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The effect of treatment with interferon-gamma on type II collagen-induced arthritis

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SUMMARY

We have investigated the effects of recombinant murine interferon-gamma (rIFN-γ) on type II collagen-induced arthritis (CA) in DBA/1 mice. Therapeutic as well as prophylactic treatment with subcutaneous rIFN-γ, at 10^5 U/mouse six times a week, inhibited the development of CA without any obvious side effects. The accompanying suppression of anti-CII antibody responses may partly explain the inhibition of CA by rIFN-γ. The possible role of the anti-inflammatory effect of systemic IFN-γ in the inhibition of CA is discussed.

Keywords interferon-gamma type II collagen arthritis

INTRODUCTION

Interferon-gamma (IFN-γ) has been found to show various properties including anti-proliferative and immunomodulatory, as well as anti-viral activity (reviews by Borden & Ball, 1981; Friedman & Vogel, 1983; Rosa & Fellous, 1984). The capacity of IFN-γ to induce and enhance the expression of class II MHC antigens on various types of cells is generally believed to be a factor in the pathogenesis of autoimmune disorders such as rheumatoid arthritis (RA) (reviews by Rosa & Fellous, 1984; Editorial, 1985). However, recent clinical studies (Obert & Hofschneider, 1985; Seitz, Manz & Franke, 1986; Lemmel *et al.*, 1987; Veys *et al.*, 1988; Cannon *et al.*, 1989) have indicated that recombinant DNA-derived human IFN-γ (rhIFN-γ) has some efficacy in the treatment of RA, although the ultimate therapeutic role has yet to be clarified.

Collagen-induced arthritis (CA) in DBA/1 mice has been shown to be similar to RA in many respects (Courtenay *et al.*, 1980; Stuart, Townes & Kang, 1984), and is widely used for the detection and evaluation of compounds with anti-inflammatory or anti-rheumatic activity (Phadke *et al.*, 1986; Paska, McDonald & Croft, 1986).

In contrast to Mauritz *et al.* (1988), who reported that locally injected recombinant rat IFN-γ exacerbates CA in mice, we have investigated the effects of systemic recombinant murine IFN-γ (referred to as rIFN-γ hereafter) administered far from the target joints in the present study. The results demonstrate an inhibitory effect of rIFN-γ on the incidence and development of CA, and may indicate a new aspect of the regulatory role of IFN-γ in chronic inflammatory diseases.

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MATERIALS AND METHODS

Mice

Female DBA/1J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used at 8–9 weeks of age.

Bovine type II collagen (CII)

Bovine CII was isolated and purified from fetal calf articular cartilage according to Miller (1972), with a slight modification as described by Kakimoto, Hirofuji & Koga (1984).

Induction of arthritis

Lyophilized CII was dissolved in 0·05 M acetic acid at 4°C, and was emulsified with Freund's complete adjuvant (FCA; Difco Laboratories, Detroit, MI). Mice were injected intradermally at the tail base with 0·1 ml of cold emulsion containing 0·2 mg of CII, and were boosted with a further 0·2 mg of CII emulsified in FCA 3 weeks later.

Evaluation of arthritis

The clinical severity of arthritis was assessed by means of a visual scoring system (Paska *et al.*, 1986). The individual paws were graded from 0 to 4, yielding a maximum possible score of 16/animal (overall lesions), as follows: 1, swelling in a single digital joint; 2, swelling in several but not all joints; 3, gross swelling of the entire paw; and 4, gross deformity and/or ankylosis. The data are presented as mean arthritis score per animal for each group.

