Analysis of impaired in vitro immunoglobulin synthesis in rheumatoid arthritis

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Abstract

Decreased immunoglobulin production in pokeweed mitogen driven lymphocyte cultures has been reported in rheumatoid arthritis (RA). Here various activators and experimental designs have been used to determine the contribution of B cells, T cells, or monocytes to this low response.

Sixty patients with RA and paired controls were studied at the onset of disease and again six months later. Concentrations of IgA, IgG, and IgM in cultures of RA peripheral blood mononuclear cells stimulated with thymus dependent activators were already decreased at the onset of the disease. Six months later RA mononuclear cells produced even lower concentrations of immunoglobulin. In contrast, stimulation with a T cell independent activator showed that RA B lymphocytes had retained normal potential to synthesise immunoglobulin.

Poor helper function was indicated by costimulation experiments and cultures of mixed mononuclear cells from patients and controls. This notion was supported also by the fact that phytohaemagglutinin induced interleukin-2 production by RA mononuclear cells was less than half of the control values. Non-specific suppressor activity was similar in RA and controls.

Monocyte functions were normal when tested by addition of indomethacin or 2-mercaptoethanol to the mitogen activated cultures.

The defect in mitogen stimulated immunoglobulin production in vitro of RA mononuclear cells thus was more pronounced with time and probably reflects impaired mediator associated help in the differentiation of B lymphocytes into immunoglobulin secreting cells.

There is ample evidence of aberrant immunofunctions in rheumatoid arthritis (RA), including serum hyperimmunoglobulinaemia and production of autoantibodies.¹ The mechanisms leading to these abnormalities are still obscure. In vitro culture studies with blood lymphocytes have shown, paradoxically, a decreased mitogen stimulated immunoglobulin secretion.²⁻⁶ The reasons for decreased immunoglobulin synthesis are unknown. It is also unknown whether these cellular mechanisms are related to B cells, T cells, or monocytes. We have extended these studies and report that this phenomenon becomes more pronounced during six months' follow up of recent onset disease. In this study

we also report on experiments designed to determine whether the decreased immunoglobulin production is due to changes in the functions of B cells, T helper or T suppressor cells, or monocytes.

Patients and methods

PATIENTS AND CONTROLS

The patient group comprised 58 patients (17 men, 41 women) with newly diagnosed RA fulfilling American Rheumatism Association criteria for definite or classical RA. The patients were aged 17 to 78 years (mean 48) with a duration of disease of two to 24 months (mean eight). At the onset of the study none of the patients had received second line antirheumatic drugs. During the next six months before the second blood sample 54 patients received intramuscular injections of sodium aurothiomalate (10, 20, 30, and 50 mg weekly up to 13 mg/kg body weight and thereafter 50 mg monthly), three patients were treated with hydroxychloroquine (300 mg daily), and one with a non-steroidal anti-inflammatory drug. If these drugs were not tolerated (12 patients) auranofin, sulphasalazine, D-penicillamine, or azathioprine was given. Fifty nine healthy laboratory and office personnel (26 men, 33 women) with a mean age of 36 (range 26-61) served as controls.

BLOOD SAMPLES

Blood samples were taken at the onset of the study and six months later. Blood was collected by venepuncture and treated with heparin (20 U/ml preservative free sodium heparin). Mononuclear cells were separated by Ficoll-Paque (Pharmacia Chemicals, Uppsala, Sweden) density gradient centrifugation and washed three times with Hanks's balanced salt solution. Cells were suspended (1×10^6 cells/ml) in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco, Scotland), glutamine 3 g/l, and gentamicin 15 mg/l (Sigma Chemical Co, USA).

CELL CULTURES

Mononuclear cells were cultured in microtitre plates of tissue culture grade (Nunc, Denmark) in 200 μ l volumes stimulated with either pokeweed mitogen diluted 1:100 (Gibco Ltd) or *Staphylococcus aureus* Cowan I 0.05% vol/vol prepared as described elsewhere.⁷ Pokeweed mitogen stimulated cocultures were set up by mixing equal numbers of RA and control mononuclear cells to give a cell density 1×10^6 /ml suspension. Hydrocortisone (The Upjohn Co,

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USA) 10^{-5} mol/l, concanavalin A (Pharmacia Chemicals, Sweden) 4 mg/l, or indomethacin (Lääketehtas Orion, Finland) 1 mg/l was added directly to cultures. Cells were cultured in a humidified CO_2 incubator at 37°C for seven days. Cultures of 1 ml volume in round bottomed 12×75 mm plastic tubes (Sterilin, England) were stimulated with 1:4 vol/vol Epstein-Barr virus containing B95-8 marmoset cell culture supernatant, filtered through a $0.45 \mu\text{m}$ nitrocellulose filter (Schleicher and Schuell, Germany), and stored for a maximum of three months in a refrigerator.

