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NF-kappa B activation *in vivo* in both host and tumour cells by the antivascular agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA)

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Abstract

5,6-Dimethylxanthenone-4-acetic acid (DMXAA), a new anticancer agent developed in this centre, has an antivascular action and causes regression of transplantable murine tumours that is mediated partially by the intratumoral production of tumour necrosis factor (TNF). DMXAA activates the nuclear factor-κB (NF-κB) transcription factor, which is involved in TNF synthesis and has also been suggested to mediate resistance to TNF. We wished to determine whether tumour cell NF-κB activation modulated the *in vitro* and *in vivo* effects of DMXAA. We compared the response of the 70Z/3 pre-B lymphoma cell line with that of its mutant 1.3E2 sub-line, which has a defective γ-subunit of IKK, the kinase that phosphorylates IκB leading to NF-κB activation. As shown by electrophoretic mobility shift assays (EMSAs), DMXAA induced *in vitro* translocation of NF-κB (p50 and p65 subunits) into the nucleus of 70Z/3 cells, but not of 1.3E2 cells. However, when the cell lines were then grown as subcutaneous tumours in mice and treated with DMXAA (25 mg/kg), activation of NF-κB was found in nuclear extracts prepared from both 70/Z3 and 1.3E2 tumours, as well as from Colon 38 tumours that were used for comparison. This suggests that DMXAA induces NF-κB responses in host components of the tumour. Tumours grown from both 70Z/3 and 1.3E2 cells were found to regress completely following DMXAA treatment. Thus, the antitumour action of DMXAA appears to be independent of the ability of the target tumour cell population to induce NF-κB expression. Moreover, activation of NF-κB in the tumour cell did not confer resistance to DMXAA-induced therapy.

Keywords: Anticancer agent; NF-kappa B; DMXAA; Antivascular; TNF

1. Introduction

The investigational anticancer agent DMXAA, developed in this centre [1], demonstrates excellent preclinical activity against subcutaneous tumours and has also shown evidence of activity in a clinical trial [2]. Its mode of activity is novel and complex, involving tumour vascular shutdown [3,4] and modulation of host immunity through the production of cytokines [5,6]. A particular feature of DMXAA's activity is its ability to stimulate production of the tumour necrosis factor-α (TNF) within the tumour micro-environment [7,8]. It also stimulates the synthesis of mRNA for interferon-α (IFN-α), interferon-inducible protein-10 (IP-10), and macrophage inhibitory protein-1α (MIP-1α) [6]. Results with TNF or TNF-receptor 1 knockout mice strongly indicate that TNF is the dominant cytokine in mediating the antitumour effects of DMXAA in Colon 38 tumours [8,9].

DMXAA activates the transcription factor nuclear factor-κB (NF-κB) in several human tumour lines in culture [10]. Since NF-κB activation has been suggested to contribute to the resistance of tumours to TNF-induced apoptosis [11], these results raise the question of whether some tumours resist the action of DMXAA by *in vivo* activation of NF-κB, thus reducing their response to DMXAA-induced TNF. We sought to address this question by comparing the responses of the wild-type 70Z/3 pre-B lymphoma line with its variant 1.3E2 sub-line that does not respond with NF-κB activation to a number of stimuli [12]. NF-κB is activated by the phosphorylation and subsequent proteolytic degradation of an inhibitory subunit, IκB, by the enzyme IκB kinase (IKK) [13]. This enzyme complex

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contains three subunits, IKKα, IKKβ and IKKγ, and in the mutant 1.3E2 lymphoma line the γ-subunit (NEMO) is absent [12]. In this study, we investigated whether tumour cell NF-kB activation modulated the *in vitro* and *in vivo* effects of DMXAA.

2. Materials and methods

2.1. Agents

DMXAA was synthesised in this laboratory [1] and was dissolved in phosphate-buffered saline and injected intraperitoneally (i.p.) at the indicated doses in a volume of 10 μl per g body weight. For *in vitro* studies, DMXAA was dissolved in α-Modified Eagle Medium (MEM) (Gibco BRL) culture medium. Recombinant murine TNF (rmTNF) was from BD PharMingen, USA.

