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Academic Dissertation

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IMMUNOGLOBULIN PRODUCTION BY CULTURED LYMPHOCYTES OF PATIENTS WITH RHEUMATOID ARTHRITIS; ASSOCIATION WITH DISEASE SEVERITY

Ilmari Jokinen

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The synthesis of immunoglobulin (Ig) in cultures of mononuclear cells from the blood of patients with recent onset rheumatoid arthritis (RA) was studied. B lymphocytes were activated with mitogenic activators, pokeweed mitogen and *Staphylococcus aureus* Cowan I, and with Epstein-Barr virus (EBV). Ig production was assayed by counting the number of Ig-secreting cells and by determining the concentration of secreted Ig in the culture medium. The patients were followed by medical examinations, radiography, and clinical laboratory tests at six month intervals for two years.

Mitogen-induced Ig synthesis by the cells of patients was significantly decreased compared to healthy subjects. The decreased responses were evident already at diagnosis before any disease-modifying anti-rheumatic medication was started. The mechanism of decreased responses were then studied by supplementing the cultures with modifying agents to examine the contribution of B cells, T cells and monocytes to the impaired Ig synthesis. Phytohaemagglutinin-induced interleukin-2 (IL-2) production was also studied. The low responses of rheumatoid lymphocytes to mitogens is, according to these experiments, due to poor helper function, possibly mediated by reduced production of IL-2.

Further, the association of the *in vitro* responses with disease outcome was studied. Impaired mitogen-induced IgM production, as well as decreased PHA-induced IL-2 release, at the onset of disease, associated with joint destructions two years later. EBV-induced Ig response was associated with the disease: cultured mononuclear cells of patients with severe disease were not able to down-regulate excessive Ig synthesis in long term cultures. When used as a test high EBV-induced Ig synthesis predicted with a high probability the development of joint erosions and a poor outcome of disease.

The *in vitro* assessment of immune functions may make it possible to develop tests which identify already in the early phase of disease the patients in risk to have destructive disease.

Key words: rheumatoid arthritis, autoimmunity, Ig synthesis, PWM, EBV, erosive disease, prediction of outcome.

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List of original publications

This thesis is based on the following publications. The papers are referred to in the text by their Roman numerals (I-V).

I. Poikonen, K., Oka, M., Möttönen, T., Jokinen, I. & Arvilommi, H. 1982: Synthesis of IgM, IgG, and IgA in rheumatoid arthritis. Ann. Rheum. Dis. 41: 607-611. http://dx.doi.org/I0.II36/ard.4I.6.607

II. Jokinen, I., Poikonen, K. & Arvilommi, H. 1985: Synthesis of human immunoglobulins in vitro: comparison of two assays of secreted immunoglobulin. - J. Immunoassay: 6: 1-9. https://doi.org/I0.I080/0I97I5285080630I7

III. Jokinen, I., Poikonen, K., Möttönen, T., Hannonen, P., Oka, M., Ilonen, J., Surcel, H-M., Karttunen, R. & Arvilommi, H. 1990: Analysis of impaired in vitro immunoglobulin synthesis in rheumatoid arthritis. - Ann. Rheum. Dis. 49: 507-511. http://dx.doi.org/I0.II36/ard.49.7.507

IV. Jokinen, E.I., Möttönen, T.T., Hannonen, P.J. & Arvilommi, H.S. 1992: Association of in vitro immune functions with the severity of the disease in rheumatoid arthritis. - Br. J. Rheumatol. (in print) https://doi.org/I0.I093/rheumatology/32.7.550

V. Jokinen, I., Möttönen, T., Hannonen, P., Mäkelä, M. & Arvilommi, H. 1992: Prediction of severe rheumatoid arthritis using Epstein-Barr virus -induced immunoglobulin synthesis by lymphocytes of patients with recent onset disease. - Manuscript (submitted) https://doi.org/I0.I093/rheumatology/33.I0.9I7

Abbreviations

AKA	antikeratin antibodies
APC	antigen presenting cells
APF	antiperinuclear factor
BCGF	B cell growth factor
C1q	complement component 1q
CD	cluster of differentiation
Con A	Concanavalin A
CRP	C-reactive protein
CTD	connective tissue disease
DMARD	disease modifying anti-rheumatic drug
EBV	Epstein-Barr virus
EBNA	Epstein-Barr virus nuclear antigen
ELISA	enzyme-linked immuno sorbent assay
ELISPOT	enzyme-linked immuno spot
HC	hydrocortisone
HLA	human leucocyte antigen
HSP	heat shock protein
IFN-γ	interferon-γ
Ig	immunoglobulin
IL-	interleukin-
IND	indomethacin
LPS	lipopolysaccharide
MC	monocyte
MHC	major histocompatibility complex
MNC	mononuclear cells
NSAID	nonsteroidal anti-inflammatory drug
PGE_2	prostaglandin-E_2
PHA	phytohemagglutinin
PFC	plaque forming cell
PWM	pokeweed mitogen
RA	rheumatoid arthritis
RF	rheumatoid factor
RHP	reversed haemolytic plaque
RIA	radioimmunoassay
SAC	*Staphylococcus aureus* Cowan I
ssDNA	single-stranded DNA
TGF	transforming growth factor
TNF	tumour necrosis factor
WaRo	Waaler-Rose

Contents

1 INTRODUCTION

The etiology of rheumatoid arthritis (RA), a systemic inflammatory disease in man, remains unknown, but there is no doubt of the role of autoimmunity in the manifestation of the disease. Characteristic to RA is hyperactivation of the humoral immune system exemplified by raised serum immunoglobulin (Ig) levels, various autoantibodies and an increased number of activated B lymphocytes in the circulation (Gupta & Talal 1985). The clinical course of RA in an individual cannot be predicted, and it varies from remitting disease to severely erosive polyarthritis (Pincus 1991).

A crude indication of B-cell function, e.g. in immunodeficiency diseases, can be obtained by measuring concentrations of serum Igs or by assessing the responses to antigens. However, the mechanisms controlling the immunological responses are very complex and in order to study the various cell interactions involved and to identify mechanisms of deviant functions, it is necessary to turn into *in vitro* systems.

Evidence suggests that B-cell hyperreactivity in RA arises as a consequence of disordered T cell functions (Harris & Epstein 1990). In this series of studies blood lymphoid cells from patients with recent-onset RA were studied *in vitro* to investigate B-lymphocyte functions and their regulation.

2 REVIEW OF LITERATURE

2.1 Rheumatoid arthritis

2.1.1 General

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease of unknown origin affecting mainly multiple joints. Extra-articular signs, such as rheumatoid nodules, vasculitis and lymph-adenopathy are present in a proportion of patients. RA affects about 1% of the population worldwide and is more common among women (prevalence 1.5 %), than men (prevalence 0.5%) (Calin 1986). Because of its high frequency and potentially severe nature, RA is one of the most important causes of disability in adults (Harris & Epstein 1990; Wolfe 1991).

2.1.2 Etiology of rheumatoid arthritis

The etiology of RA remains unknown despite intensive research. No single specific cause of RA has been found, and RA may turn out to be a disease with multiple etiology. There is currently much data on factors associated with RA including genetics, immunology, microbiology, endocrinology and metabolic events. Information has been gained on

geographically defined populations, and on socio-economical and psychological status of RA patients (Kouri 1985; Hochberg & Spector 1990). A convincing body of evidence has accumulated to suggest a central role of immunological events in the development of RA in genetically susceptible individuals (Kouri 1985; Deighton & Walker 1991). Although much of the permanent damage to the joints is exerted by nonspecific myeloid cells, the triggering mechanisms are likely to involve activation of T lymphocytes by an as yet unidentified exogenous or self antigen (Vaughan et al. 1988; Wilder 1990).

It has long been known that inherited factors are important in RA. Rheumatoid arthritis clusters in some families but the first degree relatives of probands with RA have no more than a slightly increased risk of developing the disease (Aho et al. 1986; Deighton & Walker 1991). The genetic contribution to RA cannot be greater than 30% and it is therefore likely that environmental and/or random factors are needed to trigger the disease (Walker et al. 1985; Grennan et al. 1986).

Stastny (1978) first described the association between major histocompatibility complex (MHC) class II alleles and RA. Subsequent studies have delineated the molecular basis of this association (Gregersen et al. 1987). In Caucasian populations the relative risk of RA is about four times greater in individuals with the HLA-DR4 antigens. The third hypervariable region of the DRβ-chain is the major site of sequence differences that distinguish the various DR subtypes. The third diversity region of the DRβ-chain is shared by DR4(w14), DR4(w15), DR4(w4), and also by DR1, and is involved in regulating immune recognition events in RA. It has been shown, consistent with the shared epitopes hypothesis, that a particular DRβ-sequence at residues 70-74 is strongly associated with RA. Although the 3-dimensional structure of class II molecules has yet to be defined, computer models indicate that the hypervariable region of DRβ1 may contain a region with an α-helical structure that is critical for T-cell recognition. This data suggests that the susceptibility to RA is not strictly linked to the MHC haplotype, but rather to conformational structures in MHC class II products: the presence or absence of a particular conformation of antigen-presenting molecules (Merryman et al. 1989; Rønningen et al. 1990; Wilder 1990).

MHC is clearly important in determining the susceptibility to RA, but it cannot explain some other aspects of the disease, e.g. female predomi-nance: female to male ratio is of 3:1. There is also evidence that disability may be greater among women than men. Arthritis models on rats have shown that neuroendocrine regulation, corticosteroids and

other hypothalamic-pituitary-adrenal axis hormones, are important regulators of inflammation (Sternberg et al. 1989a, 1989b; Wilder 1990). The association of RA with certain epitopes of the DR locus emphasizes the role of the immunological system, but what is the causative agent, "the triggering event"? Infection or a chronic carrier state is considered to be a likely basis, because of the immunological abnormalities that characterize the disorders. Viruses such as rubella, measles, parvo and Epstein-Barr virus (EBV) in susceptible people are the strongest candidates for triggering RA (Lydyard & Irving 1988; Venables 1988; Silman 1989; Vaughan 1989; Vaughan 1990; Silman 1991; Wilder & Crofford 1991).

Several lines of evidence show that EBV, a DNA containing virus of the herpes group, may be an important contributor to the patogenesis of RA. The main findings are as follows: B cells of RA patients are more readily infected by the virus than normal cells, which is reflected as a high number of circulating virus-infected cells. T cells of patients with RA are ineffective in inhibiting EBV-induced growth of B cells and Ig production *in vitro*. Further, RA patients have increased titers of antibodies against EBV-related antigens, especially against EBNA-1. (Epstein-Barr virus nuclear antigen)(Stierle et al. 1983; review by Fox et al. 1985; review by Lydyard & Irving 1988). However, the levels of antibodies against some EBNA-1 derived peptides in the preillness sera of RA patients are reported to be normal (Kouri et al. 1990).

Fox et al. (1985) have proposed a molecular mimicry hypothesis to explain the role of EBV in the pathogenesis of RA. In RA there are increased amounts of antibodies against EBV antigens because of the patient's inability to suppress EBV infection. These antibodies can crossreact with normal proteins, e.g. the 62kD protein in the synovium (Luka et al. 1984), and this could result in the initiation and perturbation of synovitis . In addition, crossreaction of the EBNA-1 peptide p107 (also known as p62), the major epitope of the EBV-encoded EBNA-1 antigen, with collagen and keratin has been described in RA (Sulitzeanu & Anafi 1989; Birkenfeld et al. 1990).

Recently, the interest in relating EBV and RA has increased through the concept of another molecular mimicry between viral glycoprotein gp110 and the susceptibility sequences in the β-chain of HLA DR4 and DR1 molecules. T-cell recognition of certain determinant on gp110 among subjects with HLA Dw4, -Dw14, or -DR1 may be important in determining who will develop RA and who will not (Roudier et al. 1988; Roudier et al. 1989; Vaughan 1990).

Relating mycobacteria to RA is of interest because these bacteria express stress proteins, also known as heat-shock proteins (HSP), which can induce arthritis in rats. Patients with RA have increased amounts of antibodies to HSPs from mycobacteria (Rook et al. 1990; Strober & Holoshitz 1990). T lymphocytes which carry γδ-T cell receptors and proliferate in response to mycobacterial antigens are found in the synovial fluid of RA patients (Haregewoin et al. 1989; Gaston et al. 1989). It is thus possible that γδ-T cells, in response to mycobacterial antigens, could be amplified and perpetuated by cross-reactivity with HSPs on the surface of synovial cells and therefore might be related to the genesis of RA (Young & Elliot 1989; Strober & Holoshitz 1990; Gaston 1991).

2.1.3 Immunopathology of rheumatoid arthritis

RA is an autoimmune disease characterized by several antibodies against the body's own constituents. There is little question that autoimmunity has a major role in the progression of RA, but data supporting autoimmunity as the initial cause of the disease is not convincing. Immunoglobulin and collagen are commonly implicated as the endogenous proteins of RA autoimmunity.

Rheumatoid factors (RF) are autoantibodies reacting with the Fc-portion of autologous IgG. RFs are found in the serum, synovial fluid and synovial membrane of most patients with RA. It is clear that RFs contribute to the amplification of rheumatoid synovitis through activation of complement and formation of immune complexes that are phagocytosed by PMN in synovial fluid. Although RFs in most instances precede clinical disease, their role in the pathogenesis remains obscure (Aho et al. 1985; Tuomi et al. 1988; Möttönen et al. 1988; Carson 1989; Aho et al. 1991). In RA, as well as some other arthritides, there is a reduced glycosylation pattern of IgG and this abnormally glycosylated IgG may serve as an antigen in genetically susceptible hosts (Hansson et al. 1985; Parekh et al. 1985; Hay et al. 1991).

Immunization by collagen type II (articular collagen) of certain species of animals results in polyarthritis that resembles human RA (Stuart et al. 1984). The presence of collagen autoimmunity in RA is well established, but it is not restricted to RA. Most data from several studies are consistent with the hypothesis that RA is not caused by the develop-

ment of anticollagen antibodies, but rather synovitis and destructive arthritis are amplified by anticollagen antibodies (Pereira et al. 1985; Rowley et al. 1986; Morgan et al. 1987).

The inflammatory events in RA comprise increased synovial fluid production, proliferation of cells in the synovial membrane, tissue destruction, repair, and fibrosis. Almost all currently available data support the hypothesis that there is overactivation of lymphocytes in RA. Both humoral and cell-mediated responses contribute to the inflammation in the RA joint. The T cells that accumulate in the synovial membrane have a crucial role. There is a marked dominance of helper cells in the synovial fluid and synovial membrane. Numerous plasma cells are found in the synovial tissue of RA patients because B cells proliferate and differentiate into Ig-secreting cells, a process that is mediated through cytokines derived from lymphocytes and monocytes. Inflammation is perpetuated by the formation of immune complexes and production of lymphokines. Once inside the joint, due to the attraction of chemotactic factors, neutrophils are activated by phagocytosis of cellular debris and immune complex aggregates. Neutrophils degranulate and release proteinases, products of arachinoid acid metabolism and reactive oxygen compounds (Haas et al. 1989; Harris & Epstein 1990; Strober & Holoshitz 1990). Candidates for the central role in the pathogenesis of RA are, however, MCs and macrophages which are present in the pannus. These cells are the major producers of IL-1 which is an important mediator of inflammation, and contribute to the degradation of cartilage and to bone erosion. There are several cell types with dendritic shapes in the synovial tissue. Some of these are of mesenchymal origin, while others are of lymphoid lineages: follicular dendritic cells and interdigitating cells. These cells express class II MHC proteins and produce IL-1. The production of proteinases and collagenases by fibroblast-type cells, chondrocytes and undifferen-tiated connective tissue cells result in the degradation of cartilage proteoglycan and collagen. IL-1 and PGE_2 activate osteocytes and osteoclasts which contribute to bone demineralization (Figure 1)(Allison 1988).

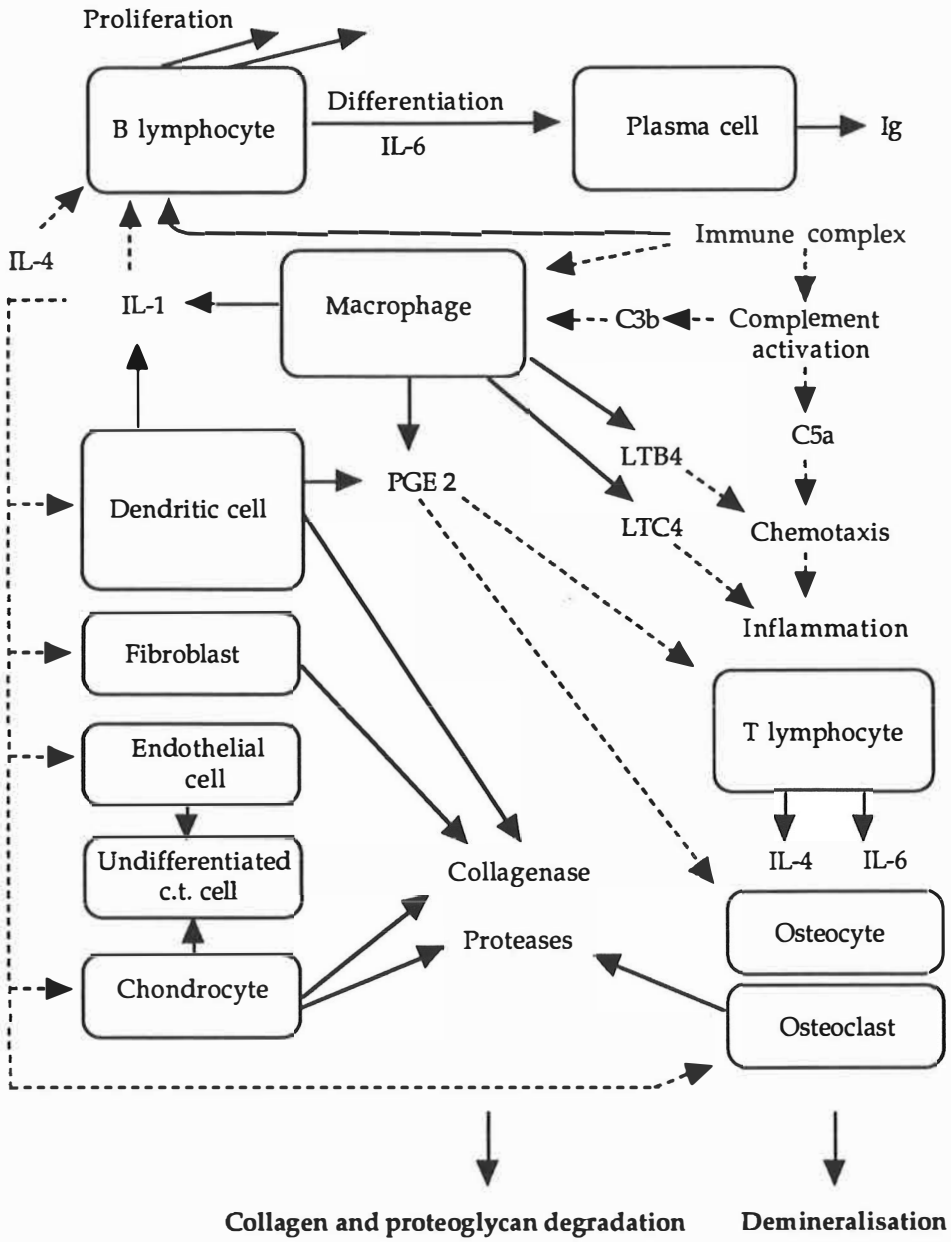

Fig 1. Interactions of cells and mediators in the pathogenesis of RA (Allison 1988; LTB4= leukotriene B4, LTC4= leukotriene C4, c.t. cell = connective tissue cell)

2.1.4 Markers of disease activity and the course of disease

Typical consequences of RA include morning stiffness, joint pain and swelling, elevated body temperatute, fatigue, anaemia, weight loss and, disability. It is difficult to assess disease activity and at present there is no single test that does this reliably (Persselin 1991). In clinical practice an opinion of disease activity is formed by a combination of information from laboratory and clinical variables, radiological assessment, and overall impression of the patient. To overcome problems of assessment several composite indices of rheumatoid activity have been developed (Harris & Epstein 1990).

The clinical course of RA cannot be predicted in an individual patient. The severeness of the disease varies from spontaneously remitting pauciarticular disease to erosive, progressive polyarthritis often with fluctuating activity of the disease. RA becomes usually symptomatic only gradually. General fatigue and malaise may be present before more specific joint symptoms appear, in most cases in metacarpophalangeal and interphalangeal joints. Joint effusions develop and the joints are painful. Irreversible destruction of cartilage occurs in the later stages of the disease (Wolfe 1991).

2.1.5 Prediction of patient outcome

RA is a heterogeneous disease; different patient subgroups have different courses. A major impediment to the treatment of RA is the inability to predict which patients will do well and which will become disabled or even succumb to disease (Bellamy 1991). Factors reported to correlate with the development of bone erosions and cartilage loss include e.g. raised blood levels of RF (especially IgA RF), circulating IgA-immune complexes, and depressed complement 1q (C1q) levels (Teittsson et al. 1984; Westend et al. 1986; Arnason et al. 1987; Eberhardt et al. 1988; Eberhardt et al. 1990b). However, only a few factors are known to predictor an unfavourable clinical outcome in early RA: female sex and positive IgA RF. Later in the disease some characteristics associate with a more unfavourable clinical course: persistently increased ESR or CRP, decreased hemoglobin, or the appearance of subcutaneous nodules (van der Heijde et al. 1988). Recently, an association between the development

of joint erosions and complex IgA-α1-antitrypsin has been reported, and a predictive value of the complex in the joint damage is suggested (Davis et al. 1991).

