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The antitumour agent 5,6-dimethylxanthenone-4-acetic acid acts *in vitro* on human mononuclear cells as a co-stimulator with other inducers of tumour necrosis factor

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Abstract

5,6-Dimethylxanthenone-4-acetic acid (DMXAA), currently in phase I trials, demonstrates excellent activity against transplantable murine tumours with established vasculature. The induction of cytokines, particularly of tumour necrosis factor (TNF), appears to be critical to its action. We investigated TNF induction by DMXAA in cultured human peripheral blood leucocytes (HPBL). TNF was measured by an enzyme-linked immunosorbent assay after 8 h, and NF-κB induction by electrophoretic mobility shift assays (EMSA) after 2 h. DMXAA (800 μg/ml) had no effect alone on TNF production but augmented, by up to 4-fold, the ability of bacterial lipopolysaccharide (LPS) to induce TNF. Previously reported results showing TNF production by DMXAA alone were traced to the presence in an earlier batch of DMXAA of a small amount of LPS, the action of which could be blocked by polymyxin B. DMXAA stimulated TNF production by deacylated LPS, which alone had little effect. An antibody (MEM-18) to the CD14 receptor, while blocking the induction of TNF by LPS, enabled DMXAA to both synthesise TNF and induce NF-κB. The structurally related drug, flavone acetic acid (FAA), did not induce TNF or synergise with anti-CD14 antibody. DMXAA strongly augmented the ability of suboptimal concentrations of interleukin-1 (IL-1) (25 ng/ml), okadaic acid (OA) (20 ng/ml) and phorbol-12-myristate-13-acetate (PMA) (5 ng/ml) to induce TNF production, suggesting that it affects multiple pathways converging on NF-κB activation. Sodium salicylate, a drug reported to inhibit the β-subunit of IκB kinase (IKK), appeared to competitively inhibit TNF production by DMXAA in the presence of anti-CD14 antibody. Taken together, the results indicate DMXAA acts *in vitro* on HPBL to co-stimulate TNF production by a wide variety of agents, and suggests that IKK is the target that mediates this action.

Keywords: Lipopolysaccharide; CD14; Interleukin-1; Okadaic acid; Phorbol ester; NF-kappaB

1. Introduction

The experimental antitumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) [1] was developed as a potent analogue of flavone acetic acid (FAA). FAA has excellent preclinical antitumour activity, but no clinical activity [2], while DMXAA has been reported in Phase I clinical trials to induce selective changes in tumour blood flow [3] and to show some evidence of clinical activity [4]. The antitumour effects of DMXAA and FAA on experimental murine tumours are dramatic and involve shutdown of the tumour vasculature [5,6] and modulation of the host immunity [7,8]. DMXAA induces a number of cytokines and chemokines in mice, including tumour necrosis factor (TNF) [9] and the antiangiogenic chemokine interferon-inducible protein-10 (IP-10) [10]. A series of studies has demonstrated that the antivascular effects of DMXAA are associated with TNF production, particularly within tumour tissue [11–13].

TNF is produced as a result of activation and nuclear localisation of the Nuclear Factor-kappa B (NF-κB) family of transcription factors, which are liberated by the phosphorylation and subsequent proteolytic degradation of the inhibitory subunit IκB. This is carried out

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by the enzyme IκB kinase (IKK), which comprises α-, β- and γ-subunits. Phosphorylation and activation of the β-subunit [14] is thought to be mediated by the NF-κB inducing kinase (NIK), which is activated by the TNF and interleukin-1 (IL-1) kinase cascades [15], and NF-κB activating kinase (NAK), which is activated by phorbol esters and growth factors [16]. Lipopolysaccharide (LPS) acts by binding (in concert with an LPS binding protein) to the CD14 receptor, a membrane-bound 55 kDa glycoprotein [17] linked to the TNF and IL-1 kinase cascades by the toll-like receptor (TLR) proteins on the plasma membrane [18,19]. DMXAA has been reported to activate the NF-κB pathway in a murine macrophage line [20].

Although TNF is synthesised by human peripheral blood leucocytes (HPBL) *in vitro* in response to LPS, conflicting data exist as to whether it is produced by DMXAA [21,22]. We have investigated further in this report the effects of DMXAA on TNF production in HPBL.

