

Reactions of primate respiratory mast cells

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Living respiratory mast cells were obtained from rhesus monkeys and maintained in short-term tissue culture preparations. The cells are detected with relative ease when lightly stained with toluidine blue by bright-field microscopy. Examination of the stained cells by dark-field microscopy provided a more striking definition of individual mast cell granules. These mast cells degranulated after in vitro exposure to Ascaris antigen, anti-IgE, or compound 48-80. The degranulation occurred as an intracellular phenomenon without other significant morphologic changes. With the use of anti-IgE, no definite correlation of respiratory mast cell reactivity with reverse passive respiratory or reverse passive cutaneous reactivity of animals was established. Intact mast cells excluded trypan blue, but degranulated mast cells were stained with trypan blue. The high percentage of mast cells reacting to 50 µg per milliliter of compound 48-80 provided a presumptive means of establishing whether or not a particular mast cell was alive subsequent to a failure to degranulate in response to another agent. Peroxidase was shown to be present in the primate respiratory mast cell granules. Cells from the monkey peritoneum were examined. Mast cells were not found by morphologic examination. There was no significant histamine content of the peritoneal cells.

Immediate-type cutaneous and respiratory reactivities to nematode antigens in rhesus monkeys have been demonstrated.¹ With the use of the technique of bronchial brush biopsy, methods for obtaining living mast cells from these animals were established.² The respiratory mast cells undergo morphologic changes consistent with degranulation following exposure in vitro to Ascaris antigen. Antisera against human IgE or IgG were sufficiently cross-reactive to rhesus immunoglobulins to result in degranulation of some respiratory mast cells.²

More recently, another in vivo immediate-type respiratory response in primates has been studied. This is an acute airway response to aerosolized anti-IgE, which has been termed reverse passive respiratory reactivity.³

The present studies had several objectives: The first of these was confirmation and extension of previous studies of the reactivity of the respiratory mast cells to anti-IgE and compound 48-80. Second, cell studies were done to determine whether the degree of in vitro cell reactivity to anti-IgE could be correlated with the presence or absence of respiratory reactivity of the intact animal to anti-IgE. Third, following exposure to anti-IgE, compound 48-80, or Ascaris

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antigen, cells were studied for evidence of cytotoxicity coincident with mast cell degranulation. Finally, the use of dark-field microscopy was evaluated as a method of study of living mast cells in respiratory tissue to determine whether it would provide greater ease of detection of mast cells and facilitate observation of the morphologic changes during degranulation.

METHODS AND MATERIALS

Animals

Three young adult rhesus monkeys were used. Two of these animals had marked cutaneous and respiratory reactivity to challenge with rabbit anti-IgE and to *Ascaris* antigen.¹ The third animal had no respiratory reactivity and less cutaneous reactivity to these types of challenge (Table I). The methodology of the respiratory challenge to anti-IgE and criteria for a positive respiratory response are described in detail elsewhere.³

Respiratory mast cells

Tissue was obtained from the respiratory tract of monkeys anesthetized with pentobarbital by the bronchial brush biopsy technique.² The animals had not received respiratory challenge with antigen or anti-IgE for at least 7 days. Following removal of the bronchial brush, the tissue was immediately teased onto glass slides and covered with tissue culture medium (medium 199, Grand Island Biological Co., Grand Island, N. Y.). Cover slips were applied and the cell preparations stored at 4° C. until use. All cells were examined within 6 hours of brush biopsy. Five minutes prior to use, a 0.0005 per cent solution of toluidine blue in tissue culture medium was added to the cell preparations. The cells were warmed to 37° C. and maintained at that temperature by a constant-temperature device (Air Curtain Incubator, Sage Instruments, Inc., White Plains, N. Y.). Reagents were added to cell preparations by a flow system previously described.² This consisted of having the cover slip suspended above the microscope slide by thin strips of petroleum jelly and gently flushing through the reagents while observing the mast cells.

