

Evaluation of a model for post-partum arthritis and the role of oestrogen in prevention of MRL-*lpr* associated rheumatic conditions

L. G. RATKAY, D. ZHANG, J. TONZETICH, J. G. LEVY* & J. D. WATERFIELD *Department of Oral Biology, Faculty of Dentistry, and *Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada*

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SUMMARY

Sixty-eight percent of female MRL-*lpr* mice developed a post-partum exacerbation of their mild spontaneous arthritis within 30 days of parturition. The flare became evident between 5 and 15 days after delivery. Histologically it was characterized by a significant increase of subsynovial inflammation and synovial hyperplasia without changes in the level of cartilage and bone erosion. Immunohistologically, marked subsynovial and frequent synovial staining of MHC class II bearing cells was noted, along with the sporadic presence of CD3, CD4, and CD43 receptor-bearing cells in the subsynovium. Injection of physiological levels (0.08 mg/kg) of estradiol on days 2, 3, 9, 15 and 20 post-partum delayed and reduced the flare to 23% of the animals. Administration of pharmacological amounts (0.4 mg/kg per day for 2 weeks following Freund's complete adjuvant injection) prevented adjuvant-enhanced arthritis, reducing the incidence from 67% to the baseline 21% level. Deleterious changes in the underlying systemic lupus erythematosus (SLE), as demonstrated by proteinuria and mortality rate increases, were elicited only by the employed pharmacological amounts of estradiol. These results indicate that the MRL-*lpr* mice might serve as a model for post-partum flare of arthritis in SLE and rheumatoid arthritis by providing an approach to study the complexity of the effects of pregnancy on autoimmune diseases, and to obtain further evidence for the involvement of oestrogen in arthritis.

Keywords arthritis post-partum flare mice oestrogen systemic lupus erythematosus

INTRODUCTION

Systemic lupus erythematosus (SLE) is a progressive connective tissue disorder accompanied by a wide spectrum of autoantibody production. The tissues/organs commonly affected are the skin, kidney, brain, vasculature, heart, and diarthroid joints. Articular disease is the most frequent early manifestation of SLE, being present in more than 90% of cases. This articular pathology is often transient, and generally less severe than that seen in rheumatoid arthritis (RA). As articular changes often precede other features of SLE, they are frequently diagnosed as RA. Notwithstanding, RA also may coexist with SLE [1].

SLE is a disease that most frequently afflicts women of childbearing age, and RA also occurs three times more frequently in women than men [2,3]. Therefore the effect of pregnancy on these diseases is of particular importance. It is

known that oestrogen levels increase during pregnancy, and decline immediately after delivery. Oestrogen has two known effects on the immune system: it suppresses antigen-specific T cell-dependent immune functions, characteristic of RA, and stimulates B cell responses, characteristic of SLE [4]. While the exacerbating effect of pregnancy in SLE is controversial [5,6], the ameliorating effect in RA is well described [7]. However, more than 90% of patients with RA remission during pregnancy develop a post-partum flare up of the disorder [8]. These findings suggest that oestrogen might influence these rheumatic conditions.

The MRL-*lpr* mouse strain has been successfully used as a model for studying both SLE and RA [9-15]. It develops a mild spontaneous form of arthritis which can be enhanced by an intradermal injection of Freund's complete adjuvant (FCA) [16]. Although the sex hormone involvement in SLE in MRL-*lpr* is well characterized [17-26], oestrogen has been studied less in respect to its effect on MRL-*lpr* arthritis [27], and has not been described in relation to pregnancy in these mice.

Correspondence: Dr L. G. Ratkay, Department of Oral Biology, Faculty of Dentistry, University of British Columbia, 2199 Wesbrook Mall, Vancouver, BC, Canada V6T 1Z3.

We noticed during routine breeding of MRL-*lpr* mice that a significant number of older female breeders developed post-partum erythema and swelling. Hence, this study examines the possibility of whether the MRL-*lpr* spontaneous arthritis could serve as a model for post-partum arthritis flare up in SLE or RA. We also investigated the effect of estradiol on the murine arthritis, using both the post-partum arthritis flare up and the adjuvant enhanced arthritis models in MRL-*lpr* mice.

