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Evidence for the presence of activated CD4 T cells with naive phenotype in the peripheral blood of patients with rheumatoid arthritis

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SUMMARY

We have investigated whether T cell activation in rheumatoid arthritis (RA) preferentially engages distinct T cell subpopulations in the peripheral blood (PB) and in the synovial fluid. We found that CD25 expression was enhanced among PB CD4 T cells of RA patients as compared with CD4 cells of patients with reactive arthritis, degenerative joint disease or of healthy controls. Within the CD4 T lymphocyte subset we found that the $CD45RO^-$ (naive) cells selectively in RA displayed higher levels of CD25 protein and of interferon-gamma mRNA expression when compared with the respective subset of all other investigated groups. These results show that in the PB of RA, but not in the PB of the other arthropathies or healthy controls, $CD45RO^-$ CD4 T lymphocytes exist which display well-defined signs of activation.

Keywords rheumatoid arthritis T cell activation T cell subsets CD25 interferon-gamma

INTRODUCTION

Understanding of the immunological mechanisms underlying certain pathologic conditions such as rheumatoid arthritis (RA) necessitates the detailed analysis of the functional repertoire of the immune system. Since T lymphocytes play a major role in immune functions by direct interaction with targets or indirectly by the production of a variety of immunomodulatory factors, many investigations have focused on the phenotypical and functional characterization of T cells present in the inflammatory sites of RA. In these studies T cells from synovial tissue have been found to produce certain cytokines [1–3] and to display an activated phenotype [4]. In addition, the use of a limited T cell receptor repertoire has been postulated [5].

With regard to the peripheral blood (PB), several reports [6–8] have demonstrated *in vivo* activation of T cells in RA. In particular, these studies suggested that T lymphocytes from the PB manifest enhanced HLA-DR expression, but no or relatively few cells display the IL-2 receptor (IL-2R) α chain CD25. Possibly due to the use of more sensitive immunofluorescence measurement, a series of recent studies demonstrated that certain functional subsets of circulating T cells of even healthy donors express CD25 in relatively high frequency [9–11]. With regard to these observations the previously reported low IL-2R expression of RA T cells has to be reconsidered. In addition, increased amounts of the secreted form of CD25 were found in RA serum and its level has been correlated with disease activity [12], again suggesting significantly increased production of this molecule in RA.

In this study we asked whether the postulated enhanced CD25 expression might be due to activation of a particular T lymphocyte subset. Since CD4 T cells have been described to be the predominant T cell subset present in rheumatoid lesions [13], we analysed the CD4 cell population in terms of activation related cellular events. The CD4 T cell population has been shown to comprise of functionally different subsets subdivided by means of the almost mutual expression of CD45 isoforms [14,15]. Accordingly, we further analysed the pattern of activation antigen and lymphokine expression in the $CD45RO^+$ (memory) and in the $CD45RO^-$ (naive) CD4 subpopulations, respectively.

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SUBJECTS AND METHODS

Patients and controls

The following patients entered the study: 15 patients with RA [16] (mean age $\pm$ s.d., 67 ± 10 years), 12 of them with knee joint effusion; seven patients with reactive arthritis (age 29 ± 8 years), six of them with knee joint effusion; 13 patients with degenerative joint disease (age 63 ± 14 years) and seven healthy controls (age 57 ± 13 years). Heparinized peripheral blood samples and, if possible, joint fluid was taken from the subjects and subjected to the analysis detailed below.

Among the RA patients, three had stage I, six had stage II, two had stage III and four had stage IV, according to the classification of Steinbroker, Traegger & Batterman [17]. Four-

teen patients had active disease (ESR > 40 mm/h, > 3 joints swollen and tender, morning stiffness > 1 h), and one had inactive disease. Nine patients were positive for rheumatoid factor (laser nephelometry). At the time of investigation, six patients received disease-modifying drugs (DMD) (two were on gold therapy, one received D-penicillamine, three were on chloroquine therapy), two patients received low-dose corticosteroid therapy ($\leqslant$ 10 mg prednisolone/day); all patients were on non-steroidal anti-inflammatory drugs (NSAIDs) (mainly diclofenac). Among the patients with reactive arthritis, four were on NSAIDs (diclofenac or oxicames), three received antibiotics (tetracycline or erythromycin). Among the patients with degenerative joint disease, seven received NSAIDs (diclofenac or ibuprofen).