Microscopy

Cell preparations were evaluated by bright-field microscopy, dark-field microscopy or by both methods. Because it was found that exposure of viable mast cells to intense light resulted in nonspecific degranulation of certain cells, prolonged observation of mast cells was avoided. The bright-field microscopy was done with a partially closed condenser. Dark-field microscopy was done with intermittent observation of cells with an avoidance of intense illumination. After these conditions were established, no nonspecific degranulation of viable mast cells by light was observed.

The criterion for considering a cell to be degranulated was that there was a marked decrease in the number of granules in the mast cell under direct microscopic examination. This was either complete degranulation or a loss of a large number of granules.

For evaluating intact mast cell morphology, slides with bronchial tissue were covered with toluidine blue in tissue culture medium and incubated at room temperature for 5 minutes. The slides were then washed with tissue culture medium, air dried, and mounted with glycerin.

Slides of bronchial tissue which were to be used for peroxidase staining were frozen immediately and stored at -80° C.

Antigens and antisera

Ascaris antigen was a preparation of *Ascaris suum* purified by Sephadex chromatography and standardized by cutaneous reactivity in *Ascaris*-sensitive animals.⁴ The standard *Ascaris* antigen (SAA) contained 0.2 mg. N per milliliter.

Anti-IgE used for in vitro reactivity of mast cells was prepared against the Fc portion of a purified myeloma protein as previously described.³

TABLE I. Characteristics of rhesus monkeys used as source of respiratory mast cells and effect of rabbit anti-IgE on viable cells

Characteristics	Animal		
	A	B	C
Reverse passive respiratory reactivity to aerosol challenge with anti-IgE ¹	Positive	Positive	Negative
Reverse cutaneous reactivity to challenge with anti-IgE*	10-4	10-5	10-2
Effect of anti-IgE on respiratory mast cells in vitro			
Concentration of anti-IgE			
1:100	7/7†	13/28	7/24
1:1,000	4/11	12/28	11/33
1:10,000	7/11	6/7	0/2
No. cells degranulated/total No. live cells observed	18/29	31/63	18/59
Per cent of cells degranulated	62	49	30

*Expressed as the highest serial tenfold dilution of rabbit antihuman IgE giving a positive cutaneous reaction.

†Expressed as number of cells degranulated per number of live cells observed.

Other reagents

Compound 48-80 was obtained from Burroughs Wellcome & Co. (Tuckahoe, N. Y.), and varying concentrations were prepared in tissue culture medium.

Trypan blue was obtained from Allied Chemical and Dye Corp. (New York, N. Y.), and a 0.5 per cent solution in tissue culture medium was prepared for addition to mast cell preparations.

Peroxidase stain

Mast cells were stained for the presence of peroxidase.⁵ The substrate solution contained 30 mg. per cent 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in Tris-buffered, phosphate-buffered saline, pH 7.6. For visualizing peroxidase, the substrate solution was prepared fresh each time, and immediately before use 3 drops of 3 per cent H₂O₂ were added to 5 ml. of the solution. Slides containing respiratory cells were flooded with the substrate solution and incubated for 5 minutes at room temperature. After the reaction, the slides were washed, dehydrated with graded alcohols, cleared, and mounted in Permount.

Histamine determination

This was done by the method of Shore, Burkhalter, and Cohn.⁶

Collection of peritoneal cells

Sterile phosphate-buffered 0.15M NaCl, 200 ml., pH 7.35, was infused into the peritoneal cavity of rhesus monkeys anesthetized with sodium pentobarbital. After 30 minutes of incubation and gentle agitation of the animal, the peritoneal fluid was withdrawn and centrifuged, and the sediment was evaluated for histamine content by chemical analysis and for mast cells by microscopic inspection of stained cells.

