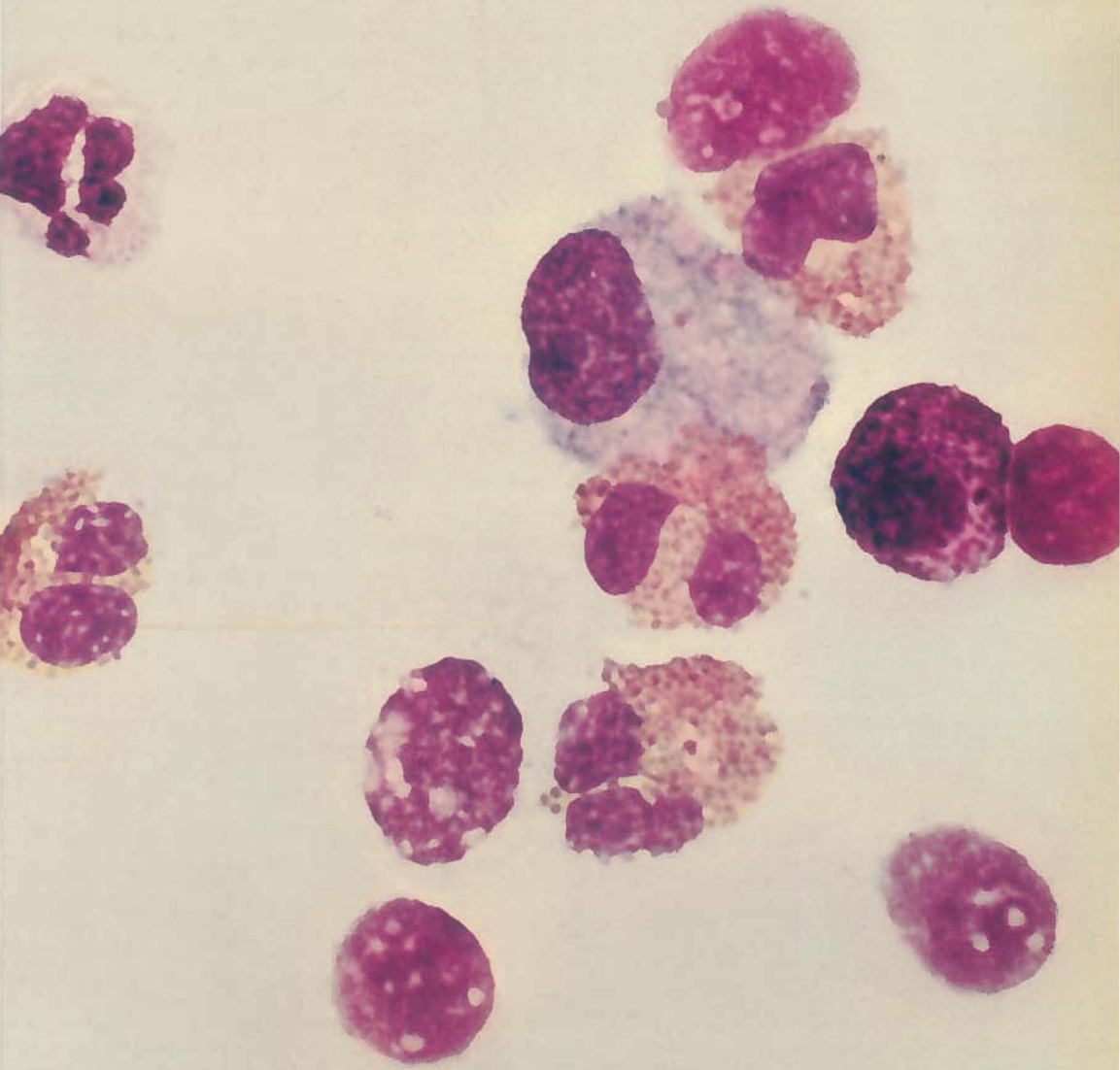
The late allergic reaction in bronchial asthma. [De allergeen-gelnduceerde laat obstructive reactie bij allergische CARA patienten]

de Monchy, Jan Gunnar Rene

The late allergic reaction in bronchial asthma



J.G.R. de Monchy

The late allergic reaction in bronchial asthma

STELLINGEN

I
De eosinofiele granulocyt speelt waarschijnlijk een oorzakelijke rol bij het ontstaan van de late allergische reactie bij huisstofmijtinhalatie.

II
De late allergische reactie berust niet alleen op een IgE-gemedieerd mechanisme.

III
Histamine is als mediatorstof tijdens de late allergische reactie van ondergeschikt belang.

IV
De dosis-response relatie voor huisstofmijtextract tijdens de vroege bronchusobstructieve reactie kan als een negatief exponentiële curve worden beschreven.

V
Er zijn aanwijzingen dat vroegtijdige verbreking van het contact tussen allergenen en het shockorgaan bij allergische individuen de progressie naar meer chronische afwijkingen kan voorkomen.

VI
In de differentiaal diagnose van osteoporose dient gesystematiseerde mastocytose te worden opgenomen.

VII
De effecten van angiotensine converting enzyme remmers op de bloeddruk zijn niet evenredig met de serumspiegel.

VIII
De toepassing van 'ongestandaardiseerde' allergeenextracten in de huispraktijk dient gestaakt te worden.

IX
Ook bij ernstige intoxicaties met parathion is houtskool-hemoperfusie niet zinvol.

X
Bij vrouwen met idiopathisch oedeem wordt een verhoogde frequentie van psychiatrische ziektebeelden gevonden.

XI
Bij patiënten met rhinitisklachten in het vroege voorjaar kan een eveneens bestaande overgevoeligheid voor steenvruchten de indicatie voor hyposensibilisatie versterken.

XII
Seborrhoisch eczeem behoort tot het atopie-syndroom.

XIII
De schade die door het 'langzaam wurgende' karakter van de bezuinigingen op het wetenschappelijk onderzoek wordt aangericht is groter dan op grond van de feitelijke beperkingen noodzakelijk is.

XIV
Het optreden van een locale ontstekingsreactie na een insectensteek is geen steekhoudend argument om als steekproef een proefsteek uit te voeren.

XV
Het verschil tussen een allergische reactie en een promotie is beter nooit dan laat.

Stellingen behorende bij het proefschrift van J.G.R. de Monchy THE LATE ALLERGIC REACTION IN BRONCHIAL ASTHMA, Groningen, 1986.

RIJKSUNIVERSITEIT TE GRONINGEN

The late allergic reaction in bronchial asthma

De allergeen-geïnduceerde laat obstructive reactie bij allergische CARA patiënten

Met een samenvatting en conclusie in het Nederlands

PROEFSCHRIFT

ter verkrijging van het doctoraat in de geneeskunde aan de
Rijks Universiteit te Groningen
Op gezag van de rector magnificus
Dr. E. Bleumink in het openbaar te verdedigen op
woensdag 10 december
des namiddags te 14.45 uur precies

door

JAN GUNNAR RENE DE MONCHY
geboren te Stockholm

krips repro meppel
1986

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Referent: Dr. H.F. Kauffman

Diagrams were made by J. Brouwer, J. Duitsch, J. Pleiter and L. Leertouwer.
The investigations described in this thesis and the printing costs were supported by the Nederlands Astma Fonds.

Ter nagedachtenis aan mijn Vader
aan Philip en Erik

Voorwoord

Dit onderzoek werd verricht binnen de afdelingen Allergologie, Longziekten en Longfunctie en in samenwerking met het Centraal Klinisch Chemisch Laboratorium van het Academisch Ziekenhuis te Groningen.

Tevens werd samengewerkt met de afdeling Klinische Chemie van de Universiteit van Uppsala (Zweden), met de afdelingen Longziekten van het Academisch Ziekenhuis te Utrecht, van het Academisch Medisch Centrum te Amsterdam en het Centraal Laboratorium van de bloedtransfusiedienst te Amsterdam.

Het onderzoek werd mede mogelijk gemaakt door een subsidie van het Nederlands Astma Fonds.

Mijn dank voor het tot stand komen van dit proefschrift gaat in eerste instantie uit naar alle patiënten en proefpersonen die bereid waren om ongemak en benauwdheid te doorstaan. Hun inzet en belangstelling voor het onderzoek hebben voor mij een belangrijke stimulans gevormd. De situering van het onderzoek binnen de Interne Kliniek (toenmalig hoofd prof. dr. E. Mandema) maakte het mogelijk om met vele collega's regelmatig te overleggen, waarvoor ik alle betrokkenen recht dankbaar ben.

Prof. dr. K. de Vries maakte het mogelijk om het onderzoek te verrichten. Beste Klaas, de intensieve interactie tussen jou en mij is niet in enkele zinnen te schetsen. Laat ik volstaan met te vermelden dat ik van jou veel meer geleerd heb dan alleen een vak. Je manier van omgaan met mensen stimuleert tot zelfstandigheid en getuigt van respect voor je medewerkers. De wijze waarop je de integratie tussen artsen en biochemici binnen de afdeling gestalte geeft, is zeker bijzonder te noemen.

Prof. dr. H.J. Sluiter, beste Henk, jouw grote belangstelling voor de CARA maakte dat je groen licht gaf voor de bronchoalveolaire lavage; een onderzoek dat bij bronchusobstructieve patiënten zeker in de beginperiode controversieel genoemd kon worden. Ik wil je oprecht danken voor de steun die ik steeds mocht ondervinden van de afdeling Longziekten. In de begin periode was dit met name in de persoon van prof. dr. H.M. Jansen. Mijn dank geldt ook de Longfunctieafdeling (hoofd: prof. dr. R. Peset Reig).

Dr. H.F. Kauffman leverde een belangrijke bijdrage aan het tot stand komen van dit proefschrift. In een vroeg stadium, Henk, doorzag jij het belang van de gedane waarnemingen en stimuleerde jij het laboratorium onderzoek. De wijze waarop jij het laboratorium van de afdeling Allergologie de afgelopen jaren gestalte hebt gegeven, verdient ieders bewondering. Het is verheugend dat bij het onderzoek naar de eosinofiele cel in vitro, thans goede vorderingen worden gemaakt.

Drs. J.B. Hemel, beste Jan, wat wij samen doorstaan hebben de laatste jaren is niet gering. Van en met je heb ik veel geleerd, ik hoop dat we ook nog in de toekomst nog veel zullen samenwerken.

Dr. J.J. Keijzer, beste Jules, met veel genoegen denk ik terug aan de 'histaminetijd'. Je kritische en intelligente geest, gepaard aan een Zuidnederlands 'Joye de vivre', wordt hier node gemist.

Dr. G.H. Koëter, beste Gerard, met de jouw eigen voortvarendheid heb je mij op belangrijke punten geholpen bij het lavageonderzoek. Ik hoop dat we in de komende tijd op vruchtbare wijze samen aan de allergische component van het CARA-ziektebeeld zullen kunnen werken.

Dr. P.L.B. Bruynzeel, beste Piet, de samenwerking met 'Utrecht' biedt ons over en weer de kans aan het CARA-onderzoek bij te dragen.

Met genoegen denk ik terug aan de vele discussies met dr. H. Meurs, drs. S. van der Heide, dr. E. Kloprogge, dr. F. Beaumont en dr. D.S. Postma.

Belangrijke hulp met het verzamelen van patiëntengegevens en het maken van berekeningen kreeg ik van mevr. J. Kamperman en mevr. H. Wartena.

Mevr. B. Schilizzi corrigeerde niet alleen de Engelse tekst, maar leverde ook inhoudelijk in toenemende mate een bijdrage aan het eosinofielen onderzoek.

In het bijzonder ben ik veel dank verschuldigd aan mevr. T. Rakers, mevr. A.R. Homan, mevr. J. Kukler, mevr. M. van Kammen, mevr. H. Faber en mevr. A. Poppinga voor het uitvoeren van het patiënt-gebonden onderzoek en aan mevr. B. v.d. Belt, dhr. F. Weeke en mevr. M. Nakken en dhr. H. Lange voor het laboratoriumonderzoek.

De inzet van mevr. J. Kruitbosch, mevr. I. Lambeck, mevr. T. Hanemaaijer en mevr. C. Koppe bij het typen en corrigeren van het proefschrift, moet met nadruk vermeld worden.

Zonder de hulp van de voortreffelijk georganiseerde verpleegafdeling B_1, onder leiding van mevr. E. Mensinga, was dit onderzoek niet mogelijk geweest.

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

General introduction

1.1. Introduction

Late allergic reactions have been documented in various tissues such as the skin (1), the lungs (2), and the nasal mucosa (3), of sensitized subjects.

Although single late allergic reactions occasionally occur, the late reaction is classically the second part of a biphasic response. While the early phase occurs some 15 minutes following an allergen contact, and subsides within one hour, the late phase starts about 3 hours after allergen contact, reaches its maximum after 7–8 hours, and may still be present after 24 hours.

The mechanism of the early phase was shown to be dependent on IgE mediated mastcell (or basophil) degranulation, but the mechanism of the late phase is largely unknown.

The purpose of the studies presented in this thesis was to investigate the mechanism of the late reaction, in the lung, focusing on the question whether this reaction was IgE or immune complex mediated, since in the literature both pathways have been advocated (4). We chose to study the *late asthmatic reaction* (LAR), since the LAR is a clinically relevant problem. Patients who exhibit a biphasic response following allergen challenge seem to have a more serious type of bronchial 'asthma' than those who have single early reactions (EAR) (2). This may be related to the observation that the LAR may induce an increase in bronchial hyperreactivity (5). The LAR is relatively easy to measure by simple lung function techniques such as the forced expiratory volume in one second (FEV_1).

In this study of the LAR three different approaches were followed:

a. As described in Chapters 2, 3 and 4, bronchoalveolar lavage (BAL) was carried out following allergen challenge. BAL was undertaken since other methods of analyzing the mechanism of the LAR, such as measurement of mediators, chemoattractants, immunoglobulins, or cellular constituents in peripheral blood, had failed to shed a clear light on this reaction. (see 1.4.7.).
b. Since the late *cutaneous* reaction was shown to be accompanied by infiltration of mainly granulocytes (6), a link between these cells and the

partially bronchospastic nature of the LAR was sought. This was done by studying the capacity of eosinophils and neutrophils to produce spasmogenic substances in vitro (chapter 2.3.).

c. In Chapter 5 'modelling' studies of the early and late asthmatic reactions are performed. The purpose of these studies was to develop a mathematical model for the bronchial obstructive reactions which could be used both to predict the severity of the reactions following allergen provocation and to analyse factors contributing to the occurrence of these reactions.

1.2.1. Chronic non-specific lung diseases (CNSLD)

About the terms asthma, bronchitis and emphysema confusion still exists. In 1959 during the Ciba-symposium (7) asthma was defined as airway obstruction which varies spontaneously or in response to treatment; chronic bronchitis was defined as chronic sputum production for at least 3 months in each year for at least 2 years, and emphysema was defined as an abnormal enlargement of the air spaces distal to bronchioles (8). In practice is it impossible to make a clear distinction between patients with asthma, bronchitis and some types of emphysema since these diagnoses are based on lung function, symptoms, and post-mortem findings, respectively. Furthermore, no mutually exclusive criteria can be found, on the contrary there is a clear overlap in symptoms, lung function and pathology between patients with these disorders (9). Orie suggested in 1960 to use the general term: '*Chronische aspecifieke respiratoire aandoeningen*' (CARA) as an overall diagnosis (10). This was not only based upon the impossibility to make a clear distinction between patients with asthma, bronchitis and some forms of emphysema, but also on the observation that patients may show these 'diseases' consecutively during their life and frequently have among their close relatives patients suffering from these 'diseases'. This emphasis on a common (genetical?) factor in asthma, bronchitis and some forms of emphysema has been referred to as the Dutch hypothesis (11).

The use of the general term CARA (English: Chronic non-specific lung diseases CNSLD), however, is only acceptable if followed by an accurate description of:

a. The clinical picture (complaints);
b. extent of reversibility of airflow obstruction;

c. degree of aspecific bronchial hyperreactivity;
d. presence, specificity, and degree of allergy;
e. complicating diseases, functional, anatomical and humoral abnormalities.

The operational definition of CNSLD used in epidemiological studies is: chronic cough with or without sputum during at least 3 months a year during at least 2 consecutive years and/or attacks of dyspnoea and/or wheezing at rest or during exercise (12). Other, e.g. infective causes, however, must have been excluded.

CNSLD is a major health problem in this country. It has been shown that the symptoms of CNSLD in men and women occur in about 30% of the population between 40 and 64 years of age. Moreover, 5–10% of the children between 6 and 16 years old have complaints of frequent or long-standing cough, attacks of dyspnoea, dyspnoea on exercise for 3 or more years in succession. In the whole population CNSLD has an incidence of 10%. It was the cause of death in 3.2% of the male population in 1981. The 'disease' results in repeated absence from work and hospitalisations (50.000/year or 10% of all hospital admissions in 1982, according to the Netherlands Central Bureau of Statistics).

1.2.2. Clinical 'picture' of the patients

The patients participating in these studies had mild to moderate complaints of wheezing, chest tightness, and coughing, with no or very limited sputum production. Most of them experienced exacerbations of their symptoms in autumn and following contact with house dust, animals, or after exercise. Their age varied from 15 to 46 years.

Most patients were well controlled with inhalation of disodium cromoglycate (DSCG) combined with ipratropium bromide and/or inhaled corticosteroids. During exacerbations they additionally used inhaled beta-mimetic drugs or theophylline.

Patients with a history suggestive of a recent bronchial infection were excluded.

None of the patients used oral steroids as a maintenance therapy. Many had complicating ailments such as rhinitis, conjunctivitis, eczema or hives. Among their close relatives were many individuals with the same atopic conditions. Base Line Forced Expiratory Volume in one second (FEV_1) varied from 50% to 122% of the predicted value. The airway obstruction

of these patients was shown to be completely or largely reversible. All had a light to moderate degree of airway hyperreactivity as defined by histamine and acetylcholine thresholds. A variable degree of house dust mite (HDM) allergy was present as defined by a wheal and flare reaction to HDM extract and specific IgE antibodies. Most patients had a blood eosinophilia and all patients were symptom free without medication during a 14 day elective admission to hospital. (Inhaled corticosteroids had been stopped 5 days and other medication at least 3 days before HDM provocation).

Since the characteristics of the patients that were selected for our studies comply with the criteria defined during the Ciba-symposium (7) for asthma, in the following chapters these patients will be called 'asthmatics'.

1.2.3. Airflow obstruction

The common denominator in CNSLD patients is diffuse airflow obstruction. Airflow obstruction can occur as a consequence of:

a. *contraction of smooth muscles.* Contraction and dilation of the smooth muscle of the bronchial tree are dependent on both neurogenic and humoral factors. The humoral factors can be divided in hormonal, enzyme-like and immunological factors. Bronchial obstruction occurs when the balance of contracting and dilating forces is disturbed.
b. *increased thickness of the airway wall.* Increased thickness of the airway wall occurs as a consequence of mucosal oedema, muscular hypertrophy and/or inflammation. This inflammation can be caused not only by bacterial infection but also by inhalation of toxic gases or by ongoing allergic stimulation.
c. *accumulation of mucus.* Accumulation of mucus may occur as a consequence of hypersecretion as e.g. in patients with hypertrophy of mucous glands and chronic sputum production. It may also, at least partially, be the result of a defect in ciliary activity as can be found in cigarette smokers.

   Since in these studies cigarette smokers and patients with recurrent bacterial infections were excluded, hypersecretion did probably not contribute to airflow obstruction (see characterization of patients).
d. *Loss of elastic recoil.* In emphysematous lungs loss of elastic retracting forces of the lung tissue is present. This results in a diminished traction on the intrapulmonary airways during expiration leading to premature

closing of the airways during expiration. These patients also have an increased compliance and an enlargement of total lung capacity. Since our patient group was selected on the presence of reversible airway obstruction and allergy, loss of elastic retracting forces in the lung tissue will hardly be of importance in this younger patient group; this is in accordance with the finding of normal compliance (after bronchodilation) and a normal total lung capacity.

1.2.4. Reversibility of airflow obstruction

Airflow obstruction can be subdivided in a reversible and an irriversible part. The size of the reversible part can be measured by performing FEV_1 before and shortly after administration of a bronchodilating drug (short-term reversibility). However, following a period of adequate 'anti-allergic' therapy even a larger improvement of FEV_1 can be obtained (long-term reversibility).

These observations have led to the hypothesis that therapeutic intervention in the chain of events ultimately leading to bronchial obstruction will be optimal only when started as early as possible in this chain. This concept was based largely on clinical grounds, but is now being supported experimentally (13, 14, 15). The pathological processes which occur early in the chain of events have a tendency to become selfperpetuating and self-amplifying. This is illustrated in many clinical situations, e.g. viral-bacterial bronchial infections promoted by insufficient expectoration may lead to increased bronchial hyperreactivity thereby promoting bronchospasm, etc.

Another example is the allergic process itself which seems to be selfperpetuating since late-phase allergic reactions destabilize the lung, not only by decreasing basal values of lung function, but also by increasing bronchial hyperreactivity as was shown in studies performed by Gökemeyer (16) and Cartier et al. (5). Moreover, histamine released during the allergic reaction increases lung permeability and thus may facilitate sensitization to new allergens (17). *Based on the afore-mentioned concepts, the treatment of CNSLD should be directed as far as possible towards the underlying mechanisms and the best bronchodilatory drug does not per se offer the best maintenance therapy.*

1.3.1. Hyperreactivity of the airways (in asthmatic patients)

Hyperreactivity of the airways is a characteristic feature of the asthmatic patient. The degree of obstruction following an allergic or non-allergic stimulus is dependent on the severity of this stimulus and the degree of airway hyperreactivity. In patients with a high degree of airway hyperreactivity a small stimulus will suffice to cause obstruction. Although there is an association between base-line FEV_1 and the degree of bronchial hyperreactivity, bronchial hyperreactivity may vary within certain limits without affecting base-line calibre. Since the patient-group was selected on a stable FEV_1 value (less than 15% variation during the day and from day to day) the relation between reactivity and base line calibre will not be discussed.

The degree of bronchial hyperreactivity can be measured with provocation tests. Defined doses of histamine, acetylcholine or metacholine can be inhaled and the response of the bronchi is measured by e.g. spirometry (FEV_1).

Bronchial hyperreactivity has not always been clearly defined, resulting in differences in interpretation. Hyperreactivity can be considered a bronchus obstructive reaction of an individual to stimuli (such as cold air, dust, fog, etc.) which in the majority of the population does not give rise to complaints. However, hyperreactivity can also be defined as the predisposition of an individual to develop the before-mentioned reaction pattern after adequate exposition. Since asthmatic patients do not always react in the same degree to stimuli such as cold air, dust, and fog, and since for example a viral infection, contact to dust and/or irritants (notably cigarette smoking) may serve as a primer, a more concise definition of bronchial hyperreactivity is needed. One possibility is to consider the measured degree of bronchial hyperreactivity as the sum of a '*primary*', genetically determined, component and a '*secondary*', acquired, component. Both on practical and theoretical grounds the distinction between a primary and a secondary component of hyperreactivity is complex. It is conceivable that all hyperreactivity is secondary. But the observation that immunologically similar individuals (with the same degree of specific antibodies) and with a comparable allergen exposition show lasting differences in bronchial hyperreativity, precludes such an hypothesis. Moreover, patients may be allergic without showing bronchial hyperreactivity or conversely have a marked bronchial hyperreactivity without signs and symptoms of allergy

(18). Thus an inborn propensity to respond with bronchial obstruction following adequate stimuli is suggestive in some patients. Although hyperreactivity is broadly distributed in the population, the bimodal distribution of the reactivity to metacholine is suggestive for a genetical control (19).

However, bronchial hyperreactivity can also be induced by recurrent bronchial infections during early childhood (20).

1.3.2. Reflex bronchoconstriction

Irrespective of the causes of bronchial hyperreactivity the phenomenon can be considered a disturbance in regulation of the bronchial conductance. This conductance is in the asthmatic group of patients largely determined by the degree of contraction of smooth muscles and the thickness of the airway wall. It has been suggested that bronchial hyperreactivity can be caused by a defect in the vagal reflex loop originating from the afferent part (irritant receptor), the central part (brainstem) or the efferent part (synaps, neuro-muscular junction) (21, 22, 23). The vagal nerve theory of bronchial hyperreactivity has become less attractive in *these* patients since atropine did not influence allergen induced increases in bronchial hyperreactivity to histamine (24).

Injury to the epithelial lining of the bronchi and concomitant retrograde stimulation of C-fibres connected to afferent nerve endings recently was suggested to be an alternative reflex loop (short cut) (25).

Such injuries to the epithelial lining could be caused by several factors such as inhaled irritants or toxic gases (SO_2, ozon) (26), but can also be caused by products from inflammatory cells such as eosinophil granulocytes (27). The marked airway inflammation which can be found in 'asthmatic' patients per se could also contribute to bronchial 'hyperreactivity' simply by reducing airway calibre (28). However, since hyperreactivity may persist even after the calibre has returned to normal, disturbances in the regulation of the bronchial tone must play an important role.

1.3.3. Hormonal regulation of bronchial tone

It could be considered that circulating catecholamines are important, at least under pathological conditions. A role for circulating catecholamines is tentative in those patients who react with bronchial obstruction after the

use of oral beta-blocking agents such as propranolol. It is also more indirectly demonstrated by the protective effect of epinephrine on the allergen induced bronchial obstructive reactions (29). Next to a decreased production of catecholamines by the adrenal medulla, for which evidence can be found in older patients (30), a disturbance at the level of the smooth muscle can be envisaged by a defect in the coupling of beta-agonist and receptor.

In asthmatic patients such a 'beta-receptor-blockade' was suggested by Szentivanyi (31). Later studies, however, showed that the beta-receptor dysfunction could be explained by the prior use of beta-agonists by these patients or possibly by the occurrence of bacterial infections (32).

Meurs and Koëter found that a disturbance in beta-adrenergic response on the lymphocytes from asthmatic patients can be secondary to the allergic process itself, and that the regulatory dysfunction was mainly localized in the post-receptor compartment (33). If the lymphocyte is an acceptable model for the bronchial smooth muscle, beta-receptor dysfunction following allergen application could be an illustration of secondary bronchial hyperreactivity.

Hormones from the adrenal cortex such as hydrocortisone may have an indirect effect on bronchial hyperreactivity by reducing inflammatory reactions; but a direct regulatory effect on bronchial conductance seems unlikely (34).

1.3.4. Defective inhibitory system in asthmatic patients

Since in normals a plateau in the dose-response curve to inhaled histamine or acetylcholine occurs, asthmatics in whom such a plateau is less obvious or absent may lack inhibitory systems present in normals.

This inhibitory effect found in normals could be caused by: epinephrine, vaso-active intestinal peptide, histamine (by H_2-receptors) or bronchodilating prostaglandins. Neither of these substances, however, seems to explain per se the phenomenon of limited bronchoconstriction (35).

Another regulatory entity is the bronchial smooth muscle itself.

1.3.5. The bronchial smooth muscle

The muscular tonus can be considered the result of a number of con-

tracting and relaxing factors. Activation of the parasympathetic nerve system can induce liberation of mediators from mast cells and leukocytes and possibly also alpha-adrenergic stimulation resulting in contraction, while beta-adrenergic stimulation and activation of the non-adrenergic inhibiting system induces relaxation.

Biochemically, the regulation of the muscular tonus can be seen as the regulation of the free Ca^{2+}-ion concentration in the cytoplasma of the smooth muscle (36). This Ca^{2+}-'metabolism' is under the influence of a number of receptor-operated processes with bidirectional control. Stimulation of the cell-activating receptors R_a for histamine or acetylcholine for example induce an 'opening' of the Ca^{2+} channels in the cell membrane and/or mobilization of the intracellular Ca^{2+} leading to an elevation of the free intracellular Ca^{2+}, which ultimately results in a contraction of the actine-myosine filaments (see Fig. 1a). Stimulation of cell-inhibiting receptors (R_i) with epinephrine or vaso-active intestinal peptide initiates, by activation of adenylate cyclase, an increase in cyclic AMP leading to a decrease in the free Ca^{2+}-ion concentration in the cell and thereby induces relaxation (see Fig. 1b).

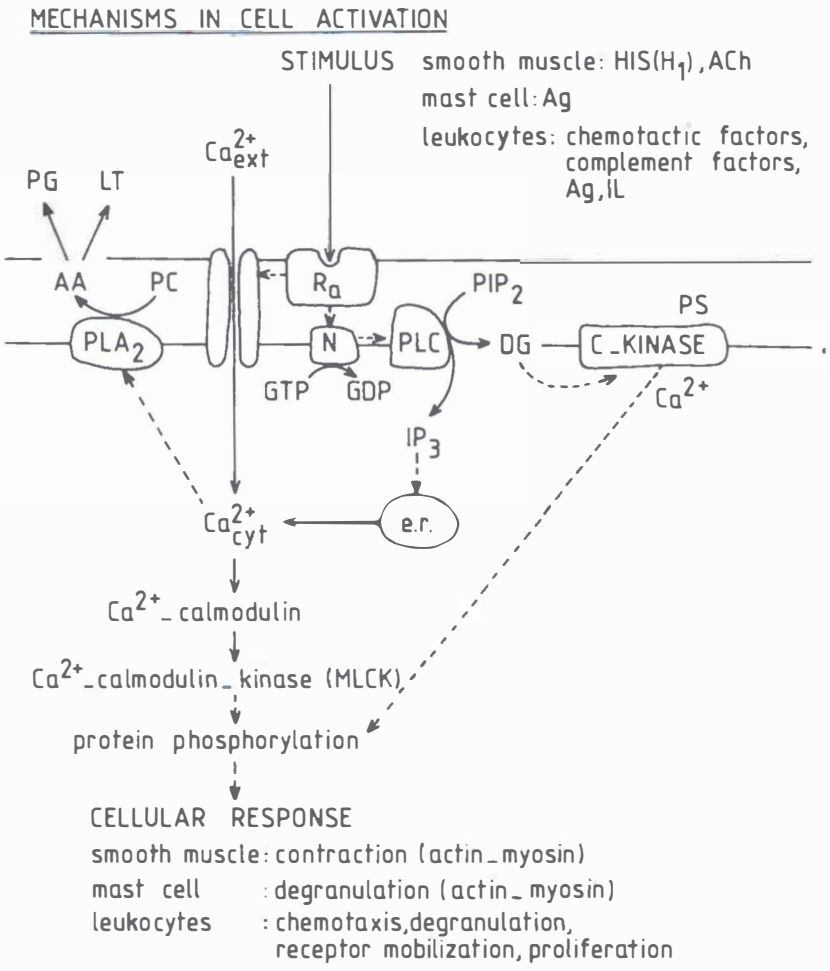
If bronchial hyperreactivity is considered to be an imbalance between these regulatory mechanisms, this can be caused by an overactivity of the activating system or by a reduced activity of the inhibiting system. Since the regulation of the mast cell is largely analogous to the regulation of the smooth muscle cell, a neurohumoral disturbance can also lead to destabilizing of mast cells, thus increasing the contractile load on the smooth muscle by liberation of histamine or leukotrienes.

In the following sub-sections on exercise-induced asthma and aspirin-induced asthma, two special types of bronchial reactivity are briefly introduced, since these reaction patterns have certain similarities with the LAR.

1.3.6. Exercise-induced 'asthma'

Exercise-induced 'asthma' (EIA) classically consists of bronchial obstruction with an onset several minutes after the termination of vigorous exercise. The obstruction usually is of short duration; there are, however, patients who require bronchodilator treatment to recover to pre-exercise lung function. Loss of water from bronchial epithelial lining fluid and sub-

sequent osmotic changes are likely to be the initiating factor in EIA (37). But the further mechanism of EIA seems to be heterogeneous since some patients do respond to cholinergic blockade while others do not. Following the reaction to exercise about 50% of the patients are less responsive to an identical exercise task performed within one hour (38). In subjects who were not refractory, cholinergic blockade prevented hyperventilation-induced bronchoconstriction indicating that in those subjects who probably did not respond by a reflex mechanism a refractory period could be induced.



In some patients, particularly in children, EIA will be followed by a 'late reaction' (3–9 hours later). This late phase is usually abolished by pretreatment with DSCG. The propensity to develop a late reaction is not determined by the severity of the disease, nor by the extent of baseline reactivity in asthmatic subjects (39). There is a correlation between the severity of the early and late reaction following exercise (39). Mediator release during EIA may have a pro-inflammatory effect by activating circulating granulocytes. An increase of neutrophilic complement receptors can be found up to 60 minutes after exercise (40). This is preceded by an increase in neutrophil chemotactic factor (NCF) (41). Thus in EIA both evidence of reflex constriction and secretion of mediators can be found.

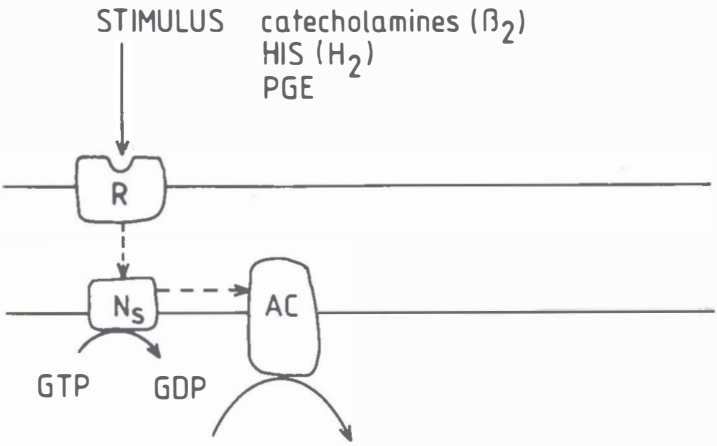
1.3.7. Aspirin-induced 'asthma'

Aspirin (acetyl salicylic acid) may evoke bronchial obstructive reactions and/or hives and angio oedema in 5 – 10% of the asthmatic patient group. These patients are characterized by:

a. age= usually 40–50 years;

Fig. 1a. Activation mechanisms in smooth muscle, mast cells and inflammatory leukocytes.
Activation of the cells is initiated through stimulation of Ca^{2+}-mobilizing receptors (Ra) by specific stimuli. Receptor stimulation is followed by at least two events: (a) Passive influx of extracellular Ca^{2+} into the cytoplasm through transmembrane Ca^{2+} channels down to a steep, 10.000-fold concentration gradient; (b) Stimulation of the phosphatidylinositol (PI) turnover. The initial event in PI-turnover is a guanine nucleotide-dependent activation of the enzyme phospholipase C (PCC) with subsequent hydrolysis of the plasma membrane-bound phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) into the intracellular second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). IP_3 releases intracellularly stored Ca from the endoplamatic reticulum (e.r.), thereby also raising the cytoplasmatic Ca^{2+} concentration ($[Ca^{2+}_{cyt}]$). DG activates the Ca^{2+}- and phospholipid-dependent protein kinase C (C-kinase). The enhanced $[Ca^{2+}_{cyt}]$ and C-kinase activation display a concerted action in the further stimulation of the cells by phosphorylation of various cellular proteins. Cytoplasmatic free Ca^{2+} ions bind to the Ca^{2+} acceptor protein, calmodulin, which may subsequently activate specific protein kinases, including myosin light chain kinase (MLCK). MLCK activation results in the phosphorylation of myosin light chains, which facilitates the interaction between actin and myosin filaments. Actin-myosin interaction causes contraction of smooth muscle and is probably involved in the degranulation of mast cells and leukocytes. DG-induced C-kinase activation is another pathway of cellular protein phosphorylation which contributes to the same physiological responses. The interrelationship between the different mechanisms is presently unclear. Influx of Ca^{2+} ions has also shown to activate arachidonic acid (AA) production by stimulation of phospholipase A_2 (PLA_2). From AA mediators such as prostaglandins (PG) and leukotrienes (LT) are formed.