Cell preparation

Mononuclear cells (MNC) were prepared by density gradient centrifugation (Ficoll–Hypaque; Pharmacia, Uppsala, Sweden). T lymphocytes were separated by rosetting with neuraminidase-treated sheep red blood cells (SRBC) followed by density gradient centrifugation and subsequent hypotonic lysis of SRBC.

Subsets of T cells were prepared by a negative-selection technique by the use of immunomagnetic beads (Dynal, Fort Lee, NJ). E^+ cells were depleted of CD8 (MoAb VIT 8) and $\gamma\delta$ TCR^+ cells ($CD4^+$ T cells) and of CD8, $\gamma\delta TCR^+$ and $CD45RO^+$ cells ($CD4^+CD45RO^-$ T cells). The respective T cell subpopulations were always > 90% pure.

Monoclonal antibodies

UCHL1 (CD45RO, IgG2a) was obtained from Dr Peter Beverley. The $\gamma\delta$ TCR MoAb was a gift from Dr R. L. H. Bolhuis. Anti-Leu3a PE (CD4, IgG1) and anti-IL-2R (CD25) PE (IgG1) were purchased from Becton Dickinson (Mountain View, CA). FITC- and PE-labelled irrelevant MoAbs (IgG2a and IgG1) were used as appropriate isotype matched controls.

Staining of cells

The cells were incubated for 10 min with human gamma globulin (1 mg/ml) to reduce unspecific staining. Cells (5×10^5 per tube) were incubated with the indicated FITC- and/or PE-labelled MoAbs, incubated for 20 min, washed three times in PBS containing 1% bovine serum albumin (BSA) and 0·1% sodium azide and subsequently fixed in 0·5% paraformaldehyde. All procedures were carried out at 4°C.

Flow cytometry

Membrane fluorescence was analysed on a standard FACScan flow cytometer supported by a Consort 30 software (Becton Dickinson). An air-cooled Argon ion laser emitting 15 mW at 488 nm served as the light source.

The method for the evaluation of three-parametric data from a combination of two-dimensional stainings was described recently [10]. Controls excluded, three different sets of stainings were needed for the flow cytometry part of this study. In all stainings the reactivity of UCHL1 FITC was plotted on the abscissa. On the ordinate, the reactivity of PE-labelled MoAbs to CD4, CD25 or the reactivity of the combination of CD4 and CD25 was recorded. From these stainings, the percentages of any population or subpopulation can be accurately calculated.

In situ *hybridization*

Total or presorted T cells (2×10^5) were coated to 8×8-mm square areas of poly-L-lysin (Sigma, St Louis, MO) treated microscopic slides (LAB-TEK^R tissue culture chamber/slides; Miles Scientific, Naperville, IL) as established by Schwarz *et al.* [18]. After formaldehyde fixation slides were frozen to −20°C until further use. For prehybridization and hybridization conditions we optimized a protocol described by others for the use on tissue sections [19]. The cells were hybridized with the ^{32}P-labelled oligonucleotide probes specific for the human IL-2 (5′GGTTGCTGTCTCATCAGCATATTCACACATG [20]) or interferon-gamma (IFN-γ) (5′ACAGTTCAGCCATCACTTGGATGAGTTCATGTATTGCTTTG [21]) transcript. Both probes were initially tested on Northern blot analysis and were found to detect the correct sized transcript in mitogen activated T cells (not shown). After overnight hybridization, the slides were washed and then exposed to radiographic film (X-omat S; Kodak, Rochester, NY) at −70°C using enhancer screens. The autoradiographs were densitometrically evaluated with an Ultrascan XL laser densitometer (LKB).

