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Intraleucocytic Immunoglobulin in Eosinophilia in Man

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Summary. The washed buffy coat smears of fifteen normal subjects and forty-three patients with eosinophilia were examined by the immunofluorescence technique for the presence of intracellular immunoglobulin. Immunofluorescence was not present in any of the normal buffy coat smears. Five of the patients with eosinophilia (including a patient with eosinophilic collagenosis) demonstrated intraleucocytic immunofluorescence which was blocked by specific unconjugated antisera. In four of these patients there was reactivity with anti-IgG but not with anti-IgA or anti-IgM, while in the one patient with eosinophilic collagenosis there was intraeosinophilic fluorescence with anti-IgA only.

INTRODUCTION

Eosinophilia is associated with such diverse diseases as drug reactions, parasitic infestations, collagen vascular disease, atopic disease and certain malignancies (Pourfar, 1968). *In vitro* and animal experiments have shown that eosinophils are phagocytic cells and that antigen–antibody complexes may be chemotaxic for eosinophils (Litt, 1961; Litt, 1962; Sabesin, 1963; Litt, 1964; Zolov and Levine, 1969; Archer, Nelson and Johnston, 1969). While this property is also shared by aggregated protein (Cohen, Sapp, Rizzo and Kostage, 1964; Cohen and Sapp, 1965), complex polysaccharides (Cohen and Sapp, 1963) and other particulate material (Kostage, Rizzo and Cohen, 1967), antigen–antibody reactions are likely to be the significant factor common to the eosinophilic states encountered clinically.

A case of extreme eosinophilia of unknown aetiology, with clinical and autopsy findings suggestive of eosinophilic collagenosis, prompted investigation into the possibility that these cells were phagocytosing antigen–antibody complexes. As the nature of the postulated antigen was unknown, an attempt was made to demonstrate immunoglobulins in the cytoplasm of the eosinophils by the fluorescent antibody technique. Subsequently, other patients with eosinophilia were studied in a similar manner.

PATIENTS AND METHODS

Buffy coat smears were prepared from fifteen patients with normal white blood cell counts and from forty-three patients with eosinophilia (over 500 eosinophils/mm^3). Venous blood was anti-coagulated with EDTA and allowed to sediment spontaneously at room temperature; the leucocyte-rich supernatant plasma was withdrawn, the cells were washed twice in phosphate buffered saline and suspended in 0·5 per cent bovine serum albumin. The cells were then spread on standard microscope slides.

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Fluorescein conjugated antisera specific for immunoglobulins G, A and M* were adsorbed with beef liver powder and diluted 1 in 4 with phosphate buffered saline. Specific fluorescent antibodies to IgG and IgA were blocked by adding the appropriate immunoglobulin for use in control tests. Immunoglobulins were separated from the sera of patients with multiple myeloma exhibiting large M peaks of either IgA or IgG. After precipitation of the globulins from the sera with 18 per cent sodium sulphate and dialysis against phosphate buffered saline, the immunoglobulins were fractionated with DEAE Sephadex as outlined by Stanworth (1960), and their specificity was confirmed by immunoelectrophoresis.

Fluorescein and protein content of the blocked and unblocked fluorescent antibodies was estimated with a Zeiss spectrophotometer using the nomogram of Wells, Miller and Nadel (1966), (Table 1). Bovine serum albumin was added to the unblocked reagents to equalize the protein content of the blocked and unblocked stains.

TABLE 1

FLUORESCEIN AND PROTEIN CONCENTRATIONS OF FLUORESCENT ANTIBODIES

Fluorescein conjugated antibodies	FITC* (μg/ml)	Protein (mg/ml)	F/P† (μg/mg)	F/P molar
Anti-IgA	27·5	2·15	12·5	5·2
Anti-IgA (blocked)	25·0	3·75	6·1	2·5
Anti-IgG	18·3	2·10	8·8	3·7
Anti-IgG (blocked)	21·0	2·60	8·0	3·4
Anti-IgM	19.3	3·40	5·7	2·3

* FITC = Fluorescein isothiocyanate.
† F/P = Fluorescein: protein ratio.

