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Cytokine production in synovial tissue of mice with collagen-induced arthritis (CIA)

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SUMMARY

The kinetics of cytokine production in arthritic limbs of mice with CIA was determined by using modified immunohistochemical techniques. Tissue cryostat sections of undecalcified whole paws were analysed for the presence of tumour necrosis factor-alpha (TNF-α), IL-6, IL-2, IL-4, IL-5 interferon-gamma (IFN-γ), transforming growth factor-beta 2 (TGF-β2) and TGF-β3. Locally produced TNF-α, IL-6 and TGF-β2 were observed within the lining layer, sublining and pannus at all stages of disease. The staining of TNF-α was particularly intense at the cartilage–pannus junction. In contrast to the monokines, IFN-γ and TGF-β3 were only expressed in scattered cells within the deeper layers of the synovia. Interestingly, IFN-γ was not present in the late phase of CIA, despite the continued presence of TNF-α and IL-6 in the pannus. Production of IL-2, IL-4 or IL-5 was not detected in any joint. The observed pattern of a relative paucity of T cell-derived cytokines and an abundance of monokines during the late phase of T cell-dependent CIA indicates that the synovial cytokine pattern previously described in rheumatoid arthritis (RA) is fully compatible with a pathogenic role of T cells. The temporal as well as spatial dissociation between expression of T cell-derived cytokines and monokines indicates that T cell-independent mechanisms may also be of importance in the triggering of monokine production during arthritis.

Keywords cytokine kinetics collagen-induced arthritis

INTRODUCTION

The regulation of cytokine production during rheumatoid arthritis (RA) has been intensively studied during the last few years. The first studies of patients in late stages of disease indicated an abundance of monokines and a relative lack of lymphokines [1–8]. In contrast to these results, studies on arthroscopic biopsies from early onset of disease have demonstrated the presence of T cell-derived cytokines, although restricted to a minority of T cells infiltrating the joint [9,10]. The results of these investigations in humans have been difficult to interpret, and have therefore led to a debate concerning the relative roles of T cells *versus* macrophages in the pathogenesis of RA. Moreover, it has been difficult to elucidate to what extent the various synovial cytokine patterns observed represent various disease entities or different phases within one and the same disease.

In contrast to the situation in RA, little is known concerning the synovial production of cytokines in relevant animal models of arthritis. However, we assume that such knowledge of the local cytokine production in experimental arthritis would be of interest, not only for increased understanding of the pathogenesis of disease, but also in order to serve as a reference for what is to be anticipated in various phases of other chronic arthritides. We have accordingly chosen to study synovial cytokine production at various time points during CIA in the DBA/l mouse.

Using a recently developed procedure for the evaluation of intracellular production of cytokines by means of immunohistochemistry and intracellular staining of cryopreserved sections from arthritic joints [11], we have obtained a new insight into the dynamics and distribution of cytokine production in a T cell-dependent mouse arthritis model.

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MATERIALS AND METHODS

Mice

Male DBA/l mice, 8–12 weeks old, originally purchased from Harlan UK Limited (Bicester, UK), were used. Mice were bred and maintained at the animal unit of the Karolinska Hospital (Stockholm, Sweden). The mice were kept in a separate animal room under climate controlled conditions with a 12-h light/dark cycle, housed in polystryrene cages containing wood shavings and fed standard rodent chow and water *ad libitum*. During the experiments six to eight mice were kept in each cage. Mouse colonies were screened and determined to be pathogen-free.

Induction of CIA

Mice were immunized intradermally at the base of the tail with a total volume of 100 μl of 150 μg rat collagen type II (prepared from

a rat chondrosarcoma as described previously [12,13]) dissolved in 0·1 M acetic acid and emulsified 1 : 1 with Freund's complete adjuvant (FCA; Difco, Detroit, MI). The mean day of onset of clinical arthritis was 30 days after immunization. Day 1 of arthritis was considered to be the day on which erythema and/or swelling was first observed.

Antibodies used for immunohistochemical analysis

MoAbs used were: anti-IL-2, S4B6; anti-IL-4, BVD4-1D11; anti-IL-5, TRFK4 and TRFK5; anti-IL-6, MP5-20F3; anti-interferon-gamma (IFN-γ), XMG12; anti-tumour necrosis factor (TNF), MP6-XT122; anti-CD4, RM4-5; anti-CD8a, 53.6.7; anti-CD11b (Mac-1), M1/70; anti-IA^q, KH116; and anti-GR-1 (myeloid differentiation antigen), RB6-8C5. These MoAbs were purchased from PharMingen (San Diego, CA).

Affinity-purified polyclonal antibodies used were: anti-transforming growth factor-beta 2 (TGF-β2), K94 and anti-TGF-β3, K95 (polyclonal antibodies were kindly provided by Dr K Miyazono, Ludwig Institute, Uppsala, Sweden).