2. Materials and methods

2.1. Incubation of HPBL with drugs

Partially purified buffy coats were purchased from Auckland Blood Centre and divided into 15-ml aliquots in 50-ml centrifuge tubes (2070 Conical Tubes, Becton Dickinson Labware, NJ, USA). All extraction operations were carried out at 7 °C to prevent clotting. Unsupplemented α-modified essential medium (α-MEM) was added to 30 ml and a 10-ml layer of Ficoll-Paque PLUS was slowly added to the bottom of the tubes. After centrifugation at 300*g* for 30 min, the upper layer was removed and the HPBL layer was carefully drawn off into a fresh 50-ml centrifuge tube. The volume was adjusted to 50 ml with unsupplemented growth medium, the cells were centrifuged at 300 g, and the HPBL were resuspended at 10^7 cells/ml in α-MEM supplemented with fetal bovine serum (10% v/v), streptomycin sulphate (100 μg/ml) and penicillin-G (100 units/ml). Cells were added either to 24-well plates (1 ml/well; Nunc, Kamstrup, Roskilde, Denmark) or to 100 mm Petri dishes (10 ml/plate) and incubated in 5% CO_2/air at 37 °C overnight. Agents (made up at twice the final concentration) were added and plates were further incubated for the appropriate times. DMXAA sodium salt (this laboratory) was dissolved in medium and protected from light [23]. FAA (National Cancer Institute, USA) was dissolved in 5% (w/v) sodium bicarbonate and diluted with medium. IL-1 (R&D Systems, MD), okadaic acid, LPS and deacylated LPS (Sigma Chemical Co., MO, USA) were dissolved in α-MEM, filter-sterilised and used immediately. The MEM-18 mouse anti-human CD14 IgG antibody was obtained from Sanbio bv, am Uden, The Netherlands, and was freed from azide before use by ultrafiltration. It was found to be free of LPS by the Endospecy ES-50M LPS quantitation system (Seikagaku Corporation, Tokyo, Japan).

2.2. Electrophoretic mobility shift assays (EMSA) protocol

The oligonucleotide (5′-AGCTTACAAGGGACTTTC), from the κ-immunoglobulin enhancer [24], was annealed with its complementary strand and labelled using an Oligonucleotide Labelling Kit (Pharmacia Biotech, Piscataway, NJ, USA). The labelled probe was centrifuged through a ProbeQuant G-50 micro-column (Pharmacia Biotech, Piscataway, NJ, USA; 1000*g*, 3 min) to remove unincorporated α-[^{32}P]-deoxycytidine triphosphate (dCTP), and diluted to an activity of 20 000 counts per minute (cpm)/μl. HPBL in tissue culture dishes (10 ml; 10^7 cells/ml) were incubated overnight before addition of DMXAA or LPS and further incubation for 2 h [20,25]. Cells were removed from tissue culture dishes and washed with ice cold phosphate-buffered solution (PBS) (250*g*, 10 min, 4 °C). The pellets were lysed in Nonidet P-40 (NP-40)-containing buffer (10 min incubation on ice), the samples centrifuged (700*g*, 5 min, 4 °C), the supernatant removed, and the nuclear pellet lysed with high salt buffer (30 min on ice). Dialysis buffer (100 μl) was added and the tubes were centrifuged (20 000*g*, 15 min, 4 °C). Supernatants containing the nuclear proteins were removed, assayed for protein content, and stored at −70 °C until assay. Nuclear extract corresponding to 5 μg of protein was combined with loading buffer (4 μl), poly dI:C (1.5 μg) and water to a total volume of 20.5 μl. Tubes were centrifuged briefly, kept on ice for 10 min and radiolabelled probe (20 000 cpm) was added. After a further 15 min on ice, loading dye (in 2 μl) was added and samples were electrophoresed. The gel was dried and exposed overnight at −70 °C to autoradiographic film (Kodak, PTY Ltd., Victoria, Australia).

2.3. Measurement of TNF

After the appropriate incubation period of HPBL with drug in 24-well plates, supernatants were removed and either assayed immediately or stored at −20 °C. TNF standards were prepared by making serial dilutions of the TNF stock solution in supplemented culture media (concentration range 10–10 000 pg/ml). Enzyme-linked immunosorbent assay (ELISA) plates were made using the OptEIA Human TNF-α Set (Pharmingen, San Diego, CA, USA). TNF standards and samples were added to the ELISA plates and the assays were carried out according to the manufacturer's directions.

