Functional capacities of T lymphocyte subsets from synovial fluid and blood in rheumatoid arthritis

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SUMMARY A reverse haemolytic plaque forming cell (PFC) assay was employed to analyse the impact of T suppressor/cytotoxic and T helper cells on B cell function in 10 patients with rheumatoid arthritis (RA). In all cases T8-enriched cells from synovial fluid and blood suppressed the pokeweed mitogen (PWM) induced IgM, IgG, and IgA secretion by autologous lymphocytes to the same degree. The suppression was partly abolished by irradiation of T8-enriched cells. T4-enriched cells from blood increased the PWM induced Ig secretion by autologous blood B cells. In six of 10 patients responses 1.2 to four times higher were obtained with T4-enriched cells from synovial fluid, but in four of 10 patients synovial fluid T4-enriched cells did not increase the PWM responses of blood B cells. T4- and T8-enriched T cells from synovial fluid comprised more Ia⁺ cells than did T cells from blood (36% v 3% and 43% v 6%). Ia⁺ T helper and suppressor/cytotoxic cells may modulate in vivo activation of synovial B cells in RA.

Key words: T helper cells, T suppressor/cytotoxic cells, Ia⁺ T lymphocytes, plaque forming cells.

Several immunological disturbances in rheumatoid arthritis (RA) indicate that B lymphocytes are intimately involved in the disease process. Thus hypergammaglobulinaemia, raised titres of auto-antibodies, and immune complexes in serum are frequently found. A number of experiments in man and in mice have shown that subsets of T lymphocytes modulate B cell function. Two such subsets, T helper/inducer cells and T suppressor/cytotoxic cells, have been characterised in man by means of mouse monoclonal antibodies (Mab) raised against surface membrane glycoproteins (T4 and T8 molecules respectively).¹

Earlier data suggest that blood T suppressor/cytotoxic cells are functionally defective in RA, assessed by in vitro stimulation of blood B lymphocytes with antigen,² pokeweed mitogen,³ and Epstein-Barr virus.⁴ RA synovial tissue is infiltrated with numerous T lymphocytes, of which most belong to the T4⁺ population, whereas few are T8⁺.^{5,6} In RA synovial fluid (SF) the numbers of

T8⁺ cells are similar to or higher than the numbers of T4⁺ cells.^{7,8} Some authors have demonstrated decreased functional activity of T suppressor/cytotoxic cells isolated from RA synovial tissue;^{9,10} in one report, however, such defects could not be shown.¹¹ Little is known about the functional activities of T4⁺ cells in synovial tissue or in SF.

The gene products of the human major histocompatibility complex have important roles in a variety of immune reactions. Class II (or Ia related) gene products are easily demonstrable in the cell membrane of haemopoietic precursor cells, B cells, and monocytes/macrophages.¹² Resting T cells display low amounts of class II gene products, whereas these molecules appear on T cells activated by mitogens, antigens, or allogeneic cells.¹² Recent studies indicate that more Ia⁺ blood T cells can be found in RA patients than in normals.¹³ In addition, Ia⁺ T cells are frequently found in RA synovial tissue^{5,6} and in RA SF.¹⁴

The purposes of the present study were to compare the functional capacities of T cell subsets isolated from synovial fluid and blood of RA patients, and to characterise the T cell subsets by means of a Mab against Ia molecules.

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Patients and methods

PATIENTS

Ten patients with definite or classical RA according to the American Rheumatism Association criteria were studied. Nine of the patients were seropositive. One patient was receiving gold salts, two penicillamine, and one prednisone (dosage 10 mg daily) at the time of study. All patients were treated with non-steroidal anti-inflammatory drugs. Non-haemorrhagic SF was obtained in all cases by therapeutic knee puncture; a blood specimen was drawn the same day.

CELLS

Mononuclear cells from blood (BMC) or from synovial fluid (SF) (SMC) were prepared by centrifugation on Ficoll/Hypaque, washed, and resuspended in RPMI 1640 (Gibco, Paisley, Scotland), supplemented with 10% fetal calf serum (FCS) (Flow, Irvine, Scotland), antibiotics, and L-glutamine exactly as described previously.¹⁵

BMC and SMC were rosetted with sheep red blood cells (SRBC) treated with 2-aminoethylisothiuronium (AET) (Sigma, St Louis, Mo., USA); T-depleted cells (B cells and monocytes/macrophages) and T-enriched cells were isolated as described previously.¹⁵

