Synthesis of IgM, IgG, and IgA in rheumatoid arthritis

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SUMMARY We studied the production of immunoglobulins by lymphocytes separated from the blood of 15 rheumatoid arthritis (RA) patients, of 12 patients suffering from other connective tissue diseases (CTD), and of 18 healthy controls. The production of IgM, IgG, and IgA in pokeweed-mitogen-stimulated cultures was measured by counting the number of plaque-forming cells (PFC) and by determining the concentration of secreted immunoglobulins by means of an enzyme immunoassay. Synthesis of immunoglobulins, particularly IgM and IgG, was lower than in other CTD patients or controls. The IgM response of RA patients was 20% and 29% (PFC and Ig concentration) that of the controls. The respective figures for IgG were 33% and 53% and for IgA 61% and 72%.

The formation of antibodies against immunoglobulins is typical of rheumatoid arthritis (RA). These antibodies are known as the rheumatoid factors. With respect to responses *in vivo* against various antigens and observed levels of serum immunoglobulins there is controversy about antibody formation in RA. However, the majority of studies have demonstrated elevated levels of IgG, IgM, and IgA.¹

Although *in-vitro* methods for studying antibody response at the cellular level have been used in laboratory animals for several years, only recently has it been possible to apply the same methodology to human cells. A major advance in this respect has been the development of plaque assays, in which the number of cells secreting various classes of immunoglobulins after stimulation with a mitogen is determined.^{2,3} Using this plaque technique and an enzyme-linked immunosorbent assay (ELISA) we studied the synthesis of IgG, IgM, and IgA in patients suffering from RA.

Materials and methods

Subjects. Fifteen patients with active, definite rheumatoid arthritis according to the ARA criteria were selected for study (Table 1). Ten of the patients were seropositive and five seronegative. None had received systemic corticosteroid therapy before test-

ing. Administration of nonsteroidal anti-inflammatory agents was not stopped because of the study. A control patient group consisted of 12 patients with reactive arthritis, scleroderma, polymyositis, or fibrositis (Table 1). Normal controls were 18 healthy subjects from the laboratory staff.

Cell isolation. Mononuclear cells were separated from 30 ml heparinised venous blood by centrifugation on Ficoll-Isopaque gradient. Cells from patients and paired normal donors were separated in parallel. Separated cells were washed 3 times in Hanks's balanced salt solution (HBSS) and resuspended in culture medium. The cell concentration was adjusted to 1×10^6 cells/ml.

Cell cultures. Cultures were prepared in 15×100 mm round-bottomed plastic tubes (Sterilin, Teddington Middlesex, England) in a final volume of 2 ml. The culture medium was RPMI-1640 (Flow Laboratories Ltd., Irvine, Scotland) supplemented with heat inactivated fetal calf serum (FCS, Flow Laboratories Ltd.), gentamicin (15 µg/ml, Schering Corporation, Kenilworth, USA), and L-glutamine (3

Table 1 Details of the patients and controls

Diagnosis	n	Female	Male	Mean age in years $\pm$ SD
RA	15	7	8	43 $\pm$ 18
Reactive arthritis	5	2	3	37 $\pm$ 13
Scleroderma	3	3	—	49 $\pm$ 11
Polymyositis	1	1	—	50
Fibrositis	3	3	—	41 $\pm$ 17
Controls	18	12	6	31 $\pm$ 8

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mg/ml). Cells were cultured with (stimulated cultures) or without (unstimulated cultures) pokeweed mitogen (PWM, Gibco, New York, USA) at a final dilution of 1:100. Cultures were incubated for 5 days in a humid atmosphere of 5% CO₂ and 95% air at 37°C. At the end of this period the cells were washed twice with 2 ml HBSS and resuspended in 1 ml of culture medium without FCS. The culture supernatants were stored at -20°C for Ig determination.

Coupling protein-A to SRBC. Protein-A of *Staphylococcus aureus* (Pharmacia Fine Chemicals, Uppsala, Sweden) was coupled to SRBC with chromic chloride.² One volume of protein-A (0.5 mg/ml) was mixed with 10 volumes of chromic chloride solution (2.5×10^{-4} M) and one volume of packed, washed SRBC and incubated at 30°C for 1 h. All reagents were prepared in 0.9% NaCl. After incubation the cells were washed 3 times in 0.9% NaCl and resuspended in HBSS.

