Lymphocyte studies in rheumatoid arthritis

II. Antibody-mediated and mitogen-induced lymphocyte cytotoxicity in synovial fluid and peripheral blood

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SUMMARY A comparison was made of the activity of synovial fluid (SF) lymphocytes with peripheral blood lymphocytes in antibody-mediated and mitogen-induced lymphocyte cytotoxicity in patients with a variety of inflammatory joint diseases. SF lymphocytes consistently showed little or no antibody-mediated cytotoxicity (AMC) although mitogen-induced cytotoxic activity was comparable with that of the peripheral blood lymphocytes. Blocking substances on the cell surface were not responsible for the lack of AMC by SF lymphocytes as preincubation at 37°C and enzyme treatment (trypsin, neuraminidase) of the cells did not restore activity. The lack of AMC by SF cells from a variety of inflammatory joint fluids demonstrates that this may be a consequence of inflammation in the joint and excludes the possibility that this is a specific property of fluids from certain conditions such as rheumatoid arthritis.

Lymphocytes thought to be involved in AMC have a characteristic surface morphology (Fc receptor positive, E rosette negative, surface immunoglobulin negative). Such lymphocytes are present in synovial fluid in comparable proportions to those in blood. Hence the absence of AMC indicates that functional assays must be used in determining the presence or absence of cells with special functions.

It is accepted that much of the tissue damage observed in the joints of patients with rheumatoid arthritis (RA) and in those with other inflammatory joint diseases is a result of cell-mediated immunity. Consequently the study of lymphocytes both by morphological and by functional techniques has attracted considerable interest. In particular the study of cytotoxic lymphocytes may be relevant not only in the pathology of the inflammatory arthritides, by causing cell damage, but also in the persistence of disease, by their ability to eradicate antigen-bearing target cells in the synovium.

Although abnormalities in the distribution of lymphocyte populations in patients with rheumatoid arthritis have been reported, especially in synovial fluids, it is not clear whether these differences are specific to RA (Winchester, 1973). Similar confusion exists with respect to tests of lymphocyte function.

Consequently peripheral blood and synovial fluid lymphocytes from patients with inflammatory arthritis (rheumatoid arthritis, ankylosing spondy-

litis, Reiter's disease, Behcet's disease, and psoriatic arthritis) were used in antibody-mediated and mitogen-induced lymphocytotoxic tests in an attempt to discover whether abnormalities shown by the synovial fluid lymphocytes were disease-specific or a consequence of the inflammatory response in the joint.

Materials and methods

TARGET CELLS

Chang human liver cells (Flow Labs) were cultured in Eagles suspension medium (Gibco Bio-Cult) supplemented with 10% fetal calf serum (FCS), 200 mmol/l glutamine, and 2 µg/ml gentamycin and buffered with 20 mmol/l Hepes and 0.035% sodium bicarbonate. The cells were shaken off as required.

ANTISERUM

AntiChang antiserum (ACh) was raised in rabbits (Panayi, 1977) and used at 10⁻³ dilution in the cytotoxic test.

MITOGENS

After preliminary experiments an optimum dose of each mitogen was selected for use: purified phytohaemagglutinin (PHA; Wellcome Laboratories) at 1 µg/ml, concanavalin A (Con A; Miles Laboratories) at 10 µg/ml, and pokeweed mitogen (PWM; Gibco Bio-Cult) at a 1/100 final dilution.

PATIENTS

Patients with rheumatoid arthritis (RA) and other inflammatory joint diseases (OID)—including Reiter's disease, Behcet's disease, psoriatic arthritis, and ankylosing spondylitis—were used whenever synovial fluid (SF) and peripheral blood could be taken simultaneously. The patients were taking a variety of drugs.

LYMPHOCYTE SEPARATION

Twenty ml of peripheral blood and SF were taken into sterile universals (Sterilin) containing 100 IU of preservative free heparin (Boots).

Heparinised blood was separated on a Triosil-Ficoll density gradient (Böyum, 1968). The mononuclear cells obtained from the interface were washed twice in Eagles medium supplemented with 10% FCS (TCM) and adjusted to $2 \cdot 10^6$ cells/ml. There were approximately 85–95% lymphocytes and 5–15% monocytes.

Heparinised SF was spun at 500 *g* for 10 min at 4°C, and the cells washed once in TCM. They were then resuspended in 10 ml TCM and separated on a Triosil-Ficoll gradient. Cells at the interface were washed twice in TCM and adjusted to $2 \cdot 10^6$ /ml. There were approximately 70–90% mononuclear and 10–30% polymorphonuclear cells. After separation the cells were 99% viable, by trypan blue exclusion.

