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Sec. Research Graduate School Committee

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ERRATA

p. 10, line 3: should read "loses" instead of "looses".
p. 14, line 3: should read "these data" instead of "this data".
p. 17, paragraph 3, line 8: "IL-1 receptor (IL-1R)" should read "IL-1 receptor antagonist (IL-1R*a*)."
p. 22, line 4: should read "pathogenic" instead of "morbidious"
p. 34, line 1: "few particularities" should read "*a* few particularities".
p. 53, line 3: "concavalin" should read "concanavalin".
p. 55, line 9: "Streptococcus pyogenus" should read "Streptococcus pyogenes".
p. 56, paragraph 3, line 3: should read "(Yocum, et al., 1986)" instead of "(Yochum, et al., 1986)"
p. 57, line 10: "S. pyogenus" should read "S. pyogenes"
p. 78, line 7: "Glacial acid" should read "Glacial acetic acid".
p. 81, line 15: "N_2HPO_4" should read "Na_2HPO_4".
p. 178, line 3: should read "derangement" instead of "disarrangement"
p. 200, line 7: "Edwards J C W and S. W L (1995)..." should read "Edwards J C W and Wilkinson L S (1995)..."
p. 243, paragraph 7: "Yochum D E (1986)..." should read "Yocum D E (1986)..."

ADDENDUM

p. 5, paragraph 3, 2nd sentence: should read: "The main proliferating cells are type B fibroblast-like lining cells, while macrophage-derived cells type A synoviocytes are mainly accumulated through recruitment of circulating cells (Mulherin et al. 1996)"
p. 14, line 7: "Based on these experiments, a pioneering study was carried out in humans. Partially humanised (chimeric) anti-TNFα antibody was administered to patients with RA (Elliott et al. 1993). The results were very encouraging as the patients demonstrated significant improvement in clinical scores and laboratory signs (Elliott et al. 1993). Similar results were obtained with a recombinant human TNF receptor-Fc fusion protein (Moreland et al. 1997)." should read "Based on these and other experiments, studies were carried out in humans (Elliott et al. 1993; Elliott et al. 1994; Moreland et al. 1997; Lorenz et al. 2000). Partially humanised (chimeric) anti-TNFα antibody (Infliximab™) and soluble TNF receptor (p75) linked to the Fc portion of human IgG1 (Etanercept™) have produced significant improvement in clinical scores and laboratory signs in patients with RA (Elliott et al. 1993; Elliott et al. 1994; Moreland et al. 1997; Lorenz et al. 2000). Both drugs are currently used in routine clinical practice in U.S.A. as single therapy or in combination with methotrexate (Bankhurst 1999; Maini et al. 1999; Weinblatt et al. 1999; Lipsky et al. 2000)."
p. 21, paragraph 4, line 3: "In Northern Europe RA is associated with some HLA DR4, DR6 and DR1 alleles, while HLA-DRB 1 alleles were identified in other RA populations." should read "In Northern Europe RA is associated with some HLA DR4, DR6 and DR1 alleles, while other HLA-DRB 1 alleles, like DR6, DR 10 and DR9 were identified in other RA populations (Willkens et al. 1982; Massardo et al. 1990)."
p. 22, paragraph 1 should read: "It is based on an assumption that MHC class II molecules are crucial in the pathogenesis of RA. Although no single HLA gene has been identified as conferring a risk of RA, the "shared epitope" hypothesis suggests that several HLA-DR molecules have a common aminogroup sequence ("shared epitope") in their peptide-binding groove (third hypervariable region of an α-helical structure). This particular sequence ensures the most efficient presentation of an unknown antigen, with appropriate complementary structure, leading to the development of an autoimmune disorder known as RA.
p. 25, line7: "interleukin (IL)-2 receptor and" should be removed.
p. 38, paragraph 1 line 3: "CIA does not exhibit any systemic features, as opposed to RA in humans." should read: "CIA does not exhibit any extra-articular features as opposed to RA in humans."
p. 57, paragraph 3, line 1: "MRL-lpr/lpr mice spontaneously develop symmetrical erosive arthritis (Andrews et al. 1978)." should read "MRL-lpr/lpr mice have a mutation in the *Fas* encoding gene that leads to defective apoptosis. (Wu et al. 1993). These mice are most commonly used as a model for SLE. Besides developing massive lymphadenopathy, multiple autoantibodies and immune-mediated tissue damage, MRL-lpr/lpr mice also spontaneously develop symmetrical erosive arthritis (Andrews et al. 1978; Hang et al. 1982)."
p. 58, paragraph 3, line 3: "The lack of MHC class II overexpression and T cell synovial infiltrate as well as the presence of a profound general impairment of the immune system resulting in glomerulonephritis, vasculitis and dermatitis contribute to the differences between MRL-lpr/lpr model and rheumatoid arthritis." should read: "The lack of MHC class II overexpression and T cell synovial infiltrate as well as the presence of a profound general dysregulation of the immune system resulting in the development of a lymphoproliferative syndrome, glomerulonephritis, vasculitis and dermatitis contribute to the differences between MRL-lpr/lpr model and rheumatoid arthritis"
p. 74, line 10: "HCl" should read "HCl 11.6M".

p. 78, last line: "pH was adjusted to 7.4."should read: "pH was adjusted to 7.4 using concentrated HCl."
p. 79, line 2: should read: "NGS (Dako, Glostrup, Denmark) 500µl"
p. 79, line 14: should read: "NBS (Serotec, Kindlington, Oxford, UK) 100µl"
p. 80, line 3: should read: "NRS (pooled rat serum from normal animals collected from clotted blood obtained by exsanguination after cardiac puncture) 500µl"
p. 81, lines 3-4 should read:
"$Na_2H_2PO_4xH_2O$ 0.15M 41.5ml
NaOH 0.6M 8.5ml"
p. 90, paragraph 1, 1st sentence should read: "Joints were fixed in 10% neutral buffered formalin for 4-6 hours at 4°C. The specimens were decalcified in EDTA decalcifying solution for 3-5 weeks at 4°C (completion of decalcification was confirmed by X-ray). (Walsh et al. 1993)
p. 91, paragraph 1 should read: The technique was modified from Walsh et al 1993 (Walsh et al. 1993). Joints were fixed in 4% paraformaldehyde for 4-6 hours at 4°C, washed in PBS/sucrose for 2 days and in 0.1M Tris buffer for 1 day at 4°C. Sucrose is a hydrophilic molecule that binds water molecules in the tissues during infiltration. It is used to minimise ice crystal formation during rapid freezing and may also partially reverse the cross-linking effect of paraformaldehyde fixation, therefore maintaining antigenicity (Hancock and Atkins 1986). The specimens were decalcified in EDTA/PVP decalcifying solution for 4-6 weeks at 4°C, changing solution twice a week (completion of decalcification was confirmed by X-ray). PVP was added to the decalcifying solution to increase the osmolality of the solution and improve tissue preservation. When fully decalcified, the joints were washed in 0.1M Tris buffer for 30 minutes and placed in 20% sucrose for 24 hours at 4°C. The joints were infiltrated with O.C.T./sucrose for 24 hours and O.C.T./DMSO/sucrose for 24 hours at 4°C. The OCT infiltration step (including addition of DMSO for better OCT penetration into the tissue) ensured easier sectioning of the frozen joints. Joints were then snap-frozen in Tissue-Tek O.C.T.® (Sakura Finetechnical, Tokyo, Japan) in liquid nitrogen and stored at - 80°C.
pp. 94-95 comments: In these techniques H_2O_2/methanol treatment was very efficient in blocking endogenous peroxidase as 3% H_2O_2 concentration was used, as opposed to the 0.3% H_2O_2 routinely used in immunohistochemical techniques. The lack of endogenous peroxidase staining was confirmed by the absence of staining in negative control sections.
p. 127, addition to section 4.2.3:
"Joint space loss: intact joint space = 0, 25% of the joint space occupied by inflammatory tissue = 1, inflammatory tissue occupies 25-50% of the joint space = 2, 50-75% of joint space occupied by inflammatory tissue = 3, less than 25% of joint space left = 4.
Fibrosis: absent = 0, fibrous tissue constitutes less than 25% of the synovium = 1, 25-50% of the synovium is represented by fibrous tissue = 2, fibrous tissue constitutes 50-75% of the synovium = 3, over 75% of the synovium is replaced by fibrous tissue = 4. "
p. 128, paragraph 2 addition: Commercially available MCA453 and NDS61 antibodies (both IgG1) were used at concentrations of 0.25µg/ml and 28µg/ml respectively. mAbs obtained from tissue cultures were also IgG1 and used as culture supernatants; therefore used at empirically determined concentrations. Every immunohistochemistry run included isotype-matched IgG1 mouse immunoglobulin (Dako, Glostrup, Denmark) as negative control at 28 µg/ml, the highest known mAb concentration used.
p. 131, paragraph 1, line 1: should read "ulcers" instead of "erosions"
p. 133, paragraph 2, line 3: "appeared at the time of the onset of arthritis and" should read "were noted at the time of onset of arthritis and"
p. 135, line 7: "Approximately $^1/_3$ of them expressed MHC class II (Figures 5.15a and 5.16)."should read "A few scattered cells expressed MHC class II molecule (Figures 4.15a and 4.16)."
p. 138, paragraph 4, first sentence: should read "There was a 6-fold increase in T cells at the time of onset as compared to the pre-arthritic stage (p=0.01) (Figure 4.9b and 4.10) accompanied by a dramatic 18-fold increase in the cells expressing IL-2 receptor (Figures 4.11b and 4.10)."
p. 156, paragraph 2, line 7 insert: "The other reason for rapidly developing ankylosis may be the young age and small joint size of the rats, similar to juvenile rheumatoid arthritis, where ankylosis is more common and occurs earlier than in adult RA (Lang et al. 1995)."
p.162, line 8 insert: "A number of cells, namely, fibroblasts, mast cells, memory B cells and some activated CD4+ T cell clones have been shown to produce NGF (Ehrhard et al. 1993; Leon et al. 1994; Torcia et al. 1996)"
p. 164, paragraph 3 addition: "Every immunohistochemistry run included the use of IgG1 mouse immunoglobulin (Dako, Glostrup, Denmark) as negative control at 11.4µg/ml, concentration equivalent to the highest concentration of the commercial mAbs used in these experiments."
Figure 5.6.legend should read: "***Identification of NGF and Trk A positive cells in CIA rat synovium.*** This figure depicts double labelling for **a)** monocytes/ macrophages and NGF (5 days after onset), **b)** T cells and NGF (1 day after onset), **c)** NK cells and NGF (3 days after onset), **d)** B cells and NGF (1 day after onset), **e)** for neutrophils and NGF (1 day after onset), **f)** monocytes/macrophages and trk A (5 days

after onset), **g)** T cells and trk A (3 days after onset), **h)** NK cells and trk A (3 days after onset), **i)** B cells and trk A (1 day after onset), **j)** for neutrophils and trk A (1 day after onset). **a-d, f-i:** NGF and Trk A - red, cell markers - green, co-localisation of both - yellow. **e, j:** nuclear staining -blue, NGF and Trk A - red, co-localisation -white/ lilac."

p. 173, paragraph 4, line 2: should read: "However, no NGF mRNA was demonstrated by reverse transcription polymerase chain reaction (RT-PCR) in macrophages in the experiments employing a model of spinal cord injury in which autologous peritoneal macrophages were grafted into the sites of the spinal cord suppression lesions (Franzen et al. 1998)."

p. 179, paragraph 4 comments: as per p.108, section 3.3.1.4.

p. 182, paragraph 3: "These data suggest the involvement of T cells in the regulation of arthritis." should read "These data suggest a role for T cells in this model of arthritis."

p. 184, after paragraph 1, the following paragraph should be inserted:

"Further studies were suggested to further investigate the role of NGF in the development of arthritis. Some of them are currently being performed as a part of another research project. The expression of NGF and its receptors in rheumatoid synovium should be studied, as it will help to determine the relevance of the findings in the animal model to the human disease. The establishment of in situ hybridization for NGF mRNA, particularly combined with immunohistochemical staining for cell surface markers, will allow the identification of the source of NGF in the synovium. The contribution of NGF to the inflammatory process in the joints can be tested in vitro by analyzing the effects of NGF stimulation and NGF blockade (with specific antibodies following non-specific stimulation) on the production of cytokines, namely TNF-α, IL-1β and IL-6, by synovial cells in synovial cell cultures. The blockade of NGF in CIA using anti-NGF antibodies may provide valuable information on the pathophysiological role of NGF in arthritis development in vivo. The changes at the microscopic level can also be assessed and compared with the untreated CIA described in this thesis."

Additional comments

Comments on the immunohistological technique:

The specimens used for synovial cell counts were cut until the full cross-section of the joint was obtained. If cells were evenly distributed, a minimum number of 25 graticules was counted for the evenly distributed cells. When cells were not evenly distributed throughout the synovium, the whole area of the synovium was counted in order to avoid sampling error. Error of measurement was minimised by using the minimum of 6 animals per group. The results are shown as mean±sem.

The expression of NGF and trk A was also studied in full cross-sections of the joints. The total synovial area and the percentage of the area stained positively were analysed using an Image Analysis System (ImagePro Software). A minimum of 6 animals per group was used. The results were expressed as mean±sem.

All counting and staining analysis were done blindly as to tested experimental group.

p. 105, section 3.3.1.1, comments: The examiner has questioned the specificity and generalisability of the CIA model since it incorporated FIA, which has been shown previously to be arthritogenic in DA rats by other researchers (Holmdahl and Kvick 1992). The candidate was aware of these studies and, therefore, when the model was being established, FIA was injected intradermally and subcutaneously in two separate rat groups in the same way as CII/FIA to control for this eventuality. The DA rats obtained from Monash Animal Services did not develop any symptoms of arthritis (either clinical or histological) after either of the FIA injections. The explanation for such discrepancy in the reaction to FIA may be the difference in the FIA composition or some genetic variations in the DA strain bred at Monash, Australia and the strain used in Europe.

p. 108, section 3.3.1.4, comments: As it was pointed out by the examiner, we have not examined the effect of the age on the development of the CIA in rats re-challenged with CII intradermally after subcutaneous injection, i.e. 16.5 week-old rats. To control for the difference in size, the amount of CII was adjusted according to the rats' weight. We have since performed experiments inducing this model of CIA in 14-week-old rats, and there is no evidence of 14-week-old rats being less susceptible to arthritis than 10-week-old rats. Other researchers have used 10-24 week-old animals in their experiments (Trentham et al. 1977). We are therefore confident that the differences seen in *sc/id* versus *id* injected rats are not due merely a reflection of the age-related variation in susceptibility to CIA. However, development of CIA in older naïve rats will be later tested in our laboratory by other researchers.

p. 128, paragraph 2 comments: Several fixatives, namely formalin, PLP and 4% formaldehyde, were tested as part of the establishment of the current protocol. Formalin was the least effective fixative in preserving antigenicity of the epitopes, while paraformaldehyde-based fixative like PLP and 4% paraformaldehyde were equal in reactive epitope preservation. PLP has earlier been shown to be suitable for OX-6 antigen detection (Stein-Oakley et al. 1991 #611]. 4% paraformaldehyde was chosen as it is easier to prepare and is presumed to better preserve cytokine antigenicity. Sucrose washes are also likely to contribute to better antigen preservation in the technique described.

p. 131, comments: The statement regarding the general features on p38 has been corrected. The statement referred to the absence of *extra-articular* rather than general symptoms, as weight loss and restricted mobility are the common features of any CIA model, including the one described in this thesis.
p. 133, comments: The time of onset of arthritis was defined as the time when animals started to demonstrate changes in walking behaviour (by which they reduce the pressure on the foot). At this time-point, no redness or swelling of the joints were evident. Histologically, at the time of onset, surprisingly few changes were seen in the joints (refer to histological description), while the investigation of the "day 10" group (approximately 2 days before arthritis onset) failed to detect any consistent changes. This allowed us to suggest that the first histological changes in the joints occurred very close to the onset of arthritis, as defined in this study, between day 10 and 12 after collagen injection.
Figure 5.6, comments: Representative examples of various time-points between 1 and 5 days after onset. The pattern of NGF and trk A expression did not vary significantly between the time-points of arthritis.

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Mechanisms Of Joint Injury In An Animal Model Of Collagen-Induced Arthritis

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

by

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TABLE OF CONTENTS

ABSTRACT

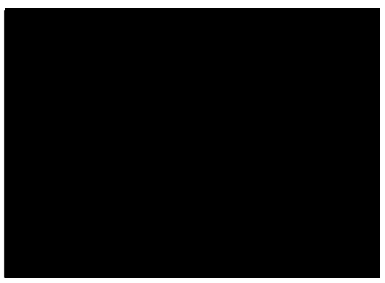
Rheumatoid arthritis (RA) is a chronic debilitating disease characterised by remitting and relapsing joint inflammation. This thesis characterizes an animal model for RA, collagen-induced arthritis (CIA), and addresses the role of nerve growth factor (NGF) in arthritis. This study elucidates the optimal conditions required for the induction of CIA with uniform clinical features. The DA rat strain was shown to have 100% susceptibility to CIA along with a predictable time of onset and location of arthritis, as opposed to the Lewis rat strain, which demonstrated lower incidence, varied onset and location. Two methods of collagen (CII) inoculation for CIA induction have been investigated, intradermal and subcutaneous. It has been shown that it is imperative for collagen to be introduced intradermally as subcutaneous injection does not result in arthritis and, furthermore, protects against subsequently induced CIA disease. It has been proposed that the different effects produced by subcutaneous and intradermal inoculations of CII may be due to the distinct cell populations in the skin and the subcutaneous layers. CIA in DA rats has been shown to develop as a severe rapidly progressing disease, in which inflammation would peak five days after the onset of arthritis and subside by day 20 leading to complete or partial joint ankylosis. These observations were confirmed histologically. By day 5 after onset an inflammatory pannus, closely resembling that in RA, was formed. Pannus incurred substantial bone erosion and cartilage loss in the joint, and erosions and joint space were later filled out with fibrous tissue. The synovium also became infiltrated with inflammatory cells. Neutrophils, monocytes, T cells and NK cells appeared in the synovium at the time of onset and their numbers increased until day 10 after onset, after which their numbers started to subside. Mast cells were found around capillaries

filled with inflammatory cells at the time of onset and disappeared or had an "empty" appearance after the onset of arthritis. It can be speculated that the recruitment of inflammatory cells to the joint may be mediated through degranulation of MCs in the synovium. It was found that, unlike in chronic RA, where T cell aggregates dominate the synovium, the proportion of T cells in CIA synovium never rose over 10% of the total leukocyte numbers. This population, however, underwent a 12-fold expansion by day 5 after onset. However, many of the T cells expressed IL-2 receptor. This data suggests the involvement of T cells in the regulation of arthritis. The absence of the T cell aggregates could be due to the lack of chronicity in CIA. Overall, the pannus formed during progression of CIA was very similar to the pannus in rheumatoid joints. Despite lacking chronicity and associated T cell aggregates, the synovial changes in CIA joints resemble the ones in early and acute RA, justifying the use of CIA as a model for the human disease. The severity of pain and inflammation in CIA suggested involvement of the neuro-endocrine system and prompted an investigation of nerve growth factor (NGF) expression in CIA joints. NGF has recently been shown to regulate a number of immune cell functions, acting through its receptors trk A and p75. NGF was absent in pre-arthritic synovium, but was significantly increased after the onset of arthritis. The study demonstrated that cells positive for NGF were monocytes/macrophages and neutrophils, while T, B and NK cells were NGF negative. NGF expression was significantly downregulated at day 20 after onset, when inflammatory infiltrate was no longer prominent and was replaced by fibrous tissue. The trk A receptor was also demonstrated on normal synoviocytes, as well as on a number of the infiltrating cells during joint inflammation. p75 was found to be expressed by peripheral nerve endings in both normal and CIA synovium. These findings suggest that NGF and its receptor trk A are abundantly expressed in

the synovium during inflammation and, therefore, may stimulate inflamatory cells in synovium to produce pro-inflammatory mediators. It is possible that NGF inhibition may prove beneficial, as NGF blockade may suppress both pain and the inflammatory process.

DECLARATION

The work presented in this thesis is original and contains no material which has been accepted for the award of any other degree or diploma in any university or other institution. To the best of my knowledge, the thesis contains no material previously published or written by another person, except where due reference is made in the text. I further certify that the writing of this thesis, results, their interpretation, the conclusions and suggestions are my own work.



Olga Bakharevski

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PUBLICATIONS

Bakharevski O, Stein-Oakley AN, Thomson NM, Ryan PFJ. Collagen-induced arthritis in rats. Contrasting effect of subcutaneous versus intradermal inoculation of type II collagen. *J Rheumatol* 1998; 25:1945-52.

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Lindsay ML, **Bakharevski O**, Ryan PFJ. The pathophysiological role of nerve growth factor in arthritis. Oral presentation at the Australian Rheumatology Association 42nd Annual Scientific Conference, Hobart, April 2000.

LIST OF ABBREVIATIONS

Abbreviation	Definition
ANOVA	Analysis of variance
APC	Antigen presenting cell
CII	Collagen type II
CIA	Collagen-induced arthritis
DA	Dark Agouti
DAB	Diaminobenzidine
DDC	Dermal dendritic cell
DIP	Distal interphalangeal
DMSO	Dimethyl sulfoxide
FIA	Freund's incomplete adjuvant
H_2O_2	Hydrogen peroxide
HLA	Human leukocyte antigen
GM-CSF	Granulocyte macrophage-colony stimulating factor
IL	Interleukin
IL-2R	Interleukin-2 receptor
LC	Langerhans cell
MC	Mast cell
MCP	Metacarpophalangeal
MTP	Metatarsophalangeal
MHC	Major histocompatibility complex
NFκB	Nuclear factor κB
NGF	Nerve growth factor

NK	Natural killer
PBS	Phosphate buffered saline
PIP	Proximal interphalangeal
PMN	Polymorphonuclear cell
RA	Rheumatoid arthritis
SEM	Standard error of the mean
TCR	T cell receptor
TGFβ	Transforming growth factor β
TNF	Tumor necrosis factor

Chapter 1

LITERATURE REVIEW

I. LITERATURE REVIEW

1.1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory multi-systemic disease. It is characterised by the symmetrical inflammation of synovial joints, but commonly other tissues also become involved. The term "rheumatoid arthritis" was first introduced by A. B. Garrod in 1859 (Garrod, 1859). Back then, this term included polyarticular osteoarthritis as well as inflammatory arthropathy (Halberg, 1994).

RA has been identified in all parts of the world. The incidence of RA varies between different populations but seems to be independent of climate, geography and altitude, suggesting a genetic predisposition to this disease (Smith, et al., 1991). The average annual incidence of RA is about 30 cases per 100,000 worldwide. RA is three times more frequent in women than in men (Smith, et al., 1991).

1.1.1 Clinical manifestations of RA

Symmetrical inflammation of the small joints of hands, wrists, and feet is a typical feature of RA (Gordon, et al., 1994). However, any synovial joint may be involved early or later on in the disease. Arthritis is accompanied by morning stiffness. Typical joint deformities develop over time. Characteristic radiographic changes include marginal bone erosions and juxta-articular osteoporosis. Late changes comprise loss of joint spaces, subluxation and ankylosis. Occasionally,

osteonecrosis may occur. Extra-articular manifestations are often observed in RA (Matteson, et al., 1994), including haematological abnormalities, rheumatoid nodules, vasculitis, Sjogren's syndrome, neurologic and ocular symptoms.

RA is a clinically polymorphic disease. There are no pathognomonic symptoms for this disease, thus, a set of diagnostic criteria has been proposed to facilitate the diagnosis. The most recent revision of these criteria by the American Rheumatism Association (ARA) is presented in Table 1.1. (Arnett, et al., 1988).

1.1.2 Morphological changes in rheumatoid joints

1.1.2.1 Rheumatoid synovium

The synovium is a thin connective tissue lining that covers the inner surface of the joint capsule and the periosteal surface of any articular bone, but which does not cover cartilage (Figure 1.1).

Two distinct layers can be distinguished in the synovium. The intima, which is in direct contact with the articular cavity, is formed by an incomplete layer of cells called synoviocytes A (macrophage-like cells) and B (fibroblast-like cells). Besides obvious morphological similarities between synoviocytes A and macrophages, and synoviocytes B and fibroblasts, some researchers have demonstrated that under certain circumstances connective tissue can be transformed into a synovial lining cell layer (Fassbender, 1983), suggesting that synoviocytes descend from monocytes/macrophages and residential tissue

Table 1.1. (Arnett, et al., 1988) The 1987 revised criteria for the classification of rheumatoid arthritis*.

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joints	At least 3 joint areas simultaneously have had soft tissue swelling or fluid observed by physician. The 14 possible areas are right or left proximal interphalangeal (PIP), metacarpophalangeal (MCP), wrist, elbow, knee, ankle, and metatarsophalangeal (MTP) joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetrical arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

* For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 of these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Designation as classic, definite, or probable rheumatoid arthritis is *not* to be made.

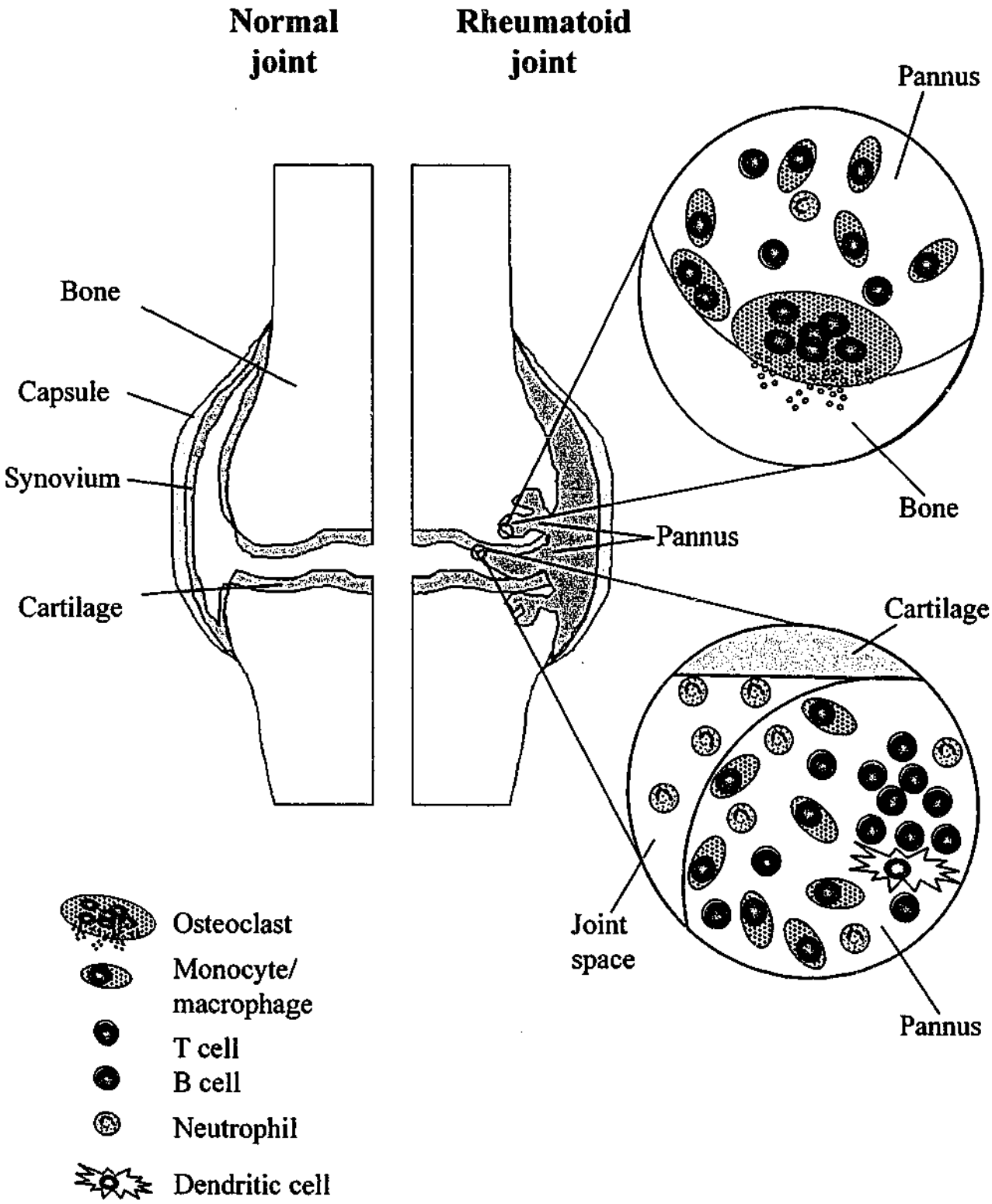


Figure 1.1. Schematic representation of the pathological changes in the joint during rheumatoid arthritis

fibroblasts. The subintima is a flat layer of fibrous or adipose tissue, relatively acellular, containing scattered blood vessels, fat cells and fibroblasts.

The synovium is believed to be the major site of inflammation in RA and to play a key role in joint destruction (Freemont, 1995). In RA the mass of synovium increases dramatically (Figure 1.1). Different factors such as hyperplasia of synoviocytes, oedema, cell infiltration, and extensive new blood vessel formation contribute to it.

1.1.2.1.1 Intimal layer hyperplasia

The synoviocytes that form the intimal layer of synovium become hypertrophic and increase in number. The main proliferating cells are type A macrophage-derived lining cells, although there is also some local proliferation of fibroblast-derived cells (Mulherin, et al., 1996). As a result, the once discontinuous layer of cells is transformed into a thick multi-cellular lining. Macrophages often constitute the most superficial layer with the cells projecting long microvilli from their surface. The macrophage-derived cell population in rheumatoid synovium is not homogenous. Macrophages of the intimal layer express almost exclusively CD68 marker, while the cells in the subintimal layer express either CD68 or CD14.

Synovial lining hyperplasia has been identified in the earliest biopsied rheumatoid lesions (Revell, 1987). In late RA the hyperplasia may extend to a depth of over ten cells.

1.1.2.1.2 Cartilage/bone-pannus junction

A unique feature of rheumatoid synovium is that it becomes locally invasive at the synovial interface with the cartilage and bone. The synovial tissue, which forms destructive finger-like processes overlying cartilage, is referred to as pannus. The pannus is unique to RA; other forms of chronic arthropathies can be destructive, but they lack this characteristic histologic appearance (Firestein, 1998). There are different opinions on the cellular composition of the pannus. Some researchers characterise them as immature mesenchymal cells which subsequently mature into fibroblasts (Fassbender, 1983). Other authors have observed different types of cells in close contact with the cartilage at the cartilage-pannus junction (Bromley, et al., 1984b). With macrophages and fibroblasts found in 76% of all sections examined, they also observed mast cells, polymorphonuclear cells (PMNs), plasma and dendritic cells constituting the invading layer of cells. Prevalence of PMNs at the cartilage-pannus junction has often been a finding in early RA (Mohr, et al., 1980; Revell, 1987), suggesting the important role of these cells in cartilage breakdown. Bone resorption by pannus is mediated through the formation of specialised cells, osteoclasts, which are observed in resorption lacunae at the areas of pannus invasion into bone in RA patients (Gravallese, et al., 1998)

1.1.2.1.3 Subintimal inflammatory infiltrate

In RA the usually hypocellular subintimal layer of the synovium becomes infiltrated by inflammatory cells. The composition of this infiltrate depends on the stage of the disease. It has been shown that in the early stages of RA PMNs are the predominant cell type (Revell, 1987). In late stages, despite the abundance of

neutrophils in the synovial fluid, it is uncommon to find significant numbers of these cells in the synovium, although numbers may increase during the flares (Freemont, 1995).

In the chronic stage of RA lymphocytes constitute the majority of the cells in the subintimal cell infiltrate. Lymphocytes may be distributed evenly in the subintimal tissue or form aggregates with distinct follicular organisation (Edwards, et al., 1995).

Lymphocytes present are predominantly T cells with CD4/CD8 ratios ranging from 4:1 to 14:1 in different specimens (Firestein, 1998). They are mostly found in close contact with HLA-DR positive cells (Klareskog, 1991). Nearly all CD4+ T cells are of the "memory" phenotype, as they are CD29+ and CD45RO+ (Cush, et al., 1991). Naive T cells are hardly found in rheumatoid synovium. A small proportion of T cells express a T cell receptor (TCR) composed of γ/δ chains rather than α/β chains, and the ratio of γ/δ TCR T cells and α/β TCR cells in rheumatoid synovium is higher than in peripheral blood (Brennan, et al., 1988; Reme, et al., 1990). It is known that γ/δ T cells, as opposed to α/β T cells, can respond in a non-MHC-restricted manner, however, their precise functions and role in RA are unclear.

B cells constitute a small population, about 1-5% of synovial cells. They are found mostly in the centres of T cell aggregates (Firestein, 1998). B cells in rheumatoid synovium differ from normal B cells, as a significant percentage of them express CD5, which is normally expressed on T cells. CD5+ cells have been

associated with production of autoantibodies, including rheumatoid factor (Shirai, et al., 1991).

Plasma cells are found diffusely distributed outside tight lymphoid clusters in rheumatoid synovium and in close proximity to the blood vessels (Brown, et al., 1995). Their numbers vary but in some cases they make up the majority of the cellular infiltrate, and even constitute the invasive front at the cartilage-pannus junction (Bromley, et al., 1984b), where their numbers were found to correlate with the total number of the infiltrating mononuclear cells (Brown, et al., 1995).

The percentage of natural killer (NK) cells, which mediate non-MHC-restricted cytotoxicity, is less than 1% within the rheumatoid synovium (Firestein, 1998). As these cells suppress a number of immune responses, including immunoglobulin production and autologous mixed leucocyte reaction (Shah, et al., 1985), their absence in rheumatoid synovium may contribute to the chronic lymphoid stimulation and, therefore, perpetuation of the inflammation.

Mast cells are present in normal synovium and constitute up to 3% of the cells (Castor, 1960). The proportion of mast cells in rheumatoid synovium is significantly higher than in normal synovium and correlates with the lymphoid infiltration of synovium (Malone, et al., 1987) and clinical parameters of the disease (Gotis-Graham, et al., 1997). Considering their ability to produce proteinases, chemotactic factors and vasoactive materials, mast cells may play an important role in the inflammatory process. Both PMNs and mast cells were

observed penetrating cartilage matrix at the cartilage-pannus junction in a few biopsy specimens (Bromley, et al., 1984a).

1.1.2.2 Cartilage in rheumatoid joints

There are two major processes leading to cartilage destruction in RA: first, destruction of cartilage by the invading synovium; second, chondrocytic chondrolysis. Synovial pannus cells either directly or indirectly stimulate the production of the matrix degrading enzymes, matrix metalloproteinases (MMPs), such as stromelysin, collagenase and others (Cawston, 1995). MMPs are found in rheumatoid synovium (Pap, et al., 2000; Yamanaka, et al., 2000), more frequently observed in cells at the cartilage-pannus junction.

The chondrocytes themselves, apart from producing the cartilage matrix, are capable of producing the degrading factors (Clegg, et al., 1999; Gowen, et al., 1984; Lefebvre. et al., 1990b). Stimulated by such cytokines as IL-1 and TNFα, they can produce nitric oxide and collagenase, causing cartilage destruction from the side of the lacunae. As many chondrocytes are found dead in the cartilage, the production of such factors may be a prodrome of the cell death (Freemont, 1995).

The attack on cartilage from both sides, i.e. from the invasive pannus and from the chondrocytes themselves, results in the erosion of the cartilage. The cartilage in the rheumatoid joints becomes irreversibly damaged and even completely lost in many areas of the joint.

1.1.2.3 Bone changes in RA

Subchondral bone is also altered by the synovial pannus in a manner similar to that observed for the cartilage (Bromley, et al., 1985; Freemont, 1995). In addition, juxta-articular bone marrow looses its haematopoietic cells and develops infiltrate similar to that of the synovium, but with a clear prevalence of macrophages. Formation and activation of multi-nucleated osteoclasts occurs (Goldring, et al., 2000). Osteoclasts then contribute to the bone erosion in RA. The damage to the bone caused during the course of RA is clearly visible as marginal erosions in the X-ray films (Bromley, et al., 1985).

Other common changes in bone in RA include osteopenia caused by generalised osteoclasis stimulated by IL-1 (Fox, et al., 2000; Gough, et al., 1998) and avascular necrosis caused by vasculitis within the arteries supplying the juxta-articular region of the bone (Freemont, 1995).

1.1.2.4 Autoantibodies in RA

Rheumatoid factor (RF) is an antibody directed against the Fc portions of the IgG molecules. RF usually belongs to the IgM class (Burastero, et al., 1988). RF is found in 90-70% of the total RA population (Bland, et al., 1963; Masi, et al., 1976), but is not specific for RA. However, it is one of the criteria in RA diagnosis (see Table 1.1) and it is a predictive factor for a severe disease course (Bland, et al., 1963; Masi, et al., 1976). Healthy individuals with RF are 40 times more likely to develop RA later in their life, than their RF-negative counterparts (Aho, et al., 1991; Jonsson, et al., 2000).

The immune response to collagen type II (CII), a major component of cartilage, represents an autoimmune reaction that might be involved in the perpetuation of inflammation in the joint. Antibodies to CII are detected in synovial fluids and synovial tissue of a significant proportion of patients with RA (Cracchiolo, et al., 1975; Tarkowski, et al., 1989). Half of these patients also demonstrate a T cell response to CII (Snowden, et al., 1997). The absence of the T cell response in the other half of the patients may indicate the development of peripheral tolerance to this cartilage component. Follow up of these distinct groups of patients will be of value to assess the role of autoimmunity to CII in RA.

Other autoantibodies found in RA patients include anti-keratin antibodies (AKA) and anti-perinuclear factor (APF) (Aho, et al., 1994). AKA is a specific, but not very sensitive marker (Hoet, et al., 1992). APF, in contrast, is sensitive, but not very specific (Hoet, et al., 1992). Most of the AKA positive sera is APF positive. AKA occurs in both RF positive and negative patients (Hoet, et al., 1992). Positive AKA or APF tests in healthy subjects indicate five times increased risk of developing RA.

1.1.3 Cytokines and growth factors in the rheumatoid joint

Cytokines are intercellular messenger molecules, which are produced by activated cells and act on other cells that express specific receptors for them. Virtually every known cytokine, at least at the mRNA level, has been identified in rheumatoid synovium (Table 1.2). Considering the variety of cells present in the synovium, this could be predicted. The varying levels of different cytokines

Table 1.2 Cytokines and their possible targets in the rheumatoid joints.

Cytokine	Source	Cell target	Effect
TNFα	Monocytes Macrophages Mast cells Fibroblasts Neutrophils T cells	T cells Chondrocytes Endothelial cells Monocyte/ macrophages Fibroblasts Osteoclasts	Proliferation, MHC class II expression Nitric oxide and MMPs production Expression of adhesion molecules Cytokine production Cytokine production Activation of bone resorption
IL-1	Monocytes Macrophages Fibroblasts Neutrophils	Endothelial cells Chondrocytes Osteoclasts Fibroblasts	Expression of adhesion molecules Stimulation of MMPs production MHC class II expression Activation of bone resorption Stimulation of MMPs production
IL-2	T cells	T cells NK cells B cells	Activation Activation Stimulation of antibody production
IL-4	T cells	T cells Osteoclasts Monocyte/ Macrophages Endothelial cells	Decrease of IL-2, IFNγ and TNF production Indirect suppression of bone resorption Reduction of pro-inflammatory cytokine (IL-1, IL-6 and TNFα) production Increase of adhesion molecule expression
IL-6	Monocytes Macrophages Fibroblasts Chondrocytes T, B cells	B cells Monocytes/ Macrophages	Stimulation of antibody production Induction of IL-1 and TNFα production
IL-8	Macrophages Chondrocytes Endothelial cells	Neutrophils T cells	Chemotaxis Chemotaxis
IL-10	Monocytes Macrophages T cells B cells	Monocyte/ Macrophages T cells	Downregulation of the MHC class II and co-stimulatory molecules Suppression of pro-inflammatory cytokine production Inhibition of IL-2, IFNγ and TNF production
IFNγ	T cells	Monocyte/ Macrophages Fibroblasts	MHC class I and II expression, activation, inhibition of IL-10 production Activation
TGFβ	Monocytes Macrophages Fibroblasts Neutrophils T cells	Synoviocytes Monocytes/ Macrophages T cells	Inhibition of proliferation and collagenase production Inhibition of the IL-1R antagonist production, stimulation of IL-1 production Suppression of proliferation
GM-CSF	Monocytes Macrophages Chondrocytes Endothelial cells	Macrophages Neutrophils Monocytes	MHC class II expression Expression of the adhesion molecules Chemotaxis Differentiation into macrophages

probably reflects the degree of the involvement of different cell types in the inflammatory processes.

Monocyte/macrophage and fibroblast-derived cytokines dominate in the rheumatoid joint. T cell derived cytokines, on contrary, are scant in synovium despite the presence of a T cell infiltrate. Their concentrations in the synovial tissue are negligible in comparison to levels produced by activated T cells *in vitro* (Bergroth, et al., 1989; Firestein, et al., 1988; Firestein, et al., 1987).

1.1.3.1 Tumour necrosis factor α (TNFα)

TNFα is one of the most abundant cytokines in rheumatoid joints, detected at both protein and mRNA levels (Di Giovine, et al., 1988; Firestein, et al., 1990a; Hopkins, et al., 1988; Saxne, et al., 1988). Monocyte/macrophage cells are the main source of TNFα in synovium (Firestein, et al., 1990a).

TNFα has a potent effect on immunocompetent cells, including increased T cell proliferation and enhancement of MHC molecule expression. TNFα also contributes to the recruitment and retention of the inflammatory cells in synovium through induction of adhesion molecules on endothelial and stromal cells and through stimulation of production of chemoattractants. TNFα is a potent inducer of a number of pro-inflammatory cytokines. The dominating role of TNFα has been demonstrated in synovial cell cultures by neutralisation experiments using specific anti-TNFα antibodies. Neutralisation of TNFα in synovial tissue cultures caused a significant down-regulation of spontaneously produced pro-

inflammatory cytokines, such as interleukin (IL)-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6 and IL-8 (Brennan, et al., 1989; Butler, et al., 1995; Haworth, et al., 1991). This data prompted *in vivo* studies testing the effect of anti-TNFα antibodies or TNFα-receptor in experimental models of arthritis (Piguet, et al., 1992; Williams, et al., 1995). Both treatments resulted in amelioration of the clinical signs of arthritis and less severe joint destruction. Based on these experiments, a pioneering study was carried out in humans. Partially humanised (chimeric) anti-TNFα antibody was administered to patients with RA (Elliott, et al., 1993). The results were very encouraging as the patients demonstrated significant improvement in clinical scores and laboratory signs (Elliott, et al., 1993). Similar results were obtained with a recombinant human TNF receptor-Fc fusion protein (Moreland, et al., 1997). Immunotherapy targeting TNFα seems to be one of the most progressive approaches to RA treatment.