In addition to difficulties in finding good predictors of severe RA, it is not self evident which measures reflect most accurately the outcome of the disease. Radiographic abnormality is most closely linked to progressive inflammatory disease and represents a generally irreversible "outcome" of the disease. The most powerful indicator of outcome may be loss of function, as measured by the Stanford Health Assessment Questionnaire function disability index (HAQ) or similar (van der Heijde et al. 1990; Wolfe 1991).

2.2 Immunoglobulin synthesis

It has long been recognized that the immune system is multifunctional, with humoral and cellular components. The different functions are attributable to cells of separate lineages. B lymphocytes are responsible for humoral immunity: the production of antibodies. T cells mount responses against antigens present on other cells of the body. In addition, a number of other nonlymphoid cells are involved in the immune defence such as macrophages, dendritic cells of the spleen, and epithelial Langerhans cells, and specialized epithelial cells of the thymus. Lymphoid cells are found in the various primary and secondary lymphoid tissues including the spleen, the lymph nodes, the Peyers' patches of the intestine, the tonsils, the thymus, and the bone marrow. A substantial fraction of the lymphocytes and the macrophages comprise the recirculating pool of cells found in the blood and in the lymph.

2.2.1 Cellular basis of immunoglobulin production

B cells are produced in the bone marrow of mammals. The differentiation of B cells can be considered to occur in two steps. The first step

hematopoietic stem cells into virgin B cells, the second step is initiated after the encounter with antigen and occurs after the cells have left the hematopoietic tissue. Five main stages have been identified: progenitor B, pre-B, immature B, mature B, and plasma cell. During the differentiation Ig genes are rearranged and the cells in different stages express a variety of markers e.g. cytoplasmic and surface Ig and antigens defined by monoclonal antibodies (CD antigens) (Figure 2) (Klein 1990).

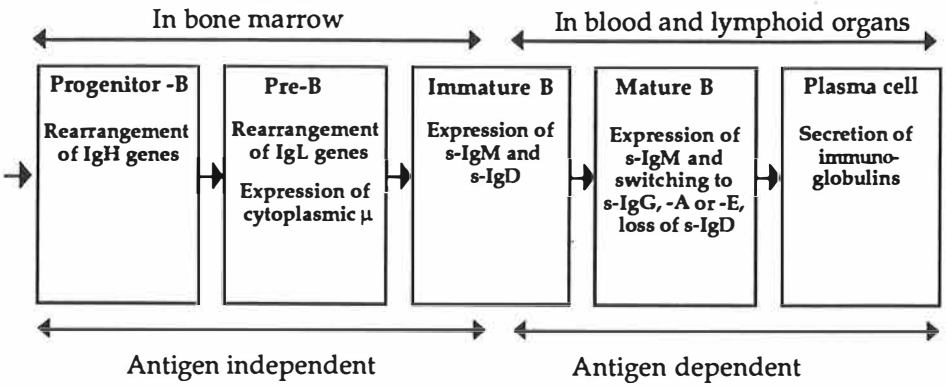

Fig. 2. Main stages in the differentiation of a B lymphocyte (Klein 1990)

Antigen-activated B cells proliferate into antigen-specific clones which further develop into plasma cells. Plasma cells synthesize and secrete large amounts of Ig. Long-lived memory cells may also be generated and these cells are later able to respond to the same antigen. Individual B lymphocytes are monospecific -i.e. they make antibody with only a single type of antigen-binding site (Klein 1990).

2.2.2 Regulation of immunoglobulin production and the role of mediators

Experimental evidence support the phenomenon of immunological suppression. The concept that there are suppressor T cells which play

suppressor T cells which play a role in the regulation of immune responses. However, there have been sharply conflicting views of the nature of the cells which mediate suppression: do they belong to a separate subpopulation or is suppression the result of the functional behavior of normally helping lymphocytes receiving a particular set of signals from other cells? Recently the role and existence of suppressor T cells has been discussed in the context of increasing data on molecules and principles involved. Attempts to formulate a new framework for understanding suppression have been done (Bloom et al. 1992), but contradictory evidence in favor of the classical concept has also been presented (Dorf et al. 1992). The issue now remains open.

The nature of the mechanism of suppression is beyond the scope of the present study, and for the sake of clarity, the regulation of Ig synthesis has been explained here according to the classical concept of helper and suppressor T cells.

During the immune response to T-dependent antigen, three distinct cell populations co-operate to induce antibody synthesis: B cells, helper T cells and the macrophages. Cognate interaction of B cells and T cells, together with soluble mediators secreted by T cells and antigen presenting cells (APC) necessary for the activation of B cell, leads to clonal expansion and maturation of B cell into Ig-producing plasma cells.

Much of the current knowledge about the regulation of B-cell functions has been obtained from studies using polyclonal B-cell activators. B-cell activities are under the control of T cells. Helper T cells induce B cells to make Ig and the other population regulating the humoral immune system consists of suppressor T cells. The mechanism of induction of suppressor T cells is heterogeneous and the final effector suppressor cells regulate the activity of the immune system through action on B cells, macrophages and, also other T cells (Unanue & Allen 1987; Ratcliffe & Klaus 1988; Klein 1990).

The regulation of B-cell responses is a complex process that involves the activities of a variety of cytokines from activated T cells and APCs. The activity of each cytokine is determined by the presence of specific receptors on responding cells. There are important differences between the regulation of human and murine B lymphocytes, especially with regard to the action of interleukin-2 (IL-2) and interleukin-4 (IL-4) (Hambling 1988; Lipsky 1989; Callard & Turner 1990). Cytokines which influence B cells were originally divided broadly into B cell growth factors and

differentiation factors. The availability of recombinant factors has led to reappraisal of their significance in regulation of B-cell functions. On the basis of *in vitro* studies it is clear that the same factors may influence all stages of B-cell maturation and that there probably are no separate growth and differentiation factors (Lipsky 1989).

A number of soluble factors have been reported to be involved in B-cell activation in man, e.g. IL-1 and a product of T cells referred to as B-cell growth factor low ($BCGF_{low}$) because it is only 12kd in molecular weight. $BCGF_{low}$ stimulates B cell to enter the cell cycle without completing mitosis. Tumor necrosis factor-α (TNF-α) which is secreted by MC enhances also B-cell activation (Hamblin 1988). Interleukin-2 facilitates the initial activation of resting B cells in man (Lipsky 1989).

In humans, IL-2 promotes the growth and differentiation of B cells, and there is little evidence that other cytokines can promote the generation of Ig secretion in the absence of T cells or their products. IL-2 is capable of facilitating the differentiation of cells that produce all isotypes of Ig and other cytokines can enhance the production of Ig, but only in the presence of IL-2 (Punnonen & Eskola 1987; Lipsky 1989).

Interleukin-4 increases the expression of class II MHC molecules and Fc-receptors for IgE in resting murine B cells and enhances the secretion of IgG_1 and IgE. In humans, IL-4 promotes T-cell proliferation and enhances IL-2 production and responsiveness. IL-4 is known to promote antibody synthesis at later stages of B-cell differentiation in man. IL-4 and IFN-γ regulate a class switch to different antibody glasses, e.g. the synthesis of IgE (Jelinek & Lipsky 1988; Hart et al. 1989). On the other hand, TGF-β (transforming growth factor β) and IL-4 inhibit responses of preactivated B cells. This implies that IL-4 acts also to limit initial B-cell activation in man (Lipsky 1989). IL-4 thus plays a complex role in the regulation of human B-cell responses, suppressing initial activation, but promoting subsequent differentiation (Lipsky 1989).

Interleukin-5 (IL-5) enhances the secretion of IgA and IgM of murine B cells and synergizes IL-4 to promote IgE secretion. In humans, IL-5 has also been implicated in IgA secretion. However, the effect of human IL-5 on B cells remains controversal and the range of activities of human and murine IL-5 may be different (Mizel 1989). Purified human B cells are able to respond to IL-5 in proliferation or differentiation only in the precence of T cells. Isotype (IgA and IgE) regulation by IL-4 and IL-5 in humans may occur through the activity of these lymphokines on T cells or, alternatively, an oblicatory signal may be provided by T cells in the response by human B cells, which is provided by LPS to murine B

cells (Callard & Turner 1990). Whether the high molecular weight (50-60 kDa) B cell growth factor ($BCGF_{high}$) is equivalent to IL-5 is unclear. $BCGF_{high}$ has a direct effect on growth of preactivated, e.g. SAC-activated, human B cells. This type of factors are classified as "BCGF-II activity" which is mediated by IL-5 in murine systems (Gordon & Guy 1987; Lipsky 1989).

Interleukin-6 (IL-6) appears to play a major role in the terminal maturation of activated B cells into Ig secreting cells. IL-6, in contrast to differentiation, has no effect on the proliferation of B cells. IL-6 enhances the generation of Ig-generating cells in an isotype non-specific manner. IL-1 and IL-6 have almost identical effects in acute inflammatory responses and it is not clear whether some functions ascribed to IL-1 might actually be mediated by IL-6 (Lipsky 1989; Hirano et al. 1990).
A summary of cytokines involved in the regulation of human B-cell responses is shown in Table 1 (Hamblin 1988; Lipsky 1989; Klein 1990).

Table 1. Cytokines in the responses of human B lymphocytes (Lipsky 1989).

Activity	Active in the absence of other cytokines	Activity	Active in the absence of other cytokines
Activation:		Differentiation:	
IL-2	+	IL-2	+
$BCGF_{low}$	+	$BCGF_{low}$	–
INF-γ	+	INF-α	–
IL-1	–	INF-γ	–
Proliferation:		IL-1	–
IL-2	+	TNF-α	–
$BCGF_{low}$	+	TNF-β	–
$BCGF_{high}$	+	IL-6	–
TNF-α	+	Suppression:	
TNF-β	+	IL-4	+
		TGF	+

Although B-cell growth and differentiation are controlled by T cell-derived factors, the macrophage-derived mediators, e.g. IL-1, also contribute to the regulation because T-cell functions are augmented by these factors. There is also evidence that B cells have a capacity to control their own growth in an autocrine fashion by IL-1-like factors or through cell contact factors (Gordon & Guy 1988; Banchereau et al. 1991).

The mechanisms by which B-cell responses are regulated *in vivo* are far from clear. Activation of B cells by T-dependent antigens occurs in proximity to the T cells and APCs in lymph nodes. In this environment the availability of antigen may be limiting. The degree of cognate T-B-interaction may be greater than *in vitro* and non-cognate mediators tend to be less important, possibly because of a shorter biological half-life of relevant molecules (Ratcliffe & Klaus 1988).

2.2.3 Polyclonal activators of immunoglobulin synthesis

In addition to antigen, a wide variety of agents, including microbes and their cellular and extracellular products and plant derived lectins, are able to induce activation and mitogenesis of lymphocytes in a polyclonal manner (Chen et al. 1982; Waldmann & Broder 1982; Vendrell et al. 1985; Räsänen et al. 1986). Mitogens and polyclonal activators serve as probes of lymphocyte activation and maturation, as a model for antigen-induced responses, and as indicators of lymphocyte function. Polyclonal activators have certain advantages over the antigen-specific system, e.g. they permit analysis of the activity of antigen-nonspecific helper and suppressor products secreted by several types of cells. Polyclonal activators are usually not genetically restricted and thus permit the coculture of cells from unrelated individuals, a characteristic of value in the analysis of MC-functions and regulatory T-cell activity in patients with immunological disorders (Waldmann & Broder 1982).

Mitogens are often polyvalent, and at least divalent, and are thus able to crosslink surface receptors. Lectins bind specifically to certain sugars borne by some of the cell surface proteins, the mitogen receptors (Table 2). Mitogenic stimulation results first in changes in the flux of monovalent cations and protein phosphorylation, accelerated protein synthesis, increased DNA synthesis, blast transformation, and finally division of lymphocytes. Many of the B cell mitogens induce differen-

tiation of lymphocytes, which results in the synthesis and secretion of polyclonal antibodies (Ashman 1984; Callard 1988; Klein 1990).

Polyclonal B-cell activators can be divided into T-cell dependent, e.g. pokeweed mitogen (PWM), and T-cell independent mitogens, e.g. *Staphylococcus aureus* bacteria and Epstein-Barr virus (EBV). When B cells are stimulated with T cell-independent agents the activation is T cell-independent, but all polyclonal cell activators, with the exception of EBV, require T cells or T cell-derived factors for the generation of Ig-secreting cells in man. When different T cell-dependent mitogens are used the polyclonal activation is not subject to the same degree of influence by T cell regulation (Waldmann & Broder 1982; Jelinek & Lipsky 1987).

2.2.3.1 Pokeweed mitogen (PWM)

PWM induces the synthesis of all major isotypes IgA, IgG and IgM, but not IgE. The four subclasses of IgG are produced in approximately the same proportions as found in serum. The commercial PWM which is isolated from the roots of *Phytolacca americana*, is a mixture of several mitogens components (Ashman 1984).

Activation of B-cell differentiation by PWM requires the presence of helper T cells, accessory cells and, direct cell interactions between B cells and helper T cells. In later stages of differentiation T cells can be replaced by a supernatant from mitogen-stimulated peripheral blood cells (conditioned medium), but not by IL-2 alone (Ceuppens & Stevens 1986). PWM-induced Ig synthesis in human system is a multistep process: induction of IL-2, release of factors from $CD4^+$-cells inducing B-cell differentiation, and further B-cell differentiation by T cell-derived helper factors resulting in the secretion of Ig in the culture supernatant (Miedema & Melief 1985; Miedema et al. 1985a). IL-2 plays a critical role and the presence of IL-2 has been shown to be essential from the initial stage of B-cell activation through the end stage of B-cell differentiation (Ceuppens & Stevens 1986; Nakagava et al. 1987).

PWM does not activate the whole B-cell population. The majority of PWM-responsive cells isolated from discontinuous density gradients are large, low-density B cells in the activated stage. PWM-responsive cells have been shown to be $sIgD^-$ cells; B cells secreting IgA or IgG come from

IgM^- precursors, whereas $sIgM^+$-cells mature almost exclusively into IgM-secreting cells. *In vitro* responses to PWM correlate with the expression of CD20 and CD21 surface antigens (Jelinek & Lipsky 1987; Callard 1988).

The mitogenic effect of PWM is not restricted to B cells and it stimulates T lymphocytes as well (Miedema et al. 1985a; Serke et al. 1987). PWM appears to induce generation of suppressor T cells that are able to inhibit the maturation of B cells. Suppression is mediated mainly via $CD8^+$ T cells, but the precursors have to be activated by suppressor inducers found among the $CD4^+$ T cells (Jones 1983; Jones 1985; Nilsson et al. 1986b; Abo et al. 1987; Suzuki & Sakane 1988; O'Gorman & Oger 1989). IL-1 released by MCs is required to support the differentiation of B cells, which is clearly demonstrated by removing MCs from the cultures (de Vries et al. 1979; Rosenberg & Lipsky 1979; Waldmann & Broder 1982).

2.2.3.2 *Staphylococcus aureus* Cowan I (SAC)

Formaldehyde and heat treated *Staphylococcus aureus* Cowan I bacteria (SAC) contain large quantities of protein A. Protein A, a cell wall component of most strains of *Staphylococcus aureus*, binds to the Fab- as well as the Fc-portion of human Ig. SAC binds to and crosslinks surface Ig with a resultant activation signal mimicking the activation by antigen. The signal provided by SAC not only delivers the initial activation but also gives sufficient stimulus for the progression of B cells into the S-phase (Kehrl et al. 1984; Jelinek & Lipsky 1987). T cell-derived lymphokines play only a minimal role in the activation and there is no obligatory need for lymphokines during the initial B-cell activation, although the lymphokines can facilitate the response when the activation is suboptimal (Waldmann & Broder 1982; Ito & Lawton 1984; Kehrl et al. 1984). Soluble factors are involved in B-cell proliferation induced by SAC, and B cells have been found to produce factors which increase their proliferation in an autocrine fashion. Analysis of the responding cell populations has revealed that both subsets, IgD^- and IgD^+,respond to SAC and T-cell factors and differentiate into approximately equal numbers of Ig-secreting cells (Jelinek & Lipsky 1987; Callard 1988).

Although the activation is T cell independent, no differentiation into Ig-secreting cells of B cells activated by SAC occurs in the absence of T cells or factors produced by T cells. IL-2 induces the proliferation as well as the differentiation of B lymphocytes in cultures of tonsillar cells (Pryjma et al. 1986; Punnonen & Viljanen 1989). IFN-γ has no effect on non-preactivated B cells *in vitro*, but it enhances proliferation induced by SAC (Peters et al. 1986). IL-4 suppresses B-cell responsiveness and it has been suggested that the primary role of IL-4, when added to SAC-activated cultures of purified B cells in the form of recombinant IL-4, may be regulating rather than enhancing (Jelinek & Lipsky 1988).

Stimulation of peripheral blood B cells with SAC and T-cell help results in the production of IgA, IgG, and IgM. In contrast to PWM, SAC does not activate suppressor T cells *in vitro*, but the Ig response induced by SAC can be inhibited by suppressor T cells (Pryjma et al. 1980; Pryjma et al. 1986).

2.2.3.3 Epstein-Barr virus (EBV)

Few agents have convincingly been shown to induce the differentiation of Ig-secreting cells in man without T-cell influences. EBV is the only stimulant found not to require T cells or T cell-derived factors for the generation of Ig-secreting cells, although some mitogens can activate B-cell proliferation independently of T cells (Waldmann & Broder 1982; Tosato & Blaese 1985).

The mitogenic response follows the binding of the virus to the EBV receptor on the surface of the cell. The receptor has been identified as the CD21 surface antigen (Callard 1988; Clark & Lane 1991). The result of activation gives rise to Ig synthesis with the distribution of isotypes not expected from serum levels. In cultures of human blood cells EBV (line B95-8 supernatant) induces synthesis of all major Ig classes, except IgE, and there is usually a predominance of IgM and an excess of IgG_3 (Jelinek & Lipsky 1987; Callard 1988).

EBV activates only a portion of the B cells; only 1-2 % of circulating surface Ig-positive B cells can be induced to secrete Ig (Tosato & Blaese 1985). In a study of Chan et al. (1986) B cells responding to EBV could be divided into three subsets according to size and expression of surface Ig-isotype. Large $sIgM/D^+$ B cells gave rise to IgM-secreting cells, small

sIgM/D$^-$ -sIgG$^+$ or sIgA$^+$ B cells gave rise to IgG- or IgA-secreting cells, and a minor population of sIgM$^+$/D$^-$ cells gave rise to IgG- or IgA-secreting cells.

Although B cells are activated and matured into Ig-secreting cells without the contribution of T cells, the Ig synthesis is subject to T-cell regulation *in vitro*. T cells from patients with infectious mononucleosis can suppress EBV-induced B-cell differentiation in cultures (Tosato et al. 1979). Also, after infection with EBV *in vitro* of peripheral MNCs from subjects with prior contact with the virus, immunoregulatory T cells are generated that inhibit the Ig secretion of activated autologous B cells in a "late suppression" fashion (Tosato et al. 1982).

In addition to a polyclonal activation of B-cells, EBV transforms B lymphocytes to immortalized lymphoblastoid lines that can be propagated for years. The induction of indefinite cellular proliferation by EBV is termed "transformation", "immortalization" or "outgrowth". The relation between the mitogenic response and the transformation event is not clear, but they can occur separately and thus are not necessarily linked to each other (Tosato & Blaese 1985). The frequency of circulating B cells that can be induced to produce Ig *in vitro* with EBV is significantly higher than the frequency of immortalizable cells (Crain et al. 1989).

2.2.3.4 Other polyclonal activators

Phytohemagglutinin (PHA) is a lectin of *Phaeseolus vulgaris*, the red kidney bean. The binding of PHA on the cell surfaces of B and T cells is roughly similar, but mitogenesis occurs mainly, though not exclusively, in T lymphocytes. Concanavalin A (Con A) which is derived from the jack bean *Canavalis ensiformis* consists of tetramer subunits; in solution Con A is mitogenic for T lymphocytes. Con A induces the development of cells able to suppress blastogenic response and maturation of B cells into Ig-secreting cells (Ashman 1984; Klein 1990).

Lipopolysaccharides (LPS) derived from the cell wall of various strains of *Escherichia coli* or *Brucella abortus* activates mouse B cells, but LPS is only a weak activator of human cells (Waldmann& Broder 1982). A great number of agents of nonmicrobial origin activates lym-

phocytes, e.g. antibodies, dextran sulphate, heavy metals, proteases, ionophores, and wheat germ agglutinin, a nonmitogenic lectin (Ashman 1984).

