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Effect of pregnancy on collagen-induced arthritis in mice

GILLIAN T. WAITES* & A. WHYTE *AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, UK*

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SUMMARY

Bovine type II collagen administered to non-pregnant female DBA/1 mice caused arthritis in 55% of animals with a mean onset time of 70 days following immunization. Of collagen-treated females subsequently becoming syngeneically pregnant before the onset of arthritis, all developed the disease within 10 days of parturition, representing an earlier onset, compared to non-pregnant females, of 41 days. This earlier onset also occurred in females with an allogeneic pregnancy, but did not occur in females resorbing their fetuses (only syngeneic pregnancies were examined). In females with arthritis at the time of conception a significant remission was observed during pregnancy followed by exacerbation post-partum. This sequence of remissions during pregnancy and exacerbations post-partum occurred with each pregnancy (parities of up to four studied). The treatment of multiparous females with collagen demonstrated that pregnancy does not provide long-term protection against the development or progression of arthritis, as such females were equally susceptible to post-partum onset of collagen-induced arthritis (CIA) and the remissions and exacerbations described above. The modifying effect of pregnancy on CIA in mice is complex and does not appear to be the result of a single pregnancy-associated phenomenon.

Keywords Pregnancy DBA/1 mice collagen-induced arthritis

INTRODUCTION

The immunological changes associated with pregnancy and with autoimmune diseases are areas of continuous investigation. There is evidence that pregnancy can modify certain autoimmune conditions (Cercere & Persellin, 1981; Østensen & Husby, 1984; Mor-Yosef *et al.*, 1984), although not always beneficially. The modulatory effect of pregnancy on diseases such as systemic lupus erythematosus and ankylosing spondylitis remains controversial, but it is generally accepted that rheumatoid arthritis (RA) subsides during pregnancy with exacerbation frequently occurring post-partum (Persellin, 1977; Cercere & Persellin, 1981; Østensen & Husby, 1983; Mor-Yosef *et al.*, 1984). If the basis of this interaction could be determined it may indicate not only a potential treatment for RA but also provide a better understanding of the processes involved in preventing rejection of the fetus and in the immunological imbalance which results in autoimmunity.

It has been suggested that factors in serum during pregnancy may be responsible for the remission of RA seen during gestation, an idea supported by the extensive evidence showing inhibition of immune and inflammatory responses by human and animal pregnancy sera (Harrison, 1976; Appelboom & Persellin, 1984). An association between pregnancy and the inflammatory response is further suggested by the finding of a number of serum factors common to both pregnant females and animals during the acute-phase of an inflammatory response (Waites & Bell, 1986). Of

Correspondence: Dr A. Whyte, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, UK.

* Present address: Department of Obstetrics and Gynecology, Clinical Sciences Building, Leicester Royal Infirmary, PO Box 65, Leicester LE2 7LX, UK.

the factors considered for their role in amelioration of RA during pregnancy, serum levels of human pregnancy-associated α_2-glycoprotein (α_2-PAG), known to be immunoregulatory both *in vitro* and *in vivo* (Thomson & Horne, 1980), show the greatest correlation with remission (Persellin *et al.*, 1982; Unger *et al.*, 1983) although this has not been substantiated by all workers (Horne *et al.*, 1979; Østensen, von Schoultz & Husby, 1983).

As part of an investigation into the anti-inflammatory properties of pregnancy-associated factors, we have examined the effect of pregnancy on collagen-induced arthritis (CIA) in mice, a model originally described in rats by Trentham, Townes & Kang (1977), and used more recently in mice by Courtenay *et al.* (1980). The condition in the mouse provides a better model than the rat since it is not complicated by the arthritogenic activity of adjuvant, and the disease process in mice shows many similarities to human RA (Trentham, 1982).

MATERIALS AND METHODS

Animals. Mice of the inbred DBA/1 (H-2q) strain were purchased from Olac (Bicester, England). DBA/1 females were mated with males of the same strain and the morning on which a vaginal plug was detected was designated day 0 post-coitum (p.c.) of pregnancy. Some females were mated with males of the C57BL/10ScSn strain (H-2b) (Olac).