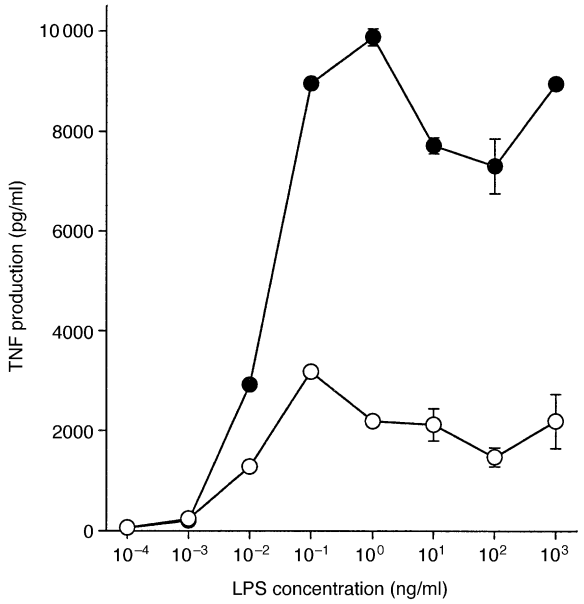

Fig. 1. Effect of 5,6-dimethylxanthenone-4-acetic acid (DMXAA) on lipopolysaccharide (LPS)-induced tumour necrosis factor (TNF) production in human peripheral blood leucocytes (HPBL) *in vitro*. HPBL were incubated (8 h) with the indicated concentrations of LPS alone (○) or in combination with DMXAA (●). Supernatants were moved and assayed for TNF content. Vertical lines represent the ranges of duplicate cultures.

3. Results

3.1. Synergy between LPS and DMXAA in the induction of TNF

Based on an initial observation that the *in vitro* production of TNF by HPBL in response to DMXAA was altered by a small concentration of LPS, we tested for possible synergy between LPS and DMXAA. LPS alone was inactive at concentrations below 1 pg/ml and induced a relatively constant amount of TNF production over the range 10–1 μg/ml (Fig. 1). DMXAA (800 μg/ml) was unable to induce TNF production alone, but caused an approximately 4-fold increase in TNF levels in response to LPS over the range of 100–1 μg/ml, and more than doubled the response at 10 pg/ml.

The CD14 receptor, present on the membranes of cells of the macrophage/monocyte lineage, mediates the effects of LPS [26] and antibodies can be prepared that bind to this receptor and prevent the action of LPS [27]. As shown in Fig. 2a, such an anti-CD14 antibody (MEM-18; 10 μl added 15 min prior to the drugs) inhibited TNF production by HPBL in response to 1 μg/ml LPS by approximately 50%, abolishing production in response to 1 ng/ml LPS. The antibody alone at this concentration caused no significant increase in TNF

