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Induction of tumour necrosis factor and interferon-γ in cultured murine splenocytes by the antivascular agent DMXAA and its metabolites

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Abstract

The induction of haemorrhagic necrosis by 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in transplantable murine tumours depends on the *in situ* synthesis of cytokines, particularly tumour necrosis factor (TNF). Since the *in vivo* action of DMXAA would be greatly clarified by the development of an *in vitro* model, we investigated whether DMXAA could induce cytokines in cultured murine splenocytes. DMXAA alone induced low amounts of TNF with an optimal concentration of 10 μg/mL and an optimal time of 4 hr. When combined with low concentrations of lipopolysaccharide, deactivated-lipopolysaccharide (dLPS) or phorbol-12-myristate-13-acetate that did not elicit TNF production alone, synergistic TNF production was obtained. DMXAA also induced interferon-γ at an optimal dose of 300 μg/mL, but the addition of dLPS had no further effect. Decreasing culture pH, although not changing the optimal concentrations for stimulation, increased both TNF and interferon-γ production in response to DMXAA. The major DMXAA metabolites, DMXAA-glucuronide and 6-hydroxy-5-methylxanthenone-4-acetic acid, did not induce either cytokine alone, in combination with dLPS or at low pH. The results indicate that DMXAA rather than a metabolite is responsible for cytokine induction and suggest that the microenvironment of the tumour may be responsible for the observed selective induction of cytokines in tumour tissue.

Keywords: Splenocytes; Lipopolysaccharide; Okadaic acid; Phorbol myristate acetate; Glucuronide

1. Introduction

DMXAA, an anticancer drug developed in this centre, has recently completed Phase I clinical trials in New Zealand and UK. It acts *in vivo* as a biological response modifier, activating through host and tumour cell components a complex series of responses that lead to shutdown of tumour blood flow and the induction of haemorrhagic necrosis of transplantable tumours [1]. It also induces the synthesis of a variety of cytokines and chemokines, including TNF, interferon-α (IFN-α), interferon-γ (IFN-γ) and interferon-inducible protein-10 (IP-10) [2–4]. Several observations suggest that these are important mediators of DMXAA-induced antitumour activity. Co-administration of anti-TNF antibody partially reverses its blood flow inhibitory and antitumour action [5]. Mice lacking genes for TNF or for TNF receptor-1 exhibit reduced responses to DMXAA [6,7]. IFNs and IP-10 are induced by DMXAA [4,8,9] and both have been reported to induce tumour haemorrhagic necrosis [10,11]. Although both tumour and host cell synthesise TNF mRNA *in vivo* in response to DMXAA, the majority of TNF protein is derived from host cells [12,13].

A previous study has demonstrated that cultured human peripheral blood leucocytes (PBL) induce TNF in response to DMXAA [14], although the free drug concentration required for TNF induction was higher than that obtainable *in vivo* in clinical trials [15]. We wished to determine whether cultured murine cells could induce cytokines in response to DMXAA at a free drug concentration comparable to that achieved *in vivo* at the maximum tolerated dose [16].

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Abbreviations: DMXAA, 5,6-dimethylxanthenone-4-acetic acid; TNF, tumour necrosis factor; IFN, interferon; IP-10, interferon-inducible protein-10; PBL, peripheral blood leucocytes; LPS, lipopolysaccharide; MEM, modified essential medium; DMXAA-G, DMXAA-glucuronide; 6-OH-MXAA, 6-hydroxy-5-methylxanthenone-4-acetic acid; dLPS, deactivated-lipopolysaccharide; PMA, phorbol-12-myristate-13-acetate; OA, okadaic acid.

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One feature of the *in vivo* induction of cytokines by DMXAA has been its delayed time course in comparison to that of lipopolysaccharide (LPS) [3]. One explanation for this longer time course would be that it acts indirectly through the generation of a metabolite. DMXAA is metabolised *in vivo* and the major metabolites are the glucuronide and hydroxyl derivatives [17]. It has been hypothesised that the biological effects of the related compound flavone acetic acid could be caused by a metabolite [18]. We have therefore compared the ability of DMXAA to induce cytokines *in vitro* with that of its two major metabolites.

2. Materials and methods

2.1. Materials

The culture medium was α-modified essential medium (αMEM) (Gibco BRL) supplemented with foetal bovine serum (10%), antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) and 2-mercaptoethanol (50 μM). DMXAA was synthesised as the sodium salt at the Auckland Cancer Society Research Centre [19]. DMXAA-glucuronide (DMXAA-G) and 6-hydroxy-5-methylxanthenone-4-acetic acid (6-OH-MXAA), determined by high performance liquid chromatography to be 99% pure, were extracted using solid phase extraction procedure as previously described [20] from urine samples from patients in the Phase I clinical trial [21]. DMXAA and its metabolites were dissolved in αMEM. LPS and dLPS from *Escherichia coli* serotype 055:B5, phorbol-12-myristate-13-acetate (PMA) and okadaic acid (OA) were purchased from Sigma Chemical Co and dissolved firstly in PBS before diluting in αMEM.