Preparation of bovine type II collagen. Articular cartilage obtained from the joints of calves, collected at autopsy from a local abbatoir, was carefully sliced from the bone using a scalpel blade, and collagen was extracted by the method of Herbage, Bouillet & Bernengo (1977) with some modifications. Briefly, following homogenization of the cartilage in liquid nitrogen, the proteoglycans were removed by stirring with 4 M guanidinium hydrochloride (BDH Chemicals Ltd, Poole) in 0·05 M Tris, pH 7·5, for 24 h at 4°C. The insoluble residue was washed with 0·5 M acetic acid, then digested with pepsin (twice crystallized, Sigma) at a ratio of 10:1 collagen:pepsin (w/w) for 24 h at 4°C. The collagen was precipitated with 0·9 M NaCl, centrifuged at 20,000 g_{max} for 1 h and redissolved in 0·05 M Tris buffer containing 0·5 M NaCl, pH 7·5. This precipitation was repeated by further addition of NaCl up to 3·5 M, centrifugation at 30,000 g_{max} for 1 h and redissolving ready for dialysis against 0·02 M disodium hydrogen orthophosphate buffer, pH 9·4. After centrifugation at 30,000 g_{max} for 1 h the collagen was dissolved in 0·5 M acetic acid and dialysed against 0·05 M acetic acid, then freeze-dried and stored in a desiccator at −20°C. The purity of the collagen preparation was assessed by electrophoresis in sodium dodecyl sulphate gels.

Induction and assessment of arthritis. Female mice were injected subcutaneously (s.c.) on day 0 in four sites on the back with a total of 100 μg of bovine type II collagen dissolved in 0·1 ml of 0·1 M acetic acid and emulsified in an equal volume of complete Freunds adjuvant (CFA; Difco, Michigan). Each animal received an intraperitoneal (i.p.) booster injection of 100 μg collagen in 0·1 ml of 0·1 M acetic acid on day 21. Female mice of the control group received two injections, the first including CFA, as above but with the vehicle (0·1 M acetic acid) alone. Some females were pregnant at the time of the booster injection. Mice were examined daily for the development of arthritis and the disease severity recorded in two ways. Firstly, micrometer measurements were made at regular intervals of the foot depth and joint width on the hind limbs. Secondly, a clinical scoring system was devised based on the visual appearance and mobility of the animal. Each limb was scored from 0 to 4, as shown in Table 1, giving a maximum score of 16 per animal. Statistical analyses were performed using the Fisher exact probability test and Student's *t*-test.

RESULTS

Type II collagen-induced arthritis in DBA/1 female mice. Micrometer measurements of foot depth and joint width correlated well with the clinical scoring system used for the assessment of arthritis (see Table 2) except in cases of severe arthritis (score of 4 per limb), where ankylosis was the main feature with foot oedema being greatly reduced (although the joint remained enlarged). For this reason most of the data are expressed in terms of the clinical scoring system. Figure 1 shows that

Table 1. Clinical scoring system devised for the assessment of type II collagen-induced arthritis in mice

Clinical score	Disease severity
0	Normal
1	Slight swelling of toes or foot or redness
2	Obvious swelling of foot or joint or both
3	Badly swollen foot and joint with oedema
4	Ankylosis and limb rigidity; reduced joint swelling and mobility of animal

Each limb is scored from 0–4 giving a maximum total of 16 per animal.

the percentage of virgin females with arthritis (Fig. 1a; closed symbols) increased steadily after the second injection up to 120 days. The mean onset time was $70{\cdot}2 \pm 21{\cdot}7$ days post-induction, i.e. after the primary injection (range 29–112 days; $n=37$). The mean clinical score also increased up to 140 days (Fig. 1b; closed symbols). In 55% of virgin females treated (37/67) there was some sign of arthritis, with 31% having a mean clinical score of >5 and therefore at least one limb with a score of >2. The physical appearance of arthritic limbs is shown in Fig. 2. All limbs were equally susceptible to arthritis and the severity of the disease (i.e. number of limbs involved and degree of swelling) at its outset was variable. Although the degree of swelling in the early or mild cases of arthritis did fluctuate, once a limb became badly inflamed or rigid the disease did not undergo spontaneous remission. Control animals received injections of acetic acid only, and no signs of arthritis were observed in these animals ($n=20$; assessed to day 207 after injection). In addition, no spontaneous development of arthritis was seen in untreated DBA/1 female mice.

