T-cell responses to viral, bacterial and protozoan antigens in rheumatoid inflammation. Selective migration of T cells to synovial tissue

K. R. Shadidi, T. Aarvak, S. Jeansson¹, J. E. Henriksen², J. B. Natvig and K. M. Thompson

The National Hospital, Institute of Immunology, Department of Rheumatology Research, Oslo, ¹Ullevål University Hospital, Department of Microbiology, Oslo and ²Diakonhjemmets Hospital, Department of Rheumatology, Oslo, Norway

Abstract

Objective. To identify any preferential or selective migration of T-cell specificities to inflamed tissues of rheumatoid arthritis (RA) patients.

Methods. Lymphocytes from peripheral blood (PB) and synovial tissue (ST) were isolated from RA patients and stimulated with a panel of crude antigen preparations from 18 bacterial, protozoan and viral sources. Proliferative responses of the T lymphocytes to each antigen and group of antigens were compared in PB and ST. Antigen-specific T-cell clones were developed and their migratory capacities towards synovial chemokines were compared.

Results. ST-derived T cells showed a small but significantly higher stimulation index (SI) to the group of intestinal bacteria compared with PB T cells. Conversely, responses of ST-derived T cells to Acanthamoeba polyphaga (AP) were both profoundly and significantly lower compared with PB-derived T cells. The viral antigens as a whole gave comparable reactivities in blood and ST. The migratory capacity of AP-specific T-cell clones towards chemokines produced by ST was profoundly poorer compared with Campylobacter jejuni- and herpes simplex virus-specific T-cell clones.

Conclusions. The results indicate a selective migration of T cells of given specificities to the inflamed rheumatoid synovium.

KEY WORDS: Rheumatoid arthritis, Antigens, Proliferation, Intestinal bacteria, Acanthamoeba polyphaga, Migration.

There is compelling evidence that T cells are of crucial importance for the development and maintenance of rheumatoid arthritis (RA) (reviewed in [1, 2]). The strong association of RA with HLA-DR molecules containing a particular conserved region of amino acids also implies an important role of T cells in RA [3, 4]. Reports have indicated a clonal expansion of T cells in the RA synovium [5, 6], and isolated T-cell clones show features that suggest that they may recognize antigens in the synovium [6].

Studies on the specificities of T cells found in RA synovial tissue (ST) can provide information concerning two major questions relating to the aetiology and pathogenesis of RA. The first concerns the potential role of pathogens as causative agents. Whether the chronic progression of RA is due to a continuing presence of the foreign antigen(s) in the joint, or whether T cells are generated that cross-react with joint autoantigens is not clear [7]. In either case one might expect to find an elevated reactivity of T cells to the antigen in the inflamed ST, compared with the blood. So far only a few antigens have been looked at in any single study. In this study we used a broad panel of common pathogens from microbial and viral sources, including a protozoan source possibly involved in RA (S. Jeansson, unpublished results).

The second question concerns the migration of T cells into the ST in RA. After primary antigen stimulation, T cells selectively express tissue-specific adhesion markers [8] and chemokine receptors [9, 10], which enable them to home to the site where their antigen is situated. We therefore studied the migratory capacity of T-cell clones specific for Acanthamoeba polyphaga (AP), herpes simplex virus (HSV) and Campylobacter jejuni towards a supernatant from a ST explant. This is of particular interest as it is known that the migration of lymphocytes to the joint can result in the formation of secondary lymphoid organ-like structures [11, 12].
The aim of this study was to compare patterns of T-cell antigen responses in ST- and peripheral blood (PB)-derived T cells from RA patients, and the migratory capacity of corresponding T-cell clones. This might identify antigens implicated in the disease pathology and throw light on the mechanisms responsible for the massive T-cell infiltration seen in RA.

Materials and methods

Patients

Samples of ST and PB were obtained from nine patients (eight female and one male) (mostly from The Diakonhjemmet Hospital and The National Hospital, Oslo, Norway) with RA fulfilling the 1987 American College of Rheumatology criteria [13]. The mean age of the patients was 59.33 yr (range 43–69 yr), the mean duration of disease was 18.00 yr (range 4–36 yr). All patients but one were seropositive for rheumatoid factor (RF). Patients with high or moderately high disease activity were included. At the time of surgery, five patients were receiving corticosteroids, two in combination with methotrexate and folate and one in combination with calcium. One patient was receiving gold salts and one patient did not receive any medication.

