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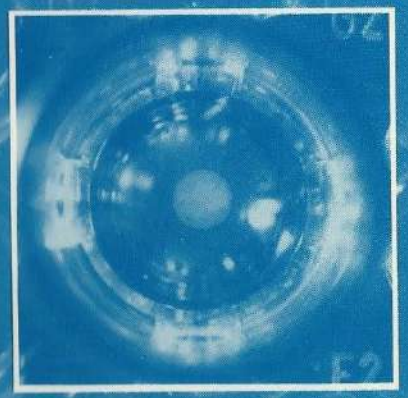
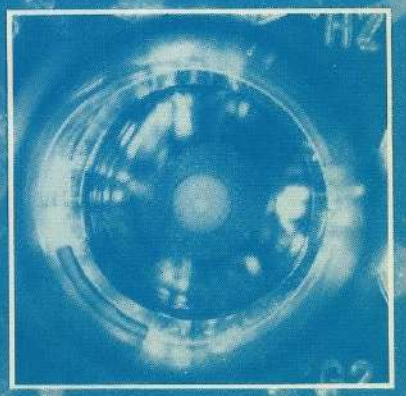
Ella M. van de Plassche-Boers DEFECTS IN CMI IN CHRONIC PURULENT RHINOSINUSITIS

DEFECTS IN CELL-MEDIATED IMMUNITY IN CHRONIC PURULENT RHINOSINUSITIS

Ella M. van de Plassche-Boers





DEFECTS IN CELL-MEDIATED IMMUNITY IN CHRONIC PURULENT RHINOSINUSITIS

Dit proefschrift werd bewerkt in het Pathologisch Instituut van de Vrije Universiteit te Amsterdam (hoofd: prof.dr. C.J.L.M. Meijer), onder supervisie van dr. H.A. Drexhage.
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VOOR FRANK, EDWIN EN ROBIN

CONTENTS

ABBREVIATIONS

BSA	bovine serum albumin
CD	cluster of differentiation
CMI	cell mediated immunity
Con A	concanavalin A
DNCB	dinitrochlorobenzene
DTH	delayed type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
FMLP	formyl-methionyl-leucyl-phenylalanine
IFNγ	γ-interferon
Ig	immunoglobulin
IL	interleukin
HLA	human leukocyte antigens
kD	kilo Dalton
LTT	lymphocyte transformation test
MALT	mucosa-associated lymphoid tissue
MHC	major histocompatibility complex
MI	migration index
MIF	migration inhibition factor
PBL	peripheral blood lymphocytes
PBS	phosphate-buffered saline
PHA	phytohaemagglutinin
PPD	purified protein derivative (tuberculin)
PWM	pokeweed mitogen
RAST	radioallergosorbent test
RLA	rat liver araginase
SI	stimulation index
SK/SD	streptokinase/streptodornase

Surface markers of immunocompetent T-lymphocytes, detected by monoclonal antibodies:

CD2 : OKT11
CD3 : OKT3, Leu-4
CD4 : OKT4, Leu-3
CD8 : OKT8, Leu-2

CHAPTER I

INTRODUCTION

I. INTRODUCTION

I.1. MICRO-ORGANISMS AND IMMUNITY IN CHRONIC PURULENT RHINOSINUSITIS

Therapy-resistant chronic purulent rhinosinusitis is a clinical problem that causes great discomfort to the patients and is responsible for considerable absenteeism.

General symptoms of chronic rhinosinusitis are variable and can be surprisingly mild. The main complaints are nasal obstruction, nasal discharge and post-nasal drip, and a characteristic periodicity of headache with pain increasing upon stooping forward and upon sudden jarring of the body (Wright, 1979b, Ballenger, 1985).

The aim of treatment is to establish adequate drainage of the sinuses by means of medical treatment (topical decongestives and systemic antibiotics) and minor surgical procedures such as antral washouts (Wright, 1979b). If further surgery is required an antrostomy according to Caldwell-Luc is usually performed (Wright, 1979b; Montgomery, Singer and Hamaker, 1985). Recently functional endoscopic surgery of the paranasal sinuses has been introduced (Stammberger, 1986; De Vries and Vuyk, 1988).

Despite these therapeutic modalities it is our experience that 10-20% of the patients continue to have problems. It should be borne in mind, however, that the department of Otorhinolaryngology of our University Hospital is a regional referral centre. Reliable data on the incidence of therapy-resistant chronic purulent rhinosinusitis are not available.

Patients suffering from the clinical symptoms of chronic purulent rhinosinusitis comprise a heterogeneous group. If patients suffering from atopic rhinosinusitis with overt allergic complaints and patients with mucociliary dyskinesia (Van der Baan et al, 1987) are excluded, a large group remains, which is still heterogeneous. In the etiology of chronic purulent rhinosinusitis in this group of patients, various factors may play a role. Firstly the role of micro-organisms has to be considered. Secondly host factors are of importance. These may be divided into local anatomical factors and immunological factors. Investigations as to the role of the immune system in the pathogenesis of this disease have so far mainly focussed on the role of antibodies

against certain micro-organisms (Turk and May, 1967; Moxon, 1981). Much less is known about the role of cell-mediated immune mechanisms.

I.2 MICRO-ORGANISMS

I.2.1. Role of micro-organisms in chronic purulent rhinosinusitis

In health, the anterior third of the nose may contain commensal micro-organisms such as various types of streptococci, Haemophilus influenzae and staphylococci (Wright, 1979a). In acute infections these commensals may rapidly increase in numbers and spread further into the respiratory tract, and since the paranasal sinuses are continuous portions of the upper respiratory tract, they usually participate in these infections (Wright, 1979b, Ballenger, 1985). There are no micro-organisms which have a special affinity for sinus infections (Ballenger, 1985).

The vast majority of acute upper respiratory tract infections are viral in origin (Wright, 1979a). Several viral respiratory pathogens may significantly alter local and/or systemic physical and immune defence mechanisms, paving the way for a secondary bacterial invasion (Veltri, Sprinkle and Ballenger, 1985). Local factors e.g. anatomical abnormalities may be of additional etiological significance and may predispose towards development of a chronic infection (Wright, 1979a).

Chronic rhinosinusitis in many cases follows an incompletely resolved acute rhinosinusitis but it may also appear insidiously (Wright, 1979b, Ballenger, 1985). The maxillary sinus is most frequently affected (Wright, 1979a, 1979b). If prolonged acute sinusitis is not adequately treated a chronic condition is easily established which may persist for several months by which time permanent damage has probably been done to the mucous membrane. Large areas of cilia-bearing cells will be destroyed and the viscous mucus is difficult to transport towards the ostia: the resulting stagnation easily gives rise to further reinfection (Wright, 1979a and b).

Relapsing of purulent infections has not only been observed in the upper but also in the lower respiratory tract (Mulder et al., 1952; Elmes, Knox and Fletcher, 1953; Franklin and Garrod, 1953; May, 1953a and b; May, 1954, Allibone, Allison and Zinnemann, 1956). In both

situations all kinds of commensal bacteria, but most notably non-capsulated H. influenzae, are considered to act as a pathogen (Mulder et al., 1952; Franklin and Garrod, 1953; May, 1953a; May, 1954; Allibone, Allison and Zinnemann, 1956; Mulder, 1956; Turk and May, 1967, see Ch. 6). It is not known why this commensal and others do behave as pathogens under these circumstances, if they indeed act as pathogens.

I.2.2. H. influenzae in chronic purulent rhinosinusitis

For about half a century since its discovery in 1892 by Pfeiffer H. influenzae has been wrongly accused of causing influenza, hence its name (Turk and May, 1967, Ch. 1).

A number of studies in the thirties involving individuals in normal health, studied in their normal environments (Turk and May, Ch. 3), revealed that H. influenzae is in fact a very common inhabitant of the healthy human upper respiratory tract, the vast majority of strains (± 95%) being non-capsulated (Mulder, 1956; Masters et al., 1958; Turk and May, 1967, Ch. 3 and 6).

The six different types of capsulated strains, detected by Pittman in 1931, are pathogens. The most frequently (± 50%) isolated type b is notorious for causing meningitis in children under 3 years of age (Alexander, 1943).

It became clear from several studies (Masters et al., 1958; Turk and May, 1967, Ch. 3) that carrier rates of non-capsulated strains of H. influenzae in healthy individuals varied between 25 and 84% of the people examined at any one occasion. Everyone carries this species from time to time, most individuals probably carry H. influenzae intermittantly in their upper respiratory tracts (Turk and May, 1967, Ch. 3). The healthy bronchial tree, however, is sterile (Brumfitt, Willoughby and Bromley, 1957; Lees and McNaught, 1959 a and b).

An association between H. influenzae and sinusitis was first suggested in 1919 by Crowe and Thacker-Neville and later by others (e.g. Mulder et al., 1952). As mentioned before, H. influenzae is also commonly present in the respiratory tract of patients with chronic bronchitis (Mulder et al., 1952; Elmes, Knox and Fletcher, 1953; Franklin and Garrod, 1953; Allibone, Allison and Zinnemann, 1956; Mulder, 1956; Brumfitt, Willoughby and Bromley, 1957; Lees and McNaught, 1959 a and b;

Turk and May, 1967, Ch. 6). It is not known whether the presence of H. influenzae is a result of the disease, or causative to the disease or due to secondary invasion from their normal habitat, viz. parts of the upper respiratory tract (Turk and May, 1967, Ch. 4 and 6).

I.3. IMMUNE SYSTEM

The defence mechanism of the body against all kinds of pathogens, the immune system, is often divided into innate or non-specific and adaptive or specific immunity. When considering the first site of contact of a pathogen with the immune system, a division into mucosal and systemic immunity can be made.

I.3.1. Mucosal immunity

A variety of both immune and non-immune mechanisms has evolved at mucosal surfaces to protect the host from invasion by pathogens and other environmental macromolecules as well as from subsequent local damage (Cumella and Ogra, 1987). Non-immune mechanisms include low pH, lysozyme and the integrity of the mucous and epithelial barriers that help prevent mucosal penetration by viruses, bacteria and other substances (Cumella and Ogra, 1987). In the respiratory tract, non-immune mechanisms also include mucociliary clearance and surfactant. The hallmark of specific mucosal immunity, however, is the production and action of secretory IgA (sIgA).

In humans, a well-defined mucosal immune system, called MALT (mucosa-associated lymphoid tissue) exists (Cumella and Ogra, 1987). This secretory system includes BALT (bronchus-associated lymphoid tissue), GALT (gut-associated lymphoid tissue, including Peyer's patches in the small intestine), the lymphoid tissues associated with the external genital tract, the conjunctiva, the mammary glands and possibly the tonsils (Cumella and Ogra, 1987; Brandtzaeg, 1988).

The local application of antigen to the upper respiratory tract often leads to an antibody response that is predominantly sIgA and may occur in the absence of a serum antibody response (Scicchitano, Ernst and Bienenstock, 1987). Respiratory tract secretions also contain

variable quantities of secretory IgM, IgG, IgE and even IgD (Bienenstock and Befus, 1980; Cumella and Ogra, 1987; Brandtzaeg, 1988).

sIgA is a dimer linked by a J-chain, produced by mucosal B-cells and provided with a secretory piece which is secreted by epithelial cells (Bienenstock and Befus, 1980; Scicchitano, Ernst and Bienenstock, 1987). sIgA protects mucosal surfaces against viruses and bacteria primarily by immune exclusion: sIgA can block specific antibody sites on microbial cell walls, thus preventing adherence to the epithelium. In this way antigen clearance is a non-inflammatory process (Cumella and Ogra, 1987). Otherwise, once antigens gain access to the circulation they have the capacity to elicit systemic immune responses in which IgG, IgM, complement and to a lesser extent IgA are involved; hence an inflammatory reaction is triggered (Cumella and Ogra, 1987; Scicchitano, Ernst and Bienenstock, 1987). Some evidence indicates that IgA also reduces inflammation at mucosal surfaces by inhibiting neutrophil chemotaxis (Scicchitano, Ernst and Bienenstock, 1987).

Secretory immunity depends on complex and only partly defined regulatory mechanisms. Regulatory events occur primarily in MALT where the B-cells proliferate under the influence of antigen stimulation. T-helper cells and MHC-class II positive macrophages, dendritic cells and epithelial cells are present in the mucosa; these cells play a role in antigen presentation (Brandtzaeg, 1988).

After stimulation the B-cells migrate through lymph and blood to other localizations of MALT all over the body, where they mature to immunoglobulin-producing cells (Brandtzaeg, 1988). This event supports the concept of a common mucosal immune system, first proposed by McDermott and Bienenstock (1979). Evidence is accumulating for a preferential homing of these B-cells due to different endothelial recognition specificities (Van der Brugge-Gamelkoorn and Kraal, 1985; Pals et al., 1989).

There is evidence for a similar migration pathway for T-cells at least in the intestine; T-cells in the respiratory tract have been poorly studied (Pals et al., 1986; Scicchitano, Ernst and Bienenstock, 1987).

Disturbance of the balance between antigen access to MALT, the non-specific defence mechanisms available at a particular site, and the local immune response subsequently evoked may result in disease. To our knowledge no study about the role of these disturbances in chronic purulent rhinosinusitis is now available.

A mucosal response will be elicited if the antigen enters the body via the mucosa. Defective mucosal barriers or other ways of entry may result in contact of the antigen with the systemic immune response.

I.3.2. Systemic immunity

Immune responses can be divided into "humoral" and "cellular" responses. The term "humoral" immune responses comprises the process of the generation of antibodies reactive with a given antigen and the consequent allergic inflammatory reactions (atopic reactions = type I, antibody-mediated cytotoxicity = type II, immune complex-mediated reactivity = type III). An example of "cellular" immune reactivity (or delayed type hypersensitivity = type IV) is the Mantoux reaction towards PPD (PPD = purified protein derivative, a cell wall component of Mycobacterium tuberculosis), which is generated by antigen-specific lymphocytes and by macrophages and macrophage-like cells (classification according to Coombs and Gell).

Lymphocytes responsible for cellular immune responses are predominantly thymus-dependent T-cells; antibody-producing cells are B-cells.

Immune responses can be divided into three stages:

1. The recognition phase, predominantly fulfilled by a special class of cells, the dendritic cells (non-lymphocytic mononuclear cells) and T-lymphocytes. In this process the antigen is recognized as foreign, processed and presented to the T-cells. Even when appropriately presented, most B-cells will not respond directly upon recognition of their antigen. A second signal is needed to trigger the B-cell; this signal is normally provided by T-cells.
2. The production phase. In response T-cells will undergo clonal expansion, while B-cells will expand and mature to antibody-secreting plasma cells. This process normally occurs in lymph nodes and spleen.
3. The effector phase, in which antigen-specific effector T-lymphocytes and antibodies are helpful to the process of antigen elimination. T-cells modulate the B-cell response, are cytotoxic or secrete soluble factors called lymphokines (e.g. IL-2, MIF, IFNγ) which trigger inflammatory reactions by attracting phagocytes (macrophages and

polymorphonuclear granulocytes) and other lymphocytes to the site of reaction. Elimination processes can further be aided by antibody and complement which can either destroy antigens or facilitate their phagocytosis.

I.4. PUTATIVE MECHANISMS OF CHRONIC RELAPSING OF PURULENT RHINOSINUSITIS

Several mechanisms possibly play a role in the disease process of purulent rhinosinusitis becoming chronic.

It is likely that the non-capsulated haemophili and other commensals are unable to establish themselves in the sinuses unless there is a breakdown of the mechanisms which normally prevent bacterial growth. But when this happens H. influenzae and other commensals of the upper respiratory tract invade the sinuses and may aggravate the inflammatory processes going on there (Mulder et al., 1952; Turk and May, 1967, Ch. 7).

The rapidity with which recurrence of infections occurs in the upper and lower airways is suggestive more of relapse than of re-infection (Turk and May, 1967, Ch. 6): throughout the whole period of a specific antibiotic therapy the sputum may remain mucoid, and yet pus and H. influenzae reappear within a week of stopping treatment, especially if there is an associated sinusitis. H. influenzae is probably responsible for the production of pus, a property which it shares with the pneumococcus, its fellow commensal of the upper respiratory tract which can also become a pathogen in the lower part of the respiratory tract (May, 1953a and b; Murdoch et al., 1959; May and May, 1963). Both H. influenzae and Streptococcus pneumoniae have the power to destroy mucin (Himmelweit, 1949).

Anti-bacterial drugs may not penetrate well into the secretions, and these, if not sterilized, can act as an extensive reservoir for organisms capable of causing relapses of infection (Turk and May, 1967, Ch. 8).

The production of toxins capable of inhibiting ciliary movement of the bronchial epithelium by H. influenzae has also been reported (Denny, 1974; Hingley et al., 1986; Sykes et al., 1987).

The pathogenicity of some bacteria, including H. influenzae and S. pneumoniae, may furthermore be related to their ability to secrete a protease specific for IgA (Kilian, Mestecky and Schrohenloher, 1979).

The possibility of defective systemic immune mechanisms as underlying cause has long been suggested (Turk and May, 1967; Ch. 6; Polmar et al., 1972). A few cases based on well-defined deficiencies in humoral immunity have been reported (Palmer, 1976), as well as cases based on immune-complex mediated reactions (Van der Zwan et al., 1977; Clarke, 1979) and on atopic reactivity (Pauwels, Verschraegen and Van der Straeten, 1980). In general however, specific antibody production is intact and anti-bacterial serum titres are either normal or raised (Tunevall, 1952, 1953a and b; Glyn, 1959; May, 1965a and b; Morgan and Wood, 1965; Burns and May, 1967), which is not surprising for commensals (Turk and May, 1967, Ch. 9). The antibodies detected by various methods (e.g. haemagglutination, complement fixation) do not appear to have any protective value (Turk and May, 1967, Ch. 9).

The role of cell-mediated immunity and the factors influencing this type of immunity in relapsing of purulent respiratory infections is not yet clear and have not been investigated extensively.

I.5 INTRODUCTION TO TESTS FOR CELLULAR IMMUNITY IN CLINICAL PRACTICE

Several types of tests are available to assess systemic cell-mediated immunity in clinical practice. The most commonly used are:

1. the quantitation of the different types of lymphocytes in peripheral blood;
2. in vivo "Mantoux-like" skin tests: the delayed type hypersensitivity (DTH) skin tests or type IV reactions;
3. functional in vitro tests, on lymphocytes and monocytes/macrophages.

ad 1. The study of lymphocyte subpopulations was made possible when it was shown that they express different cell surface markers.

Monoclonal antibodies are used to identify human peripheral T-lymphocytes and their subpopulations. Cells are incubated with the specific antibody and stained with a fluorescein-conjugated second antibody to immunoglobulin. Positive cells can be counted with a fluo-

rescent microscope. Alternatively, isolated and stained lymphocytes can be passed through a laser beam and the light emitted by the staining label detected in a sensor - a technique called flow cytometry.

A whole series of monoclonal antibodies is now available which recognize CD antigens (clusters of differentiation) expressed characteristically, but not uniquely, by lymphocytes: CD2 is the T-cell E rosette receptor, CD3 is a cluster of transmembrane proteins associated with the T-cell receptor, antibodies to CD4 recognize T-helper/inducer lymphocytes, and antibodies to CD8 T-suppressor/cytotoxic cells.

B-lymphocytes are not only identifiable by CD21 (C3d receptor) and CD22 (membrane protein) but also by the presence of surface immunoglobulin which has been synthesized by the cells. It is detected by direct immunofluorescence using fluorescein-conjugated antisera to human immunoglobulin. Lymphocytes are incubated with these conjugates, washed and examined by fluorescent microscopy, or by flow cytometry. Surface immunoglobulin is seen as a ring of fluorescence around the margin of the cell.

ad 2. In vivo delayed type hypersensitivity skin tests.

Two types of skin tests are used to detect specifically sensitized T-lymphocytes:

a. the intradermal test, in which antigen is injected into the skin;
b. the patch test, in which antigen is absorbed through the skin.

A positive intradermal skin test (example: the Mantoux test) usually appears at 48-72 hours and then fades over several days. The area of injection is red and indurated but not particularly itchy or painful. The basis of the reaction is formed by the production of lymphokines by specific T-lymphocytes upon exposure to an antigen to which the individual is already sensitized. Skin testing thus assumes that the patient has been previously sensitized to the antigen (named recall antigens, e.g. Candida antigen, streptococcal antigen).

Patch tests are essential in determining which antigen is responsible for contact dermatitis. Patch tests are usually read after 2-4 days; the time taken for a positive reaction to develop depends on the rate of absorption and can vary from 2-7 days. A positive result is indicated by a red, oedematous, itchy and indurated lesion in the area of contact.

ad 3. Functional in vitro tests.

A. The blastogenesis or proliferation of lymphocytes.

When specific lymphocytes are exposed to their corresponding antigens, they respond by changing into blast cells over a few days. This process is called lymphocyte transformation. The proliferative response is best measured by radioactive thymidine incorporation into DNA. Stimulation by antigens is specific, and prior exposure is required. Non-specific mitogens (e.g. PHA and ConA for T-cells; PWM for B-cells) can also be used, without prior exposure. Allogeneic lymphoid cells can be used in the mixed lymphocyte reaction: the stimulation is specific and MHC class II-dependent, prior exposure is hence not required.

B. Lymphokine production by specific T-cells.

Another possibility is the assessment of lymphokines produced by exposure of sensitized cells to their antigen. One of these lymphokines is the macrophage migration inhibition factor (MIF). MIF-production can be measured by the conventional capillary method (George and Vaughan, 1962) or by the microdroplet agarose assay (Harrington and Stastny, 1973; Singh and Khan, 1982). This assay uses the human monocytoid U937 cell-line as indicator cells in agarose microdroplets. Alternatively the inhibition of random neutrophil movement can be measured (LMI = leucocyte migration inhibition).

C. Quantitation of the number of phagocytes and the assessment of phagocyte functions.

Absolute numbers of lymphocytes, neutrophils and monocytes can easily be calculated from the total and differential white cell counts.

Neutrophils can be separated using the density gradient method and then be purified from the deposit by lysis of the contaminating red cells. Monocytes are harvested from the interface and then isolated by adhesion to a flat surface from which the lymphocytes may be washed away, or by additional density gradient centrifugation using Percoll.

Chemotaxis is the directional movement of cells towards a chemoattractant, usually casein or the synthetic peptide FMLP. It can be measured by the conventional "Boyden chamber" assay (Zigmond and Hirsch, 1973; Wilkinson, 1974) or by the polarization assay (Cianciolo and Snyderman, 1981). The polarization of monocytes towards chemoattractants is an early event that precedes this chemotactic response.

Phagocytosis is the ingestion of foreign material. Ingestion can be estimated by incubation of phagocytes with inert particles such as latex beads, or bacteria. Intracellular enzyme activity can be measured either by bacterial killing or by NBT dye reduction (NBT = nitroblue tetrazolium). Phagocytic cells are only able to reduce NBT if they have been activated.

Other possibilities are the chemiluminescence assay based on the ability of activated neutrophils to emit light, and the protein iodination assay, which tests the integrity of the myeloperoxidase-hydrogen peroxide killing mechanism, using ^{125}I.

I.6 AIM OF THIS STUDY

Chronic and relapsing purulent rhinosinusitis resistant to current antibiotic and surgical treatment is an ill-defined disorder. Detection of underlying mechanisms may have consequences for treatment.

The aim of the present investigation was to study cellular immune functions in patients with chronic and relapsing purulent rhinosinusitis resistant to current antibiotic and surgical treatment to establish whether defects of systemic cell-mediated immunity play a role in the pathogenetic mechanism of this disorder. Several chronic disorders, e.g. chronic mucocutaneous candidiasis (Lehner, Wilton and Ivanyi, 1972), chronic pulmonary infections (Polmar, 1976) and pyogenic staphylococcal dermatitis (Hanifin, Ray and Lobitz, 1974) are known to be accompanied by cellular immune defects, viz. microbial-antigen specific defects as assessed by DTH reactivity and MIF-production. A parallel might exist between these disorders and chronic relapsing purulent rhinosinusitis.

For patients to be included in this study, the following eligibility criteria had to be met: duration of the disease at least 18 months, a positive culture for H. influenzae, S. pneumoniae, other streptococci, or staphylococci on one or more occasions, no response to, or only temporary relief after treatment with several courses of antibiotics, failure of surgery to improve the drainage of the ethmoidal and maxillary sinuses to effect a permanent cure, normal levels of total serum IgG, IgM and IgA, total numbers of peripheral blood leucocytes and differential white cell count within normal limits, and no treatment with antibiotics and/or other drugs known to influence the immune system, at least two weeks prior to the time of testing.

The following studies were carried out:

* Non-capsulated Haemophilus influenzae is frequently found in relapsing chronic purulent rhinosinusitis. Antibody production against this commensal micro-organism has been investigated but no disturbances have been detected (Turk and May, 1967; Moxon, 1981). However, T-cell immune responses against H. influenzae have not yet been studied.
  To obtain an antigen preparation from H. influenzae which could be used for studying the cellular immune response against this micro-organism we have tested two forms of the somatic antigen, derived from non-capsulated H. influenzae (Chapter II). The most suitable form of this common H. influenzae antigen for monitoring T-cell reactivity by DTH skin testing was established. An ELISA test for estimating H. influenzae-specific antibodies was developed.

* The most suitable form of the antigen for testing being established, we used this antigen preparation to study DTH skin test reactivity towards H. influenzae in patients suffering from chronic relapsing purulent rhinosinusitis (Chapter III). Skin test data were compared with the outcomes of skin testing with Candidin and Streptokinase/Streptodornase, as well as with in vitro parameters (viz. the enumeration of total numbers of T-cells and T-cell subsets and the lymphocyte transformation tests, LTT).

