Spontaneously increased B cell growth factor and B cell differentiation factor activities in the synovial fluid of patients with rheumatoid arthritis

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SUMMARY The presence of factors implicated in B cell proliferation and differentiation was studied in synovial fluid (SF) from patients with rheumatoid arthritis (RA) and from patients with ankylosing spondylitis (AS) and traumatic joint injury. Culture with *Staphylococcus aureus* Cowan I B cell blasts showed strong B cell growth factor (BCGF) activity in the SF from patients with RA. This BCGF activity was significantly greater than that found in SF from patients with traumatic joint injury and similar to that of patients with AS. The presence of B cell differentiation factor (BCDF) for IgM(μ) in the SF from patients with RA was also demonstrated and was significantly greater than that found in SF from patients with AS and traumatic joint injury. Moreover, a significantly increased BCDF for IgG(γ) was also found in the SF from patients with RA compared with that observed in those patients with traumatic joint injury, which, however, was similar to that of patients with AS.

Key words: interleukins, cytokines.

Rheumatoid arthritis (RA) is a chronic, multisystem disease characterised by persistent immunological activity in the synovium infiltrated with mononuclear cells¹—predominantly activated T lymphocytes.² Evidence of B lymphocyte activation and differentiation into plasma cells can also be found in the inflamed synovium.³ The mechanisms which induce and maintain this altered immune response have not been identified, however.

Several steps are involved in the activation of resting B lymphocytes causing proliferation and differentiation into immunoglobulin secreting cells.⁴ It has been suggested that in the pathway of human B cell activation the initial signal of the cascade is provided by the specific interaction of antigen with the surface membrane immunoglobulin. This requirement can be partially circumvented by the use of polyclonal activators such as anti-immunoglobulin

antibodies and *Staphylococcus aureus* Cowan I. The cells then express receptors for specific growth factors, mainly secreted by T helper cells, B cell growth factor(s) (BCGF), and interleukin 2, formerly defined as T cell growth factor. Upon binding of these factors to their specific receptors the lymphocytes enter S phase and become cycling cells. Subsequently, B cell differentiation factors (BCDFs) promote the differentiation of B lymphocytes into immunoglobulin secreting cells.^{5,6}

Regardless of the advances toward defining the physiology of B cell activation, the role of BCGF and BCDF in the expansion and differentiation of B lymphocytes into immunoglobulin secreting cells in the synovium of patients with RA remains to be clarified.

In the present study we have demonstrated the presence of strong BCGF activity in the synovial fluid (SF) from patients with RA, which is independent of the interleukin 2 activity also found in this fluid. Furthermore, relevant BCDF activities for IgG, BCDF(γ), and for IgM, BCDF(μ), were also found in the SF from these patients. Finally, a strong

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correlation between BCDF(γ) activity and IgG concentrations in this fluid was demonstrated. The pathogenic significance of these findings is discussed.

Patients and methods

PATIENTS

Twenty nine patients were studied, of whom 13 had definite or classical RA according to the criteria of the American Rheumatism Association.⁷ Eight patients had suffered traumatic joint injury and eight had ankylosing spondylitis (AS) according to the New York criteria.⁸

Patients with RA (10 female, three male) ranged in age from 23 to 69 years, and duration of their disease varied from three months to 19 years; they were receiving non-steroidal anti-inflammatory drugs or analgesics. One patient had been taking 5 mg of prednisone daily for several months. Three others had been receiving gold treatment for years, while two patients had received penicillamine and another chloroquine. Patients with traumatic injuries to their knees (four men, aged 32 to 58 years) and those with AS (six men, aged 35 to 47 years) were taking non-steroidal anti-inflammatory drugs or analgesics.

PREPARATION OF SYNOVIAL FLUID

All SF samples were collected aseptically by aspiration of knee joints using a syringe and a 19 gauge needle. The fluid was centrifuged at 400 g for 10 minutes. Aliquots of the supernatants were stored at -70°C . Before use SF was thawed and diluted with RPMI 1640 medium (Gibco, Europe) to reach dilutions ranging from 1/2 to 1/640.

REAGENTS

S aureus Cowan I strain was purchased from Bethesda Research Laboratories (Garthersburg, MD). Recombinant interleukin 2 was obtained from Hoffman-La Roche (New York, NY). High molecular weight BCGF obtained from a T cell line and the 33B7.3 monoclonal antibody recognising the interleukin 2 receptor (Tac) were the generous gift of Dr J Ambrus (Laboratory of Immunoregulation, NIH, Bethesda, MD)⁹ and Dr D Olive¹⁰ respectively.

