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Original Paper

Antitumour Activity of the Novel Immune Modulator 5,6-Dimethylxanthenone-4-acetic Acid (DMXAA) in Mice Lacking the Interferon-gamma Receptor

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5,6-Dimethylxanthenone-4-acetic acid (DMXAA), a novel antitumour agent currently undergoing clinical evaluation, appears to mediate its antitumour effects through immune modulation and the production of cytokines. We used mice with a targeted disruption of the interferon-gamma (IFN-γ) receptor gene as a model to evaluate the role of the host response to IFN-γ in the antitumour action of DMXAA on colon 38 tumours. A feature of the results was that while DMXAA treatment induced both IFN-γ and tumour necrosis factor (TNF) in serum, the increase was >20-fold higher in IFN-γR$^{0/0}$ mice than in wild-type mice. In contrast, mRNA levels for IFN-γ and TNF were similar in the two mouse strains, suggesting that the concentrations of these cytokines were controlled by a post-transcriptional mechanism. Serum nitrate levels, used as a measure of nitric oxide production, were increased by DMXAA, but to a similar extent in both strains of mice. Complete regressions of colon 38 tumours were obtained in response to DMXAA in the knockout mice, although the dose required for 100% cure was higher and the reduction in tumour volume occurred more slowly than in the wild-type counterparts. The results demonstrate that the host response to IFN-γ is not essential for an antitumour response. Similar results were obtained in mice that were immunosuppressed by treatment with cyclosporin A before treatment with DMXAA. The results are consistent with the concept that the antitumour activity of DMXAA involves complex immunomodulation, probably with significant redundancy in contributing cytokines. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: DMXAA, tumour necrosis factor, interferon-gamma receptor, lipopolysaccharide, knockout, antitumour, colon 38
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INTRODUCTION

THE DEVELOPMENT of synthetic small molecules that can stimulate or amplify the host's immune response against an established tumour represents an attractive approach to cancer therapy. 5,6-Dimethylxanthenone-4-acetic acid (DMXAA), a new drug developed in the Cancer Research Laboratory, University of Auckland School of Medicine and currently in phase I clinical trial, is an example of this approach. In mice, DMXAA activates a complex series of host responses beginning with the induction of tumour vascular collapse and leading ultimately to the elimination of the tumour cells [1–3]. Many of its biological effects have been attributed to the induction of cytokines, most notably, tumour necrosis factor (TNF) and the interferons (IFN) [4]. DMXAA was originally developed as a dose-potent analogue of flavone acetic acid (FAA), which showed excellent activity against transplantable murine tumours [5], but failed to demonstrate clinical responses [6]. While both DMXAA and FAA stimulate TNF mRNA in murine cells, only DMXAA stimulates it in human cells [7–9]. DMXAA also stimulates TNF synthesis in human cells [8].

A number of cytokines are involved in the action of DMXAA and FAA in murine tumours. The early events of tumour vascular collapse and subsequent necrosis appear to be TNF mediated, as antibodies to TNF ablate FAA-induced tumour blood flow shutdown [10]. Natural killer cell activity,

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which is stimulated by these agents, is thought to play a role in tumour elimination and is mediated by IFN-α/β [11]. T cell immunity, which appears to be an important component for long-term tumour regression, is amplified by IFN-γ [12]. Studies from several laboratories supported an essential role for T cells in the long-term antitumour action induced with FAA. Cures were obtained against the Renca renal carcinoma only if FAA was administered with interleukin-2 (IL-2) [13], and *in vivo*, depletion of L3T4 and Lyt2 subpopulations abrogated the antitumour response of FAA to colon 26 carcinomas [14]. Bibby and colleagues [15] showed that FAA could induce growth delays against a panel of tumours in normal euthymic hosts, but not against tumours implanted in nude or thymectomised mice, indicating that the immune status of the host was important for the response. However, our studies using the colon 38 tumour showed that while the cure rate was lower, complete regressions were obtainable when the tumours were implanted in nude or thymectomised hosts after treatment with FAA and DMXAA [16]. Thus, while a functional T cell immunity is definitely beneficial, it appears less critical for some tumour models than for others.

FAA and the active xanthenones have been shown to upregulate the gene for IFN-γ [17], an essential cytokine in the generation of immune reactivity. It plays a vital role in the priming of macrophages [18], in the amplification of T cell immunity [19], and in the stimulation of other mediators, such as the anti-angiogenic chemokine, IP-10 [20], nitric oxide [21] and TNF [22]. Mice with a targeted disruption of the IFN-γ receptor gene (IFN-$\gamma R^{0/0}$) have been generated by Huang and co-workers [23]. Although the immune system appears to have developed normally and they have normal cytotoxic and helper T cell functions, these mice have decreased resistance to viral infections [23]. IFN-$\gamma R^{0/0}$ mice, thus represent an excellent model for evaluating the effects of IFN-γ on the host immunity, and in this report we have used IFN-$\gamma R^{0/0}$ mice to examine the role of IFN-γ on host immune cells in the antitumour action of DMXAA on colon 38 tumours.

