STEROID PULSE THERAPY FOR RHEUMATOID ARTHRITIS: EFFECT ON LYMPHOCYTE SUBSETS AND MONONUCLEAR CELL ADHESION

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SUMMARY

Eighteen patients with clinically active rheumatoid arthritis, satisfying the ARA criteria, were admitted to hospital for i.v. methylprednisolone pulse therapy. Studies of circulating lymphocyte subsets 1 h before and 24 h after pulsing were carried out together with studies on their adhesion to endothelium-containing lamina propria of porcine gut at various time points. Additionally, circulating VCAM-1 was estimated pre- and post-pulse by ELISA. We observed a marked fall (59%) in circulating mononuclear cells (P < 0.01), mainly involving T cells. However, the degree of reduction in cell adhesion did not appear to reflect change in any particular circulating subset, but was more likely due to changes in adhesion molecule expression of several subsets. No significant change in circulating VCAM-1 was observed. It would appear, therefore, that the early beneficial effect of steroid pulsing in rheumatoid arthritis coincides with a demonstrable reduction in cell adhesion to gut. This may have implications for the pathogenesis of this disease.

KEY WORDS: Rheumatoid arthritis, Adhesion molecules, Corticosteroid therapy, Lymphocyte subsets.

RHEUMATOID arthritis (RA) is characterized by chronic synovial inflammation, with invasion of the synovial membrane by leucocytes, induced and maintained by inflammatory cytokines [1, 2]. Corticosteroids have been used in the treatment of RA since 1949 [3], and are noted for their ability to induce rapid amelioration of symptoms and signs of this disease. Recently, they have been claimed to be beneficial in small doses early in the disease [4]. Corticosteroids administered by pulse are known to have many effects in active RA, such as decreasing serum interleukin-6 (IL-6), IL-8 and soluble IL-2 receptor levels [5], as well as diminishing C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and immune complex levels [6, 7]. They cause a temporary lymphocytopenia (especially of T cells), as well as fluctuations in the levels of synovial fluid and peripheral blood neutrophil levels [6].

Accumulation of polymorphonuclear cells and lymphocytes within the synovial environment in RA follows their migration from the bloodstream. Entry into the synovium is achieved via adherence to vascular endothelium, and migration into the matrix of the membrane. Adhesion molecules found on both the endothelial surface and the leucocyte surface mediate this migration, and hence play an important role in the pathogenesis of RA. On endothelial surfaces, VCAM-1, ICAM-1, CD44, P- and E-selectin, and GlyCAM-1 are present. On lymphocyte surfaces are found counter-receptors. These include the integrins VLA-4, alpha4-beta7 and LFA-1, plus, amongst others, sialated Lewis-X, sialated Lewis-A and L-selectin [8–11]. Of these, ICAM-1/LFA-1 and, perhaps more importantly, the VCAM-1/VLA-4 interactions seem to play an important role in the accumulation of lymphocytes within the synovial environment [12, 13].

Aberrant lymphocyte recirculation (‘iteropathy’) may be involved in RA, lymphocytes of mucosal origin homing pathologically to synovial joints [14]. We have previously shown that synovial fluid mononuclear cells from RA patients adhere preferentially to endothelium-containing lamina propria of porcine gut compared with lymph node tissue, and in greater proportion than peripheral blood from the same individual [15].

Little is known, however, of the in vivo effects of pulse corticosteroid therapy on adhesion molecules or lymphocyte migration pathways. We have therefore studied the constitution of mononuclear cell populations and mononuclear cell adhesion in patients with active disease before and after undergoing this form of treatment. We also studied circulating VCAM-1 since this adhesion molecule is found on endothelial cells, as well as in the circulation. In the event that pulse steroid therapy may affect its expression on endothelium, we wondered whether this might be reflected in the circulation.