Before use, the antisera were adsorbed with a film of Sephadex G-25 and mixed with an equal volume of a 1:800 dilution of a 2 per cent solution of Congo Red as described by Dr C. Teplity (Litt, personal communication). In spite of the addition of Congo Red as a counterstain, it was found necessary to treat the buffy coat smears with 0·01 N hydrochloric acid for 30 seconds prior to staining to prevent non-specific fluorescence (La Brec, 1965; Hokenson and Hansen, 1966).

The slides were stained for 30 minutes with blocked and unblocked fluorescent antibodies and washed for 1 hour in phosphate buffered saline. The slides were mounted with buffered glycerine and visualized with a Zeiss photomicroscope using ultraviolet illumination with a BG 12 exciting filter and 53/44 barrier filters.

RESULTS

The smears from the 15 normal blood samples failed to show fluorescence suggestive of specific staining for immunoglobulins (Table 2). In spite of the procedures devised to reduce non-specific staining, cells from a few patients showed a very slight degree of generalized cytoplasmic fluorescence. As the intensity of this fluorescence was similar using blocked and unblocked antiglobulins, it was felt to represent non-specific staining.

Five of the smears prepared from patients with eosinophilia showed cytoplasmic fluorescence which was negative in control tests, using blocked antisera (Table 2).

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A patient with extreme eosinophilia had marked eosinophilic cytoplasmic fluorescence with fluorescein conjugated anti-IgA only and this fluorescence was blocked by prior addition of IgA to the antisera (Fig. 1). This patient had the clinical picture of eosinophilic collagenosis (Odeberg, 1965; Pierce, Hosseinian and Constantine, 1967), with congestive heart failure, cardiomegaly and an initial leucocytosis of 20,000/mm^3 (65 per cent eosinophils). This increased during the 2½ years of his disease to a maximum leucocyte count of 60,000/mm^3 with 80 per cent eosinophils. Blast cells were never seen in the peripheral blood and there was no increase in primitive cells in the marrow. The serum immunoglobulin levels are shown in Table 3.

TABLE 2

RESULTS OF LEUCOCYTE STAINING WITH FLUORESCENT ANTIBODIES

	No. of patients	Specific cytoplasmic fluorescence		
		IgA	IgG	IgM
Eosinophilia	43	1	4	—
Controls	15	—	—	—

TABLE 3

PATIENTS WITH POSITIVE CYTOPLASMIC FLUORESCENCE

Patient	Age	Eosinophil count/mm^3	Clinical diagnosis	Serum immunoglobulins (mg/100 ml)		
				IgG	IgA	IgM
E.J.	41	22,844	Eosinophilic collagenosis	1275	600	80
C.C.	20	538	Idiopathic thrombocytopenic purpura	—	—	—
T.B.	20	586	Bronchial asthma	—	—	—
C.K.	51	2036	Bronchial asthma	—	—	—
E.C.	44	1301	Drug reaction (ampicillin)	2250	290	100

Four other patients with eosinophilia exhibited positive fluorescence when buffy coat preparations were stained for IgG (Table 2). Cells from one of these patients are shown in Fig. 2. Staining for IgA and IgM failed to demonstrate significant fluorescence.

The leucocytes of the remainder of the patients in this group failed to fluoresce significantly when stained for IgG, IgA and IgM. These smears were obtained from patients with a variety of diseases including bronchial asthma, malignancy, drug reactions, during recovery from pneumonia, thyroiditis, and rheumatoid arthritis.

