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Monoclonal antibodies to human eosinophil plasma membrane antigens enhance the secretion of eosinophil cationic protein

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SUMMARY

This study was done to examine the nature of the membrane constituents involved in the secretion of eosinophil cationic protein (ECP) from human blood eosinophils. Three mouse monoclonal antibodies were used, which showed greater binding to membrane antigens on activated, and light density eosinophils from patients with an eosinophilia, than on nonactivated or normal density eosinophils. All three antibodies (EoN4, EoN5 & EoN6) stimulated normal density human eosinophils to secrete ECP, either alone or in association with sepharose-C3b. The antibodies bound to at least two separate sites on the membrane, which were distinct from the receptors for immunoglobulins, C3b, and eosinophil activating factor. One combination of antibodies increased the amount of ECP which was secreted. The membrane antigen recognized by antibody EoN4 was a glycoprotein, molecular weight 75 kD. These findings showed that ECP secretion may be induced by a wider range of stimuli than has been previously recognized, and that the antigens recognized by these monoclonal antibodies may play an important role in the induction of eosinophil degranulation.

Keywords eosinophil monoclonal antibodies membrane antigens eosinophil cationic protein activation

INTRODUCTION

Many of the biological effects of eosinophils are now known to result from the secretion of their stored granule contents (Spry & Tai, 1984). In eosinophils, the secretory process is thought to involve at least two separate steps, including solubilization of the granule contents, followed by their release to the outside of the cell (Tai & Spry, 1980). Eosinophil secretion has been induced *in vitro* by antibody, and C3b-coated particles, and the calcium ionophore A23187 (Herman *et al.*, 1979), and eosinophil activating factor (EAF) (Thorne *et al.*, 1985). Granulocytes have a number of surface glycoproteins in common with other leucocytes (Banga *et al.*, 1984), and several which are not present on other cell types. This has been shown by direct surface labelling of both human eosinophils (Andersson & Gahmberg, 1978), and mouse eosinophils (Burgess *et al.*, 1980), and with monoclonal antibodies to human and mouse eosinophil membrane antigens (Zola *et al.*, 1981; Ball *et al.*, 1982; Foon *et al.*, 1983; Lopez, Strath & Sanderson, 1984; Lopez & Vadas, 1984; Tai *et al.*, 1985). A number of membrane constituents have been defined on cultured eosinophils by Thorne and her colleagues (Thorne & Franks, 1984), and some were shown to alter after the cells had

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interacted with immune complexes (Thorne, Oliver & Glauert, 1980; 1981; Thorne *et al.*, 1982), and parasite antigens (Thorne, Richardson & Butterworth, 1984). Although the role of plasma membrane antigens in regulating eosinophil function is not known, Lopez & Vadas (1984) have recently described the capacity of an antibody (to a 110 kD glycoprotein antigen on blood eosinophil and neutrophil plasma membranes), to increase the granulocyte-dependent killing of antibody-coated tumour cells. This suggested that some granulocyte differentiation antigens detected by monoclonal antibodies are involved in the induction of effector functions.

No lineage-specific membrane antigens have been detected on human (or mouse) eosinophils, using monoclonal antibodies, but three mouse monoclonal antibodies (EoN4, EoN5 and EoN6), have been produced which showed preferential binding to activated, and light density eosinophils, (Tai *et al.*, 1985). As these three antibodies appeared to bind principally to eosinophils which were likely to be preparing for, or undergoing secretion, they were studied further, to see whether they might bind to antigens which were associated with the induction of secretion. Attempts were made to characterize the membrane constituents recognized by these antibodies, to see whether they were known components of the plasma membrane, including receptors for IgG (Tai & Spry, 1976), IgE (Capron *et al.*, 1981), C3b and C3bi (Tai & Spry, 1980), and eosinophil activating factor (Thorne *et al.*, 1984). Stimulation of these receptors has been shown to result in the secretion of up to 30% of the eosinophil granule proteins, including eosinophil cationic protein (ECP) (Winqvist, Olofsson & Olsson, 1984), eosinophil peroxidase (Oliver, Glauert & Thorne, 1982), and histaminase (Zeiger & Colten, 1977).

It was hoped that these studies might lead to ways for regulating the extent of eosinophil secretion *in vivo*, where eosinophil granule constituents have been shown to give rise to protective effects (Butterworth *et al.*, 1979), and occasionally severe tissue injury, including eosinophilic endomyocardial disease (Tai *et al.*, 1982; Olsen & Spry, 1985).