CELL PREPARATIONS

Human tonsils were obtained at tonsillectomy from patients with chronic tonsillitis and were dispersed into single cell suspensions. Mononuclear cells were separated by the standard Hypaque-Ficoll gradient method. B cell enriched populations were obtained from mononuclear cells by rosetting out T cells twice

with sheep erythrocytes treated with aminoethyl-isothiuronium bromide, as previously described.¹¹

CELLULAR ASSAYS FOR BCGF AND BCDF ACTIVITIES

B cells were cultured for two days with *S aureus* Cowan I (1:12 500 v/v) in RPMI 1640 containing 10% fetal calf serum and 50 $\mu\text{g}/\text{ml}$ gentamicin (complete medium). After washing, the cells were recultured in complete medium at a density of 0.5×10^5 cells/well in 96-well, flat bottomed microtitre plates (Costar, Cambridge, MA) and were incubated in 100% humidity with 5% CO_2 in air at 37°C . For proliferation assays B cells were cultured in triplicate for three days in the presence of different concentrations of SF from patients or controls. In inhibitory assays 0.5×10^6 cells/well were cultured in triplicate for three days in complete medium in the presence or absence of either recombinant interleukin 2 (100 IU/ml), 20% BCGF derived from human T cell line DND, or 20% SF from patients with RA and in the presence or absence of serial dilutions of the monoclonal antibody to Tac chain. The cultures were pulsed with 37 kBq/well of [^3H]thymidine (New England Nuclear, Boston, MA) during the last 16 to 20 hours of a 72 hour culture period. Incorporation of [^3H]thymidine was measured by standard liquid scintillation counting techniques after harvesting by a Titertek cell harvester (Flow Laboratories Inc, Rockville, MD). In the differentiation assays cells were recultured for five days with varying concentrations of SF from patients and controls. Samples of SF at the same concentrations were cultured in parallel. Total IgG and IgM in culture supernatants and in serum and SF samples was measured by an enzyme linked immunosorbent assay (ELISA) as reported.¹¹

CELLULAR ASSAY FOR INTERLEUKIN 2 ACTIVITY

The assay for detection of interleukin 2 activity in SF was performed with the murine T cell line CTL-L2 as target cells¹²; 5×10^4 cells/ml were cultured for 36 hours with serial dilutions of the SF to be assayed in 96-well, flat bottomed microtitre plates. Then 37 kBq/well [^3H]thymidine was added to the cultures for the last eight hours, and the uptake was measured as described above. Interleukin 2 units were calculated by probit analysis as described by Gillis *et al.*¹²

STATISTICAL METHODS

The group's data were compared by the unpaired Mann-Whitney U test. A p value of less than 0.05 was considered to indicate a significant difference between groups. For the correlation studies linear regression analysis was used.

Results

INCREASED BCGF ACTIVITY IN SF FROM PATIENTS WITH RA

S aureus Cowan I stimulated human B lymphocytes undergo DNA synthesis in the absence of exogenous growth factor. This B cell proliferation is short lived, though it can be maintained with BCGF.¹³ The presence of this activity in SF from patients with the chronic inflammatory arthropathies RA and AS, and patients with traumatic joint injury was investigated.

Figure 1 shows that culture with *S aureus* Cowan I B cell blasts revealed relevant BCGF activity in the SF from patients with either RA, AS, or traumatic joint injury. The B cell proliferation maintaining effect of SF from patients with RA was significantly higher, however, than that found in patients with traumatic joint injury ($p<0.05$). There was no significant difference between the BCGF activity detected in SF from patients with RA and AS ($p>0.05$). It is noteworthy that the same SF preparations showed no proliferative inducer effect upon non-stimulated B cells (data not shown).

The implications of the interleukin 2 pathway in the growth of B cells have recently been reported.^{5 14}

Fig. 1 Increased B cell growth factor activity in synovial fluid (SF) from patients with rheumatoid arthritis (RA). *Staphylococcus aureus* Cowan I B cell blasts (5×10^4 cells/well) were cultured for three days in culture medium in the presence of SF from patients with RA, ankylosing spondylitis (AS), or traumatic joint injury. Proliferation was assessed by pulsing with [^3H]thymidine for the last 16 hours of culture. Data represent the mean [^3H]thymidine uptake in triplicate wells supplemented with 25% of each SF; significance: RA v traumatic joint injury (TI), $p<0.05$.

