ENHANCED CO-STIMULATORY ABILITY OF SYNOVIAL FLUID ACCESSORY CELLS IN RHEUMATOID ARTHRITIS

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SUMMARY

We have established in vitro assays that allow the examination of co-stimulatory function of rheumatoid arthritis (RA) antigen-presenting cells (APC). Synovial fluid (SF) and peripheral blood (PB) APC co-stimulatory ability was compared in the activation of peptide-specific human T-cell clones. T-cell receptor (TCR) stimulation by peptide or anti-CD3 antibody allowed the direct comparison of SF and PB APC co-stimulatory activity, separately from their ability to process antigen. SF APC from 15 RA patients consistently enhanced T-cell proliferation when compared to their PB counterparts. Moreover, increasing the numbers of PB APC present resulted in only a minor increase in T-cell proliferation, failing to achieve levels stimulated by SF APC. We propose that the enhanced co-stimulatory function of synovial APC may be a significant factor in the persistence of local immune responses in RA.

KEY WORDS: Rheumatoid arthritis, Accessory cells, Co-stimulation.

RHEUMATOID arthritis (RA) is a systemic disease with prominent inflammatory arthritis resulting from a complicated network of factors both systemically and at the site of inflammation [1]. Clinically, RA is characterized by a chronic, progressive destruction of joints. Histologically, mononuclear cell infiltration into the synovium and synovial tissue adjacent to cartilage of joints precedes bone erosion. A prominent feature of the inflamed synovium is the presence of activated immune cells, particularly CD4 T lymphocytes in association with major histocompatibility complex (MHC) class II-expressing antigen-presenting cells (APC). T-cell function has been extensively studied in RA and is known to be depressed, but less is known about APC function, and the relative importance of these cell types in the immunopathogenesis of RA is a matter of some debate [2].

Effective APC have the capacity to provide all the necessary signals required for the activation and proliferation of CD4 T cells. Firstly, generation of a ligand for the T-cell receptor (TCR). This requires the processing of exogenous antigen into immunogenic peptides and their presentation at the cell surface in association with MHC class II. Secondly, the provision of co-stimulatory signals [3]. The importance of these non-antigen-specific, non-MHC-restricted co-stimulatory signals has been highlighted by several recent studies in which TCR stimulation in the absence, or blockade of, co-stimulatory signals resulted in T-cell death or unresponsiveness to further antigenic challenge [4, 5].

The best-characterized interactions providing co-stimulation are those between B7.1 (CD80), B7.2 (CD86) expressed by APC with their T-cell ligands CD28 and CTLA-4 [6, 7]. Expression of members of the B7 family varies amongst different APC types and may be modulated by activation or environmental factors such as cytokines. Activated B and T cells express both CD80 and CD86, similar to dendritic cells (DC) and macrophages [8]. CD80 is absent from resting monocytes, but may be induced after interferon (IFN)-γ treatment [9]. Parenchymal cells generally lack co-stimulatory activity and if MHC class II molecules are induced, TCR ligation by self-peptide/MHC complexes present within tissues results in T-cell non-responsiveness. In contrast, during inflammatory reactions, the inappropriate expression of co-stimulatory molecules may arise, subsequently allowing T-cell activation [10]. Modulation in the expression of co-stimulatory molecules may therefore provide a mechanism for controlling the initiation and persistence of immune responses.

We have previously demonstrated that synovial fluid (SF) APC have enhanced ability to stimulate autologous peripheral blood (PB) T-cell responses to a range of recall antigens when compared to their PB counterparts [11]. We propose that the increased SF APC activity is associated with the ability to provide enhanced T-cell co-stimulation and suggest this may play an important role in the pathogenesis of RA. To dissect this concept in vitro and to determine its relevance to RA, we established assays where TCR ligation and co-stimulatory signals were provided independently to human CD4 T-cell clones. TCR ligation was provided by soluble specific peptide or immobilized anti-CD3 monoclonal antibody. The co-stimulatory signals essential for T-cell proliferation were provided separately by APC isolated from the SF or PB of RA patients. Using these defined assays, we have demonstrated the superior co-stimulatory ability of rheumatoid SF APC in comparison to their PB
counterparts. Upregulated co-stimulatory activity in the synovium may be important in determining the localization of inflammation to the joints in RA and provide new therapeutic targets for immune intervention.

PATIENTS AND METHODS

Paired samples of PB and SF were obtained from 15 patients with early or active RA, as defined by the American College of Rheumatism criteria [12]. The effectiveness of APC from the two sites (PB and SF) of the same individual patients was compared in each set of experiments. Patients receiving steroids or second-line therapy were excluded.