MATERIALS AND METHODS

Materials

DMXAA was synthesised in the Cancer Research Laboratory, University of Auckland School of Medicine [1] and was dissolved in 5% sodium bicarbonate. The required dose in a volume of 0.1 ml per 10 g body weight was injected intraperitoneally (i.p.). Cyclosporin A (CsA, Sandimum, Sandoz, Switzerland) as a clinical formulation was injected i.p., at 50 mg/ml per injection. Culture medium (α-MEM) was supplemented with 10% fetal calf serum, 100 μg/ml streptomycin sulphate and 100 units/ml penicillin G.

Mice

IFN-$\gamma R^{0/0}$ mice and their wild-type counterparts were obtained from Genesis Research and Development Corporation (Auckland, New Zealand) and were the offspring from the interbreeding of wild-type or IFN-$\gamma R^{0/0}$ (129/Sv/Ev×C57B1/6)F_1 mice. C3H/HeJ, C57B1/6J and (C57B1/6×DBA/2)F_1(BDF_1) hybrid mice were bred in our animal facility and were housed under conditions of constant temperature and humidity using sterile bedding and food, according to institutional ethical guidelines. All mice were used between 8 and 12 weeks of age.

Tumour implantation

Colon 38 tumours were grown subcutaneously (s.c.) by implanting 1 mm^3 fragments in the flank of anaesthetised (sodium pentobarbitone, 72 mg/kg for wild-type or IFN-$\gamma R^{0/0}$, 90 mg/kg for BDF_1, C57B1/6J and C3H/HeJ) mice. The colon 38 tumour originated from C57B1/6 ($H\text{-}2^b$) mice and grew successfully in 100% of implanted syngeneic C57B1/6J and semisyngeneic BDF_1 hosts, but 0% of allogeneic C3H/HeJ. In this series of experiments, the implantation rate of colon 38 tumours in the wild-type or IFN-$\gamma R^{0/02}$ mice was 82 and 70%, respectively. These rates were thought to reflect the percentage of the colony that carried the $H\text{-}2^b$ haplotype from the C57B1/6 parent. The rate at which the tumours became palpable and the size of the tumours in the wild-type and IFN-$\gamma R^{0/0}$ mice were similar.

Tumour growth delay and tumour necrosis determination

Experiments were initiated when tumours were approximately 5 mm in diameter, generally 9 days after implantation. Tumour-bearing mice were treated with drug i.p. and the tumours measured using callipers three times weekly thereafter. Tumour volumes were calculated as $0.52 \times a^2 b$ where a and b are the minor and major axes of the tumour. The arithmetic means were calculated for each time point, counting cured tumours as zero volume. The growth delay was determined as the difference in the number of days required for the control versus treated tumours to increase four times in volume. In order to measure necrosis, tumours were removed, fixed in formalin, sectioned and stained with haematoxylin and eosin. Tumour necrosis was quantified microscopically using a grid system as previously described [24].

Measurements of nitrate

Nitrate production *in vitro* was determined in cultures of thioglycollate-elicited peritoneal macrophage monolayers. Mice were injected i.p. with 4% thioglycollate (0.2 ml/mouse) and 3 days later, the peritoneal cavity was lavaged and the cells (10^5) dispensed in 100 μl culture medium per well of flat-bottomed 96-well plates. The plates were incubated at 37°C in an atmosphere of 5% CO_2 air for 30 min, and the wells were washed twice to remove non-adherent cells. The adherent cell monolayer was then cultured with either DMXAA (100 μg/ml) or lipopolysaccharide (LPS, *Escherichia coli* serotype 0127:B8, Sigma, St. Louis, Missouri, U.S.A., 5 μg/ml) and varying concentrations of IFN-γ (0–100 units/well) in a total volume of 200 μl culture medium. Culture supernatants were harvested after 48 h. *In vivo* production of nitrate was determined from plasma samples collected from mice treated 12 h previously with DMXAA (30 mg/kg) or LPS (175 μg/ml). Nitrate concentrations in the culture supernatants, or aliquots of plasma filtrates, were assayed using ion chromatography [25] and concentrations were derived from peak areas for each sample using the appropriate calibration curve.

Measurement of mRNA by Northern blot analysis

Total cellular mRNA was extracted from murine spleen cells using Trizol (Gibco BRL, Gaithersburg, Maryland, U.S.A.) according to the manufacturer's instructions. The RNA samples (10 μg) were fractionated by electrophoresis on a formaldehyde-denaturing 1% agarose gel and transferred overnight to a nylon membrane (Hybond N^+, Amersham, Bucks, U.K.). After UV cross-linking, the membrane was