MATERIALS AND METHODS

Animals

MRL/MpJ-*lpr/lpr* (MRL-*lpr*) mice were obtained from a breeding colony maintained in the animal facilities at the Department of Oral Biology. This colony was established from stocks originally purchased from the Jackson Labs (Bar Harbor, ME) and bred for two to nine generations. Histological analysis of different generations of these mice demonstrated no difference in the arthritic pathology of the spontaneous arthritis, in its post-partum flare up, or in the ability of FCA to enhance the disease (data not shown). The mice were kept on a standard diet with water *ad libitum*. Pregnant mice were housed one per cage. The colony was routinely screened for *Mycoplasma pulmonis*, and *Myc. arthritidis*, rodent coronaviruses (including hepatitis), and Sendai virus using the Murine ImmunoComb test (Charles River Labs, Wilmington, MA). Antibodies only against coronaviruses were detected.

Breeding protocol

Ten-week-old virgin females were mated. In the first experiment the females were separated from the males after mating, but not during the second experiment. The litters were not weaned from the mothers until the termination of the studies. At that time the female parents were 17 weeks old.

Adjuvant injection

Adjuvant injection was carried out as described previously [16]. Briefly, 13-week-old female mice were injected intradermally at two thoracic sites (selected for good lymph drainage) with 0.05 ml FCA, supplemented to 10 mg/ml with heat inactivated *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI).

Estradiol administration

Animals were randomly selected for estradiol injection. They were injected subcutaneously with 3.2 µg (0.08 mg/kg) β-estradiol-3-benzoate in olive oil (Sigma, St Louis, MO) on days 2, 3, 9, 15 and 21 after parturition. This dosage of estradiol was shown previously to be adequate to provide physiological serum oestrogen levels characteristic of pregnancy [28].

In a separate experiment a pharmacological dose of 0.4 mg/kg per day in olive oil was administered subcutaneously, starting day 0 for 14 days, to non-mated adjuvant-injected animals. Controls were injected with olive oil only.

Clinical evaluation

Clinical evaluation was carried out as described previously [16,29,30]. Mice were examined 'blind' every second day for

30 days after injection for visual appearance of arthritis and scored as positive if erythema and swelling of a fore or hind paw(s) was observed. Bimalleolar ankle width measurements were also recorded every 5 days using a micrometer.

On day 30 the experiment was terminated. Lymphadenopathy was evaluated using a subjective 0–4 non-invasive scoring scale based on palpation of the lymph nodes. The degree of lymph node involvement was rated as: 0, no palpable lymph nodes; 1, one palpable lymph node in one area; 2, seriously enlarged lymph node(s) in one area; 3, seriously enlarged lymph node(s) in both axillary and inguinal areas; and 4, massively enlarged lymph nodes in all areas.

Renal function was assessed on the basis of semiquantitative colorimetric determination of the urinary protein level, using Albustix sticks (Miles, Ontario, Canada). Protein was measured as g/l, with concentrations above 0.3 g/l considered abnormal.

Histological evaluation

After the animals were killed the joints and kidneys were dissected and fixed in 10% formalin. The joints were then decalcified for 48 h in 10% formic acid. Both tissues were subsequently processed for paraffin embedding. Serial 3–5 µm thick sections were stained with haematoxylin and eosin (H&E).

Histopathological alterations of the joints were evaluated 'blind' for the presence of the following features: 1, subsynovial inflammation; 2, synovial hyperplasia; 3, cartilage erosion and pannus formation; and 4, bone destruction. Within each parameter scores were assigned from 0 to 2 (0, no; 1, mild; and 2, severe involvement).

Kidney sections were also examined 'blind' by a pathologist for mesangial cell proliferation, crescent formation, interstitial cellular infiltration, and vasculitis. The histological changes were evaluated by a grading system from 0 to 4, where 4 corresponded to severe, 3 moderate, 2 mild and 1 minimal presence of diseased lesions.

Immunohistological evaluation

In order to identify subpopulations of cells in the affected areas and any changes during disease progression, the tarso-metatarsal joints from mice of both control and experimental groups were fresh frozen and cryosectioned. The immunohistology was carried out applying commercially available biotinylated MoAbs directed against mouse cell surface antigens: MHC class II (Ia^b) antigen and IL-2 receptor (activation markers), CD3 (pan T), and CD4 (T helper) (Life Technologies, Gaithersburg, MD). Biotin-labelled anti-CD43 (activated T) antibody was kindly provided by Dr H. Ziltener (Biomedical Sciences, University of British Columbia).