Cell separation

Peripheral blood and hyaluronidase-treated SF mononuclear cells (MC) were obtained by centrifugation on Ficoll-Paque density gradients. Whole MC populations were initially used as a source of APC, but were further purified for subsequent experiments. In these experiments, cells were obtained from PBMC/SFMC as sheep red blood cell rosette-negative cells [13] and irradiated (3000 rad) prior to use. APC populations isolated by this technique were monitored to verify depletion of autologous T cells. This was achieved by incubating APC with anti-CD3 fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody on ice for 60 min. Cells were washed three times and resuspended in ice-cold phosphate-buffered saline (PBS), then analysed using the Coulter EPICS XL cytometer and were <5% CD3+. Cells were cultured in RPMI 1640 medium supplemented with 10% human serum, non-essential amino acids (1%), HEPES (10 mM) and sodium pyruvate (Sigma), glutamine (2 mM), streptomycin (100 μg/ml) and penicillin (100 U/ml).

T-cell clones

Peptide-specific CD4+ T-cell clones HG-B9, HP-40 (DR 4 restricted) and SPY-1 (DR3 restricted) were derived by limiting cell dilution of antigen-stimulated PBMC from normal controls, and respond to PPD, tetanus toxoid peptide 830-844 (synthesized by Alta Bioscience, Birmingham University) and Mycobacterium leprae 56 kDa HSP peptide 4-13, respectively. T-cell clones were maintained by culture in growth medium containing 50 μM recombinant interleukin-2 (rIL-2) (Eurocetus) and were expanded with irradiated allogeneic PBMC together with 1 μg/ml phytohaemagglutinin (PHA) (Wellcome Diagnostics) every 2–3 weeks. For proliferation assays, clones were at least 10 days post-addition of allogeneic PBMC and washed extensively prior to use.

Proliferation assays

All assays were performed in triplicate 200 μl volumes. Cell proliferation was measured by the uptake of [3H]thymidine (0.15 μCi/well; Amersham International, Amersham) during the last 6–8 h of culture.

Peptide stimulations. T-cell clones (2 × 10⁴) were incubated for 72 h with the optimal concentration of peptide (determined in previous assays) in the absence or presence of PB or SF APC (5 × 10⁶).

Anti-CD3 stimulations. To determine the optimal concentrations of antibody for use in subsequent experiments, varying concentrations of anti-CD3 antibody, OKT3 (purified on a protein G column from tissue culture supernatants of the ATCC OKT3 cell line) in bicarbonate buffer were incubated in 96 well plates overnight at 4°C. Plates were subsequently washed twice in PBS and incubated for 30 min at 37°C with the T-cell clones (2 × 10⁵) prior to addition of varying numbers of PB T-cell-depleted accessory cells (AC) obtained from a normal control. Selected concentrations of anti-CD3 antibody determined by this assay were used in further experiments to stimulate T-cell clones in the absence or presence of either PB or SF T-cell-depleted AC.

As with the peptide-stimulated cultures, antibody-stimulated cultures were harvested after 72 h onto glass fibre filters and radioactivity was determined by liquid scintillation counting using a Wallac Betaplate counter. Data represented are the mean c.p.m. of triplicate cultures ± s.e.m.

Phenotypic analysis of APC

AC were resuspended in a total volume of 100 μl ice-cold PBS (containing 1% heat-inactivated fetal calf serum) and the optimal, pre-determined dilution of conjugated antibody. Monoclonal antibodies (IgG1) CD11c:KB90, CD13:WM-47, HLA-DR:CR3/43 were purchased from Dako, FcγR1:32.2 from Medarex and CD14:M-A8 from Serotec. After incubation on ice for 30 min, cells were washed twice and resuspended in 500 μl ice-cold PBS/HIFCS and kept on ice until examined by flow cytometry. Ratios of relative fluorescence intensity (RFI) were calculated by dividing the median channel of fluorescence (MCF) for the specific cell marker antibody by the MCF of the irrelevant control antibody.

Statistics

The non-parametric Wilcoxon signed-rank test was used for comparison of data obtained from paired observations within individuals.

