Antibody-mediated leucocyte cytotoxicity to Chang human liver cells in rheumatoid arthritis and other diseases

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Panayi, G. S. (1976). *Annals of the Rheumatic Diseases*, 35, 27–31. Antibody-mediated leucocyte cytotoxicity to Chang human liver cells in rheumatoid arthritis and other diseases. The incidence of an IgG-antibody which induces lymphocyte cytotoxicity to Chang human liver cells in culture was estimated in the sera of patients with rheumatoid arthritis, systemic lupus erythematosus, ankylosing spondylitis, psoriatic arthritis, Crohn’s disease, ulcerative colitis, and in healthy controls. It was found in 41% of control subjects and in 31% of patients with rheumatoid arthritis. None of the other patient groups differed from the control group. This may be the first demonstration of an antibody response to an antigen or antigens which is almost entirely confined to patients with rheumatoid arthritis. The possibility that an antigenic similarity exists between the rheumatoid synovial membrane and Chang cells is currently under investigation.

The possibility that rheumatoid arthritis (RA) may be due to an infective process has received much experimental attention (Barland, 1973). One way of demonstrating such a process has been to estimate the concentration of antibodies to putative pathogenic organisms in the sera of patients with RA (Laitinen, Vesikari, and Vaheri, 1972). However, no consistent differences with control populations have been established as yet. MacLennan, Loewi, and Howard (1969) described the presence of a naturally occurring IgG antibody in the sera of RA patients and of healthy control subjects which was capable of promoting lymphocyte cytotoxicity against Chang human liver cells growing as an established tissue-culture cell line.

A study is reported in which the incidence of such an antibody has been estimated in the sera and synovial fluids of patients with RA, patients with other diseases, and healthy control subjects.

Materials and methods

**TISSUE CULTURE MEDIA**

Culture of Chang liver cells was carried out in Eagle’s minimal essential medium for suspension culture (Flow Laboratories) containing 10% fetal calf serum, 20 mmol/l HEPES buffer, 100 µg/ml penicillin, and 100 µg/ml streptomycin, supplemented with 2 mmol/l glutamine and the pH adjusted to 7.2 with 4 N sodium hydroxide.

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Cytotoxic experiments were performed in tissue culture medium (TCM) consisting of Eagle’s minimal essential medium containing the same additives as the suspension medium.

**CULTURE OF CHANG CELLS**

Chang human liver cells (Flow) were grown in glass medical flats in suspension medium (S·TCM). After the cell monolayer had become confluent, cells were removed by vigorous shaking of the culture bottles, which were replenished with fresh S·TCM and incubated at 37°C until the next harvest of cells.

**PREPARATION OF HUMAN LEUCOCYTES**

Venous blood (20 ml) was collected from normal healthy subjects into plastic universal bottles (Sterilin) containing preservative-free heparin (Evans Medical; final concentration 5 IU/ml). The blood was mixed with one-quarter of its volume of 3.5% w/v Dextran T250 (Pharmacia) and incubated at 37°C for 1 hour. The leucocyte-rich plasma layer was removed, the cells washed three times in TCM by centrifugation at 1000 g for 5 minutes, and finally resuspended at the appropriate concentration in TCM.

**COLLECTION OF SERA**

Patients and healthy control subjects were bled and serum was separated, heated at 56°C for 30 minutes, and stored at −20°C. No sera were older than 6 months and most had been collected within 3 months before testing. All experiments were performed on coded samples.
COLLECTION OF SYNOVIAL FLUIDS

Fresh synovial fluids were centrifuged at 2500 g for 20 minutes at 4°C and the cell-free supernatant immediately stored at −20°C. Fluids were coded. They were heat inactivated before testing.

RHEUMATOID FACTOR

The Rose–Waaler technique was used to estimate rheumatoid factor in sera and synovial fluids.