Table 1. Effect of prophylactic treatment of rIFN-γ on the incidence of collagen-induced arthritis

Experiment	Treatment with	Incidence of arthritis				
		Day 31	Day 38	Day 48	Day 57	Day 61
A						
	PBS	4/8	5/8	6/8	ND	7/8
	rIFN-γ (10^3 U)	2/8	6/8	8/8	ND	8/8
	rIFNγ (10^4 U)	1/8	3/8	8/8	ND	8/8
	rIFNγ (10^5 U)	0/8*	0/8*	2/8	ND	5/8
	Indomethacin (0·5 mg/kg)	4/8	5/8	5/8	ND	8/8
B						
	PBS	5/8	7/8	8/8	8/8	ND
	rIFNγ (10^5 U)	0/8*	2/8*	5/8	6/8	ND
	Cyclophosphamide (7 mg/kg)	0/8*	1/8*	4/8*	5/8	ND

Mice were immunized with type II collagen (CII) twice, on days 0 and 21, and treated prophylactically with either saline, rIFN-γ, indomethacin, or cyclophosphamide at the indicated doses for 5 weeks, six times/week from day 20 as described in Materials and Methods. Incidences are expressed as number of mice with arthritis/number of mice used. Arthritis scores are shown in Fig. 1.

* Significantly different from control (PBS) $P < 0.05$.

ND, not determined.

Recombinant murine IFN-γ

rIFN-γ (8×10^6 anti-viral U/mg protein) from transfected *Escherichia coli* was provided by Genentech (South San Francisco, CA). The preparation, without any detectable amount of bacterial lipopolysaccharide (LPS), was diluted to an appropriate concentration with phosphate-buffered saline (PBS), pH 7·4 immediately before use.

Administration of rIFN-γ or other drugs

For the prophylactic schedule, 0·1 ml of appropriately diluted rIFN-γ was injected subcutaneously into the back of mice for 5 weeks six times/week starting on day 20 after the primary immunization with CII (one day before the booster immunization). Control mice received a comparable volume of PBS. Cyclophosphamide (Shionogi, Osaka, Japan) or indomethacin (Sigma Chemical Co., St Louis, MO) were administered orally according to the same schedule into mice in a reference group. Each group consisted of eight mice. For the therapeutic schedule, the mice immunized with CII were visually assessed for arthritis on day 33 after the primary immunization, and the mice with lesions in one or two out of four extremities were used for experiments. They were divided into two groups of nine mice each, so that the mean arthritis score of each group matched. A volume of 0·1 ml of either rIFN-γ or control PBS was administered subcutaneously into the backs of the mice for 4 weeks six times/week from day 33. The mice tested survived to the end of each experiment.

ELISA for antibodies to CII

Mice were bled by retro-orbital puncture and individual serum samples were collected. ELISA was used to measure CII-reactive IgG antibodies in sera. The methods were adapted from those previously described (Stuart & Dixon, 1983). Briefly, wells of flat-bottomed microplates were coated overnight at 4°C with 0·1 ml of 10 μg/ml of CII disolved in 0·05 M Tris-buffered saline, pH 7·6 (Tris-NaCl). The wells were then washed with PBS containing 0·05% Tween 20 and 1% bovine serum albumin (BSA) (PBS-T-BSA), and 0·1 ml of the sample diluted optimally with PBS-T-BSA was added. After 90-min incubation at room temperature, the wells were washed with PBS containing 0·05% Tween 20 (PBS-Tween), incubated with 0·1 ml of a 1/1000 dilution of goat anti-mouse IgG coupled to horseradish peroxidase (Cooper Biomedical, Malvern, PA) at room temperature for 90 min, washed with PBS-Tween, and developed at room temperature for 15 min with 0·2 ml of 0·4 mg/ml *o*-phenylenediamine and 0·003% H_2O_2 in 0·05 M citrate plus 0·1 M phosphate at pH 4·5. The reaction was stopped by adding 0·05 ml of 4 N sulfuric acid, and the optical density (OD) at 492 nm was measured using ELISA Reader EAR400 (SLT Instruments, Grodig, Austria). A serum pool from DBA/1 mice exhibiting typical arthritis was arbitrarily assigned a value of 100 U/ml as a reference, and was included in each assay. The amount of anti-CII IgG in a test sample was quantified in U/ml by comparison of its OD reading with a standard curve of OD *versus* concentration obtained with dilutions of the reference serum. The standard curve was fitted to the log–logit formulation described by Rodbard & Hutt (1974), without weighting, using a programmable computer.

