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Interaction between endotoxin and the antitumour agent 5,6-dimethylxanthenone-4-acetic acid in the induction of tumour necrosis factor and haemorrhagic necrosis of colon 38 tumours

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Abstract The investigational antitumour agent 5,6-dimethyl-xanthenone-4-acetic acid (5,6-MeXAA) induced dose-dependent haemorrhagic necrosis of colon 38 tumours to a similar extent to that induced using bacterial lipopolysaccharide (LPS). TNF-α activity in serum and mRNA for TNF-α in splenocytes were induced over a broad range of LPS doses, whereas with 5,6-MeXAA, induction occurred only at concentrations approaching the maximum tolerated dose. At concentrations that provided similar degrees of haemorrhagic necrosis, the levels of serum TNF-α induced using 5,6-MeXAA were 100-fold lower than those obtained with LPS, indicating that haemorrhagic necrosis was not directly correlated with TNF-α levels. There was also no correlation between the degree of tumour necrosis and the duration of growth delay. Treatment with LPS did not induce a significant delay in growth, despite extensive tumour haemorrhagic necrosis, whereas with 5,6-MeXAA, treatments that improved the cure rate did not necessarily give longer growth delays. Therapy using a combination of sub-optimal doses of both compounds was synergistic for the induction of serum TNF-α and message for TNF-α but was not synergistic for antitumour efficacy. Thus, no correlation is evident between cure rates, duration of growth delay, haemorrhagic necrosis and TNF-α induction by 5,6-MeXAA or LPS.

Key words Lipopolysaccharide · Antitumour activity · Cytokine · Synergism

Introduction

The investigational antitumour agent 5,6-dimethylxanthenone-4-acetic acid (5,6-MeXAA, NSC 640 488), now scheduled for clinical evaluation, was developed in this laboratory [44] as a more potent analogue of flavone acetic acid (FAA). FAA, synthesized by Atassi et al. [2], is highly active against transplantable murine tumours [40, 46] and human xenografts in nude mice [27] but is clinically inactive [32]. The antitumour activity of these compounds is complex and appears to involve more than the one host defence mechanism [9, 30]. Treatment of mice bearing s.c. tumours with FAA and 5,6-MeXAA results in rapid and extensive haemorrhagic necrosis of the tumours, a response attributed to tumour necrosis factor-α (TNF-α)-mediated vascular effects [36]. In vitro studies have shown that 5,6-MeXAA and FAA can act directly on macrophages, stimulating their production of TNF-α, interferons and nitric oxide [19, 31, 48] and enhancing their tumouricidal activity [10]. All of these may contribute to the overall antitumour effect. A functional T-cell population appears to be necessary for complete regression of some tumour models [6, 41], whereas in other models, the presence of T-cells, while not essential, enhances the number of complete regressions [16]. FAA has been shown to up-regulate mRNA to interferon-γ in splenic T-lymphocytes [35]. Interferon-γ is a potent priming agent for both macrophages and T-lymphocytes [7] and its production would have a powerful enhancing effect on host immunity. After FAA treatment, natural killer (NK) cells are elevated [9] through the production of interferons [31]. Although NK cells are not involved in haemorrhagic necrosis [11], they have been implicated as having a role in the long-term cure of mice [22, 50].

Many of the biological activities of 5,6-MeXAA and FAA parallel those of lipopolysaccharide (LPS): induction of cytokines, activation of macrophage tumouricidal activity, nitric oxide production and induction of haemorrhagic necrosis [42]. The toxic effects of the drugs are also similar. Furthermore, macrophages from LPS-resistant C_3H/HeJ mice are also hyporesponsive to FAA [12] and 5,6-MeXAA [18], indicating that they may activate a common biochemical pathway.

In the present study we compared the antitumour activity of 5,6-MeXAA and LPS, examining the relationship between TNF-α synthesis and antitumour effects.

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Since LPS has also been proposed as an antitumour treatment [24], we also examined the ability of LPS and 5,6-MeXAA to interact or synergise with each other, with the notion of improving the antitumour activity and therapeutic index.

Materials and methods

Materials

5,6-MeXAA (molecular weight, 304.3 Da) was synthesized in this laboratory [44], and solutions were prepared fresh for each experiment and protected from light [43]. LPS (prepared by phenol extraction of *Escherichia coli* serotype 055:BS) was obtained from Sigma (St. Louis, Mo.). The culture medium was Alpha-MEM (Gibco, Grand Island, N.Y.) supplemented with 10% foetal calf serum, penicillin (100 units/ml) and streptomycin sulphate (100 μg/ml).