Preparation of samples for immunohistochemical analysis

Animals were killed 0 h, 2–3 days, 10–12 days and 23–25 days after onset of arthritis development. The hindpaws were removed, immediately snap frozen in isopentane prechilled by liquid nitrogen, and kept at −70°C until sectioned. Horizontal sections of 8 μm thickness, encompassing the ankle and metatarsal joints, were cut and mounted on chromium potassium sulfate and gelatin-coated glass slides (Novakemi, Stockholm, Sweden), and were either fixed for 20 min in 2% formaldehyde (Sigma Chemical Co., St Louis, MO) in PBS at room temperature, or fixed in 50% acetone for 30 s followed by 100% acetone for 3 min at 4°C. All slides were subsequently stored at −70°C until required for staining.

Immunohistochemical detection of intracellular cytokine on formaldehyde-fixed frozen sections

The cryopreserved sections were stained for intracellular production of cytokines as previously described [11]. Briefly, endogenous peroxidase activity was blocked by applying 1% hydrogen peroxidase and 2% sodium azide dissolved in balanced salt solution (BSS; GIBCO Ltd, Paisley, UK) supplemented with 0·1% saponin (Riedel de Haen AG, Seelze, Germany) for 1 h at room temperature in the dark. The sections were then washed twice in BSS–saponin and thereafter blocked with avidin–saponin for 15 min and biotin–saponin for an additional 15 min (avidin/biotin blocking kit; Vector, Burlingame, CA). After three additional washes in BSS–saponin, sections were incubated overnight at room temperature in a humidified chamber with 100 μl cytokine-specific MoAb (5 μg/ml) diluted in BSS–saponin. The slides were then washed thoroughly in BSS–saponin and 100 μl of biotinylated rabbit anti-rat IgG (mouse adsorbed; Vector) 1 : 200 in BSS–saponin were applied for 45 min at room temperature. Sections were again rinsed in BSS–saponin, and 100 μl of a solution of Vectastain avidin-biotin-horseradish peroxidase (Vectastain, ABC-HP-kit; Vector) prepared according to the manufacturer's directions in BSS–saponin were applied for 45 min at room temperature. After a final wash in BSS without saponin, the substrate diaminobenzidine (Peroxidase Substrate Kit; Vector) was added. The reaction field was stopped after 10–15 min by three washes in BSS, after which sections were counterstained with Mayer's haematoxylin, and slides were mounted in a glycerin buffer.

Immunohistochemical detection of TGF-β2, TGF-β3 and cell surface markers on acetone-fixed sections

To inactivate endogenous peroxidase, the slides were treated with PBS containing 0·3% H_2O_2 for 30 min. After additional washes in PBS, the sections were incubated in a humidified chamber overnight at room temperature with 100 μl portions of unlabelled rat MoAbs, biotin-labelled mouse MoAbs or unlabelled rabbit polyclonal antibodies diluted in PBS. Thereafter, biotinylated secondary antibodies were applied and incubated for 45 min at room temperature. Sections were again rinsed in PBS, and thereafter incubated in ABC Elite complex at room temperature for 45 min. The immunocomplexes were visualized by aminoethylcarbazole, to which 0·3% hydrogen peroxidase had been added. Finally, slides were counterstained with haematoxylin.

Specificity tests

Non-specific binding of secondary antibody or of detection complex was excluded by omitting the primary antibody and by using an irrelevant antibody in each assay. In blocking experiments, antibodies were preincubated overnight at 4°C with 15–25 μg/ml of the corresponding peptides before application to tissues. Spleens from *Trypanosoma cruzi*-infected mice were used as positive controls for IL-2, IL-4 and IL-5.

Scoring system

Slides were scored blind by two independent persons. The infiltrates in the ankle as well as metatarsal joints were analysed. The following scoring system for assessment of numbers of cytokine-producing cells per cryocut section was employed: 0 = negative cells, (+) <1% positively stained cells, + = 1–10% positively stained cells, ++ = 10–25% positively stained cells, +++ = 25–50% positively stained cells, and ++++ = 50–75% positively stained cells. Data indicate the mean values from six mice per time point.

RESULTS

Distribution and kinetics of monokine-producing cells in arthritic and non-arthritic joints

To analyse the functional state of macrophages and monokine production in the joint, MoAbs with specificity against TNF and IL-6 were used. It was evident that many of the inflammatory cells stained for TNF throughout the disease process, with a decline 3 weeks after onset of disease (Table 1). TNF-positive cells were mainly localized in the lining layer of the synovial membrane, while other positive cells had a distribution in the deeper layers and throughout the pannus area at the first observation period. The cells producing TNF were detected throughout the disease process in the lining layer and sublining layer. Interestingly, the intensity of TNF staining particularly increased at the interface of cartilage and bone (Figs 1c, 2c, 3b).