ELISA MEASUREMENT OF IMMUNOGLOBULIN PRODUCTION

Supernatants from cultures were stored frozen at -20°C . Immunoglobulin concentration was determined by a double antibody sandwich enzyme linked immunosorbent assay (ELISA) method² using isotype specific trapping and alkaline phosphatase conjugated isotype specific detecting antibodies (Orion Diagnostica, Finland). Measurements were standardised with known concentrations of standard sera (Behringwerke, Germany). The absorbances were read with a Titertek Multiscan plate reader (Flow Laboratories).

PRODUCTION AND DETERMINATION OF INTERLEUKIN-2

Mononuclear cells (1.5×10^6) were cultured for 24 hours with phytohaemagglutinin (Difco Laboratories, USA) 1:100 final dilution in 1 ml of RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine 3 g/l, and gentamicin 15 mg/l. Cell free supernatants were harvested by centrifugation and stored frozen at -20°C . The samples were assayed for interleukin-2 (IL-2) by an IL-2 dependent murine cytotoxic T cell line CTLL-2⁸ or concanavalin A stimulated lymphocytes.⁹ The assays of patients with RA and controls were performed simultaneously, and the values for RA samples were calculated as a percentage of the mean of controls.

STATISTICAL METHODS

Student's *t* test and the Mann-Whitney U test were used to determine statistical significance.

Results

IMMUNOGLOBULIN SECRETION BY MITOGEN STIMULATED MONONUCLEAR CELLS

We used ELISAs to measure immunoglobulin isotypes in supernatants of seven day cultures of blood mononuclear cells at the onset of disease and six months later. Two polyclonal stimulants were used: pokeweed mitogen, a strictly monocyte and T cell dependent mitogen,^{10 11} and *S aureus* Cowan I, which is considered to be a B cell activator fairly independent of T cells. Yet the differentiation of cells activated by *S aureus* Cowan I is reported to depend on T cells.^{12 13} At the onset of the disease production of immunoglobulin was already decreased in RA and at six months the defect had become greater

in all isotypes (fig 1). Both mitogens seemed to show the defect similarly. Unstimulated RA mononuclear cells, however, produced similar amounts of immunoglobulin to those of healthy controls (fig 1).

Epstein-Barr virus, a direct B cell activator,¹¹ did not stimulate secretion of IgA and IgG, but the concentration of IgM was increased two- to 10-fold compared with unstimulated cultures. There was no significant difference between RA and control mononuclear cells in production of IgM either at the onset of the study or after six months (data not shown).

TESTS OF SUPPRESSOR AND HELPER FUNCTIONS

Hydrocortisone in physiological and pharmacological concentrations causes a marked enhancement of immunoglobulin secretion in cultures stimulated with pokeweed mitogen.¹⁴ It is suggested that this effect is due to modulation of the triggering signal for B cells by naturally occurring suppressor T cells¹⁵ or T8^+ T cells.¹⁶ Figure 2 shows the effect of 10^{-5} mol/l hydrocortisone on immunoglobulin secretion by RA and control mononuclear cells. The results are expressed as the ratio of hydrocortisone supplemented/unsupplemented cultures stimulated either with pokeweed mitogen or *S aureus* Cowan I. The relative increase of immunoglobulin secretion by hydrocortisone in pokeweed

Figure 1: Production of IgA, IgG, and IgM in cultures of seven days of blood mononuclear cells. Open bars represent healthy controls, striped bars patients with rheumatoid arthritis (RA) at the onset of the study, and black bars patients with RA six months later. The cultures were unstimulated (NIL), stimulated with pokeweed mitogen (PWM), or with *Staphylococcus aureus* Cowan I (SAC). Results are expressed as the mean (SEM), for RA $n=60$, for controls $n=57$ at the onset and $n=47$ six months later. ** $p<0.01$, *** $p<0.001$.

Figure 2: The effect of hydrocortisone 10^{-5} mol/l on pokeweed mitogen (PWM) or *Staphylococcus aureus* Cowan I (SAC) stimulated cultures of healthy subjects (open bars), patients with rheumatoid arthritis (RA) at the onset of the study (striped bars), and patients with RA after six months (black bars). The results are expressed as the mean (SEM) of the ratio calculated from the concentrations in hydrocortisone supplemented/unsupplemented culture supernatants in cultures of seven days. The numbers of subjects were $n=50$ for controls and $n=54$ for patients with RA. ** $p<0.01$.

