Immunology 1993 **79** 152–157

Prevention of pristane-induced arthritis by the oral administration of type II collagen

S. J. THOMPSON, H. S. G. THOMPSON,*† N. HARPER,* M. J. DAY, A. J. COAD, C. J. ELSON & N. A. STAINES* *Department of Pathology and Microbiology, University of Bristol and* **Immunology Section, King's College, London*

Accepted for publication 9 December 1992

SUMMARY

This is the first demonstration of a role for type II collagen in pristane-induced arthritis. Pretreatment with soluble type II collagen either lowers or raises the subsequent incidence and severity of pristane-induced arthritis. These effects are dependent upon both the dose and route of administration of the soluble type II collagen. Increasing doses of orally administered type II collagen lowered both the incidence and severity of pristane-induced arthritis. Conversely, increasing doses of intraperitoneally administered type II collagen increased both the incidence and severity of arthritis. This exacerbation of pristane-induced arthritis was accompanied by elevated B- and T-cell responses to type II collagen. These findings highlight the importance of the site at which antigen is encountered in influencing subsequent immune responses and extend the observations of the use of orally administered antigens to ameliorate experimental autoimmunity.

INTRODUCTION

Pristane-induced arthritis (PIA) was first described in 1981 when the intraperitoneal (i.p.) injection of the non-antigenic mineral oil 2,6,10,14-tetramethylpentadecane (pristane) induced an arthritis between 100 and 200 days later in approximately one-third of the treated BALB/c mice.[1] This is an immune polyarthritis which both histologically and in its pattern of Ig glycosylation is similar to human rheumatoid arthritis.[2-6] Additionally, mice with PIA have elevated T- and B-cell responses to the 65,000 MW mycobacterial heat-shock protein (hsp65) and predosing mice i.p. with hsp65 blocks the development of PIA.[7]

Although immunological reactivity to type II collagen (CII) has not been shown to be a feature of PIA in CBA/Igb mice[7] this does not exclude the possibility that cartilage collagens may influence the course of PIA in these mice. It has been shown that prior exposure to CII can block the development of collagen-induced arthritis (CIA). Originally the intravenous administration of CII was shown to prevent CIA in rats.[8] More recently it has been shown that the oral administration of CII, prior to the arthritogenic challenge, blocks the development of both CIA[9,10] and adjuvant arthritis (AA).[11]

†Present address: Kennedy Institute of Rheumatology, London, U.K.

Correspondence: Dr S. J. Thompson, Dept. of Pathology and Microbiology, The Medical School, University Walk, University of Bristol, Bristol BS8 1TD, U.K.

This study set out to determine whether immunity to cartilage collagens can influence the development of PIA. We have examined immunological reactivity to cartilage collagens and assessed the effects of administering soluble collagens type II (CII) and type IX (CIX), either orally or i.p. on the development of PIA. We report that collagen immunity is a feature of PIA in so far as soluble CII can ameliorate or exacerbate PIA dependent upon its route of administration.

MATERIALS AND METHODS

Animals

Male CBA/Igb mice aged between 4 and 8 weeks were used.[2] CBA/Igb mice were originally a gift from Professor H. S. Micklem (Department of Zoology, Edinburgh, U.K.).

Induction and histopathological assessment of arthritis

Arthritis was induced by two intraperitoneal injections of 0·5 ml of pristane (Aldrich Chemical Co., Gillingham, U.K.) 50 days apart. The mice were examined visually for the incidence of arthritis in the tarsal (ankle) joints at various time-points, and in some experiments the arthritis was assessed by measuring the tarsal joints with a micrometer. Enlarged joints ranged in size from 3·0 to 4·2 mm compared with normal joints which had a range from 2·5 to 2·8 mm. Experiments were terminated 200 days after the initial injection of pristane. After death, stifle (knee) joints were dissected out, fixed in neutral-buffered formalin and decalcified. Longitudinal sections were prepared and were stained with haematoxylin and eosin. Arthritis was assessed by a veterinary pathologist (MJD) who scored the

