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Increased limb involvement in murine collagen-induced arthritis following treatment with anti-interferon-gamma

R. O. WILLIAMS, D. G. WILLIAMS, M. FELDMANN & R. N. MAINI
Kennedy Institute of Rheumatology, incorporating Charing Cross Sunley Research Centre, London, UK

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SUMMARY

We have tested the effect of administering H22, a hamster neutralizing MoAb to murine interferon-gamma (IFN-γ) in collagen-induced arthritis. Mice were immunized with human type II collagen in adjuvant on day 1 and boosted with soluble collagen on day 21. H22 was administered (250 μg, intraperitoneally) either during the induction of arthritis (on days 0, 6, 13 and 20) or around the time of disease manifestation (on days 21, 28, 35 and 42). Control mice received either an isotype-matched non-neutralizing MoAb or saline. Both treatment regimes gave similar results. Treatment with H22 did not significantly affect the incidence of arthritis, time of onset, degree of oedema, histopathological severity, or level of anti-type II collagen IgG. However, a highly significant increase ($P < 0.01$) in the number of limbs affected by arthritis was observed in the H22-treated group, irrespective of whether the antibody was administered during the induction of arthritis, or during the time of clinical manifestation of disease. From these results it was concluded that anti-IFN-γ treatment caused an increase in the number of arthritic lesions, but did not affect the severity of each individual lesion.

Keywords interferon-gamma collagen-induced arthritis DBA/1 mice

INTRODUCTION

Much of the research that has been carried out into the roles played by cytokines in chronic inflammatory conditions such as rheumatoid arthritis (RA) has focused on the identification of these molecules in affected tissues, and on the *in vitro* properties that they exhibit. It is important to establish, however, which of these properties are important *in vivo*, since this information is critical for the development of therapeutic strategies based on the modulation of cytokine activity. For example, on the basis of *in vitro* studies, a wide range of both pro- and anti-inflammatory properties have been described for interferon-gamma (IFN-γ) [1], but it is not known which of these properties is pathologically relevant. One potentially useful approach to the study of the pathological importance of cytokines *in vivo* is to administer cytokine-specific neutralizing antibodies in particular disease situations. For example, the role of tumour necrosis factor in collagen-induced arthritis was established by administering MoAbs specific for this cytokine [2,3].

Collagen-induced arthritis demonstrates marked similarities to human RA with respect to both clinical and histological findings [4] and can therefore be used to study the effects of administering cytokines or agents capable of neutralizing cytokines *in vivo*. For example, two studies have recently been published on the effect on collagen-induced arthritis of administering recombinant IFN-γ to collagen-immunized DBA/1 mice. In the first of these studies, higher incidence, earlier onset and increased severity of arthritis were demonstrated in mice given footpad injections of recombinant IFN-γ [5]. On the other hand, systemic administration of IFN-γ was found to inhibit the development of arthritis [6]. We describe the effect of administering anti-IFN-γ MoAb systemically to heterologous type II collagen-immunized DBA/1 mice. Our results suggest that IFN-γ plays a disease-limiting role in collagen-induced arthritis which is manifest not only during the induction of arthritis but also around the time of disease expression.

Correspondence: Richard Williams, Kennedy Institute of Rheumatology, 6 Bute Gardens, London W6 7DW, UK.

MATERIALS AND METHODS

Mice

Male DBA/1 mice were purchased from Olac Ltd (Bicester, UK) and used at 10–12 weeks of age.