Mice and tumours

(C_{57}Bl/6xDBA/2)F_1 (BDF_1) hybrids, bred in the laboratory animal facility and between 8 and 12 weeks of age, were anaesthetized (sodium pentobarbital, 90 mg/kg i.p.), and 1-mm^3 cubes of colon 38 tumour were implanted s.c. in the flank. Growth-delay experiments were routinely initiated when the major diameter of the tumours had reached 5 mm. Haemorrhagic necrosis assessments were carried out on tumours where the major diameter was up to 8 mm.

Assessment of haemorrhagic necrosis

Tumour-bearing mice were injected i.p. with drug dissolved in medium (0.2 ml/20 g body weight). The tumours were removed 24 h later and fixed in 10% formalin. Fixed tumours were embedded in paraffin wax and sections were stained with haematoxylin and eosin. The section across the major diameter of the tumour was examined on a grid marked at 0.4-mm intervals and was scored for the percentage of necrosis by the grid-intersection method as previously described [3].

Growth-delay determinations

Mice bearing colon 38 tumours were treated with drug; thereafter, the tumours were measured three times weekly with callipers and tumour volumes were calculated using the formula $0.52a^2b$, where a and b are the minor and major axes of the tumour, respectively. The arithmetic mean (used to include tumours that had completely regressed) and standard error of the mean were calculated at each time point and expressed as fractions of the initial mean tumour volume. The growth delay was determined as the difference in the number of days required for the control versus treated tumours to increase 4 times in volume.

Northern-blot analysis of mRNA

RNA was extracted in a one-step method following the protocol of Chomczynski and Sacchi [20]. RNA was analyzed as previously described [19]. RNA (15 μg) was separated by electrophoresis on formaldehyde/agarose gels and then transferred to nylon filters. Filters were hybridized with a ^{32}P-labeled cDNA probe to murine TNF-α mRNA (0169, Genentech, San Francisco, Calif.). Hybridized filters were exposed to X-ray film and relative mRNA signals were quantitated by laser densitometric scanning. Equality of loading of the lanes was checked by visualization under UV light of ethidium bromide-stained 18S and 28S bands.

TNF-α bioassay

Serum TNF-α levels were measured using a standard cytotoxicity assay using actinomycin D-treated L929 cells [29]. The L929 cell line was kindly provided by Dr. R. H. Wiltrout (National Cancer Institute, Bethesda, Md., USA) and maintained in this laboratory in culture medium. L929 cells from an exponentially growing culture were seeded into flat-bottomed microwells (3×10^4 cells/well) and allowed to adhere overnight. Actinomycin D (Merck Sharp and Dohme, Rahway, N.J., USA; final concentration, 8 μg/ml) was then added, followed by serial dilutions of serum to be assayed (duplicate wells for each serum dilution), and cultures were incubated for 24 h at 37° C in an atmosphere of 5% CO_2 in air. Cell killing was assessed by a colorimetric assay using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Serva, Heidelberg, Germany) as previously described [25]. MTT (final concentration, 500 μg/ml) was added and the cultures were further incubated (1 h) to enable dark blue crystals to appear. The culture supernatant was then removed and 100 μl dimethyl sulphoxide (Prolabo, Paris, France) was added to solubilise the crystals. The absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader (MR 600, Dynatech, Alexandria, Va., USA); 1 unit of TNF-α was defined as the amount required to kill 50% of the cells.

Results

Haemorrhagic necrosis and TNF-α production

Since TNF-α appears to be an important mediator of haemorrhagic necrosis [8], we compared TNF-α production with the degree of haemorrhagic necrosis induced in colon 38 tumours using different doses of LPS or 5,6-MeXAA. The degree of haemorrhagic necrosis increased with LPS doses above 5 μg/mouse (Fig. 1 A) and with 5,6-MeXAA doses above 10 mg/kg (Fig. 1 B). Both compounds were capable of inducing nearly 100% necrosis of colon 38 tumours at their respective optimal doses (175 μg/mouse for LPS and 27.5 mg/kg for 5,6-MeXAA).