MECHANISMS IN CELL INHIBITION



activation of Ca^{2+}-efflux (Ca^{2+}-Mg^{2+} ATPase, Na^{+}-Ca^{2+} exchange),
inhibition of PIP_2 turnover,
inhibition of MLCK

CELLULAR RESPONSE

smooth muscle: relaxation
mast cell : inhibition of degranulation
leukocytes : inhibition of chemotaxis and degranulation

b. sex: frequently females;
c. presence of nasal congestion and polyps;
d. blood eosinophilia.

Not only acetyl salicylic acid, but also the structurally unrelated non-steroidal anti-inflammatory drugs (NSAIDS) and food-additives (such as the yellow dye tartrazine) may evoke the same response following ingestion.

Since all NSAIDS have in common an inhibitory effect on the enzyme cyclo-oxygenase (see 1.4.4.3), this inhibition is held responsible for the effects.

Platelets of these patients were shown to have a specific functional abnormality both in vivo and in vitro. Cyclo-oxygenase inhibitors activate these cells in a way very much the same as can be found following an IgE dependent stimulation of platelets (42). Elaborate investigations, however, have shown that the activation of platelets following contact with acetylsalicylic acid, is not a consequence of an immunological process (43). This is also illustrated by the fact that structurally similar compounds such as sodium salicylate and salicylamide (which do not inhibit cyclo-oxygenase) do not cause platelet activation in vivo and in vitro.

Important links exist between platelets and the allergic process: Platelets were shown to have functional IgE receptors and produce factors upon stimulation that increase the releasability of mastcells. Eosinophils on the other hand can activate platelet production of platelet activating factor (PAF-aceter) (43a).

Bronchial hyperreactivity may be considered a hallmark of asthma, but it is not a static entity. Effective anti-asthmatic regimens such as allergen-avoidance, disodium cromoglycate and inhaled corticosteroids have all been shown to reduce the degree of bronchial hyperreactivity. This was not the case when patients received a monotherapy

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Fig. 1b. Inhibition mechanisms in smooth muscle, mast cells and inflammatory leukocytes.
Contraction of smooth muscle and activation of mast cells and leukocytes are inhibited by receptor (R)-mediated stimulation of the plasma membrane-bound enzyme adenylate cyclase. The stimulation of adenylate cyclase involves a guanine nucleotide-dependent regulatory coupling protein (N_s) which transduces the receptor stimulus to the catalytic unit (AC). AC converts ATP to cyclic AMP, which acts as a second messenger to activate one or more CAMP-dependent protein kinases (A-kinase). Via protein phosphorylation, A-kinase activation causes efflux of Ca^{2+} from the cytoplasm and inhibition of PIP_2 turnover and MLCK. Altogether, these processes lead to relaxation of smooth muscle, inhibition of degranulation of mast cells and leukocytes and inhibition of leukocyte chemotaxis.
Berridge M.J. Scient. Am. 1985, 253: 123–134.
Rasmussen H., Barrett P.Q. Physiol. Rev. 1984, 64: 938–984.

with beta-agonists. These drugs may even increase the degree of bronchial hyperreactivity in the first weeks after they have been introduced (15).

1.4.1. Allergy

Allergy can be described as an immune response directed towards heterologous (in principle) not infectious material leading to a (reversible) injury of the host. At the first glance this definition appears permissive because it does not spell out the conditions involved, such as 'asthma', allergic rhinitis, skin rash or a drug reaction. This is correct because allergic reactions can involve any organ system in the body. On the other hand, the definition is extremely stringent in that it insists that an immunological mechanism is involved in the pathogenesis and in that auto-immunity, transplantation and tumor-immunity, and the immune response directed against bacteria and viruses are excluded. Moreover, the criterion of inflicting injury to the host presumes a detrimental effect. Clearly allergy is not restricted to IgE mediated mastcell degranulation.

Although knowledge of the immunological mechanism is very important in predicting the outcome of repeated allergen exposure, other factors also influence the clinical manifestations of allergy. First the physiochemical properties of the allergen itself. The molecular structure of allergen must be such, that an immune response can occur and the substance is able to reach target cells. Concerning inhalant allergy, particle size, gravity and density of the particles in the air are relevant. Secondly, target organ sensitivity to mediators produced by the immunological process modulates the severity of the response. Moreover, the production of mediators by effector cells such as mast cells and basophils is to a certain extent regulated by the same neurogenic and humoral factors as is the bronchial smooth muscle cell itself.

1.4.2. History

Hippocrates (460 – 357 AD) in his aphorisms already mentioned an asthma attack caused by an (at that time unknown) allergic influence. Bostock, in 1819, was the first to describe the symptoms of hay fever. This disease was subsequently known as Bostock's catarrh. In 1873 Blackley discovered that hay-fever was caused by pollen (44). As the knowledge con-

cerning allergic reactions increased, the need for a more accurate definition became urgent. In 1901 Von Behring and Kitasato introduced the term *hypersensitivity* (45). The authors indicated with this description that in certain animals a hypersensitive state towards a tetanus toxine was found that did not occur in other animals of the same species. The former group of animals was suggested not to have a protection against the toxine and to be incapable of an immunological response (immunity). In 1902 Portier and Richet introduced the concept of *anaphylaxis* where immunization was not protective but on the contrary greatly enhanced the sensitivity to relatively harmless substances, as opposed to *prophylaxis* where by immunization tolerance could be reached (46). In 1903 Arthus described local inflammatory reactions following reinjection of substances which where completely harmless at their first administration. In their classic monograph of serum sickness, published in 1905, Von Pirquet and Shick showed that repeated use of serum in the same individual was not only attended by grave risk of anaphylactic reactions, but increasingly lead to attacks of a delayed complex of symptoms known as serum sickness (47). Von Pirquet defined allergy as a situation of a changed reactivity. Moreover, Von Pirquet tried to differentiate between allergens and antigens: 'Antigen implies a substance giving rise to the production of antibody. The term allergen is more far reaching. Allergen comprise besides of antigens proper, the many protein substances which lead to no production of antibodies but to supersensitivity'.

Notwithstanding his efforts to create more clarity, the words 'hypersensitivity', 'anaphylaxis', and 'allergy' remained randomly used. Coca and Cooke tried to end this confusion by differentiating between several types of hypersensitivity (48):

1. 'normal' hypersensitivity that may occur in normal individuals, such as serum sickness;
2. 'abnormal' hypersensitivity that may only occur in a limited group of individuals. In this latter group they differentiated between 3 forms: a. anaphylaxis b. hypersensitivity to infection (for example tuberculine) and c. atopy.

Asthma, hay-fever, and later also infantile eczema, diseases subject to a 'common heriditary cause', were considered atopic ailments. Later the tendency to develop wheal and flare reactions to common environmental antigens was added to the definition of atopy.

Coca and Cooke stressed the principle difference between atopy and anaphylaxis.

An important problem remained in that sometimes no antibody was found in sensitized animals that developed an anaphylactic shock upon re-injection of the antigen. The proponents of the idea that humoral events were responsible for anaphylaxis, were opposed by others who claimed that only 'sessile' antibodies fixed on cells could be involved. The question was settled when Sir Henry Dale discovered in 1910 the pharmacologic properties of histamine (49). The interaction of allergen with (sessile) antibodies at the surface of blood or tissue cells produced histamine and seemed to provide a satisfactory explanation for the phenomenon of anaphylaxis.

The important work of Dale also made the principal difference between anaphylaxis and atopy less obvious. As did the experiments of Praustnitz and Küstner (50). Serum of the fish hypersensitive Küstner was injected into the skin of Praustnitz who was not hypersensitive to this allergen. Twenty-four hours later, fish allergen was introduced on the same place where the serum had been injected and a local reaction in the skin occurred. The transmission of this hypersensitivity was shown to be dependent on heat-labile ('reaginic') antibodies.

It was not until Gell and Coombs made their classification of hypersensitivity reactions that the terminology became unequivocal. Mastcell degranulation caused by reaginic antibodies was subsequently called type 1 allergy. However, the classification of Gell and Coombs needs to be supplemented in order to explain the late allergic reaction as will be illustrated in this thesis. Furthermore it is now clear that the same mechanisms may be responsible for both protective and harmful reactions partly depending on the antigens (allergens) and on the site of contact.

1.4.3.1. IgE

The reaginic antibodies were identified by Ishizaka (51) and Johansson (52) as IgE antibodies. The antibodies have a sedimentation coefficient of 8 S and a molecular weight of 90.000 of which approximately 12% is accounted for by carbohydrate. The molecule has the same basic four chain structure as found in other immunoglobulins: 2 light L-chains and 2 heavy H-(epsilon) chains that carry the isotopic determinants. A comparison of the molecular size of the epsilon polypeptide chain with that of the H-chain of other classes reveal it to be about the same as mu IgM but 10.000 daltons more than gamma (IgG), delta (IgD) or alpha (IgA). This is be-

cause epsilon and mu chains have 5 domains (1 variable and 4 constants) whereas the rest of the heavy chains have only 4 domains (1 variable and 3 constants).

Accumulated evidence demonstrates that the structure essential for sensitization of mast cells and basophils is localized on the carboxyl terminal F_c portion of the epsilon heavy chain (53). It is well established that reagenic antibody is heat-labile. IgE loses its ability to induce a Praustnitz-Küster reaction after heating at 56 °C for 2 – 4 hours but retains its capacity to combine with allergen. In normals IgE levels are low or absent but after infection with moulds or parasites they may be greatly enhanced.

The most important biological property of IgE antibody is its ability to sensitize homologous tissues for allergic reactions. The minimum concentrations of IgE antibodies required for sensitizing normal human skin for a positive PK reaction is in the order of 0.2 – 0.3 ng/ml.

Sensitization of human skin by IgE but not by the other immunoglobulins is supported by reverse type PK reactions. Intracutaneous injection of a minute dose (10^{-4} μg) of the antibodies specific for IgE into normal individuals results in induction of a wheal and flare reaction whereas even a 1000-fold of the antibodies specific for the other immunoglobulin classes i.e. IgG, IgM, IgA, and IgD, fails to do so (53).

IgE antibodies can not only sensitize skin but also other tissues such as lung tissue.

Mast cells and basophils carry high-affinity receptors for IgE, other cells like eosinophils have low-affinity receptors for manomeric IgE but polymeric or complexed IgE can be bound with high affinity.

1.4.3.2. Measurement of IgE

Several methods are available to measure total IgE in sera and secretions. These include non-competitive solid phase immunoassays by the use of anti-IgE coupled to an insoluble carrier and several variations of a liquid phase competitive radio-immunoassay. Serum IgE values are generally expressed in an international unit/ml (IU/ml) based on a WHO-reference standard. One IU of IgE is equivalent to approximately 2.4 ng. Total IgE values over 200 IU/ml serum in an adult population are considered elevated.

The level of IgE in respiratory secretions is positively correlated with that found in serum 54.

IgE is present in a variety of tissue fluids such as sputum, saliva, gastrointestinal fluid, and in broncho alveolar fluid (BAL fluid).

1.4.3.3. Measurement of antigen specific IgE antibody

The most commonly used tests measuring IgE antibodies are:

1. Antibody fixed to tissue mast cells and/or basophils *in vivo*: skin test and provocational challenge tests, and *in vitro*: leukocyte histamine release.
2. IgE antibody in serum can be measured *in vivo* by using the Praustnitz-Küstner test, and *in vitro* by using the radio-allergosorbent test (RAST).

Skin tests can be performed by injecting allergen intradermally. The advantage of skin testing includes ease of performance, economy, and rapidity of results. However, skin testing procedures are also subject to error because of variations in technique and the necessity of the use of a biologically unstable, often inadequately standardized allergenic extract. False positive skin reactions are observed at high allergen concentrations in some patients an may lead to erroneous diagnoses.

The RAST results correlate positively with the results of skin and challenge tests as well as *in vitro* with leukocyte histamine release. Since cell reactivity may interfere with the measuring of IgE only the RAST offers the possibility of a reproducible quantification of IgE antibodies. However, when these tests are carefully performed with the use of the same allergen preparation 10–25% of patients with positive challenge tests will have negative RAST. This indicates that the RAST is less sensitive than bioassays (55).

1.4.3.4. IgE in atopic disease

Patients with so-called atopic diseases including allergic rhinitis, allergic asthma, atopic dermatitis and urticaria commonly have moderately elevated IgE levels. In seasonal allergic rhinitis, it has been possible to demonstrate a significant positive correlation between the quantity of specific IgE antibodies and the intensity of the patients' symptoms. Such a correlation provides indirect evidence that the IgE antibodies play a role in the pathogenesis of atopic respiratory disease; it also suggests that quantification of IgE antibodies by skin testing or RAST may be useful in the assessment of IgE mediated respiratory symptoms (56). In patients with rhinitis and 'asthma' an elevated total IgE serum level is compatible with an atopic

etiology (57). However, a normal serum IgE level does not rule out a limited set of IgE-dependent allergies.

A characteristic of atopic disease is the tendency to develop IgE antibodies to a wide spectrum of environmental allergens. This tendency may be detectable during the first year of life (58).

Serum IgE is markedly elevated in approximately 90% of patients with atopic dermatitis. The degree of elevation correlates roughly with the severity of the disease (59). This strong elevation may result from non-specific effects on IgE immunoregulation, since T-cell dysfunction is also a feature of this disease. The contribution of IgE to the pathogenesis of eczema is not yet known since the pathological anatomical substrate of atopic dermatitis mainly consists of a mononuclear infiltrate. However, basophils and eosinophils have been shown to infiltrate into patch test sites where inhalant allergens had been applied to the skin of patients with eczema, suggesting interactions between all these cell types (60). Finally, IgE antibody directed towards foods, drugs, and insect venoms, may be detected in individuals who are clinically tolerant when they are challenged with the allergens. This observation suggests that other risk factors are important in establishing the significance of IgE antibodies in immunopathology and that protective mechanisms (IgG-blocking antibodies) may be operative in preventing acute anaphylactic reactions in some individuals. The regulation of the IgE response in normals and under pathological conditions will not be discussed here; this has recently been reviewed by Weller (34).

1.4.4.1. Mastcells

Mastcells were first described by Paul Ehrlich in 1879. They are widely distributed in the human body and are specially prevalent at sites that come into contact with the external environment such as the nose, lung, skin, and gastro-intestinal tract.

Nasal and lung mast cells are usually ovoid or irregularly elongate and contain a round or ovoid nucleus. Their surface contains numerous cytoplasmic projections which increase the surface area for receptors up to 3-fold. The secretory granules of mastcells which number up to 400/mature cells contain the potent, preformed chemical mediators including histamine, chemotactic factors, proteases, and proteoglycanes (61).

In rodents there are at least two populations of mastcells which differ morphologically, histochemically, biochemically, and pharmacologically (62). *Mastcells of connected tissue* are large and have granules which contain heparin as the supporting proteoglycane matrix and chymase as the predominant neutral protease. In contrast, *mastcells of the gastro-intestinal mucosa* and probably at other mucosal surfaces are smaller, contain a less sulphated proteoglycane within the granules, and a more soluble chemotryptic protease (63). Both mastcell types contain histamine though the mucosal mastcell has less of this mediator and probably does not contain 5-hydroxytryptamine. Both mastcells may be activated for mediator secretion by cross-linkage of their cell surface by IgE with bridging of IgE receptors. It is known that mucosal mastcells develop from bone marrow precursor cells under the influence of specific T-lymphocyte growth factors, one of which has been identified as interleukine-3 (64).

It is tempting to speculate that mastcells superficial to the basement membrane of the airways represent mucosal mastcells, although, histochemical techniques as yet have been unable to identify different mastcell subpopulations within the human lung. Mastcells derived from the airway surface and the alveoli may be recovered in BAL fluid where they constitute about 0.1% of the nucleated cells derived from normal lung. In sarcoidosis, extrinsic allergic alveolitis, and cryptogenic fibrosing alveolitis, the proportion of mastcells recovered in BAL is significantly increased. Recently increased numbers of mastcells have also been recovered in BAL of patients with allergic asthma (65).

1.4.4.2. Mediator production by mastcells (Preformed mediators)

When lung tissue is sensitized with anti-ragweed IgE antibodies and then exposed to ragweed allergen, histamine and a wide array of other preformed substances are released.

Among these are small proteins and peptides which have been shown to stimulate the selective migration of eosinophils and neutrophils (chemotactic factors). Activated human mastcells release a number of small acidic peptides of molecular weight of 300 - 3000 Dalton, which preferentially attract eosinophils to the site of the release. This group of peptides has been referred to as eosinophil chemotactic factor of anaphylaxis (ECF-A) (66); the precise molecular configuration of these molecules is uncertain. Specific receptors have been demonstrated on the

surface of eosinophils which, when stimulated, increase random cell movement (chemokinesis) and at higher concentration produce directed migration along a concentration gradient (chemotaxis). As the concentration of the chemotactic peptide increases, the migration of the eosinophil diminishes and finally ceases (high dose-inhibition). A high molecular weight glycoprotein (650.000 Dalton) from mastcells has also been identified that preferentially stimulates the chemotaxis of neutrophils. The immunological release of this neutrophil chemotactic activity (HMW-NCA) from human lung fragments has been documented; a similar activity with a low molecular weight has been described to originate from monocytes and T-lymphocytes in response to their IgE-dependent activation (67) (LMW-NCA).

1.4.4.3. Newly generated mediators

Calcium-dependent activation involving IgE or other mechanisms stimulate membrane phospholipase to release arachidonic acid from the membrane phospholipids.

Arachidonic acid may be oxydized along one of two pathways; the cyclo-oxygenase pathway leading to prostaglandins and related substances and the lipoxygenase pathway leading to hydroxyeicosaenoic acid (HETE) and leukotrienes (LTs) (68). Human mastcells generate almost entirely prostaglandin D_2 as their cyclo-oxygenase output of arachidonic acid (69). This mediator is a potent bronchoconstrictor being 3 – 5 times more potent than PGF_2-α, and 30 times more potent than histamine. In addition PGD_2 is a vasodilator and may potentiate the increase in capillary permeability produced by other mediators such as histamine and the sulphidopeptide leukotrienes. Other cyclo-oxygenase products may also be formed during allergic stimulation (tromboxane A_2, $PGF_2\alpha$ and PGE_2) but these mediators are not mastcell derived but generated from secondary cell types activated during mastcell degranulation.

The calcium-dependent 5-lipoxygenase pathway is the predominant route for arachidonic acid oxidation by human mastcells. 5-lipoxygenase products include 5-HETE and the dihydroxyeicosatetraenoic acid (LTB_4). Both of these mediators are chemotactic factors for neutrophils and eosinophils. LTB_4 is one of the most potent naturally occurring chemotactic agents. The 5-lipoxygenase intermediate LTA_4 may undergo adduction to form the first of a series of 6-sulphidopeptide leukotrienes

(LTC_4), which can then be metabolized to LTD_4 and LTE_4 (61). These three sulphidopeptide leukotrienes comprise the previously recognized slow reacting substance of anaphylaxis (SRS-A). The sulphidopeptide leukotrienes are among the most potent contractile agonists known for airway smooth muscle. In addition they cause vasoconstriction and increased vascular permeability. IgE-dependent activation of basophils and possibly mastcells generates an additional membrane-derived mediator recognized as PAF (70). PAF produces a wide variety of pharmacological effects, including bronchoconstriction, increased capillary leakage and leukocyte chemotaxis. Recent studies have shown that PAF may also be released by alveolar macrophages following cross-linking of IgE on their surface (71).

1.4.5.1. The eosinophil granulocyte

The eosinophil and the basophil granulocytes were the first determinable cell types recognized by Paul Ehrlich after the application of the tar dyes (analines) used to stain blood cells (72): 'The large group of cole tar dyes can be divided into two general classes. One of these dyes exemplified by Fuchsin . . . , Safranin . . . , namely the basis analine dyes . . . . The other group, the acid dyes (represents) compounds for example ammonium picrate . . . . I found that one of the granules which I have called eosinophil or alpha-granulations combines with essentially all of the acid dyes. (I have tried over 30 of these acid dyes) whereas the eosinophil granulations are not stained at all by any of the basic analine dyes.'

A second citation of Ehrlich is as follows: 'The shape of the eosinophil granules is almost in all cases round but occasionally I have observed forms in the shape of short rods with rounded ends. In contrast to the generally uniform shape of eosinophil granules is the fact that they vary considerably in their size. The cells exhibit differences in general size and shape. The granules fluctuate in number from cell to cell. Just as important are the various distribution patterns of the granules which sometimes are diffusely dispersed in the cell and other times are gathered in the nuclear hoff or specially excluded from this region' (73).

This citation evokes the image of a very active cell continuously undergoing morphological changes. The modern understanding of the eosinophil closely fits into this early description. The role of eosinophil cells in patients with allergic asthma has for a long time been obscure. Originally these cells were regarded with suspicion because of their capacity to

kill helminths, and their involvement in inflammatory processes. Later their homeostatic functions were stressed and regarded as neutralizing towards the effects of mastcell degranulation (68). A more direct role of the eosinophil cell in asthmatic patients as an inflammatory cell producing toxic products, resulting in tissue injury and inflammation, now seems more likely.

1.4.5.2. The eosinophil in vivo

In 1872 it was already shown by Leyden that in sputum of asthmatic patients so-called Charcot-Leyden-crystals (CLC) could be found that were produced by eosinophil cells (75). Also other eosinophil derived substances can be found in the sputum or serum of asthmatic patients. These substances originate from the small and large cytoplasmic granules.

Dahl found elevated concentrations of eosinophil cationic protein (ECP) in the circulation during early bronchial obstructive reactions following allergen challenge (76). Furthermore, Gleich found that major basic protein (MBP) was found in highly elevated concentrations in the sputum of asthmatic patients (77). When patients are treated sputum MBP is correlated with the improvement of lung function (78). Both MBP and ECP are known to cause tissue injury and inflammation (79).

The toxicity mediated by eosinophils may explain some of the detrimental effects observed after prolonged hypereosinophilia in humans, e.g. cardiomyopathy, neuropathy, and chronic eosinophilic pneumonia (80).

Since PAF (81), ECF-A (66) (mastcell derived), ECF (82) (T cell and macrophage derived), C5A (83), LTB_4 (84) and histamine (85) all have been shown to be chemotactic for eosinophils, blood or tissue, eosinophilia may have many different causes.

In our society, however, allergen exposure in sensitized subjects is the most frequent cause of eosinophilia.

There is an increasing amount of evidence that eosinophils are involved in 'asthma'. In a general population peripheral eosinophilia was correlated to bronchial obstruction in patients with positive skin tests (85). In steroid dependent astmatic patients elevations in eosinophil counts were shown to correlate inversely with FEV_1 and the eosinophil is the main infiltrating cell in asthma (86). Whether the eosinophil is a 'friend or foe' in asthma can not be determined from these studies; but the potential for inflammation of this cell has certainly overshadowed its homeostatic capacity.

1.4.5.3. The eosinophil in vitro

In vitro studies have also identified the eosinophil granulocyte as an active cell with potential for tissue injury. These in vitro studies are mainly concerned with the following items:

a. morphological and density properties;
b. cell receptor expression;
c. functional properties such as production of mediator substances.

After isolation of eosinophil cells by density-dependent centrifugation, a number of eosinophils of allergic asthmatic patients were shown to be lighter than normal eosinophils; they were in the 'hypodense state' (87).

The presence of hypodense eosinophil cells has been shown previously to be prominent in other diseases accompanied by blood eosinophilia, such as the hypereosinophil syndrome or the PIE syndrome (pulmonary infiltrates and eosinophila) (80).

Hypodense eosinophils were shown to be in an activated state with respect to receptor expression, cytotoxic properties and oxygen metabolism (88). Elevation of receptor expression of both eosinophils and neutrophils was found in the circulation after allergen inhalation (89).

Eosinophils were shown to produce the spasmogenic LTC_4 (90, 91).

A conclusive explanation for the presence of hypodense eosinophils is not yet present. Two possibilities are prominent:

a. Hypodense eosinophils are young cells recently released from the bone marrow. This would fit in with the observation that unripe eosinophils from the bone marrow are indeed hypodense;
b. Hypodense eosinophils could be activated cells partially degranulated. This is in concert with the higher *in vitro* activity of isolated hypodense eosinophils as can be shown by enhanced cytotoxic capacity and increased oxygen metabolism (88).

1.4.5.4. Eosinophils and neutrophils

Eosinophil cells have a number of properties comparable to those of neutrophil cells. The most important are:

A. an infiltrative capacity in tissue, following chemotactic stimuli
B. phagocytosis of non-self material associated with the production of oxygen radicals, and degranulation.

However, there are also major differences between these two cell types:

a. Next to 'normal' IgG immune complex and complement receptors activated eosinophils also have IgE immune complex receptors that play an important role in their anti-parasitic activity (88).
b. It has been clearly shown that whereas neutrophil cells can 'only' produce the chemotactic LTB_4, eosinophils have the potential for LTC_4 production (90, 91); whether this in fact occurs during allergic reactions in vivo is uncertain.
c. A striking difference between neutrophils and eosinophils *in vivo* is the response to corticosteroids. While peripheral neutrophil counts rise following administration of corticosteroids, eosinophils acutely disappear from the circulation.

1.4.6. Other cells bearing IgE receptors

In contrast to the high-affinity IgE receptor found on mastcells and basophils, other cell types have low-affinity receptors for IgE, such as T-cells, B-cells, monocytes, eosinophils and platelets. T-cells with F_c receptors for IgE participate in the regulation of IgE-specific responses. IgE receptors on platelets may be relevant since binding of IgE and allergens (or anti IgE antibodies) increases a selective platelet activation followed by the release of cytocidal mediators and generation of oxygen metabolites (43a).

IgE immune complexes activate monocytes (71) (or alveolar macrophages) to secrete mediators important in the inflammatory process including prostaglandins, leukotrienes and platelet activating factor (92).

Since allergen, IgE and alveolar macrophages are present in abundance in the lungs of allergic individuals, these cells may have a hitherto underestimated 'first line' function following allergen contact. Futhermore macrophages are important as phagocytes and as cells capable of antigen presentation, regulation of lymphocyte functions and may also have cytotoxic effects.

1.4.7. The late allergic reaction

The late allergic reaction is an inflammatory response usually occurring 3–8 hrs after allergen exposure in atopic patients. Although incidentally isolated late reactions are found, classically the late reaction is part of a biphasic response. In the skin this biphasic response was first described as

early as in 1922 by Cook (1). Since that time much work has been done on the nature of the initial phase of the allergic reaction, the early reaction, leading to the discovery of its IgE and mastcell dependent mechanism (51, 52). In 1952 Herxheimer pointed out that late bronchial reaction was of 'great practical importance' and was associated with more severe asthma than in patients without late reactions (2). Nonetheless, it was not until Pepys *et al.* (93) and Booy Noord *et al.* (94) called attention to this type of reaction, that scientific interest was aroused. Taylor (3) and later Pelikan (95) described a late reaction of the nasal mucosa. Dieges reported that during hyposensitization parallel with clinical improvement of patients, the late cutaneous reaction (LCR) diminished prior to any effect on the early cutaneous reaction (ECR) (96). Walker found the same for the LAR (97).

Following a LAR patients usually show an increase in bronchial hyperreactivity to non-specific stimuli (5). The clinical pattern of the EAR and the LAR are not identical. The LAR is of longer duration than the EAR and does not react as readily to beta-agonists as the EAR does (98). In contrast to the EAR, pretreatment with corticosteroid usually abolishes the LAR (99). Also non-steroidal anti-inflammatory drugs may offer protection against the development of the LAR (100). DSCG may protect to a certain degree against the development of both EAR and LAR (99). Recently the LAR was also shown to be responsive to xantine derivatives (101). Because of its typical occurrence 3 – 8 hrs after inhalation of allergen an IgG-immune complex mediated reaction was originally suggested by Pepys to explain the mechanism of the LAR (93). The generation of immune complexes of IgG with inhaled antigen and subsequent activation of the complement cascade by the classic route with production of anaphylatoxins C3a and C5a was suggested as a means of inducing mastcell degranulation resulting in a second phase of bronchial obstruction largely independent of IgE antibodies.

This hypothesis was mainly evaluated by studying the late response in the skin of patients with bronchopulmonary aspergillosis (ABPA). IgG, IgM and C3 deposition was shown at the site of the LCR suggesting indeed complement activation by the classical route. The marked sensitivity to corticosteroids of the LCR as well as the LAR was also considered to support the above mentioned hypothesis. However, the clinical picture in common inhalant allergic asthmatics is quite different from that in patients with ABPA. In contrast to patients with ABPA, patients with HDM

sensitive asthma do not respond to inhalation of the allergen with a rise in body temperature, malaise, joint pains, leukocytosis, and signs of infiltrative changes on the X-thorax and a restrictive type of lung function impairment.

HDM-allergic asthmatic patients react during the LAR with a sometimes protracted or recurrent obstructive reaction, with absent or minimal 'systemic' complaints. The LAR is usually followed by a blood eosinophilia 24 hrs after allergen challenge (84). Thus the mould-induced skin – or bronchial-reaction does not seem to be a good model to study HDM – induced LAR.

Moreover, elevated levels of precipitating IgG antibodies to common inhalants in patients with late reactions were seldom found in LAR patients.

Following allergen provocation usually no clear evidence of complement activation was shown: whether during the LAR renewed mastcell degranulation occurs remains a matter of controversy (89, 102–105).

To date some reports have been published suggesting IgG_4 to be a short term sensitizing antibody associated with the development of the LAR (106). Subsequent studies have not been able to confirm these observations (102, 104).

1.4.8. More about the late cutaneous reaction (LCR)

At the height of its response the LCR is characterized by erythema, warmth, oedema, pruritus and tenderness. The area involved is much more extensive than the initial wheal and flare response. In contrast to the sharp delineation of the wheal at 15 minutes, the border of the LCR is ill defined. After the peak at 6–12 hrs the lesion begins to subside and by 24–48 hrs it has disappeared except for residual petechiae, which may exist for several days. Dolovich showed that the LCR could be induced by injection of anti-IgE antibodies (107). Attempts to induce LCRs at the same site repeatedly showed that subsequent lesions were smaller indicating a relative refractory state (108). This was the case regardless of whether the LCR was induced by anti-IgE in a non-atopic patient or by allergen in an atopic patient. The size of the LCR is directly related to the size of the immediate wheal and flare reaction (109).

Solley *et al.* found in passive sensitization studies that by heat-treating serum, (thus inactivating IgE), the occurrence of the ECR but also of the LCR was blocked (6). Also was shown that by selective absorption of IgE,

the ECR and the LCR could be inhibited (4). Additional evidence that IgE was responsible for the LCR came from experiments with myeloma IgE. When serum from a ragweed-sensitive subject was mixed with myeloma IgE in proportions such as the myeloma IgE was in excess of serum IgE by 100- or 1000-fold, both the ECR and the LCR were completely blocked (6).

Injection of compound 48/80, a chemical substance known to trigger mastcell degranulation, produced reactions very similar to the typical LCR induced by IgE-containing serum and antigen (6).

However, Stahlenheim reported that injection of either anti-IgG Staphylococ protein A, or anaphylatoxins produced a long-lasting local inflammatory lesion similar to the LCR. These stimuli clearly could also activate mastcells (110).

Injection of rat peritoneal mastcell granules with disrupted membranes may induce a reaction as judged by PMN and mononuclear cell infiltration (111).

Solley *et al.* analyzed the LCR quantitatively (6). Biopsy specimens taken from 1–8 hrs showed increasing oedema and cellular infiltration as well as vessel abnormalities consisting of perivascular infiltration, hyalinisation and frank haemorrhage and necrosis in a few patients. Initially the cellular infiltrate was entirely mononuclear and limited to the perivascular tissue, but with time eosinophils, neutrophils and mononuclear cells emerged. Overall infiltrating cell population were 41.6 ± 9%, neutrophils 32.4 ± 19.2%, eosinophils 23.0 ± 13.0%, and basophils and mastcells 3 ± 1.6%.

Biopsies of the 24 hrs lesion induced in ragweed-sensitive patients showed a marked cellular infiltration that was almost entirely mononuclear.

Electromicroscopic examination of 8 hrs lesions showed 48% lymphocytes, 7% monocytes, 27% eosinophils, 9% neutrophils, 3% basophils and 1% plasma cells. Of interest was the presence of free eosinophil granules throughout the tissue.

Immunofluorescence failed to demonstrate IgG, IgM, IgA, C_3, IgE or fibrine consistingly. Subsequent investigations on the pathological mechanisms of the LCR have shown that cellular infiltration of the skin may occur in the absence of a clinically evident lesion. De Shazo *et al.* found that fibrine deposition was associated with a macroscopically manifest reaction (112).

Absence of a macroscopically manifest reaction in the presence of a microscopically found cellular infiltrate was reported by several authors (111, 112, 113). Thus infiltrated cells may need a second stimulus to induce an LCR.

1.4.9. Animal models of the LAR.

Several animal models of the LAR have been developed. Abraham (114) used sheep with naturally occurring cutaneous sensitivity to ascaris suum (AS). When these sheep inhale AS, bronchoconstriction, hyperinflation, decrease in arterial oxygen tension, and a fall in dynamic compliance occur within minutes. Sheep without cutaneous sensitivity do not develop obstruction following challenge. Pretreatment with DSCG prevented the response; moreover, following challenge an increase in plasma histamine was found. These observations suggest this response to be an immediate hypersensitivity reaction.

Some sheep may show a biphasic reaction, the reaction begins at about 5–6 h and continues to at least 8 h after challenge. The late response is a combination of hyperinflation and increased pulmonary airflow resistance.

The response was prevented by pretreatment with DSCG, or inhaled corticosteroids but insensitive to atropine; metaproterenol partially and transiently reversed the increase in airway resistance. Application of the SRS-A antagonist FPL 55712 between the early and late response produced a good protection.

Histological analysis of bronchial biopsies obtained during the LAR showed a mononuclear infiltrate, while in BAL fluid, slightly increased numbers of eosinophils and neutrophils were found. Behrens has worked out an animal model using New Zealand white rabbits immunized with Alternaria tenuis allergen (AT) (115). Serum of these rabbits was infused to age-matched non-sensitized recipients. Animals having received serum containing only anti-AT IgE showed a biphasic response following AT challenge.

The severity of the response was comparable to that of the immunized animals. Animals receiving serum containing both anti-AT IgE and anti-AT IgG showed a blunted response, while animals having received only anti-AT IgG containing serum had neither early nor late response.

Immunofluorescence studies in selected animals failed to show deposi-

tion of IgG or C_3 in airways or bloodvessels. From the animal LAR can be concluded that the reaction is an inflammatory IgE dependent response and that leukotrienes probably are important mediator substances.

1.5. Summary of the introduction

The introduction starts by stating the purpose of the study and is followed by an overall description of the applied methods. Subsequently the general term CNSLD (Dutch: CARA) is motivated, followed by a more detailed description of the type of patients participating in the study.

Next factors that might be of importance for the development of the LAR are briefly introduced. Since exercise-induced 'asthma' and aspirin-induced 'asthma' show certain similarities to the LAR, these two reaction patterns are briefly discussed. The concept of allergy then is approached from an historic point of view, followed by a description of the involved cell types and some of their mediator substances. This is followed by a description of the LAR in human beings and in animals. In the following chapters our studies concerning the contents of the BAL fluid and a mathematical modelling of the LAR patients are described as well as in vitro tests of leukotriene production by granulocytes. In the general discussion an effort is made to present a concept of the mechanism of the LAR.

