STUDIES IN EXPERIMENTAL EOSINOPHILIA

VI. Uptake of Immune Complexes by Eosinophils

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ABSTRACT

A method is described whereby immune complexes may be visualized in a single cell. Bovine serum albumin labeled with a red-fluorescing dye was joined to a rabbit antiserum labeled with a green-fluorescing dye to yield an immune complex which fluoresced yellow when illuminated by ultraviolet light. Such yellow-fluorescing immune complexes were injected into the peritoneal cavity of guinea pigs, and the peritoneal exudates were examined subsequently. Yellow fluorescent particles were seen in eosinophils obtained from guinea pigs sensitized to hemocyanin and from normal animals. Eosinophils of the blood and of the bone marrow could also take up the complexes *in vitro*. Neither antigen nor antibody alone was taken up by eosinophils, nor was a mixture of labeled antigen and labeled normal globulin. Similar observations were made with human blood eosinophils. These experiments suggest that eosinophils act as part of the defense against the pathogenic effects of certain immune complexes.

A large body of both direct and circumstantial evidence indicates an association of the eosinophil with a variety of immune events, but its functions have remained obscure. Earlier studies have demonstrated that mixtures of antigen with antibody can mediate the accumulation of eosinophils at extravascular sites (1). It was not evident whether these complexes made physical contact with the cells or whether they generated some other tactic factor. Sabesin has shown recently (2) that, following the intraperitoneal injection of ferritin into guinea pigs which have been sensitized with this antigen, eosinophils of the peritoneal exudate contain ferritin surrounded by a zone of lesser electron opacity. The intracellular aggregates have the same appearance as ferritin-antibody complexes prepared *in vitro*. These experiments present a strong case for the uptake of immune complexes by eosinophils, although the evidence for the participation of antibody rests on the relatively non-specific property of moderate electron opacity. We have used immunofluorescent techniques to study more directly the uptake of immune complexes. This paper describes a method by which both antigen and antibody may be localized within the same cell and indicates, in addition, that complexes of the two gain entry into eosinophils.

MATERIALS AND METHODS

PREPARATION OF IMMUNOFLUORESCENT REAGENTS: Protein-dye conjugates were prepared by the method of Riggs *et al*, (3). Bovine serum albumin (BSA) in 1 per cent solution was reacted for 18 hours at 4°C in 0.05 M carbonate buffer, pH 8.5, with 20 parts (w/w) of tetramethylrhodamine isothiocyanate (TMRITC) (Baltimore Biological Laboratories, Baltimore, lot 203603). Unreacted dye was removed by column chromatography on Sephadex G-50 (Pharmacia, Ltd., Upsala, Sweden), and the protein was eluted with 0.0175 M phosphate buffer,

pH 6. A rabbit antiserum against BSA (anti-BSA), containing 868 μg antibody N/ml, was reacted with fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratories lot 210643) under similar conditions and was chromatographed on diethylaminoethyl (DEAE) Sephadex A-25 for separation of free dye and isolation of the labeled globulin fraction (4). Normal rabbit serum was also conjugated with FITC in similar fashion and the labeled globulin fraction isolated. The column eluates were concentrated to an appropriate volume by adding dry Sephadex G-25 to the BSA conjugate, and Sephadex G-50 to the antiserum and normal serum conjugates. The slurry was filtered under vacuum and the paste was washed with small portions of 0.85 per cent saline. The concentrated solutions were then dialyzed against 0.01 M phosphate buffer, pH 7.3, and stored at 4°C with 1:10,000 merthiolate as preservative. Each conjugate was analyzed for dye content by spectrophotometry at 495 mμ (FITC) and 550 mμ (TMRITC). The protein content was determined spectrophotometrically at 277 mμ in 0.25 N acetic acid, and a correction applied for the absorption due to the respective dye. The BSA-TMRITC contained 1200 μg N/ml; the anti-BSA–FITC conjugate, 315 μg antibody N/ml (3020 μg Protein N/ml); and the normal rabbit globulin-FITC conjugate, 3000 μg N/ml.

The above stock conjugates were combined or diluted prior to injection. 0.04 ml BSA-TMRITC was incubated with 0.04 ml anti-BSA–FITC at room temperature for 30 minutes. This yielded a mixture containing 48 μg antigen N and 13 μg antibody N, which represents about a twenty-fold excess of the amount of antigen needed to precipitate this amount of antibody at equivalence. Just prior to injection, 0.2 ml of saline was added in order to increase the volume. A similar mixture was prepared with normal rabbit globulin-FITC in place of anti-BSA–FITC. Labeled antigen and labeled antibody were also diluted with 0.2 ml of saline prior to injection.