In the patient with eosinophilic collagenosis where most white cells were eosinophils, the number of fluorescent cells indicated that eosinophils were responsible for the fluorescence (Fig. 1). In the four other patients with a positive test, the staining pattern suggested that the nuclei of most of the positive cells were bilobed, though some were trilobed. To study these cells further, identical fields were compared after Romanowsky staining (Fig. 3). Although the acidic treatment prior to staining had disrupted the granules, most of the

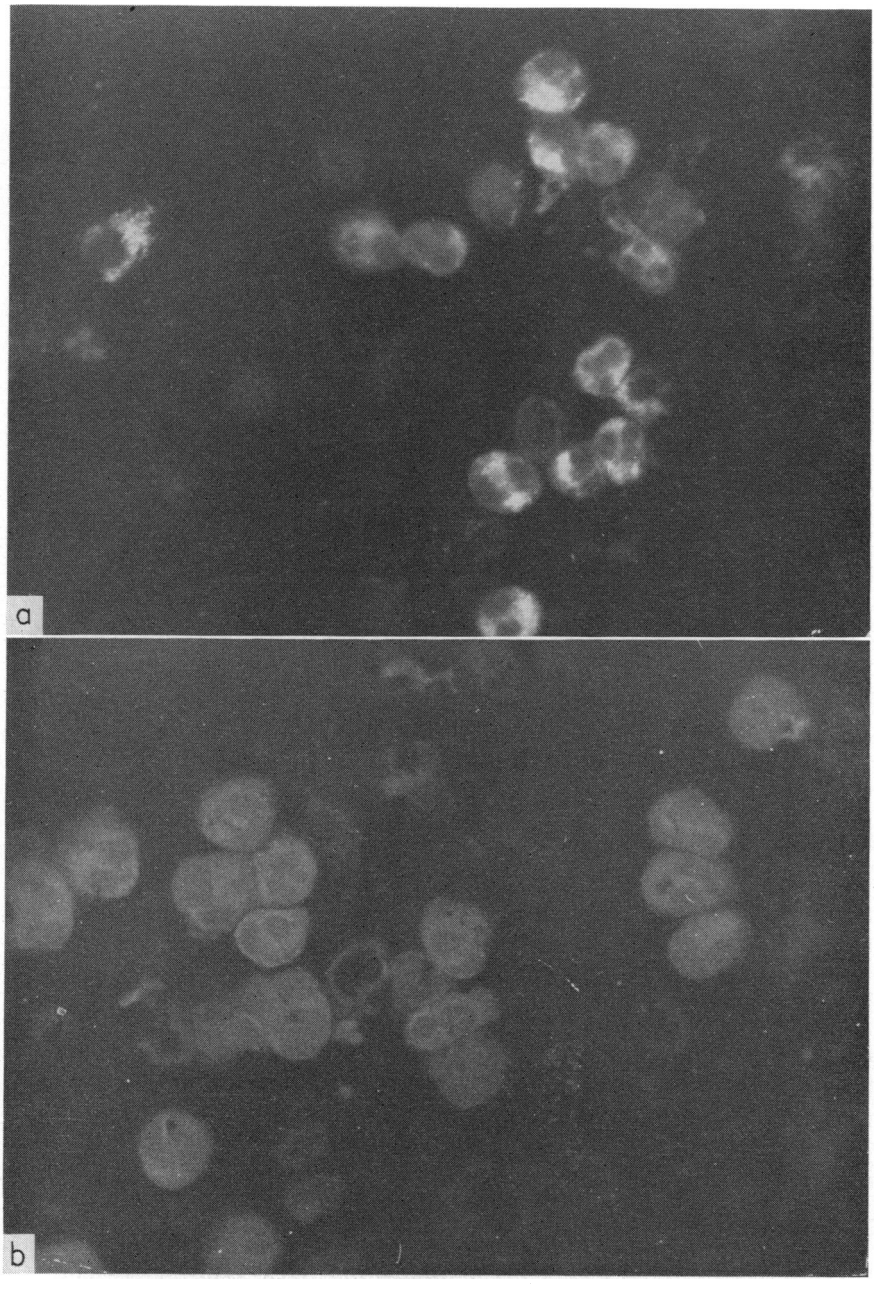

FIG. 1. Patient E.J. (Eosinophilic collagenosis) buffy coat smear stained for IgA. (a) Unblocked, (b) blocked by the addition of IgA to the fluorescent antibody.