MATERIALS AND METHODS

Patients

Eighteen patients (13 female and five male, aged 50–75 yr) who satisfied the ARA revised criteria for RA [16], with active disease, were treated with a steroid pulse either to induce benefit prior to the kick-in effect of their new disease-modifying anti-rheumatic drug (DMARD), or as intercurrent treatment whilst remaining on their existing DMARD. None were receiving immunosuppressives. Each patient received an i.v. infusion of 0.5 g methylprednisolone acetate over a 45 min period.

Blood samples

Separation of mononuclear cells [for cell adhesion studies at various time points (n = 18) (see Table I) and for immunophenotyping of separated cells pre- and
Ten fields were counted on each tissue section. Expressed as nanograms per millilitre.

Quantification was carried out by one observer (AK) using bright-field microscopy, ×250 magnification, with a graticule-equipped eyepiece. PB MC (>90% lymphocytes) adherent to lamina propria were enumerated. Adherence was expressed as the number of cells adherent per square millimetre. Latex particle adherence was subtracted from this figure to allow for background adherence. Ten fields were counted on each tissue section.

**post-pulsing (n = 8)**. Mononuclear cells (MC) were obtained from peripheral blood (PB) by density-gradient centrifugation. Viability was >90% by trypan blue exclusion. Morphologically, by Leishmann staining, >90% of the cells had the appearance of lymphocytes. Pre-pulse MC were stored overnight at 4°C in Iscove’s modified Dulbecco’s medium, and adherence assessed the following day, together with post-pulse MC. Previous experiments had established that there was no significant alteration in adherence of cells thus stored for up to 48 h.

**Whole blood samples** for immunophenotyping of circulating PB lymphocyte subsets pre- and 24 h post-steroid pulsing (n = 8). Samples (5 ml) of PB were mixed with EDTA and immunophenotyped using a flow cytometer (FACscan; Becton Dickinson). Analyses were carried out in parallel on separated mononuclear cells, as used in the adhesion assay, and whole blood (as a control for the effect of the cell separation process on the constitution of cell subsets).

**Adherence assay**

Adherence of MC from RA patients was enumerated at in vitro adherence assay using serial frozen sections of endothelium-containing gut lamina propria from porcine small intestine cut from the same block in individual experiments. The method used was as previously described [15]. Briefly, cell suspensions consisting of 250,000 separated MC were layered onto the tissue section previously encircled by a limiting wax pen mark. This was agitated on a shaking platform at 70 r.p.m. for 30 min at 4°C. The same volume and quantity of latex particles of similar diameter to MC were added, and incubation continued at room temperature. A 5 min wash was followed by the addition of mouse alkaline phosphatase anti-alkaline phosphatase (APAAP) Mab diluted 1:40 in PBS which would bind to the previously layered bridging Mab. A final wash for 5 min in PBS was carried out before the addition of substrate for the alkaline phosphatase. This was prepared by dissolving 12.5 mg naphthol phosphate sodium salt in 25 ml Veronal acetate buffer, and by adding 12.5 mg fast red TR salt and 6 mg levamisole to inhibit any endogenous phosphatase activity. The sections were rinsed in 0.9% saline and incubated with the APAAP substrate for 20 min at room temperature. Sections were counterstained briefly with haematoxylin and washed in tap water. They were mounted in aqueous mounting medium (Glycergel). All mononuclear cells adherent to lamina propria were enumerated and expressed as percentage positive or negative cells.

**Circulating VCAM-1 assay**

Plasma samples were stored at −20°C until the assay was performed on all eight cases. Human soluble VCAM-1 (sVCAM-1) was assayed using a commercially available kit (R&D Systems). One hundred microlitres of anti-sVCAM-1 horseradish peroxidase conjugate were added to each well of a 96 well plate, followed by 100 μl of 1:50 diluted plasma sample, or 100 μl of sVCAM-1 control standard. Wells were mixed gently and incubated for 1.5 h at room temperature. Well contents were then aspirated and washed five times with 300 μl of wash buffer. After aspiration, the plate was inverted and the last trace of buffer removed by tapping the plate on a paper towel. One hundred microlitres of substrate solution (tetramethylbenzidine) were added, and incubation continued at room temperature for a further 30 min. The reaction was then stopped by the addition of acid solution and the optical density of wells read using a plate reader (Dynatech MR600) set at 450 nm with a correction wavelength of 620 nm. All samples were assayed in duplicate, and compared with a standard curve. Results were expressed as nanograms per millilitre.