Blood and SF T-enriched cells were further purified by the following procedures, modified from Egeland and Lea.¹⁶ Firstly, one volume of ox red blood cells (ORBC) was mixed with one volume of rabbit antimouse Ig (RAM) (diluted 1:16 in 0.9% NaCl; Dakopatts, Copenhagen, Denmark) and 10 volumes of CrCl₃ (diluted 1:150 in 0.9% NaCl), incubated for 1 h at 30°C, washed, and resuspended (1% v/v) in RPMI with 10% FCS. Secondly, 100 µl OKT4 or OKT8 (Ortho Diagnostics, Raritan, NJ, USA), diluted 1:10 in RPMI, was added to 2×10⁶ T-enriched cells. Cells were incubated for 30 min at 4°C and washed twice. Thirdly, the T cells were incubated with 600 µl RAM-ORBC for 30 min at 37°C, centrifuged for 5 min at 72 g (to avoid unspecific rosette formation), gently resuspended, and again centrifuged on Ficoll/Hypaque for 30 min at 300 g. Interphase cells were then washed three times. The interphase cells obtained after treatment with OKT8 Mab were used as T4-enriched cells; similarly, T8-enriched cells were obtained from OKT4 treated suspensions. In some experiments RAM-ORBC rosette forming cells were deprived of ORBC by lysis with distilled water for 30 s and washed three times; these cells will be designated T4*- and T8*-enriched cells.

Half of the T4- and T8-enriched cells were

irradiated in a ¹³⁷Cs source (600 rad/min) with a dose of 2500 rad.

IDENTIFICATION OF CELLS

100 µl cells (2–5×10⁶/ml in RPMI 1640 with 10% FCS) were incubated with 25 µl OKT4, OKT8, or mouse monoclonal antihuman Ia (NEN Nuclear, England) (all diluted 1:20 in RPMI) for 30 min at 4°C, washed twice, incubated with fluorescein isothiocyanate conjugated RAM (20 µl, diluted 1:10 in RPMI) for 30 min at 4°C, and washed twice. B cells were identified as surface membrane immunoglobulin (SMIg) positive cells after staining with FITC conjugated antihuman µ, γ, α, κ, and λ chains (Dakopatts). T subsets and B cells were counted in a fluorescence microscope. Monocytes/macrophages were identified by cytocentrifuged preparations stained for α-naphthyl acetate esterase activity. Viable cells were identified by trypan blue exclusion.

GENERATION OF CONDITIONED MEDIUM CONTAINING INTERLEUKIN-2

T-enriched cells were obtained from five healthy adult volunteers. T cells were cultured in flasks (Nunc), 5×10⁶/ml in 5 ml RPMI 1640 containing 2% pooled human serum and phytohaemagglutinin (PHA) (PHA-P, Difco, USA), 1 µg/ml. Cells were cultured for 24 h at 37°C; cell free supernatants were incubated for 1 h with an equal volume of human erythrocytes (type O) to remove PHA, pooled, and stored at -20°C. The presence of interleukin-2 (IL-2) was tested as described previously.¹⁷ Briefly, human lymphoblasts (stored in liquid nitrogen) stimulated by concanavalin A were cultured for 48 h with or without 50% conditioned medium (CM); 50 µCi [¹⁴C]thymidine was added 24 h before harvest, and thymidine incorporation was measured by liquid scintillation counting. Two representative experiments showed the following: without CM, 141 and 196 cpm/10⁵ cells; with CM, 17 230 and 13 017 cpm/10⁵ cells.

GENERATION OF IMMUNOGLOBULIN SECRETING CELLS

BMC, autologous mixtures of BMC and T subsets, or mixtures of T-depleted cells and T subsets, were cultured in round bottomed microtitre plates (Nunc, Roskilde, Denmark) at a concentration of 0.5×10⁶/ml, 200 µl/well. Mixtures of cells were always based on the numbers of viable cells. Pokeweed mitogen (PWM) was added at the onset of culture (0.8 µg/ml). Cells were cultured for six days in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. After harvest, cells were washed once in minimal essential medium (Flow).

In some experiments IL-2 containing CM was added (50% v/v) at the onset of culture.

QUANTIFICATION OF IMMUNOGLOBULIN SECRETION

A reverse haemolytic plaque forming cell (PFC) assay was employed.¹⁸ Briefly, SRBC conjugated with Protein A (Pharmacia, Uppsala, Sweden) by CrCl_3 , cultured cells, antihuman IgM, IgG, or IgA (Dakopatts), and guinea pig serum were mixed in agar (supplemented with diethylaminoethyl-dextran) at 45°C. Four to eight drops of 200 μl were placed on microtitre plate lids (Nunc) and covered with slips. After 4 h at 37°C plaques were counted in indirect light at 10 $\times$ magnification. PFC were expressed per 10⁶ originally cultured cells.