Type II collagen

Type II collagen was extracted from human articular cartilage by limited pepsin digestion [7]. Briefly, femoral head cartilage was powdered in a liquid nitrogen freezer mill, then suspended in a solution of 4 M guanidine-HCl/0·05 M Tris (pH 7·5) in order to remove proteoglycans. After washing, cartilage collagens were solubilized by pepsin digestion (1 mg/ml in 0·5 M acetic acid) for 24 h at 4°C. Type II collagen was then precipitated from solution by salt fractionation, and redissolved in 0·05 M Tris/0·2 M NaCl (pH 7·4) for 24 h at 4°C, in order to inactivate

Table 1. Effect of anti-IFN-γ treatment during the induction of collagen-induced arthritis

	H22	L2-3D9	PBS
Incidence of arthritis	7/9	10/10	8/10
Day of onset (mean ± s.d.)	29 ± 5	31 ± 4	30 ± 5
Median clinical score (range)	8·5 (2–9)*	4·0 (3–8)	3·0 (2–9)
Median clinical score/limb (range)	3·0 (2–3)	3·0 (2·5–3)	3·0 (2–3)
Anti-collagen IgG (arbitrary units; mean ± s.d.)	38 ± 8	36 ± 7	33 ± 9

Mice were immunized with type II collagen/Freund's complete adjuvant (FCA) on day 1 and given H22 (anti-IFN-γ; 250 μg, intraperitoneally) on days 0, 6, 13 and 20. Controls received L2-3D9 or PBS.

* H22-treated group significantly higher than control groups ($P < 0.01$).

residual pepsin. Following removal of salts by dialysis, the collagen was lyophilized and stored at −20°C in a desiccator. No contaminating proteins were detected by SDS–PAGE.

Anti-IFN-γ MoAb

The hamster anti-murine IFN-γ MoAb used in these experiments was an IgG1 antibody designated H22. This antibody possesses potent IFN-γ-neutralizing properties, as demonstrated by its ability to inhibit 100% of both the antiviral activity and the macrophage-activating properties of the culture supernatant of the 24/G1 T cell hybridoma [8]. A control hamster MoAb of the same isotype, L2-3D9, was a non-neutralizing antibody to IL-2. Both monoclonals were generously provided to us by Dr R. D. Schreiber (Washington University Medical School, St Louis, MO) in conjunction with Celltech Ltd (Slough, UK).

Induction of arthritis

Human type II collagen was dissolved in 0·1 M acetic acid (1 mg/ml) and emulsified in an equal volume of Freund's complete adjuvant (FCA; Difco Laboratories, East Molesey, UK). The emulsion (0·2 ml) was injected intradermally at the base of the tail on day 0. On day 21 mice were given a booster injection of type II collagen (100 μg; intraperitoneally). Mice were inspected for arthritis and the clinical severity of disease was assessed using a scoring system where 0 = normal, 1 = slight swelling and/or erythema, 2 = oedematous swelling, 3 = joint rigidity. Each limb was graded in this way, giving a maximum score of 12 per animal. In addition, the degree of oedema in each affected hindpaw was monitored by measuring paw thickness using calipers. Results were expressed as the percentage increment in paw width relative to paw width at day 0.

Measurement of anti-collagen II antibodies

The amount of type II collagen-specific IgG contained in the sera on day 45 post-immunization was estimated using an ELISA. Microtitre plates were coated overnight at 4°C with type II collagen dissolved in 0·05 M Tris-buffered saline, pH 7·4 (5 μg/ml). The plates were blocked with casein in PBS (2% w/v) before incubation of test sera diluted in PBS containing Tween 20 (0·05%, v/v). A positive standard consisting of pooled serum from arthritic DBA/1 mice was included on each plate. Following washing, goat anti-mouse IgG-alkaline phosphatase conjugate (1 : 1000; Sigma, Poole, UK) was incubated for 2 h at room temperature. After further washing, the substrate (*p*-nitrophenyl phosphate; Sigma) was added and allowed to develop for approximately 1 h. Optical densities were read at 405 nm on a microplate autoreader (Biotek Instruments Inc., Winooski, VT).

Histology

Affected feet were removed at post mortem, fixed in 10% buffered formalin, then decalcified in EDTA in buffered formalin (5·5% w/v). The feet were then embedded in paraffin, sectioned and stained with haematoxylin and eosin.