baked (30 min, 78°C), and each membrane was pre-hybridised (2 h) in 7 ml hybridisation mix at 42°C. This comprised 50% formamide, 0.075 M sodium chloride, 0.05 M sodium dihydrogen phosphate, 5 μM ethylene diamine tetra-acetic acid (EDTA), 0.001% polyvinyl pyrrolidone, 0.001% bovine serum albumin, 0.001% Ficoll, 0.01 mg/ml herring sperm DNA and 0.5% sodium dodecyl sulphate (SDS). The cDNA probe to the cytokine mRNA of interest was labelled with α^{32}P-dCTP (Amersham) using a random priming kit (RTS Radprime DNA labelling system, Gibco BRL). Excess radioactivity was removed by elution through a G-50 Sephadex column and a labelled probe (10^6 cpm/ml hybridisation mix) was then added to the membrane and hybridised for 36 h at 42°C. The blots were washed twice in 2× standard saline citrate (SSC), once in 0.1% SDS for 10 min at 42°C, and finally in 0.2×SSC, 0.1% SDS for 10 min at 65°C. Blots were exposed to Kodak X-OMAT X-ray film for 3 days at −70°. After hybridisation with one probe, membranes were stripped (two washes in 300 ml 0.1×SSC, 1% SDS for 15 min at 80°C), and rehybridised with another probe. Loading of the lanes was determined from the signal obtained from hybridisation of the blot with the probe to human β-actin.

Measurement of IFN-γ and TNF

Mice were bled from the ocular sinus and the blood was allowed to clot overnight on ice. The samples were centrifuged (2000 ***g***, 30 min, 4°C) and the serum was then removed and stored at −20°C until being assayed for cytokines. IFN-γ was measured using an enzyme-linked immunosorbent assay (ELISA) protocol from PharMingen. Briefly, wells of a NUNC 'Maxisorb' plate were coated overnight at 4°C with rat antimouse IFN-γ capture antibody (R4-6A2, PharMingen). The wells were washed and 2-fold dilutions of standards and samples were titrated into the wells and incubated for 1 h at room temperature. Biotinylated anti-IFN-γ detecting antibody (XMG1.2, PharMingen) was added to the wells and incubated for 1 h at room temperature. The wells were then washed and horseradish peroxidase avidin D (Vector no. 2004) was added for 1 h at room temperature. After four further washes, *o*-phenylenediamine in 30% hydrogen peroxide was added to each well and the colour reaction allowed to develop at room temperature in the dark. The reaction was stopped with 10% HCl and the absorbance at 495 nm measured on an ELISA reader.

TNF was measured using a standard L929 cytotoxicity bioassay as described previously [26]. Briefly, L929 cells (3×10^4 cells per 100 μl of culture medium) were placed in each well of flat-bottomed 96-well plates and allowed to adhere overnight. The cells were incubated for 1 h with actinomycin D (8 μg/ml final concentration, Merck Sharpe and Dohme, Granville, NSW, Australia). Samples were added to the first row of wells to give a total volume of 300 μl and sequential 3-fold dilutions of the sample were performed over the length of the plates. Plates were then incubated for 24 h at 37°C. MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl)-2H-tetrazolium bromide; 500 μg/ml] was added and the cultures were incubated for 1 h to allow crystal formation. Supernatants were removed and the crystals were solubilised in 100 μl dimethyl sulphoxide. Absorbance at 570 nm was measured using an ELISA reader. One unit of TNF was defined by this assay as the amount reducing staining intensity of L929 cells by 50%.

RESULTS

Characterisation of the IFN-γR$^{0/0}$ and wild-type mice with respect to nitric oxide production

We first wanted to verify that the cells from IFN-γR$^{0/0}$ mice behaved as if lacking the receptor for IFN-γ. Because nitric oxide production by macrophages in response to LPS stimulation is highly dependent on priming with IFN-γ and is absent in macrophages from IFN-γR$^{0/0}$ mice [27], we investigated nitric oxide production in the IFN-γR$^{0/0}$ and wild-type mice in our colony. Thioglycollate-elicited peritoneal macrophages were cultured with LPS (5 μg/ml) together with varying concentrations of IFN-γ, and the amount of nitrate in culture supernatants was determined as a measure of nitric oxide production. The response to DMXAA (100 μg/ml) was compared in the same experiment. Macrophages from wild-type mice generated nitrate in response to LPS when IFN-γ was added to the culture (Figure 1b). No production was obtained in cultures of macrophages from IFN-γR$^{0/0}$ mice with any concentration of IFN-γ (Figure 1a). Surprisingly, DMXAA did not stimulate nitrate production in macrophages from wild-type or IFN-γR$^{0/0}$ mice at any concentration of IFN-γ. The strict dependency on priming with IFN-γ appeared to be circumvented *in vivo*, as IFN-γR$^{0/0}$ mice treated with LPS had elevated nitrate levels in the serum (Figure 2). DMXAA elevated nitrate levels to a higher extent than LPS *in vivo* (Figure 2).