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CHAPTER 2

BRONCHO-ALVEOLAR EOSINOPHILIA DURING ALLERGEN-INDUCED LATE ASTHMATIC REACTIONS

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CHAPTER 2.1

Broncho-alveolar Eosinophilia during Allergen-induced Late Asthmatic Reactions

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2.1.1. Abstract

In order to obtain information about the nature of the local inflammatory process during late asthmatic reactions after house dust mite inhalation, bronchoalveolar lavage (BAL) was performed in 19 asthmatic patients and in 5 control subjects. In 16 of the patients and in all of the control subjects, BAL was performed 6 to 7 h after allergen inhalation. Six of the patients showed early and late asthmatic reactions (LAR), 5 showed early reactions, and 5 showed no reactions. Bronchoalveolar lavage was also performed shortly after the early reaction in 5 patients with documented combined early and late reactions. In the BAL fluid of the patients with LAR, a significant eosinophilia ($0.01 < p < 0.05$) was found compared with that in all other patient groups and with that in the control subjects. This bronchoalveolar eosinophilia was accompanied by elevated eosinophil cationic protein/albumin ratio in the BAL fluid ($0.01 < p < 0.05$). These observations suggest that eosinophils and their mediators might be involved in the development of LAR after allergeen inhalation.

2.1.2. Introduction

The clinical pattern of late asthmatic reactions (LAR) is marked by a slowly progressive bronchial obstruction starting 3 to 4 h after allergen inhalation and usually reaching its maximum after 7 to 8 h, sometimes followed by recurrent nocturnal attacks (1, 2). The LAR are not accompa-

nied by fever or leucocytosis. Twenty-four hours after allergen inhalation, however, an increase in circulating eosinophils may be found (1, 3). In contrast to early asthmatic reactions (EAR), the occurrence of LAR can almost always be prevented by pretreatment with corticosteroids, orally or inhaled. Whereas beta-agonists prevent EAR, the response of LAR to these drugs is partial and of limited duration (4). Sodium cromoglycate administered prior to allergen inhalation will, in a majority of the cases, prevent EAR as well as LAR (5). This was suggested to be caused by inhibiting mast cells from releasing mediators such as histamine and chemotactic factors (6). Among the latter are the high-molecular-weight neutrophil chemotactic factor (HMW-NCF), which is possibly derived from mast cells (7), and a low-molecular-weight eosinophil chemotactic factor, which was shown to be present in human mast cells and stimulates eosinophil chemotaxis after challenge with antigen of passively IgE-sensitized human lung fragments (8). The function of the eosinophil at the site of an immediate hypersensitivity reaction has been interpreted as a dampening down of the response that elicited its arrival (9). Recently, however, more attention has been given to the agressive properties of these cells (10).

Concerning the occurrence of an immune-complex-mediated inflammation in the lung during LAR, as studied by complement split products and hemolytic activity in peripheral blood, data are conflicting (11, 12). There is also controversy whether during LAR renewed mast cell degranulation takes place (11, 13, 14). In a recent publication, we showed that elevation of plasma histamine found during LAR was not correlated to lung function, suggesting that histamine was not derived from the lung compartment (11). The HMW-NCF in peripheral blood was shown to be elevated during LAR comparable with the elevations during EAR (14). However, because the source of HMW-NCF is not shown, this does not prove renewed degranulation of mast cells during LAR. Thus, studies in peripheral blood are difficult to interpret with respect to the mechanism of LAR. The aim of this study was to investigate whether during LAR, as during late phase skin reactions, cellular infiltration occurs (15) and to obtain information about the nature of this infiltration by performing broncho-alveolar lavage (BAL). Broncho-alveolar lavage and studies of the cell populations harvested by this procedure offer information about the cell infiltration in the interstitium (16). In skilled hands, BAL causes only minimal discomfort to the patient (17).

2.1.3. Methods

Selection and Characterization of the Patients

Nineteen patients with well-documented bronchial asthma gave their informed consent to participate in this study. The patients, 15 males and 4 females 15 to 39 years of age (mean age, 23.8), were selected on the basis of clinical history of wheezing after allergen exposure, positive skin tests, and raised specific IgE for house-dust mite. All patients showed an increased bronchial hyperreactivity to histamine and acetylcholine; none of them reported aspirin idiosyncrasy (Table 1). The patients were admitted on a nonacute basis to evaluate their complaints. None of them had experienced acute asthmatic attacks for at least 2 months, and there was no history of respiratory tract infections during this period. All were nonsmokers.

Control Subjects

Five healthy nonsmoking volunteers, 3 males and 2 females 23 to 33 years of age (mean age, 26.2), participated in this study as control subjects. All control subjects had negative skin tests to house-dust mite and a negative history of asthma, rhinitis, and eczema.

Inhalation Tests

Histamine and acetylcholine were inhaled during 30 s in stepwise increasing concentrations using an open delivery system (Wiesbadener Doppelspray, 8 L/min air flow, nebulizer output approximately 0.2 ml/min) as described previously (18). A concentration causing a fall of more than 15% in forced expiratory volume in one second (FEV_1) was considered as a threshold value.

Allergen inhalation was performed by inhaling at 15-min intervals, 4 stepwise-increasing concentrations (50, 250, 1,250, and 6,250 biological units (BU)/ml) (19) of house-dust mite extract during 1 min using a Wiesbadener Doppelspray.

Table 1. Clinical data of the asthmatic patients

Patient No.	Age (yr)	Sex	Specific IgE to HDM*	Nonspecific Hyperreactivity†		FEV_1 Before Challenge	Bronchial Response to HDM Challenge‡		Moment of Bronchial Lavage
				Histamine (mg/ml)	Acetylcholine (mg/ml)	(% pred)	Early	Late	(hours after challenge)
1	23	M	>20	16	8	101	−26	−30	
2	31	M	>20	8	16	89	−67	−20	
3	20	F	>20	16	16	99	−23	−47	6−7
4	19	M	>20	8	16	98	−39	−23	
5	34	M	>20	16	16	92	−38	−41	
6	21	F	10−20	16	8	96	−70	−31	
7	22	M	>20	2	4	61	−16	+9	
8	19	M	>20	2	16	78	−23	+11	
9	22	F	>20	32	16	114	−34	+2	6−7
10	39	M	10−20	16	16	84	−22	+7	
11	39	M	10−20	32	8	81	−16	+9	
12	18	M	>20	4	8	73	−2	+21	
13	16	M	>20	16	2	91	+3	+4	
14	24	M	>20	8	64	122	+4	−3	6−7
15	19	M	>20	4	16	70	−14	−14	
16	31	F	>20	2	8	68	+1	−6	
17	29	M	>20	2	1	86	−32	−	
18 (second lavage of Patient 3)						81	−34	−	
19 (second lavage of Patient 6)						102	−50	−	2−3
20	17	M	>20	8	8	96	−39	−	
21	15	M	>20	16	64	93	−21	−	

Definition of abbreviations: HDM = house-dust mite extract; FEV_1 = forced expiratory volume in one second.

* Percentage binding of radioactivity. Normal values less than 2% binding (Phadebas RAST kit).

† Threshold values expressed as concentration (*mg/ml*) causing a fall in FEV_1 of 15% or more.

‡ Maximal response in FEV_1 expressed in percentage of initial pulmonary function, corrected for control values obtained after inhalation of coca solution.

BAL Procedure

The BAL was performed either 2 to 3 h (ante-LAR) or 6 to 7 h (LAR, EAR, control) after allergen inhalation. Both the patients and the normal

subjects were pretreated with 10 mg oxyphenonium bromide (an anticholinergic drug) subcutaneously, 10 mg diazepam intramuscularly, and local anaesthesia with lidocaine 2% applied to the nose, pharynx, trachea, and vocal cords prior to insertion of a BF-B_3-R 5.9 mm Olympus fiberoptic bronchoscope (Olympus Corp. of America, New Hyde Park, NY) in the right middle lobe in a wedge position. Lavage was then performed using 10 20-ml aliquots of saline. The fluid was aspirated and collected in plastic tubes and transported in ice to the laboratory. This procedure was approved by the Internal Review Board.

Laboratory Techniques

In the laboratory, the contents of tube 1 and 2 were pooled (Pool I) separately from the other tubes (Pool II, tubes 3 to 5; Pool III, tubes 5 to 10). Cells and fluid were separated by centrifugation (10 min, 4 °C, 200 *g*). The fluid was stored at −80 °C, and the cells of pool I were resuspended in RPMI in a concentration of $10^6 - 1.5 \times 10^6$ cell/ml. Total cell numbers were counted in a Coulter counter (Coulter Electronics, Hialeah, FL). For cytologic examination, cytospin preparations were made from the cell suspensions in a Shandon cytospin-2 cytocentrifuge (Shandon Southern Instruments, Sewickley, PA) using 200-μl aliquots, spinning at 300 *g* for 5 min. After fixation the object glasses were stained with May-Grünwald and Giemsa (3 min May-Grünwald, 4 min Giemsa).

A cell differentiation was performed by counting 500 leucocytes. The albumin concentration in the BAL fluid was measured using a Behring laser-nephelometer. Eosinophil cationic protein (ECP) was measured in Pool I using a radioimmunoassay as described previously (20). The IgE determination was done using the commercially available Phadebas-RAST kit (Pharmacia Diagnostics, Piscataway, NJ). Serum samples from all control subjects showed less than 2% binding of radioactivity.

Study Design

Patients were admitted to hospital during a 2 week period. At admission all medication was stopped; on Day 4, control solution was inhaled, and forced expiratory volume in one second (FEV_1) values were followed during the day with hourly intervals. On Day 5, house-dust mite was inhaled until a decrease in FEV_1 of 15% or more was obtained. On Days 11, 12,

or 13, the same amount of allergen was inhaled as on Day 5 followed by BAL either 6 to 7 h (16 patients) or 2 to 3 h (5 patients) after allergen inhalation.

Statistical Analysis

Statistical analysis was performed using unpaired t test.

2.1.4. Results

After house-dust mite extract inhalation, 6 patients showed both EAR and LAR prior to BAL; 5 patients showed a single EAR, and 5 showed no reaction (NR). In 5 patients with known LAR, BAL was performed prior to the expected development of LAR (ante-LAR). These patients had recovered from the early reaction before BAL; after BAL no further spirometry was carried out (see Table 1). Both normal subjects and patients with or without LAR tolerated the lavage procedure well, with only moderate discomfort; no one required treatment with corticosteroids or antibiotics afterwards. In Table 2, the fluid recovery, the harvested amount of cells, and the albumin concentration in the fluid are given; no significant differences were found between patient groups and the control group. In mean differential leukocyte counts in patients with LAR, EAR, NR, ante-LAR, and in control subjects, the percentages of eosinophils were, respectively, ($\pm$ SEM) 30 $\pm$ 7.9, 7.2 $\pm$ 3.5, 3 $\pm$ 1.3, 4 $\pm$ 2.3, and 0.6 $\pm$ 0.2. The percentage of eosinophils harvested during LAR was significantly elevated ($0.01 < p < 0.05$) compared with that in all other patient groups and the control group. The percentages of neutrophils and lymphocytes were not significantly different in the patient groups from those in the control group (Fig. 1). In 2 patients (Patients 3 and 6), BAL was performed on 2 occasions, once before an anticipated late reaction when these patients showed 4% and 2% eosinophils, respectively, and once during the late reaction showing 19% and 8% eosinophils, respectively (see Table 1 and Fig. 2).

The mean ECP/albumin ratio ($\pm$ SEM) in patients with LAR, EAR, NR, ante-LAR, and in control subjects, as illustrated in Fig. 3, were respectively, 9.6 $\mu g/\mu g \times 10^{-4} \pm 2.4$, 3.4 $\mu g/\mu g \times 10^{-4} \pm 2.3$, 2.5 $\mu g/\mu g \times 10^{-4} \pm 0.7$, 1.7 $\mu g/\mu g \times 10^{-4} \pm 0.6$, and 1.2 $\mu g/\mu g \times 10^{-4} \pm 0.6$.

Table 2. Mean total fluid recovery, harvested numbers of cells, and albumin concentration (± SEM) of patients and control subjects*

Groups	Fluid Recovery (*ml*)	Cells ($ml \times 10^5$)	Albumin (*mg/L*)
LAR†	94 ± 18.8	2.7 ± 1.0	54 ± 22.3
EAR†	119 ± 18.8	2.2 ± 0.5	78.3 ± 24
NR†	98 ± 23	1.5 ± 0.5	51.3 ± 9.1
Control†	137 ± 23	1.4 ± 0.1	45.4 ± 13
Ante-LAR‡	125 ± 6.2	1.5 ± 0.3	78 ± 17.8

Definition of abbreviations: LAR = late asthmatic reactions; EAR = early asthmatic reactions; NR = no reactions.

* No significant differences were found between patient groups and the control group.

† Lavage 6 to 7 h after allergen inhalation.

‡ Lavage 3 h after allergen inhalation.

The ECP/albumin ratio was significantly elevated ($0.01 < p < 0.05$) in the LAR group compared with that in all other groups with the exception of the EAR group.

2.1.5. Discussion

In patients showing LAR after house-dust mite inhalation, we found significantly elevated eosinophil counts in the BAL fluid. This bronchoalveolar eosinophilia was not present in patients with only EAR or showing no reaction. Moreover, if in patients who previously showed LAR a BAL was performed immediately after their early reaction, no eosinophilia was present. Thus, in the early phase of LAR, eosinophils seem to migrate to the bronchial lumen.

The presence of eosinophils during LAR was also demonstrated by the finding of elevated levels of ECP in the lavage fluid obtained from patients during LAR. These elevated ECP levels probably reflect eosinophil degranulation during LAR and not merely the elevated eosinophil numbers in the lavage fluid.

In contrast to eosinophils, no elevation in neutrophil counts was found in the fluid. This observation probably reflects the absence of increased numbers of these cells in the interstitium (16). This is in contrast to the mixed cellular infiltration shown during IgE-dependent late phase skin reactions (15). The initial observations on the late phase skin reactions of

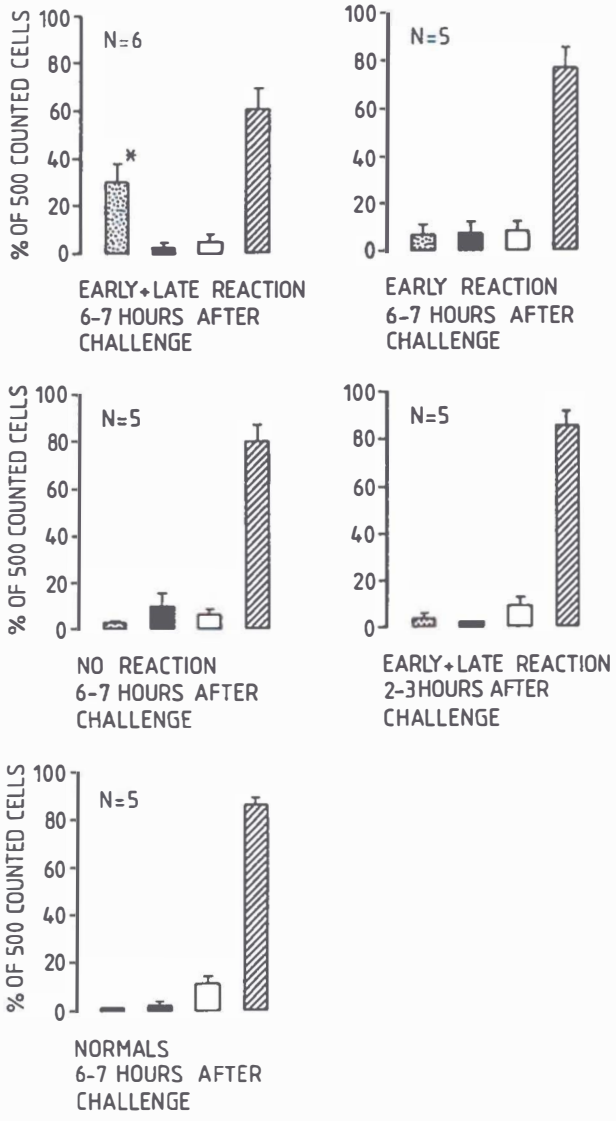

Fig. 1. Mean (± SEM) % of eosinophils (*dotted bars*), neutrophils (*solid bars*), lymphocytes (*open bars*), and alveolar macrophages (*hatched bars*) in broncho-alveolar lavage fluids (* = significant elevation in % eosinophils observed during the late allergic reactions compared with those in the control group and the other patient groups ($0.01 < p < 0.05$)).

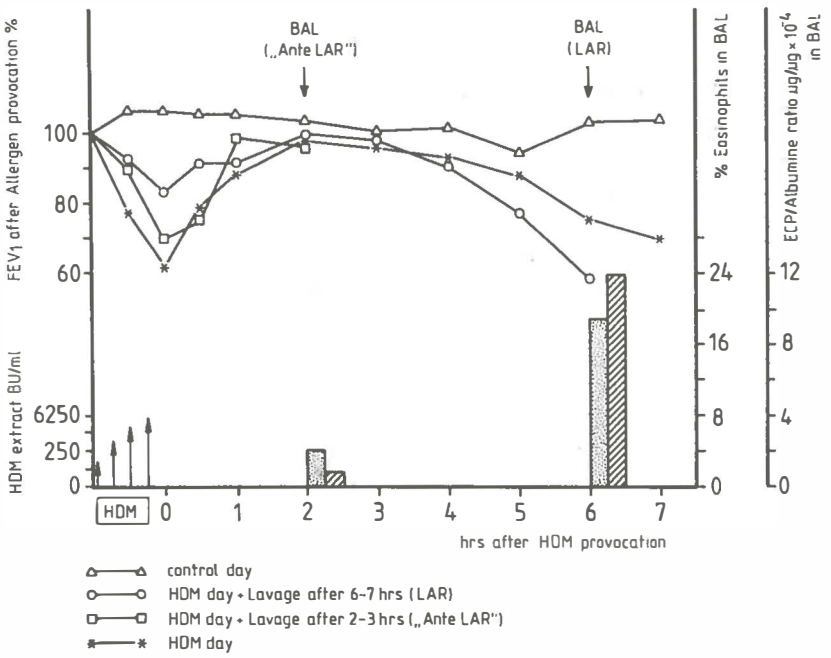

Fig. 2. Forced expiratory volume in one second (FEV_1) after inhalation of control solution or house-dust mite extract (HDM), and FEV_1 after provocation and subsequent broncho-alveolar lavage (BAL) after 2 to 3 h (ante-LAR) or after 6 to 7 h (LAR). The % eosinophils (*dotted bars*) and the ECP albumin ratio (*hatched bars*) in BAL are given (Patient 3).

Dolovich and co-workers (21), however, also showed a preponderance of eosinophils infiltrating.

There is increasing evidence that eosinophils are involved in asthma. In a general population, peripheral eosinophil numbers were correlated to bronchial obstruction in patients with positive skin tests (22). In steroid-dependent asthmatic patients, elevations in eosinophil counts were shown to correlate inversely with FEV_1 and airway conductance (23) and data from the literature suggest that the eosinophil is the main infiltrating cell in asthma (24).

Booy-Noord and co-workers (1) found that in patients with LAR as opposed to those with EAR, an increase in peripheral blood eosinophil levels could be demonstrated 24 h after allergen inhalation.

Frigas and associates (25) showed that in the sputum of patients with

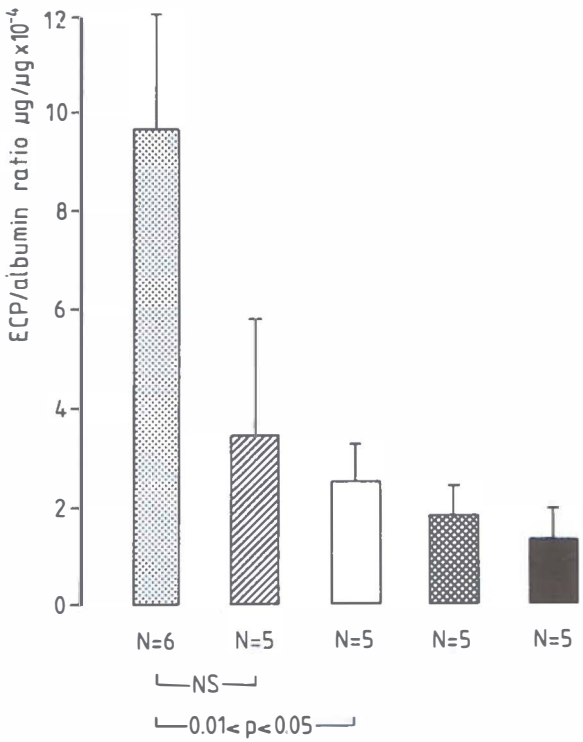

Fig. 3. The ECP/albumin ratio for patients with early and late reactions (*dotted bar*), early reactions (*hatched bar*), no reactions (*open bar*) sampled 6 to 7 h after allergen challenge, and that for patients with early and late reactions sampled 2 to 3 h after allergen challenge (*cross-hatched bar*) and control subjects sampled 6 to 7 h after allergen challenge (*solid bar*).

exacerbations of their asthma, the eosinophil-derived major basic protein was strongly elevated. The major basic protein as well as ECP are known to cause tissue injury and inflammation (10, 26 – 28). Recently, eosinophils also have been shown to produce the spasmogenic leukotriene LTC_4 after stimulation with Ca-ionophore (29, 30). Thus, infiltration and activation of eosinophils might lead to bronchospasm and inflammation.

It is tempting to suggest that the eosinophils are attracted by chemotactic substances released during mast cell degranulation in EAR. Such chemotactic substances include the low-molecular-weight eosinophil chemotactic factor (8), histamine (31), and leukotrienes (32). Because in some patients EAR is not followed by eosinophil infiltration, additional factors influencing eosinophil migration and activation must be present.

We conclude that the data obtained from local sampling in the bronchi during LAR suggest that infiltration of eosinophils rather than of neutrophils accompanies the early stages of LAR.

Acknowledgements

Supported by Grant No. 32.79.27 from the Netherlands Astma Fonds and by a grant from the Swedish Medical Research Council.

We are indebted to the department of ENT (Prof. Dr. P. E. Hoeksema, Dr. E. Edens) and to the Department of Haematology (Prof. Dr. H. O. Nieweg, Prof. Dr. M. R. Halie) for providing technical assistance. The skilful technical assistance of Mrs. B. Gritter, Mrs. M. de Bruin, and Mrs. K. Lindblad is gratefully acknowledged. We thank Mrs. J. J. Kruitbosch for preparing the manuscript.

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CHAPTER 2.2

Broncho-alveolar Lavage and the Late Asthmatic Reaction

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2.2.1. Introduction

In order to study the mechanisms of the late asthmatic reaction (LAR), several approaches are possible. Mediators, chemoattractants and complement split products can be studied in the circulation and correlated to variations in lung function. Concomitant changes in lung function and in the levels of histamine (1), for example, during the early asthmatic reaction (EAR) and LAR suggest this mediator to be derived from the lungs and can be interpreted as evidence for mast cell degranulation during the LAR. Changes in plasma histamine, however, might also be caused by increased numbers and/or vulnerability of blood basophils. These artefacts probably can be avoided by measuring histamine degradation products in, for example, hourly voided urine portions. In contrast to the EAR, during the LAR no changes in urinary N^{τ}-methylhistamine were found (2). Thus, data derived from peripheral samples during the LAR seem to be contradictory.

A different approach is made by studying the lungs directly during the LAR. This can be done by lung function techniques such as density-dependent volume flow curves, offering information on the site of the reaction but not on its mechanism (3). Studies in broncho-alveolar lavage (BAL) fluid obtained before and during the LAR may, however, provide such information.

2.2.2. The late asthmatic reaction (LAR)

The late allergic reaction in the bronchi is manifested by a slowly progressive bronchial obstructive reaction starting 3–4 h after allergen inhalation, and usually reaches its maximum after 7–8 h, sometimes followed by recurrent nocturnal attacks (2, 4, 5).

In contrast to the EAR, the occurrence of the LAR can almost always be prevented by oral or inhaled corticosteroids (6). Whereas beta-agonists prevent the EAR, the response of the LAR to these drugs is partial and of limited duration (7). Sodium cromoglycate administered prior to allergen inhalation will, in a majority of the cases, prevent the EAR as well as the LAR (6). This was suggested to be caused by inhibiting mast cells to release mediators such as histamine, leukotrienes, and chemotactic factors. Among the latter are a high-molecular-weight neutrophil chemotactic factor (HMW-NCF) and the low-molecular-weight eosinophil chemotactic factor (ECF-A), which is present in human mast cells and stimulates eosinophil chemotaxis after challenge with antigens of passively IgE-sensitized human lung fragments (8). Due to the release of these chemotactic factors during mast cell degranulation, the occurrence of the LAR is suggested to be associated with infiltration of mainly granulocytes (9). Originally the LAR was considered to be an IgG-immune-complex-mediated inflammation in the lungs comparable to the situation in allergic bronchopulmonary aspergillosis (ABPA) (10). However, since the IgE-dependent nature of the late skin reaction was clearly established (11), and since no differences in precipitating antibodies directed to common inhalant antigens were found between normal and asthmatic patients, and no other symptoms of type III reactions such as fever and leucocytosis were found, the immune complex theory has been largely abandoned.

The cellular infiltrate occurring during late skin reactions is limited and cannot account *per se* for the observed swelling (12). Assuming a similar mechanism for the late reaction in the skin and in the lung during the LAR (1), mediator release of infiltrating or of already present cells around the bronchi is likely to occur. Although some studies found evidence for mast cell degranulation during the LAR (1), we were unable to detect histamine production in hourly shed urine portions analysed for N^{τ}-methylhistamine by isotope dilution mass fragmentography during the late reaction, as opposed to the elevations in this unique histamine metabolite found during the early reaction (2). Some histamine produc-

tion may occur during the LAR (2, 13), but the quite distinct characteristics of the EAR and the LAR seem to preclude a major role for a common mediator. Alternative mediators involved in the occurrence of the LAR might be leukotrienes, prostaglandins or platelet activating factor (PAF).

In order to learn more about the nature of the LAR, histological studies of the changes in and around the bronchi seem almost inevitable.

Although the different techniques of lung biopsy have improved in recent years, problems remain regarding safety and tolerance. Lung biopsies are, therefore, not acceptable in the management of uncomplicated asthma or in research protocols concerning these patients. Histological examination of the changes in lung tissue of asthmatic patients, therefore, has been mainly limited to post-mortem material of patients who died in status asthmaticus (14), or to lung tissue adjacent to operated pulmonary tumours derived from an elderly group of patients. Although examination of these tissues may provide useful information about the changes in end-stage pulmonary disease, it is obvious that it is not representative for day-to-day asthma in the young allergic patient.

2.2.3. Broncho-alveolar lavage (BAL)

An alternative to histological examination of lung biopsies is the cytological study of fluid obtained by broncho-alveolar lavage (BAL) (15). The technique of BAL was greatly facilitated after the introduction of the flexible bronchoscope. BAL causes only moderate discomfort and no hazard to the patient, provided that an adequate patient selection and premedication have been carried out (16).

BAL originally was carried out for therapeutic rather than for diagnostic purposes (17). In 1961 Myrwik (18) described how, with the help of this technique, highly purified rabbit alveolar macrophages could be obtained. The first diagnostic studies performed in humans concerned differences between smokers and non-smokers (19). Several groups of researchers have since then intensively participated in the development of BAL, notably the group from the NIH, the Brompton Hospital group and the group in Lille.

The type of diseases which were investigated concerned primarily sarcoidosis, idiopathic pulmonary fibrosis and allergic alveolitis. In the first few years of BAL most studies were focused on the correlation between the findings in lung biopsies and the cytology of BAL fluid; usually a good

correlation was shown (15). Subsequently, more fundamental studies appeared concerning quantification of subsets of T lymphocytes, concentrations of immunoglobulins, etc., in the fluid. Additionally, BAL proved to be of use in monitoring disease activity and, in certain cases, could facilitate the diagnosis of lung diseases such as pulmonary haemosiderosis (20) and alveolar proteinosis (21).

The usefulness of BAL in the clinical situation is determined by the safety of such a procedure and the yielded information. Problems that might arise during BAL are largely due to the bronchoscopy and are the result of side-effects from local anaesthesia, faulty technique of bronchoscopy, hypoxaemia due to ventilation perfusion inequalities during the procedure, or bronchospasm. The latter point, of course, deserves special attention when bronchoscopy and subsequent lavage are performed in asthmatic patients. Some authors have described a transient febrile reaction that could be the result of the lavage procedure itself.

When adequate precautions are taken concerning patient selection and careful local anaesthesia (anticholinergics, bronchodilatation and 10 mg valium i.m.), an experienced bronchoscopist should have no trouble in performing BAL in patients with a mild degree of bronchus obstruction.

If the BAL fluid is used only for performing differential cell counts, the information obtained may be rather limited. However, it is also possible to perform further studies on the cells using immunofluorescence or to analyse the BAL fluid for such soluble constituents as immunoglobulins, immunocomplexes, mediators and complement factors, which will probably enhance the clinical usefulness of BAL.

The technique of BAL in asthmatic patients

Obviously the safety and the feasibility of BAL in patients with obstructive disorders is determined by the degree of bronchial obstruction and by the pre-lavage bronchodilation that can be obtained (22). However, perhaps even more important is that prior to bronchoscopy the patient is well informed and motivated to co-operate. After the local anaesthetic drug is applied to the nose, oropharynx, trachea and vocal cords, the bronchoscope is inserted, preferably through the nose, allowing the patient to become used to the sensation of its presence. When coughing occurs, lidocaine is applied. After the bronchoscope is placed in a wedge position in a subsegmental middle lobe a total of 200 ml (10 × 20 ml) sterile saline

is infused and aspirated immediately through a siphon under continuous suction of 100 mmHg negative pressure into a cooled polyethylene tube.

BAL in asthmatic patients has been considered cumbersome, since asthmatic patients might react with bronchospasm on the local anaesthetic drug (23). Although we did not measure lung function before and after lidocaine, we never saw any clinical manifest reaction following local application of lidocaine (final concentration in the lavage fluid approximately 50 mg/l). BAL in asthmatics has also been discouraged since the yield of fluid might be insufficient for laboratory studies. Indeed a slightly reduced recovery in patients with manifest bronchus obstruction was found. However, the recovery of fluid and cells was sufficient for cytological and biochemical analysis of the fluid obtained (24).

2.2.4. Work-up of the BAL fluid

Pooling of the samples

The BAL fluid obtained can be pooled in several ways. We chose a method which allowed us to make a distinction between samples 1 and 2 (Pool I), samples 3–5 (Pool II), and samples 6–10 (Pool III). Pool I is supposed to be derived mainly from the bronchi, while Pools II and III might represent a bronchoalveolar specimen (25).

In patients lavaged during late asthmatic reactions evidence for eosinophil infiltration was mainly found in Pool I (the bronchial sample) as compared to the bronchoalveolar samples (Table 1). In a study published by Diaz (26) bronchial eosinophilia was present in asthmatic patients and disappeared after treatment with disodium cromoglycate. No differences were found in the broncho-alveolar eosinophil content. Thus, if Pools I,

Table 1. Percentages of eosinophils, neutrophils, lymphocytes and alveolar macrophages in four patients with LAR.

	Pool I	Pool II	Pool III
Eosinophils	22 ± 9	13 ± 9	9 ± 6
Neutrophils	4 ± 4	4 ± 3	5 ± 5
Lymphocytes	6 ± 7	10 ± 10	10 ± 10
Alveolar macrophages	66 ± 15	73 ± 16	74 ± 11

II and III are not separately analysed, variations in differential cell counts may be overlooked.

Once the BAL fluid has been aspirated from the lungs, it should be collected in cooled polyethylene tubes and placed on ice. Immediately after collection, EDTA can be added in a final concentration of 2 mmol/l which reduces the viscosity of mucus, prevents degranulation of cells and stabilizes complement factors. However, when BAL is to be used in functional tests and bioassays, prior addition of EDTA might have disadvantages.

Cells and supernatants are separated by centrifugation at 4 °C, 10 min 200 g. Then the fluid is usually stored at −80 °C and the cells are resuspended in RPMI medium (with 20% autologous plasma) at concentrations of 10^6 – 1.5 × 10^6/ml.

Quantification of the cellular components

In order to obtain quantitative information about the cells harvested by BAL, several techniques can be used.

1 Total cell counts can be performed in a Coulter counter.

2 For cytological examination cytospin preparations can be made of cell suspensions in a cytocentrifuge. After fixation the cell preparations are stained with, for example, May-Grünwald Giemsa (3 min May-Grünwald, 4 min Giemsa). Differential cell counts are made counting at least 500 cells.

3 Additionally, viability tests may be carried out using Trypan Blue exclusion. Normally more than 80% cells are vital.

Measuring soluble components in lavage fluid

When measuring proteins of BAL, different methods are used employing either concentrated or non-concentrated lavage fluid. Quantification of the proteins by making use of the precipitating properties in gels (radioimmunodiffusion test, immunoelectrophoresis) is less sensitive when compared to methods using the turbidity characteristics of protein antibody immune complexes in a liquid phase (laser-nephelometer), the former making concentration of lavage fluid inevitable. Concentration methods used are lyophilization and resolubilization in a smaller volume or concentration through permeable membranes with a distinct molecular weight exclusion (diafiltration). Both methods have the disadvantage that proteins

present in these dilute solutions can be lost either by denaturation, or by adherence to membrane surfaces. In Table 2 the concentrations of albumin, IgG, IgA, IgM and C_3 are summarized for untreated lavage fluid and lavage fluid after concentration by lyophilization or diafiltration followed by reconstitution to the original volume. The results show that both methods of concentration will lead to losses of protein in a varying degree. After lyophilization loss of protein was found for albumin (3 out of 4), IgG (4 out of 4), IgA (4 out of 4) and IgM (1 out of 2). After diafiltration the loss of protein is even higher for albumin and IgG, and is also less, but to a smaller extent, for IgA and IgM. By contrast, after lyophilization the loss of C_3 is higher. These data may explain differences in concentrations of protein/albumin ratios as reported in the literature, and stress the need for measuring proteins without prior concentration as can be performed using the laser-nephelometer.

Table 2. Concentration of proteins in four samples of BAL fluids in the untreated solutions and after concentration by both lyophilization and diafiltration.

Component	Concentration method	Patient no.			
		1	2	3	4
		Concentration (mg/L)			
Albumin	Untreated	192	136	80	68
	L + R	150	212	n.m.	58
	D + R	106	96	n.m.	48
IgG	Untreated	24.6	41.0	31.5	132.0
	L + R	12.1	19.2	6.5	109.0
	D + R	17.5	19.2	n.m.	8.5
IgA	Untreated	17.3	7.0	15.5	6.0
	L + R	12.9	2.7	8.2	2.3
	D + R	7.0	3.4	2.4	4.5
IgM	Untreated	n.m.	n.m.	4.2	6.0
	L + R	n.m.	n.m.	5.4	2.3
	D + R	n.m.	n.m.	0.7	4.5
C_3	Untreated	–	–	2.44	–
	L + R	–	–	0.59	–
	D + R	–	–	1.09	–

n.m. = not measurable
L + R = lyophilization and reconstitution to starting volume
D + R = diafiltration and reconstitution
– = not done

In lung lavage fluid histamine and the unique histamine metabolite N^{τ}-methylhistamine can be directly measured with isotope dilution mass fragmentography (2).

An important and incompletely solved problem is the correction for sampling errors. One possibility is to express concentrations of the several proteins as a potassium ratio in the lavage fluid. Using potassium as a standard has the disadvantage that it may be strongly influenced by cell lysis or the presence of cell debris and does not show a constant ratio to proteins when lavage is carried out with different volumes (27). Another way of assessing dilution is to express the concentration of a component as an albumin ratio. Although influenced by inflammatory reactions in the lung, albumin, because of its reliable detection, seems to be the most suitable internal standard in asthmatic patients. We found no differences in albumin concentration of the lavage fluid of patients lavaged during late reactions and other asthmatic patients, or controls, as opposed to the increased albumin concentration in the BAL fluid of a patient lavaged during an exarcebation of APBA (28).