2.2. Cell lines

The murine pre-B lymphoma 70Z/3 and mutant 1.3E2 lines were kindly provided by Dr Gilles Courtois, Unité de Biologie Moleculaire de l'Expression Genique, Institut Pasteur, Paris, France, and cultured in α-MEM supplemented with 100 units/ml ampicillin, 100 μg/ml streptomycin, 10% (v/v) fetal calf serum (FCS) and 50 μM β-mercaptoethanol. All cell lines were maintained at 37 °C under humidified atmosphere of 5% CO_2.

2.3. Mice and tumours

C57Bl/6 mice were supplied by the Animal Resources Unit, University of Auckland, while (C57Bl/6DBA/2)F_1 (BDF_1) hybrid mice were bred at the animal facilities at the Auckland Cancer Society Research Centre. All mice were housed under constant temperature and humidity with sterile bedding, water and food according to institutional ethical guidelines.

The Colon 38 adenocarcinoma was implanted into syngeneic C57Bl/6 hosts by transferring tumour fragments (approximately 1 mm^3) subcutaneously (s.c.) into the left flank of anaesthetised (sodium pentobarbital; 82 mg/kg; i.p.) mice. Both BDF_1 and nude mice were used as hosts for the 70Z/3 and its mutant 1.3E2 line. Tumours were initiated by inoculation of 10^6 cells in a volume of 100 μl, s.c., in athymic nude mice. The tumours that developed were then excised and fragments implanted into anaesthetised BDF_1 mice for experiments. Experiments were carried out when the tumours had reached approximately 6 mm in diameter, generally 14 days after implantation, except growth inhibition studies, which were initiated when tumours were approximately 4 mm in size. Following treatment, tumours were measured thrice weekly and tumour volumes calculated as $0.52a^2 \times b$ where a and b are the minor and major axes of the tumour, respectively. The arithmetic mean (scoring complete regressions as zero tumour volume) of at least six mice per treatment group was calculated. Haemorrhagic necrosis was assessed in tumours implanted in BDF_1 and nude mice 24 h after treatment. Tumours were excised, fixed in formalin (10%), embedded in paraffin, sectioned and stained with haematoxylin and eosin. Tumour sections were examined on a grid and scored for percentage of necrosis as previously described in Ref. [14].

2.4. Cytotoxicity assay

Cell viability was assessed using the colorimetric 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [15]. 70Z/3 and 1.3E2 cells (5×10^4 cells/well) were plated in triplicates in 96-well flat-bottom plates and incubated at 37 °C for 1 h. DMXAA and rmTNF were added to the cells from separate plates at 3-fold dilutions from maximal concentrations of 1000 and 3.5 μg/ml, respectively. After incubation for 24 h, 10 μl MTT (5 mg/ml) was added and the cultures were further incubated for 1–2 h. Once MTT crystals were observed, isopropanol/HCl (100 μl) was added and the absorbance measured at 550 nm with an automated microplate reader ELx808 (Bio-Tek Instruments, Inc., USA). The IC_{50} was defined as the concentration of DMXAA or TNF that reduced specific staining by 50%.

2.5. Preparation of cytoplasmic and nuclear fractions

Mice with tumours were sacrificed by cervical dislocation at different times after DMXAA administration. The tumours were excised into 10 ml ice-cold phosphate-buffered saline, diced with a scalpel and passed through Swinning filters (Millipore, USA) to disrupt the cell fragments. The cell suspension was allowed to settle on ice, pipetted into fresh tubes and centrifuged at 2000*g* to pellet the tumour cells. 70Z/3 and 1.3E2 cells (2.5×10^7 cells per plate) were incubated with DMXAA at 0, 100 and 250 μg/ml and harvested after 2 h.

