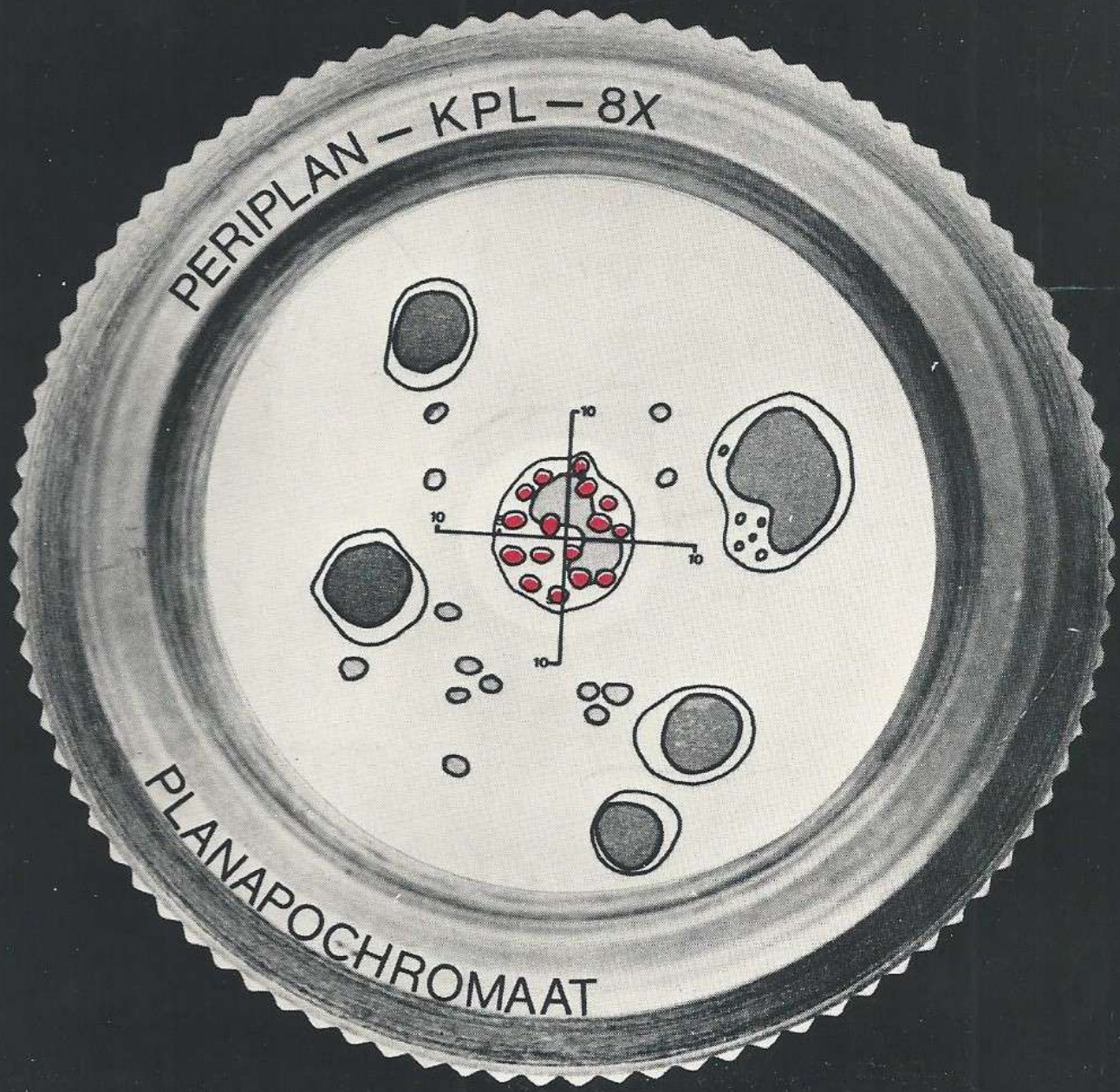


IgE ON HUMAN BASOPHILS

P.J. STALLMAN

P.J. STALLMAN – IgE ON HUMAN BASOPHILS

RODOPI

IgE ON HUMAN BASOPHILS

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van
doctor in de Geneeskunde
aan de Universiteit van Amsterdam,
op gezag van de Rector Magnificus
Dr. G. den Boef,
hoogleraar in de Faculteit der
Wiskunde & Natuurwetenschappen
in het openbaar te verdedigen in de aula der
Universiteit (tijdelijk in de Lutherse Kerk,
ingang Singel 411, hoek Spui) op
donderdag 24 februari 1977 om 16.00 precies

door

PIETER JOHAN STALLMAN

geboren te Amsterdam

Rodopi

1977

Promotor : Prof. Dr. J.J. van Loghem
Co-promotor : Dr. Th. M. Feltkamp-Vroom
Co-referent : Dr. E.E. Reerink-Brongers

This thesis was prepared at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (research director: Prof. Dr. J.J. van Loghem), in the department of Immunohistopathology and the Laboratory of Experimental and Clinical Immunology of the University of Amsterdam, Amsterdam The Netherlands.

Financial support was given by the Netherlands Asthma Foundation.

Author's present adress:

Department of Otorhinolaryngology, Wilhelmina Gasthuis, University of Amsterdam, 1e Helmersstraat 104, Amsterdam, the Netherlands.

Het zou onjuist wezen de medische wetenschap te verwijten dat zij weinig weet. De andere wetenschappen weten ook weinig. Maar zij worden niet gedwongen de schijn op te houden.

(Dag Dokter! W.F. Hermans)

voor Nineke,
die weet welke dingen werkelijk belangrijk zijn.

ACKNOWLEDGEMENTS.

I wish to express my gratitude to all those who have assisted in the completion of this thesis:

- Dr. K. Ishizaka (Johns Hopkins University, Baltimore, USA) and Dr. S. Kochwa (Mount Sinai Hospital, New York, USA) for kindly providing the IgE myeloma proteins;
- Prof. Dr. J. Swierenga (St. Antonius Hospital, Utrecht), Prof. Dr. P.J. Zuidema (Institute of tropical Hygiene, Amsterdam), Dr. E.A. van Dishoeck and Dr. R.J. van der Wal (Gen. Hospital Maarschalksbos, Baarn) and Dr. P.J. van der Werff (Amsterdam Institute of Allergic Diseases) for supplying the tissue and blood specimens;
- Prof. Dr. J.J. van Loghem for his stimulating advice and encouragement during the investigations;
- Mrs. Dr. Th.M. Feltkamp-Vroom, Mrs. Dr. E.E. Reerink-Brongers, Dr. R.C. Aalberse, Dr. Sj.Sc. Wagenaar, Mr. E.H. van Elven, M.Sc., and many others for their helpful suggestions and pleasant co-operation;
- Mrs. M. Aalbers, J.G. van Heertum, E.M. Hoorweg, J.M. Reith, G.W.M. Schipper and Mr. J. Agterberg for their skilful technical assistance;
- Mr. M.F.M. Janssen for his statistical advice;
- Mrs. J.M.Th. Knappers and J.C.G. ter Braak, M.A., for correcting the English text of the manuscripts;
- Mrs. D. Sjouwerman, Mr. C.C. Harteloh, A.F. Jans and L.R. Hafkamp for preparing the photographs and the figures;
- Mrs. G. Wolters-de Graaf and H.R. Hertroijs for their secretarial assistance.

CONTENTS

LIST OF ABBREVIATIONS

aHTLA	anti-human thymus lymphocyte antigen serum
aIgE	anti-immunoglobulin E serum
aIgE Upps	anti-immunoglobulin E from Uppsala
B	IgE-receptor complex (Chapter IX)
B_{max}	amount of IgE-receptor complexes, when all receptors are saturated with IgE
B	cell-bound IgE reacted with FlaIgE ≃ fluorescence intensity of the cells (Chapter VII)
B_{lim}	Minimal amount of cell-bound IgE reacted with FlaIgE necessary for a positive score
BasIgE	total amount of basophil-bound IgE
BSA	bovine serum albumin
$C_1(_{3c,3d,4})$	complement factor 1(3c,3d,4)
CIgE	IgE fraction from the serum of patient C
CLL	chronic lymphatic leucaemia
Co	commercial fluorescent aIgE
DAB	diaminobenzidine
EDTA	ethylenediaminotetra-acetate
FITC	fluorescein isothiocyanate
FlaIgE	sheep anti-human IgE serum labeled with FITC
HoaR-HRP	horse anti-rabbit serum labeled with HRP
HoaR-TRITC	horse anti-rabbit serum labeled with TRITC
HRP	horse radish peroxidase
HTLA	human thymus lymphocyte antigen
IFT	immunofluorescence technique
IgE	immunoglobulin E
IgA (D,G,M,)	immunoglobulin A(D,G,M)
IgND	IgE myeloma protein from patient ND
K	equilibrium constant of the reaction
k	velocity constant of the reaction
M	mole
MIgE	molecular weight of IgE
MCFT	mast cell fluorescence titer
MCIgE	total amount of mast cell-bound IgE
MDC	minimal detectable change

MEM	minimal essential medium
mol	molecules
MPV	micro photometer with a variable diaphragm
ND	patient ND, having an IgE myeloma
NHS	normal human serum
NRS	normal rabbit serum
PBS	phosphate buffered saline
PFA	paraformaldehyde
PS	patient PS, having an IgE myeloma
RTLA	rabbit thymus lymphocyte antigen
RAST	radio allergo sorbens test
RFI	relative fluorescence intensity
r	correlation coefficient
R	free IgE-receptors
SwaR-FITC	swine anti-rabbit IgE serum labeled with FITC
SEM	standard error of the mean
TRITC	tetramethyl rodamine isothiocyanate

CHAPTER I

INTRODUCTION

It is well known that antibodies (reagins) play an important role in allergic processes. In 1966 they were demonstrated to belong to a fifth class of immunoglobulins by Ishizaka et al. (6), who isolated a reagin-rich fraction out of the serum of ragweed-atopic patients, and called this new class of immunoglobulin IgE. In 1967 Johannson and Bennich (10) described a patient, having a multiple myeloma with a paraprotein, which did not belong to the known classes of immunoglobulin. This paraprotein, called IgND, appeared to be identical with IgE.

The mechanism of allergic reaction is based on the interaction between the allergen and IgE bound to basophilic granulocytes or mast cells, by which histamine and other mediators of the allergic response are released (5, 8, 13). The presence of cell-bound IgE on mast cells and basophils was demonstrated by different methods such as: autoradiography (2, 5, 7, 8), immunofluorescence technique (1, 3, 4), immunoferritine technique (1, 11, 14) and histamine release with anti-IgE (1, 8, 12).

A quantitative investigation of cell-bound IgE was performed by Ishizaka et al. (9). Basophil-bound IgE was quantitated in leukocyte suspensions, using the C_1 fixation transfer test. No significant differences were found between atopic and non-atopic donors with regard to basophil-bound IgE, and no correlation with the IgE serum level could be established. The presence of free IgE receptors on human basophils was suggested by Levy et al. (12) in passive sensitization experiments and confirmed by Ishizaka et al. (9) by means of the C_1 fixation transfer technique. A disadvantage of the forementioned quantitation method is the need to prepare basophil-enriched leukocyte suspensions.

The results of previous studies (3, 4) indicated variations in the amount of cell-bound IgE on human mast cells, by end point titration with a fluoresceinated sheep anti-human IgE serum.

Therefore the aim of this study was:

1) to develop a simple method to identify basophilic granulocytes in leukocyte suspensions;
2) to estimate the amount of basophil-bound IgE by quantitative immunofluorescence microscopy;
3) to investigate possible differences between atopic and non-atopic

subjects with regard to the amount of basophil-bound IgE;
4) to investigate the relation between IgE serum level and basophil-bound IgE, and
5) to study the presence of free receptors for IgE in passive sensitization experiments.

REFERENCES

1. Becker, K. E.; Ishizaka, T.; Ishizaka, K., and Grimley, P. M.: Surface IgE on human basophils during histamine release. J. exp. Med. 138: 394-409 (1973).
2. Callerame, M. L., and Condemi, J. J.: Demonstration of IgE on human skin mast cells using anti-IgE^{125I} and refined light microscopy. Amer. J. clin. Path. 62: 823-829 (1974).
3. Feltkamp-Vroom, Th. M.; Stallman, P. J.; Aalberse, R. C., and Reerink-Brongers, E. E.: Immunofluorescence studies on renal tissue, tonsils, adenoids, nasal polyps, and skin of atopic and nonatopic patients with special reference to IgE. Clin. Immunol. Immunopathol. 4: 392-404 (1975).
4. Feltkamp-Vroom, Th. M.; Wagenaar, Sj. Sc., and Swierenga, J.: Atopic lung disease and IgE mast cell fluorescence in bronchial tissue. (To be published).
5. Ishizaka, K., and Ishizaka, T.: Mechanisms of reaginic hypersensitivity: a review. Clin. Allergy 1: 9-24 (1971).
6. Ishizaka, K.; Ishizaka, T., and Hornbrook, M. M.: Physicochemical properties of reaginic antibody. V. Correlation of reaginic activity with γE-globulin antibody. J. Immunol. 97: 840-853 (1966).
7. Ishizaka, K.; Tomioka, H., and Ishizaka, T.: Mechanisms of passive sensitization. I. Presence of IgE and IgG molecules on human leukocytes. J. Immunol. 105: 1459-1467 (1970).
8. Ishizaka, T.; DeBernardo, R.; Tomioka, H.; Lichtenstein, L. M., and Ishizaka, K.: Identification of the basophil granulocytes as the site of allergic histamine release. J. Immunol. 108: 1000-1008 (1972).
9. Ishizaka, T.; Soto, C. S., and Ishizaka, K.: Mechanisms of passive sensitization. III. Number of IgE molecules and their receptor sites on human basophil granulocytes. J. Immunol. 111: 500-511 (1973).
10. Johannson, S. G. O., and Bennich, H.: Immunological studies of an atypical (myeloma) immunoglobulin. Immunology 13: 381-394 (1967).
11. Lawson, D.; Fewtrell, C.; Gomperts, B., and Raff, M. C.: Anti-immunoglobulin-induced histamine secretion by rat peritoneal mast cells studied by immunoferritin electron microscopy. J. exp. Med. 142: 391-402 (1975).
12. Levy, D. A., and Osler, A. G.: Studies on the mechanisms of hypersensitivity phenomena. XIV. Passive sensitization *in vitro* of human leukocytes to ragweed pollen antigen. J. Immunol. 97: 203-212 (1966).
13. Lichtenstein, L. M.: Allergy; in BACH and GOOD Clinical immunobiology, vol. 1, pp. 243-269 (Acad. Press, New York 1972).
14. Sullivan, A. L.; Grimley, P. M., and Metzger, H.: Electron microscopic localization of immunoglobulin E on the surface membrane of human basophils. J. exp. Med. 134: 1403-1416 (1971).

CHAPTER II

THE PRODUCTION AND TESTING OF THE FITC-LABELED SHEEP ANTI-HUMAN IgE

Since the studies dealing with cell-bound IgE could only be performed thanks to the availability of a specific antiserum against human IgE, the properties of this antiserum are briefly described in this chapter. More elaborate details of the production and testing of this antiserum have been described previously (1, 2).

The anti-IgE serum, used in these studies, was prepared in the Department of Immunopathology of Serum Proteins of the Central Laboratory of the Blood Transfusion Service (head: Dr. E. E. Reerink-Brongers) by Dr. R. C. Aalberse and coworkers in the following way. The anti-IgE serum was produced by immunizing sheep with fractions rich in polyclonal IgE, isolated according to Ishizaka (3) from serum of two patients with a microfilarial and a schistosomal infection, respectively. The antiserum was made monospecific for precipitation reactions by stepwise absorption with NHS (1). In Fig. 1 it is shown that the anti-IgE (aIgE) gave a reaction of identity with the IgE fraction from patient C (CIgE) and the IgE myeloma proteins ND[1] (4) and PS[1] (5) and no reaction with NHS.

The anti-IgE was conjugated with fluorescein-isothiocyanate, and the direct immunofluorescence technique was used for the demonstration of IgE on cells, either in tissues or in suspension. Fluorescein was conjugated to the immunoglobulin fraction of the unabsorbed anti-IgE. After Sephadex G 25 gel filtration the conjugate was absorbed by stepwise addition of pooled NHS in order to make the antiserum monospecific in precipitation reactions. The conjugated material was subsequently freed from proteins with F/P ratios of <1 and >4, by which a mean molar ratio of fluorescein to sheep IgG of 2.8 was obtained. This conjugate was further absorbed with pooled NHS until tanned red cells coated with IgA, IgD, IgG, IgM, and κ and λ light chains, albumin, fibrinogen, transferrin, and the complement factors C3d, C3c, C4 were no longer agglutinated. The specificity of the conjugate was ascertained by fluorescence inhibition using tonsil sections as substrate. The fluorescence was abolished by absorption with myeloma PS or by IgE-rich serum C, but not by NHS.

1. ND and PS were kindly provided by Dr. H. Bennich and Drs. S. Kochwa and K. Ishizaka, respectively.

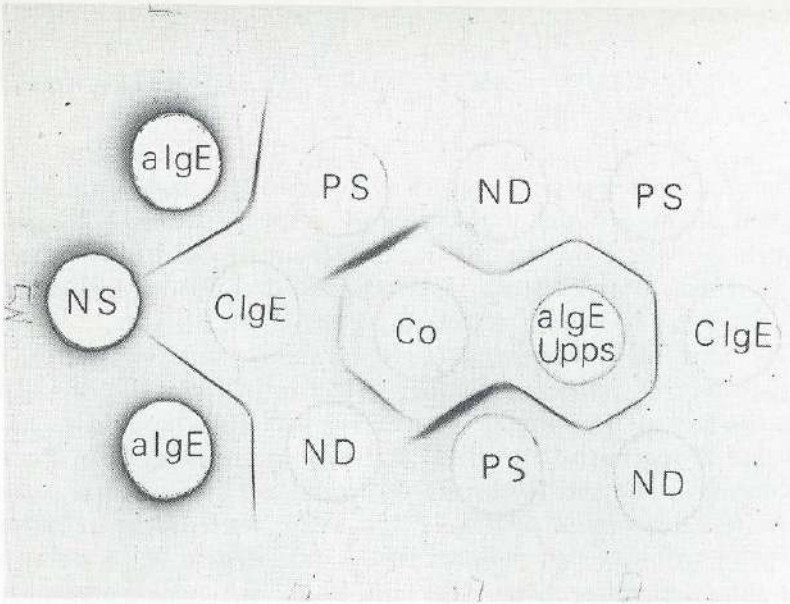


Fig. 1*. Specificity of anti-IgE, PS, IgE myeloma PS; ND, IgE myeloma ND; aIgE, anti-IgE Amsterdam; aIgE Upps, anti-IgE Uppsala; Co, fluorescent anti-IgE; CIgE, polyclonal IgE fraction (see text). A commercial fluorescent anti-IgE serum (Co) produced a precipitation line with PS, spurring over ND and CIgE. As tonsils treated with Co showed fluorescent structures that did not disappear after absorption of Co with CIgE, this antiserum was discarded from further studies. An anti-IgE serum prepared by Dr. S. G. O. Johansson (aIgE Upps) also gave a reaction of identity with ND, PS, and CIgE.

*reprinted from Clin. Immunol. Immunopathol. 4: 392-404 (1975).

REFERENCES

1. Aalberse, R. C. Immunoglobulin E, allergens and their interaction. (Rodopi, Amsterdam 1974). Thesis.
2. Feltkamp-Vroom, Th. M.; Stallman, P. J., Aalberse, R. C., and Reerink-Brongers, E. E.: Immunofluorescence studies on renal tissue, tonsils, adenoids, nasal polyps and skin of atopic and nonatopic patients with special reference to IgE. Clin. Immunol. Immunopathol. 4: 392-404 (1975).
3. Ishizaka, K.; Ishizaka, T., and Hornbrook, M. M.: Physicochemical properties of reaginic antibody. V. Correlation of reaginic activity with γE antibody. J. Immunol. 97: 840-853 (1966).
4. Johannson, S. G. O., and Bennich, H.: Immunological studies of an atypical (myeloma) immunoglobulin. Immunology 13: 381-394 (1967).
5. Ogawa, M.; Kochwa, S., Smith, C., Ishizaka, K., and McIntyre, O. R.: Clinical aspects of IgE myeloma. New Engl. J. Med. 281: 1217-1220 (1969).

CHAPTER III

IDENTIFICATION OF BASOPHILIC GRANULOCYTES IN HUMAN LEUKOCYTE SUSPENSIONS SUBJECTED TO IMMUNOFLUORESCENCE PROCEDURES WITH ANTI-IgE*

P. J. STALLMAN and THEA M. FELTKAMP-VROOM

Department of Immunohistopathology of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology, University of Amsterdam, P.O.B. 9190, Amsterdam, The Netherlands

ABSTRACT

A method is described by which human leukocyte preparations obtained by Ficoll-Isopaque centrifugation are stained and fixed in such a way that the basophilic granulocytes in these preparations can be identified when the latter are subjected to immunofluorescence procedures with anti-IgE. Capping, blocking and absorption experiments confirmed the specificity of the method.

INTRODUCTION

Antibodies against immunoglobulin E (IgE) induce the release of histamine from peripheral blood leukocytes of atopic and non-atopic individuals (8). Since the fluorescent antibody technique was not considered to be sensitive enough to detect the minute amounts of IgE on the surface of cells involved in this histamine release (18), Ishizaka et al. (9, 10) applied autoradiography. In this way authors could identify the IgE-bearing cells as basophilic granulocytes. Sullivan et al. (17) examined human leukocytes by electron microscopy. Using an anti-IgE antibody and a hybrid antibody, directed against both anti-IgE and ferritin, they detected IgE on the surface of basophilic granulocytes in a uniform diffuse pattern, in discrete patches or in polar caps. Becker et al. (2) assessed the distribution of IgE bound to the cell-surface by the fluorescent antibody technique using fluorescein conjugated anti-IgE in their studies on the relationship between the shifts in IgE distribution and the histamine release induced by either anti-IgE or ragweed antigen. By staining cell pre-

*J. immunol. Methods 10: 271-277 (1976).

parations with toluidine blue, they found that 2-3% of the cells demonstrated metachromatic granules, typical of basophilic granulocytes; the percentage agreed with that of the fluorescent cells.

Rosenbaum (14) criticized the finding of IgE-bearing lymphocytes, reported by investigators who studied chronic lymphatic leukemia (1, 12), infantile X-linked agammaglobulinaemia (5) and other diseases in percentages of up to 5%, and asked for confirmation of this finding by a reliable cell identification method. He pointed to the observation that when leukocytes are separated on a Ficoll-Isopaque gradient, basophilic granulocytes are concentrated in the top layer in percentages varying between 1 and 5 (10). Since the cells of this layer had been used in the forementioned studies, he questioned the lymphoid nature of these IgE-bearing cells. This problem and the fact that autoradiography is a less suitable technique for routine investigations led us to develop a method which allows the detection of basophilic granulocytes in leukocyte suspensions subjected to immunofluorescence procedures with anti-IgE.

MATERIALS AND METHODS

Preparation of leukocyte suspensions

Ten ml of venous blood were drawn from non-selected subjects with a heparinized syringe of the Vacutainer system (3200 KA, Becton and Dickinson, Rutherford, New Jersey) containing 143 I.U. of heparin. The blood was mixed with 2.5 ml of a 5% dextran (mol. wt. 200,000, Poviet, Amsterdam, The Netherlands) solution in 0.9% saline and the erythrocytes were allowed to sediment for 30 min at 37° C. The supernatant was mixed with 8 ml of minimal essential medium (MEM, Gibco, Grand Island, N.Y.) containing 5 I.U. of heparin/ml pH 7.4. The mixture was layered on top of 3 ml of a Ficoll-Isopaque mixture of specific gravity 1.077 in each of two tubes of 14 mm diameter, and centrifuged according to the method of Böyum (3) at 560 *g*. The interphase cells were pipetted off, centrifuged at 650 *g* for 10 min and resuspended in 0.25 ml of a washing fluid as used by Hijmans et al. (6), containing 20 ml 20% bovine serum albumin, 60 ml phosphate buffered saline 0.01 M, 8 ml 5% EDTA solution and 100 U/ml of penicillin G, pH adjusted to 6.8.

The cells were stained and fixed simultaneously by the addition of a mixture of 0.5 ml freshly prepared toluidine blue 0.2% in phosphate buffered saline (PBS), pH 7.2, and 0.5 ml paraformaldehyde

2% in 0.1 M phosphate buffer (PFA), pH 7.4 (15) respectively. After a staining and fixation period of 10 min, the cells were centrifuged for 10 min at 650 *g* and washed twice with 10 ml of Hijmans' washing fluid.

After the second washing, the cells were resuspended either in the washing fluid or in a mixture of 5% bovine serum albumin and 5% gelatin in PBS. The latter cell suspensions were put into gelatin capsules which were snapfrozen in liquid nitrogen as previously described (16) and stored in liquid nitrogen until used. The cells for the capping experiments were stained with toluidine blue but not fixed with PFA.

Antisera

The production and testing procedures of the sheep anti-human IgE serum labeled with fluorescein isothiocyanate (FlaIgE), has been described previously (4). The antiserum was used in dilutions varying from 1 : 5 to 1 : 10,000. For the blocking experiments the unlabeled sheep anti-IgE antiserum was used.

Immunofluorescence procedure on the leukocyte suspensions

To 0.1 ml aliquots of the leukocyte suspensions, 0.1 ml of the FlaIgE in serial dilutions from 1 :20 to 1 : 10,000 was added. After incubation for 60 min at 20° C, 2 ml of PBS were added and the cells were centrifuged at 650 *g* for 10 min. After a second washing with 2 ml of PBS for 15 min the cells were resuspended in 0.05 ml of Hijmans' washing fluid and put on a slide which was covered with a slip and sealed with paraffin wax. For the capping experiments the resuspended cells were kept for 60 min at 37° C before they were put on slides. For the blocking experiments incubation with FlaIgE was preceded by incubation with unlabeled aIgE in a dilution of 1 : 5 for 60 min, followed by two washings. For the absorption experiments the cell suspensions were incubated with FlaIgE that had previously been absorbed with IgE-rich serum. The titer of the latter was determined by agar precipitation, and equivalence was found in a ratio of 1 vol of IgE-rich serum to 3 vol of FlaIgE. FlaIgE absorbed with normal human serum served as control.

Immunofluorescence procedure on the frozen leukocytes

Six μm sections of the frozen leukocyte suspensions were cut at

-20° C, put on a gelatin coated slide and air-dried. The slides were then washed in 5% BSA in PBS for 5 min and incubated with FlaIgE for 60 min. After washing with PBS for 30 min one drop of a 1 : 1 (v/v) glycerine : PBS mixture was added. The slides were covered with a slip and sealed with paraffin wax.

Reading the slides

All slides were read on a Leitz Orthoplan fluorescence microscope, equipped with filter combination number 3 of the Ploemopak incident illumination equipment as described by Ploem (13) and Hijmans et al. (7). A Xenon lamp (Osram XBO 150W) was used for excitation. Ansco 200 or Tri-X-Kodak films were used for the fluorescence micrographs and Tri-X-Kodak or S.O.-Kodak films for the light microscopic micrographs.

RESULTS

Leukocyte suspensions

Some cells, their number varying between 1 and 3% in the preparations investigated, showed a strong, ring-shaped fluorescence, which was interpreted as membrane-bound fluorescence (fig. 1A). Simultaneous examination by light microscopy revealed that these cells contained the reddish purple, metachromatic granules typical of basophilic granulocytes (fig. 1B). Even when the antiserum was used in high dilution (up to 1 : 10,000), membrane-bound fluorescence was observed.

In some cell suspensions a few cells demonstrated faint fluorescence in a diffuse pattern. These cells, which were only visible when high antibody concentrations were used, did not show the metachromatic granules when examined by light microscopy. We considered them to be monocytes, on morphological grounds. We did not see fluorescent lymphocytes in our preparations.

Capping experiments on the leukocyte suspensions

The cells after incubation at 37° C showed fluorescence patterns that were dependent on the concentration of the antiserum. When the serum was used in a concentration of 1 : 10 capping was found. Both patchy patterns and capping were seen when the serum was used in a concentration of 1 : 40 to 1 : 640 (fig. 2). When diluted

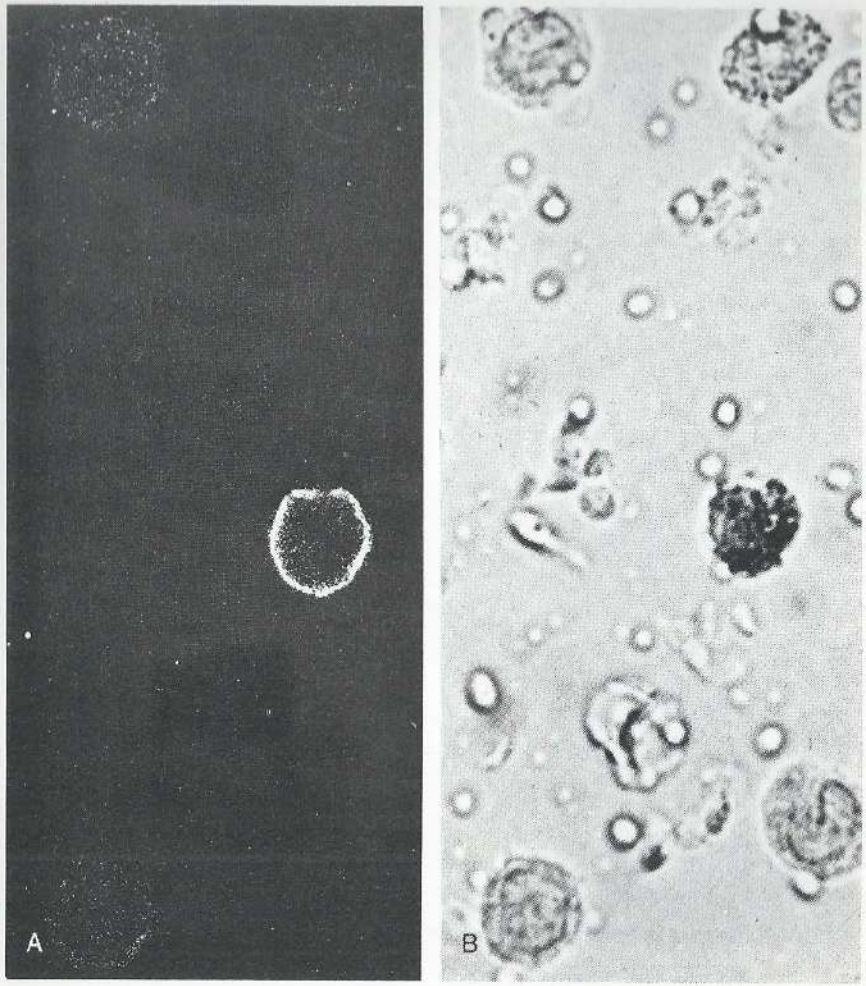
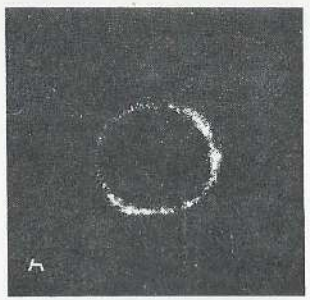


Fig. 1. A) Leukocyte suspension incubated with FlaIgE 1 : 10. Besides a cell with ring-shaped fluorescence two faintly fluorescent cells are shown (x 1000). B) Same preparation in light microscopy. The cell with the ring-shaped fluorescence shows metachromatic granules. The two faintly fluorescent cells have the appearance of monocytes (x 1000).