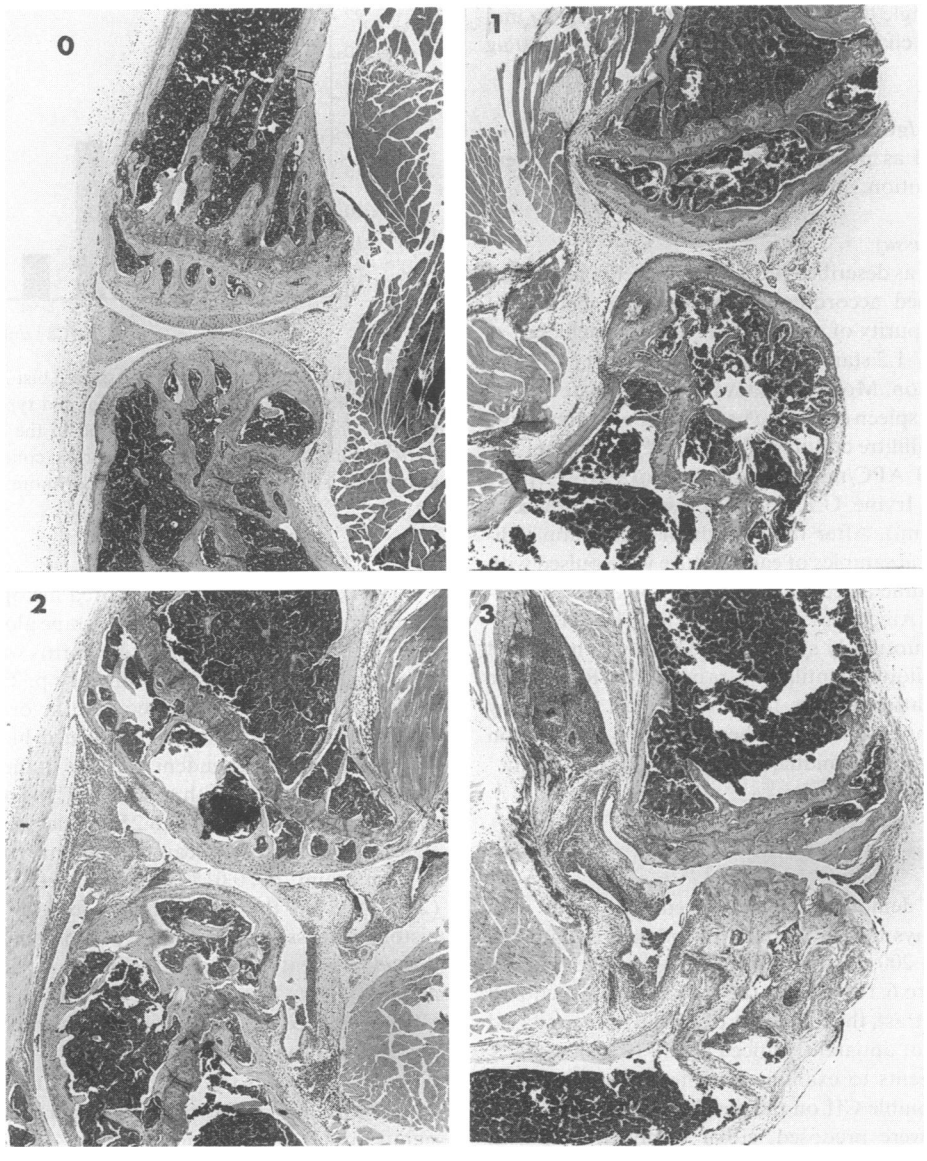

Figure 1. Haematoxylin- and eosin-stained longitudinal sections of stifle (knee) joints from normal and arthritic mice were graded for clinical severity as follows: (0) normal; (1) synovial hyperplasia with pannus formation and mild inflammation (PMN) or non-inflammatory mild articular cartilage degeneration; (2) articular cartilage degeneration with synovial hyperplasia and pannus formation—moderate to severe inflammation (PMN and macrophages); (3) articular cartilage degeneration with synovial hyperplasia and pannus formation—severe inflammation (PMN and macrophages). Significant inflammation in joint space with PMN, macrophages and debris.

sections blind for joint changes according to the following grading system (see Fig. 1):

0. Normal.
1. Synovial hyperplasia with pannus formation and mild inflammation (polymorphonuclear leucocytes—PMN) or non-inflammatory mild articular cartilage degeneration.
2. Articular cartilage degeneration with synovial hyperplasia and pannus formation. Moderate to severe inflammation (PMN and macrophages).
3. Articular cartilage degeneration with synovial hyperplasia and pannus formation. Severe inflammation (PMN and macrophages). Significant inflammation in joint space with PMN, macrophages and debris.