Nuclear and cellular proteins were prepared as previously described in Ref. [16]. Cells were lysed in cell lysis buffer (15 mM KCl, 10 mM hydroxyethylpiperazine ethanesulphonic acid (HEPES) (pH 7.6), 2 mM $MgCl_2$, 0.1 mM ethylenediaminetetra-acetic acid (EDTA), 25 μM dithiothreitrol (DTT), 25 μM phenylmethylsulphonyl fluoride (PMSF) and 0.5% Nonidet P-40) for 10 min. The nuclei pellet was then incubated in 10 μl nuclei lysis buffer (0.5 M KCl, 25 mM HEPES (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 25 μM PMSF) on ice for 30 min, and then 100 μl dialysis buffer (25 mM HEPES, pH 7.6, 0.1 mM EDTA, 1 mM DTT,

10% glycerol, 25 μM PMSF) was added. After centrifugation at 20 000*g* for 15 min, the supernatant was collected and the protein concentration was determined with Bradford reagent at 596 nm [17].

2.6. Assay for NF-κB activation

NF-κB complexes in the nuclear extracts were determined using the electrophoretic mobility shift assay (EMSA) as previously described in Ref. [16]. Briefly, the oligonucleotide (5′-AGCTTACAAGGG ACTTTC-3′) containing the NF-κB consensus binding site from the κ immunoglobulin enhancer gene [18], annealed to its complementary strand, was radiolabelled using the Klenow fragment of DNA polymerase I (Pharmacia Oligolabelling kit, Pharmacia) and [α-^{32}P]dCTP (370 mBq/ml, 10 mCi/ml, Redivue, Amersham) in a fill-in reaction for 5′-protruding ends. DNA binding reactions were carried out in a total volume of 15 μl containing 5 μg nuclear protein, 4 μl binding buffer (20 mM KCl, 12 mM HEPES, pH 7.6, 2.5 mM $MgCl_2$, 0.4 mM EDTA, 0.5 mM DTT, 25 μM PMSF, 12% glycerol) and 1.5 μg poly (dI:dC). Samples were incubated on ice for 10 min before adding ^{32}P-labelled probe (20 000 counts per minute (cpm)). Reactions were terminated by the addition of a loading dye (250 mM Tris (pH 7.5), 0.2% bromophenol blue, 0.2% xylene cyanol and 4% glycerol). Samples were loaded onto a 4% polyacrylamide gel and electrophoresed in 0.25 × TBE buffer (22.3 mM Tris, 22.2 mM borate, 0.5 mM EDTA) at 150 V for 2 h. The dried gels were exposed to autoradiography (Kodak Scientific Imaging Film) at −70 °C overnight. For supershift analyses, 1 μl of antibody was added before the addition of the oligonucleotide. Antibodies to the NF-κB family of proteins were a gift from Dr Nancy Rice, NCI, Frederick, MD, USA [19].

2.7. TNF assay

Mice were anaesthetised with halothane and bled from the ocular sinus. The blood was allowed to coagulate overnight on ice. After centrifugation of samples (2000*g*, 30 min, 4 °C), the serum was removed and stored at −70 °C. Excised tumours were homogenised in 1.5 ml of α-MEM medium using a tissue homogeniser. The homogenates were centrifuged (2000*g*, 30 min, 4 °C) and the supernatant was removed and recentrifuged (14 000*g*, 30 min at 4 °C). Serum and supernatants from tissue homogenates were kept at −70 °C until use. TNF was assayed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (OptEIA Mouse TNF kit, PharMingen, San Diego, CA, USA) according to the manufacturer's directions. Results were normalised to pg TNF produced per g of tissue.

3. Results

3.1. Activation of NF-κB in murine Colon 38 tumours by DMXAA

Translocated NF-κB was detectable in nuclear extracts prepared from subcutaneous Colon 38 as early as 30 min following the administration of DMXAA (25 mg/kg i.p.). Stronger bands were seen at 2 and 3 h following treatment (Fig. 1a) and later time-points could not be tested as the tumours became too necrotic. Striking intertumour heterogeneity was observed, and activated NF-κB was seen in only a proportion of tumours at each of the 30 min or later time-points (Table 1).

Supershift assays were carried out with nuclear proteins prepared at 30 min, 1 h and 2 h from positive tumours. At all time-points assayed, the NF-κB band was predominantly made up of p50 and p65 subunits. A small amount of supershifting was obtained with antibodies to p52 that increased in strength with nuclear extracts from later time-points (Fig. 1b). No significant supershifting was detectable with antibodies to Rel B and c-Rel family members.