2.2.5. Local alveolar eosinophilia during allergen-induced late asthmatic reactions

In order to obtain information about the nature of the local 'inflammatory' process during late asthmatic reactions following house-dust mite inhalation, BAL was performed in 19 asthmatic patients and 5 control subjects (24) (6–7 h after allergen inhalation). Six patients showed both early and late reactions (LAR) prior to BAL; five patients showed a single early reaction (EAR), and five showed no reaction (NR). In five patients with known LAR, BAL was performed prior to the expected development of LAR (ante-LAR; 2–3 h after allergen inhalation). These patients had recovered from the early reaction before the moment of BAL. Both normals and patients with or without LAR tolerated the lavage procedure well and described only moderate discomfort. No one required treatment with corticosteroids or antibiotics afterwards. No significant differences were found in the fluid recovery, the harvested amount of cells, or the albumin concentrations in BAL fluid. In mean differential leucocyte counts in patients with LAR, EAR, NR and ante-LAR and control subjects, the percentage eosinophils were, respectively, 30 ± 7.8, 7.2 ± 3.5, 3 ± 1.4, 4 ± 2.3 and 0.6 ± 2 (mean ± s.e.). The percentage of eosinophils harvested during the LAR was significantly elevated ($0.01 < p < 0.05$) as compared

to all other patient groups and control subjects.

The percentages of neutrophils and lymphocytes were not significantly different in the patient groups compared to the control subjects. Figure 1 shows the correlation between the degree of bronchial obstruction during the late asthmatic reaction and the percentage eosinophils ($r = -0.51$, $p < 0.05$) and neutrophils ($r = 0.08$, $p < 0.05$) found in the lavage fluid. When the percentage of eosinophils and neutrophils are plotted against the severity of the EAR, no significant correlations are found. In the lavage fluid the level of eosinophil cationic protein (ECP), lactoferrin and lysozyme were determined using a radioimmunoassay (29, 30, 31). A significant correlation was found between the ECP level and the degree of bronchial obstruction during the LAR (Fig. 2). However, no correlation was found with either lactoferrin, which is neutrophil derived (32), or lysozyme which may be produced by neutrophils or alveolar macrophages in addition to some production by mucus glands (Fig. 3).

The absence of neutrophils or neutrophil-dependent markers is striking

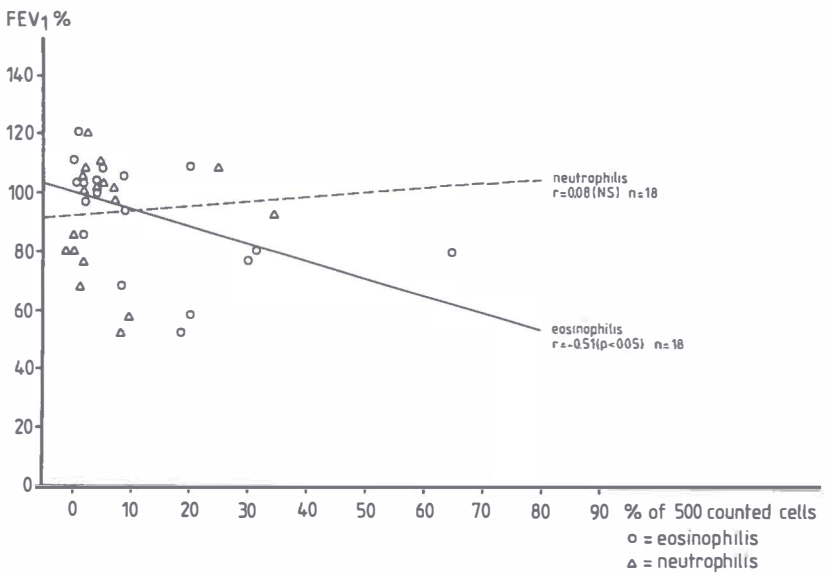

Fig. 1. Percentage eosinophils (●) and neutrophils (Δ) in BAL fluid plotted against the fall in FEV_1 during the LAR in patients lavaged 6–7 h after allergen inhalation. A significant correlation was found for the percentage of eosinophils ($r = -0.5$, $p < 0.05$).

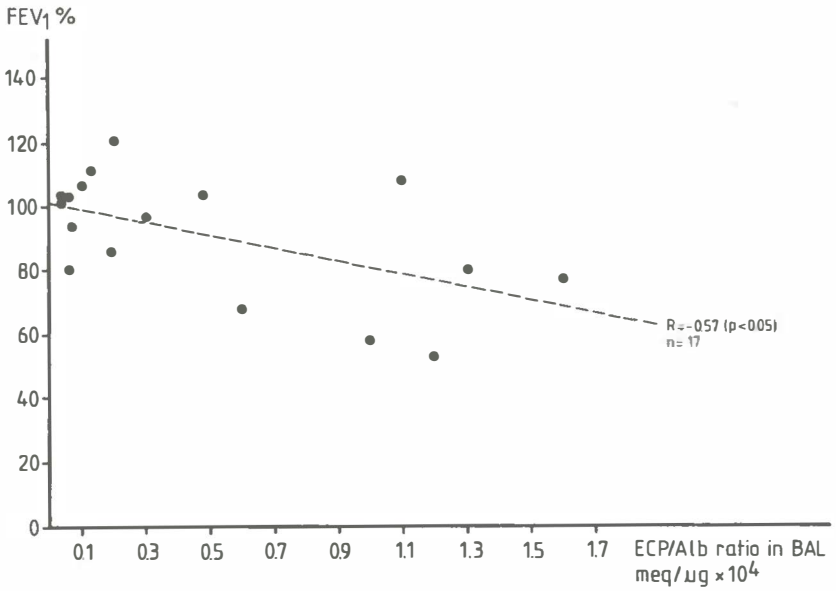

Fig. 2. ECP/albumin ratio in BAL fluid plotted against the fall in FEV_1 during the LAR in patients lavaged 6–7 h after allergen inhalation. A significant correlation was found ($r = -0.57$, $p < 0.05$).

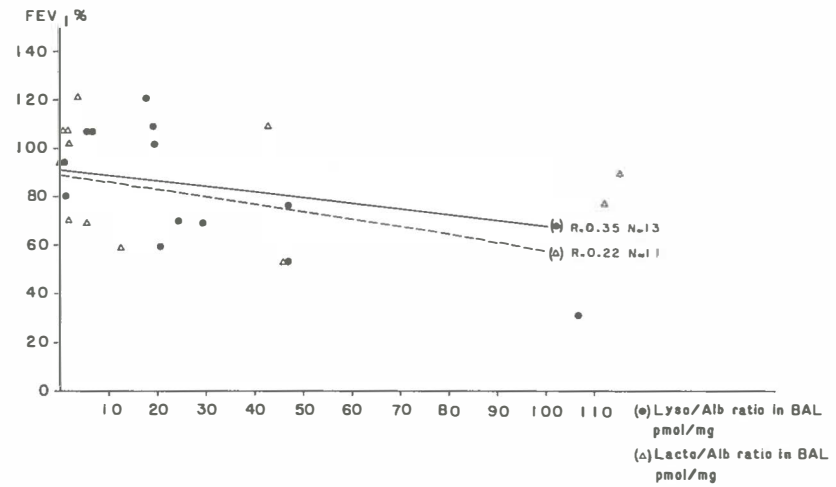

Fig. 3. Lysozyme/albumin ratio (●) and lactoferrin/albumin ratio (△) in BAL fluid plotted against the fall in FEV_1 during the LAR, in patients lavaged 6–7 h after allergen inhalation. No significant correlations were found.

since in skin late reactions a mixed cellular infiltrate is found. Possibly the type of antigen used here is of importance since BAL fluid of a patient lavaged during an exacerbation of ABPA showed a predominant infiltration of neutrophils (28). Although the LAR has been described as an inflammatory reaction of the airways because of the absence of both increased albumin levels or neutrophil cells, this 'inflammatory' reaction is clearly distinct from the reaction to micro-organisms, for example.

The eosinophil and bronchial asthma

There is increasing evidence that eosinophils are pathogenetically involved in asthma. In a general population, peripheral eosinophil numbers were correlated to bronchial obstruction in patients with positive skin tests (33). In steroid-dependent asthmatic patients elevations of eosinophil counts were shown to correlate inversely with FEV_1 and airway conductance (31). Data from the literature suggest that the eosinophil is the main infiltrating cell in asthma (14). Booy-Noord found that in patients with a LAR as opposed to those with an EAR, 24 h after allergen inhalation an increase in peripheral blood eosinophil levels could be demonstrated (4). Frigas showed that in the sputum of patients with asthma exacerbations, eosinophil-derived major basic protein (MBP) was strongly elevated (35). MBP as well as ECP are known to cause tissue injury and inflammation (36, 37, 38). Recently eosinophils have been shown to produce the spasmogenic leukotriene LTC_4 after stimulation with calcium ionophore (39, 40). Thus, infiltration and activation of eosinophils might lead to bronchospasm and 'inflammation'. If the eosinophils found in the lungs during LAR are attracted by chemotactic substances released during mast cell degranulation in the EAR, no explanation is found for the phenomenon that some patients with a strong EAR did not show eosinophil infiltration during the LAR, and additional factors influencing migration or activation must be present.

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CHAPTER 2.3

The eosinophilic granulocyte: an active participant in the late phase asthmatic reaction?

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Keywords: asthma, late phase asthmatic reaction, eosinophils, bronchoalveolar lavage, leukotrienes.

Abbreviations used:

BAL, broncho-alveolar-lavage; ECP, eosinophil-cationic-protein; MBP, major-basic-protein; HMW-NCF, high-molecular-weight neutrophil chemotactic factor; ECF, eosinophil chemotactic factor; PAF, platelet-activating-factor; f-MLP, N-formyl-methinyl-leucyl-phenylalanine; LTB_4, LTC_4, LTD_4 and LTE_4, leukotrienes B_4, C_4, D_4 and E_4; SRS-A, slow-reacting-substance of anaphylaxis; 15-HETE, 15-monohydroxyeicosatetraenoic acid; FEV_1, forced expiratory volume in 1 sec; LAR, (early and) late phase asthmatic reaction; EAR, early asthmatic reaction; NR, no asthmatic reaction; HPLC, high-performance-liquid chromatography; RIA, radioimmunoassay.

2.3.1. Abstract

The role of the eosinophilic granulocyte in immediate hypersensitivity reactions is generally believed to be a beneficial one, since this cell may phagocytose mast cell granules and inactivate certain mast cell mediators. However, it has become clear that the eosinophilic granulocyte also has potent secretory capacities, and by this property may contribute in a detrimentel way to the allergic process.

In studying the late phase allergen induced bronchoconstriction by means of broncho-alveolar-lavage (BAL) an evident infiltration of eosinophilic granulocytes in the bron-

chioli in the beginning of the late phase asthmatic reaction was noticed. Since also eosinophil cationic protein (ECP) has been reported to be elevated in the lavage fluid, an active secretory role of the eosinophil in the late phase asthmatic reaction seemed likely. Although the release of ECP and other granular proteins may contribute to epithelial damage and inflammation and thereby to an increase in bronchila hyperreactivity, they do not explain the late phase bronchoconstrictive reaction. Since leukotrienes were thought possible candidates to cause this reaction, it was decided to isolate eosinophils from human peripheral blood and to study their leukotriene synthesis pattern. To our surprise purified human eosinophils almost exclusively synthesize the strongly bronchoconstrictive leukotriene LTC_4 in considerable quantities upon in vitro stimulation with either the calcium ionophore A23187 or opsonized zymosan. These findings suggest that the eosinophil may play an active role in causing the late phase asthmatic reaction.

2.3.2. Introduction

The clinical pattern of the late phase asthmatic reaction (LAR) is marked by a slowly progressive bronchila obstruction starting 3–4 hrs after allergen inhalation and usually reaching its maximum after 7–8 hrs, sometimes followed by recurrent nocturnal attacks (2,24). The LAR is not accompanied by fever or leukocytosis. Twenty-four hrs after allergen inhalation, however, an increase in circulating eosinophils may be found (2, 7). The latter finding stimulated us to further investigate a possible active role of the eosinophilic granulocyte in the LAR.

2.3.3. The late phase reaction

Late phase reactions after allergen challenge have been reported in the skin (8, 31), in the nose (11, 27) and in the lung (1, 2, 9, 10, 23). The late phase reaction normally follows an immediate type reaction. Since during the immediate-type reaction mediator-release from mact cells takes place, the late-phase reaction is considered a mast cell-dependent reaction (14, 19). Whether repeated mast cell degranulation occurs during the LAR is not yet clear (18, 21). Mast cells have shown the capacity to release mediators which may be responsible for the cellular infiltrates seen after the immediate-type reaction. High molecular weight neutrophil chemotactic factor (HMW-NCF), which is possibly mast cell derived (26), appears in the circulation (25) and is thought to attract mainly neutrophils (9, 10, 23).

Eosinophil chemotactic factor (ECF) was shown to be present in human mast cells and is thought to attract eosinophils (17). Furthermore certain arachidonic acid metabolites like LTB_4 may be formed by the mast cell (25). This compound is thought to possess a strong chemotactic capacity for both human neutrophils (12) and eosinophils (22).

Although most reports on late phase reactions agree in that there is a cellular infiltration before the late phase reaction takes place, the infiltrating cell-types sometimes differ.

In the *skin* the initial observations on the late phase reaction showed a preponderance of infiltrating eosinophils (8). On the other hand Solley et al. (31) showed a mixed cellular infiltrate (predominantly lymphocytic but also containing eosinophils, neutrophils and basophils) during IgE-dependent late phase skin reactions.

In the *nose* late phase reactions have been reported (11, 27), but they lack a careful description of the cellular infiltrate. Pelikan (28) has reported the appearance of eosinophils in nasal smears 30–60 minutes after an immediate reaction. However, these findings were not related to the occurrence of late phase reactions.

In the *lung* little is known about the cells infiltrating in the lung tissue before and during the late phase asthmatic reaction. The presence of increased neutrophil chemotactic activity (NCA) in serum during the late phase reaction, however, strongly suggests the infiltration of neutrophils in the long tissue (9, 10, 23). The changes in NCA in serum in relation to the changes in FEV_1 during an immediate and late phase reaction are shown in Fig. 1.

We have approached the problem of the cellular infiltration during the late phase asthmatic reaction in a different way; broncho-alveolar lavage (BAL) studies were undertaken to determine which cell types would penetrate in the lung tissue (20). In 16 allergic asthma patients and 5 control subjects BAL was performed 6–7 hrs after house dust mite extract inhalation (Fig. 1).

Prior to the BAL investigations allergen inhalation tests combined with spirometric measurements (FEV_1) were performed to classify the reaction pattern of these patients. Six of these patients showed an early and late reaction (LAR), 5 patients showed an early reaction (EAR) and 5 patients showed no reaction (NR). BAL was also performed shortly after the early reaction (3 hrs) in 5 patients with documented combined early and late reactions (ante LAR). In the BAL fluid of LAR patients a significant

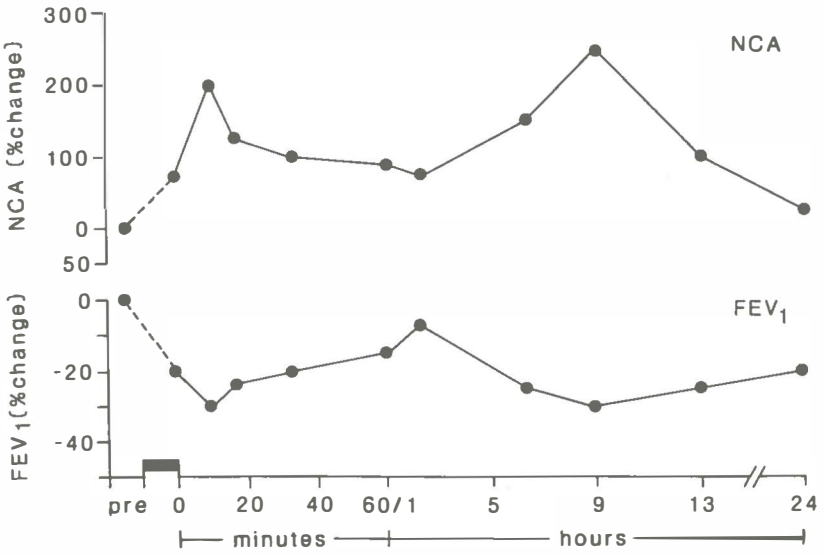

Fig. 1. Mean percentage change of serum NCA in relation to FEV_1 in 10 patients with early and late phase reactions after allergen challenge. There is a statistically significant rise in NCA activity and a statistically significant decrease in FEV_1. For further details see Durham *et al.* J. Allergy Clin. Immunol., 1984; 74: 40–60 (reproduced with permission).

eosinophilia was found ($p \leq 0.05$) compared to all other patient groups and the control subjects.

Figure 2 shows the percentage of eosinophils found in the BAL fluid of the different groups investigated.

The percentages of neutrophils, lymphocytes and alveolar macrophages were not significantly different in the patient groups compared with the control group. Also the harvested number of cells and the albumin concentration in the BAL showed no statistically significant differences between the different groups.

These results suggest that eosinophils infiltrate around the bronchioli in the beginning of the late phase asthmatic reaction.

2.3.4. Active participation of eosinophils in the late phase reaction

Besides the number of eosinophils in the BAL fluid of the different groups investigated, also the amount of eosinophil cationic protein (ECP)

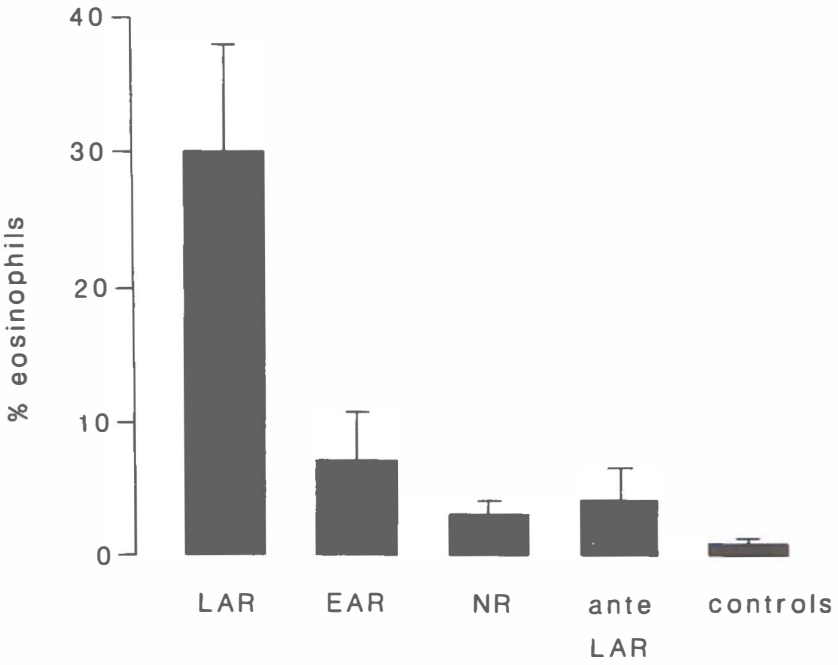

Fig. 2. Mean (± SEM) % of eosinophils in the broncho-alveolar lavage fluids of the different patient groups (LAR = early and late phase asthmatic reaction (n = 6); EAR = early phase asthmatic reaction (n = 5); NR = no asthmatic reaction (n = 5); ante-LAR = late phase asthmatic reaction in which BAL was performed three hours after allergen challenge instead of 6 - 7 hrs after allergen challenge (n = 5); controls (n = 5).

released in the BAL fluid was measured in the forementioned study (20). It could be shown that ECP was significantly elevated in the LAR group compared to the other groups. This may indicate that not only the number of eosinophils is increased (see Fig. 2), but that these cells also actively degranulate. ECP as well as another basic granular compound, major basic protein (MBP), have been shown to cause both tissue injury and inflammation (13, 32). Therefore it is tempting to suggest that the release of this kind of granular compounds may contribute to chronic inflammation and thereby to an increase in bronchial hyperreactivity. Thus these compounds and also oxygen radicals may contribute to the existence of chronic inflammation and bronchial hyperreactivity, but do not directly contribute to the bronchoconstriction during the late phase asthmatic reaction.

The reaction pattern, the duration of the late phase asthmatic reaction and the lack of histamine metabolites in urine voided during the late phase asthmatic reaction (21) suggest that other mediators than histamine are involved (16). This view is further supported by the action of certain drugs. If patients, to be challenged with allergen, are pretreated with corticosteroids this prevents the late phase but not the early phase asthmatic reaction (1). On the other hand pretreatment with beta-agonists may prevent the early phase but not the late phase asthmatic reaction (3).

This and other evidence have suggested that these mediators may be arachidonic acid metabolites, i.e. the sulfido-peptide leukotrienes LTC_4, LTD_4, and LTE_4 (formerly collectively called SRS-A: slow reacting substance of anaphylaxis).

2.3.5. Leukotriene C_4 synthesis by human eosinophils

Granulocytes, which may contribute to the late phase asthmatic reaction, are capable of synthesizing the strongly chemotactic compound LTB_4 as well as the strongly bronchoconstrictive compound LTC_4 (4, 15). Two findings stimulated us to further investigate whether eosinophils would be responsible for the LTC_4 synthesis by human granulocytes:

1. It was shown that the amount of LTC_4 synthesized by human granulocytes was strongly dependent on the percentage of eosinophils present in the granulocyte preparation (5);
2. The previously mentioned cellular infiltration of predominantly eosinophils in the beginning of the late phase asthmatic reaction (20) which suggests an active participation of this cell type in this reaction.

To see whether eosinophils are responsible for the LTC_4 synthesis by human granulocytes, neutrophils and eosinophils were isolated to a high degree of purity from a normal granulocyte preparation (33). The almost pure cell preparations were then stimulated *in vitro* by means of the calcium ionophore A23187 and leukotriene synthesis was measured by means of both reversed-phase HPLC (34) and RIA (for LTC_4 and LTB_4) (6). The results are shown in Fig. 3.

Surprisingly, Figure 3 shows that eosinophils when stimulated with the calcium ionophore A23187 almost exclusively synthesize the strongly bronchoconstrictive compound LTC_4 while neutrophils almost exclusively synthesize the strongly chemotactic compound LTB_4, its isomers and its metabolite 20-OH-LTB_4.

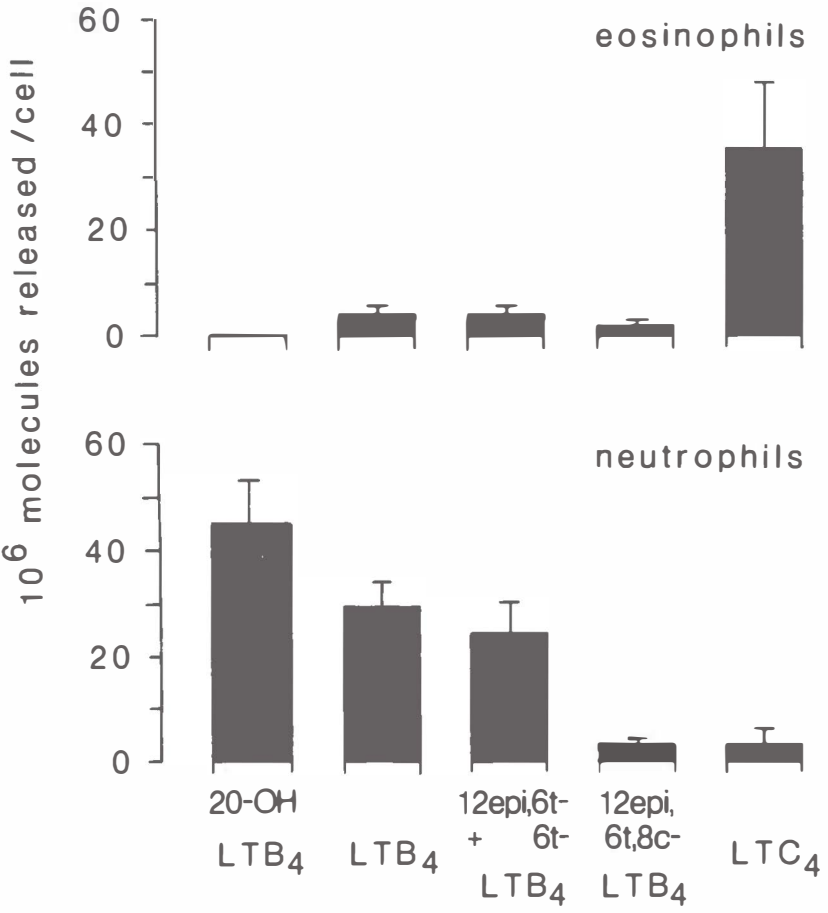

Fig. 3. Mean (± SD) leukotriene synthesis of purified human eosinophils (purity: 96 ± 4%; n = 5) and purified human neutrophils (purity: 96 ± 4%; n = 5). Leukotriene synthesis was stimulated for 5 min. by the addition of the calcium ionophore A23187 (20 μM), Ca^{2+} (2 mM), glutathione (5 mM) at pH = 7.4 and 37 °C. The leukotrienes formed were analyzed as described previously (33) by means of RP-HPLC and their number expressed as 10^6 molecules released/cell (= 1.67 pmol/10^6 cells).

Further *in vitro* studies concerning the LTC_4 synthesizing capacity of the human eosinophil have indicated that added arachidonic acid in a concentration over 80 μM inhibits the LTC_4 formation. Reversed-phase HPLC analysis showed that the inhibition of this LTC_4-synthesis coincided with an enormous increase in the synthesis of 15-LT's and

15-HETE (6). In a following series of experiments addition of increasing amounts of 15-HETE together with the calcium-ionophore A23187 showed a dose-dependent inhibition of the LTC_4 synthesis (see Fig. 4).

This finding suggested that the presence of increasing amounts of exogenous arachidonic acid may stimulate the 15-lipoxygenase activity of the eosinophil leading to strong production of 15-LT's and 15 HETE. The latter compound may strongly inhibit 5-lipoxygenase activity and consequently LTC_4 formation. Therefore 15-HETE may act as an internal regulator of 5-lipoxygenase activity.

Since A23187 is a rather unphysiological stimulus, we have looked for more physiological stimuli to induce LTC_4 formation. Of the tested physiological stimuli, ECF-A, PAF, zymosan activated serum, LTB_4, f-MLP, and opsonized zymosan, only opsonized zymosan was capable of inducing LTC_4 synthesis by the human eosinophil (5).

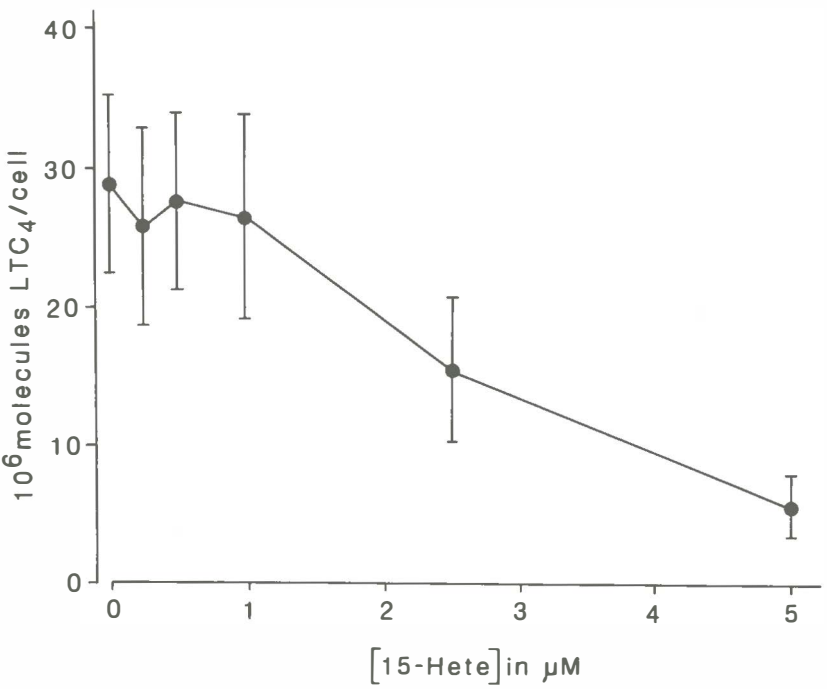

Fig. 4. Inhibition of A23187 (20 μM) induced LTC_4 synthesis by human eosinophils (purity: 78 ± 9%; n = 3; mean ± SEM) by added 15-HETE.

Table 1. Mean (± SEM) LTC_4 production by purified human eosinophils upon *in vitro* stimultion with: a) the calcium ionophore A23187 (10 μM), or b) opsonized zymosan (5 mg/ml) under optimum conditions.

Stimulus:	n:	cell purity:	LTC_4 production: (expressed in 10^6 molecules (cell)
A23187	10	87 ± 4	50 ± 7
opsonized zymosan	10	87 ± 3	8 ± 2

Reversed-phase-HPLC showed that exclusively LTC_4 was formed upon this stimulation (for quantities see Table 1).

This finding may indicate that immune complexes containing IgG, C_{3b}, and even IgE or mast cell granules may have the capacity to induce formation of this bronchoconstrictive mediator. This suggests an active participation of the eosinophil in the late phase asthmatic reaction. Moreover, this finding indicates that eosinophils can be responsible for the asthma-like symptoms in case of pulmonary eosinophilic syndromes.

From the findings presented here it can be concluded that:

1. Eosinophils penetrate into the bronchioli in the beginning of the late phase asthmatic reaction (LAR);
2. These eosinophils possibly are active or become activated since an elevation of the eosinophils derived from compound ECP was reported to be present in BAL fluid during the LAR;
3. The release of ECP and possibly other eosinophil derived compounds, such as the major basic protein (MBP) and oxygen radicals, may contribute to tissue injury and inflammation which are suggested to be causative to the increased bronchial hyperreactivity, observed after late asthmatic reactions;
4. These eosinophils may also be responsible for the bronchial obstruction observed during the LAR since they were shown to produce almost exclusively the strongly bronchoconstrictive compound LTC_4 upon stimulation with physiological stimuli *in vitro*;
5. Neutrophilic granulocytes may further stimulate the infiltration of eosinophils since they are capable of synthesizing large amounts of the strongly chemotactic compound LTB_4 upon stimulation with physiological stimuli *in vitro*.

Acknowledgements

This work was supported by grants of the 'Nederlands Astma Fonds', (grants no. 79.27 and 82.18).

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CHAPTER 3

HISTAMINE IN LATE ASTHMATIC REACTIONS FOLLOWING HOUSE-DUST MITE INHALATION

De Monchy J.G.R., Keyzer J.J., et al. Histamine in the late asthmatic reactions following house-dust mite inhalation. Agents and Actions 1985; 16: 252–5.

CHAPTER 3

Histamine in late asthmatic reactions following house-dust mite inhalation

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3.1.1. Abstract

In order to investigate the role of histamine in the late asthmatic reaction (LAR) following house-dust mite (HDM) inhalation, we studied, with hourly intervals, urinary N^{τ}-methylhistamine (an important metabolite of histamine) in 14 allergic asthmatic patients before and after broncho provocation with HDM.

Four patients showed an early asthmatic reaction (EAR), while 10 patients developed a LAR as well. In the hour following the EAR a significant increase in urinary N^{τ}-methylhistamine was observed as compared to the control day ($0.01 < p < 0.05$). During the LAR no increase of this metabolite was detected in the urine of the patients. Additionally, histamine was measured in broncho-alveolar lavage fluid (BAL fluid) obtained from 6 patients during the HDM-provoked LAR and compared to histamine levels in BAL fluid from patients without a LAR, following broncho provocation.

In the LAR group higher histamine levels were found than in the other patient and control groups. For the whole patient group no correlation was found between the degree of bronchial obstruction during the LAR and the BAL fluid histamine values. No difference was found in N^{τ}-methylhistamine in BAL fluid between patients with LAR and controls. Thus histamine metabolite studies in the urine failed to provide evidence of involvement of histamine in the LAR, while further data are needed to interpret the results of local sampling in the lung.

3.1.2. *Introduction*

When allergic asthmatic patients inhale sufficiently large doses of allergen a biphasic response in the forced expiratory volume in one second (FEV_1), may follow. It is generally accepted that the early asthmatic reaction (EAR) can be attributed to an IgE-mediated mast-cell degranulation resulting in production of mediators such as histamine and leucotrienes (1), whereas the degree of bronchial obstruction following degranulation is largely dependent on the pre-existing bronchial hyper-reactivity (e.g. histamine sensitivity) (2).

The pathogenesis of the second phase of bronchial obstruction, the late asthmatic reaction (LAR) usually starting 4–8 h after allergen is still largely unknown.

Among the mechanisms that have been proposed are deposition of immune complexes in the airways, release of mast-cell-dependent mediators and cellular infiltration. In a recent study we showed that in the initial phase of the late asthmatic reaction a cellular infiltrate consisting mainly of eosinophils was present (3). This inflammatory response, however, does not exclude the role of histamine or other mediators, since the obstruction in the airways during the LAR is partly due to bronchospasm as is illustrated by the partially broncho-dilating effect of beta-adrenergic drugs. To investigate the role of histamine in the LAR we measured N^{τ}-methylhistamine, a reliable indicator of histamine production in hourly shredded urine portions both during the EAR and the LAR (4). Additionally we measured histamine and N^{τ}-methylhistamine in bronchoalveolar lavage fluid.

3.1.3. *Patients and methods*

In this study two groups of patients were studied: group A comprised of patients in whom urine was collected on the control day and following allergen provocation, and group B comprised of patients in whom broncho-alveolar lavage was performed, following allergen inhalation (see Table 1).

All patients suffered from bronchial asthma with a history of wheezing on allergen exposure and on non-specific stimuli such as cold air or exercise. All had a positive skin test to house-dust mite and elevated anti-house-dust mite IgE, as well as an increased bronchial sensitivity to hista-

Table I. Clinical data of the patients group A comprises patients participating in the urinary N^{τ}-methylhistamine study. Group B comprises patients participating in the broncho-alveolar lavage study.

Patient	Age/sex	Histamine[a] threshold (mg/ml)	FEV_1 inhalation % predicted	Maximal decrease in FEV_1	
				EAR	LAR
Group A					
1	17 F	8	78	32	62
2	38 M	4	62	49	31
3	25 M	32	96	29	< 10
4	23 F	4	88	46	52
5	22 M	4	101	49	< 10
6	22 M	16	86	38	17
7	30 F	32	93	20	< 10
8	27 M	4	72	14	25
9	15 M	4	79	35	< 10
10	33 M	16	102	20	20
11	23 M	2	116	54	56
12	29 M	8	70	20	20
13	31 M	8	93	55	24
14	25 M	8	95	46	31
Group B					
15	23 M	16	101	26	30
16	31 M	8	89	67	20
17	20 F	16	99	23	47
18	19 M	8	98	39	23
19	34 M	16	92	38	41
20	21 F	16	96	70	31
21	22 M	2	61	16	< 10
22	19 M	2	78	23	11
23	22 F	32	114	34	< 10
24	39 M	16	84	22	< 10
25	39 M	32	81	16	< 10
26	18 M	4	73	< 10	< 10
27	16 M	16	91	< 10	< 10
28	24 M	8	112	< 10	< 10
29	79 M	4	70	14	15
30	31 F	2	68	< 10	< 10

[a] Threshold values expressed as concentration (mg/ml) causing a fall in FEV_1 of 15% or more. Maximal decrease in FEV_1 expressed in percentage of initial pulmonary function corrected for control values obtained after inhalation of coca solution.

mine. The patients were admitted on a non-acute basis to evaluate their complaints. None of them had experienced acute asthmatic attacks for at least 2 months and there was no history of respiratory tract infections during this period. All were non-smokers. Bronchial hyperreactivity was measured by serial inhalations of histamine diphosphate solution in increasing concentration using a 'Wiesbadener doppel spray'. The lowest concentration at which a fall of 15% or more was found was called the histamine threshold value.