MATERIALS AND METHODS

Eosinophils. Eosinophils were purified from the blood of patients with hypereosinophilia by sedimentation on a methylcellulose-Hypaque gradient (Tai & Spry, 1980), followed by metrizamide density gradient separation (Vadas *et al.*, 1979). Eosinophils with different densities were obtained from the 24–25% gradients (normal density, > 90% pure), the 23% gradient (intermediate density, > 80% pure), and the 20 and 22% gradients (light density, > 80% pure) (Tai *et al.*, 1985). The 20% neutrophil contamination in the light density eosinophil preparation was unimportant, as initial experiments showed they had no effect on the assay system used in the experiments reported here (data not shown). The eosinophils were washed twice in minimum essential medium (MEM)-bicarbonate pH 7·3, containing deoxyribonuclease (DNase), 30 mg/l, and 2% fetal calf serum (FCS), and then adjusted to 10^7/ml in serum-free medium.

Monoclonal anti-eosinophil antibodies. Three IgM monoclonal antibodies to eosinophil plasma membrane constituents were selected from a panel of antibodies raised in mice immunized with light density eosinophils (Tai *et al.*, 1985). They bound to both eosinophils, and neutrophils, but not to other cell types. These three antibodies bound preferentially to light density eosinophils, and were named antibodies EoN4, EoN5, and EoN6, (CDw15GUEoN4–6 according to the nomenclature of Barnard *et al.*, 1984). They were also shown to bind to a higher proportion of blood eosinophils in patients with the idiopathic hypereosinophilic syndrome, who have many activated, and degranulated eosinophils (Tai *et al.*, 1985), although it is not known at present if the antigen density per cell is greater. It was therefore hoped that these antibodies would recognize membrane components involved in activation and degranulation.

Effects of antibodies EoN4, EoN5 and EoN6 on eosinophil secretion. Antibodies EoN4, EoN5, and EoN6 were tested for their effects on ECP release, either alone or in the presence of Sepharose-C3b. Sepharose-C3b was chosen as it is one of the most potent stimuli for ECP release (Winqvist *et al.*, 1984). This was prepared by treating equal volumes of Sepharose-4B (Pharmacia, Uppsala, Sweden) with fresh normal human serum for 15 min at 37°C. The Sepharose-C3b was washed three times, and resuspended to a thick slurry (Winqvist *et al.*, 1984). Eosinophils (10^6 cells) were

incubated with 0·1 ml monoclonal antibody, and 0·1 ml Sepharaose-C3b for 1 h at 37°C. After adding 0·3 ml 1 M NaCl, to remove particle-bound cationic proteins, the cells were pelleted at 500 *g* at 4°C for 10 min, and 0·3 ml of the supernatants were removed for ECP measurements.

ECP determinations. ECP was measured using a double immunoradiometric assay, and an ^{125}I-labelled monoclonal antibody to human ECP: antibody EG2 (Tai *et al.*, 1984). The antibody was purified from ascitic fluid on DEAE Affi-Gel Blue (Bruck *et al.*, 1982). The IgG peak was concentrated to 1 mg/ml using polyethylene glycol 20,000 (Sigma Chemical Co., St Louis, Mo, USA). Iodination was performed using 1 mCi ^{125}I (Amersham, UK) and 50 μg Iodogen (Pierce & Warriner, UK) for 1 ml of EG2 antibody at room temperature for 15 min, with constant flicking (Fraker & Speck, 1978). The iodination process was terminated by transferring the solution into another tube. Two drops of 1% blue dextran were added to the solution, and the labelled antibody EG2 was separated from free ^{125}I on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). Polyvinylchloride microtitre plates were coated with 50 μl of purified antibody EG2 (5 μg/ml) in a bicarbonate buffer pH 9·6 at 37°C for 1 h, followed by three washes in phosphate buffered saline containing 0·05% Tween 20 (PBSTw). Unattached sites on the plastic plates were blocked with 50 μl of 1% gelatin for 1 h at 37°C. Then 50 μl of the test sample diluted in PBSTw (in triplicates), was added to each of the wells and incubated for a further 1 h at 37°C, and then washed as above. The amount of ECP bound was determined as follows: 50 μl ^{125}I-EG2 (5 μg/ml) was added to each well, and left at room temperature overnight. After washing thoroughly, the wells were cut out, and counted in a gamma counter (Nuclear Enterprises). The concentrations of unknown samples were read off from standard curves made using a highly purified ECP sample (kindly donated by Professor Inge Olsson). Results were expressed in terms of absolute amounts of ECP released rather than per cent released, because the extraction methods available do not solubilize all of the intracellular ECP, which could lead to inaccurate results.