Effect of pregnancy on the development of type II collagen-induced arthritis. Females were given a primary s.c. injection of collagen and 1 week later mated with males of the same strain. All females were given a booster i.p. injection of collagen as usual. Some were pregnant at this stage while others remained with the males until they became pregnant. The timing of the pregnancy was noted. The development of arthritis in these females having had one pregnancy is shown in Fig. 1 (open symbols). The percentage of primiparous females with arthritis on any given day throughout the study was significantly higher (by Fisher exact test) than that of virgin females (Fig. 1a; open symbols) and the onset of arthritis was significantly earlier in the former (primiparous females $41{\cdot}3 \pm 11{\cdot}2$ days after induction; range 22–70; $n=25$ compared to virgin females $70{\cdot}2 \pm 21{\cdot}7$ days after induction; range 29–112; $n=37$; $P<0{\cdot}001$ by Student's t-test). All primiparous females developed arthritis (of which 88% had a clinical score of >5) compared to 55% of the group of virgin females. In the majority of primiparous individuals the first signs of arthritis appeared after littering, a steady increase being observed over the first 10 days (see Fig. 3). Only rarely did arthritis develop in these females during pregnancy. There was a tendency for a higher clinical score in primiparous compared to virgin females (Fig. 1b; open symbols), particularly in the early stages of RA development, although this was not for the most part significant. Preliminary results suggest that collagen-treated DBA/1 females mated with C57BL/10ScSn males also experienced post-partum exacerbation of arthritis.

Multiparous DBA/1 females, which previously had three normal pregnancies, were also given injections of collagen and syngeneically mated. As with virgin females having one pregnancy after treatment with collagen the multiparous females developed arthritis following treatment and a 4th pregnancy. In no instance was arthritis observed in previously virgin or multiparous females during pregnancy (syngeneic only examined) after treatment with acetic acid alone ($n=10$ and 13 respectively). Spontaneous development of arthritis in untreated females following syngeneic or allogeneic pregnancy was never observed.

Effect of pregnancy on the course of established type II collagen-induced arthritis. Females with established collagen-induced arthritis had a remission of the disease during pregnancy. This occurred whether the arthritis first developed in non-pregnant females ($n=7$) or in females after a

Table 2. Relationship between clinical score and micrometer measurements of hind limbs used in assessing arthritis in mice

Clinical score†	Group size	Micrometer measurement Mean ± s.d. (mm)		% increase in measurement compared to non-pregnant female	
		Foot	Joint	Foot	Joint
0 (non-pregnant female)	59	1·84 ± 0·08 (59)	2·29 ± 0·19 (59)	—	—
0 *(acetic acid treated female)	33	1·83 ± 0·13 (66)	2·34 ± 0·21 (66)	0	2·2 NS
1	28	1·91 ± 0·16 (88)	2·37 ± 0·18 (88)	3·8	3·5
2	27	2·09 ± 0·25 (126)	2·61 ± 0·32 (126)	13·6	14·0
3	20	2·65 ± 0·52 (49)	2·97 ± 0·33 (49)	44·0	29·7
4	18	1·91 ± 0·19 (31)	2·71 ± 0·39 (31)	3·8	18·3

NS, not significant. All other increases are significant ($P < 0·001$).

* No difference was found in measurements between non-pregnant and pregnant females.

† Irrespective of the state of parity of the female (i.e. includes both virgin and previously pregnant mice).