The two compounds exhibited different dose-response profiles for the up-regulation of splenic TNF-α mRNA and the production of serum TNF-α. LPS induced similar levels of TNF-α mRNA over a broad spectrum of doses ranging from 0.1 μg/mouse up to the maximum tolerated dose of 200 μg/mouse (Fig. 1 C), whereas with 5,6-MeXAA, maximal levels of mRNA were detected following a dose of 27.5 mg/kg (Fig. 1 D). Serum TNF-α levels were measured at 1 h after LPS administration and 3 h after 5,6-MeXAA treatment, which were determined to be maximal in a separate experiment (data not shown). Induction of TNF-α by LPS occurred over the whole range of LPS doses tested (Fig. 1 E), whereas that by 5,6-MeXAA occurred over a very narrow range with an extremely sharp dose-response relationship (Fig. 1 F). At doses that gave similar levels of haemorrhagic necrosis, the serum TNF-α levels induced with 5,6-MeXAA were 100-fold lower than those induced using LPS.

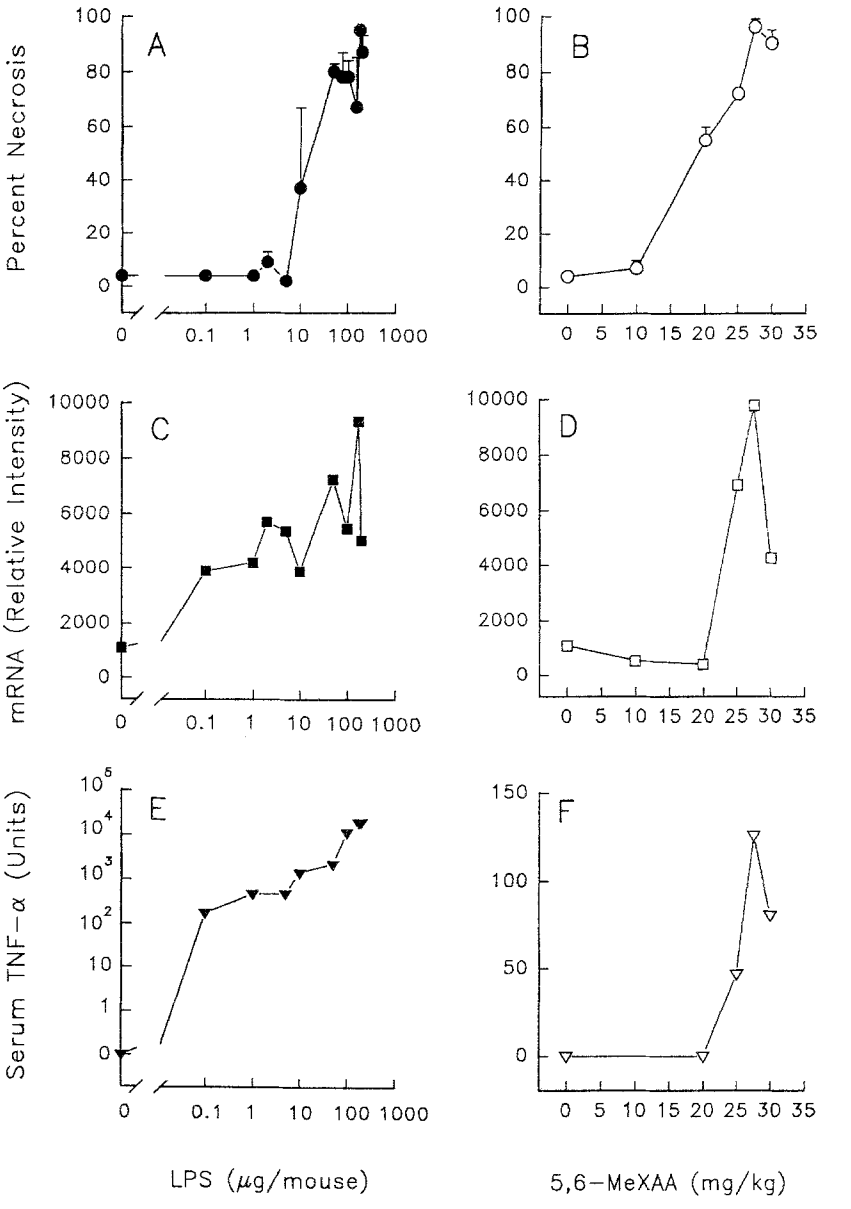

Fig. 1A–F Haemorrhagic necrosis and induction of TNF-α mRNA and serum TNF-α by LPS and 5,6-MeXAA. BDF_1 mice bearing colon 38 tumours were injected with LPS (**A, C, E**) or 5,6-MeXAA (**B, D, F**). Haemorrhagic necrosis of tumours (mean values for 5 mice/group) was assessed after 24 h (**A, B**). Up-regulation of TNF-α mRNA in total cellular mRNA isolated from 2 spleens/group at 2 h after drug treatment was determined using Northern-blot analyis (**C, D**). The TNF-α level in serum pooled from 3 mice/group was determined at 1 h after LPS administration (**E**) and 3 h after 5,6-MeXAA treatment (**F**)

Table 1 TNF-α induction and antitumour activity of 5,6-MeXAA[a]

Treatment	Dose (mg/kg)	Serum TNF-α (units)	TNF-α mRNA (relative intensity)	Percent necrosis	Growth delay (days)	Percent cures
