T Lymphocyte lines from arthritic synovial fluid: establishment and function

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SUMMARY T Lymphocyte lines have been established, with the addition of interleukin-2 (IL-2), from the synovial fluid (SF) of patients with arthritis. Characterisation of seven of these SF-derived T cell lines showed them to be T3+, T11+, and to contain a significant proportion of T8+ cells (mean 41%). The proportion of T4+ cells varied among the lines, with a mean T4+/T8+ ratio of 0.6. A significant autologous mixed lymphocyte reaction (MLR) was observed only in one of four T cell lines assayed. Two of four lines assayed showed natural killer (NK) cell-like activity, while one line displayed a significant suppressor activity, suggesting that the T cell population in SF contained NK-like cells or suppressor cells, or both, either of which may be selected in the establishment of SF-derived T cell lines.

Key words: T lymphocytes, arthritis, T cell lines.

Some arthritic diseases, such as rheumatoid arthritis (RA), are characterised by the infiltration of large numbers of lymphocytes, especially T cells, into synovial tissue. The origin and function of these T cells is unclear. Assays with monoclonal antibodies to T cell surface markers indicate that in SF there is an increased proportion of T8+ cells, generally considered to be the cytotoxic/suppressor phenotype, compared with that found in the peripheral blood (PB) of RA patients. Some functional studies of the T cells isolated from synovial tissue and/or fluid have shown suppressor activity, whereas others have shown spontaneous cytotoxic activity, and another has failed to show either.

The aim of the present study was to establish T cell lines from the SF of patients with certain arthritic diseases that would allow analysis of SF T cell function. Previous studies by us have indicated that T lymphocytes isolated from the peripheral blood of patients with acute infectious mononucleosis died rapidly when cultured in vitro. If significant numbers of T cells isolated from the SF of arthritis patients also died rapidly in vitro, meaningful assays of SF T cell function would be difficult to carry out, since T cell functions have generally been defined by in vitro assays conducted over periods ranging from 15 hours to seven days, by which time many (if not all) of the T cells may have died.

In the present study we describe the death in vitro of T cells isolated from the SF of patients with three different clinical types of arthritis, show the effect of IL-2 on their survival, report on the establishment of T cell lines from the SF of such patients, and describe functional assays of some of these lines.

Materials and methods

CULTURE MEDIA Cells were cultured in RPMI 1640 (Commonwealth Serum Laboratories, Vic.) supplemented with 10% heat-inactivated human serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Where indicated this basic medium was modified by the addition of 20% crude IL-2 (supernatant from MLA 144 T cell cultures, kindly provided by Dr A Hapel, John Curtin School of Medical Research, Canberra).

LYMPHOCYTE DONORS Mononuclear cells were isolated from the SF or PB of five patients with classical or definite RA (American Rheumatism Association criteria), one patient...
with reactive arthritis (React A) due to *Yersinia enterocolitica*, and one with psoriatic arthritis (PsA), and from the PB of four healthy controls. Most patients were receiving non-steroidal anti-inflammatory drugs. Patients receiving steroid treatment were excluded.

**LYMPHOCYTE SEPARATION**

Unfractionated mononuclear (UM) cells were isolated from the SF or heparinised PB by isopyknic centrifugation on Ficoll-Paque (Pharmacia) as described previously. UM cells were mixed with 2-aminoethylisothiouronium bromide treated sheep erythrocytes and the E rosetting (T) cell population isolated essentially as previously described. In some cases the SF was added, immediately on collection, to an equal volume of culture medium containing 40% IL-2, and all solutions used in the subsequent lymphocyte separation contained 20% IL-2.

**MARKERS OF CELL DEATH**

Certain histological changes associated with cell death, classically designated as nuclear pyknosis and karyorrhexis, occur in both necrosis and apoptosis. Necrotic cells also show karyolysis and swelling of the cytoplasm, which eventually loses its basophilia, and cell boundaries become indistinct. Apoptotic cells appear to be condensed, typically with intensely eosinophilic cytoplasm, which frequently shows surface protrusion. These protuberances separate to form roughly spherical bodies sometimes containing basophilic nuclear fragments.

One commonly employed marker of cell death, dye exclusion, is a poor indicator of cell death by apoptosis. Apoptotic cells exclude dye until they undergo secondary disintegration.

**Apoptosis**

Smears were air dried, fixed with methanol, and stained with haematoxylin and eosin. The percentage of cell death by apoptosis was estimated from counts of 500 cells in each smear by an oil immersion objective. Where it was impossible to delineate apoptotic bodies in a compact cluster, the cluster was scored as one: the counts were thus only approximate.