3.2. Activation of NF_κB in wild-type 70Z/3 and IKK-defective 1.3E2 cells in vitro

The 70Z/3 pre-B lymphoma line translocates NF-κB in response to a number of stimuli, while the variant 1.3E2 cell line, which lacks the IKKγ subunit, does not [12]. We compared the NF-κB response with DMXAA in these cell lines in culture. A 2 h exposure to DMXAA at 100 and 250 μg/ml resulted in strong NF-κB activation in the 70Z/3, but in very weak activation in the 1.3E2 cells (Fig. 2a). Supershift experiments showed that the band from the 70Z/3 cells induced with DMXAA consisted primarily of p50 and p65 heterodimers (Fig. 2b).

3.3. Activation of NF-κB in wild-type 70Z/3 and IKK-defective 1.3E2 cells in vivo

NF-κB activation was observed in nuclear proteins extracted from 70Z/3 as well as from 1.3E2 tumour tis-

Table 1
Proportion of Colon 38 tumours in C57B1/6 mice showing activated NF-κB following DMXAA administration (25 mg/kg i.p.)

Time	Number positive/number tested	%
0	0/8	0
15 min	0/4	0
30 min	2/6	33
1 h	6/9	67
2 h	7/10	70
3 h	3/5	60

NF-κB, nuclear factor-κB; DMXAA, 5,6-dimethylxanthenone-4-acetic acid.