1.1.3.2 IL-1

IL-1 is produced in high quantities in rheumatoid synovium by monocytes/macrophages (Wood, et al., 1992), and up to 10% of the cells in rheumatoid synovium were found to contain IL-1β mRNA (Firestein, et al., 1990a). A correlation was found between synovial production of IL-1, MHC class II expression and radiological progression in RA (Miyasaka, et al., 1988). IL-1 causes activation of endothelial cells with increased expression of cellular adhesion molecules, thus increasing inflammatory cell recruitment to the synovium. It stimulates prostanoid production by connective tissue and polymorphonuclear cells. IL-1 also activates osteoclasts and modulates

chondrocyte functions (Lefebvre, et al., 1990a; Shiozawa, et al., 1994). Cartilage resorption by RA synovial fluid has also been related to IL-1 levels (Hollander, et al., 1991), most likely through stimulating synovial fluid cells and chondrocytes to produce degrading matrix metalloproteinases (Saklatvala, 1981). *In vitro* experiments have demonstrated that invasion of articular cartilage by fibroblasts is stimulated by IL-1β and is inhibited by anti-IL-1 antibodies (Wang, et al., 1997). Localisation of IL-1 to the cartilage-pannus junction (Chu, et al., 1991) further supports its involvement in the joint destruction process.

1.1.3.3 IL-6

IL-6 is significantly elevated in synovial fluid and synovial tissue, as well as in the serum of RA patients (Field, et al., 1991; Firestein, et al., 1990a; Houssiau, et al., 1988). The source of IL-6 in the synovium is mainly fibroblast-like synoviocytes and, to a lesser extent, macrophages and endothelial cells (Firestein, et al., 1990a). IL-6 has been implicated as a major mediator of acute phase response in RA (Kutukculer, et al., 1998; Lacki, et al., 1997; McNiff, et al., 1995). It induces production of Acute Phase Proteins (APPs) and stimulates immunoglobulin production (Nawata, et al., 1989).

1.1.3.4 IL-2 and interferon (IFN)-γ

CD4+ T cells can be classified into distinct subsets according to the cytokines they produce (Mosmann, et al., 1986). T cells of Th1 subset produce IL-2 and IFN-γ, while Th2 clones produce IL-4, IL-6 and IL-10. Th1 cytokines promote T cell-mediated immunity, activate macrophages and induce production of pro-

inflammatory cytokines, whereas Th2 cytokines mediate immediate type hypersensitivity reaction, stimulate humoral immune response and deactivate monocytes/macrophages. Recently, a Th3 subset has been identified for cells producing TGFβ.

Despite a remarkable number of CD4+ T cells in the synovium, levels of T cell derived cytokines are scarce. There are contradictory data on the presence of IL-2 in synovial tissue and synovial fluid of RA patients. While some researchers found low levels of IL-2, others were unable to detect the cytokine (Altomonte, et al., 1992; Firestein, et al., 1988; Kutukculer, et al., 1998; Morita, et al., 1998; Steiner, et al., 1999; Wong, et al., 1996). Some have detected IL-2 mRNA, but not protein (Warren, et al., 1991). It is possible that protein IL-2 is bound to its receptors, expressed by synovial T cells, and is therefore undetectable. However it is also possible that the lack of IL-2 can be attributed to a genuine deficit of its production by T cells.

Unlike IL-2, IFNγ is readily detected in both synovial fluid and synovial tissue in RA, although at significantly lower levels than any of the "macrophage-derived" cytokines (Firestein, et al., 1990a; Firestein, et al., 1987; Hopkins, et al., 1988; Morita, et al., 1998; Murray, et al., 1998; Saxne, et al., 1988; Steiner, et al., 1999). Both CD4+ and CD8+ T cells have been identified as a source of IFNγ. IFNγ is a potent macrophage activating factor and inducer of MHC class II expression. It also activates neutrophils and promotes T and B cell differentiation. All these effects of IFNγ may contribute to synovial inflammation in RA. This is supported

by an observation in the collagen-induced arthritis model, where administration of IFNγ leads to aggravation of arthritis (Cooper, et al., 1988).

1.1.3.5 IL-4 and IL-10

IL-4 and IL-10 are regulatory cytokines that exhibit anti-inflammatory effects. They suppress pro-inflammatory cytokine production by monocytes/macrophages and IFNγ production by T cells.

IL-4, which is produced by Th2 T cell clones, mast cells and eosinophils, is barely detectable in RA synovium or synovial fluid. The data on its presence remains controversial (Miossec, et al., 1992; Miossec, et al., 1990; Morita, et al., 1998; Steiner, et al., 1999). It has been hypothesised that the lack of IL-4 in RA synovium contributes to the persistence of rheumatoid inflammation (Miossec, 1995). This hypothesis is supported by *in vitro* experiments and results obtained from animal models. Addition of IL-4 to synovial cultures strongly reduces pro-inflammatory cytokine production and increases IL-1 receptor (IL-1R) production (Chomarat, et al., 1995; Miossec, et al., 1992). Furthermore, reduced bone resorption and cartilage degradation in the presence of IL-4 has been demonstrated *in vitro* (Miossec, et al., 1994; van Roon, et al., 1995). Administration of IL-4 in the collagen-induced arthritis model resulted in amelioration of arthritis and suppression of cartilage destruction (Horsfall, et al., 1997; Joosten, et al., 1997).

IL-10 is abundantly represented in the RA joint, expressed by monocytes/ macrophages and, to lesser extent, T cells (Cohen, et al., 1995; Cush, et al., 1995;

Katsikis, et al., 1994). IL-10 is known as a major down-regulator of T cell derived cytokines and as a stimulator of the natural inhibitors of TNFα and IL-1, soluble TNF receptor and IL-1 receptor antagonist (Brennan, et al., 1995). On the other hand, IL-10 is an important factor inducing complete plasma cell differentiation and may thus contribute to the production of antibodies in the synovium. IL-10 diminishes IL-1 and TNFα production in rheumatoid cell cultures, suppresses cartilage degradation and ameliorates arthritis in the collagen-induced arthritis model (Joosten, et al., 1997; Jorgensen, et al., 1998; Kawakami, et al., 1997; Möttönen, et al., 1998; van Roon, et al., 1996; Walmsley, et al., 1996). Daily administration of IL-10 in experimental arthritis results in marked improvement suggesting that despite rather high levels of endogenous IL-10 in synovium, its exogenous administration can be of benefit in arthritis.

1.1.3.6 Chemotactic cytokines

A predominant feature of rheumatoid inflammation is the recruitment of a large number of various immunocompetent cells to the synovium. A number of chemotactic factors have been identified in rheumatoid synovium: IL-8, which predominantly recruits neutrophils; RANTES (regulated upon activation normal T cell expressed and secreted), MCP (monocyte chemoattractant protein) –1, and MIP (macrophage inflammatory protein) -1α and β, which mainly recruit T cells and monocytes (Akahoshi, et al., 1993; Brennan, et al., 1990; Deleuran, et al., 1994; Hosaka, et al., 1994; Koch, et al., 1992; Rathanaswami, et al., 1993; Villiger, et al., 1992). Continuous intraarticular infusion of IL-8 in rabbits has led to severe arthritis characterised by the infiltration of polymorphonuclear and, later, mononuclear cells in synovial tissue, and marked hypervascularization in

the synovial lining layer (Endo, et al., 1994; Endo, et al., 1991). Furthermore, administration of IL-8 neutralising antibody in established experimental arthritis almost completely blocks the infiltration of PMNs into the joints and provides protection from cartilage damage (Akahoshi, et al., 1994). Immunological blockade of either MCP-1 or RANTES in the experimental model of arthritis resulted in the amelioration of the disease (Barnes, et al., 1998; Ogata, et al., 1997), while administration of the CC chemokine receptor antagonist resulted in prominent reduction of both incidence and severity of the collagen-induced arthritis (Plater-Zyberk, et al., 1997). While a number of cytokines may contribute to cell recruitment in arthritis, the data from animal models clearly demonstrate that chemotactic cytokines play a critical role of in the development of arthritis.

1.1.3.7 Growth factors and colony-stimulating factors (CSFs)

Transforming growth factor (TGF) β, often considered an anti-inflammatory cytokine, is produced in rheumatoid synovium. TGFβ was found in rheumatoid synovial fluid (Arend, et al., 1990), and a significant proportion of synovial cells demonstrated TGFβ mRNA (Firestein, et al., 1990a). TGFβ has an inhibitory effect on the production of a number of pro-inflammatory cytokines (Arend, et al., 1990; Lotz, et al., 1990), although it has no effect when the target cells are already activated (Fava, et al., 1989). On the contrary, TGFβ may contribute to the on-going damage by recruiting inflammatory cells to the joint (Brennan, et al., 1995) and promoting fibroblast proliferation (Kendall, et al., 1997; Krummel, et al., 1988), thus contributing to pannus formation. It has been reported that rheumatoid synovial cells proliferate under serum-free conditions and that this growth was

abrogated by addition of anti-TGFβ antibodies (Goddard, et al., 1992). In an animal model of CIA exogenous TGFβ administration produced a pro-arthritic effect (Cooper, et al., 1992), suggesting that this growth factor may exert a harmful effect in arthritis.

Other growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and acidic and basic fibroblast growth factors (aFGF and bFGF), have been found in rheumatoid synovium and synovial fluid (Goddard, et al., 1992; Kusada, et al., 1993; Remmers, et al., 1990; Remmers, et al., 1991a; Sano, et al., 1990). These growth factors have been reported to be mitogenic for cultured synovial fibroblasts (Butler, et al., 1989; Remmers, et al., 1991b). PDGF was clearly the most potent stimulant of long-term growth of synoviocytes, while EGF had minimal effect. Synoviocyte proliferation was significantly inhibited by anti-PDGF antibody, thus confirming the importance of PDGF in the process of synovial expansion (Remmers, et al., 1991b). These results suggest that growth factors such as PDGF. and possibly aFGF and bFGF, play major roles in stimulating synoviocyte hyperplasia in RA.

CSFs, besides their haematopoietic role can act as cytokines at inflammation sites, serving as activators of inflammatory cells and inducers of HLA class II expression and cytokine synthesis in the target cells. Granulocyte/macrophage CSF (GM-CSF) is abundant in RA synovium at both mRNA and protein levels (Alvaro-Garcia, et al., 1991; Firestein, et al., 1990a) and is produced spontaneously by isolated RA synovial cells (Haworth, et al., 1991). Treatment with GM-CSF resulted in exacerbation of arthritis in an experimental model for

RA, while GM-CSF knockout animals had impaired cellular response to an arthritogenic stimulus and became resistant to arthritis (Campbell, et al., 1997; Campbell, et al., 1998). These findings suggest that GM-CSF is a potent accelerator of the pathological events leading to arthritis, supporting the notion that it may participate in human RA.

Thus, the prevalence of pro-inflammatory mediators in the rheumatoid synovium, together with insufficient suppressive mechanisms may lead to chronic inflammation and progressive joint destruction.

1.1.4 Aetiological factors

The cause of RA remains unclear despite intensive research in this area. Currently it is envisaged that while there is a genetic predisposition to the disease, exogenous factors also play an important role.

1.1.4.1 Genetic factors

Increased susceptibility to RA and development of more severe disease has been demonstrated in patients with particular HLA class II genes (Lanchbury, 1994; Ollier, et al., 1995). In Northern Europe RA is associated with some HLA DR4, DR6 and DR1 alleles, while HLA-DRB 1 alleles were identified in other RA populations. Comparison of the amino acid sequences of RA-associated and non-associated HLA-DR subtypes has revealed a conserved sequence in the third hypervariable region of the HLA-DR molecules associated with RA. This sequence was called the 'shared epitope'. Based on these findings, the hypothesis

of the 'shared epitope' was introduced (Gregersen, et al., 1987). It was based on the assumption that RA is initiated by some antigen presented to T cells by MHC class II of a specific phenotype. If the 'shared epitope' was not expressed by MHC class II molecules in an individual, the morbidious antigen could not be recognised by T cells, therefore, the individual had no risk of developing RA. This theory, however, has proved to be an oversimplification, as a significant proportion of the RA patients are 'shared epitope'-negative.

Other genes, such as those encoding cytokines and T cell receptors, can be also involved in the regulation of susceptibility to RA (Kingsley, et al., 1997). Female preponderance in the RA population and the results of family studies (Rigby, et al., 1991; Silman, et al., 1992) support the role of non-HLA genes in the disease development.

1.1.4.2 Exogenous antigens

Despite the clear indication that genetic factors are involved in the pathogenesis of RA, environmental factors undoubtedly play a significant role, as homozygous twin studies have demonstrated only 12-30% concordance rate for the disease (Wordsworth, et al., 1991). To date there is no unequivocal data implicating a particular microorganism in RA (Salmon, et al., 1995). A number of infectious agents have been suggested as possible candidates, e.g. bacteria, mycoplasma and viruses. *Mycobacterium tuberculosis* was suggested as an aetiological candidate as it was shown to be arthritogenic in rats (adjuvant-induced arthritis) (Pearson, 1956). Synovial T cells from patients with RA have also been shown to react with a 65kDa mycobacterial protein, which belongs to the family of heat shock

proteins (hsp) (Holoshitz, et al., 1986). However, an identical response was found in other inflammatory conditions such as pleural effusion, arguing against the aetiological role of this bacterial protei: in RA (Res, et al., 1990).

A number of viruses, including Epstein-Barr and human T cell lymphotropic virus type I (HTLV-I) were studied as aetiological factors for RA, as it was suggested that they may trigger autoimmunity (Blaschke, et al., 2000; Iwakura, et al., 1991a; Roudier, et al., 1989). An immunological cross-reactivity of viral proteins with some autoantigens, such as collagen, actin and cytokeratin, suggests a possible mechanism for the development of the autoimmunity (Baboonian, et al., 1991). However this does not explain why only a small proportion of infected individuals develops arthritis and why there is a significant proportion of patients devoid of viral infection (Niedobitek, et al., 2000; Venables, et al., 1981).

1.1.4.3 Autoantigens

The immunological changes described above and systemic nature of RA identify it as an autoimmune disease. Not surprisingly, the quest for the crucial self-antigen remains one of the major directions in the research into RA. The most popular concept implicates cartilage components as self-antigens. Collagen type II and proteoglycans are the major candidates. Type II collagen is specifically found in cartilage and some of the patients demonstrate humoral and cellular responses to its native form (Cook, et al., 1994; Morgan, et al., 1987). However, these patients represent only 10% of RA patients and have "atypical" DR3 and DR7 HLA profile (Klimiuk, et al., 1985; Morgan, 1990). Recently human cartilage proteoglycan pg39 has been proposed as a novel autoantigen (Verheijden, et al.,

1997). This proteoglycan was specifically recognised by peripheral T cells from RA patients, but not healthy individuals. In animal models both collagen type II and pg39 induce arthritis resembling the human RA. While it can be argued that autoreactivity to cartilage components may be secondary due to destruction and exposure of previously unexposed antigens, it is possible that even then these antigens may contribute to the inflammatory process in the joint.

For many years heat shock proteins (hsp), molecules widely distributed in the tissues, were believed to represent a group of self-antigens involved in the development of RA (Kaufmann, 1990; Lydyard, et al., 1990; McLean, et al., 1990). Although humoral and cellular reactivity to 65kDa hsp was detected in RA patients, it was found, after thorough investigation, to be directed against bacterial, not human sequences (Fischer, et al., 1991; Gaston, et al., 1990; Kingston, et al., 1996). Therefore, hsp are no longer considered as autoantigen candidates.

1.1.5 Pathogenetic theories

There are a number of theories of how RA develops. Each places a different emphasis on the role of different cell subsets and it may well be that elements of all are involved.

1.1.5.1 T cell hypothesis

The T cell hypothesis probably represents the most popular theory for the development of the RA (Miossec, 2000). According to this hypothesis, T cells

play the pivotal role in the initiation of arthritis and are involved in the maintenance of chronic inflammation. There is substantial evidence of the important role of T cells in RA. Firstly, the association of the particular MHC class II with RA suggests T cell involvement, since one of the major functions of the MHC class II molecules is antigen presentation to $CD4^+$ T cells. Secondly, T cells represent a significant proportion of rheumatoid synovial infiltrate. Thirdly, T cells appear activated as they express interleukin (IL)-2 receptor and MHC class II (Pitzalis, et al., 1987). Fourthly, T cells in synovium are found in close proximity to antigen-presenting cells (Fischer, et al., 1991; Gaston, et al., 1990; Kingston, et al., 1996). More recently it has been shown that autoreactive T cells are found within the inflamed rheumatoid synovium and, furthermore, that the antigens driving these T cells are cartilage derived (Fang, et al., 2000). Despite all these facts, the amount of T cell-derived cytokines produced in rheumatoid synovium is minute as compared to monocyte/macrophage derived mediators. This, however, is arguable as low levels of T cell derived cytokines may be due to the studies being carried out on the specimens obtained from patients with long-standing disease. In relatively "early" RA a number of T cell-derived cytokines were detected at higher levels (Ulfgren, et al., 1995). On the other hand, even low levels of these cytokines during the chronic stage of RA may be important in inflammation control. T cells can also participate in inflammation through cell-to-cell contact (Burger, 2000; Klareskog, et al., 1995). The efficacy of cyclosporin A therapy in RA patients also supports T cell involvement in the development of arthritis (Chang, et al., 1995; Yocum, 1993).

1.1.5.2 Mesenchymal hypothesis

The mesenchymal (or macrophage-fibroblast) theory assigns the role of the initiation of RA to T cells, but challenges the contribution of T cells to the later stages of arthritis (Firestein, 1991; Shiozawa, et al., 1992). The reasons include lack of noticeable clonal restriction and T cell proliferation, very low levels of T cell-derived cytokines and the moderate effect of T cell targeting therapies in the established disease. Several therapeutic approaches specifically targeting T cells have failed to produce a significant effect. Thus, total lymphoid irradiation and thoracic duct drainage have generally resulted in limited and temporal improvement (Firestein, et al., 1990b). Failure of treatment with anti-CD4 antibodies in RA patients seems to add to this list (Yocum, et al., 1998). However, it can be argued that the synovial concentration sufficient to produce a clinical effect has not been achieved due to restrictions imposed by wide $CD4^+$ T cell suppression in the peripheral blood.

In contrast to the limited evidence of T cell activation, there is no doubt that synovial macrophages and fibroblasts are activated. This observation is based on their morphological appearance, high surface MHC class II expression and production of significant amounts of cytokines, such as IL-1, TNFα, IL-6 and GM-CSF (Fontana, et al., 1982; Hirano, et al., 1988; Nouri, et al., 1984; Saxne, et al., 1988; Xu, et al., 1989). Synovial hyperplasia has also been found to develop due to the recruitment of mononuclear phagocytes (Henderson, et al., 1988) along with proliferation of synovial fibroblasts (Qu, et al., 1994). These mesenchymal cells, when activated and organised into an invasive front, have the capacity to destroy bone and underlying cartilage. There are a number of mechanisms that

cause cartilage and bone destruction: 1) production of proteases and glycosidases (Hamerman, et al., 1967; Harris ED, 1976), and 2) activation, by release of cytokines, of other cells, such as chondrocytes and PMNs, which produce degrading enzymes. The role of the mesenchymal cells as "workhorses" of the rheumatoid joint destruction is supported by the finding of these particular cells at the pannus/erosion interface in the majority of the RA patients. Recently, a positive correlation was demonstrated between the number of macrophages in the synovial lining and the radiological course and outcome of RA (Mulherin, et al., 1996).

1.1.5.3 Dendritic cell theory

Dendritic cells (DCs) are a population of potent antigen-presenting cells (APCs). DCs have a unique ability to stimulate autologous T cells in the absence of endogenous antigens (Inaba, et al., 1984; Knight, et al., 1988; Knight, et al., 1983; Nussenzweig, et al., 1980; Vakkila, et al., 1990). A recently proposed hypothesis (Thomas, et al., 1996) suggests that RA starts by recurrent non-specific damage to the joint that induces DC differentiation and attraction of T cells. Moreover, in the case of DCs, MHC-derived peptides can serve as autoantigens. While DC activation causes increased synthesis of MHC class II and I molecules and subsequent increase in MHC degradation, the lack of the lysosomal peptides in them, as compared to macrophages (Thomas, et al., 1993), may result in the association of MHC-derived peptides with MHC class II molecules in the endosomal compartments. Priming of the autoreactive T cells clones results in activation and expansion of these clones followed by B cell stimulation with subsequent production of autoantibodies. The humoral immune response along

with the production of T cell-derived cytokines initiates the activation of mesenchymal cells and PMNs leading to the joint destruction. In the course of this destruction an array of new self antigens become the objects of the immune reactions and contribute to the chronicity of the disease. At this stage T cell clonality is hard to observe as many T cell responses may become polyclonal and recruitment and activation of the T cells in the synovium may be due to "bystander" effects (Thomas, et al., 1996).

1.1.5.4 Mesenchymoid hypothesis

Unlike the described above "immunological" theories this one represents quite a different approach to the allegedly "autoimmune" disease. This hypothesis is based on the observation of the specific morphological characteristics of the rheumatoid synovial fibroblasts (Fassbender, 1983). Inflammatory processes in RA involve the exudation of fibrin and its deposition in the synovium. Normally, that would result in a formation of granulation and scarring. In contrast, in rheumatoid synovium, a uniform cell formation develops with relatively large, chromatin-poor nuclei and little cytoplasm. These transformed cells are called *mesenchymoid*. Mesenchymoid cells are short lived and are either destroyed or mature into fibroblasts.

The mesenchymoid hypothesis suggests that initial joint destruction occurs through the attack by the uniform, immature mesenchymoid cells. The inflammatory cells seen in the synovium at the later stages of the disease represent the result of secondary synovitis.

It can be concluded that each of the hypothetical mechanisms of RA development described above is supported by evidence. It is likely that a combination of all cell populations, including T cells, macrophages, fibroblasts and DCs, contribute to the rheumatoid synovitis. The relative contribution of each of the cell populations probably depends on the duration of the disease, and on genetic and environmental aspects.

Knowledge of the mechanisms behind joint destruction in RA will result in more specific and, presumably, more effective treatment for RA. Therefore, more studies are required to identify the exact course of the joint destruction in RA.

1.2 ANIMAL MODELS FOR RHEUMATOID ARTHRITIS

1.2.1 Introduction

The limited availability of human RA biopsy material as well as the considerable variability of the disease course from patient to patient restricts research in humans. Animal models established in inbred strains are uniform in their disease patterns and provide statistically significant sample numbers for investigation of different aspects of arthritis. Furthermore, models of experimental arthritis are the most valuable tools for potential drug trials, where new treatment strategies can be tested for their effect on the inflamed joint.

Several animal models for rheumatoid arthritis have been described. All of them share certain features with RA, but, on the other hand, none of the models exactly reflects the human disease. Six main groups of experimental polyarthritis models can be distinguished:

1) arthritis induced by intradermal injection of autoantigen suspended in mineral oil (collagen-induced arthritis);

2) arthritis induced by systemic injection of microbial components (cell wall-induced arthritides);

3) arthritis induced by intradermal injection of microbial preparations in mineral oil or by adjuvant alone (adjuvant-induced arthritis);

4) spontaneously occurring polyarthritis in some mice strains;

5) arthritis in transgenic animals;

6) in vivo model of cartilage destruction by rheumatoid synovium (synovial engraftment into SCID mouse).

1.2.2 Collagen-induced arthritis as a model for RA

1.2.2.1 Introduction

Nineteen distinct collagens have been identified in mammalian tissues (Seyer, et al., 1996). Each collagen molecule consists of three α-chains, which have a repetitive Gly-X-Y sequence (Cremer, et al., 1998). Due to their composition, all α-chains form a left-handed α-helix. The presence of Gly in every third position allows three α-chains to twist about one another to create a superhelix. The resulting structure is highly resistant to proteolysis, except by specific collagenases (Cremer, et al., 1998).

The first observation of the antigenicity of collagen was made by Steffen and Timpl in 1963 (Steffen, et al., 1963). These authors demonstrated that antibodies against connective tissue in sera of patients with rheumatoid arthritis were directed against collagen. Antibodies to collagen were later found by other researchers in sera and synovial fluid from patients with RA (Andriopoulos, et al., 1976a; Andriopoulos, et al., 1976b; Cracchiolo, et al., 1975; Michaeli, et al., 1974). Furthermore, injection of different types of collagen has been reported to elicit type-specific antibody response (Hahn, et al., 1974; Nowack, et al., 1975).

1.2.2.2 Arthritogenicity of collagen type II

Five collagens have been identified in articular cartilage, CII, CVI and CIX-CXI (Eyre, et al., 1995). CII is a homotrimer composed of α_1(II) chains. It constitutes 85% of the total collagen content in the cartilage, while the others comprise less than 10% each, and forms a backbone of cartilage heteropolymeric fibrils (Cremer, et al., 1998). Type XI collagen regulates the fibril size, while CIX may bond CII fibrils together and facilitate its interaction with proteoglycan macromolecules.

The immunogenicity of CII was discovered in 1974 (Hahn, et al., 1974) and soon its arthritogenic capacities were revealed. In 1977 Trentham and colleagues (Trentham, et al., 1977) reported production of arthritis in rats injected with collagen type II emulsified in either complete or incomplete Freund's adjuvant. The condition is now known as collagen-induced arthritis (CIA).

A number of species have been found to be susceptible to CIA (Cathcart, et al., 1986; Courtenay, et al., 1980; Trentham, et al., 1977; Yoo, et al., 1988). The discovery of CII arthritogenicity prompted studies of the properties of other collagens. CXI was found to be arthritogenic in rats but not in mice, with arthritis being less severe than that generated with CII, while CIX was not arthritogenic at all (Cremer, et al., 1995). Attempts to induce arthritis with non-cartilaginous collagens, such as type I and V were not successful (Cremer, et al., 1994; Trentham, et al., 1977).

The native form of CII seems to be essential for arthritis induction as its denatured form does not cause arthritis and is poorly immunogenic (Trentham, et al., 1977). Arthritogenic epitopes on the CII molecule have been studied using renatured peptides obtained after cyanogen bromide cleavage. In DBA/1 mice a number of peptides were recognised by the sera of the animals with arthritis induced by whole CII molecule immunisation (Terato, et al., 1985). Only one of these peptides, CB11 (122-147), was capable of arthritis induction, suggesting that it contained the major immunogenic and arthritogenic epitope for these mice. The particular arthritogenic epitope, though, may be different for different species or strains of animals. The MHC II haplotype is an important factor determining which epitope is recognised by the immune system. Thus, in B10.RIII mice, which bear H-2^r haplotype (as opposed to H-2^q haplotype in DBA/1 mice), CB11 was not arthritogenic, whilst CB8 peptide induced arthritis with an incidence comparable to the arthritis caused by whole CII molecule (Myers, et al., 1995).

The source of the collagen is important but not crucial. Chick, bovine, porcine, rat and human collagens were found to be arthritogenic (Cremer, 1988) although the incidence and severity of arthritis caused by different collagen types in different species and strains of animals varied.

1.2.2.3 Susceptibility to CIA

1.2.2.3.1 Species

As mentioned above, a number of species have been found to be susceptible to arthritis. Following the induction of CIA in rats (Trentham, et al., 1977), the disease was successfully produced in mice (Courtenay, et al., 1980). While the

histology of arthritis in mice was similar to that in rats, there were few particularities. Firstly, mice showed less response to CII emulsified in incomplete Freund's adjuvant (FIA) than in complete Freund's adjuvant (CFA). Secondly, a single injection of collagen was not sufficient to induce arthritis in mice and an intraperitoneal boost injection was required. Thirdly, delayed and variable onset, as well as variability in arthritis location, was observed in mice. Evidence of CII arthritogenicity in primates came when two teams of investigators described arthritis in squirrel, rhesus and cynomolugus monkeys after CII vaccination (Cathcart, et al., 1986; Yoo, et al., 1988). Bone erosions and cartilage destruction in monkeys were less pronounced than in rats and extraarticular symptoms, such as anorexia and loss of balance, were noted. Establishment of CIA in mice and primates has proven that the arthritogenicity of type II collagen is not restricted to the rat species.

1.2.2.3.2 Immunogenetics of CIA

Autoimmune diseases are associated with genes that regulate immune responses (Lechler, 1996). Genetic factors may not be the cause of arthritis, but certainly contribute to the predisposition and the severity of this disease (Lanchbury, 1994; Ollier, et al., 1995). It has been noticed that certain arthritides are associated with particular MHC genes. For example, ankylosing spondylitis is associated with HLA B27 (Brewerton, et al., 1973; Schlosstein, et al., 1973), while RA has been linked to HLA DR4Dw4/14, DR1 (Goronzy, et al., 1986; Nepom, et al., 1989).

Susceptibility to experimental CIA in rats and mice was found to be controlled by both MHC and non-MHC genes (Cremer, et al., 1995; Griffiths, 1988; Griffiths,

et al., 1992; Griffiths, et al., 1981; Lorentzen, et al., 1995; Nabozny, et al., 1994; Trentham, et al., 1977; Wooley, et al., 1987). Even in susceptible strains the manifestation and severity are regulated by both MHC linked and non-linked genes (Griffiths, 1988).

The importance of MHC genes could be anticipated, as these molecules play a key role in antigen presentation and CIA is known to be a T cell-dependent disease. Certain MHC haplotypes have been clearly associated with susceptibility and resistance to CIA (Griffiths, 1988). It has been shown that susceptibility is associated with the initiation of the disease, as strains resistant to active induction of CIA are susceptible to passive transfer of the disease (Griffiths, 1988). Hybrids carrying "CIA susceptible" MHC genes and "CIA-resistant" non-MHC genes develop arthritis with lower incidence (Griffiths, et al., 1984; Lorentzen, et al., 1997), demonstrating the role of non-MHC genes in arthritis development. Evidently, the balance of both MHC and non-MHC genes determine the susceptibility, severity and manifestation of CIA.

1.2.2.3.3 Gender

There is a significant female prevalence in RA, with the male to female ratio being 1:3 (Smith, et al., 1991). In CIA model in mice, males are more susceptible, while in rats females are more prone to the disease (Holmdahl, et al., 1986a). It is unlikely that oestrogens are responsible for the increased female susceptibility to RA as contraceptive pills and pregnancy have been shown to have a protective effect (Hazes, et al., 1990a; Hazes, et al., 1990b; van Zeben, et al., 1990). Likewise, administration of oestrogen suppresses CIA in both rats and mice

(Holmdahl, 1995; Holmdahl, et al., 1986a). Moreover, testosterone has been shown to promote arthritis in DBA/1 mice (Holmdahl, et al., 1992b).

The female prevalence in rat CIA may be explained by the location of the arthritis-promoting genes on the X-chromosome, as was shown by Jansson and colleagues (Jansson, et al., 1993). This was supported by the demonstration that hybrid males which received their X-chromosome from a highly susceptible mother and Y-chromosome from a moderately susceptible father, have a higher incidence of arthritis than off-spring of a highly susceptible male and a moderately susceptible female (Holmdahl, 1995). On the other hand, the phenomenon of female preponderance could be only partially related to sex chromosome-located genes, as female prevalence was less pronounced in castrated rats, pointing to sex hormone involvement (Holmdahl, 1995).

1.2.2.3.4 Flora

Bacterial flora is known to influence immune responses (Kiyono, et al., 1980; Moreau, et al., 1988). It has been shown that digestive microflora induces suppressive T cell regulation against foreign antigens (McGhee, et al., 1980; Michalek, et al., 1982; Wannemuehler, et al., 1982). Experiments with Dark Agouti (DA) rats, which are highly susceptible to CIA, have revealed that rats kept under germ-free conditions had markedly enhanced disease susceptibility and immune response to CII (Breban, et al., 1993). Immunisation of highly resistant germ-free Fisher rats also resulted in a stronger immune response to CII, although these rats did not develop arthritis. These experiments have clearly demonstrated

a modifying role of the microflora on CIA and autoimmunity to CII (Breban, et al., 1993).

1.2.2.3.5 Stress

Psychological stress has been found to influence CIA. It has been demonstrated that the stress caused by exposure to a predator, transportation or handling can profoundly suppress clinical and histological manifestation of arthritis (Rogers, et al., 1980). There is a possible explanation of this effect of psychological trauma. Stress has been shown to suppress humoral (Solomon, 1969) and cellular immune responses (Joasoo, et al., 1976) in rats and mice. This effect may be mediated through suppression of the immune response by an increase of glucocorticoid and catecholamine levels associated with stress (Monjan, et al., 1977). It has been demonstrated that stress (Fleshner, et al., 1996) as well as glucocorticoids (Araneo, et al., 1989; Daynes, et al., 1989; Daynes, et al., 1990) can switch Th1 regulated immune responses to Th2 responses. Taking in consideration the pivotal role of Th1 cells in CIA (Mauri, et al., 1996), a shift towards a Th2 response can be suggested as a mechanism of CIA suppression by stress.

1.2.2.4 Clinical manifestations of CIA

CIA manifests within 12-24 days after immunisation with CII (Caulfield, et al., 1982; DeSimone, et al., 1983; Trentham, et al., 1977). Clinical symptoms of arthritis include moderate to severe oedema and erythema over the affected joints (DeSimone, et al., 1983; Trentham, et al., 1977). Swelling increases until day 4 after onset and then reaches plateau (Takeshita, et al., 1995; Trentham, et al., 1977). Hind paw joints are more often affected than the joints of the front paws

(Takeshita, et al., 1994; Trentham, et al., 1977). X-ray examination reveals soft tissue swelling and articular bone erosions, which later progresses to bony ankylosis (Trentham, et al., 1977). CIA does not exhibit any systemic features, as opposed to RA in humans.

1.2.2.5 Histopathological features of CIA

There are no apparent histological changes in the joints before the onset of arthritis. However, weak IgG (Takeshita, et al., 1995) or fibrin deposits (Caulfield, et al., 1982) on the synovial surface have been described. At the time of onset, proliferation of the synovial lining cells and infiltration of the synovium with inflammatory cells are observed (Takeshita, et al., 1994). The infiltrate consists of various inflammatory cells, including mononuclear and polymorphonuclear cells (Caulfield, et al., 1982; Stuart, et al., 1979; Takeshita, et al., 1995; Terato, et al., 1982; Trentham, et al., 1977). The ratios of these cells in the infiltrate vary between different experiments performed by different research groups. This can be due to different strains of rats being used, or other variables such as the source of the animals, asymptomatic infections or stress, or as was shown, the morbidity rate (Takeshita, et al., 1994). At four days after onset severe destruction of the cartilage and subchondral bone by synovial pannus is evident, with various cell types including osteoclasts located at the invasive edge of the pannus (Takeshita, et al., 1995). Invasion of the pannus into the bone and cartilage is accompanied by substantial chondrocyte death and the disruption of the normal architecture of extracellular matrix of the cartilage (DeSimone, et al., 1983). The end stage of arthritis includes collapse of the joint cavity and epiphysial bone and cartilage replacement by fibrous tissue (fibrous ankylosis) (Wooley, et al., 1981).

There are very few studies investigating the exact cellular composition of the synovial tissue during CIA (Holmdahl, et al., 1988; Holmdahl, et al., 1985). The results obtained in these experiments were also hard to interpret, as the antibodies used for immunohistochemical cell identification often were reactive with more than one cell type. For example, Mac1 antibody used in mice CIA (Holmdahl, et al., 1988) is reactive with macrophages, polymorphonuclear cells and NK cells, W3/13 antibody used in rat (Holmdahl, et al., 1985) recognises T cells, neutrophils and NK cells, and W3/25 stains both macrophages and CD4+ T cells. While the authors based their assessment on the combination of immunohistochemical and morphological examination, clearly, in some cases, it would be difficult to distinguish between different cell types.

1.2.2.6 Pathogenesis of CIA

1.2.2.6.1 Humoral immunity

One of the main features of CIA is a strong activation of autoreactive B cells. It has been shown that arthritis does not develop until high levels of IgM and IgG antibodies reactive with self CII appear in the circulation (Stuart, et al., 1979; Trentham, et al., 1977; Trentham, et al., 1978b). The resistance to CIA in guinea pigs is believed to be due to a failure to induce any anti-collagen antibodies cross-reactive with self-antigen (Hernandez, et al., 1988).

At the humoral level, both antibody production and complement activation have been found to be important. Direct evidence of the arthritogenicity of anti-collagen antibodies was obtained in experiments demonstrating passive transfer of CIA. Intravenous injection of affinity purified anti-collagen IgG fraction obtained

from rats with CIA results in transient arthritis in naive recipients (Stuart, et al., 1982). The histopathologic features of this arthritis are similar to early changes in CIA, including synovial cell hyperplasia, infiltration of the synovium with inflammatory cells and appearance of neutrophils on the cartilage surface (Kerwar, et al., 1983). The arthritis, however, resolves in 4-5 days without any evidence of the permanent damage to bone and cartilage (Stuart, et al., 1982). Passive transfer of arthritis was achieved even in strains resistant to conventional CIA induction (Kerwar, et al., 1983).

Complement activation following anti-CII antibody binding to cartilage plays an important role in the development of the CIA. Immunofluorescent analysis has revealed the presence of both IgG and complement C3 on the cartilage surface after injection of anti-collagen IgG in experiments with passive CIA transfer (Kerwar, et al., 1983; Stuart, et al., 1983). Such passive transfer of arthritis was unsuccessful after complement depletion with cobra venom factor despite apparent IgG binding to the joint cartilage (Kerwar, et al., 1983). In conventionally induced CIA depletion of complement C3 with cobra venom factor resulted in a delay of the arthritis onset until C3 levels returned to normal (Kerwar, et al., 1981; Morgan, et al., 1981). Neutrophils did not accumulate in the joints of these animals and no other inflammatory changes were noted.

1.2.2.6.1.1 Rheumatoid factor

Rheumatoid factor (RF) is an antibody reactive with Fc portions of IgG (Allen, et al., 1966). High levels of RF are a typical finding in a number of autoimmune diseases, particularly, RA (Holmdahl, et al., 1987). RF has been detected in mice

with CIA (Holmdahl, et al., 1986b). Furthermore, a relationship was noticed between autoimmunity to CII and RF production (Holmdahl, et al., 1987). Holmdahl *et al.* have demonstrated an increase in serum levels of RF in mice injected with CII, but not with collagen type I or keyhole limpet hemocyanin (KLH) (Holmdahl, et al., 1987).

1.2.2.6.2 Cellular immunity

The initial experiments demonstrating the role of T cells in the CIA development were carried out by Trentham and colleagues (Trentham, et al., 1978a), demonstrating that CIA can be passively transferred to naive recipients with pooled spleen and lymph node cells from rats immunised with CII. The appearance of activated, IL-2R bearing cells in the synovium early at the onset of arthritis (Holmdahl, et al., 1988) is also suggestive of T cell involvement.

Collagen-specific T cell lines were isolated from lymph nodes of mice with CIA (Dallman, et al., 1985; Hom, et al., 1986). Intraarticular inoculation of such CII-reactive T lines resulted in arthritis with pannus formation that persisted for a few weeks (Brahn, et al., 1989). Viable cells were a requisite for arthritis induction and no humoral response to CII was required. Injection of ovalbumin-specific T cell lines did not produce arthritis.

T cell elimination studies have provided further insight into the role of T cells in CIA. Administration of anti-αβTCR antibodies at the time of onset was shown to suppress arthritis (Goldschmidt, et al., 1994), while administration of the antibody after onset reversed ongoing CIA (Goldschmidt, et al., 1991). Treatment with

anti-CD4 antibodies was effective when a prophylactic regime of administration was used (Chiocchia, et al., 1991; Goldschmidt, et al., 1994; Ranges, et al., 1985). A significant decrease in arthritis incidence and delayed onset were observed in the treated animals. Anti-CD4 treatment after the onset of arthritis was ineffective (Goldschmidt, et al., 1994). This may be due to the fact that depletion of the T cells with anti-CD4 antibody is less efficient once the T cells have been activated, as anti-αβTCR administration at the same timepoint proves to be suppressive (Goldschmidt, et al., 1994).

Severe combined immunodeficient (SCID) mice, which lack T and B cells, do not develop CIA after injection of arthritogenic CII preparation (Klareskog, et al., 1983). However, these mice were capable of developing arthritis after transfusion of spleen cells from arthritic mice which are CIA-susceptible (Williams, et al., 1992b). Intraperitoneal injection of native, but not denatured, CII was also required for arthritis development. CD4+ T cells were essential to transfer arthritis as their depletion inhibited arthritis and anti-CII antibody production (Kadowaki, et al., 1994). Depletion of CD8+ T cells, on the other hand, resulted in earlier onset of the CIA.

An analysis of TCR Vβ gene expression in rat synovium during CIA development was carried out to investigate the fine specificity of the "arthritogenic" T cells in CIA. A 60% prevalence of TCR Vβ6, 8.2 and 8.5 genes was noticed 2 days after the onset of arthritis (Erlandsson, et al., 1994). Changes in the lymph nodes at the same time did not show such marked bias. A week later there was an evident increase of the heterogenicity of the TCR Vβ expression. An important role of a

particular T cell clone which uses a limited number of Vβ chains has been also demonstrated in the mouse CIA model (Chiocchia, et al., 1991). Treatment with monoclonal antibodies against Vβ8.1,2 or Vβ5.1,2 resulted in reduced incidence of arthritis. Administration of anti-Vβ2 antibody delayed the onset, while injections of either anti-Vβ6 or anti-Vβ11 did not affect arthritis at all. These findings suggest that cells of Vβ8.1,2 and Vβ5.1,2 phenotype are critical for the anti-CII response development, while Vβ2–expressing cells may be involved in the initiation of the disease as their temporary elimination with antibodies delayed the onset. The role of T cell receptor was further studied in TCR congenic mice (Nabozny, et al., 1995). Congenic mice expressing different TCR chains had distinct arthritis phenotypes. CIA in these mice differed in regard to incidence, onset and severity of the disease.

1.2.2.6.3 Cytokines

The pattern and kinetics of cytokine production often reflects the sequence of immunological events occuring in the body. Several studies have examined the cytokine profiles in synovium and lymph nodes of rats with CIA. It has been noted that synovial expression of cytokines in CIA closely resembles that of human RA (Marinova-Mutafchieva, et al., 1997; Müssener, et al., 1997). TNFα, IL-1β and IL-6 were the most abundant cytokines found in the synovial lining layer and, particularly, at the sites of pannus formation and bone erosion (Marinova-Mutafchieva, et al., 1997; Müssener, et al., 1997). TNFα was detected at the time of onset, while IL-1β appeared only two days later (Marinova-Mutafchieva, et al., 1997), suggesting that TNFα is a primary pro-inflammatory

monokine in CIA. IFNγ producing cells were found scattered in deeper layers of the synovium, while other T cell-derived cytokines, such as IL-2 and IL-4 were not detected (Müssener, et al., 1997).

A different picture, however, was seen in the lymph nodes of animals with CIA. Along with abundant expression of TNFα (Mussener, et al., 1995), T cell cytokines were plentiful (Mauri, et al., 1996). A transient increase in IL-10, a Th2 cytokine, at day 3 after CII injection was followed by its almost complete suppression by day 6. From that point IFNγ, a Th1 cytokine, could be detected in the lymph node and its levels increased until the onset of arthritis. After onset the levels of IFNγ gradually decreased through arthritis progression and remission. Low levels of IL-4 and reappearance of IL-10 were detected throughout this period. Non-arthritic animals injected with adjuvant only presented with a predominantly Th2 type response, suggesting that the Th1 response is important in CIA development.

Studies employing exogenous cytokine administration or cytokine elimination by specific monoclonal antibodies support the observations described above. Systemic administration of TNFα resulted in increased severity of CIA (both clinical and radiological) (Brahn, et al., 1992), while intra-articular administration resulted in accelerated onset and higher incidence of the disease (Cooper, et al., 1992). Treatment with either anti-TNFα antibodies or TNF receptor-IgG fusion protein resulted in reduced incidence, arthritic score and severity of joint destruction in mice with CIA (Williams, et al., 1992a; Williams, et al., 1995). Similar results were obtained for another monokine, IL-1. Recombinant IL-1

administration resulted in increased incidence and earlier onset of arthritis, while the use of an IL-1 receptor antagonist reduced the incidence and delayed the onset of CIA (Killar, et al., 1989; Phadke, et al., 1986; Wooley, et al., 1993).