RESULTS

Morphologic changes in living mast cells

Viable respiratory mast cells stained lightly with toluidine blue and were maintained in short-term tissue culture with observation by standard light microscopy for periods of up to one hour. These cells showed no spontaneous

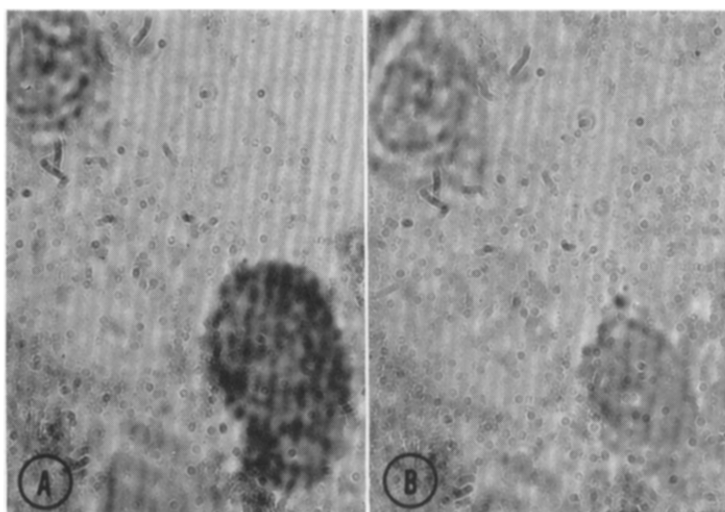


FIG. 1. Morphologic changes in respiratory mast cell in vitro seen by bright-field microscopy. **A**, before addition of *Ascaris* antigen; **B**, after addition of *Ascaris* antigen. Mast cell granules have disappeared and only the nucleus is seen. ($\times 1,600$)

morphologic changes under the conditions of the experiments. The addition of *Ascaris* antigen (1:100 or 1:1,000 SAA), anti-IgE, or compound 48-80 resulted in degranulation in many of the mast cells. This morphologic change began between 2 and 10 minutes after addition of the agent, was often rapid in its progress, and consisted of intracellular disappearance of the mast cell granules (Fig. 1). Pseudopods occasionally formed, cytoplasm with granules flowed into these pseudopods, and degranulation within the pseudopods occurred. During the observation of hundreds of degranulating mast cells, granules were at no time extruded outside of the mast cell membrane. Granules did not move from the inner portion of the cell to the cell membrane but disappeared at any location within the cell.

More than 250 cells were observed during the current experiments and were studied by one, often 2 or occasionally by 3 observers. No difference in the morphologic appearance of degranulation after exposure to *Ascaris* antigen, anti-IgE, or compound 48-80 was observed.

Intracellular motion of the mast cell granules was observed before and during the degranulation process, but no definite change in morphology or activity of granules was observed during the degranulation process. In some cells the degranulation was complete. These cells were known to be mast cells only because they were identified initially and were under continuous observation. In other mast cells the degranulation was incomplete and varied from localized degranulation to generalized degranulation with varying numbers of granules remaining.

Dark-field examination

In initial experiments, respiratory tissue was obtained in the usual manner, 0.0005 per cent toluidine blue added to the living cell preparations and a mast

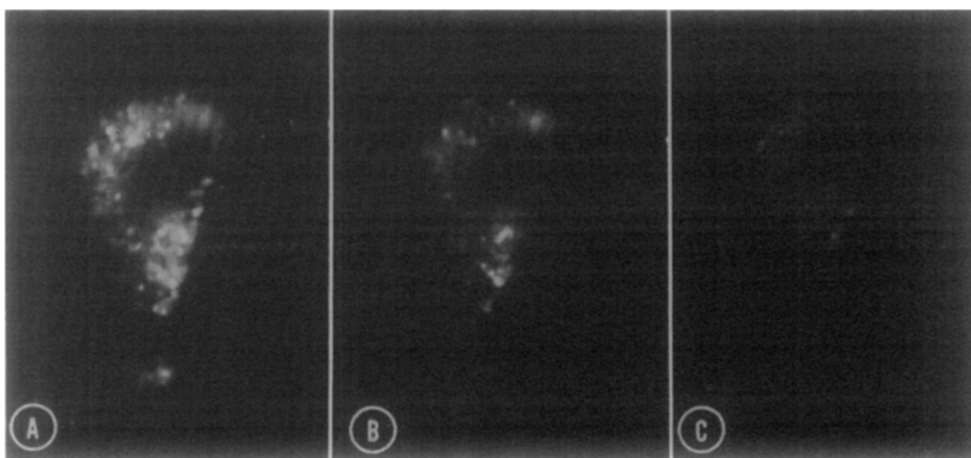


FIG. 2. Morphologic changes in respiratory mast cell in vitro seen by dark-field microscopy. **A**, Before addition of anti-IgE; **B**, 2 minutes after addition of anti-IgE; **C**, 10 minutes after addition of anti-IgE. (Original magnification X1,000.)