Control		0	1080	4±1	0	0
5,6-MeXAA	10	0	530	7±3	0	0
	20	0	400	55±5	0	0
	25	47	6920	72±2	7	20 (1/5)
	27.5	126	9770	96±3	13	40 (2/5)
	30	81	4270	90±5	13	63 (10/16)

[a] Summary of data from Figs. 1B, 1D and 1E together with the growth delay and cure rates determined from groups of 5 mice each, except at 30 mg/kg, which was determined from a group of 16 mice. Figures in parentheses represent the number cured over the number in the group

Growth delays and cure rates

Both LPS and 5,6-MeXAA were tested for induction of growth delays in the colon 38 tumour model (Fig. 2). No growth delay or long-term cure was obtainable using LPS at all doses up to the maximum tolerated dose. In contrast, 5,6-MeXAA produced growth delays and complete cures at a dose of 25 mg/kg or higher; a growth delay of 13 days was obtained with doses of 27.5 and 30 mg/kg, but a significant improvement in the number of cures was obtained using the higher dose (Table 1). A dose of 25 mg/kg produced a growth delay of 7 days (20% cure rate), whereas no growth inhibition or cure was obtained at 10 or 20 mg/kg (Table 1). Thus, although the haemorrhagic necrotic response to LPS and 5,6-MeXAA were very similar, the antitumour activity of the two compounds was quite different as determined by growth delay or cure rate in the colon 38 model.

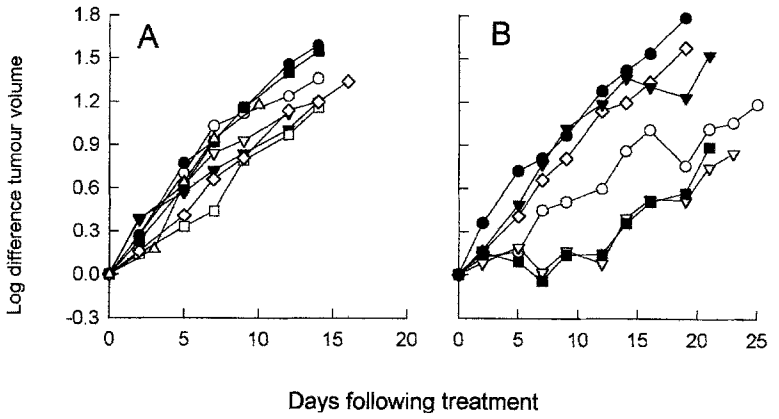

Fig. 2A, B Growth inhibition of colon 38 tumours by LPS or 5,6-MeXAA. BDF_1 mice bearing palpable colon 38 tumours were treated with **A** LPS (◇, 0; ▼, 0.1; ○, 1; ●, 2; ▽, 5; ■, 10; □, 100; △, 175 μg/mouse) or **B** 5,6-MeXAA (◇, 0; ▼, 10; ●, 20; ○, 25; ▽, 27.5; ■, 30 mg/kg) and tumour volumes were measured (mean values for 5 mice/group)