An interesting diphasic effect of the Th1 cytokine, IFNγ, in CIA has been shown by Boissier et al. (Boissier, et al., 1995). It was noted that early treatment with IFNγ neutralising antibodies resulted in reduced severity of the arthritis, while treatment after onset had an aggravating effect on CIA. This study ended the ambiguity resulting from contradictory reports on IFNγ administration in CIA (Cooper, et al., 1988; Mauritz, et al., 1988; Nakajima, et al., 1990). Clearly, IFNγ plays a multifaceted role in CIA, initially enhancing the immune processes and later down-regulating inflammation.

An apparent association of Th2 cytokines with the recovery process in CIA (see above) was supported by observations of the protective effect of IL-10, particularly in combination with IL-4, against CIA (Joosten, et al., 1997). Conversely, administration of anti-IL-10 and anti-IL-4 antibodies resulted in accelerated onset of CIA and increased severity of arthritis (Joosten, et al., 1997; Kasama, et al., 1995).

Taken together these observations suggest that while monokines are responsible for the synovial inflammation process and destruction of bone and cartilage, T cell derived cytokines play an important regulatory role in the initiation and, later, suppression of arthritis.

1.2.2.6.4 Overall mechanism

As it was described above, both humoral and cellular components of the immune response to CII are important for CIA development. Neither antibody, nor T cells alone were able to produce a "full-blown" CIA (Stuart, et al., 1982; Trentham, et al., 1978a). "Full-blown" arthritis can only be produced when both antibodies and T cells are transferred (Seki, et al., 1988).

The most probable mechanism of CIA development was described by Stuart et al. (Stuart, et al., 1988) (Figure 1.2). Immunisation with CII gives rise to both humoral and cellular response to collagen. Autoreactive antibodies are produced and accumulate in the joint where they bind to the cartilage surface (Kerwar, et al., 1983; Stuart, et al., 1983). Initial binding to intact cartilage may not be significant, but it still activates complement (Kerwar, et al., 1983; Stuart, et al., 1983). This results in chemoattraction of polymorphonuclear cells (PMNs) to the joint. Neutrophils are critical in CIA induction as their depletion completely abrogates CIA (Fava, et al., 1993). Neutrophil elastase released from PMNs seems to be the most important mediator in regard to CIA induction (Kakimoto, et al., 1995). It increases epitope exposure of CII, which is followed by increased antibody binding and complement activation (Jasin, et al., 1991). The vital role of complement in the initiation of the disease has been demonstrated by complete prevention of arthritis through treatment with cobra venom prior to the onset of arthritis, while in the established lesion no effect was found (Kerwar, et al., 1981). Complement components are chemoattractive for inflammatory cells that start to migrate to the joint.

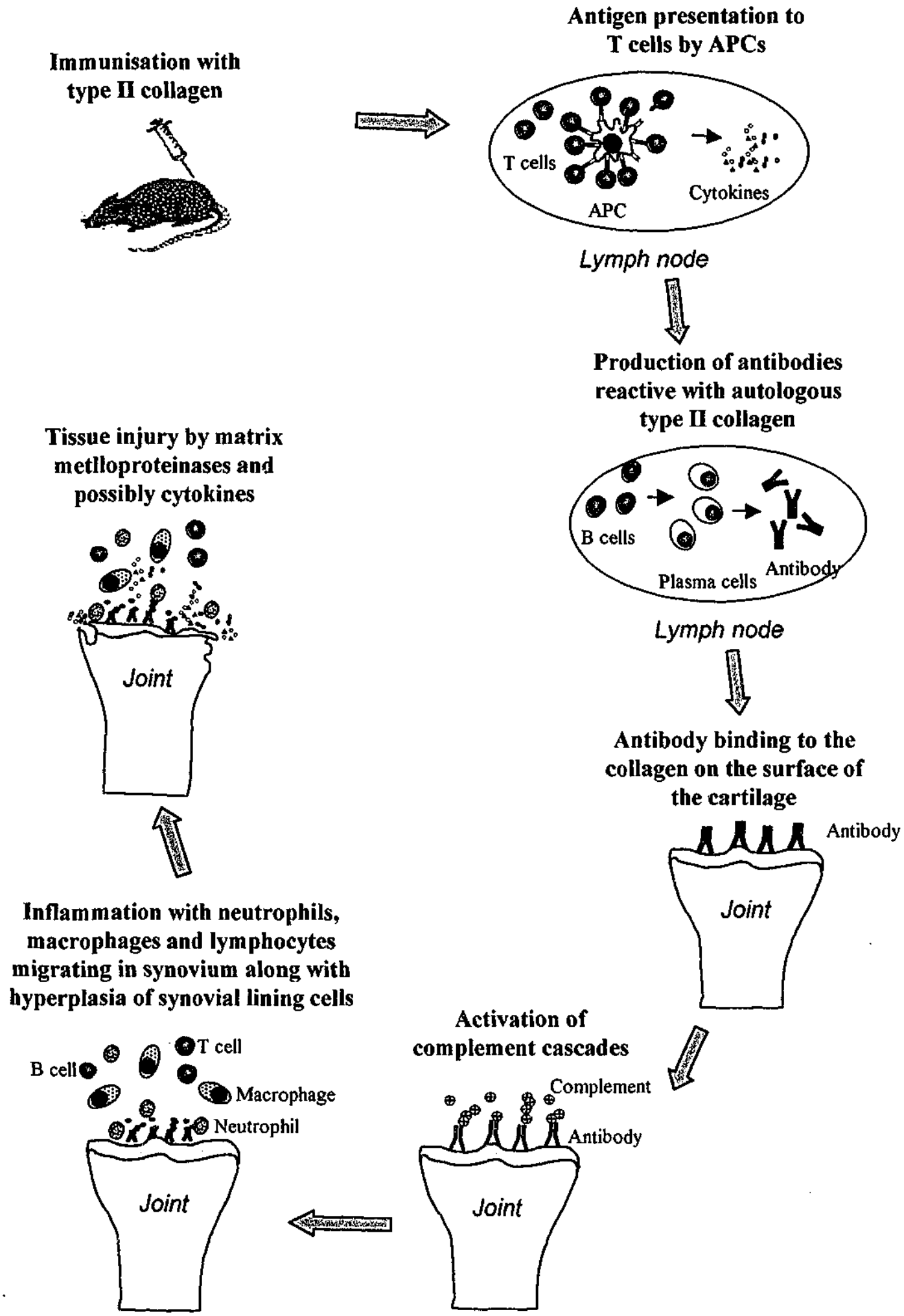


Figure1.2. Pathogenesis of collagen-induced arthritis.

Activated T cells appear early in CIA synovium (Holmdahl, et al., 1988). These cells produce a number of cytokines capable of activation of local cells, such as macrophage-like and fibroblast-like synoviocytes and chondrocytes, as well as newly recruited neutrophils and macrophages (Stuart, et al., 1988). Activated residential and infiltrating inflammatory cells produce proteinases, such as collagenases, gelatinase and proteoglycanase, which degrade proteoglycans and collagen from cartilage and bone matrix, resulting in typical bone and cartilage erosions (Stuart, et al., 1988).

1.2.2.7 CIA and RA

The relationship between human RA and CIA remains complex. Along with a number of similarities, there are some differences between the two conditions (Table 1.3). The central common element between RA and CIA is the autoimmunity to CII, a major component of the cartilage, although it can be speculated that an immune reaction to collagen may occur in RA patients as a secondary process following the destruction of the cartilage and exposure of previously hidden collagen epitopes. Nevertheless, this autoimmune process may contribute to joint destruction.

It should be noted that CIA has to be artificially induced, while RA occurs spontaneously. The course of the disease also differs significantly. RA is a chronic, remitting disease, while CIA subsides a few weeks after onset. The pattern of joint destruction, however, is strikingly similar in both RA and CIA. The synovium becomes hypertrophic and infiltrated by inflammatory cells. It

Table 1.3 Comparison of RA and CIA

Feature	RA	CIA	References
Immunogen	unknown	CII	(Maini et al. 1995) (Trentham et al. 1978)
Commencement	spontaneous	induced	(Maini et al. 1995) (Trentham et al. 1978)
Genetic predisposition	present	present	(Ollier and MacGregor 1995) (Griffiths et al. 1992)
Gender association	important	important	(Smith and Arnett 1991) (Holmdahl et al. 1986)
Onset	slow	rapid	(Morrow et al. 1999) (Trentham et al. 1977)
Course	chronic, remitting	acute, non-remitting	(Morrow et al. 1999) (Trentham et al. 1977)
Joints mainly affected	peripheral, symmetric	peripheral, symmetric	(Arnett et al. 1988) (Trentham et al. 1977)
Extra-articular lesions	present	absent	(Morrow et al. 1999) (Trentham et al. 1977)
Synovial pannus formation	present	present	(Morrow et al. 1999) (Trentham et al. 1977)
Cartilage and bone erosions	present	present	(Morrow et al. 1999) (Trentham et al. 1977)
Periarticular bone changes	osteoporosis	new bone formation	(Morrow et al. 1999) (Trentham et al. 1977)
T lymphocyte synovial infiltrates	typical	not prominent	(Edwards and S. 1995) (Holmdahl et al. 1985)
Macrophage-derived cytokines	abundant	abundant	(Saxne et al. 1988) (Marinova-Mutafchieva et al. 1997)
T cell-derived cytokines	scant	scant	(Firestein and Zvaifler 1990) (Mussener et al. 1995)
Neutrophils in synovial fluid	abundant	abundant	(Freemont 1995) (Fava et al. 1993)
Autoimmunity to CII	in some patients	compulsory	(Beard et al. 1980) (Trentham et al. 1978)
Rheumatoid factor presence	in majority of patients	present	(Masi et al. 1976) (Homdahl et al. 1986b)
NSAIDs	effective	effective	(Morrow et al. 1999) (Sloboda et al. 1981)
Prednisolone	effective	effective	(Morrow et al. 1999) (Sloboda et al. 1981)
Gold salts and chloroquine	effective	exacerbates the disease	(Morrow et al. 1999) (Sloboda et al. 1981)
Cyclophosphamide	effective	effective	(Morrow et al. 1999) (McCune et al. 1982)

forms an invasive pannus that erodes subchondral bone and cartilage. The main difference in the histological appearance of the synovium in RA and CIA is the lack of T cell infiltrate in the latter, which is probably due to the acute course in CIA. On the other hand, synovial cytokine balance is very similar in both RA and CIA, characterised by an abundance of monokines and relative paucity of T cell-derived cytokines.

Obviously, it is highly desirable to produce a model that would completely mimic human RA. Such model is not as yet available. Despite some differences from RA, CIA can be considered as one of the best models for this disease. Since it shares very important features with RA the study of CIA may give us valuable clues on the evolution of the human disease.

1.2.3 Other animal models

1.2.3.1 Adjuvant – induced arthritis

Adjuvant-induced arthritis (AIA) described by Carl Pearson in 1956 was allegedly the first model for RA (Pearson, 1956). Arthritis was induced by a single intradermal injection of dead *Mycobacterium tuberculosis* suspended in mineral oil (incomplete Freund's adjuvant). It has been shown that AIA can only be induced in rats (Glenn, et al., 1965). Hamsters, mice, guinea pigs, rabbits, dogs and monkeys have been found to be resistant. Recently, however, AIA has been induced in mice under slightly different conditions (Knight, et al., 1992): a boost injection of adjuvant was required and the latent period was found to last for about 2 months.

In rats arthritis starts 13 to 19 days after inoculation (Glenn, et al., 1965). Clinically it is characterised by symmetrical, monophasic, often severe arthritis (Klareskog, 1989). Hindlimbs are mainly involved, particularly the ankles. Arthritis starts to decline around day 20-22. Granulomas are often found in lungs and liver. The formation of granulomas is most likely due to the inoculum reaching the blood stream and becoming trapped in the inner organs (Glenn, et al., 1965).

The predominant feature of the pathological changes in AIA is periostitis and extensive periosteal new bone formation outside the joint at sites of ligament and muscle insertion (Glenn, et al., 1965). Later the inflammatory process expands to the other regions of the joints. Synovial lining cells proliferate with pannus

formation. The subintimal layer becomes infiltrated with mononuclear cells, predominantly CD4+ T cells arranged in a focal or diffuse pattern (Klareskog, 1989), but no B cells or plasma cells are found.

Although adjuvant arthritis has been extensively studied, no specific inciting or target antigen has been convincingly implicated (Taurog, et al., 1985). It has been suggested that cross-reactivity between some mycobacterial component and a rat autoantigen induces the arthritis. A T cell line, A2b, that can initiate arthritis in irradiated rats has been isolated (Holoshitz, et al., 1983). This clone, besides *M. tuberculosis*, has been shown to recognize a proteoglycan component of cartilage (van Eden, et al., 1985). The target antigen recognised by A2b T cells has been elucidated as a peptide with a relative molecular weight of 65kDa (van Eden, et al., 1988). A study in patients with RA has revealed that T cell clones from these patients reactive with M. Tuberculosis also recognize the 65kDa protein (Holoshitz, et al., 1986). The 65kDa antigen appears to be a heat shock protein (hsp) related to homologous proteins in other bacteria and higher eukaryotes. The epitope within hsp recognised by T cells has been cloned and shown to be a nonapeptide homologous to a peptide in the mammalian cartilage link protein with four of the nine amino acids being identical (van Eden, et al., 1988). Attempts to induce adjuvant arthritis with 65kDa hsp emulsified in mineral adjuvant have failed (Billingham, et al., 1990a). However, treatment with 65kDa protein has been shown to generate resistance to subsequent induction of AIA.

While the matter of the provocative agent remains unsolved, there is substantial evidence of T lymphocyte involvement in the pathogenesis of AIA. In 1983

Taurog and his colleagues (Taurog, et al., 1983) adoptively transferred arthritis to naïve rats with CD4+ T cells obtained from M. tuberculosis stimulated rats cultured in the presence of concavalin A (Con A). Stimulation with Con A, which is a non-specific T cell activator, seems to be essential as unstimulated T cells were capable of induction of arthritis only in immunosuppressed recipients. Con A, being a stimulus for IL-1, IL-2 and other cytokine production, promotes proliferation of T cells in culture (Cannon, et al., 1993a). Another indication of T cell involvement is the suppression of adjuvant arthritis after treatment with antibodies directed against molecules involved in MHC class II restricted antigen presentation to helper T lymphocytes, namely CD4, MHC class II (Billingham, et al., 1990b), α/β T cell receptor (TCR) (Yoshino, et al., 1990), and ICAM-1 (Iigo, et al., 1991).

All available evidence suggests that T lymphocytes expressing α/β TCR are responsible for the development of AIA. On the other hand, γ/δ T cells seem to play a protective role, as their depletion aggravates adjuvant arthritis (Pelegri, et al., 1996). However, it remains uncertain how T cells fulfil their function. Experiments involving arthritis transfer with labelled T cells (van den Langerijt, et al., 1994) have demonstrated the absence of these cells at the lesion site, while blood, lymph nodes, spleen and liver contained plenty of them. Although these data suggest that T lymphocytes act from outside the actual damage site, there is a possibility that a few specific (and important) T cells at the site of the lesion have been missed (Billingham, 1995).

The term "adjuvant-induced arthritis" has, for a long time, been associated only with arthritis induced by complete Freund's adjuvant, in the other words, mycobacterial components emulsified in mineral or vegetable oil. Now it has been shown that *M. tuberculosis* can be replaced by other mycobacterial strains, such as corynebacteria or nocardia (Billingham, 1995). It has been demonstrated that muramyl dipeptide (MDP), a component of mycobacterial wall and other Gram-positive bacteria, is capable of inducing AIA. It is of particular interest that MDP itself is not an antigenic substance. Coincidently, another non-antigenic compound CP 20961 suspended in mineral oil has shown arthritigenic properties, inducing arthritis identical to "classical" AIA (Chang, et al., 1980). Furthermore, arthritis induced by Freund's incomplete adjuvant (mineral oil) alone has been described in DA rats (Cannon, et al., 1993b; Kleinau, et al., 1991). This arthritis has been shown to be controlled by MHC and non-MHC genes and to be α/β T cell dependent, as with the classical AIA (Holmdahl, et al., 1992a; Lorentzen, et al., 1995). These observations question the hypothesis of the essential role of immunity to bacterial components in the development of arthritis. Thus, after years of extensive investigation, the mystery of an antigen or event leading to AIA remains unsolved.

Despite obvious differences from RA including lack of chronicity, extreme severity, and some histological dissimilarities, AIA remains a classical model of experimental polyarthritis, which is widely used in medical research.

1.2.3.2 Streptococcal cell wall-induced arthritis

Chronic arthritis in rodents can be induced by intra-articular or systemic injection of bacterial or fungal cell walls (Heymer, et al., 1982). Intra-articular injection of cell walls causes monoarthritis, while intraperitoneal administration induces polyarthritis. Streptococcal cell wall induced arthritis (SCWIA) has been studied very intensively.

SCWIA is a model of chronic erosive arthritis. It was described for the first time in 1977 (Cromartie, et al., 1977). The arthritis is induced by a single intraperitoneal injection of a sterile aqueous suspension of cells or cell walls from group A Streptococci (Streptococcus pyogenus).

Acute arthritis is observed 1 day after injection, reaching its peak in 3-5 days, and subsiding in 4-7 days. The acute phase is replaced by chronic disease from day 14-21, where exacerbations and remissions persist for a few months, progressing to fibrosis and ankylosis of the joints (Cromartie, et al., 1977; Dalldorf, et al., 1980).

Histologically, SCWIA is characterised by synovial proliferation and infiltration by granulocytes and, later, mononuclear cells. The inflammatory masses can also be seen in the bone marrow. At the chronic stage cartilage destruction and excessive new bone formation can be seen. The spleen and, to a lesser extent, liver may be affected by granuloma formation.

The humoral immune response in SCWIA is directed against bacterial proteoglycans (van den Broek, 1989). Elevated levels of these antibodies have also been found in juvenile spondylitis and rheumatoid arthritis in humans (Johnson, et al., 1984).

There is an obvious genetic predisposition to the disease, as it can be induced only in susceptible strains, namely Lewis rats (100% incidence) and Sprague Dawley rats (70% incidence) (van den Broek, 1989). It appears that susceptibility to SCWIA is controlled by non-MHC class genes, as Fisher 344 (F344) rats which share RT1.A and RT1.B loci (MHC class I and II respectively) with Lewis rats are resistant to the disease (Anderle, et al., 1979). Recently it has been suggested that reduced glucocorticoid production secondary to a defect of corticotropin releasing hormone synthesis and secretion in Lewis rats may contribute to their susceptibility to arthritis (Sternberg, et al., 1989). It has been found that female rats have a higher incidence of SCWIA than males, and castration of males or administration of estradiol results in an increased incidence of the disease (Zhang, et al., 1995).

T cells play a critical role in the chronicity of SCWIA, as only an acute phase is observed in nude (athymic) Lewis rats (Ridge, et al., 1985). Furthermore, administration of cyclosporin A inhibits chronic joint inflammation (Yochum, et al., 1986). Group A streptococcal peptidoglycan-polysaccharide complex persistence is also required for the chronicity of SCWIA (Janusz, et al., 1984). The severity of arthritis correlates with the amount of antigen present in the joint (Dalldorf, et al., 1980).

Possible mechanisms for SCWIA development may involve defective immunological tolerance to bacterial antigens (van den Broek, 1989). Immunological tolerance to gut flora has been shown to play an important role in arthritis development. F344 rats are resistant to SCWIA, but when raised in germ-free environment, they develop arthritis similar to that seen in the Lewis rats, while their counterparts, returned to ordinary non-germ free conditions, acquire resistance to the disease (van den Broek, 1989).

In an experiment studying the induction of tolerance to SCWIA, it has been revealed that pretreatment with a suspension of mycobacterial 65kDa hsp (a similar protein has been isolated from *S. pyogenus*) provides protection from the development of arthritis (van den Broek, et al., 1989). Splenic T cells can transfer this protection to a recipient subsequently challenged with SCWIA. It has been suggested that injection of the 65kDa protein induces immunosuppression that down regulates all SCW-specific immune responses (van den Broek, 1989). Probably, the same mechanism is involved in protection against adjuvant-induced arthritis. However, the phenomenon of such "shared" protective mechanism requires further investigation.

1.2.3.3 Spontaneously occurring arthritides

1.2.3.3.1. Arthritis in MRL-lpr/lpr mouse

MRL-lpr/lpr mice spontaneously develop symmetrical erosive arthritis (Andrews, et al., 1978). Macroscopically, this arthritis can be observed in approximately 20-25% of 5-6 months old MRL-lpr/lpr mice, while morphological signs of arthritis are present in 70-75% of animals (Hang, et al., 1982). Arthritis is characterised by

proliferation of synovial lining and infiltration of cartilage and bone by pannus. Synovial cell hyperplasia constitutes the initial stage of the arthritic lesion and has been described in young mice 7-13 weeks old (O'Sallivan, et al., 1985). Pannus formation with destruction of cartilage can be observed. Invading synoviocytes to a large extent display macrophage markers at the age of 14 to 16 weeks. Most of these cells are negative for MHC class II expression. The arthritic joints do not demonstrate mononuclear cell infiltrate. However, despite there being virtually no T cells in the arthritic synovium, daily administration of cyclosporin A to mice from 4 to 18 weeks old has been shown to abrogate the erosive arthritis (Mountz, et al., 1987), suggesting T cell involvement.

An interesting feature of the MRL-lpr/lpr mice is the spontaneous production of rheumatoid factor (RF) and other autoantibodies. However, there is poor correlation between circulating RF levels and the severity of arthritis (Koopman, et al., 1988). Indeed, about 25% of animals with histological changes characteristic of arthritis lacked elevated serum RF. Antibodies against collagen type II have also been found in this model, but the titres are low and they appear after the onset of the disease (Tarkowski, et al., 1986).

The lack of MHC class II overexpression and T cell synovial infiltrate as well as the presence of a profound general impairment of the immune system resulting in glomerulonephritis, vasculitis and dermatitis contribute to the differences between MRL-lpr/lpr model and rheumatoid arthritis. Nevertheless, the MRL-lpr/lpr mouse represents a particularly valuable model for investigation of synovial cell

hyperplasia, pannus formation and cartilage/bone destruction in the absence of lymphoid infiltrate.

1.2.3.3.2. Spontaneous arthritis in male DBA/1 mice

For years male DBA/1 mice have been used for collagen-induced arthritis modelling. However, recent studies (Holmdahl, et al., 1992b; Nordling, et al., 1992a) have demonstrated that 70 to 90% of ageing male DBA/1 mice develop chronic intermittent arthritis in their hind limbs. Proximal and distal interphalangeal and metatarsophalangeal joints and, occasionally, ankles have become involved. The mean severity is about 3 (on the scale of 0-3 per limb with maximum score of 12 for an animal). No females have developed arthritis.

Histologically, the early stages are characterised by synovial lining cell proliferation and mononuclear cell infiltration of the synovium. In the late stages erosions of bone and cartilage appear at the pannus-cartilage junction. No T lymphocytes have been detected and only limited MHC class II expression is described (Holmdahl, et al., 1992b).

Measurable levels of anti-collagen type II antibodies have been detected in about 25% of male mice 3 months or older, while no antibodies were detected in younger males and females (Nordling, et al., 1992b). In experiments investigating the possible arthritogenic role of these antibodies, four month old male DBA/1 mice were treated with anti-idiotopic antibodies to anti-collagen type II antibodies. The incidence of arthritis was reduced, but the severity in the diseased

animals remained the same. These results suggest that anti-collagen type II antibodies may contribute to the development of arthritis.

While the role of specific immunological reactivity in the pathogenesis of spontaneous arthritis in DBA/1 mice remains obscure, there is a genetic predisposition, as well as sex and behavioural regulation of the disease. A number of mouse strains have been studied (Holmdahl, et al., 1992b), with only DBA/1 male mice spontaneously developing arthritis. Moreover, the disease appears to be controlled by recessive genes, since F1 hybrids of DBA/1 mice with another strain B10Q have been found resistant to arthritis. Sex hormones have been proven to be of importance, as castrated mice do not develop arthritis, and disease susceptibility is restored with testosterone treatment (Holmdahl, et al., 1992b). Aggressive behaviour also seems to have a pro-arthritic effect, since housing of less than 3 males per cage completely prevents arthritis.

Arthritis in DBA/1 mice represents a very interesting model of spontaneous arthritis in animals with an otherwise normal immune system.

1.2.3.4 Arthritis in transgenic animals

Transgenic animals constitute an important tool for studying the functional effects of specific gene products *in vivo*. Recent cytokine studies in RA have suggested TNFα has a significant role in the development of the disease (Chu, et al., 1991; Elliot, et al., 1994). A transgenic mouse for human TNFα has been generated (Keffer, et al., 1991), and all these mice develop arthritis in their ankles at 3-4 weeks of age. Histological analysis of the joints has revealed proliferation of

synoviocytes with pannus formation, a subintimal infiltrate consisting of neutrophils and macrophages with a few lymphocytes, and, later, articular cartilage destruction. Arthritis in these transgenic mice can be suppressed by anti-TNFα antibody treatment. Thus, human TNFα transgenic mice clearly demonstrate the arthritogenic properties of TNFα.

Various exogenous agents have been implicated as possible mediators for RA: bacteria, mycoplasma and viruses. A high incidence of chronic arthritis has been reported in patients with human T cell leukemia virus type 1 (HTLV-1) associated myelopathy (Nishioka, et al., 1989). This observation led to the creation of a mouse strain transgenic for HTLV-1 (Iwakura, et al., 1991b), which developed arthritis. Besides joint inflammation, these transgenic mice developed thymus atrophy along with a reduced proliferative response of T cells to Concavalin A. Two transgenic lines were originally produced which differed more than 20 times in transgene mRNA expression. The incidence of arthritis was higher in the line with higher mRNA expression. Arthritis is seen in female mice from 2 to 3 months of age with 50% incidence reported by 12 months. Male mice become affected later (from 5-10 months) with a lower incidence of arthritis. Typically, ankles become affected, however, in some animals signs of arthritis have been also found in the toes and knees.

Histological examination of the joints in HTLV-transgenic mice revealed erosions of cartilage and bone associated with pannus-like tissue consisting mostly of fibroblasts and an inflammatory infiltrate. The latter was located predominantly around blood vessels, and was composed of lymphocytes, neutrophils and

macrophages. There are significant amounts of RF, as well as antibodies to CII, HSP and DNA in arthritic transgenic mice (Iwakura, et al., 1995). Antibody production was detected before the onset of arthritis, suggesting it was not induced secondarily as a result of joint inflammation.

It is known that the HTLV-1 genome encodes a characteristic protein Tax, which can activate many cellular genes including *c-fos, c-jun* (Iwakura, et al., 1994), TGFβ (Kim, et al., 1990), GM-CSF (Green, et al., 1989), IL-2 and IL-2R (Siekevitz, et al., 1987). Tax is suspected to play an important role in the development of arthritis in HTLV-1 transgenic mice. It has been shown that in arthritic joints, expression of IL-1β, IL-6, TGFβ1, and IL-2 is increased, while IL-1β, TNFα and IFNγ are weakly activated (Iwakura, et al., 1995). Both MHC class I and class II are upregulated.

Although the HTLV-1 transgenic animal model differs in some aspects from human RA, it clearly proves that HTLV-1 can cause immune system hyperreactivity and induce autoimmune arthritis.

1.2.3.5 Models employing severe combined immunodeficiency (SCID) mice

None of the above described models exactly reflect the cellular composition of the inflamed RA synovial membrane. An *in vivo* model of cartilage destruction caused by *human* rheumatoid synovium has not been available until recently. In 1983 a severe combined immunodeficient (SCID) mouse strain was generated and described (Bosma, et al., 1983). SCID mice lack T and B cells and, therefore, are

unable to reject allografts. Rheumatoid synovial graft implants into SCID mice have become a new type of model for RA.

In the first model described (Sack, et al., 1994) rheumatoid synovium was engrafted into the mouse knee joint. Implanted synovial tissue induced cartilage and bone erosions through formation of pannus. The pannus tissue was found to be rich in either human (coming from the graft), or, more often, murine macrophages and neutrophils. The attraction of murine macrophages and neutrophils to the inflammatory focus is likely to be due to the intense cytokine and chemokine production.

Interaction between RA synovium and human cartilage has been investigated in another model (Geiler, et al., 1994), in which these tissues were coimplanted subcutaneously in SCID mice. This model, presumably, better resembles the situation in rheumatoid joints. The implants have demonstrated synovial invasion into the cartilage with the invading front consisting almost exclusively of fibroblast-like cells. These findings coincide with those described in humans (Fassbender, 1983; Shiozawa, et al., 1983). Very few human lymphocytes have been detected in the implanted synovial tissue, probably due to their migration from the graft. The only murine cells discovered in human synovium were macrophages, which, however, were not found in the vicinity of cartilage erosions.

The models for RA in SCID mice provide a brilliant opportunity for investigation of molecular and cellular mechanisms of cartilage erosion by rheumatoid

synovium *in vivo*. They also might represent a useful model for new therapeutic strategies aimed at inhibiting joint destruction.

1.3 NERVE GROWTH FACTOR AND ARTHRITIS

1.3.1 Biology of nerve growth factor

NGF is a member of a protein family called neurotrophins. Apart from NGF, this family includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), NT4/5 and NT6.

NGF was discovered 50 years ago as a soluble factor stimulating neurite overgrowth from sympathetic and sensory ganglia in chick embryos (Levi-Montalcini, 1987). It was soon realized that NGF regulates the survival of certain neurone populations during ontogenesis and acts as a trophic messenger between the neurone and its target tissue. The most abundant sources of NGF include mouse (not other mammals') submaxillary gland (Levi-Montalcini, et al., 1968) and snake venom (Angeletti, et al., 1979).

A free NGF molecule consists of three subunits, α, β and γ (Figure1.3).

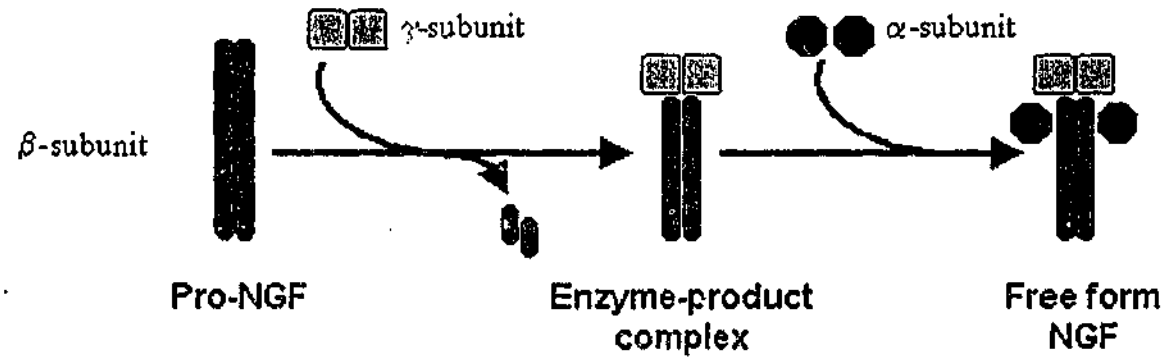


Figure 1.3. NGF synthesis *in vivo*.

The β-subunit is responsible for the biological effect of NGF (Greene, et al., 1971). It is a dimer of two identical chains bound by a non-covalent bond. Each monomer consists of 118 amino acids (Angeletti, et al., 1971). Cyanogen bromide and trypsin cleavage of β-NGF has revealed that the biologically active site is localized to residues 10-25 and 75-88, linked by a disulfide bond (Mercanti, et al., 1977). β-NGF is synthesised as pro-NGF, which is cleaved to become β-NGF by a γ-arginine esteropeptidase, which remains in the enzyme-product complex and becomes the γ-subunit (Berger, et al., 1977). The role of the α-subunit is not as yet clear. It is believed that both the α- and γ-subunits protect free NGF from proteolytic cleavage.

1.3.2 Receptors for NGF

NGF acts through its receptors, trk A and p75. Trk A, a 140 kDa tyrosine kinase, displays high affinity binding and is capable of internalizing NGF (Barbacid, 1993) (Figure 1.4).

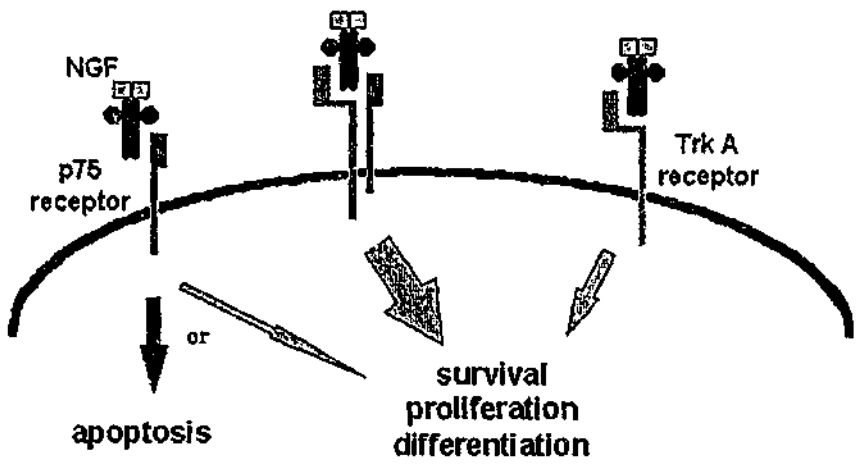


Figure 1.4. Effects of NGF receptors Trk A and p75 on target cells.

Trk A is a specific receptor for NGF, however, NT3 may also signal through it, although with less efficiency (Bothwell, 1995). Ligand binding results in trk A phosphorylation followed by a cascade of protein phosphorylations in the cell (Davies, 1997). Trk A mediates survival and cell differentiation (Davies, 1994; Lewin, et al., 1996).

p75, a 75kDa protein, binds NGF with low affinity and does not internalise NGF (Meakin, et al., 1992). p75 belongs to the TNF receptor I/Fas family of receptors (Frade, et al., 1998). Two different responses can be generated following NGF binding to p75: activation of gene transcription via the nuclear factor κB (NF-κB) and programmed cell death (apoptosis) via the c-Jun N-terminal kinase (JNK) pathway (Bothwell, 1996; Carter, et al., 1996; Casaccia-Bonnefil, et al., 1996). p75 receptor binds all neurotrophins, however, only NGF can induce apoptosis.

Trk A and p75 can be co-expressed on the cell surface. Such co-expression results in higher affinity binding to trk A (Hempstead, et al., 1991) and enhancement of survival/differentiation stimulus produced by the same amounts of NGF (Davies, et al., 1993; Lee, et al., 1994). Co-expression also increases ligand discrimination by trk A, reducing the ability of NT3 to signal through it (Benedetti, et al., 1993). The mechanism behind such events is that trk A suppresses the JNK signalling pathway of p75, while the NF-κB pathway remains unaffected (Yoon, et al., 1998).

1.3.3 NGF as a pain mediator

There is an overwhelming body of evidence demonstrating that NGF is part of a pain-signalling system. It appears that NGF maintains nociceptor sensitivity in vivo. It is normally expressed in different tissues in the body at very low concentrations (Shelton, et al., 1984). The immunological elimination of NGF, however, results in hypoalgesia and reduced responsiveness to heat and chemical irritants (McMahon, et al., 1995). Therefore, those low NGF levels seem to be required for maintenance of the "physiological" sensitivity of the pain-signalling system.

Another fact supporting NGF regulation of nociception is that trk A receptors are expressed on the small neurones of dorsal root ganglia (DRG) which are known to be responsive to nociceptive stimuli (Averill, et al., 1995).

Interesting results has been obtained involving overexpression of NGF in the body. Injection of NGF into human volunteers has resulted in disseminated pain in the internal organs after intravenous injection and local pain and hypersensitivity following subcutaneous administration (Petty, et al., 1994). A systemic hyperalgesic effect has been also observed in experiments involving chronic NGF infusions and in NGF transgenic mice (Al-Salihi, et al., 1995; Davis, et al., 1993).

NGF also seems to be one of the main mediators of inflammatory pain. First, elevated NGF levels are consistently found at the sites of inflammation (Aloe, et al., 1992b; Safieh-Garabedian, et al., 1995; Weskamp, et al., 1987). Second,

immunological sequestration of NGF (using specific antibodies) prevents local sensory hypersensitivity caused by an injection of complete Freund's adjuvant (Ma, et al., 1997; Woolf, et al., 1994). Furthermore, it has been shown that hyperalgesia caused by such pro-inflammatory cytokines as TNFα and IL-1β is mediated through an increase in NGF levels (Safieh-Garabedian, et al., 1995; Woolf, et al., 1997).

1.3.4 NGF and the immune system

The role of NGF in inflammation is not restricted to its participation in the pain-signalling pathway. There is ample evidence that NGF exerts an effect on a number of cells of the immune system (Figure 1.5). In vivo administration of NGF results in widespread increase in the size and number of mast cells in peripheral tissues (Aloe, et al., 1977), and similar changes were found in NGF transgenic mice (Getchell, et al., 1995). NGF has also been shown to promote mast cell survival in vitro (Bullock, et al., 1996). B cell antibody production can be modulated by NGF (Brodie, et al., 1994; Kimata, et al., 1991a; Kimata, et al., 1991b; Yanagida, et al., 1996). Furthermore, NGF seems to be an essential autocrine survival factor for memory B lymphocytes (Torcia, et al., 1996). NGF has been demonstrated to stimulate polymorphonuclear leukocyte migration both in vitro and in vivo (Boyle, et al., 1982; Gee, et al., 1983).

Being sensitive to NGF, some immune cells are also a source of this neurotrophin. These include mast cells, which have been shown to synthesize, store and release

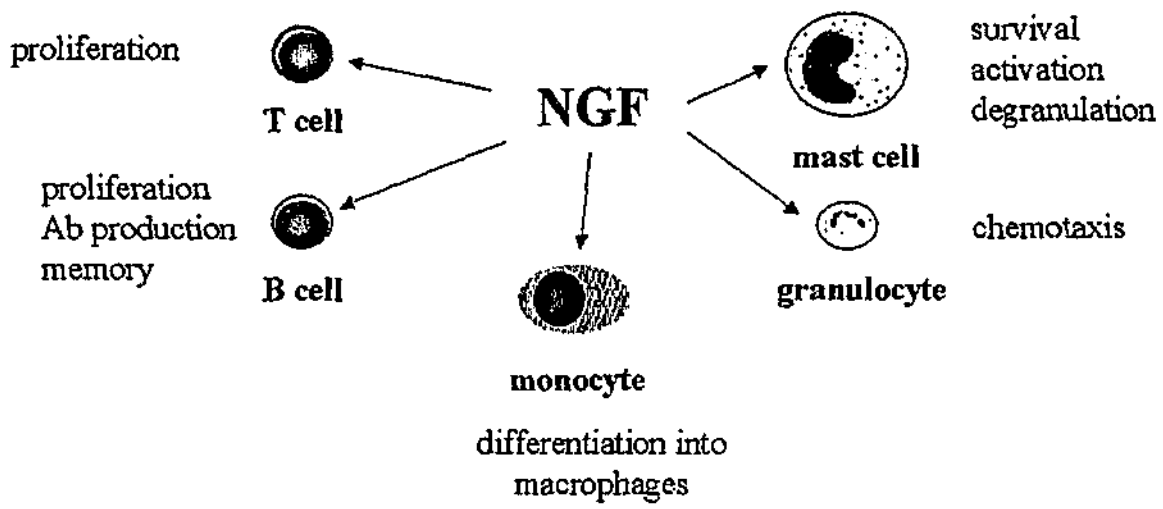


Figure 1.5. Effect of NGF on the cells of the immune system.

NGF (Leon, et al., 1994), memory B cells (Torcia, et al., 1996) and some activated $CD4^+$ T cell clones (Ehrhard, et al., 1993a).

1.3.5 NGF in arthritis

The observations of the increased concentrations of NGF at sites of inflammation along with the mounting data that immune cells are able to respond to it (see above) have led to a hypothesis that NGF can be involved in arthritis. NGF levels were studied in the sera and synovial fluids of patients with different forms of arthritides. Elevated levels of NGF were found in patients with rheumatoid arthritis, osteoarthritis, juvenile chronic arthritis, ankylosing spondylitis and psoriatic arthritis compared to normal controls (Aloe, et al., 1992a; Dicou, et al., 1993; Dicou, et al., 1996; Falcini, et al., 1996; Halliday, et al., 1998). Furthermore, a direct correlation was found between NGF serum concentrations and indices of the disease activity in juvenile chronic arthritis (Falcini, et al., 1996). Studies in transgenic arthritic mice which express the human TNFα gene show overexpression of NGF in their synovium (Aloe, et al., 1993). Such increase

in NGF is the result of inflammation caused by overexpression of TNFα, as NGF elevation following an intra-articular injection of TNFα is significantly less prominent (Aloe, et al., 1993). The most likely cytokine to be responsible for up-regulation of NGF levels is IL-1β. Injection of IL-1β, but not of TNFα, induced significant increase in NGF levels in the joints of mice with experimentally induced arthritis (Manni, et al., 1998). When the two cytokines were administered concomitantly the effect of IL-1β was enhanced. Administration of antibodies to NGF into TNFα-transgenic mice, caused reduction in mast cell numbers, pro-inflammatory substance P and histamine levels in synovium, thus demonstrating some anti-inflammatory activity (Aloe, et al., 1995). In carrageenan-induced arthritis NGF immunoreactivity in synovium was found perivascularly and in the infiltrating lymphocytes (Aloe, et al., 1992b).

Taken together this data suggests that the role of NGF may extend far beyond its functions as a mediator of inflammatory pain. It is well possible that it is involved in the maintenance of joint inflammation through its effect on the inflammatory cells. Further studies, however, are required to test this hypothesis.

1.4 AIMS OF THIS STUDY

As described in the Literature Review, despite intensive research, rheumatoid arthritis remains a disease associated with high morbidity and mortality. There are many gaps in our knowledge of the exact mechanisms that drive the disease. This study characterizes an animal model for RA, collagen-induced arthritis in the rat, analysing the mechanisms driving autoimmune joint destruction, as well as assessing the relevance of this animal model to human RA. This thesis also addresses the involvement of the neurotrophic peptide, NGF, in joint inflammation. Therefore, the specific aims of this study are:

- To establish a reproducible model of collagen-induced arthritis.

- To characterise pathological changes and analyse cell populations in the arthritic joints of animals with collagen-induced arthritis.

- To assess expression and distribution of NGF and its receptors during the development of arthritis.

Chapter 2

MATERIALS AND METHODS

II. MATERIALS AND METHODS

2.1 BUFFERS AND SOLUTIONS

ABTS substrate

ABTS (2,2' Azino-bis[3-Ethylenbenz-Thiazoline-6-sulfonic Acid], Sigma, St. Louis, Missouri, USA)	8mg
0.1 M citric acid	4.8ml
dH_2O	8ml
0.2M Na_2PO_4	3.2ml
Hydrogen peroxide 30%	1.6µl

The solution was mixed with stirring, prepared fresh before use.