Preparation of mononuclear cells (MNC)

This was carried out as described previously [14]. Briefly, ST digests and heparinized PB were washed and resuspended in phosphate-buffered saline (PBS), layered on top of Isopaque-Ficoll separation media and resuspended in phosphate-buffered saline (PBS), layered on top of Isopaque-Ficoll separation media and resuspended in PBS. All antigen concentrations were determined by optical density and stored at –70°C until use. Phytohaemagglutinin (PHA) (Sigma) was used as a proliferation control. This was dissolved in PBS to a concentration of 1 mg/ml and stored at –20°C until use.

Proliferation assays

MNC (10^5) were cultured in 96-well, round-bottom plates (Costar Cambridge, MA, USA) with DMEM F12 medium supplemented with 10% human AB serum and stimulated in triplicate.

Preparation of antigens

Inactivated mumps, rubella, varicella zoster virus (VZV) were purchased from Viral Antigens Inc. (Memphis, TN, USA). Inactivated HSV and cytomegalovirus (CMV) were kind gifts from Professor Petter Gaustad (Institute of Virology, The National Hospital, Oslo, Norway). *Borrelia afzelii, B. garinii* and *B. burgdorferi sensu stricto* (kind gifts from Dr Pål Jenum, The Public Health Institute, Oslo, Norway) antigen preparation was a kind gift from Professor Miklos Degré (Institute of Virology, The National Hospital, Oslo, Norway). *Salmonella infantis, Shigella flexneri, Klebsiella pneumoniae, Yersinia enterocolitica, Helicobacter pylori* and *C. jejuni* were kind gifts from Professor Petter Gaustad (Institute of Microbiology, The National Hospital, Oslo, Norway). Antigen preparation was performed with heat inactivation similar to that described in [16, 17]. AP was suspended in PBS with 1 mg/ml L-cysteine (Sigma, St. Louis, MO, USA). Three patients were continuously cooled in an ice–saltwater bath. The suspension was centrifuged at 4°C at 320 g for 30 min. The pellet was washed once in PBS without L-cysteine and resuspended in PBS. All antigen concentrations were determined by optical density and stored at –70°C until use. Phytohaemagglutinin (PHA) (Sigma) was used as a proliferation control. This was dissolved in PBS to a concentration of 1 mg/ml and stored at –20°C until use.

T-cell lines and clones

Cloning was carried out as described in [14], with these modifications: MNC (1 x 10^6) from a healthy donor were initially stimulated with APP, HSV, C. jejuni as described above. APP-specific clones did not cross-react with APS. Cloning was performed on day 14 by limiting dilution (0.3–1 cells/well) in 20-μl wells (Terasaki; Nunc, Roskilde, Denmark), using 1 x 10^4/ml irradiated autologous PB MNC, antigen and 20 U/ml interleukin (IL)-2. T-cell clones were expanded after 8 days to 96-well plates (Nunc) in medium containing 20 U/ml IL-2, antigen and 1 x 10^6 irradiated autologous PB MNC and later to 24-well plates (Costar).

Transmigration assays

The migratory capacities of three T-cell clones specific for HSV, three clones specific for C. jejuni and three clones specific for APP were tested using a 5-μm pore size polycarbonate tissue culture insert (Costar) in a 24-well tissue culture plate (Costar). On the ninth day after antigen stimulation, a total of 7.5 x 10^4 T cells in 100 μl was added to the top chamber of the transwell. A supernatant, obtained from an overnight culture of ST-derived cells (600 μl), or RPMI 1640 with 1% human serum as a control, was added to the bottom chamber of the transwell. Both liquids were filtered through a 22-μm filter (Millipore, Bedford, MA, USA) before use. All experiments were performed in duplicate. After a 90-min
incubation at 37°C, the migrated cells were counted with a fluorescence activated cell sorter (FACS) in 60 s and at a 60 μl/min flow rate. Prior to the transmigration assay, cell viability was determined using trypan blue staining and light microscopy. All clones exceeded 90% viability.

**Statistics**

Incorporated thymidine scores [counts per minute (c.p.m.)] for the different antigens were expressed as stimulation indices (SI = mean c.p.m. of triplicate with antigen/mean c.p.m. of background triplicate). Statistical comparisons of the differences between blood and ST responses were performed using GraphPad Instat.

