Characterization of IL-2 responsive synovial T lymphocytes in rheumatoid arthritis

II. Functional properties

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Summary. Lymphocytes from peripheral blood (PBL) and synovial fluid (SFL) were obtained from patients with rheumatoid arthritis (RA) and cloned under limiting-dilution conditions without prior activation but in the presence of exogenous interleukin (IL)-2. The precursor frequencies of such in vivo activated IL-2-responsive cells were higher in RA SFL (1/83) than in RA PBL (1/201) or normal PBL (1/377). These HLA-Dr/Ia-positive clones expressed T-cell markers CD3 and T101 and were either CD4- or CD8-positive but lacked NK markers CD11, CD16, and HNK-1. All such clones were cytotoxic for NK-sensitive K562 targets and NK-insensitive Raji cell targets. These cells, which most closely resemble non-major histocompatibility complex (MHC) restricted cytotoxic T (CTL) cells, are present with increased frequency in RA synovial fluids.

Key words: Synovial T cells – Non-MHC restricted CTL cells – Rheumatoid arthritis

Introduction

Several properties of lymphocytes isolated from the synovial fluids (SFL) and synovial tissues of patients with rheumatoid arthritis suggest that these cells have been activated in vivo. Such cells express altered adherence properties [1], cytotoxic activities [2], and surface antigens [3], compared with nonactivated lymphocytes. A variety of lymphokine activities (interleukin 1, interleukin 2, B-cell growth factors, and interferon) have also been identified in synovial fluid (SF) and synovial tissues (ST), further supporting the contention that there are local immune reactions occurring in the rheumatoid joint [4–6].

Previous studies have indicated that SFL include cells which respond to IL-2 in the absence of apparent in vitro activation, suggesting that the cells have been preactivated [7–9]. Phenotypic analysis of the surface markers of such IL-2-responsive cell lines and clonal populations derived from SF has indicated that the predominant marker pattern is CD4–CD8+, in contrast to similar lymphocyte populations obtained from autologous peripheral blood (PBL), among which 90% of the clones were CD4+CD8– [8]. The growth properties and requirements of the IL-2-dependent SFL were also found to differ under certain circumstances from those of PBL that had been isolated similarly. SFL were less sensitive to inhibitors present in SF than PBL clones were, and SFL clones grew under conditions in which PBL clones did not, e.g., on autologous irradiated SFL filler [7, 8].

The present report deals with the further characterization of IL-2-dependent SFL clones with respect to their cytotoxic activities and surface markers.

Materials and methods

Lymphocyte donors. Sterile synovial fluid and peripheral venous blood samples were obtained from four patients with definite or classical rheumatoid arthritis (RA) as defined by the criteria of the American Rheumatism Association. The patients were being treated with a variety of nonsteroidal antiinflammatory agents at the time of study. Peripheral venous blood was also obtained from healthy normal volunteers.

Lymphocyte separation. Mononuclear cells from blood (PBL) or synovial fluid (SFL) were isolated by centrifugation on a Ficoll-dextran gradient (LSM, Litton Bionetics, Kensington, Md., USA) [10]. The cells were washed in Hanks’ balanced salt solution (Gibco, Grand Island, NY, USA), and cultured in RPMI 1640 (Gibco) containing l-glutamine, 25 mM Hepes (KC Biologicals, Lenexa, Kansas, Md., USA), 100 μg/ml penicillin G, 100 μg/ml streptomycin (Gibco), 50 μg/ml gentamicin (Schering Corporation, Kenilworth, NJ, USA), and 10% fetal calf serum (FCS; BDH, Rexdale, Ontario, Canada) at 37°C in a humidified 5% CO2 atmosphere.

IL-2-responsive lymphocytes. Lymphocytes from the various sources were adjusted to 100, 200, 400, 600, 800, and 1000 cells per well in round-bottom microtiter trays and cultured in the presence of 104 autologous irradiated (50 GY) PBL in RPMI-1640 10% fetal calf serum and IL-2-containing medium (see below). Twenty-four replicate microcultures were initiated for each cell concentration and for the irradiated filler cells alone. The cultures were fed on day 7 with 100 μl IL-2-containing medium. The cultures were
microscopically scored for growth, and those at limiting dilutions [11] were subcultured in fresh IL-2-containing medium. The' clones were fed by dilution in fresh IL-2-containing media every 3 to 4 days.

Cytotoxicity assay. Mycoplasma-free K562 and Raji cells were grown in RPMI-1640, 10% FCS. The cytotoxic activity of the clones was determined in a chromium-release assay [12]. Cells were labelled by incubating 5x10^6 cells in 0.5 ml RPMI-1640, 10% FCS, containing 300 μCi Na_2^35CrO_4 (New England Nuclear, Boston Mass., USA) for 90 min at 37°C. The cells were washed twice in RPMI-1640 and resuspended to a final concentration of 5x10^4 cells/ml. In all assays 5x10^3 target cells were used and spontaneous release was less than 10%.