RESULTS

Anti-IFN-γ treatment before the onset of arthritis

The aim of this experiment was to determine whether treatment with MoAb to IFN-γ during the induction of collagen-arthritis influenced the incidence and/or severity of subsequent disease. Mice were immunized with type II collagen/FCA on day 1, then boosted with type II collagen alone on day 21. H22 was administered (250 μg in PBS, intraperitoneally) to one group of mice on days 0, 6, 13 and 20. Another group received L2-3D9 (250 μg in PBS, intraperitoneally) and a third group was given PBS alone. Each group contained 9–10 mice.

The time of onset of arthritis varied from day 20 to day 41 post-immunization (mean = day 30) and was characterized in all groups by redness and swelling in one or more limbs. There was a highly significant increase in the clinical scores at day 45 in the H22-treated mice, compared with those treated with L2-3D9 or PBS ($P < 0.01$, Mann–Whitney *U*-test; Table 1). However, the scoring system that was used took account of the number of arthritic limbs/mouse as well as the severity of arthritis in each limb (see Materials and Methods). When the clinical scores of the individual mice were corrected for the number of arthritic limbs per mouse, no significant differences were found between the groups (Table 1). On day 45, the median number of arthritic limbs per mouse in the IFN-γ-treated group was 3·0 (range 2–4). This was significantly higher ($P < 0.01$; Mann–Whitney *U*-test) than the number of arthritic limbs per mouse in the L2-3D9-treated group (median 1·5; range 1–3) or the PBS-treated group (median 1·5; range 1–3). No differences were seen between the L2-3D9-treated group and the PBS-treated group, indicating that hamster IgG1 did not modify disease outcome non-specifically. Figure 1 shows the progressive involvement of limbs over the observation period and demonstrates that the increased limb involvement in the H22-treated group was evident consis-

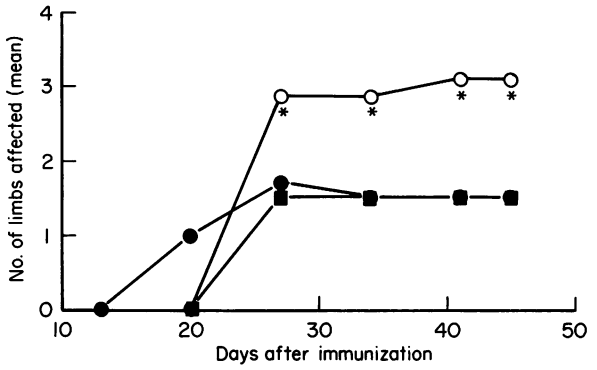

Fig. 1. Increased limb involvement in arthritic mice treated with anti-IFN-γ (H22) during the induction of collagen-induced arthritis. Mice were immunized with bovine type II collagen on day 1 and boosted on day 21. Intraperitoneal injections of H22, L2-3D9 or PBS were given on days 0, 6, 13 and 20. *$P<0.05$ (Mann–Whitney *U*-test; H22-treated *versus* L2-3D9-treated groups). ●, PBS; ■, L2-3D9; ○, H22.

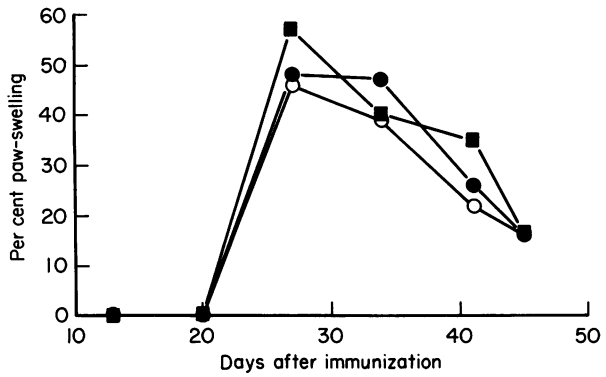

Fig. 2. Mean footpad swelling in arthritic mice treated with H22 (anti-IFN-γ) from day 0 to day 20. Mice were immunized with type II collagen/Freund's complete adjuvant (FCA) on day 1 and boosted with soluble collagen on day 21. No differences were observed between H22-treated group and L2-3D9-treated group. ●, PBS; ■, L2-3D9; ○, H22.

tently from soon after the onset of arthritis until termination of the experiment.