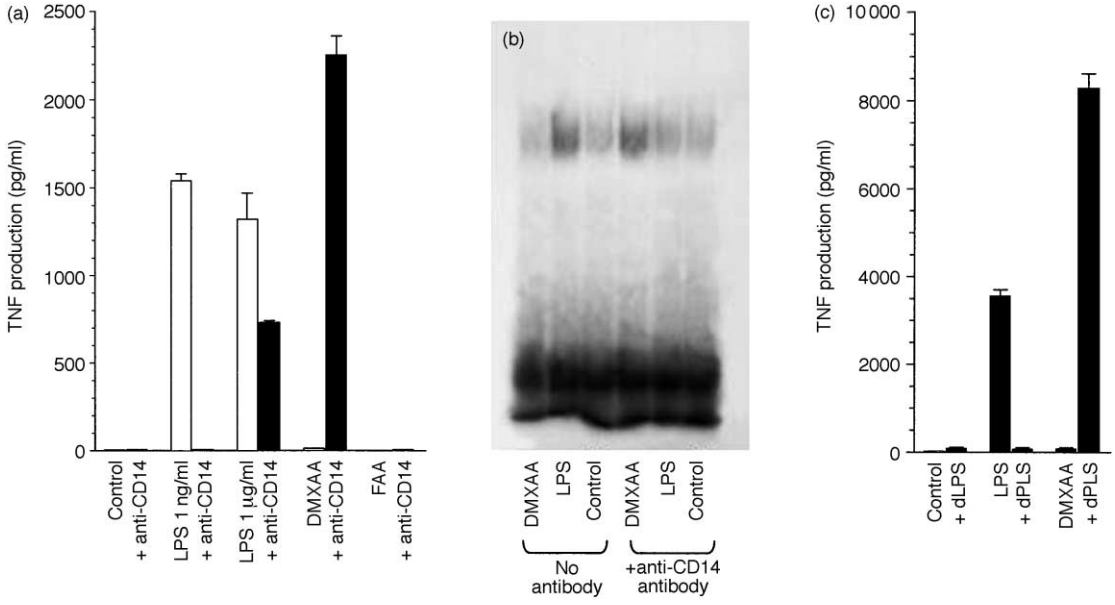

Fig. 2. (a) Effect of anti-CD14 antibodies on 5,6-dimethylxanthenone-4-acetic acid (DMXAA) and lipopolysaccharide (LPS)-induced tumour necrosis factor (TNF) production in HPBL *in vitro*. Human peripheral blood leucocytes (HPBL) were incubated (8 h) with LPS (1 ng/ml or 1 μg/ml), DMXAA (800 μg/ml) or flavone acetic acid (FAA) (800 μg/ml) in the absence (no shading) or the presence (shading) of anti-CD14 antibodies. Supernatants were removed and assayed for TNF. (b) Effect of anti-CD14 antibodies on DMXAA- and LPS-induced Nuclear Factor-kappa B (NF-κB) activation in HPBL *in vitro*. HPBL were incubated (15 min) with DMXAA (800 μg/ml), LPS (1 ng/ml) or no addition (control) in the presence or absence of anti-CD14 antibodies. Nuclear extracts were prepared and assayed for the presence of NF-κB. (c) Effects of dLPS on LPS- and DMXAA-induced TNF production in HPBL *in vitro*. HPBL were incubated (8 h) with dLPS alone (500 μg/ml), or in combination with LPS (1 ng/ml) or in combination with DMXAA (800 μg/ml).

production. Unexpectedly, anti-CD14 antibody induced high TNF production when combined with DMXAA, which alone did not induce TNF. The synergistic effect was not observed with FAA, which did not induce TNF, either alone or in the presence of anti-CD14 antibody (Fig. 2a). To confirm that the effects of DMXAA were specific, HPBL were also incubated with each of the drugs in the presence of mouse anti-human antibody to Mac-3, a cell marker of monocytes and macrophage, but this had no effect.

The induction of TNF by DMXAA is mediated by the induction of the NF-κB transcription factor [20]. The effect of the anti-CD14 antibody on the induction of NF-κB in HPBL was therefore tested using EMSA assays. NF-κB activation was not observed in untreated HPBL or in HPBL treated with anti-CD14 antibody alone. Activation was observed in response to LPS (1 ng/ml) and this was inhibited by the presence of anti-CD14 antibody. DMXAA alone (800 μg/ml) did not induce NF-κB activation, but gave a clear response in the presence of the anti-CD14 antibody (Fig. 2b).

An inactive form of LPS, deacylated LPS (dLPS), does not induce TNF alone, and competitively inhibits the induction of TNF by LPS by competing for the CD14 receptor for LPS [28]. As shown in Fig. 2c, dLPS (500 μg/ml; 15 min pre-incubation) induced TNF production only slightly above control values. dLPS also strongly reduced TNF production in response to LPS (1 ng/ml). However, a combination of dLPS (500 μg/ml; 15 min pre-incubation) with DMXAA (800 μg/ml) caused a large increase in TNF production.

3.2. Inter-individual variability in the response of HPBL to DMXAA and anti-CD14 antibody

As shown in Fig. 1, LPS at concentrations as low as 10 pg/ml synergised with DMXAA in TNF production by HPBL. Although earlier batches of DMXAA synthesised in this laboratory were previously found to be negative for LPS in the Limulus polyphemus amoebocyte lysate test, the sensitivity of this test was not sufficient to eliminate the possibility of a trace LPS contamination. A more stringent test (Endospecy LPS quantitation system) was applied, and the DMXAA used for these experiments was found, at a concentration of 800 μg/ml, to have a very low LPS concentration (0.024 pg/ml). However, the batch of DMXAA used for our previous study with HPBL [22] was found to have an LPS concentration of 5.6 pg/ml under the same conditions. Furthermore, when HPBL were exposed to this batch of DMXAA in the presence of polymyxin B (10 μg/ml), a drug which binds to LPS and prevents its interaction with the receptor [29], TNF production was abolished.