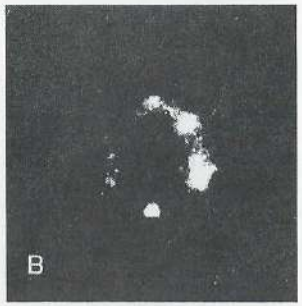
more than 1 : 640 only the ring-shaped membrane fluorescence was observed. The faintly fluorescent monocytes did not show capping.

Blocking experiments on the leukocyte suspensions

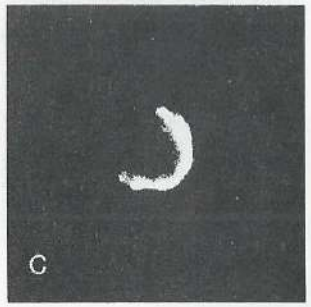
Suspensions that had been treated with unlabeled anti-IgE did not



A) Ringshaped pattern



B) patchy pattern



C) cap formation

Fig. 2. Different patterns in leukocyte suspensions incubated with FlaIgE (x 1000).

demonstrate membrane-bound fluorescence. The faintly fluorescent monocytes were seen also in these cell preparations, their fluorescence picture being unaltered.

Absorption experiments on the leukocyte suspensions

Membrane-bound fluorescence was abolished when the FlaIgE in a dilution of 1 : 20 had been absorbed with IgE-rich serum in ratios 1 : 1 and 1 : 5. At ratios of 3 : 1 and 6 : 1 (FlaIgE : IgE–rich serum) weak membrane-bound fluorescence was seen. In the suspensions prepared for these experiments monocytes could be recognized by

their faint diffuse fluorescence. Absorption of the FlaIgE with normal human serum (NHS) at any ratio did not influence the membrane-bound fluorescence.

Cryostat sections

Before applying the immunofluorescence procedure metachromatic cells were seen in a frequency of 3-5 cells per section. In contrast with cells in suspension, we encountered difficulties in interpreting results obtained with the antiserum in high dilutions. Owing to the water solubility of the toluidine blue stain, the colour of stained sections disappeared upon contact with PBS. This made it difficult to decide whether basophilic granulocytes had reacted negatively or were not detected because they had lost their stain.

DISCUSSION

The satisfactory results of our experiments prove that staining with toluidine blue and simultaneous fixation with paraformaldehyde of enriched leukocyte suspensions is a simple and reliable technique for detection of basophilic granulocytes in such suspensions subjected to immunofluorescence procedures with anti-IgE. Optimal results were obtained with simultaneous staining and fixation. The changes observed in the distribution of IgE fluorescence when the cells were incubated at 37° C are in agreement with the findings of Becker et al. (2). This means that the IgE detected is bound to the surface membrane of the basophil. In addition, the results of the blocking and absorption experiments confirm the specificity of the method. The faintly diffuse fluorescence on some other cells, considered to be monocytes, was not abolished either by blocking or by absorption procedures and was considered nonspecific. Our finding that lymphocytes were negative for membrane-bound IgE, requires further investigation. Since difficulties in interpreting negative results were encountered when frozen cell sections were tested with the antiserum in high dilutions, the use of cells in suspension is preferred. The technique requires only small amounts (10 ml) of blood. This makes it very suitable for the study of patients whose condition does not allow taking of the large quantities of blood necessary for preparation of pure suspensions of basophilic granulocytes as described by Ishizaka et al. (11).

REFERENCES

1. Aisenberg, A. C., and Block, K. J.: Immunoglobulins on the surface of neoplastic lymphocytes. New Engl. J. Med. 287: 272-276 (1972).
2. Becker, K. E.; Ishizaka, T.; Metzger, H.; Ishizaka, K., and Grimley, P. M.: Surface IgE on human basophils during histamine release. J. exp. Med. 138: 394-409 (1973).
3. Böyum, A.: Isolation of leukocytes from human blood. Scand. J. clin. Lab. Invest. 21: suppl. 97, 1-51 (1968).
4. Feltkamp-Vroom, Th. M.; Stallman, P. J.; Aalberse, R. C., and Reerink-Brongers, E. E.: Immunofluorescence studies on renal tissue, tonsils, adenoid, nasal polyps and skin of atopic and nonatopic patients with special reference to IgE. Clin. Immunol. Immunopathol. 4: 392-404 (1975).
5. Gajl-Peczalska, K. J.; Ballow, M.; Hansen, J. A., and Good, R. A.: IgE-bearing lymphocytes and atopy in a patient with X-linked infantile agammaglobulinaemia. Lancet 1: 1254 (1973).
6. Hijmans, W.; Schuit, H. R. E., and Klein, F. E.: An immunofluorescence procedure for the detection of intracellular immunoglobulins. Clin. exp. Immunol. 4: 457-472 (1969).
7. Hijmans, W.; Schuit, H. R. E.; Jongsma, A. P. M., and Ploem, J. S.: in HOLBOROW Standardization in immunofluorescence, pp 193-302 (Blackwell Sci. Publ., Oxford-Edinburgh 1970).
8. Ishizaka, T.; Ishizaka, K.; Johannson, S. G. O., and Bennich, H.: Histamine release from human leukocytes by anti-γE antibodies. J. Immunol. 102: 884-892 (1969).
9. Ishizaka, K.; Tomioka, H., and Ishizaka, T.: Mechanisms of passive sensitization. I. Presence of IgE and IgG molecules on human leukocytes. J. Immunol. 105: 1459-1467 (1970).
10. Ishizaka, T.; DeBernardo, R.; Tomioka, H.; Lichtenstein, L. M., and Ishizaka, K.: Identification of basophil granulocytes as a site of allergic histamine release. J. Immunol. 108: 1000-1008 (1972).
11. Ishizaka, T.; Soto, C. S., and Ishizaka, K.: Mechanisms of passive sensitization. III. Number of IgE molecules and their receptor sites on human basophil granulocytes. J. Immunol. 111: 500-511 (1973).
12. Piessens, W. F.; Schur, P. H.; Moloney, W. C., and Churchill, W. H.: Lymphocyte surface immunoglobulins. Distribution and frequency in lymphoproliferative diseases. New Engl. J. Med. 288: 176-180 (1973).
13. Ploem, J. S.: The use of a vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light. Z. wiss. Mikr. 68: 129-142 (1967).
14. Rosenbaum, I. T.: Basophil or IgE-bearing lymphocyte? New Engl. J. Med. 291: 678-679 (1974).
15. Smit, J. W.; Meyer, C. J. I. M.; Décary, F., and Feltkamp-Vroom, Th. M.: Paraformaldehyde fixation in immunofluorescence and immunoelectron microscopy. Preservation of tissue and cell surface membrane antigens. J. immunol. Methods 6: 93-98 (1974).
16. Steffelaar, J. W.; Graaff-Reitsma, C. B. de, and Feltkamp-Vroom, Th. M.: Immune complex detection by immunofluorescence on peripheral blood polymorphonuclear leukocytes. Clin. Exp. Immunol. 23: 272-278 (1976).
17. Sullivan, A. L.; Grimley, P. M., and Metzger, H.: Electron microscopic localization of immunoglobulin E on the surface membrane of human basophils. J. exp. Med. 134: 1403-1416 (1971).
18. Tada, T., and Ishizaka, K.: Distribution of γE-forming cells in lymphoid tissues of the human and monkey. J. Immunol. 101: 377-387 (1970).

CHAPTER IV

THE ABSENCE OF A HUMAN THYMUS LYMPHOCYTE ANTIGEN (HTLA) ON BASOPHILS AND MAST CELLS*

P. J. STALLMAN, E. H. VAN ELVEN, TH. M. FELTKAMP-VROOM and A. BRUTEL DE LA RIVIERE.

Department of Immunohistopathology and Electron Microscopy of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology, University of Amsterdam, P.O.B. 9190, The Netherlands.

ABSTRACT

Basophilic granulocytes and mast cells of different species have been reported to originate from thymocytes and other lymphocytes. Recently these observations were confirmed, when evidence was given, that thymic antigen is present on rabbit basophilic granulocytes. In the study reported here, human leukocytes were tested by the immunofluorescence technique (IFT) and the immunoelectron microscopy technique to see whether a human thymus lymphocyte antigen (HTLA) could be detected on their surface. We could demonstrate the presence of HTLA on T-lymphocytes but not on basophilic granulocytes, nor on mast cells in cryostat sections of various tissues.

INTRODUCTION

The origin of human basophilic granulocytes and mast cells is still unknown. Since Ranvier's (18) observation of *in vitro* transformation of frog lymphocytes into mast cells, thymocytes and lymphocytes have been mentioned as mast cell precursors in a number of species: rat (6), chicken (8) and man (7). More recently Ginsburg (12) observed mast cells originating in mouse thymus tissue cultures, while Ishizaka (14) described in a preliminary report the presence of IgE receptors on, and histamine containing granules in cells, that arose by transformation of cultured rat and mouse thymus cells. A more

*Scand. J. Immunol. (in press)

direct approach was reported by Day et al. (9). They found that a goat anti-rabbit thymocyte antigen serum (aRTLA) reacted with rabbit basophils, and that this resulted in the release of histamine. Both histamine-releasing activity and thymocytotoxicity disappeared after absorption with rabbit thymocytes and basophils. These results strongly suggest the presence of a common antigen on the cell surface of rabbit basophils and on thymocytes. This antigen seems to be absent on rabbit mast cells, as these cells failed to release histamine, when challenged with aRTLA. The question whether the same conditions exist in man, led us to investigate human basophils and mast cells for the presence of a human thymus lymphocyte antigen (HTLA) using an IgG fraction of rabbit anti-human thymus lymphocyte serum (aHTLA).

MATERIALS AND METHODS

Preparation of leukocyte suspensions

Leukocyte suspensions were prepared from 10 ml venous blood, made incoaguable with heparin, as previously described (22). After erythrocyte sedimentation with dextran 5%, leukocytes were separated on a Ficoll-Isopaque mixture (specific gravity 1.077). Interphase fractions were collected, stained and simultaneously fixed by adding a mixture of toluidine blue 0.1%, pH 7.2, and paraformaldehyde (PFA) 1%, pH 7.4, washed twice and resuspended in Hijmans' washing fluid (22). By using Giemsa and non-specific esterase staining methods, 75% of the cells in the interphase fractions were on average shown to be lymphocytes.

Antisera

A rabbit antiserum against human thymocytes was prepared by immunizing rabbits with human thymocytes. The resulting antiserum, which was not spontaneously specific for T-lymphocytes, was repeatedly absorbed with leukocyte-free human red cells (10), isolated human granulocytes, and chronic lymphatic leucaemia (CLL) cells isolated from different donors. The granulocytes used for absorption were obtained by leukocyte separation on Ficoll-Isopaque and contained neutrophilic and eosinophilic granulocytes, but no basophils, as the latter remain in the mononuclear fraction. The specificity after absorption was established in the IFT on isolated

human blood cells in suspension as well as in smears (lymphocyte suspensions depleted or enriched in T-cells, granulocytes, monocytes, platelets, and CLL-cells) (Table I). Moreover, its reactivity with various plasma proteins was established with a passive agglutination test (Table II). Before it was used in the IFT on cryostat tissue sections of thymus, spleen and lymph node, the aHTLA was absorbed with acetone dried human liver powder, 10 mg/ml. Nearly all lymphocytes located in the thymic cortex and medulla, the periarteriolar lymphocyte sheath and the paracortical areas showed a rim-like fluorescence with aHTLA. According to the specificity criteria, proposed by a WHO/IARC workshop (19) the preparation is considered specific for T-lymphocytes in the IFT. Elaborate details concerning the preparation and specificity testing of the aHTLA are described elsewhere (4, 5). aHTLA was used in the indirect IFT,

TABLE I
Titers of antibodies against different blood cells in the IFT, after absorption of the aHTLA with erythrocytes.

test cells	before absorption	after absorption with granulocytes, CLL cells and IgG isolation of the aHTLA.
thymocytes	640	640
CLL cells	160	10
granulocytes	160	10
monocytes	160	10
lymphocytes	320	640
thrombocytes	80	<10

Table II
Titers of antibodies against plasma proteins in the passive agglutination test, using human erythrocytes coated with various plasma proteins, after the above mentioned absorptions of the aHTLA.

IgG isolation of the aHTLA:	IgG	IgM	IgA	IgD	albumin	fibrinogen	κ	λ
before abs.	128	32	256	64	32	512	<4	<4
after abs.	<4	<4	<4	<4	<4	<4	<4	<4

CLL = Chronic Lymphatic Leucaemia

using an FITC labeled swine anti-rabbit IgG (SwaR-FITC) purchased from Dakopatts (Danmark) as a second layer. Both leukocytes and tissue sections were incubated with a fluoresceinated sheep anti-human IgE (FlaIgE). The preparation and specificity testing of this fluorescent antiserum have been previously described (11). For the double staining procedures on the tissue sections, a tetramethyl-rodamine isothiocyanate labeled horse anti-rabbit Ig (HoaR-TRITC) was used. For immunoelectron microscopic investigations horse anti-rabbit Ig was coupled to horse radish peroxidase (HoaR-HRP), according to Avrameas (1).

The immunofluorescence procedure as performed on the leukocyte suspensions

Aliquots of 50 µl of the cell suspensions were incubated for 60 minutes at room temperature with 50 µl of a twofold serial dilution of aHTLA in PBS. Final dilutions ranged from 1 : 40 to 1 : 320. Cell suspensions were washed twice with PBS, resuspended in 50 µl of the SwaR-FITC, which was diluted 1 : 80 in PBS and incubated for 60 minutes at room temperature. After being washed twice, the cells were resuspended in 25 µl of Hijmans' washing fluid, and put on a slide. A cover slip was sealed over them with paraffin wax. As a control normal rabbit serum (NRS), absorbed with human red blood cells, diluted 1 : 20 in PBS, was used. FlaIgE, diluted 1 : 20 in PBS, was used in the direct immunofluorescence technique, as described previously (22).

The immunofluorescence procedure for the tissue sections

Tissue specimens from adenoid and bronchial biopsies were quick-frozen in liquid nitrogen, 4 µm sections were cut at -20° C and air-dried. The sections were fixed in acetone for 10 minutes and washed in PBS for 5 minutes. To demonstrate the presence of HTLA on mast cells, sections were incubated with aHTLA, which had been diluted 1 : 20 and 1 : 40 in PBS, at room temperature for 30 minutes and were washed in PBS for 30 minutes. Subsequently sections were incubated for 30 minutes with HoaR-TRITC, diluted 1 : 40 in PBS, and washed again. To identify the mast cells, a triple layer technique was used by incubating the sections for 30 minutes with FlaIgE, diluted 1 : 20 in PBS, and washing with PBS. The sections were mounted with PBS/glycerin 1:1 (v/v) and a cover slip was sealed over them with paraffin wax.

Reading the slides

All slides were examined under a Leitz Orthoplan fluorescence microscope, equipped with filter combination number 3 of the Ploemopak incident light illumination equipment, as described by Ploem (17). For the examination of the slides tested in the triple layer IFT, filter combination number 4 of the Ploemopak was used as well. A mercury lamp (Philips CS 150 W) was used for excitation. The fluorescence and light microscopic micrographs were made on Kodak Tri X film.

Immunoelectron microscopy

For ultrastructural studies the leukocyte suspensions were fixed in PFA 1%, and washed twice in Hijmans' washing fluid. The cells were incubated with aHTLA, diluted 1 : 20 in PBS, at room temperature for 60 minutes. Normal rabbit serum (NRS) served as control. The cells were washed three times with PBS and incubated at room temperature for 30 minutes with the HoaR-HRP in a dilution of 1 : 160. After two washings with PBS, the peroxidase was visualized according to Graham and Karnovsky (13), followed by incubation with osmium tetroxide according to Millonig (15), dehydration in ethanol and propylene oxide and embedding in Epon. Ultrathin sections were cut with a Reichert microtome. The sections were observed with a transmission electron microscope (Philips EM 300), both without staining and after staining with saturated uranylacetate for 10 minutes.

RESULTS

IFT on the leukocyte suspensions and tissue specimens

We examined leukocyte suspensions from 5 donors, in the IFT with aHTLA. Peripheral blood leukocytes in the interphase fractions were found to be positive for HTLA in percentages ranging from 47% to 56% (mean 52%), using aHTLA in a final 1 : 40 dilution. Since routine Giemsa stainings showed approximately 75% lymphocytes in these fractions, we calculated 70% of the lymphocytes to be HTLA-positive. A HTLA-positive cell is seen in Figure 1a, while in Figure 1b the same cells are shown, as seen with light microscopy. In light microscopy basophilic granulocytes, with their striking metachromatic granules, were easily distinguished. No fluorescence was seen

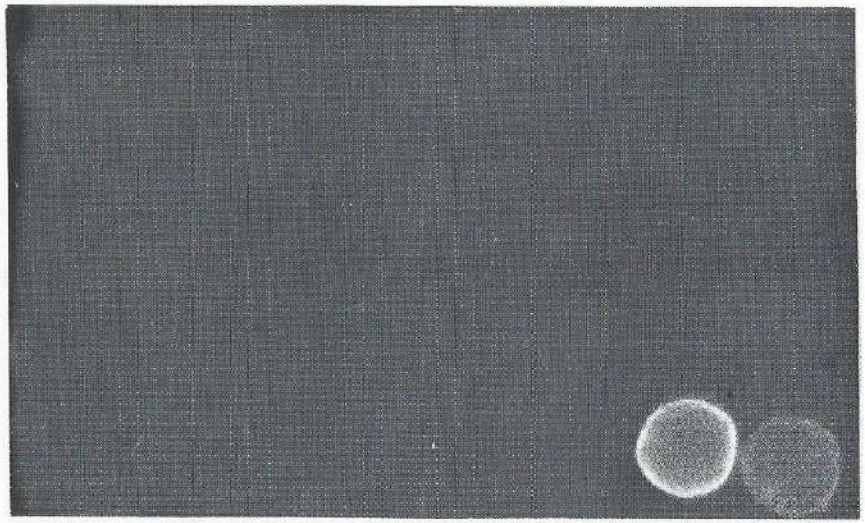


Fig. 1a: A human leukocyte suspension, incubated with rabbit anti-human thymus lymphocyte antigen (aHTLA), diluted 1 : 40, followed by incubation with swine anti-rabbit IgG-FITC, diluted 1 : 80. Membrane-bound fluorescence is to be seen on one of the cells. (x 1000)

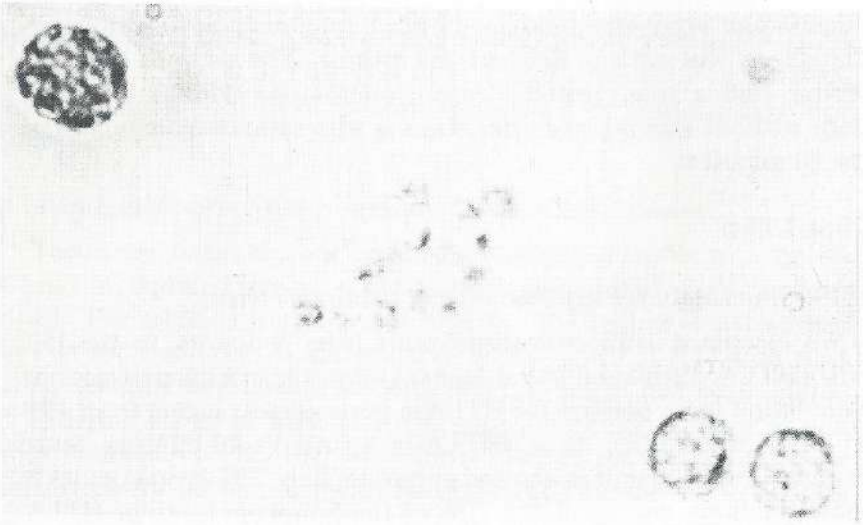


Fig. 1b: The same cells as in figure 1a, seen in light microscopy. This cell preparation was stained with toluidine blue 0.1% and fixed with PFA 1%. The basophilic granulocyte, not showing any fluorescence with aHTLA in figure 1a, is clearly identified by its metachromatic granules. The cell, demonstrating membrane-bound fluorescence, is a lymphocyte. (x 1000)

Fig. 2a: A human leukocyte suspension, incubated with sheep anti-human IgE–FITC (FlaIgE). Membrane-bound fluorescence is observed on one of the cells. (x 1000)

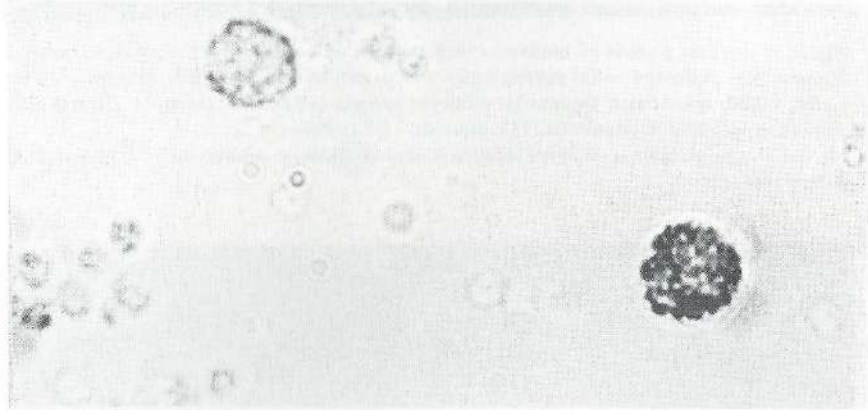


Fig. 2b: The same cells as seen in figure 2a, seen in light microscopy. This cell preparation was stained with toluidine blue 0.1% and fixed with PFA 1%. The cell, demonstrating membrane-bound fluorescence in figure 2a, is identified as a basophilic granulocyte. (x 1000)

on the basophils with aHTLA in any of the dilutions (Fig. 1a/b). Using aHTLA in a 1 : 160 dilution, there were still HTLA-positive lymphocytes to be seen. In suspensions incubated with FlaIgE, a ring-shaped fluorescence was seen, demonstrating membrane-bound IgE (Fig. 2a/b), as described previously (22). The cell membrane-

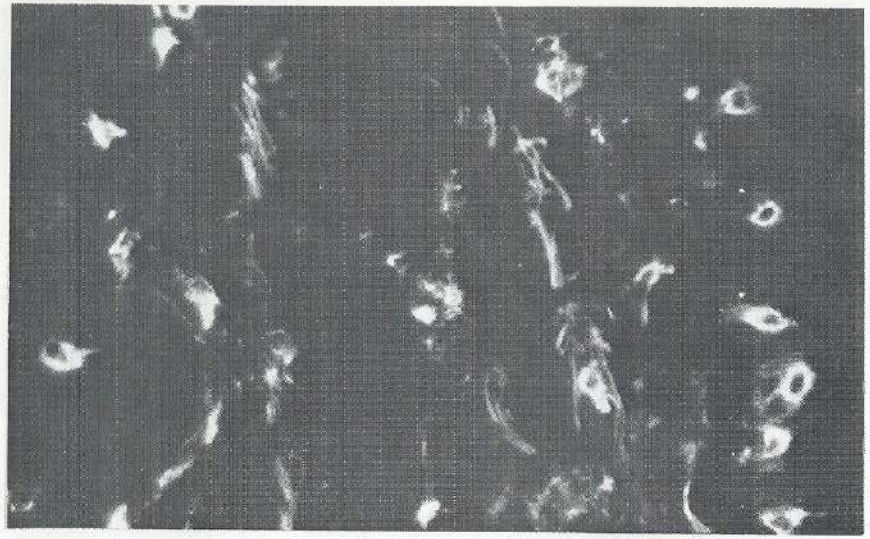


Fig. 3: A cryostat section of human bronchial tissue is incubated in a triple layer immunofluorescence technique with subsequently sheep anti-human IgE-FITC (FlaIgE), diluted 1 : 20, rabbit anti-human thymus lymphocyte antigen (aHTLA), diluted 1 : 20, and horse anti-rabbit IgG-TRITC (HoaR-TRITC), diluted 1 : 80. (x200)
Fig. 3a: The preparation is observed with the Ploemopak filter combination n° 4. FlaIgE is seen on the mast cells.

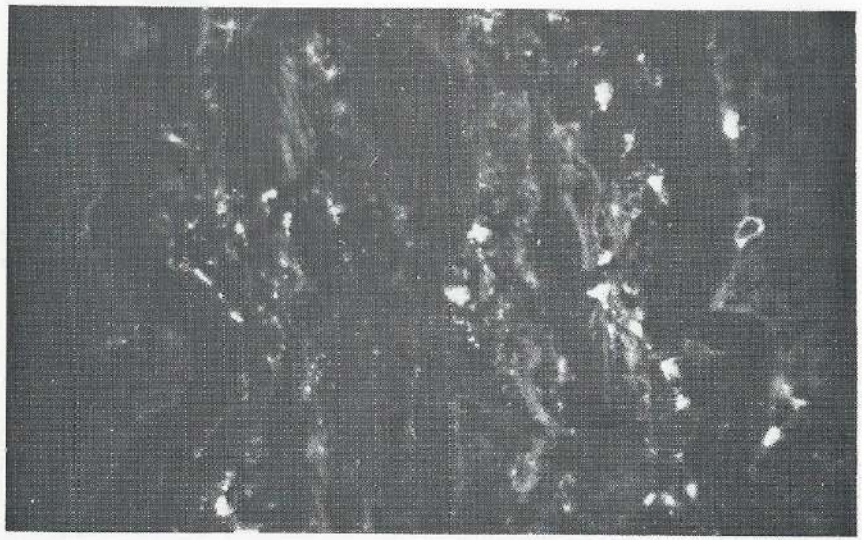


Fig. 3b: An identical field observed with the Ploemopak filter combination n° 3. aHTLA is seen on a thymus lymphocyte. Mast cells do not show any fluorescence.

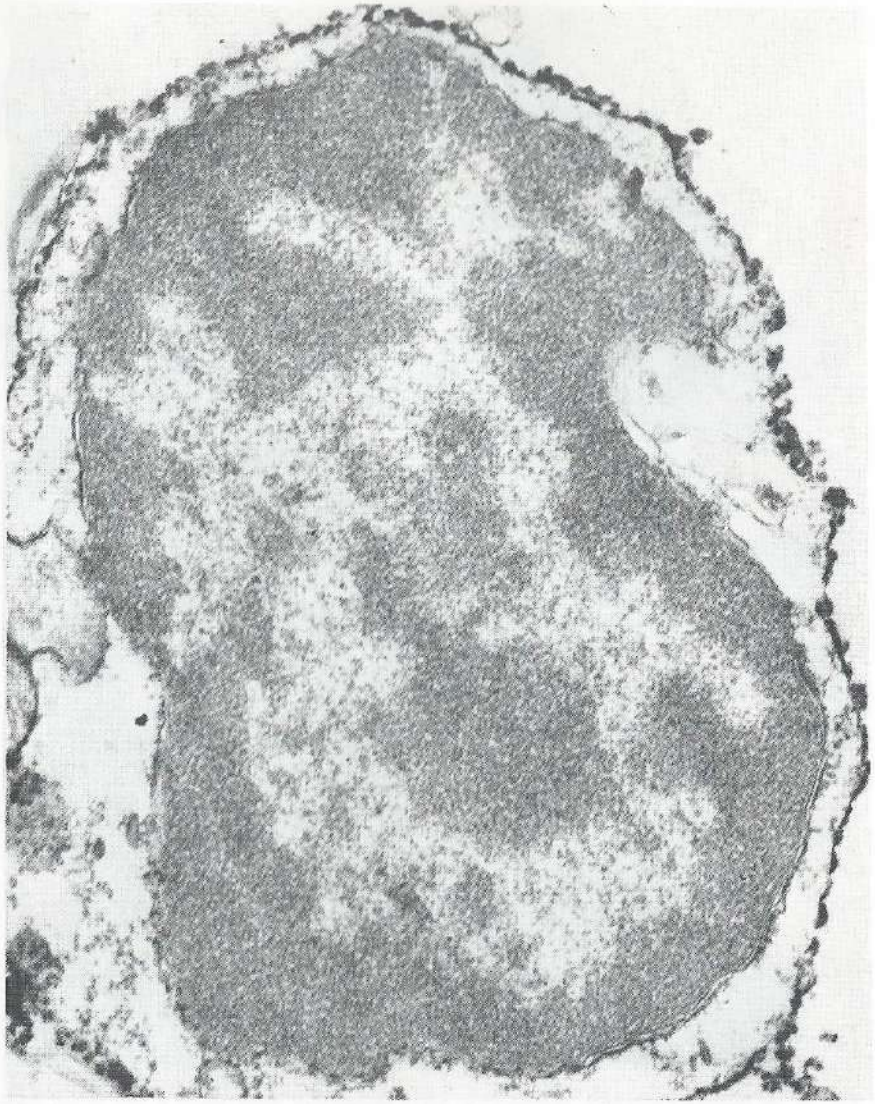


Fig. 4: A human lymphocyte after incubation with aHTLA and HoaR-HRP showing a positive peroxidase reaction on the cell surface membrane. (x 22.500)

bound IgE on mast cells (11) was used to identify these cells in a triple layer IFT. Cells positive for HTLA were seen and presumably are T-lymphocytes (Fig. 3b), but none of the IgE-positive mast cells were positive with aHTLA (Fig. 3a). Control sections, incubated with NRS, did not show any fluorescence with HoaR-TRITC.

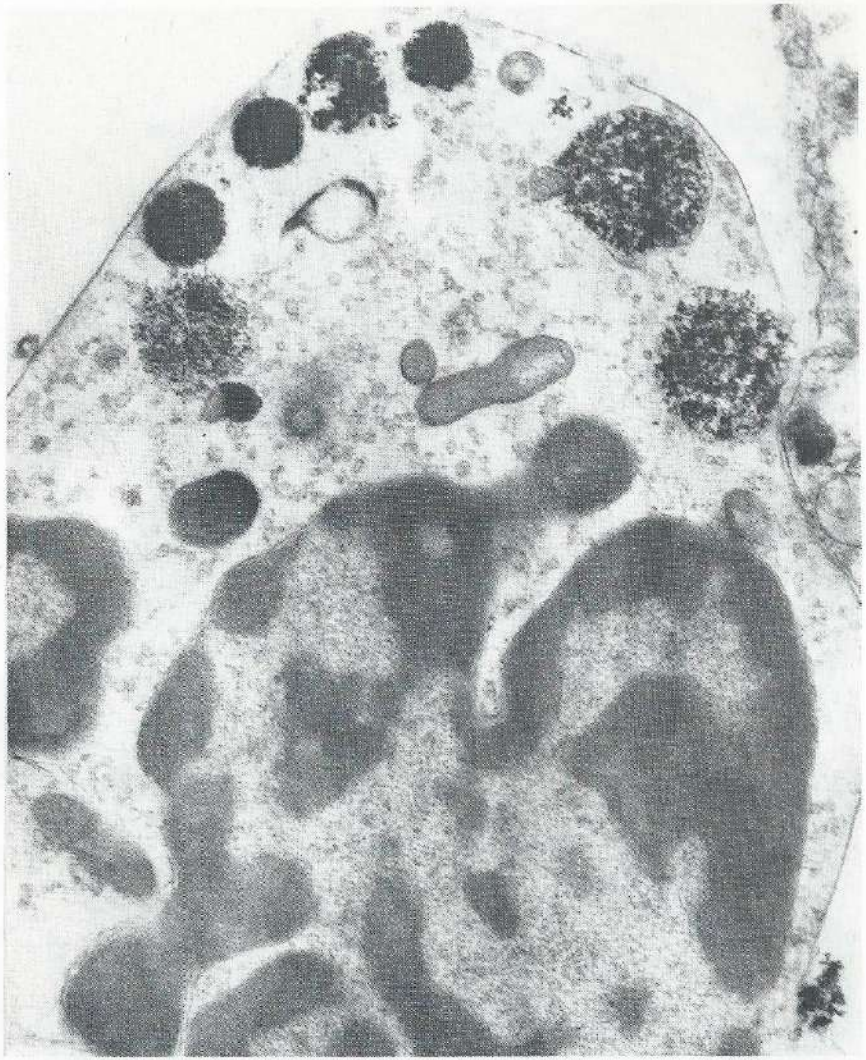


Fig. 5: A human basophilic granulocyte after incubation with aHTLA and HoaR-HRP showing no membrane-related peroxidase reaction. (x 22.500)

Immunoelectron microscopy

In the leukocyte preparations, containing lymphocytes as well as monocytes and granulocytes, all kinds of leukocytes could be easily distinguished, even without staining. Only a part of the lymphocytes showed a positive peroxidase staining (Fig. 4), indicating the

presence of HTLA on their cell surface membrane. None of the other kinds of leukocytes, including the basophilic granulocytes (Fig. 5), did show cell surface membrane related peroxidase staining. Furthermore, in preparations incubated with NRS, peroxidase staining was detected on none of the leukocytes. This demonstrates the specificity of the immunoelectron microscopic technique with aHTLA and HoaR-HRP.