cell identified by bright-field microscopy. The same field was observed after conversion of the microscope to dark-field microscopy. The mast cells showed typical polymorphism and the granules were uniform in size. With the use of dark-field illumination, the mast cell granules were bright orange, which made them easily identifiable. The orange color was dependent upon prior staining with toluidine blue. That these were mast cells was confirmed by the morphologic changes which occurred in individual cells subsequent to the addition of *Ascaris* antigen, anti-IgE, and compound 48-80. These cell changes after exposure to anti-IgE and compound 48-80, as seen by dark-field microscopy, are shown in Figs. 2, 3, and 4. The granules of a dead mast cell are shown in Fig. 5. These are more distinct than in Figs. 2 to 4 because of absence of motion of granules during photography.

The advantages of dark-field examination of mast cells in the viable respiratory tissue preparations are as follows: (1) The mast cells were more easily identified in the live cell preparations because of the bright orange color contrasting with the dark background. (2) Individual mast cell granules were more easily observed, and motion of granules was particularly well defined. (3) Because the granules were more easily observed, the intracellular degranulation process was more easily defined. Individual granules could be seen to disappear within the cell subsequent to addition of the cell-stimulating agent.

Exposure of respiratory mast cells to trypan blue

Trypan blue was added to respiratory mast cell preparations prior to addition of degranulating agents, and most mast cells were found to exclude trypan blue. Mast cells which excluded trypan blue were exposed to *Ascaris* antigen, anti-IgE, or compound 48-80 and after degranulation were exposed again to trypan blue. These degranulated cells then stained, demonstrating that the same cells which excluded trypan blue prior to degranulation did not exclude trypan blue subsequent to degranulation. Mast cells which did not degranulate following

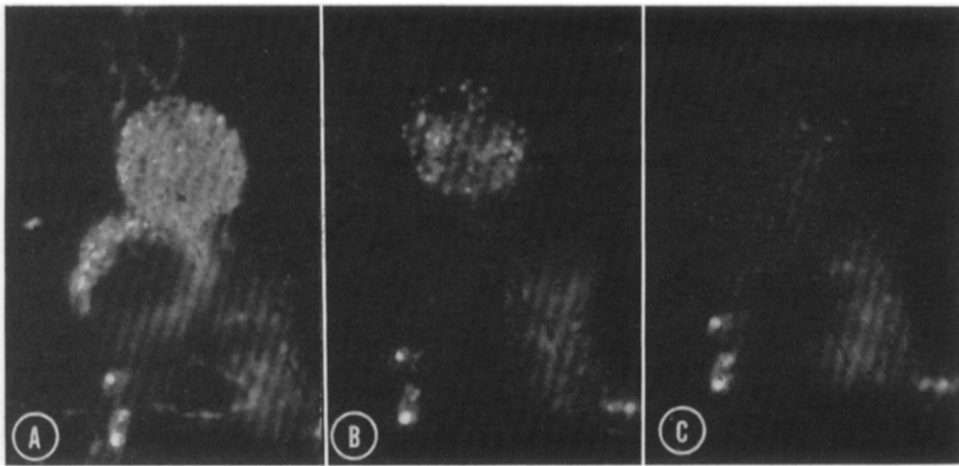


FIG. 3. Morphologic changes in 2 adjacent respiratory mast cells in vitro seen by dark-field microscopy. **A**, before addition of compound 48-80; **B**, 2 minutes after addition of compound 48-80. One partially degranulated cell remains visible; **C**, 8 minutes after addition of compound 48-80. A few granules of the upper cell are seen. (Original magnification $\times 1,000$.)

successive exposures to anti-IgE, *Ascaris* antigen, or compound 48-80 stained with trypan blue.