House-dust mite provocation was performed by inhaling with 15 min intervals 4 stepwise increasing concentrations (50, 250, 1250 and 6250 Bu/ml) of house dust mite during 1 min using a 'Wiesbadener doppel spray'.

In patients from group B broncho-alveolar lavage (BAL) was performed 6–7 h after the allergen inhalation. The technique of BAL was carried out as described (3). Briefly, after bronchodilation by subcutaneous injection of 10 mg of oxyfenonium bromide (an anticholinergic drug) and local anaesthesia with Lidocaine 2% a flexible Olympus (BF B_3-R 5.9) bronchoscope was placed in wedge position in the (right) middle lobe and 10 aliquots of 20 ml saline were injected and aspirated by gentle suction and transported on ice to the laboratory. There the contents of tube 1 and 2 were pooled in pool I. The contents of tube 3, 4, 5 in pool II and of tube 6, 7, 8, 9, 10 in pool III. Fluid from pool II was used for histamine and N^{τ}-methylhistamine determination. The procedure was approved by the Internal Review board.

Patients were admitted to hospital during a 2 week period. At admission all medication was stopped. On day 4 control solution was inhaled and FEV_1 values were followed during the day at hourly intervals. On day 5 stepwise increasing dosages of house-dust mite extract were inhaled. When a decrease of FEV_1 of more than 15% was obtained the procedure was stopped. On either day 11, 12 or 13 the same amount of allergen was inhaled as on day 5 followed by BAL. Patients from group A were admitted to hospital following the same scheme as described above. On day 4 (control day) and on day 5 (house-dust mite day) urine was collected in hourly fractions starting 2 h before and continuing up to 10 h after allergen or control solution inhalation.

To prevent bacterial growth urine was voided in polypropylene vials containing 0.5 ml of 20% chlorhexidine solution. Aliquots were stored at −20 °C until analysis. During the day when urine was collected the pa-

tients avoided foodstuffs known or suspected to contain histamine (sauerkraut, cheese or yoghurt). N^{τ}-methylhistamine in urine and histamine and N^{τ}-methylhistamine in BAL fluid were determined with isotope dilution mass fragmentography as described previously (5, 6). The normal range for urinary N^{τ}-methylhistamine is 40 ± 60 μmol/mol creatinine. All patients in group A had normal prechallenge values; normal values for BAL have not been described. Both N^{τ}-methylhistamine and histamine in BAL are expressed as albumin ratios to account for dilution during the lavage.

3.1.4. Results

Group A

In Fig. 1 the changes in urinary N^{τ}-methylhistamine excretion

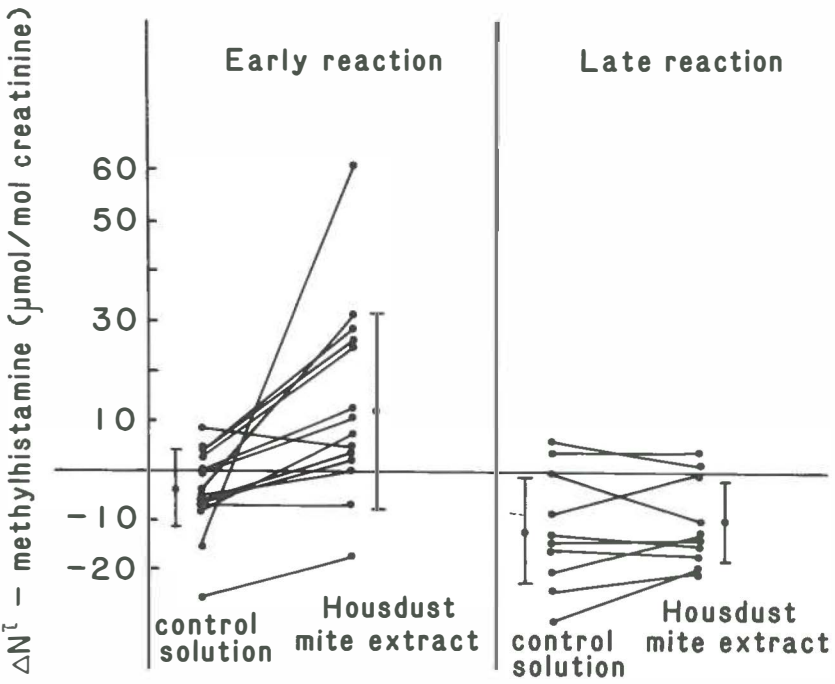

Fig. 1. Change in hourly urinary N^{τ}-methylhistamine excretion between the urines collected before and after inhalation of control solution and allergen extract. For both early and late reaction after inhalation the urines were those collected as soon as maximal decrease in FEV_1 was registered. Individual values and mean ± SD are depicted.

produced before and after inhalation of either control solution or allergen extract are shown for patients with early reactions and patients with late reactions. The depicted data regard those urine portions gathered after the 1 h period in which the maximal fall FEV_1 was observed and the corresponding time during the control day. As the inhalation started between 8 and 9 a.m. each difference in N^{τ}-methylhistamine was calculated with respect to the value obtained from the urine collected at 8 a.m. The mean increase in N^{τ}-methylhistamine (17.6 μmol/mol) following the EAR was significantly elevated compared to the corresponding values on the control day ($p < 0.05$). In the urine portions gathered during the LAR no significant differences were found, as compared to the values obtained during the corresponding time on the control solution day.

Group B

Following house-dust mite inhalation 6 patients developed an early and late asthmatic reaction (LAR), 5 patients developed the single early reaction (EAR) and 5 patients neither early nor late reaction (NR). The 5 controls did not react to house-dust mite inhalation. The mean histamine content of the BAL fluid obtained in 6 patients during actual late asthmatic reaction (LAR), (45 ± 20 (SD) pmol/mg Albumin, was significantly elevated compared to controls (10 ± 6 pmol/mg Albumin) and to EAR (10 ± 5 pmol/mg Albumin) but, not compared to NR, 30 ± 30 pmol/mg Albumin. The N^{τ}-methylhistamine level in the lavage fluid in the LAR group, 5.2 ± 3.1 pmol/mg, was not significantly different from controls, 3.6 ± 3.5. When the N^{τ}-methylhistamine values for all patients were plotted against the fall in FEV_1 during the late reaction a significant correlation was found ($R = -0.61$, $p = <0.05$). However, when the N^{τ}-methylhistamine value was plotted against the fall in FEV_1 during the preceding early reaction the same degree of correlation was found. No significant correlation was found between histamine values in the lavage fluid and the decrease in FEV_1 during either the early or late reaction.

3.1.5. Discussion

From urinary N^{τ}-methylhistamine determinations we found evidence for mast-cell degranulation during the early asthmatic reaction. No evidence was found for renewed mast-cell degranulation during the late asthmatic reaction. However, since the bronchial obstruction occurring during

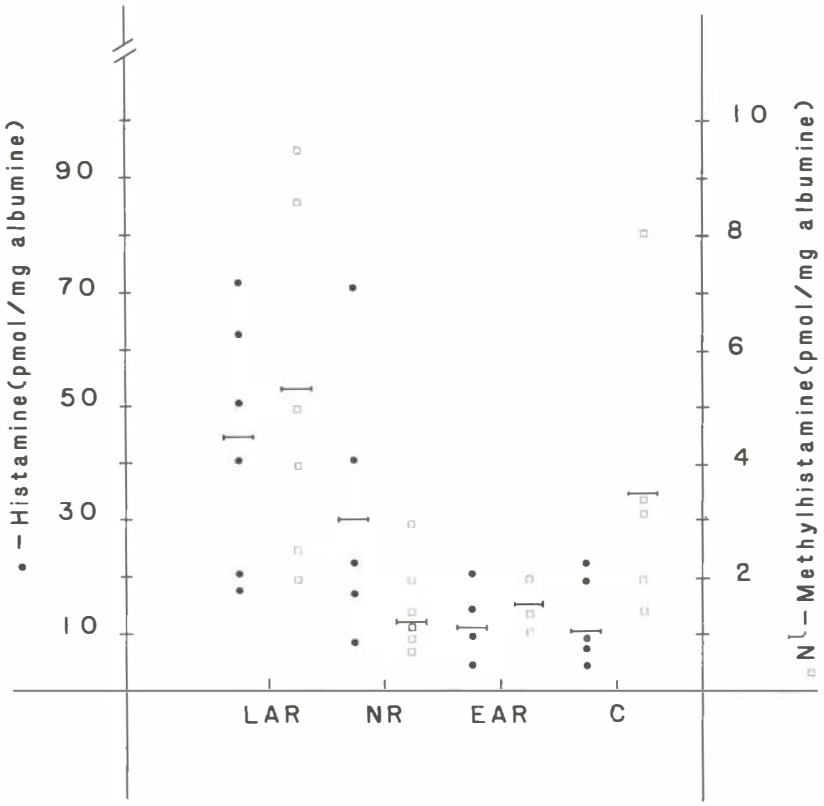

Fig. 2. Histamine and N^{τ}-methylhistamine levels in broncho-alveolar lavage fluid obtained 6–7 h after allergen provocation from patients showing a late reaction (LAR), a single early reaction (EAR) and no reaction (NR) and controls (C). To account for dilution during the lavage procedure histamine and N^{τ}-methylhistamine are given as an albumin ratio.

the LAR seems to be only partially caused by mediator release, determination of urinary N^{τ}-methylhistamine might not be a sensitive enough parameter for the detection of histamine production.

Therefore we additionally sampled directly from the lung compartment by means of BAL.

Although the concentration of histamine in the LAR group was higher than in the other group, no correlation was found between the degree of bronchial obstruction and the histamine content of the lavage fluid obtained 6–7 h after allergen provocation. Since histamine may be metabo-

lized in the lungs we additionally determined N^{τ}-methylhistamine in the lavage fluid. A significant correlation was found between concentration of N^{τ}-methylhistamine in lavage fluid and the late FEV_1 values. However, the same degree of correlation was present with the early FEV_1 values suggesting that N^{τ}-methylhistamine found in the lung during the LAR might be derived from the early reaction.

The histamine, N^{τ}-methylhistamine ratio in serum is approximately 0.7, in lavage fluid we found a ratio of approximately 10, suggesting that either histamine is degraded very slowly in the lung compartment or more likely that the relatively high histamine levels found are caused by mechanical injury to histamine-containing cells. In the latter case N^{τ}-methylhistamine might be a better parameter of histamine production *in vivo* than histamine itself.

When these BAL patients were allocated into three groups, LAR, EAR and NR, according to the degree of bronchial obstruction during the early and late phase no significant difference was found in N^{τ}-methylhistamine values of patients with LAR as compared to controls. Although production of some histamine during the LAR can not be excluded, histamine seeems not to be the main mediator of the LAR.

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CHAPTER 4

IMMUNOGLOBULINS AND COMPLEMENT SPLIT PRODUCTS IN PLASMA AND IN BRONCHOALVEOLAR LAVAGE FLUID FOLLOWING ALLERGEN PROVOCATION

CHAPTER 4

Immunoglobulins and complement split products in plasma and in bronchoalveolar lavage fluid following allergen provocation

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4.1.1. Abstract

This study describes the results of immunoglobulin and complement determination in serum and in bronchoalveolar lavage fluid (BAL fluid) following allergen provocation in allergic asthmatic patients. The patients showed either single early (EAR), dual (LAR) or no (NR) bronchial obstructive reactions following provocation with house dust mite extract (HDM). Serum total IgE and anti HDM IgE were higher in the patient groups compared to the controls. No significant differences were found in total and specific IgE levels between the patient groups. Serum total IgG, IgA, and IgM showed no differences within the patient groups nor between the patient groups and controls. The HDM specific IgG_1 (IgG_1 spec.) level was significantly ($P < 0.05$) higher in the LAR patient group compared to the EAR group. Serum total IgG, anti HDM IgG (IgG spec.) and anti HDM IgG_4 (IgG_4 spec.) did not differ significantly between the patient groups nor between the patient groups and the controls. The BAL fluid was divided in a 'bronchial' sample (pool I), a 'bronchoalveolar' sample (poool II), and an 'alveolar' sample (pool III).

In the BAL fluid no difference was found in the total IgG content between the various groups. No evidence for local IgG production was found. In the LAR group BAL fluid IgA was elevated compared to the EAR group ($P < 0.05$) in pool I and pool II. No differences were found in BAL fluid total IgE and IgE spec. between the patient

groups (pool I). C1q in the BAL fluid was decreased in the LAR group, compared to the other patient groups (pool II). Total C3 and C3a were not significantly different within the patient groups nor between patients and controls. C1q was negatively correlated to the number of cells in the same pool ($P < 0.05$) and with the number of eosinophils from pool I.

In conclusion, we found that patients with a LAR following HDM inhalation have (compared to patients with an EAR) an elevated level of serum IgG_1 spec. In the BAL fluid of these patients total IgA was found elevated whereas total IgG and total IgE and anti HDM IgG were not. Although some complement activation may occur during the LAR, an immune complex mediated reaction is unlikely to be underlying the pathogenesis of the LAR.

4.1.2. Introduction

Since the first description of the immediate type allergic reaction in the skin, the bi-phasic nature of this reaction was recognized (1). The early phase of the reaction was shown to be dependent on the presence of allergen specific reaginic antibodies, identified by Ishizaka and Johansson as IgE antibodies (2, 3). A cross-linking of these antibodies at the surface of mast cells by allergen was shown to result in the production of spasmogenic mediators such as histamine, prostaglandin D_2 and leukotriene C4 (4, 5). Such mediators contract smooth muscle in the bronchi and cause vasodilation and increase mucus production. These pathophysiologic phenomena are thought to explain sufficiently the mechanism of the early phase of the immediate type allergic reaction; the mechanism of the late phase however, remains largely unknown.

Because of its typical occurrence 3–8 hrs after inhalation of allergen an IgG immune complex mediated reaction was suggested by Pepys (6). The generation of immune complexes of IgG with inhaled antigen in the lungs and subsequent activation of the complement cascade by the classic route with production of the anaphylatoxins C3a and C5a was suggested as a means of inducing mast cell degranulation. This second phase of bronchial obstruction was supposed to be largely independent of IgE antibodies. This hypothesis was mainly evaluated by studying the late response in the skin following local injection with mould extract in patients with allergic bronchopulmonary aspergillosis (ABPA). In the skin IgG, IgM, and C3 deposition was shown during the late cutaneous reaction (LCR), indeed suggesting complement activation by the classical route. However, the

clinical picture in common inhalant allergic asthmatics is quite different from patients with ABPA. Patients with HDM sensitive asthma do not respond to inhalation of the allergen with a rise in body temperature, malaise, joint pains, leukocytosis, and signs of infiltrative changes on the chest Xray and a restrictive type of lung function impairment as patients with ABPA and extrinsic allergic alveolitis may do (7). HDM allergic asthmatic patients react during the LAR with a sometimes protracted or recurrent obstructive reaction with absent or minimal systemic complaints (7, 8). Thus mould-induced skin or bronchial reactions seem to be quite different from the HDM induced LAR. Moreover, a LAR could be induced even when low levels of IgG antibodies were found (9). Following allergen provocation usually no clear evidence of complement activation was shown and whether during the LAR renewed mast cell degranulation occurs remains a matter of controversy (10, 11, 12, 13). Solley found in skin biopsies taken during the LCR following pollen application, oedema, degranulated mast cells and infiltrating eosinophils, neutrophils, but no complement or Ig deposition. A similar pattern was found following the application of anti IgE antibodies. With heat inactivated serum (thus deplete of IgE), not only the occurrence of the ECR but also the LCR was blocked in passive sensitization studies (14, 15). It was also shown that by selective absorption of IgE, the ECR and the LCR could be inhibited (16).

The observation that, following hypo-sensitization, the IgG levels increase and usually the LAR diminishes or disappears, seems to indicate that IgG antibodies rather prevent than induce the LAR (17, 18, 19, 20). However, during immunotherapy mainly IgG_4 is produced (21) and it may be possible that other IgG subclasses such as the complement binding IgG_1, would be involved in the occurrence of the LAR. Some reports have been published suggesting IgG_4 to be a short term sensitizing antibody associated with the development of the LAR (22). Subsequent studies by others have not been able to confirm these results (10, 12). Recent work suggests that within the lungs of patients with obstructive reactions after exposure to allergen, inflammatory cells accumulate. Using BAL we showed that the late phase response was associated with the accumulation of eosinophils in the lungs (23). Metzger et al. found that BAL fluid contained both neutrophils and eosinophils during the LAR (24). Frigas et al. showed that in the sputum of patients with asthma exacerbations the eosinophil derived major basic protein (MBP) was strongly elevated (25). MBP as well as eosinophil cationic protein (ECP) are known

to cause tissue injury and inflammation (26, 27). Recently, eosinophils have been shown to produce the spasmogenic leukotriene C4 after stimulation with calcium ionophore (28) and with receptor-dependent stimuli as serum treated with Zymosan (29) and IgG coated sepharose beads (30). Thus, infiltration of eosinophils might lead to bronchospasm and inflammation. However, individuals may also show a bronchoalveolar eosinophilia without evidence of broncho-spasm. In the skin also cellular infiltration can occur following allergen application without local swelling (31) indicating that the mere presence of these cells in the skin or in the bronchi does not lead to manifest reactions. Activation of granulocytes seems to initiate their aggressive properties (32). This activated state is accompanied by the expression of immunoglobulin and complement receptors on the surface of these cells (33). During EAR and LAR also activation of granulocytes occurs (34). Thus, the presence of IgG and complement, even without a full-blown immune-complex reaction, in the environment of eosinophils might have consequences for the expression of the LAR.

In this study we chose to investigate the role of antibodies in the LAR by studying the concentrations in plasma and in BAL fluid. Since the results of studies focusing on changes in plasma levels of complement products have been contradictory, we studied the presence of complement split products in BAL fluid in order to obtain a local sampling.

4.1.3. Patients

Nineteen patients with well-documented bronchial asthma gave their informed consent for participating in this study. 15 males and 4 females, 15–30 yrs of age, mean age 23.8 yrs, were selected on the basis of a clinical history of wheezing after allergen exposure, positive skin tests and raised specific IgE for HDM. All patients showed an increased bronchial hyperreactivity to histamine and acetylcholine. None of them reported aspirin idiosyncrasy. Patients were admitted on a non-acute basis to have their complaints evaluated. None of them had experienced acute asthmatic attacks for at least two months and there was no history of respiratory tract infection during the period. All were nonsmokers. As control subjects, 5 healthy nonsmoking volunteers, 3 males and 2 females, 23–33 yrs of age, mean 26.2 yrs, were chosen. All control subjects had negative skin tests to HDM and a negative history of asthma, rhinitis or eczema. Clini-

cal data of these subjects have been decribed in detail elsewhere (23). Inhalation tests and BAL procedure were performed as described previously (34).

4.1.4. Laboratory techniques

In the laboratory, the contents of tube 1 and 2 were pooled (pool I) separately from the other tubes (pool II, tubes 3 to 5; pool III, tubes 5 to 10). Cells and fluid were separated by centrifugation 10 min, 4 C, 200 g. The fluid was then stored at −80° C and the cells were resuspended in RPMI-1640 in a concentration of 10^6 − 1.5 × 10^6 cells/ml.

Total cell numbers were counted in a Coulter counter (Coulter Electronics, Hialeah, FL). For cytological examination, cytospin preparations were made from the cell suspension in a Shandon cytospin-2 cytocentrifuge (Shandon Southern Instruments, Sewickley, PA) using 20 µl aliquots (300 g for 5 min). After fixation the glass slides were stained with May-Grünwald and Giemsa (3 min May-Grünwald, 4 min Giemsa). A cell differentiation was performed by counting 500 leukocytes. The albumin concentration in the BAL fluid as well as in the IgG and IgM content was measured without prior concentration in a Behring laser nephelometer (LM, Hoechst Behring, Frankfurt, West Germany) following the procedure for serum and cerobrospinal fluid for the patient sera and BAL fluid, respectively. The LM standard sera and LM specific anti-sera were also obtained from Behring and used according to the manufacturer's instructions. It is important to note that monomeric and secretory IgA present in BAL fluid is detected together in the LM procedure. IgE in the lavage fluid was detected using a radio-isotope technique with 100 µl of lavage fluid. IgE in the sera was detected with the isotope method. 10 µl patient serum for total IgE and 50 µl serum for anti-HDM IgE. IgG spec. and IgG_4 spec. was detected using an antigen binding test (36), IgG_1 was detected using the RAST-technique (37). Specific IgE was also detected in the lavage fluid with the standard (RAST) technique using 400 µl of the lavage fluid and overnight incubation. C1q and C3 were measured by radioimmunoassay (RIA) (38, 39). In the RIA for C3 both native C1 as well as C3 activation products containing the C3 fragment are measured. C3a was measured by a competitive RIA: binding of ^{125}I-C3 to anti-C3a-Sepharose was inhibited by C3a in samples to be tested. The lower limit of detection of this RIA is 2−3 nanogram C3a per ml of sample.

Since sampling from the lung compartment may result in different dilutions of the aspirated epithelial lining fluid, the concentration of immunoglobulins and complement components in the lavage fluid were expressed related to the albumin concentration in BAL fluid, corrected for the serum albumin level. The corrected values were calculated as follows:

$$\text{Ig corrected} = \text{Ig BAL fluid} / \frac{\text{albumin BAL fluid}}{\text{albumin serum}} \text{ or}$$

$$\text{Ig corrected} = \text{Ig BAL fluid} \times \frac{\text{albumin serum}}{\text{albumin BAL fluid}}$$

If the concentration of an immunoglobulin in the BAL fluid was compared to that in the serum, both Igs were expressed with regard to the albumin concentration in the serum and lavage and presented as a ratio.

Thus:

$$\frac{\text{Ig BAL fluid}}{\text{albumin BAL fluid}} / \frac{\text{Ig serum}}{\text{albumin serum}} = \frac{\text{Ig BAL fluid} \times \text{albumin serum}}{\text{albumin BAL fluid} \times \text{Ig serum}}$$

If this ratio is well over 1 it suggests either local production or enhanced transport from the intravascular compartment into the bronchial lumen.

4.1.5. Study design

The patients were admitted to hospital for a 2 week period. At admission all medication was stopped. On day 4 control solution was inhaled and FEV_1 values were followed during the day with hourly intervals. On day 5 house dust mite was inhaled until a decrease in FEV_1 of 15% or more was obtained. On day 11, 12, or 13 the same amount of allergen was inhaled as on day 5 followed by BAL, either 6–7 hrs (16 patients and 5 controls) or 2–3 hrs (5 patients) after allergen inhalation.

Statistical analysis was performed using Student's two-tailed t-test.

The procedure was approved of by the Internal Review Board.

4.1.6. Results

After HDM extract inhalation 6 patients showed a dual reaction (LAR) prior to BAL, 5 patients showed a single EAR, and 5 patients showed no reaction (NR). In 5 patients with known LAR, BAL was performed prior to the expected development of LAR (ante-LAR). These patients had recovered from the early reaction before BAL. After BAL no further spirometry was carried out. Both normal subjects and patients with or without LAR tolerated the lavage procedure well with only moderate discomfort.

In Fig. 1 the serum concentration of total IgE and IgE spec. are given in the several patient groups as well as in the normals. All patients showed a significantly elevated concentration of total and IgE spec. compared to the control group but no significant differences were found between the patient groups. In Fig. 2 the serum concentration of IgG spec., IgG_4 spec. and total IgG are depicted (note that the LAR and ante-LAR group are taken together since this is a serum study). In the LAR group all specific IgG antibodies tended to be higher than in the other groups. Only the differences in IgG_1 between the EAR and the combined LAR, ante-LAR group, however, were found to be significant ($P < 0.05$).

In Fig. 3 the albumin concentration in the serum and in the BAL fluid is given. No significant differences were found between the several groups.

Neither the IgG/albumin ratios nor the IgG BAL fluid/serum ratios corrected for albumin showed significant differences. The IgG BAL fluid/IgG serum ratio for all groups was close to 1, suggesting that there is no local production of IgG (Fig. 4).

In Fig. 5 the IgA levels are depicted in the same way as for IgG. No differences were found in serum IgA. In the BAL fluid IgA only in pool II a significant difference was found between the LAR group and the ante-LAR group. When IgA was expressed on a serum ratio corrected for albumin both in pool I and II significant increases in the LAR group were found. This observation suggests that during the LAR local accumulation of IgA takes place. In pool III no significant differences between the several groups were found. The effect of the LAR is clearly illustrated by two patients who had no elevated IgA levels when they were 'washed' prior to the development of the LAR (pool I IgA 4.33, 5.49, respectively), while 'washed' during the LAR elevated IgA levels were found (pool I IgA 14.08, 13.14, respectively).

In Fig. 6 the ratios are given for IgM in BAL fluid. Both for patients and controls lower mean levels were found than for IgG and IgA.

Fig. 7 shows total IgE/albumin ratios, IgE spec./albumin ratios and total IgE BAL fluid/serum ratios. No significant differences were found between the groups.

In Fig. 8 the concentrations of C3, C1q and C3a are given for pool II. In the LAR group C3 and C1q were lower and C3a was higher compared to the other groups; only the LAR C1q level was significantly different from the ante-LAR group, the NR group, and the EAR group (one-sided prob.) but not from the controls.

4.1.7. Discussion

The purpose of this study was to investigate if the occurrence of the LAR is associated with the presence of elevated levels of specific immunoglobulins, or if evidence for complement activation in the early stages of the LAR can be found. Thus, in serum as well as in BAL fluid total and specific immunoglobulins were measured as well as complement split products in BAL fluid. The relation of specific immunoglobulins with the occurrence of the LAR can be envisaged in at least two different ways: Firstly, specific IgG or IgM immunoglobulins might form immune complexes with the inhaled allergen and activate complement by the classical pathway leading to generation of C3a and C5a, thereby inducing mast cell degranulation. For such a mechanism relatively little evidence is present in the literature as was suggested before. Secondly, IgG or complement split products might play a role in the inflammatory process during the LAR in concert with infiltrating cells such as eosinophil granulocytes.

Activated eosinophils have binding sites for IgE, IgG, and complement on their surface (40, 30, 34). The enhanced expression of Ig receptors coincides with an increased metabolic activity of these cells and an increased helminthocidal capacity (33, 41). Binding of IgG to the surface of activated eosinophils was also shown to stimulate sulphidopeptide leukotriene production. And since LTCs at least in an animal model (42) were shown to be involved in the occurrence of the LAR, anti HDM IgG in the sera of patients from the LAR group might be relevant.

IgG_1 spec. in the BAL fluid of these patients is probably proportional to its serum value. In normals it was shown that IgG_1 was present in bronchial lining fluid in concentrations similar to their serum concentra-

tions and that lung and serum concentrations are directly related (43). Serum IgG_4 was higher in the LAR group but did not reach significance as was also found by Durham (12). Since BAL fluid IgG_4 was reported to be increased by local synthesis or accumulation, at least in normals, the serum data cannot be extrapolated to the BAL fluid (43). In serum and BAL fluid total IgE, and IgE spec. showed no differences between the patient groups. The BAL fluid serum ratio for IgE was low indicating that local production of IgE in the bronchi cannot explain the observed reaction patterns following bronchoprovocation. These results are in accordance with similar observations on local IgE production of a patient with ABPA. However, caution is needed in the interpretation of these data since differences in molecular weight of albumin and Igs might bias the Ig/albumin ratio (44).

IgA was elevated in the BAL fluid of patients from the LAR group, but this seems to be the consequence rather than the cause of the LAR, since no elevated IgA was found in the ante-LAR group. Moreover, since the BAL fluid/serum ratio was high, significantly elevated compared to the EAR and ante-LAR patients and to the controls, this ratio may serve as an indicator of inflammation. This agrees with the findings of Diaz, who observed that IgA decreased in the BAL fluid of allergic asthmatic patients treated with DSCG (45). In a patient with ABPA IgA was also shown to be elevated (46). In general BAL fluid IgM values were lower than BAL fluid IgG or BAL fluid IgA. Both IgA and IgM levels decrease from pool I ('bronchial' sample) to pool III ('alveolar' sample). IgG is fairly constant in pool I, II and III. BAL fluid total IgG was not elevated as can be found in inflammatory lung diseases such as sarcoidosis, cryptogenic fibrosing alveolitis, extrinsic allergic alveolitis and ABPA (46, 47). The elevated IgG levels in these diseases seem to be associated with the presence of an alveolitis component. Very recently Ita et al also reported IgG_1 anti HDM levels to be elevated in LAR patients (48).

Finally, C3, C3a, and C1q was measured in pool II. In the LAR group depressed C3 and C1q and elevated C3a was found, suggesting some classical pathway complement activation. However, only C1q was significantly different in the LAR group as compared to the ante-LAR group, the NR group, and the EAR group, but not compared to the controls.

Since C1q levels were significantly related to the number of cells in pool II and the number of eosinophils in pool I ($P < 0.05$) adherence to

inflammatory cells might be an alternative explanation for the decreased levels of C1q in the LAR group.

In conclusion, allergic asthmatic patients showing dual reactions following HDM provocation show an inflammatory and bronchospastic reaction. This reaction is characterized by increased eosinophil numbers and a corresponding ECP concentration, in the bronchial and bronchoalveolar samples (pool II), and elevated IgA. These findings underline the bronchial localization of the inflammation during the LAR. Complement consumption may occur, and play a modulating role in the inflammatory process. An immune-complex hypersensitivity reaction, however, is unlikely to be underlying the pathogenesis of the LAR.

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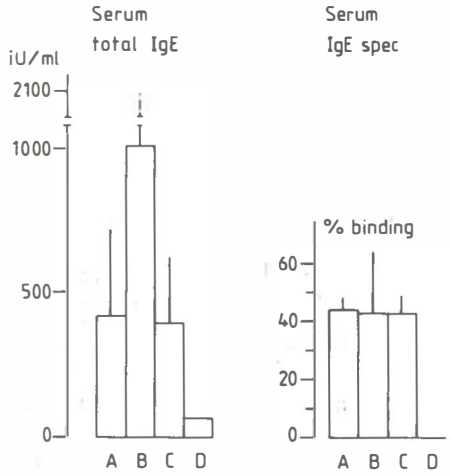

Fig. 1. Serum concentrations of total IgE and IgE spec. (for HDM). Between the patient groups no significant differences were found.

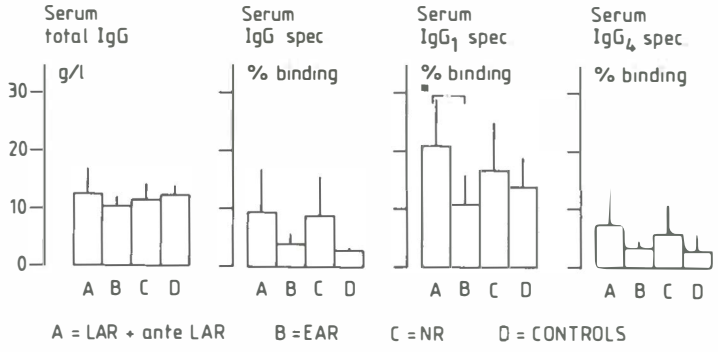

Fig. 2. Serum concentrations of total IgG, IgG spec. (for HDM), IgG_1 spec. and IgG_1 spec. The serum concentrations of IgG_1 spec. in the LAR group was significantly elevated compared to the EAR group ($P < 0.05$).

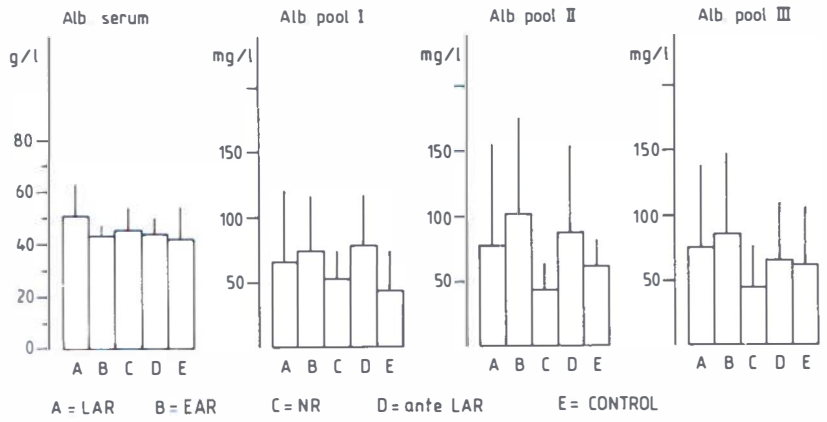

Fig. 3. Albumin concentrations in serum and BAL fluid. No significant differences were found between the groups.

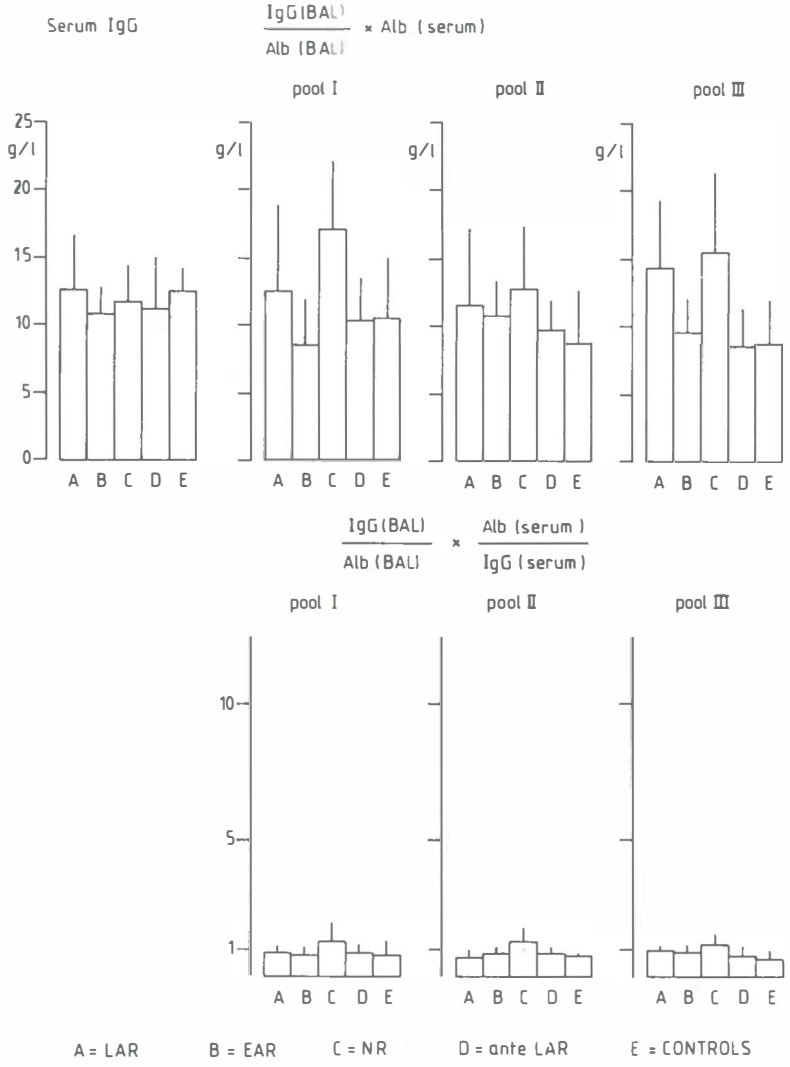

Fig. 4. Serum total IgG, BAL fluid total IgG albumin ratio and IgG BAL fluid/serum ratio corrected for albumin. No significant differences between the patient groups were found.