The distribution pattern of IL-6-positive cells was similar to that of TNF-expressing cells (Figs 1, 2 and 3). However, in contrast to TNF, the intensity of IL-6 did not increase at the cartilage–pannus junction. No IL-6- or TNF-positive cells were detected in non-arthritic synovial tissues.

Distribution and kinetics of lymphokine-producing cells in arthritic joints

MoAbs with specificity for IFN-γ, IL-2, IL-4 and IL-5 were used to analyse the functional state of lymphocytes in the joints. Scattered

Table 1. Frequency* of cells in arthritic and non-arthritic limbs recognized by antibodies specific for:

d.a.o.†	TNF	IL-6	IFN-γ	TGF-β2	TGF-β3	CD4	IA^q	Mac-1	Gr-1
0	0	0	0	(+)	0	0	(+)	+	+
2–3	+++	++	+	+	++	+	++	+++	+++
11–12	+++	+++	+	++	++	++	++	++++	++++
23–25	++	++	0	++	+	+	+	+++	+++

*0 = 0%; (+) <1%; + = 1–10%; ++ = 10–25%; +++ = 25–50%; ++++ = 50–75%.
† Days after onset of arthritis.

cells expressing IFN-γ were observed in the sublining layer, at a distance from cartilage and bone (Figs 1a and 2a). The highest frequency of IFN-γ-producing cells was noted 3–12 days after onset of disease, whereas 3 weeks after onset virtually no staining for IFN-γ was apparent (Table 1). IL-2, IL-4 or IL-5-producing cells were never recorded in any joint, but were detected in positive controls.

Distribution and kinetics of TGF-β2- and TGF-β3-producing cells in arthritic and non-arthritic joints

In synovial sections from normal joints a weak staining for TGF-β2 was observed in vascular endothelial cells, whereas no cells stained positive for TGF-β3 (Table 1). Two days after onset of disease, blood vessels and cells adjacent to tendons stained intensively positive with antibodies directed against TGF-β2 (Fig. 4a). Scattered TGF-β3-positive cells were also observed in the synovial tissue at this time point (Fig. 4b). Later on in the inflammatory process, 2 weeks after onset of disease, TGF-β2 was expressed in the lining layer and TGF-β3 in the sublining layer (Fig. 4c,d). In the late phase of CIA, an intense immunoreaction for TGF-β2 was detected throughout the pannus area, whereas scattered cells expressing TGF-β3 were observed throughout the synovia (Fig. 4e,f).

Distribution of cell surface markers

In sections from non-arthritic limbs, most lining cells in the non-proliferative synovium stained with the Mac-1 (granulocytes, macrophages and natural killer cells) and Gr-1 (granulocytes) markers. Low numbers of Mac-1 and Gr-1 staining cells were also localized in the sublining layers of the synovial tissue. A few cells in the lining layer stained with the anti-IA^q antibody. In contrast, no CD4$^+$ or CD8$^+$ cells were observed in non-arthritic joints (Table 1).

In arthritic joints, a large fraction of synovial cells were stained with Mac-1 and Gr-1. These cells were observed within the thickened synovial lining layer, within the sublining layer and in the pannus, constituting 50–75% of the total number of infiltrating cells (Table 1, Fig. 5c,d). The predominance of Mac-1$^+$ and Gr-1$^+$ cells (i.e. granulocytes and macrophages) was evident at all stages of disease, reaching its plateau 12 days after disease onset (Table 1).

Scattered cells expressing CD4 were observed throughout the disease process in the lining layer and in the deeper layers of the synovia, at a distance from cartilage and bone (Fig. 5a). Early after onset of disease <10% of the total number of inflammatory cells were CD4$^+$. The highest frequency of CD4-staining cells, approaching 15% of all infiltrating cells, was noted around day 12 after arthritis development (Table 1). Notably, very few cells staining for CD8 (<1%) were apparent within the arthritic limbs.

The number (10–25%) and distribution of IA^q-expressing cells did not change significantly in the arthritic joints throughout the observation period (Table 1, Fig. 5b). In addition to the cells within the lining layer, scattered cells reacting with anti-IA^q antibodies were also demonstrated in the deeper connective tissue and at the cartilage–pannus junction.

DISCUSSION

In this study we have demonstrated that a recently developed technique, based on immunohistochemical intracellular staining of cytokines [11], can be used to analyse cytokine-producing cells in the joints of mice with CIA. The results from these stainings indicated three major findings of substantial interest: (i) a dominant and universal presence of monokines in contrast to a paucity of T cell-derived cytokines throughout the disease process of T cell-dependent inflammatory arthritis; (ii) a difference in the cytokine pattern at various stages of disease, with T cell-derived cytokines (IFN-γ) only being expressed during the initial phase of disease and monokines being present at all stages of disease; (iii) a difference in distribution pattern of lymphokines in relation to monokines, in which cells staining for monokines, i.e. TNF and IL-6, were observed in the lining and sublining layers, whereas IFN-γ-positive cells were recorded only in the sublining layer.