Delayed-type hypersensitivity (DTH) responses to CII

The assay system was essentially the same as described previously (Phadke *et al.*, 1986). Briefly, 10 μg of CII in 10 μl of Tris-NACl were injected into one ear of each mouse tested, and the same volume of Tris-NaCl alone was injected into the other ear. The ear thickness was measured by using a micrometer (Ozaki Engineering, Tokyo, Japan), before and 24 h after injections. DTH reaction was expressed as [(thickness at 24 h − thickness before injection) in ear injected with CII] − [(thickness at 24 h − thickness before injection) in ear injected with Tris-NaCl alone].

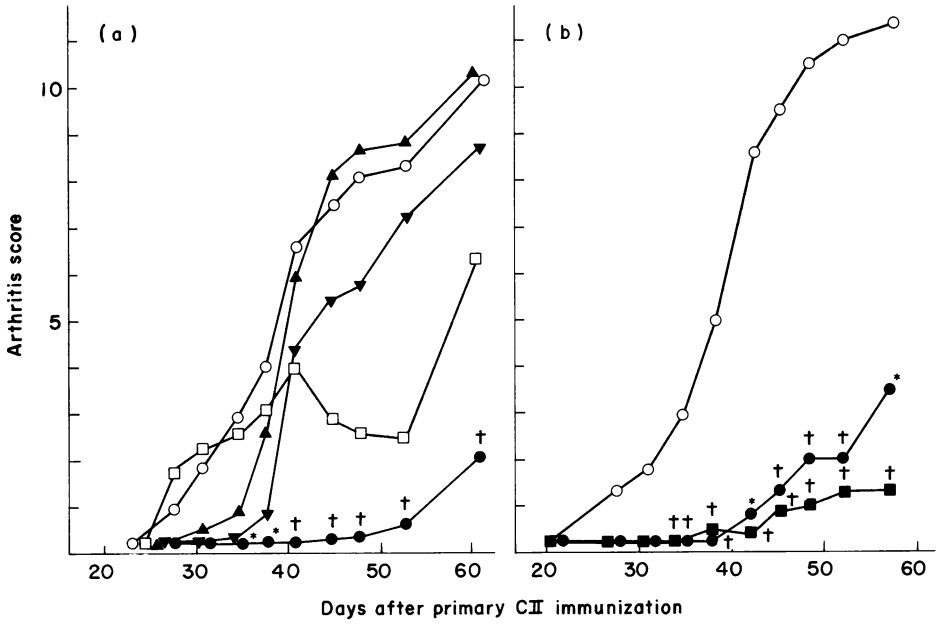

Fig. 1. Effect of prophylactic treatment of rIFN-γ on the development of CA. Mice were immunized with CII twice, on days 0 and 21, and given one of the following treatments for 5 weeks six times/week from day 20: s.c. PBS (control, ○); s.c. 10^3 U rIFN-γ (▲); s.c. 10^4 U rIFN-γ (▼); s.c. 10^5 U rIFN-γ (●); 0·5 mg/kg indomethacin *per os* (□); or 7 mg/kg cyclophosphamide *per os* (■). Mean arthritis scores of eight mice per group are shown. Statistically significant differences from control: * $P<0.05$; † $P<0.01$.

Table 2. Effect of treatment with rIFN-γ on IgG antibody and DTH responses to type II collagen (CII) in mice with collagen-induced arthritis

	Serum anti-CII IgG (U/ml)		Ear swelling (mm × 10^{-2})
Treatment with	Day 35	Day 58	Day 56
PBS	60·1 ± 20·0	97·0 ± 13·4	19·1 ± 2·6
rIFN-γ (10^5 U)	32·6 ± 10·2†	43·0 ± 20·8†	19·0 ± 2·8
Cyclophosphamide (7 mg/kg)	10·8 ± 1·8†	16·9 ± 6·1†	8·4 ± 2·1†

Mice were immunized with CII twice, on days 0 and 21, and treated prophylactically with either saline, rIFN-γ, or cyclophosphamide at the indicated doses for 5 weeks six times/week from day 20 as described in Materials and Methods. The values are mean ± s.e.m. of eight mice per group. Arthritis incidence and score are shown in Table 1, Experiment B and Fig. 1b. Significantly different from control (PBS): * $P<0.05$; † $P<0.01$.