Table 2. Mitogenic lymphocyte lectins (Klein 1990)

Mitogen	Sugar specificity	Receptor expression on cells	
		B	T
PWM	di-acetylchitobiose	+	+
PHA	N-acetyl-D-galactosamine	(+)	+
Con A	α-D-mannose, α-D-glucose	–	+

2.2.4 Assays of immunoglobulin synthesis

A variety of techniques have been developed to study polyclonal activation of human B cells. These techniques include methods to measure B-cell proliferation and those to quantitate Ig production. One must remember that proliferation *per se* does not measure the terminal differentiation of B cells into secreting plasma cells. Three main procedures to quantitate synthesis of Ig include the determination of cells containing Ig, enumeration of Ig-secreting cells, and measurement of Ig concentration.

Immunoglobulins in the cytoplasm of cultured MNCs have usually been demonstrated by fluorochrome labelled anti-Ig antibodies. The proportion of cells with cytoplasmic Ig at the end of culture period is then calculated. This technique appears to suffer from low sensitivity and a high risk of false positive results, which is due to the highly subjective assessment technique (Wangel et al. 1984).

The number of Ig-secreting cells can be counted by diluting the cells in an environment in which the Ig formed by each individual cells can be assessed. One of the most common methods, the hemolytic plaque technique, was developed by Jerne and Nordin (1963). In the original version of the technique, cells in a chamber from animals immunized with sheep erythrocytes are suspended together with an excess of sheep

red blood cells and complement. On incubation the complement together with secreted antibody against sheep red cells causes lysis of the erythrocytes surrounding the secreting cells. Direct plaques obtained in this way measure essentially IgM production because the IgM isotype is highly hemolytic. The plaque technique has given rise to a number of modifications of which the reverse hemolytic plaque assay (RHP) has become a standard method for the study of human B cell functions (Gronowicz et al. 1976; Lanzavecchia et al. 1979; Freijd & Kunori 1980; Oudenaren van et al. 1981). In the RHP assay staphylococcal protein A-coated sheep red cells are used as the indicator cells. The lymphocytes to be tested are mixed with indicator cells and, in most assays, the mixture is then incorporated in an agar layer or incubated as a fluid in a flat chamber. After incubation the plaques are developed by adding antiserum against Ig and fresh complement.

Another approach to studying the number of Ig-secreting cells, developed by Czerkinky et al. (1983) and Holt et al. (1984), is enzyme-linked immunospot assay (ELISPOT). Ig-secreting cells are incubated in a dish with a coat of immobilized antigen. The secreted Ig is captured locally and is visualized, after removal of the cells, by treatment with enzyme-labelled anti-Ig antibody. The color is developed during the enzyme reaction by incorporating the substrate in a gel poured over the dish. The colored reaction product has only limited solubility and forms spots which are easily counted. ELISPOT has some advantages over the RHP assay, e.g. there is no need to use of unstable complement.

The quantification of Ig secreted into the culture medium provides yet another approach to study the activation and differentiation of B cells. Quantitation may be performed in several ways based on anti-Ig antibodies. Radioimmunoassay (RIA) (review of method Larsen & Odell 1986), enzyme-linked immunosorbent assay (ELISA) (review of method Voller & Bidwell 1986) are among the most widely used methods. These assays are characterized by high sensitivity and a lack of subjectivity permitting the analysis of even very small concentrations of Ig. On the other hand, these assays cannot be used in the presence of serum or other biological material containing human Ig. In addition, they cannot be used when agents are present which can bind to or catabolize Ig.

2.2.5 Immunoglobulin synthesis in rheumatoid arthritis

Several studies suggest that B lymphocytes are activated in RA. Polyclonal hypergammaglobulinemia and the presence of rheumatoid factors (RFs) and other autoantibodies are characteristic to RA. The etiology of these abnormalities is unknown but they are thought to be the consequence of *in vivo* polyclonal B-cell activation (Pardo & Levinson 1983; Fox & Smith 1986; Keystone et al. 1986). Most of the Ig in serum is normally made in the lymph nodes, bone marrow, and other lymphoid tissue. The serological changes in RA may also reflect the influence of locally activated B and T cell populations in synovium, not only of activated B lymphocytes in the blood circulation. The absolute number, and also the relative proportions, of B lymphocytes in the blood of patients with RA are similar to those in healthy subjects (Burmester et al. 1978). Large numbers of B cells and plasma cells are observed in the inflamed synovium of patients and there is evidence for spontaneous Ig secretion by synovial B cells and high numbers of Ig-secreting peripheral blood cells (al-Balaghi et al. 1982; Youinou et al. 1984). The results of the study by Bell and Pinto (Bell & Pinto 1984) suggest that in RA patients with primarily articular disease have active synthesis of Ig which is limited to the synovial compartment, while patients with extra-articular features have active Ig-producing cells also in the circulation.

The most characteristic serological feature of RA is an increased level of RFs (Carson 1989). Over 80% of patients with RA are seropositive, but RFs appear also in other autoimmune diseases and also after severe infections or after vaccination. RF production may play a physiological role during the secondary immune response, probably enhancing elimination of bacterial, viral, or autologous antigens (Tarkowski et al. 1985). The role of RFs of various isotypes has been subject to a number of studies, but the pathogenic, and especially the etiological, role of RFs remains unclear. Today, intense research is focused on a subpopulation of B cells with the CD5 surface antigen. $CD5^+$ B lymphocytes produce most of the polyreactive antibodies which have anti-self and anti-idiotype reactivity. These antibodies, e.g. RF and anti-ssDNA, are predominantly IgM (Casali et al. 1987; Kipps 1989). Patients with RA have an elevated number of these cells (Lydyard et al. 1987; Plater-Zyberk & Maini 1988; Brennan et al. 1989; Smith & Olson 1990). Whether the autoantibodies produced by these cells are pathogenic is not clear. The autoantibodies produced by $CD5^+$ B cells are polyreactive and are natural antibodies with a low affinity. They are

found also in healthy people, which suggests that these autoantibodies may not be pathogenic (Becker et al. 1990; Raveche 1990). On the other hand, $CD5^+$ B cells of patients with RA have been also shown to produce monoreactive, high affinity autoantibodies (Burastero et al. 1988; Karsh et al. 1989).

Antibodies to native type II collagen are present in the sera and synovial fluid of a majority of patients with RA (Claque & Moore 1984; Morgan et al. 1987). Type II collagen is restricted to a few tissues, such as cartilage, and thus immunity is of relevance in the chronic inflammatory arthritides. A pathogenic role of anti-collagen has been suggested in a subgroup of patients, but anticollagen antibodies may only be a reflection of collagen damage (Pereira et al. 1985).

Antikeratin antibodies (AKA), which bind to the stratum corneum of rat esophagus, are found in the sera of patients with RA (Young et al. 1979; Hajiroussou et al. 1985; Youinou et al. 1985; Hoet et al. 1991). The precence of AKA is suggested to be highly specific for RA (Vincent et al. 1989; Paimela et al. 1992) and recently it has been shown that AKA precede the clinical disease (Kurki et al. 1992). An antibody reacting with cytoplasmic granules in human buccal mucosa cells, the antiperinuclear factor (APF), is also characteristic for RA (Nienhuis & Mandema 1964; Sonntag et al. 1979; Kataaha et al. 1985; Hoet et al. 1991). At present the targets of both APF and AKA are unknown, but in one study it has been suggested that EBV might be the immunogen for APF (Westgeest et al. 1989). Other autoantibodies found in the serum of RA patients include anti-ssDNA (Karsh et al. 1982), antilaminin (Lassoued et al. 1990), and anti-intermediate fiber antibodies (Kataaha et al. 1985).

2.2.6 Immunoglobulin synthesis *in vitro* of cells from rheumatoid arthritis patients

Aberrations of B-cell related functions are evident in patients with RA. These aberrations probably arise as a consequence of defects in the regulation of the immune response (reviewed by Petersen 1988). *In vitro* studies on isolated lymphoid cells from the blood or synovial tissue of patients with RA has been performed to clarify the cellular mechanisms behind the disease. Segond et al. (1979) studied the primary *in vitro* antibody response toward an antigen, TNP-hapten, in cultures of peripheral

blood lymphocytes and found a lowered response in patients as compared to healthy subjects. Poikonen et al. (I) reported low *in vitro* Ig synthesis after polyclonal stimulation by PWM in cultures of MNCs from the blood from RA patients. Several studies have confirmed that IgG and IgM synthesis by these cells is depressed (Panush et al. 1983; Pardo et al. 1984; Heilmann & Petersen 1984; Boling et al. 1987) although normal response to PWM has been reported in one study (Patel et al. 1983). The mechanisms under the reduced responsiveness to polyclonal stimulation of blood cells from patients are unclear. The specificity of secreted Ig has usually not been studied but blood B cells from RA patients are known to secrete also IgM-RF and IgG-RF in response to PWM (Patel et al. 1983; Boling et al. 1987). In addition to blood lymphocytes, the synovial cells also have been reported to respond poorly to PWM (Petersen et al. 1984).

Among the most striking immunoregulatory defects in RA are the inability of patients' lymphocytes to control EBV-induced Ig secretion (Tosato et al. 1981; Irving et al. 1985) and the impaired inhibition of proliferation of EBV-infected cells (Bardwick et al. 1980; Depper et al. 1981; Gaston et al. 1986; McChesney & Bankhurst 1986). The relation of these two phenomena is not clear. Lymphocytes from healthy donors produce increasing number of Ig-secreting cells for 10-14 days, after which a marked suppression occurs; such suppression is absent in patients with RA (Irving et al. 1985; Tosato et al. 1981). Kahan et al. (1985) have shown that the defect of T cell regulation of EBV-induced Ig synthesis is present only in certain subgroup of RA patients . This defect appears not to be characteristic to RA only but is present also in other autoimmune diseases (Shore et al. 1989). The defective regulation of lymphoblast transformation in patients with RA may be partly due to diminished generation of IFN-γand low IL-1 and IL-2 production (Hasler et al. 1983; Lotz et al. 1986a; Lotz et al. 1986b).

3 AIMS OF THE STUDY

Since there are multiple immunological abnormalities in RA, it was of interest to study the function of the cells responsible for Ig synthesis. The specific aims were:

1. To determine whether Ig production by lymphocytes from RA patients is different from the production by lymphocytes of healthy subjects.

2. When it was found to be defective, to characterize the mechanisms for the decreased production of Ig.

3. To elucidate whether the defect is associated with the severity of the disease.

4 MATERIALS AND METHODS

4.1 Patients and control subjects

There were 27 patients in study I. All had rheumatic disorders; 15 patients were suffering from active, definite RA. 18 healthy control subjects were recruited from the laboratory staff. Papers III-IV consisted of 58 patients with newly-diagnozed definite of classical RA meeting the diagnostic criteria of the American Rheumatism Association. The patients in study V were a subgroup of the ones in the study IV (45 patients). Fifty nine healthy members of the hospital and laboratory staff served as controls in papers III-V. These controls were matched as closely as possible for age and sex distribution. A summary of patients data is shown in Table 3.

Table 3. Description of the patients with RA in original papers I and III-V.

Study	No of subjects	Sex; m/f	Age ± SD; years	Mean duration of disease (range); months
I	15	8/7	43 ± 18	
III, IV	58	17/41	48 ± 16	8 (2-24)
V	45	14/31	48 ± 15	12 (2-30)

4.2 Isolation and activation of peripheral mononuclear cells (I-V)

Blood samples were drawn into heparinized tubes and MNCs were separated from venous blood by centrifugation on Ficoll-Isopaque (I, II) or commercial separation medium Ficoll-Paque (III-V). Separated cells were washed with Hanks' balanced salt solution and resuspended in culture medium. The culture medium consisted of RPMI -1640 supplemented with inactivated fetal calf serum, antibiotics, and L-glutamine. Cultures were prepared in round-bottomed plastic tubes in volumes of 1 ml (I, II, V) or in the wells of microtiter plates in volumes of 200 µl (III, IV).

The synthesis of Igs by lymphocytes was induced with polyclonal activators. Pokeweed mitogen (PWM), diluted 1:100 and SAC 0.05%, prepared as described by Forsgren et al. (1976), were used to stimulate the cells (I-IV). Hydrocortisone 10^{-5} mol/l, Con A 4 mg/l, and indomethacin (IND) 1 mg/l were added to cultures in order to modify the regulation of Ig synthesis (III). Cocultures of cells from patients and healthy subjects were set up by mixing equal numbers of the respective cells. After 7 days of culture the ratio observed/expected (O/E) of Ig concentrations was calculated as follows

$$O/E = (C_{patient} + C_{control}) : 2 \times C_{mixed} \ ,$$

where C is the concentration of Igs in the respective cultures of MNC from
the patients and controls. A ratio O/E>1 would indicate poor helper T cell function of patient cells and a ratio O/E<1 would indicate activation of suppressor cells (Jones 1981) (III).

Filtered supernatant, 20% of the volume of culture medium, from the marmoset cell line B95-8 containing EBV was added to cultures to induce Ig synthesis (III, V).

The production of IL-2 by the MNCs was studied by stimulating the cells with PHA diluted 1:100 (III).

Table 4. summarizes the agents added to the culture, their mode of action, and the expected effect on Ig synthesis.

Table 4. Summary of polyclonal stimulants of Ig synthesis of blood MNC cultures, and agents added into activated cultures to modify responses.

Agent	Mode of action	Expected effect on Ig synthesis	Reference
PWM	Polyclonal activation of B cells; activation and differentiation are T-cell- and MC-dependent	Induction of Ig synthesis	Waldmann& Broder 1982; Jelinek& Lipsky 1987
SAC	Polyclonal activation of B cells; activation is T-cell independent, differentiation is under T-cell regulation	Induction of Ig synthesis	
EBV	Polyclonal activation of B cells; activation and differentiation are T-cell independent (T-cell control of the growth of B cells in immune subjects)	Induction of Ig synthesis	
ConA	Activation of certain T suppressor precursors	PWM-system: decreased SAC-system: increased	Pryjma et al. 1986b
HC	Inhibition of $CD8^+$ lymphocytes	PWM-system: increased SAC-system: no effect	Fauci et al. 1977; Paavonen 1985
IND	Inhibition of PGE_2 synthesis by MC	Increased in RA	Goodwin et al. 1979
2-ME	Reduction of MC cell surface -SH groups	Increased in RA	McKeown et al. 1984
PWM-activated cocultures (mixed MNC cultures)	Deficient helper, or increased suppressor function affects the observed/expected ratio of Ig synthesis		Jones 1981

4.3 Hemolytic plaque assay (I, II)

The number of Ig-secreting cells , both in circulation ("spontaneous plaque forming cells") and in cell cultures, was determined by the hemolytic plaque assay and the results were expressed as the number of plaque forming cells (PFC) (I, II). The assay takes advantage of the hemolysis by guinea pig complement of sheep red blood cells coated with rabbit anti-human Igs via protein A attached on red cells. The assay is isotype specific, i.e. cells secreting IgA, IgG or IgM are quantified.

4.4 Determination of immunoglobulin concentration in supernatants of cell cultures (I-V)

The concentration of Igs in the supernatants of cell cultures were measured by the isotype specific, double antibody sandwich ELISA. Flat-bottomed polystyrene microtiter plates were coated with anti-human Ig and saturated with bovine serum albumin. Ig bound from the sample was detected with anti-human Ig conjugated to alkaline phosphatase. After incubation for 1 to 2 hours the enzyme reaction (p-nitrophenylene as the substrate) was stopped and the absorbances were read on a plate reader. The Ig content of the samples was calculated from the curve obtained with standard sera. Modifications of the assay are described in detail in the respective studies.

4.5 Interleukin-2 assay (III, IV)

Cell-free supernatants of cultures stimulated with PHA were analyzed for IL-2 activity by an IL-2 dependent murine cytotoxic cell line CTLL-2 in the National Public Health Institute, Oulu. A batch of samples was analyzed by lymphocytes stimulated with Con A. In both analyses the

results were expressed as the percentage of the activity in the samples from cultures of controls done simultaneously with the patient cultures.

4.6 Clinical and laboratory measures of disease activity (IV, V)

The patients were followed by an experienced rheumatologist (T. Möttönen, Central Hospital, Jyväskylä) for two years by clinical examination at six month intervals. Clinical and laboratory procedures included the collection of data on several measures of which the following were used in these studies: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor: Waaler-Rose (WaRo) titer, joint tenderness: Ritchie's articular index, and clinical rheumatoid activity: Mallya index. The Mallya index incorporates subjective morning stiffness (minutes), subjective pain scale (0-10 cm), grip strength (kp/cm^2), Ritchie's index, hemoglobin (E-Hb g/l) and ESR (mm/h).

4.7 Radiography (IV, V)

Radiographs of the patients´ hands and feet were taken at the beginning of the study and thereafter at six month intervals for up to two years. The number of eroded joints was counted. An overall destruction index, Larsen's index, was calculated by summing up the indices of the individual joints. The indices of the subtalar and wrist joints were corrected using a coefficient of 5.

4.8 Determination of HLA-types (IV, V)

Serologic typing for HLA-alleles was performed in the National Public Health Institute, Oulu, by standard microcytotoxicity testing. Lympho-

cytes for typing were isolated from heparinized venous blood by Ficoll-Paque density gradient centrifugation and by rosetting with 2-aminoethylisothiouronium-treated sheep red cells (Ilonen et al. 1990).

5 RESULTS

5.1 Methods for measuring immunoglobulin production in cell cultures (I, II)

Two methods of measuring Ig synthesis in cultures of peripheral blood MNCs were compared: enumeration of Ig-secreting cells and quantification of the secreted Ig in the supernatant.

Kinetic studies have shown that stimulation with PWM, and also with SAC, increases the number of Ig-secreting cells between days 4 to 6. After that a sharp decrease occurs while the concentration of Ig in supernatant keeps on increasing even after that. To compare the method of measuring of Ig secretion, the cultures of MNCs of healthy subjects were harvested on the day of maximum of PFC and part of the cells was used in the plaque assay and the rest of cells were allowed to secrete Ig in freshly changed medium for 20 hours. The number of PFC and concentration of newly secreted Ig during the incubation correlated well: r=0.88 for IgA and r =0.95 for IgG and IgM (II).

Polyclonally activated cultures are usually harvested on days 6 or 7, and either Ig accumulated in the supernatant or the number of Ig-secreting cells is determined. In study II the number of PFC and the concentration of accumulated Ig in 6 day culture supernatants had a high correlation: r=0.76 for IgA, r=0.86 for IgG, and r=0.93 for IgM.

In study I the synthesis of Ig after PWM stimulation of MNCs from patients with RA and other connective tissue disease (CTD) and from healthy subjects were determined; PFC assay was carried out and the Ig

in culture supernatants was determined. The results show that in healthy subjects as well as in patients with chronic inflammatory disease the two ways of measuring Ig synthesis gave parallel results (I).

On the basis of this comparison it is concluded that the assay of secreted Ig may be used as an alternative to the enumeration of PFC. The use of the concentration as the measure of Ig synthesis has many practical advantages over the PFC assay: problems of hemolysis and batch-to-batch variation of complement and sheep red cells and the processing of them are avoided. In addition to this, the assay is easy to standardize by using standard sera available commercially, the samples can be frozen and analyzed at the same time to overcome variation of analysis, the samples may be reanalyzed, and much larger number of samples can be processed at the same time.

5.2 Mitogen activated immunoglobulin synthesis in rheumatoid arthritis (I,III)

Peripheral blood MNCs from patients with RA or other CTD and from healthy individuals were activated with PWM and cultured for 6 days. The synthesis of IgG and IgM, determined with the PFC assay and with the concentration of Ig in supernatants, was significantly decreased in RA (I: Table 2). The synthesis of IgG and IgM in unstimulated cultures was also lower by the cells from RA patients than by the cells from healthy subjects (I: Fig. 4).

In order to study whether the lymphocytes of patients had been activated *in vivo*, and therefore died during the first days of culture without being able to respond to activation with PWM, the number of PFC of isolated blood MNCs was counted directly without culture. This spontaneous, nonactivated, synthesis of IgA, IgG and IgM was similar in patients and controls (I: Fig. 3).

The impaired production of Ig after PWM activation by cells of patients with RA was confirmed in study III (III: Fig. 1).

5.3 Analysis of impaired immunoglobulin synthesis in patients with rheumatoid arthritis (III, V)

To study the mechanisms of decreased *in vitro* Ig synthesis by lymphocytes of patients with RA, a group of 58 patients with recent onset RA were examined (III).

5.3.1 Immunoglobulin synthesis in cultures activated with polyclonal stimulants

Two polyclonal stimulants of Ig synthesis were used: PWM, a MC- and T cell-dependent mitogen, and SAC which activates B cells without T-cell help, but the response is regulated by T cells. The Ig synthesis was first assayed at the time when the patients were admitted to the hospital. The MNCs of patients with RA had a decreased response in a similar way as when activated either with PWM or SAC. Unstimulated cells from RA patients synthesized equal amounts of Ig as controls. The deficiency of Ig synthesis was confirmed six months later when the responses were reassayed (III: Fig. 1).

5.3.2 Epstein-Barr virus -induced immunoglobulin synthesis

Epstein-Barr virus, a T-cell independent B cell activator, was used in seven day cultures to induce Ig synthesis (V). There was no significant difference (t-test) between the concentration of IgA, IgG and IgM in the supernatants of patient and control cultures, suggesting that the B cells of patients retain their normal function (Table 5.).

Table 5. The EBV-induced synthesis of Igs in 7 day cultures of MNCs of patients with RA and healthy controls.