The cytokine-specific MoAbs used in the present study have been carefully selected from a panel of different MoAbs. By using these MoAbs for staining, combined with the modified techniques for fixation and permeabilization of frozen sections of arthritic joints, we have been able to detect the *in situ* production of intracellular cytokines. This intracellular staining of cytokines, confined to the Golgi apparatus, has previously been described [11], and determined to correlate well with the production of the respective cytokine mRNA. Concerning the staining of TGF-β2 or TGF-β3, it should be noted that antisera against the precursor forms of these TGF-βs were used, due to the fact that active TGF-β isoforms are so similar that the available antisera have the ability to cross-react.

The results from this investigation demonstrate an abundance of monokines, i.e. TNF and IL-6, and a relative paucity of T cell cytokines, i.e. IFN-γ, at all stages of disease. Similar results have been reported in RA, demonstrating a pronounced expression of monokines in relation to lymphokines [1–10]. These data have provoked a debate concerning the importance of T cell activation in RA. With this in mind, it is of interest to note that the cytokine staining patterns described in synovial lesions of RA are very similar to the cytokine staining patterns of synovial lesions in CIA, particularly during the late stages of this experimental disease. As CIA is well documented to be dependent on functional T cells [14–17],

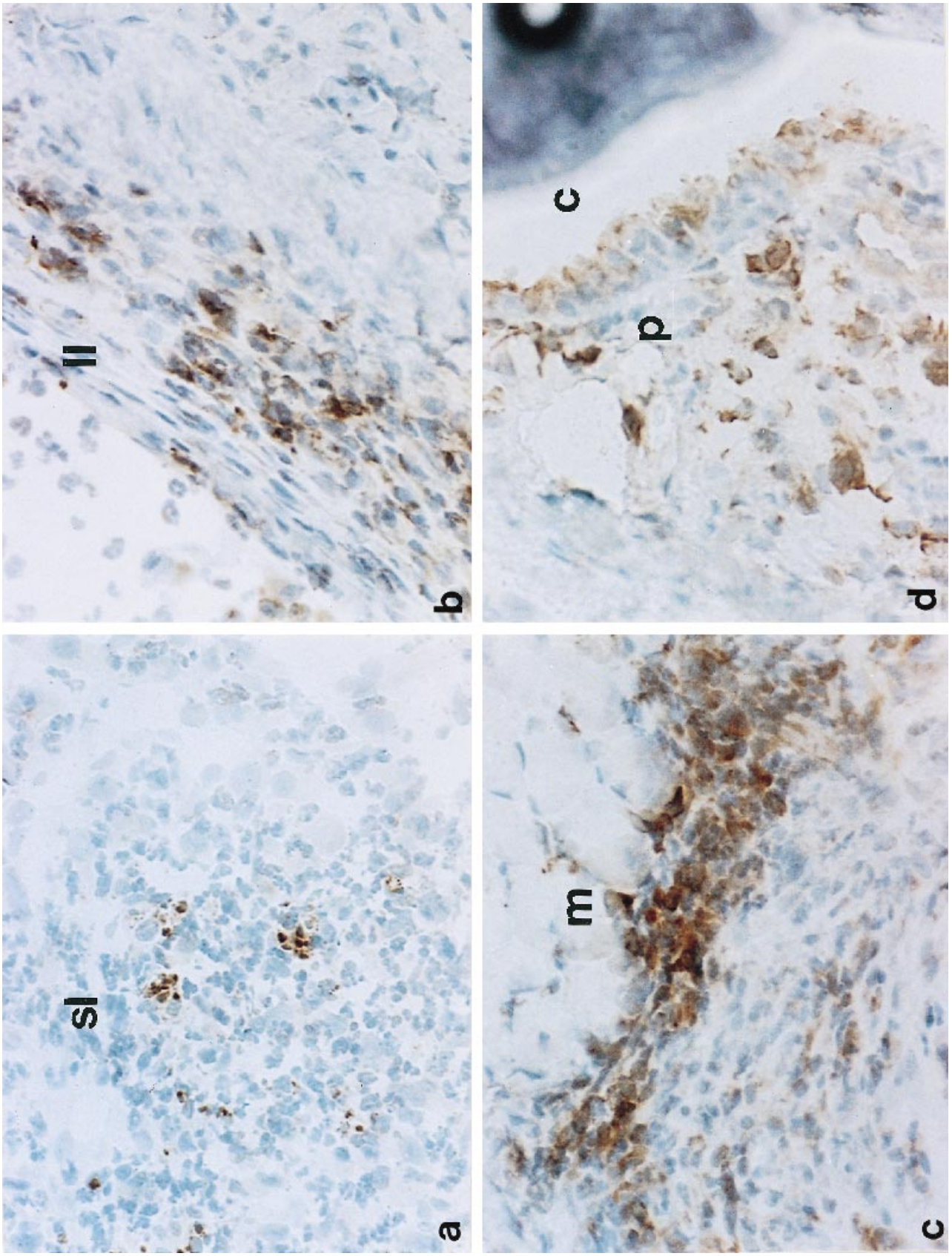


Fig 1. Intracellular immunoperoxidase staining for IFN-γ in the sublining layer (a), IL-6 in the lining layer (b), IL-6 adjacent to muscles (c), and tumour necrosis factor (TNF) at the cartilage–pannus junction (d) on synovial sections 3 days after disease onset. sl, Sublining layer; ll, lining layer; m, muscle; c, cartilage; p, pannus. (Original mag. ×250.)

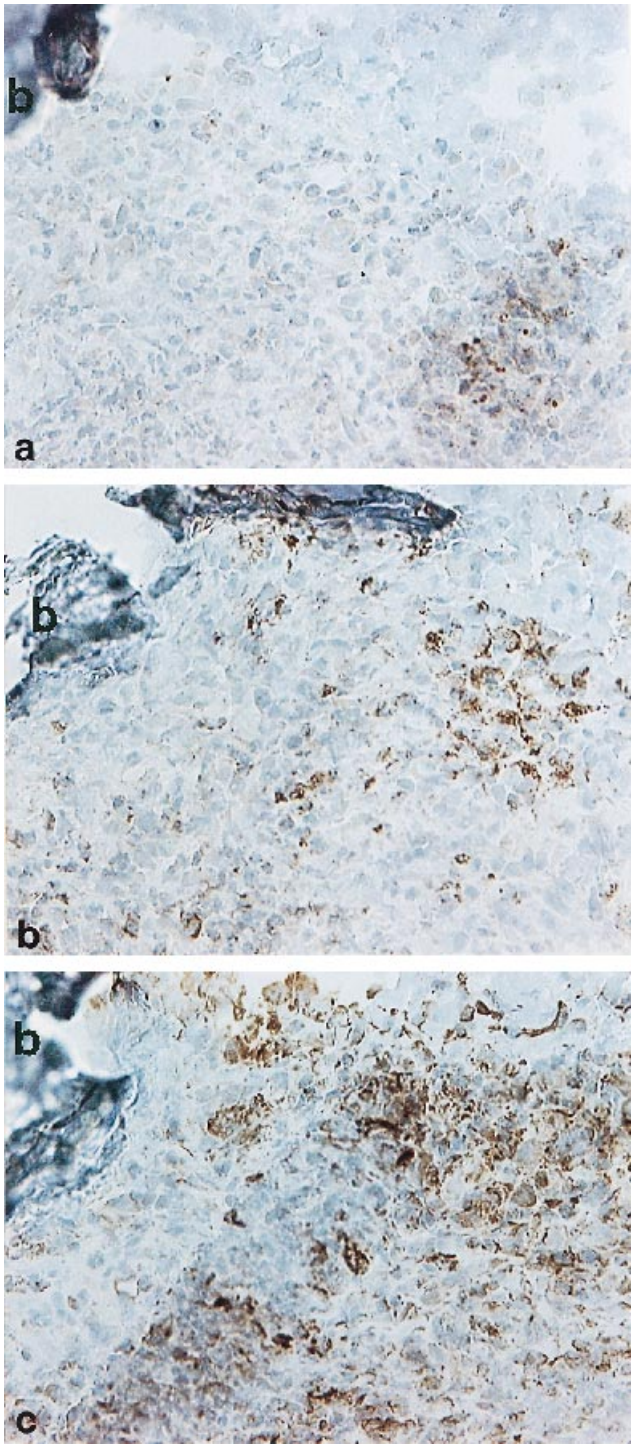


Fig. 2. Intracellular immunoperoxidase staining for IFN-γ (a), IL-6 (b) and tumour necrosis factor (TNF) (c) on synovial sequential sections 12 days after disease onset. b, Bone. (Original mag. ×250.)