Immunization and gavage of animals

Collagens were dissolved in 0·01 M ethanoic acid. In the first experiment 500 μg of CII (isolated from bovine nasal septum and papain solubilized) or CIX (isolated from rat chondrosarcoma) were administered i.p. to mice 10 days before the first injection of pristane. In a subsequent experiment mice (35/group) received CII i.p. or orally with the aid of a rigid cannula inserted via the oesophagus directly into the stomach. Each

mouse received a single dose on 5 consecutive days (up to and including the day of challenge with pristane) of 5, 50 or 500 μg CII.

ELISA for anti-CII IgG antibodies

This was performed as previously described.[12] The sera were tested at a 1/100 dilution.

Proliferative T-cell assay

This was carried out as described previously.[7] Briefly, responder T cells were enriched according to the panning method of Engleman *et al.*[13] A purity of greater than 90% was achieved as assessed by anti-Thy-1.2 staining using flow cytometry (FACScan, Becton Dickinson, Mountain View, CA). Irradiated (1000 rads) normal mouse spleen cells were used as antigen-presenting cells (APC). Two-millilitre cultures of $1{\cdot}25 \times 10^6$ purified splenic T cells with $1{\cdot}25 \times 10^6$ APC/ml, were plated out in a 24-well plate (Flow Laboratories, Irvine, U.K.), in the presence or absence of test antigen (10 μg/ml). After the periods of incubation indicated, duplicate 100 μl samples of each culture were pulsed with 2 mCi of [^{3}H]thymidine (specific activity 70–85 Ci/mM; Amersham International, Amersham, U.K.) for 6 hr. The [^{3}H]thymidine incorporated into newly synthesized DNA was measured using conventional liquid scintillation procedures. Results are presented as stimulation indices (SI = c.p.m. test divided by c.p.m. control without antigen) where positive stimulation resulted in maximal [^{3}H]thymidine uptake of ~20,000 c.p.m.

RESULTS

Incidence of PIA

Initial experiments demonstrated that predosing (i.p.) with 500 μg of CII 10 days before pristane injection increased the frequency of PIA at 200 days from 3/14 (21%), in the pristane only control group, to 6/16 (38%) in the group of mice predosed i.p. with CII. By contrast, the frequency of PIA in mice predosed with CIX i.p. was not apparently affected 3/13 (23%).

Further experiments to examine the effect of the route of administration of soluble CII on the development of PIA were carried out. Mice were predosed, either orally or i.p., with increasing amounts (5, 50 or 500 μg) of soluble CII then challenged with an otherwise arthritogenic dose of pristane. Mice were scored visually for clinical signs of arthritis. Mice predosed orally with CII had a lower frequency of arthritis at day 200 with a delayed onset as assessed visually halfway through the experiment at day 100 (i.e. 0/32 given 5×500 μg CII versus 4/35 in the pristane only control group). Conversely, mice predosed i.p. with CII had an increased frequency of arthritis at day 200 with a more rapid onset (6/35 given 5×500 μg CII i.p. at day 100). These divergent effects were dose dependent in so far as the lowest frequency of arthritis was observed in mice predosed orally with 5×500 μg of soluble CII, whilst the highest frequency of arthritis was observed in mice predosed i.p. with 5×500 μg of CII (data not shown). Since the visual differences in the frequency of arthritis were most apparent between the groups of mice that had been predosed with the highest doses of CII (5×500 μg) and the lowest doses of CII (5×5 μg) the actual frequency of arthritis in these groups of mice was determined histopathologically at day 200. The frequency of arthritis in mice predosed either orally or i.p. with the intermediate dose of CII (5×50 μg) was not confirmed histopathologically.