DISCUSSION

That mast cells originate from thymocytes and lymphocytes by transformation, is supported by findings reported in the literature (6, 7, 8). However, the mast cells or basophils in these publications were not defined, for instance by means of their enzyme content or their ultrastructural characteristics (20). Recently more direct evidence was presented by both Ishizaka (14) and Day et al. (9). Ishizaka demonstrated the development of IgE receptor bearing "mast cells" in cultured mouse and rat thymus cells. Although the question is important, whether the thymocyte preparations were pure or whether they contained macrophages originally present in the thymus itself, since evidence has also been given for mast cell differentiation from connective tissue cells (16). Day et al. (9) showed a possible origin of rabbit basophils, by giving evidence for the presence of Rabbit Thymus Lymphocyte Antigen on rabbit basophils. Day's observation of the absence of RTLA on rabbit mast cells seems to point to a basic difference between these two types of cells in rabbits. The present study reports a very direct way of testing the presence of a thymus lymphocyte antigen (HTLA) on basophilic granulocytes or mast cells. This antigen or combination of antigens can be defined by the positive reaction with an antiserum (aHTLA), which was prepared by immunizing rabbits with human thymus lymphocytes (5), and was found to be present on the T-lymphocytes in peripheral blood and in several tissues. Using immunofluorescence and immunoelectron microscopic techniques, we could not demonstrate the presence of this antigen (HTLA) either on mast cells or on basophilic granulocytes. As HTLA could be demonstrated easily on T-lymphocytes in peripheral blood cell suspensions, and a cell-membrane related antigen (IgE) could be demonstrated on basophilic granulocytes in cell suspensions treated with the same procedure, HTLA is considered to remain intact during the staining and fixation

procedure with PFA and toluidine blue. However, we cannot exclude a possible selective damage of the antigen on basophils. Approximately 70% of the lymphocytes, present in the interphase fraction, were HTLA-positive, which is in accordance with the percentages of E-rosetting cells, reported in lymphocyte suspensions after Ficoll-Isopaque separation (2, 3).

For the mast cell identification we used immunofluorescence with anti-IgE. HTLA was shown to be absent from mast cells in cryostat sections of various tissue specimens, while it could be demonstrated on lymphocytes in these sections and in other tissues (5, 21).

We may conclude therefore, that, although several reports suggest that mast cells or basophilic granulocytes derive from thymus lymphocytes in animals, we could not demonstrate a common antigen on these cells in man. The possibility however remains, that there are several specific human thymus lymphocyte antigens. One of them, against which antibodies are lacking in our antiserum, could be shared by T-lymphocytes and basophilic granulocytes.

REFERENCES

1. Avrameas, S., and Ternynck, T.: Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. Immunochemistry 8: 1175-1179 (1971).
2. Bentwich, Z., and Kunkel, H. G.: Specific properties of human B and T lymphocytes and alterations in disease; Transplant. Rev. 16: 29-50 (1973).
3. Brown, G., and Greaves, M. F.: Enumeration of absolute numbers of T and B lymphocytes in human blood. Scand. J. Immunol. 3: 161-172 (1974).
4. Brutel de la Rivière, A.; Verhoef-Karssen, P. R.; Bosma, A.; Borne, A. E. G. Kr. von dem, and Engelfriet, C. P.: Specific antisera against human blood cells applicable in the indirect immunofluorescence technique. Scand. J. Immunol. (in press).
5. Brutel de la Rivière, A.; Verhoef-Karssen, P. R.; Oers, M. H. J. van; Schoorl, R.; Feltkamp-Vroom, Th. M.; Borne, A. E. G. Kr. von dem, and Zeylemaker, W. P.: The preparation and specificity testing of a rabbit anti-human thymocyte serum. Vox Sang. (in press).
6. Combs, J. W.; Lagunoff, D., and Benditt, E. P.: Differentiation and proliferation of embryonic mast cells of the rat. J. Cell. Biol. 25: 577-592 (1965).
7. Csaba, G.; Acs, T.; Horvath, C., and Mold, K.: Genesis and function of mast cells. Mast cell and plasmacyte reaction to induced, homologous and heterologous tumours. Brit. J. Cancer 15: 327-335 (1961).
8. Dantschakoff, W.: Untersuchungen über die Entwicklung von Blut und Bindegewebe bei Vögeln. Arch. mikr. Anat., 73: 117-178 (1909).
9. Day, R. P.; Singal, D. P., and Bienenstock, J.: Presence of thymic antigen on rabbit basophils. J. Immunol. 114: 1333-1336 (1975).

10. Diepenhorst, P.: Removal of leukocytes from blood by filtration through cotton wool. (Krips Repro Publ., Meppel 1974) Thesis.
11. Feltkamp-Vroom, Th. M.; Stallman, P. J.; Aalberse, R. C., and Reerink-Brongers, E. E.: Immunofluorescence studies on renal tissue, tonsils, adenoids, nasal polyps, and skin of atopic and nonatopic patients, with special reference to IgE. Clin. Immunol. Immunopathol. 4: 392-404 (1975).
12. Ginsburg, H., and Sachs, L.: Formation of pure suspensions of mast cells in tissue culture by differentiation of lymphoid cells from the mouse thymus. J. nat. Cancer Inst. 31: 1-39 (1963).
13. Graham, R. C., Jr., and Karnovsky, M. J.: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14: 291-302 (1966).
14. Ishizaka, T.: Workshop report on structure and biology of IgE. p. 278 in BRENT & HOLBOROW, Progress in Immunology II, vol. 1. (North-Holland Publishing Company, Amsterdam 1974).
15. Millonig, G.: Advantages of a phosphate buffer for OsO_4 solutions in fixation. J. appl. Phys. 32: 1637 (1961).
16. Montagna, W., and Melaragno, H. P.: Histology and cytochemistry of human skin. III. Polymorphism and chromotropy of mast cells. J. invest. Derm. 20: 257-261 (1953).
17. Ploem, J. S.: The use of a vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light. Z. wiss. Mikr. 68 129-142 (1967).
18. Ranvier, L.: Transformation *in vitro* des cellules lymphatiques en clasmatocytes, C. R. Acad. Sci. (Paris) 112: 688 (1891).
19. Report of a WHO/IARC-sponsored workshop on human T and B cells, London, 15-17 july 1974: Identification, enumeration, and isolation of B and T lymphocytes from human peripheral blood. Scand. J. Immunol. 3: 521-532. (1974)
20. Sagher, F., and Even-Paz, Z.: Mastocytosis and the Mast Cell. Chapter XIX. The relationship of tissue mast cells to other cells. (Karger, Basel, New York 1967).
21. Schoorl, R.; Brutel de la Rivière, A.; Borne, A. E. G. Kr. von dem, and Feltkamp-Vroom, Th. M.: Identification of T- and B-lymphocytes in human breast cancer with immunohistochemical techniques. Am. J. Path. 84: 529-544 (1976).
22. Stallman, P. J., and Feltkamp-Vroom, Th. M.: Identification of basophilic granulocytes in human leukocyte suspensions subjected to immunofluorescence procedures with anti-IgE. J. immunol. Methods. 10: 271-277 (1976). (Chapter III of this thesis).

CHAPTER V

ESTIMATION OF BASOPHIL-BOUND IgE BY QUANTITATIVE IMMUNOFLUORESCENCE MICROSCOPY*

P. J. STALLMAN and R. C. AALBERSE

Department of Immunohistopathology of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology, University of Amsterdam, P.O.B. 9190, Amsterdam, The Netherlands.

ABSTRACT

By means of a previously developed basophil staining and fixation technique (19), it was possible to identify human basophilic granulocytes in leukocyte suspensions, which had been subjected to an immunofluorescence technique with anti-IgE. Using fluoresceinated anti-IgE the fluorescence intensity of the basophils was measured by means of quantitative immunofluorescence microscopy as a reflection of the IgE-load per cell. The reproduceability of this technique and the influence of the incubation time were studied. The mean fluorescence intensity of the basophils varied considerably in six donors, but the cells of two atopic patients showed the highest intensity. IgE was eluted from the cells at pH 2.5 and measured in the supernatant. A correlation was found between the amount of eluted IgE per basophil and the mean fluorescence intensity of the basophils. The number of IgE molecules per basophil was found to be in the range of 15,000 to 500,000.

INTRODUCTION

Human IgE molecules combine with basophilic granulocytes and mast cells (14, 20). In this way cells are sensitized for the release of chemical mediators upon reaction with an appropriate allergen or aIgE (3, 11, 16). The presence of IgE on human basophils was demonstrated directly, for the first time, by Ishizaka et al. (10) by means of autoradiography. To elucidate the role of these cells in atopy, it is important to know how many IgE molecules are present on the human basophil and whether cell-bound IgE is in equilibrium

*Int. Arch. Allergy (in press)

with serum IgE. This led Ishizaka et al. (13) to apply the C_1 fixation transfer technique to determine the number of cell-bound IgE molecules. We developed a method for identification by light microscopy of basophilic granulocytes, which have reacted with FlaIgE (19). When the technique for the quantitation of cell-bound IgE, as used on human tissue mast cells (8) by end point titration with FlaIgE, was applied on basophils in suspension, a high basophil fluorescence titer (1 : 2500 to 1 : 10,000) was found in almost all subjects, atopic or not. This seemed to confirm the results of Ishizaka et al. (13), that the amount of IgE on basophils from different individuals only varies to a limited extend. Experiments, however, with mast cells, which pointed to a varying IgE-load in different individuals, led us to a further investigation, i.e. to measure the fluorescence intensity by quantifying the individual cell fluorescence on basophils with a microfluorometer. In order to relate the fluorescence intensity found on human basophils to the number of cell-bound IgE molecules, elution procedures were performed on leukocyte suspensions from several donors.

MATERIALS AND METHODS

Preparation of the leukocyte suspensions

Human leukocyte suspensions were prepared as described previously (19). Ten ml of venous blood, made incoaguable with heparin, were drawn from six donors, two of which showed clinical atopy. IgE serum levels and values determined by the Radio Allergo Sorbens Test (RAST) in the serum of these donors are given in Table I. The blood was mixed with 2.5 ml of a 5% dextran solution in 0.9% saline and the erythrocytes were allowed to sediment at 37° C for 30 minutes. The supernatant was mixed with 8 ml phosphate buffered saline (PBS) pH 7.2, containing 5 IU of heparin/ml. The mixture was layered in two tubes on top of 3 ml of a Ficoll-Isopaque mixture with a specific gravity of 1.077, and centrifuged according to the method of Böyum (5). The interphase cells were removed, centrifuged at 650 *g* for 10 minutes and resuspended in 0.25 ml of the washing fluid, pH 6.8, described by Hijmans et al. (9). The cells were fixed and stained simultaneously for 10 minutes with a mixture of 0.5 ml toluidine blue 0.2% in PBS, pH 7.2, and 0.5 ml paraformaldehyde 0.2% in 0.1 M phosphate buffer (PFA), pH 7.4 (18). After that, they were centrifuged at 650 *g* for 10 minutes,

washed twice with 10 ml of Hijmans' washing fluid and resuspended in 1 ml of the latter. The percentage of basophilic granulocytes present in the cell suspensions of the donors ranged from 0.9 to 2.2%.

The quantitative immunofluorescence technique

The immunofluorescence procedure on the cell suspensions was performed as described earlier (19). The production and testing of the sheep anti-human IgE serum, labeled with fluorescein isothiocyanate (FlaIgE), used for these studies has been described previously (7). FlaIgE was used in final dilutions ranging from 1 : 40 to 1 : 1280. Unless specified otherwise, 50 μl aliquots of the cell suspension were incubated with 50 μl of the antiserum at room temperature for 60 minutes. After two washings with PBS, the cells were resuspended in 25 μl of Hijmans' washing fluid and put on glass slides. A cover slip over the cells was sealed with paraffin wax. The intensity of the fluorescence on the basophils was measured with a Leitz Orthoplan microscope equipped with a Leitz photometer attachment (MPV) as described by van Boxtel (4) and Capel (6), and expressed in arbitrary units as the relative fluorescence intensity (RFI). Before each series of measurements the system was calibrated by means of a fluorescent uranyl glass (Schott GG 17). Basophils, with their striking metachromatic granules, were identified by light microscopy and focused in the aperture of the 3.2 mm measuring diaphragm. The fluorescence of individual cells was measured by switching to incident fluorescence microscopy and immediate estimation of the intensity of the fluorescence. Thus no bleaching of the fluorescence could occur. Background fluorescence was measured in various parts of the preparation and the mean background fluorescence was calculated and subtracted from the mean value of fluorescence intensity obtained by measuring 10 basophils, unless specified otherwise.

Elution procedures

In order to make a quantitative assessment of the amount of basophil-bound IgE-molecules, we performed elution procedures on cell suspensions of the same six donors. Interphase fractions were collected from 10 ml heparin blood as described above. The unfixed cells were washed twice in Hijmans' washing fluid and resuspended in 1 ml PBS.

The total number of leukocytes was established in a Coulter counter. The percentage of basophils was established in a stained and fixed sample of the leukocytes of the same donor, which sample was also used for measuring the fluorescence intensity. After centrifugation, the unfixed cells were resuspended in 0.5 ml of an elution buffer: glycine HCl buffer, as described by Ishizaka et al. (12), and the pH was adjusted to 2.5. After incubation at 0° C for 10 minutes and a subsequent centrifugation, the supernatants were collected. The amount of IgE in the eluate and in the last washing fluid, collected in the elution procedure, was measured.

Assay of total IgE and Radio Allergo Sorbens Test (RAST)

Preparation of ^{125}I-anti-IgE.

Purified polyclonal IgE was coupled to cellulose. A column was prepared and used to isolate anti-IgE antibodies from the sheep anti-IgE as described by Aalberse et al. (1). 30 μg purified antibody was reacted with 1 mC ^{125}I (Amersham) and 20 μg chloramine T. After one minute 48 μg Na-metabisulphite was added. After addition of 100 μl normal sheep serum the mixture was filtered over 150 ml Sephadex G 200. The 7 S fractions were pooled and diluted to 500 ml with PBS containing 3 mg/ml BSA and 1 mg/ml NaN_3. Ten ml aliquots were stored frozen.

RAST.

RAST was performed as described by Wide et al. (22) with some modifications: as carrier of the allergen Sepharose was used, and the incubation medium (incubation medium 1) contained epichlorohydrin-treated dipropylene triamine to prevent non-immune IgE binding (2). For every test 50 μl of the labeled anti-IgE was used.

IgE assay.

Serum IgE was quantitated by an inhibition assay, using Sepharose-coupled IgE: 100 μl of a serum with a high IgE level (60,000 IU/ml) were added to 200 mg CNBr-activated Sepharose (Pharmacia Ltd.). After coupling and washing the Sepharose particles were resuspended in 100 ml of an incubation medium (incubation medium 2) containing 0.2% Tween-20 and 0.1 ml/ml normal sheep serum. To 0.5 ml of this suspension, 10 μl patient serum and 50 μl ^{125}I-anti-IgE were added. The mixture was incubated overnight, and the Sepharose-bound radioactivity was measured.

IgE in the eluates from basophils was quantitated in a non-competitive assay. Fifty μl anti-IgE antiserum was coupled to 100 mg CNBr-activated Sepharose. The anti-IgE-Sepharose particles were resuspended in 100 ml of incubation medium 1. To 0.5 ml of this suspension, 0.2 ml 0.1 M K_2HPO_4 and 0.2 ml eluate or a dilution of the eluate in the elution buffer were added. The mixture was incubated overnight and washed; 0.5 ml incubation medium 2 and 50 μl ^{125}I-anti-IgE were added; after further incubation overnight, the Sepharose-bound radioactivity was measured. As a reference, IgE-rich serum was diluted in the elution buffer to 0.8-6.4 IU/ml.

An exact quantitation of the IgE serum level was performed on the sera in which, using the standard inhibition assay, an amount of IgE less than 100 IU/ml was found. This was done by a non-competitive assay, similar to the IgE assay in the eluates.

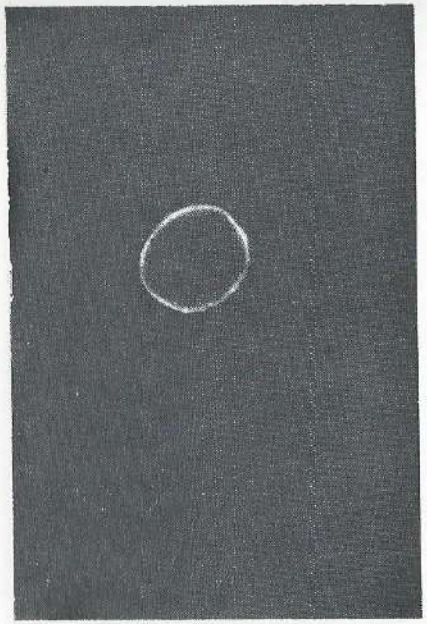
RESULTS

The intensity of the fluorescence of basophils with FlaIgE

Basophilic granulocytes in the leukocyte suspensions showed a ring-shaped fluorescence pattern after incubation with FlaIgE (Fig. 1a). The results of absorption, blocking and capping experiments, as described in a previous article (19), proved the specificity of the method and showed that we are dealing with membrane-bound fluorescence. When the same cell preparations were examined by means of light microscopy the fluorescent cells were found to contain metachromatic granules (Fig. 1b).

Quantitation of the intensity of the fluorescence

The fluorescence intensity of the basophilic granulocytes from six subjects was measured after incubation with serial dilutions of FlaIgE. Background values were subtracted and mean intensity values were calculated. These values, plotted against the FlaIgE concentration in the incubation medium are shown in Fig. 2. The intensity of the fluorescence of the basophilic granulocytes of donors 1 and 2 was much higher, thus pointing to a larger amount of cell-bound IgE. Statistical analysis showed, that the mean fluorescence intensity of the basophils, within the range tested, was linearly correlated with the logarithm of the FlaIgE concentration in the incubation medium (Fig. 3). To test the reproduceability of the method, the basophils from two blood samples from each donor were measured with a



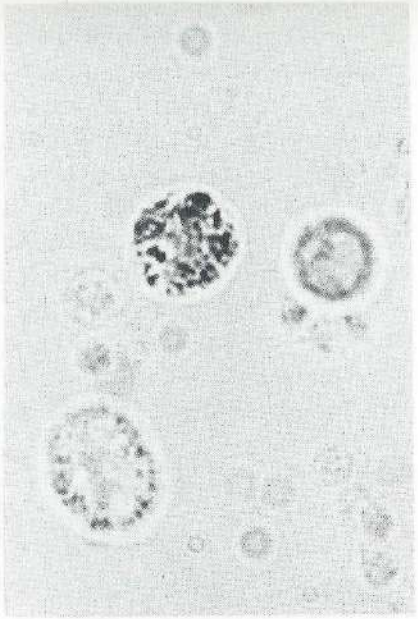


Fig. 1a: A human leukocyte suspension, incubated with sheep anti-human IgE-FITC (FlaIgE), diluted 1 : 40. Membrane-bound fluorescence is observed on one of the cells. (x 1000)

Fig. 1b: The same cells as seen in figure 1a, observed in light microscopy. This cell preparation was stained with toluidine blue 0.1% and fixed with PFA 1%. The cell, demonstrating membrane-bound fluorescence in figure 1a, is identified as a basophilic granulocyte by its metachromatic granules. (x 1000)

two-week interval. The intensity curves obtained (Fig. 3), showed great similarity. Double reciprocal plotting of the data was performed and the points of intersection with the axes were calculated (Fig. 4).

Distribution of the intensity values

The distribution of the intensity of the fluorescence of 60 basophils from the donors 2, 3 and 4, after incubation with FlaIgE, diluted 1 : 40, is shown in Fig. 5. Using the chi-square test for goodness of fit, no significant deviation from a normal distribution was found.

Incubation time

The incubation time is one of the parameters, that influence the slope of the fluorescence intensity curve, as mentioned by Sundqvist (21). To test the influence of a varying incubation time, samples of cells, incubated with FlaIgE in dilutions 1:40 and 1 : 320, were

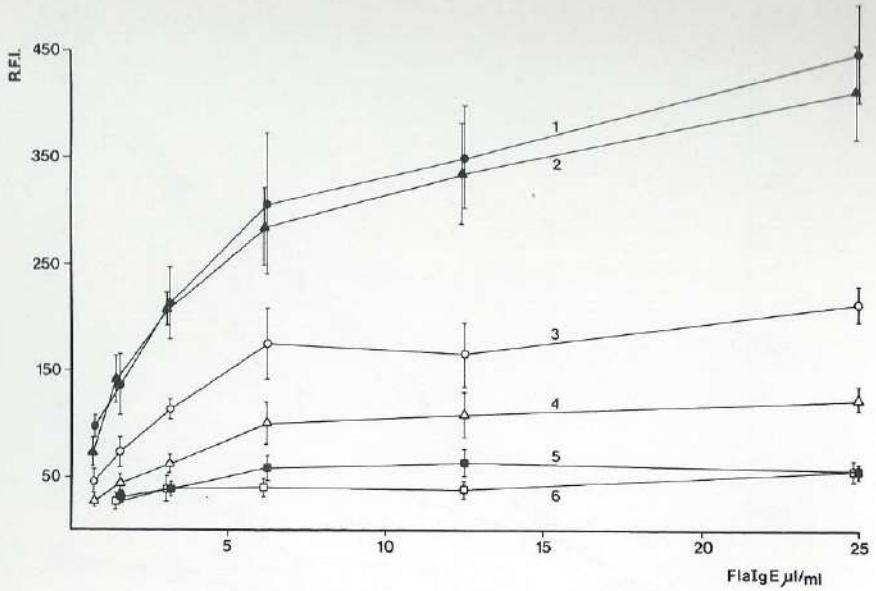


Fig. 2: The fluorescence intensity of the basophils from six subjects as measured by quantitative fluorescence microscopy, after incubation with different amounts of FlaIgE.

taken after 5, 10, 20, 30, 60 and 120 minutes (Fig. 6). The variations in the fluorescence intensity of the basophils from these donors were proportionally present in the samples taken after any duration of incubation. However, prolonged incubation did not greatly increase the fluorescence intensity. In some cases, with prolonged incubation, a higher level of unspecific fluorescence, as it was previously defined (19), was found on other cells.

Number of incubated basophils

The fluorescence intensity of the basophils did not change when the number of these cells, incubated with the same amount of antiserum, was increased up to four times.

Elution procedures

Cell-bound IgE was eluted from the basophils in cell suspensions, obtained from the six donors under investigation. IgE could be demonstrated in all eluates. The amount of eluted IgE ranged from 0.2 to 7 IU/ml. The last washing fluid never contained more than

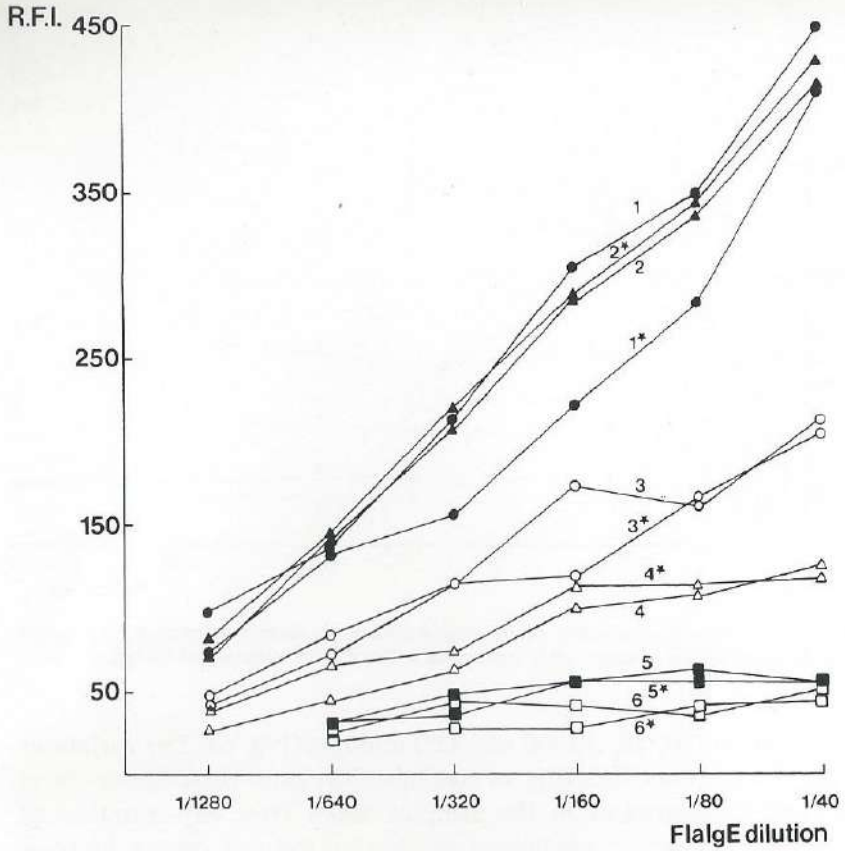


Fig. 3: The mean fluorescence intensity of the basophils from the six donors was measured with a two week interval to test the reproduceability, and was correlated to the logarithm of the amount of FlaIgE. Correlation coefficients were calculated for the various curves:
$r_1 = 0.994$, $r_1 * = 0.980$; $r_2 = 0.999$, $r_2 * = 0.999$;
$r_3 = 0.971$, $r_3 * = 0.982$; $r_4 = 0.987$. $r_4 * = 0.949$;
$r_5 = 0.848$, $r_5 * = 0.933$; $r_6 = 0.585$, $r_6 * = 0.963$.

0.02 IU/ml. The amount of IgE, eluted from 10^6 basophils of each donor, was calculated and compared with the fluorescence intensity of the basophils, measured at the same time (Fig. 7). As a parameter of the latter, the relative fluorescence intensity after incubation with FlaIgE, diluted 1 : 40, was taken. A significant correlation was established between the eluted IgE and the fluorescence intensity of the basophils.

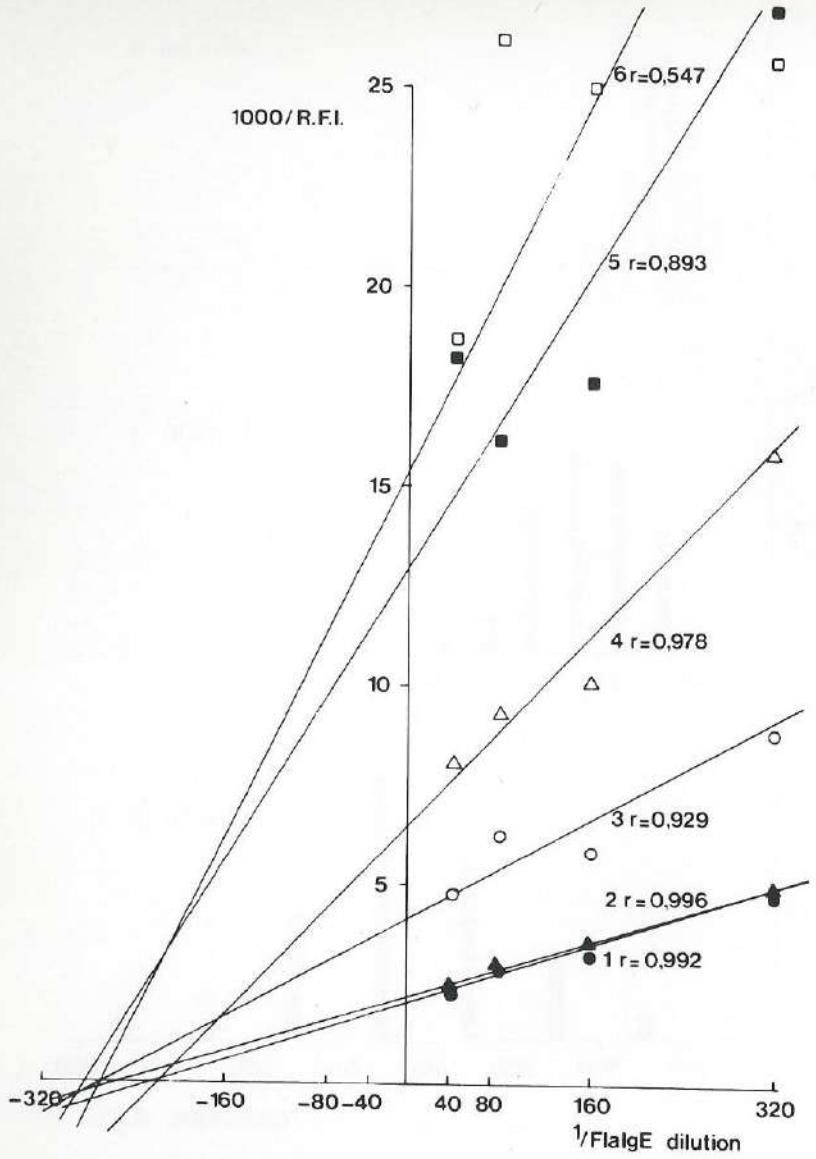


Fig. 4: Double reciprocal values of the fluorescence intensity of the basophils from the six donors and the FlaIgE-dilution in the incubation medium.