Statistical analysis

The data on antibody levels and DTH responses were analysed using analysis of variance (ANOVA), followed by Dunnet-type multiple comparison. Mean arthritis scores were assessed by using either Wilcoxon's rank sum test (between two groups), or Kruskal–Wallis test followed by Dunnet-type multiple comparison (between control group and treatment groups of more than two). Arthritis incidences were compared by the Fisher exact test.

RESULTS

The prophylactic effect of rIFNγ on the development of CA

Severe arthritis could be induced in a majority of DBA/1 mice within 1–2 weeks after the second CII immunization, which was done 21 days after primary CII immunization. Therefore, we started treatment with rIFN-γ from 1 day before the second immunization in a prophylactic schedule. Mice were given s.c. doses of 10^3, 10^4 or 10^5 U rIFN-γ for 5 weeks, six times/week. As shown (representatively) in Table 1 and Fig. 1, rIFN-γ at a dose of 10^5 U/mouse delayed the onset of arthritis and inhibited the lesion development remarkably, i.e. more efficiently than 0·5 mg/kg of indomethacin in Experiment A, and comparable to 7 mg/kg of cyclophosphamide in Experiment B in both arthritis incidence (Table 1) and score (Fig. 1). However, a sharp increase in the severity was noted after ceasing treatment. The prophylactic effect of rIFNγ was dose-dependent; doses below 10^4 U/mouse were not effective (Experiment A).

We examined anti-CII immune responses in mice treated prophylactically with 10^5 U of rIFN-γ in Experiment B. As shown in Table 2, treatment with rIFN-γ significantly suppressed the serum level of anti-CII IgG antibody, although less

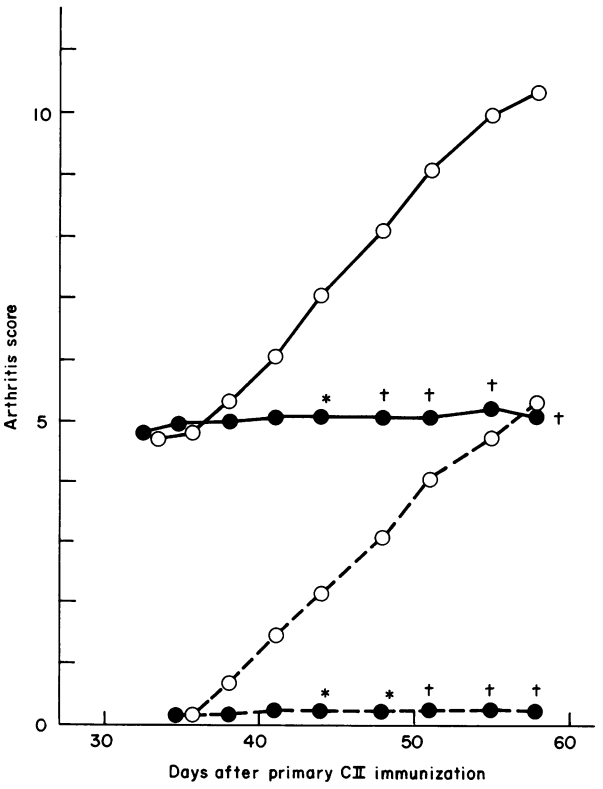

Fig. 2. Effect of therapeutic treatment of rIFN-γ on established CA. Mice were immunized with CII twice, on days 0 and 21, and selected for the therapeutic protocol on day 33 as described in Materials and Methods. They were treated subcutaneously with either PBS (control, ○) or 10^5 U rIFN-γ (●) for 4 weeks, six times/week from day 33. Dashed lines, scores for new lesions in the extremities which had been normal up to and including day 33; solid lines, scores for overall lesions. Arthritis scores are expressed as the mean of nine mice per group. Statistically significant differences from control: * $P<0.05$; † $P<0.01$.

effectively than cyclophosphamide. However, anti-CII DTH, measured by ear swelling, was not suppressed by rIFN-γ in contrast to the treatment with cyclophosphamide. Lack of inhibitory effect of rIFN-γ on anti-CII DTH was confirmed by using a radiometric ear assay (data not shown).