1% Acid alcohol

HCl	10ml
Ethanol	700ml
dH_2O	290ml

Bisbenzimide H33342

Bisbenzomide H33342 (Calbiochem®, La Jolla, CA, USA)	2.8mg
dH_2O	100ml

The solution was prepared in the fume hood and protective clothing, gloves, mask and glasses were worn.

Bovine serum albumin (BSA) (20%)

BSA powder	20mg
1x PBS	up to 10 ml

Serum was stored at -20°C and defrosted before use.

BSA (20%)/Triton X (0.3%)

BSA (20%)	10ml
Triton X-200	30µl

The solution was mixed well until Triton X was fully dissolved and then stored at -20°C.

BSA (1%)

BSA (20%)	500µl
1x PBS	9.5ml

Serum was stored at -20°C and defrosted before use.

0.1M Citric acid

Citric acid	19.2g
dH_2O	100ml

Diaminobenzidine (DAB) substrate

(DAB Immunopure Metal Enhanced Substrate Kit; Pierce, Rockford, Illinois, USA)

Diaminobenzidine	0.5ml
Buffer	4.5ml

Reagents were mixed immediately prior to use.

EDTA decalcifying solution (pH 6.95)

Tris	12.11g
EDTA	100g
dH_2O	up to 1L

Solution was stirred until EDTA was fully dissolved, pH was adjusted to 6.95 using concentrated NaOH.

EDTA/PVP decalcifying solution (pH 6.95)

Tris	12.11g
EDTA	100g
PVP-40T	75g
H_2O	up to 1L

Tris was dissolved in water with stirring, EDTA was added and allowed to dissolve completely. pH was adjusted to 6.95 using concentrated NaOH. PVP-40T was added and solution was left stirring overnight (O/N). Solution was stored at 4°C.

0.1% Eosin

1% eosin in dH_2O	10ml
$CaCl_2$	2g
dH_2O	90ml

Ethanol 50%

Ethanol absolute (100%)	500ml
dH_2O	500ml

Ethanol 70%

Ethanol absolute (100%)	700ml
dH_2O	300ml

Ethanol 90%

Ethanol absolute (100%)	900ml
dH_2O	100ml

Gelatine 20%

Gelatine	40g
dH_2O	200ml

Gelatine was added to water and was heated, with stirring, until completely dissolved. Gelatine solution was stored at 4°C and warmed up to 37 °C before use.

Harris' haematoxylin

Haematoxylin	3g
Absolute alcohol	30ml
dH_2O	600ml
Aluminium ammonium sulphate	60g
Mercuric oxide	1.5g
Glacial acid	24ml

Haematoxylin was dissolved in absolute alcohol (solution A). Aluminium ammonium sulphate was dissolved in hot distilled water (solution B). Solution A was added to Solution B, boiled and then mercuric oxide was added. The solution was cooled rapidly under running tap water (solution C). Before use solution C was filtered through Whatman filter paper and glacial acetic acid was added.

3% Hydrogen peroxide (H_2O_2)/methanol block

Methanol	400ml
H_2O_2 30%	40ml

1% milk powder in PBS with 0.05% Tween 20 (MP/PBS/Twn), pH 7.4

Milk powder	10g
PBSx10	100ml
Tween 20	0.5ml
dH_2O	1L

Solution was prepared fresh. Milk powder and Tween 20 were dissolved with stirring, pH was adjusted to 7.4.

Normal goat serum (NGS) (5%)

NGS	500µl
PBS	9.5ml
Sodium azide (10%)	10µl

Serum was stored at 4 °C.

NGS (20%)/Triton X (0.3%)

NGS	2ml
Triton X-200	30µl
PBS	8ml
Sodium azide (10%)	10µl

Solution was mixed with stirring until Triton X was fully dissolved and stored at 4°C

Normal rabbit serum (NBS) (1%)

NBS	100µl
1x PBS	9.9ml
Sodium azide (10%)	10µl

Serum was stored at 4 °C.

NBS (20%)

NBS	2ml
1x PBS	8ml
Sodium azide (10%)	10µl

Serum was stored at 4°C.

Normal rat serum/ BSA (NRS/BSA) (5%/1%)

NRS	500µl
BSA	100µl
1x PBS	9.6ml

Solution was prepared fresh, as it did not contain sodium azide.

Normal rat serum/ NBS (NRS/NBS) (5%/1%)

NRS	500µl
NBS	100µl
1x PBS	9.6ml
Sodium azide (10%)	10µl

Serum was stored at 4°C.

OCT/sucrose

OCT embedding medium	25ml
Sucrose 20%	25ml

Solution was mixed by rotating overnight at room temperature and stored at 4°C.

OCT/DMSO/sucrose

OCT	25ml
Sucrose 20%	24.5ml
DMSO	625µl

Solution was mixed by rotating overnight at room temperature and stored at 4°C.

Paraformaldehyde fixative (4%)

$Na_2H_2PO_4xH_2O$	41.5ml
NaOH	8.5ml
Paraformaldehyde	4g
dH_2O	10ml
Glucose	0.54g

Paraformaldehyde and distilled water were heated to 65°C in a fume cupboard, a few drops of 2M NaOH were added until the solution cleared and then glucose was added. $Na_2H_2PO_4xH_2O$ and NaOH were mixed and 45ml of this solution was added to 5ml paraformaldehyde mixture. Fixative was stored in a light-proof container at 4°C.

10 X PBS

NaCl	200g
N_2HPO_4	28.75g
KCl	5g
KH_2PO_4	5g
dH_2O	up to 2.5L

Solution was mixed with stirring and stored at room temperature.

PBS/gelatin (washing fluid)

20% gelatin	40ml
10x PBS	200ml
dH_2O	1760ml

Solution was stored at 4°C.

PBS/sucrose

Sucrose	7g
PBSx10	100ml
dH_2O	900ml

Solution was stored at 4°C.

PBS with 0.05% Tween 20 (PBS/Twn)

PBSx10	100ml
Tween 20	0.5ml
dH_2O	900ml

0.2M di-Sodium hydrogen orthophosphate

Na_2HPO_4	28.4g
dH_2O	100ml

Sucrose 20%

Sucrose	20g
dH_2O	1L

Solution was stored at 4°C.

Sodium bicarbonate 2%

Sodium bicarbonate	10g
dH_2O	500ml

Sodium azide 10%

Sodium azide	10g
dH_2O	100ml

Toluidine Blue solution 0.5%

Toluidine Blue O	0.25g
Acetic acid	1.5ml
dH_2O	48.5ml

Acetic acid was added to the water and mixed, then Toluidine Blue was added and the solution was mixed by stirring. The solution was stored at 4°C.

Tris buffer 0.1M, pH 6.95

Tris base	12.11g
dH_2O	988ml

pH was adjusted to 6.95 with concentrated HCl.

TritonX 0.4%

Triton X 200	200µl
PBS	50 ml

Solution was mixed by stirring until Triton X was completely dissolved

Vectabond™ reagent solution

Vectabond™ reagent	7ml
Acetone	350ml

Solution was mixed well by stirring. It was prepared fresh just prior to use.

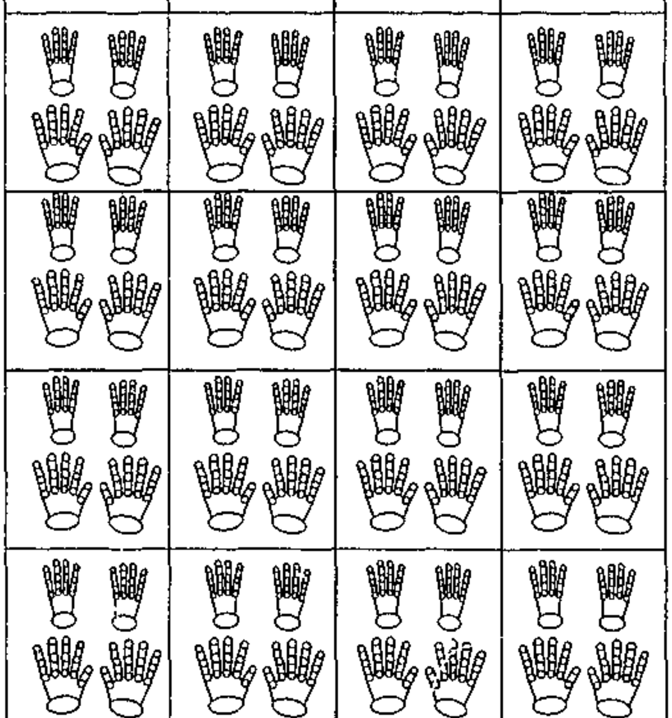
2.2 ANIMALS

Female 8-week-old Dark Agouti (DA) and 10-week-old Lewis rats were obtained from Monash Animal Services. The animals were housed 6 in each cage. Rats were kept in climate controlled environment with 12 h light/dark cycles, and fed standard rodent chow supplemented with sunflower seeds (approximately 5g per animal per day) and water *ad libitum*. Rats were given 2 weeks for acclimatisation prior to injections. During these two weeks rats were handled daily, so they became accustomed to handling.

During the course of the experiment rats were weighed and monitored daily for signs of arthritis. A special observation sheet was designed where all the data was recorded (Figure 2.1). All experimental procedures conformed to the NH&MRC code of practice and were approved by the Alfred Hospital Animal Ethics Committee.

Front side of the observation sheet **Back side of the observation sheet**

Box #			
Rat number			
Injected with			
CII/FIA	CII/FIA	CII/FIA	CII/FIA
Date of injection			



Day killed			
Comments			

Weight and behaviour

Date	Rat number			

Figure 2.1. An example of the observation sheet used for the monitoring of the rats developing arthritis.

2.3 METHODS

2.3.1 Preparation of collagen emulsion in incomplete Freund's adjuvant (CII/FIA)

The emulsion was prepared fresh prior to injections and was used within 4 hours. Lyophilised native bovine collagen type II (Sigma, Sydney, Australia) was dissolved at a concentration of 2mg/ml in 0.01M acetic acid at 4°C by overnight rotation. Solubilised collagen was emulsified 1:1 in Freund's incomplete adjuvant (FIA) (Sigma, Sydney, Australia) on ice as following: collagen solution was added slowly drop by drop into a tube containing cold (4°C) FIA while mixing on a Vortex Mixer (Ratek Instruments, Boronia, VIC, Australia). The emulsion was then placed on ice and mixed further using a 20-ml glass syringe with a 19G needle. The mixing continued until the emulsion became quite viscous and hydrophobic as determined by placing a drop of it onto water. The emulsion was then kept on ice.

2.3.2 Preparation of FIA emulsion

FIA emulsion was prepared using 0.01M acetic acid (without collagen) and FIA in a 1:1 proportion, following the protocol described above.

2.3.3 Injections

Each rat was anaesthetised by placing in a sealed perspex box which had a separate compartment containing cotton wool soaked with ether. Once asleep, the

rat was taken out and the adequate depth of anaesthesia was confirmed by the absence of corneal reflex and unresponsiveness to toe and tail pinch. During the injections anaesthesia was maintained by placing a nose cone with ether-soaked cotton wool over the rat's nose.

2.3.3.1 Intradermal (id) injections

Each animal received a total of 300µl of emulsion distributed between 5-6 sites at the base of the tail. The typical "orange skin" appearance of the injection site, along with the localisation of the bleb in the skin (which moved together with the skin), constituted the criteria for defining a successful intradermal injection. Injections were performed using a 2-ml glass syringe with a 21G needle.

2.3.3.2 Subcutaneous (sc) injections

Subcutaneous injections were performed at the base of the tail with 300µl of emulsion being distributed over 5-6 sites. The emulsion was injected into the space under the skin. No bleb formation was observed, and the skin remained intact.

2.3.4 Observation of the animals throughout the experiment

Rats were monitored daily. General behaviour, weight and appearance of the paw joints were checked and recorded (Figure 2.1). Arthritic scores (see below) were noted. Paw thickness was measured over the metatarsophalangeal and metacarpophalangeal areas using a dial gauge calliper (Mitutoyo, Japan) at the time of injection and at the time of killing.

2.3.5 Evaluation of arthritis

Arthritis was assessed using a scale (arthritic score) from 0 to 16, with a maximum of 4 for each paw (Figure 2.2). 0 = no arthritis; 1 = swelling and/or redness of one-two interphalangeal joints; 2 = involvement of three-four interphalangeal joints or one larger joint; 3 = more than four joints red/swollen; 4 = severe arthritis of the entire paw.

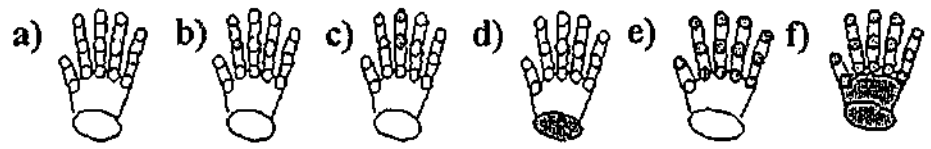


Figure 2.2. Examples of arthritic scores (as recorded in the observation sheet): **a** = 0; **b** = 1; **c,d** = 2; **e** = 3; **f** = 4.

2.3.6 Collection and processing of the joint specimens

Animals were sedated by CO_2 inhalation and injected intraperitoneally with Nembutal® (Boehringer Ingelheim, Artarmon, NSW, Australia) at 0.1ml per 100g rat's weight to achieve deep anaesthesia. Eye blink and toe pinch reflexes were checked to ensure adequate depth of anaesthesia. After exsanguination by cardiac puncture which led to animal death (later checked by opening the chest cavity), hindpaw interphalangeal joints, metatarsophalangeal joints and skin-free ankles were removed and placed in either formalin or 4% paraformaldehyde fixative.

2.3.6.1 Processing the joints for paraffin embedding

Joints were fixed in 10% neutral buffered formalin for 4-6 hours at 4°C. The specimens were decalcified in EDTA decalcifying solution for 3-5 weeks at 4°C (completion of decalcification was confirmed by X-ray). Joints were then placed in 50% ethanol for 1 hour or until further processed. The specimens were then treated in an automated tissue processor Citadel 1000 (Shandon Inc., Pittsburg, USA) as shown in Table 2.1, and embedded in paraffin. Paraffin blocks were stored at room temperature.

Table 2.1. Program used for automated tissue processing

Step	Time
Ethanol 70%	4 hours
Ethanol 90%	2 hours
Ethanol 100%	2 hours 40 minutes
Ethanol 100%	2 hours 40 minutes
Ethanol 100%	4 hours
Xylene	30 minutes
Xylene	30 minutes
Xylene	30 minutes
Paraffin wax	2 hours 10 minutes
Paraffin wax	4 hours
Total	**23 hours**

2.3.6.2 Processing of the joints for freezing in Tissue-Tek O.C.T.®:

Joints were fixed in 4% paraformaldehyde for 4-6 hours at 4°C, washed in PBS/sucrose for 2 days and in 0.1M Tris buffer for 1 day at 4°C. The specimens were decalcified in EDTA/PVP decalcifying solution for 4-6 weeks at 4°C, changing solution twice a week (completion of decalcification was confirmed by X-ray). When fully decalcified, the joints were washed in 0.1M Tris buffer for 30 minutes and placed in 20% sucrose for 24 hours at 4°C. The joints were infiltrated with O.C.T./sucrose for 24 hours and O.C.T./DMSO/sucrose for 24 hours at 4°C. Joints were then snap-frozen in Tissue-Tek O.C.T.® (Sakura Finetechnical, Tokyo, Japan) in liquid nitrogen and stored at - 80°C.

2.3.7 Collection of sera

Rats were bled by cardiac puncture under Nembutal® anaesthesia at the time of killing. Blood was collected using a 5ml syringe with a 21G needle. Blood was transferred into a tube, allowed to clot and then was spun down at 2,600 rpm for 15 minutes. Serum was collected and stored at -20°C before testing for antibodies.

2.4 STAINING TECHNIQUES

2.4.1 Haematoxylin and eosin staining (H&E)

Paraffin blocks were cooled in the –20°C freezer for 45 minutes and 5 μm paraffin sections were cut using an AS500 semi-thin microtome (Anglia Scientific, Cambridge, England). Sections were floated on 20% ethanol and then, briefly,

onto a 56°C water bath to allow them to unfold and soften the wax. Subsequently they were placed on 'SuperFrost Plus' slides (Menzel-Glaser, Germany) and allowed to adhere and dry for a few hours. Slides were then placed in a 60°C oven for half an hour to melt the wax and increase adherence, dewaxed in Histolene (Fronine Pty. Ltd., Riverstone, NSW, Australia) (2 changes, 5 minutes each), rehydrated through graded ethanols (100%, 100%, 70%) and brought to water. Slides were stained with Harris' haematoxylin for 1-2 minutes, rinsed in tap water, dipped in 1% acid alcohol, rinsed with water again and placed in 2% sodium bicarbonate for 15 seconds. After washing with tap water slides were placed in 0.1% eosin stain for 3 minutes. This was followed by a rinse in water and dehydration through graded ethanols (70%, 90%, 100%, and 100%). Slides were then dipped in Histolene (Fronine Pty. Ltd., Riverstone, NSW, Australia) (2 changes) and coverslipped using Biomount-X (Biochroma, Huntingdale, VIC, Australia).

2.4.2 Toluidine Blue staining for mast cells

Paraffin sections were cut and dewaxed in histolene as described above. After washing in two changes of 100% ethanol to remove all histolene, slides were airdried and placed in Toluidine Blue solution for 1 minute. They were subsequently rinsed in tap water, quickly dried and coverslipped. Mast cells stained bright purple, while all nuclei in the section appeared light blue in colour.

2.4.3 Vectabond™ slide coating

Slides were placed in metal or glass racks, washed thoroughly in detergent, rinsed in water and immersed in acetone for 5 minutes. Slides were then placed in Vectabond (Vector Laboratories, CA, USA) reagent for 5 minutes. After removing slides from the Vectabond reagent and draining the excess, slides were washed by dipping for 30 seconds in MQ water (without creating bubbles). Water was changed after every 5 racks of slides. Water droplets were removed from the slides by gentle tapping and slides were left to dry at room temperature or at 37°C to facilitate the process. Slides were stored in boxes at room temperature.

2.4.4 4-layer immunohistochemistry for rat frozen tissue using monoclonal antibodies for cell surface markers

Four μm thick sections of Tissue-Tek O.C.T.® (Sakura Finetechnical, Tokyo, Japan) embedded frozen joints were cut on a Cryocut 1800 cryostat (Leica Instruments GmbH, Germany) at –26°C using a steel blade, and placed on Vectabond coated slides. Slides were left to dry overnight before use. Alternatively, after drying, slides were wrapped in foil and placed in the -80°C freezer for storage. Before use slides were allowed to thaw for approximately 30 minutes.

Sections were pre-incubated with 20% NBS for half an hour to block non-specific binding of the antibodies. Slides were then drained and appropriate primary antibodies were added to the sections. The sections were incubated with these

antibodies overnight at 4°C. After washing with PBS/gelatine (all washes were performed for 5 minutes with stirring), secondary goat anti-mouse IgG antibodies (Sigma-Aldrich, St. Louis, USA) diluted 1:200 in 5%NRS/1%NBS were applied for half an hour. Subsequently, slides were washed with PBS/gelatine, dehydrated through graded ethanols (70%, 90%, 100%, 100%) and placed in 3% H_2O_2/methanol block for 15 minutes to neutralise endogenous peroxidase. Later slides were rehydrated by reversing through graded ethanols (100%, 100%, 90%, and 70%) and washed with PBS/gelatine. Tertiary antibody, rabbit anti-goat immunoglobulin (Dako, Glostrup, Denmark), was applied diluted 1:50 in 5%NRS/1%NBS for 30 minutes followed by the PBS/gelatine wash. Sections were then incubated for half an hour with the fourth antibody layer, goat peroxidase anti-peroxidase (Dako, Glostrup, Denmark) diluted 1:60 in PBS/gelatine. After another wash in PBS/gelatine, a detection substrate, diaminobenzidine (DAB) was applied for 5-20 minutes (reaction was controlled microscopically). When the desired colour was achieved the reaction was terminated by washing in tap water for 10 minutes. Sections were then counterstained in Harris' haematoxylin, dehydrated (through graded ethanols), rinsed in histolene and coverslipped using Biomount-X (Biochroma, Huntingdale, VIC, Australia). Positive cells were stained dark brown, while all nuclei were stained light blue.

2.4.5 3-layer immunohistochemistry for rat frozen tissue using polyclonal antibodies to NGF and its receptors

Four μm thick sections of Tissue-Tek O.C.T.® embedded frozen joints were prepared as described above. Sections were incubated in 0.4% Triton X for 30 minutes to penetrate cell membranes (as intracellular molecules were targeted). Following a 5-minute wash in PBS (all washes were performed for 5 minutes with stirring), slides were pre-incubated with 20% NGS/0.3% Triton X for an hour to block non-specific binding of the antibodies. Slides were then drained, rinsed in PBS and appropriate primary antibodies (diluted in 5% NGS) were added to the sections. The sections were incubated with these antibodies for 2 hours at room temperature. After washing with PBS, slides were dehydrated through graded ethanols (70%, 90%, 100%, 100%) and placed in 3% H_2O_2/methanol block for 10 minutes to neutralize endogenous peroxidase. Later slides were rehydrated by reversing through graded ethanols (100%, 100%, 90%, and 70%) and washed with PBS. Secondary antibody, biotinylated goat anti-rabbit immunoglobulin (Dako, Glostrup, Denmark), was applied diluted 1:200 in PBS for 30 minutes followed by the PBS wash. Sections were then incubated for half an hour with ABC reagent (Dako, Glostrup, Denmark). After another wash in PBS, a detection substrate, DAB was applied as described above (Section 2.4.4)

2.4.6 Double immunofluorescence

Four μm thick sections of O.C.T. embedded frozen joints were prepared as described above. Sections were incubated in 0.4% Triton X for 30 minutes to

penetrate cell membranes. Following a 5-minute wash in PBS (all washes were performed for 5 minutes with stirring), slides were pre-incubated with 20% BSA/0.3% Triton X for an hour to block non-specific binding of the antibodies. Slides were then drained, rinsed in PBS and appropriate polyclonal antibodies (anti-NGF, trk A, or p75) (diluted in 1% BSA) were added to the sections. The sections were incubated with these antibodies for 2 hours at room temperature. After washing with PBS, secondary antibody, goat anti-rabbit immunoglobulin (Dako, Glostrup, Denmark), was applied diluted 1:200 in 5%NRS/1%BSA for 30 minutes. Following another PBS wash, rabbit anti-goat Cy3-conjugated antibodies (Sigma, St. Louis, Missouri, USA) were applied diluted 1:400 in 5%NRS/1%BSA for 30 minutes. Starting from this step, all washes were performed using washing dishes covered with foil to protect slides from light exposure. After a PBS wash, 20% BSA was again applied to the sections for 20 minutes, then shaken off the slides. Monoclonal antibodies against cell surface markers were applied for 2 hours at room temperature, followed by another wash. Biotinylated goat anti-mouse immunoglobulin antibody (Dako, Glostrup, Denmark) was applied diluted 1:50 in 5%NRS/1%BSA for 30 minutes. Following another PBS wash, streptavidin-FITC complex (Cedarlane, Hornby, Ontario, Canada) was applied diluted 1:100 in PBS for 30 minutes. Slides were washed in PBS and fluorescent DNA dye Bisbenzomide H33342 (Calbiochem®, La Jolla, CA, USA) was added for 5 minutes to stain cell nuclei. Those slides were then washed again and coverslipped using Glycergel mounting medium (Dako Corporation, Carpenteria, CA, USA).

2.5 ANALYSIS OF STAINING (CELL COUNTS)

2.5.1 Mast cell count

The area of synovium was measured using an Image Analysis System (ImagePro Software). The total number of mast cells in the synovium and the number of mast cells per unit area (mm^2) were assessed.

2.5.2 Assessment of infiltrating cell subpopulations

Positively labelled synovial cells in each section were counted using a 100-square graticule at a magnification x400. Each graticule corresponded to an area of $0.15mm^2$. A minimum of 25 graticules per joint was counted for evenly distributed cells and up to 100 graticules (usually the whole synovial area) were counted for unevenly dispersed cells. The number of cells per unit area (mm^2) was assessed.

2.5.3 Evaluation of NGF and trk A staining

The total synovial area and the area stained positively with each antibody (brown colour) were measured using an Image Analysis System (ImagePro Software). The percentage of the area which was stained positively was calculated.

2.6 ELISA FOR THE DETECTION OF ANTI-COLLAGEN ANTIBODIES

One mg of lyophilized native bovine type II collagen (CII) (Sigma, Sydney, Australia) was dissolved in 100µl of 0.5M acetic acid at 4°C overnight. Later 900µl of MQ water was added. This was kept as a stock (1mg/ml) CII solution at 4°C.

Stock CII solution was diluted in PBS to 10µg/ml and ELISA plates were coated with CII overnight at 4°C. The excess CII was flicked out thoroughly and ELISA plates were incubated with MP/PBS/Twn for 2h to block non-specific binding of the antibodies. The plates were then washed 6 times with PBS/Twn. Dilutions of rat sera were applied for 4 hours at room temperature. After washing 6 times with PBS/Twn and 6 times with RO water, ELISA plates were incubated with peroxidase-conjugated secondary antibody against the appropriate rat immunoglobulin subclass diluted in MP/PBS, for 4 hours at room temperature. Twelve washes with RO water were followed by incubation with ABTS substrate for 30 minutes. The plates were read on an ELISA microplate reader (Bio-Rad, model 3550) at 415 nm wavelength. The average "background" reading from the wells containing neither antigen nor antibodies was subtracted from the reading for each test well.

2.7 STATISTICS

The data were evaluated statistically using SPSS for Windows. Kruskal-Wallis 1-Way ANOVA by rank was performed. When significant difference was detected by ANOVA, pairs were analysed by Mann-Whitney U-test.

Chapter 3

ESTABLISHMENT OF THE COLLAGEN-INDUCED ARTHRITIS MODEL IN THE RAT

III. ESTABLISHMENT OF THE COLLAGEN-INDUCED ARTHRITIS MODEL IN THE RAT

3.1 INTRODUCTION

Collagen-induced arthritis (CIA) is driven by an autoimmune response to type II collagen (CII), which is a major component of the cartilage. Autoimmunity to collagen has been demonstrated in RA patients (Bartholomew, et al., 1991; Beard, et al., 1980; Jeng, et al., 1990). Therefore, collagen-induced arthritis (CIA) may represent one of the best models for rheumatoid arthritis (RA). A study of the synovial processes occurring in CIA as part of the autoimmune response to the cartilage antigen, may provide a clue to the sequence of events in the rheumatoid joints. Rats were chosen for this study because the collagen emulsion for CIA induction in rats can be prepared with FIA, which does not contain *Mycobacteria* antigens. In mice, complete Freund's adjuvant, containing *M. tuberculosis* is required. In this case it is possible that bacterial, rather then self-cartilage antigens, may drive the immune process.

A number of rat strains are susceptible to CIA, but they all differ in regard to the clinical course of arthritis (see Chapter 1). This project aimed to characterise histopathological changes in CIA joints, with early synovial events preceding the manifestation of arthritis being of particular interest. Therefore, a strain that had 100% incidence of arthritis, with low onset variability between the animals, and

predictable location of arthritis, had to be chosen. Two rat strains known to have a high incidence of arthritis, Dark Agouti (DA) and Lewis (Cremer, et al., 1995), were tested for the reproducibility of arthritis induction.

Collagen-induced arthritis in susceptible strains of rats develops after a single intradermal (id) injection of CII emulsified in incomplete Freund's adjuvant (CII/FIA) (Trentham, et al., 1977). However, some studies claim that subcutaneous (sc) vaccination is arthritogenic as well (Carlson, et al., 1985; Hom, et al., 1986; Kobayashi, et al., 1991; Sugita, et al., 1993). Technically subcutaneous injections are easier to perform and are less traumatic for the animals. On the other hand, it has been shown that the route of administration often regulates an immune response to an antigen, thus, the same antigen can induce or protect against disease. For example, intradermal immunisation of laboratory animals with the nicotinic acetylcholine receptor and myelin basic protein results in the development of experimental myasthenia gravis and autoimmune encephalomyelitis, respectively (Bernard, et al., 1975; Lennon, et al., 1975; Levine, et al., 1976). Administration of these antigens by nasal or oral route does not result in disease, and protects against it (Li, et al., 1998a; Li, et al., 1998b; Wang, et al., 1993). Similar observations were made in CIA: CII administered nasally, orally or intravenously is not arthritogenic. Furthermore, such administration protects animals against subsequently induced CIA (al-Sabbagh, et al., 1996; Cremer, et al., 1983; Khare, et al., 1995; Kresina, et al., 1985; Staines, et al., 1996; Thompson, et al., 1985). In so far as the cutis and subcutaneous layers are situated in close proximity to each other, one would expect that immune reactions induced by intradermal and subcutaneous

immunisations to be similar. Therefore, two routes of CII administration, intradermal (id) and subcutaneous (sc), were tested.

3.2 MATERIALS AND METHODS

3.2.1 Experimental groups

10-week old female DA rats and 11-week old Lewis rats were used in this experiment. Groups of rats were injected with either CII/FIA, FIA alone, or saline. Injections were performed either intradermally or subcutaneously (Table 3.1).

Table 3.1. Animal groups

Group of rats	Rat number	Injected with	Route of injection
DA control	6	saline	id
DA id	6	CII/FIA	id
DA sc	5	CII/FIA	sc
DA sc/id	5	CII/FIA twice	sc→id
DA FIA id	6	FIA	id
DA FIA sc	6	FIA	sc
Lewis control	5	saline	id
Lewis id	10	CII/FIA	id
Lewis sc	5	CII/FIA	sc
Lewis FIA id	5	FIA	id
Lewis FIA sc	5	FIA	sc

All injections were carried out as described in Chapter 2. Rats were routinely monitored for 45 days after injections. Rats which developed severe arthritis were humanely killed at that time. Serum specimens and joints were collected from each rat at the time of death.

To analyse the possible tolerogenic effect of sc injections, the DA sc group received a second inoculation of CII/FIA intradermally 45 days after the first injection (DA sc/id group).

3.2.2 ELISA for anti-collagen antibody subtypes

An ELISA assay for anti-collagen antibody detection was performed as described in Chapter 2. Rat sera was diluted in MP/PBS/Twn 1:50 for the IgG2a assay, 1:200 for the IgG and IgG2b assays, and 1:400 for total Ig assay. To determine the subclass of antibodies to CII present in each animal, the following secondary HRPO-conjugated reagents were used: anti-rat IgG, anti-rat IgG2a antibodies (Serotec, Kindlington, Oxford, UK) and anti-rat immunoglobulin antibodies (Silenus Laboratories, Hawthorn, VIC, Australia) applied at 1:1500 dilution, and anti-rat IgG2b antibodies (Serotec, Kindlington, Oxford, UK) used at 1:200 dilution. On each plate a row of wells coated with antigen, but without rat sera was run to ensure the lack of non-specific binding of the secondary, peroxidase-conjugated antibodies. Antibody levels were measured twice in two separate runs. The results shown represent the mean of the two experiments.

3.3. RESULTS

3.3.1 Clinical signs of arthritis

3.3.1.1 Rats injected with saline or FIA

Neither DA nor Lewis rats injected with saline or FIA by either route developed arthritis. Their behaviour was normal and they gained weight steadily (Figures 3.1 and 3.2).

3.3.1.2 Rats injected with CII/FIA intradermally

3.3.1.2.1 DA rats (DA id group)

The incidence of arthritis in DA id rats was 100%. Clinical onset of arthritis occurred between 16 and 24 days (Table 3.2).

Table 3.2. Arthritis in DA rats injected with CII/FIA intradermally.

Rat strain	Rat number	Arthritis onset (days after CII injection)	Arthritic score
DA	1*	18	7
	2*	21	14
	3*	16	13
	4	24	10
	5*	18	12
	6*	18	9

* Rats with particularly severe arthritis (had to be killed at day 4 after onset of arthritis)

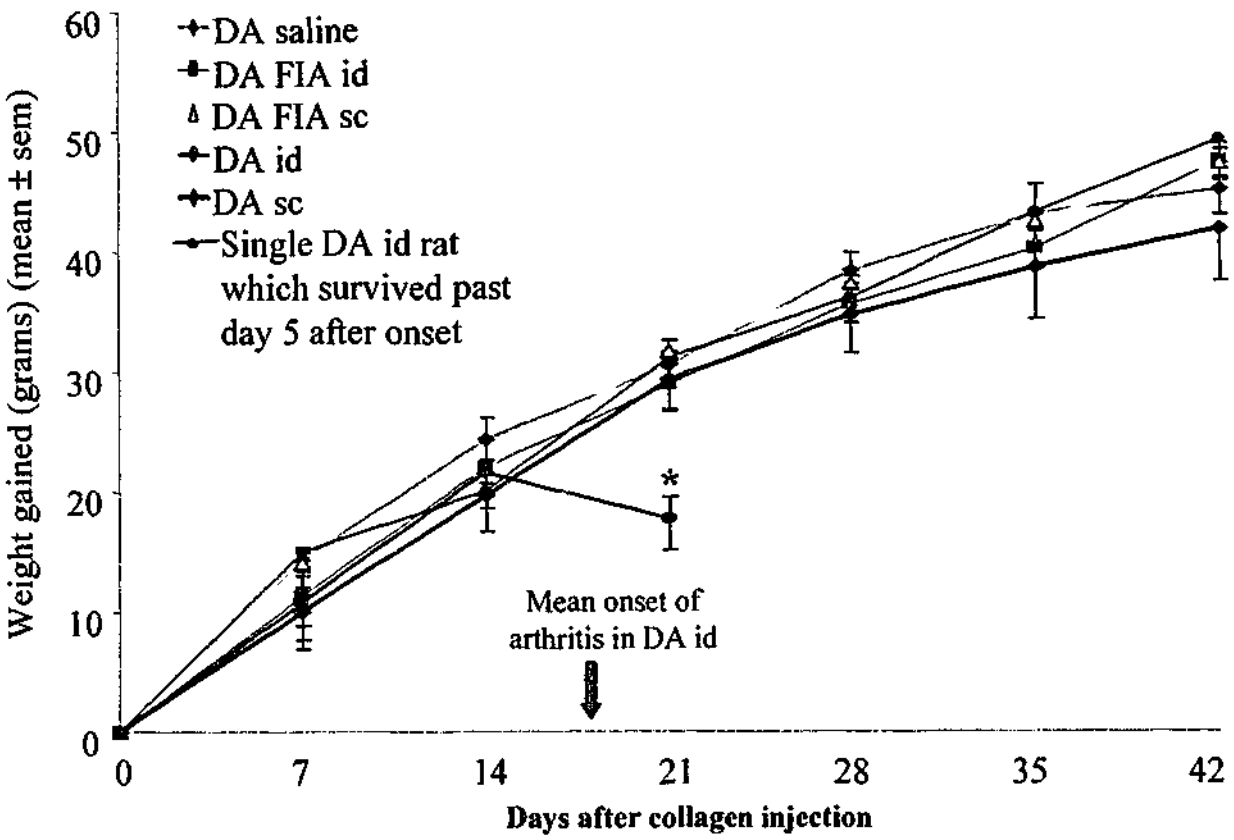


Figure 3.1 Weight changes in DA rat groups
(*$p < 0.01$ as compared to other groups at the same time-point)

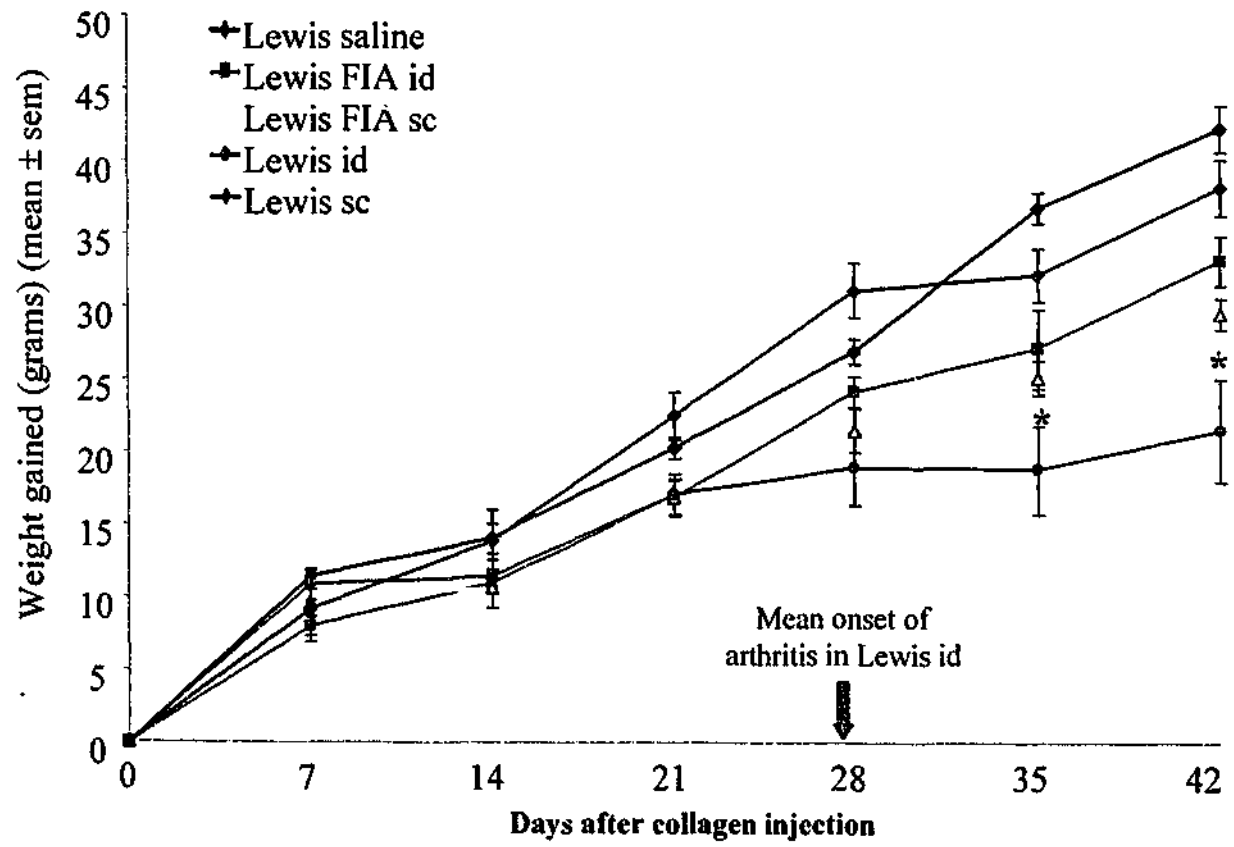


Figure 3.2 Weight changes in Lewis rat groups
(*$p < 0.01$ as compared to normal control and Lewis sc at the same time-point)

Inflammation was consistently observed in hindpaw interphalangeal (IP) joints, metatarsophalangeal joints and ankles. In a number of animals arthritis also affected wrists and frontpaw IP joints. Inflammation manifested as redness and swelling over the joints. The rats walked with a limp and had restricted mobility with the development of arthritis. In some animals extensive swelling caused ulceration of the skin over the affected joint. The arthritic score varied from 7 to 14 in these rats (Table 3.2).

In some rats the overall arthritic score was relatively low, but arthritis in one or two hindpaws was extremely severe. General symptoms such as substantial weight loss (more than 10% as compared to the time of the arthritis onset) (Figures 3.1), substantially restricted mobility (inability to use their hind paws) and vocalisation with handling were prominent in 5 out of 6 DA rats. Due to these symptoms these five rats had to be killed on day 4 after arthritis onset.

3.3.1.2.1 Lewis rats (Lewis id group)

Lewis rats injected with CII/FIA id had only an 80% incidence of arthritis. Onset of arthritis was generally at a later time than seen in DA id rats, between 19 and 44 days after CII injection (Table 3.3).

There was a different pattern of localization of arthritis as compared to DA id rats. Inflammation was seen only in ankles and tarso-metatarsal joints in Lewis rats. The arthritic score was generally low, varying from 2 to 4 (Table 3.3). There was a significant ($p < 0.01$) reduction in weight gain in this group of animals as compared to Lewis control and Lewis sc groups.

Table 3.3. Arthritis in Lewis rats injected with CII/FIA intradermally.

Rat strain	Rat number	Arthritis onset (days after CII injection)	Arthritic score
Lewis	1	-	0
	2	24	2
	3	44	4
	4	40	2
	5	25	4
	6	19	2
	7	38	4
	8	19	2
	9	-	0
	10	19	4

3.3.1.3 Rats injected with CII/FIA subcutaneously

Neither DA (DA sc) nor Lewis (Lewis sc) rats injected with the arthritogenic collagen preparation subcutaneously developed arthritis. Their behaviour remained normal and they gained weight steadily (Figures 3.1 and 3.2).

3.3.1.4 DA rats re-injected with CII/FIA intradermally after subcutaneous injection (DA sc/id group)

Subcutaneous injection of CII/FIA failed to induce arthritis. Rats were re-injected with CII/FIA 45 days later to test a possibility of the tolerogenic effect of sc inoculation.

Four out of 5 DA sc/id rats developed arthritis as compared to 5 out of 5 DA id rats. On average the onset of arthritis was delayed by up to 27 days in DA sc/id

rats as compared to rats inoculated id only (Tables 3.2 and 3.4). The arthritic scores in DA sc/id arthritic animals (5.5 ± 4.2) were significantly lower ($p<0.05$) than in DA id rats (10.8 ± 2.6) (Tables 3.2 and 3.4).

Table 3.4. Arthritis in DA rats injected with CII/FIA intradermally after subcutaneous inoculation (DA sc/id)

Rat group	Rat number	Arthritis onset (days after CII injection)	Arthritic score
DA sc/id	1	-	0
	2	30	4
	3	47	1
	4	27	6
	5	17	11

The general symptoms in DA sc/id rats were less severe. All five DA sc/id animals survived till day 45 after id CII/FIA injection and further without displaying signs of pain or distress, or significant weight loss. None of the DA sc/id rats developed skin ulceration over inflamed joints while 3 of the 5 DA id rats did. Rats were killed 70 days after the injection.

3.3.2 Histopathology

3.3.2.1 Rats injected with saline or FIA

Rat joints were studied 45 days after the injections were carried out. Hindpaw distal IP joints were studied in DA rats and ankles in Lewis rats as these particular joints were characteristically affected in the CIA model. The joints had normal

appearance with no infiltrate in the synovium or cartilage/bone damage present (Figures 3.3a-b and 3.4 a-b).

3.3.2.2 Rats injected with CII/FIA intradermally

Four of the 5 DA id rats were killed at day 4 after onset. Distal interphalangeal joints of these rats demonstrated histopathological changes characterised by synovial lining cell proliferation with extension of synovial cells over the cartilage, subchondral bone erosion and inflammatory cell infiltration of synovial sublining layer (Figure 3.3c). By day 21 after onset of arthritis, as seen in the single animal from DA id group that survived, extensive bone and cartilage destruction, fibrous ankylosis and new bone formation were observed (Figure 3.3d).

Ankles from Lewis rats were collected at day 45 after injections. Due to the variability in the time of onset, the duration of arthritis in different joints was different in different animals. Joints inflamed for 2-5 days displayed "active" inflammation, as synovium was infiltrated with polymorphonuclear and mononuclear cells (Figure 3.4c). In the ankles with duration of arthritis being 26 days, signs of fibrous ankylosis were evident (Figure 3.4d). Two of the rats that did not develop arthritis had no histopathological changes in their joints.