The tissue blocks were immersed in Histo Prep (Fischer, Fair Lawn, NJ), snap frozen in liquid nitrogen-prechilled isopentane, and stored at –70°C for a maximum of 2 weeks before cryosectioning. Sagittal sections of 8–10 µm thickness were cut on a cryostat at –25°C. The sections were attached to adhesive tape (Scotch Brand Tape 600, 3M Canada) [31,32] collected on glass slides, and stored at –20°C for a maximum of 1 week. Localization of the antibody binding was established by a biotin-streptavidin-alkaline phosphatase method. The non-specific binding sites were saturated during 30 min incubation

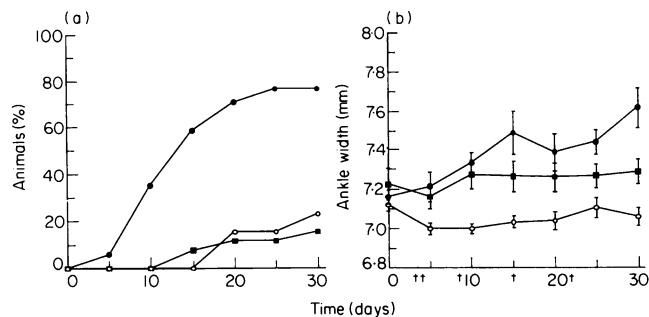


Fig. 1. Post-partum flare up and the effect of oestrogen on the arthritis of MRL-*lpr* mice. (a) Incidence of swelling and erythema. (b) Changes in bimalleolar ankle width. The 30-day post-partum period had an exacerbating effect on the clinical arthritis parameters (●). One group of animals after delivery (○) received 0.08 mg/kg estradiol at days 2, 3, 9, 15, and 21 and showed an incidence comparable to, and a severity lower than the control group with spontaneous arthritis (■).

with rabbit serum (Dako, Carpinteria, CA). After washing in Tris-buffered saline pH 7.6 (TBS), biotin-labelled antibody was applied overnight at 4°C in 1:50 dilution in TBS containing 1% bovine serum albumin (BSA). During incubation the slides were kept in a moist chamber to prevent them from drying. After rinsing with TBS, the alkaline phosphatase-conjugated streptavidin (Dako, Carpinteria, CA, and Life Technologies) was added for 30 min. After washing again in TBS the slides were treated with New Fuchsin containing TBS supplemented with 0.1% Levamisol (Sigma) according to the manufacturer's specifications. The slides were developed under the microscope for about 5 min until the desired intensity of red staining was achieved. The slides were then counterstained with Mayer's haematoxylin (Sigma), covered with Crystal Mount (Biomed, Foster City, CA) overnight at room temperature, permanently mounted with Entellan (Merck, Darmstadt, Germany) and evaluated under a light microscope. For a negative control, TBS was substituted for the primary antibody. A lymph node from the same animal as well as the bone marrow within the test section served as a positive control. Alkaline phosphatase labelling was employed, as peroxidase staining has been reported as unreliable if the method includes the use of adhesive tape. Endogenous staining was reduced by 0.1% Levamisol.

Statistical analysis

Data are expressed as mean \pm s.e.m. Statistical differences among groups were determined using the ANOVA and Bonferroni multiple comparison tests.

RESULTS

The incidence of swelling and erythema in post-partum animals during 30 days after delivery was observed in 76% (13/17) of the mice (Fig. 1a). This was confirmed in a second experiment in which 67% of the animals demonstrated similar evidence of swelling and erythema (16/24) (data not shown). The clinical onset of post-partum flare was successfully prevented (21%, 3/14) and the time of the onset delayed by the administration