Statistical analysis

To determine statistical significance the nonparametric Kruskal–Wallis test was used and the null hypothesis rejected at the $P < 0.05$ level.

RESULTS

Frequency analysis of major T lymphocyte subpopulations in RA

Single-parameter flow cytometry analysis was performed to measure the frequencies of CD3, CD4, CD45RO and CD25 (IL-2Rα, p55) positive lymphocytes. The respective mean ($\pm$s.e.m.) percentages of positive cells obtained from RA patients, reactive arthritis patients, patients with degenerative joint disease and from healthy age-matched controls are depicted in Fig. 1. Among the peripheral blood lymphocytes (PBL) of RA patients we found 75 $\pm$ 2% $CD3^+$, 44 $\pm$ 3% $CD4^+$, 45 $\pm$ 3% $CD45RO^+$ and 25 $\pm$ 3% $CD25^+$ cells. The frequencies of these major T lymphocyte subsets did not differ significantly from the respective PBL values obtained from all other three groups of subjects. The average percentage of IL-2Rα expressing PBL was marginally increased in the RA group as compared with the control groups (reactive arthritis, 16 $\pm$ 3%; degenerative joint disease 19 $\pm$ 2%; healthy controls, 19 $\pm$ 4% $CD25^+$ PBL) ($0.05 < P < 0.1$).

Furthermore, we analysed the frequency of the major T lymphocyte subsets among synovial fluid lymphocytes (SFL) derived from joint effusions of reactive arthritis and RA patients. Among SFL of RA patients we found 79 $\pm$ 4% $CD3^+$, 44 $\pm$ 5% $CD4^+$, 78 $\pm$ 4% $CD45RO^+$ and 24 $\pm$ 6% $CD25^+$ cells. The increased number of lymphocytes expressing CD45RO among SFL *versus* PBL ($P < 0.01$) was a constant finding for both RA and reactive arthritis (Fig. 1).

Selective increase of CD25 expression in peripheral blood T lymphocyte subpopulations of RA patients

In order to investigate further whether the observed marginal enhanced CD25 expression of PBL in RA may be localized within a particular T lymphocyte subset, we performed a multiparameter analysis as described previously [10]. By the use of dual-parameter analysis we found that RA CD4 T cells

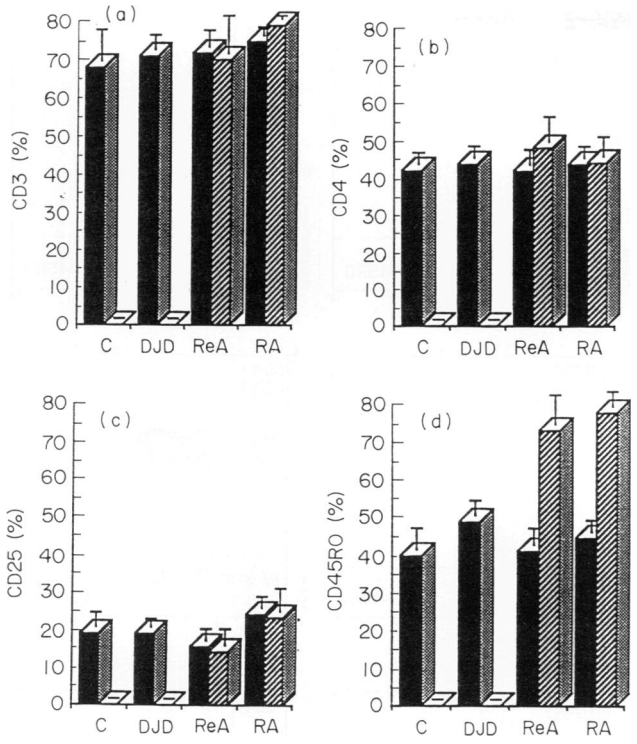

Fig. 1. Frequency analysis of functional lymphocyte subpopulations of the peripheral blood (■) and of the synovial fluid (■). MNC derived from control individuals (C), from patients with from degenerative joint disease (DJD), reactive arthritis (ReA) or rheumatoid arthritis (RA) were analysed by single-parameter flow cytometry. Means ± s.e.m. of $CD3^+$ (a), $CD4^+$ (b); $CD25^+$ (c); or $CD45RO^+$ (d) lymphocytes are given. The analysis of synovial fluid MNC was not applicable in the DJD and in the control group.