PRE-INCUBATION OF LYMPHOCYTES

Peripheral blood lymphocytes (PBL) and synovial fluid lymphocytes (SFL) were reincubated in TCM in plastic tubes (NUNC) at 37°C for 1 hour or 22 hours, and were then washed twice in TCM before being used in further experiments.

ENZYME TREATMENT OF LYMPHOCYTES

All enzyme treatments involved incubating cells in serum-free TCM at a concentration of $5\text{--}10 \times 10^6$ lymphocytes/ml. Neuraminidase (Sigma) was used at 25 IU/ml for 30 min at 37°C. Trypsin (Gibco Bio-Cult) was added to a final dilution of 0.25% for 5 min at 37°C. The cells were washed twice in TCM after treatment and adjusted to 2×10^6 viable cells/ml.

CYTOTOXIC SYSTEM

The antibody mediated cytotoxic (AMC) system has previously been described in detail (Panayi and Corrigan, 1977). In brief Chang cells were labelled with ^{51}Cr sodium chromate (Amersham) and were cultured in plastic tubes (Turner-Staynes) at a ratio of 1:20 with the lymphocytes. Specific antibody or mitogen was added as the cytotoxic promoting agent. The control cultures contained Chang cells and lymphocytes only. The cultures were incubated for 18 hours at 37°C and the amount of ^{51}Cr released into the supernatant was counted. Cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{{}^{51}\text{Cr in supernatant}}{\text{total } {}^{51}\text{Cr in culture}} \times 100$$

and

$$\text{specific release } {}^{51}\text{Cr} = \frac{\% \text{ cytotoxicity in test cultures} - \% \text{ cytotoxicity in control culture}}{\% \text{ cytotoxicity in control culture}}$$

Results

ANTIBODY-MEDIATED (AMC) AND MITOGEN-INDUCED (MIC) CYTOTOXICITY

The greatest difference in cytotoxic ability between SFL and PBL was seen in AMC. The cytotoxic activity of SFL was significantly less than that of PBL both in RA ($P < 0.001$) and in OID ($P < 0.02$) as can be seen from Figure 1.

By contrast there was no difference in MIC induced by PHA between SFL and PBL either in RA or OID (Fig. 1). In a further series of experiments it was found that MIC induced by PWM or Con-A also did not differ between SFL and PBL (Table 1).

EFFECTS OF VARIOUS TREATMENTS ON AMC

Pre-incubation

Pre-incubation of SFL or PBL at 37°C for 1 hour or 22 hours caused a slight but insignificant decrease in cytotoxic potential (Fig. 2). At no time and in no experiment did the AMC ability of SFL increase or approach that of autologous PBL after pre-incubation.

Enzyme treatment

Treatment of SFL or PBL with either neuraminidase or trypsin did not lead to any increase in AMC (Fig. 3). In some experiments, individual lymphocytes showed either increases or decreases in cytotoxicity after one or other enzyme treatment when compared to control lymphocytes which had not been exposed to the enzyme. These differences were not significant.

Fig. 1 The comparison of K-cell (antibody-mediated) cytotoxicity and PHA (phytohaemagglutinin)-induced cytotoxicity by peripheral blood (PBL) and synovial fluid lymphocytes (SFL) in patients with rheumatoid arthritis (RA) and other inflammatory joint diseases. In K-cell cytotoxicity, RA, SFL, and others SFL were significantly less compared to corresponding PBL ($P < 0.001$ and $P < 0.05$, respectively). There was no significant difference between PBL and SFL for PHA-induced cytotoxicity.

Table 1 Mitogen-induced cytotoxicity by synovial fluid (SFL) and peripheral blood lymphocytes (PBL) activated by concanavalin-A (Con-A)* 10 µg/ml or poke-weed mitogen (PWM; 1:100 dilution). Statistical analysis by paired t test comparing each pair of PBL and SFL showed no significant difference.

Disease	No. of patients	Con-A		PWM	
		PBL	SFL	PBL	SFL
RA	7	13.6±8.2	9.2±8.1	8.4±5.2	11.1±1.2
OID	5	22.5±6.5	19.7±9.6	15.0±2.9	13.5±1.3

Fig. 2 The effect on antibody-mediated cytotoxicity of pre-incubating peripheral blood lymphocytes (open columns) or synovial fluid lymphocytes (hatched columns) at 37°C for 1 hour (B) or 22 hours (C). There was no significant increase in cytotoxicity when compared with control (A).

EFFECTS OF VARIOUS TREATMENTS ON PHA-INDUCED MIC

As a control on the effects of the various treatments on AMC, the same treatments were applied to lymphocytes involved in PHA-induced MIC.