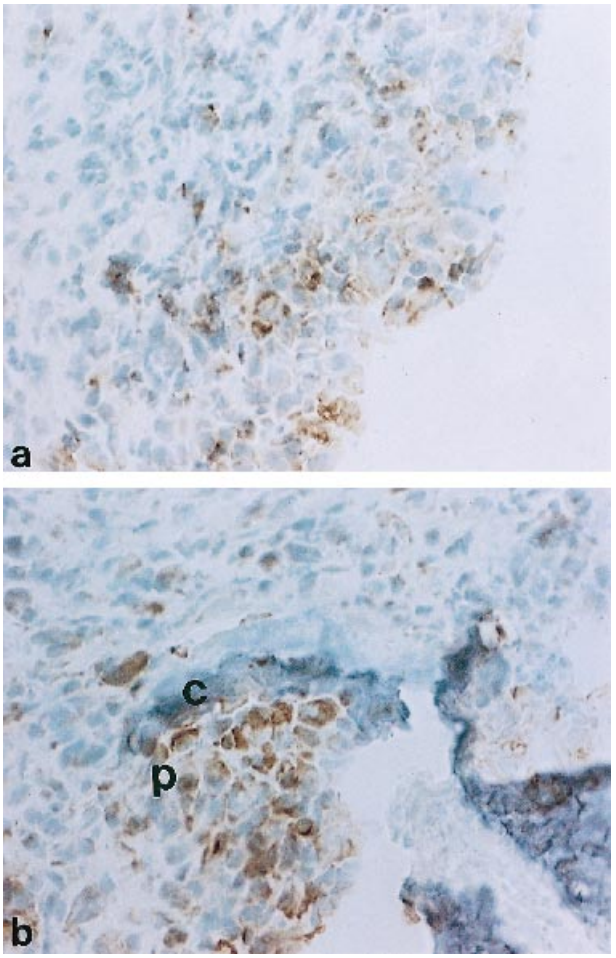


Fig. 3. Intracellular immunoperoxidase staining for IL-6 (a) and tumour necrosis factor (TNF) (b) on synovial sections 23 days after disease onset. Note the strong staining of TNF at the cartilage–pannus junction. c, Cartilage; p, pannus. (Original mag. ×250.)

the present findings thus indicate that the pattern of cytokine production observed in RA is also compatible with a functional pathogenic role of T cells in RA.

Elucidation of the kinetics of cytokine production are critical in interpretation of the clinical course of RA. The T cell-derived cytokine IFN-γ was present in substantial amounts in the early stages of disease and was virtually absent in the later stages, although clinical signs of the disease were rather similar at these time points. Conversely, production of TNF and IL-6 was recorded during all phases of the disease. This demonstrates the critical importance of a careful definition of the stage of disease, and of the importance of having arthroscopic access to synovial tissues from patients at various stages of disease development, in order to obtain specimens in which different aspects of the pathogenesis of RA can be studied. It is possible that early biopsies may provide the best chances of studying T cells of potential pathogenic importance. A better appraisal of this aspect may also explain the conflicting data concerning the expression of T cell-derived cytokines in RA synovial tissues [9,10,18–20].

More basic issues on which the present study may shed some new light, concern when and to what extent synovial inflammation in CIA is directly dependent on T cell activity, and to what extent a more autonomous monocyte/macrophage activity is involved. Thus, the present data demonstrated an early appearance of IFN-γ production in areas of the synovial tissue in which T cells were abundant. Whether the IFN-γ produced by these synovial T cells was a result of T cell activation elsewhere in the body, or was a result of T cell activation locally in the joint, cannot be addressed