Considerable inter-individual variation in TNF production by DMXAA in different HPBL samples has been reported [22]. To determine the cause of this variation, we compared TNF production by HPBL from eight donors incubated either with DMXAA and anti-CD14 antibody or with the batch of DMXAA used in the previous study [22]. TNF production (>10 pg/ml) was not detected either in the controls of any of the donors or in response to DMXAA alone. Combination of DMXAA and anti-CD14 antibody induced TNF production in all eight donors, although HPBL from one donor (P8) secreted only low amounts (Fig. 3). An excellent correlation ($r = 0.94$) was observed between TNF responses of the earlier batch of DMXAA and current batch of DMXAA supplemented with anti-CD14 antibody, suggesting that the observed variation was related to differences in the CD-14 response rather than to differences in response to DMXAA.

3.3. Effects on TNF production by cytokines

The CD14 receptor for LPS appears to interact with TLR proteins, which are part of the same superfamily as the receptor for the cytokine IL-1. Like LPS, IL-1 induces TNF production in HPBL through the induction of NF-κB [19]. We found DMXAA to cause a large stimulation of TNF production by concentrations of IL-1 that alone had little effect. At an IL-1 concentration of 30 ng/ml, DMXAA caused a 56-fold enhancement of TNF production (Fig. 4). Similar experiments with the cytokine IL-6, which induces NF-κB in some cells, but not in HPBL, were performed. No stimulation of TNF production was found in response to IL-6 alone

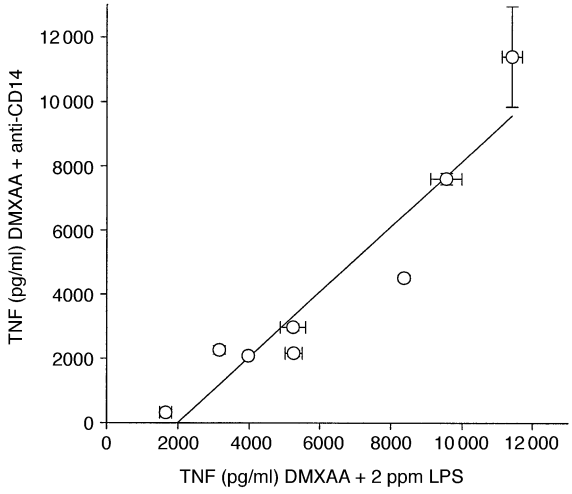

Fig. 3. Relationship between tumour necrosis factor (TNF) production in response to 5,6-dimethylxanthenone-4-acetic acid (DMXAA) (800 μg/ml) in the presence of anti-CD14 antibodies (10 μl added 15 min prior to DMXAA), and response to the DMXAA batch (800 μg/ml) used in a previous study [22] and containing 2 parts per million (ppm) LPS. Human peripheral blood leucocytes (HPBL) from eight donors were incubated with drug for 8 h. Supernatants were removed and assayed for TNF. Horizontal and vertical bars represent the ranges of duplicate cultures.

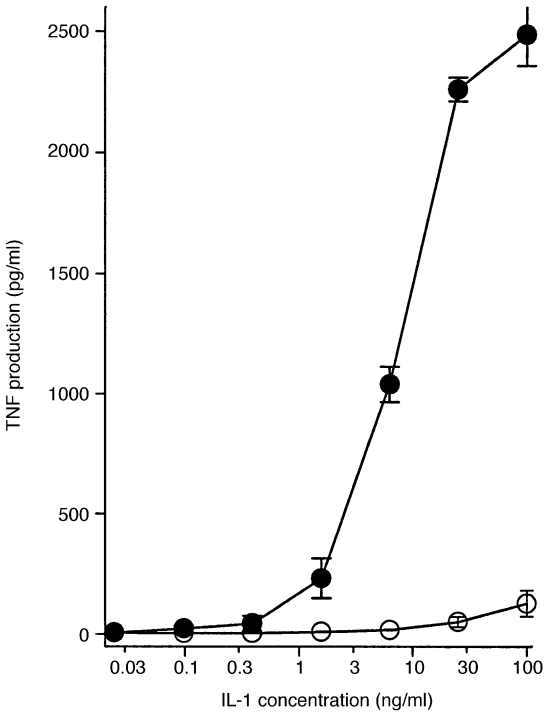

Fig. 4. Effect of 5,6-dimethylxanthenone-4-acetic acid (DMXAA) on interleukin-1 (IL-1) induced TNF production in human peripheral blood leucocytes (HPBL) *in vitro*. HPBL were incubated (8 h) with the indicated concentrations of IL-1 alone (○) or in combination with DMXAA (●). Supernatants were removed and assayed for TNF. Vertical lines represent the ranges of duplicate cultures.

at concentrations up to 1 μg/ml, and the addition of DMXAA had no effect.