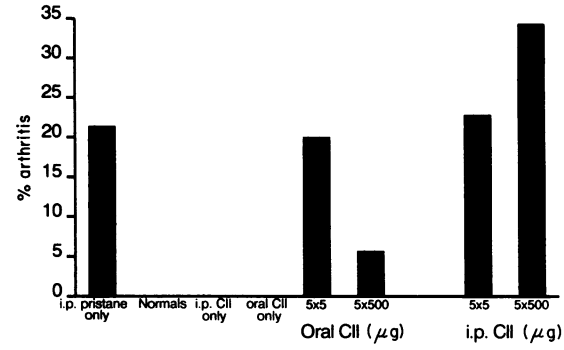

Figure 2. The frequency of arthritis assessed histologically 200 days after the first injection of pristane in control and type II collagen-predosed mice. A group of mice ($n = 20$) housed in the same facility were left untreated and used as age-matched normal controls. Mice were scored as positive if the joint had a score of 1 or more.

The frequency of arthritis scored histopathologically in the control group of mice receiving pristane alone was 6/28 (21·4%) (Fig. 2). No histological signs of arthritis were seen in mice only receiving soluble CII either orally or i.p. The differences in the incidence of PIA observed between the orally and intraperitoneally predosed groups was confirmed histologically (Fig. 2). There was a dose-dependent decrease in the incidence of PIA in mice predosed orally with soluble CII. In fact, at the higher dose (5×500 μg CII) only 2/32 (6·25%) mice developed histological signs of arthritis (significantly different from the pristane only control group, $P < 0{\cdot}015$ Fisher's Exact test) compared to 7/33 (21·2%) given the lowest dose (5×5 μg CII). By contrast, histological assessment of mice predosed i.p. with CII showed a dose-dependent increase in the incidence of PIA. That is, 12/35 animals (34·3%) predosed i.p. with the highest dose (5×500 μg CII) developed clinical signs of arthritis compared to 8/35 (22·9%) in the group given the lowest dose of CII (5×5 μg). The difference between the frequency of arthritis in animals predosed with the highest dose of collagen orally and i.p. is significant ($P < 0{\cdot}01$, Fisher's Exact).

Severity of arthritis

The severity of the arthritis was assessed by comparison of the mean histopathological score of arthritis obtained on examination of longitudinal sections from the stifle joints of arthritic mice in each group (Fig. 3). These results extend the observations on the incidence of arthritis. Normal age-matched controls, along with mice dosed orally and i.p. with soluble CII alone were all scored 0, having no microscopic joint changes. Examination of the affected stifle joints from the group of mice given pristane alone (6/28) had a mean histopathological score of $1{\cdot}47 \pm 1{\cdot}14$, $n = 6$. The severity of the arthritis was significantly lower in those mice predosed orally with the low ($0{\cdot}88 \pm 0{\cdot}63$, $n = 7$) dose of CII compared with the pristane only control group ($P < 0{\cdot}05$, Mann–Whitney U-test). Similarly, the score ($0{\cdot}65 \pm 1{\cdot}0$, $n = 2$) was clearly lower than the control group in those mice treated orally with the high dose. In contrast, the groups of mice predosed i.p. with CII, both low and high doses, both had increased mean histopathological scores over that of the pristane only control group ($1{\cdot}75 \pm 0{\cdot}92$, $n = 8$ and

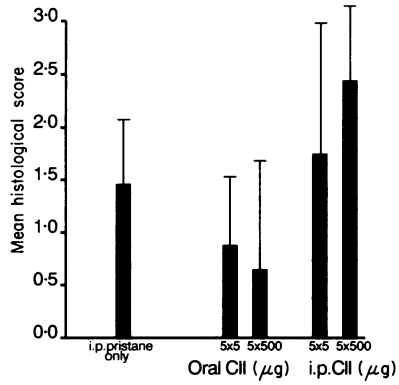

Figure 3. The mean histopathological scores of the stifle (knee) joint sections of negative control mice and the arthritic mice in the CII-predosed groups. In each experimental group, only data from arthritic mice are included in the calculations.

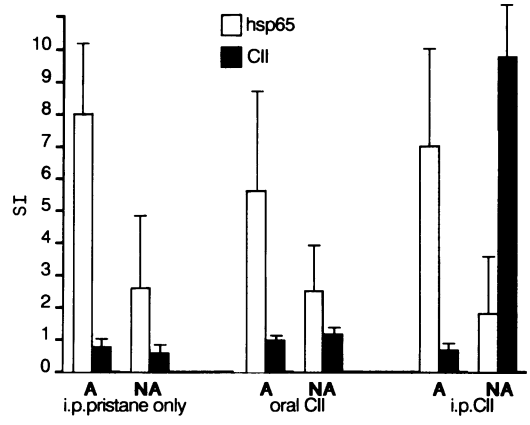

Figure 5. *In vitro* T-cell proliferative responses to hsp65 (a kind gift from Dr W. van Embden, RIVM, Bilthoven, The Netherlands) and type II collagen in arthritic (A), non-arthritic (NA), control (i.p. pristane only) and collagen predosed (5 × 500 μg CII) mice. The results are presented as the means of the stimulation indices from four mice/group.