Induction of TNF and IFN-γ in the IFN-γR$^{0/0}$ and wild-type mice

Since both TNF and IFN-γ have been implicated in the antitumour action of DMXAA and FAA, the capacity of IFN-γR$^{0/0}$ and wild-type mice to produce these cytokines was compared. Mice were treated with varying doses of DMXAA up to 75 mg/kg. Previous studies established that in normal mice the optimal dose for TNF production was 55 mg/kg, and that although this dose was higher than the maximum tolerated dose, it caused no signs of animal distress at the time of sacrifice for serum collection [26]. mRNA was extracted from splenocytes after 2 h, when mRNA production

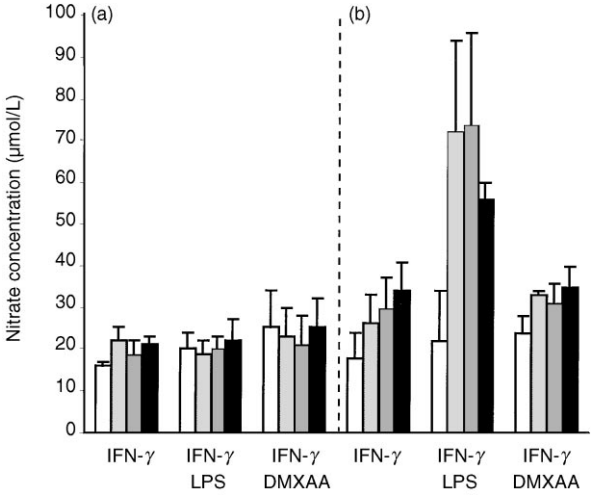

Figure 1. Production of nitrate in cultures of thioglycollate-elicited peritoneal macrophages from IFN-γR$^{0/0}$ (a) and wild-type mice (b) in response to interferon-gamma (IFN-γ), lipopolysaccharide (LPS; 5 μg/ml) or 5.6-dimethylxanthenone-4-acetic acid (DMXAA; 100 μg/ml). Open bars represent no IFN-γ and increasing concentrations of IFN-γ (10, 20, 100 units per culture) are represented by bars of increasing depth of shading. Mean ± SEM of quadruplicate cultures.

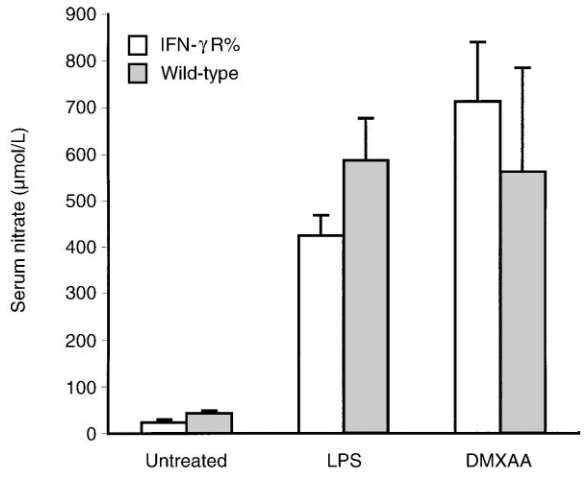

Figure 2. Serum nitrate concentrations measured in IFN-$\gamma R^{0/0}$ mice (open bars), and wild-type mice (shaded bars), untreated, or 12 h after treatment with lipopolysaccharide (LPS; 175 μg/mouse) or 5,6-dimethylxanthenone-4-acetic acid (DMXAA; 27.5 μm/kg). Mean±SEM of 3 mice per group.

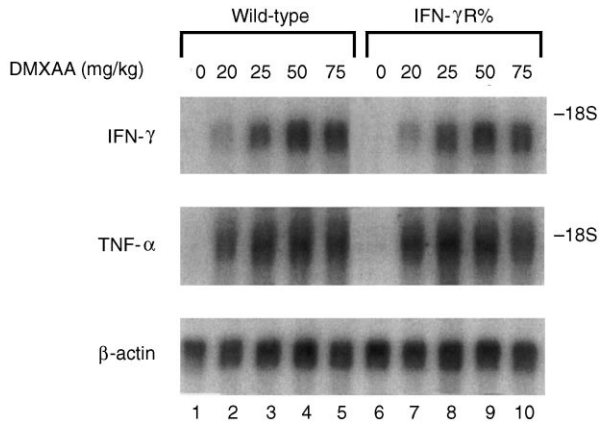

Figure 3. Induction of tumour necrosis factor (TNF) and interferon-gamma (IFN-γ) mRNA in IFN-$\gamma R^{0/0}$ and wild-type mice by 5,6-dimethylxanthenone-4-acetic acid (DMXAA). mRNA extracted from splenocytes from untreated mice or after 2 h following treatment with DMXAA at the indicated doses were analysed by Northern blotting for expression of IFN-γ and TNF mRNA. Loading of individual lanes was determined from the intensity of the β-actin signal.

for TNF and IFN-γ was maximal. Serum was collected at the same time and assayed for TNF and IFN-γ activity. Northern analysis showed that similar levels of mRNA for TNF and IFN-γ were produced in IFN-$\gamma R^{0/0}$ and wild-type mice in response to DMXAA (Figures 3 and 4). However, when cytokine activity was measured, serum from IFN-$\gamma R^{0/0}$ mice was found to have dramatically higher levels of both IFN-γ and TNF than that from wild-type mice (Figure 4). While IFN-γ activity in wild-type mice was at the limits of detection in the ELISA, levels in IFN-$\gamma R^{0/0}$ mice increased with increasing dose of DMXAA and were 10–20 times higher than those observed in wild-type mice. TNF activity in IFN-$\gamma R^{0/0}$ mice was also higher than that in wild-type mice, and increased with increasing dose of DMXAA. In wild-type mice, maximal TNF activity was obtained at 50 mg/kg and then declined with higher doses of DMXAA (Figure 4).