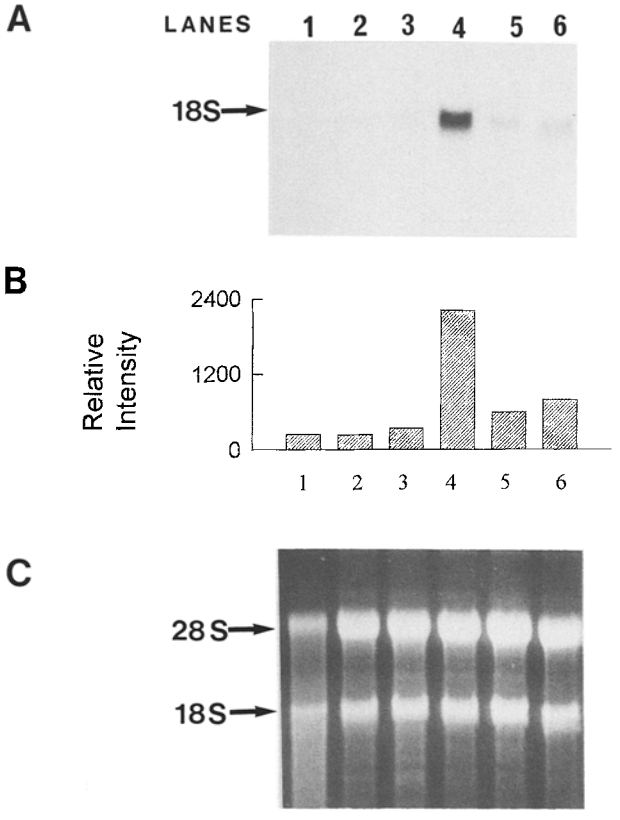

Fig. 3A–C Synergism between LPS and 5,6-MeXAA in the induction of TNF-α mRNA. Colon 38 tumour-bearing BDF_1 mice were treated with LPS and/or 5,6-MeXAA. **A** After 2 h, mRNA for TNF-α in total cellular RNA isolated from 2 spleens pooled was measured by Northern blotting. **B** Relative intensity of signals as determined by scanning densitometry. **C** UV illumination of agarose gel showing 28S and 18S mRNA and equality of lane loading (*Lane 1* control, *lane 2* 20 mg/kg 5,6-MeXAA, *lane 3* 10 mg/kg 5,6-MeXAA, *lane 4* 20 mg/kg 5,6-MeXAA plus 2 μg LPS, *lane 5* 10 mg/kg 5,6-MeXAA plus 2 μg LPS, *lane 6* 2 μg LPS)

Treatment with a combination of LPS and 5,6-MeXAA

The results summarised in Table 1 and Fig. 1 show that although both compounds have a similar ability to induce haemorrhagic necrosis, 5,6-MeXAA alone is capable of giving rise to cures. 5,6-MeXAA induced TNF-α only at doses close to the maximum tolerated dose, but LPS, on the other hand, had the advantage of being able to induce TNF-α over a very broad range of doses. We investigated the possibility of improving the performance of the individual compounds in combination therapy. Mice bearing colon 38 tumours were given a sub-optimal dose of 5,6-MeXAA combined with a low dose of LPS, and the cure rates, growth delays, haemorrhagic necrosis and TNF-α mRNA induction were measured.

Table 2 TNF-α production and antitumour response to combinations of LPS and 5,6-MeXAA[a]

Treatment (doses)		Serum TNF-α	TNF-α mRNA	Percent necrosis	Growth delay	Percent cures
LPS + 5,6-MeXAA						
0	0	0	705	4±1	0	0
1	0	52	1240	4±1	0	0
2	0	125	2040	9±4	0	0
5	0	469	3000	2±1	0	0
0	20	52	360	55±5	0	0
1	20	47	3080	86±6	0	0
2	20	4718	6680	76±9	8	0
5	20	8173	5250	74±12	0	20

[a] BDF_1 mice implanted with colon 38 (10 days) were given LPS (at the indicated doses in μg/mouse) and 5,6-MeXAA (at the indicated doses in mg/kg). Serum TNF-α (units) and TNF-α mRNA (relative intensity) were measured after 2 h from groups of 2 mice each. Antitumour activity was determined from groups of 5 mice each