* The results of the above-mentioned studies showed that defects in antigen-specific T-cell reactivity were present in patients with chronic purulent rhinosinusitis. This prompted us to a more extensive evaluation of different T-cell parameters to commensal micro-organisms (viz. H. influenzae, streptococci and Candida albicans) in chronic purulent rhinosinusitis (chapter IV). Antigen-specific DTH skin test reactivity, lymphocyte transformation tests (LTT), but particularly lymphokine production (MIF) by T-cells were assessed

and compared to each other in patients and controls (healthy volunteers).

To gain insight in the cellular immune functions of these patients, several assays had to be developed. First the most suitable form of the H. influenzae antigen for in vitro T-cell testing was established and the possibility of a direct mitogenic effect of the microbial antigens examined using cord blood. The possibility of B-cell reactivity participating in the blastogenic response was also considered. The disadvantages of the conventional MIF test were circumvented by the development of a modified microdroplet agarose assay.

* Since T-cell defects are often accompanied by impaired monocyte function, we investigated lastly monocyte chemotaxis in chronic purulent rhinosinusitis patients (Chapter V). A modification of the "polarization" assay developed in our laboratory was used (Tan et al., 1986).

The possibility of immunosuppressive serum factors playing a role in the detected defects of cell-mediated immunity in these patients, was also investigated. In particular the role of p15E, the transmembraneous part of a retroviral envelope protein, gp85 has been investigated since this factor is known to suppress the blastogenic response of lymphocytes and the chemotactic response of monocytes of animals and man (Mathes et al., 1978 and 1979; Copelan et al., 1983; Snyderman and Cianciolo, 1984).

REFERENCES

Alexander HE (1943). Experimental basis for treatment of Haemophilus influenzae infections. Am. J. Dis. Child. 66, 160-171.

Allibone EC, Allison PR and Zinneman K (1956). Significance of H. influenzae in bronchiectasis of children. Brit. Med. J. i, 1457-1460.

Baan S van der, Veerman AJP, Heidendahl GAK, Hollander W den and Feenstra L (1987). Primary ciliary dyskinesia and nasal mucociliary clearance. Respiration 52, 69-75.

Ballenger JJ (1985). Paranasal sinus infections. In: Diseases of the nose, throat, ear, head and neck. 13th edition; Lea & Febiger, Philadelphia, pp. 205-217.

Bienenstock J and Befus AD (1980). Review Mucosal Immunity. Immunology 41, 249-270.

Brandtzaeg P (1988). Immunobarriers of the mucosa of the upper respiratory and digestive pathways. Acta Otolaryngol. (Stockh.) 105, 172-180.

Van der Brugge-Gamelkoorn GJ and Kraal G (1985). The specificity of the high endothelial venule in bronchus-associated lymphoid tissue (BALT). J. Immunol. 134, 3746-3750.

Brumfitt W, Willoughby MLN and Bromley LL (1957). An evaluation of sputum examination in chronic bronchitis. Lancet ii, 1306-1308.

Burns MW and May RJ (1967). H. influenzae precipitins in the serum of patients with chronic bronchial disorders. Lancet i, 354-358.

Cianciolo GJ and Snyderman R (1981). Monocyte responsiveness to chemotactic stimuli is a property of a subpopulation of cells that can respond to multiple chemoattractants. J. Clin. Invest. 67, 60-68.

Clarke CW (1979). In vivo and in vitro reactions of Haemophilus influenzae in bronchial obstruction. Br. J. Dis. Chest 73, 373-381.

Copelan EA, Rinehart JJ, Lewis M, Mathes L, Olsen R and Sagone A (1983). The mechanism of retrovirus suppression of human T cell proliferation in vitro. J. Immunol. 131, 2017-2020.

Cumella J and Ogra PL (1987). Mucosal immune system and local immunity. In: Immunity of the ear. Ed. by J. Bernstein and P. Ogra. Raven Press, New York, pp. 135-151.

Denny FW (1974). Effect of a toxin produced by H. influenzae on ciliary respiratory epithelium. J. Inf. Dis. 129, 93-100.

Elmes PC, Knox K and Fletcher CM (1953). Sputum in chronic bronchitis; effects of antibiotics. Lancet ii, 903-906.

Franklin AW and Garrod LP (1953). Chloramphenicol treatment of bronchiectasis in children. Br. Med. J. ii, 1067-1069.

Glynn AA (1959). Antibodies to Haemophilus influenzae in chronic bronchitis. Br. Med. J. ii, 911-914.

George M and Vaughan JH (1962). In vitro cell migration as a model for delayed hypersensitivity. Proc. Soc. exp. Biol. Med. 111, 514-521.

Hanifin JM, Ray LF and Lobitz WC (1974). Immunological reactivity in dermatophytosis. Br. J. Dermatol. 90, 1-8.

Harrington JT and Stastny P (1973). Macrophage migration from an agarose droplet: development of a micromethod for assay of delayed hypersensitivity. J. Immunol. 110, 752-759.

Himmelweit F (1949). Role of bacteria in etiology of epidemic influenza. Trans. Med. Soc. Lond. 65, 438-444.

Hingley ST, Hastie AT, Kueppers F, Higgins MI and Weinbaum G (1986). Bacterial ciliostatic factors; effects on respiratory cilia. Eur. J. Respir. Dis. 69 (suppl. 146), 291-293.

Kilian M, Mestecky J and Schrohenloher RE (1979). Pathogenic species of the genus Haemophilus and Streptococcus pneumoniae produce immunoglobulin A1 protease. Infect. and Immun. 26, 143-149.

Lees AW and McNaught W (1959a). Bacteriology of lower-respiratory tract secretions, sputum and upper-respiratory tract secretions in normals and chronic bronchitis. Lancet ii, 1112-1115.

Lees AW and McNaught W (1959b). Non-tuberculous bacterial flora of sputum and of the upper and lower respiratory tract in pulmonary tuberculosis. Lancet ii, 1115-1117.

Lehner T, Wilton JMA and Ivanyi L (1972). Immunodeficiencies in chronic mucocutaneous candidiasis. Immunology 22, 775-778.

Masters PL, Brumfitt W, Mendez RL and Likar M (1958). Bacterial flora of the upper respiratory tract in Paddington families, 1952-54. Br. Med. J. i, 1200-1205.

Mathes LE, Olsen RG, Hebebrand LC, Hoover EA and Schaller JP (1978). Abrogation of lymphocyte blastogenesis by a feline leukemia virus protein. Nature (Lond.) 274, 687-689.

Mathes LE, Olsen RG, Hebebrand LC, Hoover EA, Schaller JP, Adams PW and Nichols WS (1979). Immunosuppressive properties of a virion polypeptide, a 15.000-dalton protein, from feline leukemia virus. Cancer Res. 39, 950-955.

May JR (1953a). The bacteriology of chronic bronchitis. Lancet ii, 534-537.

May JR (1953b). Antibiotics in chronic bronchitis. Lancet ii, 899-902.

May JR (1954). Pathogenic bacteria in chronic bronchitis. Lancet ii, 839-842.

May JR and May DS (1963). Bacteriology of sputum in bronchiectasis. Tubercle (Lond.) 44, 162-173.

May JR 1965a). Antibodies to Haemophilus influenzae in the sera of patients with chronic bronchitis. J. Path. Bact. 90, 163-174.

May JR (1965b). Haemophilus influenzae antigen-antibody reactions. J. Path. Bact. 90, 379-392.

McDermott MR and Bienenstock J (1979). Evidence for a common mucosal immunologic system. I. Migration of B immunoblasts into intestinal, respiratory and genital tissues. J. Immunol. 122, 1892-1898.

Montgomery W, Singer M and Hamaker R (1985). Surgical treatment of sinus infections. In: Diseases of the nose, throat, ear, head and neck by J.J. Ballenger. 13th edition; Lea & Febiger, Philadelphia, pp. 224-242.

Morgan WKC and Wood WH (1965). Antibody response to Haemophilus influenzae in obstructive airway disease. Lancet i, 1128-1131.

Moxon ER (1981). Immunology of Haemophilus influeanze infections. In: Immunology of human infection, Part I: Bacteria, Mycoplasmae, Chlamydiae and Fungi, Vol. 8, Comprehensive Immunology; eds. RA Good and SB Day, Plenum Medical Book Company, New York, London, pp. 113-138.

Mulder J, Goslings WRO, Van der Plas MC and Lopes Cardozo P (1952). Studies on the treatment with antibacterial drugs of acute and chronic mucopurulent bronchitis caused by H. influenzae. Acta med. Scand. 143, 32-49.

Mulder J (1956). Bacteriology of bronchitis. Proc. Roy. Soc. Med. 49, 773-776.

Murdoch JMcC, Leckie WJM, Downie J, Swain RHA and Gould JC (1959). An evaluation of continuous antibiotic therapy in chronic bronchitis. Br. Med. J. ii, 1277-1285.

Pals ST, Kraal G, Horst E, Groot A de, Scheper RJ, Meijer CJLM (1986). Human lymphocyte-high endothelial venule interaction: organ-selective binding of T and B lymphocyte populations to high endothelium. J. Immunol. 137, 760-763.

Pals ST, Horst E, Scheper RJ, Meijer CJLM (1989). Mechanisms of human lymphocyte migration and their role in the pathogenesis of disease. Immunol. Rev. 108, 111-133.

Pauwels R, Verschraegen G and Van der Straeten M (1980). IgE antibodies to bacteria in patients with bronchial asthma. Allergy 157, 665-669.

Polmar SH, Waldmann TA, Balestra ST, Jost MC and Terry WD (1972). Immunoglobulin E in immunologic deficiency diseases. I. Relation of IgE and IgA to respiratory tract disease in isolated IgE deficiency, IgA deficiency and ataxia telangiectasia. J. Clin. Invest. 51, 326-329.

Polmar SH (1976). Immunodeficiency and pulmonary disease. In: Lungbiology in Health and Disease, eds. CH Kirkpatrick and HY Reynolds, Vol. I, Dekker, New York, pp. 191-209.

Scicchitano R, Ernst P and Bienenstock J (1987). Respiratory tract lymphoid tissue and other effector cells. In: Immunology of the ear. Ed. by J Bernstein and P Ogra. Raven Press, New York, pp. 107-134.

Singh K and Khan A (1982). The usefulness of a monocytoid cell line (U-937) in the macrophage migration inhibition assay. J. Clin. Hemat. Oncol. 12, 29-38.

Snyderman R and Cianciolo GJ (1984). Immunosuppressive activity of the retroviral envelope protein p15E and its possible relationship to neoplasia. Immunol. Today 5, 240-244.

Stammberger H (1986a). Endoscopic endonasal surgery - concepts in treatment of recurring rhinosinusitis. I. Anatomic and pathophysiologic considerations. Otolaryngol. Head Neck Surg. 94, 143-147.

Stammberger H (1986b). Endoscopic endonasal surgery - concepts in treatment of recurring rhinosinusitis. II. Surgical technique. Otolaryngol. Head Neck Surg. 94, 147-156.

Sykes DA, Wilson R, Greenstone M, Currie DC, Steinfort C and Cole PJ (1987). Deleterious effects of purulent sputum sol on human ciliary function in vitro: at least two factors identified. Thorax 42, 256-261.

Tan IB, Drexhage HA, Scheper RJ, von Blomberg-van der Flier BME, de Haan-Meulman M, Snow GB and Balm AJM (1986). Defective monocyte chemotaxis in patients with head and neck cancer. Restorarion after treatment. Arch. Otolaryngol. Head Neck Surg. 112, 541-544.

Tunevall G (1952). Sero-bacteriological investigations of sinusitis in children. Acta Path. Microbiol. Scand. suppl. 93, 291-298.

Tunevall G (1953a). Studies on Haemophilus influenzae: Haemophilus influenzae antigens studied by gel precipitation method. Acta Path. Microbiol. Scand. 32, 193-197.

Tunevall G (1953b). Studies on Haemophilus influenzae: Complement fixation test for H. influenzae antibody. Acta Path. Microbiol. Scand. 32, 258-274.

Turk DC and May JR (1967). Haemophilus influenzae; its clinical importance. The English Universities Press Ltd. London.
Ch. 1: The influenza bacillus, pp. 1-4.
Ch. 3: Distribution of Haemophilus influenzae in healthy human communities, pp. 13-23.
Ch. 4: Haemophilus influenzae as a pathogen, pp. 24-26.
Ch. 6: Bronchial disorders, pp. 39-57.
Ch. 7: Other conditions, pp. 58-70.
Ch. 8: Chemotherapy, pp. 71-94.
Ch. 9: Immunology, pp. 95-105.

Veltri RW, Sprinkle PM and Ballenger JJ (1985). Etiology of inflammatory diseases of the upper respiratory tract. In: Diseases of the nose, throat, ear, head and neck, ed. by JJ Ballenger, 13th edition; Lea & Febiger, Philadelphia, pp. 172-198.

De Vries N and Vuyk HD (1988). Functionele endoscopische chirurgie van de neusbijholten. Ned. Tijdschr. Geneeskd. 132, 910-913.

Wilkinson PC (1974). Outline of a method for measuring chemotaxis. In: Chemotaxis and inflammation. Churchill Livingstone. Edinburg and London; pp. 169-172.

Wright D (1979a). Acute sinusitis. In: Scott-Brown's Diseases of the ear, nose and throat, 4th edition, volume 3: The nose and sinuses. Eds. J Ballantyne and J Croves. Butterworths, London, Boston, Sydney, Wellington, Durban, Toronto, 243-272.

Wright D (1979b). Chronic sinusitis. In: Scott-Brown's Diseases of the ear, nose and throat, 4th edition, volume 3: The nose and the sinuses. Eds. J. Ballantyne and J. Croves. Butterworths, London, Boston, Sydney, Wellington, Durban, Toronto, 274-313.

Zigmond SH and Hirsch JG (1973). Leucocyte locomotion and chemotaxis. New methods for evaluation and demonstration of a cell-derived chemotactic factor. J. Exp. Med. 137, 387-410.

Zwan JC van der, Kaufmann HK, Orie NMG and De Vries K (1977). Bronchoobstructive reactions with antigenic and toxic components of H. influenzae. Acta Tuberc. Pneumol. Belg. 68, 281-284.

CHAPTER II

THE USE OF SOMATIC ANTIGEN OF HAEMOPHILUS INFLUENZAE FOR THE MONITORING OF T CELL-MEDIATED SKIN TEST REACTIVITY IN MAN

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The Use of Somatic Antigen of *Haemophilus influenzae* for the Monitoring of T Cell-Mediated Skin Test Reactivity in Man

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To investigate its usefulness as a skin test antigen, *Haemophilus influenzae* somatic antigen was tested in 28 healthy individuals, both in soluble and aggregated form. All subjects were found to possess specific antibodies against *H. influenzae* of both IgG and IgM subclass, thus showing their previous exposure to this commensal micro-organism.

The somatic antigen in solution was found to be a poor antigen for eliciting a delayed hypersensitivity skin response: only 2 out of 16 subjects reacted with a positive DTH pattern.

In contrast, 25 out of 28 persons showed a positive DTH pattern when somatic antigen was used in aggregated form. Two types of DTH reaction patterns could be detected (in a ratio of approximately 3:2), viz. those with an early (24 h) and those with a late (48 h) maximal swelling. Histology of 3 early and 1 late DTH reaction showed perivascular infiltrates of mainly $T_{helper}/T_{inducer}$ lymphocytes. Hardly any basophils were seen. One negative skin test, biopsied at 6 h, showed no signs of Arthus reactivity.

It can be concluded that skin tests using the aggregated form of the somatic antigen of *H. influenzae* are useful for assaying specific T-cell-mediated reactivity in man.

Key words: *skin test – DTH reactivity – Haemophilus influenzae*

Introduction

In a number of clinical conditions, such as immunodeficiencies, cancer and autoimmunity, it is of value to measure the function of the T cell system (Spitler, 1980). Delayed-type hypersensitivity (DTH) skin tests employing antigens derived from micro-organisms such as *M. tuberculosis* (e.g. PPD), streptococci (e.g. SK-SD) and *Candida albicans* are used for this purpose (Spitler, 1980).

Unencapsulated *Haemophilus influenzae* also contains T cell-mediated immunity-inducing somatic antigens (Hoeksma, 1972). In this paper we report the usefulness of

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such antigens for testing T cell reactivity in man, as *H. influenzae* bacteria are practically commensal, colonizing our respiratory tract (Turk and May, 1967; Moxon, 1981). Man is the only host in which it is known to be naturally prevalent (Moxon, 1981).

Earlier experiments from our laboratory carried out with rats showed that DTH skin reactions with this somatic antigen could only be obtained when it was used in aggregated form; the antigen lost its DTH activity in solution (Drexhage and Oort, 1977).

The results reported here were obtained by testing 28 healthy individuals, using the antigen in both aggregated and soluble form. To establish whether contact with *H. influenzae* had indeed taken place in our subjects, specific antibody titres were estimated by means of an ELISA technique.

Materials and Methods

Subjects

Twenty-eight healthy laboratory staff members, 10 females and 18 males, aged 24–44 years, median 33 years, volunteered. Blood samples for estimating *H. influenzae*-specific antibody titres were taken immediately before skin testing, to avoid possible influences from the skin test itself (Thestrup-Pedersen, 1974). Fifteen cord blood samples from healthy neonates born after uncomplicated pregnancy and delivery served as negative controls.

Skin test antigens

Somatic *H. influenzae* antigen was isolated using a combination of the techniques of Platt (1939) and Tunevall (1953). Unencapsulated bacteria, obtained as a lyophilized strain (α5424) from the Institut Pasteur (Paris, France) were grown overnight in serum broth, plated on saponin agar and harvested after 24 h at 37°C. The bacteria were washed once in saline (15 min at $3600 \times g$), heat-killed in 0.5% phenol in saline (60 min at 56°C) and washed thrice in saline (15 min at $3600 \times g$). The pellet was mixed with 1% Na_2CO_3 (pH 10.5) and stored at −20°C until further use. After thawing, the bacteria were mechanically disrupted using a Potter-Elvehjem tube for 30 min at 37°C and the solution centrifuged for 20 min at $20{,}000 \times g$. The pellet (consisting of undisrupted bacteria and cell debris) was discarded and the pH of the clear, viscous supernatant was adjusted to 6.5 with 5 N acetic acid, whereafter a precipitate was formed which was spun down at $20{,}000 \times g$ (20 min). The precipitate obtained was redissolved in 1% Na_2CO_3, the pH readjusted to 6.5 with 5 N acetic acid and centrifuged for 20 min at $20{,}000 \times g$. The resulting pellet was resuspended in distilled water, freeze-dried and stored at 4°C.

The aggregated form of this somatic antigen was prepared by suspending 10 mg of freeze-dried material in 5 ml phosphate-buffered saline (PBS) at pH 6.5 followed by sonication for 10 min (or more, if necessary to dissolve the material) at 0°C (Sonifier B12, Branson Sonic Power Cie., Danbury, CT, U.S.A.). To obtain particles of approximately the size of bacteria, 5 ml 2% glutaraldehyde in PBS was slowly

added to the suspension, followed by 5 min sonication at 0°C. The suspension was left standing at room temperature for 30 min, with occasionally stirring, then washed twice in distilled water (20 min, 3600 × *g*), and freeze-dried. Aliquots of 0.5 mg aggregated material per ml 0.5% phenol in saline were checked for sterility and prepared for use as skin test antigen (ampouled). The aggregated form of somatic antigen prepared in this way has proven to be an excellent skin test antigen in rats (Drexhage and Oort, 1977).

We are well aware that the production of aggregated somatic antigen described does not guarantee an antigen preparation with standard particle size and properties. For use in our 28 subjects we employed only 2 batches with equal potency in a rat skin test model (described earlier by Drexhage and Oort, 1977).

Skin tests

Delayed responsiveness was tested by intradermal injection of 0.1 ml of each antigen preparation in the forearm. The skin reactions were read at 30 min, 6, 24, 48 and 72 h and the diameter of the induration, expressed as the average of 2 measurements at right angles, was recorded.

H. influenzae antibody titres

Antibody titres were estimated by means of a modification of the ELISA technique, as described for anti-penicillin antibodies by De Haan et al. (1979); using 100 µl volumes in U-shaped microtitre plates (Linbro, Flow Lab., U.S.A.). Rabbit anti-human IgG and IgM were purchased from Dako (Copenhagen). Horseradish peroxidase-conjugated sheep anti-rabbit immunoglobulin G was a gift from Dr. D.M. Boorsma. 5-Amino 2-hydroxybenzoic acid in distilled water (pH 6.0) + 0.005% H_2O_2 were used as colour substrate. All dilutions were prepared in PBS (pH 7.4) containing 0.05% Tween 20 and 1% bovine serum albumin (BSA). Optimal conditions and dilutions for all the steps involved in the procedure were established by checkerboard titrations. After each incubation except the last, the plates were washed 5 times with deionized water containing 0.05% Tween 20.

Optimal coating conditions were found to be 10 µg/ml *H. influenzae* antigen in 0.1 M sodium carbonate buffer (pH 9.6, containing 0.02% NaN_3), for 2 h at 37°C. All subsequent incubations were for 30 min at 37°C. The end point of the reaction was read after the final incubation with substrate for 1 h at room temperature, followed by 16 h at 4°C, and expressed as the titre = $-^2\log$ dilution of the serum tested.

In absorption experiments, 2 vols. of serum were mixed with 1 vol. of washed packed *H. influenzae* and incubated overnight at 4°C. The bacteria were removed by centrifugation (15 min, 3600 × *g*) and the titre of the supernatant was estimated.

Skin biopsies

Six of our volunteers consented to a biopsy of the skin test. 4 mm punch biopsies were taken at 6, 24 or 48 h after injection of the aggregated somatic antigen. A second skin test was left untouched to establish the time course of the reaction. Half of the biopsied material was overlayed with OCT Compound (Tissue-Tek®, Miles Lab., U.S.A.) in small aluminium tins and snap-frozen in liquid nitrogen. The other half was fixed in 2% glutaraldehyde in 0.9 M phosphate buffer (pH 7.4) and processed as described by Dvorak et al. (1974). After embedding in Epon, 1 µm semi-thin sections were cut and stained with 1% toluidine blue to establish the presence of basophils.

Of the frozen material, 5 µm cryostat sections were mounted on poly-L-lysine-coated slides, air-dried and fixed in acetone, and used for immunohistochemical demonstration of surface IgG and IgM, T cell antigens, class II MHC antigens, OKT6 antigen and C3 and C1q, as described by Taylor et al. (1983). Before incubation with the various antisera, slides were washed in 3 changes of 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 30 min. All procedures were carried out at room temperature. The following antisera were used in the appropriate dilutions between 1 : 30 and 1 : 400 : FITC-conjugated rabbit anti-human IgG, IgM, C3 and C1q (Dako); mouse anti-human T cell: Leu1, Leu3a (Becton Dickinson) and OKT8 (Ortho), mouse anti-human Ia and OKT6 (Ortho) and anti-B cell: Leu10 (Becton Dickinson). All dilutions were made in PBS with 0.5% bovine serum albumin. After incubation for 1 h in these antisera, slides were washed 3 times in PBS (30 min). The anti-Ig-treated preparations were further incubated with a swine anti-rabbit IgG peroxidase conjugate (Dako); the anti-T cell-, anti-Ia-, anti-OKT6- and anti-B cell-treated preparations were incubated with a goat anti-mouse IgG peroxidase conjugate (Dako). Subsequently sections were rinsed 3 times in PBS and stained for peroxidase activity, using 3,3′-diaminobenzidine-tetra-HCl (Sigma, U.S.A.) in 0.5 mg/ml Tris-HCl pH 7.4 containing 0.1% H_2O_2 as a substrate. 0.05% $CuSO_4$ in 0.9 M NaCl was added to the slides for 5 min to intensify the label. After rinsing in tap water, sections were briefly counterstained with haematoxylin, rinsed again in tap water, dehydrated through graduated alcohols and mounted in Malinol (Chroma-Gesellschaft, F.R.G.). Control slides for each step were included in the procedure.

Results

ELISA

Fig. 1 shows the IgG and IgM titres detected in our 28 healthy subjects and in 15 cord blood samples. All 28 healthy individuals and in fact all healthy persons tested so far (over 50) were found to possess *H. influenzae*-specific IgG titres as well as IgM titres, although the latter were at lower levels. In cord blood only antibodies of IgG class were found; IgM antibodies were not detectable. This IgG is probably of maternal origin and transplacentally transferred.

The ELISA titres were found to be reproducible and the specificity was checked by absorption of some sera with unencapsulated *H. influenzae*. After 1 adsorption, specific IgG titres were reduced to 1/8–1/16 of the original titre, while specific IgM became undetectable.

The antibody titres of 6 of our subjects were studied longitudinally. Changes in titres were not observed over a period of 1 year in 4, and over a period of 3 years in 2 persons. None of these persons had overt acute infections with *H. influenzae*

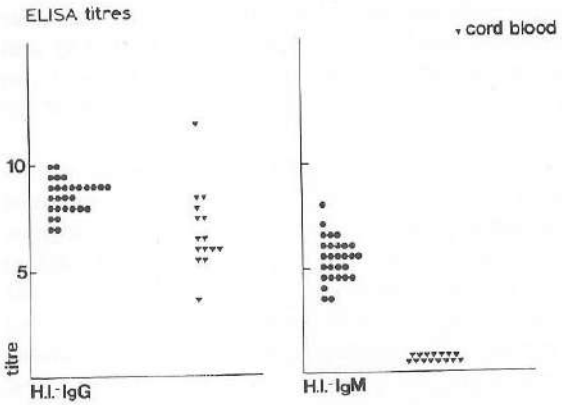

Fig. 1. IgG and IgM antibody titres measured by ELISA against unencapsulated *H. influenzae* bacteria in 28 healthy individuals (●) and 15 cord blood samples (▼).

during these follow-up periods. IgG and IgM titres were thus apparently stationary having reached optimal levels, as can be expected for commensal micro-organisms.