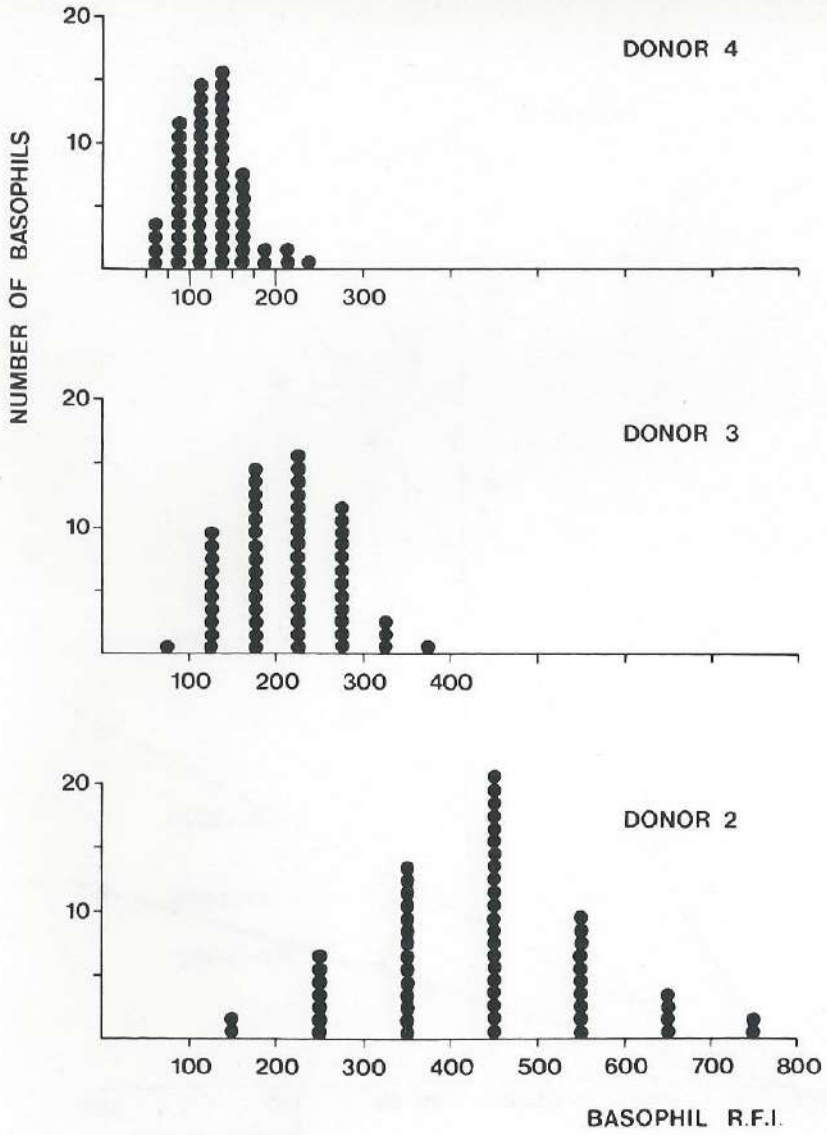


Fig. 5: Distribution of the fluorescence intensity of 60 basophils from three donors incubated with FlaIgE, diluted 1 : 40.

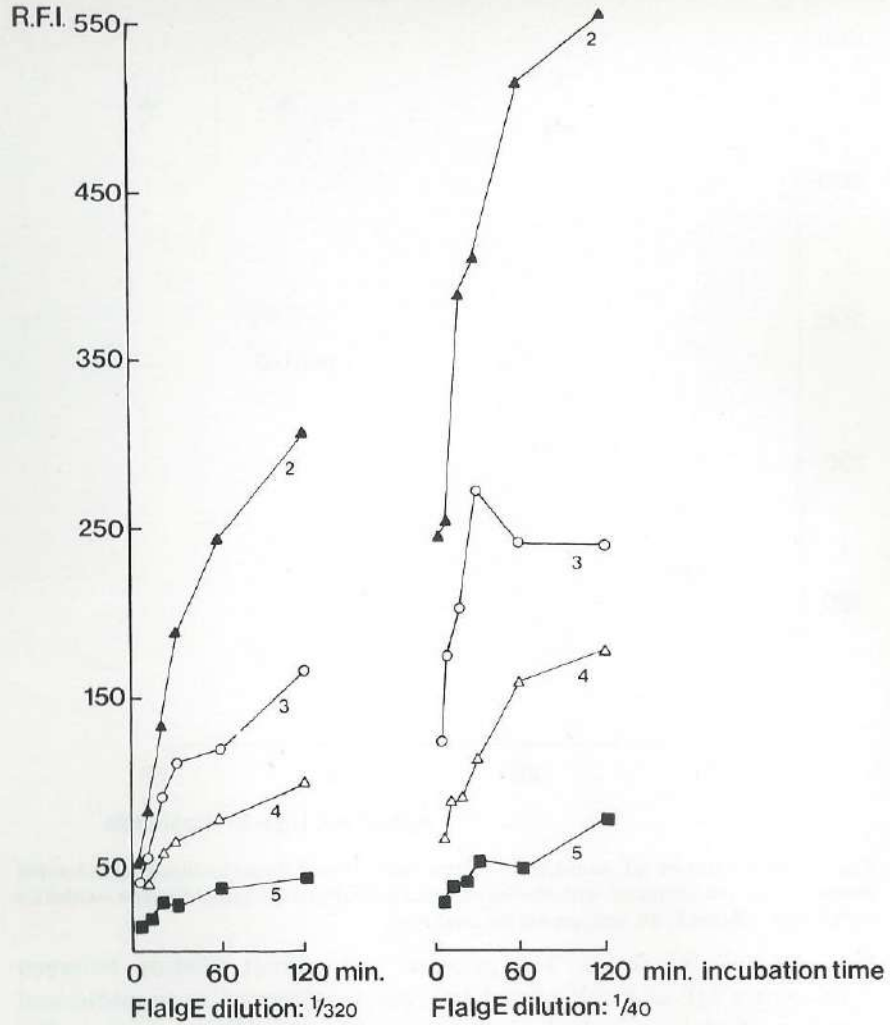


Fig. 6: The influence of the incubation time on the mean fluorescence intensity of the basophils from four donors incubated with FlaIgE, diluted 1 : 320 and 1 : 40.

Total serum IgE and cell-bound IgE

Sera were obtained from the six donors simultaneously with heparin blood, used for the fluorescence intensity measurements of the basophils. In these sera total IgE and antigen specific IgE, as determined by RAST, were measured (Table I). The two atopic donors both showed a high fluorescence intensity of the basophils

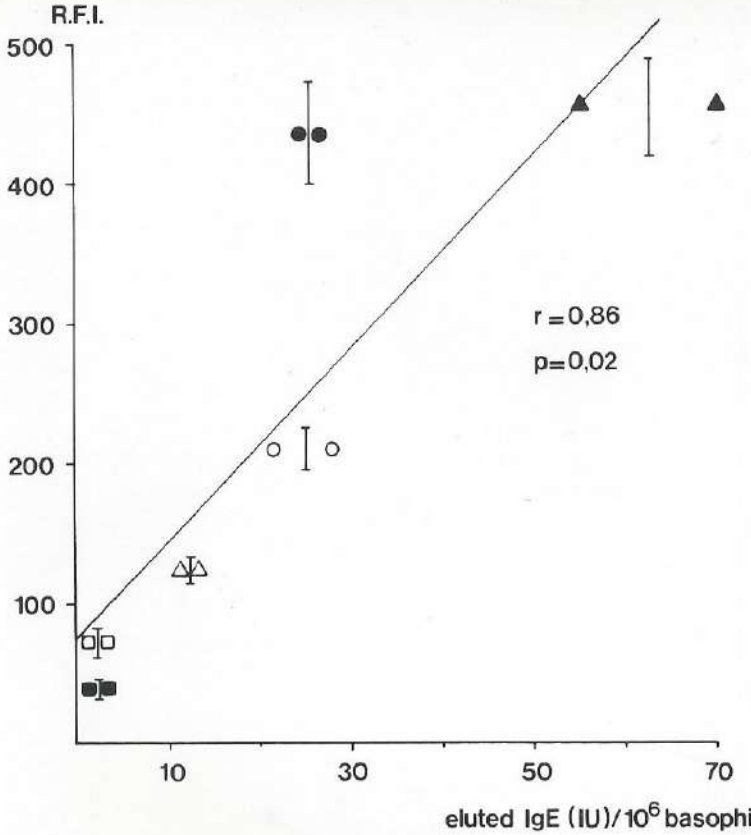


Fig. 7: The amount of IgE eluted from 10^6 basophils of each donor is calculated in a duplo measurement, and compared with the fluorescence intensity of the basophils, after incubation with FlaIgE, diluted 1: 40, measured at the same time.

and elevated IgE levels. The presence of a direct relation between total serum IgE and cell-bound IgE was investigated in an additional number of donors, which will be included in future studies. The fluorescence intensity, measured after incubation of the basophils with FlaIgE, diluted 1 : 40, was compared with the IgE serum levels of these donors. Using the Spearman rank test, a significant rank correlation between the IgE serum level and the fluorescence intensity of the basophils could be established ($p < 0.01$). However, no linear correlation was found (Fig. 8). In this group no distinction was made between atopic and non-atopic subjects, as only the influence of the IgE serum level on the fluorescence intensity of the basophils was studied.

Table I
Total serum IgE and RAST scores of the six donors

Donor	total IgE IU/ml	RAST score house dust	RAST score pollen
1	450	23	0
2	500	38	4
3	58	0	0
4	26	0	0
5	4	0	0
6	6	0	0

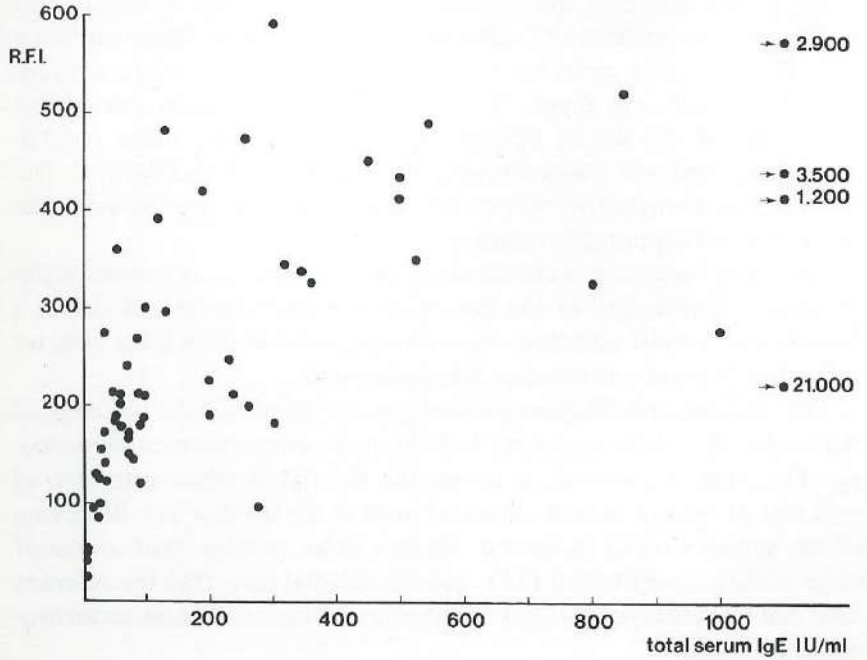


Fig. 8: The fluorescence intensity of the basophils from 61 donors, incubated with FlaIgE, diluted 1 : 40, was compared with the total IgE serum levels of these donors.

DISCUSSION

The results indicate that quantitation of cell-membrane fluorescence with FlaIgE on basophilic granulocytes is possible at the single cell level with the identification technique using toluidine blue and paraformaldehyde. After treatment with paraformaldehyde, redistribution of IgE on the basophil cell-membrane was not observed. This fixation procedure is also supposed to counteract the influence of cell metabolism on cell-membrane antigens. In the applied direct IFT with FlaIgE, the fluorescence intensity of the basophils of all donors decreased with decreasing concentrations of FlaIgE, and no prozone effect was present. There was a linear correlation between the mean fluorescence intensity of the basophils and the logarithm of the FlaIgE concentration. Therefore, in following experiments, the fluorescence intensity of basophils, incubated with only one concentration of the FlaIgE, will be considered representative.

It is important to compare these results with those obtained by Sundqvist (21) in a general study on the fluorescence of the membrane antigens. He analysed the different reaction patterns in various test systems for quantitation of cell-membrane antigens per cell-membrane unit. He found that, apart from the individual properties of the antigenic system itself, the shape of the fluorescence intensity curves was dependent on the use of direct or indirect IFT, temperature, incubation time, antibody concentration and possibly cell metabolism. The influence of temperature was mainly expressed in his procedure by the occurrence of capping phenomena.

Although variations were observed in the diameter as well as in the fluorescence intensity of the basophilic granulocytes of each donor a statistically normal distribution was found and therefore there were no indications for the existence of subpopulations.

Our staining and fixation method greatly simplifies the focusing of the basophils, which is so time consuming in phase-contrast microscopy. Focusing is essential, as measuring the fluorescence intensity of cells out of focus can lead to uncertainty of the results (21). Bleaching of the samples being measured, which can be an important source of error during quantitation (15), can be avoided now that the relevant cells can be identified by light microscopy by means of their metachromatic granules.

Differences in cell-bound fluorescence on basophils, incubated with the same amount of FlaIgE, can theoretically be ascribed to

differences in: 1) accessibility of the IgE molecules on the cell surface, 2) the number of IgE molecules per basophil, or 3) the number of basophils per incubation. Obviously, the cells are not saturated with anti-IgE antibodies at the highest concentration of FlaIgE used. This might indicate that a limiting amount of FlaIgE is used. In that case the fluorescence per cell depends on the number of cells tested. However, the fluorescence intensity was found to be independent of the number of basophils per incubation over a fourfold range. This indicates an excess of FlaIgE and an insignificant depletion by the basophils of the FlaIgE in the incubation medium. Under these conditions, antibody binding to the cells is linearly related to the number of IgE molecules per cell, provided that the affinity of the antibodies for cell-bound IgE is constant. If the IgE molecules on the cells of one donor are less accessible for the antibodies than those on the cells of another donor with the same number of IgE molecules per cell, this would result in a lower fluorescence intensity. From the slope of the FlaIgE dose-fluorescence response curves (Fig. 4), however, no differences in affinity could be demonstrated. Moreover, the elution experiments confirmed, that the IgE-load per basophil differs for different subjects. The amount of eluted IgE in the supernatant varied between 0.2 and 7 IU per 10^5 basophils, present in the leukocyte suspension. A significant correlation was established between the eluted IgE per basophil and the fluorescence intensity of the basophil (Fig. 7). From the amount of eluted IgE, an estimation of the number of IgE molecules on human basophils can be made, since elution, even at pH 4, as performed by Ishizaka et al. (12), has proved to remove all cell-bound IgE. In our method we estimate the number of IgE molecules per basophil to range from 15,000 to 500,000, when calculated with the formula: 1 IU IgE eluted per 10^5 basophils = 2.4×10^{-9} (g IgE) . 6×10^{23} / 2×10^5 (M IgE) = 7.2×10^9 mol IgE per 10^5 basophils. We conclude, that the amount of cell-bound IgE on basophilic granulocytes of different donors varies considerably. These results are in contrast with the results of Ishizaka et al. (13), who, using the C_1 fixation transfer technique, found the average number of IgE molecules per basophil to range from 10,000 to 40,000 both in atopic and in non-atopic donors. It is uncertain what factors influence the amount of basophil-bound IgE, as measured by our method. The IgE serum level has a correlation with the basophil-bound IgE, as may be expected, but a direct relation could not be established (Fig. 8). An explanation for the variations in

the amount of basophil-bound IgE may be a varying number of receptor sites for IgE on the basophilic granulocytes of different individuals, or a higher affinity of these receptors for IgE. After incubation of leukocyte suspensions with IgE myeloma protein, the number of basophil-bound IgE molecules was increased significantly in Ishizaka's experiments (13). The total number of receptor sites for IgE was estimated to range from 30,000 to 100,000, which is less than the number of basophil-bound IgE molecules found in some of our donors. Our results suggest a correlation between atopy and the fluorescence intensity of the basophils, although the number of donors in this study is too small to draw definite conclusions. In order to further investigate this correlation, studies on the basophilic granulocytes of atopic patients are in progress.

REFERENCES

1. Aalberse, R. C.; Brummelhuis, H. G. J., and Reerink-Brongers, E. E.: The purification of human polyclonal IgE by immunosorption. Immunochemistry 10: 295-303 (1973).
2. Aalberse, R. C.: Immunoglobulin E, allergens and their interaction. (Rodopi, Amsterdam 1974). Thesis.
3. Becker, K. E.; Ishizaka, T.; Metzger, H.; Ishizaka, K., and Grimley, P. M.: Surface IgE on human basophils during histamine release. J. exp. Med. 138: 394-409 (1973).
4. Boxtel, C. J. van: Platelet autoantibodies, methodological and clinical studies. (Aemstelstad, Amsterdam 1972). Thesis.
5. Böyum, A.: Isolation of leukocytes from human blood. Scand. J. clin. Lab. Invest. 21: suppl. 97, 1-51 (1968).
6. Çapel, P. J. A.: A quantitative immunofluorescence method based on the covalent coupling of protein to Sepharose beads. J. immunol. Methods 5: 165-178 (1974).
7. Feltkamp-Vroom, Th. M.; Stallman, P. J.; Aalberse, R. C., and Reerink-Brongers, E. E.: Immunofluorescence studies on renal tissue, tonsils, adenoids, nasal polyps and skin of atopic and nonatopic patients with special reference to IgE. Clin. Immunol. Immunopathol. 4: 392-404 (1975).
8. Feltkamp-Vroom, Th. M.; Wagenaar, Sj. Sc., and Swierenga, J.: Atopic lung disease and IgE mast cell fluorescence in bronchial tissue. (To be published).
9. Hijmans, W.; Schuit, H. R. E., and Klein, F. E.: An immunofluorescence procedure for the detection of intracellular immunoglobulins. Clin. exp. Immunol. 4: 457-472 (1969).
10. Ishizaka, K.; Tomioka, H., and Ishizaka, T.: Mechanisms of passive sensitization. I. Presence of IgE and IgG molecules on human leukocytes. J. Immunol. 105: 1459-1467 (1970).
11. Ishizaka, T.; DeBernardo, R.; Tomioka, H.; Lichtenstein, L. M., and Ishizaka, K.: Identification of basophil granulocytes as a site of allergic histamine release. J. Immunol. 108: 1000-1008 (1972).
12. Ishizaka, T., and Ishizaka, K.: Mechanisms of passive sensitization. IV. Dissociation of IgE molecules from basophil receptors at acid pH. J. Immunol. 112: 1078-1084 (1974).
13. Ishizaka, T.; Soto, C. S., and Ishizaka, K.: Mechanisms of passive sensitization. III. Number of IgE molecules and their receptor sites on human basophil granulocytes. J. Immunol. 111: 500-511 (1973).
14. Ishizaka, T.; Tomioka, H., and Ishizaka, K.: Degranulation of human basophil leukocytes by anti-IgE antibody. J. Immunol. 106: 705-710 (1971).
15. Jongsma, A. P.; Hijmans, W., and Ploem, J. S.: Quantitative immunofluorescence. Standardization and calibration in microfluorometry. Histochemie 25: 329-343 (1971).
16. Levy, D. A., and Osler, A. G.: Studies on the mechanisms of hypersensitivity phenomena. J. Immunol. 97: 203-212 (1966).
17. Lichtenstein, L. M.: Allergy; in BACH and GOOD Clinical immunobiology, vol. 1, pp. 243-269 (Acad. Press, New York 1972).
18. Smit, J. W.; Meijer, C. J. L. M.; Décary, F., and Feltkamp-Vroom, Th. M.: Paraformaldehyde fixation in immunofluorescence and immunoelectron microscopy. J. immunol. Methods 6: 93-98 (1974).
19. Stallman, P. J., and Feltkamp-Vroom, Th. M.: Identification of basophilic granulocytes in human leukocyte suspensions subjected to immunofluorescence procedures with anti-IgE. J. immunol. Methods 10: 271-277 (1976), (Chapter III of this thesis).
20. Sullivan, A. L.; Grimley, P. M., and Metzger, H.: Electron microscopic localization of immunoglobulin E on the surface membrane of human basophils. J. exp. Med. 134: 1403-1416 (1971).
21. Sundqvist, K. G.: Quantitation of cell-membrane antigens at the single cell level. I. Microfluorometric analysis of reaction patterns in various test systems, with special reference to the mobility of the cell-membrane. Scand. J. Immunol. 2: 479-494 (1973).
22. Wide, L.; Bennich, H., and Johansson, S. G. O.: Diagnosis of allergy by an *in vitro* test for allergen antibodies. Lancet *ii*: 1105-1107 (1967).

CHAPTER VI

QUANTITATION OF BASOPHIL-BOUND IgE IN ATOPIC AND NON-ATOPIC SUBJECTS*

P. J. STALLMAN and R. C. AALBERSE

Department of Immunohistopathology of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology, University of Amsterdam; P.O.B. 9190, Amsterdam, The Netherlands.

ABSTRACT

Basophil-bound IgE was measured by quantitative immunofluorescence microscopy in atopic subjects and in healthy controls. A correlation was found between IgE serum level and basophil-bound IgE. The basophils from the atopic patients, both from those with a low and those with an increased serum IgE, showed a significantly higher fluorescence intensity than the basophils from the respective controls. Our results indicate, that both atopy and the IgE level of the serum determine the amount of basophil-bound IgE.

INTRODUCTION

Antibodies belonging to the IgE class can bind to mast cells and basophilic granulocytes (8). The reaction of cell membrane-bound IgE with an appropriate allergen leads to the release of several substances, such as histamine, which mediate the allergic response. After the presence of IgE bound on human basophils had been demonstrated by Ishizaka et al. (9) by means of autoradiography, the question was raised how much IgE is present on the basophilic granulocytes, and whether there is a relation between the amount of IgE present on these cells, and atopy. Using the C_1 fixation transfer test, the number of cell-bound IgE molecules was measured by Ishizaka et al. (10) on the basophils of 13 subjects. In that study the number of basophil-bound IgE molecules was estimated to range from 10,000 to 40,000, while no correlation could be established between the

*Int. Arch. Allergy (in press).

number of IgE molecules bound, and either the serum level of IgE or atopy. Recently, however, Conroy et al. (5, 11) reported a correlation between the IgE level of the serum and the amount of IgE eluted from the basophils. By means of reversed anaphylaxis, the amount of basophil-bound IgE was estimated in patients with chronic urticaria by Greaves et al. (7). They could not find evidence for a quantitative difference of IgE bound to the basophils of the patients and to those of the controls.

In a previous study we were able to determine the amount of basophil-bound IgE in a small group of donors by quantitative immunofluorescence microscopy (13). The results suggested a correlation between atopy and the intensity of the IgE-fluorescence on the basophilic granulocytes. Elution experiments confirmed that the fluorescence intensity of the basophils is correlated with the amount of basophil-bound IgE (13). In this study we investigated the cells of atopic subjects and controls with low as well as increased IgE serum levels, to assess the influence of both the IgE level of the serum and atopy on basophil-bound IgE.

MATERIALS AND METHODS

Selection of the subjects

Heparinized blood and serum samples were obtained from the subjects, who were divided in four groups:
Group I: atopic patients with an IgE serum level ⩾ 200 IU/ml,
Group II: atopic patients with an IgE serum level ⩽ 100 IU/ml,
Group III: control subjects with an IgE serum level ⩽ 100 IU/ml and
Group IV: control subjects with an IgE serum level ⩾ 200 IU/ml.

The distribution of age and sex in the subjects of the four groups is given in table I. The diagnosis of atopy was based on clinical grounds: anamnese, family history and clinical symptoms (at least two of these had to be positive for atopy), combined with a positive result in the Radio Allergo Sorbens Test (RAST) or skin reaction. Since the patients in Group II form only a small subpopulation of atopic patients, they were preselected for the combination of a low IgE serum level and a positive RAST score in routine diagnostic measurements.

The atopic patients were being treated in the St. Antonius Hospital (Utrecht) and the Institute of Allergic Diseases (Amsterdam). Control Group III consisted of healthy donors (Red Cross

TABLE I. Distribution of age and sex in the various groups of donors.

Group	Age				Sex	
	0-10 years	11-30 years	31-50 years	⩾51 years	m.	f.
1: atopics, IgE serum level ⩾ 200 IU/ml.	6	4	4	0	10	4
2: atopics, IgE serum level ⩽ 100 IU/ml.	0	6	4	4	6	8
3: controls, IgE serum level ⩽ 100 IU/ml.	4	8	5	2	10	9
4: controls, IgE serum level ⩾ 200 IU/ml.	2	4	2	2	7	3

Blood Bank, Amsterdam) and of children undergoing tonsillectomy in the Maarschalksbos Hospital (Baarn). Control group IV consisted of healthy blood donors and of one patient with a Schistosoma infection, who was treated at the Institute of Tropical Hygiene (Amsterdam).

Preparation of the leukocyte suspensions

Leukocyte suspensions were prepared from 10 ml heparinized blood, as previously described (13, 14). After erythrocyte sedimentation with dextran, the leukocytes were separated on Ficoll-Isopaque. The interphase fractions were collected, stained and fixed with toluidine blue 0.1% and paraformaldehyde 1%.

Quantitative immunofluorescence technique

The direct immunofluorescence procedure, applied to cell suspensions, has been previously described (14), and so has the production and testing of the sheep anti-human IgE serum, labeled with fluorescein isothiocyanate (FlaIgE), used in these experiments (6). Aliquots of the cell suspension were incubated at room temperature for 60

minutes, with the FlaIgE in final dilutions ranging from 1 : 40 to 1 : 320. The fluorescence intensity of the basophils was measured, as previously described (13), with a Leitz Orthoplan microscope equipped with a Leitz photometer attachment, and expressed in arbitrary units as the relative fluorescence intensity (RFI). Basophilic granulocytes were identified by light microscopy, and the fluorescence intensity of single cells was measured. Contrary to the previously described quantitation procedure, the fluorescence intensity of an additional 50 basophils was measured in the leukocyte suspensions incubated with FlaIgE, diluted 1 : 40. Mean values were calculated and mean background measurements subtracted.

The assay of total IgE and the Radio Allergo Sorbens Test (RAST)

Total serum IgE was quantitated in the department of Immunopathology of Serum Proteins by an inhibition assay, using Sepharose-coupled IgE (1, 2). A non-competitive assay (13) was applied to the sera in which, using the inhibition assay, an amount of IgE of less than 100 IU/ml was found. The RAST was performed as described by Wide et al. (16) with slight modifications (13). All subjects were routinely tested for the presence of IgE antibodies against grasspollen and housedust allergen, and sera were tested for the presence of IgE antibodies against other allergens (house mite, human dander, cat dander) if there was a clinical indication to do so.

RESULTS

Basophil fluorescence with FlaIgE

After incubation with FlaIgE, the basophilic granulocytes showed a ring-shaped fluorescence pattern (Fig. 1a). The basophils could be easily detected in light microscopy by their metachromatic granules (Fig. 1b).

Quantitation of the fluorescence intensity

The mean fluorescence intensity of the basophils, obtained after incubation with each dilution of FlaIgE, was calculated for each donor and background values were subtracted. In a previous study it was shown, that the intensity of the fluorescence on the basophilic granulocytes decreased with decreasing concentrations of FlaIgE in the incubation medium (13). The intensity of the fluorescence

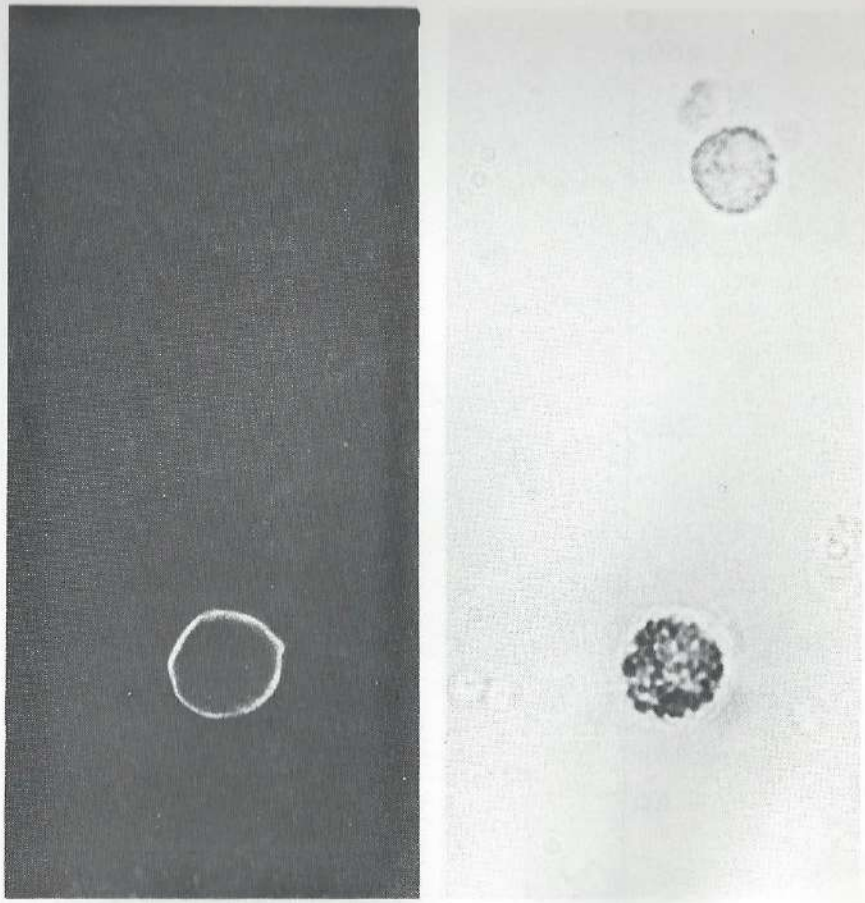


Fig. 1a: A human leukocyte suspension, incubated with sheep anti-human IgE-FITC (FlaIgE), diluted 1 : 40. Membrane-bound fluorescence is observed on one of the cells. (x 1000)

Fig. 1b: The same cells as seen in figure 1a, observed in light microscopy. This cell preparation was stained with toluidine blue 0.1% and fixed with PFA 1%. The cell, demonstrating membrane-bound fluorescence in figure 1a, is identified as a basophilic granulocyte by its metachromatic granules. (x 1000)

showed a linear correlation with the logarithm of the FlaIgE concentration over the range tested. To establish the dose-response curves of the basophils from the subjects of the four groups used in this study, the mean intensity of the fluorescence on the basophils, incubated with FlaIgE in various dilutions, was calculated for the whole group

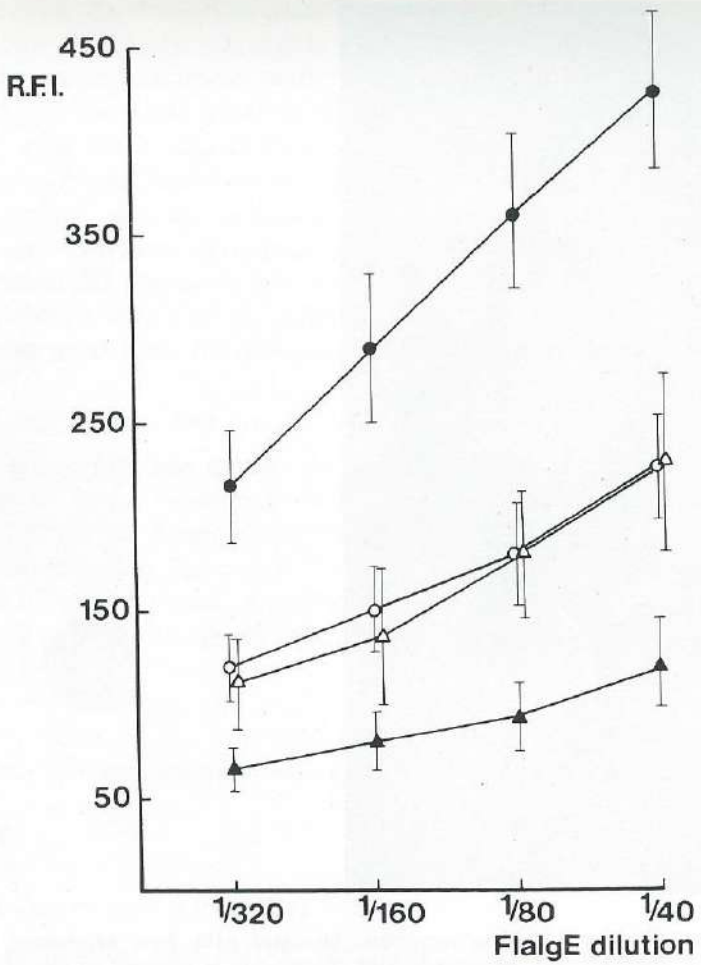


Fig. 2: The mean fluorescence intensity (± 2x standard error of the mean) of the basophils from all subjects in each of the four groups. The leukocytes were incubated with FlaIgE in twofold dilutions for 60 minutes. The fluorescence intensity was measured by quantitative fluorescence microscopy and background values were subtraced. The linear regression was determined.

