×.

DISCUSSION

HRA arguably represents the best candidate for a lymphokine capable of provoking basophil mediator release during delayed onset immunologic reactions *in vivo*. We have demonstrated here that preparations containing HRA activity can induce a pattern of noncytotoxic basophil degranulation similar by ultrastructure to that observed in response to specific antigen (17), C5a (18), or mannitol (19). Basophils exposed to any of these agents *in vitro* undergo conventional exocytosis: the direct fusion of membranes surrounding individual cytoplasmic granules with the plasma membrane. Whether HRA can also induce nonexocytotic, vesicular transport of granule contents, for example, if used at lower concentration or in concert with other agents, remains an open question. The precise mechanism by which HRA preparations provoke basophil degranulation and mediator release also remain to be defined. For example, until basophil preparations of 100% purity are available, we cannot prove that HRA causes degranulation by a direct effect on basophilic leukocytes.

HRA preparations also contain BCF, and these activities may reside on very similar or identical molecules. Although this study was not designed to evaluate chemokinetic or chemotactic effects of HRA, we noticed that those individual basophils in populations exposed to HRA that did not exhibit degranulation developed an elongate cellular process reminiscent of the uropod-like structure we described previously in motile guinea pig basophils (28). We have reported that individual guinea pig basophils rarely if ever exhibit ultrastructural evidence both of granule exocytosis and motility (28, 29). Human basophils exposed to HRA also exhibit either degranulation or polarization, but not both, suggesting that these cells may not be able simultaneously to crawl and to degranulate. Phase microscopic observations of living human basophils are also consistent with this possibility (30).

In addition to defining the effects of HRA on basophil morphology, this study illustrates the excellent preservation of basophil ultrastructure achieved with the Percoll method of basophil purification. According to our previous classification of basophil morphology in "control" preparations of cells isolated over Ficoll–Hypaque (17), the unstimulated cells examined in this report would be characterized as type I, i.e., cells whose granules are filled uniformly with electron dense material. Similar results have been obtained recently with basophils isolated by a method based on the affinity of the cells to columns containing specific antigen (31, 32). By contrast, basophils isolated by a Ficoll–Hypaque method contained many cells whose granules appeared partially depleted of granule content (17). While these changes may have reflected the condition of the basophils *in vivo* (33), the possibility that they in part reflected effects of the Ficoll–Hypaque isolation method cannot be excluded.

ACKNOWLEDGMENTS

This work was supported by USPHS Grants CA 28834 and AI 12621.

REFERENCES

1. Dvorak, H. F., and Dvorak, A. M., *Human Pathol.* **3**, 454, 1972.
2. Askenase, P. W., *Prog. Allergy* **23**, 199, 1977.
3. Dvorak, A. M., *In* "Immediate Hypersensitivity, Modern Concepts and Developments" (M. K. Bach, Ed.), p. 369. Dekker, New York, 1978.
4. Galli, S. J., and Dvorak, H. F., *In* "Cellular, Molecular and Clinical Aspects of Allergic Disorders" (S. Gupta and R. A. Good, Eds.), p. 1. Plenum, New York, 1979.
5. Dvorak H. F., Galli, S. J., and Dvorak, A. M., *Int. Rev. Exp. Pathol.* **21**, 119, 1980.
6. Galli, S. J., Dvorak, A. M., and Dvorak, H. F., *Prog. Allergy* **34**, 1, 1984.
7. Dvorak, H. F., Mihm, M. C., Jr., Dvorak, A. M., Johnson, R. A., Manseau, E. J., Morgan, E., and Colvin, R. B., *Lab. Invest.* **31**, 111, 1974.
8. Dvorak, A. M., Mihm, M. C., and Dvorak, H. F., *J. Immunol.* **116**, 687, 1976.
9. Dvorak, H. F., Mihm, M. C., Jr., Dvorak, A. M., Barnes, B. A., Manseau, E. J., and Galli, S. J., *J. Exp. Med.* **150**, 322, 1979.
10. Dvorak, H. F., and Dvorak, A. M., *Clin. Haematol.* **4**, 651, 1975.
11. Boetcher, D. A., and Leonard, E. J., *Immunol. Comm.* **2**, 421, 1973.
12. Ward, P. A., Dvorak, H. F., Cohen, S., Yoshida, T., Data, R., and Selvaggio, S. S., *J. Immunol.* **114**, 1523, 1975.
13. Lett-Brown, M. A., Boetcher, D. A., and Leonard, E. J., *J. Immunol.* **117**, 246, 1976.
14. Thueson, D. O., Speck, L. S., Lett-Brown, M. A., and Grant, J. A., *J. Immunol.* **123**, 626, 1979.
15. Thueson, D. O., Speck, L. S., Lett-Brown, M. A., and Grant, J. A., *J. Immunol.* **123**, 633, 1979.
16. Thueson, D. O., Plank, D. E., Lett-Brown, M. A., and Grant, J. A. *In* "Human Lymphokines: Biological Immune Response Modifers" (A. Khan and N. O. Hill, Eds.), p. 229. Academic Press, New York, 1982.
17. Dvorak, A. M., Newball, H. H., Dvorak, H. F., and Lichtenstein, L. M., *Lab. Invest.* **43**, 126, 1980.
18. Dvorak, A. M., Lett-Brown, M., Thueson, D., and Grant, J. A., *J. Immunol.* **126**, 523, 1981.
19. Findlay, S. R., Dvorak, A. M., Kagey-Sobotka, A., and Lichtenstein, L. M., *J. Clin. Invest.* **67**, 1604, 1981.
20. Palade, G., *Science* **189**, 347, 1975.
21. Dvorak, A. M., Hammond, M. E., Morgan, E., Orenstein, N. S., Galli, S. J., and Dvorak, H. F., *Lab. Invest.* **42**, 263, 1980.
22. Raghuprasad, P. K., *J. Immunol.* **129**, 2128, 1982.
23. Siraganian, R. P., *J. Immunol Methods* **1**, 283, 1975.
24. Karnovsky, M. J., *J. Cell Biol.* **35**, 213, 1967.
25. Danen, E., Goldstein, L., Marikovsky, Y., and Skutelsky, E., *J. Ultrastruct. Res.* **38**, 500, 1972.
26. Dvorak, A. M., Hammond, M. E., Dvorak, H. F., and Karnovsky, M. J., *Lab. Invest.* **27**, 561, 1972.
27. Pruzansky, J. J., Chunghsin, T., Krajewski, D. V., Zeiss, C. R., and Patterson, R., *Immunology* **47**, 41, 1982.
28. Galli, S. J., Dvorak, A. M., Hammond, M. E., Morgan, E., Galli, A. S., and Dvorak, H. F., *J. Immunol.* **126**, 1066, 1981.
29. Dvorak, A. M., Osage, J. E., Dvorak, H. F., and Galli, S. J., *Lab. Invest.* **45**, 58, 1981.
30. Hastie, R., *Clin. Exp. Immunol.* **8**, 45, 1971.
31. MacGlashan, D. W., Jr., and Lichtenstein, L. M., *J. Immunol.* **124**, 2519, 1980.
32. MacGlashan, D. W., Jr., Lichtenstein, L. M., Galli, S. J., Dvorak, A. M., and Dvorak, H. F., *In* "Cell Separation: Methods and Selected Applications" Edited by (T. G. Pretlow, II and T. Pretlow, Eds.), p. 301. Academic Press, New York, 1982.
33. Dvorak, A. M., and Monahan, R. A., *Arch. Pathol. Lab. Med.* **106**, 145, 1982.

Received December 12, 1983; accepted with revision March 15, 1984.