Skin tests

The somatic antigen of *H. influenzae* was used as skin test antigen both in the aggregated and in the soluble form. Fig. 2 shows the results obtained with SA in the soluble form. Only 2 of 16 persons tested reacted with a delayed hypersensitivity pattern; 14 showed a weak maximal swelling at around 6 h, which we regarded as a non-specific inflammatory response of the skin test site, due to the injection of foreign material. A wheal and flare reaction at 30 min was not detectable in any of the individuals tested.

Fig. 3 shows the results obtained with SA in aggregated form. 25 of our 28 volunteers now reacted with a delayed-type pattern, and only 3 showed an aspecific

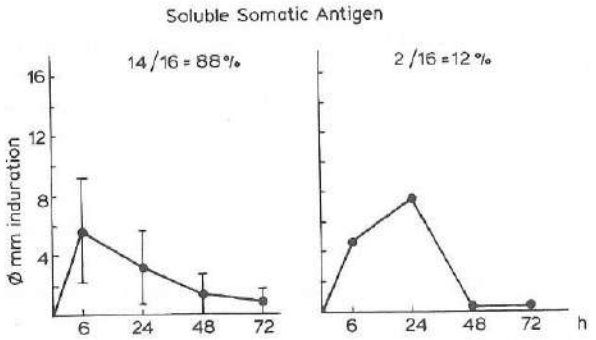

Fig. 2. Skin test patterns obtained with somatic antigen in soluble form in 16 healthy individuals. Left panel shows the weak response seen at 6 h in 14 of the subjects (mean ± SEM bars). Right panel shows delayed hypersensitivity response seen at 24 h in 2 individuals.

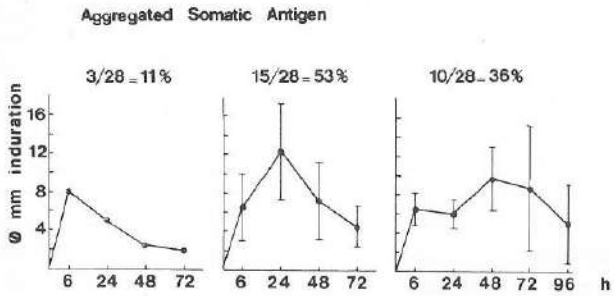

Fig. 3. Skin test patterns obtained with the aggregated form of somatic antigen in 28 healthy individuals. Left panel shows weak non-specific 6 h response; centre panel shows a delayed pattern peaking at 24 h; right panel shows a delayed pattern with maximal induration at 48–72 h.

reaction. In 15 of the positive responders there was a maximal swelling at 24 h, whereas 10 showed a maximal reaction at 48 to even 72 h. Only 1 person had slight indications of a wheal and flare reaction at 30 min.

No differences in strength or reaction pattern were observed between females and males. Repeated skin testing in 8 persons (up to 4 times) with intervals of at least 2 months showed skin test reactivity to be consistently positive in 6 persons, and consistently negative in 2. This constancy of response has been reported by several investigators regarding other skin test antigens, e.g. PPD (Holt et al., 1976), histoplasmin (Graybill and Alford, 1976) and for tetanus, diphteria, streptococcus, tuberculin, candida, trichophyton and proteus antigens (Lesourd and Winters, 1982).

Skin biopsies

Two biopsies were taken at 6 h in persons whose reactions were maximal at 24 h. Some perivascular infiltration was evident, mainly consisting of class II MHC-positive dendritic cells. Some Leu3a$^+$ cells were also present, but hardly any OKT8$^+$ cells. In the epidermis OKT6$^+$ dendritic cells could clearly be seen, mainly localized near the dermis. It is important to note that in the one person with a negative skin test who was also biopsied at 6 h, similar infiltrates were seen. Signs of immune complex deposition could, however, not be detected by immunofluorescence techniques. Polymorphonuclear cells were absent.

Three biopsies were taken at 24 h, when the reactions reached their maximum. The perivascular infiltrate was now predominantly formed by T_{helper} cells. In between these cells class II MHC$^+$ dendritic cells could again be seen closely contacting these lymphocytes. Only a few OKT8$^+$ cells ($T_{suppressor/cytotoxic}$) and round Leu10$^+$ cells (B cells) were present. The epidermal OKT6$^+$ dendritic cells were again clearly present in approximately equal numbers as at 6 h; they now tended to be located in the upper layers of the dermis. Jones-Motes reactivity could be excluded, since hardly any basophils were present in the semi-thin sections.

The one biopsy taken at 48 h from a person whose skin test was maximal at this time showed the same pattern as described for the reactions maximal at 24 h. It is noteworthy that at this time $T_{suppressor/cytotoxic}$ cells (OKT8$^+$) were present in substantial numbers.

Discussion

The results presented in this study clearly indicate the usefulness of somatic antigen prepared from *Haemophilus influenzae* for the assessment of T cell reactivity in man in an in vivo skin test assay.

Twenty-five of our 28 healthy volunteers showed positive delayed hypersensitivity skin reactions when the antigen was used in the aggregated form. The T cell nature of the reaction was confirmed in immune histology in 6 persons. The soluble form of somatic antigen was poor in eliciting delayed-type skin reactivity.

Earlier experiments in rats had already shown the importance of the antigenic form (Drexhage and Oort, 1977), and the results in humans are entirely in accordance with those of the rats. We detected 2 types of DTH reactivity: one with an early maximum at 24 h and the other with a late maximum at 48–72 h. They appeared in a ratio of 3:2 in our group of volunteers. The existence of different types of DTH patterns has also been described for mycobacterial antigens in sensitized mice (Rook, 1978; Rook and Stanford, 1979). In these animals, either an early maximum at 18–23 h or a late maximum at 40–48 h was observed. Depletion of B cells did not affect these reactivity patterns, but anti-Thy1,2 treatment did, indicating the T cell nature of both maxima (Rook and Stanford, 1979). The authors presumed that the difference in these patterns was based on the presence of cytotoxic T cells in the late-type response, while these cells were absent in the early-type response. Our histological results from skin tests with somatic antigen of *H. influenzae*, finding substantial numbers of $OKT8^+$ cells in the reaction maximal at 48 h, support this view.

The early maximum at 24 h showed no signs of Jones-Mote reactivity, since hardly any basophils could be detected. Besides, the induration of the skin test site, although decreased at 48 h as compared to 24 h, was still clearly present in all individuals. Furthermore, we never saw signs of immediate wheal and flare reactions after repeated skin testing. Thus none of the characteristics described by Katz (1978) for Jones-Mote reactivity were present.

Three of our 28 healthy individuals did not show any delayed responsiveness to aggregated somatic antigen; their skin test showed a weak maximal swelling at around 6 h. We regard such a pattern of responsiveness as a non-specific inflammatory response to the injected material, since such reactivity to the antigen can also be observed in non-sensitized rats (Drexhage and Oort, 1977). In addition, Arthus reactivity was excluded by histological examination.

There are a few possible explanations for the negativity of the skin test in those persons, in whom contact with *H. influenzae* was proven by means of a positive ELISA. Skin test-blocking antibodies may be present as has been described by Roupe and Strannegard (1972) and Bernhard et al. (1972) for DNCB skin tests. In the rat model this blocking mechanism was shown to exist for *H. influenzae* somatic antigen (Drexhage and Oort, 1977), but we did not investigate this possibility in our 3 skin test-negative individuals. Selective T cell defects for the somatic antigen, similar to those described for candida and mycobacterial antigens (Metcalf et al., 1981), may be another possibility. Such selective T cell defects for somatic antigen

were indeed detected in 15 out of 21 patients with chronically relapsing purulent rhinosinusitis. Details of this study have already been published elsewhere (Drexhage et al., 1983).

Skin tests are time-consuming and unpleasant for patients. In vitro tests having a good correlation with the in vivo skin test would be useful. [^{3}H]Thymidine incorporation in, and lymphokine production by, lymphocytes upon contact with antigen are often used for this purpose. Reports on the correlation of these in vitro parameters of CMI with those of the skin test are highly controversial (Astor et al., 1973; Clausen, 1973; Fleer et al., 1976; Lewinsky et al., 1977; Räsänen, 1980).

At present we are carrying out experiments to study the correlation between the outcomes of the DTH skin test, the lymphocyte transformation test and macrophage migration inhibition factor (MIF) production upon stimulation with somatic antigen.

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References

Astor, S.H., L.E. Spitler, O.L. Frick and H.H. Fudenberg, 1973, J. Immunol. 110, 1174.
Bernhard, J.D., S.S. Rosenfeld and E. Klein, 1972, J. Med. 3, 313.
Clausen, J.E., 1973, J. Immunol. 110, 546.
De Haan, P., D.M. Boorsma and G.L. Kalsbeek, 1979, Allergy 34, 111.
Drexhage, H.A., 1977, 'Allergische' Reacties op Bacteriële Antigenen, Academical Thesis (Free University, Amsterdam).
Drexhage, H.A. and J. Oort, 1977, Clin. Exp. Immunol. 28, 280.
Drexhage, H.A., E.M. Van de Plassche, M. Kokjé and J.A. Leezenberg, 1983, Clin. Immunol. Immunopathol. 28, 218.
Dvorak, H.F., M.C. Mihm Jr., A.M. Dvorak, R.A. Johnson, E.J. Manseau, E. Morgan and R.B. Colvin, 1974, Lab. Invest. 31, 111.
Fleer, A., M. Van der Hart, B.J.Th. Blok and P.Th.A. Schellekens, 1976, Eur. J. Immunol. 6, 163.
Graybill, J.R. and R.H. Alford, 1976, Clin. Exp. Immunol. 25, 28.
Hoeksma, A., 1972, Antibodies and Delayed Type Skin Reactions with Respect to the Serological Types of Haemophilus influenzae, Academical Thesis (University of Utrecht).
Holt, P.G., P.J. Fimmel, W.N. Bartholomaeus, L.M. Roberts, M.K. Tandon and D. Keast, 1976, Int. Arch. Allergy Appl. Immunol. 51, 560.
Katz, S.I., 1978, J. Invest. Dermatol. 71, 70.
Lesourd, B. and W.D. Winters, 1982, Clin. Exp. Immunol. 50, 635.
Lewinsky, U.H., G.M. Mavligit, J.U. Gutterman and E.M. Hersch, 1977, Clin. Immunol. Immunopathol. 7, 87.
Metcalf, J.F., J.F. John Jr., G.B. Wilson, H.H. Fudenberg and R.A. Harley, 1981, Am. J. Med. 71, 485.
Moxon, E.R., 1981, in: Immunology of Human Infection, Part I: Bacteria, Mycoplasmae, Chlamydiae and Fungi, Vol. 8, Comprehensive Immunology, eds. R.A. Good and S.B. Day (Plenum Medical Book Company, New York and London) p. 113.

Platt, A.E., 1939, Aust. J. Exp. Biol. Med. Sci. 17, 19.
Räsänen, L., 1980, Clin. Exp. Immunol. 40, 566.
Rook, G.A.W., 1978, Nature (London) 271, 64.
Rook, G.A.W. and J.L. Stanford, 1979, Parasite Immunol. 1, 111.
Roupe, G. and O. Strannegard, 1972, Int. Arch. Allergy Appl. Immunol. 43, 691.
Spitler, L.E., 1980, in: Manual of Clinical Immunology, 2nd edn., eds. N.R. Rose and H. Friedman (American Society for Microbiology, Washington, DC) p. 200.
Taylor, C.R., F.M. Hoffman, R.L. Modlin and T.H. Rea, 1983, J. Cutaneous Pathol. 10, 145.
Thestrup-Pedersen, K., 1974, Immunology 27, 965.
Tunevall, G., 1953, Acta Pathol. Microbiol. Scand. 32, 258.
Turk, D.C. and J.R. May, 1967, Haemophilus influenzae, Its Clinical Importance (English Universities Press, London).

CHAPTER III

SELECTIVE T-CELL DEFECTS TO HAEMOPHILUS INFLUENZAE IN CHRONIC PURULENT INFECTIONS OF THE UPPER RESPIRATORY TRACT

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SUMMARY

Delayed type hypersensitivity (DTH) skin test reactivity to a somatic antigen of Haemophilus influenzae was studied in 36 patients with unexplained, chronically relapsing, purulent upper respiratory tract infections. Only 8 showed a DTH reactivity comparable to that of healthy controls. A majority - 24 patients - had a defective DTH response, whereas 4 showed exaggerated reactivity leading to necrosis of the test site and general feelings of malaise. Not only was the DTH reactivity to somatic H. influenzae antigen affected, but also that to streptokinase/streptodornase and candidal antigen in most cases, though to a lesser extent. Skin test reactivity to the mitogen PHA was normal as were the DTH skin test reactivities in 4 out of 5 control patients with mucous atopic rhinosinusitis and 2 cases of nasal suppuration due to disturbed mucociliary transport. In addition a high incidence of atopic skin tests and thyroid autoimmunity was evident in patients as well as in their first-degree relatives. These results suggest that unexplained, chronically relapsing upper respiratory tract infections might be based on restricted T-cell defects to H. influenzae, streptococcal, and candidal antigens. Such defects are reminiscent of the T-cell immune disorders to fungi playing a role in some cases of chronic mucocutaneous candidiasis.

INTRODUCTION

Commensal bacteria, such as unencapsulated Haemophilus influenzae, but also streptococci and pneumococci, are considered to act as pathogens in chronically relapsing, purulent infections of the upper and lower respiratory tracts (Mulder et al., 1952; Turk and May, 1967). Such disorders are often observed in ENT and pediatric practice.

Depending on the stage of the disease, the location of the infective focus, sampling methods, and culture conditions (Haas et al., 1977), the frequency of isolation of H. influenzae and also of the other pathogens ranges from 25 to 100% (Turk and May, 1967). The special characteristics making these organisms important pathogens are largely unknown, though a few cases have been reported based on well-defined deficiencies in

humoral immunity (Polmar, 1976). In general antibody production is intact and high titres of precipitating antibodies are found (Burns and May, 1967).

A further proposed mechanism facilitating H. influenzae respiratory tract colonization is the production by the bacteria of a factor hampering ciliary movement of the bronchial epithelium (Denny, 1974). Antigen-antibody complex-mediated reactions (Van der Zwan et al., 1977; Clarke, 1979) as well as atopic reactivity (Pauwels, Verschraegen and van der Straeten, 1980) have also been suggested as contributing to the pathogenic mechanisms. The role of the thymus-dependent cell-mediated immunity (CMI) is not clear. Somatic antigen of H. influenzae is a potent stimulator of the T-cell system (Drexhage and Oort, 1977), and it is therefore likely that CMI plays a part in these infective disorders. Moreover overt disturbances in CMI - as in Di George syndrome - are accompanied by H. influenzae infections of the respiratory tract.

These notions have led us to study delayed skin test reactivity to a somatic antigen of H. influenzae in 36 patients with chronically relapsing, purulent infections of the upper respiratory tract. The patients were selected on the basis of unexplained chronic suppuration. None of them had gross disturbances in mucociliary transport or deficiencies in H. influenzae specific IgG, IgM, or IgA production. In all, current surgical procedures as well as several courses of antibiotic treatment had not effected a permanent cure.

PATIENTS AND METHODS

Patients

Skin tests were performed on groups of patients in the following categories.

a) Chronically relapsing, purulent rhinosinusitis. This group included 36 patients (24 females, 12 males; ages 15 to 69 years, median 40 years) with unexplained chronic suppuration of the upper respiratory tract, on one occasion complicated by a middle ear and mastoid infection. All of them showed or had shown positive bacterial cultures on one or more

occasions; H. influenzae was present in about 50% of the cases, Streptococcus pneumoniae in about 40%, and other streptococci and staphylococci in about 20%, and in 2 separate cases Escherichia coli and Branhamella were isolated. Duration of disease varied from 18 months up to approximately 40 years. All of the patients had been treated with several courses of antibiotics, which had given only temporary relief. All had been operated upon to improve drainage of the maxillary and ethmoidal sinuses; in all instances the operation had failed to cure them permanently. There were no gross disturbances in mucociliary transport.

b) Atopic mucous rhinosinusitis. This control group included five patients (four females, one male; age 23 to 40 years, median 30 years) all of whom showed strong atopic reactivity to allergens such as grass pollen and house dust (mite), both in skin tests and in in vitro (radioallergo sorbent test, RAST). Three of them had markedly elevated levels of IgE. All had complaints of seasonal or perennial episodes of mucous rhinitis; purulence was very infrequently found.

c) Disturbed nasal mucociliary clearance. This control group included two patients (one female, one male; ages 25 and 33 years) with heavy suppuration of the upper respiratory tract. Both had a positive culture for H. influenzae.

The mucociliary transport rate in the nose had been measured radiographically using Te-albumin.

d) Healthy individuals. This group included 20 healthy laboratory staff (7 females, 13 males; age 24 to 44 years, median 33 years) with a negative personal and family history for atopy and autoimmunity. When comparisons are made with the patient group, it must be borne in mind that these controls do not match entirely for either age or sex. Other groups of healthy individuals have not yet been studied as we thought it more appropriate to skin test only healthy laboratory staff in this stage of the survey.

Serum immunoglobulins and H. influenzae antibody titres.

The concentration of serum IgG, IgM and IgA was measured by means of commercially available kits. Antibody titres to H. influenzae were estimated by means of a modification of the ELISA-technique, as described for anti-penicillin antibodies by De Haan, Boorsma and Kalsbeek,

1979; using 100 µl volumes in U-shaped microtitreplates (Linbro, Flow Lab. Inc. USA). Rabbit anti-human IgG and IgM were purchased from DAKO (Copenhagen). Horseradish peroxidase conjugated sheep-anti-rabbit IgG was a gift from Dr. D.M. Boorsma, Dept. of Dermatology. 5-Amino 2-hydroxybenzoic acid in distilled water (pH 6.0) + 0.005% H_2O_2 was used as colour substrate. All dilutions were made in PBS (pH 7.4) containing 0.05% Tween 20 and 1% bovine serum albumin (BSA). Optimal conditions and dilutions for all the steps involved in the procedure were established by checkerboard titrations. After each incubation except the last, the plates were washed 5 times with deionized water containing 0.05% Tween 20.

Optimal coating conditions were found to be 10µg/ml H. influenzae antigen in 0.1M sodiumcarbonate buffer (pH 9.6, containing 0.02% NaN3), during 2 hr at 37°C. All subsequent incubations were 30 min at 37°C. The end point of the reaction was read after the final incubation with substrate for 1 hour at room temperature, followed by 16 hr at 4°C, and expressed as the titre = -2 log dilution of the serum tested.

In absorption experiments, two volumes of serum were mixed with one volume of washed packed H. influenzae and incubated overnight at 4°C. The bacteria were removed by centrifugation (15 min. 3600 g) and the titre of the supernatant was estimated.

Skin test antigens

Somatic H. influenzae antigen was isolated using a combination of the techniques of Platt and Tunevall as described before in Drexhage and Oort (1977): H. influenzae bacteria, kept as a lyophilized strain (α-5424, Institut Pasteur, Paris, France), were grown overnight in serum broth, thereafter plated on saponin-agar, and harvested after 24 hr of culture. The bacteria were heat-killed (56°C, 60 min.) in 0.5% phenol in saline, washed three times in saline (15 min, 3600 g), freeze-dried in distilled water, and stored at 4°C. One milligram of the freeze-dried material was mixed with 10 ml 1% Na_2CO_3, pH 10.5, and vigorously shaken at 21°C for 30 min, and thereafter centrifuged for 20 min at 20,000 g. The pellet was discarded and the pH of the clear supernatant was adjusted to 6.5 with 1 N HCl, whereafter a precipitate was formed which was spun down at 20,000 g (20 min). The pellet was redissolved in 1%

Na_2CO_3 and again treated with 1 N HCl to bring the pH down to 6.5, centrifuged, resuspended in distilled water, freeze-dried and stored at 4°C. The somatic H. influenzae antigen obtained in this way proved to be a suitable antigen for stimulating T-cells in rats and in particular the aggregated form of this material is an excellent DTH skin test antigen (Drexhage and Oort, 1977).

The aggregated form was prepared by suspending 10 mg of freeze-dried material in 5 ml phosphate-buffered saline at pH 6.5, thereafter sonicating it for 10 min at 0°C (Sonifier B 12, Branson Sonic Power Cie, Danbury, Conn.) to obtain particles approximately the size of bacteria. The suspension was further treated by adding 5 ml 2% glutaraldehyde in PBS and vigorously shaken for 30 min at room temperature. It was washed two times in distilled water (20 min, 3600 g) and freeze-dried. A suspension of 500 µg aggregated material per milliliter 0.5% phenol in saline was prepared for use as skin test antigen.

We are well aware that the described production of aggregated somatic antigen does not guarantee an antigen preparation with standard properties and particle size, thus giving rise to substantial differences in skin test assays. To circumvent this problem we used only two batches with equal potency in a rat skin test model (Drexhage and Oort, 1977).

A commercially available preparation of 1% candidal antigen (HAL allergens, Haarlem, The Netherlands) and a commercially available preparation of 100 U of streptokinase/streptodornase per milliliter solvent (Varidase, Lederle, Wayne, Mich.) were also used as skin test antigens, as was PHA, which was obtained from Wellcome Laboratories (Kent, United Kingdom) and used at a strength of 5 µg/ml saline.

Delayed responsiveness was tested by intradermal injection of 0.1 ml of each antigen preparation in the forearm. The skin reactions were read at 30 min, 6, 24, 48, and 72 hr and the diameter of induration was recorded.

Enumeration of total blood T-lymphocytes and T-lymphocyte subsets.

The percentage of E-rosette-forming cells in Ficoll-Isopaque isolated and cryopreserved blood lymphoid cell suspensions was estimated by methods described before (Von Blomberg et al., 1980). In addition the

percentage of T-lymphocytes and T-lymphocyte subsets was determined by reacting the lymphoid cell suspensions with monoclonal antibodies against pan T-cells (OKT3, Orthoclone), helper T-cells (OKT4), suppressor/cytotoxic T-cells (OKT8) and E rosette receptors (OKT11) (Ortho, Raritan, N.J.) and staining with a fluorescein-isothiocyanate-conjugated second immunoglobulin as indicated by the manufacturer. Two hundred cells were counted in a fluorescent microscope; the tests were done in duplicate.

Absolute numbers of T-cells and T-cell subsets were calculated by multiplication from the total peripheral lymphocyte counts.

T-lymphocyte proliferative responsiveness.

Lymphocyte cultures in the presence of 20% pooled human serum, were carried out on Ficoll-Isopaque isolated blood lymphoid cells. The lymphoid cells were cultured in Linbro/Titertek microplates (Flow, Irvine, Scotland) using 0.15 ml Hepes-buffered RPMI 1640 (Gibco, Glasgow, Scotland) supplemented with antibiotics. In time- and dose-response curves with cells of five healthy controls the optimal antigen concentration for DNA synthesis to occur was found to be 50 µg somatic H. influenzae antigen/ml of culture fluid. The optimal time of culture was 6 days. The optimal cell concentration was 2×10^5 cells per well. The results are expressed in terms of the stimulation indices (SI), i.e. the ratio of the uptake of [^{3}H]thymidine in antigen-stimulated versus control cultures. The SI of lymphoid cells isolated from neonatal cord blood were measured to determine the nonspecific mitogenic capacity of somatic antigen. On three occasions the responder lymphoid cells were depleted of B-cells by incubating them on plastic petri dishes coated with Ig (panning technique). This procedure almost completely depleted our suspensions of B-cells (13 → 2%). The SI of these depleted suspensions reached the same or even higher levels, indicating the T-cell nature of the test. Proliferative responsiveness to PHA was also measured using a 3-day culture of 4×10^4 cells with 5-hr [^{3}H]thymidine pulse.

RESULTS

Approximately half of the patients with relapsing, chronic suppuration of the upper respiratory tract showed other immunological disorders, such as atopic skin test reactivity to common allergens as grass pollen and house dust mite, but also in some cases to pneumococcal and staphylococcal antigens as well as to the soluble form of H. influenzae somatic antigen. In seven there was a combination with thyroid autoimmunity, including thyroid atrophy treated with thyroxin, recurrent simple sporadic goiter which had been treated by surgery, transient attacks of Graves' disease, and a case of positive thyroid autoantibodies without overt thyroid disease. Recurrent dermatophytosis was present in five. Most of the patients had first-degree relatives with histories of chronic respiratory diseases and thyroid autoimmunity (table 1).