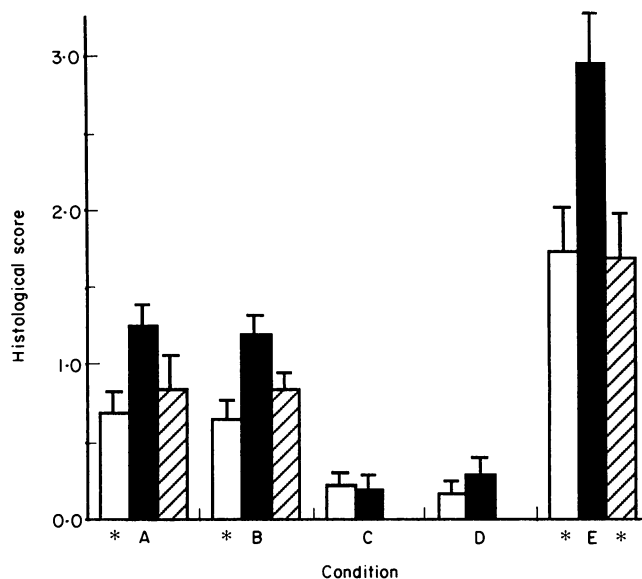


Fig. 2. Histopathological evaluation of the joints 30 days after delivery. The histological scores were determined as described in Materials and Methods. A, Subsynovial inflammation; B, synovial hyperplasia; C, pannus formation and cartilage erosion; D, bone destruction; E, overall histological score. *The corresponding value is significantly different from the post-partum arthritis group's value ($P < 0.05$, ANOVA and Bonferroni multiple comparison test). □, Spontaneous arthritis ($n = 23$); ■, post-partum arthritis ($n = 20$); ▨, post-partum arthritis + estradiol ($n = 13$).

of physiological amounts of estradiol. This frequency seen in estradiol-injected animals was comparable to the frequency of clinical signs of arthritis onset in non-mated, age-matched female MRL-*lpr* mice, which developed spontaneous arthritis (23%, 5/22). Bimalleolar ankle measurements, which quantify the severity of the swelling, showed similar patterns (Fig. 1b), although there was a remission of swelling in some of the cases in all three groups.

Subsynovial inflammation, synovial hypertrophy, and the overall histological scores increased significantly post-partum ($P < 0.05$) (Fig. 2). However, there was no significant increase in cartilage erosion, pannus formation, or bone destruction. Administration of physiological doses of estradiol significantly lowered the overall histological score ($P = 0.034$), and prevented cartilage erosion, pannus formation, and bone destruction. It also decreased somewhat the level of inflammation and synovial hypertrophy.

Figure 3 demonstrates the extent of subsynovial inflammation and synovial hypertrophy. Figure 3a depicts the subsynovial infiltration of MHC class II-positive cells. Cells also expressed the MHC II antigen at certain areas of the synovial lining (Fig. 3b). There was a sporadic, but significant presence of CD3- and CD43-expressing cells, as well as occasional cells staining for the CD4 marker (Fig. 3c). Anti-IL-2R MoAbs were rarely detected in the test or the positive control sections.

The post-partum period, or the short-term administration of physiological amounts of estradiol, did not significantly alter any systemic disease manifestations, recorded at the termination of the experiment. These mice developed a marked

lymphadenopathy, significant proteinuria, glomerulonephritis, and autoantibody levels (Table 1).

The effect of pharmacological amounts of estradiol (0.4 mg/kg) was also investigated on the development of adjuvant-enhanced arthritis in female MRL-lpr mice. Thirteen-week-old mice received this dose daily for 2 weeks starting on the day of the FCA injection. This dose prevented the adjuvant enhancement of disease, as indicated by the similar frequency of clinical arthritis observed in treated and control animals (Table 2). The severity of swelling was significantly lower than in adjuvant-injected mice ($P = 0.012$). Histological analysis revealed a significant degree of prevention in all the pathological parameters examined, including the overall score ($P < 0.05$). This dose, however, appears to increase the mortality level to 34%, compared with 22% which is characteristic of this strain at 4 months old (Table 3). In the survivors this was accompanied by a significant decrease of lymphadenopathy ($P \leq 0.02$), and a marked increase of proteinuria in some animals, resulting in a non-significant ($P \geq 0.183$) increase in the mean proteinuria level.

DISCUSSION

The hormonal changes associated with pregnancy affect even the healthy mother, and can seriously alter the course of autoimmune diseases. Pregnancy causes flare up in active SLE. While it ameliorates RA during gestation, it exacerbates the disease after delivery [33]. Pregnancy and lactation also increase the dangers of the side effects that accompany the standard treatment in these diseases. Therefore, it is deemed imperative to develop animal models which allow the study of the mechanisms involved in these complex situations and test treatment modalities which could be used without endangering the health of both the mother and the fetus or breast-fed newborn.

The most frequently used model for studying the effect of pregnancy in arthritis is collagen-induced arthritis. The pre-partum remission and post-partum exacerbation, as well as the acceleration of the onset of the disease by pregnancy, were described in a study that was conducted in a small number of DBA/1 mice with type II collagen-induced arthritis [34]. Recently it was reported that pregnancy prevented pristane-induced arthritis [35] and proteoglycan-induced arthritis in mice [36]. As there are differences in both strain and in model in the reaction to pregnancy, it is important to investigate the effect of pregnancy in alternative model systems.