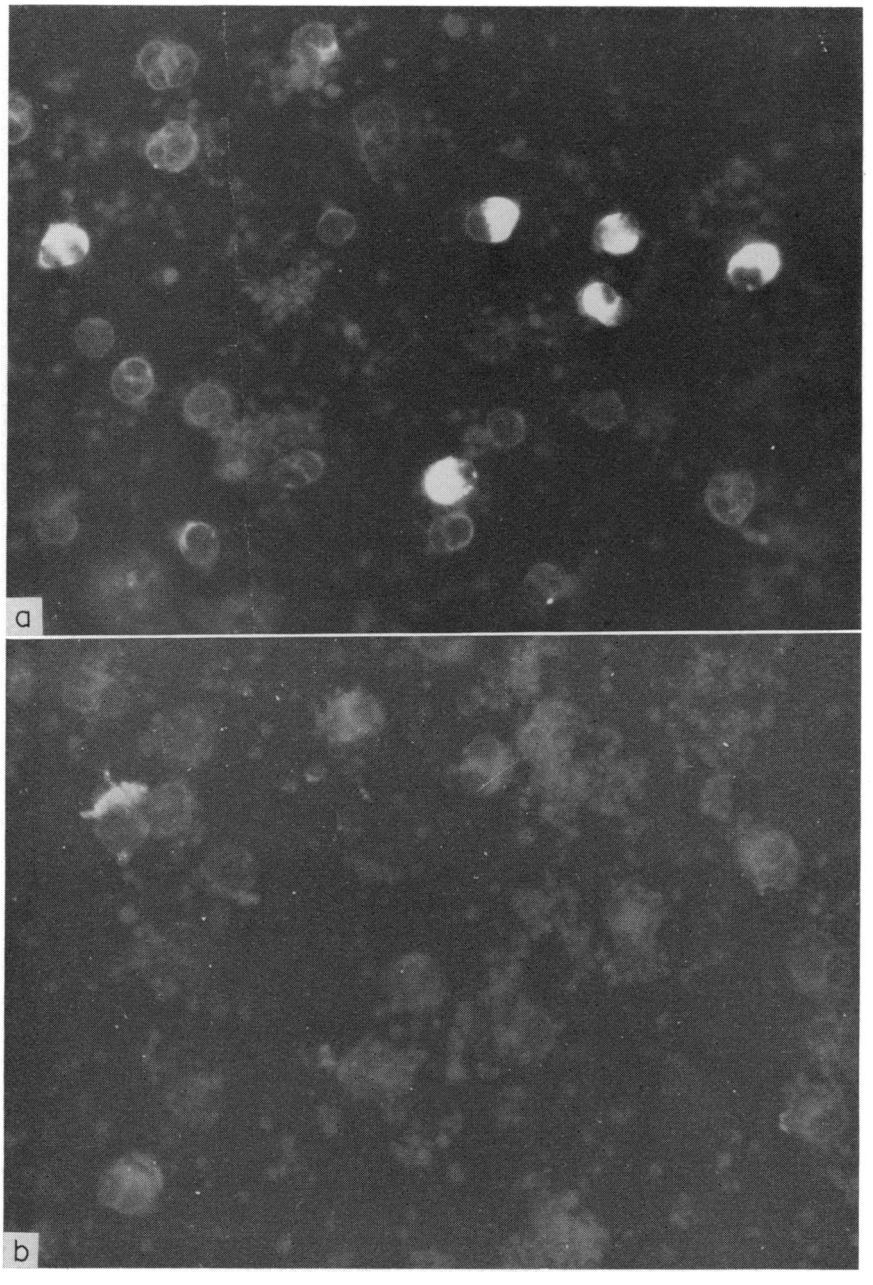

FIG. 2. Patient C.C. (idiopathic thrombocytopenic purpura) buffy coat smear stained for IgG. (a) Unblocked, (b) blocked by the addition of IgG to the fluorescent antibody.