Table 1
Other immune disorders in chronic purulent rhinosinusitis.

	percentage incidence
Atopic reactivity to grass pollen, house dust mite, staphylococcal, pneumococcal, and H. Influenzae allergens with raised IgE	52
	20
Autoimmune thyroid disease	19
Recurrent dermatophytosis	14
Positive family history for	
Chronic respiratory disease	50
Autoimmune thyroid disease	40

The levels of serum immunoglobulins were found perfectly normal (fig. 1a) in practically all patients except for a slightly raised IgG, IgM and IgA in a few. Only one case (a female, aged 22 years) out of 36 tested showed a lowered serum IgA. This prevalence of partial IgA deficiency of around 2-3 % is comparable to that found in a normal healthy population (Hayward, 1977). With regard to specific antibodies of IgG and IgM class to H. influenzae, again near normal levels were found (fig. 1b), indicating that antibody production to one of the

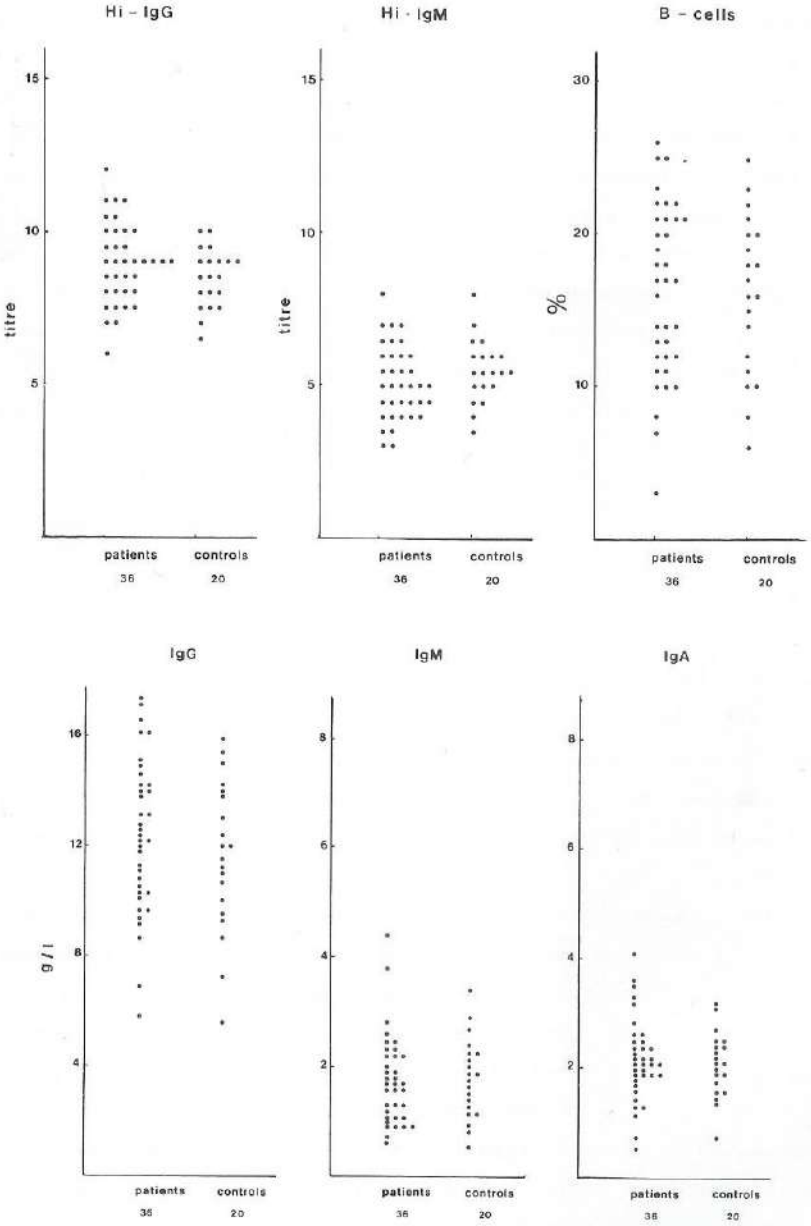

Fig. 1. The serum immunoglobulin levels (fig. 1a), the H. influenzae-specific antibodies (fig. 1b) and the circulating s-Ig$^+$-cells = B-cells (fig. 1c) of patients with relapsing purulent rhinosinusitis and healthy controls. Generally normal values were found.

important microbes colonizing the respiratory tract of the patient was completely intact. Such observations of a well functioning B-cell system in patients with relapsing rhinosinusitis have been reported by others before (Burns and May, 1967). The intact B-cell responsiveness was further illustrated by the normal percentages of B-cells (fig. 1c) found in the circulation of practically all patients; only one out of 36 (a female, aged 24) showed a lower percentage (3%). She had normal serum IgG, IgM and IgA, but a raised IgE (105 U/ml).

Aggregated somatic H. influenzae antigen produced four patterns of skin test reactivity which are listed in fig. 2. Fifty percent of the healthy controls showed DTH reactivity with a maximum swelling at 24-36 hr, whereas 40% had a later DTH reaction with the maximal skin induration at 48-72 hr. We regarded these responses as T-cell mediated, because similar reactions occur in rats sensitized by T-cell transfers (Drexhage and Oort, 1977). Two of the healthy controls (10%) did not show any delayed responsiveness to somatic antigen; they had a reaction with a weak maximal swelling at around 6 hr. We regarded such a pattern of responsiveness as a non-specific inflammatory response to the injected material, because such reactivity can also be observed in nonsensitized experimental animals (Drexhage and Oort, 1977). The two types of DTH reactivity observed in our healthy controls - the early and late peaking form - have also been described for mycobacterial antigens (Rook, 1978). In particular the early peaking DTH reactivity was suggested to reflect T-cell-mediated host defence against mycobacteria (Rook and Stanford, 1979).

Delayed hypersensitivity patterns of skin test reactivity were only found in 22% of patients with chronic suppuration of the upper respiratory tract: 24 of them (67%) showed no DTH reactivity at all. Four showed a marked enhanced reactivity not only to somatic H. influenze antigen, but also to candidal antigen or streptokinase/streptodornase. In these 4 the test site showed strong induration and blister formation which started 24 hr after antigen injection. During the exaggerated response, the patients suffered from general reactions including malaise, fever up to 40°C, and myalgia. The skin test reaction evoked a strong anamnestic response of the nasal mucosa - in the form of a mucous rhinitis - and of the cervical lymph nodes, which became tender and swollen. The nasal and pharyngeal lesions of these 4 patients were characterized by necrosis rather than by suppuration alone. Such hyper-

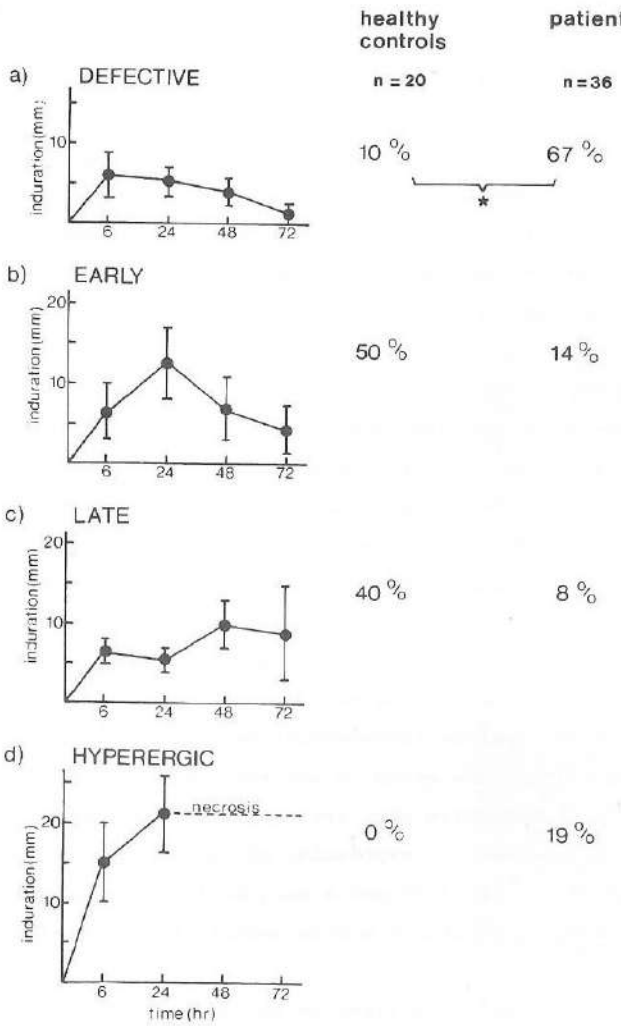

Fig. 2. The skin test reactivity to aggregated somatic H. influenzae antigen in patients with chronically relapsing suppuration of the upper respiratory tract as compared to that of healthy controls. 50 µg of the antigen was injected intracutaneously in the forearm; the diameter of induration (in mm) was recorded at 6, 24, 48 and 72 hr after injection. Four patterns of responsiveness could be detected (a-d). A statistically significant difference (X^2 test, $p<0.01$) in the incidence of defects in DTH responsiveness between patients and controls was detected.

ergic, necrotic skin test reactions were never encountered in the group of healthy controls (fig. 2).

Four out of five patients with nonpurulent atopic rhinitis showed normal DTH reactivity (early peaking form), as did the two other control patients with heavy suppuration due to impaired mucociliary transport.

Skin tests with streptokinase/streptodornase and candidal antigen gave maximal swelling of the test site 48 hr after antigen injection. Data recorded at this time are given in fig. 3. Using these antigens again a considerable number of the patients showed a weakened DTH reactivity which was the most prominent with the streptococcal antigen. There was no correlation between absence of DTH reactivity to somatic H. influenzae antigen and negativity to either streptokinase/streptodornase or candidal antigen; in fact the three patients who showed normal DTH reactivity to somatic H. influenzae antigen (fig. 2) were clearly negative for streptokinase/streptodornase and candidal antigen. Delayed skin test reactivity to the mitogen PHA was intact in all patients and controls tested, thus the detected antigen-specific DTH defects cannot be ascribed to a general impairment of delayed inflammatory responsiveness.

The number of T cells did not differ significantly between the patient group and controls, but the percentages of circulating lymphoid cells, showing the T-11 marker (E-receptor) was found diminished in our patients, particularly in the group of patients with defective DTH skin tests (fig. 4, 33% of defective skin test responders showed percentages lower than 60). In contrast the expression of the T-3 marker was practically normal (only 3 out of 35 showed a marginally lower expression). This discrepancy again indicates a subtle defect in the T-cell system of the patients.

The OKT4/OKT8 ratio was calculated to get an impression of the balance between T-helper and T-suppressor function (fig. 4). Elevated ratios (over 2.1) were noted both in the skin test normals (25%) as well as in the skin test defective group (42%). In the latter it appeared by further exploration that those with a raised OKT4/OKT8 ratio were mostly the ones who additionally had atopic skin tests and an elevated serum IgE.

The DNA synthesis of peripheral lymphoid cells in response to somatic H. influenzae antigen and PHA was also tested. Somatic antigen appeared to have slight mitogenic capacity as it stimulated cord blood

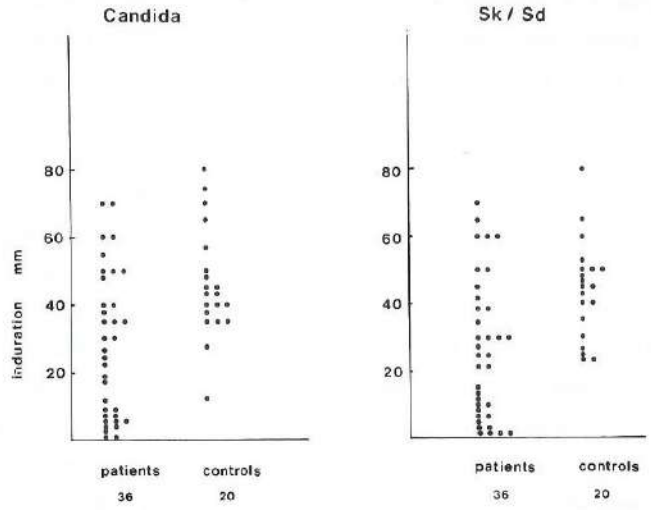

Fig. 3. The DTH skin test reactivities to streptokinase/streptodornase (10 U) and to candidal extract (1% solution) of patients with chronically relapsing upper respiratory tract infections and of healthy controls. The diameter of induration was recorded 48 hr after antigen injection. A considerable number of patients showed a weakened DTH reactivity to these antigens.

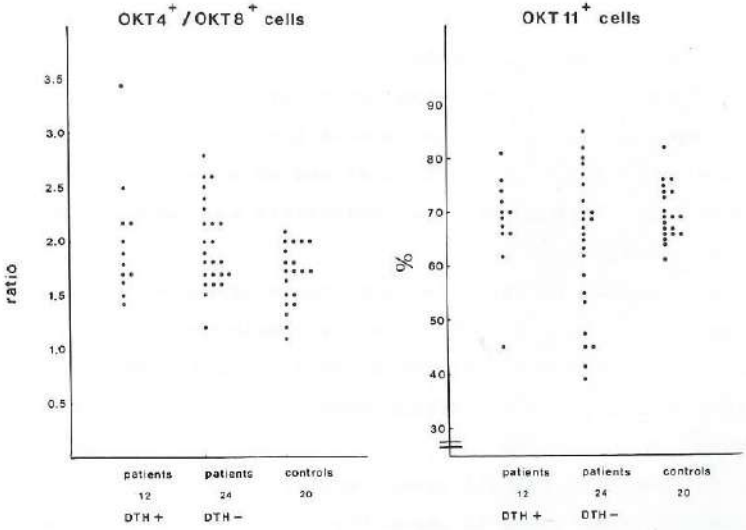

Fig. 4. The percentages of circulating OKT11-positive cells and the OKT4+ to OKT8+ cell ratio in patients with relapsing purulent rhinosinusitis and healthy controls. In the skin test defective group 33% of the patients showed a defective expression of the T11-marker, whereas 42% had raised T-helper to T-suppressor/cytotoxic cell ratios.

cells with SI up to 2.5. Eighty percent of the 20 healthy controls showed SI higher than those obtained with cord blood cells (range 2.5-15), which probably indicates the ongoing stimulation with ubiquitous H. influenzae antigen in a normal population. Some of the controls were followed, through time, and strong fluctuations of SI were found ranging from positive to even negative indicating a rapid disappearance from and appearance in the circulation of the antigen-specific lymphoid cells.

Eighty percent of the chronic purulent rhinosinusitis patients showed SI equal to or even higher than those of the healthy controls (range 2.5-40). This indicates that T-cell proliferative responsiveness is in general intact and that the high SI probably reflect the very high exposure rates to H. influenzae in some of the patients. There were, however, three patients with persistent negative responses, tests being performed over a period of 6 months. All three were heavily infected with H. influenzae. PHA responsiveness was found to be normal in all controls and patients tested.

DISCUSSION

All patients in this series had chronic, recurrent, purulent infections of the upper respiratory tract colonized by predominantly H. influenzae and streptococci and yet in a considerable proportion T-cell responsiveness to somatic antigen of H. influenzae and to streptokinase/streptodornase as measured by DTH skin test reactivity was found to be lower than those of healthy controls.

These findings are in agreement with those of Polmar (1976), who studied a group of patients with chronic sinopulmonary disease, which also lacked delayed hypersensitivity to candida, trichophyton, streptokinase/streptodornase, PPD, or histoplasmin but reacted weakly to DNCB sensitization and challenge.

Though the subject is still controversial (Rook and Stanford, 1979), delayed skin test reactivity is generally regarded as reflecting the state of protective immunity to chronic bacterial infections (Mackaness, 1968; Lagrange and Closs, 1979; Berger and Blanden, 1981). An absent DTH skin test reactivity to somatic H. influenzae antigen and to streptokinase/streptodornase may therefore be a strong indication of a high susceptibility to these bacteria.

We did not find defective DTH responsiveness in four out of five atopic control patients with mucous, practically noninfected rhinitis. This indicates a relationship between suppuration and defects in DTH reactivity, the latter being either a cause or a consequence of the purulent infection. There are, however, two arguments against the idea that they are a consequence: firstly, because we found that two patients with severe purulent rhinitis due to disturbed mucociliary clearance had normal DTH reactivities and, secondly, the DTH defects were still present in the patients after a course of antibiotic treatment, which had given temporary relief (see table II).

Table II
The skin test before and after a six-week course of doxacycline in 6 patients with relapsing purulent infections of the upper respiratory tract.

	Pretreatment	Posttreatment
Skin test defect to candidin in:	3/6 (50%)	4/5 (80%)
to Sk/Sd in:	4/6 (66%0	4/6 (66%)

The here-described cases of restricted DTH defects to H. influenzae antigen are reminiscent of the immune defects in chronic mucocutaneous candidiasis and other dermatophytoses. In these conditions impaired DTH responsiveness to mainly candidal antigens (Kirkpatrick, 1971; Lehner, Wilton and Ivanyi, 1972) and Trychophyton rubrum has been described (Hanifin, Ray and Lobitz, 1974). In fact chronic infections of the upper respiratory tract seem closely related to chronic candidiasis and other dermatophytoses: firstly, because these disorders are often complicated by purulent infections of the respiratory tract (Valdimarsson et al., 1970; Kirkpatrick, 1971; Louie and Goldberg, 1972); secondly, impaired DTH reactivity to streptokinase/streptodornase has been described in chronic candidiasis (Lehner, Wilton and Ivanyi, 1972); thirdly, a considerable number of our patients showed impaired reactivity to candidal antigen; and fourthly, five had had periods of chronic derma-

tophytosis. The underlying abnormalities of the immune system in chronic candidiasis are very heterogeneous, forming a spectrum, but in the majority the T-cell system is affected, involving disturbed T-cell cytotoxicity and impaired production of lymphokines. T-cell proliferative responsiveness is mostly affected to a lesser extent (Lehner, Wilton and Ivanyi, 1972). In analogy three of our patients showed consistently negative H. influenzae specific lymphocyte proliferation, but in the majority it was found to be intact.

Most remarkable was also the high incidence of other immune disorders such as thyroid autoimmunity and atopic skin reactions to a whole range of allergens. Such a high prevalence of immune disorders in patients with chronic purulent sputum production has been reported before (Turner-Warwick and Cole, 1982), and also in chronic mucocutaneous candidiasis there is a high incidence of autoimmune disease and atopy (Wuepper and Fudenberg, 1967; Kirkpatrick, 1971; Jones, Reinhardt and Rinaldi, 1974).

Another striking abnormality in T-cell function was the disturbed ratio of OKT4+/OKT8+ cells. It is tempting to speculate that such an imbalance in T-cell regulatory function must lead to uncontrolled exaggerated IgE immunoglobulin synthesis (Moreno, 1982), which underlies most forms of atopy. It might also bring about the escape of autoimmune B-cell clones (Cooke and Lydyard, 1981) giving rise to the thyroid autoimmunity found in a considerable proportion of our patients. Disturbed ratios of T-gamma/Tμ (CD4+/CD8+) cells have been reported in atopic disease (Canonica et al., 1979) as well as in thyroid autoimmunity (Okita, Row and Volpé, 1981).

The cause of the defects in T-cell function is speculative. Viruses, notably measles and influenza virus, are able to suppress immune T-cell function and thus pave the way for commensal micro-organisms (Editorial, i, 1982). Well known are the episodes of acute H. influenzae pneumonia accompanying epidemics of influenza (Couch, 1981). Whether chronic H. influenzae respiratory infection might evolve due to more sustained T-cell defects after virus infection is unknown. In our group 1 patient (male, 38 years) had a clear history of influenza followed by episodes of chronic purulent infections of the upper respiratory tract. However, it must be borne in mind that this patient also had a positive family history for chronic respiratory diseases.

Genetic influences probably play a very important role, this is

indicated by the high incidence of chronic respiratory diseases and other immunologic disorders in the first-degree relatives of the patients. Moreover, it is now well accepted that T-cell-mediated immunity to micro-organisms such as M. leprae is governed by HLA-DR structures coded for by the major histocompatibility complex (Editorial ii, 1982; Van Eden et al., 1982).

In children with chronically relapsing upper respiratory tract infections beneficial effects have been described of the drug levamisole (Van Eygen et al., 1976); levamisole treatment (150 mg, once weekly) has been started in eight of our patients with restricted DTH defects to H. influenzae. One of them could not take the drug for longer than a month due to adverse side effects; seven have completed a six-months course. Five out of seven showed clinical improvement which was substantiated by a positive DTH skin test in four out of six cases tested.

Levamisole has been suggested to enhance secretion of thymic humoral factors (Seki et al., 1982) and very recently beneficial effects have been reported of synthetic serum thymus factor in partial immunodeficiencies (Bordigoni et al., 1982).

In conclusion this study indicates that abnormalities in cell-mediated immunity to H. influenzae and also to streptococci form a basis for at least some chronically relapsing upper respiratory tract infections. Such infections are often found in combination with other immune disorders such as atopic skin reactions and thyroid autoimmunity, and they are reminiscent of the T-cell disorders playing a role in chronic mucocutaneous candidiasis and other dermatophytoses.

REFERENCES

Berger ML and Blanden RV (1981). The T-lymphocyte in infectious pathology. Path. Res. Pract. 171, 128-141.

Blomberg, BME von, Glerum J, Croles JJ, Stam J and Drexhage HA (1980). Harmful effects of i.v. C. parvum given at the same time as cyclophosphamide in patients with squamous cell carcinoma of the bronchus. Br. J. Cancer 41, 609-617.

Bordigoni P, Faure G, Bene MC, Dardenne M, Bach JF, Duheille J and Olive D (1982). Improvement of cellular immunity and IgA production in immunodeficient children after treatment with synthetic serum thymic factor (FTS). Lancet ii, 293-297.

Burns MW and May JR (1967). H. influenzae precipitins in the serum of patients with chronic bronchial disorders. Lancet i, 354-358.

Canonica GW, Mingari MC, Melioli G, Combatti M and Moretta L (1979). Imbalances of T-cell subpopulations in patients with atopic diseases and effect of specific immunotherapy. J. Immunol. 123, 2669-2672.

Clarke CW (1979). In vivo and in vitro reactions to antigens of H. influenzae in bronchial obstruction. Br. J. Dis. Chest 73, 373-381.

Cooke A, Lydyard PM (1981). The role of T-cells in autoimmune diseases. Pathol. Res. Pract. 171, 171-196.

Couch RB (1981). The effects of influenza on host defence. J. Infect. Dis. 144, 284-291.

Denny FW (1974). Effect of a toxin produced by H. influenzae on ciliated respiratory epithelium. J. Inf. Dis. 129, 93-100.

Drexhage HA and Oort (1977). Skintest reactivity to H. influenzae antigens as an outcome of the antigen structure and the balance between humoral and cell mediated immunity in rats. Clin. Exp. Immunol. 28, 280-288.

Drexhage HA, Botazzo GF, Doniach D, Bitensky L and Chayen J (1980). Evidence for thyroid-growth-stimulating immunoglobulins in some goitrous thyroid disease. Lancet ii, 287-292.

Drexhage HA, Botazzo GF, Bitensky L, Chayen J and Doniach D (1981). Thyroid growth-blocking antibodies in primary myxoedema. Nature 289, 594-596.

Eden W van, Vries RRP de, D'Amaro J, Schreuder I, Leiker DL and Rood JJ van (1982). HLA-DR associated genetic control of the type of leprosy in a population from Surinam. Human Immunol. 4, 343-350.

Editorial (1982). How does influenza virus pave the way for bacteria. Lancet i, 485-486.

Editorial (1982). Genetics of resistance to infection. Lancet ii, 1446-1447.

Eygen M van, Znamensky PY, Heck E and Raymaekers I (1976). Levamisole in prevention of recurrent upper respiratory tract infections in children. Lancet i, 382-385.

Haan P de, Boorsma DH and Kalsbeek GL (1979). Penicillin hypersensitivity. Determination and classification of anti-penicillin antibodies by the ELISA. Allergy 34, 111-119.

Haas H, Morris JF, Samson S, Kilbourn JP and Kim PJ (1977). Bacterial flora of the respiratory tract in chronic bronchitis: comparison of transtracheal, fiberbronchoscopic, and oropharyngeal sampling methods. Amer. Rev. Respir. Dis. 116, 41-47.

Hanifin JM, Ray LF and Lobitz WC (1974). Immunological reactivity in dermatophytosis. Br. J. Dermatol. 90, 1-8.

Hayward AR (1977). Immunodeficiency. Edward Arnold, London.

Jones HE, Reinhardt JH and Rinaldi MG (1974). Immunologic susceptibility to chronic dermatophytosis. Arch. Dermatol. 110, 213-220.

Kirkpatrick CH (1971). Chronic mucocutaneous candidiasis: model-building in cellular immunity. Ann. Intern. Med. 74, 995-978.

Lagrange PH and Closs O (1979). Protective immunity to chronic bacterial infection. Scand. J. Immunol. 10, 285-290.

Lehner T, Wilton JMA, Ivanyi L (1972). Immunodeficiencies in chronic mucocutaneous candidiasis. Immunology 22, 775-778.

Louie JS and Goldberg LS (1972). Lymphocyte-monocyte defect associated with anergy and recurrent infections. Clin. Exp. Immunol. 11, 469-474.

Mackaness GB (1968). The immunology of antituberculous immunity. Am. Rev. Respir. Dis. 97, 337-344.

Moreno C (1982). Tolerance. In: Clinical Aspects of Immunology, Eds. PJ Lachmann and DK Peters, 4th ed., Vol. I, pp. 199-242. Blackwell, Oxford.

Mulder J, Goslings WRO, Plas MC van der and Lopes Cardozo P (1952). Studies on the treatment with antibacterial drugs of acute and chronic mucopurulent bronchitis caused by Hemophilus influenzae. Acta Med. Scand. 143, 32-49.

Okita N, Row VV and Volpé R (1981). Suppressor T-lymphocyte deficiency in Graves' disease and Hashimoto's thyroiditis. J. Clin. Endocrinol. Metab. 52, 528-533.

Pauwels R, Verschraegen G and Van der Straeten M (1980). IgE antibodies to bacteria in patients with bronchial asthma. Allergy 157, 655-669.

Polmar SH (1976). Immunodeficiency and pulmonary disease. In: Lungbiology in Health and Disease, eds. CH Kirkpatrick and HY Reynolds, Vol. I, Dekker, New York; pp. 191-209.

Rook GAW (1978). Three forms of delayed skin-test response evoked by mycobacteria. Nature 271, 64-65.

Rook GAW, Stanford JL (1979). The relevance to protection of three forms of delayed skin-test response evoked by M. leprae and other mycobacteria in mice. Correlation with the classical work in the guinea pig. Parasite Immunology 1, 111-1123.

Seki H, Yokoi T, Kubo M, Moriya N, Miyawaki T, Nagaoki T, Miura M and Taniguchi N (1982). Induction of E-rosette-promoting factor in human plasma by levamisole: an assessment in a patient with partial DiGeorge syndrome. Scand. J. Immunol. 15, 141-148.

Turk DC and May JR (1967). Haemophilus influenzae, its clinical importance. English University Press, London.

Turner-Warwick M and Cole P (1982). Lung disease. In: Clinical Aspects of Immunology, Eds. PJ Lachmann and DP Peters, 4th ed., Vol. II, pp. 822-852. Blackwell Scientific, Oxford.

Valdimarsson H, Holt L, Riches HRC and Hobbs JR (1970). Lymphocyte abnormality in chronic mucocutaneous candidiasis. Lancet i, 1259-1261.