STATISTICS

Data were analysed by means of the Mann-Whitney test, the Wilcoxon test, and the Spearman rank coefficient test. *p* Values <0.05 were considered significant.

Results

CELLULAR COMPOSITIONS

Fewer T4⁺ cells and more T8⁺ cells were found among SMC than among BMC (*p*<0.05) (Table 1), in agreement with previous results.^{7,8} SMC contained more Ia⁺ cells than BMC (Table 1), confirming earlier findings.¹⁴

T-enriched cells from blood and SF contained

Table 1 Cellular composition of BMC, SMC, and T cell subsets in RA*

	T4 ⁺ (%)	T8 ⁺ (%)	Ia ⁺ (%)
BMC	60 (53-68)	28 (21-40)	17 (7-41)
SMC	45 (21-83)	50 (31-56)	63 (43-70)
Blood T4-enriched cells [‡]	94 (86-98)	3 (0-4)	3 (0-12)
SF T4-enriched cells [‡]	95 (88-97)	3 (0-4)	36 (12-49)
Blood T8-enriched cells [§]	5 (2-6)	89 (85-95)	6 (0-15)
SF T8-enriched cells	7 (2-10)	87 (82-93)	43 (16-69)

*BMC and SMC were analysed in 10 patients, T subsets in seven patients. Medians are shown with ranges in parentheses. The yields of T-enriched cells (as determined by E rosette formation) by AET treatment of mononuclear cells were: blood, 76% (65-80); SF, 73% (55-88). The viability of all subsets surpassed 90%.

[‡]The yield of T4⁺ cells obtained from T-enriched cells: 73% (50-89).

[§]Yield: 65% (43-85).

^{||}Yield: 62% (42-70).

^{||}Yield: 67% (40-79).

>90% E rosetting cells, <1% SMiG positive cells, and <1% monocytes/macrophages. The purities of T4- and T8-enriched cells are shown in Table 1. Approximately one quarter of T cells were lost during separation with AET-SRBC, and a further third were lost preparing T4- and T8- enriched cells (Table 1).

Approximately 40% of SF T subsets were Ia⁺ (Table 1), as compared with approximately 5% of blood T subsets (*p*<0.01).

The compositions of T4⁺- and T8⁺-enriched cells obtained from RAM-ORBC rosetting cells were similar to those of T4- and T8-enriched cells (data not shown); the yields, however, were only about one half.

FUNCTIONAL ACTIVITIES OF T8-ENRICHED CELLS

Firstly, T8-enriched cells were cultured with blood T-depleted cells. In all cases the PWM induced secretion of IgM, IgG, and IgA was low (Table 2), comparable to the results obtained with unstimulated cells or with T-depleted cells cultured alone. Similar experiments were carried out with irradiated T8-enriched cells; again, the response to PWM was low (data not shown). Next, T8-enriched cells were added to BMC as shown in Table 2. Blood and SF T8-enriched cells suppressed the PWM-driven Ig secretion to similar degrees. Irradiated T8-enriched cells were less suppressive (Table 2); the responses obtained with irradiated T8-enriched cells were two to four times higher than the results with experiments using non-irradiated cells. The differences, however, did not reach statistical significance.

The suppressive capacity of T8-enriched cells did not correlate with the numbers of Ia⁺ cells.

In three patients, blood and SF T8⁺-enriched cells were assayed as described above. The suppressive activity of these cells was similar to that of T8-enriched cells (data not shown).

The recovery of cells after PWM stimulation was decreased by addition of T8-enriched cells, though to a lesser degree compared with PFC formation (Table 2).

FUNCTIONAL CAPACITIES OF T4-ENRICHED CELLS

Fig. 1 shows that addition of blood T4-enriched cells to T-depleted cells reconstituted the PWM induced IgM, IgG, and IgA responses. In comparison, addition of T4-enriched cells from SF in six of 10 patients resulted in responses 1.2 to four times higher than those seen with blood T4-enriched cells (Fig. 1). In four of 10 patients SF T4-enriched cells did not increase PWM induced Ig secretion (Fig. 1). The results in these four patients did not correlate