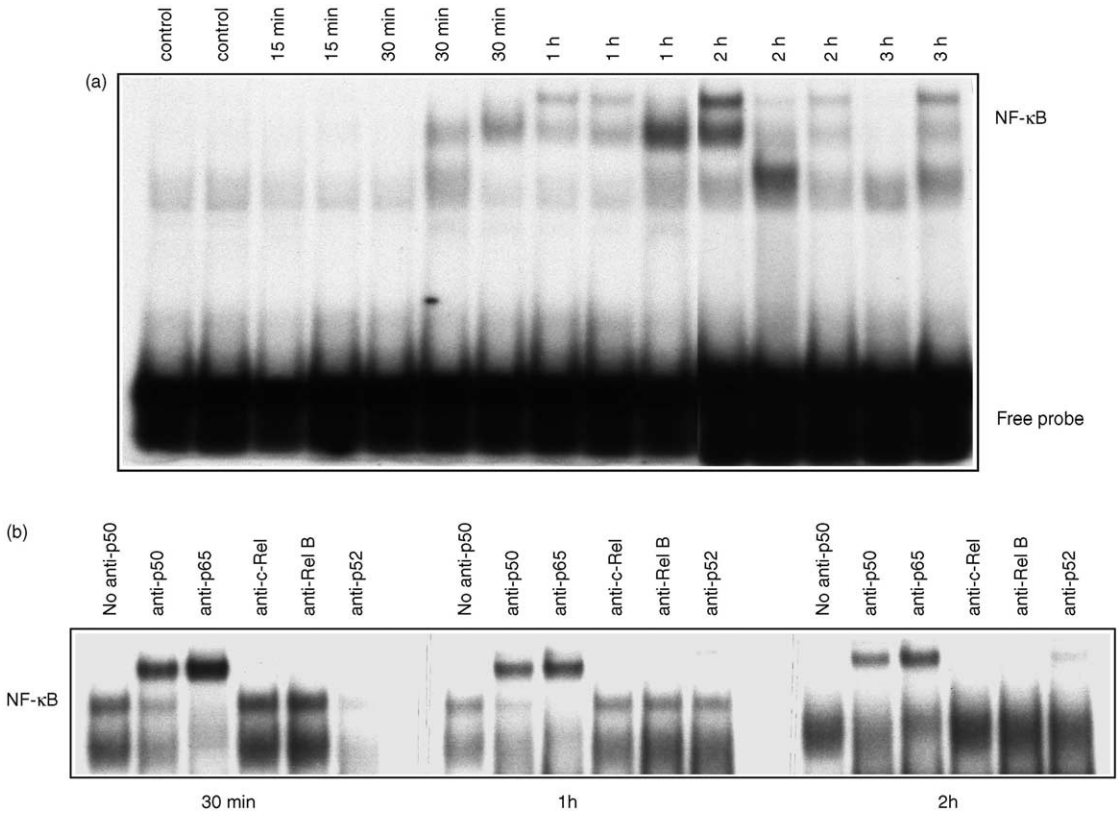

Fig. 1. Electrophoretic mobility shift assay (EMSA) probed for nuclear factor-κB (NF-κB) of nuclear extracts from individual Colon 38 tumours excised from untreated control mice, or at the indicated times after treatment with 5,6-dimethyl-xanthenone-4-acetic acid (DMXAA) at 25 mg/kg (a); supershift analyses of the subunits in the band in protein extracts from Colon 38 tumour at 30 min, 1 h and 2 h after treatment (b).

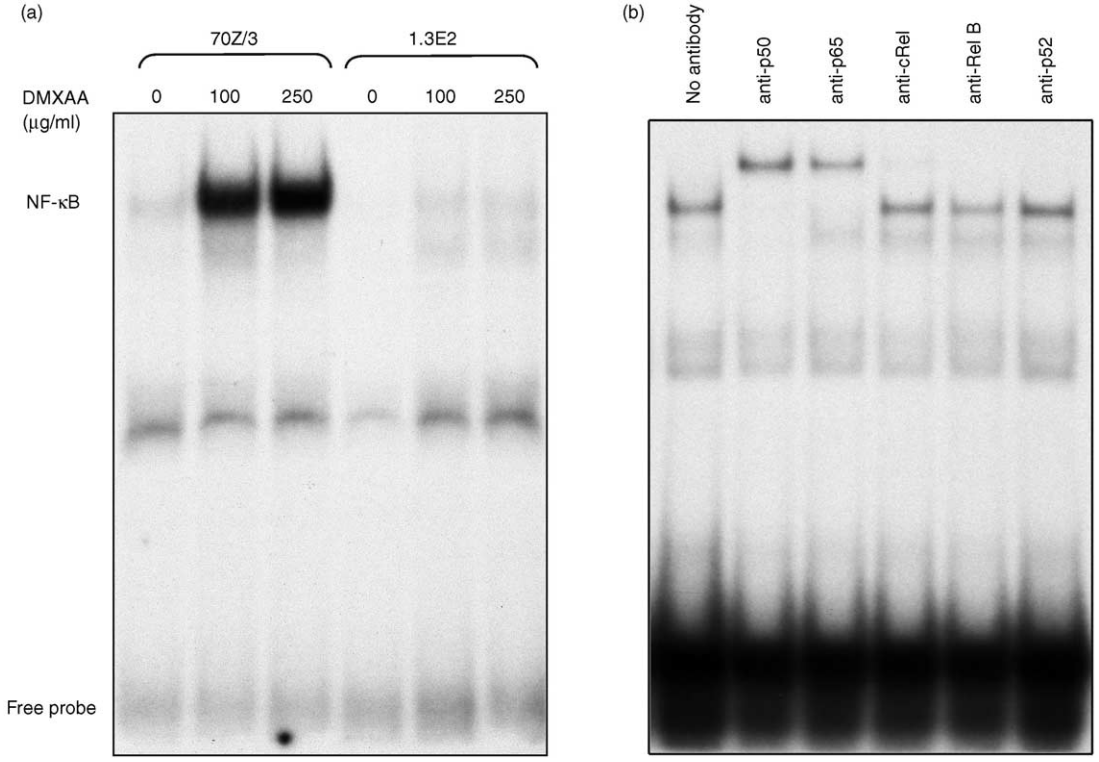

Fig. 2. EMSA of nuclear extracts from 70Z/3 and 1.3E2 cells treated with 0, 100 and 250 μg/ml DMXAA for 2 h and probed for NF-κB binding (a); supershift analyses of the band of nuclear extracts from 70Z/3 cells treated with DMXAA at 250 μg/ml for 2 h (b).

sues following DMXAA treatment (Fig. 3a and b). Evidence of heterogeneity in response among individual tumours was observed, but was not as pronounced as that in Colon 38 tumours (results not shown). Bands from nuclear extracts of 70Z/3 tumours appeared generally stronger than those from 1.3E2 tumours, but because of the heterogeneity of response, there was no significant difference between the two types of tumours. Supershift analyses showed that the p65 and p50 dimers were primarily translocated in 70Z/3 or 1.3E2 tumour nuclear extracts (Fig. 3c and d).