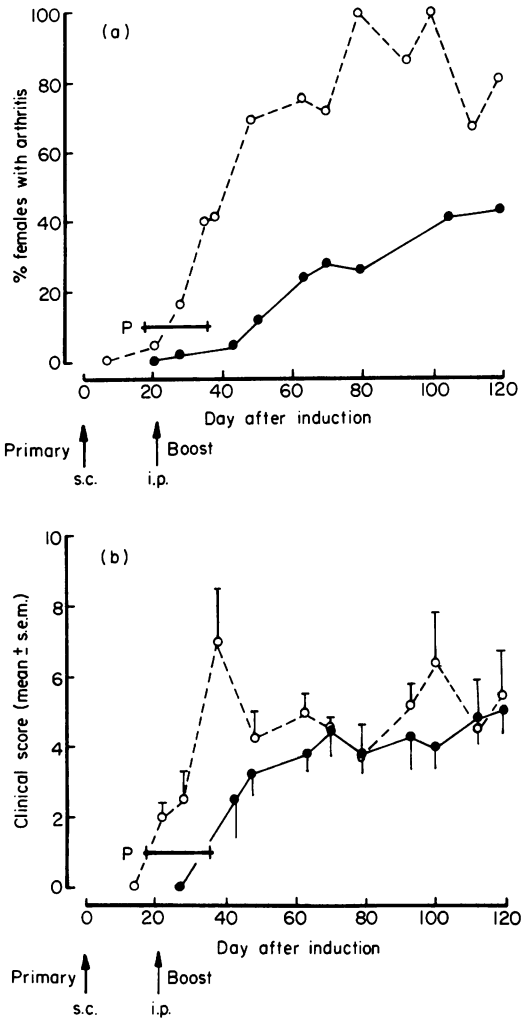

Fig. 1. Development of type II collagen-induced arthritis (CIA) in DBA/1 (H-2q) female mice. Animals were given a primary s.c. injection of 100 μg type II collagen dissolved in 0·1 M acetic acid and emulsified in CFA and an i.p. booster injection 21 days later in acetic acid without adjuvant. Results are expressed as (a) the percentage of females with arthritis, assessed by the clinical scoring system (see Table 1) and (b) the mean clinical score within arthritic animals. (●) Development in non-pregnant females (Fig. 1a, $n=67$; Fig. 1b, $n=37$); (○) development in primiparous females (Fig. 1a and 1b, $n=25$). These latter females were placed with males of the same strain from 1 week after the primary injection until they became pregnant. The booster injection was given on day 21 regardless of whether the female was pregnant or not. The percentage of primiparous females with arthritis is significantly higher than that of non-pregnant females by the Fisher exact probability test from day 28 after induction (day 28 $P=0{\cdot}018$; day 63 $P=0{\cdot}007$; day 70 $P=0{\cdot}029$; day 79 $P=0{\cdot}0002$). P = duration of pregnancy ± s.e.m.

single pregnancy ($n=11$), but was most evident in the latter due to the increased severity of arthritis at the onset of the second pregnancy. Figure 3 shows the progression of arthritis in primiparous females during and after a second pregnancy. The clinical score of arthritic animals decreased progressively throughout the second pregnancy (day 0 p.c. $7{\cdot}5 \pm 2{\cdot}67$; day 18 p.c. $2 \pm 1{\cdot}19$; $n=11$; $P<0{\cdot}001$ by Student's t-test), after which an exacerbation was seen to occur predominantly over the first 10 days post-partum as in the first pregnancy (day 18 p.c. $2 \pm 1{\cdot}19$; day 11 post-partum $7{\cdot}8 \pm 3{\cdot}5$;