The two-tailed paired Student’s t-test was used when the data sets of PB and ST results could be paired because the correlation between the columns was significant and the data sets had Gaussian distribution. The Wilcoxon matched pair test was used when the data sets could not be paired according to the criterion mentioned above, but the data sets did not have Gaussian distribution. Student’s unpaired t-test, Welch corrected, was used when the data sets could not be paired according to the criterion mentioned above, the data sets had Gaussian distribution, but the data sets did not have equal standard deviation (s.d.). The Mann–Whitney test was used when the data sets could not be paired according to the criterion mentioned above and the data sets did not have Gaussian distribution. Differences were considered statistically significant for $P < 0.05$. All data are presented as mean ± standard error of the mean (S.E.M.).

The percentage of migrated cells in the transwell assay was calculated using the equation:

$$\frac{[\text{cells migrated towards ST supernatant} - \text{cells migrated towards control medium}] \times 100}{\text{total cell number}}$$

**Results**

Reactivity of T lymphocytes in ST and PB against various micro-organisms

The SI of the responses to all antigens in PB- and ST-derived T lymphocytes are shown as the ratio ST/PB (Fig. 1). For each bacterium there was a higher response in the ST-derived T cells than the PB, but only for the bacterium *S. flexneri* did the difference between the SI in the ST- and PB-derived lymphocytes reach a significant level ($P = 0.034$).

In contrast, the ST-derived T cells were almost non-responsive to AP antigens, while PB-derived T cells showed a clear and strong response. The responses were significantly stronger in PB-derived T cells using both the supernatant and the pellet fraction of the amoeba ($P = 0.009$ and $0.046$, respectively). All patients except one showed positive antibody titres against AP in serum (data not shown). The T cells derived from the patient with no antibody titre against AP also showed no proliferation against the APP antigens and only very modest proliferation against APS antigens in blood-derived T cells (Fig. 1).

![Fig. 1. The ST/PB proliferation ratio of all antigens in individual patients (SI for the proliferation of ST-derived T cells in response to different antigens divided by SI for the proliferation of PB-derived T cells in response to the same antigen). The cut-off line is drawn to show where SI in ST is equal to SI in PB.](image-url)
The viral antigens generally gave higher responses than the other antigens both in ST and PB samples. The differences in responses between ST- and PB-derived lymphocytes for individual viruses were not significant with one exception, VZV, which gave a slight but significantly higher proliferation of ST-derived T lymphocytes ($P = 0.016$; Fig. 1). All patients included in this study responded to CMV, but the responses can be divided into two different groups. Five patients responded strongly to the antigen in PB but not in ST, and four patients had strong responses in the ST-derived T cells and poorer responses in the PB-derived T cells. All but two patients were positive for antibodies against CMV in serum (data not shown).

Three different genotypes of *B. burgdorferi* were tested: *B. afzelii*, *B. garinii* and *B. burgdorferi sensu stricto*. T-cell proliferation to *B. afzelii* and *B. garinii* was significantly higher in tissues compared with blood ($P = 0.042$ and $0.037$, respectively). However, *B. burgdorferi sensu stricto* did not induce a different response in ST- as compared with PB-derived lymphocytes. No specific antibodies against the three *B. burgdorferi* genotypes were found in any of the patients (data not shown).

The mycobacterium crude antigen BCG was also used for T-cell proliferation. BCG gave approximately equal SI in PB- and ST-derived T cells.

PHA was used as a control for T-cell proliferation. Although PB-derived T cells, as expected, proliferated more to the PHA stimulation than the ST-derived T cells, the difference was not significant (Table 1).

**Reactivity of T lymphocytes in ST and PB against different groups of pathogens**

We grouped the antigens into different categories: intestinal bacteria, virus, *Borrelia burgdorferi* and AP, and compared the SI responses within each group (Table 1). The mean SI for ST-derived T cells to antigens in the intestinal bacteria group was 3.67 compared with 2.17 in blood-derived T cells ($P = 0.025$). In contrast, for antigens in the AP group the mean SI was 1.03 for ST T cells compared with 0.47 in blood-derived T cells ($P = 0.0003$). There were no significant differences in mean SI between blood and tissue responses to antigens in the viral group.