Wuepper KD and Fudenberg HH (1967). Moniliasis, 'autoimmune' polyendocrinopathy, and immunologic family study. Clin. Exp. Immunol. 2, 71-82.

Zwan JC van der, Kaufmann HK, Orie NMG and Vries K de (1977). Bronchoobstructive reactions with antigenic and toxic components of H. influenzae. Acta Tuberc. Pneumol. Belg. 68, 281-284.

CHAPTER IV

PARAMETERS OF T CELL MEDIATED IMMUNITY TO COMMENSAL MICRO-ORGANISMS IN PATIENTS WITH CHRONIC PURULENT RHINOSINUSITIS:

A COMPARISON BETWEEN DELAYED TYPE HYPERSENSITIVITY SKIN TEST, LYMPHOCYTE TRANSFORMATION TEST AND MACROPHAGE MIGRATION INHIBITION FACTOR ASSAY

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Parameters of T cell mediated immunity to commensal micro-organisms in patients with chronic purulent rhinosinusitis: a comparison between delayed type hypersensitivity skin test, lymphocyte transformation test and macrophage migration inhibition factor assay

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SUMMARY

In 75 patients with unexplained chronic purulent rhinosinusitis T cell mediated immunity to three micro-organisms frequently colonizing the human upper respiratory tract, viz. *Haemophilus influenzae*, streptococci and *Candida albicans*, was assessed. Delayed type hypersensitivity (DTH) skin test reactivity was measured *in vivo*, whereas the blastogenic responsiveness (lymphocyte transformation test; LTT) and lymphokine production (e.g. migration inhibition factor; MIF) of the lymphocytes upon antigen stimulation were measured *in vitro*. MIF was assayed with a recently developed test system using the human monocytoid cell-line U937 as indicator cells in agarose microdroplets. Two-thirds of the 75 patients tested showed a defective DTH response to one or more of the microbial antigens; this contrasted to the findings in 25 healthy subjects, of whom over 90% showed a positive DTH reaction to any of the three antigens. PHA skin tests were entirely normal in both patients and healthy controls. Microbial antigen-specific LTT responses fluctuated considerably in time from strongly positive to negative and vice versa in healthy individuals as well as in patients. In general however, blastogenic responses in patients were comparable to or even higher than those of healthy persons. In the MIF assay, lymphocytes of all healthy individuals tested showed production of MIF upon stimulation with all three antigens; this again contrasted to two-thirds of the patients, whose lymphocytes showed a defective MIF production. Fluctuations of MIF-production in time could not be established and a very good correlation existed between the data obtained in the MIF assay and those of the DTH skin tests. These results indicate that apart from skin testing, the MIF assay seems to be the most suitable parameter to assess defects in T cell reactivity towards microbial antigens. These defects exist in two-thirds of our patients suffering from chronic purulent rhinosinusitis.

Keywords DTH skin test LTT MIF assay commensal microbial antigens chronic purulent rhinosinusitis

INTRODUCTION

In many clinical situations delayed type hypersensitivity (DTH) skin tests with antigens derived

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from micro-organisms are used to assess cell-mediated immunity (Spitler, 1980). However, although they provide valuable information, skin tests are unpleasant for patients as well as time-consuming, since frequent reading is necessary to obtain optimal information (Drexhage *et al.* 1983; van de Plassche-Boers, Drexhage & Kokjé-Kleingeld, 1985). Occasionally skin tests are contra-indicated, e.g. in hypersensitive persons and in some cases of atopic dermatitis (Spitler, 1980). Besides, skin tests affect the immunological status of the patient (Thestrup-Pedersen, 1974; Tosca, Parker & Turk, 1981), and may induce (further) sensitization. Often additional or confirmatory information is highly desirable. It is therefore not surprising that a variety of tests *in vitro* for cellular immunity have been developed, such as ^{3}H-thymidine incorporation (LTT) and lymphokine production (MIF) by lymphocytes upon contact with antigen.

Measurement *in vitro* of MIF production is considered to be the best correlate of the DTH reaction *in vivo* (Thor *et al.*, 1968; Valdimarsson *et al.*, 1970; Clausen, 1973; Senyk & Hadley, 1973), but the classical capillary method as first described by George & Vaughan (1962) has several disadvantages (large samples of blood required, laborious test procedures, and poor reproducibility, due to biological variability caused by the use of guinea pig peritoneal exudate cells). For this reason, many authors prefer the LTT, in spite of the use of radioactivity. Reports on the correlation between skin test and LTT results, however, are controversial (Valdimarsson *et al.*, 1970; Senyk & Hadley, 1973; Holt *et al.*, 1976; van de Fleer *et al.*, 1976; Lewinski *et al.*, 1977; Räsänen, 1980).

Recently, the disadvantages of the classical MIF test were circumvented by the development of a modified microdroplet agarose assay (Harrington & Stastny, 1973) using the human monocytoid U937 cell line as indicator cells (Singh & Khan, 1982).

In our department, we are investigating the role of cell-mediated immunity to commensal micro-organisms in patients suffering from unexplained chronic purulent rhinosinusitis. A remarkable number of these patients shows microbe-specific T cell defects, as indicated by the high incidence of negative DTH skin tests to either somatic *H. influenzae* antigen, streptokinase/streptodornase (Sk/Sd) or Candidin (=extracts of *Candida albicans*). Microbe-specific B cell responsiveness, e.g. measured as antibody production to *H. influenzae*, is entirely normal (Drexhage *et al.*, 1986), also indicating that all persons have been in contact with this commensal microbe (Drexhage *et al.*, 1983; 1986).

In this paper, we report the outcome of a comparison of DTH skin tests, LTT and MIF tests in 75 patients with chronical infections of the upper respiratory tract and 30 healthy hospital staff members.

MATERIALS AND METHODS

Subjects. Seventy-five patients (48 females and 27 males, ages 13–73 years, median 38 years) with unexplained chronically relapsing purulent rhinosinusitis, out-patients of the ENT-department of our hospital, were included in this study on criteria as described earlier (Drexhage *et al.*, 1983; 1986).

Thirty healthy hospital staff members (11 females and 19 males, age 24–44 years, median 33 years) with a negative personal and family history for atopy and autoimmunity volunteered as controls

Fifteen cord blood samples from healthy neonates born after uncomplicated pregnancy and delivery also served as controls.

Antigens. The following test antigens were used (see also Drexhage *et al.*, 1986): somatic *H. influenzae* antigen, prepared as described elsewhere (Drexhage *et al.*, 1983; van de Plassche-Boers *et al.*, 1985); and three commercially available preparations: viz 1% Candidal antigen (HAL allergens, Haarlem, the Netherlands) (=Candidin); 100 U streptokinase/streptodornase (Varidase, Lederle, Wayne, M, USA); phytohaemagglutinin (PHA) at a strength of 5 μg/ml saline (Wellcome Lab., Kent, UK).

Skin tests. Delayed responsiveness was tested by intradermal injection of 0·1 ml of each antigen preparation in the forearm. The skin reactions were read at 30 min, 6, 24 and 48 h and the diameter of the induration, expressed as the average of two measurements at right angles, was recorded.

before skin testing. Ficoll-Isopaque density-gradient centrifugation was used to isolate mononuclear cells. The cells were washed twice with Hanks' balanced salt solution.

Cells, 2×10^5 per well, were cultured five-fold over 6 days on round-bottomed Linbro : Titertek microtitre plates (Flow, Irvine, Scotland) in 0·15 ml Hepes-buffered RPMI 1640 containing glutamine (Gibco, Glasgow, Scotland), supplemented with 20% pooled human serum and antibiotics. A 5 h ^{3}H-thymidine pulse was used. Results are expressed as stimulation indices: SI = the ratio of ^{3}H-thymidine uptake in antigen-stimulated versus control cultures. Unless stated otherwise, the maximal SI is recorded for each individual, regardless of the antigen concentration at which it was reached. Antigen concentrations to stimulate the cultures ranged from 0·1–100 μg/ml for *H. influenzae* whole bacteria and soluble somatic antigen and from 0·1–150 μg/ml for candidal antigen.

To measure the blastogenic responsiveness towards PHA, 4×10^4 cells per well were cultured for 3 days; mitogen dilutions used were 1 : 250, 1 : 500 and 1 : 1000. The SI of lymphoid cells, isolated from neonatal cord blood cells, were measured to establish non-specific mitogenic capacity of bacterial antigens.

Some cell suspensions were depleted of B cells by means of the panning-technique described by Wysocki & Sato (1978) before measuring the blastogenic responsiveness.

Macrophage migration inhibition factor test. MIF production was estimated with an indirect microdroplet agarose assay. Throughout the test the medium used was Hepes-buffered RPMI 1640 containing glutamine and supplemented with antibiotics and 10% fetal calf serum (FCS).

Peripheral mononuclear cells ($2{\cdot}5 \times 10^6$) isolated as described for the LTT, were cultured in 15 ml conical tubes in 1 ml of the medium described above. Antigens were added to obtain final concentrations of 0, 5, 10 and 25 μg/ml for *H. influenzae* and Candidal antigen, and 1, 5 and 25 iu/ml for Varidase. One culture was prepared for each antigen concentration. Supernatants were also prepared using the mitogen Concanavalin A (Con A, Sigma, St Louis, USA) in a final concentration of 5 μg/ml. Supernatants were harvested (10 min 2000 *g*) after 3 days of culture (37°C, 5% CO_2 in air) and stored at −20°C until testing for MIF activity.

The agarose microdroplet assay was performed according to Thurman *et al.* (1983) but using the human monocytoid U937 cell-line as indicator cells (Singh, 1982). The U937 cell-line was kindly provided by Dr G. Garotta (Hoffmann La Roche, Basel, Switzerland). The cell-line was maintained by propagation in the above-mentioned medium, cultured at 37°C in 5% CO_2 in air. Cells were harvested in the logarithmic growth phase, counted, washed (10 min, 200 *g*), resuspended in a small volume of medium and transferred to a 1·5 ml conical tube, and concentrated by centrifugation (10 min, 200 *g*). The cell pellet was resuspended at room temperature (2×10^7 cells/ml) in a 0·2% agarose solution. This solution was prepared by dissolving 20 mg sea plaque agarose of low gelling temperature (Marine Colloids, Rockland, USA) in 1 ml phosphate-buffered saline (pH 7·4) at 120°C and diluting it ten times with medium, before adding the cells.

From this cell suspension, 1 μl droplets were centrally placed in the wells of flat-bottomed microtitreplates (Nunc, Denmark) using a Hamilton Repeating Dispenser with a 0·05 ml gas-tight syringe (Hamilton, Reno, USA). The droplets were left to solidify at 4°C for 10–20 min, and carefully overlaid with 0·1 ml of thawed supernatant diluted 1 : 1 with fresh medium. Each supernatant was tested five times. After incubation of the covered plates for 21 h at 37°C, and 5% CO_2 in air, migration areas (cell migration area minus area of the agarose droplet) were computed using a projection microscope and a graphic tablet, connected to a computer. MIF production was expressed as % migration inhibition:

$$\text{MI} = 100 - 100 \times \frac{\text{mean migration area in antigen-stimulated cultures}}{\text{mean migration area in medium}}\%$$

RESULTS

DTH skin tests. The results of skin testing 75 patients with chronic purulent rhinosinusitis with

Table 1. Defective skin test reactivity in 75 patients with chronic purulent rhinosinusitis to *Haemophilus influenzae*, *Candida albicans* and streptococci

Antigen	Number	%	Total	
H. influenzae	4	5		
Candidin	9	12	24	Defective for 1 antigen = 32%
Sk/Sd	11	15		
H. influenzae + Candidin	5	7		
H. influenzae + Sk/Sd	2	3	23	Defective for 2 antigens = 31%
Candidin + Sk/Sd	16	21		
H. influenzae + Candidin + Sk/Sd	3	4	3	Defective for 3 antigens = 4%
Total			50	67%

Of a total of 225 skin tests, 79 (35%) in patients were defective.
Of a total of 56 skin tests 3 (5%) in controls were defective.

used produced two types of reactivity: a positive DTH responsiveness with a maximal swelling at 24–48 h, and a defective response lacking this swelling (for further details see Drexhage *et al.* 1983; 1985; van de Plassche-Boers *et al.*, 1985). Fifty out of the 75 patients showed a defective skin test response to one or more of the antigens used, while over 90% of our healthy subjects had positive DTH reactions to all three antigens. Forty patients and 10 healthy individuals were also skin tested with PHA, and they all showed a normal DTH response.

These results indicate that the defective DTH skin test reactivity is antigen-specific and does not reflect a state of general impairment of delayed inflammatory responsiveness. Although the age-span of patients and controls is not similar, age-related differences could not be detected in the number of defective responses.

LTT. Since the opinions differ about the most suitable form of microbial antigens for use in the LTT (Zabriskie & Falk, 1970; Nilsson & Möller, 1972; Räsänen, 1980) we compared a soluble somatic antigen prepared from *H. influenzae* to whole bacteria. The results were highly comparable (correlation coefficient $r = 0{\cdot}88$); SI obtained with the soluble antigen usually were somewhat lower. Similar results have been described for other micro-organisms and their soluble antigens (Räsänen, 1980).

To examine the possibility that the SI found were due to a mitogenic effect of the antigens on the lymphocytes, dose-response curves were established in 15 cord blood samples. Soluble somatic exerted only weak stimulatory effects, SI exceeding this range ($> 2{\cdot}7$) were considered to represent a positive blastogenic response. The direct mitogenic effect of whole bacteria was somewhat greater, SI up to 4·5 being found, so we used soluble somatic antigen in the LTT throughout this study. These mitogenic effects of *H. influenzae* antigens are entirely in accordance with reports regarding, e.g. *Candida*, tubercle bacilli, and streptococci (Räsänen, 1980). The soluble Candidin we used also exerted only a very weak mitogenic effect, of SI up to 2·5.

The use of lymphocytes either fresh or after freezing and thawing, did not influence the outcome of the SI. Repeated testing of the same batch of lymphocytes on different occasions showed a good reproducibility of SI.

To examine whether T cells or B cells are responsible for the blastogenic response, experiments were carried out in which the lymphocyte population was depleted of B cells by means of a 'panning' technique. After panning, B cells decreased from 20% to 2%, whereas the percentage of T cells increased accordingly. The proliferative response was entirely intact and SI were slightly increased after panning. These results support the view that the *H. influenzae* somatic antigen specific blastogenic response is a T cell effect.

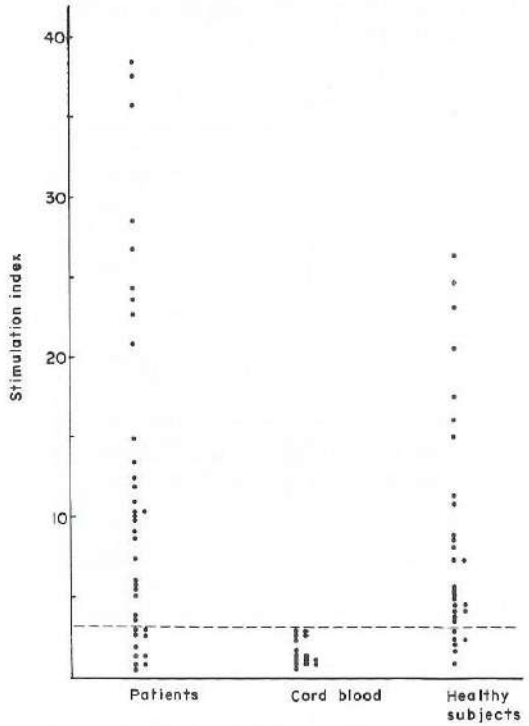

Fig. 1. The blastogenic responsiveness to soluble somatic *H. influenzae* antigen. The maximal stimulation indices of 37 patients with chronic purulent rhinosinusitis, 30 healthy subjects and 15 cord blood samples are shown. The dotted line indicates the upper limit of a mitogenic effect; values exceeding this limit represent a positive response.

and the whole micro-organism. The antigen concentration at which the optimum SI was reached was found to vary between individuals in the range of 1–15 μg/ml for whole *H. influenzae* and 10–50 μg/ml for somatic antigen. Figure 1 shows the maximal SI obtained with soluble somatic antigen in 37 patients and 30 healthy individuals.

Two-thirds of our healthy subjects had positive SI (up to 26·5), which probably reflects the continuous stimulation with commensal *H. influenzae* in a normal population. A similar proportion of the patients showed positive SI, the actual values being equal to or even higher than those of the healthy controls (up to 38·6); this probably reflects the higher exposure rate to *H. influenzae* in patients with chronic purulent rhinosinusitis. Using Candidin, entirely comparable results were obtained (data not shown).

The PHA-specific blastogenic responsiveness was measured in 30 patients and 25 healthy persons, and found to be entirely normal in all patients.

The LTT of eight healthy individuals and 10 patients were followed through time over a period of at least 6 months with intervals of at least 1 month to avoid possible influences from the skin test antigens (Thestrup-Pedersen, 1974; Tosca *et al.*, 1981). In all cases, great fluctuations of the SI were encountered, ranging from strongly positive to negative and vice versa, indicating a rapid disappearance from and appearance in the circulation of these antigen-specific T lymphocytes.

Three patients had a persistently negative blastogenic response on repeated testing over a period of 6 months, in spite of their being heavily infected with *H. influenzae*. This persistent negativity might be an indication of defective *H. influenzae*-specific T cell response in these few patients, but in general the LTT results indicate that antigen-specific T cell response is normal in nearly all the

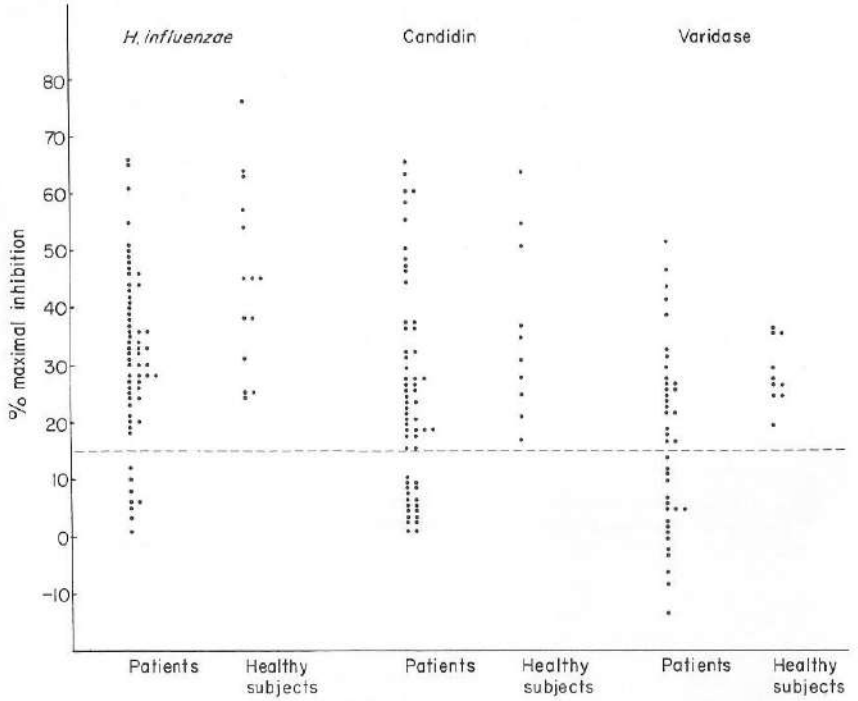

Fig. 2. The macrophage migration inhibition factor assay using *H. influenzae*, Candidin and Varidase in patients with chronic purulent rhinosinusitis and healthy subjects. The maximal % migration inhibition is given individually for each antigen. Data are shown from 60 patients and 14 healthy persons using *H. influenzae*; from 60 patients and 10 healthy persons using Candidin, and from 40 patients and 10 healthy individuals using Varidase. The dotted line represents the lower limit of values found in healthy subjects; lower values than that are considered to be negative.

MIF assay. To produce MIF-containing supernatants, whole *H. influenzae* bacteria were used. Reproducible MIF production was obtained. This is in agreement with several reports which, notwithstanding the variety of both antigens and indicator-systems used, agree upon the fact that either whole bacteria or a particulated form of the soluble antigen gives the best results in migration inhibition tests (Søborg & Bendixen, 1967; Zabriskie & Falk, 1970; Gorski, 1974; Räsänen, 1980). The U937 indicator cells provided very reproducible results; the intra-experimental variation in the migration of the cells was found to be small, so 10% inhibition was considered to be significant.

Fifteen healthy individuals were tested for all three antigens in three concentrations each. All persons, including two with a negative skin test for *H. influenzae* antigen, showed inhibition exceeding 15% at one or more concentrations used. Hence, we regarded a MIF test as positive when a percentage of inhibition of at least 15% was found at one or more of the antigen concentrations.

MIF tests with *H. influenzae* and Candidin were carried out in 60 patients, with Sk/Sd in 40 patients. The results are shown in Fig. 2. Again, two-thirds of our patients showed antigen-specific T cell defects to one or more of the antigens. In contrast, all persons showed a clearly positive lymphokine production upon stimulation of the lymphocytes with the mitogen Con A.

Repeated testing with intervals of at least 1 month in four healthy subjects and 12 patients showed no fluctuations in MIF-production.

Correlation between skin test, LTT and MIF-assay. Since LTT results were found to fluctuate with time, and skin tests and MIF assays do not do so, it is not surprising that a correlation could

Table 2. The correlation between the DTH skin test reactivity and the MIF assay using *H. influenzae*, Candidin and Varidase, in patients with chronic purulent rhinosinusitis

H. influenzae			Skin test		
	MIF	+	49	3	$\chi^2 = 33{\cdot}3$
		−	1	7	$P < 0{\cdot}001$
Candidin			Skin test		
	MIF	+	41	1	$\chi^2 = 46{\cdot}4$
		−	2	16	$P < 0{\cdot}001$
Streptokinase/ Streptodornase (Varidase)			Skin test		
			+	−	
	MIF	+	18	3	$\chi^2 = 15{\cdot}3$
		−	5	15	$P < 0{\cdot}001$

Data from 60 patients are recorded for *H. influenzae* and Candidin, and from 40 patients for Varidase. Only positivity or negativity of the test is indicated.

results of skin tests and MIF-assay. Table 2 shows the correlations between skin test reactivity and MIF assay for the three antigens tested. As can be seen, a very good correlation was found; out of 160 combinations tested, only 15 cases were found in which skin tests and MIF results differed. It must be noted, however, that either positivity or negativity of the tests is regarded in this comparison; a clear correlation between the diameter of the induration in the skin test and the maximal inhibition in the MIF-test could not be detected.

DISCUSSION

In this paper the results of a study of the microbe-specific cell-mediated immune responsiveness in 75 patients with chronic purulent rhinosinusitis are presented. These results are entirely in accordance with those reported earlier for a much smaller group of patients (Drexhage *et al.*, 1983). Two-thirds of our 75 patients showed a defective DTH skin response towards one or more of the commensal microbial antigens tested. Such a negative skin test was only rarely encountered in healthy volunteers. The PHA skin test was normal in all cases, which illustrates the antigen-specificity of the defect. The possibility that the patients had not been in contact with the microbe could be ruled out by the fact that all patients possessed *H. influenzae*-binding antibodies (Drexhage *et al.*, 1986) and in general had a positive LTT when using microbial antigens.

Although providing valuable information, skin tests are not entirely without a risk; we encountered adverse local and even generalized reactions (e.g. fever) in a few patients. To establish the value of tests *in vitro* in cell-mediated immunity, we compared the results of both LTT and MIF-test in our patients and healthy subjects.

In contrast to the skin test, in the LTT only three of our patients showed defective responses, when tested with the same microbial antigens. A considerable number of the patients had stimulation indices even higher than those of the healthy subjects, indicating an intact and even enhanced response to the continuous stimulation by the commensal micro-organisms. PHA blastogenic responsiveness was also normal in all cases. Time-course studies of blastogenic responsiveness in eight of our healthy individuals showed strong fluctuations from positive to negative. Similar fluctuations in the LTT to other microbial antigens have been reported by

With our modification of the MIF-test, all healthy individuals tested so far, including two with a negative skin test for one of the microbes, showed a positive MIF production to all three micro-organisms tested. Of the patients, two-thirds showed a defective MIF production upon stimulation with one or more of the antigens tested, again in an antigen-selective way, (all were positive to the mitogen Con A), and in accordance with the data obtained with the DTH skin tests.

In contrast to the LTT, a high degree of correlation was found between the results of the MIF test and of the skin test; in 90% of the cases tested (patients and healthy subjects), both tests were entirely in accordance with each other.

Regarding the discordance between these two tests in a relatively small number of persons, several explanations are possible. A negative skin test accompanied by a positive MIF test could be caused by the presence of blocking antibodies as has been described for DNCB (Roupe & Strannegard, 1972; Bernhard, Rosenfeld & Klein, 1972) and *H. influenzae* skin tests (Drexhage & Oort, 1977). A second possibility is the use of a higher antigenic strength for skin testing; this might very well render it positive as has been reported for e.g. PPD (Clausen, 1973). However, in view of the adverse general reactions we encountered in some patients, we are rather reluctant to try this. There is a third possibility: one of the other lymphokine factors involved in the cascade of events leading to a positive skin test might be defective: e.g. IL-2, γ-IFN, skin reactive factor or monocyte chemotactic factor. In view of the generally undisturbed blastogenic response, defective IL-2 production is highly unlikely. The production of MIF and γ-IFN is closely related but sometimes dissociated (Weiser *et al.*, 1984); isolated antigen-selective defects of γ-IFN production have been described (e.g. Virezilier *et al.*, 1978).

In conclusion our data clearly indicate that apart from the DTH skin test, the MIF assay seems useful in assessing the defects in T cell mediated immunity towards commensal micro-organisms in patients with chronic purulent rhinosinusitis. The lack of correlation between LTT and MIF assay in these patients firstly stresses the subtle nature of the microbe-selective defects, and secondly indicates that antigen-specific blastogenesis and lymphokine production must be considered as separate functions.