Interaction of Fluorescent Conjugates

WITH PERITONEAL EXUDATE CELLS: In order to increase the likelihood of collision between eosinophils and test materials, recipient guinea pigs having a large population of peritoneal eosinophils were used. The eosinophilia was induced by 12 to 15 weekly injections of 2.5 mg of hemocyanin per dose (5). 24 hours following the last sensitizing injection, fluorescent conjugates were injected into the peritoneal cavity. 60 minutes later, the peritoneal exudate cells were harvested with 15 ml of isotonic saline as the lavaging fluid. The cell suspension was immediately centrifuged at 800 RPM in a Servall SS-1 centrifuge; the pellet was resuspended in 3 drops of saline, and coverslip smears were prepared.

WITH WHITE BLOOD CELLS: Blood from normal guinea pigs was collected in a plastic syringe moistened with heparin by cardiac puncture, and centrifuged for 30 minutes at 3000 RPM. The buffy coat was suspended in 2 ml of autologous serum and incubated in roller tubes for 1 hour at 37°C with 0.05 ml BSA-TMRITC and 0.05 ml of anti-BSA–FITC (the cells were exposed thereby to soluble complexes formed at twenty-fold antigen excess). After incubation, the cells were washed twice with 5 ml of saline to remove extracellular conjugate; coverslip smears were then prepared.

Blood from a patient with a drug sensitivity and a 50 per cent peripheral eosinophilia was obtained by venipuncture. The buffy coat was handled in the same manner as the guinea pig material, except that it was incubated with antigen alone, antibody alone, and antigen + normal globulin, in addition to incubation with antigen-antibody complex.

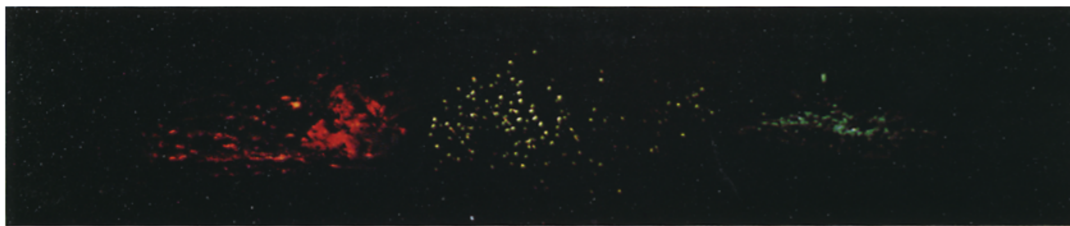
WITH BONE MARROW CELLS: A 5 x 2 mm fragment of femoral bone marrow from a normal guinea pig was agitated in 10 ml of heparinized (0.02 mg/ml) saline to provide a suspension of cells. The heavier clumps were removed by a 1-minute spin at 500 RPM and the remaining cells were concentrated by centrifugation for 5 minutes at 1000 RPM. The pellet was suspended in 2 ml of autologous serum and incubated in the same fashion as the blood cells.

PREPARATION OF SMEARS: Smears were fixed in methanol for 5 minutes, air-dried, and mounted with 10 per cent glycerin in phosphate-buffered saline, pH 7.3. The smears were examined by ultraviolet illumination with a UG-1 exciter filter and a Zeiss No. 41 barrier filter. Microphotographs were made on Kodak High Speed Ektachrome Film, Daylight Type, with 5 mm BG-12 exciter filter and Zeiss No. 47 barrier filter.

Satisfactory fixation of the smears in methanol made it possible to observe the same cell first under ultraviolet illumination, and then under tungsten illumination following staining by a conventional hematologic stain. After selected microscopic fields were photographed under ultraviolet illumination, a map was drawn with a camera lucida, showing cell outlines and the location of intracellular fluorescence. The coverslips were then demounted, washed in water, air-dried, and stained with Wright's stain. Particular cells were relocated with the aid of the camera lucida map and with the photographs. The morphology of the cell was restudied and a photograph taken under tungsten illumination, using Kodak High Speed Ektachrome Film, Type B.