CYTOTOXIC SYSTEM

$^{51}$Cr-labelling

Chang cells were obtained by shaking of culture bottles and 10$^2$ cells were washed three times by centrifugation at 1000 g for 5 minutes at 4°C. After the final centrifugation, the supernatant was removed carefully so as not to disturb the cell pellet. To this was added one drop of fetal calf serum and 0·1 ml of $^{51}$Cr-sodium chromate (Radiochemical Centre, Amersham; specific gravity 1 mCi/5 μg Cr in 1 ml) and then washed four times with TCM and adjusted to a concentration of 36 × 10$^3$/ml in TCM.

Cytotoxic test

The cytotoxic test was performed in 2·5 ml plastic flat-bottomed tubes (Turner–Staynes). To each tube was added in turn: 100 μl radio-labelled Chang cells, 100 μl white blood cells at the original blood concentration, and 200 μl of an appropriate dilution of serum or synovial fluid in TCM. Control tubes contained Chang cells, white blood cells or lymphocytes, and TCM. The tubes were incubated at 30° angle for 16 hours at 37°C. All samples were tested in triplicate. After the incubation period the tubes were centrifuged at 500 g for 5 minutes and 200 μl of the supernatant removed. The radioactivity of the tube containing 200 μl of the supernatant (T1) and of the tube containing cells and the remainder of the supernatant (T2) was counted in a Packard well-type γ-counter.

Calculation of cytotoxicity

From the radioactivity (counts per minute) in tubes T1 and T2 the per cent. release of $^{51}$Cr-sodium chromate was calculated as follows:

$$\frac{2 \times T1}{T1 + T2} \times 100$$

radioactivity released into supernatant

$\frac{\text{total radioactivity of Chang cells}}{\times 100}.$

The specific cytotoxicity in the presence of serum or synovial fluid was calculated as:

($\%$ radioactivity released in the presence of serum or synovial fluid) − ($\%$ radioactivity released in the presence of TCM).

STATISTICAL ANALYSIS

(i) Student’s ‘t’ test for independent samples was used to assess whether the cytotoxicity caused by a serum or synovial fluid was significantly different from the release caused by leucocytes or lymphocytes in the presence of TCM alone. A significant difference was taken to indicate the presence of anti-Chang antibody in that serum or synovial fluid sample.

(ii) The various patient groups were compared among themselves and with the control healthy group for significant differences in the incidence of anti-Chang antibody by the $\chi^2$ test.

Results

PREVALENCE OF ANTI-CHANG ANTIBODY IN VARIOUS DISEASES

Because of the possibility that immune complexes might inhibit the cytotoxic system, all sera were tested at a final dilution of 1/32 (MacLennan and others, 1969). A total of 186 sera were tested for the presence of leucocyte cytotoxic-promoting anti-Chang antibody and the results are shown in Table I. Of 49 sera from control patients 2 (4·1 %) possessed leucocyte cytotoxic-promoting activity. The controls consisted of patients with osteoarthritis from the same clinic as the patients with RA, ankylosing spondylitis, and psoriatic arthritis. Of sera from 74 patients with RA, 23 (31 %) were able to induce leucocyte cytotoxicity of Chang cells. This result is statistically significant ($\chi^2 = 6·959; n = 1; P < 0·01$). Three out of 24 patients with systemic lupus erythematosus had cytotoxic-promoting activity but neither in this group nor in the other disease categories (ankylosing spondylitis, psoriatic arthritis, and inflammatory bowel disease) did the difference from control subjects reach significant levels.

Although the group of patients with RA and psoriatic arthritis were age- and sex-matched with respect to the control group, this was not possible with the other disease categories. Thus patients with systemic lupus erythematosus were almost entirely female (20 of 24), while 27 of 28 patients with ankylosing spondylitis were male.