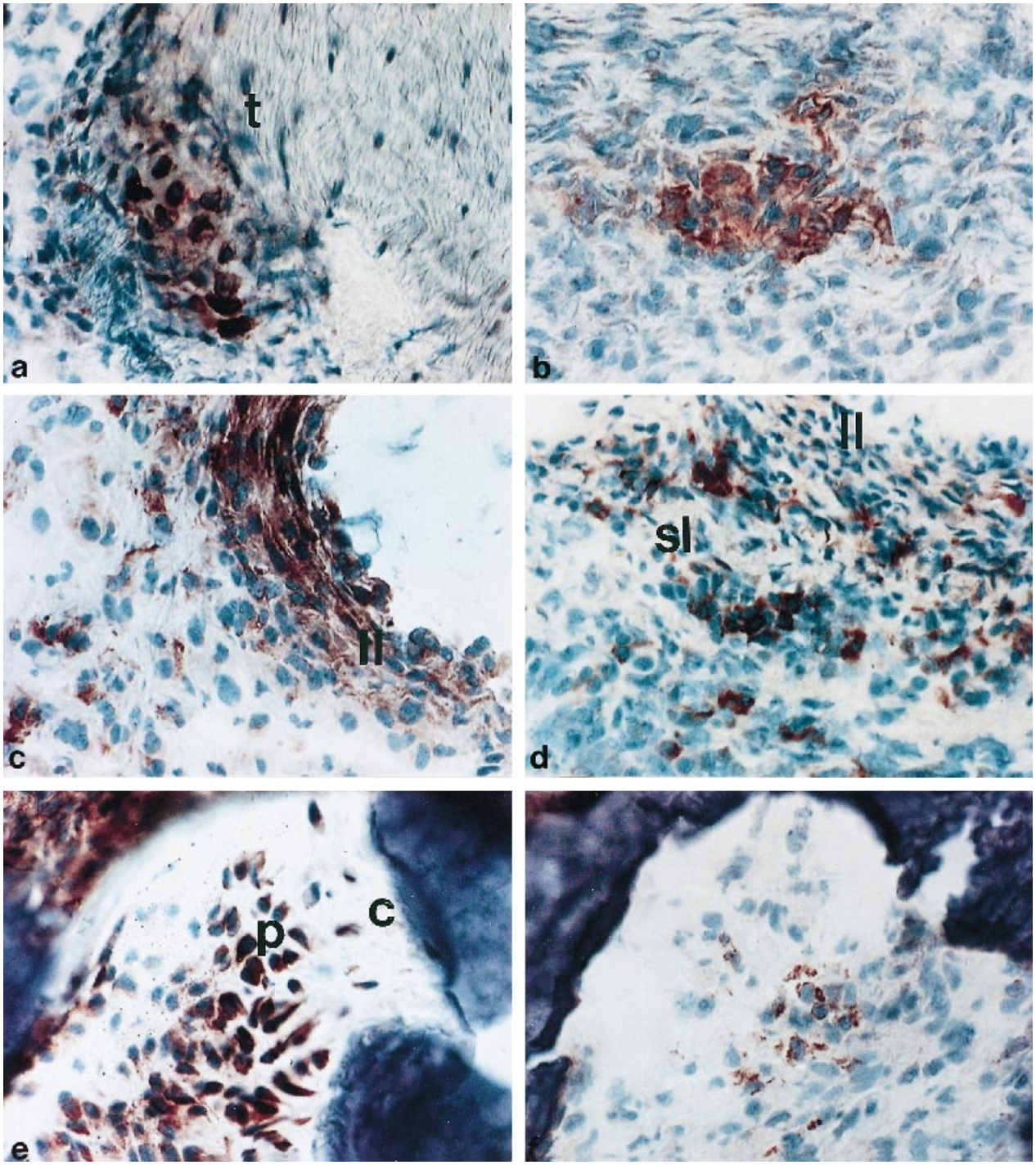
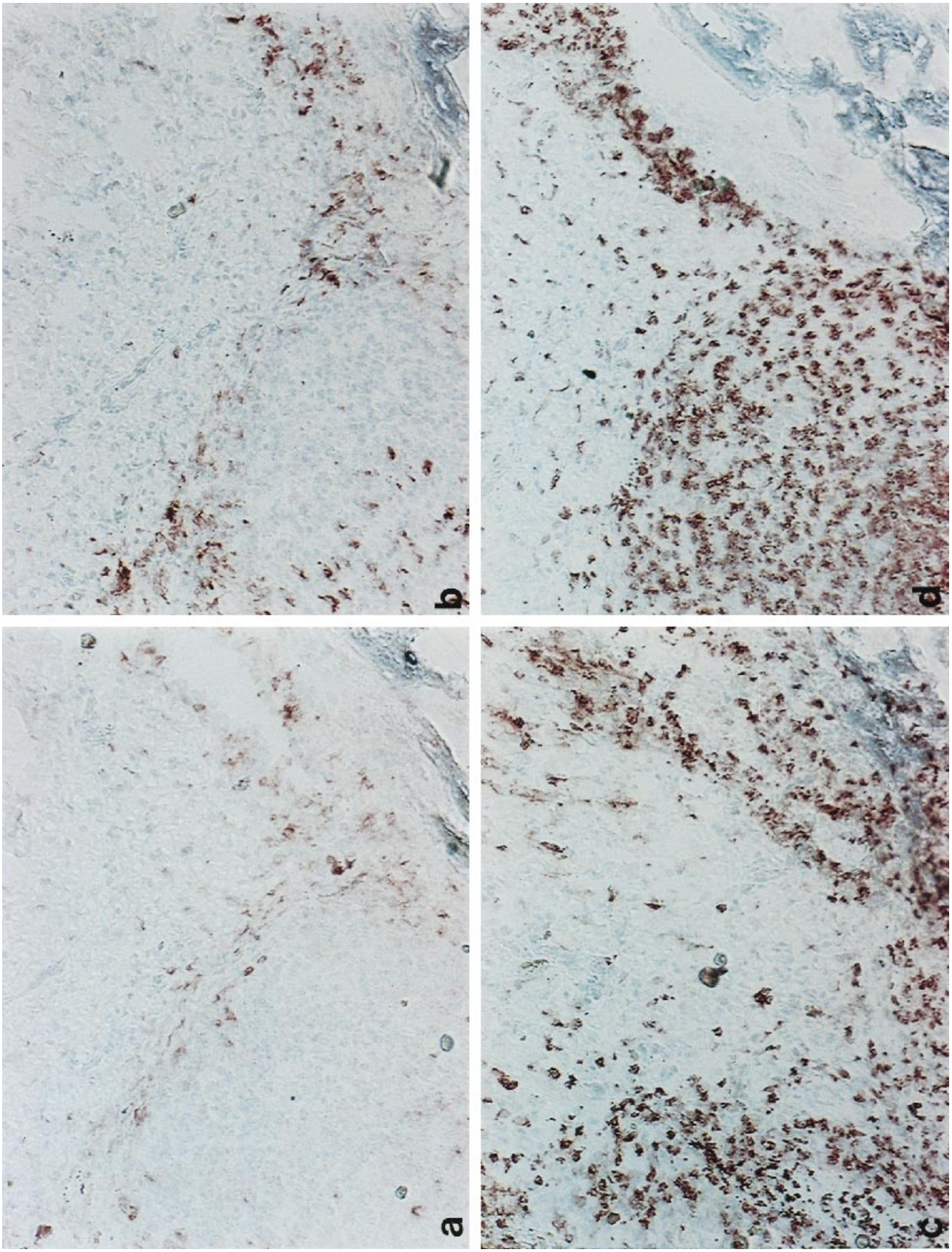