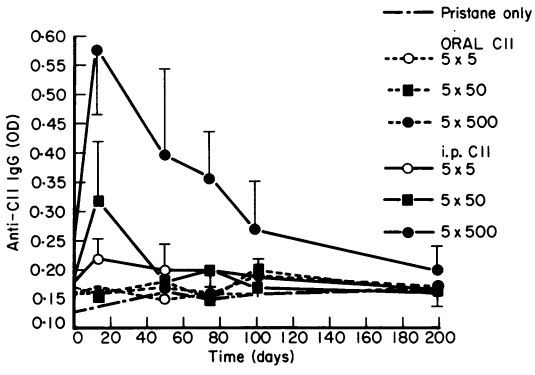

Figure 4. The levels of anti-type II collagen IgG in the sera of mice from the control and collagen-predosed groups over the 200-day period of the study.

2·44 ± 0·68, $n = 12$ respectively) ($P < 0{\cdot}05$, Mann–Whitney *U*-test).

Humoral immune responses to CII

The levels of anti-CII IgG in the sera of mice from the various experimental groups over the 200-day period of study are shown in Fig. 4. The oral administration of CII did not, at the doses used, cause any histopathological signs of arthritis, nor did it induce a detectable serum anti-CII IgG response after pristane injection. By contrast, mice that had been predosed i.p. with CII had increasing, dose-dependent, antibody responses that peaked around 14 days after the first pristane injection.

Cellular immune responses

We have previously reported that CBA/Igb mice with PIA develop elevated humoral and cellular responses to hsp65 as well as T-cell responses to undefined joint antigens present in aqueous extracts of murine knee joints.[7] We examined the *in vitro* T-cell proliferative responses to hsp65 and to CII in mice predosed orally or i.p. with the high dose of CII (5 × 500 μg). Cellular responses to concanavalin A (Con A) or irrelevent antigens, such as bovine serum albumin (BSA), were not enhanced or impaired in any of the groups tested (data not shown). In agreement with our previous findings,[7] mice developing arthritic symptoms (A) had elevated *in vitro* proliferative responses to hsp65 in comparison to mice that did not develop arthritis (NA) (Fig. 5). The oral administration of CII did not significantly affect these responses. We and others[7,14] have previously failed, as here, to detect proliferative T cell responses to CII in mice with PIA. In contrast, in the group of mice predosed i.p. with CII, mice that did not develop arthritis had greatly elevated *in vitro* proliferative responses to CII but the *in vitro* proliferative responses to hsp65 were unaffected by predosing with CII.

DISCUSSION

We have demonstrated that while detectable T- or B-cell immunity to CII is not a feature of PIA, in these mice, predosing with CII, but not CIX, influences the subsequent development of PIA. This influence on PIA is critically dependent upon both the dose and route of administration of CII. That is increasing doses of orally administered CII lowered the incidence and severity of PIA. In contrast increasing doses of i.p. administered CII increased the incidence and severity of PIA.

Mice predosed orally with CII exhibited a lower incidence of PIA. They did not have levels of either anti-CII serum IgG antibodies or *in vitro* proliferative T-cell responses that were raised above control pristane-immunized mice. However, the elevated incidence of PIA observed in the mice predosed i.p. with CII is associated with the presence of raised levels of anti-CII serum IgG antibodies. It is conceivable that this immunity to CII may have established an initial joint lesion which in turn led to the earlier onset of full pathology. The detection of *in vitro* proliferative T-cell responses to CII in the non-arthritic mice in this group further raises the possibility that there was a corresponding T-cell response in their arthritic counterparts and that these T cells may have migrated from the periphery to the site of the lesion. However, the alternative explanation that the

T cells proliferating to CII in the non-arthritic mice may be conferring some protection against PIA cannot be excluded.