Reactivity of mast cells to compound 48-80, anti-IgE, and ragweed antigen

Anti-IgE. Rabbit antihuman IgE at three different concentrations was added to mast cells to determine whether or not significant differences in degranulation would result. Differences in activity were also looked for using mast cells from animals having different respiratory and cutaneous reactivity to anti-IgE. The results are shown in Table I. It might be suggested that mast cells from Animal C, which had no respiratory reactivity and minimal cutaneous reactivity to anti-IgE, were less reactive to anti-IgE. However, the results shown in Table I are interpreted as showing no consistent, reproducible differences in reactivity of the cells from different animals to several concentrations of anti-IgE.

In all experiments in which mast cells were exposed to reactants other than compound 48-80 and did not degranulate, the viability of unreacted cells was determined by the addition of compound 48-80, and a cell was recorded as negative only if viability was demonstrated by ability to react to compound 48-80 at a concentration of 50 μg per milliliter.

Compound 48-80. Respiratory mast cells from 3 monkeys were exposed to three concentrations of compound 48-80 to determine whether degranulation of all cells occurred at any of these three concentrations. The results (Table II) indicated that a progressively higher percentage of degranulation occurred with increasing concentrations of compound 48-80, and degranulation was almost complete at the 50 μg per milliliter concentration.

Ragweed antigen. All 3 animals had negative reactivity to intracutaneous testing with 1:1,000 or 1:10,000 concentrations of ragweed antigen. Exposure

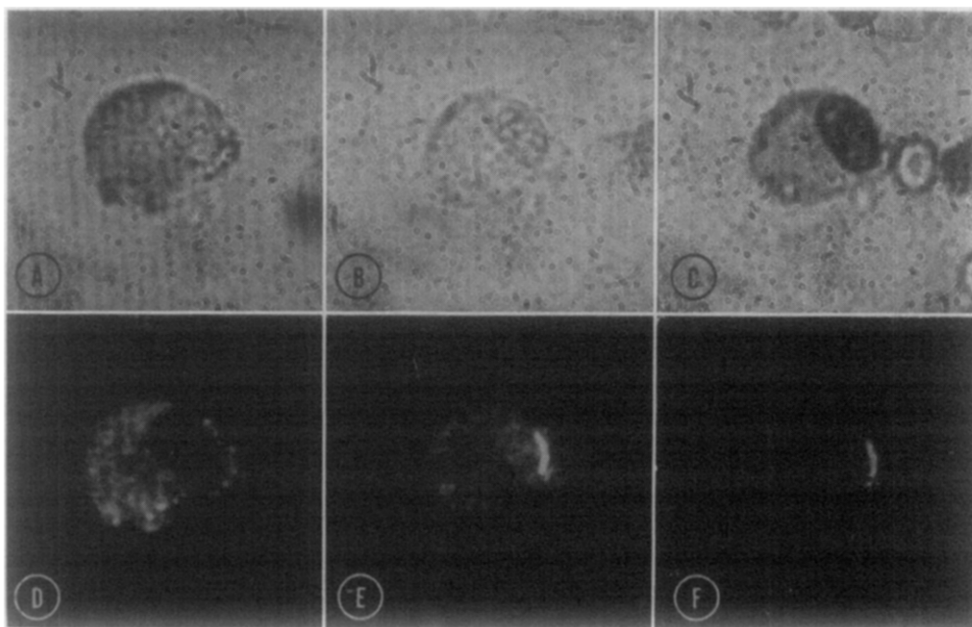


FIG. 4. Morphologic changes in the same respiratory mast cell in vitro seen by both bright-field and dark-field microscopy. **A, B, and C,** Bright-field illumination; **A,** before anti-IgE; **B,** 8 minutes after addition of anti-IgE; **C,** after addition of trypan blue. **D, E, and F,** dark-field illumination; **D,** before anti-IgE; **E,** 2 minutes after addition of anti-IgE; **F,** 8 minutes after addition of anti-IgE. (Original magnification X1,250.)

of mast cells to a 1:10,000 dilution of ragweed antigen resulted in degranulation of 5 of 46 mast cells (Table III).