2.2. Mouse experiments

C57Bl/6 mice were bred at the Animal Research Unit, Auckland University, and were housed under conditions of constant temperature, lighting and humidity. All experiments used male mice, 8–12 weeks old, and conformed to local institutional guidelines. Murine PBL were derived from mice that had been anaesthetised in a halothane-filled chamber. Blood was withdrawn by cardiac puncture and transferred to a heparinised tube. A layer (10 mL) of Ficoll-Paque™ PLUS (Amersham Biosciences AB) was slowly added to the bottom of the tube that contained 30 mL of blood. After centrifugation at 1300 *g* for 30 min, the upper layer was removed and the layer of PBL was carefully drawn off into a fresh 50 mL tube. The volume was adjusted to 50 mL with unsupplemented culture medium, the cells were collected by centrifugation at 3000 rpm and resuspended in culture medium at 10^7 cells/mL. Splenocytes were obtained from mice following cervical dislocation. Spleens were removed and the cells squeezed out from the capsule into 10 mL culture medium. Spleen cells were aspirated to form a single cell suspension and red blood cells were removed by osmotic lysis. Nucleated cells were counted using a haemocytometer and the cell concentration was adjusted to 3×10^7 cells/mL.

Colon 38 tumours were implanted subcutaneously into mice and allowed to grow to a diameter of approximately 6 mm. Mice were injected intraperitoneally with drug in a volume of 0.2 mL per 20 g body weight. Mice were killed 24 hr after treatment and tumours were removed, fixed in formalin, sectioned and stained with haematoxylin and eosin. The percentage of tissue sections that were necrotic was assessed using a grid system as previously described [22].

2.3. Culture techniques

Murine PBL and splenocytes were cultured in flat-bottomed 96-well plates at 1×10^7 cells/mL and 3×10^6 cells/well, respectively, with drugs in a total volume of 200 μL of culture medium in a humidified incubator at 37° with an atmosphere of 5% carbon dioxide in air. After the appropriate incubation period, which was 4 hr for the majority of the experiments, 150 μL of the supernatant from each well was removed and stored at −20° until analysed for cytokines. Triplicate wells were set up for each test condition and the mean ± SEM calculated. In pH dependence studies, drug was first added to cultures and pH was then adjusted by appropriate addition of HCl or NaOH before dispensing into 96-well plates. The cultures were then incubated in the normal way.

2.4. Measurement of TNF and IFN-γ

Commercially available ELISA kits (OptEIA Mouse TNF-α Kit, Catalogue No. 555268 and OptEIA Mouse IFN-γ Kit, Catalogue No. 555138, PharMingen), were used according to the manufacturer's instructions to measure TNF and IFN-γ.

3. Results

3.1. TNF production in response to DMXAA

Murine PBL were incubated for 4 hr at a range of DMXAA concentrations (10, 30 or 300 μg/mL) with or without the addition of LPS (10 μg/mL) as a co-inducer, according to the procedure used previously for human PBL [14]. No TNF production was detected with DMXAA alone and only marginal TNF synthesis (13 pg/mL) was detected in the presence of LPS at a concentration of 10 μg/mL (results not shown). As an alternative source of leucocytes, murine splenocytes were incubated for 4 hr with a range of DMXAA concentrations. Considerably higher TNF activity (180 pg/mL) was found at an optimal

