A longitudinal study of *in vitro* tests for lymphocyte function in rheumatoid arthritis

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SUMMARY *In vitro* tests of lymphocyte function have been performed in 61 patients with 'classical' or 'definite' rheumatoid arthritis. *In vitro* lymphocyte function was assessed by lymphocyte transformation responses to phytohaemagglutinin (PHA), Pokeweed mitogen (PWM), *Candida* antigen, and herpes simplex type I (HSV1). Follow up data were available after 6 months of treatment in 32 of these patients. Spontaneous lymphocyte transformation was assessed in all patients. Results obtained in patients with rheumatoid arthritis were compared to those seen in a normal control population. Disease activity of patients with rheumatoid arthritis was assessed using standard clinical methods.

Lymphocytes from patients with rheumatoid arthritis showed a similar degree of spontaneous transformation to that seen in normal subjects. In contrast, lymphocytes from patients with rheumatoid arthritis responded less well to PHA and *Candida* and HSV1 antigens when compared to normal patients. In patients with rheumatoid arthritis the response to PWM was markedly enhanced compared to normals. Clinical improvement was noted in 19 of the 32 patients seen at follow up, all of whom had received gold or penicillamine therapy. The abnormal responses of PHA and PWM seen before treatment became normal in those patients who improved clinically. The responses to *Candida* and HSV1 antigens not only returned to normal following treatment but were increased above those seen in normal controls. A statistically significant association was seen between clinical improvement and improvement of *in vitro* tests of lymphocyte function.

Classical rheumatoid arthritis is characterised histologically by the presence of rheumatoid granulomata which can be regarded as a manifestation of a cell-mediated immune response directed against an, as yet unknown, antigen. Previously published observations on cell-mediated immunity in rheumatoid arthritis are conflicting (Menard *et al.*, 1973; Waxman *et al.*, 1973; Lance and Knight, 1974; Messner, 1974; Sheldon *et al.*, 1974; Hepburn *et al.*, 1976). Some studies show a normal response to PHA while others show that the response to this and other mitogens and antigens is impaired. One report demonstrated an increased rate of spontaneous transformation in lymphocytes from patients with rheumatoid arthritis (Menard *et al.*, 1973). One possible explanation for the discrepant results

previously reported is that the majority of studies have been made without sequential observations and in addition little attempt has been made to correlate abnormal responses to the patient's clinical state. We have carried out a longitudinal study on patients with rheumatoid arthritis to determine whether or not a correlation exists between some *in vitro* tests of lymphocyte function and clinical disease activity.

Patients and methods

Sixty-one patients with ARA 'classical' or 'definite' rheumatoid arthritis were studied. In 32 of these patients observations were made before the institution of either gold or penicillamine therapy and again after 6 months of treatment. Rheumatoid disease activity was assessed by measurement of handgrip strength, aggregate proximal interphalangeal joint circumference, duration of morning stiffness, haemoglobin level, and erythrocyte sedimentation rate. Gold was administered in the form

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of 50 mg Myochrysine intramuscularly at weekly intervals for the first 20 weeks and at monthly intervals thereafter. D-penicillamine was given in a dosage of 250 mg daily with monthly increments of 250 mg to a maintenance dosage of 250 mg tid. At the time when clinical observations were made blood samples were drawn for *in vitro* testing of lymphocyte function. Control studies were performed on blood taken from 30 apparently healthy volunteers.

Lymphocytes were separated from whole blood by a standard Ficoll Hypaque method. Lymphocyte concentrations were adjusted to 1×10^6 cells/ml. for PHA and PWM responses and to 2×10^6 cells/ml. for HSV1 and *Candida*. Cells were suspended in RPMI 1640 (Grand Island Biological Co., Burlington, Ontario, Canada). Cell suspensions were distributed into flat bottomed microtitre plates. Mitogens or antigens were added to cell suspensions in triplicate. The cells were cultured for 144 hours for both mitogens and antigens. Six hours before harvesting $10 \mu\text{l}$ of ^3H thymidine was added to the cultures (concentration $20 \mu\text{Ci/ml}$, specific activity 40 mCi/mmol). Viability of cultures was routinely determined before harvesting which was performed using a multi-sample semi-automated harvester (Skatron, Lierbyen, Norway).