●–● = atopic patients (serum IgE ⩾ 200 IU/ml), r = 0.999;
○–○ = atopic patients (serum IgE ⩽ 100 IU/ml), r = 0.995;
▲–▲ = healthy controls (serum IgE ⩽ 100 IU/ml), r = 0.981;
△–△ = controls (serum IgE ⩾ 200 IU/ml), r = 0.990.

The percentual decrease, using decreasing concentrations of FlaIgE, was calculated for the individual subject (the fluorescence intensity after incubation with FlaIgE diluted 1 : 40 was taken as a 100%-value). Mean values were:

●–● = 100%, 80%, 68%, 54%; ○–○ = 100%, 83%, 66%, 50%;
▲–▲ = 100%, 78%, 66%, 56%; △–△ = 100%, 80%, 60%, 50%.

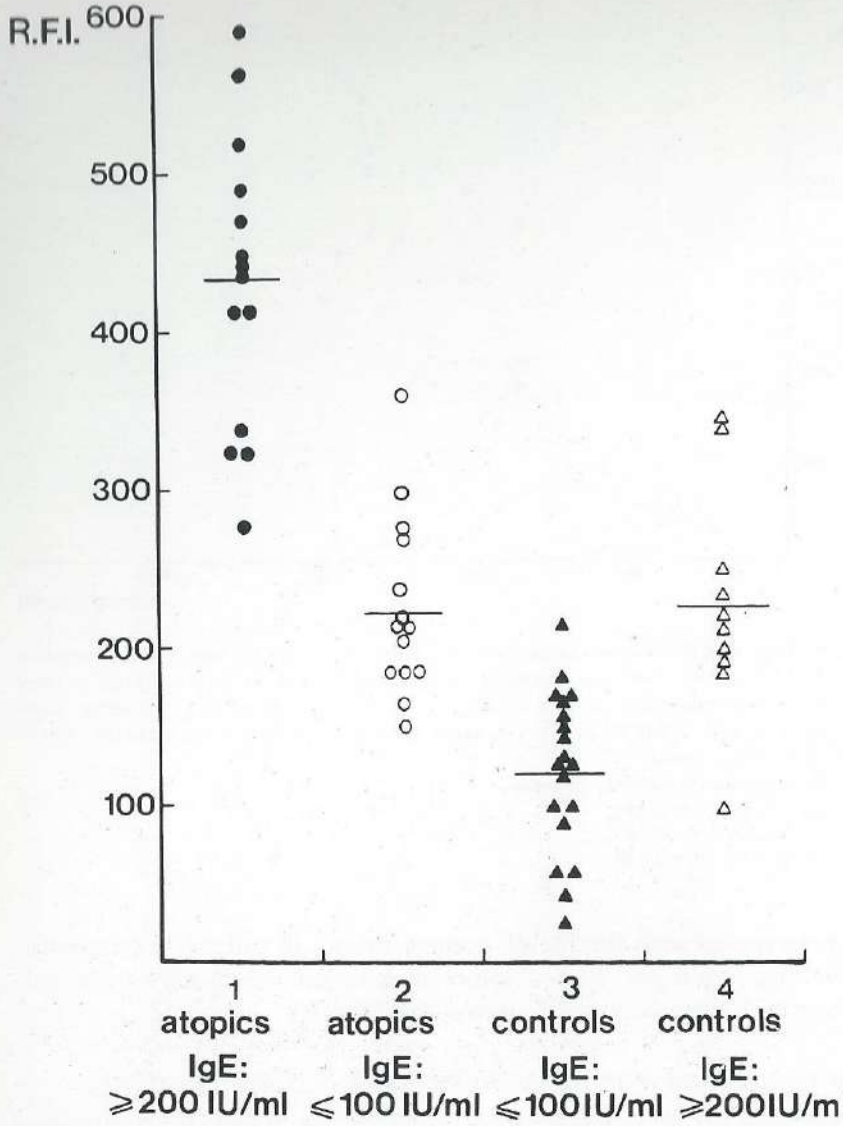


Fig. 3: The mean fluorescence intensity of the basophils from 57 subjects after incubation with FlaIgE, diluted 1 : 40.

● = atopics (serum IgE ⩾ 200 IU/ml);
○ = atopics (serum IgE ⩽ 100 IU/ml);
▲ = controls (serum IgE ⩽ 100 IU/ml);
△ = controls (serum IgE ⩾ 200 IU/ml).

Student t test analysis gives significant differences between various groups (I vs II: $p < 0.001$; I vs III: $p < 0.001$; I vs IV: $p < 0.001$; III vs IV: $p < 0.001$; II vs IV not significant).

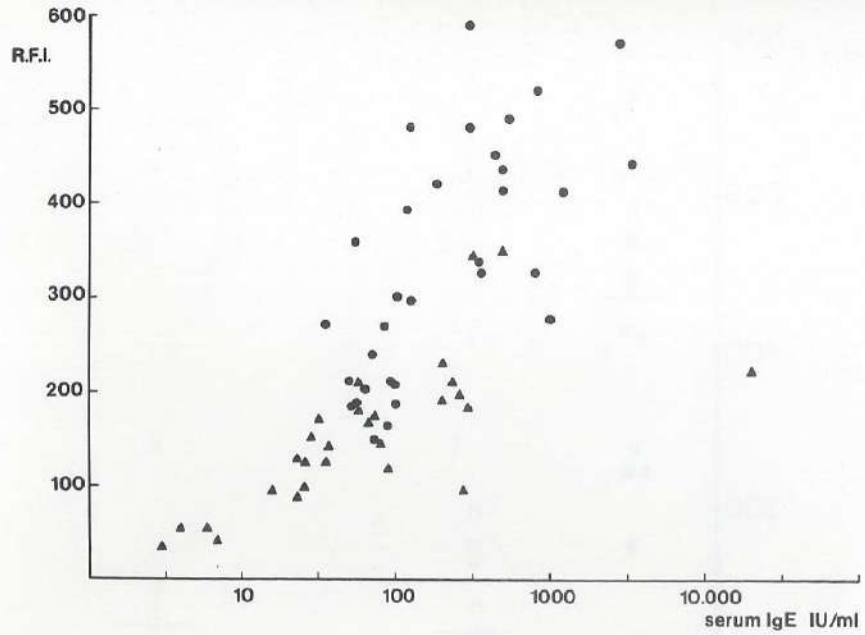


Fig. 4: The mean fluorescence intensity of the basophils from 61 subjects after incubation with FlaIgE, diluted 1 : 40, was compared with the logarithm of the total serum IgE in these subjects. Apart from the subjects already mentioned, 4 subjects with IgE serum levels ranging from 100 to 200 IU/ml were included also in this diagram. Spearman rank correlation: 0.7721, $p < 0.001$.
The linear regression was determined:
all subjects – r = 0.691,
• =all atopics – r = 0.685,
▲ =all controls – r = 0.730.

and compared with the FlaIgE concentration. In order to exclude the possibility, that the higher values dominated the mean values, the percentual decrease was calculated also (Fig. 2).

The fluorescence intensity of the basophils in relation to atopy

Because no differences were found in the shape of the fluorescence intensity curves with FlaIgE, we used the fluorescence intensity of the basophils, incubated with FlaIgE, diluted 1 : 40, to compare the four groups of subjects (Fig. 3). For statistical analysis the Student t test was used. A significant difference ($p < 0.001$) could be established between the fluorescence intensity values of the basophils from the atopic subjects in group I (serum IgE $\geqslant$ 200 IU/ml)

and the control Groups III (serum IgE $\leqslant$ 100 IU/ml) and IV (serum IgE $\geqslant$ 200 IU/ml). The fluorescence intensity of the basophils from the atopic patients with an IgE serum level $\leqslant$ 100 IU/ml (Group II) was significantly lower than the fluorescence intensity of the atopic subjects in Group I ($p < 0.001$), but significantly higher than the fluorescence intensity of the controls with an IgE serum level $\leqslant$ 100 IU/ml ($p < 0.001$). Comparison between the fluorescence intensity of the basophils from 13 atopic patients and 13 controls, matched for the IgE level of their sera, also showed a significantly higher amount of IgE on the basophils from atopics ($p < 0.01$). No significant difference was observed between Group II and Group IV.

The fluorescence intensity of the basophils in relation to the total serum IgE

When we compared the two groups of atopic patients, the influence of the IgE serum level was already demonstrated, but this influence was also present in the non-atopics. The basophils from the control subjects with an IgE serum level $\geqslant$ 200 IU/ml had a significantly higher fluorescence intensity than those from the controls with a low IgE serum level. To assess the relation between the serum IgE and basophil-bound IgE in the individual subject, the logarithms of serum IgE and the fluorescence intensity of the basophils were compared for all subjects (Fig. 4). A highly significant correlation was found, using the Spearman rank test ($p < 0.001$), but it is clear that the range of serum IgE values from the subjects, with the same fluorescence intensity of the basophils, is very wide. The linear correlation between the logarithm of the IgE serum level and basophil-bound IgE was 0.7 in the whole group, which was approximately the same in the separate groups of atopics and controls (Fig. 4).

DISCUSSION

The results of earlier experiments (13) indicated a considerable variation in the amount of IgE per basophil and suggested a correlation between atopy and the amount of basophil-bound IgE. In the present study the fluorescence intensity of the basophils was found again to vary among the different subjects. The amount of IgE on the basophils fitted in the range, previously established in 2 atopic and 4 control subjects (15,000 to 500,000 IgE molecules per basophil). We

also confirmed previous findings, i.e. that atopic subjects showed a higher amount of basophil-bound IgE than non-atopic subjects. However, the question should be raised now, whether this is an effect of the atopy itself or merely of the IgE serum level, since the majority of the atopic patients have an elevated serum IgE (4). Healthy controls, on the other hand, mostly have an IgE serum level ⩽ 100 IU/ml (12).

To study the influence of both atopy and serum IgE upon basophil-bound IgE, the forementioned four groups were formed, by selecting atopic patients with a low IgE serum level (about 10% of the population of atopics) and the rare non-atopic controls with an elevated IgE serum level. Concerning the atopy, a significant difference was found between basophil-bound IgE in atopics with an IgE serum level ⩾ 200 IU/ml and controls with an elevated IgE serum level. Moreover, atopic patients with an IgE serum level ⩽ 100 IU/ml showed a significantly higher amount of basophil-bound IgE than the relevant control group. This difference in the amount of cell-bound IgE could be observed also in individual IgE-matched atopics and controls. However, the influence of the IgE serum level cannot be ignored. Within the population of atopics, the basophils from the subjects with an elevated serum IgE (Group I) showed a significantly higher fluorescence intensity than those from atopics with a low IgE level (Group II). The same difference could be seen in the two control groups. The effect of the IgE serum level upon basophil-bound IgE was expressed also in the individual subject by a highly significant rank correlation, but the fluorescence intensity had only a weak linear correlation with the logarithm of the serum IgE concentration. Conroy et al. (5) recently measured the IgE in the supernatants after acid elution of the leukocytes and found a direct relation between serum IgE and the amount of IgE, eluted per basophil (11). A correlation between basophil-bound IgE and serum IgE could not be found by Ishizaka et al. (10), Sullivan et al. (15), who applied immunoelectron microscopy, nor by Assem (3), who measured cell-bound IgE in leukocyte suspensions by means of a radio immunosorbent technique. And neither these authors (10, 15, 3) nor Greaves et al. (7) found a clear correlation between the amount of basophil-bound IgE and atopy.

Our results indicate, that both atopy and the IgE serum level determine the amount of basophil-bound IgE. Atopy is thought to be reflected in the amount of cell-bound IgE by having an effect on

either the number of IgE-receptors or on the affinity of the latter to IgE. The influence of serum IgE might be explained by assuming an equilibrium between cell-bound and free IgE. Ishizaka et al. (10) found a 1.1- to 7-fold increase of basophil-bound IgE, measured with the C_1 fixation transfer technique, after incubation of the basophils with IgE myeloma protein. In our preliminary experiments in passive sensitization, however, we did not find an increase of the fluorescence intensity of the basophils from subjects with a low serum IgE after incubation with the same myeloma protein. Further study is required, therefore, to see whether an increase in basophil-bound IgE can be obtained by sensitization with serum from atopic patients or by changing the incubation conditions.

REFERENCES

1. Aalberse, R. C.; Brummelhuis, H. G. J., and Reerink-Brongers, E. E.: The purification of human polyclonal IgE by immunosorption. Immunochemistry 10: 295-303 (1973).
2. Aalberse, R. C.: Immunoglobulin E, allergens and their interaction. (Rodopi, Amsterdam 1974). Thesis.
3. Assem, E. S. K.: Leukocyte-bound IgE and cell reactivity in asthma. Allergol. et Immunopath. II: 41-46 (1974).
4. Berg, T., and Johansson, S. G. O.: IgE concentrations in children with atopic diseases. A clinical study. Int. Arch. Allergy 36: 219-232 (1969).
5. Conroy, M. C., and Lichtenstein, L. M.: Measurement of IgE binding to human leukocytes. Fed. Proc. 35: 809 (1976).
6. Feltkamp-Vroom, Th. M.; Stallman, P. J.; Aalberse, R. C., and Reerink-Brongers, E. E.: Immunofluorescence studies on renal tissue, tonsils, adenoids, nasal polyps, and skin of atopic and nonatopic patients with special reference to IgE. Clin. Immunol. Immunopathol. 4: 392-404 (1975).
7. Greaves, M. W.; Plummer, V. M.; McLaughlan, P., and Stanworth, D. R.: Serum and cell-bound IgE in chronic urticaria. Clin. Allergy 4: 265-271 (1974).
8. Ishizaka, K., and Ishizaka, T.: Mechanisms of reagenic hypersensitivity: a review. Clin. Allergy 1: 9-24 (1971).
9. Ishizaka, K.; Tomioka, H., and Ishizaka, T.: Mechanisms of passive sensitization. I. Presence of IgE and IgG molecules on human leukocytes. J. Immunol. 105: 1459-1467 (1970).
10. Ishizaka, T.; Soto, C. S., and Ishizaka, K.: Mechanisms of passive sensitization. III. Number of IgE molecules and their receptor sites on human basophil granulocytes. J. Immunol. 111: 500-511 (1973).
11. Ishizaka, T., and Ishizaka, K.: Immunological events at the surface of basophil granulocytes and mast cells which induce degranulation. Boerhave Symposion, Leiden. The Netherlands (1976).

12. Kjellman, N. I. M.; Johansson, S. G. O., and Roth, A.: Serum IgE levels in healthy children quantified by a sandwich technique (PRIST). Clin. Allergy 6: 51-59 (1976).
13. Stallman, P. J., and Aalberse, R. C.: Estimation of basophil-bound IgE by quantitative immunofluorescence microscopy. Int. Arch. Allergy (in press). (Chapter V of this thesis).
14. Stallman, P. J., and Feltkamp-Vroom, Th. M.: Identification of basophilic granulocytes in human leukocyte suspensions subjected to immunofluorescence procedures with anti-IgE. J. immunol. Methods 10: 271-277 (1976) (Chapter III of this thesis).
15. Sullivan, A. L.; Grimley, P. M., and Metzger, H.: Electron microscopic localisation of immunoglobulin E on the surface membrane of human basophils. J. exp. Med. 134: 1403-1416 (1971).
16. Wide, L.; Bennich, H., and Johansson, S. G. O.: Diagnosis of allergy by an *in vitro* test for allergen antibodies. Lancet ii. 1105-1107 (1967).

CHAPTER VII

CELL-BOUND IgE ON HUMAN MAST CELLS AND BASOPHILIC GRANULOCYTES IN ATOPIC AND NON-ATOPIC SUBJECTS*

P. J. STALLMAN, SJ. SC. WAGENAAR, J. SWIERENGA, R. J. VAN DER WAL and TH. M. FELTKAMP-VROOM

Department of Immunohistopathology of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology, University of Amsterdam, P.O.B. 9190, Amsterdam;
Department of Pathology of the St. Antonius Hospital, Utrecht;
Clinic for Lung Disease of the St. Antonius Hospital, Utrecht;
Clinic for Otorhinolaryngology of the Maarschalksbos Hospital, Baarn;
Department of Pathology of the Slotervaart Hospital, Amsterdam, The Netherlands

ABSTRACT

In previous studies basophil-bound IgE was measured by means of quantitative immunofluorescence microscopy. But as mast cells are of particular importance in local atopic reactions, we thought it necessary to investigate the IgE-load on both cell types. The amount of mast cell-bound IgE can be estimated in a semi-quantitative way by end point titration with a fluoresceinated sheep anti-human IgE (FlaIgE). The amount of mast cell-bound IgE was estimated this way in tonsils, adenoids, bronchial and tracheal tissues, and was compared with the amount of IgE on the basophils. A correlation was found between the IgE-load on mast cells and on basophils. Atopic patients had a higher mast cell fluorescence titer (MCFT) than non-atopic controls. It was demonstrated that, as on basophils, the IgE level of the serum contributes to this difference.

*Int. Arch. Allergy (in press)

INTRODUCTION

The interaction between immunoglobulin E, fixed on mast cells, and an allergen is one of the mechanisms (16) known to initiate the release of the mediators of allergy (9). The presence of IgE on human mast cells was demonstrated by Ishizaka et al. (8) and Callerame et al. (2) by means of autoradiography. The presence of this immunoglobulin was also demonstrated on mast cells in several tissues by means of the immunofluorescence technique with fluoresceinated anti-IgE (4). The intensity of the fluorescence of the mast cells seemed to vary, thus indicating differences in the amount of cell-bound IgE. This was considered to be confirmed by experiments, determining the amount of IgE on mast cells in human bronchial biopsy specimens semi-quantitatively by end point titration with FlaIgE (5). In atopic patients, most of them having a high IgE serum level, a high mast cell fluorescence titer (MCFT) was found.

Previously, we described a method to identify human basophils in leukocyte suspensions (12) and to quantify the fluorescence intensity of the basophils after incubation with FlaIgE (10). This fluorescence intensity correlated with the amount of IgE per basophil, as could be concluded from elution experiments (10). Studies of the basophils from atopic and non-atopic subjects showed differences in the fluorescence intensity (11). The amount of basophil-bound IgE was found to correlate with the IgE level of the serum and with the presence or absence of atopy.

Since especially mast cells are involved in local atopic reactions, we considered it necessary to compare the amount of IgE on mast cells with that on basophils. We also investigated whether both IgE serum level and atopy do affect mast cells as well as basophils, with regard to the amount of cell-bound IgE.

MATERIALS AND METHODS

Selection of the patients

For the determination of the mast cell fluorescence titer (MCFT), tissues were obtained from the following subjects:

- bronchial biopsy specimens were taken from 15 atopic patients, attending the St. Antonius Hospital in Utrecht;
- adenoid and tonsillar tissues were obtained from 10 atopic children undergoing adeno(tonsillec)tomy at the Maarschalksbos Hospital in Baarn;
- control bronchial tissues were obtained from 15 patients suffering from miscellaneous lung diseases like bronchitis (5), bronchiectasia (1), lung tuberculosis (1), sarcoidosis (1), bronchial carcinoma (6) and metastases of a mammary carcinoma (1);
- control adenoid and tonsillar tissues were obtained from 30 non-atopic children.

TABLE I — Nature of the tissues, used to determine the mast cell fluorescence titer.

	Bronchial tissue	Adenoid	Adenoid + tonsil	Total
Atopic subjects	15	6	4	25
Non-atopic subjects	15	12	18	45

The diagnosis of atopy was based on clinical grounds (anamnesis, family history and clinical symptoms: at least two of these had to be positive for the diagnosis of atopy) combined with a positive RAST score or skin reaction. From 19 of the atopic subjects and 18 of the controls heparinized blood could be obtained for the preparation of the leukocyte suspensions to determine the amount of basophil-bound IgE as previously described (12). The nature of the tissues investigated in the atopic and non-atopic subjects is summarized in Table I. The distribution of age and sex of the subjects is given in Table II.

TABLE II — Distribution of age and sex in the subjects studied. ( ) = in these subjects basophil-bound IgE was also determined.

	0-10 years	11-30 years	31-50 years	⩾51 years	Total	m	f
Atopic Subjects	15(11)	6(4)	2(2)	2(2)	25(19)	15	10
Non-atopic Subjects	27(6)	8(5)	6(3)	4(4)	45(18)	29	16

Immunofluorescence experiments on tissues

Biopsy samples were obtained from the tracheal and/or bronchial mucosa, adenoid and tonsillar tissue, snapfrozen and stored in liquid nitrogen as described earlier (4). Four μm sections were cut in a cryostat at -20° C, dried and rinsed in phosphate buffered saline (PBS) for a short time. They were dried again, fixed in acetone for 10 minutes and incubated with FlaIgE for 60 minutes. The production and specificity testing of this antiserum has been described previously (4). The sections were washed in PBS for 30 minutes and mounted with glycerol/PBS 1:1 v/v. Serial twofold dilutions of 1:40 up to 1:640 of the FlaIgE were used for the determination of the fluorescence intensity of the peripheral rim of the mast cells. The mast cell fluorescence titer (MCFT), defined as the reciprocal value of the highest dilution at which positive mast cell fluorescence was seen, was determined. Sometimes a weakly positive mast cell could still be observed, when an antiserum dilution was used at which the large majority of the mast cells had become negative. These cells were ignored in titer determination. A similar distribution of the fluorescence intensity with FlaIgE had previously been found with regard to basophils (10). In assessing the MCFT, neither the subject, whose tissues were investigated, nor the dilution of the FlaIgE was known to the observer. Fluorescence microscopy and photography on Kodak Tri X film was performed as described earlier (4). In those cases in which no mast cell fluorescence was observed with the highest concentration of FlaIgE, an adjacent section was stained with toluidine blue 1% in 0.05 M acetate buffer pH 4, to make sure by light microscopy, that mast cells were present.

Quantitative immunofluorescence technique on basophilic granulocytes

Leukocyte suspensions were prepared from 10 ml of heparinized blood as previously described (10, 11, 12). After erythrocyte sedimentation and separation on Ficoll-Isopaque, interphase fractions were collected. The cells were stained and simultaneously fixed with toluidine blue and paraformaldehyde, and incubated with FlaIgE, diluted 1:40, for 60 minutes. The intensity of the fluorescence of the single basophils was measured (10), and expressed in arbitrary units as the relative fluorescence intensity (RFI). Mean values were calculated and mean background measurements subtracted.

The assay of total serum IgE and the radio allergo sorbens test (RAST)

Total serum IgE was measured by an inhibition assay (10). A more sensitive non-competitive assay (10) was applied to the sera, in which, using the inhibition assay, an amount of IgE of less than 100 IU/ml was found. The RAST was performed, as described by Wide et al. (17), with some modifications (10), in order to determine the presence of IgE antibodies against grasspollen and house dust. The presence of IgE antibodies against other allergens (house mite, human dander, cat dander) was tested when an allergic reaction to another allergen was suspected.

RESULTS

The mast cell fluorescence titer in relation to atopy

After incubation with FlaIgE, the typical round or spindle shaped fluorescence pattern of the mast cells was observed in the large majority of the sections (Fig. 1). If no mast cell fluorescence was

Fig. 1: Mast cell fluorescence pattern in a human tonsil of an atopic subject after incubation with FlaIgE, diluted 1:40. (x 325)

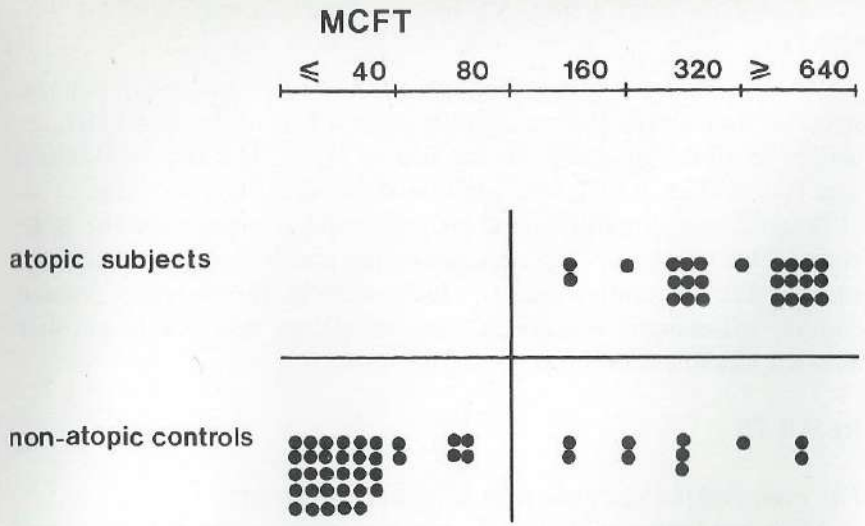


Fig. 2: The mast cell fluorescence titers of 25 atopic subjects and 45 controls. Statistical analysis, using the chi-square test gives a significant difference ($p < 0.001$).

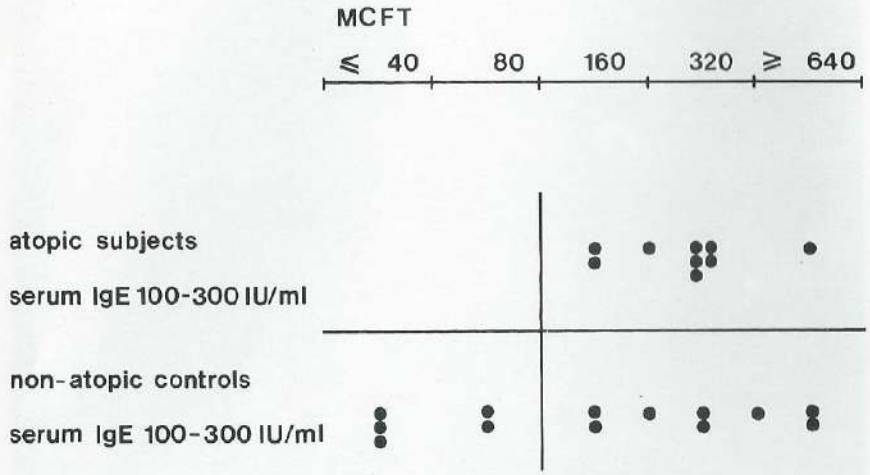


Fig. 3: The mast cell fluorescence titers in atopic and non-atopic subjects, both with an IgE serum level ranging from 100-300 IU/ml (mean atopics: 199 IU/ml; mean non-atopics: 191 IU/ml). Fisher's exact test: $p < 0.05$.

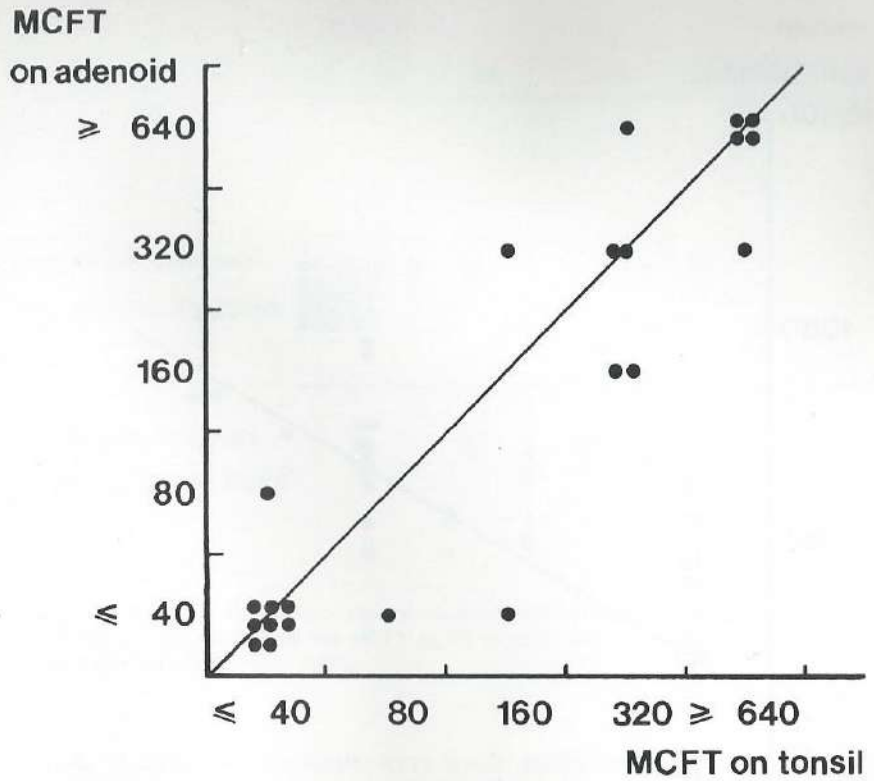


Fig. 4: The mast cell fluorescence titers determined on the adenoid tissues, compared with those found on the tonsillar tissues from 22 subjects.

seen, toluidine blue staining proved the presence of mast cells. The mast cell fluorescence titer (MCFT) in the tissues of the atopic and control subjects was determined by the use of serial dilutions of FlaIgE (Fig. 2). Using the chi-square test, a highly significant difference ($p < 0.001$) was established between the MCFT of the atopic subjects and that of the non-atopic controls. The atopics demonstrated a higher MCFT than the non-atopics, but they also had a higher IgE serum level. The latter has previously been shown to have the effect of increasing the amount of cell-bound IgE on basophils (11).

Therefore, atopic and non-atopic subjects, with IgE serum levels in the same range (100-300 IU/ml), were compared for the MCFT (Fig. 3). Statistical analysis, using Fisher's exact test, showed that the

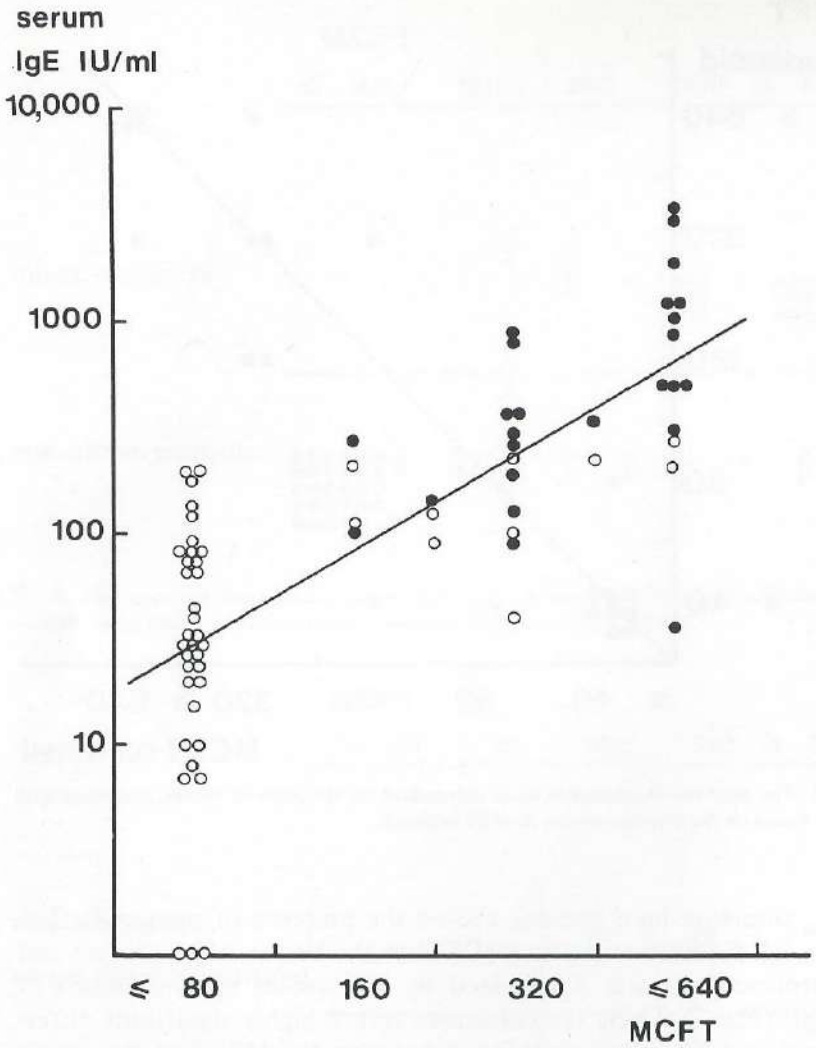


Fig. 5: The relation between the mast cell fluorescence titer and the total serum IgE in 25 atopic subjects (•) and 45 controls (o). The linear correlation between log serum IgE and log titer had a correlation coefficient (r) of 0.7367.

absence of low mast cell fluorescence titers in the atopic group was significant ($p < 0.05$).