Table 2 Functional capacities of T8-enriched cells from 10 RA patients*

Cell combinations	PWM	IgM-PFC	IgG-PFC	IgA-PFC	Recovery (%)
BMC	—	85 (0–259)	221 (0–382)	316 (0–570)	23 (11–36)
BMC	+	2264 (655–4508)	2375 (616–10412)	1742 (460–8904)	45 (22–63)
T-depleted cells	+	55 (0–251)	165 (0–363)	40 (0–85)	9 (5–16)
T-depleted cells + blood T8-enriched cells [†]	+	221 (8–365)	366 (14–491)	65 (0–197)	20 (13–28)
T-depleted cells + SF T8-enriched cells [†]	+	85 (0–304)	291 (80–582)	154 (72–360)	25 (5–33)
BMC + blood T8-enriched cells	+	415 (278–2630)	508 (166–3004)	411 (160–1920)	27 (15–36)
BMC + irradiated blood T8-enriched cells	+	1080 (245–3844)	2111 (550–4175)	962 (333–4110)	35 (17–44)
BMC + SF T8-enriched cells	+	321 (0–504)	250 (0–602)	308 (0–366)	28 (7–52)
BMC + irradiated SF T8-enriched cells	+	915 (165–5500)	1260 (355–6408)	933 (115–3675)	31 (8–58)

*BMC, autologous mixtures of 50% T-depleted cells and 50% T8-enriched cells, or autologous mixtures of 50% BMC and 50% T8-enriched cells were cultured for six days with PWM. Data represent PFC/10⁶ originally cultured cells (median and range); recovery of cells was calculated from the numbers of viable cells after six days' culture.

[†]Parallel experiments were performed using irradiated T8-enriched cells with similar results.

Fig. 1 Functional capacities of blood and SF T4-enriched cells. Blood T-depleted cells were cocultured with autologous T4-enriched cells for six days in the presence of PWM. Ordinate: PFC/10⁶ originally cultured cells.

with the numbers of contaminating T8⁺ cells among T4-enriched cells. Irradiation of SF T4-enriched cells in these four cases did not alter the low responses. No correlation was found between drug therapy and functional activity of T4-enriched cells.

In parallel experiments T4-enriched cells were added to unseparated BMC. Table 3 shows that the highest responses to PWM were obtained by adding SF T4-enriched cells; interestingly, even SF T4-

enriched cells that did not increase the responses of T-depleted cells showed this ability to stimulate BMC (demonstrated in all three patients studied). The capacity of SF T4-enriched cells to increase the Ig secretion of T-depleted cells did not correlate with the number of Ia⁺ cells.

The ability of irradiated T4-enriched cells to increase Ig secretion was lower compared with results from experiments with non-irradiated cells. This effect was most pronounced in cultures of T-depleted cells (reduction to approximately 40%) (data not shown), whereas the responses in cultures of BMC were decreased to lower degrees (reduction to approximately 80%) (Table 3).

In three patients T4*-enriched cells from blood and SF were used. The PWM responses were similar to those obtained with T4-enriched cells (data not shown).

Previous experiments showed that addition of CM containing IL-2 (30–50% v/v) increased the PWM PFC responses of B cells cultured with helper T cells 1.5 to three times.¹⁹ Therefore, the impact of CM containing IL-2 on Ig secretion was evaluated in four selected patients as shown in Table 4. In two of these the PWM responses of cultures containing SF T4-enriched cells were increased to approximately the same degree by IL-2 as that observed in cultures containing blood T4-enriched cells. In the two other patients low responses of cultures containing SF T4-enriched cells were not increased by IL-2.

Table 3 Functional capacities of T4-enriched cells from seven RA patients*

Cell combinations	PWM	IgM-PFC	IgG-PFC	IgA-PFC
BMC	—	105 (0–259)	206 (0–340)	316 (0–540)
BMC	+	1721 (655–3585)	2700 (616–10412)	1495 (460–8904)
BMC+blood T4-enriched cells	+	1456 (425–4088)	1400 (250–2192)	1248 (240–6608)
BMC+irradiated blood T4-enriched cells	+	1008 (440–7336)	1225 (314–6384)	1140 (212–8120)
BMC+SF T4-enriched cells	+	2576 (455–8960)	5740 [‡] (1248–12152)	1736 (506–12880)
BMC+irradiated SF T4-enriched cells	+	2128 (360–7304)	2176 [‡] (403–5147)	1064 (430–3920)

*Autologous mixtures of 50% BMC and 50% T4-enriched cells were cultured for six days with PWM. Data represent PFC/10⁶ originally cultured cells (medians and ranges).
[‡]p<0.05 when compared with BMC cocultured with blood T4-enriched cells.
[‡]p<0.05 when compared with BMC cocultured with SF T4-enriched cells.