Fig. 4. Representative micrographs of synovial tissue illustrating staining patterns for: (a) transforming growth factor-beta 2 (TGF-β2)-producing cells adjacent to tendons 3 days after disease onset; (b) TGF-β3-producing cells 3 days after disease onset; (c) TGF-β2-producing cells in the lining layer 12 days after disease onset; (d) scattered TGF-β3-producing cells in the sublining layer 12 days after disease onset; (e) TGF-β2-producing cells at the cartilage–pannus junction 23 days after disease onset; and (f) TGF-β3-producing cells 23 days after disease onset. t, Tendon; ll, lining layer; sl, sublining layer; c, cartilage; p, pannus. (Original mag. ×250.)

Fig. 5. Immunoperoxidase staining with anti-CD4 (a), -IA^q (b), -CD11b (c), and -GR-1 (d) antibodies on synovial sequential sections, 12 days after arthritis development in DBA/1 mice. All sections were counterstained with haematoxylin. (Original mag. ×100.)


b
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by the present study. It is likely that the IFN-γ produced by these T cells contributes to the triggering of TNF and IL-6 in adjacent cells. However, it appears more unlikely that the intense TNF and IL-6 expression that was observed in the pannus region, in close proximity to the cartilage, would depend only on the action of T cell-derived cytokines such as IFN-γ. Few, if any, T cells were apparent in the pannus, and there was a gradient of TNF and IL-6 production that was particularly intense at the cartilage–pannus junction. In addition, the production of TNF and IL-6 persisted and expanded during the course of CIA, whereas the production of IFN-γ was very limited during the late phase of disease. This spatial as well as temporal discrepancy between TNF and IL-6 *versus* IFN-γ expression in the synovium could indicate that factors other than T cell-derived molecules may contribute to the stimulation of TNF and IL-6 production. In this context it is interesting to note that several extracellular matrix components (ECM) from cartilage have been demonstrated to activate macrophages to production of both monokines and other inflammatory mediators *in vivo* as well as *in vitro* [21,22]. We have also recently demonstrated the capacity of native collagen type II to induce TNF production both *in vivo* and *in vitro* in DA rats [23]. An attractive possibility emerging from these earlier *in vitro* experiments and the present descriptive immunopathology is that molecules released from destroyed cartilage may directly bind to macrophages and other cells in the synovium, thereby stimulating these cells to produce monokines and other inflammatory mediators.

The lack of IL-4 and IL-5 in contrast to the production of IFN-γ in the arthritic paws indicate that CIA might be linked to a Th1 cytokine production pattern. A recent study has reported that IL-12, a cytokine that stimulates IFN-γ production and the development of Th1 responses, plays an important disease-promoting role in CIA [24]. Moreover, it has been demonstrated that the Th2-linked cytokines IL-4 and IL-10, which are involved in the suppression of Th1 responses, have profound ameliorative effects on experimental arthritis [25–27]. Taken together, all these studies provide evidence that CIA might be associated with an apparent Th1 lymphokine response.

Finally, we examined the production of TGF-β2 and TGF-β3, and noted that the staining of TGF-β in CIA in mice exhibited many similarities to the situation in RA in man [28] and CIA in rats [29]. TGF-β has a number of immunosuppressive properties and has been shown to counteract the proinflammatory effects of TNF and IL-1 [30]. Moreover, it has been demonstrated that systemic administration of TGF-β to rodents protects against induction of arthritis [31,32]. The presence of TGF-β in the synovia may thus reflect the induction of a feedback system which contributes to the down-regulation of other proinflammatory events. However, it is quite possible that there is not enough TGF-β produced to suppress excessive TNF and IL-6 production.

In conclusion, we believe that the present methodology for analysing local cytokine production in inflammatory lesions will be of interest for comparative studies of natural disease courses in CIA and in human RA. The methodology will also be of interest for future studies aimed at monitoring the effect of various immunotherapies on local inflammation and the cytokine production therein.

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