Administration of H22 did not influence the number of mice which developed arthritis, or the time of onset of disease (Table 1). Furthermore, no differences between the groups were observed with respect to the degree of oedema, as determined by the mean increase in paw thickness (Fig. 2). Histological examination of limbs from samples of four mice per group, all of which had demonstrated clinical arthritis for the same number of days, revealed similar patterns of synovitis, cartilage/bone erosion, and fibrosis in all three groups. Levels of anti-type II collagen IgG in the sera collected from mice on day 45 were measured by ELISA. No differences between the groups were found (Table 1).

Anti-IFN-γ treatment after the onset of arthritis

To determine the effect of anti-IFN-γ treatment on ongoing arthritis, H22 was injected (250 μg, intraperitoneally) on days 21, 28, 35 and 42. Control mice were injected with L2-3D9 (250 μg, intraperitoneally) or saline. There were 9–10 mice per group. As in the previous experiment, mice were immunized with type II collagen in adjuvant on day 1, then boosted with soluble collagen on day 21. The mean time of onset of arthritis was 31 days after immunization (range 27–45 days). Administration of H22 yielded similar results to those obtained in the previous experiment. No significant differences in the incidence or time of onset of arthritis were found between the three groups (Table 2). The progression of arthritis, in terms of swelling, was similar for the three groups over the observation period (Fig. 3). Furthermore, no histopathological differences were seen between the groups, and the levels of anti-type II collagen IgG were similar in all three groups (Table 2). However, it can be seen from Fig. 4 that, as in the previous experiment, the number of limbs per mouse affected by arthritis was higher in the H22-treated group (median 3; range 2–4) than in the L2-3D9-treated group (median 1; range 1–3) and the PBS-treated group (median 2; range 1–3). This difference was highly significant ($P<0.01$; Mann–Whitney *U*-test). From these results it was concluded that treatment with H22 caused an increase in the number of arthritic lesions/mouse, but did not affect the clinical or pathological severity of individual lesions. Similarly, time of onset of arthritis and level of circulating anti-collagen IgG were not affected by anti-IFN-γ treatment.

DISCUSSION

A number of lines of evidence point to the involvement of $CD4^+$ T cells in the pathogenesis of both murine collagen-induced arthritis and human RA. First, $CD4^+$ T cells may be found in the affected joints of mice with collagen-induced arthritis [9] and patients with RA [10]. Second, treatment with anti-CD4 MoAb inhibits the development of collagen-induced arthritis [11] and may be beneficial in RA [12]. In view of the marked localized up-regulation of class II MHC expression that is seen in RA [10,13] and collagen-induced arthritis [9], it has also been proposed that T cell-derived cytokines, particularly IFN-γ, contribute to both disease processes, since MHC expression, as a marker of up-regulated antigen-presenting function, is thought to be an important factor in the development of autoimmunity [13,14]. In support of this hypothesis, it has been demonstrated that thyroid epithelial cells, which are normally MHC class II negative, not only express MHC class II antigens but are also capable of presenting antigens if derived from patients with Graves' disease [15,16]. Furthermore, the ectopic expression of either IFN-γ or class II MHC molecules in the insulin-producing pancreatic β cells of transgenic mice resulted in the development of insulin-dependent diabetes mellitus [17]. Subsequent studies showed that diabetes induced by ectopically expressed IFN-γ was immune-mediated, involving inflammatory cell infiltration and destruction of pancreatic islets by lymphocytes, suggesting that IFN-γ may alone provide the stimulus necessary for the initiation of autoimmune disease [18]. In collagen-induced arthritis, IFN-γ injected locally into the footpads of collagen-immunized DBA/1 mice resulted in (i) enhanced MHC expression in the synovium, and (ii) increased incidence and severity of arthritis, providing further evidence for the local role of IFN-γ and MHC expression in autoimmune disease [5].