Fig. 2 Lack of correlation between B cell growth factor (BCGF) and interleukin 2 activities in synovial fluid (SF) from patients with rheumatoid arthritis (RA). Correlation between BCGF and interleukin 2 activities was studied in SF from 10 patients with RA. BCGF activity was measured as described in Fig. 1. Interleukin 2 activity was measured by the proliferation induced by serial dilutions of the SF on CTL-L2 cells, and was transformed to units/ml by probit analysis. IL-2=interleukin 2.

Therefore, the strong B lymphocyte, proliferation maintaining effect observed in SF from patients with RA could be due to the presence of interleukin 2 molecules in this fluid. Several studies have considered this question. Figure 2 shows that interleukin 2 activity was demonstrated in SF from patients with RA. Next, the correlation between the BCGF and interleukin 2 activities present in the SF from patients with RA was investigated, but no correlation was found ($p>0.05$).

It has been shown that monoclonal antibodies against the Tac chain of the interleukin 2 receptor can inhibit the proliferative response of *S aureus* Cowan I activated B cells to interleukin 2.¹⁴ Thus we also studied the possibility that the BCGF activity found in SF from patients with RA could be inhibited by adding anti-Tac monoclonal antibody to the *S aureus* Cowan I B cell blast culture. Only slight inhibition of the B cell proliferation maintaining effect shown by the SF was found upon adding anti-Tac to the culture. Similar concentrations of the monoclonal antibody fully abrogated the proliferative effect of a recombinant interleukin 2 preparation and had no effect upon the blastogenesis of high molecular weight BCGF, however (Fig. 3).

ENHANCED BCDF(γ) AND BCDF(μ) ACTIVITIES IN SF FROM PATIENTS WITH RA It has been claimed that different BCDF molecules could be implicated in the differentiation of B

Fig. 3 Monoclonal antibody to the Tac chain of the interleukin 2 receptor cannot inhibit the strong B cell growth factor (BCGF) activity present in synovial fluid (SF) from patients with rheumatoid arthritis (RA). *Staphylococcus aureus* Cowan I B cell blasts (5×10^4 cells/well) were cultured in triplicate for three days in complete medium in presence or absence of recombinant interleukin 2 (100 IU/ml), 20% BCGF derived from the human T cell line DND, or 20% SF from patients with RA, plus a monoclonal antibody to the Tac chain. The antibody had no effect on baseline B cell proliferation. The percentage of interleukin for each preparation was calculated as $[(PA - PP) \times 100] / PA$, where PA and PP are proliferation in the absence and presence, respectively, of monoclonal antibody. The amount of DNA synthesis was determined. IL-2r=recombinant interleukin 2.

lymphocytes to diverse immunoglobulin class secreting cells.¹⁵ We investigated the BCDF(γ) and BCDF(μ) activities present in SF from the three groups of patients dealt with in this study.

Figure 4 shows that the strong IgM differentiation

inducing effect upon B cells preactivated 'in vitro' with *S aureus* Cowan I was found in SF from patients with RA. This BCDF(μ) activity was significantly more marked than that of the SF from patients with AS and traumatic joint injury ($p < 0.01$). Moreover, a significantly increased BCDF(γ) activity was found in SF from patients with RA compared with that observed in those patients with traumatic arthritis ($p < 0.05$). There was, however, no significant difference between BCDF(γ) activity present in SF from patients with RA and AS, nor between that of patients with traumatic joint injury and AS ($p > 0.05$) (Fig. 5). It is noteworthy that the same SF preparations showed no differentiation inducing effect upon non-stimulated B cells (data not shown).

As we were aware that a characteristic feature of RA is enhanced immunoglobulin concentrations in SF we assessed the possible statistical association between the BCDF(γ) and BCDF(μ) activities and the IgG and IgM concentrations present in serum and in SF from patients with RA. Interestingly, a strong correlation between the concentration of IgG and the BCDF(γ) activity present in SF from these patients was shown (Fig. 6). We found no correlation between serum IgG concentrations and the BCDF(γ) activity present in SF. Correlation was also lacking between the amount of IgM, whether in serum or in SF, and BCDF(μ) activity found in the above mentioned SF.