**Dye-excluding cells**

A 20 μl cell suspension was diluted with 20 μl 0-5% aqueous trypan blue and the stained and non-stained cells were counted in a haemocytometer.

**STUDIES ON THE RESPONSE TO IL-2**

T Cells from the SF of patients were isolated either in the presence or absence of 20% IL-2 and subsequently maintained in culture media supplemented with 20% IL-2 or in unsupplemented media respectively. Twenty-four hours after seeding the percentage of dye-excluding cells and of apoptotic cells was determined in each culture.

**ESTABLISHMENT OF SF-DERIVED T CELL LINES**

T Cells were isolated from the SF of patients in the presence of 20% IL-2 and seeded into Linbro tissue culture plates (2 ml/well) at 2.5×10⁶ cells/ml in culture medium supplemented with 20% IL-2. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Culture medium was replaced approximately every four days until proliferation was evident, at which time the cultures were split 1:2 with new culture medium supplemented with 20% IL-2 and with 10⁴ irradiated (8000 cGy) autologous Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs)/ml. Subsequent splits were made approximately every four days with culture media supplemented with 20% IL-2 and irradiated autologous LCLs.

**CHARACTERISATION OF CELL SURFACE MARKERS**

Cell markers were assayed by means of monoclonal antibodies (MoAb) (Ortho-mune, Ortho Diagnostic Systems, Raritan, NJ) and fluorescein-labelled goat antimouse IgG antibody (Kirkegaard and Perry Laboratories, Gaithersburgh, MD) in conjunction with a Becton-Dickinson FACS IV cell sorter, according to the manufacturer’s instructions (Ortho-mune). The assays were carried out on some freshly isolated SF T cells, on the established SF-derived T cell lines after they had been established for greater than four weeks, and on a number of previously characterised cell lines with known markers.

**AUTOLOGOUS MIXED LYMPHOCYTE REACTION**

The autologous mixed lymphocyte reaction (AMLR) was carried out in 96-well round-bottomed microtitre plates (Disposable Products, Brisbane, Australia) in a chequerboard design with 1.0–1.5×10⁵ responder cells and an equal number of mitomycin C treated (40 μg/ml cells for 20 minutes at 37°C) stimulator cells in a final volume of 200 μl per well. All cultures were incubated at 37°C in 5% CO₂ in air for six days. 1 μCi/well of [³H]thymidine (New England Nuclear, Boston, Mass) was added 18–20 hours before harvest. The cells were harvested with a multisample harvester (Skatron) and the incorporation of [³H]thymidine determined by standard liquid scintillation counting techniques.
Results were analysed as mean counts per minute (cpm) in quadruplicate cultures minus the background cpm in responder cells when incubated alone. An AMLR was carried out on the SF-derived T cell lines after they had been established for at least four weeks. T Cells from the SF-derived lines, PB UM cells, and allogeneic LCLs from two healthy donors were used alternatively as responders or stimulators in the assays. Phytohaemagglutinin (PHA-P, Difco, Michigan) responsiveness was also assayed.

**CHROMIUM RELEASE ASSAY FOR T CELL CYTOTOXICITY**

The assay was carried out as previously described. Briefly, 100 μl effector cells (10⁶ cells/ml) were added to 100 μl chromium-51 labelled target cells (10⁵ cells/ml) in microtitre plates, the plates were lightly centrifuged (200 g, 5 min, 37°C), and incubated for five hours at 37°C. After centrifugation 100 μl of supernatant was harvested and counted for radioactivity. Assays were carried out in triplicate and the percentage specific cell lysis calculated from mean values by:

\[
\text{test release - spontaneous release \times 100}
\]

\[
\text{maximum release - spontaneous release}
\]

where test release was that obtained from target cells incubated with effector cells, spontaneous release was that from target cells incubated alone, and maximum release was that from target cells incubated in the presence of 1% sodium dodecyl sulphate. Target cells were autologous LCLs, allogeneic LCLs from other arthritis patients, and the natural killer cell (NK) sensitive K562 cell line.

**ASSAY FOR T CELL SUPPRESSOR ACTIVITY**

Cells from the SF-derived T cell lines from patient 5 or patient 7 were incubated at various concentrations with autologous PB UM responder cells (5×10⁴ cells/well) and mitomycin C treated (40 μg/ml cells for 20 min at 37°C) stimulator cells (5×10⁵ cells/well or 1×10⁸ cells/well) in 96-well flat-bottomed microtitre plates in a final volume of 200 μl/well. The cells used as stimulators consisted of equal numbers of allogeneic LCLs from two healthy donors. All cultures were incubated at 37°C in 5% CO₂ in air for five or seven days. 1 μCi/well of [³H]thymidine was added eight hours before harvest. The cells were harvested with a multiscan harvester and counted. Results are expressed as mean cpm in quadruplicate wells minus the background cpm in the autologous PB UM responder cells incubated alone.