In the light of these observations, we have determined the effect of *in vivo* systemic administration of a neutralizing antibody to IFN-γ on the outcome of collagen-induced arthritis.

Table 2. Effect of anti-IFN-γ treatment on ongoing arthritis

	H22	L2-3D9	PBS
Incidence of arthritis	9/10	9/10	8/9
Day of onset (mean ± s.d.)	29 ± 4	33 ± 7	33 ± 6
Median clinical score (range)	9·0 (6–12)*	3·0 (2–9)	4·5 (3–9)
Median clinical score/limb (range)	3·0 (2·5–4)	3·0 (2–3)	2·75 (2–3)
Anti-collagen IgG (arbitrary units; mean ± s.d.)	33 ± 12	36 ± 8	33 ± 9

Mice were treated with MoAb (250 μg, intraperitoneally) on days 21, 28, 35 and 42.
* H22-treated group significantly higher than control groups ($P < 0.01$).

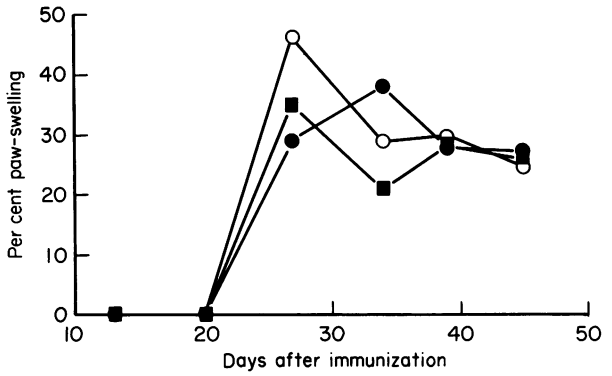

Fig. 3. Lack of effect on footpad swelling of H22 (anti-IFN-γ), administered around the time of clinical manifestation of collagen-induced arthritis. Antibodies were given on days 21, 28, 35 and 42. ●, PBS; ■, L2-3D9; ○, H22.

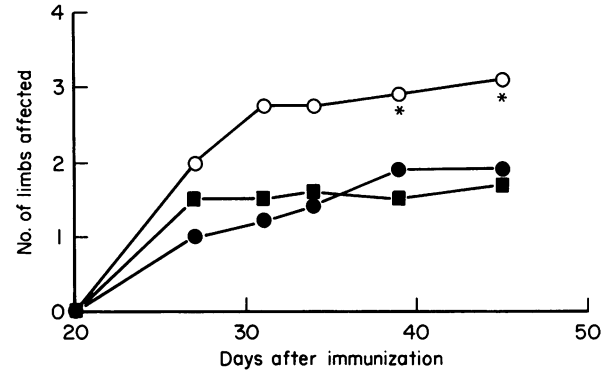

Fig. 4. Increased limb involvement in arthritic mice treated with anti-IFN-γ MoAb (H22) during the time of disease manifestation. Antibodies were given on days 21, 28, 35 and 42. *$P < 0.05$ (Mann–Whitney *U*-test; H22-treated *versus* L2-3D9-treated groups). ●, PBS; ■, L2-3D9; ○, H22.

Treatment took the form of weekly intraperitoneal injections of H22, given either during the induction of arthritis (days 0–21) or during the time of disease manifestation (days 21–42). Anti-IFN-γ treatment resulted in increased limb involvement, irrespective of whether the antibody was administered early or late in the disease. Timing of arthritis, incidence, swelling and histological severity were not significantly affected by anti-IFN-γ treatment.