RESULTS

SFMC induce greater proliferation of peptide-specific T-cell clones

MC populations isolated from the SF and PB of four RA patients were examined for their ability to induce the proliferation of peptide-specific T-cell clones HG-B9 and SPY-1 (Fig. 1a). HG-B9 (panel A) and SPY-1 (panel B) proliferated minimally when cultured with PBMC or SFMC in the absence of specific peptide. Both T-cell clones proliferated strongly when cultured with soluble, specific peptide and SFMC. T-cell proliferative responses were considerably less marked when each clone was co-stimulated by PBMC isolated from the same patients. Although levels of peptide-specific proliferation varied between T-cell...
clones and patients, SFMC consistently enhanced responses when compared to their PBMC counterparts.

**MC autologous T-cell depletion does not alter SF APC-augmented co-stimulation**

Although demonstrating a contrast in the ability of SFMC and PBMC to induce the proliferation of peptide-specific T-cell clones, the previous assay did not address the possible contribution of autologous T cells, present within the MC populations, to these responses. To clarify this issue, MC were depleted of autologous T cells by sheep red blood cell (srbc) rosetting prior to use as a source of co-stimulation. The efficiency of srbc rosetting was monitored by flow cytometry using antibodies directed against CD3. This showed that T-cell-depleted MC contained <5% CD3-positive cells. The superior ability of T-cell-depleted SFMC (from a further three RA patients) to sustain peptide-specific T-cell proliferation is illustrated in Fig. 1b. In the absence of either TCR stimulation by specific peptide or co-stimulation provided by APC, T-cell clones did not proliferate. When co-stimulated by SF APC, T-cell clones cultured with specific peptide now proliferated. As before, with the use of PB APC as co-stimulators, levels of T-cell proliferation achieved were always less than those stimulated by their SF APC counterparts.

**TCR stimulation via the anti-CD3 antibody, OKT3**

Recent studies have demonstrated the ability of T cells stimulated with anti-CD3 antibody to proliferate in response to bystander APC providing only co-stimulatory signals (trans-co-stimulation) [14]. To
**Comparison of the co-stimulatory capacity of SF and PB APC**

A representative example of the co-stimulatory ability of SF and PB APC is illustrated in Fig. 3a. Again, as in the previous assay, TCR ligation by a single concentration of anti-CD3 antibody (10 μg/ml) in the absence of APC failed to initiate T-cell proliferation. Addition of SF APC did, however, induce a dose-dependent increase in T-cell proliferation. In contrast, PB APC from the same RA patient sustained minimal T-cell proliferation. Increasing the numbers of PB APC present resulted in only a minor increase in the levels of T-cell proliferation observed. The effect of increasing TCR stimulation, at a fixed dose of RA APC, was also examined and, at each concentration of anti-CD3 antibody used, the superior
co-stimulatory ability of SF APC was confirmed (Fig. 3b). The superior co-stimulatory ability of SF APC from three additional RA patients has been demonstrated using a range of anti-CD3 concentrations and varying APC cell numbers. In these experiments, purified normal PB T cells replaced T-cell clones as the responding population (data not shown).

**SF APC provide enhanced T-cell co-stimulation**
In order to establish that the enhanced co-stimulatory ability of SF APC was not a restricted phenomenon, a further eight RA patients were examined using a single concentration of APC and fixed dose of anti-CD3 antibody (Fig. 4). Both the magnitudes of the response and the difference between SF and PB APC-induced responses showed considerable variation between patients. However, in each case, the SF APC-related responses were significantly greater than their PB APC counterparts ($P = 0.0078$, Wilcoxon signed-rank test).

**TABLE I**
<table>
<thead>
<tr>
<th>Surface antigen</th>
<th>Mean ratio of PB to SF accessory cell RFI*</th>
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<tr>
<td>CD11c</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>CD13</td>
<td>1.1 ± 0.7</td>
</tr>
<tr>
<td>CD14</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>FcγR1</td>
<td>1.6 ± 1.2</td>
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<tr>
<td>ICAM-1</td>
<td>1.4 ± 0.5</td>
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<tr>
<td>HLA-DR</td>
<td>2.7 ± 0.6</td>
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*RFI, relative fluorescence intensity (see Materials and methods).

**Phenotypic analysis of RA APC**
To address the possibility that the contrast in RA APC co-stimulatory ability was associated with the presence or absence of a critical co-stimulatory APC population, SF and PB APC were analysed phenotypically in a parallel study. FACS analysis indicated no obvious differences in the expression of a variety of monocyte and activation markers, including CD14, and CD13, between SF and PB APC (Table I). SF APC did, however, express higher levels of MHC class II than PB APC. These results suggest that the enhanced co-stimulatory ability of SF APC is not related to the presence of a unique accessory cell subset, but rather to an alteration in the co-stimulatory signals provided by these cells.