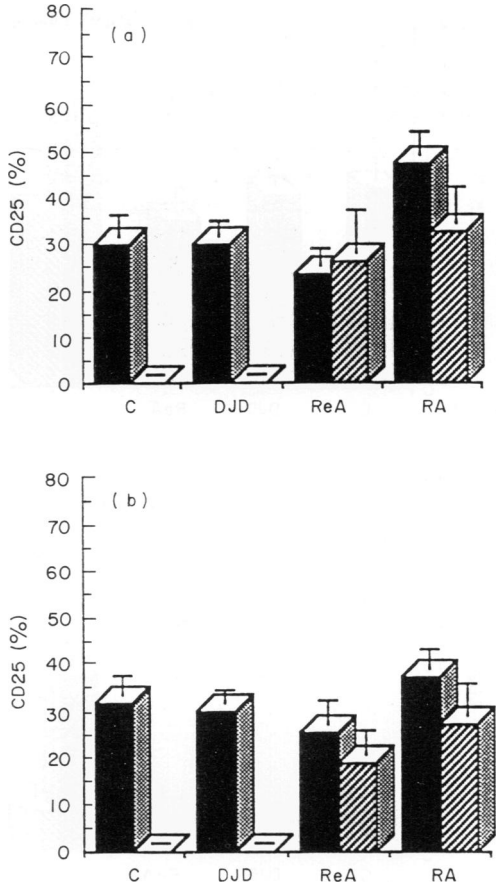

Fig. 2. Frequency analysis of CD25 expression (means ± s.e.m.) in the $CD4^+$ (a) and in the $CD45RO^+$ (b) lymphocyte subpopulations as calculated from dual-parameter flow cytometry. The sequence of the investigated groups and the used symbols are the same as in Fig. 1.

contained a higher percentage of $CD25^+$ cells (47 ± 5%) than CD4 T cells from the control groups reactive arthritis (23 ± 4%), degenerative joint disease (30 ± 3%) or healthy controls (29 ± 5%) ($P < 0.01$). The frequency of CD25 expression in the total $CD45RO^+$ (37 ± 4%) or in the total $CD45RO^-$ (14 ± 2%) lymphocyte subset of RA did not differ significantly between RA patients and control groups (Fig. 2).

In order to define further the sub-subset containing the highest proportion of $CD25^+$ cells we performed a three parametric analysis. For this purpose, we subdivided the $CD4^+$ subpopulation into $CD4^+CD45RO^+$ (memory helper) and $CD4^+CD45RO^-$ (naive helper) T cell subpopulations and calculated the frequency of CD25 expression as a third parameter. The proportion of $CD45RO^+$ or $CD45RO^-$ cells among CD4 T cells was the same in RA and control groups, respectively (data not shown). However, we found that the increased percentage of $CD25^+$ cells in the RA $CD4^+$ T cell population (Fig. 2) is predominantly related to an increased proportion of IL-2Rα expression in the $CD4^+CD45RO^-$ (naive helper) T lymphocyte subpopulation (RA, 38 ± 5% *versus* reactive arthritis, 8 ± 2%; degenerative joint disease, 7 ± 2%; healthy controls, 9 ± 3% $CD25^+$ cells) ($P < 0.01$). In addition, the $CD4^+CD45RO^+$ T cell subset of RA PBL comprises an increased proportion of $CD25^+$ lymphocytes (RA, 55 ± 5% *versus* reactive arthritis, 36 ± 5%; degenerative joint disease, 41 ± 3%; healthy controls, 43 ± 5% $CD25^+$ cells) ($P < 0.05$; Fig. 3).