Examination of mast cells for peroxidase.

This study was originated with the intent to demonstrate IgE on the surface of mast cells by using the immunoglobulin-peroxidase bridge method.⁵ In initial control studies in which mast cells were stained for peroxidase, it was found that the mast cell granules stained for peroxidase (Fig. 6). To confirm that these were mast cells, they were examined by dark-field microscopy, and the cells had the typical appearance of monkey respiratory mast cells, except for the absence of the orange hue seen after staining with very dilute toluidine blue. Peroxidase has been reported in mast cells of other species.⁷

Monkey peritoneal mast cells

Studies were done to determine whether a sufficient number of monkey peritoneal mast cells could be obtained to provide a larger supply of cells for both microscopic observation and studies of antigen- or anti-IgE-induced histamine release. Cells from peritoneal lavage were obtained from 2 separate animals, and no monkey peritoneal mast cells were identified. The histamine content of aliquots of monkey peritoneal cells containing 1×10^6 and 3.5×10^5

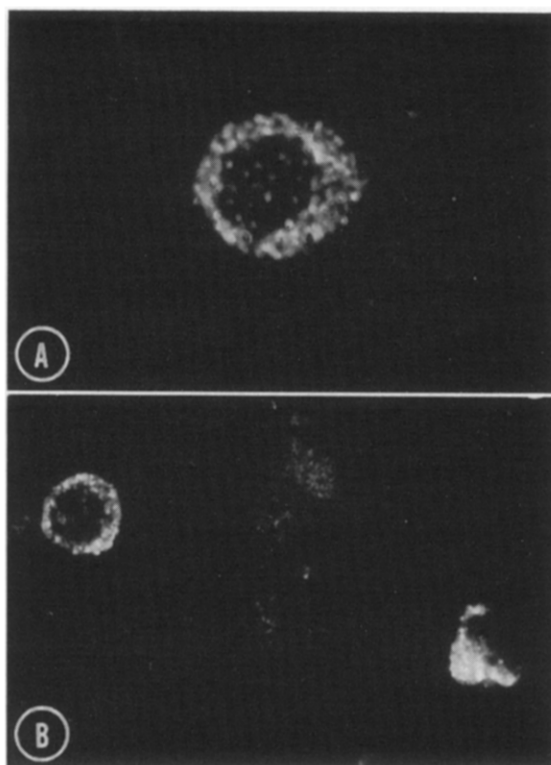


FIG. 5. Photomicrographs of dead mast cells with dark-field microscopy showing clearly defined granules. (A, $\times 1,250$; B, $\times 640$.)

cells, respectively, was determined and was not measurable by the method used. In contrast, rat peritoneal lavage producing 1×10^6 cells had $0.31 \mu\text{g}$ of histamine and approximately 4 per cent mast cells. These results suggested that the peritoneal cavity of the monkey is not a good source of material for quantitative studies of monkey mast cells and histamine release.

DISCUSSION

Studies of respiratory mast cells from rhesus monkeys have demonstrated the following: These primate mast cells can be obtained consistently and maintained for at least 6 hours in short-term tissue culture conditions. The mast cells may be obtained from the same animal on many different occasions without death of the animal.