Group	n	IgA	IgG	IgM
RA	43	187±266	527±532	662±1552
Controls	49	185±257	432±494	750±1260

The results are expressed as the mean concentration (μg/l) ± SD.

5.3.3 Suppressor and helper functions

Hydrocortisone (HC), when added to PWM-activated cultures of MNC, causes an enhanced Ig synthesis by inhibition of suppressor T cell activity. The increase of IgA, IgG and IgM was similar in cultures of cells from patients and controls. The decreased Ig synthesis of RA cells was not restored to the level of controls by the addition of HC as expected in the case of excessive activity of suppressor T cells in RA. In SAC-induced cultures, in contrast to PWM, HC did not enhance Ig synthesis by control cells and the production by the cells of patients was decreased (III: Fig. 2).

Concanavalin A is reported to stimulate precursors of suppressor T cells which then maturate into effectors by PWM and suppression of Ig syn-thesis occurs. No statistically significant differences were noted between patient and control cultures activated simultaneously with both PWM and Con A. In SAC-stimulated cultures Con A induced increased Ig synthesis by control cells. This increase was weaker in cultures of RA cells and completely absent when assayed at 6 months (III: Fig. 3).

Cells from patients and control subjects were mixed and cultured together (coculture) in the presence of PWM. The concentration of Ig in the cocultures was compared with the concentrations in culture of cells from each donor separately (as explained in the section: Material and methods of the present study). The ratio O/E (observed/expected) of cultures was calculated. O/E values at onset varied from 0.96 to 1.06 depending on the isotype analyzed, and from 1.12 to 1.22 when analysed at 6 months. The results showed no activation of suppressors but suggested impaired helper activity of T cells in RA (III: Table 1).

5.3.4 Interleukin-2 production

The ability of lymphocytes to secrete IL-2 was investigated by stimulating the cells in cultures with PHA. Interleukin-2 is a lymphokine which is produced principally by helper/inducer T cell subset and is essential for sequential release of other mediators of B-cell differentiation. Cells of patients with RA produced smaller amounts of IL-2 activity compared to the cells from healthy subjects, 85 % at onset and 42% at six months (significantly different from controls, $p<0.01$) of the mean synthesis by the cells of controls (III: Table 2).

5.3.5 Monocyte functions in cultures

Addition of indomethacin (IND), an inhibitor of prostaglandin-E_2 (PGE_2), affected similarly the Ig synthesis by the cells of patients and controls. In cultures of MNCs the MCs are an important source of prostaglandins, especially PGE_2, which contribute to Ig synthesis. The results suggest that the reduction of Ig synthesis by patient cells was not due to altered prostaglandin metabolism of MCs.

2-mercaptoethanol (2-ME) is a reducing agent which modifies sulphydryl (SH) groups on the surface of MCs. In this study 2-ME re-stored neither the low responses to PWM nor to SAC-stimulation (III). Restoring of the synthesis of Ig would be expected if sulphydryl oxidation of the surface of monocytes were the cause of the low responsiveness of cells of RA patients.

The results of the experiments to examine the mechanisms of low Ig responses of cells from the patients with RA are summarized in the Table 6.

Table 6. Tests for *in vitro* function of B cells, T cells and MCs from the blood of patients with RA.

Measure	Stimulant	Result; RA vs. control	Interpretation
Ig-synthesis	EBV	No difference	B-cell function normal
	PWM + HC	No difference	No excessive suppressor activity
	PWM + ConA	No difference	No excessive suppressor activity
	PWM in coculture	O/E values ≥ 1	Deficient help
	PWM + IND	No difference	MC-function normal
	PWM + 2-ME	No effect	MC-function normal
IL-2	PHA	Low synthesis in RA	May contribute to poor helper function

5.4 Association between mitogen-activated immunoglobulin synthesis and severity of disease (IV)

5.4.1 Clinical examination

Patients were followed by medical examination, radiography, and laboratory measurements for two years at six month intervals. The development of erosions of joints was selected as a long term estimate of severity because joint destruction is irreversible. The development of erosions

after the two year follow-up period was used as the basis for grouping the patients (IV).

5.4.2. IgM synthesis in patients with erosive and nonerosive disease

PWM-activated MNCs from patients with erosive disease synthesized lower amounts of IgM than the cells from patients without eroded joints. The difference between patient groups was statistically significant both at the onset of disease and after six months of treatment (IV: Fig. 1). Similarly, IgM synthesis in cultures of RA cells stimulated with SAC was decreased in patients with erosive disease compared to nonerosive disease (IV: Fig. 2). The association between production of Ig and the development of erosions was also analysed with regard to the costimulation experiments with SAC and Con A. The synthesis of IgM by the cells from patients with erosive RA was decreased compared to the noneroded group (IV: Fig. 3).

5.4.3 Interleukin-2 synthesis

At six months the PHA-induced release of IL-2 of MNC of patients with erosive disease was significantly decreased compared to the cells of patients with nonerosive disease. PHA-induced release of IL-2 by the cells of patients with eroded joints was 36% (±10%, n=32), and by the cells from nonerosive disease 55% (±20%, n=15) of the value of the cells from control subjects.The difference between the patient groups in terms of IL-2 synthesis was significant ($p<0.05$)(IV: Table 3).

5.5 Epstein-Barr virus-induced immunoglobulin synthesis in long term cultures

Cells of 45 patients with RA of recent (16 untreated and 29 treated for six months) and of 41 healthy control subjects were cultured for four weeks in the presence of EBV. Supernatants of cultures were harvested weekly and replaced with fresh medium without virus, and the Ig concentrations were assayed (V).

EBV-induced synthesis of all Ig isotypes, IgA, IgG, and IgM. The Ig synthesis of the controls reached maximum during the second week of culture and after that the concentrations in the supernatants decreased. On average, patients exhibited "lack of late suppression": the synthesis after the second week of culture kept on increasing. The IgM concentration in cultures of MNC from RA patients was highest at the fourth week, but the production of IgA and IgG was moderately decreased (V: Fig. 1).

5.6 Association between Epstein-Barr virus-induced immunoglobulin synthesis and severity of RA (V)

Because the EBV-driven synthesis by the MNC of RA patients was deviant as compared to controls, it was of interest to study whether the abnormal responses had any association with the severity of disease. Therefore, the patients were followed with clinical, laboratory and radiological examination for two years and the Ig synthesis was correlated with the activity and the outcome of the disease.

5.6.1 Immunoglobulin synthesis by patients with erosive and nonerosive disease

The patients were grouped on the basis of appearance of erosions after the two year follow-up; the Ig synthesis, early in the disease, of these groups were compared. Ig synthesis of the MNCs of controls and the

noneroded patients did not differ. In contrast, the concentration of Ig in cultures during the third and fourth week by patients with erosive disease was significantly raised (V: Fig. 2).

5.6.2 Association between Epstein-Barr virus-induced immunoglobulin synthesis and disease activity

To gain insight into the association between the disease and EBV-induced Ig synthesis, the patients were grouped according to the level of IgM synthesis during the fourth week of culture: normal or high responders. Synthesis was regarded as "high" if the concentration during the fourth week exceeded the mean synthesis of Ig by control cells by more than 2SD, otherwise as "normal". High EBV-induced IgM synthesis was associated with variables of articular involvement (development of erosions, the number of eroded joints and Larsen's polyarticular index) assessed at two years (V: Table 2). The difference between ESR and the Mallya index of patients with high IgM synthesis (mean ESR 30.9 mm/h, mean Mallya index 2.4) and normal IgM synthesis (mean ESR 16.5 mm/h, mean Mallya index 1,9) was statistically significant ($p<0.05$). Other measures of disease activity (CRP, WaRo-titer and Ritchie index) were also associated with IgM synthesis but the difference was not sta-tistically significant (V: Table 2).

5.6.3 Association between development of erosions and high immunoglobulin synthesis (V)

Immunoglobulin synthesis during the fourth week of culture was used to group patients to high responders and normal responders, as described above, and Ig synthesis early in the disease was used to predict the development of erosions two years later. By using high Ig synthesis as a test it was possible to identify a subgroup of patients with erosive disease. The recognition was highly specific (specificity 93.9%-100.0%)

and had a high positive predictive value: PV_{pos} 90.0% for IgA, 100.0% for IgG, and 93.3% for IgM. Sensitivity of the test ranged depending on isotype from 30.0% (IgA and IgG) to 46.7% (IgM) (V: Table 4)

6 DISCUSSION

6.1 Regulation of mitogen-induced immunoglobulin synthesis

In study I the PWM-induced Ig synthesis by MNCs of the blood of RA patients was decreased. This finding has been later confirmed by several studies, by us (III) and others (Panush et al. 1983; Heilmann & Petersen 1984; Pardo et al. 1984; Boling et al. 1987). The mechanisms of the decreased responses in *in vitro* cultures can be due to any cells in the culture: B lymphocytes, T cells, and MCs.

The function of B cells were assayed by stimulating seven day cultures with EBV, a T-cell independent B-cell activator (III, V). No difference between patients and controls was observed indicating normal function of B cells. This stands in contrast to the data of Irving et al. (1985), who found lower rates of IgM synthesis by RA lymphocytes compared to controls. However, normal function of B lymphocytes in RA has been reported also by others (Segond et al. 1979; Fawcett et al. 1988).

T cells can cause the low response because the activation and differentiation of B cells is under the control of T helper and T suppressor lymphocytes. The presence of a high number of active suppressors or, on the other hand , activation of precursors of suppressor cells in the culture may cause reduced Ig synthesis. PWM is known to activate $CD8^+$ suppressor cells and/or $CD4^+$ suppressor inducer cells (Nilsson et al. 1986b) and thus it is not possible to separate the function of existing effector suppressor T cells and the effect of suppressors induced by PWM

from their precursors. It has been shown that the regulation by T cells of the response in SAC-induced cultures is different from PWM-induced cultures: activation by SAC of B cells is T-cell independent and SAC does not activate suppressor T cells (Pryjma et al. 1980; Pryjma et al. 1986). Despite differences in mechanisms both stimulants show similar decreased Ig synthesis. Supplementation of cultures with HC to remove the effect of suppressor cells, mainly $CD8^+$ cells (Haynes & Fauci 1979; Paavonen 1985), and cocultures of patient and control cells demonstrated that excessive activity of suppressor cells in RA was not the reason for deficient Ig synthesis. This is supported by the results of Nilson et al (1986a), who reported that $CD8^+$ lymphocytes from the blood and synovium of patients with RA had the same degree of suppressor activity as the cells from healthy subjects.

Monocytes are important producers of PGE_2. PGs can exert various effects on T-cell and B-cell functions, e.g. PGE_2 inhibits the generation of Ig-secreting cells in response to SAC (Goodwin & Webb 1980; Ceuppens & Goodwin 1982; Staite & Panayi 1984). Indomethacin, an inhibitor of PG synthesis, had similar effects on Ig synthesis of RA and control cultures activated with PWM and SAC indicating that prostaglandin metabolism of monocytes from RA patients is not the reason of abnormal Ig synthesis. Further, addition of 2-ME into cultures activated with PWM and SAC failed to restore impaired Ig synthesis. McKeon et al. (1984) reported that the accessory cell function of peripheral blood MCs is dependent on free SH-groups on the cell surface, and that SH-dependent mechanisms are responsible for depressed MC-functions of cells from patients with RA. In the present study no enhancing effect by 2-ME in cultures of MNCs from RA patients was noted. The reason of the disagreement with the study of McKeon et al. (1984) is not clear. The experiments of study III show that MCs are not to blame for the low mitogen-induced Ig response by MNCs in RA.

Poor help from the T cells, e.g. an altered production of factors of B-cell activation and maturation, may contribute to low Ig synthesis. This contention is supported by several pieces of evidence. First, cocultures of cells from RA patients and controls showed a higher than 1.0 index of observed/expected ratio of Ig synthesis indicating poor help of RA T cells (Jones 1981). Second, an indication of deficient help is reduced IL-2 secretion by MNCs from RA patients. IL-2 is produced mainly by $CD4^+$ T lymphocytes (Moretta 1985). In both, PWM- and SAC-induced cultures, IL-2 is an absolute requirement for B-cell differentiation into Ig-secreting cell (Miedema et al. 1985a-b; Punnonen & Viljanen 1989). The

physiological role of IL-2 in the regulation of B-cell function is somewhat controversial but IL-2 in man is the major cytokine that promotes both growth and differentiation of B cells (Lipsky 1989; Lipsky et al. 1989; Punnonen & Viljanen 1989). Finally, when Con A was added to cultures stimulated with SAC, cells from controls increased their Ig synthesis, while cells from RA patients responded by decreased synthesis compared to cultures without Con A. This may also be explained by a low production of IL-2 because Con A induces secretion of IL-2 and other cytokines (Pryjma et al. 1980; Oben et al. 1989).

It may also be relevant that a specific loss of the T cell suppressor-inducer subset $CD4^+CD45R^+$ (detected by the antibody 2H4) occurs in RA (Emery et al. 1987). These cells are believed to be the main IL-2-producing subset among $CD4^+$ T cells after activation by mitogens (Salmon et al. 1988). Besides low production of cytokines by RA lymphocytes in culture, deficient responsiveness of patient cells to mediators could also explain the decreased Ig responses. Our experiments do not provide data for analyzing these possibilities.

The most probable mechanism of decreased Ig synthesis by the MNCs from RA patients is thus poor helper function of T cells. This deficient regulation may be mediated via disturbed cytokine elaboration, where IL-2 plays the central role. Several IL-2-related disturbances of RA lymphocytes have been reported (Allison et al. 1988; Lipsky et al. 1989). IL-2 synthesis by RA T cells after mitogen induction has been found decreased compared to synthesis by the cells of healthy subjects (Combe et al. 1987; Nouri & Panayi 1987; Waalen et al. 1987; Kitas et al. 1988; Bernier et al. 1989). Furthermore, the presence of soluble IL-2 receptors and IL-2 inhibitors in the serum of patients has been reported (Kashiwado et al. 1987; Keystone et al. 1988; Symons et al. 1988; Smith & Roberts-Thomson 1989). The expression of IL-2 receptor on RA lymphocytes has been reported decreased (Emery et al. 1988) or increased (Vaisberg & Scheinberg 1990).

Drugs commonly used in treatment of patients with RA affect the *in vitro* synthesis of Ig; the observed low responsiveness by mitogen activation might also be due to medication. The patients in the study were assayed for their polyclonal Ig synthesis before any disease modifying anti-rheumatic drugs (DMARD) were administered at the onset of clinical disease, and again six months later when the patients had been treated. The responses of the patients were reduced already before DMARD were started and thus the treatment as the cause of low Ig synthesis is unlikely. This is supported by the results of Boling et al. (1987);

patients who had been without remittive drugs for 4 weeks showed similar deficient responses to polyclonal stimulants as in the present study. However, the use of nonsteroidal anti-inflammatory drugs (NSAID) during the early stages of the disease might have altered the Ig pro-duction *in vitro*. This is not likely because data collected during the dis-ease showed that patients not receiving drugs, including NSAID, did not produce higher amounts of Ig, and patients receiving NSAID along with other medication responded similarly to those not receiving NSAID.

6.2 Epstein-Barr virus-induced Ig synthesis

During the second week of culture the Ig concentration in cultures of RA and control MNCs increased and after two weeks the level of Ig in control cultures began to decrease. Ig synthesis in RA cultures increased dramatically during the third week; during the fourth week of culture the synthesis of IgM by RA cells still kept on increasing, while the elaboration of IgM by controls was almost undetectable (V). This "lack of late suppression" of Ig synthesis in RA has been demonstrated earlier by PFC (Tosato et al. 1981) and by measuring Igs in culture medium (Irving et al. 1985). EBV, strain B-95-8, infects specifically human B lymphocytes, and infection is followed by a polyclonal activation of these cells. Later during the culture T-cell mediated suppression of Ig synthesis may occur (Tosato & Blaese 1985). The turning off of Ig synthesis is believed to be due to induction of T suppressor cells which specifically affect EBV-infected B cells. The mechanism of this defect in RA was not studied here but it has been suggested that the suppression is mediated by impaired IL-1-dependent synthesis of INF-γand IL-2 (Lotz et al. 1986a, 1986b).

Another defect of the function of lymphoid cells in RA with regard to EBV is the inability of these cells to control EBV-induced B-cell outgrowth, as measured by regression of long term cultures (Depper et al. 1981; McChesney & Bankhurst 1986). EBV-specific cytotoxic T cells develop in cultures from normal individuals and these cells mediate the regression. It is unclear whether the absence of these cells in RA, or a deficient function of them, may be related to inability of RA mononuclear cells to turn off EBV-induced Ig synthesis.

Some of the patients in the present study were treated for six months with DMARD, and this might have affected on the EBV-induced responses *in vitro*. Responses of patients receiving drugs, however, were similar to the responses of patients who had not received medication. Irving et al. (1985) also reported that drug treatment did not affect EBV-induced IgM synthesis.

6.3 Association between immunoglobulin synthesis and disease activity and prediction of outcome

In study IV the relationship between mitogen-stimulated Ig responses of blood MNC in RA patients and the clinical manifestations of RA was investigated. The main finding was that the patients who later in the course of the disease have severe joint damage have low *in vitro* IgM responses to polyclonal stimulation already at the beginning of the clinical disease.There are only a few studies concerning disease activity and mitogen activated *in vitro* Ig-responses in RA. Association between *in vitro* responses and disease activity, such as ESR or morning stiffness, or titer of RFs, have been studied but the studies have not included a sufficiently long follow-up and evaluation of prognostic value (Panush et al. 1983; Pardo et al. 1984).

The association of the immune defects, which appear as impaired mitogen-induced IgM synthesis, mainly with erosions and not significantly with other parameters of disease activity might seem to be coincidental. However, repeated tests after six months' interval demonstrated the same association. This association suggests that the patients with underlying immunological disturbances may be destined to develop erosive disease despite the fluctuations of inflammatory activity. The development of erosions may be considered to be as a cumulative, and the currently most reliable measure of disease progression, while the other measures of disease activity fluctuate with drug treatment and time (van der Heijde et al. 1988).

Only low IgM production is clearly associated with the severity of the disease although a decreased production of IgA and IgG were noted. It may be of importance that a subpopulation of B cells, $CD5^+$ B cells, which have been implicated in RA and other autoimmune diseases,

produce IgM and antibodies of the IgM isotype (Casali et al. 1987; Lydyard et al. 1987; Karsh et al. 1989; Kipps 1989).

Low PHA-induced IL-2 production by peripheral MNCs was also associated with the erosive form of the disease. Mitogen-stimulated IL-2 release by blood lymphocytes of RA patients has been reported to decrease (Combe et al. 1987; Kitas et al. 1988; Smith & Roberts-Thomson 1989), to be normal (Lemm & Warnatz 1986; Nouri & Panayi 1987), or to increase (McKenna et al. 1986; Wolf & Hall 1987). Reports on the relationship between the activity of the disease and the synthesis of IL-2 generally suggest that there is a reduction of IL-2 production in active disease (Nouri & Panayi 1987; Kitas et al. 1988; Bernier et al. 1989), but there are also conflicting reports (McKenna et al. 1986; Combe et al. 1987). The status of IL-2 related effects in RA remains a matter of controversy, confounded by the possible adsorption of IL-2 by lymphocyte receptors and the presence of inhibitors of IL-2 production and activity, especially within synovial fluid (review Lipsky et al. 1989)

Enhanced EBV-induced production of Ig by MNCs of patients with RA is associated with subsequent severe disease and predicts the development of erosions in RA (V). The response to EBV may reflect a generalized immunological disturbance in patients with RA, which is in line with the observation that the group of patients with the most severe disease exhibit increased responses. It is highly unlikely that the abnormalities in controlling the response to EBV could be the due to drug treatment, as there were no differences between the untreated patients and patients receiving DMARD.

An alternative explanation would suggest an etiologic role for EBV in about one third of RA patients. EBV has been linked to RA because of several immunological abnormalities in host responses against the virus (see Review of literature). The results of study V provide more evidence connecting EBV with RA.

Kahan et al. (1985) reported that EBV-related immunoregulatory defects do not exist in all patients with RA: about half of their patients had similar suppressor functions of Ig synthesis as healthy controls and the rest of patients had defective suppressor function. This is in agreement with the present results and strengthens the notion that this defect is not typical of all patients with RA. The defect in handling EBV is not specific to RA and has been reported also in other autoimmune diseases (Shore et al. 1989).

Several studies have shown that the development of erosions, the strongest indicator of an unfavourable outcome, takes place early in the course of RA. Almost 90% of the erosions evolve within two years of the onset of symptoms (Eberhardt et al. 1990a; Wolfe 1991). However, it is difficult to identify patients who will develop severe erosive disease. The associations of various biochemical or immunological parameters with severe disease are weak, controversial, or unconfirmed (van der Heijde et al. 1988). Perhaps in the future the Stanford Health Assessment Questionnaire or the Arthritis Impact Measurement Scales offer better means of predicting the outcome of RA (Bellamy 1991; Wolfe 1991). At present, however, there is a need for a test which can be used early during the disease to select the patients, who would benefit from aggressive anti-rheumatic therapy (Katz 1990). Based on the methodology described in studies IV and V, it might be possible to develop *in vitro* tests which could identify with a high probability the patients who will have a severe form of disease.