Precursor-frequency estimates of cytotoxic cells. Cultures from the limiting-dilution assays described above were tested for cytotoxic activity on day 14 of culture. The cultures were washed twice in RPMI-1640, 10% FCS, and resuspended in 200 μl of the same medium; 100 μl of the suspension was added to 5x10^5 labelled target cells in 100 μl in a V-bottom microtiter tray. The plates were centrifuged at 80 g for 3 min, incubated at 37°C for 4 h and centrifuged at 400 g for 10 min. A 100 μl sample of the cell-free supernatant was removed and assayed for radioactivity. Positive wells were defined as those in which ^51Cr release exceeded third standard deviations from the mean of the release value observed for control cultures which had received only irradiated filler cells and IL-2 during the limiting-dilution assay.

IL-2 sources. A single batch of IL-2-containing medium was used for the limiting-dilution analysis. The IL-2-containing medium was produced by pulse-stimulating pooled human tonsil cell suspensions with 0.1% phytohemagglutinin-P (PHA) (Difco, Detroit, Mich., USA) for 4 h, washing the cells three times in fresh lectin-free media containing 1% FCS and culturing at 2x10^6 cells/ml for 36 h. The supernatants were collected by centrifugation, aliquoted, and stored at -20°C until required. Partially purified human IL-2 (Electro Nucleonics, Fairfield, NJ, USA) was used to maintain the clones.

IL-2 assay. IL-2 was assayed using the IL-2-dependent cell line CTLL-2 in a modification of the microassay of Gillis et al. [13]. Briefly, 5x10^5 cells were cultured for 48 h in the presence of doubling dilutions of the test samples, pulse-labelled for the final 4 h of culture with 0.2 μCi [3H]-thymidine (Amersham, Oakville, Ontario, Canada) and harvested onto glass filter papers. The level of radioactivity incorporated was assessed by liquid-scintillation spectrometry. The levels of IL-2 were determined by comparing test samples with a laboratory IL-2 standard which was included in each IL-2 assay.

Cell-surface marker analysis. Markers analysis was performed using the Immunobead and Quantigen systems of Bio-Rad Laboratories, Mississauga, Ontario, Canada. These systems employ polycrylamide beads of two different colors, having covalently bound antibodies to human immunoglobulin or T-cell antigen TIO1 [14] in the Quantigen system, or to CD4 and CD8 [15] in the Quantigen T4/T8 system. These beads are mixed with the cell sample, centrifuged and incubated at 37°C, after which rosetted cells are enumerated microscopically. Since these systems have marker beads of two different colors, it is possible to enumerate two markers simultaneously on a given sample.

For the remainder of the markers employed, i.e., CD3 [15], CD16 [16], HNK-1 [17], and CD11 [18], a modified immunobead system was employed. Immunobeads coated with rabbit anti-mouse immunoglobulins (Bio-Rad) were preincubated with a predetermined optimal amount of one of the above antisera for 1 h, washed three times by centrifugation and used in the rosetting assay described for the Quantigen systems. Preliminary experiments comparing immunofluorescence and immunobead rosetting had indicated that the methods were of comparable sensitivity.

Results

The curves depicting the proportion of nonresponding cultures (fmr) versus no. of cells per culture were linear on a semilog plot and passed through 1.0 for zero cell input (Fig. 1). These results are compatible with single-hit phenomena [11]. Consequently it is justified to make precursor-frequency estimates from these curves. For four patients where paired samples were available, the frequency of IL-2-responsive cells in RA SFL (1/83) was higher than that observed for autologous RA PBL (1/201) samples, which was itself greater than that found for normal PBL (1/377); (Table 1). These differences were apparent on

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Precursor frequency for IL-2-responsiveness</th>
<th>K562 cytotoxicity</th>
<th>Raji cytotoxicity</th>
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<tr>
<td>I</td>
<td>RA PBL</td>
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<td>1/202</td>
</tr>
<tr>
<td>I</td>
<td>RA SFL</td>
<td>1/101</td>
<td>1/101</td>
</tr>
<tr>
<td>II</td>
<td>RA PBL</td>
<td>1/180</td>
<td>1/180</td>
</tr>
<tr>
<td>III</td>
<td>RA PBL</td>
<td>1/70</td>
<td>1/70</td>
</tr>
<tr>
<td>IV</td>
<td>RA PBL</td>
<td>1/201</td>
<td>1/201</td>
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<tr>
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<td>1/80</td>
<td>1/80</td>
</tr>
<tr>
<td>VI</td>
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<td>1/390</td>
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<tr>
<td>VII</td>
<td>RA PBL</td>
<td>1/340</td>
<td>1/300</td>
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Fig. 1. Titration curves of spontaneously IL-2-responsive lymphocytes derived from normal PBL (□), RA PBL (×), and RA SFL (▲). The indicated numbers of responder cells were cultured in the presence of IL-2 and 10^5 autologous irradiated PBL. The cultures were scored for growth on day 14. Identical curves in the same cultures were obtained for cytotoxicity on day 14.