RESULTS

Preliminary experiments conducted on footpads of guinea pigs revealed foci of green and red fluorescence at the site of deposition of anti-BSA–FITC



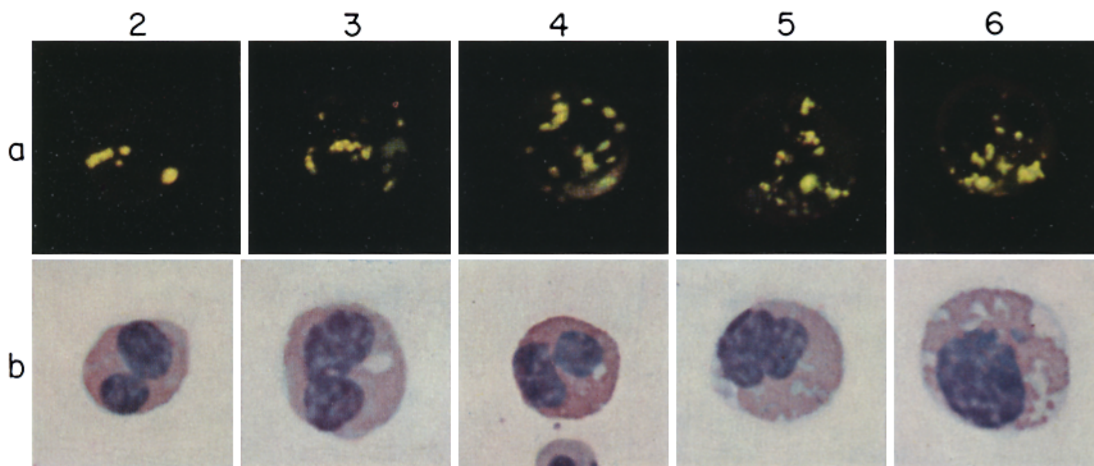


FIGURE 1 Cross-section of guinea pig footpad 30 minutes after the subcutaneous injection of BSA and anti-BSA. The BSA was labeled with TMRITC and fluoresced red-orange under ultraviolet illumination (left side of section). The anti-BSA was labeled with FITC and fluoresced green (right side of section). In the zone of intersection of antigen with antibody (center of section), deposits which fluoresce yellow may be seen and represent antigen-antibody complexes. (At this magnification, all three zones could not be encompassed in one field; the figure is a composite of three adjacent fields). × 165.

FIGURES 2 to 5 Eosinophils from peritoneal exudates of guinea pigs 1 hour after exposure *in vivo* to doubly-labeled BSA-antibody complexes. Row *a*: When viewed under ultraviolet illumination, the cells are visible by virtue of the steel gray autofluorescence of their granules. The brighter deposits colored yellow-green represent intracellular immune complexes. Row *b*: The same cells viewed after Wright's staining. One can distinguish agranular areas which correspond, in general, to the location of the fluorescent deposits seen in Row *a*. Wright's stain. × 1530.

FIGURE 6 Unmatched cells from similar exudates. In Fig. 6*a*, a large cluster of immune complexes is present in the cytoplasm of an eosinophil. Fig. 6*b* shows an eosinophil with many agranular areas. Wright's stain. × 1530.

and BSA–TMRITC, respectively (Fig. 1). In areas where the two colors merged, a new color, yellow, appeared and usually took the form of aggregates of yellow particles. If the two labeled reagents were mixed *in vitro* and viewed in ultraviolet light, the mixture appeared yellow in test tubes, in smears, and at the site of injection into footpads.

The results obtained when cells reacted with labeled proteins depended primarily on whether the antigen or antibody was available to the cells alone or in combination. After the intraperitoneal

injection of BSA–TMRITC alone, fluorescent particles with an orange-red hue were detected in the cytoplasm of peritoneal mononuclear cells within an hour (Table I). Similarly, green fluorescence was noted in mononuclears when only anti-BSA–FITC was introduced into the peritoneal cavity. In neither instance was specific fluorescence visible in eosinophils. However, when a mixture of labeled antigen and antibody was injected, fluorescent particles appeared in eosinophils as well (Figs. 2 to 6, row *a*). The particles were predominantly yellow, with hues ranging from yellow-green to yellow-white. The size ranged from barely discernible points of light to areas several microns in their largest dimension. The edges were usually discrete but the shape of the particles varied; round, oval, and irregular forms were seen. Between 10 per cent and 50 per cent of the eosinophils in an exudate contained at least one such inclusion, and some cells contained from two to more than twenty yellow spots. The inclusions could be distinguished readily from the specific granules of the cell by their shape, size, number, and color. Although these yellow particles were distributed randomly in all parts of the cytoplasm, they appeared, at times, to overlie the nucleus and frequently surrounded the nucleus to give the appearance of a figure eight. Fluorescent intracytoplasmic inclusions were also seen in mononuclear cells, where they manifested a greener coloration, were smaller and more numerous, and were homogeneously distributed in the cytoplasm. In one guinea pig, samples of peritoneal exudate were obtained every 5 minutes in the first hour after injection of labeled complexes. To provide sufficient volume for sampling, 25 ml of lavage fluid was introduced 4 minutes after injection of the complex. Intracytoplasmic yellow inclusions in eosinophils were noted in the 10 minute sample and in each subsequent sample.