The MRL-lpr mouse strain is a well studied model of SLE. It carries a dysfunctional *Fas* gene, resulting in the lack of thymic apoptosis, and the proliferation of CD3⁺, CD4⁻, CD8⁻ lymphocytes [37]. This genetic defect results in an early onset of SLE-like symptoms, including immune complex glomerulonephritis, anti-nuclear and anti-extracellular matrix autoantibodies, rheumatoid factors, and vasculitis with a 50% mortality rate at 5.5 months of age [38]. It is the only described spontaneous SLE model which also develops an arthritis. SLE symptoms and some early histopathological signs of arthritis are already present in 2-month-old animals [39].

The present study shows that this spontaneous form of arthritis in animals mated at 10 weeks of age is significantly exacerbated during the post-partum period. The reason this

finding was not previously noticed is most probably that the MRL-lpr mice are generally bred at a younger age to avoid the severity of the SLE, thereby dampening the intensity of the flare up of arthritis. This age-dependence, reflecting the time required to reach a certain stage of underlying disease, was also noted in this strain during the development of the adjuvant-enhanced arthritis model (manuscript submitted). It is of interest that in both cases approximately 2/3 of the animals developed exacerbated arthritis. This indicates the presence of an underlying component of the disease at this age that can be accelerated by certain factors. The flare-up occurred as early as 5 days after delivery, with a mean onset of 13 days, and resulted in a massive subsynovial inflammatory infiltrate. There was, however, no considerable cartilage or bone destruction by day 30 post-partum. We are planning to examine the longer-term effect of the flare-up on the severity of arthritis histopathology in post-partum *versus* non-mated female mice.

The ameliorating effect of pregnancy on RA has been attributed to physiological factors, including oestrogen, progesterone, prolactin [40,41], pregnancy-associated proteins as immunosuppressors (α -2 glycoprotein), suppression of cell-mediated immunity, and alterations in the glycosylation of IgG [8] acting as possible mediators [42]. In post-menopausal women oestrogen treatment of active RA increased bone mineral density [43]. The effect of oestrogen containing contraceptives has been described as well [44]. However, there is no clear evidence whether the use of oral contraceptives provides protection against RA. This inconclusiveness is probably due to differences in patient selection, as European hospital-based studies contradicted US population-based findings.

Oestrogen is a probable factor in the controversial effect of the pill. Although it has been previously reported that female sex hormones have only a minimal therapeutic effect on RA [45], the latest controlled study showed that adjunct therapy with oestrogen, when applied to post-menopausal RA, can be effective if the resulting serum oestrogen levels are adequate [46]. It is noteworthy that most of the pills contain both oestrogen and progesterone [47].

Holmdahl *et al.* proposed that the remission of RA during pregnancy is primarily due to oestrogen, and that progesterone might potentiate this event. It is clear that progesterone alone had no effect in preventing type II collagen-induced arthritis in mice [48]. Mattsson *et al.* suggested that post-partum flare-up is due to both the fall in the elevated pregnancy steroid levels and the surge in prolactin release after delivery [49]. Holmdahl *et al.* described the role of oestrogen in RA prevention as model- and strain-dependent [50]. Schlaghecke *et al.* reported the preventive effect of oestrogen on adjuvant arthritis in rats [51]. The MRL-lpr arthritis models could serve as alternatives to the above models, since there is a need to study the described model and strain dependency of the oestrogen effect. Here we report the preventive effect physiological doses of estradiol have on the post-partum flare-up in spontaneous arthritis and the effect pharmacological doses have on adjuvant-enhanced arthritis in MRL-lpr mice.

These data extend and support the concept of oestrogen influence in RA, and provide a new spontaneous model for studying post-partum flare-up. Further investigations are needed to study the possible involvement of other factors, and to develop a strategy for safe therapeutic intervention.