**TABLE I** The coefficient of variation was 3.9%. All experiments were performed coded and read double blind.

**Immunophenotyping of adherent cells**

The adherence technique was followed as above, except that sections were fixed with acetone for 10 min at 4°C, instead of glutaraldehyde, to prevent cross-linking of mononuclear cell surface antigens. Sections were air dried and washed in PBS for 5 min. Normal rabbit serum (NRS), diluted 1:5 in PBS, was overlayed on each section. Excess serum was tapped off and 50 μl of mouse monoclonal antibody (Mab) to human CD3, CD4, CD8, CD19 and HLA-DR (all previously diluted 1:50 in PBS) were layered onto the sections. Triplicate samples were assayed, as well as three negative controls. After 30 min incubation, sections were washed for 5 min in PBS and incubated with 50 μl of rabbit anti-mouse Mab, dilution 1:20, for 30 min at room temperature. A 5 min wash was followed by the addition of mouse alkaline phosphatase anti-alkaline phosphatase (APAAP) Mab diluted 1:40 in PBS which would bind to the previously layered bridging Mab. A final wash for 5 min in PBS was carried out before the addition of substrate for the alkaline phosphatase. This was prepared by dissolving 12.5 mg naphthol phosphate sodium salt in 25 ml Veronal acetate buffer, and by adding 12.5 mg fast red TR salt and 6 mg levamisole to inhibit any endogenous phosphatase activity. The sections were rinsed in 0.9% saline and incubated with the APAAP substrate for 20 min at room temperature. Sections were counterstained briefly with haematoxylin and washed in tap water. They were mounted in aqueous mounting medium (Glycergel). All mononuclear cells adherent to lamina propria were enumerated and expressed as percentage positive or negative cells.

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**TABLE I** Numbers of patients whose cell adherence was enumerated at the various time points shown

<table>
<thead>
<tr>
<th>Time points for venepuncture relative to steroid pulsing</th>
<th>Numbers of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>−24, −23, −18, −1 h, +1, +6, +24 h, +2 weeks</td>
<td>1</td>
</tr>
<tr>
<td>−24 h, −1 h, +1 h, +24 h</td>
<td>3</td>
</tr>
<tr>
<td>−1 h, +1 h, +24 h</td>
<td>5</td>
</tr>
<tr>
<td>−1 h, +24 h</td>
<td>9</td>
</tr>
<tr>
<td>−1 h, +24 h (inclusive total)</td>
<td>18</td>
</tr>
</tbody>
</table>

**Immunophenotyping of adherent cells**

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affected than B cells, in particular the CD4⁺ T-cell subset. When the relative proportions of circulating mononuclear subsets were compared, although there was a trend for a reduction in CD4⁺ T-helper cells, this did not reach significance. Likewise, in the case of separated cells, there was no significant change in any of the populations studied (data not shown).

The qualitative effect of steroid pulsing on the adherent cell population was studied by APAAP staining (see Table III). Apart from a just significant increase in the proportion of adherent cells expressing HLA-DR, there was no significant change in CD3, CD4, CD8 or CD19 expressing cells.

Circulating soluble VCAM-1 was determined pre- and 24 h post-pulse (n = 8). The mean and s.e. for pre- (−1 h) and post-pulse (24 h) was 842 ± 50 ng/ml and 813 ± 81 ng/ml. Clearly, there was no significant change.