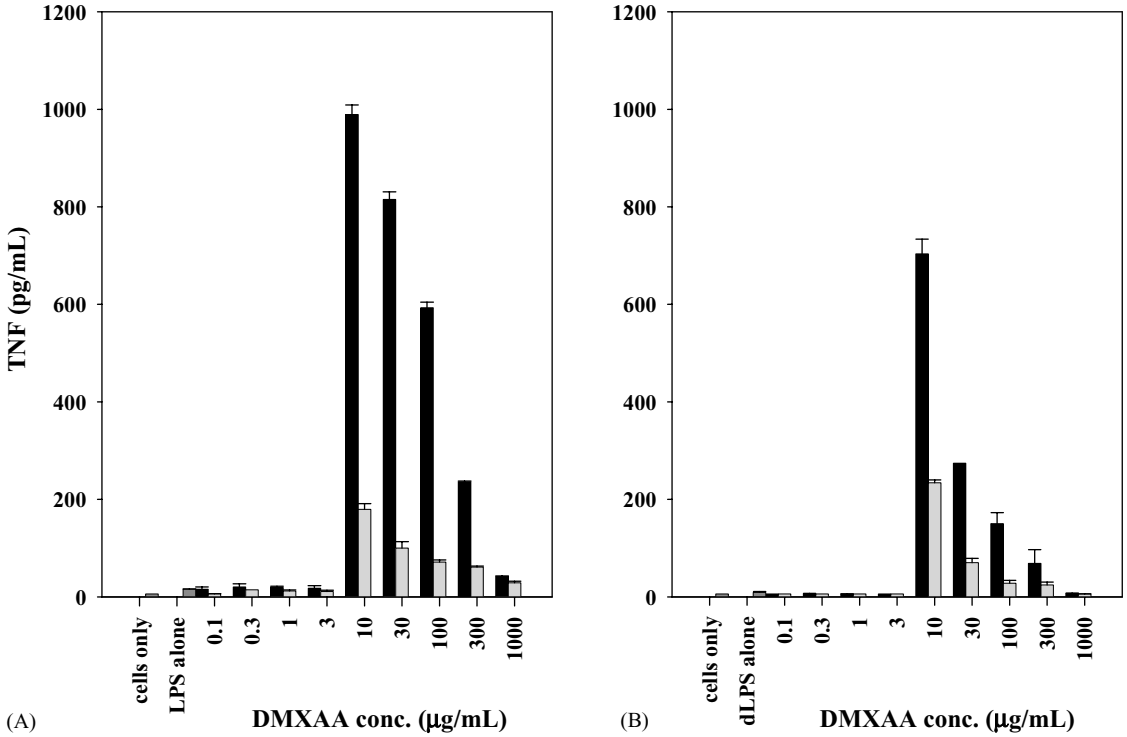

Fig. 1. TNF produced in splenocyte cultures cultured 4 hr after the addition of varying concentrations of DMXAA alone (white bars) or in combination (black bars) with (A) LPS (10 μg/mL) or (B) dLPS (10 μg/mL). Mean ± SEM of triplicate cultures.

drug concentration of 10 μg/mL (Fig. 1). TNF induction was not observed at concentrations below 10 μg/mL and was also reduced above 10 μg/mL. Activity was enhanced approximately 10-fold by addition of LPS (Fig. 1A) and 6-fold by addition of dLPS (Fig. 1B) as co-stimulators. A time course indicated an optimal incubation time of 4–8 hr (Fig. 2). The DMXAA concentration (Fig. 1A and B) and time dependencies (Fig. 2A and B) in the presence of the co-stimulators were similar to those of DMXAA alone.

Studies with human PBL indicated that a number of co-stimulatory agents stimulated the *in vitro* production of TNF apart from LPS, dLPS [14]. Using a DMXAA concentration of 10 μg/mL, we therefore compared the dependence of the response on concentrations of LPS, dLPS, PMA and OA (Fig. 3). LPS demonstrated the largest effect, while OA showed only a marginal effect (Fig. 3). Interleukin (IL)-1α (0.1–100 ng/mL) and IFN-γ (30–10,000 ng/mL) failed to co-stimulate TNF production in the presence of

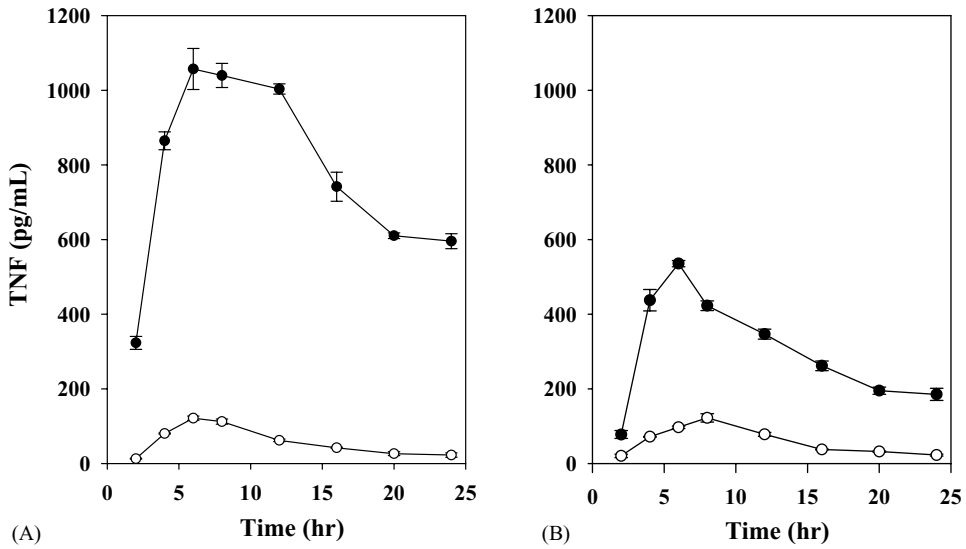

Fig. 2. Time course for TNF production by murine splenocytes cultured (A) with DMXAA (10 μg/mL) alone (O) or in combination with 10 μg/mL LPS (●) or (B) with 10 μg/mL dLPS Mean ± SEM of triplicate cultures.