In Fig. 4 we show staining examples of PBL derived from a healthy control (Fig. 4a–d) and from an RA patient (Fig. 4e–f). Although both samples had comparable CD25 expression of total CD4 (49% *versus* 38%) or $CD4^+CD45RO^+$ T cells (47% *versus* 43%), selectively the RA patient showed remarkable CD25 expression in the $CD4^+CD45RO^-$ subset (35% *versus* 13% of the cells being positive). Due to the prevalence of $CD45RO^-$ lymphocytes in this particular patient, the subpopulation expressing CD25 and not CD45RO is directly visible from the CD45RO *versus* CD25 double staining (Fig. 4g). Furthermore, the fluorescence intensities of CD25 expression of the $CD45RO^-$ and $CD45RO^+$ lymphocyte subsets are essentially comparable in this patient (Fig. 4g).

The pattern of CD25 expression in the major subpopulations of SF T lymphocytes

As had been mentioned above, both RA and reactive arthritis SF lymphocytes are highly enriched for CD45RO-expressing cells (Fig. 1). The proportion of CD25-expressing cells among $CD4^+$ (RA, 32 ± 8% *versus* reactive arthritis 26 ± 9%), $CD45RO^+$ (25 ± 5 *versus* 19 ± 5%) or $CD45RO^-$ (7 ± 2 *versus* 6 ± 2%) SF T cells was essentially comparable for RA and reactive arthritis patients (Fig. 1). Likewise, the analysis of the

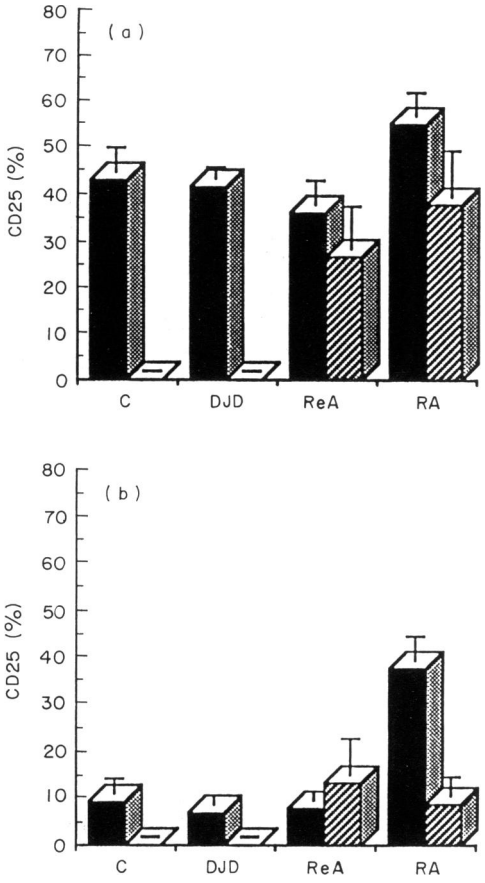

Fig. 3. Frequency analysis of CD25 expression (means ± s.e.m.) in the $CD4^+CD45RO^+$ (a) and in the $CD4^+CD45RO^-$ (b) T lymphocyte subpopulations. Data were calculated from triple-parameter flow cytometry, as previously described [10]. Symbols and abbreviations as in Fig. 1.

sub-subpopulations revealed no significant difference in the frequency of CD25 expression for the $CD4^+CD45RO^+$ (RA, 37 ± 10% *versus* reactive arthritis, 27 ± 9%) or for the $CD4^+CD45RO^-$ (9 ± 4 *versus* 13 ± 8%) T cell subpopulations.