Antitumour activity of DMXAA in the IFN-$\gamma R^{0/0}$ and wild-type mice

The antitumour activity of DMXAA in IFN-$\gamma R^{0/0}$ and wild-type mice against the colon 38 tumour was compared. Results of a representative experiment are shown in Figure 5. Mice were treated with DMXAA when tumours were approximately 5 mm in diameter, and in the wild-type mice receiving 22.5 or 25 mg/kg, 100% of the tumours regressed completely within 15 days. In the wild-type mice receiving 20 mg/kg, 83% of the tumours regressed with an overall 15 day growth delay (Figure 5a; Table 1). In the IFN-$\gamma R^{0/0}$ mice, DMXAA at doses of 20 and 22.5 mg/kg produced growth delays of 4 and 5 days, respectively, and cure rates of 40 and 75%, respectively (Figure 5b; Table 1). All the mice in the group receiving 25 mg/kg were cured, but the tumours required over 25 days to completely regress (Figure 5b). Thus, DMXAA reproducibly induced an effective anti-tumour response in IFN-$\gamma R^{0/0}$ mice, but a higher dose was required to obtain 100% tumour regression and the rate at which the tumours regressed was slower than that in wild-type mice. Induction of haemorrhagic necrosis 24 h after treatment with DMXAA, an early antitumour response, was similar in both the IFN-$\gamma R^{0/0}$ and the wild-type mice (Table 1).

Effect of CsA on the antitumour efficacy of DMXAA

We used a second approach to modulating the host immune activity by examining the effect of CsA on the DMXAA-induced antitumour response. CsA is used clinically for the control of graft rejection and is thought to be highly immunosuppressive of T cell function [28]. Recent data suggest that CsA exerts its immunosuppression of T cell functions through the inhibition of cytokine secretion [29]. Mice were implanted with colon 38 tumours, and on the day of implantation and for 2 consecutive days after implantation, CsA was administered i.p. (50 mg/kg per injection). This CsA treatment regime did not affect the implantation and growth rate of colon 38 tumours in syngeneic or semisyngeneic hosts, or in the IFN-$\gamma R^{0/0}$ and their wild-type counterparts.

Table 1. *Antitumour responses to* 5,6-*dimethylxanthenone*-4-*acetic acid (DMXAA) in IFN-$\gamma R^{0/0}$ and wild-type mice*

		IFN-$\gamma R^{0/0}$ mice			Wild-type mice		
Treatment	Dose (mg/kg)	Growth delay (days)*	Cures (%)*	Necrosis (%)†	Growth delay (days)*	Cures (%)*	Necrosis (%)†
Untreated		0	0	8 ± 6	0	0	9 ± 5
DMXAA	20	4	40	99 ± 1	15	83	97 ± 2
	22.5	5	75	98 ± 2		100	99 ± 1
	25		100	99 ± 1		100	100 ± 0

*Growth delays and cures are from Figure 5. †Haemorrhagic necrosis was measured at 24 h and expressed as the mean ± standard error of three to six mice.