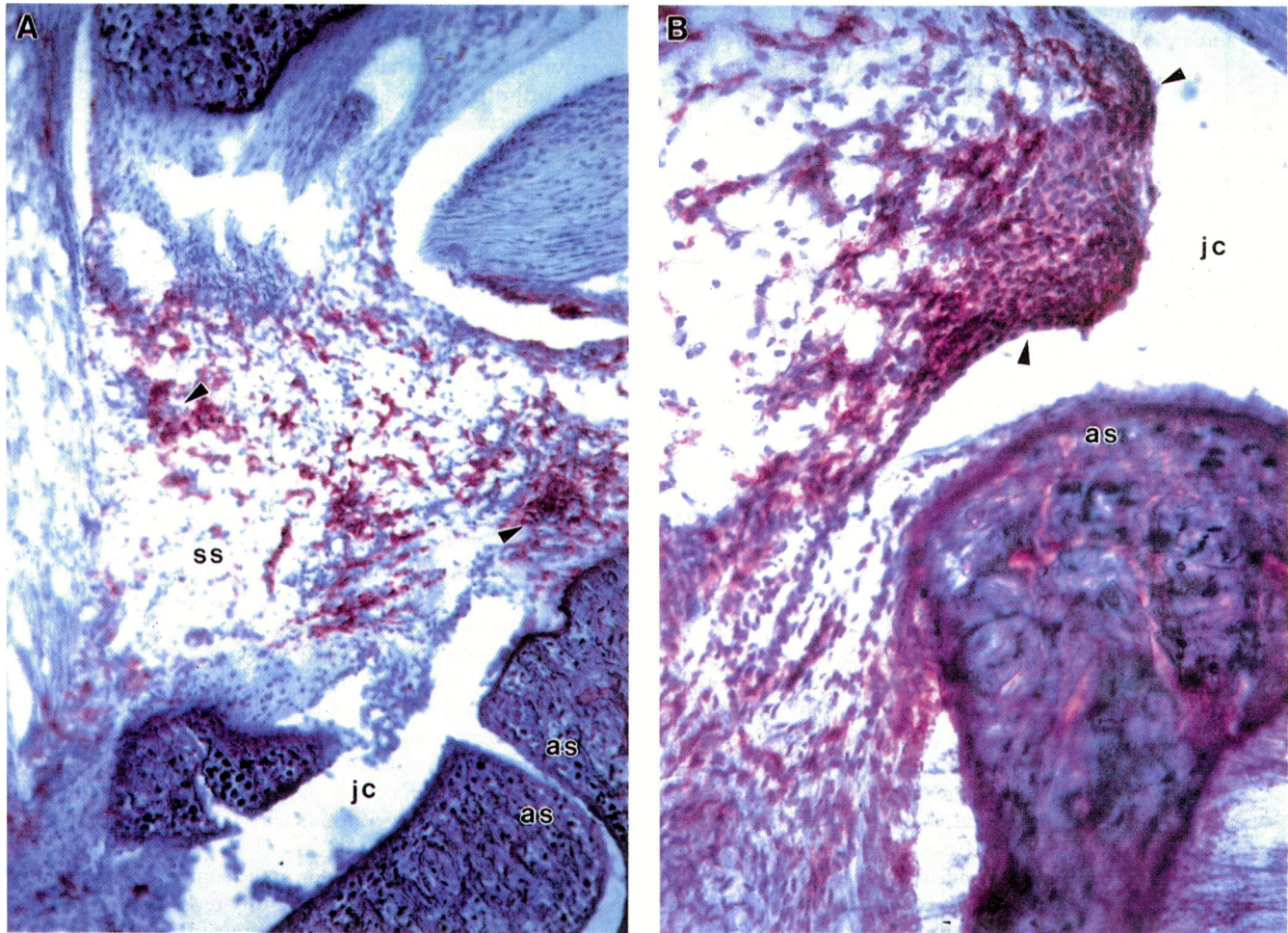


Fig. 3. Immunohistological characterization of post-partum arthritis in MRL-*lpr* mice. Frozen sections of the tarso-metatarsal joints of 4-month-old female MRL-*lpr* mice (30 days post-partum) were stained with an alkaline-phosphatase avidin-biotin method. (a) The inflamed subsynovial tissue is infiltrated with anti-MHC class II antibody-stained cells (arrowheads) (New Fuchsin counterstained with Mayer's haematoxylin, $\times 110$). (b) Hyperplastic synovial lining cells express the MHC class II marker (arrowheads) (New Fuchsin counterstained with Mayer's haematoxylin, $\times 220$). (c) Anti-CD4 antibody staining cells infiltrated the highly expanded subsynovial tissue (arrowheads) (New Fuchsin counterstained with Mayer's haematoxylin, $\times 110$). (d) A control animal showing the normal thickness of synovial lining and the lack of infiltrating cells in the subsynovium. No staining is detected with anti-CD4 antibodies (New Fuchsin counterstained with Mayer's haematoxylin, $\times 110$). as, articular surface; jc, joint cavity; sh, synovial hyperplasia; ss, subsynovium. Arrowheads define areas where cells stained positively.