3.4. Effects of DMXAA on TNF production by phorbol-12-myristate-13-acetate (PMA) and okadaic acid (OA)

PMA, an activator of protein kinase C, induces TNF synthesis in HPBL [30], probably by a mechanism involving NAK [16]. PMA alone at concentrations below 20 ng/ml had no substantial effect on TNF production by HPBL (Fig. 5a), but in combination with DMXAA (800 μg/ml) induced a high degree of TNF production. OA, an inhibitor of protein phosphatases PP1 and PP2A, stimulates TNF production in HPBL by increasing the degree of phosphorylation of cellular proteins [31,32]. OA at concentrations up to 20 ng/ml had only a small effect on TNF production by HPBL, but this was substantially increased by co-incubation with DMXAA (800 μg/ml) (Fig. 5b). At a higher concentration (100 ng/ml), OA alone induced TNF synthesis in HPBL and DMXAA reduced this effect (Fig. 5b), probably by increasing toxicity.

3.5. Effect of salicylate

Sodium salicylate has been reported to inhibit the production of TNF in response to LPS and cytokines by a mechanism that involves inhibition of the release of

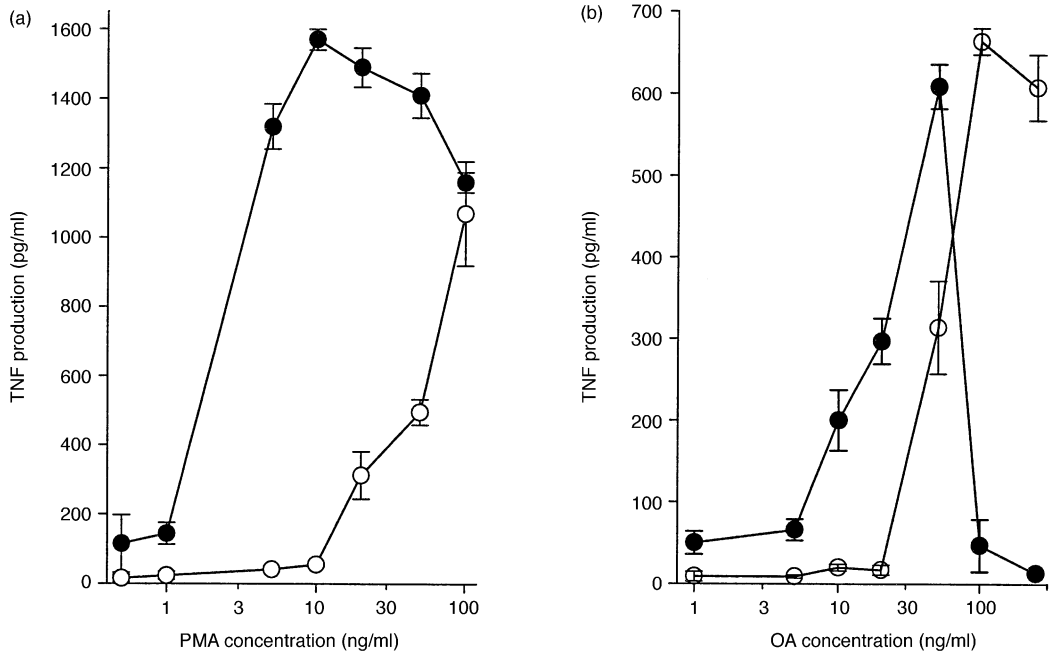

Fig. 5. Effect of 5,6-dimethylxanthenone-4-acetic acid (DMXAA) on tumour necrosis factor (TNF) production in human peripheral blood leucocytes (HPBL) *in vitro* in response to (a) phorbol-12-myristate-13-acetate (PMA) and (b) okadaic acid. HPBL were incubated (8 h) with the indicated concentrations of drug either alone (○) or in combination with DMXAA (●). Supernatants were removed and assayed for TNF. Vertical lines represent the ranges of duplicate cultures.