Fluorescent studies with pairs of antibodies. Eosinophils (1×10^6) were incubated with 100 μl of the first monoclonal antibody for 30 min at 4°C. The cells were washed three times, and then incubated with 100 μl rhodamine (TRITC)-linked goat antimouse IgG (Sigma Chemical Co., St Louis, MO, USA) diluted in 5% normal human serum for another 30 min. After three washes, 100 μl of the second monoclonal antibody was added, followed by fluorescein (FITC)-linked goat anti-mouse IgG (Nordic, Belgium). The cells were mounted in FCS, and examined by ultraviolet microscopy. Two hundred cells were counted, and the proportions of single, and double-stained cells were scored.

IgG, IgE, and EAF binding sites. The monoclonal antibodies were tested for their capacity to inhibit the binding to eosinophils of purified polyclonal and monoclonal IgG, and IgE (kindly provided by Drs T.G. Merrett & H.S. Spiegelberg, and EAF (kindly prepared and tested for its capacity to enhance eosinophil dependent killing of the schistosomula of *Schistosoma mansoni* by Dr Kareen Thorne (Veith & Butterworth, 1983; Thorne *et al.*, 1985). Eosinophils (2×10^6) were preincubated with the monoclonal antibodies at 4°C for 1 h. Then 2 μg of ^{125}I-IgG or ^{125}I-IgE were added without washing for a further hour at 4°C. The cells were centrifuged through phthalate cushions (Capron *et al.*, 1984), and counted in a gamma counter. Controls included preincubating eosinophils with an unrelated monoclonal antibody and medium containing 10% normal human serum. Eosinophils were preincubated with two different preparations of EAF (251 and 299) diluted 1/10, at 4°C for 30 min, washed, followed by antibodies EoN4, EoN5, or EoN6 for another 30 min. Then either FITC-rabbit antimouse antibody, or ^{125}I-sheep antimouse antibody (250,000 ct/min) were added, and incubated for another 30 min. The proportions of fluorescent cells were scored among at least 200 cells, and the cell-bound radioactivity was counted in a gamma counter.

Eosinophil membrane antigens bound by the antibodies. Eosinophil membrane fractions were prepared by lysing equal volumes of packed purified eosinophils in 10 mM Tris-HCl pH 7·4, 1 mM EDTA, 0·15 M NaCl, 1% Nomidet 40 (NP-40), and 1 mM phenylmethylsulphonyl fluoride (PMSF), (NP-40 buffer) at 4°C for 30 min. Unlysed cells, and nuclear debris, were pelleted at 1,000 *g* for 5 min, and the membrane components in the supernatant were sedimented at 1×10^5 *g* for 30 min. Membrane components bound by antibodies EoN4, EoN5 and EoN6 were isolated by affinity chromatography. Affigel-10 (Biorad Labs, CA, USA) was coupled with the monoclonal antibody according to the manufacturer's instructions. Five milligrams of antibody, which had been purified

from ascitic fluid on Sephacryl S-200 (Pharmacia, Uppsala, Sweden), was coupled to 1 ml of packed gel at 4°C for 4 h. The gel was washed free of uncoupled protein in 0·1 M $NaHCO_3$ pH 8·0. Active esters remaining on the beads were blocked with 1 M ethanolamine-HCl pH 8·0. The gel slurry was washed free of reactants with PBS, and then packed into a 1 ml Econocolumn (Biorad Labs, CA, USA), and stored in PBS/0·1% azide at 4°C until required. Before use, the column was pre-eluted with 3 M $MgCl_2$, and then equilibrated in NP-40 buffer. One microlitre of the eosinophil membrane preparation was loaded onto the column, and recirculated 10 times. After washing the column with 40 times the bed volume, the bound protein was eluted in the first 2 ml. The column was washed with three bed volumes of 1 M NaCl in 0·1 M $NaHCO_3$ pH 8·0, and stored in PS/0·1% azide at 4°C. The eluted proteins were analysed on 10% SDS-PAGE under reducing conditions (Laemmli, 1970), and stained with Coomassie Brilliant Blue.