3.4. *TNF- and DMXAA-induced responses in 70Z/3 and 1.3E2 tumours* in vitro

The effects of TNF and DMXAA on the proliferation of cultured 70Z/3 and 1.3E2 cells were determined by an MTT assay. The IC_{50} for DMXAA was 12 μg/ml in each case. Growth of both cell lines was unaffected by exposure to TNF up to a concentration of 3.5 μg/ml.

3.5. *DMXAA-induced responses in 70Z/3 and 1.3E2 tumours* in vivo

Tumours developing from inoculation of both 70Z/3 and 1.3E2 cells were found to be sensitive to DMXAA (25 mg/kg), with induction of complete regression in each case (Fig. 4). Similar degrees of tumour haemorrhagic necrosis were observed for each tumour, but 70Z/3 tumours expressed higher amounts of TNF after DMXAA than 1.3E2 tumours (Table 2).

4. Discussion

We demonstrate here that IKKγ is essential for the efficient *in vitro* induction of NF-κB nuclear translocation in 70Z/3 cells by the antivascular drug DMXAA. Such induction requires phosphorylation of the inhibitory IκB protein by the IKK complex, which is ineffective in the 1.3E2 cell line because of its lack of the γ-subunit (NEMO) [12]. The results are consistent with the hypothesis that we have previously advanced that the biochemical mechanism for the action of DMXAA involves activation of the IKK complex. This is based on the report that the β-subunit of the complex is a biochemical target for salicylic acid [20], combined with the finding that salicylic acid competitively inhibits the induction of TNF synthesis by DMXAA in cultured human peripheral blood leucocytes [21].

We have also demonstrated here that DMXAA activates NF-κB *in vivo* in subcutaneous Colon 38 tumours. Activation was evident as early as 30 min after treatment,