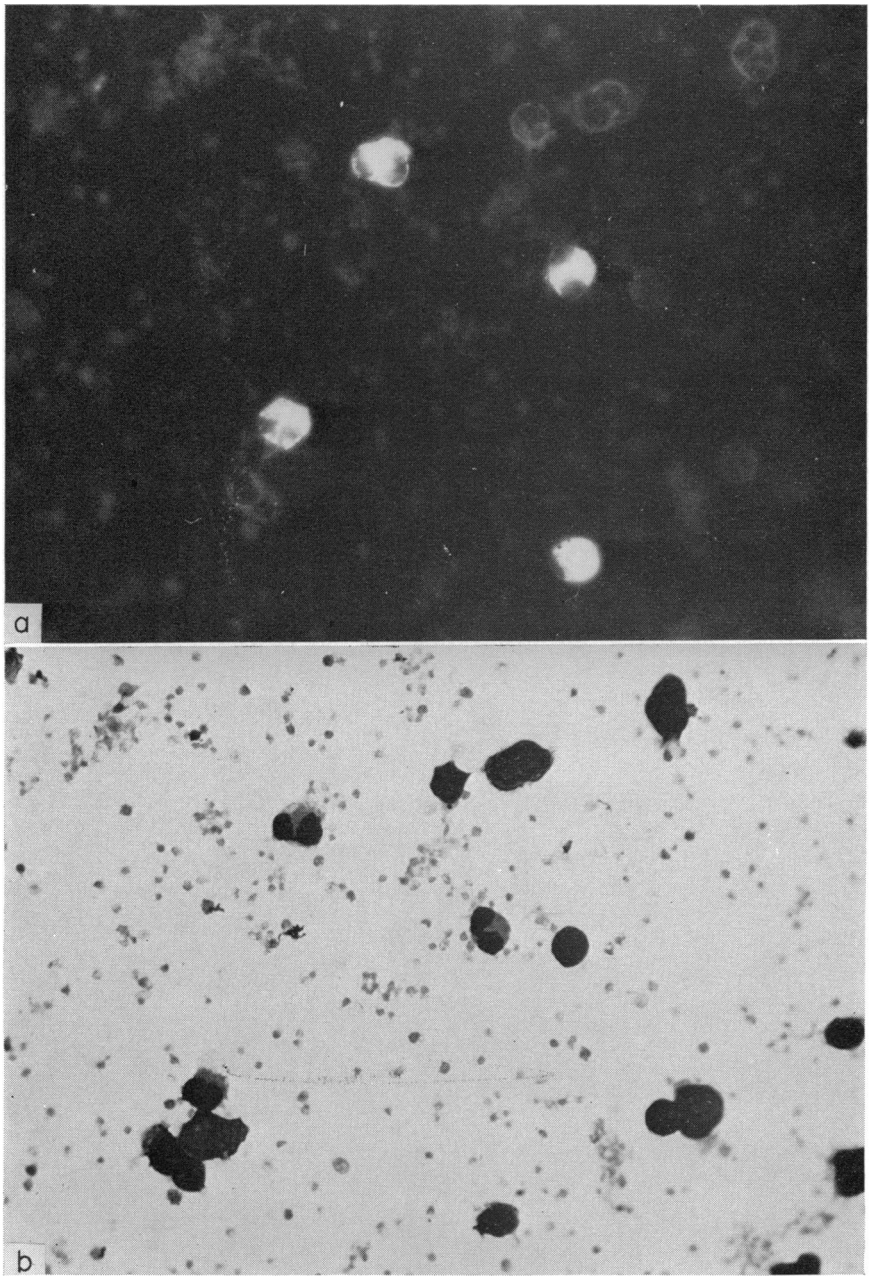

FIG. 3. Fluorescent cells in identical field restrained with Romanowsky stains for identification in Patient E.C. Three of the fluorescent cells are bilobed.

nuclei of fluorescing cells were bilobed, indicating that many of them were eosinophils. However, the presence of some fluorescent trilobed cells suggested that some could be neutrophils. No fluorescing lymphocytes or monocytes were identified.