7 SUMMARY AND CONCLUSIONS

Rheumatoid arthritis is a systemic chronic inflammatory disease characterized by several immunological abnormalities, e.g. local or systemic B-cell hyperactivity as evidenced by hypergammaglobulinemia and wide spectrum of autoantibodies. B cells are controlled by other lymphoid and nonlymphoid cells as well as mediators released by them. In the present *in vitro* study, the B-cell responses in terms of Ig production to polyclonal stimulators were examined in blood MNCs from patients with RA and controls. In addition, the regulation of the responses and the association to the disease severity were analyzed. The results showed that:

1. Ig synthesis induced by mitogenic polyclonal activators was significantly reduced in RA as compared to healthy subjects.
2. Reduced responses were due to poor help from T lymphocytes of B-cell maturation. This effect may be mediated by low IL-2 production. The function of B cells and monocytes was normal, and there was no excessive suppressor cell activity in cultures of cells from patients with RA.
3. Low IgM synthesis, detectable already at the onset of the disease, was associated with joint destruction two years later. Furthermore, PHA-induced IL-2 synthesis by the cells of patients with erosive disease was reduced compared to the cells of patients without eroded joints.
4. EBV-induced Ig synthesis was also associated with the outcome of disease. The virus-activated cells of patients with erosive disease produced high concentrations of Igs in long term cultures. Enhanced Ig synthesis early in the disease predicted the development of joint

erosions and a poor outcome with high probability.

The results of the present study implicate that it might be possible to use, already in the early phase of the disease, *in vitro* tests of immune function to identify the patients at risk of developing destructive RA.

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Jyväskylä, November 1992 Ilmari Jokinen

Yhteenveto

Nivelreumapotilaiden viljeltyjen lymfosyyttien immunoglobuliinin tuotto ja sen yhteys taudin vakavuuteen

Nivelreuma on systeeminen tulehdustauti, jota sairastaa n. 1% väestöstä. Sairaudelle on tyypillistä niveltulehdus, joka useimmiten havaitaan ensiksi käsien ja jalkojen pienissä nivelissä. Taudin etenemistä on vaikea ennustaa, sillä nivelreuma on heterogeeninen tauti: osa potilaista parantuu spontaanisti ja osalla tulehdus tuhoaa nivelten rakenteita ja johtaa toimintakyvyn menetykseen. Hoito perustuu paljolti kokemuksen kautta saatuun tietoon, koska huolimatta pitkäaikaisesta tutkimuksesta taudin perussyy on vieläkin tuntematon. On kuitenkin selvää, että sairauden taustalla on immunologisen puolustusjärjestelmän häiriöitä. Nivelreuma lasketaankin kuuluvaksi autoimmuunitauteihin. Tyypillisiä immunologisia piirteitä nivelreumassa ovat mm. hypergammaglobulinemia ja monenlaiset kehon omien rakenteiden kanssa reagoivat autovasta-aineet, mm. reumatekijä.

Tässä työssä tutkittiin nivelreumapotilaiden verenkierrosta eristettyjen lymfosyyttien toimintaa soluviljelmissä. Verestä eristettyjä lymfosyyttejä käsiteltiin B-lymfosyytteihin vaikuttavilla polyklonaalisilla aktivaattoreilla ja mitattiin solujen immunoglobuliinin tuottoa. Lisäksi tutkittiin immunoglobuliinituoton säätelyyn osallistuvien soluryhmien toimintaa ja liukoisia välittäjäaineita. Lymfosyyttikokeet tehtiin sairauden alussa ennen varsinaisen reumalääkityksen aloittamista ja toisen kerran puolen vuoden kuluttua. Olisi tärkeää voida osoittaa pahaa nivelreumaa sairastavat potilaat, ja siksi tutkittiin myös solukokeiden tulosten yhteyttä taudin vakavuuteen. Potilaita seurattiin kahden vuoden ajan reumalääkärin vastaanotolla, jolloin tehtiin myös kliinisiä laboratoriokokeita sekä tutkittiin röntgenkuvin käsien ja jalkojen nivelet. Tutkimuksen keskeiset tulokset ovat seuraavat:

1. Nivelreumapotilaiden veren lymfosyyttien mitogeenisilla aktivaattoreilla, pokeweed mitogeenilla ja *Staphylococcus aureus* Cowan I -bakteerilla, aiheutettu immunoglobuliinisynteesi on merkitsevästi alentunut verrattuna terveisiin henkilöihin.
2. Alentuneen synteesin syy on potilaiden T-lymfosyyttien huonontunut kyky auttaa B-lymfosyyttien immunoglobuliinin tuottoa. Huonontuneeseen aputoimintaan liittyy häiriötä T-lymfosyyttien välittäjäaineiden tuotossa.
3. Alentunut synteesi liittyy taudin luonteeseen: vaste oli eniten alentunut niillä potilailla, joille kahden vuoden seurannan aikana kehittyi

nivelten eroosioita.

4. Epstein-Barrin -viruksella aiheutettu immunoglobuliinisynteesi, vastakohtana em. aktivaattoreille, on merkitsevästi kohonnut erosiivista tautia sairastavilla potilailla verrattuna terveisiin henkilöihin ja potilaisiin, joille seurannan aikana ei syntynyt nivelten eroosioita.
5. Korkea Epstein-Barrin viruksella aiheutettu immunoglobuliinisynteesi kliinisen taudin alkuvaiheessa osoittaa yli 90% todennäköisyydellä ne potilaat, jotka sairastavat vakavinta nivelreumaa.

Tässä työssä käytettyjen menetelmien pohjalta saattaisi olla mahdollista kehittää lymfosyyttien toimintoja mittaavia testejä, joilla nivelreumaa sairastavien potilaiden heterogeenisestä joukosta olisi mahdollista taudin alkuvaiheessa jo ennen pahojen niveltuhojen syntymistä tunnistaa vakavaa tautia sairastavat, aggressiivisesta hoidosta eniten hyötyvät potilaat.

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ORIGINAL PAPERS

I

Synthesis of IgM, IgG, and IgA in rheumatoid arthritis

by

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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 testing. 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., Irwine, 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 ± SD*
RA	15	7	8	43 ± 18
Reactive arthritis	5	2	3	37 ± 13
Scleroderma	3	3	—	49 ± 11
Polymyositis	1	1	—	50
Fibrositis	3	3	—	41 ± 17
Controls	18	12	6	31 ± 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% CO_2 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 ($2{\cdot}5 \times 10^{-4}$ 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)/10^6 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 $0{\cdot}5 \times 10^{-3}$ M $MgCl_2$, 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

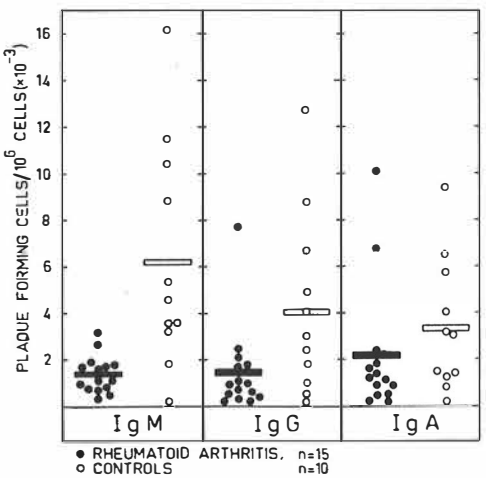

Fig. 1 *Immunoglobulin secreting cells in PWM-stimulated lymphocyte cultures. Results are expressed as plaque-forming cells (PFC)/10^6 viable cells determined by trypan blue exlusion (at the moment of plating). Bars represent arithmetic mean. The values of RA patients are significantly different from the controls for IgM ($p<0{\cdot}001$) and IgG ($p<0{\cdot}05$) by Student's t test.*

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

Group	n	PFC/10⁶ 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

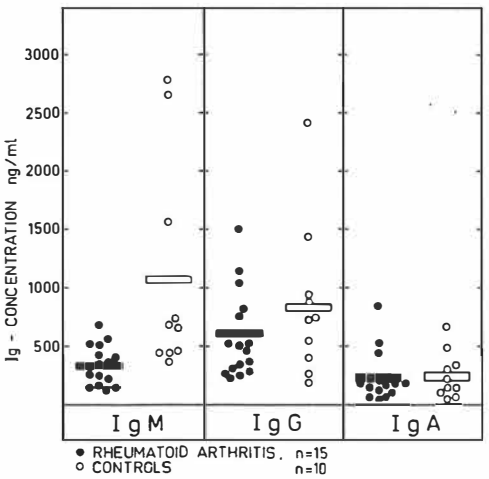

Fig. 2 *Immunoglobulin concentrations in the PWM stimulated culture supernatants determined by ELISA. Bars represent arithmetic mean. The values for RA patients are significantly different from healthy controls only for IgM (p<0·01, Student's t test).*

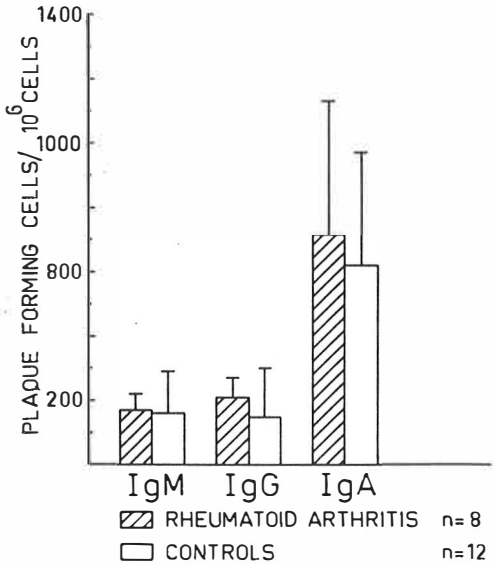

Fig. 3 *Number of cells spontaneously secreting immunoglobulin in the peripheral blood of RA patients and healthy controls. Results are expressed as plaque-forming cells (PFC)/10⁶ viable cells. Bars represent standard deviation. There is no significant difference between RA patients and the controls.*

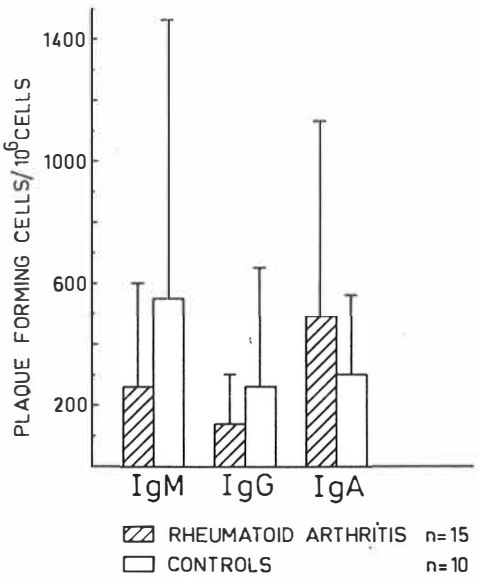

Fig. 4 *Immunoglobulin-secreting cells in unstimulated lymphocyte cultures. Results are expressed as plaque-forming cells (PFC)/10^6 viable cells. Bars represent standard deviation. The difference between RA patients and healthy controls is not significant.*

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 Panush[6] and Highton *et al.*[7] using PWM, and Haines and Hough[8] using staphylococcal protein A reported depressed response of RA cells in transformation tests. Earlier Lance and Knight[9] 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 report[10] 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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II

Synthesis of human immunoglobulins in vitro: comparison of two assays of secreted immunoglobulin

by

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SYNTHESIS OF HUMAN IMMUNOGLOBULINS IN VITRO: COMPARISON OF TWO ASSAYS OF SECRETED IMMUNOGLOBULIN

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ABSTRACT

One consequence of B-lymphocyte activation is immunoglobulin production, which can be quantitated by various techniques. We have compared assay of plaque forming cell (PFC) and determination of immunoglobulins by ELISA in culture supernatants of human lymphocytes stimulated with pokeweed mitogen and with Staphylococcus aureus. These assays correlated well ($r > 0.77$) in all major immunoglobulin classes studied.

The close correlation suggests that determination of secreted immunoglobulins by ELISA may be substituted for the PFC assay.

(KEY WORDS: Immunoglobulin synthesis in vitro)

INTRODUCTION

Activation of B-lymphocytes results in production of antibodies. In vitro this can be measured 1) by counting the cells with intracytoplasmic immunoglobulins (1,2), 2) by counting the cells secreting immunoglobulins with a plaque forming cell assay (PFC) (3,4,5) or 3) by determining immunoglobulins secreted into the culture medium. The latter can be performed with sensitive techniques like enzyme-linked immunosorbent assay (ELISA) (6,7), radioimmunoassay (8,9,10,11) or nephelometry (12). These methods for assessing B-lymphocyte function differ considerably in ease of performance, accuracy, and scope for observer errors.

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Each method may measure different aspects of B cell function and it has been claimed that measurements of PFC and immunoglobulins do not correlate (12,13). Our earlier work on in vitro antibody synthesis suggested that PFC assay and immunoglobulin determinations might indeed give similar results (14). The present study was therefore conducted to compare systematically these techniques and to determine whether the PFC assay could be replaced by ELISA, a method with many practical advantages.

MATERIAL AND METHODS

Cell Suspensions

Peripheral blood mononuclear cells from twelve healthy donors were separated by Ficoll-Isopaque centrifugation (15) and washed twice with Hanks balanced salt solution (HBSS). In order to detach the cytophilic antibodies the cells were incubated in HBSS at 37°C for 30 min. Thereafter the cells were washed once with HBSS and resuspended in RPMI 1640 (Flow Laboratories Ltd., Irwine, Scotland) supplemented with 10 mmol/l HEPES, gentamicin 70 mg/L (Schering Corp. Kenilworth, USA) and L-glutamine 0.3 g/L, and 10% heat inactivated fetal calf serum (FCS, Flow Laboratories). The number of cells was adjusted to 10^9/L.

Mitogens and Cell Cultures

Duplicate cell cultures were set up in 16x100 mm culture grade round bottomed plastic tubes (Sterilin Ltd., Teddington, England) in a volume of 1 ml with or without pokeweed mitogen (PWM, Gibco Co. New York, USA) 1:100 final dilution, or Staphylococcus aureus Cowan I 0.05v/v% (16). The cultures were incubated for 6 days in a humid atmosphere of 5% CO_2 and 95% air at 37°C. After incubation the duplicate cultures were pooled and the cells pelleted. The supernatants were stored at -20°C until assayed by ELISA.

Secondary Cultures

On day 6 the cells from the initial cultures were washed with HBSS and suspended in 2 ml of medium without FCS. A 1 ml aliquot of the

suspension was used for PFC assay and the rest incubated for 20 hours at 37°C. The supernatants were made cell free and frozen.

Plaque Assay

The number of Ig-secreting cells was determined by haemolytic plaque assay (5,14) using sheep red blood cells coupled to Protein-A by chromic chloride method (17).

Enzyme-Linked Immunosorbent Assay

The concentration of immunoglobulins in culture supernatants was determined by microplate ELISA. A double antibody sandwich method for quantitation of IgA, IgG and IgM was used (7,14). The antisera and alkaline phoshatase conjugates were from Orion Diagnostica, Helsinki, Finland.

RESULTS

Long Term Cultures

Time course studies have shown that in our lymphocyte cultures plaque forming cell maximum is on day six or seven, although the concentration of secreted immunoglobulins in the medium increases further beyond this time (data not shown). Supernatants were collected on the sixth day of culture and PFC were assayed. Fig.1 shows PFC/culture plotted against the concentration of immunoglobulin measured by ELISA in the corresponding culture supernatants. PFC calculated per 10^6 viable cells were similar as PFC calculated per culture. The plot contains all cultures regardless of the stimulant used. The range of PFC/culture was for IgM: 135-29,200, for IgG: 270-60,600 and for IgA: 60-22,000. Immunoglobulin concentrations ranged for IgM: 23-13,500 µg/L, for IgG: 89-12,000 µg/L and for IgA: 36- 2,300 µg/L.

Secondary Cultures

We expected that, due to timing of secondary cultures, even closer correlation would exist between the amount of secreted

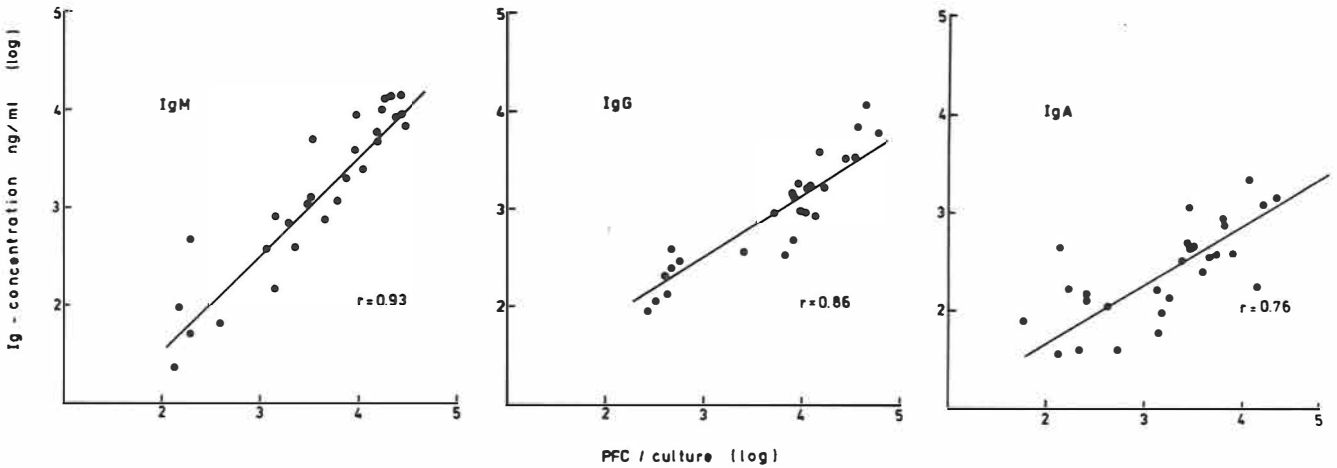

FIGURE 1. Correlation of PFC with concentration of secreted immunoglobulins in six day cultures ($p < 0.001$).

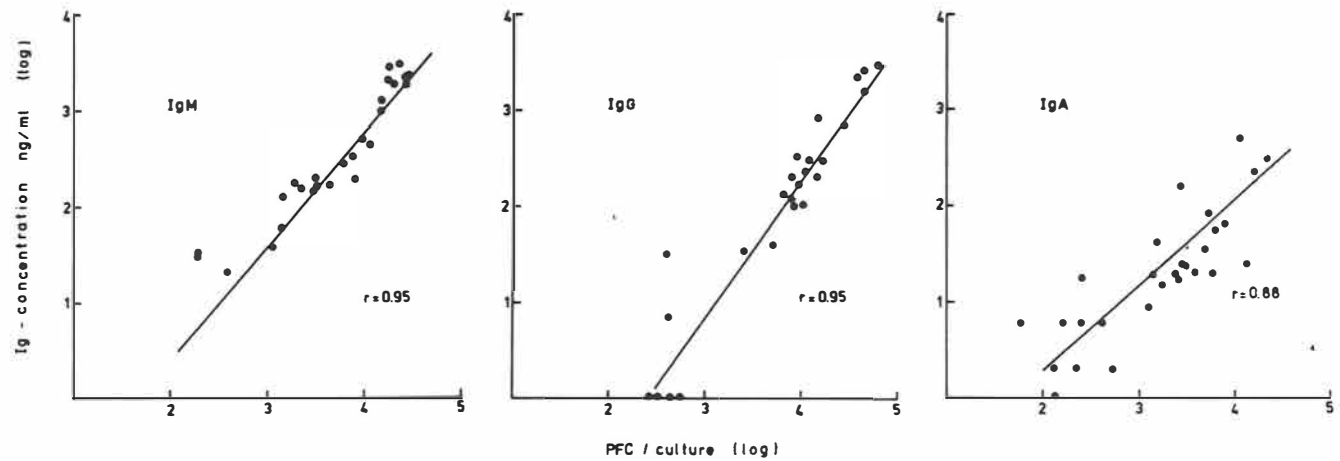

FIGURE 2. Correlation of PFC with concentration of immunoglobulins in secondary short term culture supernatants ($p < 0.001$).

immunoglobulins in those supernatants and the number of PFC. Fig.2 is a plot of the number of PFC/culture against the concentration of immunoglobulins in a 20 h culture set up at the time of the PFC assay. The concentrations of immunoglobulins were, IgM: 0-2,870 µg/L, IgG: 0-3,000 µg/L and IgA: 0-500 µg/L.

DISCUSSION

The purpose of this study was to demonstrate whether the PFC assay can be replaced by measurement of immunoglobulin concentration in culture media in studies of *in vitro* immunoglobulin synthesis. We found that the concentrations in both the short and long term culture supernatants correlate well with the number of PFC. Other workers have reported an absence of correlation (12,13). It is unlikely, however, that these differences can be explained by differing PFC assay techniques (20). There may have been different kinetics of cellular events or differences of sample timing between the laboratories. It has been shown (18,19) that before day 4 virtually no PFC are found. The number of PFC reaches its maximum around day 6 and thereafter decreases rapidly. During that time immunoglobulins accumulate in the culture supernatant and the concentration is still increasing when the number of PFC is already decreasing. It is therefore clear that PFC and immunoglobulin concentrations can not correlate after the PFC peak. In the present study the comparisons were made before or at the peak.