INCIDENCE OF ANTI-CHANG CELL ANTIBODY IN RA

The incidence of anti-Chang cell antibody was determined in the following three classes of RA. (1) Patients with RA who either were receiving intra-muscular sodium aurothiomalate for between 3 months to 2 years or were ingesting D-penicillamine

Table I  Incidence of leucocyte cytotoxic-promoting anti-Chang cell antibody in various diseases

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. tested</th>
<th>Presence of anti-Chang antibody</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Controls</td>
<td>49</td>
<td>2</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>74</td>
<td>23</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>Ankylosing spondylitis</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>Psoriatic arthritis</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Gastrointestinal disease*</td>
<td>17</td>
<td>0</td>
</tr>
</tbody>
</table>

*8 patients with Crohn’s disease; 9 patients with ulcerative colitis.
cytotoxicity produced when rabbit anti-Chang antiserum is added to Chang cells in culture. In those RA sera and synovial fluids which already possessed anti-Chang activity the addition of rabbit anti-Chang antiserum merely produced an increase in specific cytotoxicity. One synovial fluid and three sera which did not possess intrinsic anti-Chang activity, inhibited at dilutions of 1/8 and 1/16 the cytotoxicity mediated by rabbit anti-Chang antiserum.

None of the sera from patients with systemic lupus erythematosus, ankylosing spondylitis, gastrointestinal disease, and from the controls inhibited cytotoxicity induced by rabbit antiserum in the presence of leucocytes at the test dilution of 1/32.

**Discussion**

The incidence of an IgG antibody which promotes lymphocyte cytotoxicity to Chang human liver cells in culture was estimated in the sera of patients with RA, patients with other inflammatory diseases of joints or bowel, and in healthy controls. The noteworthy feature of the results was the demonstration that 31% of patients with RA possessed an antibody capable of promoting leucocyte cytotoxicity to Chang cells compared to 4.1% of control subjects. Patients with systemic lupus erythematosus, ankylosing spondylitis, psoriatic arthritis, Crohn's disease, and ulcerative colitis did not differ significantly from control patients. This appears to be the first demonstration of a humoral immune response in RA to an exogenous antigen which is almost entirely confined to patients with the disease. This result could be an artefact based on the hypergammaglobulinaemia commonly found in RA, but other studies on the incidence of viral antibodies in patients with RA appear not to be complicated by this phenomenon. In systemic lupus erythematosus, on the other hand, the increased incidence of various viral antibodies has been explained as being due to a generalized, heightened antibody response (Phillips, Christian, and Hirshaut, 1973). Hence it is of particular interest that the patients with systemic lupus erythematosus did not show an increase in leucocyte cytotoxic-promoting anti-Chang antibody.

It is known that about 14% of patients with RA and about 74% of patients with systemic lupus erythematosus possess antibodies cytotoxic to human lympho-

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Table II  Incidence of leucocyte cytotoxic-promoting anti-Chang cell antibody in rheumatoid arthritis

<table>
<thead>
<tr>
<th>RA group</th>
<th>No. tested</th>
<th>Presence of anti-Chang antibody</th>
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<tr>
<td>Sodium aurothiomalate/D-penicillamine</td>
<td>37</td>
<td></td>
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<tr>
<td>RA seropositive</td>
<td>20</td>
<td></td>
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<tr>
<td>RA seronegative</td>
<td>17</td>
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<tr>
<td></td>
<td></td>
<td>No.</td>
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<td></td>
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<td>12</td>
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<td>5</td>
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</tbody>
</table>

**Figure** Comparison of the leucocyte cytotoxic-promoting activity of paired synovial fluids (SF) and sera (S) in rheumatoid arthritis

from 4 months to 16 months. (2) Patients with RA who were seropositive for rheumatoid factor. (3) Patients with RA who were seronegative for rheumatoid factor. The results are shown in Table II. The prevalence of anti-Chang activity in the three classes was similar.

No relationship was found with the erythrocyte sedimentation rate, duration of disease, age, or sex of the patients.