Cultures of unstimulated cells from the same patient were made at all times in order to determine the rate of spontaneous transformation. Maximal stimulation responses were obtained by selecting optimum doses of both mitogens and antigens by preparing dose response curves. Doses of antigens and mitogens were tested in the following ranges:—PHA (Difco Laboratories, Detroit, Michigan, USA) $0\text{--}100 \mu\text{g/ml}$ —mean optimum dose $14 \mu\text{g/ml}$; PWM $0\text{--}50 \mu\text{g/ml}$ —mean optimum dose $25 \mu\text{g/ml}$; *Candida* antigen (Hollister Stier Laboratories, Mississauga, Ontario, Canada) $0\text{--}1:5000$ dilution stock solution—mean optimum dosage $1:2000$. The HSV1 antigen was prepared by treatment of an infected monkey cell culture (Vero) with 0.3% propiolactone (Russell, 1974).

After harvesting, the fibre discs were removed, placed in 10 ml of scintillation fluid and the emissions counted in a Beckman L.S. 230 scintillation counter. Results were expressed as the long of the ratio between stimulated and unstimulated cells from the same subject.

Statistics

The significance of lymphocyte responsiveness to mitogens and antigens comparing normals against pretreatment rheumatoid patients, normals *versus* post-treatment rheumatoid patients, and rheumatoid patients pretreatment *versus* post-treatment

was calculated using sample tests of proportions. The relationship between laboratory variables to clinical status was tested using binomial distribution tables with a probability of success of 0.5 .

Results

SPONTANEOUS TRANSFORMATION

A wide range of spontaneous transformations was seen in patients with rheumatoid arthritis and in normal controls. No statistically significant difference was detected between the results obtained in both groups.

LYMPHOCYTE RESPONSIVENESS TO MITOGENS

The PHA responses of lymphocytes in patients with rheumatoid arthritis was significantly less than the PHA response in the 30 normal control subjects (Fig. 1). Only 2 normal subjects had a $\log_{10}$ transformation index of less than 1. Seventeen patients with rheumatoid arthritis had a depressed response

Fig. 1 $\log_{10}$ transformation indices to PHA in 61 randomly selected patients with RA compared to 30 normal controls

($P < 0.01$). Follow up data were available after 6 months' treatment with either gold or penicillamine in 33 patients and there was a trend towards normalisation of the responses in patients with rheumatoid arthritis. At this time only 5 of 33 patients had a depressed PHA response. There was a statistically significant difference ($P < 0.05$) between PHA responses seen in patients before treatment and after treatment (Fig. 2). There was no significant difference between PHA responses in the normal group and in the 33 patients seen with rheumatoid arthritis at follow up. Patients with rheumatoid arthritis had lymphocyte transformation responses to PWM which were significantly elevated ($P < 0.001$) over those levels seen in normal control subjects (Fig. 3). At follow up there had been a normalisation of the PWM response in the 32 patients studied which was significantly different ($P < 0.001$) from pre-treatment results (Fig. 4). There was no statistically significant difference between PWM response of the normal control population and the 32 patients seen

with rheumatoid arthritis at follow up. Successful treatment of rheumatoid arthritis tended to enhance *in vitro* lymphocyte responsiveness to PHA whereas the response to PWM was reduced.

LYMPHOCYTE RESPONSES TO ANTIGENS

Lymphocyte responses to HSV1 and *Candida* antigens in patients with rheumatoid arthritis were reduced when compared with normal controls ($P < 0.05$). At follow up the 31 patients seen after 6 months' treatment with gold or penicillamine demonstrated an increase in the lymphocyte responsiveness to these 2 antigens and a significant difference ($P < 0.01$) existed between responses before and after treatment. A striking feature was that the post treatment response to *Candida* and HSV1 was enhanced when compared to the normal control group.

RELATIONSHIP BETWEEN CLINICAL IMPROVEMENT AND IMPROVEMENT IN LABORATORY VARIABLES

Of the 61 patients with rheumatoid arthritis seen initially, 32 patients were available for follow up after 6 months' treatment with either gold or penicillamine therapy. At this time 19 patients had shown clinical improvement and 13 remained unchanged. Of the 12 clinically improved patients whose initial response to PHA was depressed 11 showed improvement to normal levels at follow up (Fig. 5). This

Fig. 2 Changes in PHA responses seen in 33 patients with RA initially and at 6 month follow up

Fig. 3 Comparison of PWM transformation indices in 32 normal controls, 59 randomly selected patients with RA, and 32 of these patients seen at follow up

Fig. 4 Changes in PWM responses in 32 patients with RA, initially and at 6 month follow up

Fig. 5 Changes in PHA responses, initially and at 6 month follow up, in the 19 patients with RA who showed clinical evidence of improvement

correlation is significant ($P < 0.05$). In contrast, in the clinically unchanged group improvement in PHA response occurred in 2 out of the 3 patients in whom it had been suppressed (Fig. 6). Similar changes were seen in response to *Candida* and HSV1 antigens ($P < 0.05$). The PWM response tended to decline to normal levels with clinical improvement ($P < 0.05$).