ELISA overcomes many problems associated with PFC assay. The difficulties with target SRBC and antibody-mediated complement lysis are avoided. Supernatant samples can be stored for batch assay and can be reassayed Furthermore the measurement is objective and easily automated. When there are, however, only few immunoglobulin secreting cells, ELISA is not sensitive enough and PFC assay can be more useful. In addition, no costly instruments are needed for the PFC assay.

The present results suggest that assay of secreted immunoglobulins may be used as an alternative to PFC assay. This study was, however, limited to healthy individuals and it is possible that cells from patients with immune abnormalities would behave differently. It is also not certain that all the stimulants will activate B cells in the same way as PWM or Staphylococcus aureus used in this study. Furthermore, immunoglobulin subclass production may have variable effects on the PFC assay and ELISA. These points are presently under investigation.

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III

Analysis of impaired in vitro immunoglobulin synthesis in rheumatoid arthritis

by

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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. Nonspecific 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.

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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 hydroxycholoroquine (300 mg daily), and one with a non-steroidal anti-inflammatory drug. If these drugs were not tolerated (12 patients) auranofin, sulphasalazine, D-penicillinamine, 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 µl 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,

USA) 10^{-5} mol/l, concanavalin A (Pharmacia Chemicals, Sweden) 4 mg/l, or indomethacin (Lääketehdas Orion, Finland) 1 mg/l was added directly to cultures. Cells were cultured in a humified 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 μm 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) method[2] 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 ($1·5\times10^6$) 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-2[8] 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 cells[15] 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

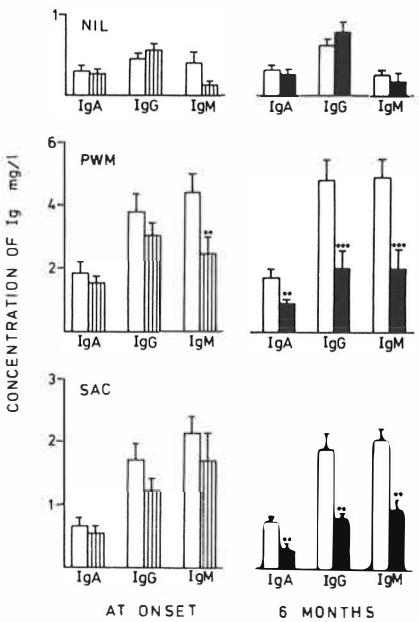

*Figure 1: Production of IgA, IgG, and IgM in cultures of seven days of blood mononuclear cells. Open bars represent healthy controls, striped bars patients with rheumatoid arthritis (RA) at the onset of the study, and black bars patients with RA six months later. The cultures were unstimulated (NIL), 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.*

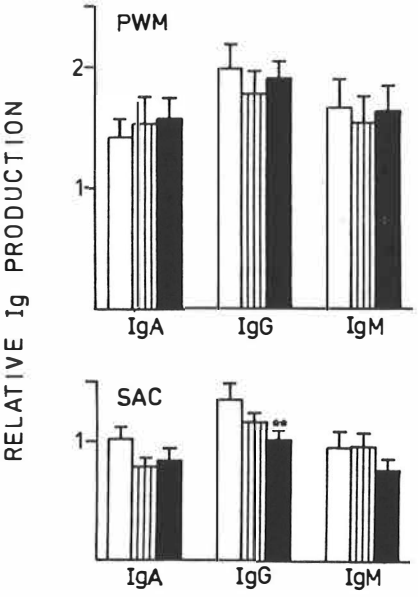

*Figure 2: The effect of hydrocortisone 10^{-5} mol/l on pokeweed mitogen (PWM) or Staphylococcus aureus Cowan I (SAC) stimulated cultures of healthy subjects (open bars), patients with rheumatoid arthritis (RA) at the onset of the study (striped bars), and patients with RA after six months (black bars). 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.*

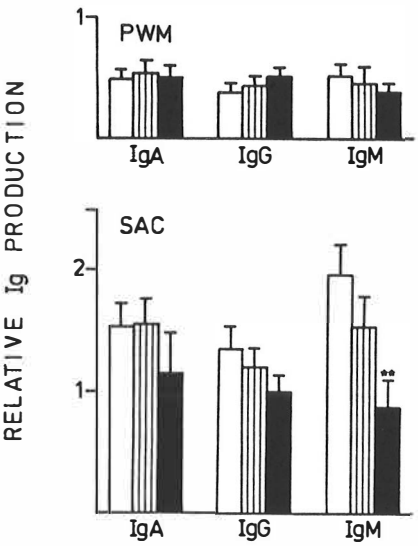

*Figure 3: The effect of concanavalin A costimulation on the immunoglobin production stimulated with pokeweed mitogen (PWM) or Staphylococcus aureus Cowan I (SAC) in mononuclear cell cultures of controls (open bars), patients with rheumatoid arthritis (RA) at the onset of the study (striped bars), and 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 sulphydryl 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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IV

Association of in vitro immune functions with the severity of the disease in rheumatoid arthritis

by

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Association of *in vitro* immune functions with the severity of the disease in rheumatoid arthritis

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SUMMARY

The production of immunoglobulins *in vitro* by lymphocytes from rheumatoid patients has been earlier shown to be defective. This report describes a two year follow-up study which shows that this defect is associated with the severity of RA. Pokeweed mitogen and *Staphylococcus aureus* Cowan I were used to stimulate *in vitro* immunoglobulin production by lymphocytes from patients with recent onset rheumatoid arthritis, and the relationship of responses to clinical characteristics were studied. Impaired polyclonal IgM synthesis, already detectable at the onset of disease, associated with joint destructions observed after a two year follow-up period. Further, phytohemagglutinin-induced interleukin-2 release by the cells of patients with erosive disease was found to be reduced compared to cells from patients without eroded joints. The results indicate that altered immune functions - manifested as decreased production of IgM and IL-2 - in rheumatoid arthritis are involved in the progression of the disease and affect the outcome of patients and, thus, represent an unfavorable prognostic feature.

Running title: *In vitro* Ig response in RA

Key words: rheumatoid arthritis, *in vitro* Ig synthesis, joint destruction, interleukin-2, association with unfavorable prognosis

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease in which hyperresponsiveness of the humoral immune system is evidenced by the production of rheumatoid factors and the frequent occurrence of hypergammaglobulinemia (1). In contrast to several studies (2-5) suggesting activation of B lymphocytes in the blood and synovial fluid of RA patients, other studies (7-10) show that polyclonal mitogen-driven humoral responses are decreased. In an earlier study, we analyzed the mechanisms of this impairment and showed that mediator-associated help is impaired, while B-cell, monocyte and suppressor functions remain intact (11). The relationship of these abnormalities to the pathogenesis or the manifestations of the disease are largely unclarified.

Since the clinical course of the disease in an individual patient is unpredictable, tests capable of identifying those patients who will develop destructive disease would be useful to rheumatologists. Some tests or indicators have been reported to correlate with poor clinical outcome (12, 13), but none has emerged as a clinically useful prognostic measure. Therefore, we carried out a follow-up investigation on a carefully studied group of patients to determine if the defects mentioned above might possibly have any prognostic significance.

Here we report an association of this defect with the severity of RA. We studied ***in vitro*** humoral responses induced by pokeweed mitogen (PWM) and *Staphylococcus aureus* Cowan I (SAC) of blood lymphocytes from healthy subjects and from recent onset RA patients before anti-rheumatic drugs were initiated. The responses were assayed again six months later. Simultaneously, the clinical activity of the disease was assessed and the patients were followed for up to 24 months. Our results suggest that the defects observed in mitogen-driven polyclonal IgM responses of the blood lymphocytes from RA patients reflect inflammatory processes resulting in destructive changes in joints and, thus, represent an unfavorable prognostic measure.

MATERIAL AND METHODS

Patients and controls

The patient group consisted of 58 newly-diagnosed rheumatoid arthritis patients, 17 males and 41 females, fulfilling American Rheumatism Association (ARA) criteria for definite or classical RA (14). The patients ranged from 17 to 78 (mean 48) years of age and the duration of disease, from 2 to 24 (mean 8) months. At the onset of the study, none of the patients had received second line anti-rheumatic drugs. During the next six month period, 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). Two patient were treated with hydroxychloroquine (300 mg daily); and two, with nonsteroidal anti-inflammatory drugs only. In those cases of intolerance or unresponsiveness, auranofin (n=6), sulfasalazine (n=19), D-penicillinamine (n=8) or azathioprine (n=2) were administered. The control group consisted of healthy laboratory and office personnel, 26 males and 33 females, ranging from 26 to 61 (mean 36) years of age.

Clinical and radiological examination

The patients were examined at diagnosis, at six months and at 24 months. Clinical rheumatoid activity was estimated by morning stiffness, pain scale, grip strength, Richie's articular index (15), E-HB, and ESR. The Mallya index (16) was calculated using these parameters. Hand and foot x-rays were taken at the onset, at six months and again at 24 months. The number of eroded joints were counted and an overall destruction index (Larsen's index) was calculated (17).

Blood samples

Blood samples were taken at diagnosis and again six months later. Control and patient samples were processed always simultaneusly. Blood was collected by venipuncture and heparinized (20 U/ml preservative-free sodium heparin). Peripheral blood mononuclear cells (MNC) were separated by Ficoll-Paque (Pharmacia chemicals, Sweden) density gradient centrifugation and washed three times with Hanks balanced salt solution. Cells were suspended at a concentration of $1x10^6$ cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS, Gibco Ltd., Scotland), glutamine at 3 g/L and Gentamicin at 15 mg/L (Sigma Chemical Co., USA).

Cell cultures

In microtiter plates of tissue culture grade (Nunc, Denmark), MNC were cultured in 200 µl volumes. The cultures were stimulated with either PWM diluted 1:100 (Gibco Ltd., Scotland) or SAC 0.05% vol/vol prepared as described elsewhere (18). In one series of experiments, Concanavalin A (ConA, Pharmacia Chemicals, Sweden) at 4 mg/L was added to SAC-activated cultures. Cells were cultured in a humified CO_2 incubator at 37 ^{o}C for 7 days.

ELISA measurements of Ig production

Supernatants from cultures were stored at -20^{o}C. Ig concentrations were determined by the double antibody sandwich enzyme-linked immunosorbent assay method (19), using isotype-specific trapping and alkaline phosphatase-conjugated, isotype-specific detecting antibodies (Orion Diagnostica, Finland). Measurements were standardized with known concentration standard sera (Behringwerke, Germany). The absorbances were determined using a Titertek Multiskan plate reader (Flow Laboratories Ltd.).

Production and determination of interleukin-2

At a concentration of 1.5 x 10^{6}/ml, MNC were cultured for 24 hours with phytohemagglutinin (PHA, Difco Laboratories, USA) at a final dilution of 1:100 in 1 ml of RPMI-1640 medium supplemented as described above. Cell-free supernatants were harvested by centrifugation and stored at -20 ^{o}C. The samples were assayed for IL-2 activity by means of the IL-2 dependent murine cytotoxic T-cell line, CTLL-2, (20) or ConA-stimulated lymphocytes (21). Control and RA patient samples were analyzed simultaneously, and the values for RA patients were calculated as the percentage of the mean of controls.

HLA-typing

Serological HLA-typing was performed by using standard two stage microcytotoxicity test by commercial antisera. B cells for DR typing were enriched from heparinized blood by density gradient centrifugation and rosetting with AET-treated sheep red blood cells. A panel of local and commercial antisera were used for defining various antigens (22).

Statistical methods

The Student's t-test was used to determine the statistical significance. Chi-square-statistics were used in analyzing grouped material.

RESULTS

In the first analysis, the number of radiologically-detected joint erosions two years after the onset of clinical disease was used as the measure of the severity of disease. The clinical, radiological and laboratory data of our patients have been published earlier (23, 24). At diagnosis, 41 patients had no eroded joints, and 17 patients had from one to nine (mean 1.0) eroded joints. Two years later, 18 of the 41 patients were still free of erosions while the remaining patients had from one to 37 (mean 6.5) eroded joints. The patients were grouped according to the presence or absence of erosions. The immune functions of these groups, measured at diagnosis and six months later, were then compared.

PWM- and SAC-induced IgM synthesis

PWM-induced IgM synthesis by MNC of RA patients was decreased, compared to that of healthy controls. The association of responses with the presence of joint erosions is shown in Figure 1. The elaboration of IgM by the patients with eroded joints was found to be markedly depressed compared to controls and to the patients without erosions, both at the beginning of clinical disease and six months later.

The responses of lymphocytes from RA patients and healthy subjects to another polyclonal B cell activator, SAC, are illustrated in Figure 2. When assayed at diagnosis, the responses of patients were reduced compared to those of controls. The patients with erosive disease had the lowest responses. When assayed again six months later, the difference between the patient groups was still evident and more pronounced.

Co-stimulation with SAC and ConA

In this set of experiments, ConA was added directly to cultures activated with SAC. Generally speaking, ConA had no effect on the secretion of immunoglobulins by control MNC when compared to activation with SAC alone. The addition of ConA to SAC-activated cultures of cells from RA patients, however, resulted in depressed Ig synthesis. As seen in Figure 3., patients with erosive disease elaborated less IgM than control subjects and patients with no eroded joints.

Polyclonal synthesis of IgA and IgG

The PWM-, SAC- and SAC+ConA-induced synthesis of IgA and IgG by RA patient MNC were lower than that by control blood lymphocytes. Neither at diagnosis nor at six months was association of IgA or IgG responses with the erosions of joints observed. The IgA and IgG responses are shown in Table 1.

Association of Ig synthesis with other clinical and demographic parameters

In order to analyze the association of Ig synthesis with other parameters of disease activity, the patients were divided into two groups according to PWM-induced IgM concentrations. One group fell inside the confidence interval of the control mean; and the second, under it. The second group was comprised of patients with the most pronounced defect. The laboratory and clinical parameters of these two groups, assessed at the end of the follow-up period, are outlined in Table 2. As expected, a statistically significant association was found with Larsens' polyarticular index, and also with global assesment. No other parameters of disease activity or the duration of symptoms were found to be significantly different in these two groups of patients. Moreover, sex, age or the precence of HLA-DR4 or -B27 antigens were not found to be related to depressed responses.

PHA-induced IL-2 production

PHA-stimulated release of IL-2 activity by MNC of rheumatoid patients was reduced compared to that by MNC of control subjects. Moreover, this phenomenon was associated with the erosiveness of the disease. As can be seen in Table 3, less IL-2 was detected in the cultures of cells from patients with erosive disease compared to that generated in the cultures of cells from patients with non-eroded joints. The difference between the groups of patients reached statistical significance ($p<0.05$) at six months.

Association with new erosions

At the time of diagnosis, 41 of the patients had no erosions. Of them, 18 remained nonerosive, and 23 developed erosions during the follow-up period. When analyzed as above, the differences in IgM and IL-2 production remained similar to those in the whole material (data not shown). However, due to variation and smaller numbers, statistical significance was reached in the case of PWM only.

DISCUSSION

In this longitudinal study, we investigated the relationship of clinical manifestations in RA and mitogen-driven humoral responses by blood MNC. Our main finding was that for those patients who later in the course of the disease sustained severe joint damage, low IgM *in vitro* responses to polyclonal stimulation were detectable already at the beginning of the clinical disease.

Only a few studies have been undertaken to investigate disease activity and spontaneous *in vivo* (2, 5) or mitogen-stimulated *in vitro* humoral immune responses in rheumatoid arthritis (8, 9, 25). These studies reveal a weak association of *in vitro* responses with clinical variables (such as titer of rheumatoid factor, ESR or morning stiffness) that reflect disease activity. None of the studies, however, involve long term follow-up investigations; and none address the question of the prognostic value of these immunologic disturbances. The design of the present investigation enabled us to correlate the immunological parameters measured at the beginning of the disease with the outcome of the patients assessed two years later. The association of the immune defects mainly with erosions, and not with other parameters of disease activity, might at first sight suggest that the association is only coincidental. Arguing against such a stance, however, is the fact that tests repeated at six month interval, demonstrated the same association. Furthermore, one might justifiably consider the development of erosions as a cumulative and, in the long run, most reliable measure of disease progression as opposed to, for example, other parameters measuring disease activity, which fluctuate with drug treatment and time. Thus, this association would suggest that those patients with the gravest underlying immunological disturbances may be destined to develop erosive disease, despite the occasional remissions brought about by treatment. The data do not, however, imply that there necessarily is a causal relationship.

Consistent with the findings of decreased Ig synthesis is low PHA-induced IL-2 production by peripheral blood cells associated with erosiveness of disease. Mitogen-stimulated IL-2 release by blood lymphocytes from RA patients has been described by various investigators as decreased (26-28), as normal (29, 30), or as increased (31, 32). These contradictions might be explained by such methodological differences as the type of assay, choice of activating agents,

the concentrations of activators and culture conditions. Reports on the relationship between the activity of disease and the synthesis of IL-2 are also contradictory (27-30), but usually reduced IL-2 production during active disease is suggested. In addition to abnormal IL-2 production, studies on IL-2 receptor expression on lymphocytes, occurrence of soluble IL-2 receptors (33) and IL-2 inhibitors in serum and synovial fluid (34-36) indicate the importance of IL-2-related abnormalities in the developments of manifestations of RA.

The decreased production of IgM could be a direct consequence of disease activity. Alternatively, one might argue it to be a consequence of drug therapy. The latter possibility is improbable, as the decreased IgM responses were observed at the onset of the study, before disease-modifying drugs were administered. Moreover, although the drug treatment of the two patient groups was similar, the differences in IgM production remained evident. On the other hand, one must consider the possibility that the use of nonsteroidal anti-inflammatory drugs (NSAID) during early stages of disease might have altered IgM production.This also unlikely, since data (not shown) collected later during the disease, show that patients not given drugs (including NSAID) did not produce higher concentrations of IgM. Subjects receiving NSAID, along with other medication, responded similarly to those patients receiving no NSAID. Thus, NSAID as the sole explanation of low IgM responsiveness seems unlikely.

It is intriguing that although decreased production of IgA, IgG, and IgM is evident in RA, only lowered IgM responsiveness is clearly associated with the severity of the disease. It may be of importance that a subpopulation of B cells, CD5-positive cells (37), has been recently implicated in RA and other autoimmune diseases. These cells have been shown to produce IgM and antibodies of IgM isotype (38-42). Whatever the mechanism(s), it is remarkable that in this study the defect, already detectable at the onset of the disease, was most pronounced in the patients who later, despite treatment, developed severe erosive disease. These results emphasize the heterogeneity of RA and further indicate that the underlying disturbances in immune functions may, in part, predict the outcome of RA patients. Considering that the long term prognosis in RA is still distressingly poor and the treatment of the disease is far from satisfactory, that population of patients who will develop severe disease needs to be identified during the early phase of disease. Our data strongly suggest that tests on immune functions could be developed further to possibly establish which RA patients will require a more

aggressive therapeutic protocol.

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Table 1.

In vitro IgA and IgG synthesis by lymphocytes from the blood of RA patients and healthy controls

Stimulus and Ig-isotype	Controls	p	RA patients	
			Non eroded	Eroded
PWM IgA				
- at diagnosis	1.8 ± 0.4	ns	1.3 ± 0.3	1.5 ± 0.3
n=	58		18	40
- at 6 months	1.7 ± 0.2	0.001	0.6 ± 0.1	1.0 ± 0.2
n=	46		18	41
PWM IgG				
- at diagnosis	3.8 ± 0.6	ns	3.5 ± 0.8	2.8 ± 0.5
n=	57		18	40
- at 6 months	4.8 ± 0.7	0.001	1.9 ± 0.5	1.9 ± 0.3
n=	47		18	41
SAC IgA				
- at diagnosis	0.7 ± 0.1	ns	0.5 ± 0.1	0.5 ± 0.1
n=	56		17	39
- at 6 months	0.6 ± 0.1	0.01	0.3 ± 0.1	0.4 ± 0.1
n=	47		18	41
SAC IgG				
- at diagnosis	1.8 ± 0.3	ns	1.0 ± 0.2	1.2 ± 0.2
n=	56		17	39
- at 6 months	1.7 ± 0.2	0.01	0.9 ± 0.2	1.0 ± 0.2
n=	47		18	41
SAC+CONA IgA				
- at diagnosis	1.1 ± 0.2	0.05	0.7 ± 0.2	0.3 ± 0.1
n=	22		9	15
- at 6 months	0.8 ± 0.1	0.01	0.2 ± 0.1	0.3 ± 0.1
n=	11		7	13
SAC+CONA IgG				
- at diagnosis	1.8 ± 0.4	0.05	1.1 ± 0.2	0.9 ± 0.2
n=	22		7	13
- at 6 months	1.4 ± 0.1	0.05	1.0 ± 0.3	0.9 ± 0.2
n=	11		7	13

Results are reported as the concentration of Ig (mg/L) in seven day cultures stimulated as indicated at diagnosis and six months later. The values are expressed as the mean ± sem.

p indicates the statistical significance of the difference between controls and the mean of all RA patients. The differences between patient groups did not reach statistical significance.

Table 2.

Association of PWM induced synthesis of IgM with demographic parameters and clinical parameters assessed two years after the onset of clinical disease.