Both adenoid and tonsils were obtained from 22 subjects and the MCFT of both organs from the same subject were compared (Fig. 4). In only one case a titer difference of more than one titer step was found. In this case sections were made again and no difference between the MCFT on both organs was found.

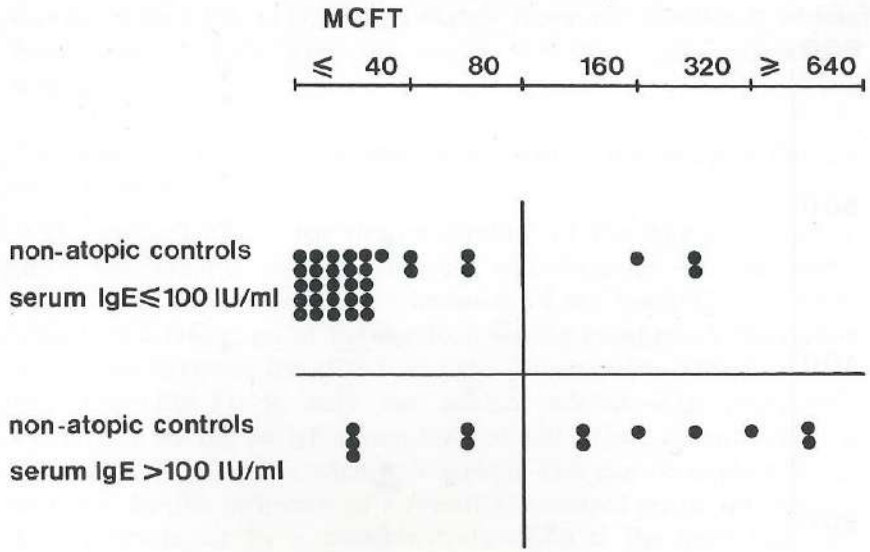


Fig. 6: The mast cell fluorescence titer of 33 non-atopic controls with an IgE serum level ≤ 100 IU/ml, compared with the MCFT of 12 controls with an IgE serum level > 100 IU/ml. Fisher's exact test: $p < 0.002$.

The mast cell fluorescence titer in relation to the IgE serum level

The relation between the mast cell fluorescence titer and the amount of IgE in the serum is shown in Fig. 5 for both atopic and non-atopic subjects: a positive correlation was found. The influence of the IgE serum level on cell-bound IgE was also demonstrated by comparing non-atopics with an IgE serum level of less than 100 IU/ml, with non-atopics with an elevated serum IgE. A significantly higher MCFT was found in the latter group, using Fisher's exact test (Fig. 6; $p < 0.002$). This influence of the IgE serum level accounts for the majority of the high mast cell fluorescence titers found in the control group in Fig. 2.

Since the IgE serum level was less than 100 IU/ml in only 3 atopic patients, no statistical analysis could be done to confirm that a smaller amount of IgE is bound on cells from atopics with a low IgE serum level, which has previously been found to be the case with

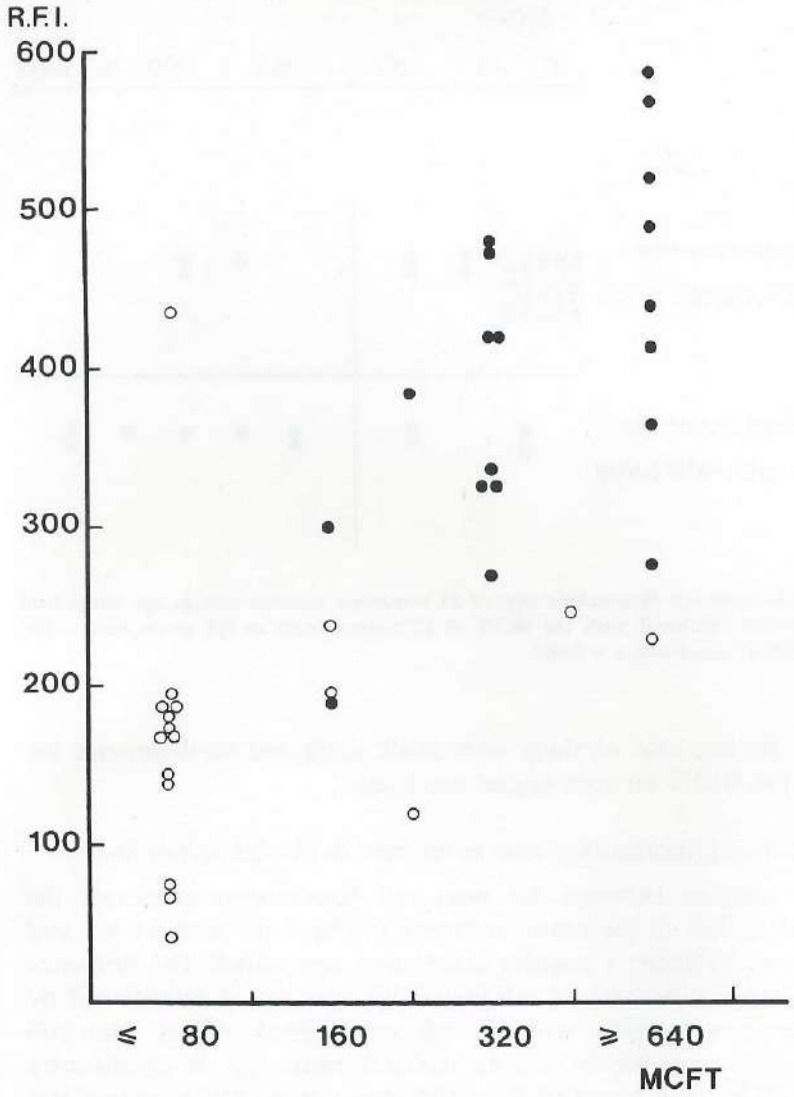


Fig. 7: The relation between the mast cell fluorescence titer and the fluorescence intensity of the basophils (RFI) from 19 atopic (•) and 18 control (o) subjects. Spearman rank correlation coefficient: 0.7612, $p < 0.0005$.

regard to basophils (11). Unfortunately it was not possible to obtain tissue samples from more subjects in this small subpopulation of atopics.

The mast cell fluorescence titer in relation to the basophil fluorescence intensity

Heparinized blood, for the preparation of the leukocyte suspensions, was obtained from 37 subjects, whose tissues were also investigated. The mean fluorescence intensity of the basophils was determined, as a reflection of the IgE-load on the basophil. A correlation was found between the MCFT and the fluorescence intensity of the basophils (Fig. 7). In only one control subject with a bronchial carcinoma, having an IgE serum level of 220 IU/ml, basophil fluorescence and MCFT were widely divergent. This can be explained, for instance, by the influence of a recently increased serum IgE level on the basophils, or by a possible dysfunction of the bronchial mast cells.

DISCUSSION

By means of the direct immunofluorescence technique, IgE was demonstrated on mast cells in tissue sections from various organs from atopic and non-atopic subjects. An estimation of the amount of IgE on these cells was made by end point titration with the fluoresceinated anti-IgE (FlaIgE).

The presence of IgE on mast cells was reported by others (2, 7, 8), but they did not find evidence for differences in the amount of IgE. Variations in the fluorescence intensity of skin mast cells were observed by Takahashi et al. (13), investigating mast cell-bound IgE in patients with atopic dermatitis. In the present study it could be confirmed that the mast cell fluorescence titers in atopic patients are higher than those of the non-atopics, as was found previously (5). The IgE level of the serum, previously shown to affect basophil-bound IgE (11), was also demonstrated to influence the MCFT in non-atopic subjects. Due to the small number of atopics with a low serum IgE in our series, this relation could not be investigated in atopics. In the whole group of atopics and non-atopics a correlation was established between MCFT and IgE serum level. This correlation was not found by Takahashi et al. (13).

Do higher mast cell fluorescence titers indicate a larger amount of mast cell-bound IgE and how is the relation between the fluorescence intensity and the amount of IgE on the mast cells? The reaction between cell-bound IgE and FlaIgE is dependent on the number of cell-bound IgE molecules, on the concentration of FlaIgE and on an equilibrium constant K. The conditions we assume for the immunofluorescence procedure on sections are the following: a) an increase in the volume of the FlaIgE, used for incubation, does not result in an increase in fluorescence; b) fluorescence is not dependent on the number of mast cells in the section; c) prolonged incubation does not induce an increase of the fluorescence intensity. Under those conditions there is an excess of FlaIgE-antibodies, and therefore, the concentration of the antiserum does not diminish significantly by binding antibodies to mast cell-bound IgE. In this case the following relation between antiserum dilution and mast cell-bound IgE can be derived from the law of mass action:

$$\frac{1}{[\text{FlaIgE}]} = \frac{K.\text{MCIgE}}{B} - K$$

B is the amount of mast cell-bound IgE (MCIgE) reacted with FlaIgE. In end point titration FlaIgE is diluted till B reaches B_{lim}, which is the minimal amount of MCIgE reacted with FlaIgE, necessary for a positive score. The reciprocal value of this dilution is called the end point titer (MCFT). This titer was found to correlate linearly with the amount of MCIgE according to the formula:

$$\text{MCFT} = \frac{K.\text{MCIgE}}{B_{lim}} - K \text{ (fig. 8)}$$

A high K-value will cause a fast rise in titer with only a slight increase in the amount of mast cell-bound IgE. The K-value is probably determined by both the affinity of the antiserum and the accessibility of cell-bound IgE in the tissue section.

Our results point to a correlation between the amount of IgE on mast cells and on basophils. The IgE-load on the basophils was estimated in a different way, i.e. direct quantitation of the immunofluorescence at a constant antiserum dilution. In this situation, the fluorescence intensity of the basophils (B) depends on the amount of basophil-bound IgE (BasIgE), the concentration of FlaIgE and a K-value:

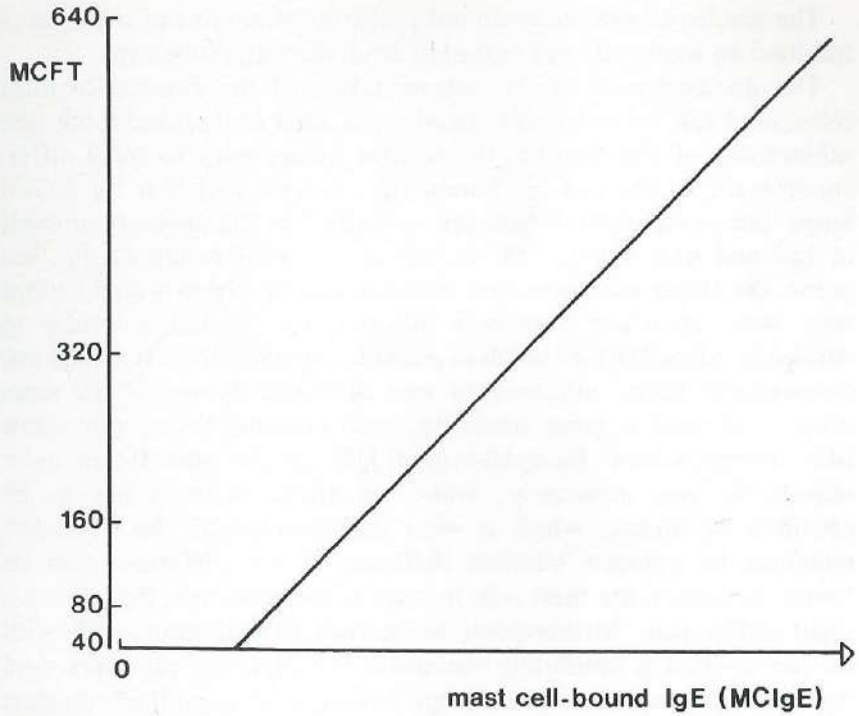


Fig. 8: The theoretical relation between mast cell fluorescence titer and mast cell-bound IgE (MCIgE).

$$B = \text{BasIgE}. \frac{K.[\text{FlaIgE}]}{1+K.[\text{FlaIgE}]}$$

This linear correlation between the fluorescence intensity and the amount of IgE bound to the basophils had been confirmed by the results of previous elution experiments (10).

Since the existence of villi of the mast cell-surface membrane has been described in literature (1, 3, 6, 14), it may be considered whether variations in IgE-load are due to an increase of the cell-surface membrane or to a greater density of IgE-receptors. As no correlation between atopic disease and the tendency to villi formation was observed (14, 15), studies to investigate the mast cell-bound IgE at the ultrastructural level will be undertaken. With regard to basophil-bound IgE, preliminary experiments, using horse radish peroxidase labeled anti-IgE, indicated variations in the density of IgE molecules on the cell-surface membrane in immunoelectron microscopy. No significant differences in the number of villi were observed.

The results of this study do not point to differences in the relative IgE-load on mast cells and basophils from the same subjects.

The disadvantages of the determination of the amount of mast cell-bound IgE by end point titration, as used in this study, are: the subjectivity of the method, the relative insensitivity to small differences in the amount of cell-bound IgE, and the fact that we do not know the exact relation between variations of the absolute amount of IgE and titer differences. In this study, with regard to the first point, the slides were observed without knowing from which patient they were or which antiserum dilution was applied, in order to eliminate subjectivity as much as possible. Furthermore, the mast cell fluorescence titers, obtained in two different tissues of the same subject, showed a great similarity, thus pointing to reproduceable titer determination. Basophil-bound IgE can be quantitated more objectively and accurately, while no tissue material has to be obtained by biopsy, which is what makes diagnostic MCFT determination in younger children difficult. If any difference can be found by testing the mast cells instead of the basophils, this will be a slight difference; furthermore, estimation of cell-bound IgE with neither method is absolutely conclusive for diagnostic purposes. And therefore, although the local allergic processes are most likely to start in the tissues on the mast cell surface, the examination of the basophilic granulocytes to determine the amount of cell-bound IgE seems to be justified and preferable, when the technical facilities for this determination are present. However, if for some other reason a biopsy has to be performed, e.g. for histopathologic investigation of atopic dermatitis or nasal polyps, cell-bound IgE can be determined by end point titration on mast cells in tissue sections.

REFERENCES

1. Bowyer, A.: Observations on the granularity of mast cells in human skin. Acta derm.-venereol. 48: 574-577 (1968).
2. Callerame, M. L., and Condemi, J. J.: Demonstration of IgE on human skin mast cells using anti-IgE^{125}I and refined light microscopy. Amer. J. clin. Path. 62: 823-829 (1974).
3. Dobbins, W. O.; Tomasini, J. T., and Rollins, E. L.: Electron and light microscopic identification of the mast cell of the gastrointestinal tract. Gastroenterology 56: 268-279 (1969).
4. Feltkamp-Vroom, Th. M.; Stallman, P. J.; Aalberse, R. C., and Reerink-Brongers, E. E.: Immunofluorescence studies on renal tissue, tonsils,

adenoids, nasal polyps, and skin of atopic and nonatopic patients with special reference to IgE. Clin. Immunol. Immunopathol. 4: 392-404 (1975).
5. Feltkamp-Vroom, Th. M.; Wagenaar, Sj. Sc., and Swierenga, J.: Atopic lung disease and IgE mast cell fluorescence in bronchial tissue. (To be published).
6. Fernando, N. V. P., and Movat, H. Z.: The fine structure of connective tissue. III. The mast cell. Exptl. Molec. Pathol. 2: 450-463 (1963).
7. Hubscher, T.; Bootello, A., and Eisen, A. H.: Human skin target cells: Nature and fate in the immediate hypersensitivity reaction. J. Allergy 53: 150-157 (1974).
8. Ishizaka, K., and Ishizaka, T.: Mechanisms of reaginic hypersensitivity: a review. Clin. Allergy 1: 9-24 (1971).
9. Lichtenstein, L. M.: Allergy, in BACH and GOOD Clinical immunobiology, vol. 1. pp. 243-269. (Acad. Press, New York 1972).
10. Stallman, P. J., and Aalberse, R. C.: Estimation of basophil-bound IgE by quantitative immunofluorescence microscopy. Int. Arch. Allergy (in press). (Chapter V of this thesis).
11. Stallman, P. J., and Aalberse, R. C.: Quantitation of basophil-bound IgE in atopic and non-atopic subjects. Int. Arch. Allergy (in press). (Chapter VI of this thesis).
12. Stallman, P. J., and Feltkamp-Vroom, Th. M.: Identification of basophilic granulocytes in human leukocyte suspensions subjected to immunofluorescence procedures with anti-IgE. J. immunol. Methods 10: 271-277 (1976). (Chapter III of this thesis).
13. Takahashi, I., and Anan, S.: Studies on IgE bound cells in cutaneous lesion of atopic determatis (Japanese). Jap. J. Allergol. 23: 348-353 + 392 (1974). Excerpta Med. 12: 471 (1975).
14. Trotter, C. M., and Orr, T. C. S.: A fine structure study of some cellular components in allergic reactions. I. Degranulation of human mast cells in allergic asthma and perennial rhinitis. Clin. Allergy 3: 411-425 (1973).
15. Trotter, C. M., and Orr, T. C. S.: A fine structure study of some cellular components in allergic reactions. II. Mast cells in normal and atopic human skin. Clin. Allergy 4: 421-433 (1974).
16. Warren, S. L.: A new look at type I immediate hypersensitivity immune reactions. Ann. of Allergy 36: 337-341 (1976).
17. Wide, L.; Bennich, H., and Johansson, S. G. O.: Diagnosis of allergy by an *in vitro* test for allergen antibodies. Lancet ii: 1105-1107 (1976).

CHAPTER VIII

ELECTRON MICROSCOPIC STUDIES ON HUMAN BASOPHILS FROM ATOPIC AND NON-ATOPIC SUBJECTS, USING HORSE RADISH PEROXIDASE LABELED ANTI-IgE*

E. H. VAN ELVEN, P. J. STALLMAN and PH. C. BRUHL, with the technical assistance of J. G. VAN HEERTUM.

Department of Electron Microscopy and Immunohistopathology of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology, University of Amsterdam, P.O.B. 9190, Amsterdam, The Netherlands

ABSTRACT

In previous studies, quantitative differences in cell-bound IgE on the basophils from various donors were measured by means of a quantitative immunofluorescence technique.

In this study, cell-bound IgE was demonstrated on basophilic granulocytes by immunoelectron microscopy, using horse radish peroxidase labeled anti-IgE. The distribution of IgE on the basophils was studied in both atopic and non-atopic subjects. The intensity of the peroxidase staining on the basophils varied and appeared to correlate with the amount of cell-bound IgE, as estimated by quantitative immunofluorescence.

INTRODUCTION

In previous studies (14, 17) a description was given of a combined staining and fixation method, by which basophilic granulocytes can be identified in leukocyte suspensions, after the immunofluorescence technique has been applied. The fluorescence pattern of these paraformaldehyde (PFA) fixed cells, was ring-shaped, which indicates membrane-bound fluorescence. On unfixed basophils capping and patching was observed. Quantitation of the fluorescence intensity showed differences between various individuals, which depended on both atopy and the IgE serum level (14, 15). The question arose, whether the observed variations in the amount of basophil-bound IgE

* Int. Arch Allergy (in press)

were due to a varying density of IgE-receptors on the cell-surface membrane of the basophils or to variations in the size of the cell membrane. The latter possibility was supported by the observation of ridges and villi on basophils (7, 22) and mast cells (11, 20, 21), although no correlation was found between the number of villi and atopy.

Another point of interest is the IgE distribution over the cell surface. In our studies a regular ring-shaped fluorescence was observed, while Sullivan et al. (18), using a hybrid antibody against IgE and ferritin in immunoelectron microscopy, found the ferritin to be either distributed in patches around the entire circumference of the basophils or to be restricted to only part of the circumference. The pattern depended on the incubation temperature.

In this study we investigated the distribution of IgE on the cell membrane of the basophils from atopic and non-atopic subjects, using the immunoperoxidase technique at the ultrastructural level. Differences in peroxidase reaction on the basophils were compared with the intensity values found with the quantitative immunofluorescence technique. The morphology of the basophils was also studied.

MATERIALS AND METHODS

Preparation of the leukocyte suspensions and quantitative immunofluorescence microscopy

Thirty ml of venous blood were obtained in heparin from 2 atopic subjects (atopy was confirmed by clinical symptoms, history and the results of the radio allergo sorbens test (RAST)) and 6 healthy controls. A 3 ml leukocyte suspension was prepared by Ficoll-Isopaque centrifugation (17); 1 ml was stained and fixed with toluidine blue and paraformaldehyde (PFA) and incubated with fluorescein isothiocyanate labeled sheep anti-human IgE (FlaIgE), diluted 1 : 40, to assess the intensity of the fluorescence of the basophils as previously described (14, 15). Two ml of the leukocyte suspension were used for immunoelectron microscopic investigation.

Antisera

The production and testing of the fluoresceinated sheep anti-human IgE (FlaIgE) has been described previously (5). In order to study cell-bound IgE in electron microscopy, an IgG fraction was

prepared from sheep anti-human IgE of another badge of the serum from the same sheep. Two ml of this fraction were absorbed twice on a Sepharose 4B column (25 ml) coupled to normal human serum according to Cuatrecasas (4). This fraction was coupled to horse radish peroxidase (ShaHIgE-HRP) according to Avrameas (1). In agar precipitation, ShaHIgE-HRP showed a precipitation line with our standard polyclonal IgE-rich serum. Equivalence was found in a ratio of one volume of IgE-rich serum to 9 volumes of ShaHIgE-HRP. Specificity testing of the antiserum was performed. Tanned red cells, coated with IgA, IgG, IgM, κ and λ light chains, albumin, transferrin and fibrinogen, were not agglutinated by the antiserum, diluted 1 : 4 to 1 : 64. Furthermore, the antiserum was tested on 4 μm cryostat sections of tonsillar tissue from an atopic subject. Sections were incubated with the antiserum, twofold diluted 1 : 10 to 1 : 40 at room temperature for 60 minutes and washed in phosphate buffered saline (PBS) for 30 minutes. To demonstrate peroxidase activity, the sections were incubated with diaminobenzidine-H_2O_2 (7.5 mg DAB in 10 ml PBS + 100 μl H_2O_2 0.3%) for 5 minutes, washed twice in PBS and treated with osmiumtetroxide for 10 seconds (12). After being washed twice in PBS, the sections were dehydrated in graded solutions of alcohol and xylol, and mounted in malinol. Mast cells could be seen in the sections as rounded or spindle-shaped cells with a rim of peroxidase activity. No positive lymphocytes or other structures were seen, not even in the sections incubated with the 1 : 10 dilution of the antiserum. This indicates the absence of antibodies to IgM, IgD, collagen and other tissue components in the antiserum.

In other experiments the antiserum was absorbed with an IgE-rich serum, both at the equivalence point (ShaHIgE-HRP : IgE-rich serum = 9 : 1) and in a ratio of 3 : 1. ShaHIgE-HRP absorbed with normal human serum (NHS) served as a control. Sections of tonsillar tissue incubated with the absorbed antiserum at the equivalence point showed very weak mast cell staining, while this staining was not seen when the antiserum absorbed in a 3 : 1 ratio was used. Basophilic granulocytes from 2 subjects were also incubated with the absorbed antiserum in a 1 : 40 final dilution of the ShaHIgE-HRP. In immunoelectron microscopy no peroxidase activity was seen on the basophils treated with the antiserum, absorbed in a 3 : 1 ratio. In some of the cells, incubated with the antiserum, absorbed at the equivalence point, a very faint, linear peroxidase activity was observed. On the basophils,

incubated with the antiserum absorbed with NHS, a similar peroxidase activity was seen as in the preparations treated with the unabsorbed ShaHIgE-HRP.

Immunoelectron microscopy

Two ml of the leukocyte suspension were fixed with PFA 1% for 15 minutes (13) and washed: once in Hijmans' washing fluid (9) and once in PBS. The cells were incubated with the ShaHIgE-HRP, diluted 1 : 40 in PBS, at room temperature for 60 minutes, followed by 2 washings with PBS for 10 minutes. The peroxidase activity was visualized according to Graham and Karnovsky (6), by incubation with diaminobenzidine-H_2O_2 (7.5 mg DAB in 10 ml PBS + 100 μl H_2O_2 0.3%) for 5 minutes, followed by 2 washings with PBS. Subsequently the cells were postfixed with 1% osmiumtetroxide in 0.1 M phosphate buffer for 60 minutes (12). Cell preparations were dehydrated in graded solutions of ethanol and propyleneoxide and embedded in Epon. All incubations and washings were performed at room temperature and standard conditions were applied to the cells of all donors. Sections were cut on a Reichert microtome and stained with a saturated solution of uranylacetate in distilled water for 20 minutes and finally coated with carbon.

For morphological investigation, leukocyte suspensions were prepared from 2 atopic and 2 non-atopic subjects, in the forementioned way. After being washed twice in Hijmans' washing fluid, cells were fixed for several hours in a paraformaldehyde-glutaraldehyde fixative in phosphate buffer, pH 7.4, according to Karnovsky (10) washed in 0.1 M phosphate buffer, and postfixed by incubation in OsO_4 1% for 60 minutes. Cells were dehydrated in graded solutions of ethanol and propyleneoxide and embedded in Epon. Ultrathin sections were cut, stained with the uranylacetate solution for 20 minutes, followed by staining with a solution of leadhydroxide for 10 minutes, and finally coated with carbon. All preparations were studied with a transmission electron microscope (Philips EM 300). From all donors approximately 10 basophils were investigated, which could be distinguished because of their characteristic granules (22). The mean intensity of the peroxidase activity of the basophils was judged subjectively as: +++ strongly positive; ++ positive; + moderately positive; ± weakly positive; – negative. All preparations were observed and scored without knowing from which subject they came. Representative cells were photographed on Kodak film.

Total serum IgE assay and radio allergo sorbens test (RAST)

The IgE serum level was determined as described previously (14). Specific antibodies to grass pollen and house dust were demonstrated with the RAST (14).

RESULTS

Basophilic granulocytes, containing the specific electron-dense granules (22), were observed in electron microscopy in the cell suspensions of all donors but one. In the leukocyte suspension of donor F.E., no such cells were found, However, peroxidase positive cells were present in the same proportion as basophils in the other subjects, and the nucleus of these cells had the same shape. Probably these cells were degranulated basophils, but this could not be ascertained.

Along the cell-surface membrane of the basophilic granulocytes, peroxidase activity was observed (Fig. 1, 2). Some mononuclear cells of subject H.R. also showed a weakly positive peroxidase staining. However, in the cell suspensions from the other donors investigated, peroxidase activity was only seen on the basophils. Approximately 10 basophils were investigated from all donors. Along the circumference of the single basophil no differences in the intensity of the peroxidase activity were seen. Between the basophils in a single subject the intensity of the peroxidase activity was found to vary marginally. This phenomenon was also observed when the basophils were studied by means of immunofluorescence microscopy (14). However, the differences between the various subjects were much more pronounced (Fig. 1, 2, 3).

The mean intensity of the peroxidase staining with ShaHIgE-HRP was compared with the fluorescence intensity of the basophils from the same subject, the IgE serum level and the presence or absence of atopy (Table I).

The distribution of the peroxidase was also found to vary: in all subjects with a peroxidase activity on their basophils ranging from moderately to strongly positive, it was homogeneous along the cell-membrane over the entire circumference of the cell (Fig. 1, 2). On the basophils, which were weakly positive, peroxidase activity was distributed in small spots over the cell circumference (Fig. 3). Capping was not observed.

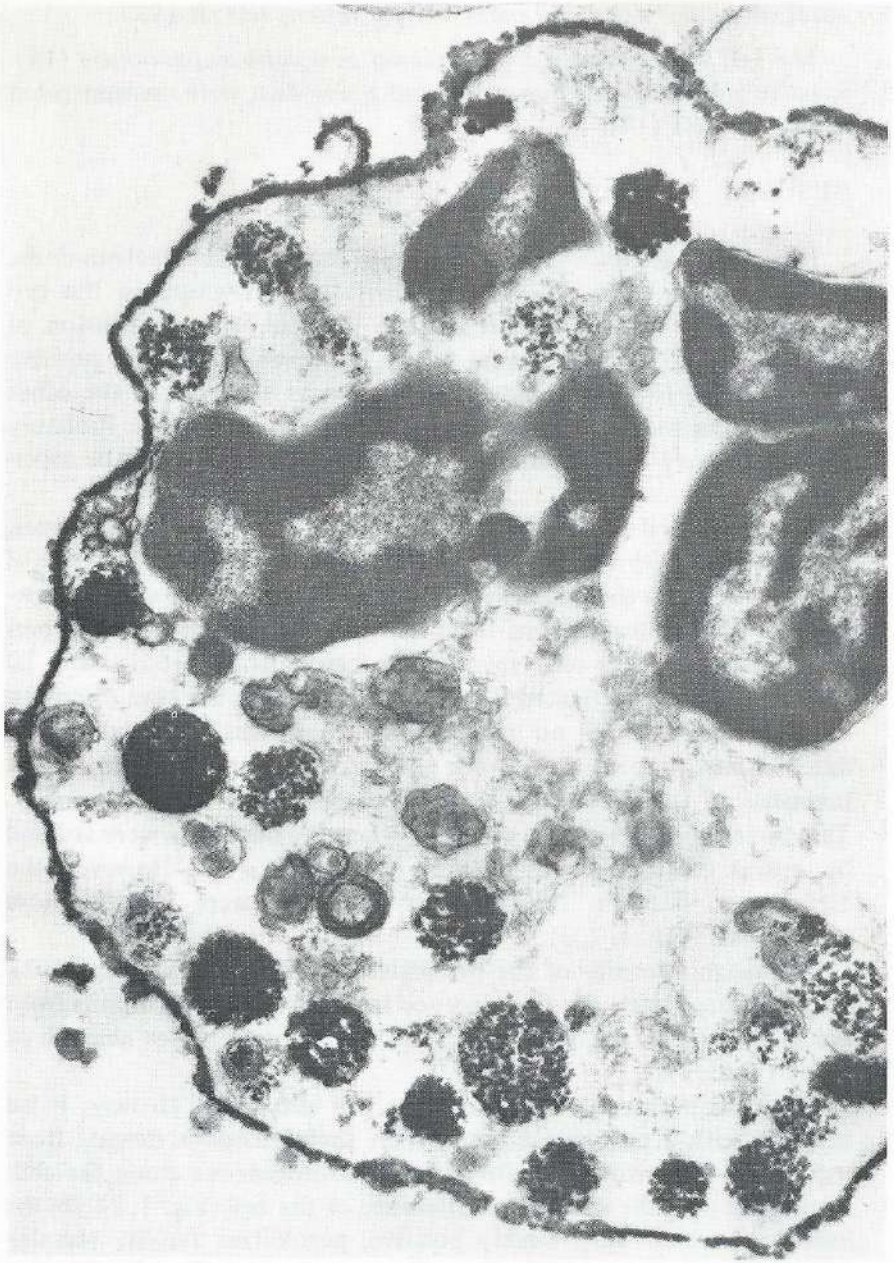


Fig. 1: A basophilic granulocyte fixed in 1% paraformaldehyde and incubated with ShaHIgE-HRP, diluted 1 : 40 in PBS, showing a strongly positive (+++) membrane staining. (x 22.500)

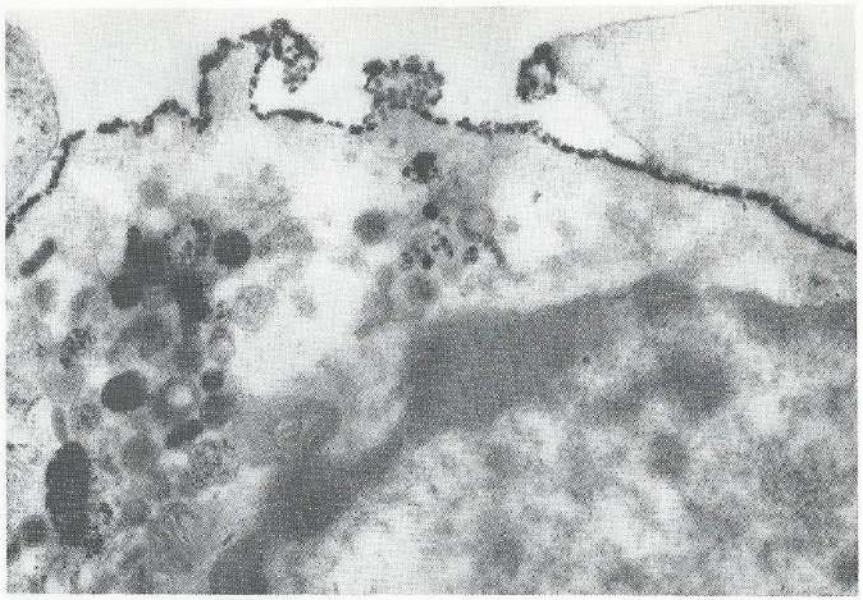


Fig. 2: Part of the cell circumference of a basophilic granulocyte, fixed in 1% paraformaldehyde and incubated with ShaHIgE-HRP, diluted 1 : 40 in PBS, showing a positive (++) membrane staining. (x 22.500)

TABLE I — Allergy parameters of the various subjects, and the relative amount of IgE on their basophilic granulocytes.