Plaque assay. The number of Ig-secreting cells was determined before culture (spontaneous plaque-forming cells) and at the end of culture period by a haemolytic plaque assay.³ Noble agar (0.5% in HBSS containing 0.05% DEAE-dextran, Pharmacia Fine Chemicals) was heated to boiling and allowed to equilibrate at 46°C in a water bath. To 700 µl of agar was added 25 µl protein-A coupled SRBC (30% suspension), 25 µl rabbit antihuman immunoglobulin (µ-, δ-, or α-specific, diluted 1:30 in saline, Dako-immunoglobulins, Copenhagen, Denmark), 25 µl guinea-pig complement (diluted 1:3 in saline), and 100 µl cell suspension. This was then mixed and 3 separate 0.2 ml drops of the mixture were pipetted into a plastic Petri dish. A 22 × 32 mm glass coverslip was immediately placed over each drop, producing a thin layer of gel.

Plates were then incubated at 37°C for 4 to 6 hours. Plaques were counted using a stereo microscope. The number of antibody secreting cells was expressed as plaque forming cells (PFC)/10⁶ viable cells as determined by trypan blue exclusion at the time of harvesting.

Ig-determination by enzyme-linked immunosorbent assay (ELISA). A double sandwich ELISA method was used for IgM, IgG, and IgA in the culture supernatants.^{4,5} Microtitre plates (Dynatech Laboratories Ltd., Sussex, England) were coated with antihuman IgM, IgG, or IgA antisera (Orion Diagnostica, Helsinki, Finland). The antisera were diluted 1:5000–1:10 000 in 0.05 M carbonate buffer, pH 9.2, and 150 µl was added to each well. The plates were incubated at 4°C overnight and then washed 3 times with phosphate buffered saline (PBS), pH 7.4, containing 0.25% Tween 20. The supernatants from lymphocyte cultures were diluted 1:10–1:80 in culture medium, and 150 µl was added to each well. The

plates were then incubated at 37°C for 1 hour, washed 3 times, and 150 µl of the alkaline phosphatase conjugated antihuman immunoglobulin serum (Orion Diagnostica), diluted 1:200 in PBS, was then added to each well.

After 1 h incubation at 37°C the plates were washed 3 times and 150 µl p-nitrophenylphosphate (1 mg/ml, Sigma Chemical Company, Saint Louis, USA) in 1 M diethanolamine-HCl buffer, pH 9.8, containing 0.5×10^{-3} M MgCl₂, was added to each well. The plates were then incubated at 37°C. After 1 h the enzymatic reaction was stopped by adding 50 µl 3 M NaOH, and absorbance at 405 nm was measured with a Titertek Multiscan plate reader (Flow Laboratories Ltd.).

The immunoglobulin content of the samples was calculated from standard curves obtained with standard sera (Behringwerke, Marburg, Germany).

Results

IgM, IgG, and IgA producing cells after PWM stimulation. Lymphocytes cultured in the presence of PWM for 5 days were harvested, and plaque assays were performed to determine the number of Ig-producing cells. The diagram in Fig. 1 shows the number of Ig-producing cells in 15 RA patients and 11 normal controls. The number of the controls used

Fig. 1 Immunoglobulin secreting cells in PWM-stimulated lymphocyte cultures. Results are expressed as plaque-forming cells (PFC)/10⁶ viable cells determined by trypan blue exclusion (at the moment of plating). Bars represent arithmetic mean. The values of RA patients are significantly different from the controls for IgM ($p < 0.001$) and IgG ($p < 0.05$) by Student's *t* test.

Table 2 Plaque-forming cells and Ig-concentrations in pokeweed-stimulated lymphocyte cultures

Group	n	PFC/10 ⁶ cells			Ig concentration, ng/ml		
		IgM	IgG	IgA	IgM	IgG	IgA
RA patients	15	1381±786**	1461±1867†††	2167±2819	330±180††	600±380**	230±220
Other patients	12	5359±4796	5359±6612	4444±4849	1080±1020	1425±1525	560±855
Controls	18	7039±5440	4374±3362	3573±2665	1140±1125	1135±1355	320±380

Results expressed as arithmetic mean ± SD.
†Significantly different from controls, $p<0.001$.
††Significantly different from controls, $p<0.01$.
*Significantly different from other patients, $p<0.01$.
**Significantly different from other patients, $p<0.05$.

was only 11 because in some experiments the same control served for more than one patient sample. The number of IgM-producing cells among rheumatoid lymphocytes was highly significantly less ($p<0.001$) than for controls. The difference between rheumatoid and normal lymphocytes for numbers of IgG-producing cells and total plaque-forming cells was also significant ($p<0.05$). Numbers of IgA-forming cells were not statistically different.