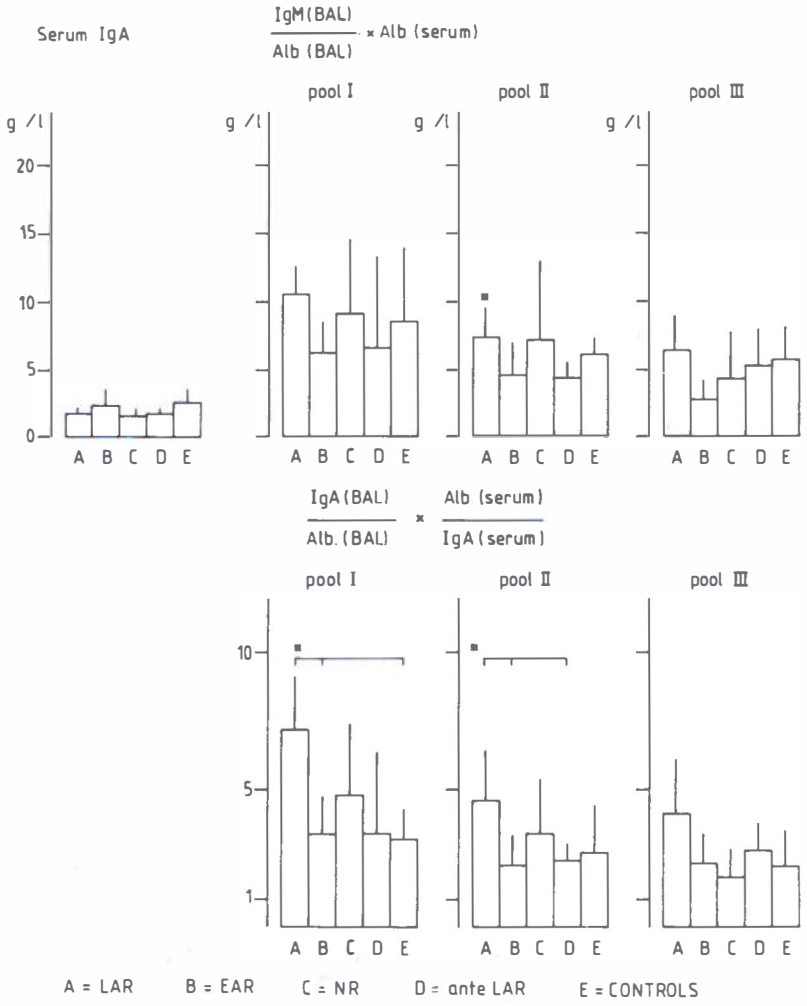

Fig. 5. Serum total IgA, BAL fluid total IgA albumin ratio and total IgA BAL fluid/serum ratio corrected for albumin. In pool I and II IgA for the LAR group was significantly elevated ($P < 0.05$).

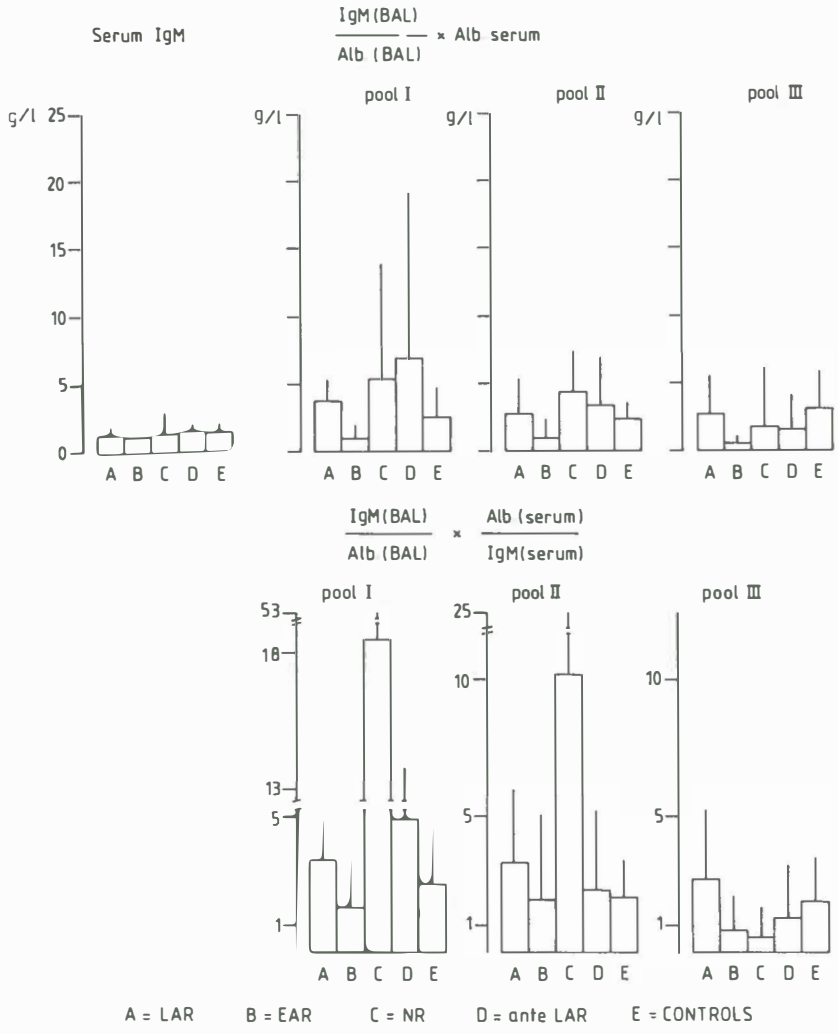

Fig. 6. Serum total IgM, BAL fluid total IgM albumin ratio and total IgM BAL fluid/serum ratio corrected for albumin in pool I and II.

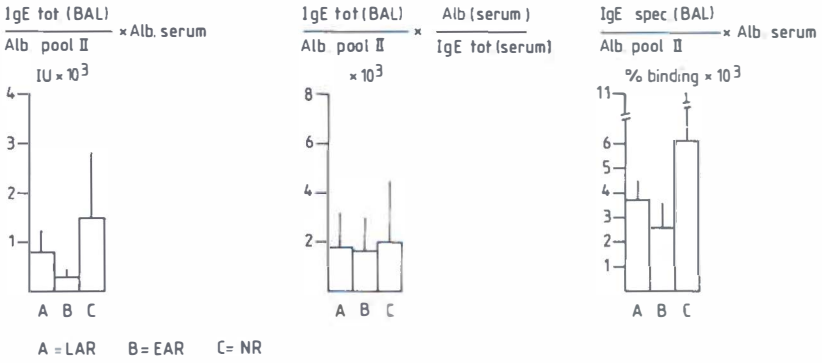

Fig. 7. BAL fluid total IgE/albumin ratio, total IgE BAL fluid/serum ratio and BAL fluid IgE spec. (for HDM)/albumin ratio. No significant differences were found between the patient groups.

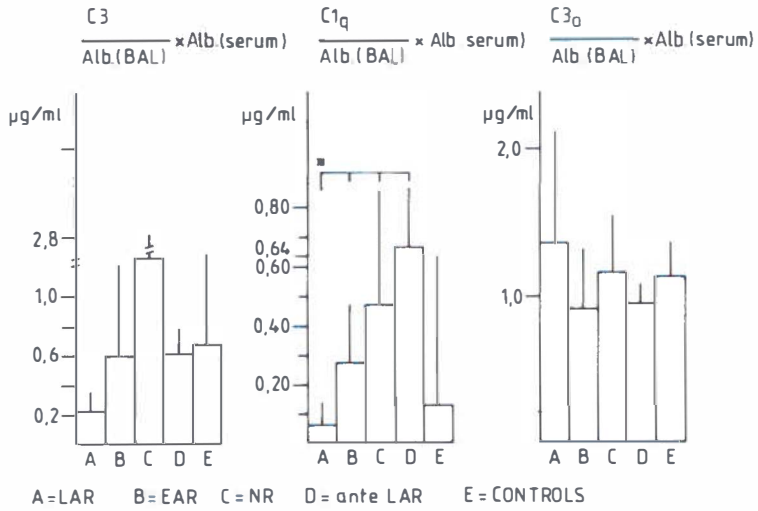

Fig. 8. BAL fluid C3 (corrected for albumin), BAL fluid C1q (corrected for albumin), and BAL fluid C3a (corrected for albumin), pool II. The levels of BAL fluid C1q were significantly depressed in the LAR group compared to the patient group ($P < 0.05$), but not compared to controls.

CHAPTER 5

MODELLING THE EARLY AND LATE ALLERGIC ASTHMATIC REACTIONS

CHAPTER 5.1

Modelling the early and late allergic asthmatic reactions

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5.1. Developing the mathematical model

5.1.1. Abstract

In this paper a method is described to yield multiple linear regression (MLR) models for early and late allergic asthmatic reactions using the data obtained from commonly applied tests for bronchial asthma.

The purpose and hypotheses underlying the study are described and linear regression and variable transformation are introduced briefly. The use of transformations is stressed, and some simple transformation functions are explained. Next the patient material, and the selection and definition of the variables used in constructing the model are discussed.

As independent variables were chosen: age, sex, total IgE, IgE specific for house dust mite (IgE spec), eosinophilic cells (Eos), histamine sensitivity (PC_{15} Hist), acetylcholine sensitivity (PC_{15} Ach), vital capacity (VC), forced expiratory volume in 1 second (FEV_1), specific work of breathing (W_{sp}), early cutaneous reaction (ECR), late cutaneous reaction (LCR).

Special attention is paid to the specification of the variables that are to be predicted. These are: sensitivity to house dust mite antigen as judged by lung function (FEV_1) during early (PD_{20} EAR) and late (PD_{20} LAR) allergic asthmatic reactions.

Chapter 5.2. is dedicated to the experimental design of the models, the optimization and the evaluation of the models.

5.1.2. Introduction

The occurrence of bronchial obstructive (asthmatic) reactions following allergen inhalation is considered to be dependent on several factors. Both the severity and the type of the reaction (early, late or combined) may differ largely among individual patients.

The purpose of this part of the study was to develop a mathematical model to predict occurrence and severity of both asthmatic reactions, constructed from relevant variables with known values.

The advantage of predicting allergic asthmatic reactions from other measured variables lies in the relative ease with which those variables can be measured compared to determining the size of the asthmatic reaction itself. This presupposes that early and late allergic asthmatic reactions are, at least to some extent, in fact predictable if other relevant variables are known. The second purpose of the study is to assess the quality of the model and the relative importance of the model-constructing variables. Knowing the relative importance of variables in a prediction model may allow a better understanding of the mechanisms underlying these allergic reactions.

Allergic asthmatic reactions can be monitored by plotting the degree of bronchial obstruction as a function of time.

The degree of obstruction is measured by spirometry and in particular the FEV_1 is a frequently used parameter. A clinically significant decrease in FEV_1 during the first hour following allergen challenge is called the early asthmatic reaction (EAR) while a significant decrease after 3 to 8 hours is in this study defined as the late asthmatic reaction (LAR).

The construction of a set of two equations predicting the degree of reaction during EAR and LAR separately is the main objective of this part of the study.

We chose MLR as a modelling technique, in order to fit the degree of EAR and LAR to a number of independent variables.

The choice of MLR implies the assumption that the estimation of the severity of EAR and LAR can in fact be based on linear models.

The variable-to-be-explained (asthmatic reaction) must for both equations be formulated and defined in such a way that this assumption is best met.

For both models (EAR, LAR) the same techniques are used, initially the same variables are introduced, and similar expressions for the depen-

dent variables are defined.

A set of variables that were considered relevant for the EAR and LAR was used for model construction. A preliminary selection was based on medical expertise, including also variables whose relevance was not certain. The selection was based on pathophysiological considerations.

After the construction of the model some procedure to test its merits and restrictions is required. The following chapter (5.2) will be largely dedicated to the problem of evaluation.

In the paragraphs below details will be given about three major elements of construction of the models following a brief introduction to linear models:

- description of the independent variables
- defining the formulae for the EAR and LAR
- finding the best transformations to be used for the variables in the model.

5.1.3. Multiple linear regression

5.1.3.1. Principles

In this study modelling the allergic asthmatic reactions means finding for both reactions separately a formula consisting of variables and constants in which the observed data can be fitted. From this formula the expected values for EAR and LAR can be calculated (estimated). This modelling is done by an MLR. In this technique the variable that has to be estimated, called the dependent variable, is assumed to be built up from additive parts of the other, predictor or independent, variables plus a constant part, which is called the intercept.

$$y = y_0 + \beta_1.x_1 + \beta_2.x_2 + \ldots + \beta_j x_j + \ldots + \beta_p.x_p$$

where y = dependent variable
x_j = independent variable
p = number of independent variables
y_0 = intercept
β_j = regression coefficient of variable x_j $(j=1 \ldots p)$

The model is called linear because a graph of every separate x against

y would represent a straight line. Of course, because of biological and measurement variation, every individual patient will show a departure from the model, even when the theoretical model is correct. This mismatch is called the residual ϵi.

$$y_i = y_0 + \beta_1.x_{i1} + \beta_2.x_{i2} + \ldots + \beta_p.x_{ip} + \epsilon_i \ (i=1\ldots n)$$

where i = a subscript identifying the individual patient
n = number of patients

For every patient the variable y and all x-variables are measured and known. It is our aim to estimate the β-parameters. The estimators of the parameters are denoted by corresponding Latin letters:

$$y_i = b_0 + b_1.x_{i1} + b_2.x_{i2} + \ldots + b_p.x_{ip} + e_i$$

It is assumed that the b-parameters have the same value for all patients.

The standard method to estimate the b – parameters is the least-squares-method. This method chooses b-parameters such that the sum of the squared residuals e_i is at minimum. In this sense the formula

$$y = b_0 + b_1.x_{i1} + b_2x_{i2} + \ldots + b_p.x_{ip}$$

fits the patient-data best.

For details on the least-squares-method or multiple regression, see for instance Green (1), Wonnacott & Wonnacott (2) or other statistics textbooks.

5.1.3.2. Transformations and curve models

In order to perform MLR the relation between the dependent and the independent variables must be linear. In this paragraph some easily applicable rules are proposed to transform simple curves into straight lines. Some examples of frequently occurring functions are:

A) a linear relationship.
An example of the function is illustrated in Figure 1a. The general formula is given by:

$$y = y_0 + b.x$$

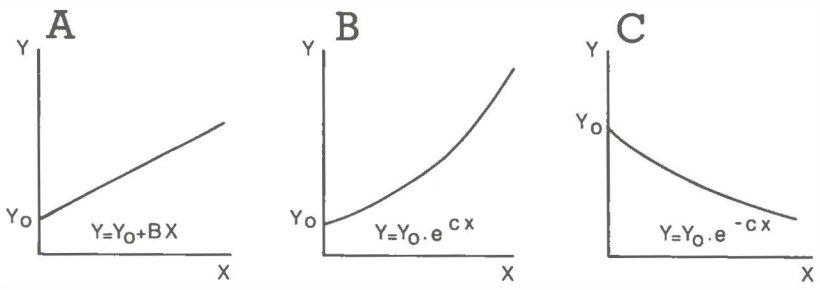

Fig. 1. The relationship between two variables (x, y) may be approached by relatively simple mathematical functions.
A) a linear relationship (y is proportional to x if $y_0 = 0$);
B) an exponential relationship (the increase in y is proportional to the value of y);
C) a negative exponential relationship (the decrease in y is proportional to the value of y).

Y is said to be proportional to x if $y_0 = 0$. If an increase or decrease in a variable x of a fixed amount Δx corresponds to a change of another fixed amount Δy in a variable y, independent of the value of x (or y), a linear relationship exists between x and y.

B) an exponential relationship.

An example of the curve is shown in Figure 1b. Characteristics are a 'fixed starting point' to the left and an increasing steepness to the right. The formula is:

$$y = y_0.e^{c.x} \qquad (c > 0)$$

This shape is expected if the increase in y is proportional to the value of y. An example is cellgrowth in time: the increase in the number of cells during one mitosis cycle equals the number of cells before the mitosis.

The formula implies a linear relation between the natural logarithm of y (ln(y)) and x:

$$\ln(y) = c.x + \ln(y_0)$$

After defining $y' = \ln(y)$ we get:

$$y' = c.x + \ln(y_0)$$

Thus, in those cases where one expects the value of a variable y to grow ever steeper with increasing variable x, a logarithmic transformation may change the curve into a straight line.

C) a negative exponential relationship.
The shape of the curve is shown in Figure 1c. Characteristics are a 'fixed starting point' to the left and a horizontal asymptote to the right. The general formula is:

$$y = y_0.e^{-c.x} \qquad (c>0)$$

Such a curve can be expected if y decreases by a constant fraction of y during every fixed interval of x. Simple elimination of a drug from plasma represents a negatively exponential relationship (y = concentration of drug, x = time), and, in general, every interrelation characterized by a 'halftime'. The formula implies a linear relation between the natural logarithm (ln(y)) and x:

$$\ln(y) = -c.x + \ln(y_0)$$

Again, $y' = \ln(y)$ leaves:

$$y' = -c.x + \ln(y_0)$$

which is a linear relation.
Thus, if one knows a variable y to decrease, from a certain initial value, becoming ever flatter with increasing variable x, a logarithmic transformation may straighten the curve.

In the following sections discussing the relation between broncho-obstructive reaction and allergen dose, and between independent variables and the dependent ones, the transformations as we described will be referred to.

5.1.4. Data

5.1.4.1. The choice of the independent variables

The occurrence of asthmatic reactions following allergen challenge was shown to be associated with the presence of positive immediate type skin

tests to the applied allergen (3). However, patients with identical skin titers may show a different reaction pattern, not only in the type of reaction, but also in the severity of the reaction. Prior studies of our group showed that, apart from the skin sensitivity or the amount of specific IgE, the degree of bronchial hyperreactivity influences the severity of the EAR (4). These data were confirmed and extended by Killian (5) and Hargreave (6). However, other factors might also influence the occurrence and severity of the EAR and LAR. Age, for example may be of importance, since children show a higher frequency of LAR than adults (7, 8). The influence of sex was studied, since sex hormones influence the immunological response (9). Next to IgE spec, IgE tot may be of importance since non-antigen specific antibodies might interfere with the action of IgE spec (10). Furthermore, the peripheral eosinophil count seems to be associated with the severity of asthma in general (11) and the occurrence of the LAR in particular (12). Eosinophil cells were found elevated in the lungs during the LAR and 24 hours later in peripheral blood (13). Two different parameters of bronchial hyperreactivity (PC_{15} Hist and PC_{15} Ach) were included in this study. The response to both agents usually shows a high degree of correlation, however, PC_{15} Ach has a somewhat greater sensitivity in the upper range. As parameters of baseline bronchial obstruction, spirometry was used: (VC, FEV_1) and the Wsp.

In addition to IgE spec we measured the ECR as a parameter for atopic sensitization and also LCR (5 hours after allergen application), since the LCR may be correlated to the LAR (14).

5.1.4.2. The features

a. Patients

In this study the data of 29 allergic asthmatic patients were studied retrospectively. In Chapter 5.2 the clinical data of the patients are given. All patients were clinically evaluated according to the same protocol.

b. Inhalation provocation

On the fifth day inhalation with control solution was carried out and followed for eight hours by an hourly spirometric measurement.

When patients showed a greater variation than 15% of base-line in

FEV_1 during the day, they were excluded from the study. On the sixth day HDM extract was inhaled using an open delivery system with a Wiesbadener Doppelspray (8 l/min airflow, nebuliser output approx. 0.2 ml/min). Four five-fold stepwise increasing concentrations (10, 50, 250 and 1250 biological units (BU)) were inhaled during 1 minute with 15 minutes interval. Since the amount of allergen delivered is assumed to be cumulative, patients received an approximate total dose of 10, 60, 310 and 1560 BU of HDM extract (Diephuis Laboratories, Groningen, The Netherlands). If a drop of more than 20% from initial value of FEV_1 occurred, the inhalation procedure was stopped. The bronchial response was followed for 8 hours, the lowest FEV_1 during the first hour after allergen inhalation was taken as a measure of the EAR, while the lowest FEV_1 between 3 and 8 hours after allergen inhalation as compared to control day was taken as a measure of the LAR.

5.1.4.3. The independent variables

The following features were measured:

a. age (years)
b. sex
c. total IgE (IU)
   determined by radio-immuno-assay (RIA)
d. IgE specific for HDM,
   determined by the radio-allergo-sorbent-test (RAST)
   The scoring system is given below:

anti-IgE adsorbed (%)	score
2	0
2 – 5	1
5 – 10	2
10 – 20	3
20	4

e. eosinophilic cells,
   number of cells counted in 1/11 mm^3 of blood
f. sensitivity to histamine inhalation:
   dose (mg) that induced a decrease of FEV_1 of 15% from initial value

g. sensitivity to acetylcholine inhalation:
dose (mg) that induced a decrease of FEV_1 of 15% from initial value

h. VC (slow inspiratory),
calculated as a percentage of the mean value of the healthy population, corrected for age and height

i. FEV_1,
calculated as a percentage of the mean value of the healthy population, corrected for age and height

j. Wsp,
the Wsp was measured on a routine basis by plotting oesophageal pressure against expired volume. The surface of the thus obtained loop is a measure for the Wsp (J/l)

k. ECR, measured after 15 minutes.
Scoring system is given below

diameter of wheal (mm)		score
early	late	
7.5	10	0
7.5 – 10.0	10 – 20	1
10.0 – 12.5	20 – 30	2
> 12.5	> 30	3

l. LCR, measured after 5 hours.
Scoring system is given under k.

The models were constructed from the features mentioned above. As indicated before, the following features were also measured:

m. FEV^c_1, measured every hour, from ± 8.00 a.m. till 5.00 p.m. on a control day and expressed with regard to the initial value ($FEV^c_{1(i)}$).

n. allergen dose biological units (BU) during bronchial challenge (10, 60, 310, 1560 BU cumulative dose).

o. FEV^p_1 measured every hour from ± 08.00 a.m. till 5.00 p.m. on a provocation day, expressed with regard to the initial value ($FEV^p_{1(i)}$).

The bronchial response to provocation ($\Delta FEV_1 max$) was derived from the maximum difference between control and provocation day for the same clock-time and calculated as follows:

$$\Delta FEV_{1,\,max} = 100 \cdot \max \left[\frac{FEV^{c}_{1(t)}}{FEV^{c}_{1(i)}} - \frac{FEV^{p}_{1(t)}}{FEV^{p}_{1(i)}} \right]$$

For the early response $0 \text{ min} < t < 60 \text{ min}$
For the late response $3 \text{ hours} < t \leq 8 \text{ hours}$

5.1.4.4. The dependent variables

Since the allergic reactions in the patients followed different doses of allergen, the severity of the EAR and LAR have to be corrected for the inhaled dose of allergen. Thus the dependent variables are constructed from ΔFEV_1 max and the cumulative allergen dose during challenge 10 – 60 – 310 – 1560 BU.

This can be done by accepting the dose that is required to develop a fixed degree of bronchial obstruction as a measure for the severity of the reaction.

The determination of such a provocative dose (e.g. PD_{20})* has to be determined from intrapolation of measured degrees of bronchial obstruction and the known corresponding doses of allergen.

Since the severity of the LAR was only measured after a cumulative allergen dose (that being required for the EAR) it was assumed that the best practical approximation for the LAR PD_{20} value is as follows: PD_{20} LAR is an interpolation between two points. Point 1 is defined by dose 0 BU and the corrected initial FEV_1 ($FEV_{1(0)}$) being 1 by definition. Point 2 is defined by the administered cumulative dose of allergen and the minimal 'FEV_1' (for calculation see 5.1.4.6.).

In order to be able to compare the model of the EAR and the LAR, the PD_{20} value for the EAR was also calculated from the $FEV_{1(0)}$ (=1) and the dose 0 BU of allergen and the minimal "FEV_1" following provocation, with the corresponding cumulative dose.

Such an estimation of a PD_{20} value, however, requires knowledge of the dose-response relationship.

* PD_{20} = the dose required for 20% decrease in FEV_1.

5.1.4.5. Dose dependence of the allergic reactions

In order to study the dose-response relationship for the EAR, 12 patients were selected with similar allergen sensitivity, as assessed by the same first allergen dose that induced an early reaction of at least 20%. The allergen dose and the corresponding decreases in FEV_1 were plotted for each patient (see Figure 2).

The four most obvious dose-response relations are:

Linear decrease in FEV_1 with increasing allergen dose

A linear decrease is theoretically improbable because it cannot be expected that the rate of decrease is constant at every level of FEV_1, especially not if FEV_1 approaches zero. However, it is possible that a linear approximation suffices for moderate decreases of FEV_1 (see Figure 3a).

Sigmoidal decrease in FEV_1 with increasing allergen dose

A sigmoidal decrease, characterized by a small reaction lag, followed by a steep descent that flattens out, seems theoretically the most plausible: at least a minimum allergen dose is required to get enough IgE cross-linking

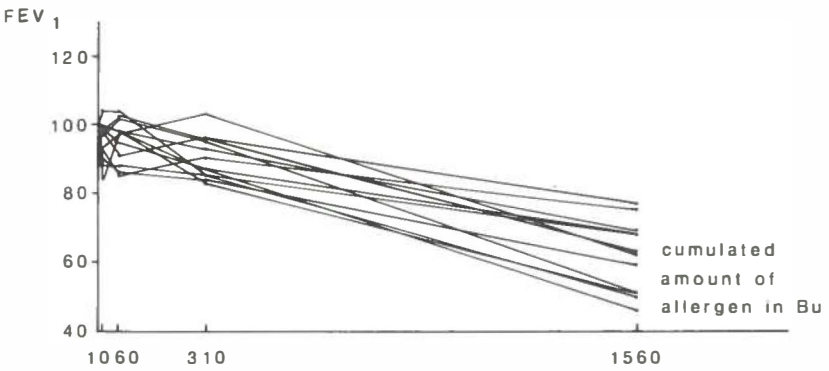

Fig. 2. For 12 patients selected for matching allergy as assessed by the same first allergen dose that induced an EAR of at least 20%, the dose-response relationship for each individual patient is shown. The cumulated amount of allergen (BU) is on the x-axis and on the y-axis is the corresponding decrease in FEV_1 as a percentage of the initial value and corrected for the control day.

on the surface of mast cells for the EAR, giving a plateau in the beginning of the dose-response curve. When FEV_1 has dropped heavily, a further increase in allergen dose will probably only marginally add to the response. This will cause the curve to flatten out.

A drawback of this approach is that, besides the steepness of the curve, the length of the plateau must be estimated.

If these are both known, a logit model might be applied. Since in the steep part of the curve too few measuring points are present this type of transformation will not be discussed here.

Exponential decrease in FEV_1 with increasing dose

The curve shows roughly the same shape as the preceding one, without, however, the plateau in the first part. The justification for this model is, that the plateau is very small with respect to the rest of the dose-response curve, and, secondly, that this is a simpler model, thus requiring fewer points of measurement (see Figure 3b).

Linear decrease in FEV_1 with logarithmic transformation of dose

Traditionally, in the dose-response relationship the dose is logarithmically transformed (see Figure 3c). This may straighten part of the curve to a certain extent, but it is fundamentally impossible that a really straight line results. A logarithmic transformation would only linearize a curve of the following formula:

$$e^{k \cdot \text{response}} = \text{dose}$$

or

$$k \cdot \text{response} = \ln(\text{dose})$$

This implies a vertical asymptote for dose 0; however, the response for dose 0 was defined as 1 ($FEV_{1(0)}$). Thus logarithmic transformation is not adequate.

From the figure it is clear that, although the approximation may be reasonably good for the last part of the curve, in essence this dose-response curve does not comply with the mentioned requirements. This reasoning holds even if the plateau is neglected. Because, for practical reasons (dose finding), challenge doses are usually increased with a constant factor in-

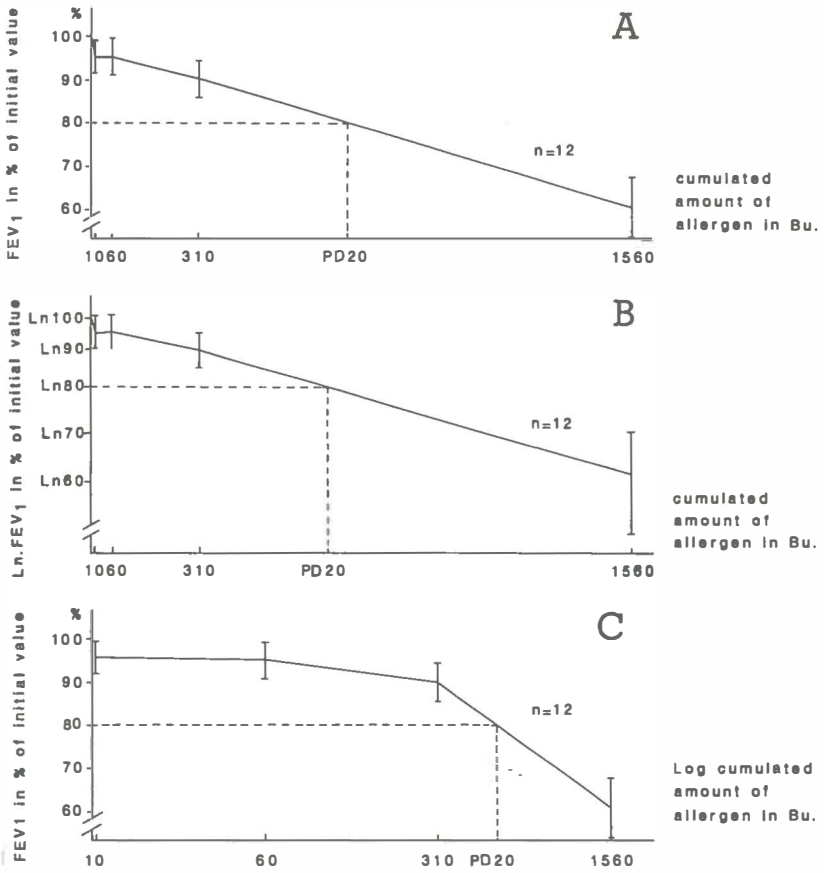

Fig. 3. For 12 patients selected for matching allergy as assessed by the same first allergen dose that induced an EAR of at least 20%, the dose-response relationship is shown. On the x-axis the cumulated amount of allergen (BU); on the y-axis the corresponding mean decrease in FEV_1 as a percentage of the initial value ($\pm$ SD) and corrected for the control day.

A) decrease in FEV_1 with allergen dose;

B) a logarithmic transformation of the y-axis with dose;

C) a logarithmically transformed x-axis with decrease in FEV_1.

A significantly better r value was found for transformation B as compared to both transformation A and C.

stead of a constant amount, logarithmic transformation of the dose axis results in a regular dose pattern. In our opinion this advantage does not counterbalance the inflation of the plateau that results and the fundamental inadequacy of this transformation.

Correlation coefficients were calculated for a linear (2a) and a (negative) exponential (2b) approach. The correlation coefficients found for the exponential transformation were significantly ($p = 0.001$) better than the ones calculated for the linear approach (see Figure 3 and Table 1). From these results was concluded that a negative exponential relation fits the data reasonably well.

It was impossible to construct a similar dose-response curve for the late reaction from the material; therefore this reaction is supposed to behave similarly.

5.1.4.6. Derivation of the dependent variables (Response defined for allergen dose and minimal FEV_1)

If indeed a negative exponential function adequately describes the dose-response relation for the allergic reaction in the bronchi then the formula:

Table 1. Mean correlation coefficients (± SD) for transformed and untransformed dose (BU) and response (FEV in % of corresponding value during control day corrected for the initial value). A significantly better mean correlation-coefficient was found for the dose/ln response transformation as compared to the two other mean correlation coefficients (two tailed student T test). See also figure 3.

	0 BU	10 BU	60 BU	310 BU	1560 BU	r dose/resp		r dose/ln resp		r ln dose/resp
1	100	93	85	90	75	− 0,8403		− 0,8614		− 0,7995
2	100	93	97	103	62	− 0,9270		− 0,9414		− 0,5995
3	100	90	86	84	59	− 0,9491		− 0,9687		− 0,8638
4	100	88	88	85	68	− 0,9080		− 0,9331		− 0,8357
5	100	98	91	96	63	− 0,9672		− 0,9728		− 0,7854
6	100	100	98	93	69	− 0,9993	p = 0,01	− 0,9998	p = 0,0001	− 0,8748
7	100	84	98	87	68	− 0,8694		− 0,8943		− 0,5951
8	100	97	102	95	51	− 0,9897		− 0,9893		− 0,7772
9	100	100	98	83	51	− 0,9878		− 0,9963		− 0,9146
10	100	98	103	96	77	− 0,9794		− 0,9832		− 0,7791
11	100	104	104	85	50	− 0,9836		− 0,9940		− 0,9081
12	100	99	95	87	46	− 0,9985		− 0,9992		− 0,8760
				mean		− 0,9499		− 0,9611		− 0,8007

$$y = y_0 . e^{-cx}$$

or

$$\text{minimal 'FEV}_1\text{'} = \text{FEV}_{1(0)} . e^{-\delta \cdot \text{dose}}$$

formalizes this concept.

minimal 'FEV$_1$'	=	the lowest FEV$_1$ measured during the early or late reaction = 1−(ΔFEV$_1$max/100)
FEV$_{1(0)}$	=	corrected initial value, being 1 by definition
δ	=	a constant specific for each patient
dose	=	the delivered dose of allergen (BU)

A logarithmic transformation of the formula will result in a linear relation:

$$\ln (\text{minimal 'FEV}_1\text{'}) = -\delta . \text{dose}$$

So δ is a measure of the dose dependence of the ln (minimal 'FEV$_1$') for a certain patient. In other words, δ is a measure for the intensity of the reaction, or of the angle of the dose-response graph.

An alternative measure is PD_{20}. As mentioned before it is defined as the dose necessary to get a minimal 'FEV$_1$' of 0.8. It is easily derived from δ as follows:

$$e^{-\delta \cdot PD_{20}} = 0.8$$

$$-\delta \cdot PD_{20} = \ln (0.8)$$

$$PD_{20} = \frac{-\ln (0.8)}{\delta}$$

So PD_{20} is inversely proportional to δ and may be considered a possible measure for the specific reaction. There is a certain similarity between antigen dose versus minimal FEV$_1$ and histamine (acetylcholine) dose versus minimal FEV$_1$, so it seemed appropriate to use a similar expression for both kinds of reaction. For histamine and acetylcholine it has become general practice to use PC_{15}'s or PC_{20}'s. Therefore its antigen analogon PD_{20} is preferred to the alternative δ.

5.1.4.7. Calculation of the dependent variables

Since ln (minimal FEV_1) = $-\delta \cdot$dose

$$\delta = \frac{-\ln (\text{minimal FEV})}{\text{dose}}$$

By substituting δ in the PD_{20} formula, PD_{20} may be written as

$$PD_{20} = \frac{\ln (0.8)\ \text{dose}}{\ln (\text{minimal FEV})}$$

or

$$PD_{20} = \frac{(-0.223 \times \text{dose})}{\ln (1-(\Delta FEV_{1,\,max}/100))}$$

For the EAR the PD_{20} calculated by application of this formula correlates well with PD_{20} values obtained by linear intrapolation ($r=0.98$, $\Delta FEV_1 max > 20$, $n=23$) (see Figure 4). This was also the case when the forelast allergen dose and the corresponding FEV_1 values were used to calculate the 'PD'_{20} value (for $10 < \Delta FEV_1 max < 20$; $r = 0.94$, $n = 10$). Assuming a comparable dose-response relation for EAR and LAR, the severity of the EAR and LAR can be expressed both as a PD_{20} value based on the observed minimal FEV_1 and the delivered dose of allergen. In conclusion the dependent variables have thus been defined and can be applied in the MLR equations.

5.1.5. Specifying the MLR-model for EAR and LAR

The independent or explaining variables (age, sex, etc.) will be entered successively into the MLR-formula after transformation. Transformations that are commonly applied to make the variables approximately normally distributed were used for the predictor variables.

Two separate but analogous models were made for EAR and LAR. By analogous is meant, that the same variable-transformations are used for the models and that they are built by MLR. So in the following both reactions will be discussed together.