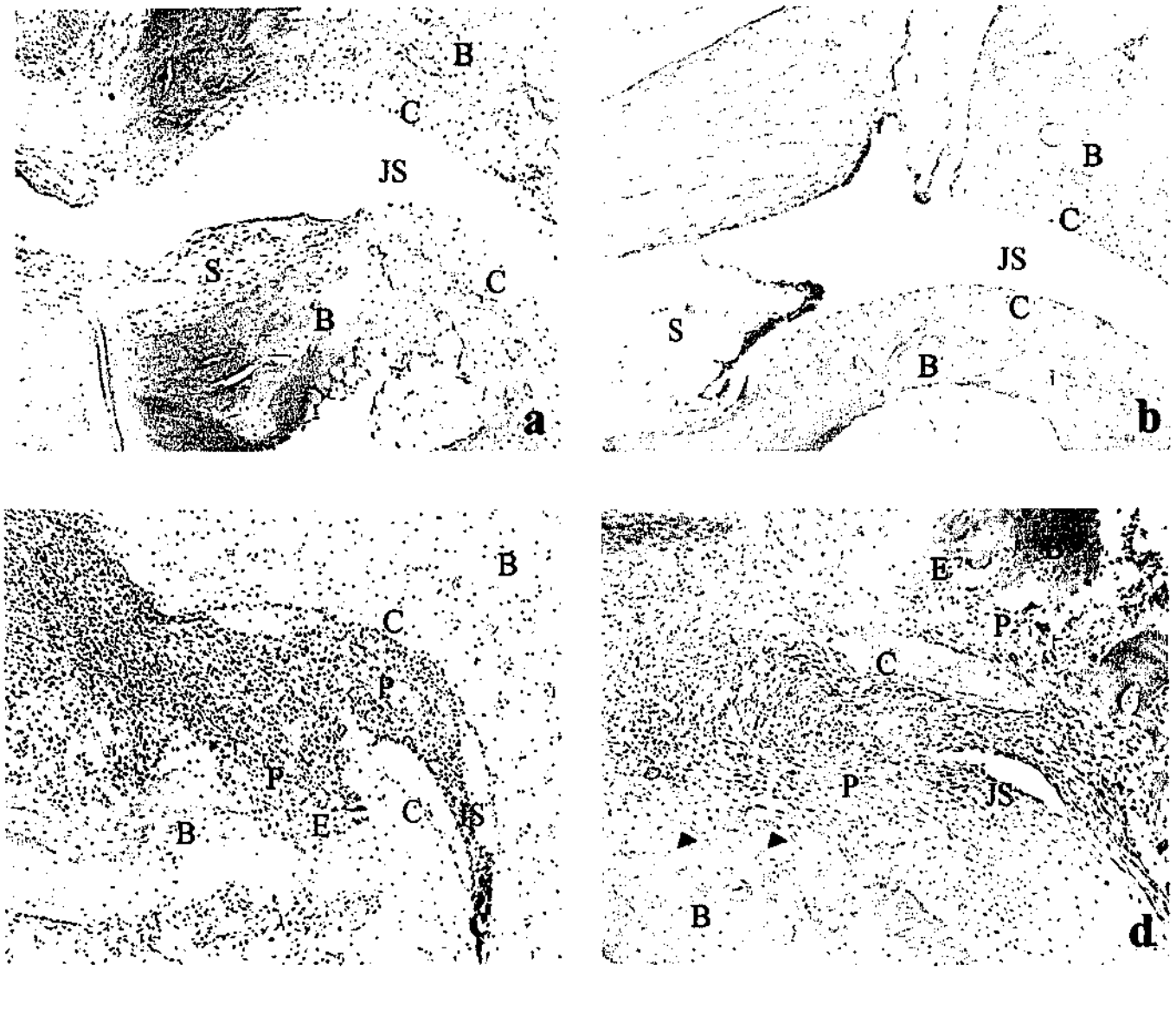


Figure 3.3. Haematoxylin and eosin staining of the distal interphalangeal (DIP) joints of DA rats: **a** normal joint; **b** FIA rat joint; **c** CII/FIA joint at day 4 after onset of arthritis; **d** CII/FIA joint at day 21 after onset of arthritis. (**B** - bone, **C** - cartilage, **S** - synovium, **P** - synovial pannus, **E** - erosion, **JS** - joint space, arrows - new bone formation)

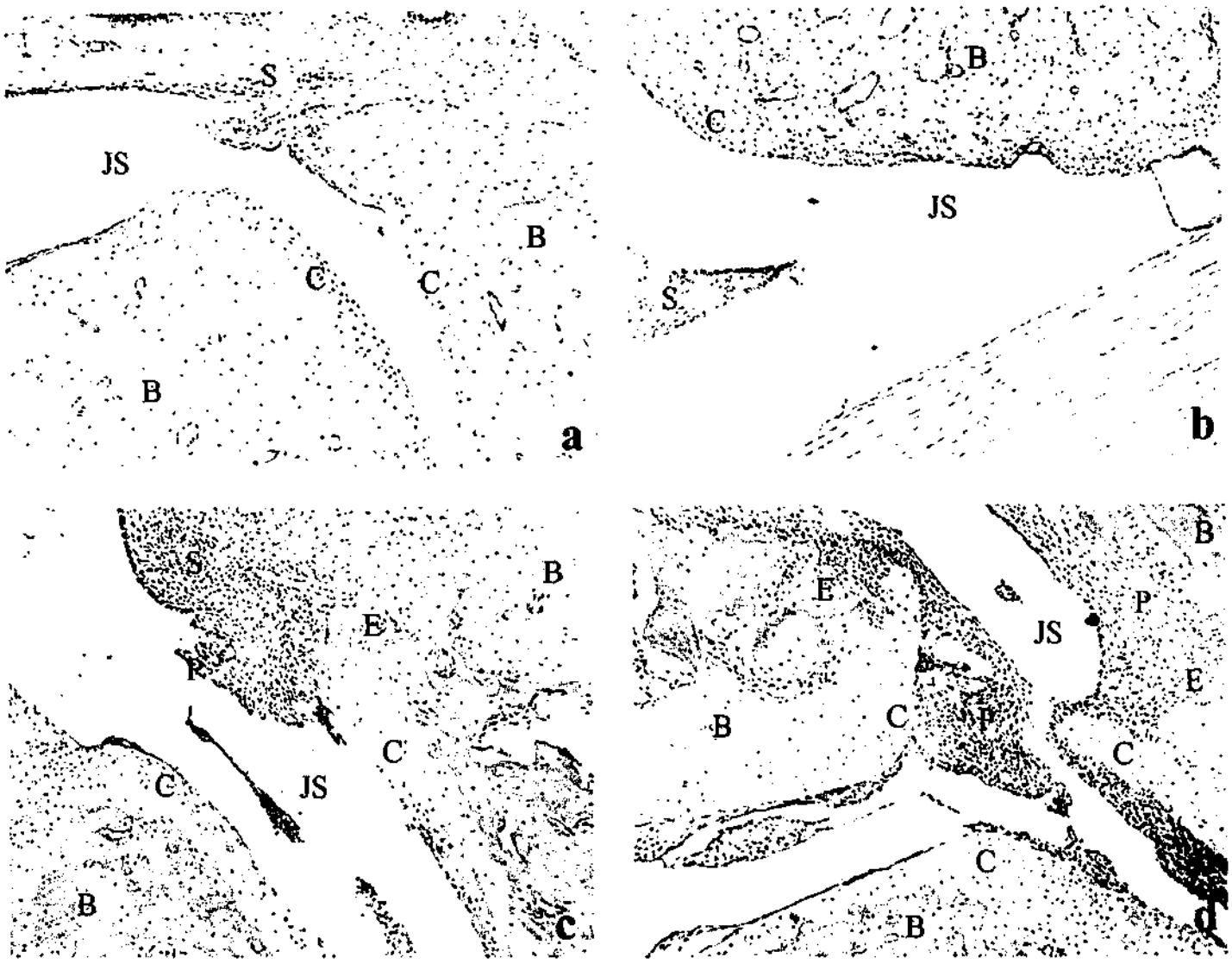


Figure 3.4. Haematoxylin and eosin staining of the ankle joints of Lewis rats: **a** normal joint; **b** FIA rat joint; **c** CII/FIA joint at day 5 after onset; **d** CII/FIA joint at day 26 after onset of arthritis. (**B** - bone, **C** - cartilage, **P** - synovial pannus, **E** - erosion, **JS** - joint space)

3.3.2.3 Rats injected with CII/FIA subcutaneously

Microscopically the appearance of the joints from Lewis rats that received CII/FIA sc was indistinguishable from normal controls (Figure 3.5).

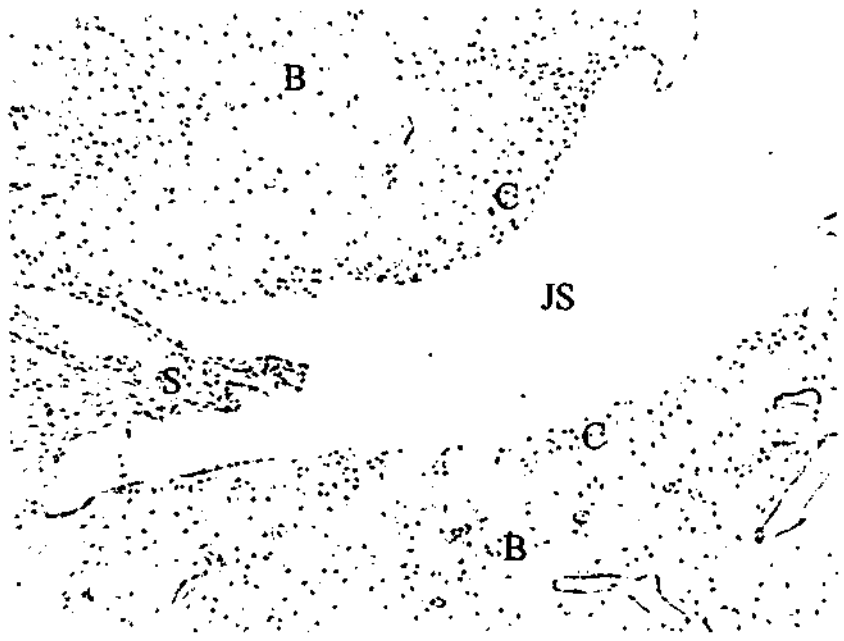
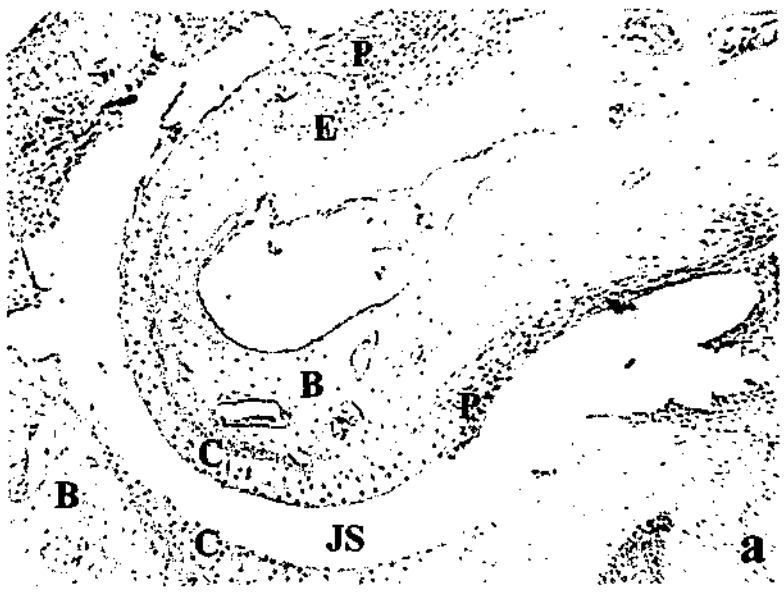


Figure 3.5. Haematoxylin and eosin staining of a section from the ankle of Lewis rat injected with CII/FIA subcutaneously (**B** - bone, **C** - cartilage, **S** - synovium, **JS** - joint space)

3.3.2.4 DA sc/id rats

Histomorphology of the affected distal IP (DIP) joints of sc/id rats at day 36 after onset of arthritis showed prominent fibrotic changes (Figure 3.6). The degree of damage varied between the DIP joints. In some joints only minor damage to bone and cartilage was present with the joint space remaining intact (Figure 3.6a). In the others, changes were similar to those found in the DA id rat that survived till day 21 after onset of arthritis (Figure 3.6b), with significant destruction of cartilage and bone epiphyses, and replacement by fibrous tissue.



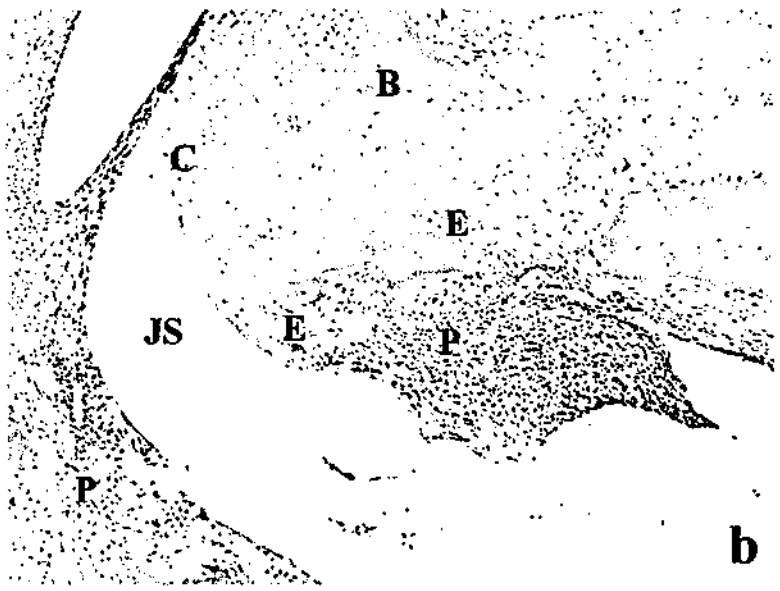


Figure 3.6. Haematoxylin and eosin staining of DIP joints of sc/id DA rats at day 36 after onset of arthritis. **a** mild changes; **b** moderate fibrous ankylosis with substantial bone erosion. (**B** - bone, **C** - cartilage, **P** - synovial pannus, **E** - erosion, **JS** - joint space)

3.3.4 Humoral immune response to CII in id, sc and sc/id rats

The levels of different subclasses of anti-collagen type II antibodies (total immunoglobulin (Ig), IgG, IgG1, IgG2a and IgG2b) in Lewis rats are shown in Figure 3.7, and in DA rats, in Figure 3.8. All groups that received CII/FIA had significantly elevated levels of anti-collagen antibodies compared to control groups ($p < 0.005$) (Figures 3.7 and 3.8).

Antibody levels obtained after intradermal and subcutaneous inoculations were compared in Lewis rats, as the DA sc group was re-injected with CII/FIA intradermally. Lewis rats injected with CII/FIA intradermally produced significantly higher levels of total Ig, IgG, IgG2a and IgG2b anti-collagen antibodies than subcutaneously inoculated animals. The levels of IgG1 were similar in both groups (Figure 3.7).

The comparison of antibody concentrations in DA sc/id rats versus DA id rats, demonstrated similar levels of all antibody subtypes studied, except for the IgG2b, isotype which was significantly lower in DA sc/id animals (Figure 3.8).

No correlation was found between the presence or absence of arthritis and the levels of any of the antibody subclasses examined.

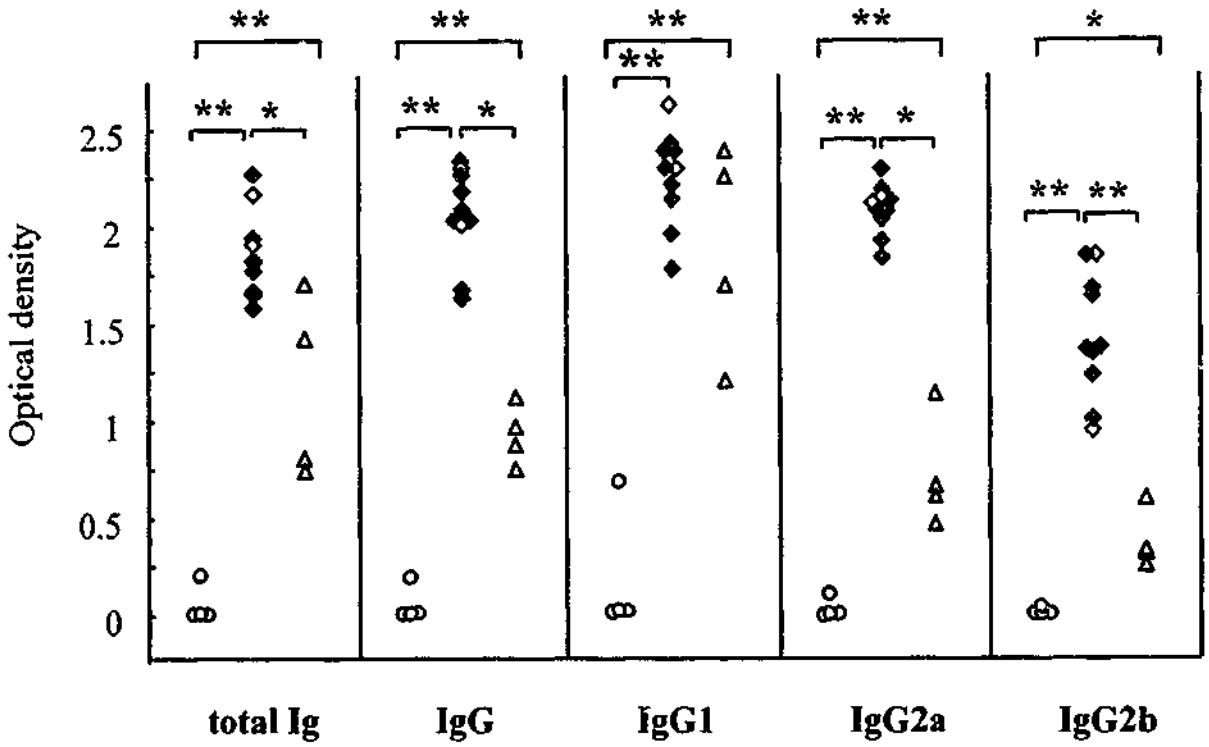


Figure 3.7 Production of different anti-type II collagen antibody subtypes in groups of Lewis rats. O - Lewis control; ◆ - Lewis i.d.; Δ - Lewis s.c. (clear symbols represent non-arthritic animals, shaded symbols represent arthritic animals).

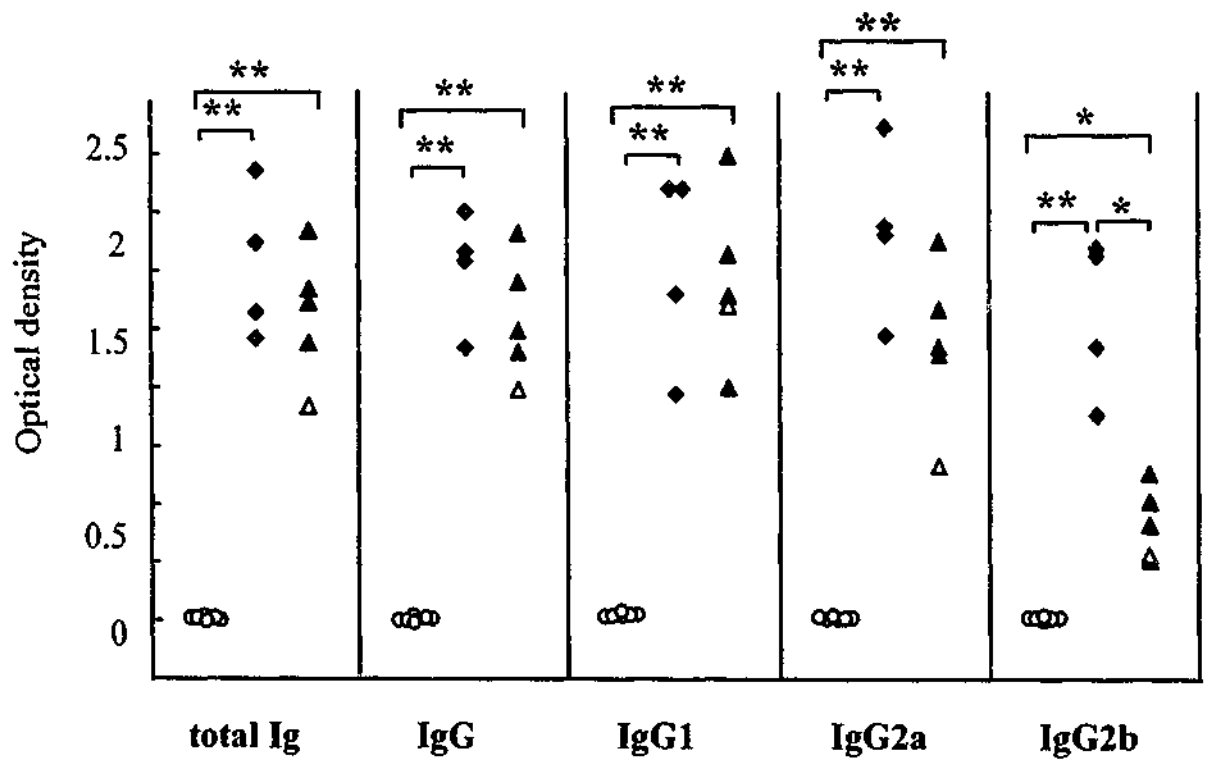


Figure 3.8 Production of different anti-type II collagen antibody subtypes in groups of DA rats. O - DA control; ◆ - DA i.d.; ▲ - DA s.c./i.d. (clear symbols represent non-arthritic animals, shaded symbols represent arthritic animals).

3.4 DISCUSSION

Collagen-induced arthritis was successfully established in both DA and Lewis rat strains. We found that DA rats were suitable as a CIA model for the purposes of this study as they had 100% incidence of arthritis with obligatory involvement of all hindpaw distal IP joints and a predictable time of onset. In contrast, Lewis rats presented with asymmetrical arthritis in their ankles and with highly variable times of onset. This made them unsuitable for studies of pre-clinical events, as the development of arthritis in any particular ankle would be impossible to predict. Our results have also demonstrated that despite the anatomical vicinity of the cutis and subcutaneous layers, the immunological responses elicited by intradermal and subcutaneous injections of CII/FIA preparation differ dramatically. While id injection of CII/FIA resulted in 80-100% incidence of arthritis, none of the rats inoculated sc developed arthritis. Microscopic examination confirmed the absence of arthritis in sc injected animals, while in id inoculated rats, typical erosive arthritis was observed. As both strains failed to develop arthritis after sc injections of CII/FIA, it can be suggested that the difference in responses to intradermal and subcutaneous inoculations of the same antigen is not restricted to one particular rat strain, but may be a general phenomenon. This is further supported by observations in other experimental arthritis models, where sc vaccinations with otherwise arthritogenic substances do not result in arthritis (Glenn, et al., 1965; Stuart, et al., 1979).

The differences in immune responses is unlikely to be due to the longer persistence of antigen in the dermis, since Glenn and Gray (Glenn, et al., 1965)

have found that removal of the injection site a few hours after inoculation does not prevent arthritis development. We propose that the difference in immunological responses to CII injected id and sc may be due to the distinct cell populations in the skin and the subcutaneous layers. While the epidermis and dermis of the skin constitute a cell-rich cutaneous immune system (Figure 3.10), the subcutaneous layer, consisting of loose connective tissue and fat, plays a minor role in immune processes.

It is proposed that after the intradermal injection, there is a release of pre-made IL-1 from epidermal keratinocytes (Kupper, 1990; Williams, et al., 1996). This is followed by activation of a variety of residential skin cells and production of inflammatory cytokines (Williams, et al., 1996). Furthermore, CII introduced intradermally is presented to immunocompetent cells by Langerhans cells (LCs) and dermal dendritic cells (DDCs) which are known as the most potent antigen-presenting cells (APCs). Furthermore, any epidermal injury results in release of pre-made IL-1α and activation of epidermal and dermal residential cells (Kupper, 1990; Williams, et al., 1996). Activated epidermal keratinocytes and dermal fibroblasts produce a number of cytokines, chemokines and growth factors (Williams, et al., 1996). These cytokines and growth factors are capable of attracting immunocompetent cells to the site of collagen injection, as well as promoting the differentiation of LCs and DDCs (Heufler, et al., 1988; Kupper, 1990; Witmer-Pack, et al., 1987), and their migration to the draining lymph nodes (Cumberbatch, et al., 1995; de Kossodo, et al., 1995; Kimber, et al., 1992).

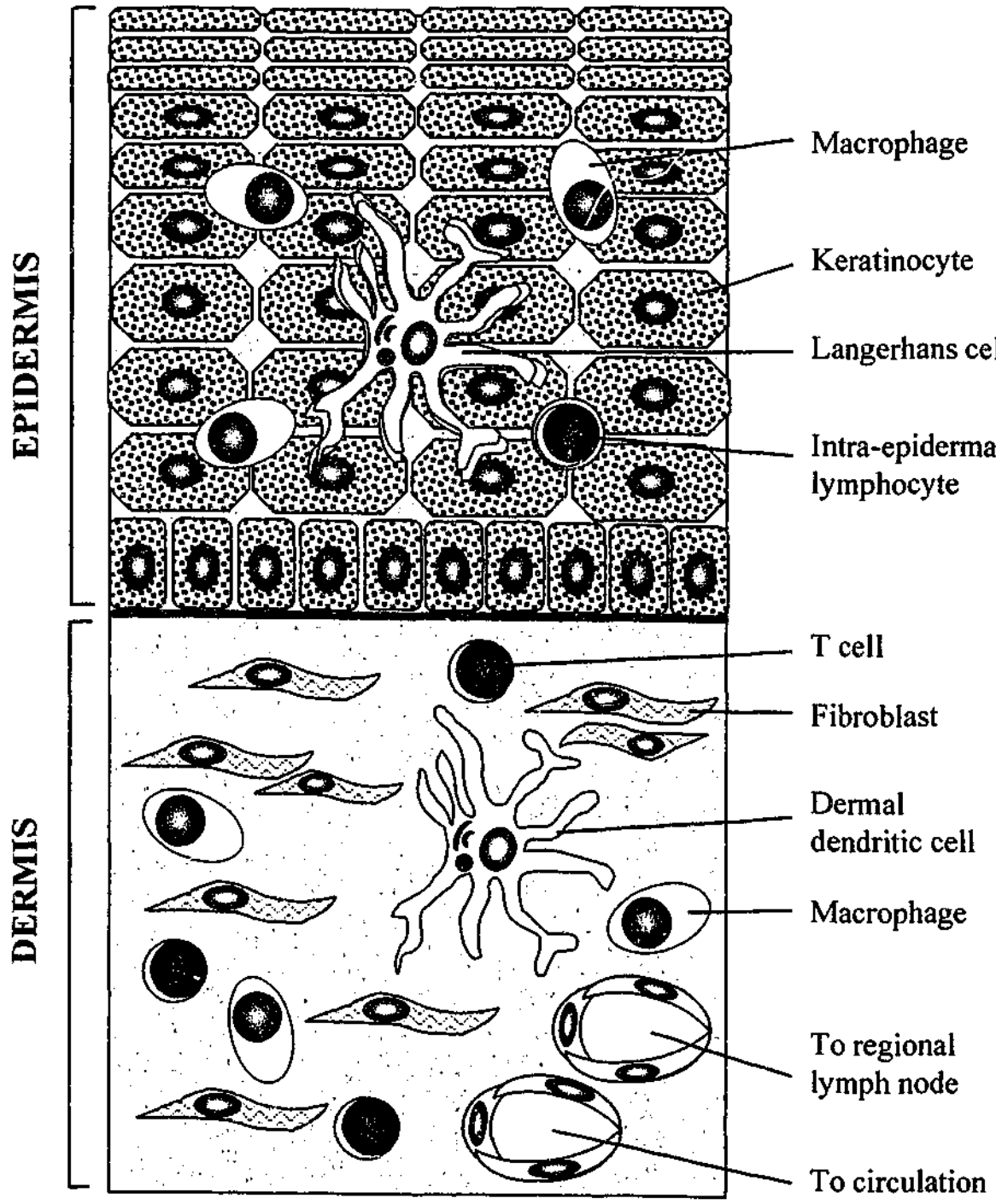


Figure 3.10. Cellular components of the cutaneous immune system.

Since only dendritic cells (DCs) but not macrophages are capable of activation of self-reactive T cell clones (Inaba, et al., 1984; Knight, et al., 1988; Knight, et al., 1983; Nussenzweig, et al., 1980; Vakkila, et al., 1990), presentation of CII by DCs may be required for the development of autoimmune arthritis. After autoreactive T cell clones are activated they can recognize collagen presented by macrophages and B cells and may promote anti-CII antibody production (Figure 3.11). Thus, the absence of arthritis in rats inoculated sc with CII may result from inefficient presentation of collagen by macrophages, which are the only APCs represented in the subcutaneous layer, and the lack of cytokine stimulation due to the absence of the keratinocytes in this layer. This may also explain the amelioration of subsequently induced CIA, as inefficient antigen presentation has been shown to tolerize specific T cells to the antigen (Jenkins, et al., 1987; Quill, et al., 1987; Sloan-Loncaster, et al., 1994; Williams, et al., 1990). The delayed onset and amelioration of CIA in sc/id injected rats may represent a partial reversal of the tolerance.

Paths of antigen presentation have also been found to regulate the production of antibody subtypes. It has been shown that DCs induce IgG2a and IgG1 responses, while macrophages stimulate IgG1 and IgE production (De Becker, et al., 1994; Sornasse, et al., 1992). Significant prevalence of IgG2a and IgG2b antibody subtypes in id rats compared to sc animals along with similar IgG1 levels, may illustrate presentation of CII in these groups by DCs and macrophages, respectively.

Skin injury
(collagen type II + adjuvant)

↓

Release of pre-made IL-1α from keratinocytes

↓

Activation of keratinocytes and fibroblasts

↓

Production of TNFα, IL-1, IL-6, IL-8, GM-CSF and M-CSF

↓

Activation of Langerhans cells and dermal dendritic cells

↓

Migration of activated dendritic cells to regional lymph nodes

↓

Presentation of type II collagen (CII) to T cells

↓

Activated CII-specific T cells stimulate production of anti-CII antibodies by B cells

Figure 3.11 Hypothetical mechanism of CIA initiation

In sc/id rats IgG2b levels were found to be significantly lower than in id rats. Suppressed IgG2b responses have been previously demonstrated by others in animals pretreated with an antigen intravenously, orally and nasally (Nagler-Anderson, et al., 1986; Thompson, et al., 1988; Wilbanks, et al., 1990). As intravenous, oral and nasal administration of collagen has been shown to suppress subsequent CIA (Cremer, et al., 1983; Nagler-Anderson, et al., 1986; Staines, et al., 1996; Thompson, et al., 1985), it is proposed that suppressed IgG2b production may be associated with the initial antigen presentation by a "tolerogenic" route.

In conclusion, the collagen-induced arthritis model has been established in rats. DA rats were found to be a highly susceptible strain with predictable location and time of onset. The histological features of arthritis resemble those seen in rheumatoid arthritis in humans and will be described in detail in Chapter 4.

This study has convincingly demonstrated that intradermal injection of collagen is pivotal for the induction of CIA, while subcutaneous inoculation is not arthritogenic. Our results allow us to hypothesize that presentation of CII after id injection is carried out by LCs and DDCs in the environment of diverse inflammatory cytokine stimulation produced by keratinocytes and dermal fibroblasts, resulting in autoimmune CIA.

The possibility of similar mechanisms being involved in initiation of RA and CIA can not be discarded. It has been recently proposed that repeated non-specific cytokine stimulation along with DCs differentiation and activation in synovium

eventually leads to RA development in genetically predisposed individuals (Thomas, et al., 1996). Further studies investigating the precise mechanisms of CIA development will thus provide potentially valuable data on the pathogenesis of autoimmune arthritides in humans as well as in animals.

Chapter 4

DEVELOPMENT OF THE COLLAGEN-INDUCED ARTHRITIS MODEL IN THE DA RAT

IV. DEVELOPMENT OF COLLAGEN-INDUCED ARTHRITIS IN THE DA RAT

4.1 INTRODUCTION

Despite its wide use over the last 23 years, the description of some of the aspects of the CIA model still remains obscure. There is incomplete and, at times, contradictory data on the sequence of synovial events in CIA, particularly on the composition of the inflammatory synovial infiltrate, which is one of the most important elements of joint inflammation. Some researchers have described a prevalence of mononuclear cells, often without further specification (Erlandsson, et al., 1994; Terato, et al., 1982; Trentham, et al., 1977), while others have reported polymorphonuclear cell dominance (Holmdahl, et al., 1988; Holmdahl, et al., 1991). These discrepancies can be based on a number of factors, including the use of different animal strains or species, as well as the different collagen type II (CII) source and preparation.

This Chapter describes in detail the development of joint injury in the CIA model using female DA rats, in which CIA was induced by a single intradermal injection of bovine CII emulsified in incomplete Freund's adjuvant. The progression of clinical symptoms of CIA and corresponding histopathological changes are described. The inflammatory cell populations infiltrating the joints during CIA are also analysed.

4.2. MATERIALS AND METHODS

4.2.1 Animals

Female 10-week old DA rats were used in this experiment.

4.2.2 Study Groups

4.2.2.1 CIA groups

Groups of 6 to 8 rats were injected intradermally with CII/FIA at the base of the tail (refer to Chapter 2). Rats were killed at different time-points (Table 4.1).

Table 4.1 Rat groups

Time-point	Number of CIA animals	Number of control animals
Day 2	8	4
Day 5	8	4
Day 8	8	-
Day 10	7	4
Onset (day 13)	6	4
Day 1 after onset	6	-
Day 3 after onset	6	-
Day 5 after onset	6	4
Day 10 after onset	6	4
Day 20 after onset	6	4

4.2.2.2 Control groups

Control groups, each consisting of 4 rats, were injected with FIA emulsion intradermally at the base of the tail (refer to Chapter 2). Each control group corresponded to a time-point of CIA (Table 4.1).

One group of 6 normal, injection-free animals constituted a "baseline" control.

4.2.3 Histopathology

Sections were assessed in a "blind" way to ensure objectivity. The following changes were measured and graded from 0 to 4:

Infiltrate: absent = 0, mild =1, moderate = 2, moderate/severe = 3, severe = 4.

Pannus: absent = 0, occupying less than 25% of the joint space = 1, occupying 25-50% of the joint space = 2, occupying 50-75% of joint space = 3, occupying over 75% of the joint space = 4.

Cartilage loss: none = 0, superficial loss = 1, loss of under 1/3 of the cartilage = 2, loss of 1/3 - 2/3 of the cartilage = 3, the loss of 2/3 of the cartilage and over = 4.

Bone loss: none = 0, superficial lacunae associated with newly formed osteoclasts = 1, loss of under 1/3 of the epiphysial bone = 2, loss of 1/3 - 2/3 of the epiphysial bone = 3, the loss of 2/3 of the epiphysial bone and over = 4.

The score for each group was determined as a mean of the scores for all animals in the group.

4.2.4 Immunohistochemistry

4.2.4.1 Monoclonal antibodies

Mouse monoclonal antibodies (mAbs) against different leucocyte surface antigens were used in this study (Table 4.2).

Table 4.2 Specificity of monoclonal antibodies

mAbs	Specificity	Source
ED-1	Rat monocytes and most macrophages	Cell line - a kind gift from Dr. C. Dijkstra
ED-2	Rat residential tissue macrophages	Cell line - a kind gift from Dr. C. Dijkstra
OX-6	Rat MHC class II antigen – expressed on monocytes/ macrophages, dendritic cells, B cells and activated T cells	Cell line - a kind gift from Prof. A. Williams
MCA453	Rat α/β T cell receptor	Serotec, U.K.
NDS61	Interleukin (IL)-2 receptor – expressed on activated T cells, B cells and NK cells	Serotec, U.K.
W3/25	Rat CD4 – expressed on "helper" T cells, activated monocytes/macrophages and dendritic cells	Cell line - a kind gift from Prof. A. Williams
OX-8	Rat CD8 – expressed on "cytotoxic/suppressor" T cells and on 10-80% of natural killer (NK) cells	Cell line - a kind gift from Prof. A. Williams
3.2.3	Rat NK cell	Cell line obtained from ECACC*
OX-33	Rat CD45 – expressed on B cells	Cell line obtained from ECACC*

* European Centre for Animal and Cell Culture

4.3 RESULTS

4.3.1 Clinical characteristics

4.3.1.1 Normal control and FIA control rats

Normal rats and rats from all FIA control groups had no signs of arthritis, joint pain or discomfort. Their general behaviour remained normal.

4.3.1.2 CIA rats

Rats injected with CII/FIA did not display any signs of arthritis until its onset, which in this cohort of rats occurred between day 12 and 14 after CII/FIA injection. Arthritis was first detected in the small interphalangeal joints of hind paws, always affecting both paws. In 30% of rats one or two ankles became involved. Arthritic scores at onset were already between 4 and 8 (Figure 4.1). Signs of arthritis included swelling and redness over the joints. Rats developed a limp. At this stage the animals still maintained their ability to move around freely.

Over the following three days significantly more joints became involved ($p = 0.001$) as compared to the time of onset (Figure 4.1), often including all hind paw joints and ankles. One ankle was involved in 23% of the animals, while both ankles were inflamed in 72%. In 70% of the animals front paw joints (interphalangeal, metacarpophalangeal, or the whole front paw including wrist) became involved. Oedema over the affected joints increased dramatically ($p<0.005$) as compared to the onset (Figure 4.2). In 29 out of 56 rats swelling was

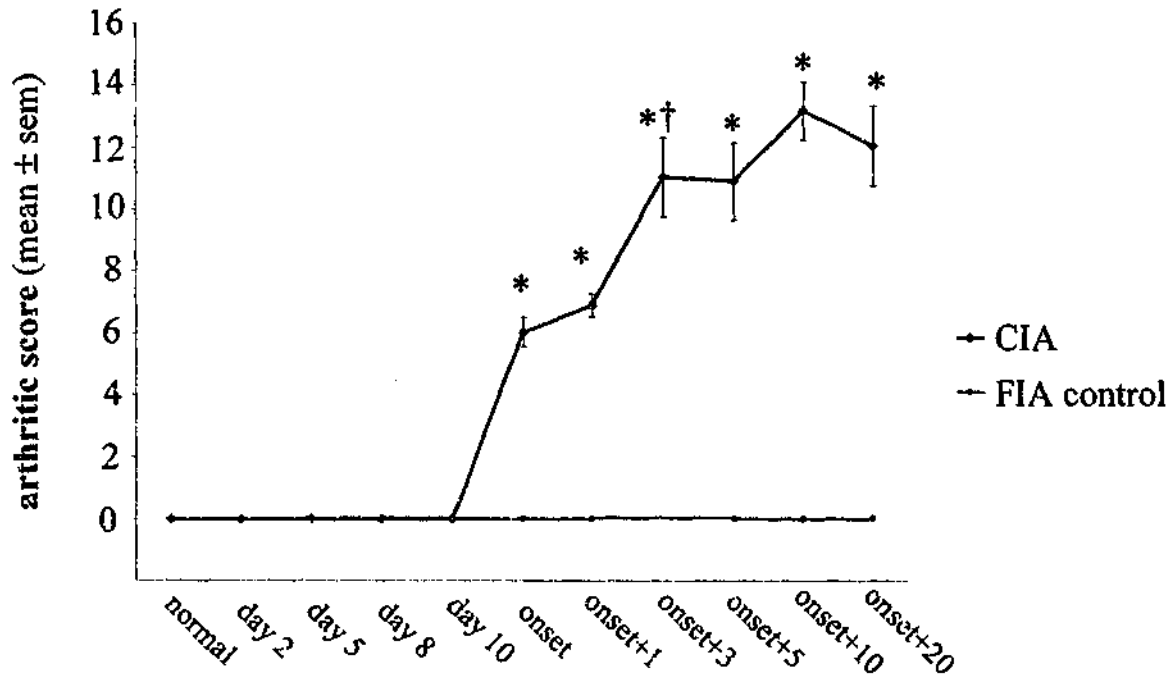


Figure 4.1. Arthritic scores in CIA and FIA control rats
* $p = 0.001$ as compared to normal or FIA control rats
† $p = 0.001$ as compared to onset

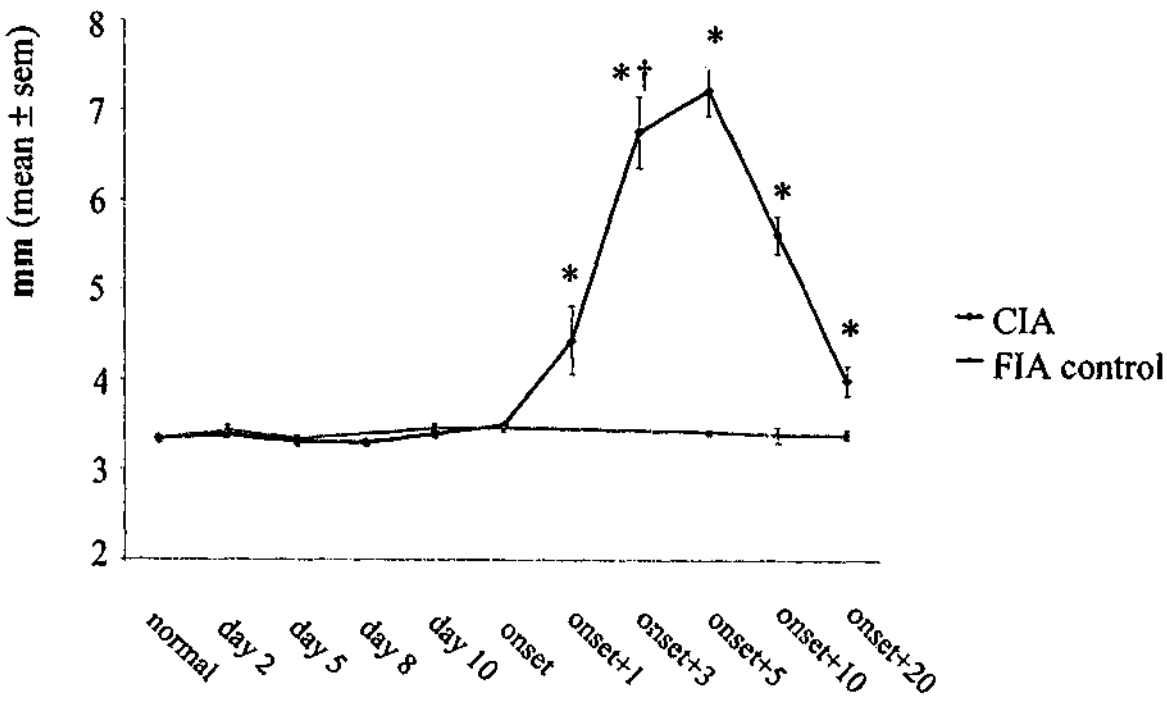


Figure 4.2. Hindpaw thickness in CIA and FIA control rats
* $p \leq 0.02$ as compared to normal or FIA control rats
† $p < 0.005$ as compared to onset

so severe that erosions appeared on the overstretched skin. At this stage of the disease rats had general signs of illness and their mobility was greatly restricted. Some of the animals displayed signs of distress, such as dramatic weight loss (over 15%), rough coat, eye discharge and vocalisation on handling. These rats were killed.