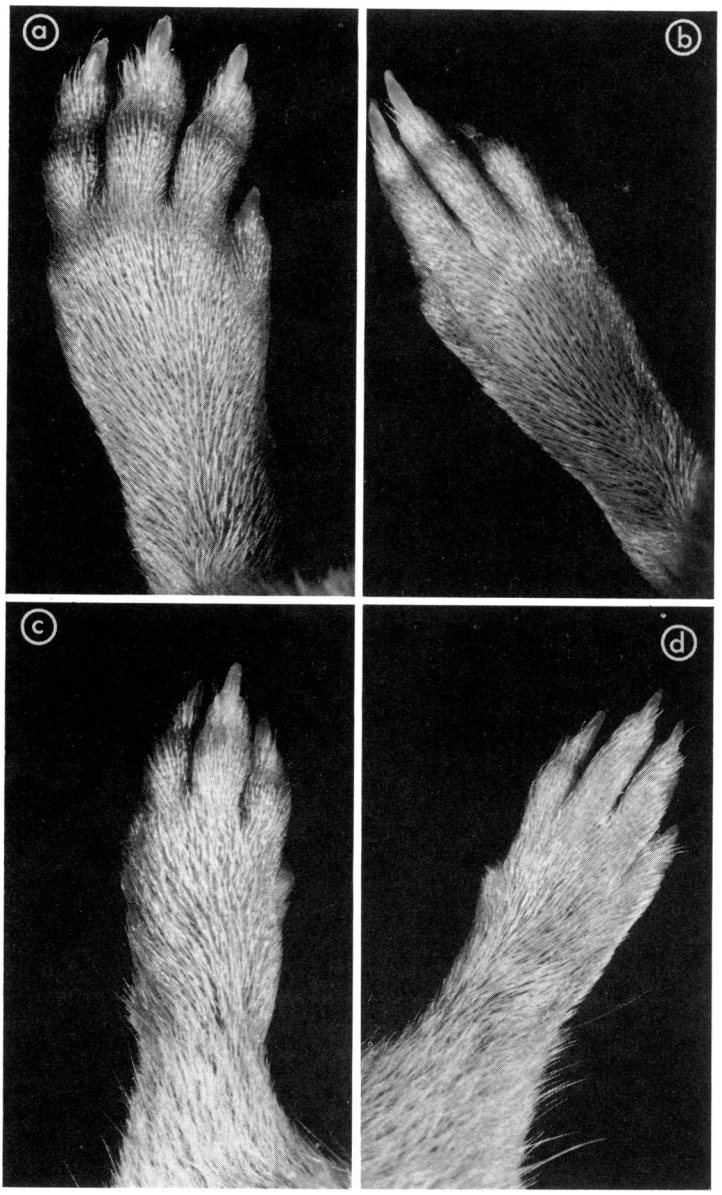

Fig. 2. (a) Hind limb of a female mouse with type II collagen-induced arthritis. The toes, foot and joints appeared red and inflamed and this would be classified as clinical score 3. (b) Normal hind limb of an untreated female mouse age-matched with (a) above. (c) Arthritic front limb of a collagen-treated female (clinical score 3), with overall swelling and redness compared to the equivalent front limb of an untreated female (d). Magnification × 6.

$P < 0{\cdot}001$). Remission was seen in all females having a successful second pregnancy, although symptoms rarely disappeared completely. Furthermore, this pattern of remission and exacerbation occurred in all subsequent pregnances so far examined, the greatest parity studied being four. Females experiencing three pregnancies before collagen treatment showed similar patterns of remission and exacerbation of arthritis as a result of subsequent pregnancies. Those animals with ankylosis did not appear to show remission in these joints as they were presumably too severely affected by the disease process.