Figure 3: The effect of concanavalin A costimulation on the immunoglobulin production stimulated with pokeweed mitogen (PWM) or *Staphylococcus aureus* Cowan I (SAC) in mononuclear cell cultures of controls (open bars), patients with rheumatoid arthritis (RA) at the onset of the study (striped bars), and patients with RA six months later (black bars). The results are expressed as the mean (SEM) of the ratio concanavalin A costimulated/unstimulated immunoglobulin production in culture supernatants of seven days. The numbers of subjects were $n=34$ for controls and $n=23$ for patients with RA. ** $p<0.01$.

mitogen stimulation was similar in RA and control mononuclear cells. When hydrocortisone was added to RA mononuclear cells the reduced immunoglobulin secretion was not restored to the level of controls.

In contrast with pokeweed mitogen stimulated cultures, the addition of hydrocortisone to cultures stimulated with *S aureus* Cowan I did not enhance immunoglobulin secretion. Interestingly, the mononuclear cells of the patients produced less immunoglobulin than the controls; this was most noticeable for IgG. This defect was greater later in the course of the disease (fig 2).

Concanavalin A may also be used to study non-specific suppressor activity. It is claimed to suppress pokeweed mitogen stimulated immunoglobulin synthesis by triggering suppressor precursor cells, which are then converted into effectors by pokeweed mitogen.¹⁷ As seen in fig 3 concanavalin A reduced equally pokeweed mitogen driven immunoglobulin secretion by both the RA and control mononuclear cells. Again, *S aureus* Cowan I, costimulated with concanavalin A, produced quite a different pattern. Instead of inducing suppression, concanavalin A increased immunoglobulin secretion 35 to 100% in control mononuclear cells (fig 3). In RA, however, this increase was not as prominent and indeed, at six months did not occur at all. A difference between RA and control responses was found in all immunoglobulin classes but reached statistical significance for IgM.

Mononuclear cells from patients with RA and healthy controls were stimulated with pokeweed mitogen and cocultured in equal numbers to find out whether decreased immunoglobulin synthesis was due to defective helper function or increased suppression. The concentration of immunoglobulin obtained in the coculture was compared with the mean concentration in cultures from each donor separately, and the ratio of observed to expected was calculated. Ratios of observed/expected over 1.0 would point to subnormal helper function, whereas ratios below 1.0 would be due to activation of suppressor T cells.^{18,19} The mean observed/expected values at onset were close to unity, but after six months the values increased to 1.12 for IgA and 1.22 for IgG and IgM, suggesting poor helper effect in RA (table 1).

INTERLEUKIN-2 PRODUCTION

Disturbances in the production of soluble mediators of cellular cooperation might be another reason for impaired immunoglobulin

Table 1: Mean observed/expected values for immunoglobulin production in pokeweed mitogen stimulated cocultures of control mononuclear cells with those from another control (control) and for mononuclear cells from a patient with rheumatoid arthritis with control mononuclear cells (RA)

Group	n	Observed/expected		
		IgA	IgG	IgM
Control	30	0.99	0.95	0.86
RA—at onset	54	0.96	0.97	1.06
—at 6 months	43	1.12	1.22	1.22

Table 2: Phytohaemagglutinin stimulated interleukin-2 production in mononuclear cell cultures of controls and patients with rheumatoid arthritis (RA). The results are expressed as a percentage of the mean of controls (SD)

Group	n	Interleukin-2
Control	52	100 (193)
RA—at onset	53	85 (155)
—at 6 months	49	42 (60)*

*Significantly different from controls, $p < 0.01$.

production. We chose to investigate IL-2, a mediator of T cell origin, because of its central role in cellular activation and differentiation. Supernatants from phytohaemagglutinin stimulated 24 hour mononuclear cell cultures of RA at the onset of the study contained 15% less IL-2 than controls (table 2). The amount of IL-2 found in culture supernatants of RA mononuclear cells was even lower after six months, being only 42% of the concentration of IL-2 in controls. The phytohaemagglutinin stimulated IL-2 production by controls, when the results in count per minute were converted to U/ml using an IL-2 standard of known concentration, was similar to those reported earlier.²⁰

TESTS OF MONOCYTE FUNCTION

Monocytes are the major source of prostaglandins, which among other things influence immunoglobulin synthesis.^{21 22} Therefore, defects in the production of prostaglandins might contribute to the decreased immunoglobulin response in RA. If this were the case, prostaglandin inhibitors would decrease immunoglobulin secretion by healthy lymphocytes more than that by RA lymphocytes. When indomethacin was added to cultures of mononuclear cells the effect was similar in RA and control cells stimulated by pokeweed mitogen or *S aureus* Cowan I (data not shown), with no significant difference.