Pre-incubation

There was a slight decrease in MIC after 1 hour pre-incubation at 37°C which decreased further after 22 hours pre-incubation. However, at no time was there a significant difference between the cytotoxic ability of PBL and SFL in this system (Fig. 4).

Enzyme treatment

Essentially the same results were obtained with MIC as with AMC (Fig. 5) except for 1 patient whose SFL showed a significant decrease in cytotoxicity after incubation in neuraminidase.

Discussion

This comparative study of PBL and SFL activity in two cytotoxic assays has shown that while the responses are equivalent in the MIC system the SFL response in AMC is greatly reduced. These results were obtained with specimens from patients with a range of inflammatory joint diseases, suggesting that any differences seen between SFL and PBL were not disease-specific but may be merely the consequence of chronic inflammation in the synovial cavity.

Fig. 3 The effect of pre-incubating blood or synovial fluid lymphocytes from patients with rheumatoid arthritis with neuraminidase (25 IU/ml) or trypsin (0.25% w/v) on K-cell cytotoxicity. Control lymphocytes were cells incubated for the same time in tissue-culture medium alone. Enzymatic pre-incubation did not increase cytotoxicity

Fig. 4 The effect of mitogen-induced cytotoxicity (phytohaemagglutinin 1 µg/ml) of pre-incubating peripheral blood lymphocytes (open columns) or synovial fluid lymphocytes (hatched columns) at 37°C for 1 hour (B) or 22 hours (C). There was no significant increase in cytotoxicity when compared with control (A)

Similar conclusions have been made on morphological criteria concerning the distribution of T and B cells in synovial fluids (van der Putte *et al.*, 1976). This is of some interest since little is known about the lymphocyte composition of chronic inflammatory lesions.

Studies of inflammatory SF have shown that T

cells are the predominant lymphocyte. A sub-population of T cells are shown to be the effector cell in a MIC system involving Chang cell target cells (Hersey *et al.*, 1976; Nelson *et al.*, 1976). Therefore these studies confirm that functionally such a sub-population of T cells exists in inflammatory joint effusions. Although SFL show decreased stimulation by mitogens when compared with PBL (Panayi, 1973), MIC activity is comparable to that of PBL. This suggests that mitogen binding to the surface of SFL and activation of the cytotoxic cell is normal and that the deficient response of SFL in mitogen-induced transformation may involve other aspects of cell metabolism, such as induction of the necessary signal following ligand-binding or different T cell subpopulations. Studies are in progress which attempt to answer this question.

The situation with respect to AMC is different. The effector cells involved in an AMC system with Chang cells as target cells are Fc receptor positive but E-rosette and surface immunoglobulin negative lymphocytes which have been called K cells (Sany *et al.*, 1976). Functional assay of K cells, in PBL populations, has shown that they form less than 0.5% of the total lymphocyte population (Panayi and Corrigan, 1976). Since 10% of PBL have the morphological characteristics of K cells it is obvious that there is a disparity between the morphological and the functional characteristics of lymphocyte sub-

Blood

Fig. 5 The effect of pre-incubating blood or synovial fluid lymphocytes from patients with rheumatoid arthritis with neuraminidase (25 IU/ml) or trypsin (0.25% w/v) on PHA-induced cytotoxicity. Control lymphocytes were cells incubated for the same time in tissue culture medium alone. Enzymatic pre-incubation did not increase cytotoxicity

populations. This study has shown that there is little or no AMC activity in inflammatory synovial fluids. Similar results have been obtained by Diaz-Jouanen *et al.* (1976) using chicken red blood cells sensitised with rabbit antibody as target cells. However, the effector cells involved in this system may be different from those involved in killing Chang cells (Nelson, *et al.*, 1976) so that the relevance of those findings to this study are not clear.

The lack of AMC by SFL was not due to adsorbed substances being present on the cell surface, as has been hypothesised (McGill and Twinn 1977; Stratton 1972), since pre-incubation of SFL at 37°C, or enzyme treatment did not restore cytotoxic activity. Diaz-Jouanen *et al.* (1976) were also unable to restore cytotoxicity by pre-incubation at 37°C. In systemic lupus erythematosus pre-incubation at 37°C will restore AMC by removal of adsorbed

substances (Feldman *et al.*, 1976). Yet SF contain normal proportions of cells with the morphological characteristics of K cells (Winchester *et al.*, 1974). Hence it may be concluded that despite the morphological evidence functional K cells are absent from inflammatory SF. It may be speculated whether such cells are preferentially localised in the synovial membrane where they may be involved in the destruction of antibody-coated target cells.

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