NF-κB [33]. Further work has shown that salicylate specifically inhibits the β-subunit of IKK [34]. 2-Hydroxyphenylacetic acid has a structural similarity to both salicylic acid and to part of the DMXAA molecule (Fig. 6a–c), raising the question of whether these compounds would inhibit the action of DMXAA. We tested the effects of salicylate and 2-hydroxyphenylacetic on the ability of DMXAA (800 μg/ml), in combination with anti-CD14 antibody, to induce TNF, and found a 50% inhibition at 3.2 and 3.3 mM, respectively, and 100% inhibition for both compounds at 15 mM (data not shown). We investigated the effects of salicylate in further detail, testing it at concentrations from 2 to 15 mM on the effect of DMXAA over the concentration range 400–1600 μg/ml (Fig. 6d). DMXAA alone provided a dose–response curve that was best fit by a sigmoidal function. The shapes of the DMXAA dose–response curves in the presence of salicylate were also described best by sigmoidal functions based on competitive inhibition kinetics (Fig. 6d).

4. Discussion

The results demonstrate that DMXAA stimulates TNF synthesis in cultured human leucocytes by amplifying a signal induced by another agent, rather than by inducing it in its own right. DMXAA increases the level of TNF production by LPS above that inducible by LPS alone (Fig. 1). Significantly, DMXAA synergises with an anti-CD14 (LPS receptor) antibody, which itself blocks TNF production by LPS (Fig. 2a). It similarly synergises with deacylated LPS, which provides only a minimal response alone and blocks TNF production by LPS (Fig. 2c). DMXAA augments, by up to 56-fold, the TNF response to the cytokine IL-1 (Fig. 4), although it fails to induce a response to another inflammatory cytokine, IL-6. However, IL-6 does not induce TNF by itself and is known to inhibit the induction of TNF by LPS [35]. DMXAA synergises with two drugs that induce TNF by pathways that do not include surface receptors, the protein kinase C inducer PMA and the protein phosphatase inhibitor OA (Fig. 5).

The induction of TNF by DMXAA *in vitro* has previously been shown to vary considerably with different donors [22]. Using a very sensitive LPS detection method, we have shown here that the DMXAA batch used in that study contained sufficient LPS (approximately 20 fM final concentration) to synergise with DMXAA and to account for the observed TNF production. The results in Fig. 3 demonstrate that the combination of DMXAA with anti-CD14 antibody restores TNF production in multiple donors to levels very similar to those produced by the DMXAA batch used in these studies, but maintains the variation among the individual donors. The strong correlation ($r = 0.94$)