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REFERENCES

Bernhard, J.D., Rosenfeld, S.S. & Klein, E. (1973) Blocking of delayed hypersensitivity by humoral antibody in an in vivo mouse transfer assay using sensitized guinea pig cells. *Cell. Immunol.* **8**, 408.

Clausen, J.E. (1973) Migration inhibitory effect of cell-free supernatants from tuberculin-stimulated cultures of human mononuclear leukocytes demonstrated by two-step MIF agarose assay. *J. Immunol.* **110**, 546.

Drexhage, H.A., van de Plassche-Boers, E.M., Kokjé-Kleingeld, M. & Leezenberg, J.A. (1983) Abnormalities in cell-mediated immune functions to *Haemophilus influenzae* in chronic purulent infections of the upper respiratory tract. *Clin. Immunol. Immunopath.* **28**, 218.

Drexhage, H.A., van de Plassche-Boers, E.M., Kokjé-Kleingeld M. & Leezenberg, J.A. (1986) Subtle antigen-selective T-cell defects as a cause of relapsing purulent infections of the upper respiratory tract. In: *Recent Advances in Primary and* Aiuti, F. Rosen & M. D. Cooper) p. 395. Serono Symposia Publications, Raven Press, New York.

Drexhage, H.A. & Oort, J. (1977) Skin test reactivity to *H. influenzae* antigens as an outcome of the antigen structure and the balance between humoral and cell-mediated immunity in rats. *Clin. exp. Immunol.* **28**, 280.

George, M. & Vaughan, J.H. (1962) In vitro cell migration as a model for delayed hypersensitivity. *Proc. Soc. exp. Biol. Med.* **111**, 514.

Gorski, A.J. (1974) Superiority of corpuscular BCG to soluble PPD antigen in the leucocyte migration assay. *Clin exp. Immunol.* **18**, 149.

Graybill, J.J.R. & Alford, R.H. (1976) Variability of sequential studies of lymphocyte blastogenesis in normal adults. *Clin. exp. Immunol.* **25**, 28.

Harrington, J.T. & Stastny, P. (1973) Macrophage migration from an agarose droplet: development of a micromethod for assay of delayed hypersensiti-

ROBERTS, L.M., TANDON, M.K. & KEAST, D. (1976) Dissociation of correlates of cellular immunity in man: functional heterogeneity within the antigen-reactive cell population. *Int. Archs. Allergy appl. Immun.* **51,** 560.

LEWINSKI, U.H., MAVLIGIT, G.M., GUTTERMAN, J.U. & HERSH, E.M. (1977) Interaction between repeated skin testing with recall antigens and temporal fluctuations of in vitro lymphocyte blastogenesis in cancer patients. *Clin. Immunol. Immunopath.* **7,** 77.

NILSSON, B.S. & MÖLLER, G. (1972) Reactivity of human lymphocytes to aggregated and nonaggregated PPD-tuberculin. *Cellular Immunology* **5,** 555.

RÄSÄNEN, L. (1980) Skin test reactivity and in vitro responses to microbes and microbial antigens. *Clin. Exp. Immunol.* **40,** 566.

ROUPE, F. & STRANNEGARD, O. (1972) The influence of antibody on the induction and elicitation of allergic contact dermatitis. *Int. Arch. Allergy Appl. Immunol.* **43,** 691.

SENYK, G. & HADLEY, W.K. (1973) In vitro correlates of delayed hypersensitivity in man: ambiguity of polymorphonuclear neutrophils as indicator cells in leukocyte migration test. *Infect. Immun.* **8,** 370.

SØBORG, M. & BENDIXEN, G. (1967) Human lymphocyte migration as a parameter of hypersensitivity. *Acta Med. Scand.* **181,** 247.

SINGH, K. & KHAN, A. (1982) The usefulness of a monocytoid cell line (U-937) in the macrophage migration inhibition assay. *J. Clin. Hemat. and Oncol.* **12,** 29.

SPITLER, L.E. (1980) Delayed hypersensitivity skin testing. In: *Manual of Clinical Immunology*, 2nd edn, (Ed. by N.R. Rose & H. Friedman) p. 200. American Society for Microbiology, Washington DC.

THESTRUP-PEDERSEN, K. (1974) Temporary suppression of lymphocyte transformation after tuberculin skin testing. *Immunology* **27,** 965.

THOR, D.E., JUREZIZ, R.E., VEACH, S.R., MILLER, E. & DRAY, S. (1968) Cell migration inhibition factor released by antigen from human peripheral lymphocytes. *Nature* **219,** 755.

THURMAN, G.B., STULL, H.B., MILLER, P.J., STEVENSON, H.C. & OLDMAN, R.K. (1983) Utilization of purified human monocytes in the agarose droplet assay for measuring migration inhibitory factors. *J. Immunol. Meth.* **65,** 41.

TOSCA, N., PARKER, D. & TURK, J.L. (1981) The effect of a delayed hypersensitivity skin reaction on in vitro parameters of cell-mediated immunity. *Cell. Immunol.* **62,** 28.

VALDIMARSSON, H., HOLT, L., RICHES, H.R.C. & HOBBS, J.R. (1970) Lymphocyte abnormality in chronic mucocutaneous candidiasis. *Lancet* **i,** 1259.

VAN DE PLASSCHE-BOERS, E.M., DREXHAGE, H.A. & KOKJÉ-KLEINGELD, M. (1985) The use of somatic antigen of *Haemophilus influenzae* for the monitoring of T-cell mediated skin test reactivity in man. *J. Immunol. Methods* **83,** 353.

VAN DE FLEER A., HART M., BLOK-SCHUT, B.J.TH. & SCHELLEKENS, P.TH.A. (1976) Correlation of PPD and BCG-induced leukocyte migration inhibition, delayed cutaneous hypersensitivity, lymphocyte transformation in vitro and humoral antibodies to PPD in man. *Eur. J. Immunol.* **6,** 163.

VIRELIZIER, J-L., LENOIR, G. & GRISCELLI, C. (1978) Persistent Epstein–Barr virus infection in a child with hypergammaglobulinaemia and immunoblastic proliferation associated with a selective defect in immune interferon secretion. *Lancet* **ii,** 231.

WEISER, W.Y., REMOLD, H.G., BLOCK, L.H. & DAVID, J.R. (1984) Dissociation of human-interferon-gamma-like activity from migration- inhibition factor. *Cell. Immunol* **88,** 109.

WYSOCKI, L.J. & SATO, V.L. (1978) 'Panning' for lymphocytes: a method for cell selection. *Proc. natn Acad. Sci. USA.* **75,** 2844.

ZABRISKIE, J.B. & FALK, R.E. (1970) In vitro reactivity of lymphocytes to particulate and soluble antigens. *Nature* **226,** 943.

CHAPTER V

ABNORMAL MONOCYTE CHEMOTAXIS IN PATIENTS WITH CHRONIC PURULENT RHINOSINUSITIS:

AN EFFECT OF RETROVIRAL P15E-RELATED FACTORS IN SERUM

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Abnormal monocyte chemotaxis in patients with chronic purulent rhinosinusitis: an effect of retroviral p15E-related factors in serum

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SUMMARY

Earlier we reported that about 60% of patients suffering from unexplained relapsing of chronic purulent rhinosinusitis show a defective T cell-mediated immunity to commensal microorganisms of the upper respiratory tract. The monocyte chemotactic responsiveness was assessed in 40 of these patients by means of the polarization assay. Impaired FMLP-induced monocyte polarization was found in 26 of the 40 patients tested. The defective chemotactic responsiveness could be explained by a p15E-related factor detectable in the serum of the patients: addition of serum fractions < 25 kD to healthy donor monocytes resulted in an inhibition of polarization; a monoclonal antibody directed against p15E neutralized this inhibitory effect. In individual patients, a decreased monocyte polarization correlated well with the presence of this p15E-related factor in serum, as well as with defective T cell reactivity.

Keywords monocyte chemotactic responsiveness polarization assay p15E-related factors chronic purulent rhinosinusitis

INTRODUCTION

A disorder frequently encountered in ENT and pediatric practice, is a relapsing of chronic purulent rhinosinusitis, in spite of several courses of antibiotics and surgical intervention to improve drainage of the sinuses. As reported earlier in detail, 60% of these patients show a defective T cell mediated immunity to commensal microorganisms of the upper respiratory tract (Drexhage *et al.*, 1983). The T cell impairment was detected using delayed type hypersensitivity (DTH) skin testing and the macrophage migration inhibition factor (MIF) production by peripheral lymphocytes; both tests showed a faulty reaction to somatic antigens prepared from *Haemophilus influenzae*, to *Candida albicans* and to *streptococci* (Drexhage *et al.*, 1983 & 1986; v.d. Plassche-Boers *et al.*, 1985 & 1986). Other T cell functions, viz. the blastogenic responsiveness towards these antigens and the number of peripheral T cells, were normal (v.d. Plassche-Boers *et al.*, 1986), indicating that the microbe-specific T cell functions were only partially defective.

An impaired function of monocytes often accompanies T cell abnormalities; this has been well documented in atopic dermatitis (Furukawa & Altman 1978; Snyderman, Rogers & Buckley 1977; Ternowitz & Herlin 1986), in the human immunodeficiency virus (HIV)-caused syndromes LAS and AIDS (Smith *et al.*, 1984; Poli *et al.*, 1985) and in immunodeficiencies accompanying various types of malignancies (Currie & Hedley 1977; Snyderman, Seigler & Meadows 1977; Snyderman *et al.*, 1978; Balm *et al.*, 1982; Tan *et al.*, 1986a).

The presence in serum of factors capable of inhibiting the function of both lymphocytes and monocytes has been reported, particularly in malignancies. Factors of Mr < 25 kD capable of inhibiting Il-2 production and monocyte chemotactic responsiveness were detected in serum, urine and cancerous effusions of cats (Mathes *et al.*, 1978 & 1979), mice (Cianciolo *et al.*, 1980) and men (Cianciolo *et al.*, 1981; Balm *et al.*, 1984; Tan *et al.*, 1986a & b). These factors appeared to share structural homology with the feline and murine retroviral transmembrane protein p15E (Cianciolo *et al.*, 1981) as could be shown by adsorption studies using monoclonal antibodies against this immunosuppressive viral protein.

The hydrophobic transmembrane protein p15E is highly conserved among many type C and type D retroviruses. A structural homology between p15E and transmembrane components of other retroviruses has recently been described (Cianciolo, Kipnis & Snyderman 1984). A number of human cell-lines derived from lymphocytic and monocytic neoplasms as well as normal PHA-stimulated lymphoblasts were found to produce p15E-like factors (Cianciolo, Phipps & Snyderman, 1984). Apparently p15E-like factors can be endogenously produced by lymphocytes, monocytes and squamous epithelial cells, moreover the factors could also be detected by immunohistochemical methods in epithelial cells overlaying areas of

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inflammation (Tan *et al.*, 1987), and in normal thymic epithelial cells (unpublished observations). This suggests that the p15E-like factors play a role in normal immune regulation.

In this paper we report on defects in chemotactic responsiveness of monocytes in the above-mentioned patients with a relapsing of chronic purulent rhinosinusitis. The presence of p15E-related factors in serum, capable of depressing monocyte chemotactic responsiveness was established also. The chemotactic responsiveness was determined using the 'polarization' assay; the polarization of human monocytes toward chemoattractants is an early event that precedes their chemotactic response (Cianciolo & Snyderman, 1981). The conventional 'Boyden chamber' assay has some disadvantages such as laborious and time-consuming test procedures, and the requirement of special equipment and a relatively large blood sample. These disadvantages are overcome by the 'polarization' assay (Cianciolo & Snyderman, 1981); a modification of this test developed in our laboratory was used (Tan *et al.*, 1986a). The assay correlates well with the Boyden chamber method (Cianciolo & Snyderman, 1981; Tan *et al.*, 1986a).

MATERIALS AND METHODS

Patients

Forty patients, 16 males and 24 females, aged 20–60 years, (median 42 years) with a relapsing of unexplained purulent rhinosinusitis were studied. Criteria for inclusion in the study were a positive culture for *H. influenzae*, *S. pneumoniae*, other streptococci, or staphylococci on one or more occasions, no response to, or only temporary relief after, treatment with several courses of antibiotics, failure of surgery to improve the drainage of the ethmoidal and maxillary sinuses to give a permanent cure, no detectable gross disturbances in mucociliary transport (exclusion of Kartagener and related syndromes, v.d. Baan *et al.*, 1987), duration of the disease of at least 18 months, normal levels of total serum IgG, IgM and IgA, total numbers of peripheral blood leucocytes and differential white cell count within normal limits, normal or slightly raised *H. influenzae*-specific IgG and IgM antibody titres (which are detectable in all healthy individuals tested, v.d. Plassche-Boers *et al.*, 1985 & 1986) and no treatment with antibiotics and/or other drugs known to influence the immune system, at the time of testing.

Healthy controls

Twenty-five healthy hospital staff members, 12 males and 13 females, aged 22–40 years (median 31 years), with a negative personal and family history of respiratory disorders, atopy and autoimmunity, volunteered as controls.

Parameters of T cell functions

DTH skin test reactivity towards three commensal microbial antigens, a somatic antigen derived from *H. influenzae*, Candidin and Streptokinase/Streptodornase, was measured as described previously (v.d. Plassche-Boers *et al.*, 1985). MIF-production of peripheral lymphocytes upon stimulation with these three antigens was estimated as described in detail elsewhere (v.d. Plassche-Boers *et al.*, 1986).

Isolation of Mononuclear Leucocytes

Blood samples were obtained by venipuncture and mixed (9 : 1, v : v) with trisodium citrate 2-hydrate. The mononuclear leucocyte (monocyte) fraction was isolated by Ficoll Paque density gradient centrifugation. After isolation, the cells were washed three times in phosphate-buffered saline (PBS), pH 7·4, containing 0·38% trisodium citrate 2-hydrate and 0·5% bovine serum albumin (BSA) and counted in a haemocytometer. The number of monocytes was determined in suspension employing positive staining with non-specific esterase (Mullink *et al.*, 1979). The percentage of NSE-positive cells varied from 5–25%. An enrichment for the monocytes in the Ficoll Paque isolated fraction was obtained by Percoll gradient centrifugation (Pertoft *et al.*, 1980). After washing, the pellet containing the monocytes was resuspended in Medium 199 (pH 7·0 and containing 0·03% L-glutamine, 1% BSA and 0·084% $NaHCO_3$) and carefully underlayed with equal volumes of Percoll 1·063 and 1·067. After centrifugation (40 min 450 *g*) the cells were collected from the interface, washed twice in Medium 199 (10 min 500 *g*) and counted: the suspension now contained 40–60% NSE-positive cells.

Polarization assay

The Cianciolo & Snyderman (1981) assay was performed with slight modifications (Tan *et al.*, 1986a). Aliquots (0·2 ml) of the Percoll purified cell suspension containing $0{\cdot}2 \times 10^6$ monocytes were added to 12–75 mm polypropylene tubes (Falcon Labware Division of Becton Dickinson Co., Oxford, California, USA) containing 0·05 ml of either Medium 199 or N-formyl-methionyl-leucyl-phenylalanine (FMLP) in Medium 199, to reach a final concentration of 10 nM. All experiments were carried out in duplicate. The tubes were incubated at 37°C in a waterbath for 15 min. The incubation was stopped by addition of 0·25 ml ice-cold 10% formaldehyde in 0·05% PBS (pH 7·2). The cell suspensions were kept at 4°C until counting in a haemocytometer using an ordinary light microscope (magnification 250 ×). The test was read 'blindly' by two people; 200 cells were counted from each tube. A cell was 'polarized' if any of the following occurred: elongated or triangular shape, broadened lamellopodia, membrane ruffling. The percentage of polarized monocytes was calculated as follows:

$$\frac{\%\text{ total cells polarized}}{\%\text{ NSE-positive cells}} \times 100\%$$

Lymphocytes do not exhibit any polarization activity toward FMLP (Cianciolo & Snyderman, 1981; Wilkinson, 1986). A good correlation has been found in our laboratory between the chemotactic responsiveness towards casein in the Boyden chamber assay and the percentage of polarized monocytes when using FMLP; it is suggested that this validates the polarization assay (Tan *et al.*, 1986a).

Serum fractionation

Sera were collected from the patients by venipuncture and diluted 1 : 1 in saline. These dilutions were subjected to ultrafiltration through Amicon CF25 centriflo cones (Amicon Corp., Danvers, USA) for 15 min at 700 *g* (molecular weight 'cut off point' 25 kD). The residues were resuspended and stored at −70°C until further use.

Adsorption experiments

Adsorption experiments were carried out with serum fractions by incubation with monoclonal antibodies in a final dilution of 1 : 200 at 4°C for 16 h, followed by Amicon ultrafiltration to

remove formed complexes. The whole procedure was repeated. The monoclonal antibodies used were anti-p15E (4F5-IgG2a, kindly provided by Dr. C. J. M. Melief, Central Laboratory of the Blood Transfusion Service, Division Tumour Biology, Plesmanlaan 121, Amsterdam, The Netherlands) and a monoclonal with the same isotype, but a specificity for an unrelated antigen: anti-rat liver araginase (anti-RLA, IgG2a).

Inhibition of polarization

The ability of the serum fractions to inhibit FMLP-induced polarization of healthy donor monocytes was determined by incubating the monocytes (1×10^6/ml) for 15 min at 37°C either with FMLP alone or with FMLP in combination with a serum fraction (final dilution 1 : 60). Addition of serum fractions alone to donor monocytes did not affect the polarization. The polarization test was performed as described above, and the percentage of inhibition of FMLP-induced polarization minus spontaneous polarization caused by addition of the serum fractions calculated.

RESULTS

T cell functions

In the group of 40 patients reported here, antigen-specific T cell defects towards somatic antigen from *H. influenzae*, to Candidin and to Streptokinase/Streptodornase could again be detected in about 60% of cases. Twenty-four of the 40 patients showed a defective DTH skin test as well as a faulty MIF-production. The outcomes of both assays correlated extremely well, and in 38 patients $\chi^2 = 31{\cdot}9$; $P < 0{\cdot}001$. Fifteen patients were defective in both assays towards one of the antigens, eight patients towards two of the antigens and one patient towards all three antigens. Two exceptions were encountered. In one patient normal skin tests to all three antigens were accompanied by faulty MIF-production towards two of the antigens (*H. influenzae* and Sk/Sd). In another patient a defective skin test was found towards Candidin, and a faulty MIF-production was recorded towards *H. influenzae*.

Monocyte polarization

Total numbers of peripheral blood monocytes were within the normal range in all patients ($5{\cdot}80 \times 10^4$ monocytes/ml blood, mean 30×10^6). For details see Balm *et al.*, 1982.

Figure 1 shows the results of the polarization assay in 40 patients and 25 healthy controls. The percentage spontaneous polarization was within similar ranges for both patients and controls (7–20%). This is in agreement with results reported earlier by Tan *et al.*, (1986a) (controls: $13{\cdot}5 \pm 6{\cdot}5$%; patients $11{\cdot}9 \pm 5{\cdot}4$%). However, the FMLP-induced polarization was decreased in 26 of the 40 patients, when compared to the values found in healthy controls: patients $28{\cdot}1 \pm 8{\cdot}8$%; controls $38{\cdot}7 \pm 7{\cdot}3$%; FMLP-induced minus spontaneous polarization: in controls $25{\cdot}2 \pm 4{\cdot}6$%, in patients $16{\cdot}1 \pm 9{\cdot}3$%.

A good correlation was found between a decreased monocyte polarization assay and the presence of defective DTH skin test and MIF test results. In 37 of the 40 patients the results of monocyte function and T cell parameters were concordant ($\chi^2 = 27{\cdot}8$, $P < 0{\cdot}001$). In two patients with normal values of monocyte polarization, DTH skin tests and MIF-production were defective. In one patient the T cell parameters were normal, but the polarization values were decreased.

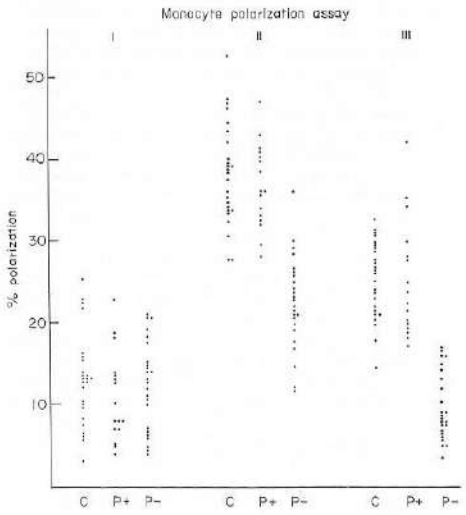

Fig. 1. Monocyte polarization assay. I, unstimulated (spontaneous) polarization; II, FMLP-stimulated polarization; III, II − I. C, healthy controls ($n = 25$); P+, patients with normal T cell functions ($n = 16$); P−, patients with decreased T cell functions ($n = 24$).

Table 1. Effect of serum fractions < 25 kD on the polarization of healthy donor monocytes

	% Inhibition of polarization			
	1:3*	1:6	1:12	1:24
Healthy individuals: $n = 12$	17·2 ± 10·4	16·6 ± 12·2	19·9 ± 8·6	17·2 ± 9·4
Patients A: $n = 7$	20·0 ± 10·7	23·6 ± 9·6	18·0 ± 11·8	19·3 ± 11·1
Patients B: $n = 17$	49·7 ± 17·7	41·7 ± 12·8	35·4 ± 17·5	17·5 ± 11·2

* Dilution of serum fraction.

Final dilutions in assay: 10 × higher.

For each concentration mean % of inhibition of polarization ± s.d. is given.

Patients A: have normal values of % polarization of their monocytes.

Patients B: 14 of the 17 patients have a decreased % polarization of their own monocytes.

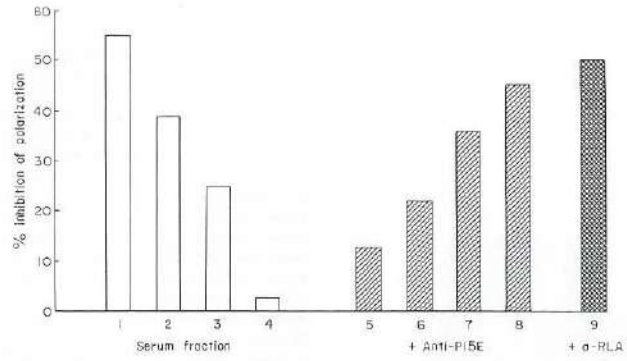

Fig. 2. Effect of adsorption on the polarization of healthy donor monocytes. All results shown were obtained by simultaneous testing of serum fractions from one patient: 1 to 4 shows the effect of different dilutions of serum fractions < 25 kD on donor monocyte polarization. Serum dilutions used: 1 = 1 : 3 (final dilution in assay 1 : 30), 2 = 1 : 6, 3 = 1 : 12, 4 = 1 : 24. In 5 to 8 the effect of adsorption of serum fraction 1 (= dilution 1 : 3) with different dilutions of anti-p15E is shown. Dilution of anti-p15E 5 = 1 : 3, 6 = 1 : 6, 7 = 1 : 12, 8 = 1 : 24, 9 shows the lack of effect of adsorption of serum fraction 1 with monoclonal antibody of the same isotype, but specificity for the unrelated antigen rat liver araginase.

Low-molecular weight inhibitory factors

Sera from 24 patients and 12 controls were ultrafiltrated on Amicon filters to yield fractions of Mr < 25 kD, which were tested in four dilutions on healthy donor monocytes for the presence of inhibitory factors. In the serum fractions from healthy individuals, some inhibitory effect was detectable, but dose-response relationships could not be established (Table 1). The level of inhibition varied between individuals, up to a maximum of 35% (inter-individual variation), when tested on the same donor monocytes. Testing of the same serum fractions on monocytes from different donors also revealed fluctuations in the level of inhibition, (intra-individual variation), but values never exceeded the inter-individual variation of 35%. Values of inhibition < 35% were thus considered to be insignificant in further experiments.

The effect of the serum fractions from seven patients was entirely comparable to healthy controls: inhibition did not exceed the 35% level. The serum fractions from 17 patients showed higher levels of inhibition of FMLP-stimulated polarization of healthy donor monocytes and dose-response effects were clearly detectable (Table 1). This indicates that factors < 25 kD capable of inhibiting monocyte polarization were present in their sera. Since in nearly all patients a 1 : 3 dilution of the fractions resulted in the highest percentage of inhibition, this dilution was used in further experiments.

Of the 17 patients possessing low molecular weight inhibitory factors, 14 belonged to the group whose own monocytes showed a decreased polarization and three patients had a normal polarization test. The seven patients without low molecular weight inhibitory factors all had a normal polarization test. Consequently, a good correlation was found in individual patients between the presence in serum of high levels of inhibitory factors < 25 kD and a disturbed chemotactic responsiveness of circulating monocytes ($\chi^2 = 13{\cdot}8$, $P < 0{\cdot}01$).

Adsorption of serum fractions with anti-p15E

The serum fractions < 25 kD from three healthy individuals and five patients with decreased monocyte polarization were adsorbed with four dilutions of a monoclonal antibody directed against p15E, as well as with a monoclonal directed against an unrelated antigen, rat liver araginase. In controls, effects from anti-p15E treatments were not detectable, but in patients this treatment had clear neutralizing effects in all cases. A representative example of the results obtained in the patients is shown in Fig. 2. In all five patients tested, the anti-p15E treatment of the serum fractions resulted in a dose-dependent decrease of the inhibiting effect, whereas anti-RLA had no effect at all. Since in practically all patients the optimal effect was seen when using anti-p15E in a 1 : 3 dilution, a larger group of patients and controls was tested using this concentration of anti-p15E.