**Comparison of sera and synovial fluids for anti-Chang activity**

Synovial fluid and serum were collected from each patient at the same time. Ten matched pairs of fluid and serum were tested at various dilutions for cytotoxicity. Five pairs showed no cytotoxic activity either in synovial fluid or serum and this result agreed with the previous report of testing serum at 1/32 dilution alone. The five other pairs showed variable leucocyte cytotoxic-promoting activity (Figure). Except at the two highest concentrations synovial fluids and sera apparently possessed equal cytotoxic activity. Where synovial fluids showed higher cytotoxicity than sera these differences were not statistically significant. It is of interest that some of the samples still showed highly significant cytotoxicity even at the highest dilution tested (1/128).

**Detection of immune complexes**

It has been reported (Jewell and MacLennan, 1973) that immune complexes can inhibit the leucocyte
cytes, some of which are related to HL-A transplantation antigens (Mittal and others, 1970). These antibodies are cold-reacting (Ooi and others, 1974). The fact that the cytotoxic tests were performed at 37°C and that patients with systemic lupus erythematosus did not show an increased incidence of anti-Chang antibodies in their sera probably indicate that the antibody demonstrated was not directed against HL-A antigens which are known to occur on Chang cells.

The presence of antibody in the serum of RA patients did not correlate with drug intake, presence or absence of rheumatoid factor, duration of disease, or disease activity as measured by erythrocyte sedimentation rate. However, this was a retrospective study and a prospective investigation is being carried out at the present time to investigate the relationship between clinical status of disease activity and presence of anti-Chang antibody.

Immune complexes and aggregated IgG have been shown to be capable of inhibiting cytotoxicity induced by lymphocytes in the presence of lymphocyte cytotoxic-promoting IgG antibody (Jewell and MacLennan, 1973). Experiments performed on ten paired samples of sera and synovial fluids showed evidence of such inhibition in one synovial fluid and three sera. It could be therefore that the incidence of anti-Chang antibody was falsely low in the patients with RA since it is known that patients may possess circulating immune complexes (Williams, Panayi, and Lessof, 1975). In those fluids and sera which possessed intrinsic anti-Chang activity it was not possible to detect the presence of complexes because of the nature of the experimental system. Thus it appears that inhibition of antibody-induced leucocyte cytotoxicity of Chang cells as a test for the presence of immune complexes should be used with caution in RA.

The fact that sera at dilutions of 1/32 from patients with systemic lupus erythematosus, ankylosing spondylitis, inflammatory bowel disease, psoriatic arthritis, and from healthy controls did not inhibit the cytotoxicity of Chang cells induced by rabbit antiserum and leucocytes indicates that sera did not possess immune complexes or aggregates of immunoglobulin in sufficient amounts to inhibit the system. Hence failure to demonstrate naturally occurring anti-Chang antibody in these sera probably means that the antibody is absent from the sera at that dilution.

It is difficult to interpret the significance of these results. It seems that patients with RA have been immunized with an antigen which cross-reacts with an antigen present on Chang cells, but an antigenic relationship between Chang cells and the rheumatoid synovial membrane is not known at present. It is known that transformed tumour cells can express embryonal antigens (Baldwin and others, 1974) and it is also known that there is increased concentration of circulating carcino-embryonic antigen in RA (Unger, Panayi, and Lessof, 1974) so that the antigenic relationship between Chang cells and rheumatoid synovium may reside in the possession of common or cross-reacting embryonal antigens. This possibility is currently under investigation.

Furthermore, since virus may be involved in the establishment of the transformed state necessary for the initiation of a continuous cell culture line (Mallucci, 1971), and since virus may be involved in the pathogenesis of RA, it may be that Chang cells and rheumatoid synovium share virally-induced antigens as the result of infection with similar viruses.

Preliminary results indicate that the anti-Chang antibody in rheumatoid sera cannot be absorbed out with adult human liver powder, muscle homogenate, human peripheral blood Buffy coat, or pooled human plasma but can be absorbed out with homogenized rheumatoid synovial membrane (G. S. Panayi and A. Unger, unpublished observations). Further studies are in progress which attempt to establish whether there is any antigenic similarity between rheumatoid synovial membrane and Chang cells and which is unique to them.

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