The histopathological analysis shows that oral administration of CII prior to the arthritogenic challenge with pristane not only decreases the incidence of PIA but also dramatically decreases the severity of any observed arthritis. Conversely, the intraperitoneal injection of CII before pristane challenge not only increases the incidence of PIA but also increases the severity of the arthritis.

The demonstration here that prior exposure to CII has profound effects on the development of PIA suggests that cartilage autoreactivity is involved in the pathogenesis of PIA even though T- and B-cell immunity to cartilage collagens is not ordinarily detectable in CBA/Igb mice with PIA.[7] It has been reported that oral administration of type I collagen (CI) in addition to CII can suppress the development of adjuvant arthritis.[11] The present study cannot exclude the possibility of an effect of CI administration on the development of PIA. The opposing effects of CII on PIA observed when it is administered orally or i.p. highlight the importance of the context in which antigen is first seen. That orally administered CII lowers the incidence and severity of PIA is consistent with several studies that have demonstrated the efficacy of orally administered autoantigens in blocking the development of the experimental autoimmune diseases, CIA,[9,10] experimental allergic encephalomyelitis,[15-17] experimental autoimmne uveoretinitis,[18] AA[11] and the diabetes in NOD mice.[19] Of particular relevance is that PIA is now the third example, CIA and AA being the others, of an experimental arthropathy in which the oral administration of CII has been shown to suppress the development of arthritis. The histological revelation of a less severe arthritis, in arthritic mice that had been predosed orally with CII, is particularly interesting as it had previously been thought that once CIA developed in mice predosed orally with CII that the arthritis was as severe as that seen in control mice,[10] although we have seen small changes in the levels of acute phase proteins in rats predosed orally with CII in CIA (H. S. G. Thompson, N. Harper and N. A. Staines, unpublished observation). One distinction, however, with this study is that the blocking of PIA is increased with increasing doses of CII whereas increasing doses of orally administered CII are less efficacious at blocking both CIA[9,10] and AA.[11]

The observed exacerbation of PIA, with respect to both the incidence and severity of arthritis, in mice predosed with CII i.p. may be a synergistic effect similar to the reported synergy between CIA and AA which was based on the observation of a particularly severe arthritis in rats receiving both antibodies to CII from rats with CIA and Con A-stimulated lymph node cells from syngeneic rats with AA.[20] It is already known that PIA is a T-cell-mediated disease (ref. 7; S. J. Thompson, M. J. Day, A. J. Coad and C. J. Elson, unpublished observations) and we have shown that the mice predosed i.p. with CII produce antibodies to CII. We therefore suggest that these two components are acting synergistically to accelerate the onset and severity of PIA. Further indirect evidence supporting a synergistic relationship between the effect of i.p. administered CII and pristane comes from the observation that the rate of onset of CIA in DBA/1 mice can be accelerated by predosing i.p. with pristane.[4]

We have not yet characterized the mechanisms involved in both the blocking and the acceleration of the development of PIA; however the simplest explanation for both these observations is that immunity to CII is involved in the pathogenesis of PIA and that consequently the specific suppression of this immunity ameliorates the arthritis while the enhancement of this immunity exacerbates the arthritis. Finally, this study further demonstrates the potential of the specific use of orally administered autoantigens in the therapy of autoimmune diseases.[21]

ACKNOWLEDGMENTS

This work was supported by the Arthritis and Rheumatism Council, U.K.