Starting from day 6-7 after the onset, arthritis began to subside and the rats' general behaviour slowly reversed to normal. No more joints become involved (Figure 4.1) and significant ($p=0.001$) reduction in joint swelling was observed at day 10 after onset (Figure 4.2).

By day 20 after the onset of arthritis, surviving rats fully regained their mobility and their behaviour appeared normal. Redness over the joints disappeared. Paw thickness, although significantly reduced as compared to day 10 after the onset ($p = 0.001$), still remained higher than in normal animals ($p = 0.02$) (Figure 4.2). This was probably due to post-inflammatory changes in the soft tissue, rather than acute inflammation. Signs of total or partial ankylosis were observed in interphalangeal joints and in ankles, respectively.

4.3.2 Histopathology

4.3.2.1 Normal joints

Normal joint structure was observed in normal DA rats (Figure 4.3a). Joint space was intact; synovial lining consisted of a discontinuous layer of cells varying in thickness from 0 to 3 cells with the sublining layer consisting of loose connective

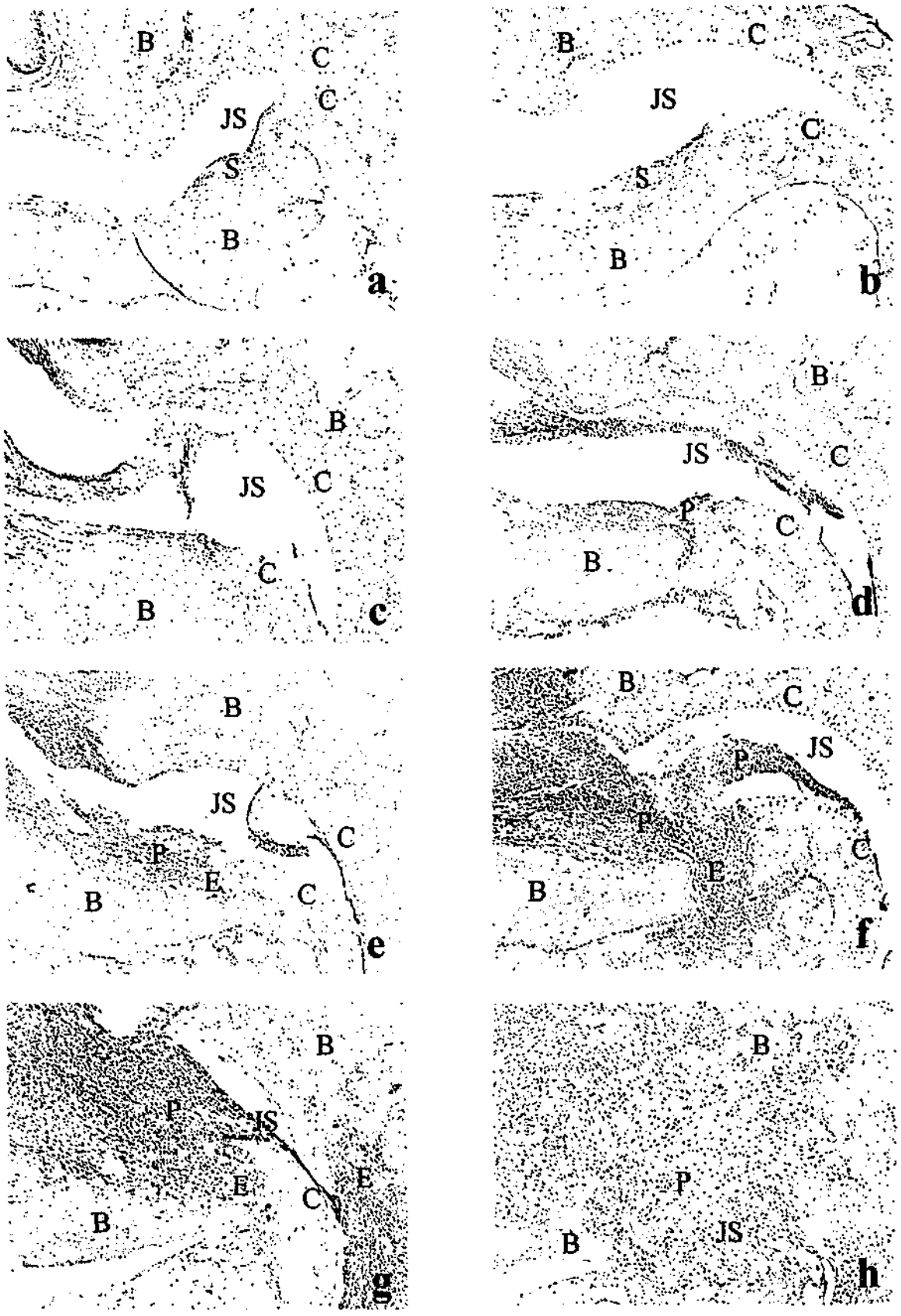


Figure 4.3. Haematoxylin and eosin staining of the DIP joints of DA rats: **a** normal joint; **b** CIA joint before onset; **c** CIA joint at onset; **d** CIA joint at day 1 after onset; **e** CIA joint at day 3 after onset; **f** CIA joint at day 5 after onset; **g** CIA joint at day 10 after onset; **h** CIA joint at day 20 after onset; **i** FIA control corresponding to CIA onset.

(**B** - bone, **C** - cartilage, **S**- synovium, **P** - synovial pannus, **E** - erosion, **JS** - joint space)

tissue and the absence of infiltrate. Bone and cartilage were undamaged (Table 4.3).

4.3.2.2 CIA model

Normal joint architecture was observed in CIA rats before the onset of arthritis at all time-points examined (Figure 4.3b). The first histological changes in the joints appeared at the time of the onset of arthritis and were characterised by the influx of inflammatory cells into the synovium and the joint space, mainly neutrophils along with some mononuclear cells (Figure 4.3c). Some neutrophils were seen, adhered to the cartilage surface. At day 1 after the onset, the infiltrate expanded and synovial lining hypertrophy and hyperplasia were noted (Figure 4.3d). More neutrophils were found on the cartilage surface. Formation of the invasive pannus overgrowing the cartilage and invading the bone was seen at day 3 after onset (Figure 4.3e). At this time-point initial stages of bone erosion and cartilage loss from the surface were noted. This was followed by further increase in the density of the inflammatory infiltrate, and further pannus expansion and intrusion into subchondral bone by day 5 after onset (Figure 4.3f). Progressive cartilage and bone loss and narrowing of the joint space were seen at day 10 after onset, while infiltration of synovium with inflammatory cells began to subside (Figure 4.3g). By day 20 after onset vast cartilage and epiphysial bone erosions were seen. A dramatic reduction in the number of inflammatory cells in the synovium was noted, the infiltrate being replaced by cells with fibroblast-like appearance. Typically, the joint space was completely lost and filled with fibrous tissue (Figure 4.3h). The synovial infiltrate, pannus, cartilage and bone loss were scored as described in Chapter 2. Results are shown in Table 4.3.

Table 4.3. Histopathological changes in CIA joints

Histological changes	normal	day 2	day 5	day 8	day 10	onset	onset+1	onset+3	onset+5	onset+10	onset+20
Infiltrate	0	0	0	0	0	1.58±0.33*	2.36±0.13*†	3.50±0.18*†	3.92±0.08*	3.07±0.30*†	1.33±0.40*†
Pannus	0	0	0	0	0	0.75±0.11*	1.21±0.10*†	1.75±0.17*†	1.75±0.17*	3.43±0.20*†	4*
Cartilage loss	0	0	0	0	0	0	0.64±0.10*	1.25±0.017*	1.50±0.18*	2.57±0.20*†	3.9±0.08*†
Bone loss	0	0	0	0	0	0	0.46±0.16*	1.17±0.11*†	1.50±0.18*	2.71±0.18*†	3.83±0.17*†
Fibrosis	0	0	0	0	0	0	0	0.75±0.25	0.33±0.21	2.29±0.29*†	3.67±0.21*†
Joint space loss	0	0	0	0	0	0.58±0.20	0.93±0.07*	1.33±0.21*	1.25±0.11*	2.86±0.26*†	3.92±0.08*†

All scores are shown as mean ± sem

* $p \leq 0.035$ as compared to normal or FIA controls

† $p \leq 0.035$ as compared to previous time-point

4.3.2.3 FIA controls

Joints of animals from FIA control groups demonstrated normal joint architecture at all time-points studied (Figure 4.3i)

4.3.3 Synovial cell populations

4.3.3.1 Normal joints

The main cell type found in normal joints was the $ED1^+/ED2^+$ residential tissue macrophage (Figures 4.4a, 4.5, 4.6a, 4.7). Approximately $^1/_3$ of them expressed MHC class II (Figures 4.15a and 4.16). Besides these, only cells with fibroblast-like morphology and some mast cells (MCs) (Figures 4.8, 4.9a, 4.10, 4.11a-14a 4.18a and 4.19) were seen.

4.3.3.2 CIA model

In the CIA model, synovial cell composition in the joints studied remained the same as in normal joints until the onset of arthritis (Figures 4.5, 4.7-8, 4.10, 4.16 and 4.19).

$ED1^+$ monocyte/macrophages increased three-fold at onset ($p=0.02$) (Figures 4.4b and 4.5). At this stage $ED1^+$ cells composed 68% of all infiltrating leucocytes (Figure 4.17). At day 5 after onset $ED1^+$ cells constituted 42.9% (Figure 4.17) as other cell populations expanded. $ED1^+$ cells were found at the bone-invading edge of the synovium (Figure 4.4c). These cells had multiple nuclei and appeared to be osteoclasts (Romas, et al., 2000). At day 20 after onset $ED1^+$ monocyte/

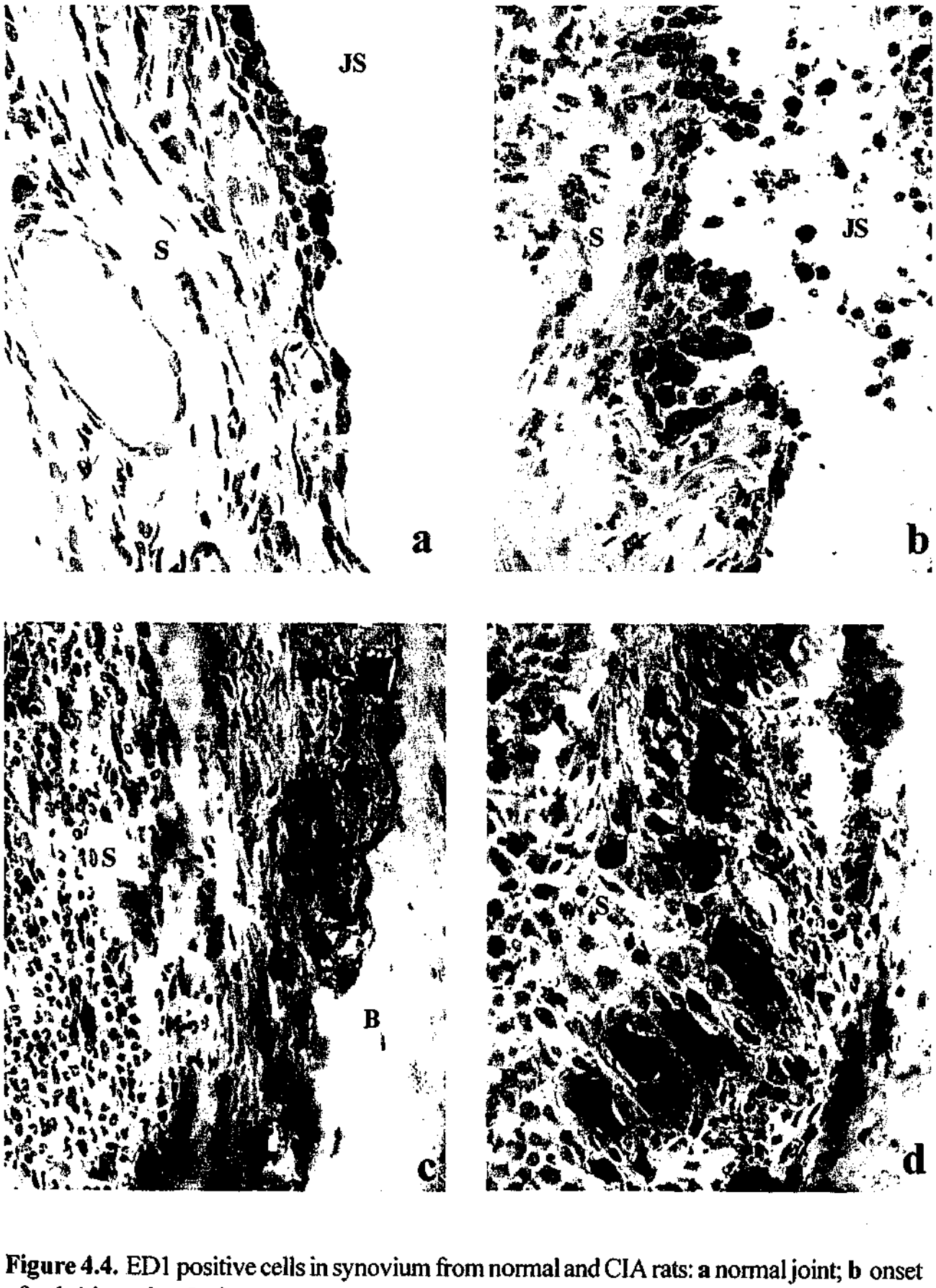


Figure 4.4. ED1 positive cells in synovium from normal and CIA rats: **a** normal joint; **b** onset of arthritis; **c** day 5 after onset; **d** day 20 after onset. (S - synovium, B - bone, JS - joint space, arrows - osteoclasts)

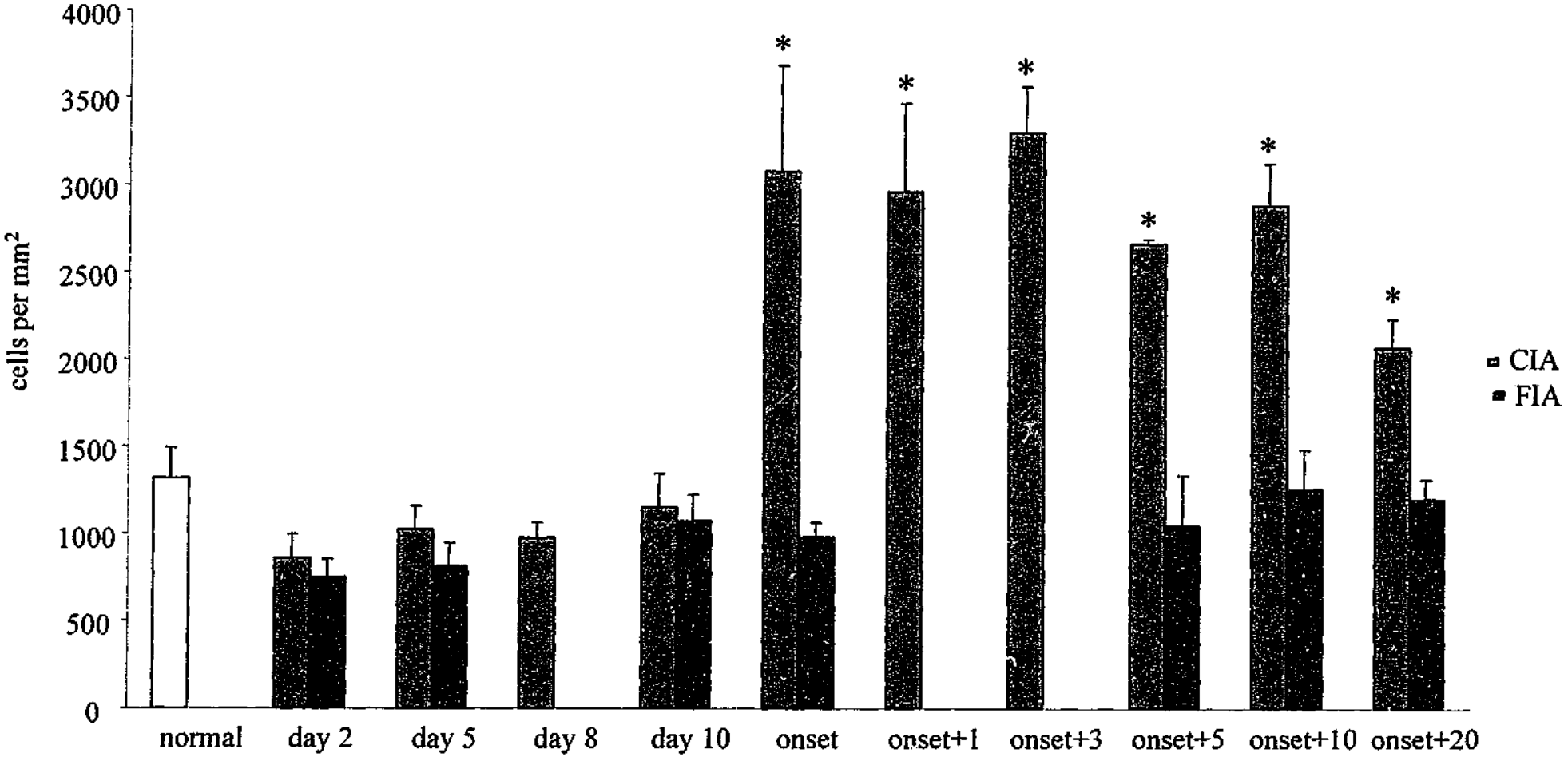


Figure 4.5 ED1$^+$ cell numbers in synovium in the course of CIA as compared to FIA controls

* $p \leq 0.05$ as compared to normal and corresponding FIA control

macrophages became the dominating leucocyte subset again, constituting 77.4% of total leukocyte population (Figures 4.4d and 4.17). Many multinucleated osteoclastic cells were also seen at this stage.

The number of $ED2^+$ cells remained constant at the time of arthritis onset (Figures 4.6b and 4.7). By day 5 after onset the number of $ED2^+$ cells per unit area dropped significantly ($p=0.02$) (Figures 4.6c and 4.7) and remained low until day 20 after onset (Figures 4.6d and 4.7).

Neutrophils (identified by their distinct nuclei) were the second largest population in the synovium at onset, constituting 20.1% of all leucocytes (Figure 4.17). Their numbers rose from 2 (in the normal joint) to 1000 per mm^2 ($p \leq 0.04$) (Figure 4.8). As arthritis progressed, reaching its peak at day 5 after onset, there was a further 2.5-fold increase ($p \leq 0.4$) in the number of neutrophils in the joint (Figure 4.8). The proportion of neutrophils rose up to 43.3% (Figure 4.17). By day 20 after onset there was a significant decrease in neutrophil numbers in synovium ($p=0.002$) (Figure 4.8). Their proportion in the total leucocyte mass dropped to 5.8% (Figure 4.17).

There was a dramatic 6-fold increase in T cells at the time of onset as compared to the pre-arthritic stage ($p=0.01$) (Figure 4.9b and 4.10) with about half of the cells being activated as they expressed IL-2 receptor (Figures 4.11b and 4.10). T cells constituted 5.4% of all leucocytes (Figure 4.17). Approximately one third of the lymphocytes were CD8 positive (Figures 4.12b and 4.10). Staining for CD4 antigen was hard to interpret as virtually all macrophages expressed the antigen.

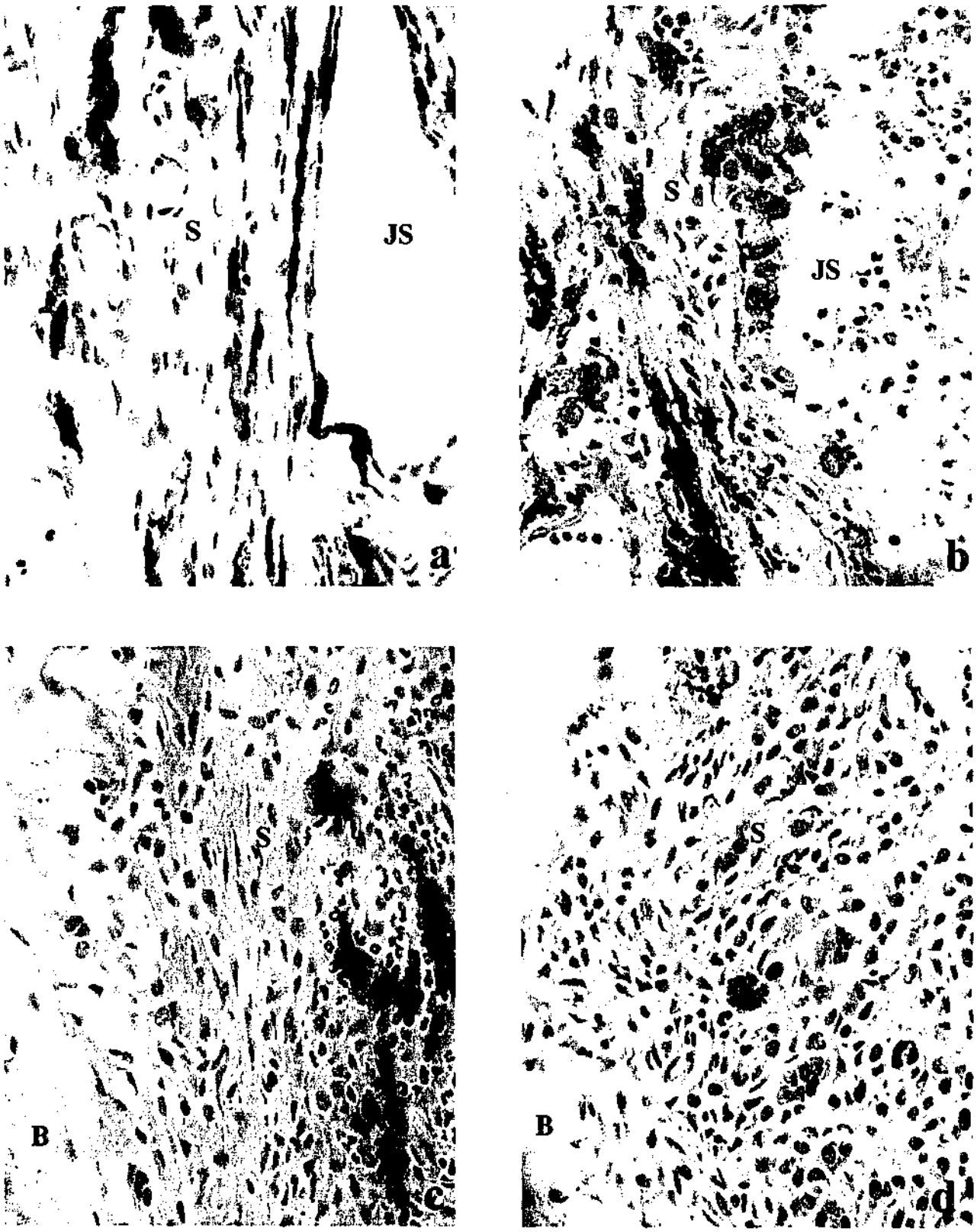


Figure 4.6. ED2 positive cells in synovium of normal and CIA rats: **a** normal joint; **b** onset of arthritis; **c** day 5 after onset; **d** day 20 after onset. (S - synovium, B - bone, JS - joint space)

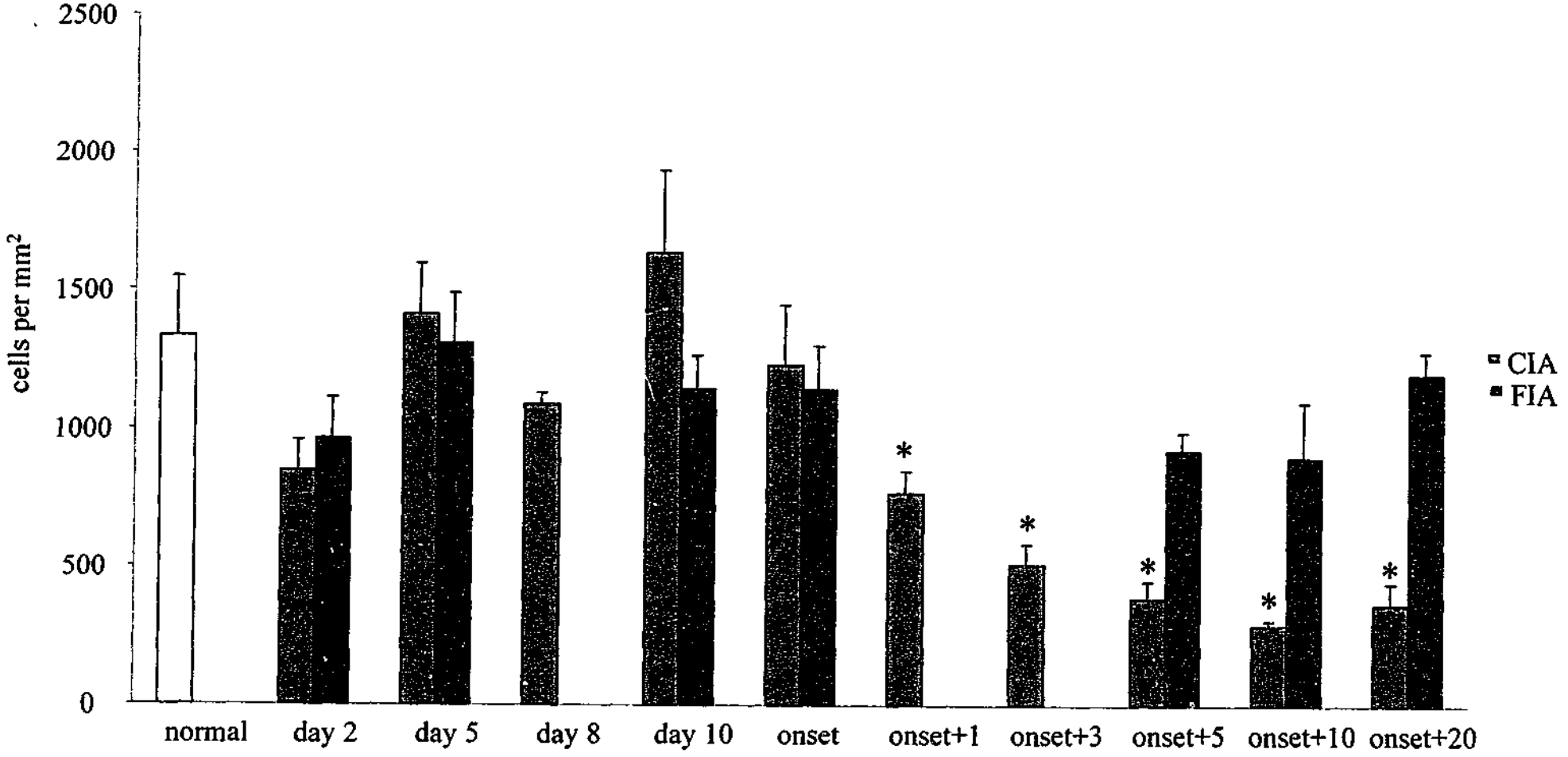


Figure 4.7 ED2$^+$ cell numbers in synovium in the course of CIA as compared to FIA controls

* $p \leq 0.05$ as compared to normal and corresponding FIA control

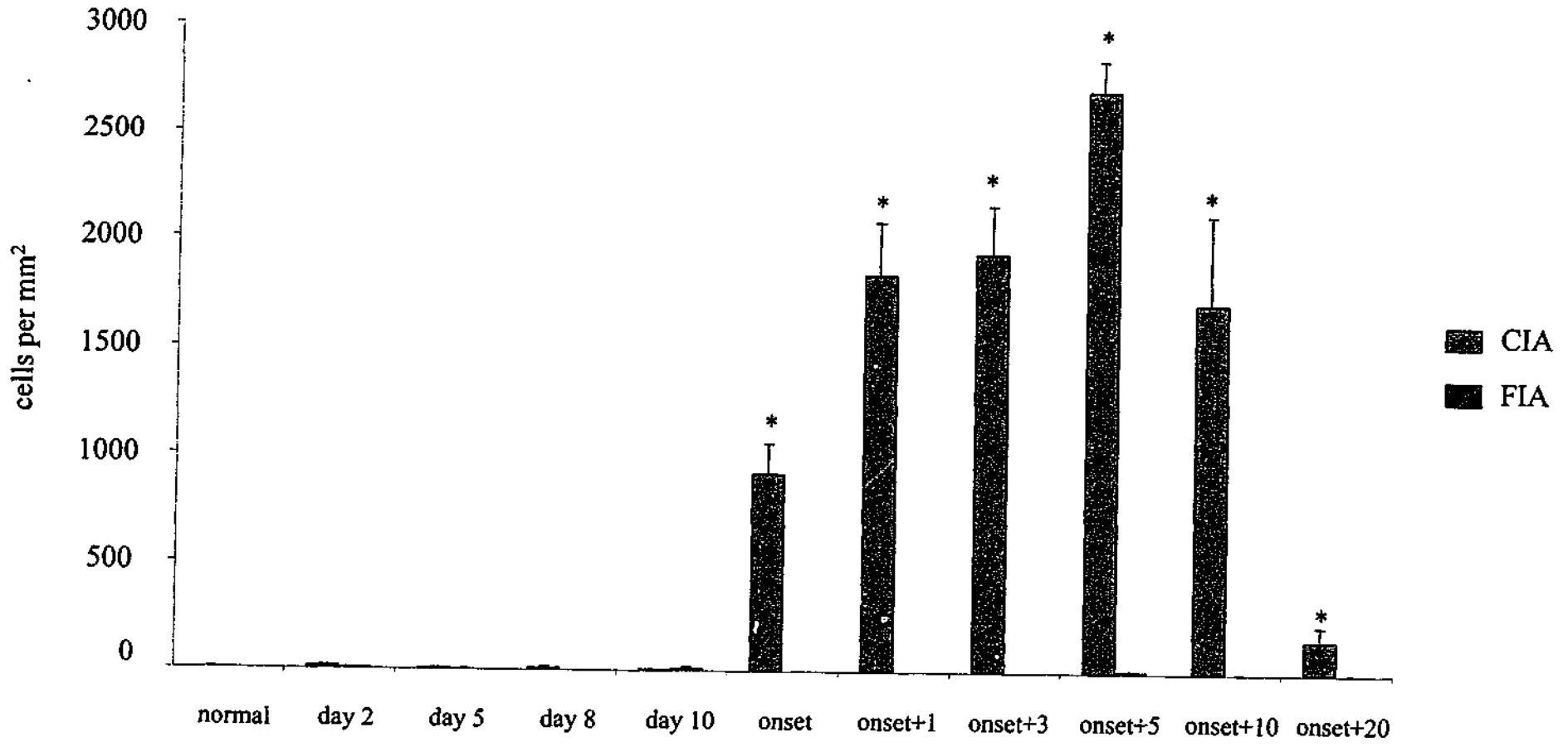


Figure 4.8. The number of neutrophils in the synovium of CIA and FIA control rats

* $p \leq 0.04$ as compared to normal or FIA control

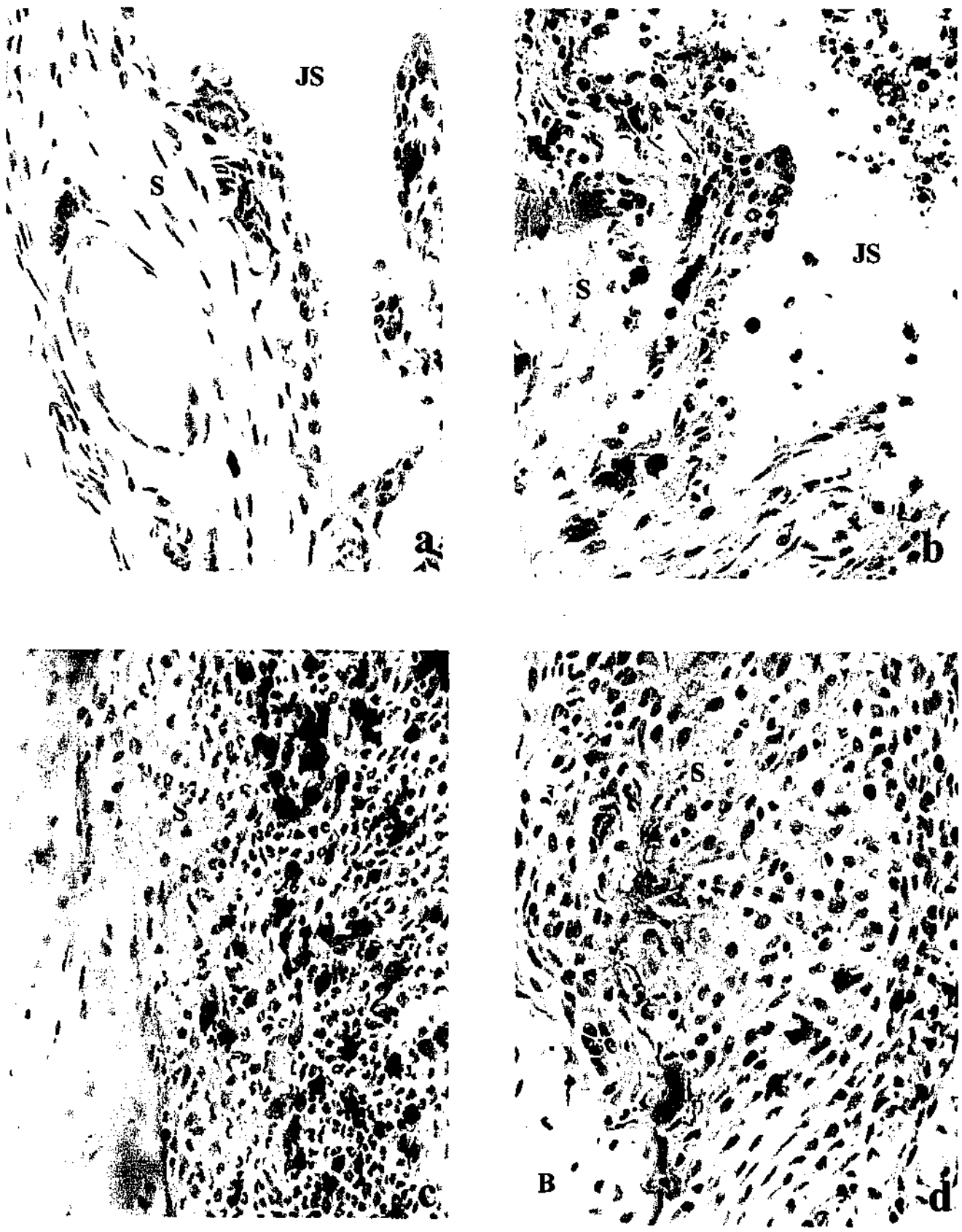


Figure 4.9. T cells in synovium from normal and CIA rats: **a** normal joint; **b** onset of arthritis; **c** day 5 after onset; **d** day 20 after onset (S - synovium, B - bone, JS - joint space)

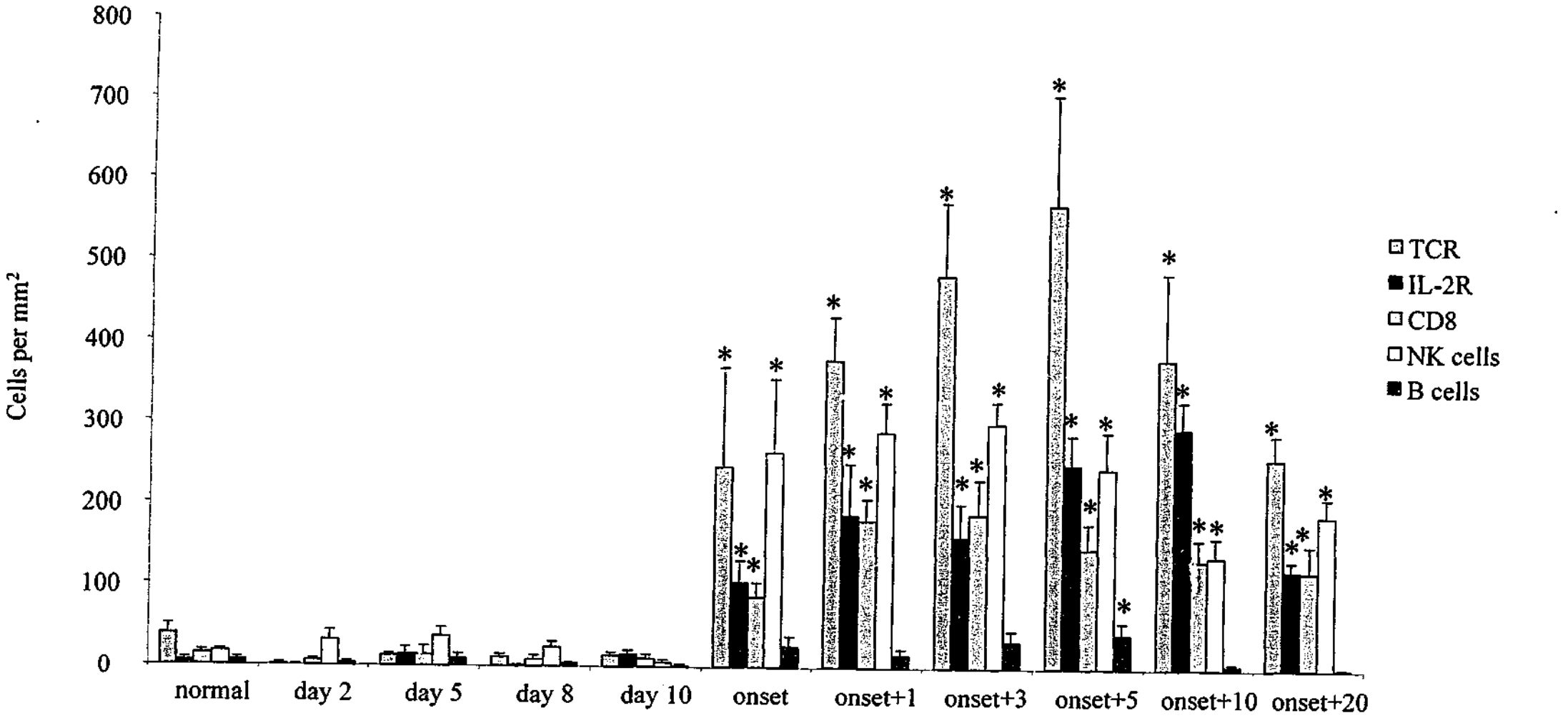


Figure 4.10 Lymphocyte subsets in the course of CIA

* $p \leq 0.05$ as compared to normal control

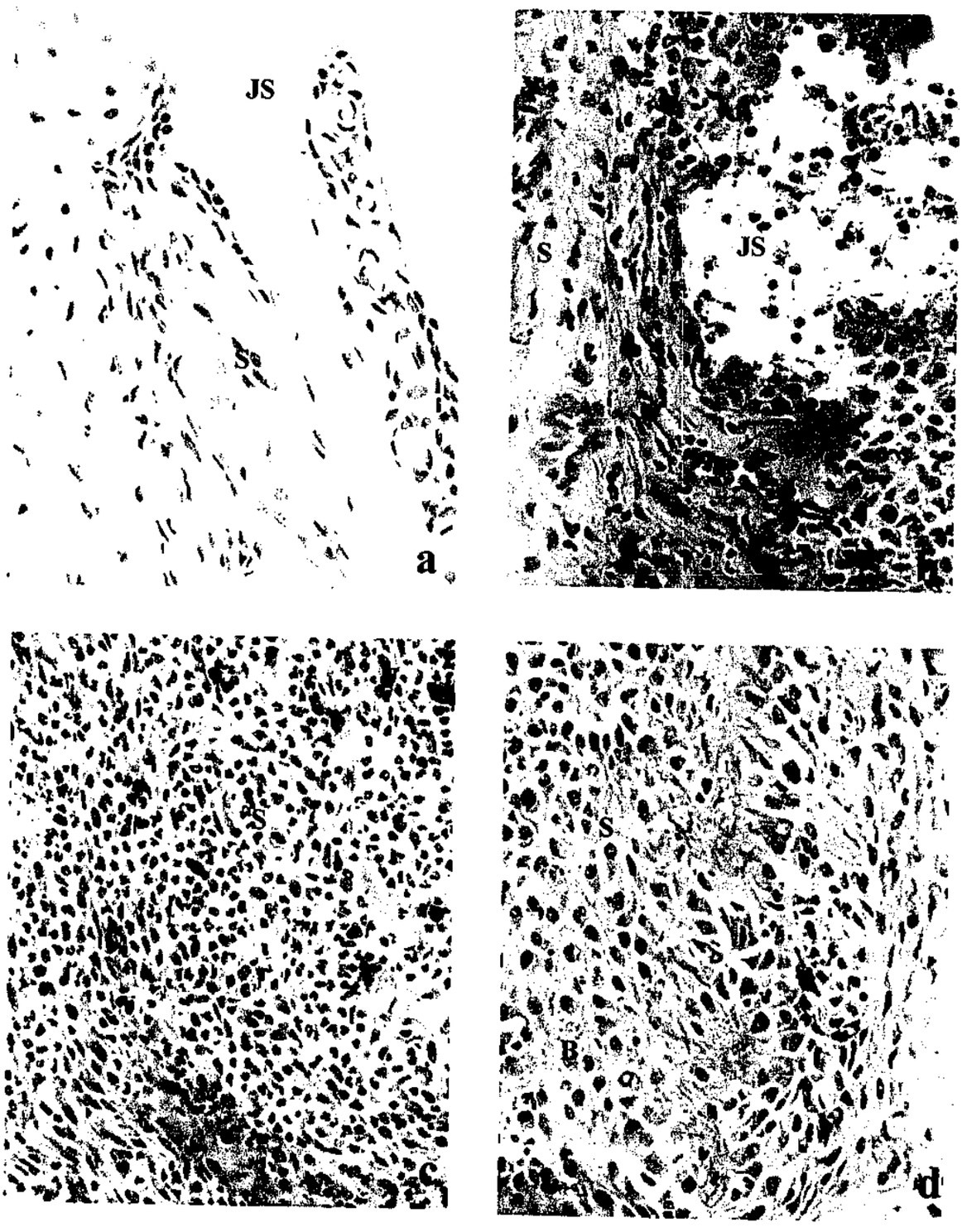


Figure 4.11. IL-2 receptor positive cells in synovium from normal and CIA rats: **a** normal joint; **b** onset of arthritis; **c** day 5 after onset; **d** day 20 after onset. (S - synovium, B - bone, JS - joint space)

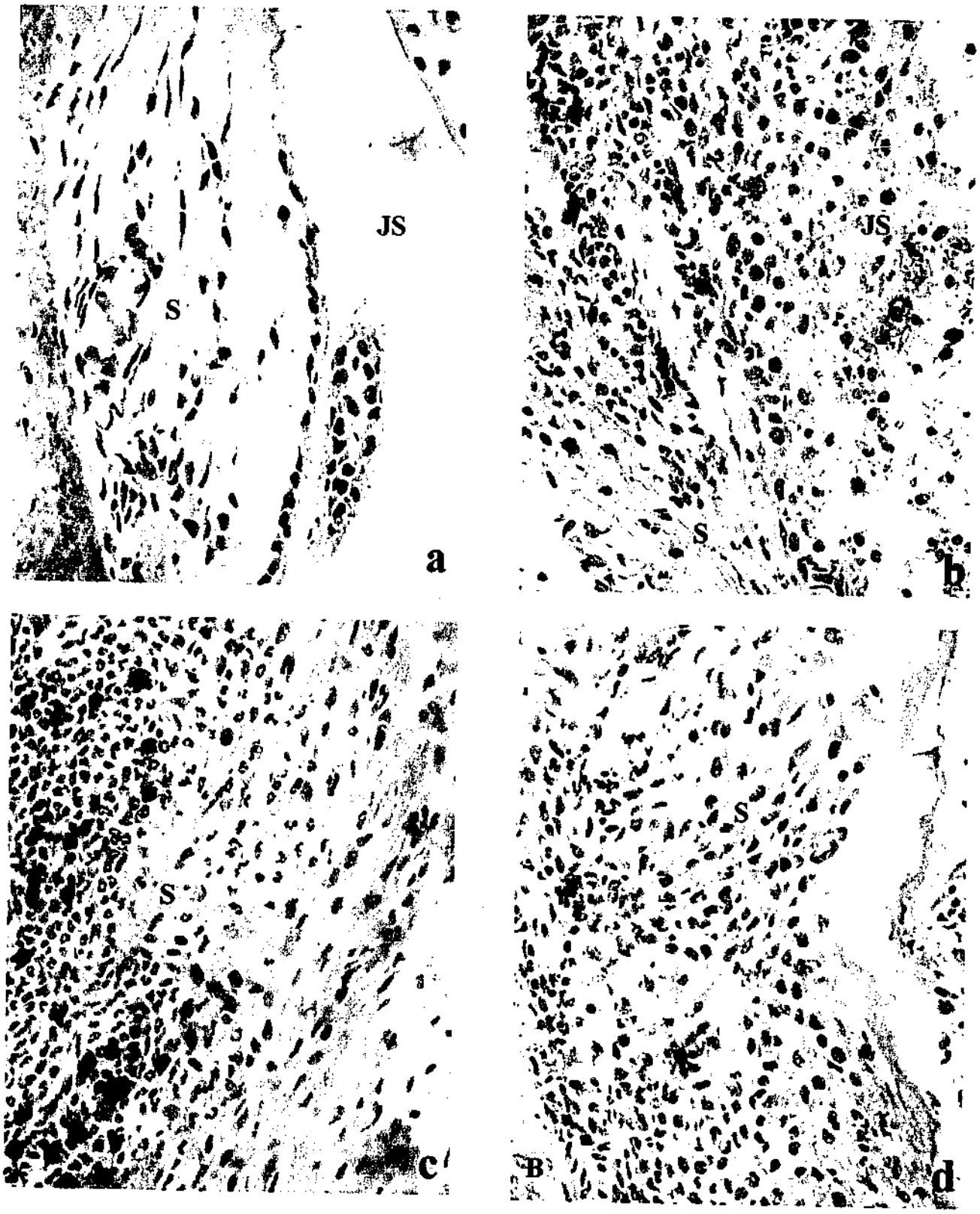


Figure 4.12. CD8+ cells in synovium from normal and CIA rats: **a** normal joint; **b** onset of arthritis; **c** day 5 after onset; **d** day 20 after onset. (S - synovium, B - bone, JS - joint space)

By day 5 after onset there was a further 2-fold increase in T cells in the synovium (Figure 4.10), although their relative number as compared to other leucocytes remained low (9.2%) (Figure 4.17). T cells, approximately half of them bearing IL-2 receptors (Figure 4.10), were found scattered throughout the synovium rather than in aggregates (Figures 4.9c and 4.11c). There was no increase in $CD8^+$ cells as compared to the time of onset (Figures 4.10 and 4.12c). By day 20 after onset there was a reduction in T cell numbers (Figures 4.9d and 4.10). They constituted 9.65% of all leucocytes present in synovium (Figure 4.17). Approximately half of these cells expressed IL-2 receptor (Figures 4.10 and 4.11d). The number of CD8 positive cells did not change as compared to day 5 after onset (Figures 4.10 and 4.12d).