TABLE I

Phagocytosis of Fluorescent Protein by Leucocytes of Guinea Pigs

Material injected			Color of test material	Recipient		Source of cells	Proportion of guinea pigs showing phagocytosis	
BSA–TMRITC	Anti-BSA–FITC	NRG–FITC		HCY-sens.	Normal		By eosinophils	By mononuclears
+			Red-orange	+		Peritoneal	0/4	4/4
	+		Green	+		Peritoneal	0/4	4/4
+	+		Yellow	+		Peritoneal	5/5	5/5*
+	+		Yellow		+	Peritoneal	4/5	5/5*
+		+	Yellow	+		Peritoneal	0/4	4/4*
+	+		Yellow		+	Blood	4/4	4/4*
+	+		Yellow		+	Bone marrow	4/4	4/4*

Cells were exposed to test materials for 60 minutes, except those from one of the sensitized guinea pigs exposed to labeled complexes, in which case samples were taken every 5 minutes. Abbreviations: BSA-TMRITC, bovine serum albumin labeled with tetramethylrhodamine isothiocyanate; Anti-BSA–FITC, rabbit anti-BSA labeled with fluorescein isothiocyanate; NRG-FITC, normal rabbit serum globulin labeled with fluorescein isothiocyanate; HCY-sens., sensitized by 12 to 15 weekly injections of 2.5 mg of hemocyanin each.

* Color of intracytoplasmic inclusions was green-yellow.

These results were obtained in animals sensitized to hemocyanin and exposed to the test materials 1 day following the last sensitizing injection. In order to ascertain whether this activity of eosinophils was peculiar to hypersensitized animals, the experiments were repeated in normal guinea pigs, in whose peritoneal cavities small numbers of eosinophils reside regularly (5). It was found that peritoneal eosinophils obtained from normal animals also took up the yellow fluorescent material presented to them *in vivo*. (Table I)

In an effort to determine at what point the cell acquires the ability to take up immune complexes, *in vitro* experiments were carried out with white blood cells obtained from the buffy coat of whole blood and with bone marrow cells in suspension. After incubation with the labeled immune com-

plexes, eosinophils of the blood were seen to contain particulate yellow fluorescence. Similarly treated bone marrow eosinophils also contained yellow fluorescent material after 1 hour of incubation (Table I).

Human eosinophils derived from blood buffy coat exhibited similar reactivities. They did not contain specific fluorescent material when either the antigen or the antibody was presented to them *in vitro*. However, fluorescent yellow particles were seen in the cells after a 1 hour incubation with the doubly-labeled complexes. No fluorescence was observed in human eosinophils when labeled normal globulin was substituted for the antibody.

There is little difficulty in recognizing eosinophils by darkfield ultraviolet illumination. They stand out by virtue of the steel gray autofluorescence of their granules and the characteristic, usually bilobate, nucleus. Verification of the identity of selected cells was provided by staining subsequently with conventional methods; cells in which yellow fluorescent material had been detected showed the typical morphology of eosinophils when Wright's stain was employed (Figs. 2 to 5, row *b*). The Wright-stained preparations revealed, in addition, that the areas of the cell in which fluorescence had been seen were devoid of granules and were characterized by a pale blue color, which is typical of the cytoplasm of this cell. There was a striking correspondence between the sites of fluorescence localization and these "holes" in the cytoplasm. Agranular areas were noted in many cells which had not been singled out during the observations of fluorescence; these cells presumably also contained immune complexes (Fig. 6*b*).

A yellow fluorescent solution also resulted when BSA–TMRITC was mixed with normal rabbit globulin-FITC. This mixture was identical with the yellow immune complexes, except for the absence of antibody. When this mixture was introduced into the peritoneal cavity of guinea pigs, none of the eosinophils subsequently isolated contained yellow fluorescence. These results are similar to those obtained *in vitro* with human eosinophils.