Inhibition of new lesion formation by rIFN-γ administered therapeutically

We administered 10^5 U of rIFN-γ subcutaneously, starting 12 days after the second injection with CII, into CA mice with established lesions in one or two out of four extremities. As shown in Fig. 2, appearance and development of new lesions in the other two to three extremities were almost completely inhibited by treatment with rIFN-γ, while mean arthritis scores for the overall lesions remained constant, indicating that the lesions, once established, were not influenced by rIFN-γ. Upon cessation of treatment, new lesions occur rapidly (data not shown).

DISCUSSION

Our results demonstrate for the first time the inhibitory effect of rIFN-γ on the development of CA in DBA/1 mice: daily systemic injection of rIFN-γ at 10^5 U/mouse delayed the onset and reduced the progression of CA markedly. Furthermore, rIFN-γ suppressed the development of anti-CII antibody responses significantly.

Mauritz *et al.* (1988) injected rIFN-γ subcutaneously into paws on one side of the body of CII-immunized DBA/1 mice, and showed an early onset and severe development of CA in the paws in which the rIFN-γ preparation was injected, but not in the paws on the other side of the body. The discrepancy between their results and ours seems to be due to differences in routes of administering IFN-γ, rather than the different species used as sources of IFN-γ, because we have also observed the exacerbation of CA by the intra-paw, but not dorsal subcutaneous injection of rIFN-γ, according to their immunization and treatment schedule (unpublished data). These results, taken together, may be related to the recent findings by Heremans *et al.* (1987) concerning the complexity of the modulating effects of IFN-γ on LPS-induced inflammation, in that local IFN-γ acts as a positive factor, whereas systemic or circulating IFN-γ is anti-inflammatory. An anti-inflammatory effect of IFN-γ administered systemically has been also shown in the case of carrageenin-induced paw oedema in mice, although partially purified human IFN-γ was used (Mecs *et al.*, 1984). Thus, we consider the anti-inflammatory activity of systemic IFN-γ to be one of the mechanisms responsible for the inhibition of CA by rIFN-γ demonstrated in the present study.

Although there was no influence on DTH responses, serum anti-CII antibody level suppression by rIFN-γ was significant. This might also partly explain the inhibitory effect of rIFN-γ on CA, because the development of CA is known to depend largely on the IgG antibody responses to CII (Trentham *et al.*, 1978; Stuart & Dixon, 1983). Alternatively, it may be that reduced anti-CII antibody is a consequence of diminished inflammatory activity with a resultant reduction in cartilage destruction and CII release. However, the regulation of humoral immune responses by rIFN-γ seems to be somewhat different from that of inflammatory responses, since Mauritz *et al.* (1988) have also found the suppression of anti-CII antibody responses accompanying exacerbation of CA by intra-paw injection of IFN-γ.

It has been indicated that rhIFN-γ has some beneficial effects in the treatment of RA (Obert & Hofschneider, 1985; Seitz *et al.*, 1986; Lemmel *et al.*, 1987; Veys *et al.*, 1988; Cannon *et al.*, 1989), although most of the clinical variables were not significantly different from placebo-control in the most recent double-blind trials (Veys *et al.*, 1988; Cannon *et al.*, 1989). The present study provides, at the level of an animal model, some evidence for the clinical efficacy of IFN-γ.

REFERENCES

BORDEN, E.C. & BALL, L.A. (1981) Interferons: biochemical, cell growth inhibitory, and immunological effects. *Prog. Hematol.* **12,** 299.

CANNON, G.W., PINCUS, S.H., EMKEY, R.D., DENES, A., COHEN, S.A., FREDERICK, W., SAWAY, P.A., JAFFER, A.M., WEAVER, A.L., COGEN, L. & SCHINDLER, J.D. (1989) Double-blind trial of recombinant γ-interferon versus placebo in the treatment of rheumatoid arthritis. *Arthritis Rheum.* **32,** 964.