The pattern of lymphokine (IFN-γ, IL-2) gene expression in peripheral blood T lymphocyte subsets in RA

In order to investigate activation-related cytokine gene expression in RA T cell subpopulations we performed *in situ* hybridizations with oligoprobes specific for IFN-γ and IL-2 mRNA. As controls we used T cells from patients with degenerative joint disease ($n=2$), reactive arthritis ($n=1$) and healthy controls ($n=2$). The experimental design uses a defined number of cells coated to an area of defined size on a microscopic slide. Therefore, autoradiograms can be quantified in a densitometer and the obtained signals can be quantified on a per-cell basis [17].

All hybridizations for IFN-γ or IL-2 mRNA, respectively, were performed in one experiment under identical conditions for all cell populations and patient groups. Figure 5 shows the densitometry evaluation of the respective autoradiographic intensities. Unfractionated T cells from RA patients hybridized significantly stronger than controls to the oligoprobes specific

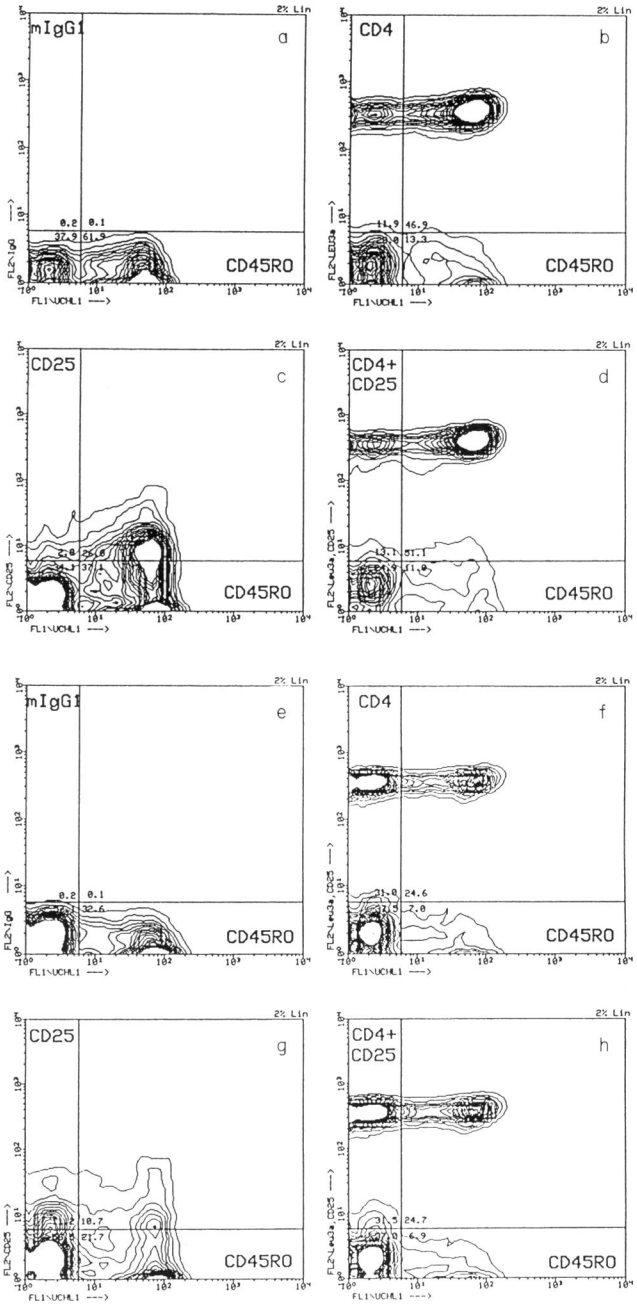

Fig. 4. Flow cytometry analysis of three parametric data as obtained from a healthy control individual (a–d) and from an RA patient (e–h). The method and the required antibody combinations have been described elsewhere [10]. The CD of the stained MoAbs are indicated. The percentage of $CD25^+$ lymphocytes calculates to 22% (29%) for the RA patient (% $CD25^+$ cells of control) 38% (40%) for total CD4 T cells, 43% (47%) for $CD4^+CD45RO^+$ and 35% (13%) for $CD4^+CD45RO^-$ T cells. The weak positive population in the upper left quadrant of plot g disappears in the respective quadrant of plot h, indicating that most of the $CD25^+CD45RO^-$ lymphocytes are $CD4^+$ T cells.