Subjects	T.M.	A.H.	C.L.	J.A.	C.H.	F.E.	H.R.	A.V.
Clinical atopy	–	–	–	–	–	–*	+	+
IgE serum level (IU/ml)	3	4	65	89	37	268	290	500
RAST-score (against house dust/pollen)	–/–	–/–	–/–	–/–	–/–	–/–	–/+++	+++/–
Fluorescence intensity of the basophils (RFI)	47	53	123	132	171	224	294	396
Peroxidase intensity of the basophils in immunoelectron microscopy	±	±	+	+++	++	+**	+++	+++

* – recently shown hypersensitivity to insect stings.

** – only "degranulated basophils" present in the preparation.

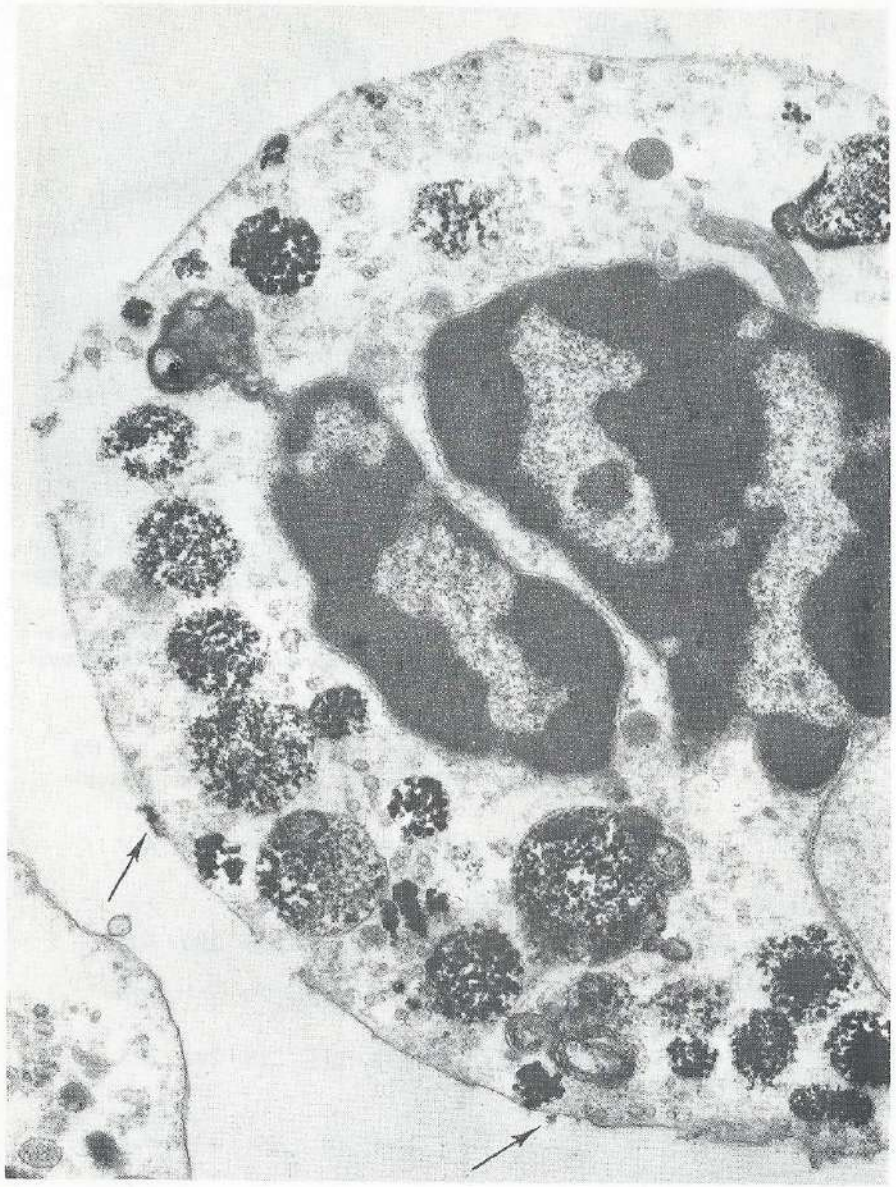


Fig. 3: A basophilic granulocyte, fixed in 1% paraformaldehyde and incubated with ShaHIgE-HRP, diluted 1 : 40 in PBS showing a weakly positive (±) spotty membrane staining (arrows). (x 22.500)

With regard to morphology, villi of the cell-membrane of the PFA-fixed cells were observed incidentally. Since the morphological details of the basophils, which had strongly reacted with the ShaHIgE-HRP, did not come forward in a satisfactory way, cells fixed in Karnovsky's fixation fluid were also examined. Villi were not thought to contribute significantly to an augmentation of the cell-surface of basophils fixed in this way either. Furthermore, no differences in villi-formation were observed between the basophils of the atopic and those of the non-atopic subjects.

DISCUSSION

IgE bound to the cell-surface of basophilic granulocytes could be detected in immunoelectron microscopy, using PFA-fixed leukocytes. Membrane-bound IgE was absent on the surface of any other kind of cells in the suspension, except in the cell suspension of one subject, in which mononuclear cells, probably monocytes, were seen, that were positive for peroxidase. Monocytes, weakly positive for FlaIgE, were observed in a previous study (17), and this reaction appeared to be aspecific in blocking and absorption experiments (17). That the peroxidase staining of the basophil cell-membrane is not an aspecific reaction was demonstrated by absorption experiments and by the results of previous experiments, using anti-human thymus lymphocyte antigen serum and horse anti-rabbit serum labeled with HRP (16).

Since no eosinophilic granulocytes were present in the preparations investigated in immunoelectron microscopy, the results of Hubscher (8), who demonstrated membrane-bound IgE on human eosinophils in electron microscopy by means of an indirect immunoferritin technique, could not be confirmed. Eosinophilic granylocytes, however, when observed in our other studies using quantitative immunofluorescence microscopy, were always negative for IgE. Also in tissue sections, eosinophils proved to be negative for IgE (5).

The question, whether the differences in the fluorescence intensity of the basophils, found previously (14), were due to variations in the density of the IgE molecules or to variations in the size of the cell-surface membrane, may be answered in favour of the first possibility or of a combination of the two causes. No differences in villi formation were observed between the basophils of the various subjects, neither did villi formation contribute significantly to the dimension

of the cell surface. However, it must be noted, that the cells were isolated prior to fixation, and, although different methods of fixation were applied, the cell morphology can have changed as a result of the isolation procedure. This problem can be overcome by direct fixation of whole blood in the paraformaldehyde-glutaraldehyde fixative, as was done with thrombocytes (unpublished results). However, applying this fixation method, the identification of the rare basophils will be an insurmountable problem, because cells can not be separated after this procedure.

On the other hand, the intensity of the peroxidase activity along the cell-surface membrane of the various subjects varied considerably. The quantitation of the intensity of the peroxidase activity in electron microscopy remains subjective, but the differences were obvious (Fig. 1, 2, 3) and these were found to correspond roughly with the amount of basophil-bound IgE, as estimated by quantitative immunofluorescence microscopy and with the IgE serum level of the subjects (Table I).

The distribution of IgE along the cell-surface membrane was linear in most of the subjects. The spotty pattern, observed in the subjects T.M. and A.H., is thought to represent either the minimal detection level for membrane-bound IgE or the distribution of the molecules, when only a minimal amount of IgE is present. From previous elution experiments (14), it can be calculated that in these 2 subjects approximately 15.000 molecules IgE are bound to 1 basophil. When distributed evenly, this would mean 15.000 molecules per 300 μm^2 (ϕ of a basophil $\simeq$ 10 μm) (18). From these figures one can expect that per 1 μm membrane in a section of 0.1 μm thickness, roughly 5 IgE molecules are present.

The linear pattern is in accordance with the results of Becker et al. (2) with monovalent anti-IgE. Apparently the fixation with PFA prevents redistribution of IgE after incubation with the antiserum (3), while after incubation of non-fixed cells at 0^0C still some redistribution occurs, although this was not found by Sundqvist (19). This possibility could explain the contrast between the forementioned linear configuration and the patchy distribution found by Sullivan et al. (18). In that study basophils from 3 non-atopic individuals showed considerable variations in the amount of surface-bound IgE, although no correlation with the IgE serum level was found. In view of the serum IgE values of the subjects in that study, it is not likely that the patchy pattern was caused by minimal

amounts of IgE on the basophils of those 3 subjects.

Our conclusion is, that IgE is distributed linearly on the human basophils along the cell-surface membrane or in spots, when minimal amounts of IgE are present. Variations between the subjects, as observed with both the immunofluorescence and the immunoperoxidase techniques, are, at least partly, caused by a varying density of IgE molecules on the cell-surface membrane. An increase in the size of the cell-surface membrane of the basophils from atopic subjects was not observed in the isolated and fixed cells, but *in vivo* differences can not be excluded.

REFERENCES

1. Avrameas, S., and Ternynck, T.: Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. Immunochemistry 8: 1175-1179 (1971).
2. Becker, K. E.; Ishizaka, T.; Metzger, H.; Ishizaka, K., and Grimley, Ph. M.: Surface IgE on human basophils during histamine release. J. exp. Med. 138: 394-409 (1973).
3. Biberfeld, P.; Biberfeld, G.; Molnar, Z., and Fragaeus, A.: Fixation of cell-bound antibody in the membrane immunofluorescence test. J. immunol. Methods 4: 135-148 (1974).
4. Cuatrecasas, P.: Protein purification by affinity chromatography. Derivazations of agarose and polyacrylamide beads. J. biol. Chem. 245: 3059-3065 (1970).
5. Feltkamp-Vroom, Th. M.; Stallman, P. J.; Aalberse, R. C., and Reerink-Brongers, E. E.: Immunofluorescence studies on renal tissue, tonsils, adenoids, nasal polyps, and skin of atopic and nonatopic patients with special reference to IgE. Clin. Immunol. Immunopathol. 4: 392-404 (1975).
6. Graham, R. C., Jr., and Karnovsky, M. J.: The early stages of absorption of injected horse radish peroxidase in proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14: 291-302 (1966).
7. Hastie, R.: A study of the ultrastructure of human basophil leukocytes. Lab. Invest. 31: 223-231 (1974).
8. Hubscher, T.: Role of the eosinophil in the allergic reactions. I. EDI – An Eosinophil-Derived Inhibitor of histamine release. J. Immunol. 114: 1379-1388 (1975).
9. Hijmans, W.; Schuit, H. R. E., and Klein, F. E.: An immunofluorescence procedure for the detection of intracellular immunoglobulins. Clin. exp. Immunol. 4: 457-472 (1969).
10. Karnovsky, M. J.: A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell. Biol. 27: 137a-138a (1965).
11. Lawson, D.; Fewtrell, C.; Gomperts, B., and Raff, M. C.: Anti-immuno-

globulin-induced histamine secretion by rat peritoneal mast cells studied by immunoferritin electron microscopy. J. exp. Med. 142: 391-402 (1975).
12. Millonig, G.: Advantages of a phosphate buffer for OsO_4 solutions in fixation. J. appl. Phys. 32: 1637 (1961).
13. Smit, J. W.; Meijer, C. J. L. M.; Décary, F., and Feltkamp-Vroom, Th. M.: Paraformaldehyde fixation in immunofluorescence and immunoelectron microscopy. Preservation of tissue and cell surface membrane antigens. J. immunol. Methods 6: 93-98 (1974).
14. Stallman, P. J., and Aalberse, R. C.: Estimation of basophil-bound IgE by quantitative immunofluorescence microscopy. Int. Arch. Allergy (in press) (Chapter V of this thesis).
15. Stallman, P. J., and Aalberse, R. C.: Quantitation of basophil-bound IgE in atopic and non-atopic subjects. Int. Arch. Allergy (in press) (Chapter VI of this thesis).
16. Stallman, P. J.; Elven, E. H. van; Feltkamp-Vroom, Th. M., and Brutel de la Rivière, A.: The absence of a Human Thymus Lymphocyte Antigen (HTLA) on basophils and mast cells. Scand. J. Immunol. (in press) (Chapter IV of this thesis).
17. Stallman, P. J., and Feltkamp-Vroom, Th. M.: Identification of basophilic granulocytes in human leukocyte suspensions subjected to immunofluorescence procedures with anti-IgE. J. immunol. Methods 10: 271-277 (1976) (Chapter III of this thesis).
18. Sullivan, A. L.; Grimley, Ph. M., and Metzger, H.: Electron microscopic localization of immunoglobulin E on the surface membrane of human basophils. J. exp. Med. 134: 1403-1416 (1971).
19. Sundqvist, K. G.: Quantitation of cell membrane antigens at the single cell level. I. Microfluorometric analysis of reaction patterns in various test systems, with special reference to the mobility of the cell membrane. Scand. J. Immunol. 2: 479-494 (1973).
20. Trotter, C. M., and Orr, T. S. C.: A fine structure study of some cellular components in allergic reactions. I. Degranulation of human mast cells in allergic asthma and perennial rhinitis. Clin. Allergy 3: 411-425 (1973).
21 Trotter, C. M., and Orr, T. S. C.: A fine structure study of some cellular components in allergic reactions. II. Mast cells in normal and atopic human skin. Clin. Allergy 4: 421-433 (1974).
22. Zucker-Franklin, D.: Electron microscopic study of human basophils. Blood 29: 878-890 (1967).

CHAPTER IX

EXPERIMENTS ON THE PASSIVE SENSITIZATION OF HUMAN BASOPHILS, USING QUANTITATIVE IMMUNOFLUORESCENCE MICROSCOPY*

P. J. STALLMAN, R. C. AALBERSE, PH. C. BRUHL and E. H. VAN ELVEN

Department of Immunohistopathology of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology, University of Amsterdam, P.O.B. 9190, Amsterdam, The Netherlands.

ABSTRACT

In previous experiments a correlation was found between the amount of IgE on human basophils and the IgE serum level. The results of these experiments are shown to fit a free exchange model with an approximately constant K-value. We investigated whether the free IgE-receptors on the basophils, which should be present according to this model, could be saturated by incubating the cell suspensions with IgE myeloma protein or with sera from patients with an elevated IgE serum level. Various incubation conditions were applied in sensitizing the leukocytes, and cells from both atopic and non-atopic subjects were used for testing, but no increase in basophil-bound IgE could be measured with the quantitative immunofluorescence technique. Nor could we demonstrate a significant dissociation of cell-bound IgE in a period of 24 hours. Therefore another hypothesis is put foreward, in which receptor turnover is taken into account: we assumed that the free receptors on the basophils had a shorter functional half-life, than the half-life of the IgE-receptor complexes. This model would explain the contradictory results mentioned above.

INTRODUCTION

Immunoglobulin E can bind to basophils and mast cells (15), and

* Int. Arch Allergy (in press)

incubated with the antiserum absorbed with NHS, a similar peroxidase activity was seen as in the preparations treated with the unabsorbed ShaHIgE-HRP.

Immunoelectron microscopy

Two ml of the leukocyte suspension were fixed with PFA 1% for 15 minutes (13) and washed: once in Hijmans' washing fluid (9) and once in PBS. The cells were incubated with the ShaHIgE-HRP, diluted 1 : 40 in PBS, at room temperature for 60 minutes, followed by 2 washings with PBS for 10 minutes. The peroxidase activity was visualized according to Graham and Karnovsky (6), by incubation with diaminobenzidine-H_2O_2 (7.5 mg DAB in 10 ml PBS + 100 μl H_2O_2 0.3%) for 5 minutes, followed by 2 washings with PBS. Subsequently the cells were postfixed with 1% osmiumtetroxide in 0.1 M phosphate buffer for 60 minutes (12). Cell preparations were dehydrated in graded solutions of ethanol and propyleneoxide and embedded in Epon. All incubations and washings were performed at room temperature and standard conditions were applied to the cells of all donors. Sections were cut on a Reichert microtome and stained with a saturated solution of uranylacetate in distilled water for 20 minutes and finally coated with carbon.

For morphological investigation, leukocyte suspensions were prepared from 2 atopic and 2 non-atopic subjects, in the forementioned way. After being washed twice in Hijmans' washing fluid, cells were fixed for several hours in a paraformaldehyde-glutaraldehyde fixative in phosphate buffer, pH 7.4, according to Karnovsky (10) washed in 0.1 M phosphate buffer, and postfixed by incubation in OsO_4 1% for 60 minutes. Cells were dehydrated in graded solutions of ethanol and propyleneoxide and embedded in Epon. Ultrathin sections were cut, stained with the uranylacetate solution for 20 minutes, followed by staining with a solution of leadhydroxide for 10 minutes, and finally coated with carbon. All preparations were studied with a transmission electron microscope (Philips EM 300). From all donors approximately 10 basophils were investigated, which could be distinguished because of their characteristic granules (22). The mean intensity of the peroxidase activity of the basophils was judged subjectively as: +++ strongly positive; ++ positive; + moderately positive; ± weakly positive; – negative. All preparations were observed and scored without knowing from which subject they came. Representative cells were photographed on Kodak film.

Total serum IgE assay and radio allergo sorbens test (RAST)

The IgE serum level was determined as described previously (14). Specific antibodies to grass pollen and house dust were demonstrated with the RAST (14).

RESULTS

Basophilic granulocytes, containing the specific electron-dense granules (22), were observed in electron microscopy in the cell suspensions of all donors but one. In the leukocyte suspension of donor F.E., no such cells were found, However, peroxidase positive cells were present in the same proportion as basophils in the other subjects, and the nucleus of these cells had the same shape. Probably these cells were degranulated basophils, but this could not be ascertained.

Along the cell-surface membrane of the basophilic granulocytes, peroxidase activity was observed (Fig. 1, 2). Some mononuclear cells of subject H.R. also showed a weakly positive peroxidase staining. However, in the cell suspensions from the other donors investigated, peroxidase activity was only seen on the basophils. Approximately 10 basophils were investigated from all donors. Along the circumference of the single basophil no differences in the intensity of the peroxidase activity were seen. Between the basophils in a single subject the intensity of the peroxidase activity was found to vary marginally. This phenomenon was also observed when the basophils were studied by means of immunofluorescence microscopy (14). However, the differences between the various subjects were much more pronounced (Fig. 1, 2, 3).

The mean intensity of the peroxidase staining with ShaHIgE-HRP was compared with the fluorescence intensity of the basophils from the same subject, the IgE serum level and the presence or absence of atopy (Table I).

The distribution of the peroxidase was also found to vary: in all subjects with a peroxidase activity on their basophils ranging from moderately to strongly positive, it was homogeneous along the cell-membrane over the entire circumference of the cell (Fig. 1, 2). On the basophils, which were weakly positive, peroxidase activity was distributed in small spots over the cell circumference (Fig. 3). Capping was not observed.

cence intensity of 30 basophils was measured and expressed as the Relative Fluorescence Intensity (RFI). The mean background values were assessed. Mean values of the fluorescence intensity of the basophils minus background values (± two times the standard error of the mean). are given in the various tables.

IgE assay

The amount of IgE in the washing fluids, obtained between the sensitization and the fixation procedure, was measured by a non-competitive assay (18). The total serum IgE of the various subjects was determined as previously described (18).

RESULTS

The relation between cell-bound and free IgE

The relation between serum IgE and basophil-bound IgE has been

TABLE Ia. The mean fluorescence intensity of the basophils from 4 atopic subjects. The cells were sensitized by incubation at 0°C for 10 minutes.

Subjects	N.G.	J.M.	M.H.	B.M.
IgE serum level – IU/ml	22	27	35	40
Unsensitized cells fixed with PFA	194(±14)*	277(±30)	195(±20)	215(±26)
Incubation with:				
Tris-A buffer	241(±22)	298(±37)	250(±22)	254(±39)
donor's own serum	219(±26)	323(±37)	228(±19)	234(±30)
myeloma IgE 30,000 IU/ml	224(±28)	308(±29)	236(±35)	243(±33)
myeloma IgE 10,000 IU/ml	218(±27)	306(±30)	244(±30)	206(±34)
myeloma IgE 3,500 IU/ml	207(±27)	260(±30)	233(±26)	226(±32)
serum from a schistosoma patient 10,000 IU/ml	232(±28)	295(±37)	237(±29)	226(±30)
serum from an atopic patient 2,500 IU/ml	208(±26)	280(±27)	218(±21)	202(±32)

* ( ) = ± 2x standard error of the mean

TABLE Ib. The mean fluorescence intensity of the basophils from 4 healthy subjects. The cells were sensitized by incubation at 0°C for 10 minutes.

Subjects	P.S.	J.A.	E.R.	G.L.
IgE serum level – IU/ml	28	30	14	25
Unsensitized sells fixed with PFA	111(± 8)*	181(±21)	101(±12)	125(±18)
Incubation with:				
Tris-A buffer	120(± 9)	198(±22)	105(±16)	115(±10)
donor's own serum	141(±10)	182(±20)	97(±12)	106(±13)
myeloma IgE 30,000 IU/ml	138(±10)	191(±27)	97(±13)	130(±13)
myeloma IgE 10,000 IU/ml	124(± 9)	197(±24)	97(±14)	131(±17)
myeloma IgE 3,500 IU/ml	135(± 9)	210(±22)	116(±12)	118(±16)
serum from a schistosoma patient 10,000 IU/ml	129(± 9)	179(±21)	n.d.	129(±12)
serum from an atopic patient 2,500 IU/ml	117(± 8)	197(±30)	n.d.	141(±15)

* ( ) = ± 2x standard error of the mean
n.d. = not done

described by Ishizaka et al. (9) as a free exchange model with the formula:

$$K = \frac{\text{(receptors combined)}}{\text{(serum IgE) (free receptors)}}$$

The fluorescence intensity values of the basophils from 29 non-atopic controls, subjects of a previous study (19) and their total serum IgE values were used to see whether these data would fit this unsaturated free exchange model (Fig. 1), and a correlation of 0.803 was found. The K-value was calculated to be approximately:

$$K = 0.039 \times [\text{IU IgE/ml}]^{-1} =$$
$$= 0.039 \times [10^3 \times 1.2 \times 10^{-14}]^{-1}\ M^{-1} =$$
$$= 3.0 \times 10^9\ M^{-1}$$

(1 IU IgE = 2.4×10^{-9} gram; molecular weight of IgE = 2×10^5 (7); 1 IU IgE/1 = 1.2×10^{-14} M).

The total amount of IgE-receptors was estimated to be 244 RFI,

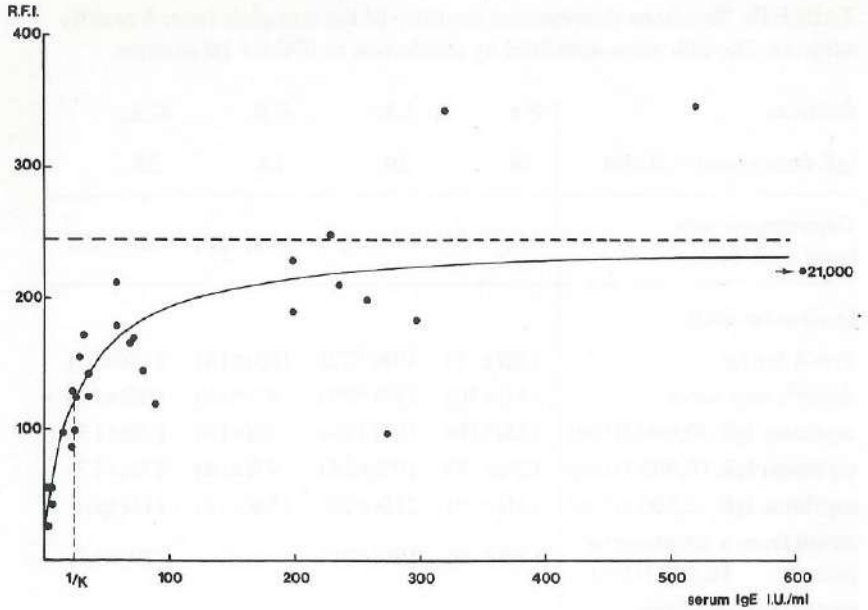


Fig. 1. The Relative Fluorescence Intensity (RFI) of the basophils from 29 non-atopic subjects was compared with the IgE level of the serum of these subjects. The best fitting curve, according to the free exchange model, was calculated using the method of least squares. A correlation coefficient of 0.803 was established*. The asymptote indicates the maximal IgE-load on basophils of non-atopic controls according to this model. The IgE serum level, corresponding with a half-maximal IgE-load, reflects the reciprocal equilibrium constant of the reaction (1/K).

$$* \; r^2 = \frac{\Sigma(y_i - y)^2 - \Sigma(y_i - \frac{x_i}{ax_i + b})^2}{\Sigma(y_i - y)^2}$$

TABLE IIa. The mean fluorescence intensity of the basophils from 4 atopic subjects. The cells were sensitized by incubation at 37°C for 60 minutes.

Subjects	N.G.	J.M.	M.H.	B.M.
IgE serum level – IU/ml	22	27	35	40
Unsensitized cells fixed with PFA	194(±14)*	277(±30)	195(±20)	215(±26)
Incubation with:				
Tris-A buffer	191(±20)	268(±29)	213(±23)	188(±27)
donor's own serum	195(±18)	270(±30)	197(±20)	171(±33)
myeloma IgE 30,000 IU/ml	160(±12)	268(±30)	205(±26)	188(±29)
myeloma IgE 10,000 IU/ml	175(±12)	258(±25)	208(±20)	183(±26)
myeloma IgE 3,500 IU/ml	167(±12)	278(±35)	208(±27)	169(±25)
serum from a schistosoma patient 10,000 IU/ml	185(±13)	243(±32)	205(±21)	195(±20)
serum from an atopic patient 2,500 IU/ml	182(±13)	261(±27)	205(±23)	209(±27)

* = ± 2x standard error of the mean

which corresponds with 180,000 IgE molecules per basophil in the elution technique (18).

Passive sensitization experiments

When the relation between cell-bound and free IgE would fit this model, only half of the receptors would be occupied at an IgE serum level of 26 IU/ml. Thus one would expect an increase in basophil-bound IgE after incubation in an IgE-rich medium, if the donor has a low IgE serum level. In atopic patients, the relation between cell-bound and free IgE did not fit the data as found for non-atopic donors; a higher fluorescence intensity of the basophils was found than was expected. This could be caused, for instance, by differences in the amount or the affinity of the IgE-receptors. However, when free IgE-receptors are present in atopic subjects, they are most likely present on the basophils of atopics with a low IgE serum level.

Leukocyte suspensions from 4 atopic subjects and 4 healthy con-

TABLE IIb. The mean fluorescence intensity of the basophils from 4 healthy controls. The cells were sensitized by incubation at 37°C for 60 minutes.

Subjects	P.S.	J.A.	E.R.	G.L.
IgE serum level – IU/ml	28	30	14	25
Unsensitized cells fixed with PFA	111(± 8)*	181(±21)	101(±12)	125(±18)
Incubation with:				
Tris-A buffer	104(± 7)	173(±24)	102(±17)	132(±17)
donor's own serum	91(± 7)	165(±25)	87(±11)	130(±18)
myeloma IgE 30,000 IU/ml	96(± 7)	175(±21)	91(±13)	128(±18)
myeloma IgE 10,000 IU/ml	97(± 7)	190(±25)	101(±13)	153(±23)
myeloma IgE 3,500 IU/ml	110(± 8)	188(±24)	91(±11)	132(±20)
serum from a schistosoma patient 10,000 IU/ml	94(± 7)	201(±30)	103(±12)	123(±13)
serum from an atopic patient 2,500 IU/ml	105(± 7)	184(±19)	85(±14)	126(±16)

* = ± 2x standard error of the mean

trols, all with an IgE serum level of less than 50 IU/ml, matched for age, sex and IgE serum level, were incubated with the myeloma IgE, in various dilutions, and with the various sera, at 0° C for 10 minutes (Table Ia, Ib) or at 37° C for 60 minutes (Table IIa, IIb). Applying the method of analysis of variance, no significant increase in the fluorescence intensity was observed under these incubation conditions.

As indicated by the values for the standard error of the mean, the minimal detectable change (MDC), testing individual comparisons with the q-statistic, was estimated to be approximately 25% (MDC = q. SEM; q is determined by: range of values/standard deviation). The fluorescence intensity values of the basophils of some of the subjects are in a slightly higher range after incubation at 0° C for 10 minutes, than after direct fixation of the cells or after incubation at 37° C for 60 minutes. However, no significant difference was found between the values obtained after incubation of the cells with Tris-A buffer or the donor's own serum, and those obtained after incubation in an IgE-rich medium.

TABLE III. IgE content in the washing fluids obtained after passive sensitization of the basophils from 4 atopic and 4 non-atopic subjects; with myeloma IgE in 3 dilutions.