**Results**

**DEATH OF SF CELLS IN VITRO AND RESPONSE TO IL-2**

Smears prepared from cultures of SF T cells incubated in the absence of IL-2 showed that these cultures contained an increasing number of condensed cells with intensely eosinophilic cytoplasms, basophilic nuclear fragments, and surface protuberances. These are histological markers associated with cell death by apoptosis, which remained at a low level in cultures containing 20% IL-2. The mean percentage (±SD) of cell death by apoptosis in individual cultures of SF T cells 24 h after isolation from four patients and incubated in the absence of IL-2 was 10-0±1.3, while it was significantly lower, 3.1±1.6, in cultures containing 20% IL-2 (p<0.01).

**CELL SURFACE CHARACTERISATION OF ESTABLISHED SF T CELL LINES**

Table 1 shows the percentage of T cells from established SF-derived T cell lines and of some freshly isolated SF T cells reacting with MoAb. The

<table>
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<th>Patient (diagnosis)</th>
<th>Cells reacting with MoAb (%)</th>
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<tr>
<td></td>
<td>T3⁺</td>
</tr>
<tr>
<td>SF-derived T cell lines</td>
<td></td>
</tr>
<tr>
<td>1 (React A)</td>
<td>43-0</td>
</tr>
<tr>
<td>2 (RA)</td>
<td>73-3</td>
</tr>
<tr>
<td>3 (RA)</td>
<td>47-5</td>
</tr>
<tr>
<td>4 (RA)</td>
<td>74-9</td>
</tr>
<tr>
<td>5 (PsA)</td>
<td>68-4</td>
</tr>
<tr>
<td>6 (RA)</td>
<td>78-4</td>
</tr>
<tr>
<td>7 (RA)</td>
<td>63-4</td>
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<tr>
<td>Freshly isolated SF cells</td>
<td></td>
</tr>
<tr>
<td>2 (RA)</td>
<td>87-5</td>
</tr>
<tr>
<td>3 (RA)</td>
<td>85-5</td>
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percentage of cells from the established lines that were T3+ and T11+ was generally high. The mean percentage of cells that were T8+, indicative of the cytotoxic/suppressor population, was 41%, while the mean percentage of cells that were T4+, indicative of the helper population, was 23%. In three lines the T4+/T8+ ratio was approximately 1, while in the other lines the T8+ cells predominated over T4+ cells. The mean T4+/T8+ ratio was approximately 0-6. Some lines were Leu 11a+, a marker indicative of NK cell phenotype, though the proportion was ≈25%. In contrast, the mean T4+/T8+ ratio of two populations of freshly isolated SF T cells was 2-15.

FUNCTIONAL STUDIES OF ESTABLISHED SF T CELL LINES

Only one of four established SF derived T cell lines assayed showed a significant stimulation in AMLR. Mitomycin treated T cells from the line derived from the SF of patient 5 (PsA) stimulated an AMLR of 5062±218 (mean±SD of quadruplicate cultures) with autologous PB UM cells. Mitomycin C treated T cells from the SF-derived lines from patients 1 (React A), 4 (RA), and 7 (RA) did not stimulate a significant AMLR with autologous PB UM cells. Mitomycin C treated autologous PB UM cells did not stimulate a significant AMLR when T cells from any of the SF-derived lines were used as responders. Cells from the SF-derived T cell lines did not respond significantly to PHA-P.

Table 2 shows that, where tested, established SF-derived T cell lines displayed insignificant or weak cytotoxicity to autologous and allogeneic LCLs (not HLA related) but in some cases were strongly cytotoxic to the NK-sensitive K562 cell line.

One of the established SF-derived T cell lines showed high levels of suppressor activity (Fig. 1 shows patient 5), whereas another showed little or no suppression (patient 7, not shown).