Statistical analyses. Differences in percentage eosinophils stained by fluorescent antibodies were compared using Student's *t*-test on arcsin transformed mean data.

RESULTS

First, experiments were done to see whether antibodies EoN4, EoN5, and EoN6 induced ECP secretion from purified blood eosinophils. As can be seen in Table 1, all three antibodies induced ECP secretion from normal density eosinophils, and antibody EoN5 was the most stimulatory. Any effect of the antibodies on ECP secretion from light density eosinophils was masked by their higher spontaneous release of ECP. All three antibodies also enhanced ECP secretion from normal density eosinophils stimulated with Sepharose-C3b, and antibody EoN6 was the most effective. Again, any effect on light density eosinophils was masked by higher ECP secretion from the controls. The antibodies caused clumping, but did not alter the viability of normal or light density eosinophils, which remained >90% viable.

The capacity of antibodies EoN4, EoN5 and EoN6 to induce ECP secretion from normal density eosinophils in the presence of Sepharose-Crb was shown to be related to the antibody concentration (Fig. 1), and in a time course study, secretion was maximal at 1 h, or later (Fig. 2).

Table 1. The effects of antibodies EoN4, EoN5, and EoN6 on ECP secretion from normal and light density blood eosinophils, with or without Sepharose-C3b

Antibody added	ECP secreted, ng/10^6 eosinophils (mean ± s.d., four experiments)	
	Normal density eosinophils	Light density eosinophils
Without Sepharose-C3b		
No antibody	111 ± 27	1400 ± 700
Antibody EoN4	600 ± 300†	1700 ± 800
Antibody EoN5	3350 ± 2600*	2400 ± 700
Antibody EoN6	1150 ± 1600†	1900 ± 500
Antibody EoN1‡	190 ± 20†	1100 ± 600
With Sepharose-C3b		
No antibody	419 ± 36	2350 ± 350
Antibody EoN4	1750 ± 900†	2950 ± 1060
Antibody EoN5	1250 ± 800†	3400 ± 800
Antibody EoN6	4750 ± 1760*	2800 ± 600
Antibody EoN1‡	300 ± 61	1900 ± 450

Significantly greater than cultures without antibody: *$P < 0.005$; †$P < 0.02$.

‡ Control IgM monoclonal antibody which binds to eosinophils.

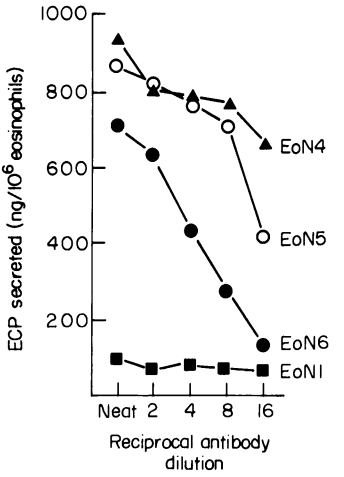

Fig. 1. The effects of serial dilutions of antibody EoN4 (▲), EoN5 (○), and EoN6 (●) on ECP secretion, in the presence of Sepharose-C3b. All three monoclonal antibodies stimulated ECP release, in a dose-dependent manner. EoN1 (■) is a control IgM anti-eosinophil antibody.

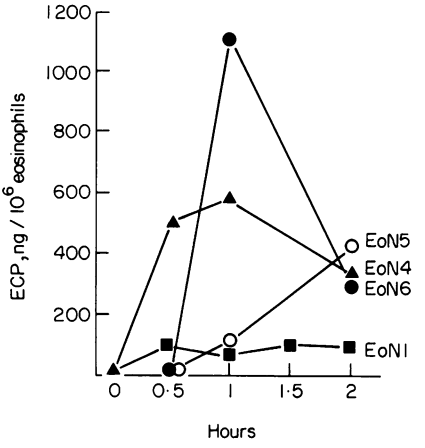

Fig. 2. The effects of EoN4 (▲), EoN5 (○) and EoN6 (●) on ECP secretion, in the presence of Sepharose-C3b, as a function of time. Maximal release occurred at or after 1 h. EoN1 (■) is a control IgM anti-eosinophil antibody.

Experiments were then done to see whether pairs of antibodies were more effective than single antibodies in enhancing ECP secretion from normal density eosinophils in the presence of Sepharose-C3b. Antibody EoN5 with antibody EoN6 induced a greater secretion of ECP than either antibody alone (Table 2).