	IgM synthesis at diagnosis			IgM synthesis at 6 months		
	Group 1 (low) n=40	Group 2 (normal) n=15	p	Group 1 (low) n=45	Group 2 (normal) n=11	p
Demographic parameters:						
Age (years)	47.1	46.9	ns	49.2	43.9	ns
Sex male : female	12 : 28	2 : 13	ns	14 : 31	1 : 10	ns
HLA DR4 pos : neg	22 : 17	8 : 7	ns	25 : 19	5 : 6	ns
HLA B27 pos : neg	6 : 33	3 : 12	ns	8 : 36	2 : 9	ns
Duration of symptoms (months)	9.0	7.0	ns	14.2	14.9	ns
Clinical parameters at two years:						
ESR (mm)	20.0	28.4	ns	24.8	19.2	ns
CRP (mg/l)	17.4	24.7	ns	20.6	20.1	ns
Waaler-Rose titer	228	192	ns	230	101	ns
Number of clinically active joints	8.2	10.4	ns	9.7	7.6	ns
Mallya index	2.0	2.2	ns	2.1	1.9	ns
Ritchie index	4.8	6.1	ns	5.4	4.7	ns
Development of erosions:						
eroded : not eroded	32 : 8	7 : 8	0.01	34 : 11	5 : 6	0.05
Number of eroded joints	4.8	2.8	ns	5.3	2.2	0.01
Larsens' index	17.0	12.5	ns	21.0	6.8	0.01
Global assessment:						
severe or mild disease : remission	34 : 6	12 : 3	ns	40 : 5	7 : 4	0.05

Figures represent the mean values or, when appropriate, number of patients. P is the statistical significance between groups.

Patient groups: Group 1 includes the patients with IgM synthesis under (low responses), and Group 2 within (normal responses), the confidence interval of the mean of IgM synthesis by controls.

Table 3.

PHA-induced release of IL-2 by blood MNC from control subjects and RA patients.

	Control	p	RA patients	
			No erosions	With erosions
At diagnosis	100	0.1	64 ± 16	59 ± 11
n=	52		14	36
At 6 months	100	0.01	55 ± 20	36 ± 10 *
n=	38		15	32

The results are expressed as the percentage of the mean of controls ± sem.
p indicates the statistical significance of the difference between controls and the mean of RA patients.
* The difference between patient groups $p < 0.05$

Legends for Figures

Figure 1.

The concentration of IgM in supernatants of seven day cultures of peripheral blood MNC stimulated with PWM. Blood samples were collected first from 58 healthy control subjects (open bars) and from 58 RA patients (hatched bars: patients without radiologically detected bony erosions, black bars: patients with eroded joints) at diagnosis and again six months later. The values represent the mean of the group + s.e.m.. The symbols above the bars for control subjects indicate the statistical significance between control subjects and all RA patients; the symbols above the bars for patients indicate the significance between the patient groups (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Figure 2.

The concentration of IgM in blood MNC cultures stimulated with SAC at diagnosis and after the six month follow-up period. The open bars represent control subjects; hatched bars, the RA patients without eroded joints; and black bars, the patients with erosions. The data are expressed as means+s.e.m. The symbols for statistical significance are as described in Figure 1.

Figure 3.

The synthesis of IgM by control subjects' and by RA patients' blood MNC cultured for seven days with SAC+ConA. Blood samples were collected at diagnosis and six months later. Open bars represent control subjects; hatched bars, the patients without erosions; and black bars, the patients with eroded joints. The data are expressed as the mean+s.e.m. The symbols for statistical significance are as described in Figure 1.

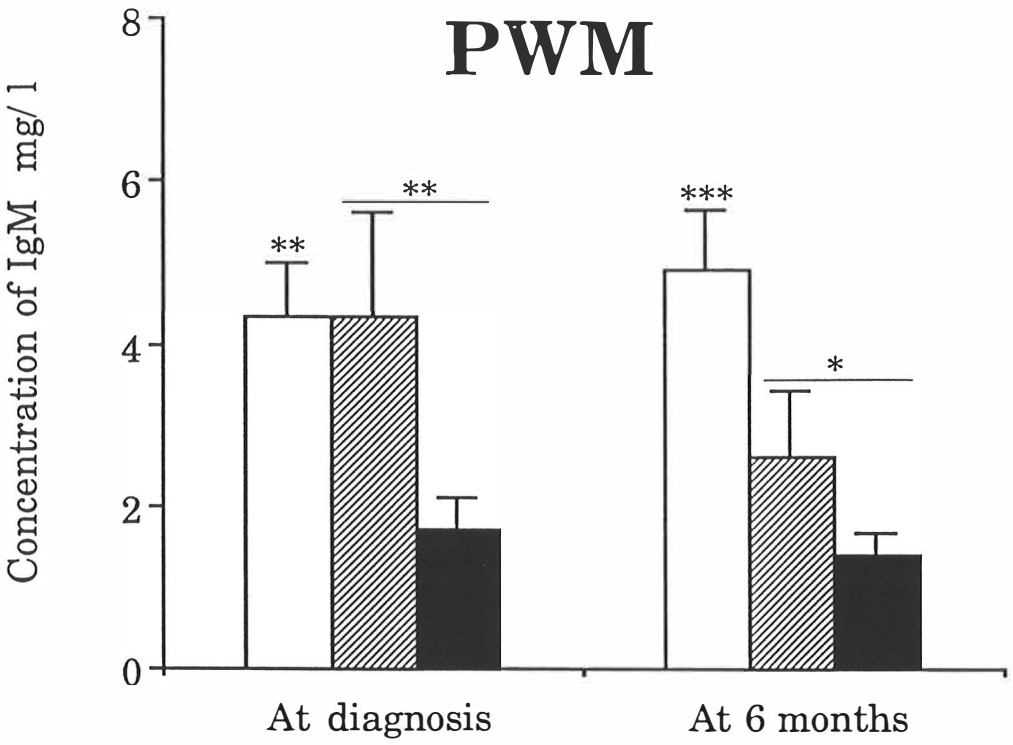

Figure 1.

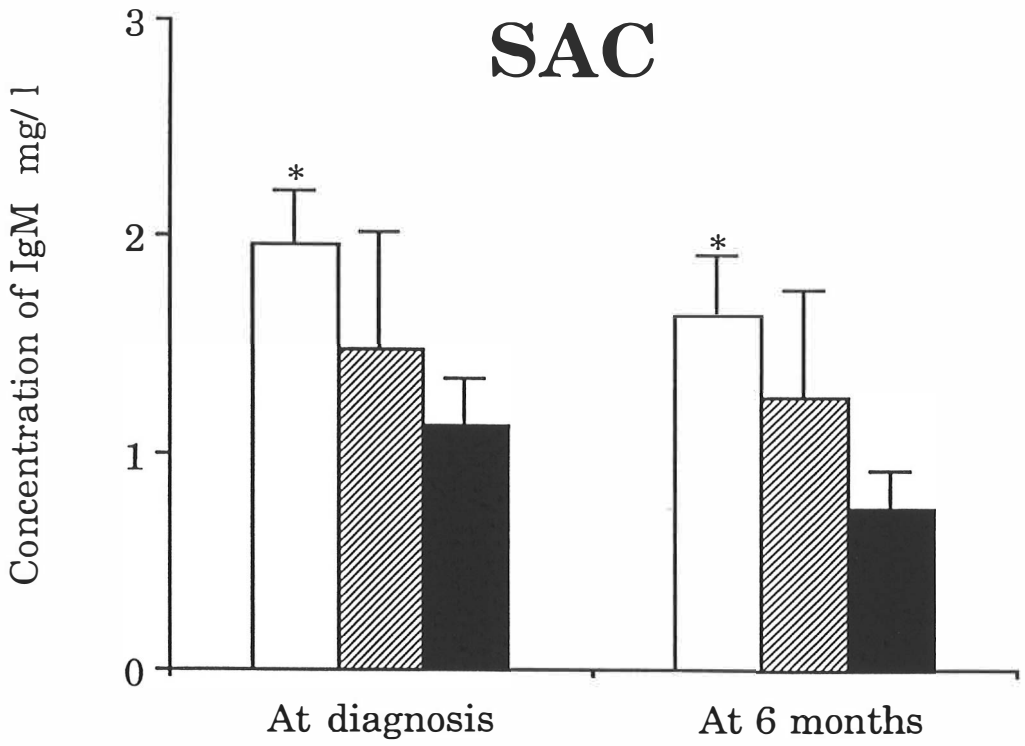

Figure 2.

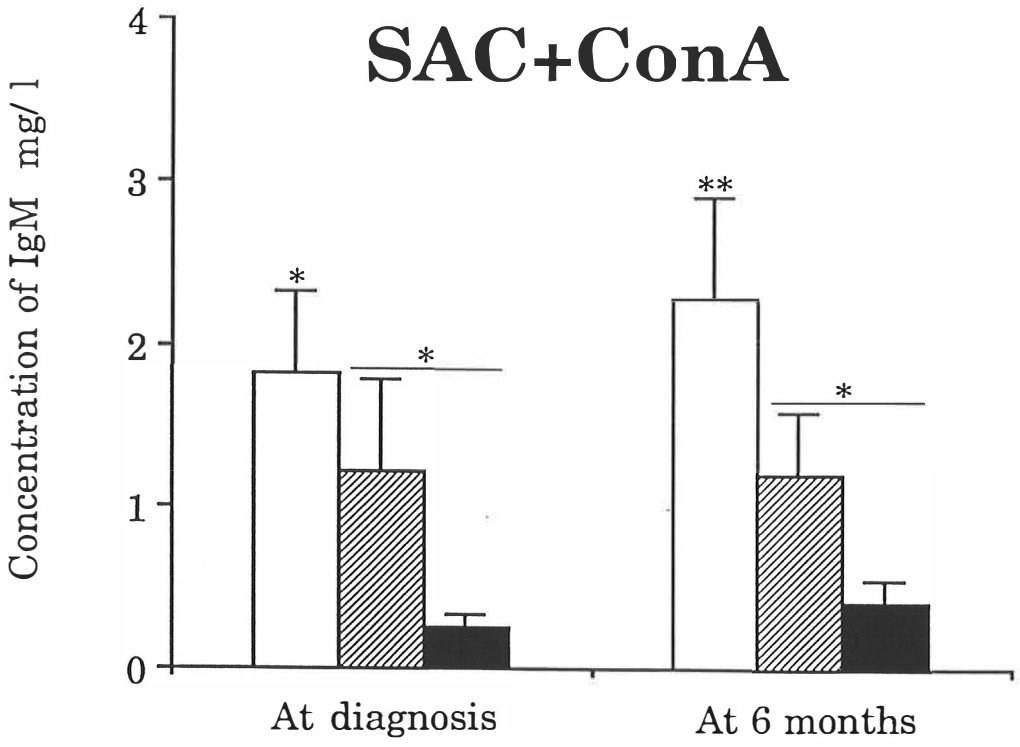
SAC+ConA
Concentration of IgM mg/l
4
3
2
1
0
*
*
**
*
At diagnosis
At 6 months

Figure 3.

V

Prediction of severe rheumatoid arthritis using Epstein-Barr virus -induced immunoglobulin synthesis by lymphocytes of patients with recent onset disease

by

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PREDICTION OF SEVERE RHEUMATOID ARTHRITIS USING EPSTEIN-BARR VIRUS-INDUCED IMMUNOGLOBULIN SYNTHESIS BY LYMPHOCYTES OF PATIENTS WITH RECENT ONSET DISEASE.

Running head: Prediction of severe RA

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ABSTRACT

Background Effective means of identifying those patients with rheumatoid arthritis (RA) who will later develop destructive disease are lacking. In search of such means we investigated whether disturbances in Epstein-Barr virus (EBV) -induced immunoglobulin synthesis, seen early in the disease, associated with the severity of RA, seen two years later.

Methods We studied prospectively 45 patients with recent onset RA and, simultaneously, 41 healthy individuals as controls. Early in the disease, blood lymphocytes were cultured in the presence of EBV for 4 weeks, and the supernatant immunoglobulins were assayed weekly. To assess the severity of disease, clinical, laboratory, and radiological evaluations were performed every six months for two years. The association of increased immunoglobulin production with the severity of RA and its usefulness as a predictive measure were then analyzed.

Results During the follow-up period, 30 of the original 45 RA patients developed erosive disease. At onset, these 30 patients with erosive disease did not differ from the 15 with non-erosive disease when assessed by several parameters. However, EBV-induced production of immunoglobulin was found to be significantly higher in the erosive compared to the non-erosive group of patients ($p<0.001$). Further, using immunoglobulin synthesis as a test, it was possible to identify a subgroup of 9 to 14 patients, depending on the immunoglobulin isotype studied, who would later develop severe erosive disease (PV_{pos} =90-100 %).

Conclusion High EBV-induced production of immunoglobulin early in RA associates with the later development of severe disease, particularly with joint erosions. This feature identifies, with over 90% likelihood, the third of patients who will develop most severe disease.

Key words: rheumatoid arthritis, Epstein-Barr virus, erosive disease, severity of disease, autoimmunity, prediction of outcome, immunoglobulin synthesis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, incapacitating disease affecting 1 to 3 percent of most populations. Even though a common disease, its cause remains unknown. It is likely that RA is a disease of multiple etiology. Genetic factors contribute significantly to the susceptibility of individual patients [1]. However, environmental factors (most likely virus(es)) are also postulated to be important triggers in the etiology of the disease [2-4]. One of the triggering candidates is Epstein-Barr virus (EBV) [2,5-8]. Raised titers of antibodies against EBV capsid and nuclear antigens as well as a high incidence of antibodies to rheumatoid arthritis nuclear antigen (RANA) are characteristic serological findings. Another abnormal host response against EBV in RA patients is increased synthesis of immunoglobulins in cultures of EBV-stimulated lymphocytes [9,10].

Just as the etiology of RA is unknown, effective means of predicting the outcome of patients or of altering the prognosis of disease by treatment also remain unknown. Therefore, the importance of identifying, as early as possible, those patients who will later develop destructive disease and who would need more aggressive therapy is apparent. Unfortunately, clinically reliable laboratory tests or indicators toward that end have not yet been described, despite intensive research efforts. According to a recent review [11], some of the less controversial prognostic features include female sex, positive rheumatoid factor, long-standing increased ESR and CRP values, decreased haemoglobin and presence of subcutaneous nodules. However, association of these parameters with poor prognosis has not been strong or consistent enough to be generally useful in predicting the fate of an individual patient [5,12,13].

We were interested in determining whether reactions to EBV - a potential etiologic agent - would associate with the severity of RA. The *in vitro* responses of lymphocytes from patients with recent onset RA to EBV were determined and related to the outcome of patients two years later.

PATIENTS AND METHODS

In this study, 45 patients with classical or definite rheumatoid arthritis, meeting the criteria of the American Rheumatism Association for diagnosis [14] were evaluated. As controls, 41 healthy subjects were simultaneously studied. The patients were admitted to the hospital after mean 9 months duration of symptoms. They had received no disease modifying anti-rheumatic drugs before recruiting to the study. Of the original 45 patients, 16 were tested for EBV-induced Ig synthesis at the time of admission and 29 patients 6 months later. The treatment and characteristics of the patients and the control subjects at the time of sample acquisition are given in Table 1.

Clinical and laboratory evaluation. The patients were evaluated at the start and then every six months for a two-year period using clinical and laboratory measures. Clinical activity of the disease was estimated, using the multivariate analysis system, including two subjective measures (morning stiffness and pain scale), semi-objective measures (grip strength and Ritchie's articular index) [15] and objective measures (E-HB and ESR), as described by Mallya and Mace (Mallya index) [16]. CRP was assayed by quantitative immunoprecipitation.

Radiological evaluation. Radiological examination of hands, wrists and feet were performed at the start, after one year and after two years of study. The number of eroded joints was counted at each visit. An overall destruction index of joints (polyarticular Larsens´-index) [17] was calculated; the indices of subtalar and wrist joints were corrected, using a coefficient of 5 as described in detail earlier [18].

Rheumatoid factor serology. Rheumatoid factor in the sera of patients was measured by the Waaler-Rose test in microtiter plates with U-shaped wells every six months. Titers with values of 1/64 or more were regarded as positive.

Serology of EBV. A commercial enzyme immunoassay (DuPont Company, Billerica, MA, U.S.A.), employing viral antigen on the solid phase, was used for determination of both IgG and IgM class antibodies in the serum of all patients and control individuals.This test gives a cut-off OD value above which the sera are considered positive. For increased sensitivity, however, we tested all the sera giving ODs less than 10% above the cut-off limit by immunofluorescence. In this method[19], cells of a chronically EBV-infected cell line were used as antigen and stained using the patient sera as primary antibodies. The samples were then blindly read under fluorescent microscope by two experienced observers.

HLA-DR typing. Mononuclear cells were isolated from heparinized peripheral blood by Lymphoprep (Nyegaard, Oslo, Norway) gradient and B lymphocytes were further enriched by depletion of T cells with 2-aminoethylisothiouronium bromine treated sheep red blood cells. HLA-DR antigens were determined by standard two stage micro lymphocytotoxicity method and commercial tissue typing trays (Biotest AG, Frankfurt, Germany) [20].

Cell cultures. Mononuclear cells (MNC) from heparinized vein blood samples were obtained by passage through Ficoll-Paque density gradient. The cells were washed and suspended at 10^6/ml in RPMI-1640 medium supplemented with 10% FCS, glutamine and Gentamicin. Aliquots of one ml each, containing 106 MNC, were dispensed into 10 ml cell culture tubes (Sterilin, UK). An EBV-containing supernatant in a 200 µl volume (as described below), was then added. Cultures of infected cells were set up in duplicate. Cells were cultured at 37°C in an atmosphere of 5% CO_2 and 95% humidity. At 7 days intervals of culture, the tubes were centrifuged at 400 x g for 5 minutes, supernatants were removed and duplicate supernatants were pooled. The cells were resuspended in 1 ml of fresh supplemented RPMI-1640 medium without virus. EBV-activated cultures of MNC from RA patients were always established simultaneously with EBV-activated cultures of MNC from controls.

The EBV used for B cell transformation was obtained from culture supernatants of the marmoset cell line, B95-8, filtered through a 0.22 μm Millipore filter. The virus-containing supernatant was a generous gift of professor George Klein, Karolinska Institutet, Stockholm, Sweden.

Determination of immunoglobulin. Supernatants of cultures were stored frozen at -20°C. Immunoglobulin excreted into the culture medium was assayed, using an isotype specific,
double antibody sandwich ELISA method as described earlier[21]. Briefly, microtiter plates were coated with swine antihuman IgA, IgG, and IgM antisera (Orion Diagnostica, Helsinki, Finland), diluted 1:1000. After overnight incubation at 4°C and washing, culture supernatants, diluted 1:15-1:150 in culture medium, were added into the wells. The plates were incubated at 37°C for 1 h, washed, and alkaline phosphatase conjugated antihuman immunoglobulin serum (Orion Diagnostica) was then added. After another 1 h incubation at 37°C, the substrate (p-nitrophenylphosphate 1mg/ml, Sigma Chemical Company, Saint Louis, U.S.A.) was pipetted into the wells. The enzymatic reaction was stopped with NaOH and absorbance at 405 nm was measured with Titertek Multiscan plate reader (Flow Laboratories Ltd, Irwine, Scotland). The immunoglobulin content was calculated from standard curves obtained with standard sera (Behringwerke, Marburg, Germany).

Statistics. Statistical analyses included Student's t-test and κ^2 statistics. Predictive values were calculated as described by Vecchio [22].

RESULTS

The patients described in this report belong to a study group, the clinical, radiological and laboratory data of which have been published earlier [18,23]. At the time of blood sample early in the disease, 28 patients had no eroded joints, while 17 already had erosions. Two years later, 15 of the original 45 patients were still free of erosions, and seven were in remission. Some parameters of disease activity at the beginning and end of follow-up are presented in Tables 2 and 3.

All except two patients and one control subject had had prior EBV infection, based on antibody determinations. The in vitro responses of these three subjects, however, were similar to those of EBV-immune individuals. Therefore, these two patients and the control subject were included for the purpose of analyses in this report.

Kinetics of immunoglobulin secretion in lymphocyte cultures

Patient and healthy control lymphocytes were cultured for 4 weeks, with the culture supernatants being harvested at 7 day intervals and assayed by enzyme immunoassay. Each assay sample thus represented the amount of immunoglobulin accumulated in the supernatant during any given seven day test period.

First, in analyzing the kinetics of various Ig isotypes production (Fig. 1), we confirmed earlier findings of down-regulation or late suppression of IgM in healthy controls. Moreover, we observed the lack of such responsiveness in patients after 14 days of culture also confirming earlier observations. Furthermore, our results show that the same holds true for IgG. The production of IgA by patient lymphocytes, however, began to decrease after two weeks, even though the IgA levels were still higher than those seen in control cultures.

The kinetics of EBV-activated responses were similar for patients treated and for patients not treated with disease modifying anti-rheumatic drugs (data not shown) This suggests that, in fact, treatment has no effect on EBV-

stimulated responses.
During this series of investigations, we used two batches of EBV strain B95-8 and observed variation in their capacity to activate lymphocytes. This produced two sets of results with different levels of Ig concentrations. However, when analyzed separately, each set exhibited similar response patterns as shown in Fig. 1. To eliminate the influence of such variation in our experiments, the concentrations of Ig synthesized by RA MNC were compared to the mean concentration of the control samples tested simultaneously. The results were then expressed as relative concentrations (RA/control).