In two separate experiments, 5,6-MeXAA (10 mg/kg) combined with LPS (1–5 μg/mouse) did not produce a TNF-α message above that induced by LPS alone. However, a combination of 20 mg/kg 5,6-MeXAA with LPS at 2 or 5 μg/mouse produced levels of TNF-α message greater than the sum of the amounts produced by each of the drugs alone. The results illustrated in Fig. 3 show synergism in the amount of TNF-α mRNA induced using 2 μg LPS with 20 mg/kg 5,6-MeXAA but not with 10 mg/kg 5,6-MeXAA. Synergism with LPS using 5,6-MeXAA at 20 mg/kg in the induction of message and serum TNF-α was observed in three separate experiments, and the results of one representative experiment are shown in Fig. 4 and Table 2. The only growth delay that was obtained occurred using the combination of 20 mg/kg 5,6-MeXAA with LPS at 2 μg/

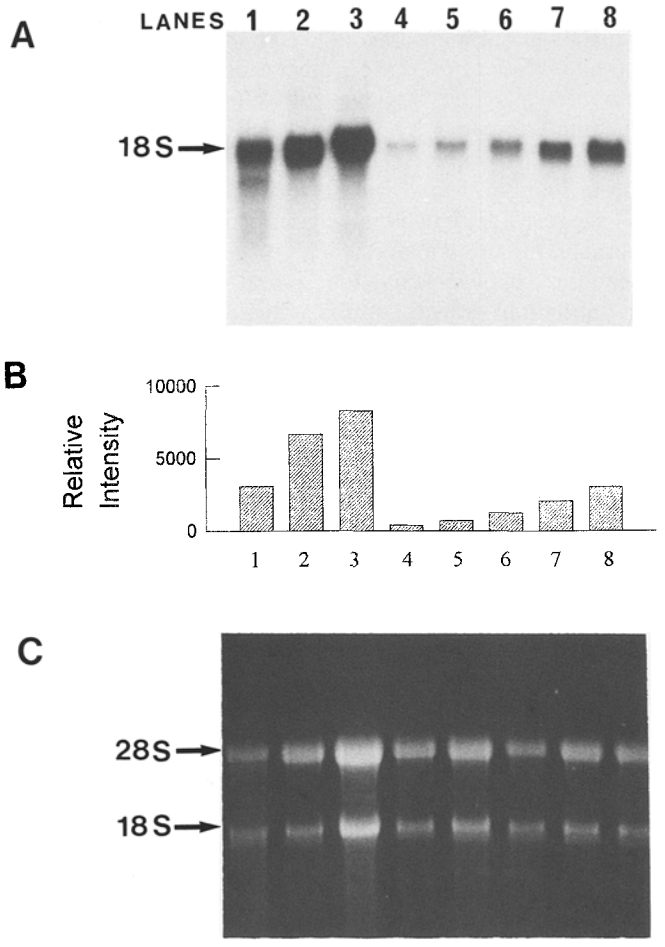

Fig. 4A–C TNF-α mRNA induced using 5,6-MeXAA (20 mg/kg) and different doses of LPS. **A** Northern blots of TNF-α mRNA isolated from splenocytes from colon 38-bearing BDF_1 mice (2/group) at 2 h after treatment. **B** Relative intensity of the signals. **C** Loading of the lanes as determined by 28S and 18S bands (*Lane 1* 20 mg/kg 5,6-MeXAA plus 1 μg LPS, *lane 2* 20 mg/kg 5,6-MeXAA plus 2 μg LPS, *lane 3* 20 mg/kg 5,6-MeXAA plus 5 μg LPS, *lane 4* 20 mg/kg 5,6-MeXAA only, *lane 5* control, *lane 6* LPS only at 1 μg/mouse, *lane 7* LPS only at 2 μg/mouse, *lane 8* LPS only at 5 μ/mouse)