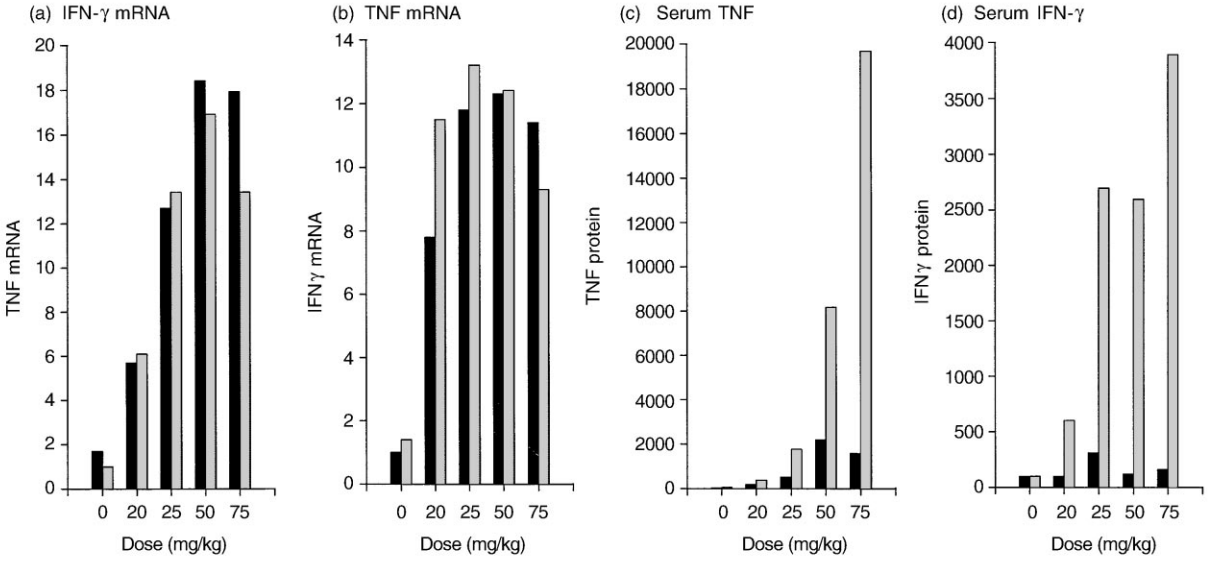

Figure 4. Comparison of interferon-gamma (IFN-γ) mRNA (a), tumour necrosis factor (TNF) mRNA (b), serum TNF-γ (c) and serum INF-γ activity (d) induced in wild-type mice (solid bars) and IFN-γR$^{0/0}$ mice (shaded bars) by 5,6-dimethylxanthenone-4-acetic acid (DMXAA) at the indicated doses. Values for mRNA represent relative intensities of signals measured from Northern blots (Figure 3). Values for TNF represent units determined using the L929 bioassay. Values for IFN-γ represent pg/ml as determined by enzyme-linked immunosorbent assay.

However, it was sufficient to inhibit allograft rejection, allowing growth of colon 38 tumours in 40% of allogeneic C3H/HeJ mice that had been treated with CsA, as compared with 0% of untreated allogeneic hosts. The growth of colon 38 tumours after treatment with DMXAA at 22.5 mg/kg in IFN-γR$^{0/0}$ and wild-type mice with or without CsA treatment was then compared. The antitumour action of DMXAA against colon 38 tumours was not diminished in CsA-treated mice (Figure 6). Following DMXAA administration (22.5 mg/kg) 100% of the tumours in the wild-type mice regressed, whether the mice had received CsA treatment or not (Figure 6a). In the IFN-γR$^{0/0}$ mice, DMXAA (22.5 mg/kg) induced a 66% cure rate and a growth delay of 9 days in mice without CsA treatment, compared with a cure rate of 60% and a 5 day growth delay in the CsA-treated mice (Figure 6b).

We also examined the effect of CsA on the antitumour action of DMXAA at 27.5 mg/kg on the colon 38 tumours growing in syngeneic C57B1/6J mice. No effect was obtained with CsA treatment on the antitumour response of DMXAA induced on colon 38 tumours in syngeneic C57B1/6J hosts (data not shown).

The effect of CsA on serum TNF production in response to DMXAA (25 mg/kg) was measured. Mice were injected with CsA (50 mg/kg) on days 0, 1 and 2 and TNF activity was measured 2 h after DMXAA administration. The response in wild-type mice was 1400 ± 170 and 515 ± 210 units in control and CsA-treated mice, respectively. The response in IFN-γR$^{0/0}$ mice was 270 ± 29 and 379 ± 41 units in control and CsA-treated mice, respectively. CsA may therefore have caused some attenuation of TNF response in wild-type mice and this was also observed at 20 mg/kg (data not shown). There was no effect of CsA in IFN-γR$^{0/0}$ mice.

DISCUSSION

In the present study, IFN-γR$^{0/0}$ mice [23] were used to evaluate the role of the host response to IFN-γ in the antitumour action of DMXAA. Complete regressions of colon 38 tumours were obtained in the knockout mice, although a marginally higher dose was required. Since it is thought that the tumour response to DMXAA, at least with respect to the induction of haemorrhagic necrosis, is a result of a host-mediated effect on tumour blood flow [3], this result indicates that host IFN-γ receptors are not required for this response. We cannot exclude the possibility that IFN-γ produced by host cells in response to DMXAA might affect the growth of the tumour, since the IFN-γ receptor status of colon 38 tumours is not known. Resolution of this question would require the use of IFN-γ knockout mice.

The observed complete tumour regressions induced by DMXAA in the IFN-γR$^{0/0}$ mice are surprising in view of the important role that IFN-γ has in the priming of macrophages and the generation of immune responses. With the considerable redundancy that is observed with cytokine functions, it is possible that in the IFN-γR$^{0/0}$ mice, other cytokines are compensating for IFN-γ. This is consistent with the observation that IFN-γR$^{0/0}$ mice appear to develop normally, with normal lymphocyte subpopulations and no obvious abnormalities [23].

The DMXAA-induced reduction in colon 38 tumour volume occurred more slowly in the IFN-γR$^{0/0}$ knockout mice than in the wild-type mice (Figure 5). This slower rate of tumour regression did not result from reduced haemorrhagic necrosis at the early stages of the antitumour response, as more than 95% necrosis was obtained in both the wild-type and the knockout mice at all doses of DMXAA tested above 20 mg/kg. IFN-γ is known to activate macrophage phagocytosis [30] and it is possible that the slower clearance of necrotic tumour tissue in IFN-γR$^{0/0}$ mice is a result of suboptimal activation of macrophage function. Such an explanation is consistent with the reduced activation of nitric oxide synthesis by macrophages from IFN-γR$^{0/0}$ mice (Figure 1).