Table 1. Systemic parameters in post-partum flare up of arthritis in MRL-*lpr* mice

Animals*	Spontaneous arthritis	Post-partum arthritis	Post-partum arthritis + estradiol	P_1, P_2
Mortality	7/32 22%	5/21 24%	3/14 21%	
Weight (g)	40 \pm 0.94 (23)	43 \pm 0.89 (8)	42 \pm 1.07 (12)	0.210, 1.000
Lymphadenopathy†	3.13 \pm 0.24 (23)	2.75 \pm 0.37 (8)	2.75 \pm 0.35 (12)	1.000, 1.000
Proteinuria†	1.23 \pm 0.23 (23)	1.40 \pm 0.41 (8)	1.02 \pm 0.35 (12)	1.000, 1.000
Kidney histology	6.75 \pm 0.93 (12)	5.00 \pm 1.34 (5)	NE	

* Data are presented as the mean \pm s.e.m. of the measurement at the end of the experiment. The number of animals is listed in parentheses.

† See Materials and Methods.

P_1 , ANOVA and Bonferroni multiple comparison test results at P significance level between spontaneous arthritis and post-partum arthritis groups; P_2 , ANOVA and Bonferroni multiple comparison test results at P significance level between post-partum arthritis with estradiol-injected and post-partum arthritis groups; NE, not examined.