Subjects	N.G.*	J.M.*	M.H.*	B.M.*	P.S.	J.A.	E.R.	G.L.
After sensitization with myeloma IgE 30,000 IU/ml:								
(IgE) in the second washing fluid – IU/ml	18	18	18	21	34	29	23	33
(IgE) in the third washing fluid – IU/ml	5	6	7	6	2	9	6	8
After sensitization with myeloma IgE 10,000 IU/ml:								
(IgE) in the second washing fluid – IU/ml	6	12	11	14	8	8	10	21
(IgE) in the third washing fluid – IU/ml	2	4	4	4	1	4	4	6
After sensitization with myeloma IgE 3,500 IU/ml:								
(IgE) in the second washing fluid – IU/ml	3	4	6	9	4	4	6	6
(IgE) in the third washing fluid – IU/ml	1	2	2	2	1	2	2	3

* = atopic subject

The amount of IgE in the washing fluid, obtained by washing three times after the sensitization procedure, was measured (Table III) and ranged from 1 to 9 IU IgE/ml in the last washing. When the amounts of IgE in the second and the third washing were compared, IgE, which was initially bound, seemed to be released. However, it is not likely that IgE, which is bound to the IgE-receptor on the basophil, is washed off,

TABLE IV. The mean fluorescence intensity of the basophils from 3 atopic and 3 non-atopic subjects. The cells were pre-incubated in Tris-A buffer at 4°C for 24 hours, and sensitized by incubation at 0°C for 10 minutes.

Subjects	J.M.*	M.H.*	B.M.*	J.A.	E.R.	G.L.
IgE serum level – IU/ml	27	35	40	30	14	25
Unsensitized cells fixed with PFA	277 (±30)**	195 (±20)	215 (±26)	181 (±21)	101 (±12)	125 (±18)
After pre-incubation, incubation with:						
Tris-A buffer	264 (±27)	204 (±27)	224 (±34)	153 (±18)	103 (±14)	156 (±18)
myeloma IgE 30,000 IU/ml	265 (±28)	204 (±24)	234 (±37)	157 (±22)	96 (±14)	158 (±16)
serum from an atopic patient 2,500 IU/ml	n.d.	n.d.	n.d.	n.d.	n.d.	151 (±18)

* = atopic subject
** ( ) = ± 2x standard error of the mean
n.d. = not done

since the amount of basophil-bound IgE did not decrease after incubation of the leukocytes in Tris-A buffer for 24 hours (Table IV). We also investigated a possible effect of the presence of both EDTA and Heparin in the incubation medium (13, 14). In 3 subjects, with a small amount of basophil-bound IgE, no increase could be measured, although various incubation conditions were applied (Table Va, Vb).

TABLE Va. The mean fluorescence intensity of the basophils from 3 healthy controls. The cells were sensitized by incubation at 37°C for 90 minutes, in the presence of 0.005 M EDTA and Heparin 1 IU/ml in the incubation medium.

Subjects	K.W.	A.H.	T.M.
IgE serum level – IU/ml	32	4	3
Unsensitized cells fixed with PFA	95(±12)*	50(± 6)	43(± 4)
Incubation with:			
Tris-A buffer	109(±17)	37(± 4)	33(± 4)
donor's own serum	96(±14)	42(± 8)	33(± 4)
myeloma IgE 30,000 IU/ml	97(±14)	52(±12)	41(± 6)
serum from a schistosoma patient 10,000 IU/ml	106(±18)	47(±14)	45(± 4)
serum from an atopic patient 2,500 IU/ml	87(±10)	47(± 4)	56(±10)

* ( ) = ± 2x standard error of the mean

TABLE Vb. The mean fluorescence intensity of the basophils from 3 healthy controls. The cells were sensitized by incubation at 37°C for 90 minutes, in the presence of 0.01 M EDTA in the incubation medium.

Subjects	K.W.	A.H.	T.M.
IgE serum level – IU/ml	32	4	3
Unsensitized cells fixed with PFA	95(±12)*	50(± 6)	43(±4)
Incubation with:			
Tris-A buffer	106(±16)	43(± 5)	35(±4)
donor's own serum	103(±16)	37(± 5)	33(±5)
myeloma IgE 30,000 IU/ml	103(±16)	55(±10)	43(±6)
serum from a schistosoma patient 10,000 IU/ml	111(±15)	47(± 6)	47(±5)
serum from an atopic patient 2,500 IU/ml	96(±10)	44(± 6)	47(±6)

* ( ) = ± 2x standard error of the mean

DISCUSSION

The relation between basophil-bound and free IgE corresponds to an unsaturated free exchange model. In this model the number of IgE-receptors on the basophils of the various subjects is assumed to be the same, with a similar affinity for IgE. The values for the fluorescence intensity of the basophils, as a measure for the IgE-load, from 29 non-atopic donors and their IgE serum values, were demonstrated not to disagree with the model: a correlation of 0.803 was found (Fig. 1). The calculated K-value was 3×10^9 M^{-1}, which fits in the range found by Ishizaka et al. (9): 0.1×10^9 to 10×10^9 M^{-1}. The total number of IgE-receptors per basophil in non-atopic subjects was calculated to be approximately 180,000 according to this model.

The observations of others seem to sustain this model:

1. the correlation between basophil-bound IgE and serum IgE level, as found by Conroy et al. (3, 8);
2. the increase of the amount of basophil-bound IgE after incubation with myeloma IgE, found by Ishizaka et al. (9) using the C_1 fixation transfer test, by Sullivan et al. (21) in immunoelectron microscopy and by Ishizaka et al. (9, 10) using autoradiography;
3. the inhibition of passive sensitization of human lung fragments by saturation of the IgE-receptors with IgE-rich serum (5), pointing to free IgE-receptors on human mast cells;
4. the passive sensitization of human leukocytes with patient serum in histamine release experiments (12, 16, 23).

These findings all point to a free exchange model with a certain amount of unsaturated IgE-receptors on the basophils.

Therefore, it was surprising, that, although various incubation conditions, mentioned in literature (9, 12, 13, 14), were applied, no increase in basophil-bound IgE could be measured after sensitization with either myeloma IgE or with IgE-rich sera. Nor could a significant dissociation of basophil-bound IgE be demonstrated by incubating the cells in Tris-A buffer for 24 hours, which contrasts with the 10% decrease per hour found on rat basophilic leukemia cells (2).

What could be the reason for this failure to demonstrate free IgE-receptors?

1) It can be that our quantitation method is not sufficiently accurate or sensitive to detect small changes in cell-bound IgE. However,

1.5- to 7-fold increase after passive sensitization with myeloma IgE as measured by Ishizaka et al. (9), would certainly have been detected. We estimate that it must be possible to detect a 25% increase or decrease. The results of the passive sensitization experiments (12) can be explained by an increase of cell-bound IgE of less than 25%, since, per basophil in the incubation medium only 100 molecules of allergen, of which presumably only a fraction really binds, are necessary for histamine release (15). Furthermore, in 12 out of 14 subjects, tested by Levy (11), whose leukocytes had shown increased sensitivity to the allergen after passive sensitization, no shift in the anti-IgE dose-response curve could be demonstrated. Presumably, a very small number of IgE molecules per cell is sufficient for sensitization. Also the preliminary experiments we performed (22), showed histamine release after passive sensitization and allergen challenge, without increase of basophil-bound IgE, as measured by quantitative immunofluorescence microscopy. The quantitative aspects of the results in immunoelectron microscopy and autoradiography are difficult to interpret.

2) It can be the sensitization conditions do not resemble the *in vivo* situation to such an extent, that an increase in cell-bound IgE can be obtained.

– The incubation time may be too short to obtain a detectable increase in cell-bound IgE. However, in Ishizaka's experiments (9) cells were incubated with the myeloma IgE for only 10 minutes. It is possible, that after binding of IgE to free receptors, some time is required for stabilization of the IgE-receptor complexes. This effect, corresponding to the distinction made by Lichtenstein (15) between absorption and fixation of IgE to basophils, can be caused, for instance, by a multivalent interaction between IgE and receptor. In that case IgE, bound *in vitro* after short time sensitization, would dissociate much faster than the IgE bound to the basophils *in vivo*. The results of our IgE determinations in the washing fluid, obtained after *in vitro* sensitization with myeloma IgE, contrasting with the absence of measurable dissociation of IgE bound to the basophils *in vivo*, sustain this possibility.

In the experiments of Ishizaka et al. (9), this loosely bound IgE could have been measured, whereas in our experiments, in which the cells were washed more extensively, this loosely bound IgE was removed. It should be noted, that in our experiments also one incubation with toluidine blue/PFA and two more washings were

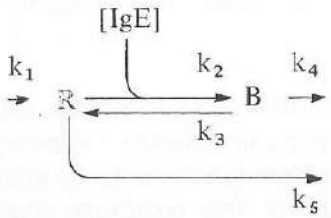
performed before the addition of the FlaIgE, thus minimizing the amount of soluble IgE in the actual measurement. Studies will be undertaken with cells being cultured in the presence or absence of IgE for several days, to further investigate the forementioned points.

– A decrease of cell metabolism prevents binding of IgE to the receptor. This is unlikely, since cells can be sensitized and release histamine under the same conditions (12, 13).

3) Possibly the results of the sensitization studies and those demonstrating binding of ^{125}I-IgE to the basophils do not reflect the presence of free receptors, but an exchange of IgE molecules. This possibility contrasts with the inhibition of passive sensitization of lung fragments by pre-incubation with IgE-rich serum (5).

If we suppose, that there is no detectable amount of free IgE-receptors on the basophils, how can this be reconciled with the dose-response relationship between cell-bound and free IgE, found to fit the unsaturated free exchange model?

Another hypothetical model is proposed, with the following equations:



$$\frac{dB}{dt} = 0 \rightarrow R\,[IgE]k_2 = B(k_3 + k_4)$$

$$\frac{dR}{dt} = 0 \rightarrow k_1 + Bk_3 = R[IgE]k_2 + Rk_5$$

Elimination of R:

$$B = \frac{k_1 k_2 [IgE]}{[IgE]k_2 k_4 + k_3 k_5 + k_4 k_5}$$

$$\frac{1}{B} = \frac{k_4}{k_1} + \frac{k_5(k_3 + k_4)}{k_1 k_2} \quad \frac{1}{[IgE]}$$

$$B_{max} = \frac{k_1}{k_4}$$

(R = free receptors; B = IgE-receptor complex; [IgE] = serum IgE.) In this alternative model, receptors for IgE (=R) are formed continuously on the surface membrane of the basophilic granulocytes with a reaction constant k_1. These receptors lose their function to bind IgE to the cell with a reaction constant k_5, unless IgE is bound to the free receptor, resulting in an IgE-receptor complex (=B), with a reaction constant k_2. This complex can either dissociate into a free receptor and IgE (k_3), or it can be shedded from the cell surface (k_4). Thus, the functional half-life of the free receptor is shorter than that of the IgE-receptor complex. In this model there is a dose-response relationship between the amount of IgE in the serum and basophil-bound IgE, although the amount of free IgE-receptors may be undetectable. The properties of the solubilized IgE-receptors from rat basophilic leukemia cells (17), i.e. a highly unstable free receptor with a functional half-life of $\simeq$ 10 minutes, support this assumption. Newman et al. (17) also found considerable protection of the receptor, when the latter was saturated with IgE. The enhancement of the ^{125}I-IgE binding to rat mast cell receptors by protease inhibitors (1) suggests that a protease in the plasma membrane interferes with the IgE-receptor, and possibly destructs it, before IgE can bind.

Also when receptor synthesis is such a fast process, that in fact not the velocity of the synthesis, but the total number of receptor sites is constant, the relation between serum and cell-bound IgE answers to the forementioned formula. In that case we must assume that the membranes of basophils with a small IgE-load are largely occupied by inactive receptors.

The relation between serum and cell-bound IgE from atopic subjects, which did not fit the unsaturated free exchange model, can easily be fitted in this new model. If we assume, for instance, that the rate of receptor synthesis or the rate of complex shedding can vary, then the amount of IgE, bound with a similar IgE serum level, can increase.

We conclude that the present model can explain the results in the experiments on the quantitation of basophil-bound IgE (18, 19):

a) the IgE serum level influences the amount of basophil-bound IgE;
b) atopy itself effects the amount of basophil-bound IgE;
c) no free IgE-receptors are detectable on the basophils.

REFERENCES

1. Bach, M. K.; Bach, S.; Brashler, J. R.; Ishizaka, T., and Ishizaka, K.: Protease inhibitors enhance ^{125}I-IgE binding to cell-free "receptors" from mast cells. Fed. Proc. 35: 808 (1976).
2. Carson, D. A.; Kulczycki, A., Jr., and Metzger, H.: Interaction of IgE with rat basophilic leukemia cells. III. Release of intact receptors on cell-free particles. J. Immunol. 114: 158-160 (1975).
3. Conroy, M. C., and Lichtenstein, L. M.: Measurement of IgE binding to human leukocytes. Fed. Proc. 35: 809 (1976).
4. Feltkamp-Vroom, Th. M.; Stallman, P. J.; Aalberse, R. C., and Reerink-Brongers, E. E.: Immunofluorescence studies on renal tissue, tonsils, adenoids, nasal polyps, and skin of atopic and nonatopic patients with special reference to IgE. Clin. Immunol. Immunopathol. 4: 392-404 (1975).
5. Godfrey, R. C., and Gradidge, C. F.: Allergic sensitization of human lung fragments prevented by saturation of IgE binding sites. Nature 259: 484-486 (1976).
6. Hijmans, W.; Schuit, H. R. E., and Klein, F. E.: An immunofluorescence procedure for the detection of intracellular immunoglobulins. Clin. exp. Immunol. 4: 457-472 (1969).
7. Ishizaka, K.; Ishizaka, T., and Hornbrook, M. M.: Physicochemical properties of reaginic antibody. V. Correlation of reaginic activity with γE-globulin antibody. J. Immunol. 97: 840-853 (1966).
8. Ishizaka, T., and Ishizaka, K.: Immunological events at the surface of basophil granulocytes and mast cells which induce degranulation. Boerhave Symposion, Leiden, The Netherlands (1976).
9. Ishizaka, T.; Soto, C. S., and Ishizaka, K.: Mechanisms of passive sensitization. III. Number of IgE molecules and their receptor sites on human basophil granulocytes. J. Immunol. 111: 500-511 (1973).
10. Ishizaka, T., and Ishizaka, K.: Mechanisms of passive sensitization. IV. Dissociation of IgE molecules from basophil receptors at acid pH. J. Immunol. 112: 1078-1084 (1974).
11. Levy, D. A.: Structure, function and quantitation of IgE; general discussion of session II; in ISHIZAKA and DAYTON The biological role of the immunoglobulin E system, p. 69 (U.S. Department of Health, Education, and Welfare, etc., Bethesda, Maryland, USA 1972).
12. Levy, D. A., and Osler, A. G.: Studies on the mechanisms of hypersensitivity phenomena. XIV. Passive sensitization *in vitro* of human leukocytes to ragweed pollen antigen. J. Immunol. 97: 203-212 (1966).
13. Levy, D. A., and Osler, A. G.: Studies on the mechanisms of hypersensitivity phenomena. XV. Enhancement of passive sensitization of human leukocytes by heparin. J. Immunol. 99: 1062-1067 (1967).
14. Levy, D. A., and Osler, A. G.: Enhancement by heparin of passive sensitization *in vitro* of human leukocytes. J. Allergy 37: 112 (1966).
15. Lichtenstein, L. M.: Allergy, in BACH and GOOD Clinical Immunobiology, vol. 1, pp. 243-269 (Acad. Press, New York 1972).

16. Middleton, E., Jr.: *In vitro* passive transfer of atopic hypersensitivity. Proc. Soc. exp. Biol. (N.Y.) 104: 245-247 (1960).
17. Newman, S. A.; Rossi, G., and Metzger, H.: Assay and some properties of solubilized cell receptor for IgE. Fed. Proc. 35: 808 (1976).
18. Stallman, P. J., and Aalberse, R. C.: Estimation of basophil-bound IgE by quantitative immunofluorescence microscopy. Int. Arch. Allergy (in press) (Chapter V of this thesis).
19. Stallman, P. J., and Aalberse, R. C.: Quantitation of basophil-bound IgE in atopic and non-atopic subjects. Int. Arch. Allergy (in press). (Chapter VI of this thesis).
20. Stallman, P. J., and Feltkamp-Vroom, Th. M.: Identification of basophilic granulocytes in human leukocyte suspensions subjected to immunofluorescence procedures with anti-IgE. J. immunol. Methods 10: 271-277 (1976). (Chapter III of this thesis).
21. Sullivan, A. L.; Grimley, P. M., and Metzger, H.: Electron microscopic localization of immunoglobulin E on the surface membrane of human basophils. J. exp. Med. 134: 1403-1416 (1971).
22. Toorenenbergen, A. W. van; Aalberse, R. C., and Reerink-Brongers, E. E.: Passive sensitization, histamine release, and immunofluorescence studies on human leukocytes (in preparation).
23. Van Arsdel, P. P., Jr., and Sells, C. J.: Antigenic histamine release from passively sensitized human leukocytes. Science 141: 1190-1191 (1963).

SUMMARY

Antibodies belonging to the immunoglobulin E class play an important part in allergic disease. The reaction between an allergen and IgE, bound to mast cells or basophilic granulocytes, initiates the release of histamine and other mediators of the allergic response.

In chapter I a short introduction is given, summarizing the various methods used by others to demonstrate the presence of IgE on basophilic granulocytes and on mast cells. A quantitative determination of the amount of basophil-bound IgE, however, was only possible with either insensitive or complicated methods. Therefore, the aim of this study was: to develop a method to identify human basophilic granulocytes in leukocyte suspensions and to estimate the amount of IgE bound to these basophils, by means of quantitative immunofluorescence microscopy.

In chapter II the production and testing of the fluorescein isothiocyanate labeled sheep anti-human IgE (FlaIgE), used in these studies, is briefly described.

In chapter III a method is described by which human leukocyte preparations, obtained by Ficoll-Isopaque centrifugation, are stained and fixed in such a way that basophilic granulocytes in these preparations can be easily identified, when the suspensions are subjected to immunofluorescence procedures with FlaIgE. Capping, blocking and absorption experiments confirmed the specificity of the method.

Chapter IV shows how the forementioned basophil identification technique is used, in order to study the presence of a common antigen on human basophils, mast cells and thymus lymphocytes, Basophilic granulocytes and mast cells of different species have been reported to originate from thymocytes and other lymphocytes, presumably T-lymphocytes. Recently, this theory was sustained, when evidence was produced that a thymic antigen is present on rabbit basophilic granulocytes. In the investigations described in this chapter, human leukocytes were tested by the immunofluorescence technique and the immunoelectron microscopy technique to see whether a human thymus lymphocyte antigen (HTLA) could be detected on their surface. We could demonstrate the presence of HTLA on T-lymphocytes, but, neither on basophilic granulocytes nor on mast cells in various tissues.

By means of the forementioned basophil staining and fixation technique, it was possible to identify basophilic granulocytes in leukocyte

suspensions subjected to the immunofluorescence technique with FlaIgE. In chapter V the way of measuring the fluorescence intensity of the basophils by means of quantitative immunofluorescence microscopy, as a reflection of the IgE-load per cell, is described. The reproduceability of this technique and the influence of the incubation time were studied. The mean fluorescence intensity of the basophils varied considerably in six donors, but the cells of two atopic patients showed the highest intensity. IgE was eluted from the cells at pH 2.5 and measured in the supernatant. A correlation was found between the amount of IgE eluted per basophil and the mean fluorescence intensity of the basophils. The number of IgE molecules per basophil was found to range from 15,000 to 500,000.

In chapter VI is described, how this technique was used for studying basophil-bound IgE in 28 atopic subjects and in 29 healthy controls. The fluorescence intensity of the basophils from the atopic patients, both with a low and with an increased serum IgE, was significantly higher than the fluorescence intensity of those from the controls. A significant correlation was also found between the IgE serum level and the amount of basophil-bound IgE.

In chapter VII the method is described, by which the amount of mast cell-bound IgE can be determined semi-quantitatively by end point titration with FlaIgE on cryostat sections of bronchial tissues, tonsils and adenoids. In 37 subjects, mast cell-bound IgE could be compared with the amount of IgE on the basophils, assessed by quantitative immunofluorescence microscopy. A correlation between the IgE-load on both cell types was found, and the influence of the IgE-serum level and atopy on mast cell-bound IgE and on basophil-bound IgE was shown to be similar.

In chapter VIII results are presented to answer the question,whether the variation in the amount of cell-bound IgE is due either to differences in the density of the IgE molecules on the surface of the basophils, or to variations in the surface of the cell-membrane e.g. by villi formation. In immunoelectron microscopy cell-bound IgE was demonstrated on basophilic granulocytes using horse radish peroxidase labeled anti-IgE. The distribution of IgE on the basophils was studied in atopic and non-atopic subjects. The intensity of the peroxidase reaction on the basophils seemed to agree with the amount of cell-bound IgE, assessed by quantitative immunofluorescence microscopy. Villi were observed incidentally and they did not contribute significantly to an augmentation of the cell surface.

Chapter IX deals with the relation between the IgE receptors on the basophils and the IgE level of the serum. In the previous chapters the correlation found between the amount of IgE on basophils and the IgE serum level is mentioned. In this chapter, these results are shown to fit a free exchange model with an approximately constant K-value. We investigated whether free IgE receptors on basophils – which should be present according to this model – could be saturated by incubating the cell suspensions with IgE myeloma protein or with sera from patients with an elevated IgE serum level. Various incubation conditions were applied in sensitizing the leukocytes, and cells from both atopic and non-atopic subjects were used for testing, but no increase in basophil-bound IgE could be measured with the quantitative immunofluorescence technique. Neither have we been able to demonstrate a significant dissociation of cell-bound IgE after incubation in an IgE-free medium for a period of 24 hours.

Finally, another hypothetical model is discussed, in which receptor turnover is taken into account: a shorter functional half-life of the free receptors on the basophils than the half-life of the IgE-receptor complexes is assumed. With this model we could explain the foremen-tioned contradictory results.

SAMENVATTING

Antistoffen van de immunoglobuline E klasse spelen een belangrijke rol bij vele allergische aandoeningen van het onmiddelijke type. IgE antistoffen kunnen specifiek gericht zijn tegen verschillende allergenen zoals graspollen, huisstof, huidschilfers van mens en dier en vele andere. Deze IgE antistoffen komen bij de mens zowel vrij in het bloed als gebonden aan cellen voor, aan de basofiele granulocyten in het perifere bloed en aan de mestcellen in de weefsels. Wanneer celgebonden antistoffen reageren met het allergeen waartegen ze gericht zijn, heeft dit tot gevolg dat uit de cel verschillende stoffen vrijkomen, onder andere histamine, die de symptomen bij een allergische reactie mede te weeg brengen. In de cellen zijn deze stoffen deels opgeslagen in specifieke korrels, waardoor zowel de mestcel als de basofiele granulocyt gekenmerkt worden.

De aanwezigheid van IgE op deze cellen is met verschillende methodes (autoradiografie, immunofluorescentie techniek, immunoferritine techniek en histamine release met anti-IgE) aangetoond. Een kort overzicht van deze methodes wordt gegeven in de inleiding, Hoofdstuk I. Ook worden in dit hoofdstuk de bevindingen van Ishizaka en anderen genoemd, die de hoeveelheid celgebonden IgE bepaalden door na te gaan hoeveel complement gebonden werd wanneer een overmaat anti-IgE werd toegevoegd aan een suspensie van witte bloedcellen waarin het aantal basofiele leukocyten bekend was. Hoe meer complement per basofiele cel gebonden werd, hoe meer IgE aanwezig geacht werd op deze cellen (C_1 fixation transfer test). Omdat de basofiele granulocyten minder dan 1% van de leukocyten in het perifere bloed uitmaken, is het nodig de bepaling te verrichten met suspensies verrijkt aan basofiele cellen.

Naar aanleiding van deze onderzoekingen stelden wij ons tot doel een methode te ontwikkelen waarmee de basofiele granulocyten tussen de andere leukocyten in herkend kunnen worden en tevens de hoeveelheid IgE per individuele basofiele cel kan worden vastgesteld.

In hoofdstuk II wordt de wijze beschreven waarop bij het schaap anti-humaan IgE serum werd opgewekt, werd gekoppeld aan fluoresceine isothiocyanaat en vervolgens werd getest. Dit antiserum (FlaIgE) wordt in de hierna beschreven experimenten gebruikt.

In Hoofdstuk III wordt de methode om basofiele granulocyten te identificieren in leukocyten suspensies beschreven. Humane leukocyten verkregen door celscheiding over Ficoll-Isopaque worden tegelijkertijd gekleurd met toluidine blauw, dat de specifieke korrels van de basofiele cellen kleurt, en gefixeerd met paraformaldehyde. Het blijkt dat met deze procedure de kleuring van de korrels behouden blijft, wanneer vervolgens aan deze cellen FlaIgE wordt toegevoegd. Op deze wijze wordt het celgebonden IgE op de nu herkenbare basofielen aangetoond. De specificiteit van de immunofluorescentie reactie wordt aangetoond door blokkerings- en absorptieproeven, evenals door "capping" experimenten.

In Hoofdstuk IV wordt een andere toepassing gegeven van bovengenoemde techniek om basofielen te herkennen, wanneer wordt nagegaan of er een gemeenschappelijk antigeen is op humane basofielen, mestcellen en thymus lymfocyten. Voor de mens en verscheidene diersoorten wordt wel verondersteld dat basofielen en mestcellen voortkomen uit thymocyten en lymfocyten. Onlangs kreeg deze theorie steun, toen de aanwezigheid van een thymus antigeen op basofiele leukocyten van konijnen werd aangetoond. In de onderzoekingen beschreven in dit hoofdstuk werd met behulp van de immunofluorescentie techniek en van immunoelectronen microscopie nagegaan of er een humaan thymuslymfocyten-antigeen (HTLA) waargenomen kon worden op het oppervlak van humane leukocyten. Wij toonden de aanwezigheid van HTLA op T-lymfocyten aan, maar niet op basofiele granulocyten noch op mestcellen in verscheidene weefsels.

We keren terug tot het celgebonden IgE in Hoofdstuk V. De eerder genoemde herkennings methode van basofiele leukocyten maakte het mogelijk de fluorescentie intensiteit van de basofielen te meten, nadat ze geïncubeerd waren met FlaIgE. Deze meetmethode werd quantitative immunofluorescentie microscopie genoemd. De basofielen werden met doorvallend licht opgezocht en binnen het meetdiafragma ingesteld. Hierna werd de intensiteit van de fluorescentie gemeten, objectief en zonder dat de fluorescentie kon uitdoven. Bij 6 personen werd van ieder de gemiddelde fluorescentie intensiteit per basofiele granulocyt gemeten. Deze bleek aanzienlijk te variëren en op de basofielen van 2 atopische donoren het hoogst te zijn. De reproduceerbaarheid van de meting en de invloed van diverse factoren, o.a. de incubatietijd, werden onderzocht. Ook werd door zuurbehandeling het IgE van de cellen van deze 6 donoren losgemaakt (elutiemethode). Het in de bovenstaande vloeistof vrijgekomen IgE werd gemeten en berekend per basofiel aanwezig in de oorspronkelijke suspensie. Er werd een correlatie gevonden tussen de hoeveel-

heid IgE geëlueerd per basofiel en de gemiddelde fluorescentie intensiteit. Zo kon ook geschat worden dat de hoeveelheid IgE moleculen per basofiel bij verschillende personen varieerde tussen de 15.000 en 500.000.

In Hoofdstuk VI wordt het onderzoek beschreven naar het basofiel-gebonden IgE van 28 atopische personen en 29 niet atopische controles. De fluorescentie intensiteit van de basofielen van atopici, zowel van die met een laag als van die met een hoog serum-IgE-gehalte, was hoger dan de fluorescentie intensiteit van de basofielen van de controles. Het basofielgebonden IgE bleek ook samen te hangen met het IgE-gehalte in het serum. Bij atopici met een laag IgE-gehalte in het serum was de fluorescentie intensiteit van de basofielen lager dan bij atopici met een hoog serum-IgE, maar toch aanzienlijk hoger dan bij controle personen met hetzelfde lage IgE-gehalte in het serum.

Bij de lokale atopische reacties zijn echter vooral de mestcellen belangrijk. Daarom wordt in Hoofdstuk VII de IgE-belading van basofielen en mestcellen van dezelfde personen onderzocht en vergeleken. De hoeveelheid IgE gebonden aan mestcellen kan worden gemeten met een semiquantitatieve methode: op vriescoupes van bronchiaal weefsel, tonsillen en adenoid wordt de immunofluorescentie techniek toegepast met een verdunningsreeks van het FlaIgE. De hoogste verdunning van het antiserum waarbij nog mestcelfluorescentie te zien was, bleek een maat te geven voor de hoeveelheid mestcelgebonden IgE. Atopische patienten hadden een grotere hoeveelheid mestcelgebonden IgE dan niet atopische personen. Evenals bij de basofielen bleek ook bij de mestcellen het serum-IgE-gehalte van invloed te zijn op de hoeveelheid celgebonden IgE. Bij 37 personen konden we de hoeveelheid mestcelgebonden IgE vergelijken met de IgE-belading van de basofielen. Er werd een overeenkomst gevonden tussen de mate van IgE-belading op de twee soorten cellen.

In Hoofdstuk VIII komt de vraag aan de orde of de verschillen in celgebonden IgE veroorzaakt worden door verschillen in dichtheid van de IgE moleculen op het oppervlak van de basofiel, of door toename van het oppervlak van de celmembraan, bijvoorbeeld door villi-vorming. IgE gebonden aan de basofiele granulocyt werd aangetoond met immunoelectronen microscopie met behulp van een met mierikswortel-peroxidase gelabeld anti-IgE. De verdeling van het IgE op de basofielen werd onderzocht bij atopische en niet atopische personen. De intensiteit van de peroxidasereactie op de basofielen

bleek overeen te komen met de hoeveelheid basofielgebonden IgE gemeten met quantitatieve immunofluorescentie microscopie. Villi werden zelden waargenomen en droegen niet in belangrijke mate bij aan de hoeveelheid celmembraanoppervlak.

Hoofdstuk IX behandelt de relatie tussen de IgE-receptoren op de basofiele leukocyt en het vrije serum-IgE. In de voorgaande hoofdstukken werd de correlatie beschreven die gevonden werd tussen de hoeveelheid IgE op basofielen en de IgE-spiegel in het serum. In dit hoofdstuk wordt getoond dat deze resultaten passen in een vrij uitwisselingsmodel met een ongeveer konstante K-waarde. We onderzochten of vrije IgE-receptoren op de basofiel, die volgens dit model aanwezig zouden zijn, verzadigd konden worden door de celsuspensies te incuberen met myeloom-IgE of met sera van patiënten met een verhoogd IgE-gehalte. Leukocyten van zowel atopische als niet atopische personen werden op deze wijze behandeld onder verschillende incubatie-omstandigheden, maar met quantitatieve immunofluorescentie microscopie kon geen toename van het basofielgebonden IgE gemeten worden. Voorts kon geen dissociatie van celgebonden IgE worden aangetoond na 24 uur incubatie in een IgE-vrij medium. Op grond van deze bevindingen wordt een ander hypothetisch model ontwikkeld waarin rekening wordt gehouden met de receptorturnover: hierbij wordt aangenomen dat de funktionele halfwaarde tijd van de vrije receptor op de celmembraan korter is dan die van de receptoren waaraan IgE gebonden is. Dit model voor het verband tussen IgE en de IgE-receptor op basofiele granulocyten kan de genoemde tegenstrijdige resultaten verklaren.


PERIPLAN – KPL – 8X
PLANAPOCHROMAAT