**DISCUSSION**
The presence of activated CD4⁺ T cells in close association with MHC II-expressing APC is a prominent feature of RA synovitis. Analysis of APC function in RA has largely focused on assays such as allogeneic MLR where TCR stimulation and co-stimulation are provided together by the same APC. The most consistent factor with these assays has been the enhanced responses of SFMC compared to corresponding PBMC. In this study, we provide new evidence that this contrast is independent of RA T-cell function or APC antigen-presenting capacity, and demonstrate that SF APC co-stimulatory function is upregulated.

Although delivery of both TCR stimulation and co-stimulatory signals by the same APC has been shown to be the most efficient way of inducing T-cell proliferation [15], T cells can respond to their independent provision [16]. Human T-cell clones expressing MHC class II molecules, and able to present peptide [17], can provide a mechanism of examining the co-stimulatory function of APC in isolation. Initial experiments in this study using peptides able to bind directly to cell surface MHC class II molecules demonstrated that enhanced T-cell responses observed in the presence of SF APC were independent of their antigen-processing ability. However, the possibility arose that during these experiments, in addition to providing co-stimulatory signals, RA APC may have been able to bind peptide and provide TCR signals. As SF APC used in this study and others [18] express high levels of MHC class II molecules, this may have influenced T-cell proliferative responses. To avoid this complication, in subsequent experiments TCR stimulation was provided by a known quantity of immobilized anti-CD3 antibody.

Titration of both PB APC isolated from a normal control and anti-CD3 antibody concentrations induced a dose-dependent increase in T-cell proliferation. This ability of human T-cell clones to proliferate in response to the independent provision of co-stimulation and TCR ligation is in agreement with separate recent studies of human T-cell activation [19, 20]. Our results and accumulating evidence from several other groups [21] also suggest that a critical balance exists between
TCR and co-stimulatory signals required to induce optimal T-cell proliferation. These reports, like ours, suggest that the magnitude of a response may be influenced by the intensity of the co-stimulatory as well as the TCR stimulus. Although data presented here are in terms of T-cell proliferation, which was variable and sometimes unexpectedly low, we have also shown that the contrasting co-stimulatory ability of RA APC extends to the production of IL-2 and IFN-γ (manuscript in preparation).

Results presented in this study differ from previous reports as we specifically demonstrate variability in the co-stimulatory function of RA APC. At a fixed level of TCR stimulus, increasing numbers of SF APC enhanced T-cell proliferation dose dependently. In contrast, PB APC from the same patient, even when present in large numbers, co-stimulated minimal T-cell responses. The failure of increasing numbers of PB APC to augment T-cell proliferation suggests that rather than PB APC lacking a critical population of cells with co-stimulatory ability, SF APC provide qualitatively greater co-stimulatory signals. This is reinforced by our phenotypic data which do not suggest any obvious difference in the cell types present within PB and SF APC populations. The increased co-stimulatory ability of SF APC seen here is in agreement with the recent studies of Looney et al. [22] in which SF fibroblasts, unlike control fibroblasts, provided T-cell co-stimulation. This suggests that factors present within the inflamed synovium may be important in the upregulated co-stimulatory ability of SF cells observed in both studies. In addition to the enhanced co-stimulatory activity of SF APC, the limited co-stimulatory ability of PB APC may suggest that they have not received such local activation, preventing optimal T-cell activation. Alternatively, depressed PB APC co-stimulatory ability may be regarded as protective in terms of a systemic disease like RA. This is important as a longitudinal study of reactive arthritis patients demonstrated that PBMC responses to the triggering organism were depressed during the acute disease, but increased significantly on recovery [23]. Moreover, our recent studies using normal PB T cells as responders demonstrate that RA PB APC co-stimulatory function appears to be depressed when compared with controls.

This study has highlighted the enhanced functional ability of SF APC to provide co-stimulation essential for T-cell proliferation. The clinical relevance of this is clearer than the mechanisms. We propose that the enhanced co-stimulatory ability of SF APC may play an important role in the pathogenesis of the synovial immune response and its localization to the joint. This phenomenon is independent of specific joint antigens, whether these are exogenous or autoantigens. The in vitro assays described here, capable of assessing the co-stimulatory ability of RA APC, will now enable us to dissect further the mechanisms involved and compare this with normal APC function. This, in turn, may allow future therapeutic intervention and modulation of disease activity in RA.

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