The cell preparations are particularly suitable for observation of morphologic changes of individual mast cells, and a variety of stimuli have resulted in these cell changes. The question of whether a mast cell not responding to an antigen is nonreactive or dead may be answered by the effect of compound 48-80 on the cell. Since over 90 per cent of cells degranulate visibly with $50 \mu\text{g}$ of compound 48-80 per milliliter, this concentration of reagent was used as test of viability. A cell nonreactive to an immunologic agent is exposed to $50 \mu\text{g}$ per milliliter of compound 48-80, and subsequent

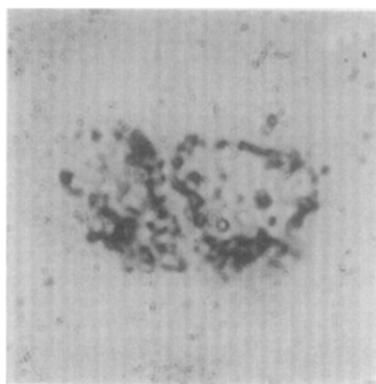


FIG. 6. Two adjacent mast cells stained for peroxidase. (X1,600.)

TABLE II. Changes in rhesus respiratory mast cells after exposure to compound 48-80

Concentration of compound 48-80 (ug/ml.)	Effect on respiratory mast cells*			Total
	Animal A	Animal B	Animal C	
5	0/4	8/17	0/9	8/30
10	6/11	7/8	3/3	16/22
50	3/3	53/61	36/36	92/100

*Expressed as cells degranulated per total number of cells observed.

TABLE III. Changes in rhesus respiratory mast cells after exposure to 1:10,000 ragweed antigen

Effect on respiratory mast cells*			
Animal A	Animal B	Animal C	Total
4/30	1/9	0/7	5/46

*Expressed as cells degranulated per total number of living cells observed.

degranulation is considered as evidence of viability. This is based on the assumption that the intracellular degranulation of a mast cell is an active process requiring a living cell.

Limitations of this primate respiratory mast cell system are that, under the conditions used, only one cell is usually present in any microscopic field, and only a few (3 to 5) cells in adjacent microscopic fields can be observed for degranulation during an individual experiment. This results in limitation of the usefulness of this system for studies of materials which might inhibit mast cell degranulation. Prior to initiation of these experiments, it was considered possible that close correlations between the respiratory reactivity, cutaneous reactivity, and the per cent degranulation of mast cells from an individual animal might be apparent. While such correlations may be possible in the future with different immunologic reactants, they were not established in these studies.

The degranulation of a small percentage of respiratory mast cells to ragweed antigen in the absence of cutaneous reactivity to this material is due

either to the occurrence of nonspecific reactivity of these cells in the in vitro conditions or to a small degree of specific immunologic reactivity, probably the former.

The examination of live mast cells under dark-field illumination provides a useful method of observing these cells. The individual mast cell granules were particularly well defined with dark-field illumination, and photography of degranulating cells resulted in somewhat better definition of mast cell granules than did bright-field microscopy. Examination of tissue mast cells by dark-field microscopy has previously been reported.⁸

In the current series of studies, the intracellular disappearance of mast cell granules was again observed. Pseudopod formation and a flow of granules was occasionally observed, but no extrusion of granules from the cytoplasm has been seen in the hundreds of degranulating mast cells observed. Morphologic observations of rat peritoneal cells⁹⁻¹¹ have been described as showing increased activity of intracellular granules, swelling of cells, a bubbling appearance of the granular cytoplasm, and extrusion of granules from the cells. With the possible exception of some cell enlargement and an occasional suggestion of increased activity of intracellular granules, no characteristic change other than progressive intracellular disappearance of granules was seen in these or previous experiments.

Staining of mast cells by trypan blue after degranulation may be interpreted as evidence of cell death or may reflect a change in permeability of the cell membrane subsequent to the degranulation process. The question of cell survival after degranulation remains unanswered.

The results of these experiments with respiratory mast cells may be compared with the recent description of degranulation of human basophil leukocytes from atopic subjects after exposure to grass pollen antigen.¹² In the latter experiments, cells were observed by phase contrast microscopy. Similarities in morphologic changes between the primate mast cells and the human basophils were the latent period prior to onset of cell changes,^{2, 12} the intracellular disappearance of granules in mast cells and basophils (although occasionally small particles were seen discharging from basophils¹²), and the intracellular appearance of vesicles at the sites of disappearance of the granules in both types of cells. The basophils were reported to show more changes in mobility and spreading¹² than were observed in the mast cell reactions.

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