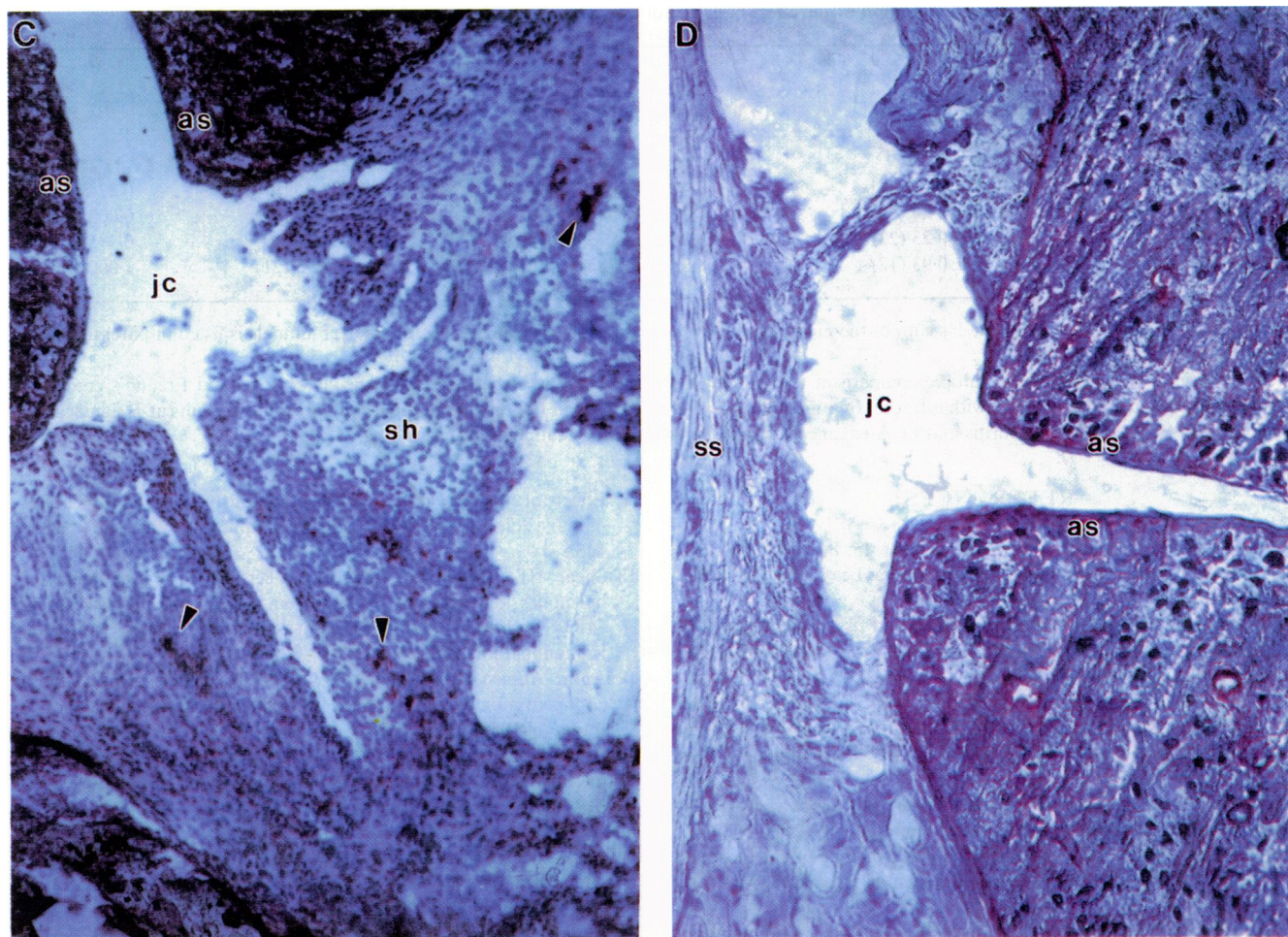


Fig. 3. (Continued).

Table 2. Evaluation of the effect of estradiol on adjuvant-enhanced arthritis in MRL-lpr mice

Animals*	Spontaneous arthritis	FCA-enhanced arthritis	FCA-enhanced arthritis + estradiol	P_1, P_2
Swelling	4/26 15%	34/51 67%	4/19 21%	
Ankle width† increase	0.10 ± 0.04 (17)	0.30 ± 0.05 (38)	0.08 ± 0.05 (19)	1.000, 0.012
Subsynovial inflammation	0.70 ± 0.13 (23)	1.16 ± 0.09 (51)	0.47 ± 0.16 (19)	0.789, 0.015
Synovial hyperplasia	0.65 ± 0.12 (23)	1.27 ± 0.09 (51)	0.89 ± 0.07 (19)	0.518, 0.000
Pannus formation and cartilage erosion	0.22 ± 0.09 (23)	0.63 ± 0.10 (51)	0.05 ± 0.05 (19)	1.000, 0.019
Bone destruction	0.17 ± 0.08 (23)	0.55 ± 0.10 (51)	0.26 ± 0.10 (19)	1.000, 0.041
Overall score of histology	1.74 ± 0.29 (23)	3.61 ± 0.31 (51)	1.68 ± 0.28 (19)	1.000, 0.000

* Data are presented as the mean ± s.e.m. of the measurement at the end of the experiment. The number of animals is listed in parentheses.

† Means maximum increase in bimalleolar ankle width during the experiment.

P_1 , ANOVA and Bonferroni multiple comparison test results at P significance level between spontaneous arthritis and Freund's complete adjuvant (FCA)-enhanced arthritis with estradiol treatment groups; P_2 , ANOVA and Bonferroni multiple comparison test results at P significance level between FCA-enhanced arthritis and FCA-enhanced arthritis with estradiol treatment groups.

Table 3. Evaluation of the effect of estradiol on systemic parameters in MRL-*lpr* mice

Animals*	Spontaneous arthritis	FCA-enhanced arthritis	FCA-enhanced arthritis + estradiol	P_1, P_2
Mortality	7/32 22%	16/69 23%	10/29 34%	
Weight (g)	40 ± 1.0 (23)	41 ± 0.8 (39)	42 ± 1.4 (19)	0.824, 1.000
Lymphadenopathy†	3.13 ± 0.24 (23)	2.77 ± 0.22 (39)	1.79 ± 0.27 (19)	0.003, 0.020
Proteinuria†	1.23 ± 0.23 (23)	1.05 ± 0.15 (39)	2.69 ± 1.41 (19)	0.395, 0.183
Kidney histology†	6.75 ± 0.93 (12)	8.38 ± 0.75 (21)	7.27 ± 0.95 (21)	1.000, 1.000

* Data are presented as the mean ± s.e.m. of the measurement at the end of the experiment. The number of animals is listed in parentheses.

† See Materials and Methods.

P_1 , ANOVA and Bonferroni multiple comparison test results at P significance level between spontaneous arthritis and Freund's complete adjuvant (FCA)-enhanced arthritis with estradiol treatment groups; P_2 , ANOVA and Bonferroni multiple comparison test results at P significance level between FCA-enhanced arthritis and FCA-enhanced arthritis with estradiol treatment groups.

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