Figure 3 shows the effects of such anti-p15E treatment in 12 controls and 24 patients. Serum fractions < 25 kD which had insignificant effects on monocyte polarization (< 35% inhibition), were not affected by the anti-p15E treatment. These serum fractions were obtained from the healthy individuals and from three patients with normal T cell functions and monocyte polarization.

The serum fractions < 25 kD obtained from 21 patients which had significant inhibitory effects on donor monocyte polarization could clearly be neutralized by anti-p15E in all cases. All of these patients had a decreased polarization of their own monocytes and T cell defects.

DISCUSSION

Earlier we reported data on DTH and MIF defects in patients suffering from a relapsing of purulent rhinosinusitis refractory to antibiotics and surgical treatment. (Drexhage *et al.*, 1983 & 1986; V.D. Plassche-Boers *et al.*, 1985 & 1986). In this paper we

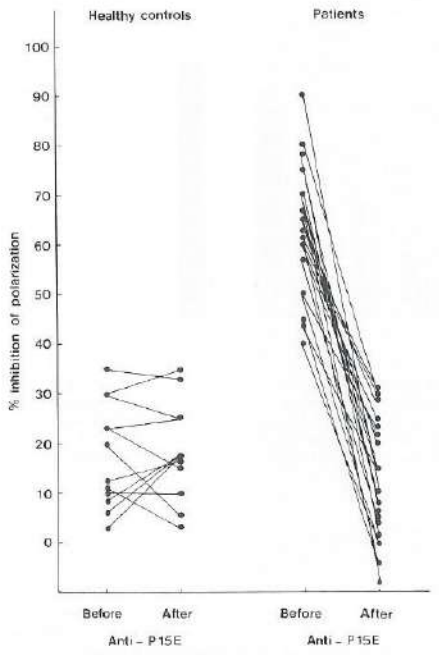

Fig. 3. The % inhibition of healthy donor monocyte polarization caused by serum fractions < 25 kD before and after adsorption with anti-p15E. Serum fractions (1:3) were adsorbed with anti-p15E (1:3). Controls: $n=12$. Patients: $n=21$ with disturbed polarization of their own monocytes.

report that the chemotactic responsiveness of monocytes, as measured by the polarization assay, is also defective in about 60% of these patients. A concordance between the presence of decreased monocyte chemotaxis and a defective T cell function was established.

A possible cause for the defective immune responsiveness was found in the presence of inhibitory factors in the patients' serum, which appeared to be p15E-related. Such increased levels of serum factors inhibiting monocyte chemotaxis have been well documented in patients suffering from thermal injuries (Altman, Furukawa & Klebanoff, 1977), several types of tumour (Pike & Snyderman, 1976; Maderazo, Anton & Ward, 1978; Snyderman, Pike & Cianciolo, 1980; Cianciolo *et al.*, 1981 & 1984a; Snyderman & Cianciolo, 1984; Balm *et al.*, 1984; Tan *et al.*, 1986b) and have also been detected in sera of HIV-seronegative homosexuals with a high risk for AIDS (Tas, Drexhage & Goudsmit 1988).

The origin of the p15E-related factors in chronic purulent rhinosinusitis is speculative. The putative possibilities range from exogenous infection with an as yet unknown retrovirus possessing envelope substances that share structural homology with p15E (it is worthy to note that several of our patients clearly indicate a virus infection just prior to the onset of their chronic upper respiratory tract complaints), to an endogenous production of the factors; it is known that p15E-related factors can be produced by lymphocytes, monocytes, thymic and mucosal epithelial cells (Tan, 1986; Tan *et al.*, 1987).

The mechanisms by which p15E inhibits FMLP-induced monocyte polarization are also speculative but interference of p15E with receptors influencing motility seems likely. Specific receptors for the N-formylated peptides have been detected on the surface of human monocytes (Weinberg, Muscato & Niedel, 1981; Benyunes & Snyderman, 1981; Snyderman & Pike, 1984). Monocytes that do respond to FMLP are also capable of responding to other N-formylated oligopeptides, as well as to other chemoattractants such as zysmosan-activated human serum (AHS) and lymphocyte-derived chemotactic factor (LDCF). Apparently, responsive monocytes share receptors for different chemotactic stimuli (Cianciolo & Snyderman, 1981). This view is supported by the findings that the p15E-like factors in human serum are capable of inhibiting the polarization induced by FMLP as well as by AHS and LCDF (Cianciolo *et al.*, 1981). On the other hand, it has been reported that on neutrophils, oligopeptide receptors appear to be different from receptors for other chemoattractants (Snyderman & Pike, 1981). In this respect it is worth noting that the FMLP-induced neutrophil chemotactic responsiveness is disturbed independently of that of the monocytes in our patients (unpublished observations) and that this has also been reported in tumour patients (Cianciolo *et al.*, 1981).

p15E-like factors are also known to influence the function of T cells. For instance, they have been reported to inhibit IL-2 production of normal T cells *in vitro* (Copelan *et al.*, 1983) and the factor is produced in mitogen-driven lymphocytic transformation assays (Cianciolo, Phipps & Snyderman, 1984). It is possible that the factors are also capable of affecting other T cell functions. Our observation of a good correlation between decreased DTH skin tests/MIF production and the presence of p15E-related factors in serum supports such a view. Another possibility might be that the p15E-disturbed monocyte functions have consequences for the functioning of the T cells (via antigen presentation, IL-1 production etc.). CKS-17, a synthetic peptide which corresponds to part of the highly conserved retroviral p15E region of homology, was reported to inhibit IL-2-dependent lymphocyte proliferation (Cianciolo *et al.*, 1985). We are now investigating the effect of this peptide on the FMLP-induced polarization of monocytes.

In conclusion, our results clearly indicate a relationship between the presence of p15E-like factors in serum and a disturbed immune function in patients suffering from a therapy refractory form of chronic purulent rhinosinusitis. In a pilot study, thymic hormone treatment was given to improve the immune responsiveness of the patients. Preliminary results indicate an effectiveness: a disappearance of p15E-like factors from the serum was found with concomitant clinical improvement. A controlled clinical trial involving a larger group of patients is presently being carried out.

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REFERENCES

Altman, L.C., Furukawa, C.T. & Klebanoff, S.J. (1977) Depressed mononuclear leukocyte chemotaxis in thermally injured patients. *J. Immunol.* **119**, 199.

v.d. Baan, S., Veerman, A.J.P., Heidendahl, G.A.K., den Hollander, W. & Feenstra, L. (1987) Primary ciliary dyskinesia and nasal mucociliary clearance. *Respiration*, **52**, 69.

Balm, A.J.M., Drexhage, H.A., v Blomberg-vd Flier, B.M.E. & Snow, G.B. (1982) Mononuclear phagocyte function in head and neck cancer: NBT-dye reduction, maturation and migration of peripheral blood monocytes. *Laryngoscope* **92**, 810.

Balm, A.J.M., v Blomberg-vd Flier, B.M.E., Drexhage, H.A., de Haan-Meulman, M. & Snow, G.B. (1984) Mononuclear phagocyte function in head and neck cancer: Depression of murine macrophage accumulation by low molecular weight factors derived from head and neck carcinomas. *Laryngoscope* **94**, 223.

Benyunes, M.C. & Snyderman, R. (1984) Characterization of an oligopeptide chemoattractant receptor on human blood monocytes using a new radioligand. *Blood* **63**, 588.

Cianciolo, G.J., Matthews, T.J., Bolognesi, D.P. & Snyderman, R. (1980) Macrophage accumulation in mice is inhibited by low molecular weight products from murine leukemia viruses. *J. Immunol.* **124**, 2900.

Cianciolo, G.J. & Snyderman, R. (1981) Monocyte responsiveness to chemotactic stimuli is a property of a subpopulation of cells that can respond to multiple chemoattractants. *J. Clin. Invest.* **67**, 60.

Cianciolo, G.J., Hunter, J., Silva, J., Haskill, J.S. & Snyderman, R. (1981) Inhibitors of monocyte responses to chemotaxins are present in human cancerous effusions and react with monoclonal antibodies to the P15(E) structural protein of retroviruses. *J. Clin. Invest.* **68**, 831.

Cianciolo, G.J., Phipps, D. & Snyderman, R. (1984) Human malignant and mitogen-transformed cells contain retroviral P15E related antigen. *J. Exp. Med.* **159**, 964.

Cianciolo, G.J., Kipnis, R.J. & Snyderman, R. (1984) Similarity between p15E of murine and feline leukaemia viruses and p21 of HTLV. *Nature* **311**, 515.

Cianciolo, G.J., Copeland, T.D., Oroszlan, S. & Snyderman, R. (1985) Inhibition of lymphocyte proliferation by a synthetic peptide homologous to retroviral envelope proteins. *Science* **230**, 453.

Cianciolo, G.J. (1986) Anti-inflammatory proteins associated with human and murine neoplasms. *Biochim. Biophys. Acta* **865**, 69.

Copelan, E.A., Rinehart, J.J., Lewis, M., Mathes, L., Olsen, R. & Sagone, A. (1983) The mechanism of retrovirus suppression of human T-cell proliferation *in vitro*. *J. Immunol.* **131**, 2017.

Currie, G.A. & Hedley, D.W. (1977) Monocytes and macrophages in malignant melanoma. I. Peripheral blood macrophage precursors. *Br. J. Cancer* **36**, 1.

Drexhage, H.A., v.d. Plasschie-Boers, E.M., Kokjé-Kleingeld, M. & Leezenberg, J.A. (1983) Abnormalities in cell-mediated immune functions to *Haemophilus influenzae* in chronic purulent infections of the upper respiratory tract. *Clin. Immunol. Immunopath.* **28**, 218.

Drexhage, H.A., v.d. Plasschie-Boers, E.M., Kokjé-Kleingeld, M. & Leezenberg, J.A. (1986) Subtle antigen-selective T-cell defects as a cause of relapsing purulent infections of the upper respiratory tract. In: *Recent Advances in Primary and Acquired Immunodeficiencies vol. 28* (ed by F. Aiuti, F. Rosen & M. D. Cooper) p. 395. Serono Symposia Publications, Raven Press, New York.

Furukawa, C.T. & Altman, L.C. (1978) Defective monocyte and polymorphonuclear leukocyte chemotaxis in atopic disease. *J. Allergy Clin. Immunol.* **61**, 288.

Maderazo, E.G., Anton, T.F. & Ward, P.A. (1978) Serum-associated inhibition of leukotaxis in humans with cancer. *Clin. Immunol. Immunopath.* **9**, 166.

Mathes, L.E., Olsen, R.G., Hebebrand, L.C., Hoover, E.A. & Schaller, J.P. (1978) Abrogation of lymphocyte blastogenesis by a feline leukaemia virus protein. *Nature* **274**, 687.

Mathes, L.E., Olsen, R.G., Hebebrand, L.C., Hoover, E.A., Schaller, J.P., Adams, P.W. & Nichols, W.S. (1979) Immunosuppressive properties of a virion polypeptide, a 15,000 Dalton protein, from feline leukaemia virus. *Cancer Res.* **39**, 950.

Mullink, H., v Blomberg-vd Flier, M., Wilders, M., Drexhage, H.A. & Alons, C.L. (1979) A simple cytochemical method for distinguishing EAC rosettes formed by lymphocytes and monocytes. *J. Immunol. Meth.* **29**, 133.

Pertoft, H., Johnsson, A., Wärmegård, B. & Seljelid, R. (1980) Separation of human monocytes on density gradients of Percoll. *J. Immunol. Meth.* **33**, 221.

Pike, M.C. & Snyderman, R. (1976) Depression of macrophage function by a factor produced by neoplasms: A mechanism for abrogation of immune surveillance. *J. Immunol.* **117**, 1243.

v.d. Plasschie-Boers, E.M., Drexhage, H.A. & Kokjé-Kleingeld, M. (1985) The use of somatic antigen of *Haemophilus influenzae* for the monitoring of T cell mediated skin test reactivity in man. *J. Immunol. Meth.* **83**, 353.

v.d. Plasschie-Boers, E.M., Drexhage, H.A., Kokjé-Kleingeld, M. & Leezenberg, J.A. (1986) Parameters of T cell mediated immunity to commensal micro-organisms in patients with chronic purulent rhinosinusitis: A comparison between delayed type hypersensitivity skin test, lymphocyte transformation test and macrophage migration inhibition factor assay. *Clin. exp. Immunol.* **66**, 516.

Poli, G., Botazzi, B., Acero, R., Bersani, L., Rossi, V., Introna, M., Lazzarin, A., Galli, M. & Mantovani, A. (1985) Monocyte function in intravenous drug abusers with lymphadenopathy syndrome and in patients with acquired immunodeficiency syndrome: selective impairment of chemotaxis. *Clin. exp. Immunol.* **62**, 136.

Smith, P.D., Ohura, K., Masur, H., Lane, H.C., Fauci, A.S. & Wahl, S.M. (1984) Monocyte function in the acquired immune deficiency syndrome. Defective chemotaxis. *J. Clin. Invest.* **74**, 2121.

Snyderman, R., Rogers, E. & Buckley, R.H. (1977) Abnormalities of leukotaxis in atopic dermatitis. *J. Allergy Clin. Immunol.* **60**, 121.

Snyderman, R., Seigler, H.F. & Meadows, L. (1977) Abnormalities of monocyte chemotaxis in patients with melanoma: Effects of immunotherapy and tumor removal. *J. Natl. Cancer Inst.* **58**, 37.

Snyderman, R., Meadows, L., Holder, W. & Wells, S. (1978) Abnormality of monocyte chemotaxis in patients with breast cancer: Evidence for a tumor mediated effect. *J. Natl. Cancer Inst.* **60**, 737.

Snyderman, R., Pike, M.C. & Cianciolo, G.J. (1980) An inhibitor of macrophage accumulation produced by neoplasms: Its role in abrogating host resistance to cancer. In: *Mononuclear Phagocytes. Functional Aspects. part I.* (ed. by R. van Furth), p. 569. Martinus Nijhoff Publishers, The Hague, Boston, London.

Snyderman, R. & Cianciolo, G.J. (1984) Immunosuppressive activity of the retroviral envelope protein P15E and its possible relationship to neoplasia. *Immunology Today* **5**, 240.

Snyderman, R. & Pike, M.C. (1984) Chemoattractant receptors on phagocytic cells. *Ann. Rev. Immunol.* **2**, 257:281.

Tan, I.B., Drexhage, H.A., Scheper, R.J., v Blomberg-vd Flier, B.M.E., de Haan-Meulman, M., Snow, G.B. & Balm, A.J.M. (1986a) Defective monocyte chemotaxis in patients with head and neck cancer. Restoration after treatment. *Arch. Otolaryngol. Head Neck Surg.* **112**, 541.

Tan, I.B., Drexhage, H.A., Scheper, R.J., v Blomberg-vd Flier, B.M.E., de Haan-Meulman, M., Snow, G.B. & Balm, A.J.M. (1986b) Immunosuppressive retroviral P15E-related factors in head and neck carcinomas. *Arch. Otolaryngol. Head Neck Surg.* **112**, 942.

Tan, I.B. (1986) Retroviral p15-E-related factors in head and neck cancer. Thesis, Free University, Amsterdam.

Tan, I.B., Drexhage, H.A., Mullink, H., Henzen-Logmans, S.C., de Haan-Meulman, M., Snow, G.B. & Balm, A.J.M. (1987) Immunohistochemical detection of retroviral-p15E-related material in carci-

nomas of the head and neck. *Otolaryngol. Head Neck Surg.* **96**, 251.

TAS, M., DREXHAGE, H.A. & GOUDSMIT, J. (1988) A monocyte chemotaxis inhibiting factor in serum of HIV infected men shares epitopes with the HIV transmembrane protein GP41. *Clin. exp. Immunol.* **71**, 13.

TERNOWITZ, T. & HERLIN, T. (1986) Defective monocyte and polymorphonuclear leukocyte chemotaxis and clinical characteristics in atopic dermatitis. *Arch. Dermatol. Res.* **278**, 454.

WEINBERG, J.B., MUSCATO, J.J. & NIEDEL, J. (1981) Monocyte chemotactic peptide receptor. *J. Clin. Invest.* **68**, 621.

WILKINSON, P.C. (1986) The locomotor capacity of human lymphocytes and its enhancement by cell growth. *Immunology* **57**, 281.

CHAPTER VI

GENERAL DISCUSSION

GENERAL DISCUSSION

Several conclusions can be drawn from our immune function studies in patients with a relapsing of purulent rhinosinusitis and unresponsiveness to current antibiotic and surgical therapy regimens.

- Defects in systemic cell-mediated immunity were found in 2/3 of the patients:
  a. DTH skin test reactivity towards three commensal micro-organisms colonizing the upper respiratory tract, viz. H. influenzae, streptococci and Candida albicans, showed defective responses.
  b. Microbe-specific MIF-production by the T-lymphocytes of these patients was also defective.
  c. The monocyte chemotactic response to FMLP - measured in the polarization assay - was disturbed. In individual patients the disturbed monocyte polarization correlated well with the microbe-specific CMI defects established in skin test and MIF-production assay.
  d. Some results indicate a discrepancy between the expression of CD3 (OKT3) and CD2 (OKT11) markers on lymphoid cells. Normal numbers of peripheral T cells were found in the patients, except for a slightly raised CD4/CD8 ratio in 36% of them. This disturbance correlated to the "atopic" state of the patient, viz. elevated IgE levels. Mitogen-induced as well as microbial antigen-induced lymphocyte proliferative responses were in general not defective.
- These defects in CMI might be explained by the presence of a p15E-like immunosuppressive factor detected in the serum of this 2/3 of the patients.
- In 1/3 of the patients the above-mentioned CMI functions were found to be intact. Although elevated serum IgE levels and atopic skin test reactivity to common allergens were often present in these patients they did not suffer from overt atopic complaints.
- B cell functions were intact in the patients: the percentage of circulating B cells was within the normal range, immunoglobulin G, A and M levels in serum were not decreased and H. influenzae-specific antibody titres were either normal or even enhanced.

A combination of defective T-cell reactivity and elevated serum IgE levels has also been reported in relapsing pyogenic dermatitis caused by S. aureus (Hanifin and Lobitz, 1977). Elevated T helper-inducer/T suppressor-cytotoxic cell ratios as judged by CD4/CD8 expression, were also frequently detected in our patients. Elevated IgE is known to be associated with raised numbers of T helper cells.

Genetic influences might play a role in therapy-resistant chronic purulent rhinosinusitis. This is indicated by the high incidence of autoimmunity and atopic skin tests in the patients and further supported by the high incidence of chronic respiratory diseases in their first-degree relatives (Ch. III). However, a pilot study on the incidence of genetic markers in our patients indicated no preference for any particular marker studied (unpublished results).

To investigate the possibility of disturbed neutrophil functions contributing to the development of chronic purulent rhinosinusitis, a pilot study was carried out in 16 patients. E. coli killing capacity was estimated according to the method described by Peck (1985) and was found to be entirely normal. Neutrophil chemotaxis was estimated in the polarization assay. Disturbed FMLP-induced polarization was detectable in several patients, but this defect neither correlated with antigen-specific T-cell reactivity (DTH skin test and MIF), nor with disturbed monocyte polarization, nor with elevated serum IgE-levels. Apparently neutrophil chemotaxis can be disturbed independently. This has also been reported in tumour patients (Cianciolo et al., 1981).

The detected disturbances in CMI might be explained by the presence of an immunosuppressive low molecular weight factor in the circulation of the patients. This factor shares homology with an envelope protein of murine and feline leukaemia virus, viz. with p15E (Cianciolo et al., 1981). This p15E is the transmembraneous part of an envelope protein, gp 85 (Mathes et al., 1979; Snyderman and Cianciolo, 1984) and can be held responsible for the previously detected immunosuppressive effects of the retroviruses preceding the tumour growth in the animals (Mathes et al., 1978 and 1979; Cianciolo et al., 1980). The species specificity of these immunosuppressive effects of murine and feline retroviral p15E is restricted: it also suppresses the blastogenic response of lymphocytes and the chemotactic response of monocytes of other animals as well as of

man (Mathes et al., 1979; Copelan et al., 1983; Cianciolo, Kipnis and Snyderman, 1984; Snyderman and Cianciolo, 1984).

Mammalian cells are capable of producing immunosuppressive peptides sharing homology with retroviral p15E (Cianciolo et al., 1983, Cianciolo, Phipps and Snyderman, 1984). These p15E-like factors were first detected by Cianciolo and Snyderman studying murine mammary carcinoma-derived factors influencing macrophage activity (Cianciolo, Herberman and Snyderman, 1980; Cianciolo et al., 1980). It was possible to isolate these factors directly from the tumours, but they also appeared to be present in plasma and urine of mice bearing the neoplasms (Snyderman, Pike and Cianciolo, 1980). Balm et al. (1984) were able to isolate the p15E-like factors from head and neck carcinomas of man, whereas Tan et al. (1986) detected these immunosuppressive factors in the circulation of head and neck cancer patients. Various malignant human cell-lines, including the monocytoid cell-line U937, are also capable of producing p15E-like factors (Cianciolo, Phipps and Snyderman, 1984) and the U937 cell-line is used in our laboratory as source of the immunosuppressive factors.

Not only malignant cells were found to be capable of producing p15E-like factors, non-malignant cells also appeared to contain p15E-like material. In immunohistochemical studies, particularly thymus epithelial cells, epithelial cells overlaying areas of inflammation and spleen marginal zone macrophages were found positive for these factors (Tan et al., 1987; M. Tas, unpublished results). It is thus not surprising that normal human DNA contains copies of a sequence coding for a peptide at least partially homologous to retroviral p15E (Repaske et al., 1983 and 1985). This homology exists in a sequence coding for 26 amino-acids which sequence is well conserved in evolution, and shares homology with sequences of envelope proteins of several other retroviruses, e.g. p21 of HTLV-1, gp42 of HIV (Cianciolo, Kipnis and Snyderman, 1984; Patarca and Haseltine, 1984). Recently a synthetic peptide has been constructed consisting of the first 16 amino-acids of the 26 amino-acid retroviral sequence: CKS-17 (Cianciolo et al., 1985). CKS-17 was found to suppress FMLP-induced monocyte polarization as well as in vitro IL-2 production by healthy donor monocytes (Cianciolo et al., 1985; Oostendorp, unpublished observations). Taken together, these data suggest that normal human DNA clearly encodes for an immunosuppressive p15E-like factor, and it is tempting to speculate that this p15E-like peptide produced by

normal tissue cells is a physiological factor playing a role in immunoregulation. Our finding that normal thymus cells were positive for p15E-like material may indicate that the immunosuppressive factor belongs to the family of immunoregulatory thymic hormones. It is an open question where the production spot of the p15E-like factors in our patients may be. Immunohistochemical studies are now in progress to further investigate this point.

A common denominator for the selective defects in the CMI of the patients could well be a basic disturbance in antigen-presentation and accessory cell function. There are various class II MHC positive cells capable of acting as antigen-presenting accessory cells in T-cell mediated immune functions. The dendritic accessory cells represent a family of cells joined by their accessory function for resting T-lymphocytes (Steinman et al., 1983; Austyn, 1987; Peters, Ruhl and Friedrichs, 1987), in part also for B-lymphocytes (Peters, Ruhl and Friedrichs, 1987), and their morphological appearance. The family includes the Langerhans cell from the skin, the veiled cell from the afferent lymph and the interdigitating cell from T-cell areas in lymphoid tissues (Austyn, 1987).

Dendritic cells seem essential to induce immune responses in previously resting T-cells. Sensitized T-cells can respond to antigen that is presented by any antigen-presenting cell. It therefore seems likely that, in addition to presenting antigen, dendritic cells can give a unique activation signal to resting T-cells that may not be required by activated cells (Austyn, 1987). Very low numbers of dendritic cells are sufficient to trigger high numbers of lymphocytes to respond (Peters, Ruhl and Friedrichs, 1987).

Dendritic cells can physically associate ("cluster") with T-cells in a unique manner that is independent of antigen or MHC-compatibility. In contrast other accessory cells cluster with T-blasts only in the presence of the specific antigen and relevant MHC. The clustering process of the dendritic cells precedes T-cell activation. The clusters are enriched for antigen-specific T-cells in antigen responses. It is within the clusters that T-cells proliferate and secrete lymphokines (Austyn, 1987).

The presence and function of dendritic cells in the lesions of Crohn's disease, thyroid autoimmunity, Type I diabetes and rheumatoid arthritis, have been extensively studied in our department during the

last few years (Wilders et al., 1984; Voorbij and Drexhage, 1985; Kabel et al., 1987, 1988; Kabel, 1989; van Dinther et al., 1989).

Very recently we found that dendritic cells cultured from the peripheral blood of patients with thyroid autoimmune diseases showed a decreased capacity to cluster with allogeneic lymphocytes (Kabel, 1989).

In patients with relapsing purulent rhinosinusitis such investigations are now in progress.

Since the CMI in patients suffering from a relapsing of purulent rhinosinusitis is likely to be depressed by the presence of a p15E-like "hormonal" factor, immunotherapy with immunostimulatory hormonal factors, called "thymic hormones" is an obvious choice. In neonates thymic hormones induce a terminal differentiation in T-cells, and are able to stimulate T-lymphocytes to produce lymphokines. In adults the administration of thymic hormones has also been described as being effective in improving CMI functions and low levels of endogenous thymic hormones can be detected in several secondary immunodeficiency states as well as in viral infections and other states of impaired adult host defence.

It is to be hoped that similar studies will ultimately contribute to refinements in the treatment of relapsing chronic purulent rhinosinusitis. In patients with demonstrable defects of CMI addition of immunotherapy to the current treatment could be considered.

Preliminary observations in patients with relapsing purulent rhinosinusitis using the thymic hormone preparation "thymostimulin" (TP-1^{R}, Serono) suggest a beneficial effect on the clinical course of the disease (subjective improvement, diminution of mucopurulent nasal discharge and a decrease in positive bacterial culture rates from the nose) and an improved function of the defective CMI (enhanced monocyte polarization, disappearance of the p15E-like factors from the circulation, improved DTH skin tests and MIF-production). These beneficial preliminary results are now confirmed by a double-blind cross-over, placebo-controlled trial with TP-1.