The intensity of the asthmatic reaction, independent of allergen dose, (PD_{20}) is the modelling aim.

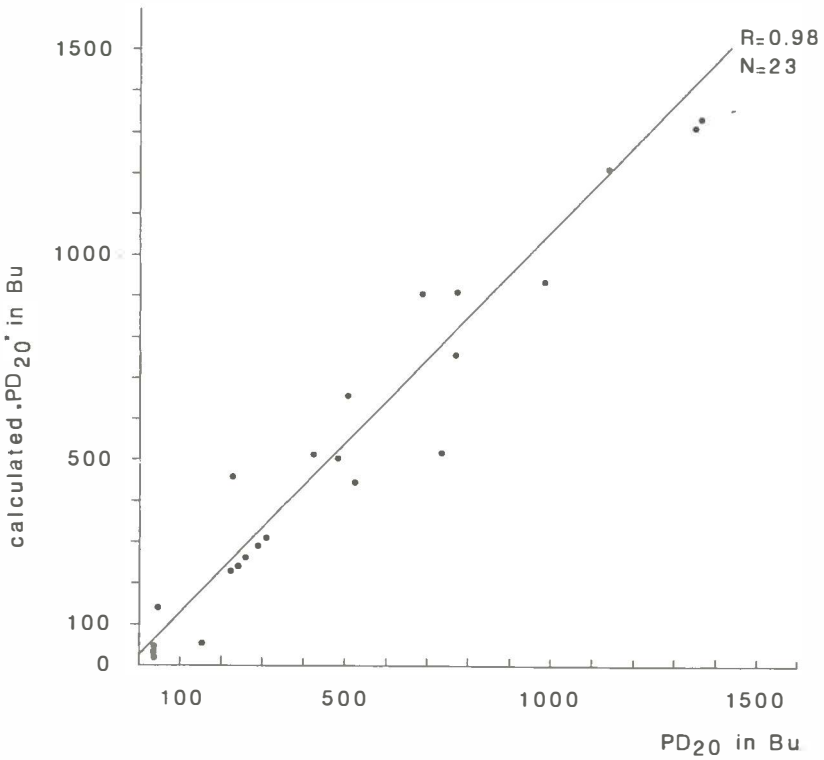

Fig. 4. Correlation between the calculated 'PD_{20}' value based on a negative exponential dose-response relationship for the EAR and the PD_{20} value measured by linear interpolation. A high degree of correlation was found ($r = 0.98$; $p = 0.0005$).

The more 'asthmatic' a patient is, the lower will be his PD_{20}, approaching, but never actually reaching, zero.

As discussed above, in such cases a negative exponential relationship can be assumed, and a logarithmic transformation of PD_{20} will give a linear relationship with the 'severity of the disease'. Thus, the logarithmically transformed PD_{20}, ln (PD_{20}), is considered the best transformation for the dependent variable.

Optimally all transformations should be based on medical expertise and not on the data themselves to get the best prospective power. However, in this study, also a more heuristic approach based on normal distribution of the residuals and linearity was used in the choice of the transformation of the variables.

The resulting model formula can be read from Table 2.

The regression coefficients and the relevance of the predictor variables will be tested in Chapter 5.2.

5.1.6. Discussion

An effort was made to fit the size of the EAR and LAR in mathematical 'models'. Since the factors contributing to the development of these bron-

Table 2. Transformation of the independent variables.

Untransformed variable	dimension	transformed variable
1. age	yr	x_1 = age
2. sex 0 = 1 0 = 2	–	x_2 = sex
3. total IgE	IU	x_3 = ln (IgE_{tot})
4. IgE specific to HDM	– (RAST score)	x_4 = IgE_{spec}
5. eosinophil cells	$\frac{no}{vol}$	x_5 = ln (eos)
6. PC_{15} histamine	$\frac{mg}{ml}$	x_6 = ln (PC_{15} Hist)
7. PC_{15} acetylcholine	$\frac{mg}{ml}$	x_7 = ln (PC_{15} Ach)
8. forced expiratory volume in one second	% of predicted value	x_8 = FEV_1
9. vital capacity	% of predicted value	x_9 = VC
10. specific work of breathing	% of predicted value	x_{10} = Wsp
11. early cutaneous reaction	– (score)	x_{11} = ECR
12. late cutaneous reaction	– (score)	x_{12} = LCR

chial obstructive reactions are only partially known and cannot be measured directly, a formal statistical approach was chosen using data from commonly applied tests for bronchial asthma.

In order to apply MLR, however, the variables had to be defined. The dependent variables i.e. the obstructive reactions, can be measured in terms of variation in FEV_1 as a function of time (early and late reactions) when a single allergen concentration is given. Since in this study four fivefold increasing allergen concentrations were used to provoke the reaction, the amount of allergen causing a decrease of 20% in FEV_1 was taken as a measure of the asthmatic reaction ('PD_{20}'). In order to compare patients with different PD_{20} values the dose-response relationship for allergen provocation first had to be worked out.

A dose ln (response) transformation was shown to be preferable to an untransformed or ln (dose) response transformed relationship for the EAR.

This indicates that the untransformed curve can be described as a power of e. (However, the difference with the linear dose response relation for the studied part of the graph is small).

In order to compare the influence of the independent variables on the EAR and LAR the severity of these reactions had to be expressed identically. For the EAR a 'PD_{20}' value calculated from the initial value of FEV_1, the 'minimal FEV_1' and the corresponding dose of allergen was shown to be highly correlated to a PD_{20} value obtained from the conventional linear interpolation. When the same dose response relation for the EAR is assumed for the LAR, the LAR can also be expressed as a 'PD_{20}' value. Finally, conventional transformations of the independent variables were proposed to enter in a linear equation between dependent and independent variables.

When trying to model biological systems like the early and late allergic asthmatic reactions, one is confronted with the limited knowledge of the underlying principles that determine these systems.

It is often not possible to find a physiologically based exact mathematical description of (parts of) such a system. In this study the authors propose a model that is based on prior medical knowledge and some experimental data. Although the model obtained may not be optimal, it may still add substantially to our ability to predict the allergic asthmatic reactions. It must be stressed, however, that the value of this model must be justified by its application in practice. In Chapter 5.2 the application of

the model to patient data will be discussed, and its strengths and weaknesses will be shown.

Acknowledgements

We are indepted to Drs J.E. Jonker, department of Clinical Methodology, University Hospital Groningen and to Dr L.Th. van der Weele, Computer Center, University Groningen.

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CHAPTER 5.2

Modelling the early and late allergic asthmatic reactions

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5.2. Prediction of the early and late reaction

5.2.1. Abstract

In this part of the study the multiple linear regression MLR models that were developed for the bronchial obstructive reaction following house dust mite inhalation were evaluated.

As proposed in Chapter 5.1, the dependent variables for the early reaction (EAR) and the late reaction (LAR) were expressed as ln 'PD_{20}' values, whereas transformations of twelve features measured prior to allergen challenge served as independent variables.

Firstly the regression coefficients of the predictor variables for EAR and LAR separately were determined using data of 29 allergic asthmatic patients. Estimation of the size of the EAR based on the obtained MLR equation showed a significant correlation with the observed PD_{20} value ($r=0.87$, 76% of the variation explained).

Secondly using stepwise regression the relative importance of the predictor variables in the model was determined.

Consequently a new model was constructed based only on those variables that offered more information than 'noise'. This reduced model proved to be almost as good as the complete model ($r=0.84$, 71% of the variation explained).

With a Leave-One-Out-Method (LOOM) the reduced model was shown to have a considerably higher predictive power (58% explained) than the full model (25% explained).

For the LAR, the correlation between the calculated ln ('PD_{20}') with the ln (PD_{20}) measured during allergen provocation was 0.86 (75% of the variation was

explained). Again variable selection hardly influenced the explanatory power of the model ($r=0.85$, 72% of the variation explained).

With LOOM the full model explained only 2%, whereas the reduced model explained 50%.

The correlation between the size of the EAR and the LAR was 0.47 ($p=0.005$).

In this group of house dust mite sensitive asthmatic patients the early reaction could be predicted mainly by taking into account indices of bronchial hyperreactivity, the number of eosinophils and pre-challenge bronchial obstruction.

The occurrence of the LAR could be predicted in the same population mainly by taking into account the severity of the EAR and/or the degree of bronchial hyperreactivity and the number of eosinophil cells prior to allergen challenge.

The number of eosinophils was more closely related to the LAR than to the EAR.

5.2.2. Introduction

In the first part of this study (5.1) an MLR equation was proposed as a model for the EAR and LAR.

The bronchial dose response relation for the EAR was shown to fit a logarithmic function, and a 'PD_{20}' value based on interpolation of the 'lung function' for dose is 0 and the final dose of allergen was proven to be a good measure for the size of the EAR. We assumed that the late asthmatic reaction (LAR) could be expressed similarly. Transformations for twelve independent variables expected to be relevant for the size of EAR and LAR, were proposed in order to approach a linear relationship with the transformed dependent variables: ln 'PD_{20}' early and ln 'PD_{20}' late.

In this part of the study the regression coefficient of the predictor variables was estimated for the EAR and LAR separately, using the data of 29 allergic asthmatic patients obtained prior to allergen challenge and the subsequent bronchial response. The quality of the model was then evaluated.

This was done in three different ways:

Resubstitution into the full model

By comparing the size of EAR and LAR calculated from the MLR equation with the observed values.

Resubstitution into a reduced model

After selection of the most relevant variables by stepwise regression a reduced MLR model is made. This technique gives an understanding of the relative importance of the independent variables in the regression equation.

Leave-One-Out-Method (LOOM)

By testing the 'predictive' value of the model using the LOOM. This also enables a calculation of the uncertainty of the prediction of the EAR and LAR.

5.2.2.1. Factors influencing the occurrence of EAR and LAR

The house dust mite induced EAR has only been described in patients with elevated amounts of allergen specific IgE.

Gökemeyer (2), Killian (3), Cockcroft (4), Neyens (5) and Keyzer (6) all have shown that the severity of the early asthmatic reaction is not only dependent on the presence and level of specific IgE antibodies, but also on the degree of bronchial hyperreactivity.

As to the late asthmatic reaction much less is known. Antigen specific IgE at least in late cutaneous reaction (LCR) was shown to be an essential prerequisite to develop the reaction (7) and the early cutaneous reaction (ECR) might have a 'gate keeper' function in the development of the LCR (8). Thus in these studies the association of EAR and LAR was also studied.

Although some studies have suggested a relation between specific IgG antibodies and the LAR (9) this could not be confirmed by later studies (10).

Gökemeyer (2) studying house dust-induced reactions found that the occurrence of a late asthmatic reaction was correlated to the degree of bronchial hyperreactivity as well as (weakly) to the late skin reaction. In the group of patients showing a late asthmatic reaction a higher blood eosinophilia was found. Since data from the literature suggest that the eosinophil cell is associated with the LAR, we evaluated the influence of the number of eosinophils as the severity of the LAR (2, 11, 12). In Gökemeyer's thesis (2) patients from the LAR group required less allergen to elicit

an EAR than those who did not show a LAR following allergen challenge. The pre-challenge FEV_1 (% predicted) in the LAR group was lower than in the patients who had only developed an EAR. However, the relative importance and the interrelationship of all these factors was not studied.

Table 1. Clinical data.

Patient + no	Age	Sex (1 = m, 2 = F)	Total IgE	Anti HDM IgE (rasclass)	Eosinophilis x 11/mm³	PC_{15} Hist	PC_{15} Ach
1	29	1	803	4	21	1.55	0.96
2	23	1	163	4	42	11.27	4.98
3	21	2	782	3	30	11.64	5.36
4	15	1	500	4	44	7.47	29.36
5	16	1	620	4	44	20.79	10.19
6	25	1	89	4	18	4.73	2.00
7	16	2	1650	3	19	0.98	0.59
8	24	2	41	2	32	2.10	6.51
9	39	1	803	3	48	32.00	6.30
10	31	1	130	4	16	6.52	12.00
11	19	1	1100	4	18	0.74	12.38
12	25	1	170	4	36	5.77	11.67
13	19	2	1755	3	43	4.00	14.10
14	20	1	900	4	38	1.60	1.46
15	37	1	264	4	22	5.25	14.40
16	23	2	1850	4	28	13.77	20.58
17	35	2	324	4	35	0.49	1.45
18	40	1	71	4	22	1.42	8.09
19	16	2	650	4	41	1.59	1.46
20	22	2	803	0	8	28.29	8.89
21	21	1	675	3	14	19.52	22.67
22	18	2	1497	4	24	16.83	27.29
23	24	2	2836	4	100	3.14	30.40
24	15	1	170	4	14	3.04	10.84
25	31	2	1160	4	26	1.18	6.20
26	35	1	5408	4	20	3.17	1.23
27	32	2	597	2	10	64.00	53.64
28	16	1	1336	4	49	64.00	52.89
29	46	1	110	0	2	64.00	16.50
mean ± sd	25.3 ± 8.3	18♂ 11♀	939 ± 1086	3.5 ± 3.2	29.8 ± 18.6	13.8 ± 19.2	13.6 ± 13.9

5.2.3. Patients and methods

From patients admitted to the hospital for an elective evaluation of their asthma between July 1981 and July 1984, 29 were selected with a history of perennial asthma usually with exacerbations in autumn, and a positive

VC % pred.	FEV_1 % pred.	Wsp % pred.	Early cutaneous reaction no of plus signs	Late cutaneous reaction no of plus signs	Δ FEV_1 early	Δ FEV_1 late	Dose HDM in B.U.
113	83	147	3.0	3.0	23	26	310
107	106	48	2.5	3.0	54	57	1560
99	100	86	3.0	3.0	37	40	1560
92	75	126	3.0	1.0	24	28	1560
86	67	194	3.0	0	68	41	1560
112	91	183	3.0	2.0	50	31	1560
88	56	130	2.5	3.0	32	38	310
97	91	120	3.0	0	32	68	1560
104	73	132	2.0	3.0	28	28	60
101	82	100	3.0	3.0	59	23	1560
105	48	217	2.5	0	53	60	1560
96	94	39	2.5	2.0	31	18	1560
107	86	150	3.0	3.0	40	32	60
85	80	142	3.0	3.0	26	47	60
100	105	111	2.5	3.0	32	28	1560
98	95	83	3.0	3.0	32	52	1560
87	60	186	2.5	3.0	32	48	60
97	60	244	2.5	3.0	34	20	310
79	63	168	2.5	3.0	28	46	310
107	110	43	2.0	0	37	16	1560
106	88	95	2.0	0	10	16	1560
88	100	50	2.5	3.0	1	16	1560
79	77	168	2.0	2.0	45	48	1560
78	67	112	2.0	3.0	31	13	1560
99	57	209	2.5	3.0	34	10	310
60	52	230	2.0	0	17	5	1560
72	79	95	3.0	0	6	4	1560
94	85	93	3.0	3.0	6	4	1560
97	105	90	1.0	0	3	2	1560
94.2% ± 12.5	80% ± 17,8	130.7% ± 56	2.6 ± 4.9	2.0 ± 1.4	31 ± 17	30 ± 18	1138 ± 645

skin test to house dust mite extract (Diephuis Allergen Laboratory, Groningen, The Netherlands). The mean age of the patients was 25.3 yr (s.d. ± 8.6 yr), 17 were male, 12 female. See Table 1. All 29 patients had been investigated according to exactly the same protocol. On day 1, 2 and 3 all medication was withheld. In the selected group of patients this did not result in a decline in FEV_1 of more than 15%. On day 4 plasma samples were drawn for total and specific IgE and eosinophil counts. Skin tests were performed as well as histamine (1–32 mg/ml) and acetylcholine (1–256 mg/ml) challenge and determination of the specific work of breathing. On day 5 a control fluid inhalation was carried out followed by FEV_1 determination every 10 minutes for the first hour and every hour up until 8 hours after inhalation. During the control day variations not greater than 15% occurred in this patient group. On day 6 allergen challenge was carried out by inhaling five-fold stepwise increasing concentrations of house dust mite extract (50, 250, 1250, 6250 biological units per milliliter (BU/ml) with 15 minutes interval. If a fall of more than 20% from the initial value occurred the inhalation was stopped. The lowest FEV_1 during the first hour was taken as a measure of the EAR while the lowest FEV_1 between 3 and 8 hours after allergen inhalation, compared to control day, was taken as a measure of the late asthmatic reaction. As explained before, LAR and EAR were expressed as ln 'PD_{20}''s.

$$\ln\ (\text{'PD'}_{20}) = \ln \left[\frac{(-0{,}223 \times \text{dose})}{\ln\ (1-(\Delta FEV_{1,\ max}/100))} \right]$$

where $\Delta FEV_1 max$ = the maximal difference between FEV_1 during control day and the test day at the same time of the day, corrected for the initial value (see 5.1.).

In order to study the influence of the dose, the LAR and EAR were also expressed independently of the dose as: ln $[1-(\Delta FEV_1 max/100).]$

5.2.4. Statistics

5.2.4.1. Multiple linear regression (see also 5.1.).

The usual way to establish the validity of an MLR model is to check if it applies to the data it was constructed from. In the model discussed here one may use the multiple correlation coefficient to estimate the agree-

ment of the PD_{20}'s calculated directly from the size of the asthmatic reaction and the allergen dose inhaled, with the 'PD_{20}''s predicted according to the MLR model.

However, as the model was constructed so that it optimally fits the data under study, one may get a too optimistic impression of the future performance of the model. This can be caused by irrelevant variables in the model. In order to achieve an optimal model, variable selection is carried out.

5.2.4.2. Variable selection

Every measurement consists of information and random fluctuation (noise). Differences in measurements between two patients can thus be divided into a fundamental and an accidental part. Some measurements that are completely redundant offer no information at all about the problem at hand and only bring in noise. It will be clear, that models incorporating these variables may fit the data, but will not accurately predict future outcomes; these models are over-defined as explained above.
So it may be useful to select only informative variables for a reduced model, thus making the model less detailed, but more suitable for future use. Since fewer measurements are required for a reduced model, costs and effort in new studies can be limited.
Several methods for variable selection are available.
In this study the method of Stepwise Regression was employed.
This method starts with a model consisting only of an intercept and the explaining variable that correlates best with the dependent variable (see Table 2). The other explaining variables are successively added to the model in the order of their explanatory power. Here the explanatory power is the reduction of the differences between model and dataset, that is, reduction of residual variance. If a new variable does not contribute to the explanatory power of the whole model the selection of variables is not continued. Moreover, after every selection-step an elimination-step may follow if the least explaining variable in the model could be dispended with without reduction of the power of the model.

The level of significance for selection and elimination (given as F-ratio's) can be chosen by the investigator. In this study $F_{\text{for selection}}$ was 0.6 and $F_{\text{for elimination}}$ was 0.5 (see Table 2).

A more formal introduction into Variable Selection is given by Hocking (13) and Thompson (14, 15).

5.2.4.3. The Leave-One-Out-Method (LOOM)

The best way to get an unbiased impression of the predictive value of a model is to sample new data. This may be too time-consuming and costly. Since a model tends to improve if it is based on more data, dividing available data into a set for model construction (training set) and an evaluation set (test set) necessarily leaves much valuable information unused for model construction. An alternative, more economical and almost unbiased validation check can be obtained if a model is made, using all patient data except one, and this model is tested on the patient that was left out. By using a scheme of comparing the ln PD_{20} of every patient with the predicted 'ln PD_{20}', using all other patients to construct the model, an impression can be obtained of the model validity, because then every model is independent of the test patient.

Of course, the models used for testing are slightly different for each patient, because with every patient a different dataset determines the model. However, if the model is good, it will be stable to these dataset fluctuations. This LOOM was used to validate the model studied in this paper. It was performed using the CLAS program (16).

LOOM is further described by Lachenbruch (17), Lachenbruch and Mickey (18), and Snapinn and Knoke (19, 20).

All calculations were performed using the Control Data Corporation Cyber 170/760 computer of the Groningen University. The ARTHUR program for multivariate data analysis comes from Harper (21). The CLAS program for multiple regression and multivariate classification and evaluation, is presently being developed (16).

5.2.5. Results

All patients tolerated the allergen inhalation without complication, although in some patients a marked reaction required intervention with adrenergic drugs delivered by inhalation. None of the patients had to be treated with oral or parenteral medication.

The mean decrease in FEV was 31% ± 17% for the early, and 30% ± 18% for the late reactions as compared to the corresponding values during the control day (see Table 1).

5.2.5.1. The EAR

Determination of the regression coefficients and resubstitution into the full model.

An estimation of the size of the EAR was made using Multiple Linear Regression, as described in Part I of this series, using the formula:

ln 'PD_{20}' EAR = 8.3
+ 0.34 ln (PC_{15} Hist)
+ 0.30 ln (PC_{15} Ach)
− 1.4 ln (Eos)
− 0.35 VC
− 0.52 LCR
+ 0.037 FEV_1
+ IgE spec.
− 0.0035 Wsp
− 0.60 ECR
+ 0.063 ln (IgE tot.)
− 0.018 Age
+ 0.71 sex.

The multiple correlation coefficient with ln PD_{20} calculated directly from FEV_1max and allergen dose was 0.87 ($p=0.001$), i.e. 76% of the variation in ln "PD_{20}" between different patients could be explained by the model. The significance of the correlation was $p=0.004$.

Bivariate correlation coefficients

In Table 2 the bivariate correlation coefficients for the explaining variables with the EAR are shown. For the EAR a significant correlation was found with ln (PC_{15} Hist), ln (PC_{15} Ach), Wsp, ln (Eos), LCR and FEV_1.

Determination of the regression coefficients and resubstitution into a reduced model

Starting with the 'intercept' and the explaining variable that offered the highest bivariate correlation coefficient (ln PC_{15} Hist) stepwise regression was applied. After selection of the variable FEV_1, ln PC_{15} Hist was removed from the model, since the same and more information could be der-

Table 2. Bivariate correlation (with P-value) and F-level after variable selection for the EAR and twelve independent variables.

	ln 'PD_{20}' EARLY		
	Bivariate correlations		variable selection
	corr. coeff.	prob. value	F-level
ln PC_{15} histamine	0.59	0.001	5.8
ln PC_{15} acetylcholine	0.55	0.002	1.5
ln Eosinophils	−0.44	0.018	1.4
late cutaneous reaction	−0.41	0.027	1.1
FEV_1 (% predicted)	0.40	0.031	0.9
IgE spec.	−0.30	0.110	0.8
Wsp (% predicted)	−0.47	0.010	<0.6
early cutaneous reaction	−0.24	0.210	<0.6
VC (% predicted)	−0.20	0.312	<0.6
ln IgE total	0.02	0.922	<0.6
Sex	−0.06	0.742	<0.6
Age	−0.04	0.854	<0.6

ived from other selected variables (e.g. PC_{15} Ach) in combination (see The Leave-One-Out-Method). The reduced model finally looked as follows:

$$\begin{aligned} \text{ln '}PD_{20}\text{' EAR} = {} & 8.4 \\ & + 0.50 \text{ ln } (PC_{15} \text{ Ach}) \\ & - 1.3 \text{ ln (Eos)} \\ & - 0.053 \text{ VC} \\ & - 0.39 \text{ LCR} \\ & + 0.053\ FEV_1 \\ & + 0.74 \text{ IgE spec.} \end{aligned}$$

The correlation coefficient for this reduced model was not notably lower than for the full model: $r=0.84$ (71% of variation explained). The significance of the correlation was $p=0.001$ (see Figure 1).

The Leave-One-Out-Method (LOOM)

Evaluation of the reduced model using LOOM showed 58% of the variation to be explainable. When LOOM was applied to the full model only 25% of the variation was explainable.

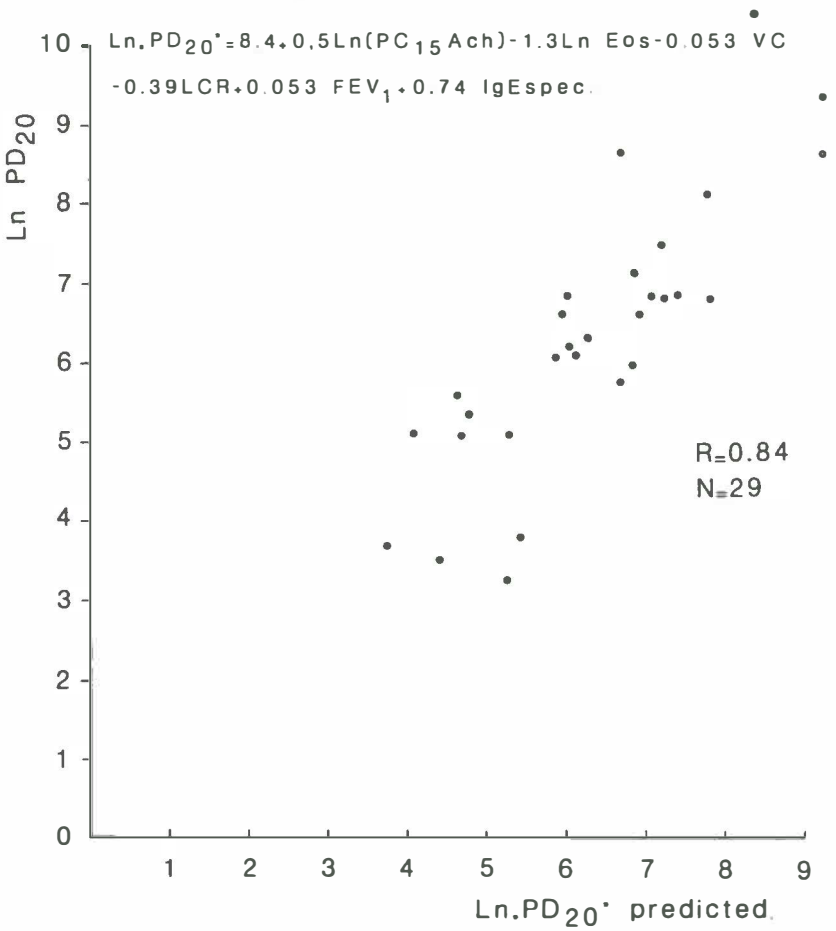


Fig. 1. Correlation between the estimated early ln 'PD_{20}' derived from the reduced formula and the observed ln PD_{20} following allergen inhalation.

Dose dependency of the EAR

If the severity of the EAR was expressed regardless of the dose (ln minimal FEV_1 = ln $1-(\Delta FEV_1 max/100)$ none of the variables showed a significant bivariate correlation with ln early reaction.

The size of the EAR was not correlated to the administered dose, however, if the correlations with all other variables were removed by calculating a semi-partial correlation coefficient, a significant value of $r=0.72$ ($p=0.004$) was found.

5.2.5.2. The LAR

Determination of regression coefficients and resubstitution into the full model

Using an MLR formula as described previously a multiple correlation coefficient of 0.86 was found (75% of the variation explained. The significance of the correlation was $p=0.006$).

ln 'PD_{20}' (LAR) = 10
+ 0.43 ln PC_{15} Hist
+ 0.33 PC_{15} Ach
– 1.5 (ln Eos)
– 0.036 VC
+ 0.90 IgE spec
– 0.40 LCR
+ 0.022 FEV_1
– 0.0027 Wsp
– 0.36 ECR
+ 0.058 ln (IgE tot)
– 0.0092 Age
– 0.066 sex

Bivariate correlation coefficients

In Table 3 the correlation coefficients are given between the LAR (as ln 'PD_{20}') and the explaining variables. Significant correlations were found with ln (PD_{15} Hist), ln (Eos), ln (PC_{15} Ach) and LCR.

Determination of the regression coefficients and resubstitution into a reduced model

Starting with the intercept and the variable that showed the highest bivariate correlation coefficient with ln 'PD_{20}' LAR (ln PC_{15} Hist) step-

Table 3. Bivariate correlation (with P-value) and F-level after variable selection for the LAR and twelve independent variables.

	ln 'PD_{20}' LATE		
	Bivariate correlations		variable selection
	corr. coeff.	prob. value	F-level
ln PC_{15} histamine	0.60	0.001	6.0
ln PC_{15} acetylcholine	0.53	0.004	1.6
ln Eosinophils	- 0.54	0.003	2.9
late cutaneous reaction	- 0.47	0.010	0.7
FEV_1 (% predicted)	0.28	0.136	0.6
IgE spec.	- 0.34	0.071	1.0
Wsp (% predicted)	- 0.32	0.088	< 0.6
early cutaneous reaction	- 0.30	0.112	< 0.6
VC (% predicted)	- 0.20	0.304	1.5
ln IgE total	0.10	0.702	< 0.6
Sex	- 0.23	0.240	< 0.6
Age	0.10	0.584	< 0.6

wise regression was performed resulting in the following 'reduced' model (see Figure 2).

ln 'PD_{20} (LAR)' = 10.4
+ 0.52 ln PC_{15} Hist
− 1.5 ln (Eos)
+ 0.37 ln (PC_{15} Ach)
− 0.027 VC
+ 0.69 IgE spec
− 0.3 LCR

This reduced model gave a correlation coefficient of 0.84 (69% of the variation explained. The significance of the correlation was p=0.001).

The Leave-One-Out-Method (LOOM)

Using the LOOM on the reduced model, still 50% of the variation could be explained. When LOOM was applied to the full model only 2% was explainable.

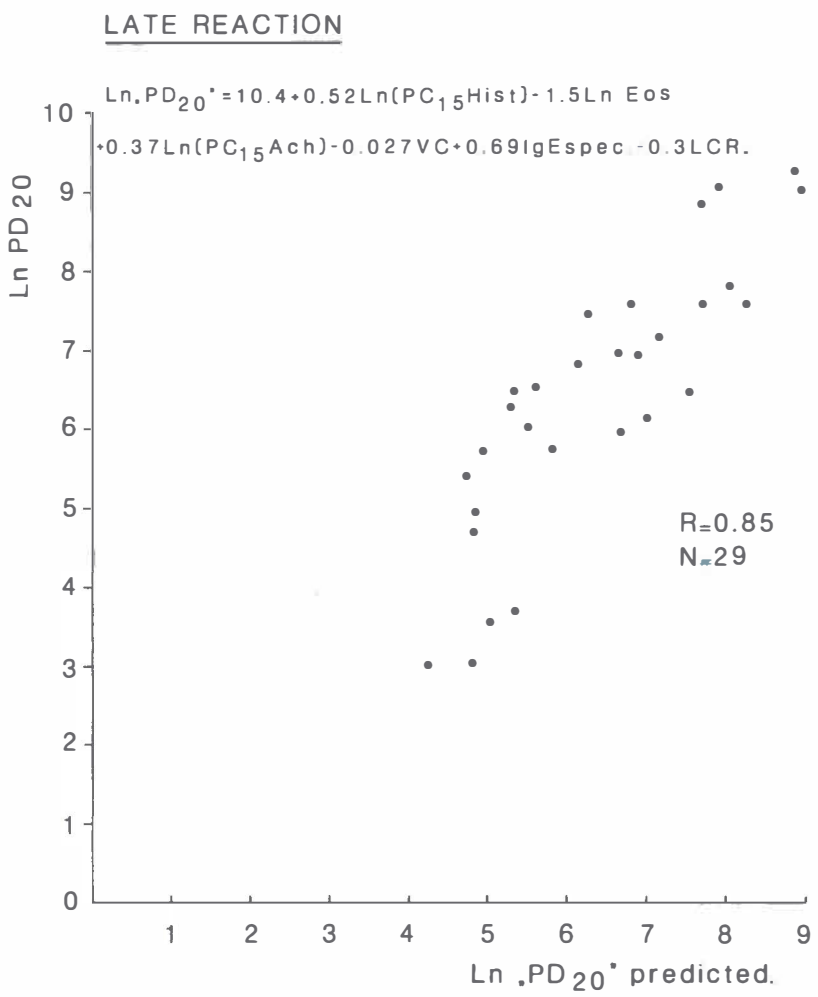


Fig. 2. Correlation between the estimated late ln 'PD_{20}' derived from the reduced formula and the observed ln PD_{20} following allergen inhalation.

Dose dependency of the LAR

If the LAR was expressed regardless of the dose (ln minimal FEV_1 = ln $1-(\Delta FEV_1 max/100)$ a significant correlation was found with ln minimal FEV_1 (early) r = 0.47 (p=0.009), ln eosinophils r=0.45 (p=0.015) and PC_{15} Hist r=0.44 (p=0.018).

The size of the LAR was not correlated to the administered dose; this was neither the case when the correlations with all other variables were removed by calculating a semi-partial correlation coefficient.

However, if the dose was omitted from the LOOM model (reduced formula) only 27% of the variation was explained! (see Figure 3).

The association of the severity of the EAR on the LAR

If a model for the LAR ln ('PD_{20}') was made including the ln 'PD_{20}' of the EAR a reduced model showed an overall correlation of r=0.92, which is higher than a model for the LAR without the ln 'PD_{20}' EAR (r=0.85). The percentage of the variation explained with the LOOM of the above mentioned model was 73% as opposed to 50% by the model without the ln 'PD_{20}' early (see Figure 3).

The contribution of the eosinophil number on the quality of the model for the LAR

If the number of eosinophils was eliminated from the complete model the overall correlation coefficient was reduced to r=0.67 (45% of the variation explained).

Using stepwise regression only PC_{15} Hist and the late skin reaction were selected.

Thus: ln 'PD_{20}' (LAR) = 6.2
+ 0.6 ln (PC_{15} Hist)
− 0.4 LCR.

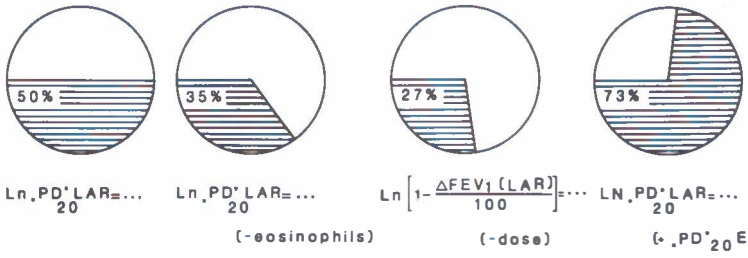
The percentage of the variation explained with the LOOM model of the above mentioned formula was 35%, as opposed to 50% by a formula including the number of eosinophils (see Figure 3).

The number of eosinophils was associated with IgE spec (p=0.001), the ECR (p=0.008) and inversely with age (p=0.05).

5.2.6. Discussion

In 1959 Tiffeneau (22) suggested that knowledge of the skin sensitivity to allergen and the non-specific bronchial hyperreactivity of patients would enable prediction of the sensitivity to inhaled allergen. Indeed

Fig. 3. Prediction of the LAR reduction of the residual sum of squares by applying several models for the LAR.



Gökemeyer (2) and later Cockcroft (4) showed that the early asthmatic reaction was correlated to these two factors. The purpose of this study was to evaluate also the importance of other commonly applied tests for bronchial asthma in predicting the severity of EAR and LAR. Since different concentrations of allergen were applied during bronchoprovocation, the pulmonary dose-response relationship for allergen had to be worked out.

In part I of this study a model was proposed for the early and the late asthmatic reaction in which the dose-response relation for the EAR was shown to be adequately approximated by a negatively exponential curve.

We assumed the dose-response relation for the LAR to be identical to the dose response relation of the EAR. This assumption however, is disputable since in 5.2.5.2. it was shown that the LAR is relatively less dose-dependent than the EAR. So a better understanding of the dose-response relation of the LAR might improve the quality of the model.

To verify the assumed linearity of the underlying models, the Durbin-Watson statistic was calculated. This statistic detects the presence of non-random differences between calculated and real ln (PD_{20})'s. Ideally the statistic should be about 2 in this setting. The value found for the LAR (reduced model) was 2.16 which is not significant at level 5%.

For the EAR (reduced model) the value was 1.45 which is marginally significant at level 5% if tested one-sidedly.

These results can be interpreted as confirming the LAR model, but they suggest that the EAR model is not yet optimal. Nevertheless, the predicted values for EAR and LAR were highly significantly correlated to the observed values.