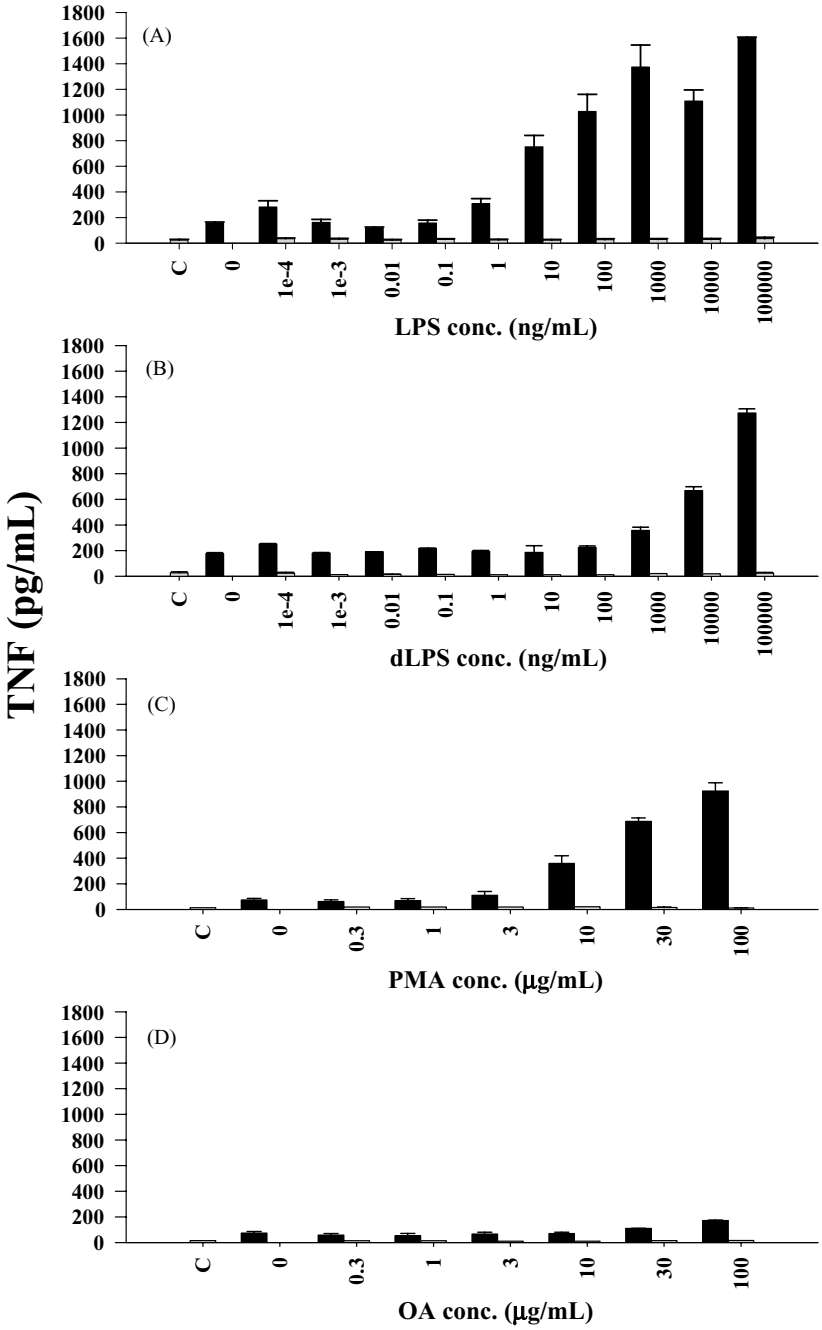

Fig. 3. TNF production by splenocytes cultured 4 hr with varying concentrations of (A) LPS, (B) dLPS, (C) PMA, (D) OA alone (grey bars) or in combination with DMXAA (10 μg/mL) (black bars). "C" denotes control cultures of cells only without drug. Mean ± SEM of triplicate cultures.

DMXAA (data not shown). None of the agents induced TNF in the splenocytes when added alone over the concentration range tested.

3.2. IFN-γ production in response to DMXAA

In order to determine whether DMXAA induced IFN-γ, cultures of murine splenocytes were incubated for 4 hr with a range of DMXAA concentrations. IFN-γ production was detectable at a drug concentration of 10 μg/mL and was maximal at 300 μg/mL, as compared with 10 μg/mL for TNF production. The addition of dLPS did not increase IFN-γ production in response to DMXAA (Fig. 4A).

3.3. Activity of DMXAA metabolites in vitro

We investigated whether the two stable metabolites of DMXAA were able to stimulate cytokine production in culture. Murine splenocytes were cultured with varying concentrations of DMXAA, 6-OH-MXAA or DMXAA-G, with and without dLPS (10 μg/mL), for 4 hr and the culture supernatants were assayed for TNF and IFN-γ. Neither