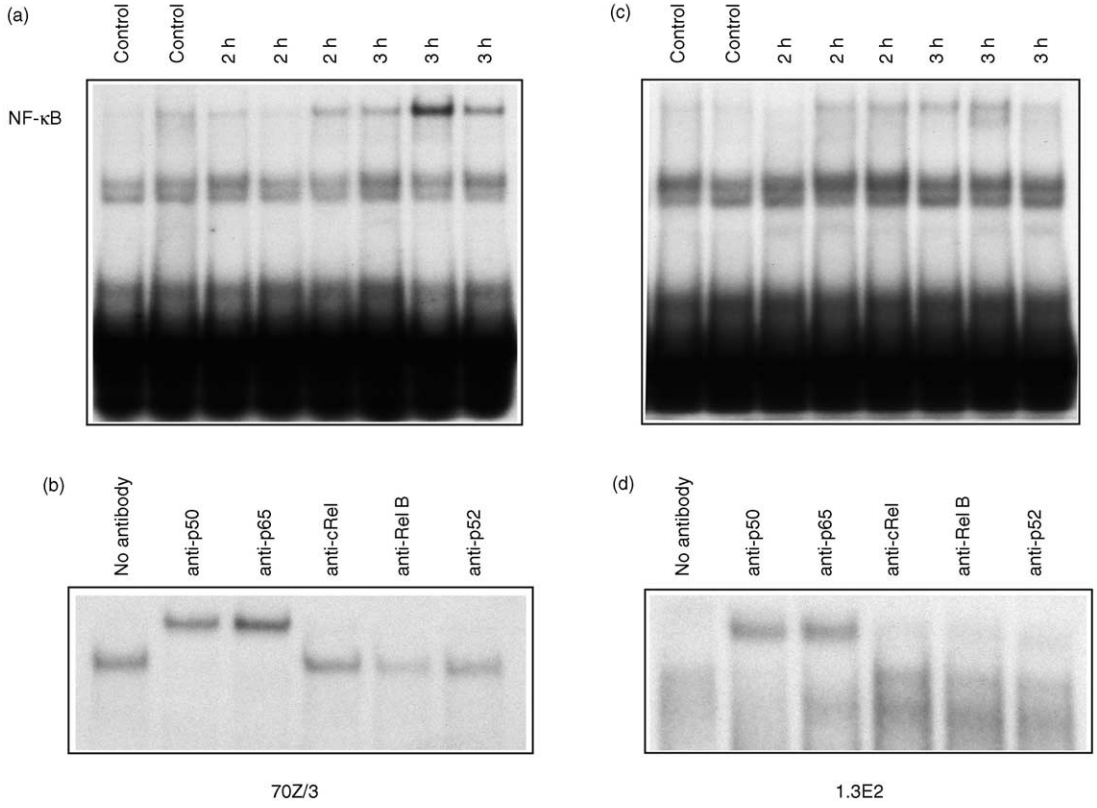

Fig. 3. EMSA probed for NF-κB in nuclear extracts from 70Z/3 (a) and 1.3E2 tumours (b) untreated or at indicated times following DMXAA (25 mg/kg) administration. Supershift analyses of the 2 h treated sample from 70Z/3 (c) and 1.3E2 tumours (d).

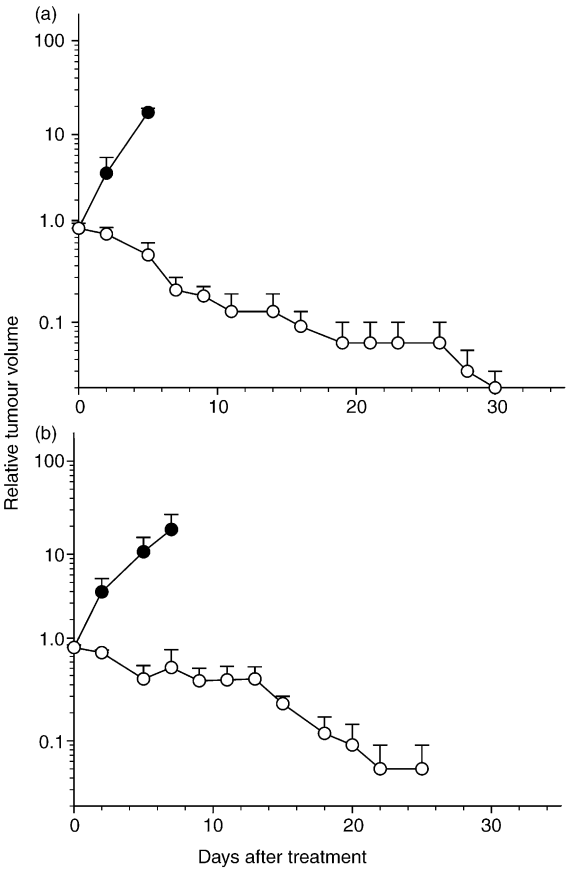

Fig. 4. Growth of 70Z/3 (a) or 1.3E2 (b) tumours untreated (●) or following DMXAA (25 mg/kg) treatment (○).

with the classical p65/p50 dimer being the dominant form translocated (Fig. 1). There was demonstrable heterogeneity in the response of individual mice at each time-point (Table 1) and one possible explanation is that it reflects different time-courses of NF-κB activation and repression among individual tumours. NF-κB activation is a transient phenomenon and is rapidly turned off as IκB is resynthesised [22].

When 1.3E2 cells were grown as subcutaneous tumours in BDF_1 mice and treated with DMXAA, evidence of activation of NF-κB was observed in the nuclear extracts prepared from the tumours (Fig. 3). Since 1.3E2 cells cannot produce it, host cells within the tumour such as tumour vascular endothelial cells or infiltrating host leucocytes are likely to be responsible. DMXAA upregulates NF-κB translocation in leucocytes *in vitro* [21] and may therefore have a similar effect on host tumour infiltrating cells *in vivo*. DMXAA induces apoptosis of tumour vascular endothelial cells in Colon 38 tumours, starting within 30 min of DMXAA administration [23] and a number of pro-apoptotic responses are under NF-κB control [24]. NF-κB activation within vascular endothelial cells may therefore also contribute to the response.