NK cells ($3.2.3^+$) underwent a 6-fold increase ($p=0.01$) at the time of onset (Figures 4.10 and 4.13b) and represented 5.8% of all the leucocytes in synovium (Figure 4.17). The number of NK cells per unit area remained elevated at day 5 and 20 after onset of arthritis (Figures 4.10 and 4.13c-d), where they constituted 2.7 and 7.1 % of all leucocytes present in synovium, respectively (Figure 4.17).

Few scattered B cells (0.6%) were seen in the CIA synovium at the time of onset (Figures 4.14b). By day 5 after onset B cells increased 7-fold as compared to pre-arthritic stages ($p=0.01$) (Figures 4.10 and 4.14c), although their number per unit area and proportion as compared to other leucocytes remained low: 42 cells per mm^2 and 0.7%, respectively (Figures 4.10 and 4.17). By day 20 after onset there was a reduction in B cell numbers (Figures 4.10 and 4.14d). They constituted 0.05% of total number of the leucocytes present in synovium (Figure 4.17).

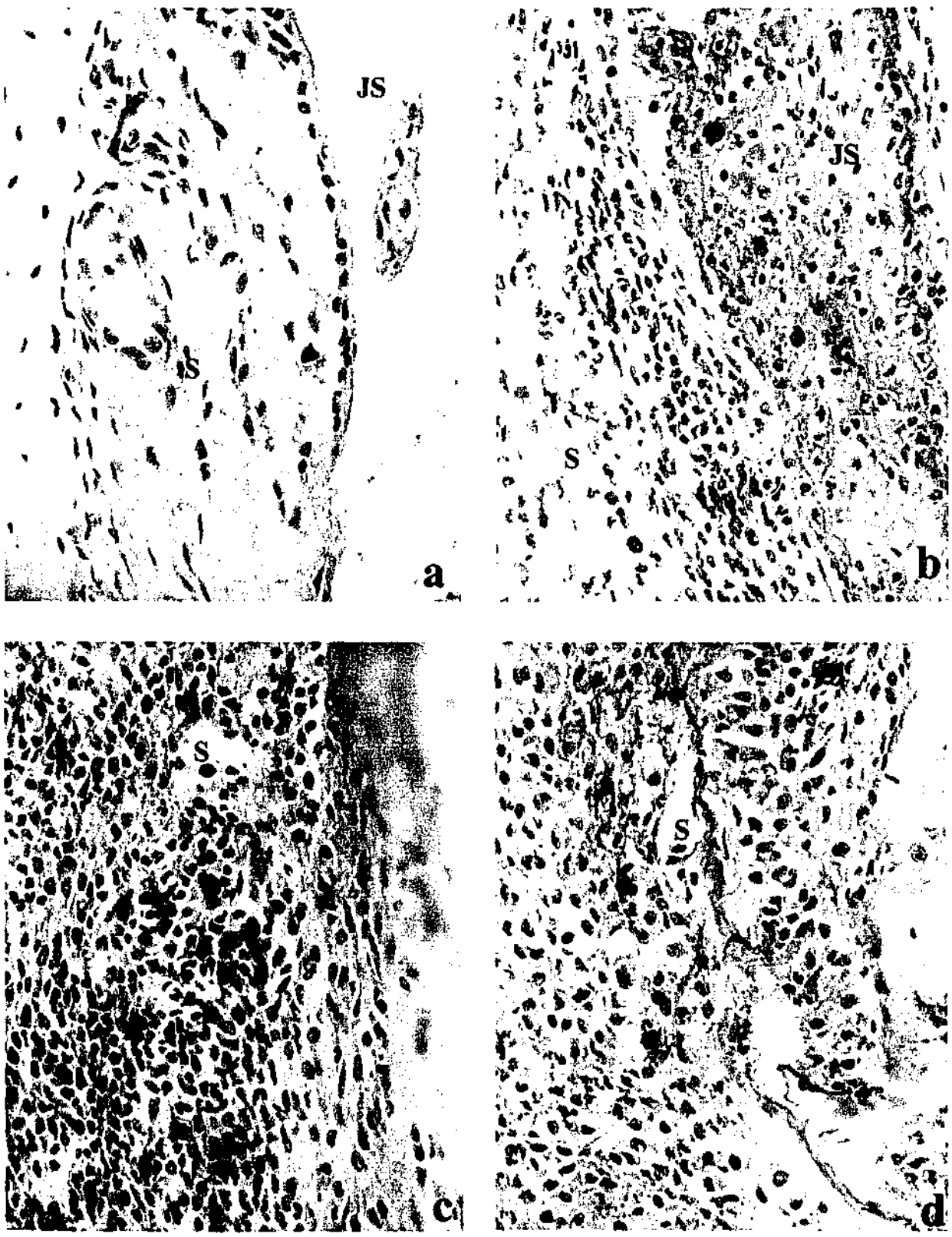


Figure 4.13. NK cells in synovium from normal and CIA rats: **a** normal joint; **b** onset of arthritis; **c** day 5 after onset; **d** day 20 after onset. (S - synovium, B - bone, JS - joint space)

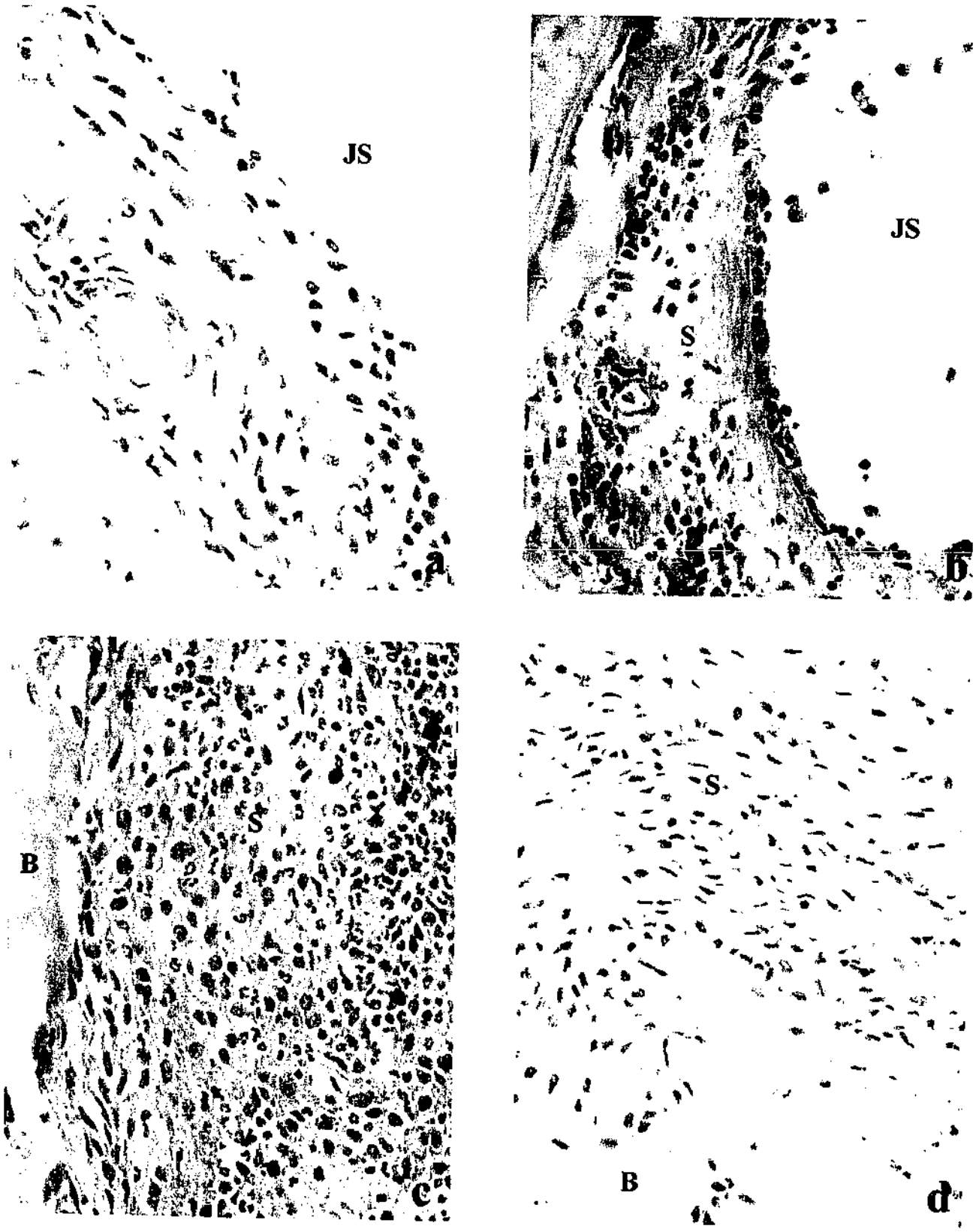


Figure 4.14. B cells in synovium from normal and CIA rats: **a** normal joint; **b** onset of arthritis; **c** day 5 after onset; **d** day 20 after onset. (S - synovium, B - bone, JS - joint space)

At the time of onset MHC class II expression in synovium increased 3 to 5 fold as compared to normal joints (p=0.01) (Figures 4.15b and 4.16). The MHC class II expression reached its maximum at day 3 after arthritis onset and remained elevated until day 20 after onset (Figures 4.15c-d and 4.16). The majority of $ED1^+$ cells, excluding osteoclasts, bore MHC class II receptors.

The number of MCs did not change at the time of onset, but the pattern of their distribution changed from being scattered in the synovium (in pre-arthritic joints) to being assembled around the capillaries with the newly recruited neutrophils (Figure 4.18b). There was a significant reduction in MC numbers per unit area ($p<0.01$) starting from day 1 after onset (Figure 4.19). The cells could either not be seen at all or "empty" cell shadows were observed (Figure 4.18c). The numbers of synovial MCs remained low until day 20 after onset with many cells appearing "empty" (Figures 4.18d and 4.19).

4.3.3.3 FIA controls

FIA controls did not differ significantly in their synovial cell composition from the normal controls at any of the time-points examined (Figures 4.12-3, 4.16 and 4.20).

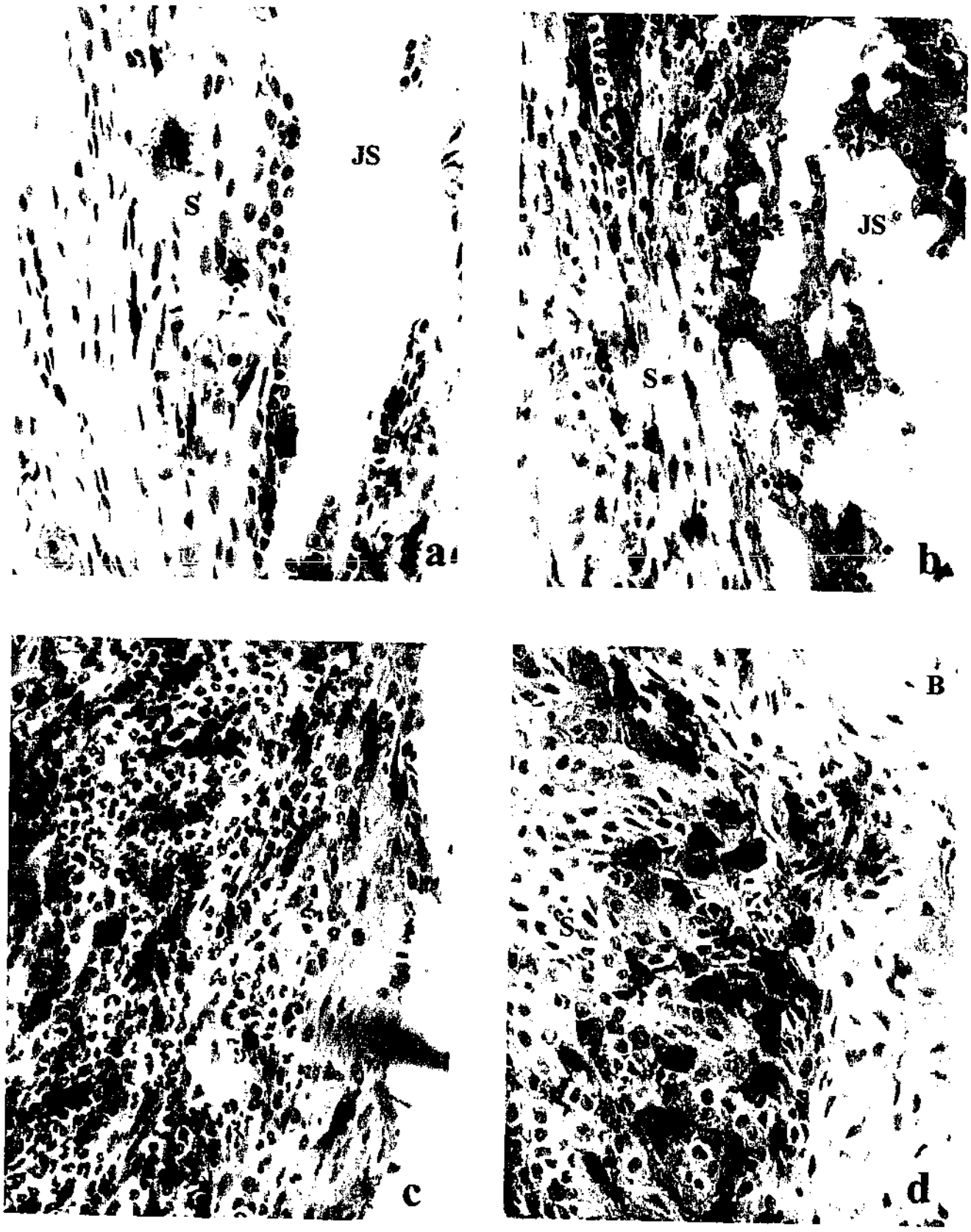


Figure 4.15. MHC class II positive cells in synovium from normal and CIA rats: **a** normal joint; **b** onset of arthritis; **c** day 5 after onset; **d** day 20 after onset. (S - synovium, B - bone, JS - joint space)

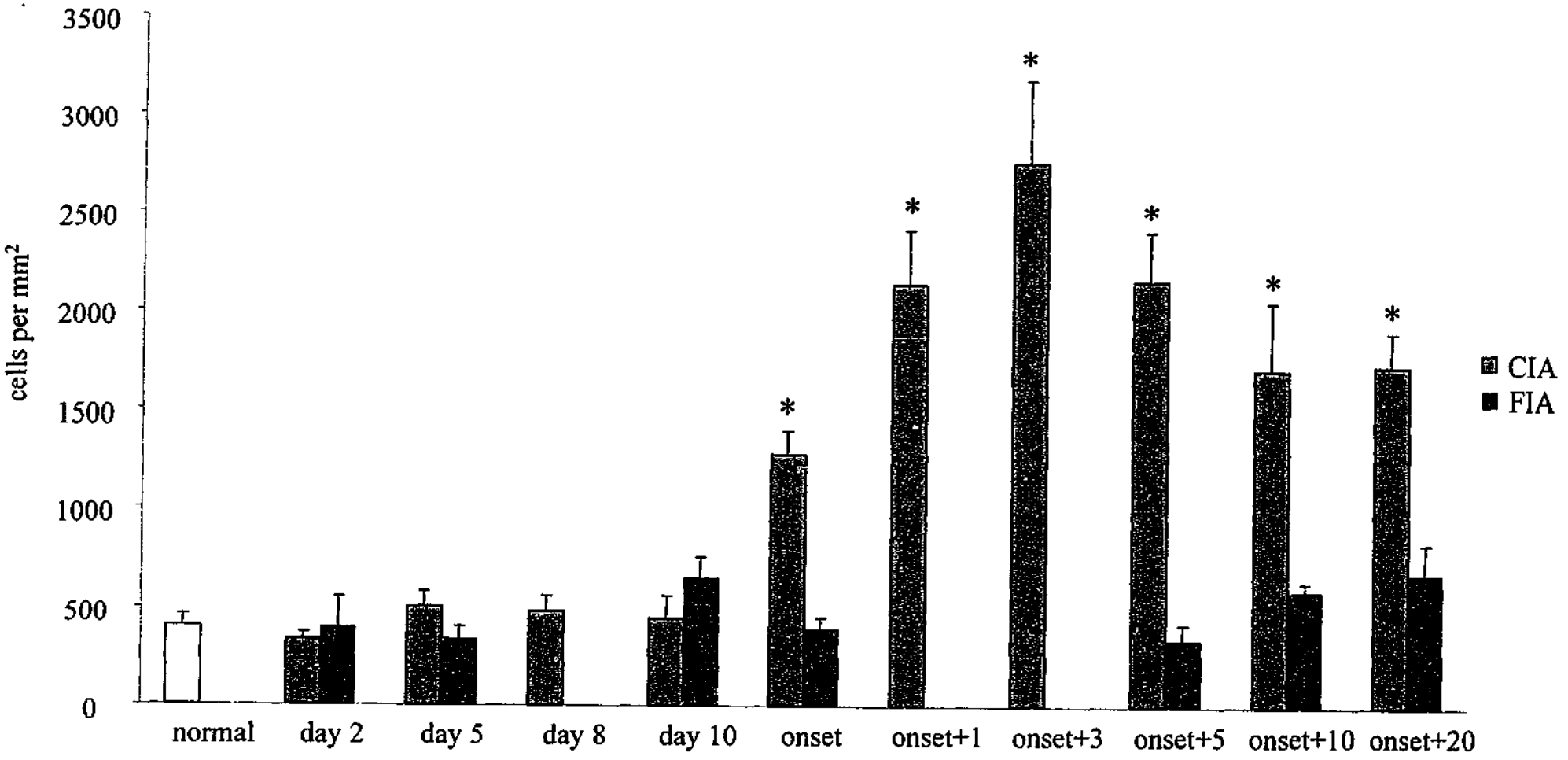


Figure 4.16 MHC class II positive cells in synovium in the course of CIA as compared to FIA controls

* $p \leq 0.05$ as compared to normal and corresponding FIA control

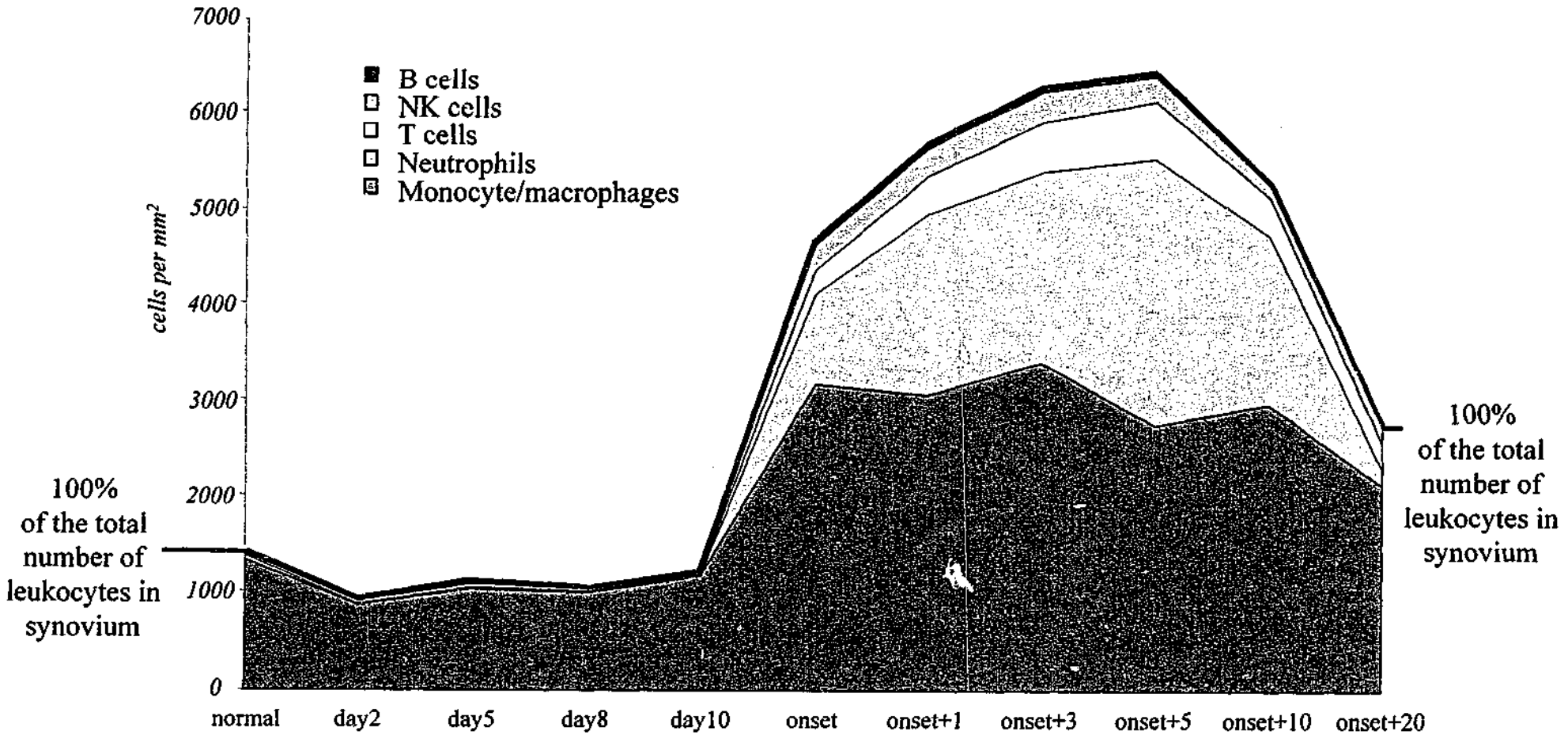


Figure 4.17 Proportion of different leucocyte populations in CIA synovium.

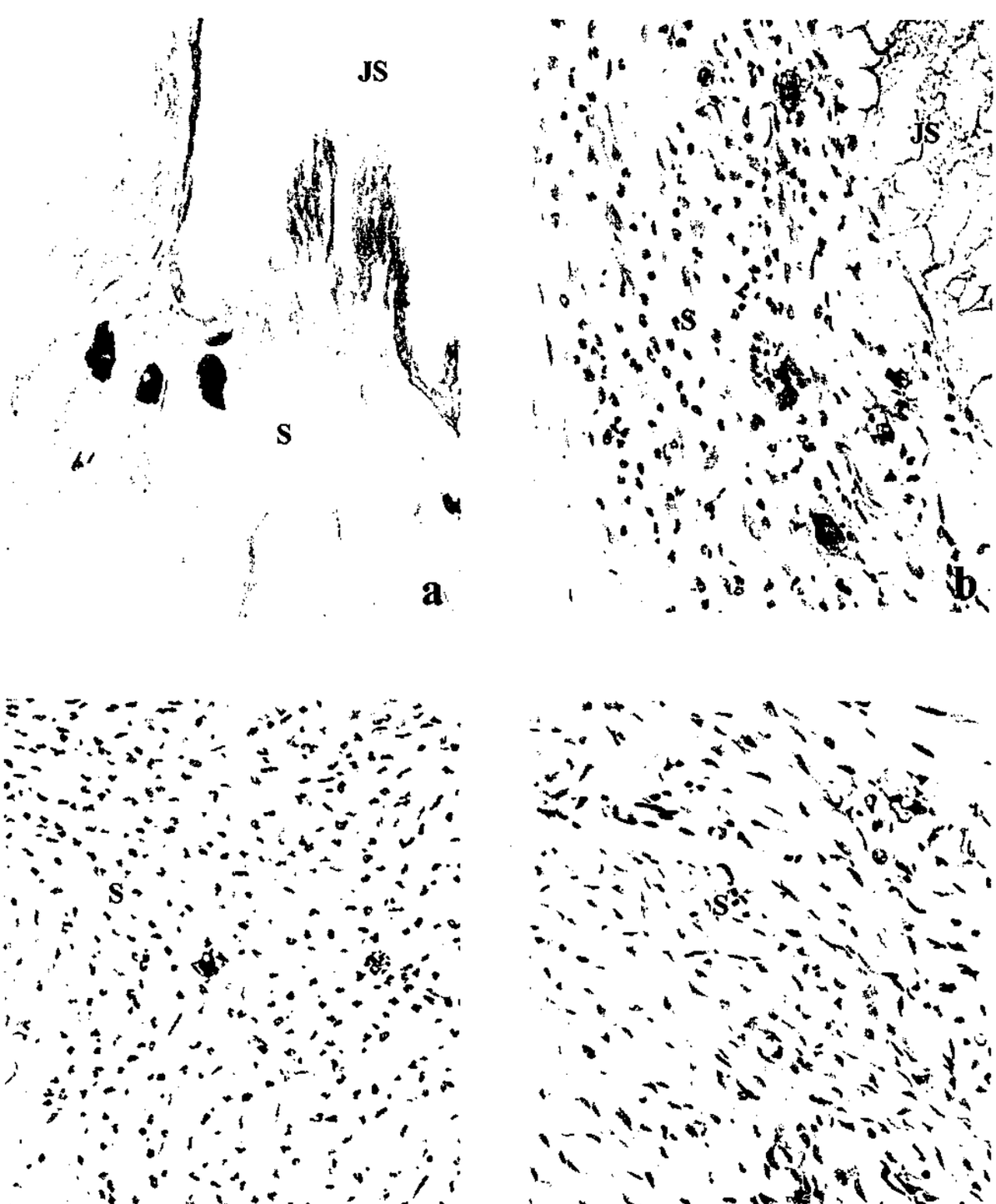


Figure 4.18. Mast cells in synovium from normal and CIA rats: **a** normal joint; **b** onset of arthritis; **c** day 5 after onset; **d** day 20 after onset. (S - synovium, JS - joint space).

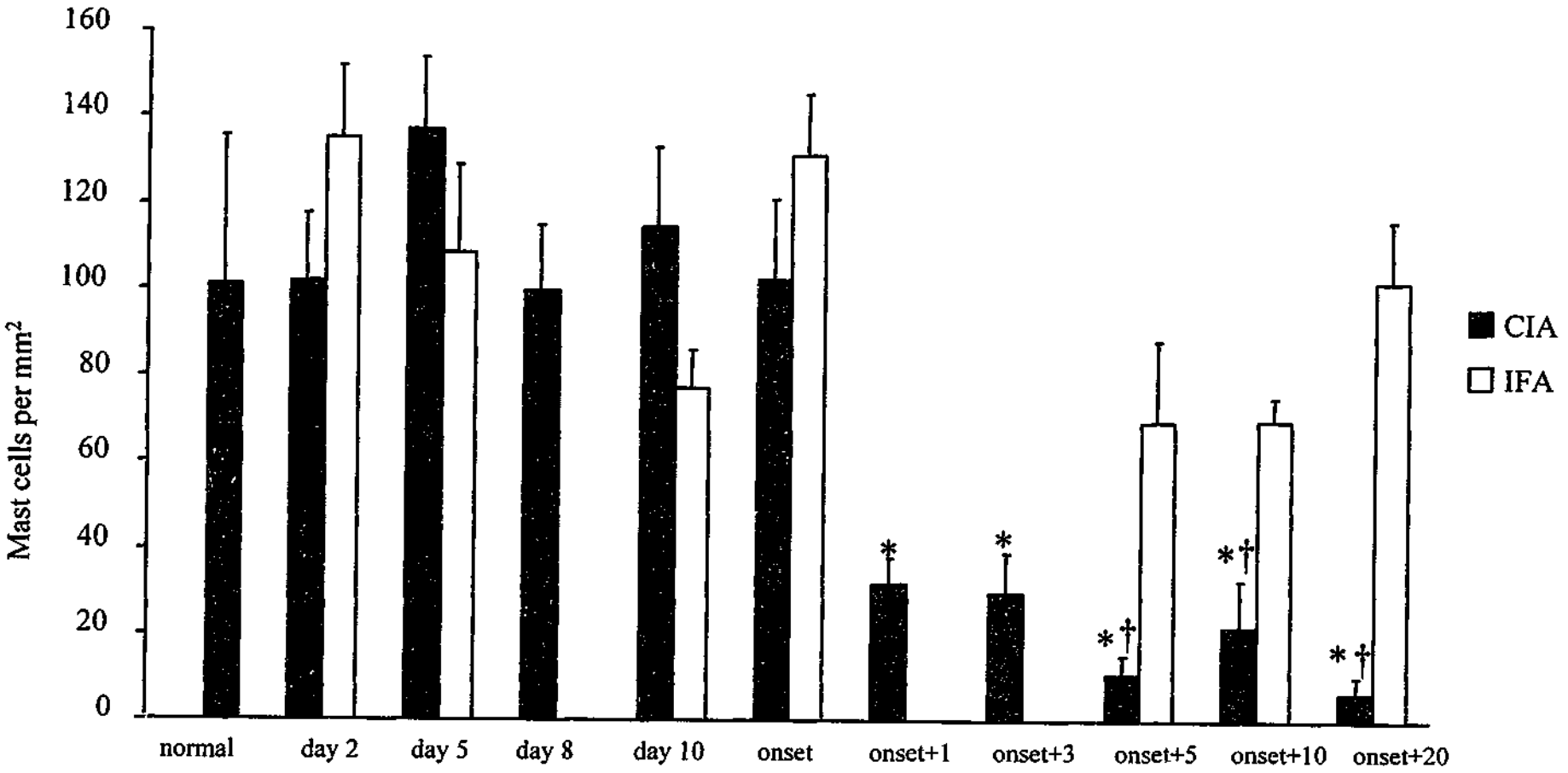


Figure 4.19 Mast cell numbers in synovium in the course of CIA as compared to FIA controls

* $p < 0.005$ as compared to the day of onset group
† $p < 0.05$ as compared to the appropriate FIA control group

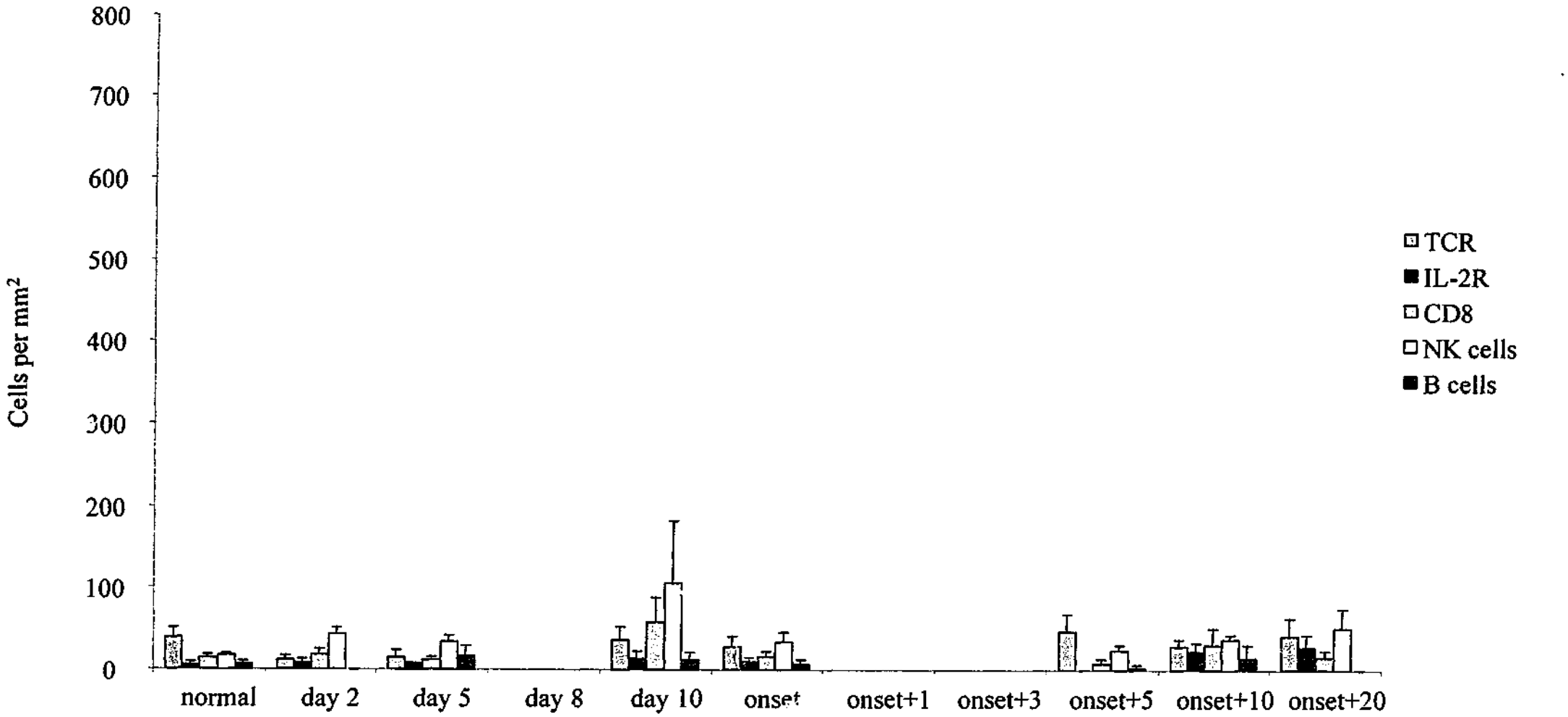


Figure 4.20 Lymphocyte subsets in the FIA control group

4.4 DISCUSSION

CIA, as established in female DA rats, is a reliable model with a 100% incidence of arthritis. One of the important features of this model was the consistent location of arthritis in distal interphalangeal joints of hind paws. This, together with quite uniform timing of the onset, made the model suitable for studying pre-arthritic, as well as arthritic, stages of CIA.

Arthritis was characterised by an acute onset with arthritis rapidly spreading to many joints and becoming severe. The severity of arthritis was one of the disadvantages of the model, as around 52% of the animals had to be killed before day 5 after onset. In the surviving animals, arthritis quickly progressed into ankylosis within 20 days. Although ankylosis is typical in the late stages of human RA, unlike CIA, it develops over the years, not days, probably due to its chronic, remitting course (Gordon, et al., 1998). The acute course of CIA is, therefore, an advantage as it allows observation of joint changes within a much shorter time frame.

The histopathological findings correlated well with macroscopic observations. The histopathological evaluation of the rat joints before the onset failed to reveal any significant changes. Onset of arthritis was consistent with an acute inflammatory process, as an influx of fluid and inflammatory cells was observed. The infiltrate increased until day 5 after the onset in agreement with an augmentation of the severity of arthritis. Infiltrate reduction was also consistent with recovery from arthritis. Formation of a pannus was observed as early as day

3 after the onset. Over the next 17 days the pannus expanded and invaded bone and cartilage. Pannus development and bone and cartilage erosions were very similar to the ones seen in RA (Firestein, 1998).

Swift recruitment of inflammatory cells to the joint may be mediated through several mechanisms. It is known that the initial step of CIA development involves binding of autoreactive anti-collagen antibodies to the cartilage surface (Kerwar, et al., 1983; Stuart, et al., 1983). Complement activation then results in recruitment of inflammatory cells to the joint (Kerwar, et al., 1983; Stuart, et al., 1983) and, perhaps, activation of the MCs (Jasin, 1987; Kasajima, et al., 1986). In this study MCs were found in close vicinity of capillaries filled with neutrophils at the time of onset, and then disappeared or had an "empty" appearance, suggestive of degranulation (Claman, et al., 1986), after the onset of arthritis. It can be speculated that degranulation of MCs can contribute to the inflammatory cell recruitment, as well as leucocyte and fibroblast activation and, both directly and indirectly, cartilage and bone matrix breakdown (Gruber, et al., 1988; Lees, et al., 1994; Qu, et al., 1995; Tetlow, et al., 1998; Zenmyo, et al., 1995).

Neutrophils, which are absent in the normal joint, accounted for 20% of the total leucocyte population at the time of onset, becoming a dominant cell type by day 5 after onset and reducing to only 5.8% by day 20 after onset. Their numbers appeared to correlate with the severity of arthritis. The fact that neutrophils, which produce a number of cartilage-degrading enzymes (Jasin, et al., 1991; Kakimoto, et al., 1995), were consistently found on the surface of the cartilage suggests that they play an important role in augmentation of the exposure of collagen type II

(Jasin, et al., 1991). As a result, more antigenic epitopes become available for antibody binding leading to an increase of complement activation and, thus, promotion of inflammation and joint destruction.

Monocyte/macrophages dominated the synovial infiltrate at all stages of CIA except at the peak of arthritis at day 5 after onset, when they were surpassed by neutrophils. Monocyte/macrophages together with fibroblasts formed the pannus tissue. Typically, they were present at the interface of the pannus with the bone. It is likely that monocyte/macrophages directly participated in cartilage/bone destruction via production of a variety of metalloproteinases (Ahrens, et al., 1996; Gibbs, et al., 1999; McCachren, et al., 1990; Shah, et al., 1995) and possibly served as precursors for ED-1^+ osteoclasts. In the later stages, macrophages, as well as MCs (Qu, et al., 1994; Skopouli, et al., 1998; Tetlow, et al., 1995), could be the source of fibrogenic growth factors involved in tissue repair (Gruber, et al., 1997; Levi-Schaffer, et al., 1995; Zhang, et al., 1999) and, therefore, could promote fibrous ankylosis of the joints. Our observations suggest that an increase of monocyte (ED1^+ cell) numbers in synovium occurred due to their recruitment rather than proliferation of the residential mononuclear cells, because in expanding synovium the number of the ED-2^+ cells per area unit decreased significantly ($p=0.01$). Approximately $^1/_3$ of synovial monocyte/ macrophages were MHC class II positive before the onset of arthritis. Almost all monocyte/macrophages expressed MHC class II after onset, which indicated their activated state.

There was a prominent expansion of T cells in the CIA synovium. Although their numbers constituted merely $^1/_5$ of that of monocyte/macrophages, their population expanded 16-fold. Many of the T cells were activated, as denoted by the expression of IL-2 receptor. This may indicate the involvement of these cells in the regulation of arthritis *in situ*. Unlike in RA, where T cells are found in aggregates (Janossy, et al., 1981), in CIA the T cells were seen scattered throughout the synovium. Such an absence of T cell aggregates in the synovium could be due to the lack of chronicity in CIA.

$CD8^+$ cells were abundant in CIA synovium, representing approximately one third of the T cells and this was similar to the pattern seen in the "immunologically active" RA synovium (Kurosaka, et al., 1983). It is possible that $CD8^+$ cells regulate the immune process *in situ* (Tada, et al., 1996) and mediate the resolution of the inflammatory processes in the joint after the acute phase. There was also a significant increase in NK cell numbers, which may have contributed to the increase in $CD8^+$ cell numbers. The role of NK cells in CIA is unclear. However, these cells may also be involved in the regulation of arthritis (Jansson, et al., 2000).

B cells were poorly represented in the CIA synovium. This was probably due to the acute nature of CIA (Berek, et al., 1997) in which the majority of antibody production most likely occurs in the lymph nodes draining the collagen injection sites.

In summary, this Chapter characterised the CIA model in DA rats. It provided consecutive description of events occurring in the inflamed joint. Uniform onset, clinical and histological changes make the CIA model in DA rats convenient for laboratory studies. The marked histological similarity of the erosive changes in the joints makes CIA a very appropriate model for RA. Although the CIA model induced by heterologous CII lacks chronicity and, therefore, differs in infiltrate composition from RA, the inflammatory changes in CIA joints resemble the ones in early and acute RA (Cush, et al., 1991; Kurosaka, et al., 1983), justifying the use of CIA as a model for the human disease. Its acute course and the rapid progression of joint damage allow studies of different aspects of arthritis, as well as pharmaceutical studies, to be carried out within a reasonable time frame.