Table 4 Influence of conditioned medium (CM) containing IL-2 on PWM induced Ig secretion*

Patient No	Blood T4-enriched cells added		SF T4-enriched cells added	
	–CM	+CM	–CM	+CM
1	2072	5480	8512	10 136
2	6720	9541	7144	8 500
3	896	2225	0	0
4	1400	2533	160	224

*Autologous mixtures of 50% blood T-depleted cells and T4-enriched cells were cultured for six days with PWM with or without CM (50% v/v). Data represent IgM-PFC/10⁶ originally cultured cells. Similar data were obtained in the case of IgG-PFC and IgA-PFC (not shown).

Discussion

In this study of RA patients Mab were used to isolate T cell subpopulations bearing T4 and T8 molecules. About 50% of the T subsets were recovered after the separation procedure. T4-enriched cells were contaminated with a few T8⁺ cells (and vice versa). Thymocytes may express both T4 and T8 molecules,²⁰ but it has been assumed that mature T cells belong exclusively to T4⁺ or T8⁺ subsets.²⁰ In myasthenia gravis low numbers of T4⁺T8⁺ cells can be found in the blood.²¹ Further studies using double fluorescence techniques are needed to discover whether or not RA synovial T cells comprise doubly marked cells.

The activity of synovial suppressor/cytotoxic T cells in RA has been evaluated in several studies.^{9–11 22} Most studies employed whole T cell populations. In normals, blood T8⁺ cells suppress

PWM induced Ig secretion¹ and antigen induced antibody secretion,²³ though a few T8⁺ cell lines do not display suppressive/cytotoxic activity.²⁴ Our results show that T8-enriched cells from blood and synovial fluid of RA patients suppress polyclonal Ig secretion of autologous B cells to similar degrees. The suppression was partly abolished by irradiation, which is compatible with the previously reported radiosensitivity of concanavalin A induced T suppressor cells.^{25 26} Recent data indicate that T8⁺ T cells encompass at least two suppressor/cytotoxic subpopulations, one radiosensitive and another radioresistant.²⁷

The activity of synovial T helper cells in RA has been assessed in only a few studies. Recently, McCain showed that RA SF T cells contained potent helper cells as measured by PWM-driven Ig secretion of allogeneic B cells.²⁸ The data of Egeland *et al*¹¹ and Ranki *et al*,²² however, showed a high degree of interindividual variation. In normals, T4⁺ T cells increase the PWM-driven Ig secretion¹ and the antigen induced antibody formation²⁰ by B cells, though a few T4⁺ T cell lines are cytotoxic.²⁹ In this study T4 enriched cells from blood of RA patients increased the polyclonal Ig secretion of autologous blood B cells. In six of 10 patients SF T4-enriched cells displayed a more pronounced helper activity than T4-enriched cells from the blood. In four of 10 patients, however, SF T4-enriched cells had no helper activity towards B cells. In all three of these patients so studied SF T4-enriched cells increased the responses of BMC containing T subsets as well as B cells. This discrepancy was not due to lack of IL-2 in the cultures (see Table 4); lack of expression of receptors for IL-2 on SF T cells is unlikely, but further studies are in progress to evaluate this aspect. The possibility that SF T4⁺ T cells were

suppressive/cytotoxic in some cases can be ruled out (see Table 3). Rather, the findings may be explained by heterogeneity within the T4⁺ cell population. Recent studies indicate that T4⁺ T cells in normals can be divided into at least two subgroups as determined by Mab;³⁰ one subgroup (T4⁺ JRA-TQI-) is mandatory for the induction of helper activity.³⁰ Thus the reversal of low helper activity of T4⁺ SF T cells in cultures containing blood T4⁺ cells (see Table 3) may be explained by the presence of this subgroup among RA blood T cells.³¹ This study showed that irradiated T4⁺ T cells displayed impaired helper activity compared with non-irradiated cells. The heterogeneity of T4⁺ cells may also influence the radiosensitivity of T4⁺ cells.^{32,33}

Earlier studies have shown that RA synovial T cells comprise more Ia⁺ cells than do blood T cells.^{5,14} This study showed that about one third of SF T4⁺ cells and one half of SF T8⁺ cells were Ia⁺. In healthy humans both T4⁺Ia⁺ and T4⁺Ia⁻ subsets are required for optimal Ig production by polyclonally activated B cells.³⁴ It has recently been proposed that T8⁺Ia⁺ T cells amplify the suppressive/cytotoxic activities of T8⁺Ia⁻ cells.³⁵ Thus Ia⁺ T helper and suppressor/cytotoxic cells in RA may modulate the activity of B lymphocytes in inflamed tissue.

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