The binding of pairs of antibodies to eosinophil membrane antigens

In order to see whether the three antibodies bound to one or more epitopes, they were interacted with light density eosinophils, and fluorescein- and rhodamine-labelled antibodies were then used to see how frequently eosinophils were single and double stained. A high proportion of eosinophils were double stained, suggesting that the antibodies were recognizing different epitopes (Table 3).

Table 2. Measurements of the synergistic effects of pairs of antibodies on ECP secretion from normal density eosinophils, in the presence of Sepharose-C3b. Culture supernatants of antibodies were used undiluted

Combinations of antibodies	ECP secreted, ng/10^6 eosinophils mean ± s.d., three experiments
Without antibodies	
Medium alone	84 ± 10
With Sepharose-C3b	178 ± 2
With single antibodies	
EoN4	586 ± 78
EoN5	115 ± 2
EoN6	1170 ± 108
With pairs of antibodies	
EoN4 & EoN5	272 ± 12
EoN4 & EoN6	272 ± 12
EoN5 & EoN6	3035 ± 732*

* significantly greater than either antibody alone: $P < 0{\cdot}01$.

Table 3. The per cent of eosinophils double and single stained by fluorescent antibodies EoN4, EoN5 and EoN6

Antibodies (A + B)	Per cent Eosinophils stained with: A alone	B alone	double-stained (mean ± s.d., four experiments)
EoN4 with 106*	80 ± 10	None	None
EoN4 with EoN5	80 ± 12†	50 ± 10	60 ± 10†
EoN4 with EoN6	78 ± 6	60 ± 5	67 ± 8
EoN5 with EoN6	48 ± 9	64 ± 3	58 ± 6

* An irrelevant monoclonal antibody.
† Significantly different from each other, $P < 0{\cdot}025$.

Table 4. The binding ^{125}I-IgG and ^{125}I-IgE to eosinophils preincubated with antibodies EoN4, EoN5, and EoN6. The quantities of isotope bound were not reduced

	IgG		Myeloma IgE		Polyclonal IgE	
	ct/min	per cent bound	ct/min	per cent bound	ct/min	per cent bound
Without eosinophils:						
Medium alone	250	0·15	345	0·19	113	0·04
With eosinophils:						
Medium alone	14537	8·74	1139	0·63	1603	0·67
Antibody 106*	21303	12·81	1120	0·62	1715	0·72
Antibody EoN4	14641	8·81	1260	0·72	1657	0·70
Antibody EoN5	15329	9·22	1025	0·56	2338	0·98
Antibody EoN6	16174	9·73	1135	0·63	1991	0·84

* An unrelated monoclonal antibody.

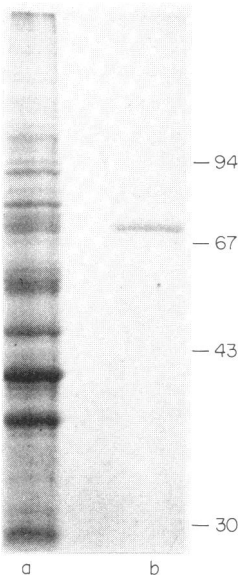

Fig. 3. 10% SDS-polyacrylamide gel showing eosinophil membrane constituents (a) unfractionated (b) after elution from an affinity column coupled with antibody EoN4. Coomassie blue stain. Molecular weight markers were: phosphorylase B (94), bovine serum albumen (67), ovalbumen (43), carbonic anhydrase (30), chymotrypsinogen (20), and lysozyme (14). The component bound by antibody EoN4 was 75 kD.

Monoclonal antibodies and binding sites for EAF, IgG, and IgE

Pretreating eosinophils with EAF, did not inhibit the binding of antibodies EoN4, EoN5, or EoN6 (data not shown). When eosinophils were preincubated with antibodies EoN4, EoN5, or EoN6 the amount of radiolabelled IgG, myeloma IgE, or polyclonal IgE which bound to the eosinophils was not reduced (Table 4), suggesting that the monoclonal antibodies were not recognizing the Fc receptors for IgG, or IgE.

Isolation of membrane antigens

Antibody EoN4 bound to a plasma membrane protein molecular weight about 75 kD (Fig. 3). The antigens recognized by antibodies EoN5 and EoN6 appeared to be less stable, as they could not be visualied by direct immunoblotting, or after concentration and elution from affinity columns.