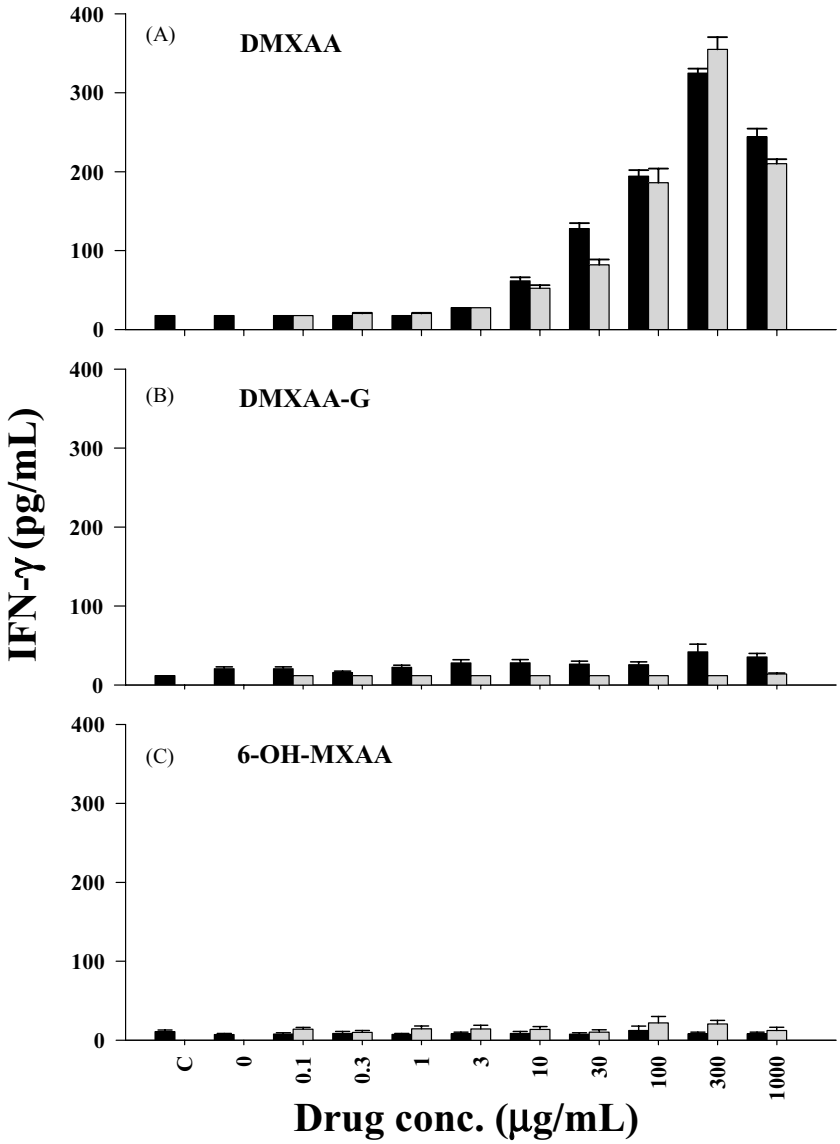

Fig. 4. IFN-γ production after 4 hr in the same cultures as for Fig. 5, stimulated with varying concentrations of (A) DMXAA, (B) DMXAA-G or (C) 6-OH-MXAA alone (grey bars) or in combination with dLPS at 10 μg/mL (black bars). "C" denotes control cultures of cells only without drug. Mean ± SEM of triplicate cultures.

metabolite, either alone or in combination with dLPS, was found to induce significant amounts of TNF (Fig. 5B and C) or IFN-γ (Fig. 4B and C).

3.4. Effect of pH on cytokine induction by DMXAA

DMXAA-G is unstable at a pH of above pH 7.0 and a possible reason for its lack of activity is that it is degraded during the incubation. We therefore lowered the starting pH of the culture media to pH 6.3, where DMXAA-G is relatively stable, and re-examined its ability to induce cytokines. Surprisingly, DMXAA alone induced high amounts of TNF, and in contrast to the results obtained at a pH of 7.2 (Figs. 1 and 2) this was not increased by the addition of dLPS (Fig. 6A). We therefore measured the pH dependence of TNF and IFN-γ induction by DMXAA and found that both TNF and IFN-γ production increased with decreasing pH of the culture medium (Fig. 7). The optimal DMXAA concentrations of 10 μg/mL for TNF production and 300 μg/mL for IFN-γ production were independent of pH. DMXAA-G, either alone or in combination with dLPS, was unable to induce TNF and IFN-γ at the lower pH (Fig. 6).

3.5. Induction of haemorrhagic necrosis by DMXAA-G

The *in vivo* activity of DMXAA-G was tested by its ability to induce haemorrhagic necrosis of Colon 38 tumours at a dose of 25 mg/kg (56 μmol/kg) and 37.6 mg/kg (82 μmol/kg) in comparison to DMXAA at a dose of 25 mg/kg (82 μmol/kg). The degree of haemorrhagic necrosis 24 hr after treatment with either dose of DMXAA-G was 5 ± 2%, as compared with 4 ± 1% for