DISCUSSION

We have found that in 18 cases i.v. pulse methylprednisolone therapy administered during active RA significantly reduced the adhesion of PB MC to endothelium-containing lamina propria of porcine gut by > 55%. The reduction was not observed at 1 h post-pulsing, although in one case studied in greater detail it was seen by 6 h. The maximum reduction was observed at 24 h and, in the aforementioned case, had returned to pre-pulse levels by 2 weeks.

The question naturally arises whether this observation can be explained by a quantitative change in the cells used in the assay, as a result of pulsing. We feel that this is not the explanation, although there was a reduction in the concentration and relative proportion of circulating CD4⁺ T cells in PB, but not in separated MC as applied in the adherence assay. Pulse methylprednisolone therapy has been shown by others to cause lymphocytopenia, affecting T cells in particular, maximal at 6 h but returning to pre-treatment levels by 24 h, although not significantly altering circulating subsets [5, 6]. We found that, apart from a reduction in the numbers of CD4⁺ T cells, methylprednisolone did not significantly reduce both the concentration and relative proportion of any circulating subset. Therefore, the reduction in adhesion was unlikely to have been due to a fall in the concentration of any particular subset of mononuclear cells, but was more likely due to an effect on the adhesion phenomenon itself, i.e. a quantitative or qualitative effect on adhesion molecule expression on the cells involved. Clearly, this would seem to be a profitable area for future study. Pertinent to this, recent work into cell adhesion molecule expression on synovial vascular endothelium, in patients with RA undergoing identical pulse therapy to our patients, has shown a substantial decrease in E-selectin and a smaller reduction in ICAM-1 expression, revealing a potential mechanism for the inhibition of neutrophil entry into inflamed RA joints [17].

Fig. 1.—Effect of corticosteroid pulse on adherence of mononuclear cells (MC) of several patients at the time points indicated. P < 0.001 at 24 h compared to pre-pulse.
Fig. 2.—Effect of corticosteroid pulse on adherence at multiple time points in one patient.

### TABLE II

<table>
<thead>
<tr>
<th>Immunophenotype</th>
<th>Cell nos pre-pulse (×10^9/l)</th>
<th>Cell nos post-pulse (×10^9/l)</th>
<th>% change in cell nos (×10^9/l)</th>
<th>Pre-pulse (%)</th>
<th>Post-pulse (%)</th>
<th>Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>1.13</td>
<td>0.71</td>
<td>−37.2</td>
<td>80 (3)</td>
<td>74 (4)</td>
<td>−6</td>
</tr>
<tr>
<td>CD3</td>
<td>1.08</td>
<td>0.61</td>
<td>−43.5</td>
<td>74 (3)</td>
<td>63 (3)</td>
<td>−11</td>
</tr>
<tr>
<td>CD4</td>
<td>0.67</td>
<td>0.35</td>
<td>−47.5</td>
<td>47 (5)</td>
<td>36 (4)</td>
<td>−11</td>
</tr>
<tr>
<td>CD8</td>
<td>0.4</td>
<td>0.29</td>
<td>−27.5</td>
<td>28 (5)</td>
<td>31 (5)</td>
<td>+3</td>
</tr>
<tr>
<td>CD19</td>
<td>0.22</td>
<td>0.22</td>
<td>0</td>
<td>13 (3)</td>
<td>19 (4)</td>
<td>+6</td>
</tr>
<tr>
<td>CD44</td>
<td>1.41</td>
<td>0.96</td>
<td>−32</td>
<td>97 (1)</td>
<td>96 (1)</td>
<td>−1</td>
</tr>
<tr>
<td>CD45RA</td>
<td>0.87</td>
<td>0.63</td>
<td>−27.6</td>
<td>58 (5)</td>
<td>62 (4)</td>
<td>+4</td>
</tr>
<tr>
<td>CD45RO</td>
<td>0.44</td>
<td>0.28</td>
<td>−36.4</td>
<td>34 (4)</td>
<td>30 (4)</td>
<td>−4</td>
</tr>
<tr>
<td>HLA-DR+</td>
<td>0.33</td>
<td>0.32</td>
<td>−0.04</td>
<td>24 (2)</td>
<td>31 (3)</td>
<td>+7</td>
</tr>
</tbody>
</table>