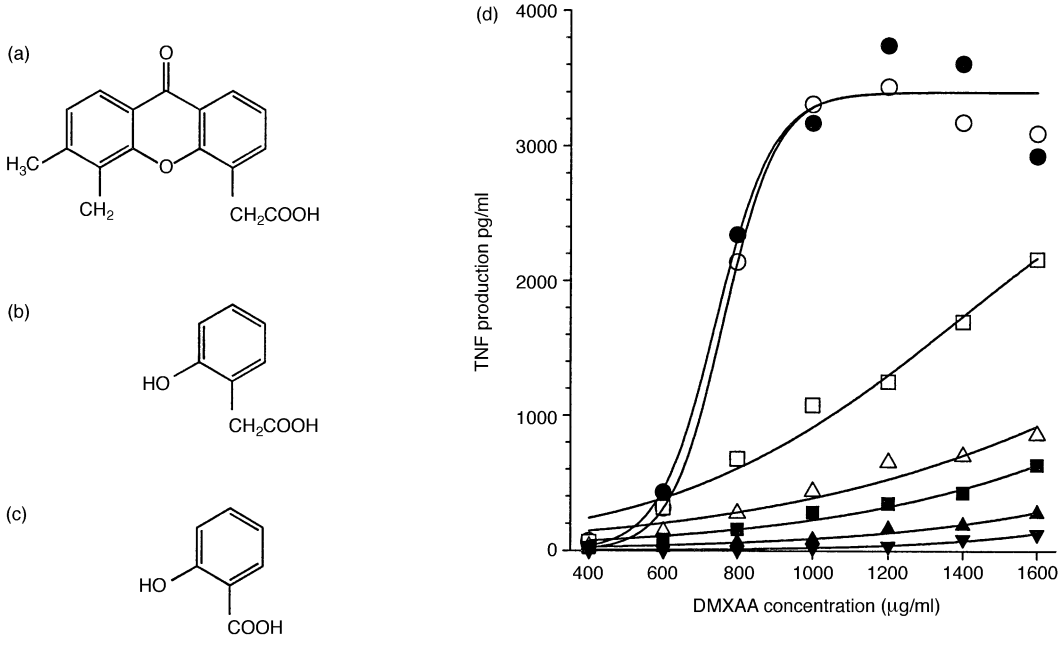

Fig. 6. (a) Structure of 5,6-dimethylxanthenone-4-acetic acid (DMXAA); (b) structure of 2-hydroxyphenylacetic acid; (c) structure of salicylic acid; (d) effect of sodium salicylate on tumour necrosis factor (TNF) production in human peripheral blood leucocytes (HPBL) in response to the indicated concentrations of DMXAA, in the presence of anti-CD14 antibody. HPBL were pre-incubated with antibodies for 15 min before addition of the indicated concentrations of DMXAA together with sodium salicylate at concentrations of 2 mM (○), 4 mM (□), 6 mM (△), 8 mM (■), 10 mM (▲) or 15 mM (▼). Control cells received DMXAA alone (●). Cultures were incubated for 8 h and assayed for TNF production (each point represents one experiment). Sigmoidal expressions were fitted to the data, with minima of 0 and maxima of 3200. Correlation coefficients were in the range 0.96–0.99.

between TNF production for the two data sets suggests that heterogeneity exists at the level of TNF response. Such heterogeneity may reflect differences in the leucocyte populations involved in the response, and these have not yet been characterised. It is noteworthy that the lack of *in vitro* activity of the drug FAA reported earlier [22] is confirmed in this study using FAA in the presence of anti-CD14 antibody (Fig. 2a).

DMXAA in the presence of anti-CD14 antibody induces NF-κB activation (Fig. 2b). LPS, IL-1, PMA and OA are all thought to mediate their effects on TNF synthesis through the activation of the NF-κB family of transcription factors. NF-κB proteins are normally sequestered in the cytoplasm by IκB inhibitory proteins, but phosphorylation of IκB by the cellular kinase IKK causes dissociation of NF-κB, localisation in the nucleus and initiation of transcription of the *TNF* (and other) genes. CD14, the receptor for LPS, lacks an intracellular binding domain, but can interact in the plasma membrane with TLR proteins [18], forming complexes with signalling components such as the TNF-receptor-associated factor (TRAF) which are common to the TNF and IL-1 signalling pathway [19]. This in turn leads to the assembly of a series of adapter proteins that includes NIK, an activator of IKK. PMA, an activator of protein kinase C, induces the production of TNF through the activation, by some forms of PKC, of NAK [16]. IκB phosphorylation is controlled by the balance between phosphorylation by IKK and dephosphorylation by protein phosphatases [36,37]. The induction of NF-κB by okadaic acid occurs by inhibition of IKK dephosphorylation by a protein phosphatase 2A [31,36].

Since DMXAA synergises with drugs acting in two different pathways leading to IKK phosphorylation, involving NIK and NAK, respectively, the possibility that the active (phosphorylated) form of IKK is the biochemical target for DMXAA must be considered. Because salicylate is an inhibitor of IKK and appears to bind specifically to the β-subunit of IKK [34], we tested salicylate and found that it inhibited the action of DMXAA (Fig. 6). The shape of the family of dose–response curves generated by different concentrations of salicylate was consistent with it functioning as a competitive rather than a non-competitive inhibitor (Fig. 6). Furthermore, the salicylate analogue 2-hydroxyphenylacetic acid, which models the acidic half of DMXAA, was an equally effective inhibitor (result not shown). Thus, the data are consistent with DMXAA and salicylate competing for the same binding site, with one causing a stimulation of activity and one an inhibition. One possible mechanism is that DMXAA binds to the phosphorylated form of IKK to induce an allosteric change that increases its activity. This is consistent with the dose-response to DMXAA in the presence of a constant concentration of a second signal (anti-CD14 antibody), which is best fitted by a sigmoidal function. The feasibility of such a mechanism is supported by the finding that a peptide corresponding to the C-terminal sequence of IκB-α can bind to an allosteric site within the IKK enzyme and greatly increase its phosphorylation activity [38].

Since DMXAA is currently being considered for further clinical trials, there are important implications of the current results for the use of DMXAA in humans. We have evidence (M. Philpott, L.-M. Ching, B.C. Baguley, manuscript in preparation) that DMXAA requires a second signal for *in vitro* production of TNF by murine leucocytes, and that depletion of circulating LPS in mice reduces the *in vivo* TNF response to DMXAA. This suggests that a second signal might be required for a TNF response in humans. Circulating LPS is known to be generally low (<10 pg/ml) in humans [39] and may be insufficient to provide an adequate second signal. The clinical co-administration of second signals for DMXAA action is an important consideration for the future development of this novel drug.

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