Association of immunoglobulin synthesis with the development of erosions

For this purpose, the patients were grouped according to the level of IgM at 4 week culture as follows: high responders with Ig levels exceeding that of control mean + 2 SD, and normal responders with levels below that threshold. The groups of high and normal responders consisted of 15 and 30 patients, respectively. Patients were then followed for two years. The clinical and laboratory parameters at the beginning and at the end of the follow-up period demonstrate that erosive disease developed significantly more often in the group of high responders compared to normal responders (93% and 53%, respectively) (Table 2). A clear association of high IgM synthesis was found with the articular parameters: development of erosions, the number of eroded joints and Larsens' index. High IgM synthesis was associated also with elevated ESR and high Mallya index at two years.
This finding was analyzed in more detail. Patients were classified according to the presence or absence of erosions at the end of the follow-up period.The presence of erosions was selected as a basis for classifying the patients, since development of erosions can be assessed objectively. Further, the development of erosions represents a cumulative indicator of severe disease not influenced by temporary remissions. Comparisons of patient groups and controls are shown in Fig. 2. The noneroded group and controls showed no significant difference while the eroded patients had higher

responses. The differences between patient groups reached statistical significance in IgA and IgG at 2, 3 and 4 weeks of culture; statistical differences in IgM responses were observed during week 3 and week 4.
Some of the patients already had erosions at the onset of the study. Therefore, it was important to re-analyze the association between EBV-induced Ig and erosions by using data on new erosions only. The association remained similar (data not shown).
Only about half of the patients later to become eroded were high responders in our EBV-test. This raised the question of whether the disease in erosive, EBV-test-positive patients was different from that in erosive, EBV-test-negative ones. Similar analysis as that presented in Table 2 (data not shown) revealed no statistically significant differences. Nevertheless, there was a clear trend towards milder disease in the latter group.
The records of clinical and laboratory parameters obtained at the onset of clinical disease were also reviewed to determine if any of them associated with the development of erosions (Table 3). The only significant association was with EBV-induced Ig synthesis.

EBV induced immunoglobulin synthesis as predictor of erosions

To address the question of whether the EBV-induced immunoglobulin synthesis early at the onset of the disease had value as a prognostic tool in recognizing patients with poor outcome, ie. with the appearance of erosions, the patients were grouped into high and normal responders as explained above. Fig. 3 illustrates, by flow chart, the value of the EBV-IgM test as a predictor of erosions. Development of erosions and calculated values for predictiveness, sensitivity and specificity for high IgA, IgG or IgM synthesis are presented in Table 4. Using Ig synthesis as an indicator, it was possible to identify a group of patients who, later, after two years, became eroded. This recognition was highly specific (93,3%) and had high predictive value (PV_{pos} =93,3%). Sensitivity of the test ranged from 30% in IgA and IgG to 47% in IgM. When the cut-off limit of Ig synthesis was lowered to the mean of controls + 1SD, the sensitivity was increased to 33-50%. Specificity decreased slightly to 89-93%.

DISCUSSION

We have shown in this study that high production of immunoglobulins by patient lymphocytes in response to EBV stimulation is associated with later severe disease and predicts the development of erosions in RA. By this feature, it might be possible to identify with high probability the population of patients destined to suffer the most devastating effects of the disease. This finding is made even more intriguing by the fact that it connects EBV, a strongly suspected etiologic agent, with severe disease.

There are at least two alternative explanations for the association of EBV with severe RA. Firstly, the response to EBV may merely reflect a generalized immunological disturbance of patients with RA and may, in fact, implicate nothing about etiology. That the group of patients with the most severe disease exhibit increased responsiveness would concur with this notion. Furthermore, our study has also shown that mitogen-induced synthesis of immunoglobulin, another measure of immune function, is associated with severe RA, although far more weakly than is shown here[24]. On the other hand, during the early phases of disease when the EBV-test was carried out, the markers of disease activity were similar in our groups of patients, revealing no grave, underlying immune disturbances in those patients positive for the test. It may be argued that the abnormalities in controlling the response to EBV could be the consequence of drug treatment. This again is highly unlikely, as no differences were found between the untreated patients and patients receiving disease-modifying drugs.

The alternative explanation would suggest an etiologic role for EBV in a third of patients suffering from RA. EBV has been linked to RA because of several immunological abnormalities in host responses against the virus: EBV-specific antibodies [25,26], increased number of circulating B cells infected by EBV [27,28], diminished cytotoxic T-cell responses and uncontrolled B cell responses induced by EBV [29-32]. Moreover, recent evidence suggests

molecular mimicry between EBV glycoprotein and HLA class II molecules in genetically susceptible hosts and also between the virus and a collagen component [33-36]. The present findings add to this list of growing evidence connecting EBV with RA. Although they can be taken as suggestive at best, we feel that the biology and varying properties of EBV-strains, the sequence of infection with various strains, and the handling of the viruses by the host epithelia or lymphocytes may be important factors in determining the fate of the
patient [37-39].

The cellular mechanisms involved in the control of EBV-induced synthesis of immunoglobulins by B lymphocytes are not fully established. EBV, when added to cultures of lymphocytes, activates B cells to proliferate and differentiate into Ig-producing cells. T cells influence this process and can also suppress B cell function [9,40]. The reason for the failure of RA cells to turn off Ig synthesis may be poor cytotoxic T cell function [29,30] or disturbances in mediator function [41,42]. Kahan et al. have reported that EBV related immunoregulatory defects do not exist in all patients with RA [43]. In their study, 18/47 patients had similar suppressor functions as healthy controls and 21/47 showed defective function. This substantiates the present results and further strengthens the hypothesis that this defect is not typical of all patients with RA. Neither is it specific to RA since it has been reported in two other autoimmune diseases; in some patients with systemic lupus erythematosus and with progressive systemic scleroderma, but not in patients with psoriatic arthritis, ankylosing spondylitis or cystic fibrosis [44,45].

Several recent studies have shown that the development of erosions, the hallmark of unfavourable outcome, takes place early in the course of RA. Almost 90% of erosions occur within 2 years of the onset of symptoms. However, identifying those patients who will develop severe erosive disease has remained largely problematic. The associations of various biochemical or immunological parameters with severe disease are weak, controversial or unconfirmed

[11,46,47]. Perhaps more efficient use, in the future, of the Health Assessment Questionnaire and the Arthritis Impact Measurement Scales may offer better means of predicting the outcome of RA (reviewed by Bellamy) [48]. Meanwhile, however, there is a great demand for a test able to select those patients who will need more aggressive therapy early during the disease.

As Harris concluded his recent review [5], "We have underestimated the morbidity and mortality of rheumatoid arthritis. Our goals must be to intervene with focused but less toxic drugs as early as feasible in the disease process". We would propose that patients´ responses to EBV might offer a basis for a test which would allow us to efficiently reach that goal.

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Table 1.

The subjects in the study

	RA patients	Healthy controls
Number	45	41
Mean age (years); range	47.8; 17-79	41.0; 22-57
Sex female : male	31:14	26:15
EBV serology pos. : neg.	43:2	40:1
Treatment:*		
untreated	16	
aurothiomalate	17	
oral auranofin	3	
hydroxychloroquine	3	
sulfasalazine	4	
D-penicillinamine	1	
NSAID only	1	

*At the time point of testing EBV-induced immunoglobulin synthesis

Table 2.

Parameters of patients grouped according to the synthesis early in the disease of IgM during the 4th week of EBV-induced lymphocyte culture.

	High synthesis*	Normal synthesis	p
Number	15	30	
Age (years at onset)	54,9 ± 14,5	47,5 ± 14,6	ns
Sex female : male	11:4	20:10	ns
HLADR4 pos.:neg.	6:9	18:12	ns
Early in the disease:			
ESR (mm/h)	36,2 ± 33,5	23,2 ± 21,3	ns
CRP (mg/L)	28,9 ± 37,5	13,2 ± 22,3	ns
WaRo (titer)	124 ± 264	253 ± 462	ns
Ritchie index	12,5 ± 4,2	10,3 ± 5,0	ns
Mallya index	2,4 ± 0,7	2,2 ± 0,5	ns
Erosions			
eroded: not eroded	5:10	12:18	ns
Number of eroded joints	2,4 ± 5,1	0,9 ± 1,8	ns
Larsens index	10,3 ± 16,8	5,2 ± 8,0	ns
Two years later:			
ESR (mm/h)	30,9 ± 31,6	16,5 ± 14,3	<0.05
CRP (mg/L)	26,0 ± 34,2	17,8 ± 21,6	ns
WaRo (titer)	229 ± 288	163 ± 246	ns
Ritchie index	7,1 ± 5,7	4,5 ± 4,3	ns
Mallya index	2,4 ± 0,8	1,9 ± 0,7	<0.05
Erosions			
eroded:not eroded	14:1	16:14	<0.01
Number of eroded joints	8,1 ± 9,3	2,8 ± 3,4	<0.01
Larsens index	32,2 ± 45,5	10,3 ± 11,5	<0.05

*Synthesis is regarded as "high" when the concentration exceeded by 2SD the mean synthesis of Ig by control cells.
The results are expressed as the mean ± SD. The grouped data are analysed using k^2-statistics.

Table 3.

Some demographic and clinical parameters of RA patients at the onset of disease. The patients are grouped according to the development of eroded joints at the end of the two year follow-up period.

	Patients with erosions (n= 30)	Patients without erosions (n=15)	p
Age (years)	53.0 ± 15.5	43.9 ± 11.6	ns
Sex female : male	21:9	10:5	ns
HLADR4 pos. : neg.	14:16	11:4	ns
ESR (mm/h)	42.0 ± 27.0	37.0 ± 24.5	ns
CRP (mg/l)	27.5 ± 30.0	23.3 ± 31.6	ns
WaRo (titer)	329 ± 531	476 ± 1108	ns
Ritchie index	10.9 ± 4.7	9.1 ± 4.9	ns
Mallya index	2.6 ± 0.5	2.4 ± 0.6	ns
IgM (relative concentration) in the 4th week culture	10.3 ± 13.4	0.97 ± 1.47	<0.001

The results are expressed as the mean ± SD.

Table 4.

The predictive value of EBV-induced immunoglobulin synthesis in the development of erosions in RA.

Synthesis of Ig		Development of erosions			Predictivity		Sensitivity	Specificity
		Yes	No	p	PVpos	PVneg		
IgA	High	9	1	0.05	90,0%	40,0%	30,0%	93,9%
	Normal	21	14					
IgG	High	9	0	0.01	100,0%	41,7%	30,0%	100,0%
	Normal	21	15					
IgM	High	14	1	0.01	93,3%	46,7%	46,7%	93,3%
	Normal	16	14					

Immunoglobulin synthesis during the fourth week in EBV-induced cultures. Synthesis is regarded as "high" when the concentration exceeded by 2SD the mean synthesis of Ig by control cells. The grouped data are analysed using κ^2-statistics.

Legends for illustrations:

Figure 1.

The synthesis of immunoglobulin in EBV-activated cultures of blood MNC from healthy control subjects and the patients with RA. The results represent the mean concentration ± S.E.M. of IgA, IgG and IgM mg/l in culture supernatants of cultures activated with one of two batches of EBV (n=22 patients and n=23 controls). The immunoglobulin was produced during the week before harvesting. Open symbols represent controls; closed symbols, patients.(* $p<0.05$ and ** $p<0.001$).

Figure 2.

EBV-activated synthesis of IgA, IgG and IgM in cultures of blood MNC of RA patients who two years later had or did not have erosive disease. The results are expressed as relative concentration of immunoglobulin (RA/control) ± S.E.M. Open bars represent the controls; shaded bars represent the patients who remained uneroded; and the black bars represent patients who had eroded joints by the two year check. Symbols on the top of white bars: difference between patients and controls, symbols on the top of black bars: difference between the patient groups (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

Figure 3.

Distribution of patients and controls according to the EBV-induced synthesis of IgM during the fourth week of culture established early in the disease. The IgM synthesis is regarded as "above normal" if the concentration in supernatant exceeded by 2 SD the mean concentration of control supernatants. The number of control individuals is less than 41 because fourth week IgM values were not available in 5 cases.

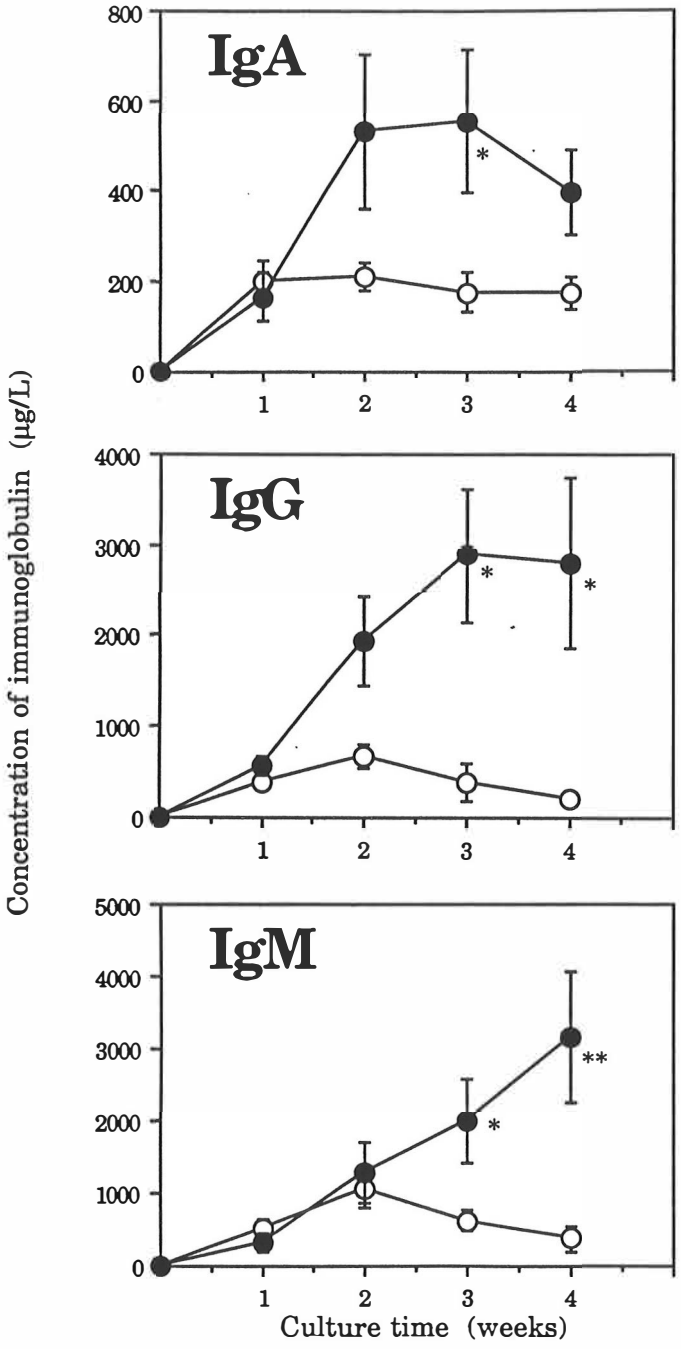
IgA
IgG
IgM
800
600
400
200
0
4000
3000
2000
1000
5000
1
2
3
4
*
**
Concentration of immunoglobulin (μg/L)
Culture time (weeks)

Figure 1.

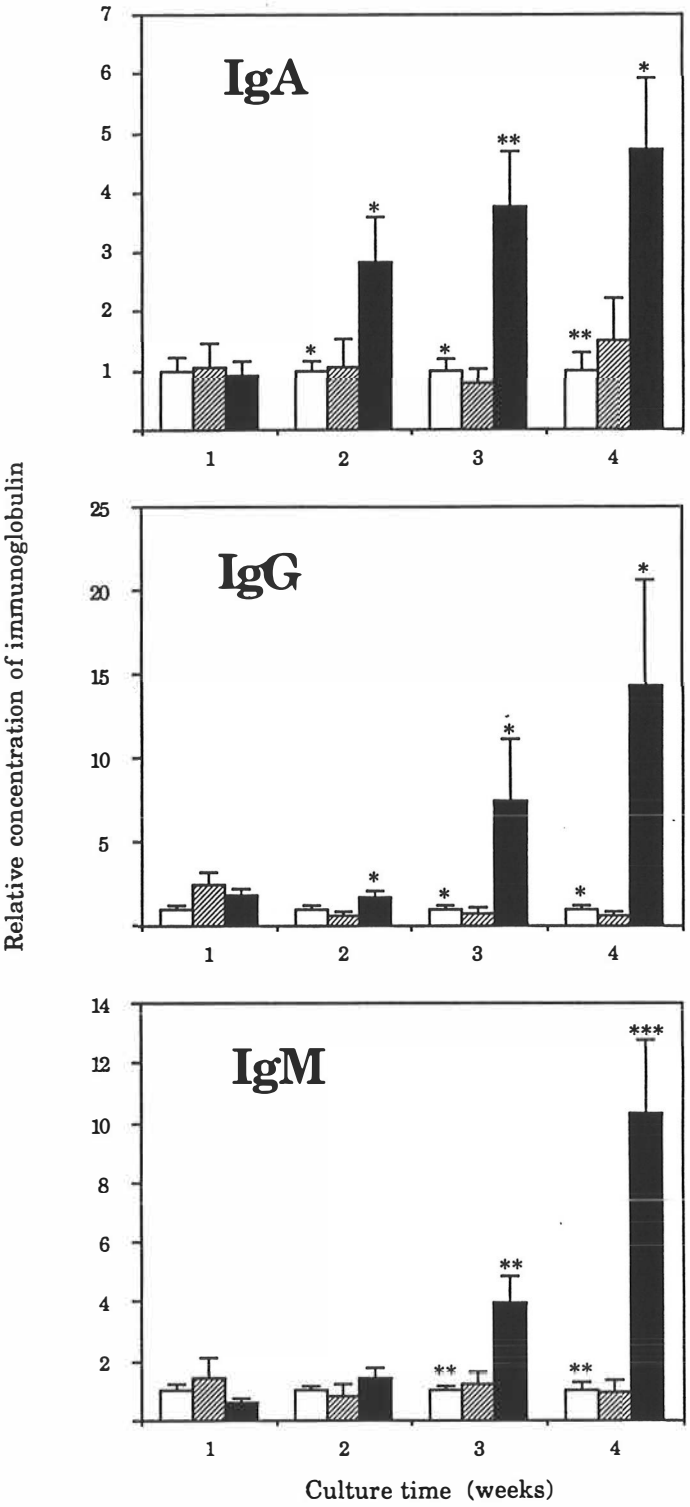
IgA
IgG
IgM
Relative concentration of immunoglobulin
Culture time (weeks)
1
2
3
4

Figure 2.

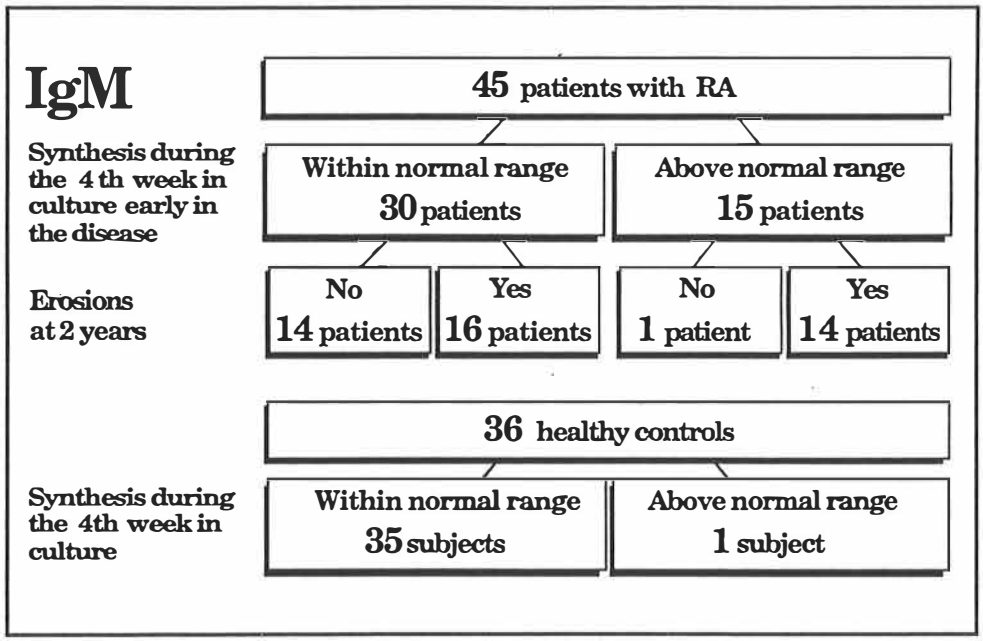
IgM
45 patients with RA
Synthesis during the 4 th week in culture early in the disease
Within normal range
30 patients
Above normal range
15 patients
Erosions at 2 years
No
14 patients
Yes
16 patients
No
1 patient
Yes
14 patients
36 healthy controls
Synthesis during the 4th week in culture
Within normal range
35 subjects
Above normal range
1 subject

Figure 3.