A particular feature of the results is that while induction of mRNA for IFN-γ in response to DMXAA occurred to similar

extents in wild-type and IFN-$\gamma R^{0/0}$ mice (Figure 3), serum IFN-γ activity was more than 20-fold higher in the knockout mice. While very low levels of biologically active IFN-γ were present in the serum of wild-type mice, the amount of cytokine activity in IFN-$\gamma R^{0/0}$ mice increased with increasing doses of DMXAA (Figure 4). The observations suggest that IFN-γ induces a post-transcriptional feedback regulation on its own production in normal mice that is absent in IFN-$\gamma R^{0/0}$ mice. The results are consistent with the ability of IFN-γ to downregulate its own receptor β-chain expression, resulting in cellular desensitisation [31]. However, IFN-γ can also upregulate its own expression in human cells [32] with elevation of mRNA levels being paralleled by enhanced secretion of biologically active cytokines [33].

TNF activity in IFN-$\gamma R^{0/0}$ mice also increased with increasing DMXAA dose, and was 2–20 times higher than in wild-type mice (Figure 4), despite similar induction of TNF mRNA (Figure 3). This suggests that IFN-γ exerts a post-transcriptional control over TNF as well as IFN-γ production. TNF production in the wild-type mice in response to DMXAA was similar to that shown previously for normal BDF_1 mice [26], with maximal production detected at a dose of 50 mg/kg, above which TNF levels decreased with increasing dose. The higher production of TNF and IFN-γ in DMXAA-treated IFN-$\gamma R^{0/0}$ mice contrasts with the lowered cytokine profiles reported for IFN-$\gamma R^{0/0}$ mice [22] and IFN-γ knockout mice during endotoxaemia [34]. Different pathways of cytokine activation may explain the differences between DMXAA and endotoxin. DMXAA does not induce the whole spectrum of anti-inflammatory cytokines that is activated in response to LPS [35] and IFNs and several chemokines are induced to a greater extent. Moreover, DMXAA signal transduction does not involve tyrosine phosphorylation of MAP kinases, which is induced by LPS [35].

DMXAA-induced increases in serum nitrate levels, an indication of nitric oxide synthase activity, were similar in IFN-$\gamma R^{0/0}$ and wild-type mice (Figure 2). Thus, IFN-γ does not appear to exert feedback control on nitric oxide synthesis as it does with IFN-γ and TNF. The source of nitric oxide produced in response to DMXAA in the *in vivo* studies is not yet known, but may involve liver cells [36], vascular smooth muscle cells [37], or macrophages activated by induced TNF [38]. Serum nitrate levels following LPS administration were

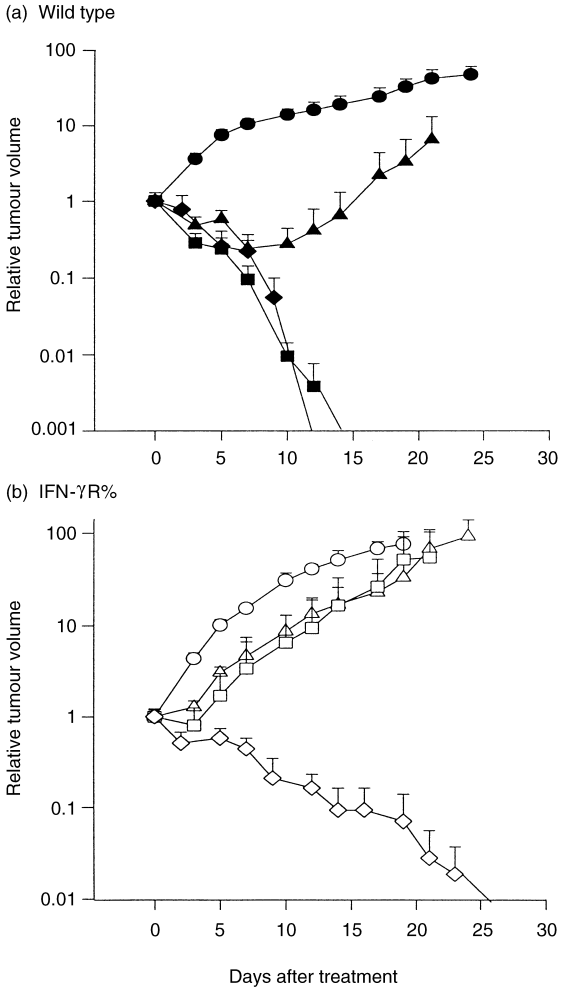

Figure 5. Growth of colon 38 tumours in wild-type (a), or IFN-$\gamma R^{0/0}$ mice (b), without treatment (●, ○), or following treatment with 5,6-dimethylxanthenone-4-acetic acid (DMXAA) at 20 (▲, △), 22.5 (□, ■), or 25 mg/kg (◆, ◇). Mean±standard error from five to six mice per group.