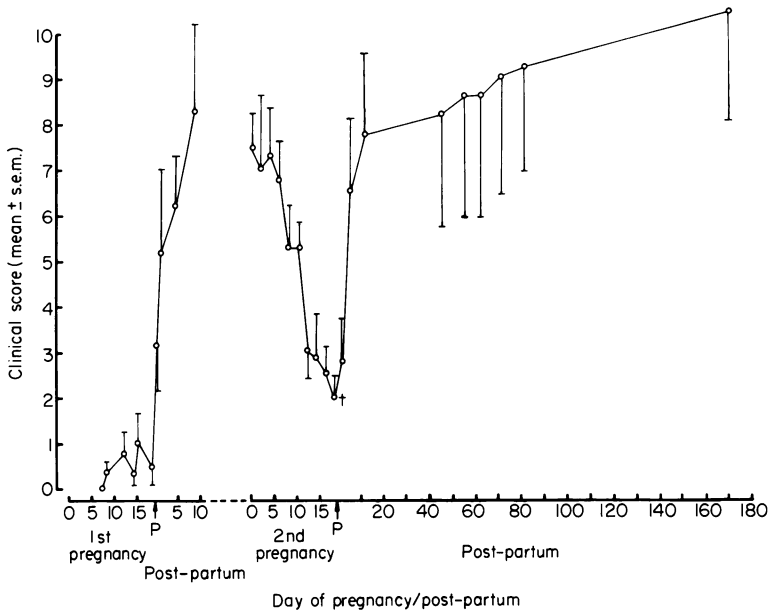

Fig. 3. The development and progression of arthritis in collagen-treated DBA/1 female mice during two pregnancies ($n = 11$). Results are plotted as a mean clinical score against the day of pregnancy (day of plug = day 0), aligning gestation dates at the start of each pregnancy irrespective of the day post-induction. Arthritis is seen to worsen after the first pregnancy, remit during the second, and increase even further after the end of the second pregnancy. In females having more than two pregnancies this pattern of remission and post-partum exacerbation continued (data not shown), and it also occurred in virgin females with collagen-induced arthritis established before mating. Non-pregnant females treated with collagen showed a steady increase in the clinical score of arthritis and no remission (see Fig. 1b). P = parturition. Note change of scale after second parturition. * Decrease is significant ($P < 0{\cdot}001$) by Student's *t*-test.

DISCUSSION

This study demonstrates that pregnancy can modify the development and progression of collagen-induced arthritis (CIA) in mice. Using the method originally described by Courtenay *et al.* (1980) it was found that s.c. injection of bovine type II collagen into female mice of the DBA/1 (H-2q) strain resulted in the development of arthritis in 55% of animals, with a mean onset time of 70 days after the primary injection. Susceptibility of mice to CIA has been shown to be H-2 linked (Wooley *et al.*, 1981), with the DBA/1 strain being of the more susceptible H-2q haplotype. Holmdahl *et al.* (1986) recently reported that autologous anti-type II collagen antibody responses are only seen in H-2q mice whereas those with p, w3, w5 and w17 haplotypes show only weak responses. They suggested that small modifications in the Ia chain of major histocompatibility complex (MHC) Class II determinants may be associated with susceptibility to arthritis.

Our findings support those of previous workers (Courtenay *et al.*, 1980; Wooley *et al.*, 1981) taking into account the fact that fewer females have been reported to develop arthritis, and then after a longer induction period, than males (Courtenay *et al.*, 1980; Holmdahl *et al.*, 1985), although the reason for this is not known. The precise relevance of CIA in mice to rheumatoid arthritis (RA) in humans is unclear, but these diseases do share a number of clinical, histological, immunological and genetic features (Wooley *et al.*, 1981; Trentham, 1982). The humoral immune response to collagen is thought to be important in CIA and RA, but although immunoglobulin preparations from the sera of arthritic mice have been shown to induce disease in other mice of the same strain (Stuart & Dixon, 1983), anticollagen antibody levels in serum do not reflect disease severity (Holmdahl *et al.*, 1985).

Pregnancy was found to have two major effects on CIA in mice. Firstly, in females with clinical symptoms of arthritis at the time of conception, a remission was seen during gestation, irrespective of the parity of the animal. Secondly, collagen-treated females with or without clinical symptoms of arthritis at the time of conception experienced post-partum exacerbation of the disease. In treated but non-arthritic females having their first pregnancy this represented an earlier onset and increased severity of arthritis compared to treated virgin females. These findings are not dissimilar to RA in women where, not only do 75% experience a remission during pregnancy and more than 90% an exacerbation post-partum, but cases have also been reported of onset of the disease after pregnancy (Cercere & Persellin, 1981).