REFERENCES

Austyn JM (1987). Lymphoid dendritic cells. Immunology 62, 161-170.

Balm AJM, von Blomberg-van der Flier BME, Drexhage HA, De Haan-Meulman M and Snow GB (1984). Mononuclear phagocyte function in head and neck cancer. Depression of murine macrophage accumulation by low molecular weight factors derived from head and neck carcinomas. Laryngoscope 94, 223-227.

Cianciolo GJ, Matthews TJ, Bolognesi DF and Snyderman R (1980). Macrophage accumulation in mice is inhibited by low molecular weight products from murine leukemia viruses. J. Immunol. 124, 2900-2905.

Cianciolo GJ, Herberman RB and Snyderman R (1980). Depression of murine macrophage accumulation by low molecular weight factors derived from spontaneous mammary carcinomas. J. Natl. Cancer Inst. 65, 829-834.

Cianciolo GJ, Hunter J, Silva J, Haskill JS and Snyderman R (1981). Inhibitors of monocyte responses to chemotaxins are present in human cancerous effusions and react with monoclonal antibodies to the P15(E) structural protein of retroviruses. J. Clin. Invest. 68, 831-844.

Cianciolo GJ, Lostrom ME, Tam M and Snyderman R (1983). Murine malignant cells synthesize a 19,000-dalton protein that is physicochemically and antigenically related to the immunosuppressive retroviral protein, P15E. J. Exp. Med. 158, 885-900.

Cianciolo GJ, Phipps D and Snyderman R (1984). Human malignant and mitogen-transformed cells contain retroviral P15E-related antigen. J. Exp. Med. 159, 964-969.

Cianciolo GJ, Kipnis RJ and Snyderman R (1984). Similarity between p15E of murine and feline leukaemia viruses and p21 of HTLV. Nature 311, 515.

Cianciolo GJ, Copeland TD, Oroszlan S and Snyderman R (1985). Inhibition of lymphocyte proliferation by a synthetic peptide homologous to retroviral envelope proteins. Science 230, 453-455.

Copelan EA, Rinehart JJ, Lewis M, Mathes L, Olsen R and Sagone A (1983). The mechanism of retrovirus suppression of human T cell proliferation in vitro. J. Immunol. 131, 2017-2020.

Van Dinther-Janssen ACHM, Pals ST, Scheper RJ, Breedveld F and Meijer CJLM (1989). Dendritic cells and high endothelial venules in the rheumatoid synovial membrane. (Accepted for publication, J. Rheumatol.).

Hanifin JM and Lobitz WC (1977). Newer concepts of atopic dermatitis. Arch. Dermatol. 113, 663-670.

Kabel PJ, Voorbij HAM, Van der Gaag RD, Wiersinga WM, De Haan M and Drexhage HA (1987). Dendritic cells in autoimmune thyroid disease. Acta Endocrinol. (Copenh.) 1987, suppl. 281, 42-48.

Kabel PJ, Voorbij HAM, De Haan M, Van der Gaag RD and Drexhage HA (1988). Intrathyroidal dendritic cells. J. Clin. Endocrin. Metab. 66, 199-207.

Kabel PJ (1989). Dendritic cells and intrathyroidal lymphoid tissue in autoimmune thyroid disease. Thesis, Free University, Amsterdam.

Mathes LE, Olsen RG, Hebebrand LC, Hoover EA and Schaller JP (1978). Abrogation of lymphocyte blastogenesis by a feline leukemia virus protein. Nature (Lond.) 274, 687-689.

Mathes LE, Olsen RG, Hebebrand LC, Hoover EA, Schaller JP, Adams PW and Nichols WS (1979). Immunosuppressive properties of a virion polypeptide, a 15.000-dalton protein, from feline leukemia virus. Cancer Res. 39, 950-955.

Patarca R and Haseltine WA (1984). Similarities among retrovirus proteins. Nature 312, 496.

Peck R (1985). A one-plate assay for macrophage bactericidal activity. J. Immunol. Meth. 82, 131-140.

Peters JH, Ruhl S and Friedrichs D (1987). Veiled accessory cells deduced from monocytes. Immunobiol. 176, 154-166.

Repaske R, O'Neill RR, Steele PE and Martin MA (1983). Characterization and partial nucleotide sequence of endogenous type C retrovirus segments in human chromosomal DNA. Proc. Natl. Acad. Sci. USA 80, 678-682.

Repaske R, Steele PE, O'Neill RR, Rabson AB and Martin MA (1985). Nucleotide sequence of a full length human endogenous retroviral segment. J. Virology 54, 764-772.

Snyderman R, Pike MC and Cianciolo GJ (1980). An inhibitor of macrophage accumulation produced by neoplasms: its role in abrogating host resistance to cancer. In: Mononuclear phagocytes. Functional aspects, part 1, ed. R. van Furth. Martinus Nijhoff publishers, The Hague, Boston, London, pp. 569-592.

Snyderman R and Cianciolo GJ (1984). Immunosuppressive activity of the retroviral envelope protein P15E and its possible relationship to neoplasia. Immunology Today 5, 240-244.

Steinman RM, Gutchinov B, Witmer MD and Nussenzweig MC (1983). Dendritic cells are the principle stimulators of the primary mixed leucocyte reaction in mice. J. Exp. Med. 157, 613-627.

Tan IB, Drexhage HA, Scheper RJ, von Blomberg-van der Flier BME, de Haan-Meulman M, Snow GB and Balm AJM (1986). Immunosuppressive retroviral P15E-related factors in head and neck carcinonomas. Arch. Otolaryngol. Head & Neck Surg. 112, 942-945.

Tan IB, Drexhage HA, Mullink R, Henzen-Logmans S, de Haan-Meulman M, Snow GB and Balm AJM (1987). Immunohistochemical detection of retroviral P15E related material in carcinomas of the head and neck. Otolaryngol. Head & Neck Surg. 96, 251-255.

Voorbij HAM and Drexhage HA (1985). Antigen presenting dendritic cells in the early phases of the spontaneous autoimmune thyroiditis of the BB/W rat. J. Endocrinol. 104 (suppl.) 97.

Wilders MM, Drexhage HA, Kokjé M, Verspaget HW and Meuwissen SGM (1984). Veiled cells in chronic inflammatory bowel disease. Clin. Exp. Immunol. 55, 377-387.

SUMMARY

Relapsing chronic purulent rhinosinusitis is an ill-defined disorder with variable complaints. Resistance against current antibiotic treatment and surgery is regularly encountered.

Pathogenetic mechanisms underlying this disorder are largely unknown, but commensal micro-organisms e.g. non-capsulated H. influenzae, streptococci and Candida albicans, are suspected to play an important role. These normal inhabitants of parts of the upper respiratory tract are known to become pathogens when invading the sinuses and the bronchial tree. Several decades ago it was already established that antibody levels to these micro-organisms are usually either normal or enhanced in patients with chronic upper respiratory tract infections, but apparently they are not protective. If immune defects do play a part, the antibody levels do indicate that B-cell functions are not affected. Systemic cell-mediated immunity had not yet been extensively investigated in relapsing purulent rhinosinusitis.

The aim of this study was to establish whether defects in systemic cell-mediated immunity could be detected which might contribute to the pathogenetic mechanisms responsible for the therapy-resistant form of chronic purulent rhinosinusitis.

The following parameters were used:

a. DTH skin test reactivity, lymphocyte blastogenic responsiveness and MIF-production towards antigens derived from H. influenzae, streptococci and Candida albicans.
b. To study monocyte chemotaxis, the FMLP-induced monocyte polarization.
c. The presence of low molecular weight p15E-like factors in serum since these factors possibly affect cellular immune functions.
d. Enumeration of total numbers of peripheral blood lymphocytes and monocytes as well as T-cell subsets and B-cells.
e. To monitor B-cell functions, serum immunoglobulin levels and H. influenzae-specific antibody titres.

In chapter II the usefulness of somatic H. influenzae antigen for monitoring T-cell reactivity in DTH skin testing was investigated in healthy volunteers. The common antigen of all haemophili was extracted from a non-capsulated strain and tested in both soluble and aggregated form. An ELISA method was developed for estimating H. influenzae-specific antibody titres in these individuals. In the serum of all individu-

als tested both IgG and IgM antibodies to H. influenzae were detectable. Soluble somatic antigen turned out to be a poor antigen for eliciting a DTH skin test response, since only 2 out of 16 healthy persons tested showed a positive reaction. In contrast, 25 out of 28 individuals showed a positive DTH reaction when tested with aggregated somatic H. influenzae antigen. Two types of reaction were encountered in a ratio of 3:2 viz. with an early (24 hr) and with a late (48 hr) maximal swelling. Histological examination showed perivascular infiltrates of mainly T-helper/inducer lymphocytes.

These results indicate that the aggregated form of somatic H. influenzae antigen is useful for DTH skin testing in man.

In chapter III, DTH skin test reactivity towards commensal microbial antigens (viz. aggregated somatic antigen derived from H. influenzae, commercially available streptokinase/streptodornase derived from streptococci and candidal antigen) was studied in 36 patients with chronically relapsing purulent upper respiratory tract infections.

Over 90% of the healthy controls showed DTH reactivity, but in two-thirds of the patients a defective DTH reactivity was encountered, whereas four showed hyperergic reactivity leading to necrosis of the test site and general feelings of malaise. A negative lymphocyte blastogenic response to H. influenzae was found in only three of our patients. CD4+/CD8+ ratios were elevated in about 40% of the patients. A high incidence of atopic skin tests and (thyroid) autoimmunity was evident in patients as well as in their first-degree relatives.

These results suggested that unexplained chronically relapsing rhinosinusitis might be based on restricted T-cell defects towards commensal micro-organisms such as H. influenzae, streptococci and Candida albicans. Therefore T-cell parameters were extensively investigated in a larger group of 75 patients. In chapter IV, a comparison was made between antigen-specific DTH skin test reactivity, lymphocyte blastogenic responsiveness (LTT) and MIF-production in these 75 patients. For the latter assay an adaptation of the microdroplet agarose assay using U937 indicator cells was developed.

Two-thirds of the patients showed a defective DTH response towards one or more of the microbial antigens (somatic H. influenzae antigen, SK/SD and candidal antigen), whereas over 90% of the healthy controls had a positive DTH reactivity to any of the three antigens. Microbial antigen specific LTT responses fluctuated considerably in time in both

patients and healthy controls. In general, however, blastogenic responses were intact in the patients and comparable to or even higher than the SI of the healthy individuals. In the MIF-assay, lymphocytes of all healthy individuals showed production of MIF upon stimulation with all three antigens; this again contrasted to two-thirds of the patients whose lymphocytes showed a defective MIF-production. Fluctuations of MIF-production in time could not be established and a very good correlation existed between the data obtained in the MIF-assay and those of the DTH skin tests.

These results indicate that apart from skin testing in vivo, the MIF-assay seems to be the most suitable in vitro parameter to assess defects in T-cell reactivity towards commensal microbial antigens. These partial T-cell defects thus existed in about two-thirds of our patients suffering from chronic purulent rhinosinusitis.

It is well documented that an impaired function of monocytes often accompanies T-cell defects. Therefore the monocyte chemotactic responsiveness was assessed in 40 patients with chronic purulent rhinosinusitis employing the polarization assay (Chapter V). Impaired FMLP-induced monocyte polarization was found in 26 out of 40 patients tested. The defective chemotactic responsiveness might be explained by the presence of a p15E-related factor in the serum of these patients: addition of serum fractions < 25 kD to healthy donor monocytes caused inhibition of polarization; a monoclonal antibody directed against p15E neutralized this inhibitory effect. A decreased monocyte polarization correlated well with the presence of p15E-related factors in serum, as well as with defective antigen-specific T-cell reactivity (DTH and MIF).

p15E is the hydrophobic transmembrane protein of murine and feline leukemia viruses and can cause immunosuppressive effects. p15E shares structural homology with envelope components of several retroviruses. Malignant as well as non-malignant mammalian cells are capable of producing p15E-like factors which is not surprising since human DNA contains copies of a sequence which is well conserved in evolution and is coding for 26 amino acids of the immunosuppressive factor. This factor may play a role in hormonal immunoregulation of the T-cell response. A recently constructed synthetic peptide CKS-17 which contains 16 amino acids of the homologous region of the various retroviruses and the endogenous sequence, was found to be capable of suppressing monocyte polarization and in vitro IL-2 production.

A basic disturbance underlying the here described selective defects in CMI of the patients could well be an impairment in antigen presentation and accessory cell function of dendritic cells. T-cells are known to proliferate and secrete lymphokines after clustering with dendritic cells. Preliminary results suggest that the p15E-like serum factors in patients with chronic purulent rhinosinusitis are capable of hampering the cluster capacity of healthy dendritic cells. Further studies are necessary to elucidate these mechanisms.

In view of the presence of immunosuppressive p15E in the circulation of patients suffering from chronic purulent rhinosinusitis, immunotherapy is an obvious choice.

SAMENVATTING

Recidiverende chronische purulente rhinosinusitis is een slecht gedefinieerd ziektebeeld met uiteenlopende klachten. Resistentie tegen gangbare behandelingen met antibioticakuren en chirurgische ingrepen komt regelmatig voor.

De aan dit ziektebeeld ten grondslag liggende pathogenetische mechanismen zijn nog grotendeels onbekend; commensale microörganismen zoals H. influenzae, streptococcen en Candida albicans worden er van verdacht een rol te spelen. Van deze normaal in (bepaalde delen van) de bovenste luchtwegen voorkomende microörganismen is bekend dat ze pathogeen worden wanneer ze de sinussen en de bronchiaalboom binnendringen. Reeds enkele tientallen jaren is bekend dat de antistoffen tegen deze microörganismen in het algemeen normaal of zelfs verhoogd zijn bij patiënten met infecties van de bovenste luchtwegen; klaarblijkelijk bieden deze antistoffen geen bescherming. Mochten immuundefecten een rol spelen, dan suggereren de antistoftiters dat de B-celfuncties niet zijn aangedaan. De rol van de cellulaire immuniteit is nog niet uitgebreid onderzocht bij recidiverende chronische purulente rhinosinusitis.

Het doel van dit onderzoek was vast te stellen of stoornissen in de systemische cellulaire immuniteit konden worden aangetoond die bij zouden kunnen dragen aan de pathogenetische mechanismen die verantwoordelijk zijn voor de therapie-resistente vorm van chronische purulente rhinosinusitis.

Onderstaande parameters werden gebruikt:

a. de huidtestreactiviteit van het vertraagde type, de lymfocytenblasttransformatietest en de MIF-productie, t.o.v. uit H. influenzae, streptococcen en Candida albicans geëxtraheerde antigenen, werd bepaald.
b. Om de chemotaxis van monocyten te bestuderen werd de polarisatie van monocyten na stimulatie met FMLP bepaald.
c. De aanwezigheid van laag-moleculaire p15E-achtige factoren in serum werd nagegaan, daar deze factoren de cellulaire immuunfuncties kunnen remmen.
d. De totale aantallen lymfocyten en monocyten in het perifere bloed werden geteld en de verdeling over T-cel subgroepen en B-cellen vastgesteld.

e. B-celfuncties werden nagegaan aan de hand van de titers van de verschillende klassen immuunglobulinen in serum, en de H. influenzae-specifieke antistof titers.

In hoofdstuk II wordt gerapporteerd over de bruikbaarheid van somatisch antigeen uit H. influenzae bij het screenen van de T-celreactiviteit d.m.v. huidtesten van het vertraagde type. Het gemeenschappelijke antigeen van alle Haemophilus stammen werd geëxtraheerd uit een ongekapselde stam en getest in oplosbare en geaggregeerde toestand in de huid van gezonde vrijwilligers.

Tevens werd een ELISA-methode ontwikkeld voor het bepalen van specifieke antistoffen tegen H. influenzae. In de sera van alle geteste gezonde vrijwilligers waren tegen H. influenzae gerichte IgG en IgM antistoffen aantoonbaar.

Somatisch H. influenzae antigeen bleek in oplosbare vorm ongeschikt te zijn voor het opwekken van vertraagd type overgevoeligheid: van de 16 geteste personen vertoonden slechts 2 een positieve huidreactie. Daarentegen hadden 25 van de 28 geteste vrijwilligers een positieve vertraagd type huidreactie bij gebruik van de geaggregeerde vorm van het somatisch H. influenzae antigeen. Er werden 2 typen reacties in de verhouding 3:2 waargenomen, nl. een reactietype met een vroege (24 uur) en één met een late (48 uur) maximale zwelling. Bij histologisch onderzoek bleken voornamelijk T-helper/T-inducer lymfocyten in de infiltraten aanwezig te zijn.

Uit deze resultaten blijkt dat somatisch antigeen uit H. influenzae in geaggregeerde vorm geschikt is voor het meten van huidtestreactiviteit van het vertraagde type bij mensen.

In hoofdstuk III werd bij 36 aan recidiverende chronische purulente rhinosinusitis lijdende patienten de huidtestreactiviteit van het vertraagde type t.o.v. commensale microbiële antigenen bestudeerd. Gebruik werd gemaakt van geaggregeerd somatisch H. influenzae antigeen, en in de handel verkrijgbare antigenen uit streptococcen en Candida albicans.

De huidtestreactiviteit van het vertraagde type was bij meer dan 90% van de controles positief; bij 2/3 van de patiënten echter afwezig, terwijl bij 4 patiënten een hypererge reactie voorkwam die leidde tot necrose van de plaats van de huidtest en algemene malaise. De lymfocyten-blasttransformatietest t.o.v. H. influenzae bleek bij slechts 3 patiënten negatief te zijn. De ratio CD4+/CD8+ cellen was bij 40% van de

patiënten verhoogd. Atopische huidtestreactiviteit en autoimmuniteit kwamen zowel bij de patiënten als bij hun eerstegraads bloedverwanten vrij veel voor.

Deze uitkomsten suggereren dat onverklaarde recidiverende chronische purulente rhinosinusitis gebaseerd zou kunnen zijn op selectieve stoornissen van T-cellen t.o.v. commensale microörganismen zoals H. influenzae, streptococcen en Candida. Daarom werden T-cel parameters uitgebreider onderzocht bij een grotere groep van 75 patiënten. In hoofdstuk IV worden de uitkomsten vergeleken van de antigeen-specifieke vertraagd type huidtestreactiviteit (DTH), de lymfocyten-blasttransformatietest (LTT) en de MIF-productie. Voor deze laatste parameter werd een test ontwikkeld die gebruik maakt van U937-indicatorcellen in agarose microdruppels.

De vertraagd type huidtestreactiviteit t.o.v. één of meer microbiële antigenen was gestoord bij 2/3 van de patiënten, terwijl de huidtestreactiviteit bij meer dan 90% van de gezonde controles positief was voor elk van deze 3 antigenen. De lymfocyten-blasttransformatiereactiviteit t.o.v. de 3 microbiële antigenen bleek in de tijd aanzienlijk te fluctueren bij patiënten en controles. In het algemeen was de blastogene reactiviteit van de lymfocyten bij de patiënten intact, de S.I.-waarden waren vergelijkbaar met die van de gezonde controles of zelfs hoger.

De lymfocyten van alle geteste gezonde individuen bleken in staat tot MIF-productie na stimulatie met de 3 antigenen. Bij 2/3 van de patiënten werd een gestoorde MIF-productie vastgesteld. Fluctuatie van de MIF-productie in de tijd kon niet worden vastgesteld. De uitkomsten van de MIF-test en die van de DTH-huidtestreactiviteit bleken zeer goed met elkaar te correleren.

Deze uitkomsten wijzen er op dat naast huidtesten in vivo, de MIF-test de meest geschikte in vitro parameter blijkt te zijn voor het aantonen van stoornissen in de cellulaire immuniteit t.o.v. commensale microörganismen in voornoemde patientengroep.

Uit de literatuur is bekend dat cellulaire immuunstoornissen vaak gepaard gaan met verminderd functioneren van de monocyten. Daarom werd bij 40 patiënten met recidiverende chronische purulente rhinosinusitis de chemotactische reactiviteit van de monocyten bepaald, gebruik makend van de polarisatietest (hoofdstuk V). Bij 26 van de 40 geteste patiënten werd een verminderde polarisatie van de monocyten na stimulatie met FMLP gevonden. Deze verminderde chemotactische reactiviteit van de monocyten

kon verklaard worden door de aanwezigheid van een aan p15E-verwante factor in het serum van de patiënten. De polarisatie van de monocyten van gezonde individuen bleek geremd te kunnen worden door toevoeging van laag-moleculaire (< 25 kD) patiëntenserumfracties; deze remming was op te heffen door een tegen p15E gerichte monoclonale antistof. Een verminderde polariseerbaarheid van de monocyten bleek goed te correleren met de aanwezigheid van p15E-achtige factoren, en met gestoorde antigeen-specifieke T-celreactiviteit (DTH en MIF).

p15E is het hydrofobe transmembraaneiwit van muize- en katteleukemievirussen, en bezit immuunsuppressieve eigenschappen. Er bestaat structurele homologie tussen p15E en celwandcomponenten van verschillende retrovirussen. Menselijk DNA blijkt copieën te bevatten van een tijdens de evolutie goed geconserveerde sequentie die codeert voor een gedeelte van het p15E, nl. 26 aminozuren; het is daarom niet verbazingwekkend dat zowel maligne als niet-maligne zoogdiercellen in staat blijken te zijn p15E-achtige factoren te produceren. Deze immuunsuppressieve factor speelt mogelijk een rol bij hormonale immuunregulatie van de cellulaire immuunrespons. Een recent vervaardigd synthetisch peptide CKS-17, dat 16 aminozuren van de genoemde sequentie bevat, blijkt in staat te zijn de polarisatie van monocyten en de in vitro productie van IL-2 te remmen.

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CURRICULUM VITAE

Geboren 1945; eindexamen HBS-B 1962, kandidaats examen geneeskunde VU 1965; diploma medisch analiste 1967.
Van 1965 tot 1970 achtereenvolgens werkzaam op het Laboratorium voor Medische Diagnostiek en de afdeling Biochemie van de VU. Doctoraal examen vrije studierichting geneeskunde VU 1977.
1970-1976 student-assistentschap Chemische Fysiologie t.b.v. practica voor medische en tandheelkunde studenten.
Van april 1979 tot oktober 1985 halftijds wetenschappelijk medewerkster aan het Pathologisch Instituut van de VU, afd. Klinische Immunologie. De laatste drie jaar van deze periode gesubsidieerd door het NAF (Nederlands Astma Fonds). In deze periode werd het experimentele werk voor dit proefschrift uitgevoerd.
Sinds augustus 1978 voor enkele uren verbonden aan de Laboratoriumschool in Beverwijk als docente medische vakken.
Thans als docente Anatomie, Fysiologie, Pathologie en Immunologie verbonden aan de Hogeschool Alkmaar, sector Techniek-Chemie; het Bakhuis Roozeboom Instituut (opleidingen voor middelbare laboratorium medewerkers) te Beverwijk en de sector techniek afdeling HLO van de Hogeschool van Amsterdam.
Sinds 1966 gehuwd, 2 kinderen geboren in 1972 en 1975.

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van Ella M. van de Plassche-Boers, Amsterdam, 8 december 1989

Promotores: Prof.dr. C.J.L.M. Meijer
Prof.dr. G.B. Snow
Copromotor: Dr. H.A. Drexhage
Referent: Prof.dr. T. Sminia

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1. Geaggregeerd somatisch antigeen uit Haemophilus influenzae is geschikt voor het meten van vertraagd type overgevoeligheid in de menselijke huid (dit proefschrift).

2. De MIF-test is de beste in vitro parameter voor T-celfuncties bij chronische purulente rhinosinusitis (dit proefschrift).

3. De gevoeligheid van de MIF-test wordt aanzienlijk verhoogd door te testen met 3 antigeenconcentraties (dit proefschrift).

4. Groei-blokkerende antistoffen spelen een rol bij de atrofische vorm van endemisch cretinisme (S.C. Boyages et al., 1989, Lancet ii, 529-532).

5. Broncho-alveolaire lavage kan een bijdrage leveren aan de diagnostiek maar niet aan de prognostiek van longziekten.

6. In tegenstelling met wat door de opstellers beoogd is, heeft het classificeren van T-cel non-Hodgkin lymfomen volgens de "updated" Kiel klassificatie géén prognostische relevantie (Suchi et al., 1987, J. Clin. Pathol. 995-1015).

7. Bij anderszins onverklaarbaar hoge waarden van cholesterol en triglyceriden in serum dient de mogelijkheid van pinda(kaas)verslaving overwogen te worden (N.R. Scheier, 1989, JAMA 262, 500-501).

8. Gezien de betrekkelijke eenvoud en de grote gevoeligheid van de Polymerase Chain Reactie, valt een kettingreactie van diagnostische toepassingen te verwachten.

9. Synthetisch vervaardigde apidaecinen vormen mogelijk de nieuwe generatie antibiotica.

10. In het licht van de UV-doorlaatbaarheid van halogeenlampen en het daarmee samenhangende verhoogde risico voor melanomen, dienen deze lampen voorzien te worden van filters.

11. Het toekomstig welzijn van de patient is gebaat bij het invoeren en bijhouden van een medisch paspoort vanaf de geboorte.

12. Het vervangen van dierproeven door in vitro technieken betekent voorlopig nog slechts vermindering van proefdiergebruik.

13. Extensiveren van het hoger (beroeps)onderwijs leidt tot meer uitval van studenten.

14. Invoering van een gezamenlijke propedeuse voor HBO en WO is de eerste stap bij het bevorderen van doorstroming, korte opleidingen en gezamenlijke tweede fase-opleidingen.

15. Het hebben van een achternaam die begint met voorvoegsels als de, van of van de(r) heeft een negatief effect op de citatie score.