Chapter 5

NERVE GROWTH FACTOR IN CIA

V. NERVE GROWTH FACTOR IN CIA

5.1 INTRODUCTION

Synovial inflammation in RA is usually considered to be a process that involves residential synovial cells and infiltrating immunocompetent cells. However, it has been recently established that the neuro-endocrine system may contribute to the regulation of pathological processes in the joint (Wilder, 1995). Nerve growth factor (NGF), a member of the neurotrophin family, may play an important role in inflammation. In adult mammals, under normal conditions, NGF is produced in minute amounts in peripheral tissues and acts as a trophic factor for sensory neurones. During inflammation, its levels increase dramatically (Aloe, et al., 1992b; Safieh-Garabedian, et al., 1995; Weskamp, et al., 1987). There is increasing evidence that circulating NGF levels are elevated in patients with autoimmune diseases, such as multiple sclerosis and systemic lupus erythematosus (Aloe, et al., 1994). An increase in NGF concentration was also seen in the sera and synovial fluids of patients with rheumatoid arthritis, osteoarthritis, juvenile chronic arthritis, ankylosing spondylitis and psoriatic arthritis as compared to normal controls (Aloe, et al., 1992a; Dicou, et al., 1993; Dicou, et al., 1996; Falcini, et al., 1996; Halliday, et al., 1998). Furthermore, a direct correlation was found between NGF serum levels and indices of the disease activity in juvenile chronic arthritis (Falcini, et al., 1996). The source of NGF in arthritis has not been demonstrated.

This chapter describes the synovial expression of NGF and its receptors, trk A and p75, in an experimental CIA model at different stages of the development of erosive arthritis.

5.2 MATERIALS AND METHODS

5.2.1 Experimental groups

NGF and NGF receptor expression was examined in joints from the groups of rats with CIA described in Chapter 3. Table 5.1 lists the experimental groups studied in this chapter and the number of animals in each group.

Table 5.1 Experimental groups

Group	n
Normal control	4
CIA onset	6
CIA day 5 after onset	6
CIA day 10 after onset	6
CIA day 20 after onset	6

5.2.2 Immunohistochemistry

5.2.2.1 Polyclonal antibodies

Anti-NGF and anti-trk A antibodies were obtained from Santa Cruz (U.S.A.) and used at concentrations 0.5 μg/ml and 0.125 μg/ml, respectively, while anti-p75 antibody was a kind gift from Prof. M. Chao (U.S.A.) and was applied at 1:4000

dilution. Appropriate dilutions of rabbit immunoglobulin (Dako, Denmark) constituted the negative control.

5.2.3 Double immunofluorescence

Double immunofluorescence allowed us to identify cells positive for NGF and trk A using specific cell-marker antibodies. NGF and trk A were visualised using red fluorescent dye, while various cell markers (see below) were depicted by green fluorescence. Double positive cells appeared as yellow. There was no specific marker available for neutrophils, therefore, they were identified using a blue DNA dye, which highlighted their characteristic polymorphic nuclei.

The monoclonal antibodies (mAbs) used included: antibodies directed against α/β T cells, MCA453 (Serotec, UK), and against NK cells, NKR-P1 (Cedarlane, Canada), and were used at concentrations of 11.4μg/ml and 4μg/ml, respectively. All the other mAbs were used as supernatants from cell cultures with their concentrations being determined empirically as supernatant dilutions. The OX-33 cell line (B cell marker) was obtained from the European Centre for Animal and Cell Culture, Salisbury, England and used at 1:400 dilution. The ED-1 cell line (common monocyte and macrophage marker) was a kind gift from Dr. C. Dijkstra (Dijkstra, et al., 1987). ED-1 antibodies were diluted prior to use 1:5.

5.3 RESULTS

5.3.1 Immunohistochemistry

5.3.1.1 NGF expression

NGF was undetectable in normal joints (Figures 5.1 and 5.2a). The first positive cells were seen at the time of onset, when many of the cells infiltrating the synovium, both mononuclear cells and neutrophils, appeared to be positive (Figures 5.1 and 5.2b). The number of NGF-positive cells remained high throughout the course of inflammation with fibroblast-like synoviocytes becoming positive as well. Extracellular staining was also noted by day 5 after onset of arthritis (Figure 5.2c). NGF was still detected in the synovium at day 20 after onset of arthritis (Figure 5.2d). However, NGF was present mainly in the sparse inflammatory foci at that time, and the positively stained area was significantly lower than at both day 5 and 10 after onset (Figure 5.2).

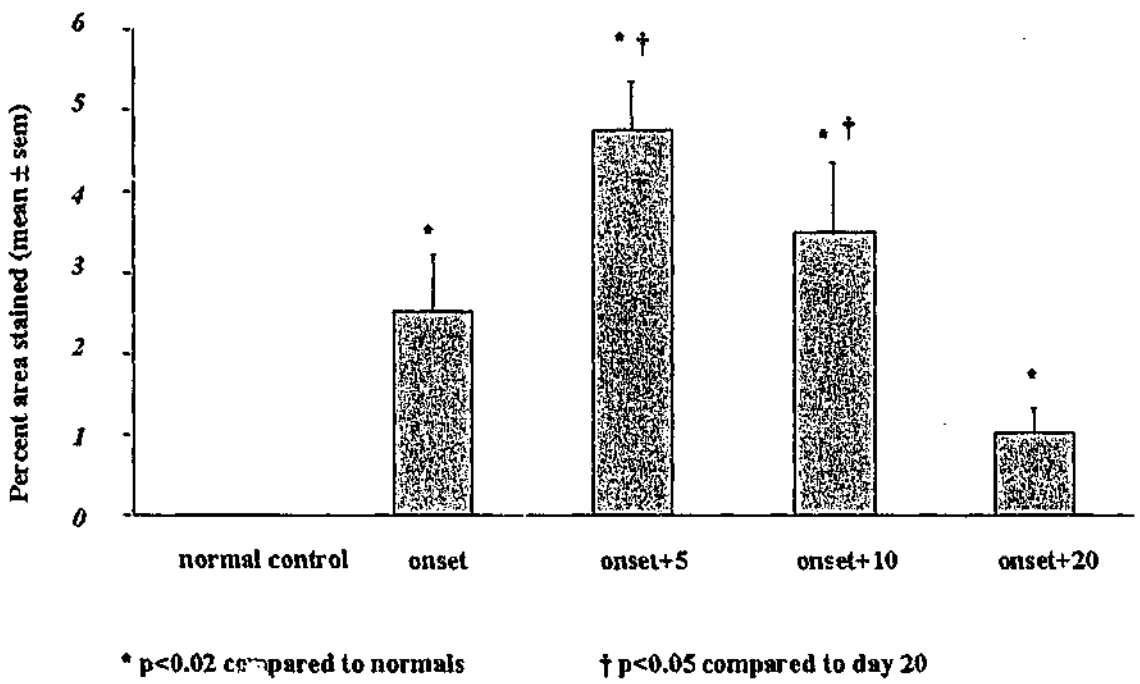


Figure 5.1. Percentage of the synovial area stained positively for NGF

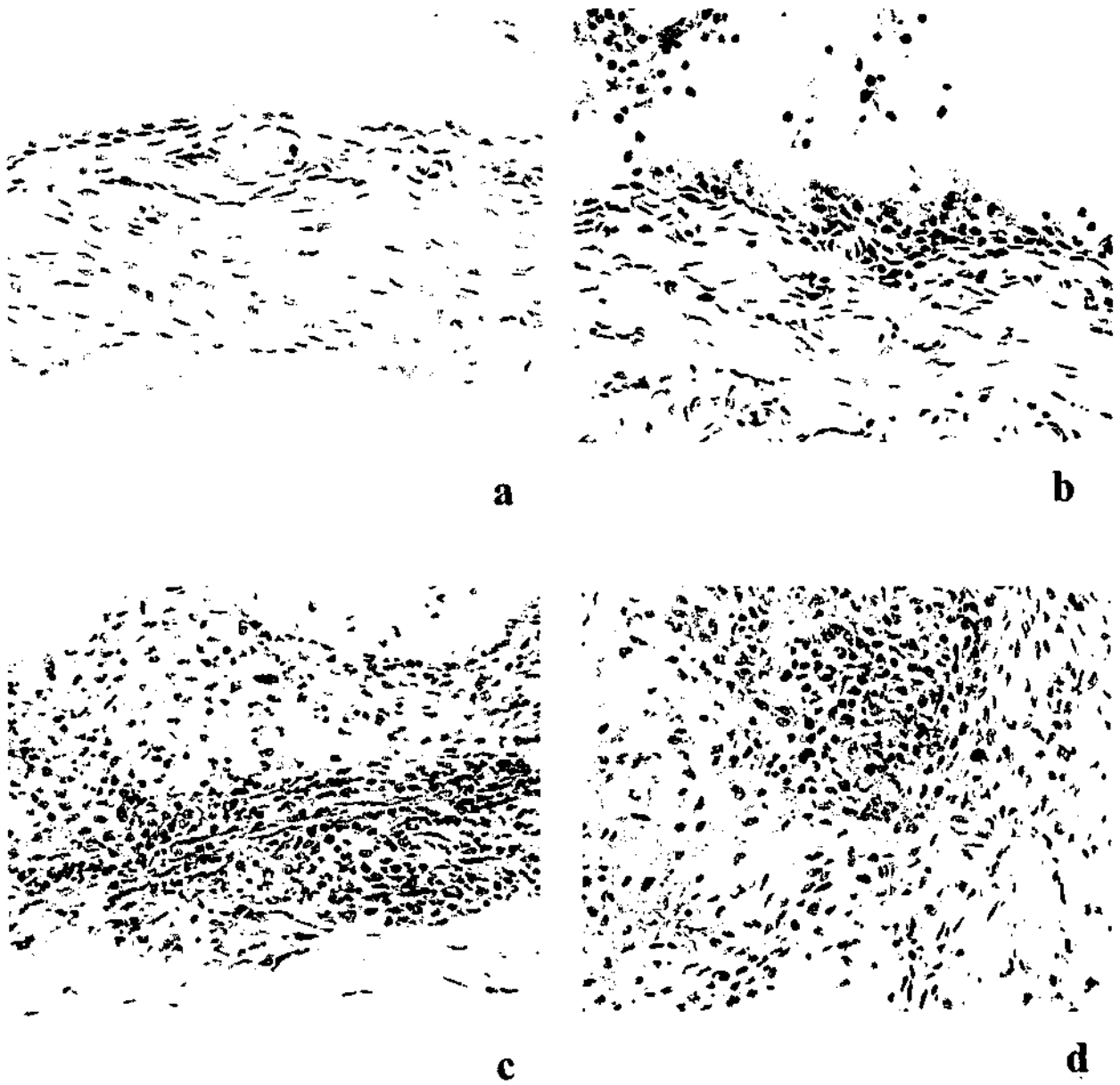


Figure 5.2. NGF expression in rat synovium: **a** in normal joint; **b** at the time of CIA onset; **c** 5 days after onset and **d** 20 days after onset.

5.3.1.2 Trk A expression

Unlike NGF, trk A was found on normal synoviocytes (Figures 5.3 and 5.4a). At the time of onset of arthritis many of the cells infiltrating the synovium were also positive (Figure 5.4b). Trk A was expressed on many synovial cells (mononuclear cells, neutrophils and fibroblast-like cells) throughout the course of arthritis (Figure 5.4c-d). The percentage of positively stained area in the synovium did not change significantly from the normal to the inflamed joint (Figure 5.3). However, the enormous increase (6-10 fold) in the synovial mass during CIA should be considered, suggesting an impressive rise in the overall number of trk A-bearing cells in the synovium during the course of arthritis.

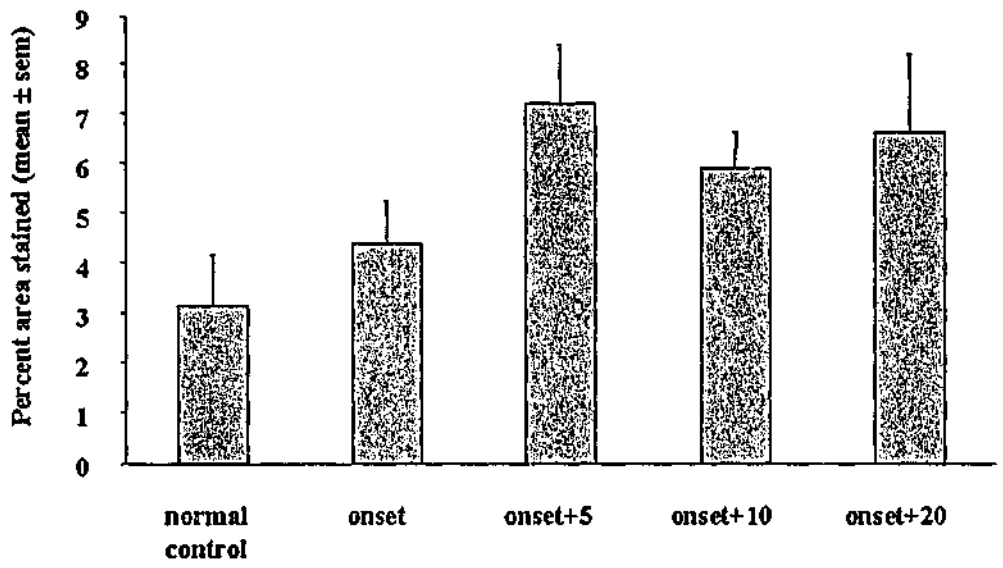


Figure 5.3. Percentage of the synovial area stained positively for trk A.

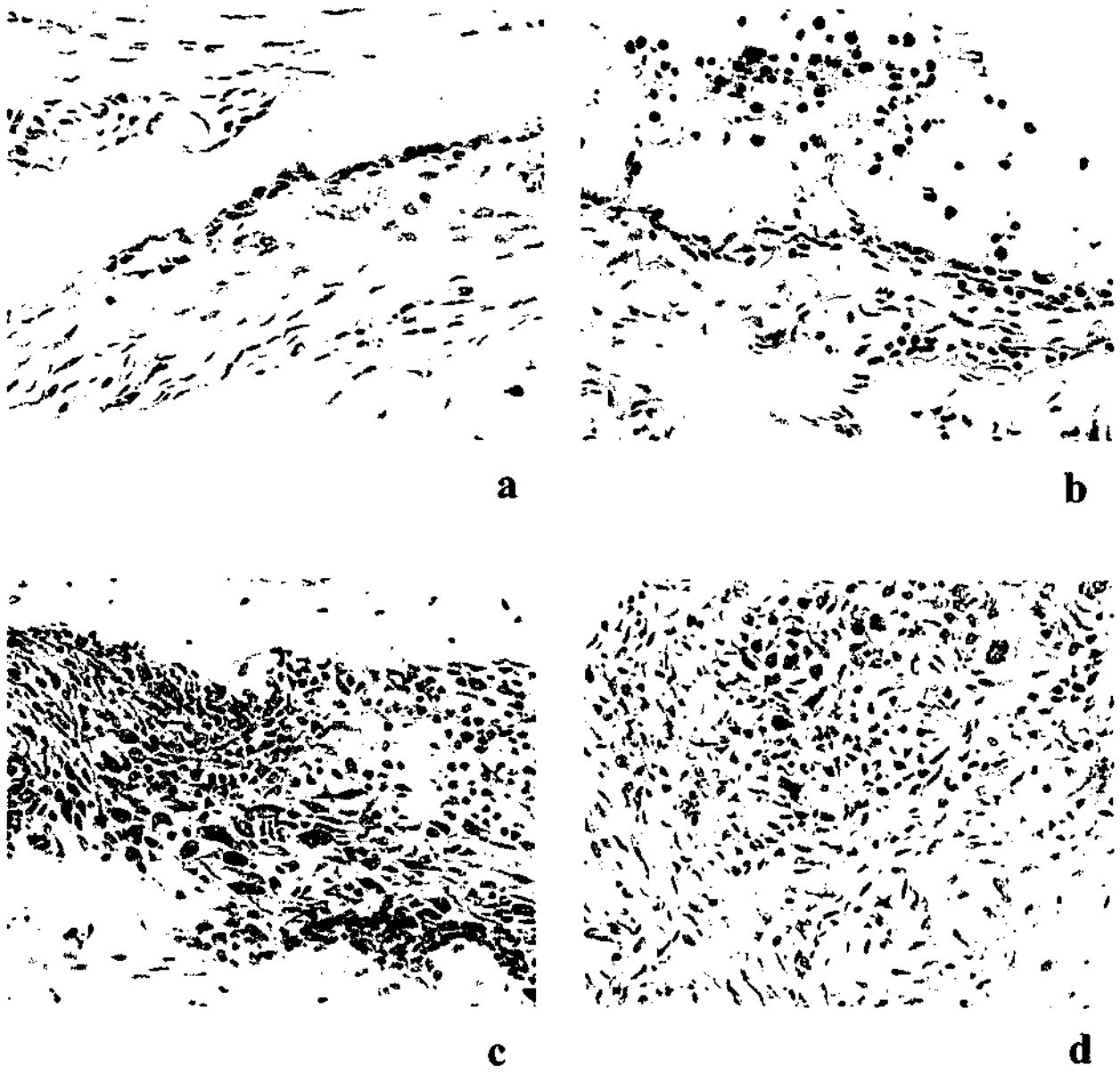


Figure 5.4. Trk A expression in rat synovium: **a** in normal joint; **b** at the time of CIA onset; **c** 5 days after onset and **d** 20 days after onset.

5.3.1.3 p75 expression

In normal synovium p75 was found to be expressed on free nerve endings and perivascular nerves (Figure 5.5a). The staining pattern did not change through the course of CIA (Figure 5.5 b-d).

5.3.2 Double immunofluorescence

5.3.2.1 NGF

NGF expression by different cell types was studied in the synovium of the distal interphalangeal joints in CIA rats at different stages of arthritis development. Figure 5.6a demonstrates localisation of NGF to monocytes/macrophages, with double positive cells seen in yellow. T cells, B cells and NK cells were negative for this factor (Figure 5.6b-d). Neutrophils were found to express NGF, as many of the polymorphonuclear cells were stained positively for this factor (white/lilac) (Figure 5.6 e).

5.3.2.2 Trk A

In normal synovium trk A was expressed on both type A ($ED1^-$ fibroblast-like) and type B ($ED1^+$ macrophage-like) synoviocytes. Sections were stained with ED1 antibody and anti-trk A antibody and both double positive (yellow) and $ED1^-/NGF^+$ (red) cells were observed (Figure 5.7), suggesting both types of synoviocytes express this factor.

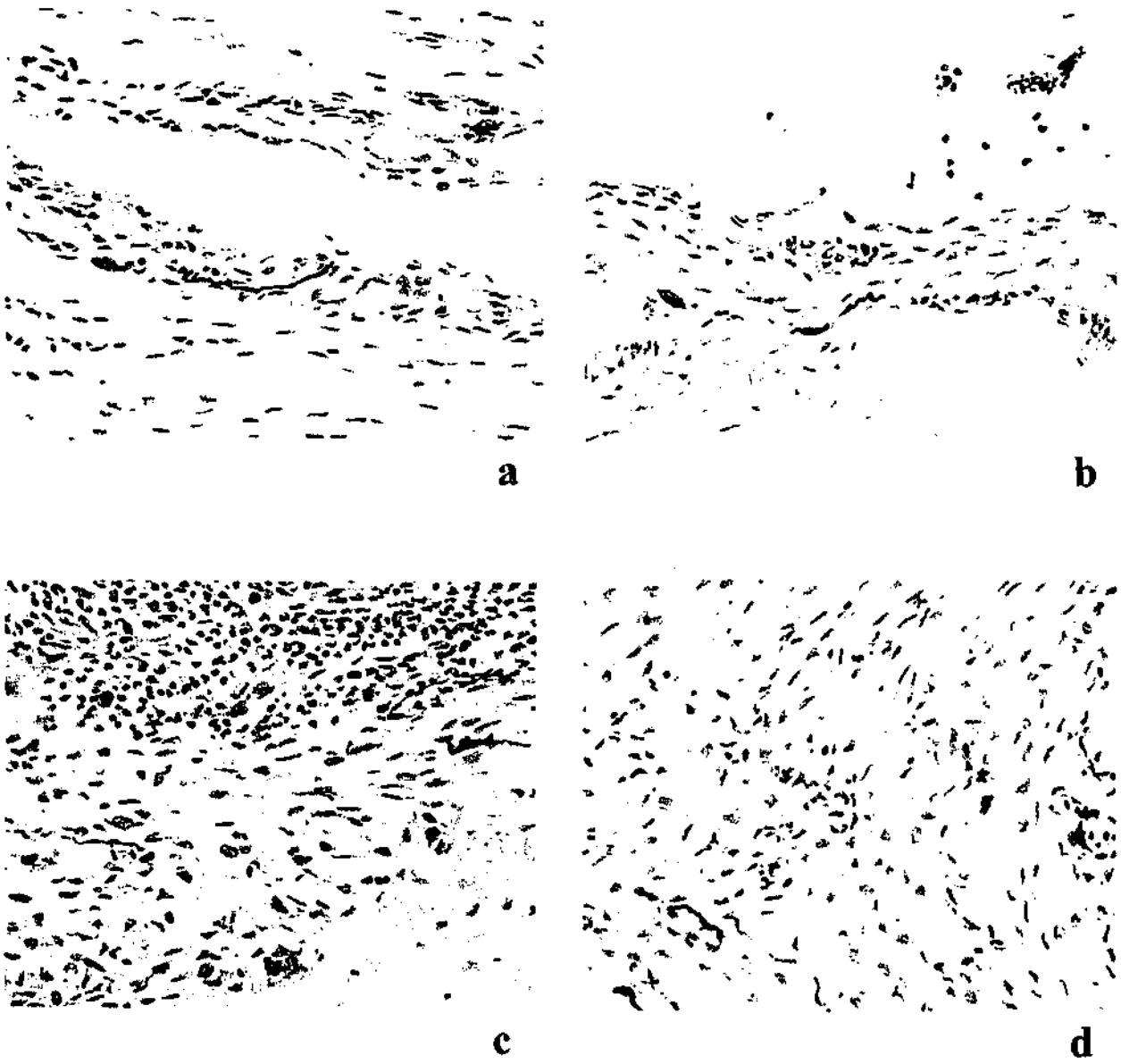


Figure 5.5. p75 expression in rat synovium: **a** in normal joint; **b** at the time of CIA onset; **c** 5 days after onset and **d** 20 days after onset.

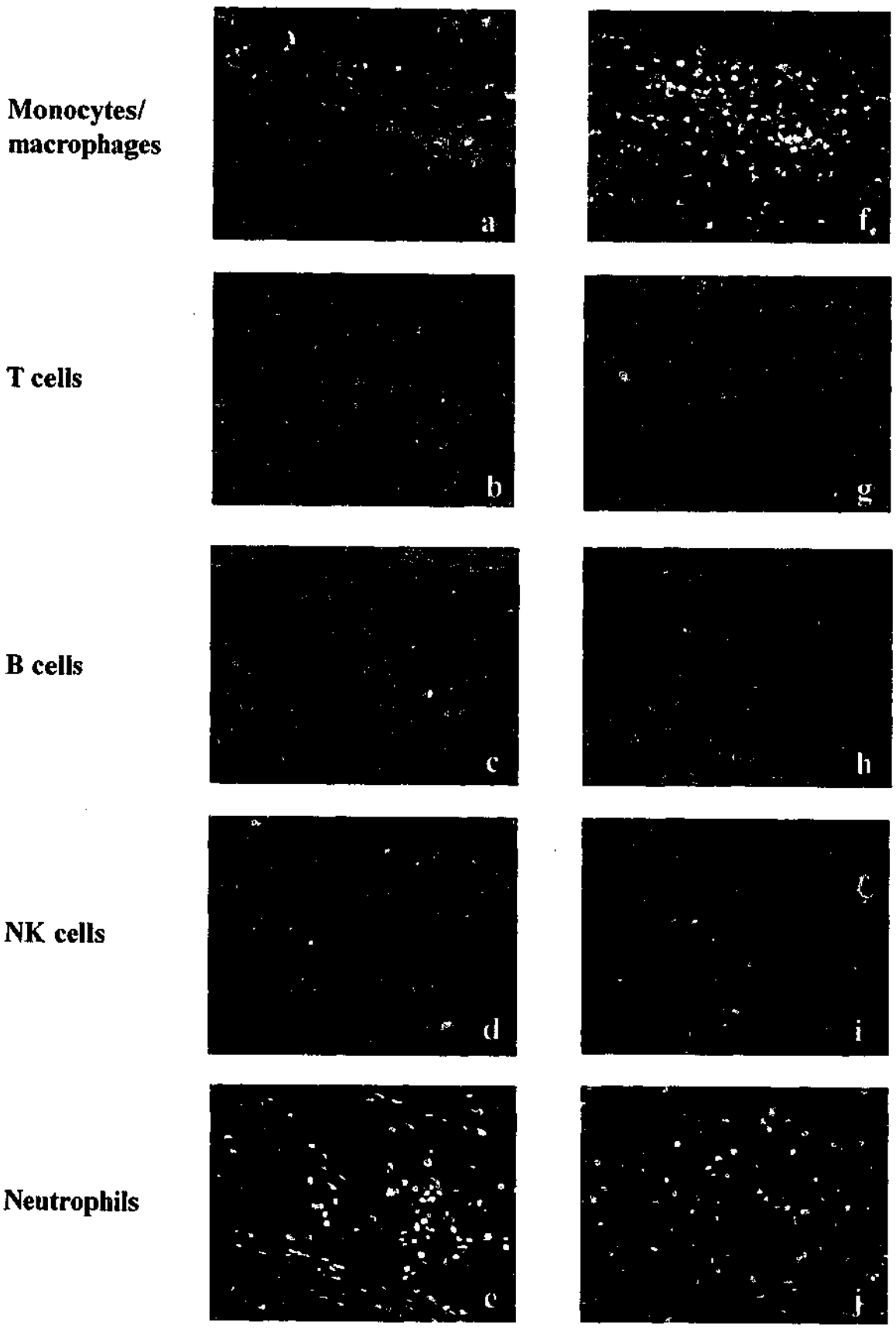


Figure 5.6. Double immunofluorescence: identification of NGF and Trk A positive cells in CIA rat synovium.
a-d, f-i: NGF and Trk A - red, cell markers - green, co-localisation of both - yellow. **e, j:** nuclear staining -blue, NGF and Trk A - red, co-localisation - white/ lilac.

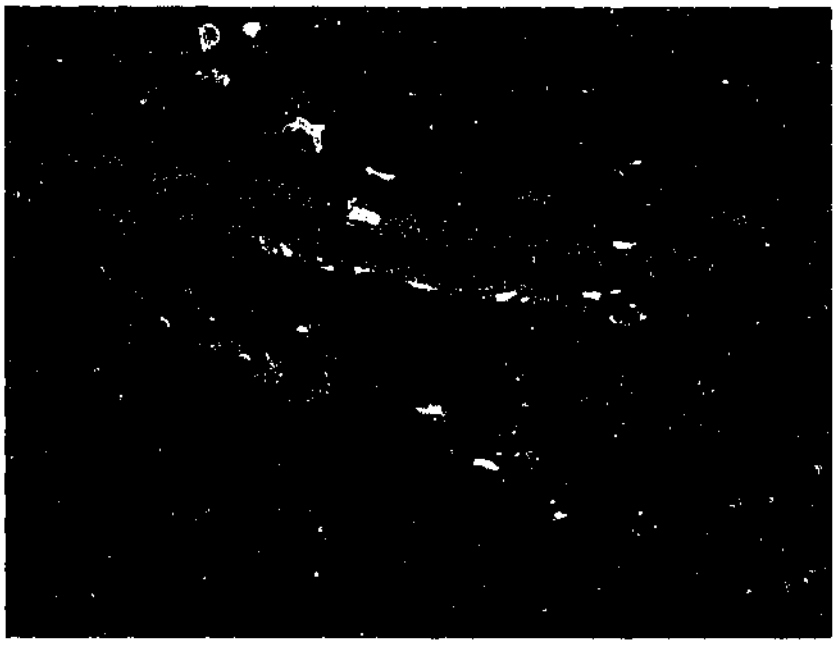


Figure 5.7. Double immunofluorescent labelling with anti-trk A and ED-1 antibodies in normal synovium. Both macrophage-like synoviocytes (yellow) and fibroblast-like synoviocytes (red) are positive for trk A.

In the arthritic synovium monocytes/macrophages were also positive for trk A (Figure 5.6f). T cells, B cells and NK cells lack this receptor (Figure 5.6g-i). Neutrophils were also found to express trk A receptor (Figure 5.6j).

5.4 DISCUSSION

Inflammation is a complex process involving cooperation between immune, endocrine and nervous systems in an effort to maintain corporal homeostasis in the case of tissue damage (Wilder et al., 1995). Inflammation is often associated with pain, which, at least partially, is mediated through activation of nociceptive neurones. Some sensory neurones can, in turn, directly participate in inflammation through release of pro-inflammatory neuropeptides, such as substance P or calcitonin gene-related peptide (CGRP) (Louis et al., 1989). NGF represents a unique mediator that, being produced in the peripheral tissues, can act on both neuronal and immune cells (Aloe et al, 1994). It mediates inflammatory hyperalgesia by decreasing the pain threshold of nociceptive neurones and, at the same time, may activate those

inflammatory cells that express receptors for it (Ma, et al., 1997).

Our results have demonstrated the appearance of NGF-positive cells in the synovium during the course of the experimental erosive arthritis, CIA. We have also shown that normal synovial cells express trk A receptor and that during joint inflammation a significant number of the infiltrating cells bear the trk A receptor as well.

We were able to identify the cells positive for NGF as being monocytes/macrophages and neutrophils. The cellular and extracellular pattern of NGF distribution in synovium during CIA suggests that the source of NGF is within the inflamed synovium. This is in contrast with the perivascular distribution of NGF found in carrageenan-induced arthritis model (Aloe, et al., 1992b). It should be noted, however, that apart from NGF-producing cells, NGF bound to the cell surface receptors could have been depicted using an immunohistochemical technique. Therefore, the exact source of NGF in synovium can be only identified by *in situ* hybridisation, a technique that will allow NGF mRNA detection.

Macrophages have been previously shown to be positive for NGF by immunohistochemistry (Liu, et al., 1994; Marcinkiewicz, et al., 1999). However, in the experiments with grafted autologous peritoneal macrophages, no NGF mRNA was detected, using reverse transcription polymerase chain reaction (RT-PCR), in these cells (Franzen, et al., 1998). Neutrophils were also found to be positive for NGF in our experiment. Similar results were obtained earlier by Liu

et al., who observed NGF protein expression by neutrophils in human brain abscesses (Liu, et al., 1994). NGF mRNA expression has not been studied in these cells.

Another potential source of NGF in the synovium may be fibroblasts (Thoenen, et al., 1988), consistent with our observations that cells with a fibroblast appearance were positive for NGF.

In CIA joints, T and B cells were found to be negative for NGF. This is in contrast to earlier reports that these cells produce NGF (Ehrhard, et al., 1993a; Santambrogio, et al., 1994; Torcia, et al., 1996). However, it should be noted that NGF production by T cells was observed only in activated murine T cell clones and, even under these conditions, some clones did not produce NGF (Ehrhard, et al., 1993a; Torcia, et al., 1996). Similarly, NGF expression by B cells was observed in cultured B cells (Torcia, et al., 1996). In CIA the lack of NGF expression by T and B cells seems to be a general pattern, as we did not find any NGF expression by these cells in the lymph nodes of the same animals (experiments performed by Ms. M. L. Lindsay under my supervision).

Unlike NGF, trk A was expressed in normal synovium by both type A and type B synoviocytes, suggesting these cells are sensitive to NGF. Furthermore, during inflammation, a significant number of monocytes/macrophages and neutrophils were found to express trk A. Some of these observations are in line with the data obtained by other researchers in different experiments. It has been demonstrated that isolated murine peritoneal macrophages express trk A and show evidence of

enhanced phagocytosis and IL-1 production (Susaki, et al., 1996) upon stimulation with NGF. Similar results were obtained in human blood monocytes, which were also shown to express functional trk A receptor (Ehrhard, et al., 1993b). Trk A receptor expression by neutrophils has been shown in brain abscesses (Liu, et al., 1994). Such expression is consistent with a number of effects exerted by NGF on these cells, such as chemotaxis, enhanced phagocytosis and superoxide production, and suppression of apoptosis (Boyle, et al., 1982; Gee, et al., 1983; Gruber, et al., 1990; Karnan, et al., 1991).

Trk A was not expressed on T cells, which was consistent with findings in human T cells (Torcia, et al., 1996). Although trk A expression was described in some activated murine T cells clones (Ehrhard, et al., 1993a; Otten, et al., 1989), this was not a consistent finding, with other researchers also failing to observe trk A receptor expression on isolated human T cells (Torcia, et al., 1996).

Synovial B cells in CIA are also devoid of trk A. Other researchers, however, have described expression of trk A on memory B cells and demonstrated that NGF was essential for their survival (Torcia, et al., 1996). Perhaps, there is a lack of memory B cells in synovium, which could be one of the reasons for the absence of trk A expression on synovial B cells.

p75 was not found on any inflammatory cell type studied. It is likely that immune cells do not normally express this receptor, as the lack of p75 expression on macrophages and T cells had previously been described by others (Ehrhard, et al.,

1993b; Susaki, et al., 1996). It has also been shown that in these cells trk A was capable of effective NGF signalling (Susaki, et al., 1996).

NGF has been shown to regulate a number of immune cell functions both in vitro and in vivo, acting through trk A as an activation stimulus. Our observation of the increased expression of NGF in the CIA joints, along with trk A receptor expression on monocytes/macrophages, neutrophils and, possibly, fibroblasts, suggests that NGF may play an important role in the development of arthritis.

Macrophages and fibroblasts have been shown to be the main source of a number of pro-inflammatory cytokines, which are believed to be of major importance in the maintenance of joint inflammation (Badolato, et al., 1996; Butler, et al., 1995; Chu, et al., 1992; Klareskog, et al., 1995). These cells together with neutrophils are the main producers of a variety of the destructive enzymes, such as matrix metalloproteinases, other proteases and glycosidases (Hamerman, et al., 1967; Harris ED, 1976). Activation of monocytes/macrophages, neutrophils and fibroblasts by NGF in CIA synovium, through trk A receptor, may lead to exacerbation of the inflammatory process and extensive joint destruction.

The similarities in the morphological manifestation of CIA to human RA, suggest that similar processes may take place in the rheumatoid synovium. This hypothesis is supported by findings of elevated NGF levels in the synovial fluid of patients with various forms of arthritis. Therefore, it is possible that in some patients, therapies directed at NGF inhibition may prove beneficial, as NGF blockade may suppress both pain and the inflammatory process.

Chapter 6

SUMMARY AND CONCLUSIONS

VI. SUMMARY AND CONCLUSIONS

Rheumatoid arthritis (RA) is a chronic debilitating disease characterised by remitting and relapsing joint inflammation. Apart from being a cause of tenacious articular pain, this inflammation results in disarrangement of normal joint architecture, joint deformation and subsequent loss of function. Despite major improvements in the management of pain and inflammation in patients with RA, it is still unclear what are its causative factors, pathogenetic mechanisms and how it can be cured.

Animal models allow us to simulate human diseases, study the processes occurring in the diseased organs and also test new treatment strategies. It is therefore imperative that the model shares as many characteristics as possible with the disease.

Collagen-induced arthritis (CIA) is an animal model for RA. It shares unique histopathological features with RA. In both diseases an invasive tongue of synovial tissue, which is known as pannus, is formed. This pannus invades bone at the cartilage/bone junction resulting in the formation of characteristic subchondral bone erosions. Despite the fact that the model of CIA has been established for over two decades, there is contradictory data in regard to the frequency of arthritis, histopathological changes and composition of the inflammatory infiltrate. This project elucidated the optimal conditions required for the induction of CIA occurring in 100% of treated animals with uniform onset and location. Such features were essential to investigate joint changes before the onset of arthritis. This thesis also describes changes in CIA joints throughout the course of the disease, including histopathological features and the identification of the inflammatory cells in the

synovial infiltrate. The potential role of NGF in joint inflammation was also analysed.

Two rat strains, DA and Lewis, were tested to find the most suitable strain for CIA. DA rats, when injected intradermally with bovine collagen type II (CII) developed symmetrical arthritis in smaller peripheral joints, in a pattern similar to the one seen in RA. In contrast, in Lewis rats, medium size joints became affected, often asymmetrically. DA rats demonstrated 100% susceptibility to CIA along with a predictable time of onset, whereas the time of onset varied in Lewis rats and only 80% of animals developed CIA.

In the literature published, two possible methods of collagen injection for CIA induction were described, intradermal and subcutaneous. This study demonstrated that it is imperative for the collagen to be introduced intradermally. Subcutaneous injection did not result in arthritis in either rat strain. The macroscopic observations were confirmed histologically.

Knowing that CII administered nasally, orally or intravenously does not produce arthritis, but can protect animals against subsequently induced CIA, we challenged subcutaneously inoculated DA rats with an intradermal injection of CII. The outcome was lower susceptibility to CIA and an ameliorated disease course.

We propose that the different effects produced by subcutaneous and intradermal inoculations of CII may be due to the distinct cell populations in the skin and the subcutaneous layers. The skin epidermis and dermis constitute a cell-rich cutaneous immune system. There are a number of antigen-presenting cells (APCs), including

macrophages and, more importantly, Langerhans cells (LC) and dermal dendritic cells (DDC). In addition, any epidermal injury, including injection of collagen in adjuvant, results in release of pre-formed IL-1α from keratinocytes which initiates production of a number of cytokines and growth factors, in turn promoting LC and DDC differentiation and migration to the draining lymph nodes. Such dendritic cells (but not macrophages) are capable of activation of self-reactive T cell clones, initiating a cascade of events leading to the attack on autologous cartilage and to pannus-driven joint destruction.

In subcutaneously inoculated animals the absence of arthritis may result from inefficient presentation of collagen by macrophages, which are the only APCs present in the subcutaneous layer, and the lack of cytokine stimulation. Such inefficient presentation of the antigen may further tolerize self-reacting T cells resulting in amelioration of subsequently induced CIA. The finding of significant prevalence of IgG2a and IgG2b anti-CII antibody subtypes, which are typical for the immune responses mediated through DCs, in intradermally injected rats as compared to subcutaneously inoculated animals indirectly supported this hypothesis. This is consistent with a recently proposed theory for pathogenesis of RA that suggests a leading role of the DCs in RA induction and development.

Once the DA rat strain was chosen as the most suitable model for our study and the route of CII inoculation established, the analysis of the joint changes was carried out at different time-points of CIA before and after the onset of arthritis. Rats injected with adjuvant alone and normal rats were used as controls.

CIA, in DA rats, was characterised by an acute onset at day 12-14 after collagen injection and was a very severe disease. Arthritis progressed quickly, reaching its peak at day 5 after onset and subsiding with the development of joint ankylosis by day 20. This pattern differs from a remitting arthritis in humans, however the resolution into ankylosis is also typical of RA. The acute course of CIA can be an advantage allowing observation of joint changes within a shorter time frame.

No macroscopic or histological changes were found in the joints before the onset of arthritis. The time of onset was characterised by an influx of fluid and inflammatory cells. The infiltrate increased, reaching its maximum at day 5 after onset. By this day the pannus was well formed. The pannus expanded and continually destroyed bone and cartilage until day 20 after onset. The inflammatory infiltrate almost resolved by day 20 and was replaced by fibrous tissue. The pannus formation and bone and cartilage erosions were very similar to the changes seen in rheumatoid joints.

Neutrophils, which are absent in normal joints, were the first cells to appear in the synovium at the time of onset. Other cells, such as monocytes, T cells and NK cells also emerged, though in much lesser numbers. Mast cells were found around capillaries filled with inflammatory cells at the time of onset and disappeared or had an "empty" appearance after the onset of arthritis. It can be speculated that the recruitment of inflammatory cells to the joint may be mediated through degranulation of mast cells in the synovium.

By day 3-5 after onset of arthritis monocyte/macrophages and fibroblasts formed the pannus tissue. Monocyte/macrophages and osteoclasts were almost exclusively seen at the invading front of the pannus. Monocyte/macrophage numbers in the synovium

were increased perhaps due to recruitment rather than proliferation of the ED-1^+/ED-2^- subpopulation. Around $^2/_3$ of all monocyte/macrophages were activated as they expressed MHC class II.

Neutrophils were consistently found on the surface of the cartilage, as well as in the deeper layers of the synovial infiltrate, suggesting their role in augmentation of the exposure of cartilage antigens. Neutrophil numbers appeared to correlate with the severity of arthritis. They almost completely disappeared by day 20 after onset of arthritis, when fibrous ankylosis developed.

Unlike chronic RA, where T cell aggregates dominate the synovium, the proportion of T cells in CIA synovium never rose over 10% of total leukocyte numbers. However, this population underwent a 12-fold expansion by day 5 after onset. Many of the T cells expressed IL-2 receptor. This data suggests the involvement of T cells in the regulation of arthritis. The absence of the T cell aggregates seen typically in the RA synovium could be due to the lack of chronicity in CIA. $CD8^+$ cells were commonly seen in the CIA synovium. There was also a significant increase in NK cells, which were probably contributing to the increase in $CD8^+$ cell numbers. Very few B cells were seen in the synovium, probably because of the acute course of arthritis and the production of antibodies in the lymph nodes.

Overall, the pannus formed during progression of CIA was very similar to the pannus in rheumatoid joints. Despite lacking chronicity and associated T cell aggregates, the synovial changes in CIA joints resemble the ones in early and acute RA, justifying the use of the CIA as a model for the human disease.

The severity of pain and inflammation in CIA together with the apparent involvement of mast cells at the time of onset suggested involvement of the neuro-endocrine system and prompted an investigation of nerve growth factor (NGF) expression in CIA joints. NGF is a member of the neurotrophin family, which in adult animals is produced in increased amounts at inflammatory sites. NGF has recently been shown to regulate a number of immune cell functions, acting through its receptors trk A and p75.

NGF was absent in pre-arthritic synovium, but was significantly increased after the onset of arthritis. The study demonstrated that cells positive for NGF were monocytes/macrophages and neutrophils, while T, B and NK cells were negative. NGF expression was significantly down regulated at day 20 after onset, when the inflammatory infiltrate was no longer prominent and was replaced by fibrous tissue.

It was found that normal synovial cells, both type A and type B, express trk A receptor and that during joint inflammation a significant number of the infiltrating cells, including monocytes/macrophages and neutrophils, bear trk A receptor. p75 was found to be expressed by peripheral nerve endings in the normal synovium. The pattern of its expression did not change during CIA. The reported findings suggest that NGF may play an important role in the development of arthritis.

Similarities in the morphological manifestation of CIA to human RA suggest that similar processes may take place in rheumatoid synovium. Elevated NGF levels in the synovial fluid of patients with various forms of arthritis, support this hypothesis. Therefore, it is possible that in some patients, therapies directed at NGF inhibition

may prove beneficial, as NGF blockade may suppress both pain and the inflammatory process.

In summary, this thesis describes the optimal conditions for CIA induction and examines the development of joint injury in CIA. Two different routes of collagen administration are discussed: arthritogenic intradermal route and protective subcutaneous route. A hypothesis explaining the difference in the effects produced by these two routes of collagen administration is proposed. Consecutive histological changes in the CIA joints are described and the analysis of inflammatory cell populations is carried out. The thesis also shows that a neurotrophic factor NGF and its receptors are present in the inflamed synovium, suggesting that this growth factor may contribute to the development of arthritis. These findings have prompted a number of studies, which aim to further elucidate the role of NGF in CIA and RA. If NGF is implicated as a contributing factor to joint inflammation, its blockade may be considered as another approach to the future treatment of RA.

Chapter 7

BIBLIOGRAPHY

VII. BIBLIOGRAPHY

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