for IFN-γ (Fig. 4a) and IL-2 mRNA (for both, $P<0.05$) (Fig. 4b). However, for $CD4^+$ RA T cells we calculated no statistically significant difference to control CD4 T cells both for IFN-γ or IL-2 mRNA, respectively. Interestingly, the naive CD4 ($CD4^+CD45RO^-$) T cell subpopulation of RA patients gave a

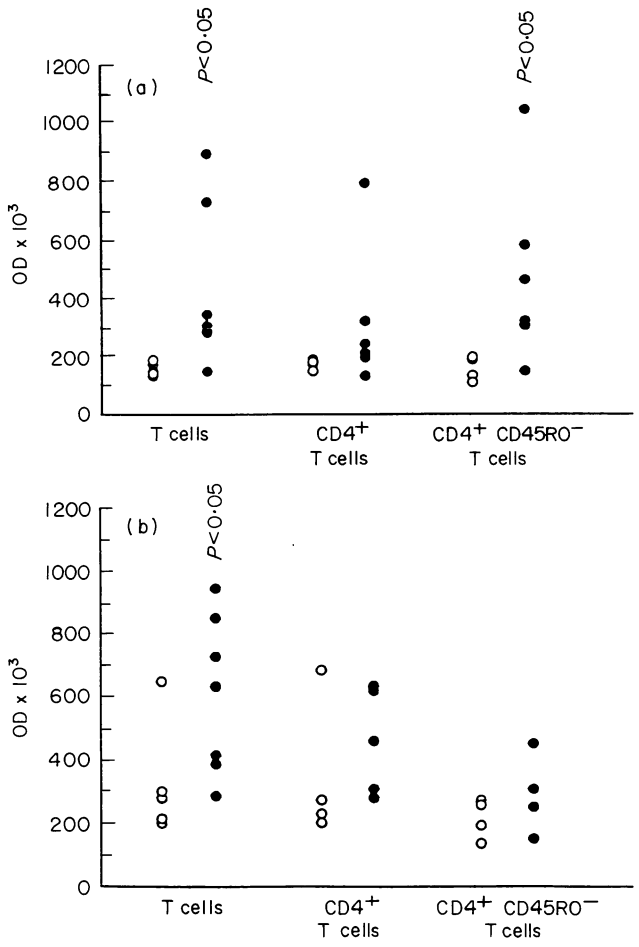

Fig. 5. Densitometric results of *in situ* hybridizations for IFN-γ (a) or IL-2 mRNA (b) expression in functional T cell subpopulations from the peripheral blood of RA patients (closed circles) and controls (open circles). Oligonucleotide probes specific for IFN-γ (Fig. 4a) or IL-2 (Fig. 4b) (b) mRNA were hybridized as described. Autoradiography intensities are given in arbitrary units of optical density (OD).

significantly stronger signal ($P < 0{\cdot}05$) for IFN-γ mRNA than the controls (Fig. 4a).

DISCUSSION

A long period of research in rheumatology has provided evidence that infectious agents [22,23] as well as autoimmunity [2,24–27] may play an important role in the pathogenesis of RA. Several reports have documented that activated T cells are present in both in the PB and in the SF membrane of RA patients [6,28,29], indicating that they may be involved in pathogenetic events. In order to deduce which T cell subpopulation(s) in particular may be activated, we analysed CD25 expression and T cell-specific cytokine expression (IFN-γ and IL-2) in subsets and sub-subsets of T lymphocytes.