Discussion

Current knowledge of RA pathogenesis suggests that this disorder is highly complex. Monokines and lymphokines accumulate in the synovial effusions to produce local and systemic effects. Interleukin 1¹⁶ and interleukin 2¹⁷ were found in synovial fluids from a significant proportion of patients with RA. Rapid differentiation of B lymphocytes to plasma cells has also been demonstrated in the rheumatoid

Fig. 4 Spontaneously enhanced B cell differentiation factor μ (BCDF(μ)) activity in synovial fluid (SF) from patients with rheumatoid arthritis (RA). *Staphylococcus aureus* Cowan I. B cell blasts (5×10^4 cells/well) were cultured in complete medium in the presence of SF from patients with RA, ankylosing spondylitis, or traumatic arthritis for five days. IgM production was determined on day 5 using an enzyme linked immunosorbent assay (ELISA). Data represent the mean in triplicate wells supplemented with 25% of each SF.

Fig. 5 Spontaneously enhanced B cell differentiation factor γ (BCDF(γ)) activity in synovial fluid (SF) from patients with rheumatoid arthritis (RA). *Staphylococcus aureus* Cowan I B cell blasts (5×10^4 cells/well) were cultured in complete medium in the presence of SF from patients with RA, ankylosing spondylitis, or traumatic arthritis for five days. IgG production was determined on day 5 using an enzyme linked immunosorbent assay (ELISA). Data represent the mean in triplicate wells supplemented with 25% of each SF.

Fig. 6 Close correlation between IgG concentrations and B cell differentiation factor γ (BCDF(γ)) activity present in synovial fluid (SF) from patients with rheumatoid arthritis (RA) is found. SF IgG was determined by standard nephelometric techniques and is expressed as mg/l. BCDF(γ) activity in SF was measured as described in Fig. 5.

synovium.¹⁸ This, together with the recent descriptions of cytokines that modulate the growth and differentiation of B lymphocytes, has led us to focus our interest on the presence of immunoregulatory B cell factors in the SF of patients with RA. Thus a bioassay to measure the replication and immunoglobulin secretion of responsive *S aureus* Cowan I activated B cells was used. With this experimental system we found BCGF and BCDF(γ) in all joint fluids examined, including those from patients with AS and traumatic joint injury, though in significantly higher levels in RA and AS than in traumatic joint injury. BCDF(μ) activity, however, was significantly more marked in SF from patients with RA than in SF from those with AS and traumatic joint injury. One potential pitfall of this study could relate to

the bioassay employed to test B cell stimulatory activities because B cell blasts proliferate in the presence of both T and B cell growth factor. Nevertheless, some reports have shown that, by this assay, BCGF activity can be segregated from interleukin 2 activity.^{5 14} In the present study, specific approaches to remedy those technical difficulties have been applied. The results obtained strongly suggest that the intense BCGF activity present in these fluids is not induced by interleukin 2. The following reasons support this contention. Firstly, there was no significant correlation between BCGF and interleukin 2 activities present in the SF from these individuals. Secondly, the BCGF activity present in the SF was only slightly inhibited by the addition to the culture medium of anti-interleukin 2 receptor monoclonal antibodies that fully abrogate the proliferative effect of high concentrations of recombinant interleukin 2. Further studies will be necessary to define the predominant growth factor present in the SF as at least four different lymphokines—lymphotoxin, interleukin 4, and two BCGFs—and two monokines—tumour necrosis factor α and interleukin 1—have now been identified as enhancers of human B cell proliferation.^{6 9 19 20} The strong BCDF(γ) and BCDF(μ) activities shown to be present in SF from patients with RA are also of considerable interest. The presence of these factors can be related to the rapid differentiation of B lymphocytes to immunoglobulin secretory cells observed in synovium from patients with RA.¹⁸ The significant correlation between immunoglobulin concentration and BCDF(γ) activity in the SF also indicated the possible pathogenetic role of these factors in inducing and maintaining the altered immune response found in the synovium. Recent studies by some of the authors of the present report describe a significant correlation between BCDF(γ) production by lymphocytes and

the IgG concentrations and anti-DNA antibodies in the sera of patients with systemic lupus erythematosus.²¹ These findings further support the in vivo role of BCDF under both physiological and pathological conditions.

In addition, the existence of relevant amounts of these immunoregulatory activities in the SF from patients with AS, as well as from those with RA, also suggests the pathogenetic implication of the molecules present in the SF in these inflammatory arthropathies. Further studies must be performed to analyse the possible association between the levels of these BCGF, BCDF(γ), and BCDF(μ) activities and the clinical course of the disease.

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