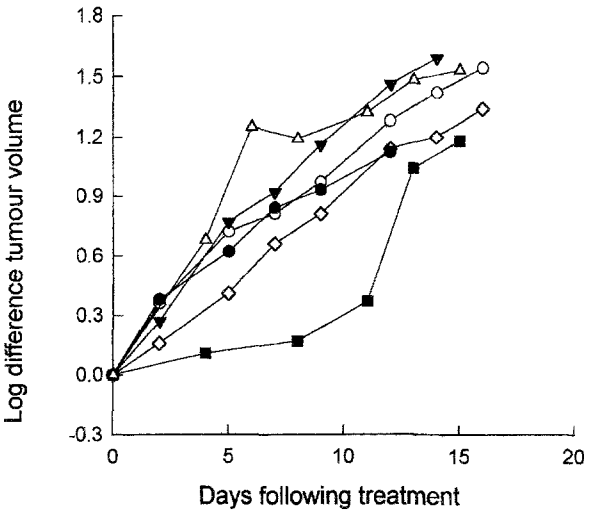

Fig. 5 Growth of colon 38 tumours after combination therapy with 5,6-MeXAA and LPS. BDF_1 mice with 10-day-old colon 38 tumours were treated with 5,6-MeXAA and/or LPS and tumour volumes were measured thrice weekly. ◇, control; ○, 20 mg/kg 5,6-MeXAA; ▼, 2 μg LPS; ■, 20 mg/kg 5,6-MeXAA plus 2 μg LPS; ●, 5 μg LPS; △, 20 mg/kg 5,6-MeXAA plus 5 μg LPS

mouse, which gave a delay of 8 days (Fig. 5). Surprisingly, no growth delay was obtained using a higher dose of LPS (5 μg) combined with 5,6-MeXAA (20 mg/kg), although this combination was synergistic in the induction of TNF-α. A 20% cure rate was obtained at combinations of 20 mg/kg 5,6-MeXAA plus LPS at 5 μg/mouse (Table 2). Thus, no advantage in antitumour efficacy was obtained by using combinations of sub-optimal doses of the two compounds.

Discussion

LPS and 5,6-MeXAA, two compounds with vastly different physicochemical properties, share a common ability to induce TNF-α (Fig. 1). LPS induces TNF-α over a very broad dose range, in contrast to the extremely steep dose response for induction of TNF-α by 5,6-MeXAA, which occurs only at doses close to the maximum tolerated dose (Fig. 1). The difference in the dose-response profiles may reflect the presence of host binding proteins. LPS is a large complex molecule that activates host cells through binding of specific surface receptors [34, 51]. The presence of LPS-binding proteins (LPB) in the serum potentiates the response to LPS significantly. LPB complexes with free endotoxin and these complexes then interact with the CD-14 receptor [33, 45], which has been shown to be extremely sensitive, enabling minute quantities of LPS to be stimulatory. In the absence of LPB, a more than 1000-fold increase in LPS concentration is necessary for cell activation [33]. The complexing of LPS to LPB and the activation of macrophages through the CD-14 receptor system could account for the ability of LPS to be stimulatory over such a broad range of doses. 5,6-MeXAA, on the other hand, is a low-molecular-weight compound that would be expected to diffuse easily into tissues and cells and is cleared with a mean transit time of 4.2 h in vivo [37].

The activity of TNF-α induced by 5,6-MeXAA is much lower than that induced by LPS. Either LPS stimulates a greater number of cells than does 5,6-MeXAA or LPS is capable of evoking a higher degree of TNF-α production per cell than is 5,6-MeXAA. The latter explanation appears more likely, since comparisons in vitro using homogeneous cell lines show greater levels of stimulation by LPS [19]. Although serum TNF-α activity is lower, 5,6-MeXAA induces a similar or higher degree of haemorrhagic necrosis than does LPS at the respective optimal antitumour doses (Fig. 1). TNF-α has been identified as the primary mediator of LPS-induced haemorrhagic necrosis [8], and antibodies to TNF-α will inhibit some of the antitumour effects induced by FAA [41], a compound analogous to 5,6-MeXAA.

The precise role of TNF-α in the initiation of haemorrhagic necrosis has not been completely elucidated. TNF-α has been shown to be directly cytotoxic against a variety of tumour lines in vitro [39, 47]. Detection of its activity requires exposure periods of greater than 12 h, and as haemorrhagic necrosis is observable within 2–4 h of drug administration [5, 46], direct killing of tumour cells by TNF-α is unlikely to be the major mechanism involved. Vascular effects of TNF-α are more likely to play an important role in the initiation of haemorrhagic necrosis, particularly since these compounds are active only against tumours with an established vasculature [53]. Like FAA [28, 52], 5,6-MeXAA has been shown to inhibit tumour blood flow within 15 min of drug administration [54], and pre-treatment of mice with antibodies to TNF-α will inhibit this effect [36]. Zwi and co-workers [52] determined that the tumour ischaemia resulting from drug-induced tumour vascular collapse was sufficient to account for a significant proportion but not all of the cell death observed after treatment with FAA. Other immune mechanisms must therefore be operational for the elimination of the remaining tumour cells to prevent regrowth. In this regard, it is of interest that although LPS induced greater amounts of TNF-α and a similar degree of necrosis, only 5,6-MeXAA gave measurable growth delays and complete regression of colon 38 tumours (Fig. 2). After LPS treatment, the tumour cells that have survived the initial haemorrhagic necrotic response appear not to be eliminated and are capable of rapidly regenerating.