Our results clearly demonstrate that single-parametric immunocytology using CD4, CD45RO or CD25 as markers for functional T cell subsets may not be sufficiently sensitive to find specific differences between the PB lymphocytes of RA and the PB lymphocytes of the subjects with reactive arthritis or degenerative joint disease or healthy controls (Fig. 1). However, using double staining experiments, we found that in RA a subpopulation of peripheral blood CD4 T lymphocytes express CD25 (Fig. 2). This percentage is significantly higher than that observed in reactive arthritis, degenerative joint disease or healthy controls. However, we and others have found that a considerable proportion of CD25 positive CD4$^+$ T cells is also present among PBL of healthy individuals (Fig. 2) [9–11]. Therefore, the increased frequency of CD25 positive CD4 T cells in RA is a quantitative rather than a qualitative difference.

In order to further characterize the sub-subset with the most pronounced CD25 expression, we investigated whether the increased abundance of CD25 expression among CD4 RA T cells may correlate with the CD45RO$^+$ (memory) CD4 phenotype or possibly with the CD45RO$^-$ (naive) CD4 T cell phenotype. By using a triple-parameter flow cytometry analysis we found that the CD45RO$^-$CD4 T cells showed the highest increase in the proportion of CD25$^+$ T cells as compared with the control groups. The importance of this finding is underlined by the fact that CD25 expression in normal individuals is almost exclusively found in the memory CD4 T cell subset (Fig. 3) [10,11]. Thus, the abundant expression of CD25 on CD45RO$^-$ CD4 T cells seems to distinguish specifically RA PBL from peripheral blood T cells of patients with reactive arthritis or degenerative joint disease as well as of healthy control individuals. In addition, the CD45RO$^+$CD4 RA T cells also showed a significantly increased percentage of CD25$^+$ T cells.

Since some reports stated that synovial fluid T cells from RA may express CD25 [28], we were further interested to see whether this CD25 expression could be found on cells of a particular T cell subset derived from synovial effusions of RA patients. In accordance with published data [30,31] we found that SF T cells from RA and reactive arthritis predominantly display a CD45RO$^+$ phenotype (Fig. 1) which has been proposed to go along with an enhanced ability of these cells to migrate into sites of inflammation [30]. Among CD4 SF T cells CD25$^+$ cells predominantly co-express CD45RO rather than CD45RA both in RA and reactive arthritis. Therefore, it seems conceivable that the CD45RO$^-$CD25$^+$CD4 RA T cells found in the PB either do not enter inflamed joints or, alternatively, convert their CD45 isoform expression before being released to the SF.

During early T cell activation—at least in *in vitro* systems—the increasing CD25 expression is paralleled by the initiation of cytokine gene expression such as IFN-γ and IL-2 [32]. In this study we demonstrate that CD45RO$^-$CD4 T cells isolated from the peripheral blood of RA patients express significant levels of IFN-γ mRNA (Fig. 4a). These findings, in conjunction with the increased CD25 expression, strongly suggest that in the peripheral blood of RA the naive CD45RO$^-$CD4 T cell subpopulation comprises a fraction of activated T lymphocytes. This interpretation is supported by the finding that IFN-γ has been detected in substantial amounts in the PB of RA patients but not in the PB of reactive arthritis patients [33] and that the level of IFN-γ mRNA closely reflects the expression of the respective protein [32].

At the moment it is tempting to speculate about a specific pathogenetic importance of the CD25 expressing CD45RO$^-$-CD4 T cell subset in RA. In analogy to our observations recent findings suggest that activated CD45RA$^+$CD4 T cells are also present in the peripheral blood of bone marrow transplant recipients [34]. Therefore, the presence of activated naive CD4 T

cells may be a common feature of several pathophysiologic states. However, it has been shown that CD25 expression and IFN-γ transcription are initiated already during the first hours of T cell activation whereas CD45 isotype conversion takes place only after several days of *in vitro* stimulation [35]. With regard to this model of T cell activation it is possible that the naive CD4 subset in RA may comprise recently activated T cells specific for a putative pathogen or autoantigen which plays an important role in perpetuating the disease progress.

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