There are many physiological and immunological changes associated with pregnancy which could be responsible for the clinical modifications of autoimmune or inflammatory conditions during gestation. A number of serum proteins have been shown to alter both during pregnancy and during an inflammatory response (Waites & Bell, 1986) implying some common regulatory process(es) between the two physiological conditions and attempts have been made to explain the remission of arthritis seen during pregnancy in terms of inhibitory factors in the serum. There are a number of proteins of interest in relation to the remission of RA including an anti-inflammatory substance termed pregnancy-associated prostaglandin synthetase inhibitor (PAPSI) described by Mortimer *et al.* (1985), and an endogenous anti-inflammatory substance lipocortin (Wallner *et al.*, 1986) the production of which is controlled by circulating steroids and therefore may be increased during pregnancy. Phadke *et al.* (1986) have suggested that there is enhanced production of interleukins 1 and 2 by mononuclear cells in type II collagen arthritis models. Since the aetiology of RA is unknown it is difficult to hypothesize how amelioration of the disease may occur. Recent studies in humans have underlined the importance of HLA-DR (MHC class II) antigen expression in the development of RA (Clot & Sany, 1984; Lee *et al.*, 1985), and enhancement of HLA-DR expression is a feature of rheumatoid joints (Ridley *et al.*, 1986). In mice, CIA can be suppressed or delayed by pre-treatment with anti-IA (≡HLA-DR) antisera (Wooley *et al.*, 1985). Also, Carter *et al.* (1984) have demonstrated increased IgG-Fc receptor expression on monocytes from RA patients compared to controls, suggesting that inhibition of Fc receptor expression may result in remission of RA. A human pregnancy-associated protein (α_2-PAG) has been shown to be capable of inhibiting both Fc receptor and HLA-DR antigen expression (Persellin & Rhodes, 1981), while its rodent counterparts are thought to interact with lectin receptors bound to the membrane of macrophages causing similar inhibition of receptor functions and antigen expression (Porstmann *et al.*, 1986).

Suppression of CIA in mice may also involve interference with expression of the L3T4 antigen (equivalent to OKT4/leu 3 in man), which is expressed on T cells responsible for class II MHC-restricted functions and has recently been suggested to be important in the development of CIA (Holmdahl *et al.*, 1985; Ranges, Sriram & Cooper, 1985). Ranges *et al.* (1985) have demonstrated that administration of monoclonal antibody *in vivo* to the L3T4 antigen to collagen-treated mice results in a reduced incidence of CIA. Whether L3T4 expression is altered during pregnancy is not known.

It would seem reasonable to suggest that pregnancy-associated soluble factors could explain the suppression of RA/CIA during gestation and that their disappearance post-partum would result in a return of symptoms. However, why arthritis should be worse post-partum and how the onset of disease noted in some humans and the earlier onset and severity noted in collagen-treated mice after parturition in this study can be explained is more difficult to envisage. It is possible that in these latter cases (especially in collagen-treated mice) preclinical disease existed and that pregnancy merely exacerbated this rather than causing it. In agreement with our work, a recent study by Hirahara *et al.* (1986) also reports a markedly rapid progression of CIA in mice post-partum compared to non-pregnant immunized females. It is possible that the unmasking of antigens, important in the development of RA, post-partum may result in a secondary immune response being mounted, which would characteristically be larger than the primary response. More work is needed to explain both the exacerbation of RA/CIA noted post-partum and also the increased susceptibility of primiparous mice to collagen.

In our studies remission of CIA in mice during pregnancy is progressive with the greatest suppression being in the third trimester, although it is important to remember that suppression of

CIA may not be immediate. In a small number of unsuccessful syngeneic pregnancies it was found that arthritic females resorbing their fetuses experienced some degree of remission of arthritis apparently related to the duration of gestation before resorption. This suggests that any serum factor or pregnancy-associated event inducing remission of arthritis must be present in the first half of pregnancy, although possibly not reaching full potential until mid to late gestation.

These females also seemed to experience exacerbation of arthritis following resorption. In contrast, preliminary results show that collagen-treated females resorbing their embryos in a first pregnancy (syngeneic) before development of arthritis did not experience the same earlier onset of arthritis noted after parturition in similar females whose pregnancies were completed (G. T. Waites & A. Whyte, unpublished work). Whether this indicates that exacerbation of CIA is an event dependent upon parturition is not yet known. The preliminary findings of similar effects of allogeneic pregnancy on CIA suggest that any relevant serum component or other pregnancy-associated event must be independent of paternal strain. The fact that pregnancy merely provides a temporary remission of the disease is seen not only by the return of CIA post-partum but also by the fact that multiparity prior to collagen treatment gives no additional protection to the female against arthritis.

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