It has been shown by Berendt and co-workers [4] that LPS will induce haemorrhagic necrosis in most tumours, but complete regressions occurred only with immunogenic tumours that had stimulated a concomitant T-cell immunity in immunocompetent hosts [4]. In tumours that did regress, haemorrhagic necrosis that preceded tumour regression was necessary to create conditions that were conductive to the functioning of the T-cell immunity. Previous studies from our laboratory have indicated that the colon 38 tumour is not highly immunogenic and does not evoke a long-term T-cell immunity [16], and the results of the present study showing that LPS does not induce cures in the colon 38 model would be compatible with the work reported by Berendt and colleagues [4]. The ability of 5,6-MeXAA to induce a high colon 38 cure rate following tumour haemorrhagic necrosis (Table 1) suggests that 5,6-MeXAA stimulates additional host mechanisms that are capable of eliminating the surviving tumour cells and preventing regrowth. As cures are obtainable in T-cell-deficient and athymic nude mice, albeit at a lower rate than in euthymic controls [16], some of these additional host cytotoxic mechanisms must be T-cell-independent. Possible candidates are natural killer (NK) cells and tumouricidal macrophages, both of which have been shown to be enhanced in response to FAA [9, 10, 50] and to 5,6-MeXAA and its analogues [13–15].

It is evident from the emerging body of literature that the mechanism of action of FAA and its analogues is extremely complex and involves multiple contributory mechanisms. The relative degree of importance of each of the factors may vary depending on the tumour model. Although we have shown that regression of colon 38 tumours can occur in T-cell-deficient mice [16], other workers using different tumour models have shown a stricter T-cell dependency [6, 41]. Similarly, work by Wiltrout and colleagues [50] and Damia and co-workers [22] has indicated a role for NK cells in long-term regressions, but Pratesi and co-workers [41] suggest a lack of involvement of NK cells after FAA treatment. In our own laboratory, cortisone pre-treatment to inhibit macrophage activation was effective in inhibiting the antitumour effects of FAA against the Lewis lung carcinoma and a spontaneous adenocarcinoma but had no effect in some other tumour models [17]. The variability of responses appear to reside in the different relationship between the tumour types and the host immune system [18].

Whereas binding of LPS to its receptor has been shown to stimulate rapid phosphorylation of mitogen-activated protein (MAP) kinases, leading to gene activation [23, 49], the activation pathway of 5,6-MeXAA is not yet understood. Although 5,6-MeXAA and LPS share the ability to induce TNF-α (Fig. 1) and nitric oxide synthesis [48], there are activities where the two agents do not appear to overlap. LPS has been shown not to be capable of inducing cytokine production by T-cells [1], whereas other workers have demonstrated induction of interferon-γ mRNA in splenic T-lymphocytes in response to FAA [35] and another active XAA analogue [26]. Our studies comparing the antitumour activity of the two compounds also suggest than 5,6-MeXAA has a broader immunomodulating ability than LPS. One possible explanation is that 5,6-MeXAA induces cytokine production in both macrophage and lymphocyte populations, whereas LPS acts on macrophages only.

In conclusion, the results we obtained with LPS and 5,6-MeXAA indicate that the induction of tumour haemorrhagic necrosis, serum TNF-α activity, tumour growth delays and cure rates do not correlate with each other (Table 1). Thus, each of these different indicators of antitumour activity appears to measure the result of a different complex of interacting factors. Synergism between LPS and TNF-α [21] and between FAA and TNF-α [38] has previously been documented. We have shown that although LPS and 5,6-MeXAA synergise in the induction of TNF-α, combination therapy using low, sub-optimal doses of each compound fails to provide improved regression rates or growth delays over those obtainable with 5,6-MeXAA alone (Table 2). These results may be relevant to the design of future clinical trials of 5,6-MeXAA.

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