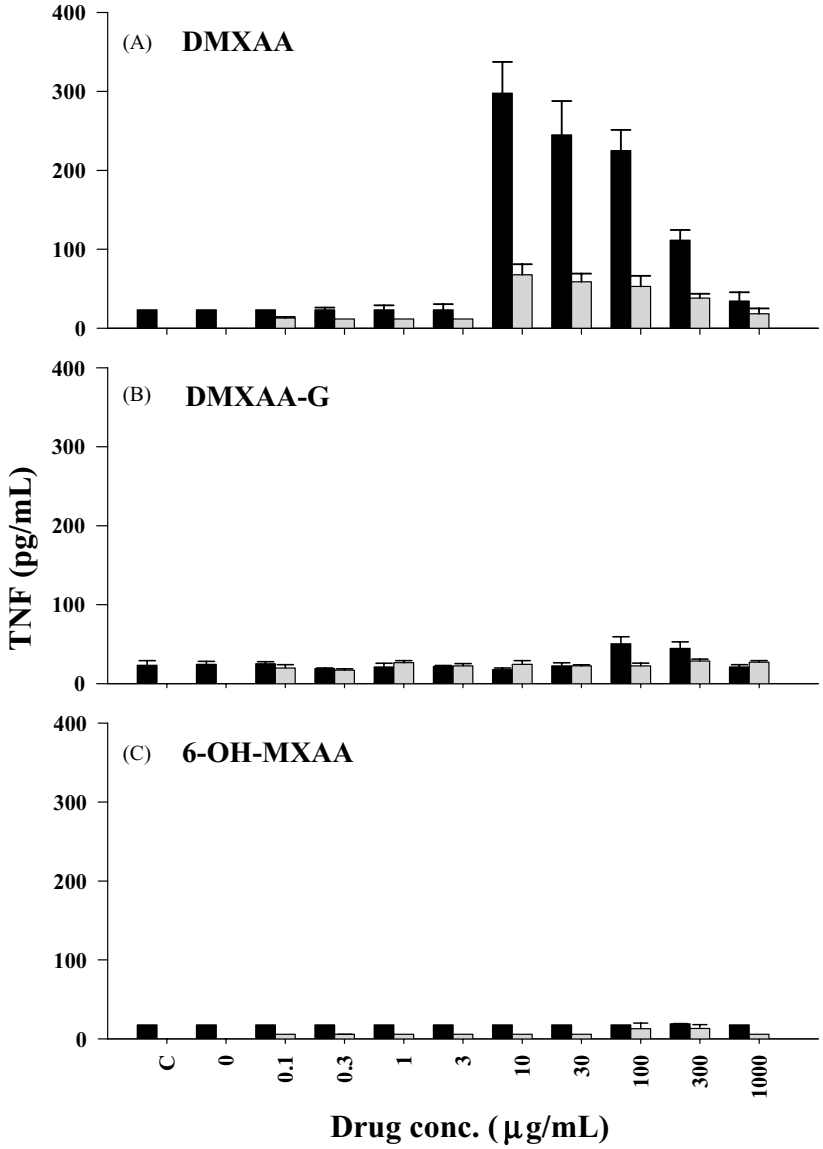

Fig. 5. TNF production by splenocytes cultured 4 hr with varying concentrations of (A) DMXAA, (B) DMXAA-G or (C) 6-OH-MXAA alone (grey bars) or in combination with dLPS at 10 μg/mL (black bars). "C" denotes control cultures of cells only without drug. Mean ± SEM of triplicate cultures.

tumours from untreated mice and 95 ± 2% for DMXAA-treated tumours, indicating that DMXAA-G was inactive *in vivo*.

4. Discussion

We demonstrate here that DMXAA, although almost unable to stimulate TNF production in murine PBL *in vitro*, is able to do so in cultures of mouse splenocytes. An unusual but extremely reproducible dose–response relationship was observed in splenocytes (Fig. 1), with optimal TNF production at a DMXAA concentration of 10 μg/mL. No production was observed at lower concentrations and progressively lower TNF production was also observed at higher concentrations. Following administration of DMXAA to mice at its maximum tolerated dose, the maximal plasma concentration is 600 μM, corresponding to a free drug concentration of 30 μM (9 μg/mL) [17]. Thus, the current results provide a basis for the observed *in vivo* increase in plasma TNF in response to DMXAA [2]. The reason for the lack of a TNF response in cultured murine PBL is not known, but is relevant to the observation that human PBL do not produce TNF when exposed to DMXAA at free drug concentrations up to the maximum achieved in Phase I clinical trials [15].