DISCUSSION

The introduction of immunohistochemical techniques has made it possible to locate either antigens or antibodies in cells. An antigen can be located with a fluorescent antibody (6), while an antibody can be located if the specific antigen is applied before the fluorescent antibody (7). However, it has not proved possible to demonstrate the coexistence of both antigen and antibody in the same cell, because the deposition, during the staining procedure, of a labeled conjugate on either member of the original antigen-antibody complex necessarily interferes with any subsequent attempts to demonstrate the other member of the pair. In such an instance, antigen may be demonstrated by the usual technique, while antibody may be implicated indirectly by the immunofluorescent demonstration of host globulin (some of which presumably is specific antibody) in a neighboring histologic section (8). Alternatively, specific antibody may be stained in a neighboring section by the use of fluorescent antigen. The latter method usually results in lesser fluorescence (compared with the "sandwich" technique). Neither method can localize both reactants in the same cell. The present paper describes a method by which immune complexes may be detected histologically by virtue of a unique fluorescent color. The combination of a red-fluorescing antigen with a green-fluorescing antibody produced a complex which fluoresces yellow both *in vitro* and *in vivo*. *In vivo*, generation of the yellow color could be seen in the zone of intersection of the green and red colors; moreover, the yellow complex had a particulate form, probably as a result of microprecipitation. Since the yellow color appeared only when the red antigen and green antibody were mixed, it can be attributed to an immune complex.[1] If one wishes to study the active participation of the host, this method is not suitable. But in situations in which a particular event involves the participation of antigen combined with antibody, and in which the event may be initiated by passive transfer techniques, this method of visualizing immune complexes may have a general usefulness.

These experiments also show that eosinophils take up immune complexes, since particulate yellow fluorescence could be seen in these cells. Since the background was devoid of any extracellular fluorescence, the fluorescence associated with the cells must have been either inside the

[1] In the instance when the labeled globulin is normal, the yellow color is not particulate, nor does it indicate an immune complex.

cells or bound to their surface. The tendency for the fluorescence to localize away from the periphery of the cell and in the plane of focus of nucleus and granules clearly places the material within the cell.

It is unlikely that these findings were due to any methodological artifact. The complications which arise from freezing, embedding, and sectioning were obviated by extremely simple processing of the exudate following removal from the animal. Non-specific staining did not present any problems, since the animals had been presented with labeled materials and *in vitro* staining procedures were unnecessary. Earlier experiments in this laboratory with conventional direct and indirect immunohistochemical techniques had failed to reveal the presence of either antigen or antibody in the eosinophils of experimentally induced peritoneal exudates. The work was rendered particularly difficult by problems of non-specific staining. The eosinophil has a notorious proclivity to combine non-specifically with fluorescent conjugates, and the various maneuvers to eliminate this (*e.g.* by tissue powder absorption, or by column chromatography on DEAE-cellulose) do not always prove satisfactory in work with this cell. Direct tracing techniques avoid this problem.

Since the (exudate) experiments were performed *in vivo*, there is reason to consider the results as having physiologic importance. Sensitization is clearly not a prerequisite for phagocytosis by eosinophils. Furthermore, this property is already present in the bone marrow, the source of most of these cells, which implies that bone marrow eosinophils are functionally mature in terms of phagocytic activity. In earlier papers, it was shown that immune complexes, formed with either serum (1) or tissue (9) antibody, acted as an eosinophilotactic stimulus. Circumstantial evidence was reviewed which suggested that the circumstances in which one encounters eosinophilia are those in which immune complexes are probably available. The present experiments extend these observations to indicate what these cells do once they arrive at the scene of an immune event; they incorporate immune complexes, the material which attracts them. These findings are in accord with the electron microscopic observations reported by Sabesin (2), but the evidence for the entry into eosinophils of the antibody moiety of the complex is of a more specific nature. In addition, the present data represent the first indication that complexes of a soluble nature are phagocytized by eosinophils. Indeed, it has not been possible, heretofore, to document the intracellular localization of such substances in any cell, except by indirect methods.[2]

It has become increasingly apparent that soluble immune complexes play an important role in the initiation of a number of pathologic states (10). It is now evident that eosinophilic leucocytes are endowed with the capacity to wall off these noxious agents by phagocytizing them and, thus, seem to be involved in defensive maneuvers. Neutrophils (11) and mononuclears (12) can perform the same function. The distinguishing features of the eosinophil's role *vis-à-vis* immune complexes will be the subject of a forthcoming communication.

This work was supported by grant E-4716 from the United States Public Health Service and grant 506 from the Greater Boston Chapter of the Massachusetts Heart Association.

The author is an Established Investigator of the American Heart Association. This work was initiated during the tenure of a Fellowship from the Helen Hay Whitney Foundation.

The author is indebted to Miss Daisy Killer and Miss Elisabeth Tappolet for excellent technical assistance, and to Mr. Leo Goodman for assistance with the microphotography.

Received for publication, July 21, 1963.

[2] Since this paper was first submitted, Archer and Hirsch (13) have reported observations made by phase microscopy which indicate that horse eosinophils can phagocytize antigen-antibody *precipitates*.

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