COURTENAY, J.S., DALLMAN, M.J., DAYAN, A.D., MARTIN, A. & MOSEDALE, B. (1980) Immunization against heterologous type II collagen induces arthritis in mice. *Nature,* **283,** 666.

EDITORIAL (1985) What triggers autoimmunity? *Lancet,* **i,** 78.

FRIEDMAN, R.M. & VOGEL, S.N. (1983) Interferons with special emphasis on the immune system. *Adv. Immunol.* **34,** 97.

HEREMANS, H., DIJKMANS, R., SOBIS, H., VANDEKERCKHOVE, F. & BILLIAU, A. (1987) Regulation by interferons of the local inflammatory response to bacterial lipopolysaccharide. *J. Immunol.* **138,** 4175.

KAKIMOTO, K., HIROFUJI, T. & KOGA, T. (1984) Specificity of anti-type II collagen antibody response in rats. *Clin. exp. Immunol.* **57,** 57.

LEMMEL, E.M., FRANKE, M., GAUS, W., HARTL, P.W., HOFSCHNEIDER, P.H., MIEHLKE, K., MACHALKE, K. & OBERT, H.J. (1987) Results of a phase-II clinical trial on treatment of rheumatoid arthritis with recombinant interferon-gamma. *Rheumatol. Int.* **7,** 127.

MAURITZ, N.J., HOLMDAHL, R., JONSSON, R., VAN DER MEIDE, P.H., SCHEYNIUS, A. & KLARESKOG, L. (1988) Treatment with gamma-interferon triggers the onset of collagen arthritis in mice. *Arthritis Rheum.* **31,** 1297.

MECS, I., KOLTAI, M., TOTH, S. & AGOSTON, E. (1984) The anti-inflammatory effect of human interferons in mice. *Contrib. Oncol.* **20,** 221.

MILLER, E.J. (1972) Structural studies on cartilage collagen employing limited cleavage and solubilization with pepsin. *Biochemistry*, **11,** 4903.

OBERT, H.J. & HOFSCHNEIDER, P.H. (1985) Interferon bei chronischer Polyarthritis. *Dtsch. med. Wschr.* **110,** 1766.

PASKA, W., MCDONALD, K.J. & CROFT, M. (1986) Studies on type II collagen induced arthritis in mice. *Agents Actions*, **18,** 413.

PHADKE, K., FOUTS, R.L., PARRISH, J.E. & BUTLER, L.D. (1985) Evaluation of the effects of various anti-arthritic drugs on type II collagen-induced mouse arthritis model. *Immunopharmacology*, **10,** 51.

RODBARD, D. & HUTT, D.M. (1974) Statistical analysis of radioimmunoassays and immunoradiometric (labeled antibody) assays. In *Radioimmunoassays and Related Procedures in Medicine, Vol. I.* (ed. by International Atomic Energy Agency) p. 165. Unibub, Lanham.

ROSA, F. & FELLOUS, M. (1984) The effect of gamma-interferon on MHC antigens. *Immunol. Today*, **5,** 261.

SEITZ, M., MANZ, G. & FRANKE, M. (1986) Einsatz von recombinantem Human-Interferon Gamma bei Patienten mit rheumatoider Arthritis. *Z. Rheumatol.* **45,** 93.

STUART, J.M. & DIXON, F.J. (1983) Serum transfer of collagen induced arthritis in mice. *J. exp. Med.* **158,** 378.

STUART, J.M., TOWNES, A.S. & KANG, A.H. (1984) Collagen autoimmune arthritis. *Annu. Rev. Immunol.* **2,** 199.

TRENTHAM, D.E., TOWNES, A.S., KANG, A.H. & DAVID, J.R. (1978) Humoral and cellular sensitivity to collagen in type II collagen-induced arthritis in rats. *J. clin. Invest.* **61,** 89.

VEYS, E.M., MIELANTS, H., VERBRUGGEN, G., GROSCLAUDE, J.P. & SCHINDLER, J.D. (1988) Interferon gamma in rheumatoid arthritis: a double blind study comparing human recombinant interferon gamma with placebo. *J. Rheumatol.* **15,** 570.