When NF-κB activation was compared in 70Z/3 and 1.3E2 tumours, the degree of activation appeared to be greater in 70Z/3 tumours (Fig. 3), although definitive evidence that tumour cells activated NF-κB in response to DMXAA was not obtained. Indirect evidence for NF-κB activation in tumour cells can be inferred from measurement of TNF production, which is under control of the NF-κB promoter. Experiments with Colon 38 tumours growing in TNF knockout mice have indicated that tumour cells synthesise TNF in response to DMXAA, although the amount produced is lower than that by host cells [8]. DMXAA-induced intratumoral TNF levels were significantly higher ($p<0.05$) in mice with 70Z/3 tumours than in mice with 1.3E2 tumours (Table 2), consistent with the hypothesis that 70Z/3 tumours synthesise TNF *in vivo*. However, 70Z/3 cells did not produce TNF in culture in response to DMXAA (unpublished data) and an alternative explanation is that the increased TNF production reflects an

Table 2
TNF production and haemorrhagic necrosis in 70Z/3 and 1.3E2 tumours

	70Z/3	1.3E2
TNF activity (pg/g)		
Serum: untreated	50±0	59±6.4
DMXAA, 25 mg/kg	347±201	156±18
DMXAA, 50 mg/kg	944±100	401±118
Tumour: untreated	193±64	74±13
DMXAA, 25 mg/kg	339±176	310±108
DMXAA, 50 mg/kg	816±18	246±29
Haemorrhagic necrosis (%)		
BDF_1 mice: untreated	2±1	6±4
DMXAA, 25 mg/kg	100±0	80±20
Nude mice: untreated	23±9	23±14
DMXAA, 22.5 mg/kg	99±0.3	99±0.3

Haemorrhagic necrosis was assessed after 24 h and TNF was measured 3 h after treatment. Mean±standard error of the mean (SEM) of three mice per group. TNF, tumour necrosis factor-α.

indirect effect whereby 70Z/3 tumours are more effective than 1.3E2 in priming host cell TNF synthesis. Different tumours have previously been shown to vary in their ability to prime the activation of tumoricidal macrophages by DMXAA [25].

DMXAA induced regression of tumours derived from both 70Z/3 and 1.3E2 cells, raising the question of whether regression was caused by a direct cytotoxic effect of DMXAA on tumour cells, by an indirect effect of induced TNF on tumour cells [26], or by the direct and indirect effects of DMXAA on the host vasculature [27]. The concentration of DMXAA required to inhibit cell growth (38 μM) was above the free plasma concentration of DMXAA obtained *in vivo,* which was approximately 18 μM [28]. Similarly, both cell lines were insensitive to rmTNF at concentrations up to 3.5 μg/ml, much higher than that obtained in plasma (<2 ng/ml) following *in vivo* administration of DMXAA [29]. Thus, the results are consistent with the hypothesis that DMXAA causes regression of these tumours through its effects on host cells.

NF-κB activation has been suggested to contribute to resistance of tumours to chemotherapy-, radiation- and TNF-induced apoptosis [11]. The observation here that DMXAA induces complete regressions of 1.3E2 and 70Z/3 tumours *in vivo* (Fig. 4) indicates that the lack of a tumour cell's ability to mount a NF-κB response does not affect its sensitivity to DMXAA. Several studies show that DMXAA combines productively with radiation and cytotoxic drugs in murine tumours [30] supporting the concept that if NF-κB is activated in response to DMXAA, it does not prevent the action of cytotoxic agents. Preliminary experiments (results not shown) indicate that tumours grown from 70Z/3 and 1.3E2 cells have similar responses to *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide dihydrochloride (DACA), a topoisomerase poison [31]. Other studies have shown that stable inhibition of NF-κB does not increase sensitivity to cytotoxic drugs [32]. Importantly, our results establish that NF-κB activation in tumours by DMXAA does not lead to resistance. Rather, it reflects activation of cytokine production, a critical component of the antitumour action of DMXAA.

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