REFERENCES

1. Potter M. & Wax J.S. (1981) Genetics of susceptibility to pristane-induced plasmacytomas in BALB/cAn: reduced susceptibility in BALB/cJ with a brief description of pristane-induced arthritis. *J. Immunol.* **127,** 1591.
2. Bedwell A.E., Elson C.J. & Hinton C.E. (1987) Immunological involvement in the pathogenesis of pristane-induced arthritis. *Scand. J. Immunol.* **25,** 393.
3. Hopkins S.J., Freemont A.J. & Jayson M.I.V. (1984) Pristane induced arthritis in BALB/c mice. I. Clinical and histological features of the arthropathy. *Rheumatol. Int.* **5,** 21.
4. Rook G., Thompson S., Buckley M., Elson C., Brealey R., Lambert C., Whyte T. & Rademacher T. (1991) The role of oil and agalactosyl IgG in the induction of arthritis in rodent models. *Eur. J. Immunol.* **21,** 1027.
5. Hitsumoto Y., Thompson S.J., Zhang Y.W., Rook G.A.W. & Elson C.J. (1992) Relationship between interleukin 6, agalactosyl IgG and pristane induced arthritis. *Autoimmunity*, **11,** 247.
6. Thompson S.J., Hitsumoto Y., Zhang Y.W., Rook G.A.W. & Elson C.J. (1992) Agalactosyl IgG in pristane-induced arthritis II. Pregnancy affects the incidence and severity of arthritis and the glycosylation status of IgG. *Clin. exp. Immunol.* **89,** 434.
7. Thompson S.J., Rook G.A.W., Brealey R.J., van der Zee R. & Elson C.J. (1990) Autoimmune reactions to heat shock proteins in pristane-induced arthritis. *Eur. J. Immunol.* **20,** 2479.
8. Staines N.A., Hardingham T.E., Smith M. & Henderson B. (1981) Collagen-induced arthritis in the rat: modification of immune and arthritic responses by free collagen and immune anti-collagen anti-serum. *Immunology*, **44,** 737.
9. Thompson H.S.G. & Staines N.A. (1986) Gastric administration of type II collagen delays the onset and severity of collagen-induced arthritis in rats. *Clin. exp. Immunol.* **64,** 581.
10. Nagler-Anderson C., Bober L.A., Robinson M.E., Siskind G.W. & Thorbeke G.J. (1986) Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen. *Proc. natl. Acad. Sci. U.S.A.* **83,** 7443.
11. Zhang Z.J., Lee C.S.Y., Lider O. & Weiner H.L. (1990) Suppression of adjuvant arthritis in Lewis rats by oral administration of type II collagen. *J. Immunol.* **145,** 2489.
12. Staines N.A., Ekong T.A.N., Thompson H.S.G., Isaacs A.B., Loryman B., Major P.J., Hobbs S.M. & Devey M.E. (1990) Low affinity antibodies against collagen type II are associated with pathology in collagen-induced arthritis in mice. *J. Autoimmunity*, **3,** 643.
13. Engleman E.G., Benike C.J., Grumet C. & Evans R.L. (1981) Activation of human T lymphocyte subsets: helper and suppressor/cytotoxic T cells recognize and respond to distinct histocompatibility antigens. *J. Immunol.* **127,** 2124.
14. Chapdelaine J.M., Whalen J.D. & Wooley P.H. (1991) Pristane induced arthritis. II. Genetic regulation in F1 hybrid mice and cellular immune abnormalities following pristane injection. *Autoimmunity*, **8,** 215.

15. BITAR D.M. & WHITACRE C.C. (1988) Suppression of experimental autoimmune encephalomyelitis by the oral administration of myelin basic protein. *Cell Immunol.* **112,** 364.
16. HIGGINS P.J. & WEINER H.L. (1988) Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein and its fragments. *J. Immunol.* **140,** 440.
17. BROD S.A., AL-SABBAGH A., SOBEL R.A., HAFLER D.A. & WEINER H.L. (1991) Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin antigens: IV. Suppression of chronic relapsing disease in the Lewis rat and strain 13 guinea pig. *Ann. Neurol.* **29,** 615.
18. NUSSENBLATT R.B., CASPI R.R., MAHDI R., CHAN C.C., ROBERGE F., LIDER O. & WEINER H.L. (1990) Inhibition of S-antigen induced experimental autoimmune uveoretinitis by oral induction of tolerance with S-antigen. *J. Immunol.* **144,** 1689.
19. ZHANG Z.J., DAVIDSON L., EISENBARTH G. & WEINER H.L. (1991) Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. *Proc. natl. Acad. Sci U.S.A.* **88,** 10252.
20. TAUROG J.D., KERWAR S.S., MCREYNOLDS R.A., SANDBERG G.P., LEARY S.L. & MAHOWALD M.L. (1985) Synergy between adjuvant arthritis and collagen-induced arthritis in rats. *J. exp. Med.* **162,** 962.
21. THOMPSON H.S.G. & STAINES N.A. (1991) Could specific oral tolerance be a therapy for autoimmune disease? *Immunol. Today*, **11,** 396.