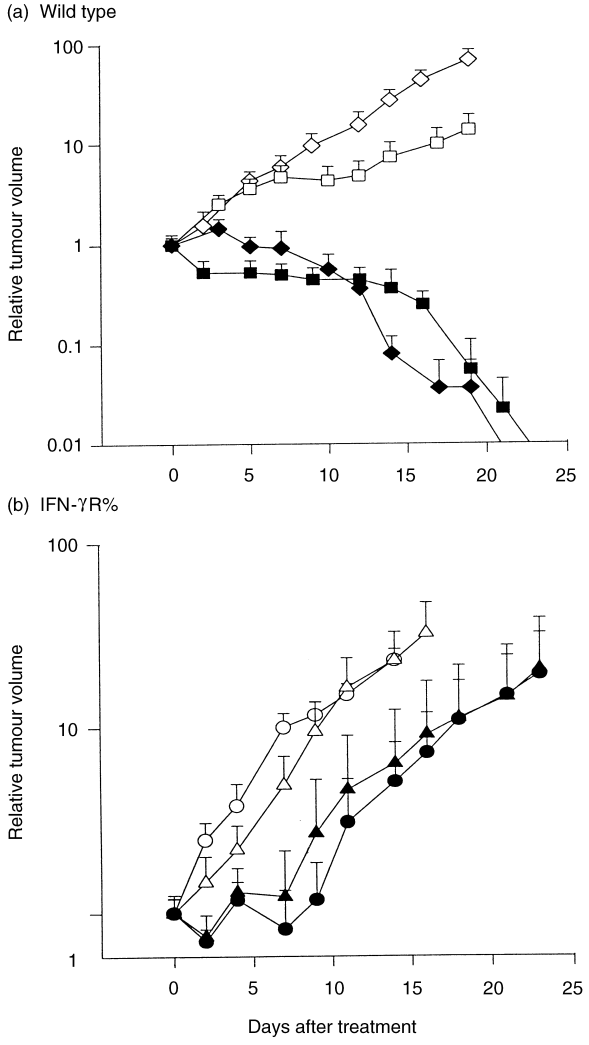

Figure 6. Effect of cyclosporin A (CsA) treatment on the growth of colon 38 tumours, untreated (open symbols) or following 5,6-dimethylxanthenone-4-acetic acid (DMXAA) at 22.5 mg/kg (solid symbols). (a) wild-type mice, no CsA (◇, ◆); CsA treated (□, ■). (b) IFN-$\gamma R^{0/0}$ mice, no CsA (○, ●); CsA treated (◇, ◆) five to six mice per group.

lower in IFN-$\gamma R^{0/0}$ mice (Figure 2) but the difference was not significant. In contrast, another study has shown significantly lower nitrate levels in IFN-γ knockout mice than in wild-type mice following LPS administration [39]. The reasons for this discrepancy are not clear. Synthesis of nitrate was not observed in cultures of macrophages from IFN-$\gamma R^{0/0}$ mice with LPS stimulation, even with the addition of IFN-γ (Figure 1). The results provide evidence that cells from the IFN-$\gamma R^{0/0}$ mice are incapable of responding to IFN-γ, confirming other results [27] that nitric oxide production by macrophages in culture in response to LPS is strictly dependent on priming by IFN-γ. Interestingly, DMXAA did not induce nitric oxide in cultures of macrophages, even in the presence of IFN-γ (Figure 1). Previously, we found that DMXAA stimulated nitric oxide synthesis only if the macrophages had been activated *in vivo* with *Bacillus Calmette-Guerin* [40].

We used CsA treatment as another model of immunosuppression, to compare the antitumour efficacy of DMXAA against the colon 38 tumour, and found no diminution of antitumour effect in CsA-treated IFN-$\gamma R^{0/0}$, wild-type and C57B1/6 animals as compared with animals not receiving CsA (Figure 6). Although a decrease in T cell numbers in the thymus or spleen was not observed (data not shown), the CsA treatment regime was sufficient to overcome allograft rejection in 40% of allogeneic H-2^k C3H/HeJ mice when implanted with the H-2^b colon 38 tumour. The CsA dosage intensity in these studies (three injections, 50 mg/kg) was well above that (three injections, 10 mg/kg) shown to be immunosuppressive and to induce significant levels of CsA in plasma [41]. It is, therefore, unlikely that the lack of effect by CsA on the antitumour action of DMXAA is due to an insufficient dose of CsA.

In conclusion, our studies show that DMXAA is capable of generating an effective response against the colon 38 adenocarcinoma in mice that have been compromised either by genetic disruption of the IFN-γ receptor gene or by pharmacological suppression using CsA. The results are consistent with DMXAA having a complex mechanism of immunomodulatory action involving a number of contributing factors. Deletion of one component may lead to a decrease in efficiency, but does not completely abolish the entire response, as the other components may still be able to compensate. Because many patients may be immunocompromised through disease or medication, the complex mechanism of action of DMXAA could be an advantage for immunomodulatory therapy.

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