The question of the validity of xenogeneic tissue has been addressed by others in respect to rodent/human tissues. Thus, human alpha4-beta7 integrin-expressing lymphocytes, like their murine counterparts, have been shown to adhere to murine MAdCAM-1 [18], suggesting that this addressin is conserved in both species. Human MAdCAM-1 selectively binds both murine and human lymphocyte cell lines expressing alpha4-beta7, and Meca-367 inhibits the adherence of alpha4-beta7-expressing murine lymphocytes to human MAdCAM-1 as well as to their own species [19]. Cross-species reactivity has been shown for mouse anti-human E-selectin Mab which inhibited porcine leucocyte adherence to human as well as to porcine E-selectin [20]. The authors suggest that the immunological similarities between pig and human E-selectin, in terms of structure and function in particular, plus conserved sequence homologies, are also suggestive of such a cross-reactivity [20, 21]. It is conceivable that such similarities may also exist for other porcine adhesion molecules, leading to cross-reactivity with equivalent human adhesion molecules.

The lack of effect on circulating VCAM-1 levels does not of itself preclude the possibility of an effect

### TABLE III

<table>
<thead>
<tr>
<th>Immunophenotype</th>
<th>Pre-pulse</th>
<th>Post-pulse</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>71</td>
<td>67</td>
<td>NS</td>
</tr>
<tr>
<td>CD4</td>
<td>59</td>
<td>55</td>
<td>NS</td>
</tr>
<tr>
<td>CD8</td>
<td>37</td>
<td>31</td>
<td>NS</td>
</tr>
<tr>
<td>CD19</td>
<td>22</td>
<td>24</td>
<td>NS</td>
</tr>
<tr>
<td>HLA-DR+</td>
<td>51</td>
<td>66</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

on endothelially expressed VCAM-1. It would have been interesting to have investigated other endothelial molecules before and after pulsing, e.g. ICAM-1 and CD44.

Corticosteroids bind to their cellular receptors to form complexes and then move within the nucleus triggering special corticosteroid-sensitive target genes. These, in turn, have been shown to stimulate transcription, as well as inhibiting gene expression [22]. Therefore, it seems likely that methylprednisolone may have a direct effect on either the expression of adhesion molecules by downregulating their transcription and
subsequent protein synthesis or, alternatively, exerting a more general effect, such as inhibition or downregulation of RNA function in the cell. Glucocorticoids have been shown to inhibit upregulation by tumour necrosis factor-\( \alpha \) of ICAM-1 expression in synovial fibroblasts [23]. Dexamethasone has been shown to inhibit RNA transcription of the IL-2 gene and increase the degradation of IL-2 mRNA [24]. It also inhibits E-selectin expression in a dose-dependent fashion [25]. Methylprednisolone at a dose of 30 mg daily has been shown to downregulate expression of LFA-1 [26]. Topical steroids administered to allergic patients have been shown to inhibit local elevation of IL-1\( \beta \), GM-CSF and the chemokines IL-8, MIP-1 alpha and RANTES [27]. However, our data have demonstrated an effect of steroid pulsing on \textit{ex vivo} cell adhesion during active RA that, to our knowledge, has not previously been demonstrated. We feel that this is likely to play an important role in the expression of active disease.

With further knowledge of the mechanisms involved, other treatment modalities directed specifically at reduction of cell adhesion would suggest themselves as worthy of exploration, hopefully thereby harnessing some of the beneficial effects of corticosteroid therapy whilst avoiding the side-effects.

\textbf{References}