For comparison we also collected samples from patients with other connective tissue diseases (Table 1). Table 2 shows the number of plaque-forming cells after PWM-stimulated culture of lymphocytes from RA patients, other CTD patients, and all the healthy controls. Fewer cells synthesising IgM or IgG were detected in rheumatoid blood than in blood from patients with other CTD or the controls. The IgM response of RA patients was 20% and 29% (PFC and Ig concentration) that of the controls. The

figures for IgG were 33% and 53% and those for IgA 61% and 72%. The results cannot be explained by possible differences in cell survival, since cell viability counts after culture were similar in all groups (data not shown).

Concentration of immunoglobulins in culture supernatants. The sensitive ELISA method for detecting IgM, IgG, and IgA in the supernatants of 5-day lymphocyte cultures confirmed the above results (Fig. 2, Table 2).

Number of cells secreting IgM, IgG, or IgA spontaneously or in unstimulated cultures. Two kinds of control experiments were carried out to exclude some possible causes for the decreased synthesis of

Fig. 2 Immunoglobulin concentrations in the PWM stimulated culture supernatants determined by ELISA. Bars represent arithmetic mean. The values for RA patients are significantly different from healthy controls only for IgM ($p<0.01$, Student's *t* test).

Fig. 3 Number of cells spontaneously secreting immunoglobulin in the peripheral blood of RA patients and healthy controls. Results are expressed as plaque-forming cells (PFC)/10⁶ viable cells. Bars represent standard deviation. There is no significant difference between RA patients and the controls.

Fig. 4 Immunoglobulin-secreting cells in unstimulated lymphocyte cultures. Results are expressed as plaque-forming cells (PFC)/10⁶ viable cells. Bars represent standard deviation. The difference between RA patients and healthy controls is not significant.

immunoglobulins in RA. We reasoned that lymphocytes of RA patients might be activated *in vivo*, so that cells were producing antibodies from the beginning of the culture and died out before the plaque assay. This was checked by investigating the number of cells secreting immunoglobulins spontaneously, that is, by assaying the plaque-forming cells without culture. As can be seen in Fig. 3 no increase in the number of spontaneous plaques was found in RA compared to controls.

Another possible explanation was decreased sensitivity or abnormal reactivity of RA lymphocytes to the stimulant PWM. This again is unlikely, because in cultures not stimulated with PWM the same trend of decreased production of IgM and IgG was obvious (Fig. 4), though the differences were not statistically significant. If hyporesponsiveness was restricted to PWM only, one would expect equal numbers of plaques by RA and control lymphocytes in unstimulated cultures.

Discussion

This study clearly shows that the blood lymphocytes of RA patients have a decreased production of immunoglobulins, especially of IgM and IgG.

The response of RA lymphocytes to mitogens or other polyclonal stimulants has been extensively studied by lymphocyte transformation, and decreased responses have been found. Of special relevance to the present study is the work done on stimulators thought to act mainly on B lymphocytes. Thus Lloyd and Panush⁶ and Highton *et al.*⁷ using PWM, and Haines and Hough⁸ using staphylococcal protein A reported depressed response of RA cells in transformation tests. Earlier Lance and Knight⁹ found a decreased response to PWM with RA lymphocytes, but almost as low a response was obtained with cells of osteoarthritis patients. However, activation of lymphocytes into antibody production or blast transformation as well as thymidine incorporation are different phenomena, which are separable. In a recent report¹⁰ on primary antibody response to an antigen, trinitrophenyl (TNP) hapten, depressed anti-TNP IgM secretion was found.

Several explanations of depressed production of antibodies may be envisaged, including defects in B cells, monocytes, or T helper cells, increased activity of T suppressor cells, and the operation of humoral factors. The data of Segond *et al.*¹⁰ suggest normal B cell function in RA. Concanavalin-A-induced suppressor activity as well as other tests for suppressor function are also reported to be normal in RA lymphocytes.^{10, 11} Thus it remains to be discovered what mechanisms are responsible for the depressed antibody synthesis we have demonstrated.

There is an apparent contradiction between these results and the high levels of antibodies detected in rheumatoid sera. Decreased antibody production should result in low levels of immunoglobulins in serum. A similar discrepancy was reported in patients with SLE whose peripheral blood B cells showed impaired immunoglobulin synthesis after PWM stimulation.^{12, 13} However, it should be kept in mind that *in-vitro* studies are performed on blood lymphocytes. It may well be that lymphocytes actively synthesising immunoglobulins are present in other sites such as synovial tissue. Furthermore, all the mechanisms regulating antibody response *in vivo* may not be present in cell cultures.

As already discussed, the explanation for the impairment of antibody synthesis in RA is not clear. We would like to think that in RA, possibly after polyclonal stimulation,¹⁴ a too vigorous antibody response takes place. The immune system thus has to use powerful mechanisms of regulation to avoid immunological exhaustion. What we see *in vitro* would be consistent with this state of affairs.

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