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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.

*Lymphocyte blastogenic responsiveness.* Heparinized blood was collected by venepuncture just

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, $2 \times 10^5$ 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 $\mu$g/ml for *H. influenzae* whole bacteria and soluble somatic antigen and from 0·1–150 $\mu$g/ml for candidal antigen.

To measure the blastogenic responsiveness towards PHA, $4 \times 10^4$ 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 $\mu$g/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 $\mu$g/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 ($2 \times 10^7$ 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 $\mu$l 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:

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

## RESULTS

*DTH skin tests.* The results of skin testing 75 patients with chronic purulent rhinosinusitis with the three microbial antigens are summarized in Table 1. As has been described before the antigens

**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.

In dose-response studies, bell-shaped curves were obtained with both soluble somatic antigen

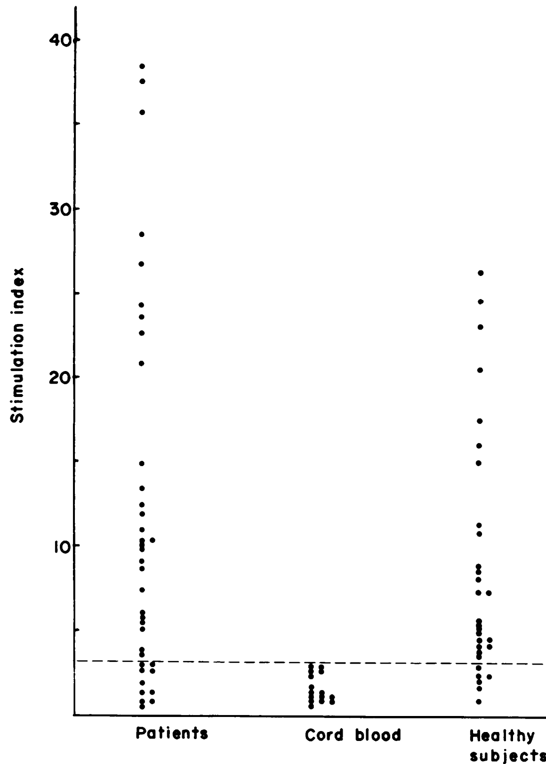


**Fig. 1.** The blastogenic responsiveness to soluble somatic *H. influenzae* antigen. The maximal stimulation indices of 37 patients with chronic purulent rhinosinusitis, 30 healthy subjects and 15 cord blood samples are shown. The dotted line indicates the upper limit of a mitogenic effect; values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

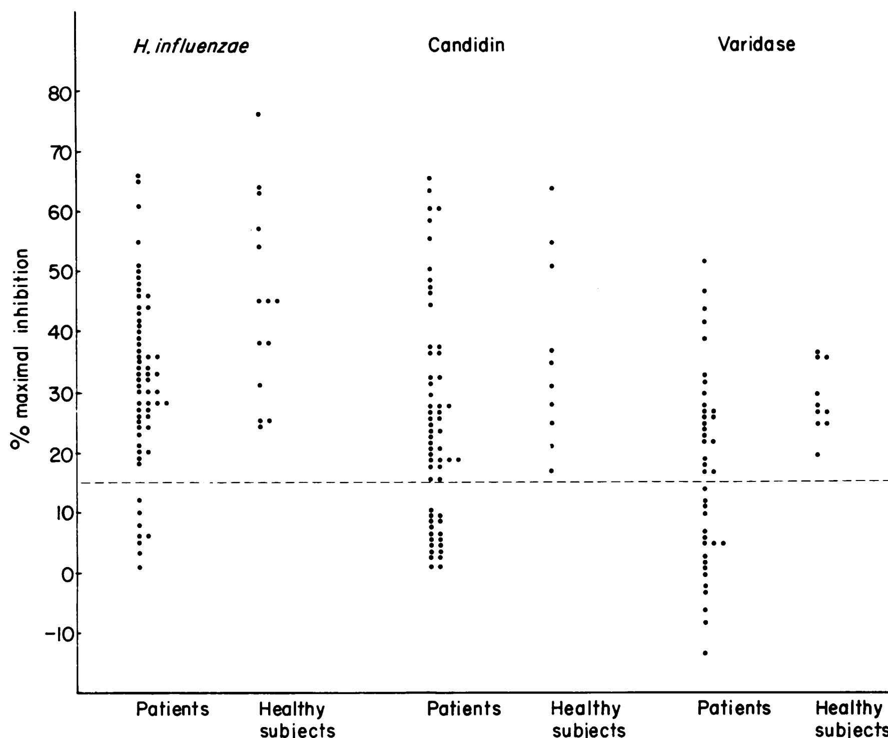


**Fig. 2.** The macrophage migration inhibition factor assay using *H. influenzae*, Candidin and Varidase in patients with chronic purulent rhinosinusitis and healthy subjects. The maximal % migration inhibition is given individually for each antigen. Data are shown from 60 patients and 14 healthy persons 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 not be detected between skin test reactivity and blastogenic response, which is in contrast to the

**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

| | | | Skin test | | |
|---|---|---|---|---|---|
| *H. influenzae* | | | | | |
| | MIF | + | 49 | 3 | $\chi^2=33.3$ |
| | | − | 1 | 7 | $P<0.001$ |
| Candidin | | | Skin test | | |
| | MIF | + | 41 | 1 | $\chi^2=46.4$ |
| | | − | 2 | 16 | $P<0.001$ |
| Streptokinase/ Streptodornase (Varidase) | | | Skin test | | |
| | | | + | − | |
| | MIF | + | 18 | 3 | $\chi^2=15.3$ |
| | | − | 5 | 15 | $P<0.001$ |

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 Graybill & Alford (1976).

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, $\gamma$-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 $\gamma$-IFN is closely related but sometimes dissociated (Weiser *et al.*, 1984); isolated antigen-selective defects of $\gamma$-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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