Discussion

The death in vitro of T lymphocytes isolated from the synovial fluid of arthritis patients was significantly reduced by the addition of IL-2 (p<0.01) and

Table 2  Cytotoxicity of established SF-derived T cell lines

<table>
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<tr>
<th>Patient (diagnosis)</th>
<th>Specific lysis (%)</th>
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<tr>
<td></td>
<td>Autologous LCLs</td>
<td>Allogeneic LCLs</td>
<td>K562</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 (RA)</td>
<td></td>
<td>3-5</td>
<td>7-8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 (PsA)</td>
<td></td>
<td>2-7</td>
<td>19-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 (RA)</td>
<td>6-8</td>
<td>15-5</td>
<td>61-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 (RA)</td>
<td>2-9</td>
<td>≤1</td>
<td>61-0</td>
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Fig. 1  [3H]Thymidine uptake (cpm) by autologous PB UM responder cells (5×10⁴ cells/well) incubated for five or seven days with mitomycin C treated stimulated cells at 5×10⁵ or 1×10⁶ cells/well in the presence of increasing concentrations of cells from a SF-derived T cell line (patient 5). [3H]Thymidine uptake was high if SF T cells were absent but decreased with increasing concentrations of SF T cells. [3H]Thymidine uptake was low if stimulator cells were omitted.
allowed SF-derived T cell lines to be readily established. SF T cell lines were established from greater than 75% of the patients. The use of IL-2 to generate SF T cell lines has also been recently reported, when such lines were established from approximately 25% of RA patients.

IL-2 support of the in vitro growth of T cells isolated from the SF of arthritis patients was not unexpected. SF T cells show characteristics associated with activated T cells, and IL-2 has been shown to support the growth of activated T lymphocytes. IL-2 like material has been shown in the synovial fluid of RA patients. The kinetics of the death of SF T cells in vitro after their isolation and culture in IL-2 free medium were similar to those previously described for infectious mononucleosis derived T cell lines after the removal of IL-2.

As expected, all of the established SF T cell lines that we characterised with T MoAb were T3+ and, where tested, T11+. All lines contained a significant proportion of cells that were T8+, while the proportion of T4+ cells varied among the lines, with the T8+ population predominating in over half of the lines (four of seven, T4+/T8+ ratio ≤0.5) and equal proportions of T4+ and T8+ in the remainder (three of seven, T4+/T8+ ratio approximately 1). T8+ Cells also predominate in other SF T cell lines established recently. A number of studies have shown a higher proportion of T8+ T cells in the synovial fluid of RA patients than in the peripheral blood of either RA patients or normal controls. However, although the proportion of T8+ cells in the established lines was more often higher than that of the T4+ cells, the reverse was the case in the two freshly isolated SF T cell populations that subsequently gave rise to T cell lines. This suggests that T8+ cells may be selected against T4+ cells during the establishment of these lines, possibly arising from differences in responsiveness to IL-2, indicating that the T cell population in the SF-derived lines did not represent that in the SF in vivo.

The cells from only one of four established SF T cell lines showed a significant AMLR when they were used as stimulators. There was no significant AMLR when the cells from the cells lines were used as responders. This is not unexpected, as it has been shown that freshly isolated SF T cells are poor responders in the AMLR and that the previously observed stimulation of autologous or allogeneic PB lymphocytes by SF UM cells appears to be mediated largely by the non-T cell population. Moreover, a T cell-T cell AMLR has been described in the PB, where the presence of activated T cells is responsible for the generation of T cells bearing the suppressor phenotype. This may explain why the line derived from the psoriatic arthritis patient showed an AMLR (Table 2, patient 5) and suppressor activity (Fig. 1). Furthermore, these SF-derived lines are presumably polyclonal.

A number of previously reported studies showed a NK cell-like activity associated with SF lymphocyte populations. Some of the established SF T cell lines described contained a population of cells that were Leu 11a+ and were cytotoxic to the NK-sensitive K562 cell line, suggesting that these lines contained NK-like cells. However, none of the established SF T cell lines were significantly cytotoxic to either autologous or allogeneic LCLs.

One of the SF T cell lines displayed significant suppressor activity, indicating the presence of suppressor cells, while another SF T cell line displayed little suppressor activity. Although MoAb analysis indicated a significant T8+ population, generally considered to be the cytotoxic-suppressor phenotype, the proportion did not necessarily reflect functional activity. This may be due to differences in the proportions of cells that were activated or to the presence of functionally different subsets bearing particular T cell markers: for example, it has been shown that T4+ cells can also have suppressor activities.

The studies reported here suggest that the T cell population in the SF of arthritis patients contains NK-like cells or suppressor cells, or both, either of which can be selected in the establishment of SF-derived T cell lines. However, they also indicate that the T cell population in these lines does not represent that found in the SF in vivo, and thus the functional activity and antigen reactivity of these cell lines may reflect neither the types of immune reactions occurring in the rheumatoid joint nor their relative importance but rather the responsiveness of certain cells to IL-2. It will be necessary to clone cells early from the SF in order to define the functional profile of the in vivo T cell population.

We would like to thank Mr. G. Robert for technical assistance and the National Health and Medical Research Council of Australia for financial support.

References