The ability of DMXAA to induce TNF synthesis in cultured splenocytes was greatly enhanced by addition of LPS, dLPS, PMA or OA (Figs. 1–3). The targets of these agents, such as Toll-like receptors, protein kinase C and protein phosphatase 2A, might be acting by stimulating intermediate pathways, as suggested by similar results using cultures of human PBL exposed to higher DMXAA concentrations [14]. The murine and human systems are

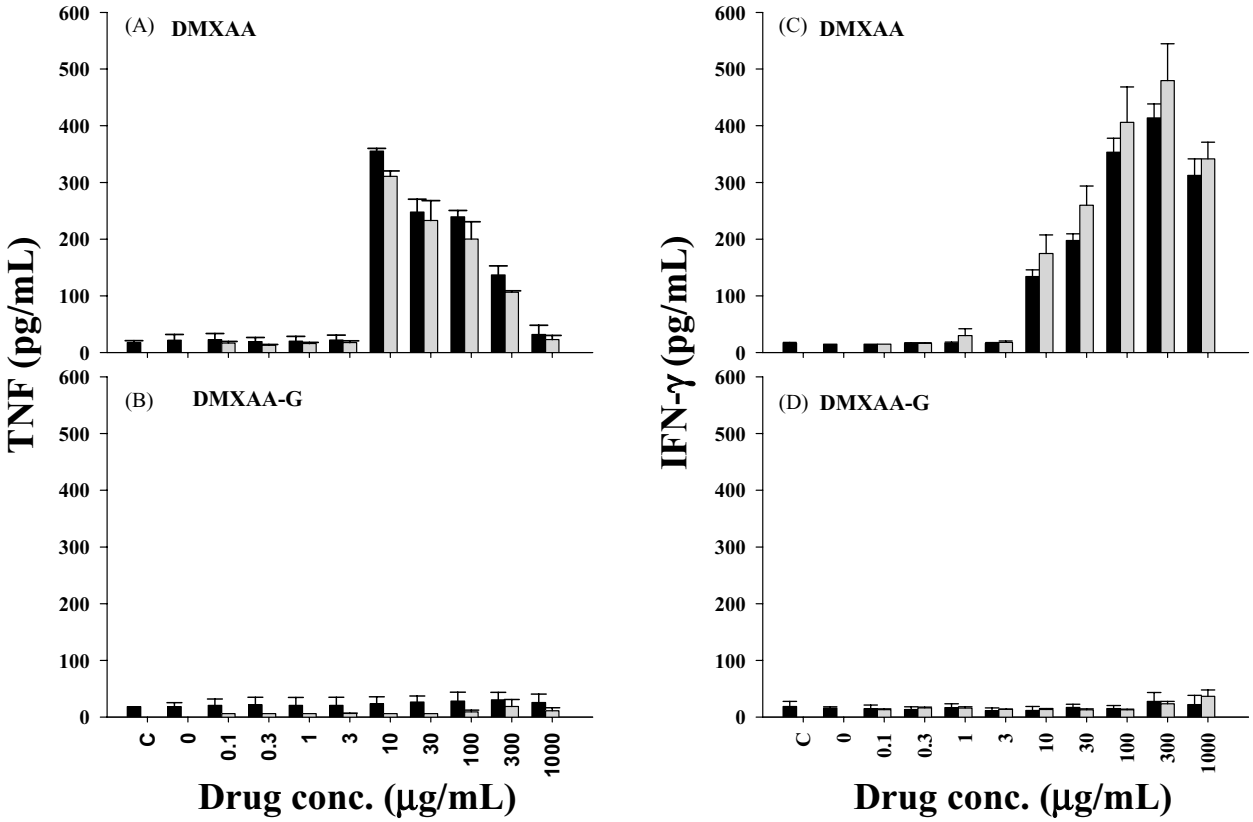

Fig. 6. TNF or IFN-γ production in cultures produced after 4 hr in response to varying concentrations of DMXAA (A and C) or DMXAA-G (B and D) alone (grey bars) or in combination with dLPS (10 μg/mL, black bars) at pH 6.3. "C" denotes control cultures of cells only without drug. Mean ± SEM of triplicate cultures.

not identical in their responses, and LPS alone can stimulate *in vitro* TNF production in the latter. However, LPS can stimulate murine peritoneal macrophages to produce TNF [3], suggesting that this difference may reside in the degree of differentiation of the macrophages.

DMXAA also induced IFN-γ in cultured murine splenocytes, but maximal production occurred at a concentration of 300 μg/mL (Fig. 6), in contrast to the optimal concentration of 10 μg/mL for TNF. Furthermore, the addition of dLPS, which stimulated TNF production, had no effect on IFN-γ production. These results suggest either that the pathways to TNF and IFN-γ production are different or that the cells producing IFN-γ are different from those producing TNF. We have not yet characterised which cell types in the splenocyte population are responsible for TNF production. Macrophages and monocytes are considered to be the primary producers of TNF, while IFN-γ is produced primarily by activated natural killer cells and T lymphocytes. However, T lymphocytes, B lymphocytes and natural killer cells are capable of producing TNF in response to viruses, parasites and tumours [23] and macrophages can produce IFN-γ under some circumstances [24].