The models, however, made so as to best fit the data, might only be ap-

plicable to the present data set. Since we wanted to test the predictive value of these models we calculated the predicted 'PD_{20}' using a LOOM and correlated it to the observed PD_{20} values. This decreased the percentage of the variation that was explained, as was to be expected, thus showing the conventional model validation based on the multiple correlation coefficient to be optimistically biased. However, still highly significant correlation coefficients were found.

The accuracy of the 'PD_{20}''s calculated by the model is hard to express in a single score. A linear regression model's accuracy is depending on the values of all independent variables that are measured. For patients with all measured variables equal to the mean value of each variable in this data set, the confidence interval of the calculated ln '(PD_{20})' was $\pm$ 0.402 for the LAR (reduced model) and ln '(PD_{20})' $\pm$ 0.394 for the EAR (reduced model), with a probability of 95%. This means that in this case the 'PD_{20}' is accurate to a factor of exp (0.4) = 1.5 with 95% probability. However, it must be noted, that the further the values of the measured variables deviate from the means, the less accurate the prediction will be.

We found that bronchial hyperreactivity and to a lesser degree the number of eosinophils and the pre-challenge bronchial obstruction give information on the size of the EAR. Since our patients were selected on the presence of a positive early skin test to HDM extract the role of the skin test and of specific IgE is underestimated in this model.

A large part of the variation is still not explained by the model. Apart from defects in the model and variations in the measurement of the variables this might indicate that other, still unknown factors influence the occurrence of the EAR. Such factors might include reactivity of mastcells or other target cells, specific IgG immunoglobulines, etc.

As for the late asthmatic reactions, the highest correlation was found with ln (PC_{15} Hist) the size of the EAR, and ln (Eos); in contrast to the EAR no dose-dependency could be shown. This might be in accordance with the data of van Lookeren Campagne (23) who showed that allergen dose reduction, in a series of bronchial challenges in children with initially dual reaction, first led to a disappearance of the EAR and only with further dose reduction also of the LAR. However, the predictive value of an MLR formula using a PD_{20} value for the LAR was better than a formula not accounting for the delivered dose of allergen (see Fig. 3). The data might suggest that the LAR itself is not dose-dependent but dependent on the severity of the EAR which is dose-dependent. Next to the degree of

bronchial hyperreactivity and the size of the EAR, the number of eosinophilic cells prior to allergen challenge is an important variable for the LAR.

The percentage of the variation that could be predicted with the LOOM was reduced by ± 15% if the eosinophils were omitted. A relation between the eosinophils and the LAR is suggested by several reports. Booy-Noord noted in 1971 (20) that a peripheral eosinophilia occurred in patients showing a LAR to inhaled house dust 24 hours after challenge. Gökemeyer (2) found in patients with dual allergic reactions to house dust, higher eosinophil counts than in patients with single reactions. We recently reported that during the LAR broncho-alveolar eosinophilia occurs which is accompanied by an elevated level of eosinophil cationic protein in the lavage. This was not found in patients with single early reactions or patients with late reactions lavaged prior to the development of the late reaction (21).

Bruynzeel (24) and several other authors have shown that eosinophils are capable of producing the highly spasmogenic leukotriene LTC_4. The leukotrienes seem to be mediators of the LAR in several animals (25) and possibly in man. It is remarkable that the number of eosinophils was highly significantly correlated to the amount of specific IgE suggesting that the eosinophilia that occurs in these patients is generated by an IgE-dependent system. Since some patients show eosinophilia without late reactions, other variables, e.g. parameters of cell activation, must probably also be taken into account to optimize the models proposed here. The general applicability of the models may be negatively influenced by the fact that only HDM sensitive 'admitted' patients without any medication were included in this study. It would be worthwhile to perform comparable studies on a group of out-patients with and without HDM allergy.

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CHAPTER 6

GENERAL DISCUSSION

CHAPTER 6

General discussion

The purpose of this thesis was to investigate the mechanism of the LAR.

Of the several hypotheses that have been put forward to explain this reaction two are the most prominent: (1, 2, 3)

a. The LAR is an IgG-Antigen (immune complex) dependent phenomenon involving activation of the complement cascade, leading to mast cell degranulation and attraction of inflammatory cells (neutrophils, possibly also eosinophils and mononuclear cells). This hypothesis was mainly evaluated in the LCR following injection of fungal antigens (1).
b. The LAR is like the EAR an IgE, and probably also mast cell dependent phenomenon. But as opposed to the EAR the LAR is not merely mast cell mediator dependent since it is accompanied by local infiltration of leukocytes (eosinophils. neutrophils (3)). This hypothesis was mainly based on studies of the pollen induced late cutaneous reaction (LCR) (2).

Several studies have been carried out to obtain evidence for one of these hypotheses in the *lung*, following allergen challenge (4, 5, 6, 7, 8).

These studies, based on sampling of peripheral blood following allergen challenge have been contradictory, and have failed to shed a clear light on the mechanism of the LAR. This thesis presents a new approach by sampling directly from the lung compartment, following allergen (house-dust-mite) challenge. Two other approaches are also presented. These comprise mathematical 'modelling' studies of the EAR and LAR, and *in vitro* investigations on the function of eosinophils and neutrophils.

Although late reactions are known in several tissues (9, 10), some more easily accessible than the lungs, we chose to study the LAR since:

a. Extrapolation from one organ to another may be difficult.
b. The LAR is a clinically relevant problem.
c. In functional terms the LAR is relatively easy to quantify.

However, monitoring the LAR also has several disadvantages:

a. A LAR is for patients tiring and somewhat unpleasant; the procedure is rather time consuming since optimal results can only be obtained when spirometry following allergen provocation is compared to the spirometric values during a control day.
b. If unintentionally a severe LAR is induced, unpleasant and potentially dangerous situations may arise.
c. By increasing bronchial hyperreactivity, severe late reactions may give rise to a (transient) increase in bronchial symptoms (11).
d. The study of the late phase reactions in the lungs does not allow an easy acces to material suitable for histological investigations.

Thus, as mentioned, the LAR has mainly been studied by indirect parameters such as serum levels of immunoglobulins, plasma or urine histamine, or histamine metabolites, plasma levels of chemotactic substances, complement split products, and by the study of activation of peripheral leukocytes following allergen challenge (5, 6, 7, 8, 12).

Since the technique of bronchoalveolar lavage was introduced, cytological studies in bronchial asthma have become possible. Bronchoalveolar lavage causes only moderate discomfort and no hazard to the patients, provided that adequate patient selection and premedication have been carried out (13). Most studies using BAL concern sarcoidosis, idiopathic pulmonary fibrosis and allergic alveolitis. Several studies have been published concerning the correlation between the findings in lung biopsies and the cytology of the BAL fluid (14). Usually a good correlation was shown. When we started performing these studies in asthmatic patients, even following bronchoprovocation, very little was known about the acceptability and possible complications of such an intervention. BAL was said to be impossible in this type of patients 'since it might induce bronchoconstriction', 'the local anaesthetic was not tolerated by asthmatic patients', and/or 'the recovery of fluids during the BAL procedure would not be sufficient to allow any scientific analysis of the obtained material'. Both from our studies (see Chapter 2.1) and from the literature (15) these pessimistic views have not been shown to be realistic. When adequate precautions are taken concerning patient selection, local anaesthesia and pretreatment with an anticholinergic and a sedative, almost every patient tolerated the BAL procedure well. Even in patients with a manifest bronchial obstruction prior to lavage, the recovery of bronchoalveolar lavage fluid (BAL fluid) was sufficient.

In patients with a LAR a local bronchoalveolar eosinophilia was found

(see Chapter 2). This eosinophilia was not present in patients who had only experienced an EAR when lavage was carried out 6 – 7 hrs after allergen inhalation. Moreover, when BAL was carried out 2 – 3 hrs after bronchial challenge in patients who had previously been shown to develop a LAR, no eosinophilia was present. In the LAR group the bronchoalveolar eosinophilia was accompanied by a rise in the eosinophil cationic protein (ECP) in the BAL fluid, probably indicating an increased metabolic activity of these cells. No evidence was found for neutrophil infiltration during the LAR, neither by the analysis of differential cell counts nor by detection of lactoferrin, a neutrophil derived substance, nor by measurement of lysozyme, which may be produced by neutrophils or alveolar macrophages in addition to some production by mucus glands.

In 2.3. is shown that eosinophil cells, in contrast to neutrophil granulocytes, produced a spasmogenic LTC_4 upon stimulation with Zymosan. Since asthmatic patients have been shown to be hypersensitive to leukotrienes (16), infiltration of eosinophils and activation of these cells may be crucial to the development of the LAR.

As the eosinophil was shown to be the main infiltrating cell in asthma (17), and since Frigas (18) showed that in the sputum of patients with asthma exacerbations the eosinophil derived major basic protein (MBP) was strongly elevated, the LAR may serve as a laboratory model for day to day asthma.

The clinical pattern of the LAR is quite different from the EAR (see 1.4.7), but also major biochemical differences can be found.

As shown previously, the EAR is not accompanied by cell infiltration but seems to be strongly mediator-dependent. We found rises in urinary N^t-methylhistamine following the EAR, indicating production of histamine during the EAR, but during the LAR no increase in urinary N^t-methylhistamine was found as reported in Chapter 3. In bronchoalveolar lavage fluid of LAR patients, *higher histamine levels* were found than in the other patient groups. Since histamine in the BAL-fluid may be derived from mechanical injury to mast cells additionally the histamine metabolite N^t-methylhistamine was measured which is a better parameter for histamine production *in vivo* than histamine itself (19). In the LAR group N^t-methylhistamine was not different from controls. This suggests that although some histamine may be produced during the LAR, histamine probably is not the main mediator substance.

Togias et al. found during the late reaction in the nose (20), histamine,

kinines and kininogens and leukotrienes. During the early reaction the same mediators were found, but also *prostaglandin* D_2. Since prostaglandin D_2 is mast cell-dependent, the absence of the latter mediator during the LAR may indicate that histamine elevation during the LAR may not be derived from mast cells but from other histamine-containing cells such as basophil granulocytes. During the late reaction, basophils could be attracted by chemotactic substances and produce mediators directly by binding of IgE and allergen to their surface. Histamine release could also occur by intervention of eosinophils in an IgG-dependent fashion since cationic proteins increase basophil releasability (21). Asthmatic patients have a higher percentage of ('light') eosinophils than normals (22), and hypodense eosinophils produce more LTC_4 than normodense eosinophils (23). The eosinophils from asthmatic patients recently were shown to produce more LTC_4 upon stimulation than normal eosinophils (24).

The question remains, how the eosinophil is 'drawn' into the allergic process. This could happen as a result of chemotactic stimulation by mast cell derived substances or by *direct* activation of IgE receptors on the surface of eosinophils without intervention of (products from) other cells. Several facts argue against such a direct involvement of eosinophils:

a. Stimulation of IgE receptors (with anti-IgE) on eosinophils did not produce an ECP response (while anti IgG did) (25);
b. Stimulation with IgG of eosinophils enhanced LTC_4 production of these cells; this suggests the presence of IgG receptors on the cell surface, which may be a consequence of the contact with chemotactic substances (26);
c. Asthmatic patients without allergen contact or without LAR following allergen challenge have only a marginally increased number of eosinophils (2 – 3%) in BAL fluid, whereas following the LAR a mean of 30% was found (see Chapter 2). Some type of chemotactic stimulus must be present being responsible for this local eosinophilia.

It is thus likely, that prior to local inflammatory and spasmogenic activities of eosinophil cells, chemotaxis from blood and bone marrow occurs. The attraction of eosinophils could be a result of mast cell degranulation since Kay has shown in 1971 that following IgE-mediated stimulation an eosinophil leukocyte chemotactic factor was liberated from human lung (low molecular weight eosinophil chemotactic factor) (27). Other mast cell-derived factors such as platelet activating factor (PAF) are also chemotactic for eosinophils (28). Only indirect arguments can be found

for the involvement of mast cells in eosinophil attraction during the LAR:

A good correlation between the severity of the EAR and the LAR was found in man (see Chapter 5.2) and in an animal model (29). Also in the skin the early and the late phase are correlated (30). Moreover, compound 48/80, a mast cell degranulator, has been shown to induce (relatively small) late cutaneous reactions (LCR). Moreover, if the skin is challenged with allergen at a site previously injected with compound 48/80, both the ECR (early cutaneous reaction) and the LCR are reduced compared to a non-pretreated skin site (31). Finally, also mast cell granules can induce an LCR (32).

However, also arguments against mast cell degranulation as the initial trigger for eosinophil infiltration and the late phase reaction can be found:

a. Beta-agonists are potent mast cell stabilizing compounds (*in vitro*) yet they do not seem to adequately prevent the LAR when given prior to allergen challenge (33). It must be noted, however, that beta-agonists may inhibit only the liberation of preformed mediators such as histamine (34).
b. Holgate found that sodium cromoglycate partially inhibited the airway and plasma histamine responses following allergen challenge in asthmatic patients, but totally inhibited the increases in neutrophil chemotactic factor (NCF). Salbutamol completely inhibited all responses (35). Nevertheless DSCG is more effective in preventing the LAR than beta-mimetic agonists (33);
c. One of the most potent eosinophil chemotactic substances present, is PAF (28). PAF can be derived from mast cells, but also from the alveolar macrophages in much larger amounts present in the lung. Alveolar macrophages (AMs) have IgE receptors, thus also the IgE dependence of the LAR could be explained. However, since IgE receptors on alveolar macrophages are of the low affinity type (36), IgE will bind preferentially to mast cells and basophils. Neutrophil chemotactic activity (NCA), clearly elevated during both EAR and LAR, can be derived from AMs but also from mast cells. However, since NCA was found to be of the high molecular type (HMW)-NCA, the mast cell origin is likely (37).

   Recently plasma HMW-NCA rise following allergen challenge was shown to coincide with a rise in two low molecular weight eosinophil chemotactic factors (LMW-ECF) (38) which could also be derived from mast cells or from macrophages (39).

An argument against the involvement of AM as the sole activator of eosinophils during the LAR is found in the observation that during the LAR no elevation of lysozyme was found in the BAL fluid (see 2.2.). In conclusion, the mast cell is still the most likely source of eosinophil chemotaxis during the LAR. Possibly mast cell heterogeneity between connective tissue mast cells and BAL mast cells may be important. For example, DSCG and the new compound Nedocromil both have been shown to be very effective in inhibiting histamine release from BAL mast cells but much less so in mast cells from dispersed lung (40, 41). DSCG and Nedocromil, however, are not only active on mast cells but also seem to affect eosinophils; this is illustrated by a recent report stating that IgE dependent stimulation of basophils was accompanied by activation of bystander eosinophils (C3b and IgG receptor expression) and that this activation could be blocked by DSCG or Nedocromil (42).

No evidence was found for neutrophil infiltration during the LAR to support the immune complex theory of the LAR (see Chapter 2). Neutrophil infiltration in the lungs usually is associated with a much more extensive tissue injury and occurs during the acute respiratory distress syndrome, the acute phase of extrinsic allergic alveolitis and ABPA (43). Metzger, however, also found eosinophils and neutrophils prior to the LAR. Since he used a *mould allergen* (Alternaria) to induce the late response, his results probably are not directly comparable to the LAR induced by HDM extract. Concerning the immune complex theory of the LAR very little evidence is present from the literature to support such an hypothesis. However, specific (complement binding) IgG_1 antibody level was higher in the serum of LAR patients (see Chapter 4). Since the level of IgG_1 in the BAL fluid was reported to be proportional to the serum level, this slightly elevated IgG_1 might be responsible for the slightly higher complement consumption found in the LAR group compared to the other patients but not compared to the controls. Thus, no convincing evidence for an Arthus phenomenon was found. Since in the BAL fluid of LAR patients an increased number of eosinophils was present, binding of complement products as C1q to these cells might also be an explanation for the observed variation in complement split products. In the BAL fluid of LAR patients elevated IgA levels and an elevated IgA BAL fluid/serum ratio was present, resulting from either local production or active transport. Increased IgA levels in symptomatic asthmatic patients have been found by others (44). Whether this increase in BAL fluid IgA represents

a 'defence' against allergic attacks is presently unknown. IgG levels in the BAL fluid of LAR patients were not increased, while the IgM level is difficult to interpret because of large standard deviations. The observation that eosinophils (see Chapter 2.2.) and IgA are most clearly elevated in pools I and II, underlines the bronchial localization of the LAR.

Finally, the modelling studies presented in Chapter 5, showed the dose-response relation for the EAR to be reasonably well approximated by a negatively exponential curve. This curve allows a calculation of the PD_{20} value for decreases in FEV_1 of both more than 20% and less than 20% ($10 < FEV_1 < 20\%$). A multiple linear regression model (MLR) based on the results of commonly applied tests for bronchial asthma was developed for the EAR and LAR. This predicted the reaction following allergen challenge reasonably well. Next to bronchial hyperreactivity, the number of eosinophils prior to allergen challenge proved to be an important predicting variable (notably for the LAR). The number of blood-eosinophils was significantly ($p = 0.001$) correlated to the serum level of HDM specific IgE. This might also indicate that blood and bronchial eosinophilia in these patients is triggered by IgE dependent mechanisms. A schematic representation of the events that might lead to the LAR is given in Fig. 1.

6.2. Conclusion

Three different approaches to studying the mechanism of the LAR following HDM provocation were employed in this thesis.

a. Cytological and biochemical studies of BAL fluid following bronchoprovocation with house dust mite extract.
b. *In vitro* studies of eosinophils and neutrophils.
c. Mathematical 'modelling' of the EAR and LAR using the results of commonly applied tests for bronchial asthma.

From these studies it can be concluded that the LAR is a bronchial inflammatory response to inhaled allergen. It is characterized by infiltration of eosinophils and probably production of both spasmogenic (LTC_4) and inflammatory (ECP) mediators. This is accompanied by an elevated IgA, and marginally elevated histamine levels. Some complement conversion may occur. Although the local eosinophilia probably is mast cell dependent, no difference in specific IgE were found between the LAR and EAR patients.

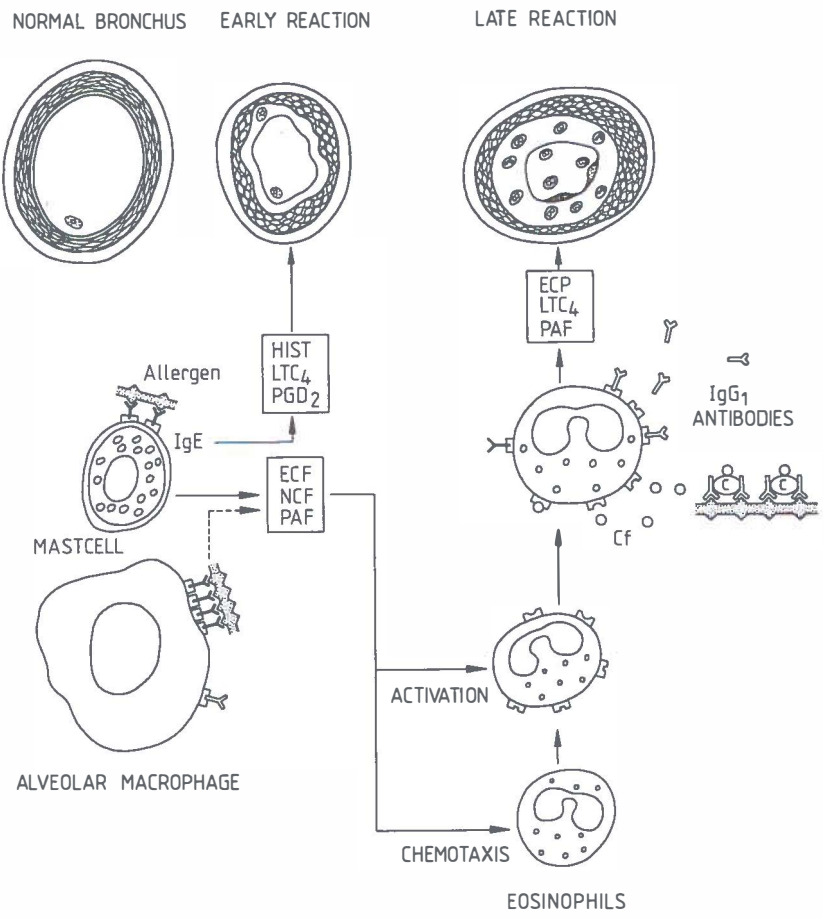

Fig. 1. A schematic representation of the proposed hypothesis. Allergen binds by IgE to mastcells, this results in relase of histamine, leukotriene C_4 prostaglandin D_2 thereby including the 'spasmogenic' early reaction. Release of chemotactic mediators (eosinophil chemotactic factor, neutrophil chemotactic factor, platelet activating factor and others) from mastcells and possibly alveolar macrophages results in infiltration and activation of mainly eosiniphil granulocytes.

Activation of eosinophils enhances IgG and complement receptor expression. Binding of IgG_1, and or complement fragments (Cf) probably stimulates the metabolic activity of eosinophils thereby promoting production of eosinophil cationic protein, leukotriene C_4 and platelet activating factor, that could induce an inflammatory late phase reaction.

The presence of complement conversion products and elevated levels of IgG1 spec in the BAL fluid of LAR patients could have a modulating effect on the metabolic activity of eosinophils.

Thus the mechanism of the LAR cannot be described solely by one of the hypersensitivity reaction types according to Gell and Coombs.

The above described inflammatory response during the LAR thus could be described as an extended type-I hypersensitivity reaction. Further *in vivo* and *in vitro* studies are required to validate the importance of eosinophils, IgG, and complement in the development of the LAR.

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6.3. Summary

In this thesis a number of studies are described concerning the mechanism of the late allergic reactions in the bronchi (LAR). In Chapter 1 a general introduction is given. Since the late allergic reaction was studied in patients with chronic non-specific lung disease (CNSLD, Dutch: CARA = chronische aspecifieke respiratore aandoeningen), this entity is first briefly described (1.2.1.) followed by a characterization of the patients participating in the studies (1.2.3.). In 1.3.1. the hyperreactive phenomena considered relevant to this patient group are introduced. Two special types

of bronchial reactivity (exercise induced asthma (1.3.6.), and aspirin-induced asthma (1.3.7.)) are introduced since these conditions bear certain similarities to allergen-induced bronchoconstriction. In Paragraph 1.4.1., allergy is introduced and defined, followed by an historical overview of allergy including some considerations on the term 'atopy'. Paragraph 1.4.3. contains basic facts of IgE molecules and the measurement of IgE in serum and secretions. Since our studies indicate that both mast cells and eosinophils are effector cells of the immediate type hypersensitivity reaction, these two cell types are introduced in 1.4.4. and 1.4.5. This is followed by an introduction on the late allergic reaction (LAR) in the bronchi, nose, skin, and in animals models (1.4.7.–1.4.9.).

In Paragraph 2.1. is described that during house dust mite (HDM) induced LAR, eosinophil cells infiltrate the bronchial lumen and that this infiltration is accompanied by elevation of the eosinophil derived cationic protein (ECP). Eosinophils and ECP were not found in subjects with only early reactions (EAR), without reactions (NR) nor in controls. When patients with a previously documented LAR were lavaged shortly after the EAR, no increase in eosinophils or in ECP was found. Paragraph 2.2. describes in detail the technique of BAL and the techniques of handling BAL fluid. Furthermore, evidence for eosinophil and neutrophil infiltration during the LAR is discussed.

In Paragraph 2.3. the eosinophil's capacity to produce the spasmogenic sulphidopeptide leukotriene LTC_4 is shown in *in vitro* studies. Neutrophils produce predominantly the chemotactic leukotriene LTB_4. In Chapter 3 the role of histamine in the LAR is discussed. The urinary concentration of the histamine metabolite N^t-methylhistamine was elevated following the EAR but not following the LAR. In BAL fluid, histamine in the LAR group was higher than in other patient groups. This could be caused by mechanical injury to infiltrated histamine-containing cells during the BAL. Thus, N^t-methylhistamine was also measured in BAL fluid. Since no difference in N^t-methylhistamine was found between patients with a LAR and controls following allergen challenge, histamine was thought to be unlikely as an important mediator of the LAR.

In Chapter 4 the relation between the LAR and the level of HDM specific and total immunoglobulins in serum and BAL fluid as well as complement split products in BAL fluid are studied with regard to the immune complex theory of the LAR. No difference in total serum IgE, IgG, IgA, and IgM were found between patients with a LAR and other patient groups.

Of the specific immunoglobulins IgE, IgG, IgG_1 and IgG_4, only IgG_1 was higher in the serum of LAR patients.

Of the complement split products C3, C3c, and C1q, only C1q was significantly depressed in the LAR group compared to the other patient groups. This suggested some complement activation during the LAR, or could be caused by binding of C1q to infiltrating cells since no difference was found between the LAR group and the controls, no support for an Arthus phenomenon during the LAR was obtained. In Chapter 5 a method is described to obtain multiple linear regression models (MLR) for EAR and LAR using the results of commonly applied tests for bronchial asthma. In 5.1. multiple linear regression (MLR) and variable transformation are introduced briefly, followed by a definition of the variables to be predicted: sensitivity to HDM allergen as judged by lung function (FEV_1) during the EAR (PD_{20} EAR) and during the LAR (PD_{20} LAR). In Paragraph 5.2. the regression coefficient of the predictor variables in the MLR formula is calculated using the data of 29 allergic asthmatic patients. Subsequently, the estimation of the size of the EAR and the LAR is correlated to the observed values for EAR and LAR. For both reactions a significant correlation was found. Using stepwise regression the relative importance of the predictor variables in the model was determined. This showed that certain variables did not contribute to the prediction of the asthmatic reactions. Thus, a reduced model was made with only these variables that offered more information than 'noise'. Finally, the predictive value of the models was tested with the leave-one-out method (LOOM). The predictive value of the reduced models showed to be better than the predictive value of the complete model. In this group of asthmatic patients the EAR could be predicted mainly by taking into account indices of bronchial hyperreactivity, number of eosinophils, and pre-challenge bronchial obstruction. The occurrence of the LAR could be predicted by taking into account the severity of the EAR and/or the degree of bronchial hyperreactivity and the number of eosinophils prior to allergen challenge. The number of eosinophils was more closely related to the severity of the LAR than of the EAR.

6.4. Samenvatting

In dit proefschrift wordt een aantal onderzoekingen beschreven aan-

gaande het mechanisme van de late allergische reactie in de longen (Late Asthmatic Reaction, LAR).

Hoofdstuk 1 begint met een algemene inleiding. Aangezien de late reactie bestudeerd werd bij patiënten met chronische aspecifieke longaandoeningen (CARA) wordt eerst dit begrip beschreven (1.2.1.). Hierna wordt een algemene beschrijving gegeven van het type patiënten die participeerden in het onderzoek (1.2.3.).

In paragraaf 1.3.1. wordt het begrip bronchiale hyperreactiviteit, voorzover relevant voor de onderzochte patiëntengroep, geïntroduceerd. Twee bijzondere vormen van bronchiale hyperreactiviteit: door lichaamsinspanning veroorzaakte bronchusobstructie, paragraaf 1.3.6., en aspirinegeïnduceerde bronchusobstructie (1.3.7.) worden besproken aangezien deze reactietypen een bepaalde overeenkomst vertonen met de door allergeen veroorzaakte bronchusobstructie.

In paragraaf 1.4.1. wordt het begrip allergie geïntroduceerd en gedefinieerd. Dit wordt gevolgd door een historisch overzicht en enkele overwegingen betreffende het begrip atopie. Paragraaf 1.4.3. bevat basiskennis over IgE moleculen en de meting van IgE in serum en in secreta. Aangezien onze onderzoekingen erop wijzen dat zowel mestcellen als eosinofiele cellen effector cellen zijn van het onmiddellijke type overgevoeligheid, worden deze beide celtypen geïntroduceerd in 1.4.4. en 1.4.5.

Hoofdstuk 1 eindigt met een inleiding over de late asthmatische reactie (Late Asthmatic Reaction, LAR) en late allergische reacties in de neus, de huid en in diermodellen (1.4.7. tot 1.4.9.).

In hoofdstuk 2.1. wordt beschreven dat, gedurende een door huisstofmijten veroorzaakte late allergische reactie, infiltratie van eosinofiele cellen in de bronchus optreedt en dat deze infiltratie gepaard gaat met een verhoging van de spiegel van het 'eosinophil cationic protein' (ECP) in de broncho alveolaire lavage (BAL)-vloeistof. Patiënten die alleen een vroege bronchusobstructieve reactie hadden doorgemaakt (Early Asthmatic Reaction, EAR) en patiënten die geen reactie hadden doorgemaakt, vertoonden geen verhoogde waarde van eosinofielen of ECP. Wanneer bij patiënten, waarbij tevoren was aangetoond dat zij na allergeeninhalatie late reacties kregen, een BAL werd uitgevoerd direct na de EAR, werd geen toename in de eosinofiele cellen of in ECP gevonden.

Paragraaf 2.2. beschrijft in detail de techniek van de BAL en hoe de BAL-vloeistof gehanteerd werd.

In paragraaf 2.3. wordt het vermogen van de eosinofiele cel om spasmogene leukotriëne LTC_4 te produceren besproken. Naar aanleiding van *in vitro* studies van neutrofiele granulocyten werd aangetoond dat deze cellen voornamelijk het chemotactische leukotriëne LTB_4 produceren.

Hoofdstuk 4 betreft de rol van histamine in de LAR. De concentratie van urinair N^t-methylhistamine (een belangrijke histaminemetaboliet) was verhoogd direkt na de EAR, maar niet tijdens de LAR. In BAL vloeistof van LAR patiënten werd een hogere histaminespiegel gevonden dan in de andere patiëntengroepen; dit lijkt in strijd met de waarnemingen in urine maar zou kunnen worden veroorzaakt door een beschadiging van histamine-bevattende cellen tijdens de bronchuslavage. Bovendien is aangetoond dat tijdens late reactie een lichte toename van histaminebevattende mestcellen optreedt; derhalve werd N^t-methylhistamine ook gemeten in de BAL vloeistof. Aangezien geen verschil in concentratie van N^t-methylhistamine in de BAL vloeistof werd gevonden tussen patiënten met een LAR en kontrolepersonen na allergeen-provocatie, lijkt histamine geen belangrijke mediatorstof te zijn bij de late reactie.

In hoofdstuk 4 wordt de relatie tussen de LAR en specifieke en totale immuunglobulinespiegels in serum en in BAL vloeistof bestudeerd, evenals de aanwezigheid van complementafbraakprodukten. Deze onderzoekingen werden verricht naar aanleiding van de immuuncomplex theorie van de LAR. Tussen patiënten met een LAR en de andere patiëntengroepen werd geen verschil in totaal serum IgE, IgG, IgA, en IgM gevonden. Van de specifieke immuunglobulines IgE, IgG, IgG_1 en IgG_4 was alleen IgG_1 hoger in het serum van de LAR patiënten. In de BAL vloeistof van LAR patiënten werd een verhoogde totaal IgA spiegel gevonden. Totaal een specifiek IgE waren niet verschillend. Van de complementafbraakprodukten C3c, C1q en C3 was alleen C1q verlaagd in de LAR groep vergeleken met de andere patiëntengroepen. Dit zou kunnen wijzen op een lichte mate van complementactivatie tijdens de LAR, maar aangezien geen verschil gevonden werd tussen de LAR groep en de kontrole groep wijzen de gevonden waarnemingen niet op het optreden van immuuncomplex fenomeen tijdens de LAR (Arthusfenomeen).

De daling in C1q was negatief gecorreleerd aan het aantal cellen in de BAL vloeistof. De afname van C1q zou derhalve kunnen worden veroorzaakt door binding aan infiltrerende cellen tijdens de LAR.

In hoofdstuk 5 wordt een methode beschreven om multipele lineaire regressie (MLR) modellen te maken voor de EAR en de LAR gebaseerd

op een aantal bij CARA gebruikelijke laboratoriumbepalingen.

In 5.1. wordt het begrip MLR en de transformatie van variabelen kort geïntroduceerd, gevolgd door een definitie van de te voorspellen variabelen; deze waren: de gevoeligheid voor huisstofmijtallergeen, zoals gemeten wordt aan de hand van de longfunctie (FEV_1). Deze gevoeligheid werd uitgedrukt als een z.g. PD_{20} waarde (zowel voor de EAR als voor de LAR).

In paragraaf 5.2. zijn de regressiecoëfficiënten van de voorspellende variabelen in de MLR formule berekend, gebaseerd op de gegevens van 29 CARA patiënten. Vervolgens werd de sterkte van de EAR en de LAR die gemeten was, vergeleken met de voorspelde waarden. Voor beide reacties werd een significante correlatie gevonden. Met behulp van 'stepwise regression' werd het relatieve belang van de voorspellende variabelen in het model bepaald. Hierbij werd aangetoond dat bepaalde variabelen niet bijdroegen tot de voorspelling van de bronchusobstructieve reacties. Derhalve werd een gereduceerd model gemaakt waarbij alleen die variabelen werden opgenomen die meer informatie dan 'ruis' opleverden.

Tenslotte werd de voorspellende waarde van de modellen getest met de leave-one-out methode (LOOM). De voorspellende waarde van de gereduceerde modellen bleek beter te zijn dan de voorspellende waarde van het complete model. In deze groep CARA patiënten kon de EAR voornamelijk voorspeld worden door rekening te houden met de mate van bronchiale hyperreactiviteit, het aantal eosinofiele cellen en de voor de provocatie bestaande mate van bronchusobstructie. Het optreden van de LAR kon worden voorspeld door rekening te houden met de sterkte van de EAR, de mate van bronchiale hyperreactiviteit en het aantal eosinofiele cellen voor allergeen-provocatie. Het aantal eosinofiele cellen vertoonde een hogere correlatie met de sterkte van de LAR dan met de sterkte van de EAR. Het aantal eosinofiele cellen was op zijn beurt weer hoog gecorreleerd met het specifieke IgE, hetgeen erop zou kunnen wijzen dat de eosinofilie door een IgE-afhankelijk mechanisme werd veroorzaakt.

6.5. Conclusie

Drie verschillende methoden om het mechanisme van de LAR te bestuderen worden in dit proefschrift beschreven.

a. Cytologisch en biochemisch onderzoek van BAL vloeistof verkregen na

bronchoprovocatie met huisstofmijt-extract.

b. *In vitro* onderzoek van eosinofiele en neutrofiele cellen.

c. Mathematische modellen van de EAR en LAR gebaseerd op de resultaten van bij CARA gebruikelijke laboratoriumonderzoekingen.

Op grond van deze studies werd geconcludeerd dat de LAR een bronchiale ontsteking is veroorzaakt door inhalatie van allergeen. De ontsteking wordt gekarakteriseerd door infiltratie van eosinofiele cellen en waarschijnlijk lokale produktie van zowel spasmogenen (LTC_4) als ontstekingsmediatoren (ECP). De locale aanwezigheid van IgGl en complement omzettingsproducten zou bij LAR patiënten een modulerende werking kunnen hebben op de metabole aktiviteit van eosinofiele cellen.

Samenvattend kan het klinisch beeld van de LAR niet alleen worden verklaard op grond van het vrijkomen van factoren uit mestcellen. De reactie wordt mogelijk ook beïnvloed door aanwezigheid van allergeen specifiek IgG, of complement *omzettings*producten. Het mechanisme van de LAR kan derhalve niet volledig worden beschreven aan de hand van een van de allergische reactietypen van Gell en Coombs.

De bovenbeschreven ontstekingsreactie tijdens de LAR zou derhalve beschreven kunnen worden als een uitgebreide type-I overgevoeligheidsreactie. Verdere *in vivo* en *in vitro* onderzoekingen zijn nodig om het belang van eosinofiele cellen, IgG en complementfactoren bij de ontwikkeling van de LAR te kunnen beoordelen.