**Reduced migratory capacity of AP-specific T-cell clones**

As the AP-derived antigens were the only antigens that T cells from ST did not react to, we wanted to study the ability of AP-specific T-cell clones as compared with *C. jejuni*- and HSV-specific T-cell clones to migrate towards a supernatant from an overnight culture of ST. The experiment was repeated three times with supernatant from different RA patients. The mean values from one representative experiment are shown in Fig. 2. Of the cells specific for AP, 9% migrated through the transwell filter towards the synovial chemoattractant. For comparison, 51% of cells reacting to *C. jejuni* and 46% of cells specific for HSV migrated through the transwell filters. These findings indicate a very low migratory capacity of AP-specific T cells compared with T cells specific for *C. jejuni* and HSV against supernatant produced by ST cells.

**Discussion**

The results show that there are significant differences in reactivities to various antigens when comparing responses between ST- and PB-derived T cells. The fact that these responses may be either stronger for the ST-derived cells (intestinal bacteria, *B. burgdorferi*) or stronger for the PB-derived cells (*Acanthamoeba*) suggests that this is not merely a general difference in reactivity due to the origin of the lymphocytes or differences in the antigen-presenting cell population.

The large number of specificities present in ST T cells suggests that the majority of T cells probably home to the ST in a non-antigen-specific manner. In autoimmune diseases where the autoantigens are known, only a very low frequency of T cells specific for the autoantigen are present at the site of pathology. Only about 1:7000 of
the T cells in PB in multiple sclerosis patients respond to the myelin basic protein, and 1:450 respond in cerebral fluid [18]. In reactive arthritis, the frequency of bacterial antigen-specific T cells is between 1:3000 and 1:10000 in synovial fluid [19].

A particularly interesting finding in this study was that AP antigens were the only antigens that induced significantly higher proliferation in the PB than in the ST. It has recently been described that ST from RA patients stain much more frequently for AP epitopes than do controls (S. Jeansson et al., unpublished observations). Increasing titres of AP-specific IgM antibodies correlate with disease activity in RA patients and a persistent AP infection is often seen in RA patients (S. Jeansson et al., unpublished data). If there is an involvement of AP in RA, it is somewhat curious that we found considerably less reactivity in the ST-derived T cells compared with the blood. Either AP-specific T cells do not migrate to the ST, or upon arrival they are anergized or deleted by peripheral tolerance mechanisms. In either case this may contribute to an environment that does not effectively clear AP antigens from the ST of RA patients.

To study further this phenomenon, we then compared the migratory properties of T-cell clones specific for AP, HSV and C. jejuni against a supernatant from an overnight culture of ST from a RA patient. The HSV- and C. jejuni-specific cells showed 4–5-fold higher capacity to migrate towards the supernatant than AP-specific T cells. Reports show that in RA, T cells infiltrating the synovium resemble in many ways T cells found in gut lymphoid organs [20, 21]. The migration was, however, shown only to be partially mediated by currently known homing receptors [22]. Our findings indicate that the activation of AP-specific T cells may lead them to express a pattern of cell surface markers, e.g. chemokine receptors, that inhibits their migration into the ST.

All the viruses induced high proliferation in the lymphocytes derived from both ST and PB. There were, however, no significant differences in the SI caused by these antigens when analysed as a group.

Two different genotypes of B. burgdorferi antigens induced high proliferation in T cells from ST, but the patients included in this study were all seronegative for antibodies against the spirochaete. It has previously been shown that both naive and memory T cells from uninfected persons can proliferate in response to B. burgdorferi stimulation [15]. The proliferation is not due to a proteoglycan, is sensitive to proteinase K and is dependent on antigen processing. The study concluded that the T-cell response from normal individuals to B. burgdorferi is more likely to be due to multiple antigen epitopes than to a superantigen response. Our findings that T cells from uninfected patients proliferated to the B. burgdorferi antigens cannot be used to distinguish between a superantigen or a regular antigen response. There is also reported wide cross-reactivity between B. burgdorferi and ‘arthritogenic enteric bacteria’ such as Salmonella, Shigella and Yersinia [23], which could explain our findings.

In conclusion, T-cell proliferation in response to most of the antigens tested was observed in both ST and PB. There is, therefore, no direct evidence that any of these antigens are involved or responsible for the disease initiation and/or maintenance. However, the lack of AP-specific T cells in the ST and the increased response to intestinal bacteria suggests that there are selective mechanisms involved in the migration of T cells to the joint that can be related to the specificity of the T cell. Future characterization of the T cells generated in response to AP and intestinal bacteria may give an indication as to the chemotactic mechanisms involved in promoting or hindering the T-cell infiltration of ST in RA. Such studies are currently being performed [24].

References

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