A decrease in the pH of the culture medium increased production of both TNF and IFN-γ in splenocytes in response to DMXAA (Fig. 7). Since tumour tissue is known to have a low extracellular pH as a result of anaerobic metabolism [25], this pH dependence could contribute to the selective effects of DMXAA in tumours. One possible mechanism for this effect is increased cellular uptake, since DMXAA accumulation by ECV304 human bladder carcinoma cells has been shown to increase at lower pH [26]. However, this explanation would not explain why there was no change in the optimal DMXAA concentration for TNF production (Fig. 7) and would also not explain why the dependence on the co-stimulatory activity of dLPS was lost at lower pH (Fig. 6). We suggest that at low pH, the co-stimulatory effect of dLPS is replaced by a pH-sensitive co-stimulatory effect. It has been reported that a proton-sensing G-protein-coupled cellular receptor, which stimulates inositol phosphate production, is inactive at pH 7.8 and is fully activated at pH 6.6 [27]. Such a pathway might help to explain the observations that a low external pH stimulates elevation of IL-8 expression by human ovarian carcinoma cells [28] and increases nitric oxide production in response to LPS in cultured rat macrophages [29].

The results argue strongly that DMXAA, rather than one or its metabolites, is the active antitumour agent. The major metabolites 6-OH-MXAA and DMXAA-G had no detectable cytokine inducing abilities *in vitro* and DMXAA-G, the major metabolite, was also unable to induce haemorrhagic tumour necrosis *in vivo*. Insufficient amounts of the

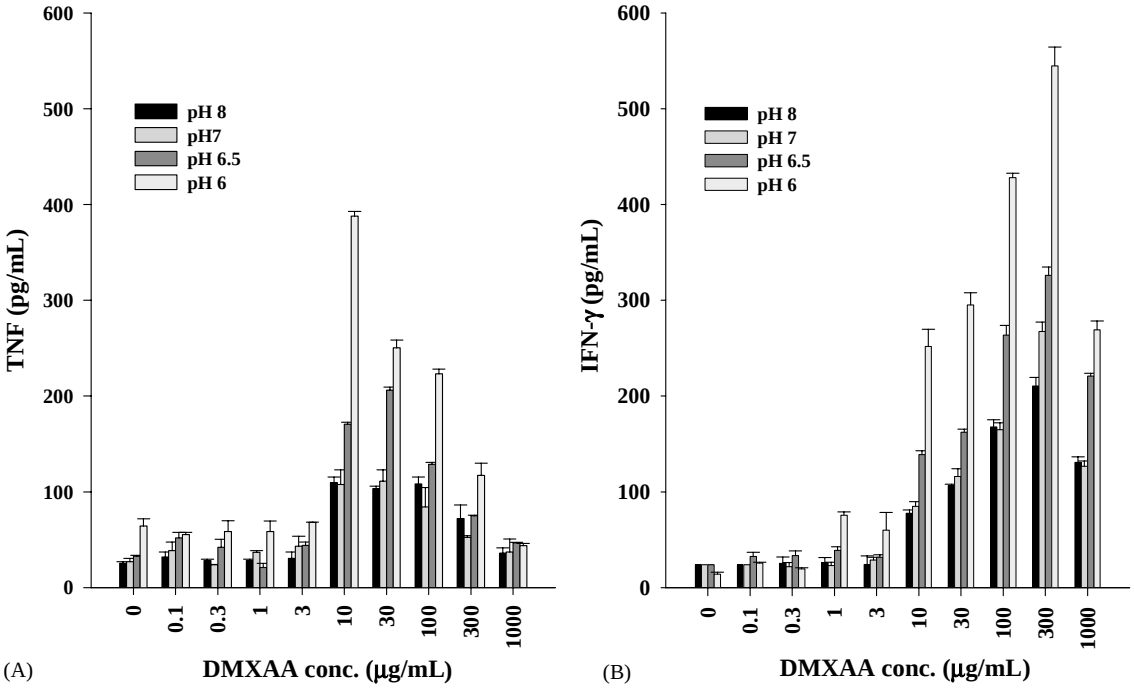

Fig. 7. TNF (A) or IFN-γ (B) production at 4 hr in response to DMXAA (10 μg/mL) using culture medium adjusted to pH 8.0, 7.0, 6.5 or 6.0 before incubation.

minor metabolite were extracted from patients' urine samples to allow it to be tested *in vivo*.

In conclusion, the current results provide an *in vitro* model that helps to explain a number of features of the *in vivo* action of DMXAA. The sudden onset of TNF production by splenocytes as the external concentration of DMXAA is increased (Fig. 2) helps to explain the steep *in vivo* dose dependence of both the antitumour activity and host toxicity of DMXAA. While DMXAA appears to have direct effect against the tumour vasculature [6], these effects are amplified by the production of TNF within the tumour tissue, resulting in prolonged blood flow inhibition, vascular collapse and haemorrhagic necrosis. The important role of TNF is emphasised by the results with TNF knockout mice [13] and with TNF receptor-1 knockout mice [7]. The ability of other co-stimulatory signals and a low extracellular pH to enhance TNF production provides a theoretical basis for the selective induction within tumour tissue. IFN-γ induction after DMXAA treatment occurs to a greater extent systemically than within tumour tissue [4] and may be important in boosting immunity necessary for the long-term tumour regressions [30]. The principles established here for mice will help our understanding of how the novel properties of DMXAA might best be exploited in a clinical situation.

Acknowledgments

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