Discussion

The results of our study support those previously published which suggest that *in vitro* lymphocyte responsiveness to both mitogens and antigens is abnormal in patients with rheumatoid arthritis (Menard *et al.*, 1973; Waxman *et al.*, 1973; Lane and Knight, 1974; Messner, 1974; Sheldon *et al.*, 1974; Hepburn *et al.*, 1976). In addition, by means of a longitudinal study in 32 of the 61 patients originally studied, we have been able to demonstrate that patients receiving treatment undergo normalisation of their lymphocyte responsiveness to both mitogens and antigens and that this shows good correlation

with improvement in the patients' clinical state. We have been unable to confirm the previous report that spontaneous lymphocyte transformation is enhanced in patients with rheumatoid arthritis (Menard *et al.*, 1973). Any group of patients suffering from rheumatoid arthritis will show a great range of disease activity and this may indeed account for some of the apparent discrepancies between previous reports of studies of lymphocyte function in rheumatoid arthritis. Our initial group of patients consisted of 61 patients taken at random from our clinics. Although this group contained patients who had been on long-term management for their disease and were in clinical remission, an attempt was made to include in this group all patients with newly diagnosed rheumatoid arthritis before institution of therapy other than routine anti-inflammatory drugs. Thus a considerable number of patients in our study had active disease at the time of their initial evaluation. At follow up every attempt was made to include patients who had previously been seen with active disease and in the interim period had received treatment with either gold or penicillamine. Included

Fig. 6 Changes in PHA responses, initially and at 6 month follow up, in the 13 patients with RA who were clinically unchanged throughout the study

in this group, however, were also some patients who had been in clinical remission throughout the 6 months' period. It is therefore important to note that in our study, improvement in laboratory variables of lymphocyte function in rheumatoid arthritis appeared to relate to the institution of active therapy for rheumatoid arthritis. In particular, those patients who were studied sequentially tended to be suffering from severe progressive disease which required either the institution of either gold or penicillamine therapy. Thus the abnormal lymphocyte response to PHA, *Candida*, and HSV1 antigens in patients in this group may reflect the severity of their disease, as may indeed be the enhanced response seen to PWM. Clinical improvement was associated with normalisation of all these responses and in the case of both *Candida* and HSV1 a marked enhanced response was seen following treatment. It is impossible to determine that this improvement in *in vitro* tests of lymphocyte function is the direct effect of the institution of gold or penicillamine therapy or a direct consequence of the patient's overall clinical improvement. The possibility that gold and penicillamine may have an effect on

lymphocyte function which is independent of their effects on rheumatoid disease activity exists. We have been unable to produce any *in vitro* evidence of this although Harth *et al.* (1976) has been able to demonstrate an apparent suppression of lymphocyte response to PHA after incubation with gold salts *in vitro*.

The interpretation of the *in vitro* responsiveness of lymphocytes to PWM and other antigens in man is the subject of some debate. The PHA response appears to be largely a reflection of T-cell function. PWM may also be an indicator of B-cell activity. T cells are probably primarily responsible for the reaction to *Candida* and HSV1 antigens. If this interpretation is applied to our data, the results are fully congruous in that restoration of T-cell function as reflected by improvement in PHA response is associated with a return towards normal of the PWM response. Improvement in T-cell function could also be held to account for the increase in magnitude of the response to HSV1 and *Candida* antigens.

Our study would suggest that patients with active rheumatoid arthritis do have significantly impaired lymphocyte responses as tested in *in vitro* methods and that this abnormality is directly related to clinical disease state. These abnormal responses appear to be reversible and after 6 months' follow up in a longitudinal study we have been able to demonstrate an improvement in T-cell function which is associated with clinical improvement to the extent that patients with inactive rheumatoid arthritis had responses indistinguishable from those seen in normal controls.

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