DISCUSSION

This study has shown that three monoclonal antibodies to antigens preferentially expressed on the plasma membrane of human blood eosinophils were able to induce the secretion of ECP. Several lines of evidence suggested that the monoclonal antibodies recognized structures distinct from receptors for IgG, IgE, C3b, and EAF. As the antibodies led to functional alterations in eosinophils, it seemed probable that the structures recognized by antibodies were involved in the regulation of eosinophil granule secretion.

These three antibodies were selected because they bound most strongly to light density eosinophils from patients with the hypereosinophilic syndrome. Previously, it had been shown that these antibodies showed greater binding to normal blood eosinophils, after they had been stimulated with EAF (Tai *et al.*, 1985). This suggested that the membrane components recognized

by these antibodies were present in larger amounts on eosinophils which were undergoing secretion, supporting the view that they were important components of the secretory response.

The membrane component detected by antibody EoN4 was partially purified, and shown to be 75 kD, smaller than the 95 kD antigen described by Foon *et al.* (1983). The components recognized by the other two antibodies (EoN5 & EoN6) were not stable during the isolation steps. Another granulocyte-specific monoclonal antibody (WEM-G1) has been described (Lopez & Vadas, 1984). It bound to a 100 kD glycoprotein in the plasma membrane of human neutrophils, and increased the capacity of eosinophils, and neutrophils to induce antibody-dependent cytoxicity to P815 mastocytoma cells.

It was of interest that antibody WEM-G1, and the antibodies described here were all of the IgM class, suggesting that multipoint cross-linkage of plasma membrane components might be involved in the induction of granulocyte secretion. This has been shown with monoclonal antibodies to pancreatic exocrine cells, which only induced secretion after they had been cross-linked with a second antibody (Podesta *et al.*, 1983). Although the monoclonal antibodies caused eosinophil aggregation, other IgM antbodies, which also bound to the plasma membrane of eosinophils (Tai *et al.*, 1985), also induced eosinophils to clump (data not shown), but they did not induce eosinophil secretion, showing that secretion was not simply the result of IgM antibodies binding. This confirmed the view that the membrane structures recognized by the three antibodies were directly involved in stimulating secretion. In addition, other monoclonal antibodies to granulocyte membrane antigens have been described, including antibody NCD-1, which had the effect of reducing neutrophil secretion (Cotter & Henson, 1984). The finding that these antibodies induced a greater secretion of ECP from normal density eosinophils than from light density cells would suggest that the light density cells were already stimulated *in vivo* and hence relatively unresponsive to *in vitro* stimulation.

A number of experiments suggested that antibodies EoN4, EoN5, and EoN6 bound to separate structures in the plasma membrane of human blood eosinophils. Double-labelling experiments showed that the three antibodies recognized different epitopes, and a small number of eosinophils appeared to only express the antigen detected by antibody EoN4. However, experiments which were designed the assess the possible synergistic effects of the antibodies on eosinophil secretion were difficult to interpret. EoN5 with EoN6 was clearly synergistic, but EoN4 with EoN5, or EoN6 resulted in a significant reduction ($P < 0{\cdot}02$) in ECP released than when the antibodies were used singly. At present, we do not have an explanation for this.

The membrane component recognized by antibody EoN4, was sufficiently stable to enable it to be isolated using affinity columns coated with the antibody. However, direct immunoprecipitation, and immunoblotting have not been successful. This might have been due to proteolysis caused by the neutral proteases present within granulocytes (Davis *et al.*, 1984; Hibbs, Mainardi & Kang, 1982). The biochemical properties of the antigens have not yet been assessed directly, but the antigen detected by antibody EoN4 could be detected after methanol, glutaraldehyde, or formaldehyde fixation, and it could be demonstrated in paraffin embedded sections by immunocytochemistry. This suggested that it was a glycoprotein. As lectins can induce neutrophil secretion (Perez & Ong, 1984), carbohydrates may be an important component of the sites where secretion is initiated in the plasma membrane.

It was concluded that human eosinophils possess several granulocyte-specific plasma membrane differentiation antigens which may initiate the secretion of the crystalloid granule basic proteins. As these basic proteins have been found to be toxic to some parasites, and host tissues, it is possible that these membrane structures play an important role in the induction of eosinophil-dependent inflammatory reactions.

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