The number of arthritic limbs/mouse is an important marker of disease severity, and our results provide evidence in favour of a disease-limiting role for IFN-γ in collagen-induced arthritis. A similar result has been reported in another experimentally induced autoimmune disease, experimental allergic encephalomyelitis, where administration of anti-IFN-γ was found to cause pronounced exacerbation of disease [19]. Our results are also consistent with the finding that systemic administration of IFN-γ to mice before the onset of collagen-arthritis inhibited the development of arthritic lesions, and treatment after the onset of clinical disease prevented new lesion development, but did not affect existing lesions [6]. Such observations may have important implications for the therapy of human RA, since this disease, like collagen-induced arthritis, is often characterized by a period of disease establishment, during which time progressive joint involvement is common. It is possible, therefore, that the most appropriate time for IFN-γ therapy is during this early phase of the disease, as this may limit the number of joints that subsequently become involved.

The mechanism by which anti-IFN-γ may be acting so as to increase limb involvement is unknown, but in view of the disease-promoting properties of locally active IFN-γ, the disease-suppressing properties are likely to involve systemic factors. IFN-γ is a highly pleiotropic cytokine, having a number of properties capable of acting systemically to inhibit the development of arthritis. For example, IFN-γ has an antiproliferative effect on a variety of cell types, including lymphocytes [20]. IFN-γ is also inhibitory for prostaglandin E_2 and collagenase synthesis [21], but promotes the synthesis of C1 inhibitor, an important inhibitor of the complement system [22]. Furthermore, IFN-γ inhibits the secretion of pituitary-derived hormones, such as adrenocorticotropin, growth hormone, as well as prolactin [23], which is believed to play a role in murine collagen-induced arthritis [24]. Increased lesion development appeared to occur independently of antibody-mediated mechanisms, since levels of anti-collagen IgG were unaffected by treatment, although we cannot rule out the possibility that a qualitative change in the antibody response had occurred, such as a shift in the relative proportions of different immunoglobulin isotypes or a change in the kinetics of the response.

Pro- and anti-inflammatory properties have been attributed to both IFN-γ and anti-IFN-γ in murine arthritis. For example, in one report the effect of systemic administration of either recombinant IFN-γ or anti-IFN-γ MoAb was tested in the rat adjuvant arthritis model. Administration of IFN-γ 1 day before immunization caused exacerbation of arthritis, but administration 1–2 days after immunization caused suppression of disease.

Administration of anti-IFN-γ MoAb, up to day 8 after immunization, suppressed arthritis, but administration from day 12 to day 24 resulted in increased severity [25]. In another report, systemic administration of this same anti-IFN-γ MoAb was shown to inhibit the development of arthritis, but the beneficial effects were dose-dependent, with high doses causing exacerbation of disease [26]. It is concluded from these findings that the timing, dosage and especially the route of administration are of critical importance in determining the effects of IFN-γ or anti-IFN-γ treatment on arthritis. It is possible that at sites of inflammation the pro-inflammatory properties of IFN-γ predominate, whereas critical concentrations of circulating IFN-γ are anti-inflammatory. It is interesting to note, for example, that systemic administration of IFN-γ to DBA/1 mice resulted in inhibition of collagen-arthritis [6], whereas injection into the feet caused increased incidence and severity of arthritis, particularly in the limbs into which the cytokine had been injected [5].

It is apparent that IFN-γ is a cytokine capable of both up-regulating and down-regulating the pathogenic processes involved in collagen-induced arthritis and that it is possible experimentally to manipulate circulating levels of the cytokine and so affect the outcome of disease. However, the difficulty in predicting the outcome of IFN-γ/anti-IFN-γ treatment calls for caution with respect to the application of this particular therapeutic approach to human disease. Further research will be required if we are to elucidate the precise roles played by IFN-γ and other cytokines *in vivo*, but such research may prove vital to our understanding of the function of these molecules and to our understanding of the pathogenesis of collagen-arthritis and perhaps of RA.

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