It has been claimed that the accessory function in in vitro IgG secretion of RA monocytes is impaired and that this impairment may be restored by addition of 2-mercaptoethanol, which modifies cell surface sulphhydryl groups.²³ We therefore tested 2-mercaptoethanol in cultures driven by pokeweed mitogen and *S aureus* Cowan I but found no enhancing effect (data not shown).

Discussion

Rheumatoid arthritis is a disease of unknown cause with features of autoimmunity. In vitro tests of RA lymphocytes have shown perturbed immunofunctions.¹ In contrast with the increased serum immunoglobulin concentrations in RA, mitogen driven immunoglobulin production has been reported to be depressed.²⁻⁶ Here we have shown that this defect in immunoglobulin production is already present at the onset of disease and more pronounced during the disease. Furthermore, our experiments suggest impaired mediator production as the most plausible mechanism for this defect.

Synthesis of immunoglobulin by B lymphocytes/plasma cells after stimulation with pokeweed mitogen is under the control of T helper

and T suppressor cells and is also monocyte dependent.^{10 11} Thus the noted defect may result from disturbances in any of these individual cell subsets. *S aureus* Cowan I does not activate T suppressor cells, but full differentiation into plasma cells depends on T cell cooperation.^{12 13 24} The fact that both mitogens, despite differences in the T suppressor control, show an equal defect in immunoglobulin production by RA lymphocytes suggests that T suppressors are not the cause. Indeed we were unable to show changes in suppressor functions of RA cells in more direct tests with hydrocortisone or concanavalin A in pokeweed mitogen cultures or with cocultures of RA and control cells. Similarly, B lymphocytes of patients with RA seem to have retained their normal potential to produce immunoglobulin as synthesis of immunoglobulin, driven by Epstein-Barr virus, is unimpaired. This is in agreement with an earlier report.²⁵ Furthermore, our experiments on monocyte function also gave negative results. Secretion of prostaglandins by monocytes may regulate B cell growth and differentiation by routes which are not yet completely clear.²² Inhibition of prostaglandin synthesis by indomethacin had similar effects on immunoglobulin secretion in patients and controls, indicating normal function of RA monocytes. Hence we conclude that B cells, monocytes, and T suppressor cells show no abnormalities in our tests, though we realise that all possible defects in these subsets of cells cannot be excluded with these experiments.

Helper functions, on the other hand, seem abnormal in this study. Firstly, we found that IL-2 secretion induced by phytohaemagglutinin was significantly reduced compared with controls, in agreement with earlier studies.^{26 27} Increased IL-2 production in RA has also been reported, however.^{28 29} The reduction of IL-2 secretion became more obvious at the same time as the reduction of immunoglobulin production during six months' follow up. Secondly, in cocultures of RA and control cells high observed/expected values indicate that low pokeweed mitogen responses of RA cells are augmented by help from control cells or factors. Thirdly, concanavalin A in the cultures stimulated with *S aureus* Cowan I increased the production of immunoglobulin in controls in this study. It is known that concanavalin A induces secretion of IL-2 and probably other mediators as well. It has also been shown that IL-2, and even more so mediators in T cell supernatants, stimulate proliferation and differentiation of B cells activated with *S aureus* Cowan I.¹² Therefore the concanavalin A induced increase of immunoglobulin production might most simply be explained by the effects of increased mediators on the differentiation of cells activated by *S aureus* Cowan I. That this increase does not occur in RA suggests defective mediator associated help or a lack of cell subsets responsible for their production. Cells bearing CD4 are thought to contain the principal IL-2 producing lymphocytes.³⁰ The only cells among CD4⁺ lymphocytes able to produce IL-2 are of the phenotype CD4⁺2H4⁺4B4⁻.³¹⁻³³ Recently, a specific loss of this

subset has been shown to occur in RA, which may be relevant.^{34 35} A weak proliferation of the true helper subset CD4⁺2H4⁺4H4⁺ owing to depressed IL-2 production may lead to low activation and especially poor production of differentiation factors for B cells. It should be pointed out, however, that it is unclear which cells are responsible for mediator production after concanavalin A or *S aureus* Cowan I stimulation.

Thus we conclude that the most probable mechanism for the defect in in vitro immunoglobulin synthesis in RA is impaired production of IL-2 and, possibly, other mediators. What then might be the cause of abnormal mediator function is another question. Our findings show that during the first six months of the disease the defect in immunoglobulin synthesis becomes more pronounced, which suggests an association with the progress of the disease or, alternatively, with the drug treatment. At the onset of the study the patients did not receive any second line antirheumatic drugs but, nevertheless, showed depressed immunoglobulin production. Non-steroidal anti-inflammatory drugs might have been used, however. The effect of these drugs is currently under investigation in our laboratory.

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