DISCUSSION

The causation of eosinophilia and the function of the eosinophil is still poorly understood. Animal experiments have shown that injection of native globulin fails to produce eosinophilia, whereas aggregated or denatured γ-globulin or antigen–antibody complexes produce local eosinophilia (Cohen *et al.*, 1964; Cohen and Sapp, 1965). Recent observations by Spiers and Turner (1969) have indicated that the stimulus for eosinophilia resides primarily in antigenic material, and production or administration of specific antibody is inhibitory both for the immune response and for eosinophilia. While many substances such as fibrin and fibrinogen (Archer, 1968), mucopolysaccharides (Cohen and Sapp, 1963), glycoproteins and even latex particles (Kostage *et al.*, 1967), are capable of inducing eosinophilia, clinically eosinophilia is commonly seen in diseases associated with antigen–antibody reactions.

Eosinophils are phagocytic cells, although they do not appear to be as active as neutrophils in this regard (Cline, Hanifin and Lehrer, 1968; Morton, Moran, Dimitrov and Falor, 1966). Intracytoplasmic vacuoles have been found in eosinophils and are more prevalent in cells of patients with allergies and in conditions involving tissue damage (Connell, 1968). Degranulation occurs during the process of phagocytosis (Archer and Hirsch, 1963). Proteolytic enzymes are probably released into phagocytic vacuoles during this process, resulting in digestion of phagocytosed material (Kelenyi, 1968).

In the present study, five of forty-three patients with eosinophilia demonstrated positive fluorescence for immunoglobulins in the cytoplasm of leucocytes. No fluorescence was seen in the smears of fifteen patients with normal leucocyte counts.

Interpretation of the significance of the cytoplasmic fluorescence is complicated by the fact that leucocytes may bind fluorescent antibodies non-specifically (Beutner, 1961; Rappaport, 1964). However, specificity of the fluorescence seen in this study is suggested by successful blocking of the antiglobulins, by the absence of fluorescence in some cells in the positive smears, and by the production of positive fluorescence by certain fluorescent antibodies and not by others.

If the fluorescence is specific, its distribution suggests that immunoglobulin molecules are present in the cytoplasm of the leucocytes.

As granulocytes are phagocytic cells, the implication of the demonstration of immunoglobulins in their cytoplasm is that they were phagocytosed. *In vitro* studies have shown that native γ-globulin is not phagocytosed by these cells. However, there is phagocytosis of γ-globulin which has been aggregated, denatured, or complexed to antigen (Cohen *et al.*, 1964; Cohen and Sapp, 1965). Conceivably the cytoplasmic fluorescence could be produced by immunoglobulin molecules which were partially metabolized or degraded *in vivo*. This appears to be unlikely, as the antigenic sites required to combine with the fluorescent antibodies must have been relatively intact. The possibility that immunoglobulin molecules may have aggregated and been phagocytosed *in vitro*, during the process of preparing the buffy coat smears, cannot be excluded but appears unlikely.

The most likely interpretation and the one most compatible with known functions of eosinophils and granulocytes is that the fluorescence was produced by immunoglobulin

that was complexed to antigen and the antigen–antibody complexes were then phagocytosed. Whether these antigen–antibody complexes were chemotactic to eosinophils or induced the eosinophilia is of course unknown. While antigen–antibody complexes would be expected to be confined to phagocytic lysosomes, the pattern of fluorescence in some cases suggested a more generalized cytoplasmic distribution. It is possible that acidic treatment distorted the pattern of distribution of immunoglobulin in the cells to produce the more generalized fluorescence.

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