Biochemical Pharmacology, Vol. 43, No. 11, pp. 2401–2406, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

NITRIC OXIDE PRODUCTION IN ENDOTOXIN-RESISTANT C_3H/HeJ MICE STIMULATED WITH FLAVONE-8-ACETIC ACID AND XANTHENONE-4-ACETIC ACID ANALOGUES

LINDY L. THOMSEN, LAI-MING CHING, WAYNE R. JOSEPH, BRUCE C. BAGULEY* and JOHN B. GAVIN

Cancer Research Laboratory and Department of Pathology, University of Auckland School of Medicine, Auckland, New Zealand

(*Received* 16 *December* 1991; *accepted* 27 *February* 1992)

Abstract—The production of nitric oxide in endotoxin-resistant C_3H/HeJ mice in response to flavone-8-acetic acid (FAA), derivatives of xanthenone-4-acetic (XAA), endotoxin and recombinant human tumour necrosis factor-α (TNF-α) was investigated and compared with the induction of haemorrhagic necrosis in subcutaneous M16/C tumours. FAA and XAA analogues stimulated nitric oxide production both *in vitro* (activated macrophages) and *in vivo* (plasma nitrate elevation) in both C_3H/HeJ and C_3H/HeN mice (5,6-dimethyl-XAA > 5-methyl-XAA > FAA > XAA > 8-methyl-XAA). Recombinant human TNF-α stimulated nitric oxide production equally from both murine strains while endotoxin stimulated nitric oxide production only by C_3H/HeN mice. The extent of induction of haemorrhagic necrosis in tumour-bearing mice treated with FAA, 5,6-dimethyl XAA or endotoxin paralleled the effects on nitric oxide production, showing a differential between the two strains of mice only in the case of endotoxin.

Flavone-8-acetic acid (FAA†) is an antitumour agent with excellent activity against a broad range of experimental solid tumours [1] but which has failed to demonstrate clinical activity as a single agent [2]. Xanthenone-4-acetic acid (XAA) and its analogues are structurally related to FAA, and some of its derivatives show antitumour activity similar to FAA in experimental systems [3, 4]. These compounds induce haemorrhagic necrosis in experimental murine tumours [3–5] probably as a result of vascular changes including a decrease in tumour blood flow [6–8]. The effect may be mediated by tumour necrosis factor-α (TNF-α) [9], the production of which is stimulated by FAA [10] and XAA analogues.‡ However, blood flow effects are unlikely to be the sole cause of tumour regression [7] and other cytotoxic mechanisms appear to be involved. Investigations in this laboratory have identified the macrophage as a possible mediator of the action of these agents [11, 12]. A striking relationship has been found between the stimulation of nitric oxide production by activated murine macrophages and the antitumour activity of FAA, XAA, 5-MeXAA, 5,6-MeXAA and 8-MeXAA [13]. Furthermore, the correlation found for these compounds between antitumour activity and elevation of plasma nitrate concentrations in mice [14] suggests that reactive nitrogen intermediates are formed *in vivo* and play a role in the mechanism of action of these compounds.

One feature of the action of FAA is that it resembles that of endotoxin. Both induce tumour haemorrhagic necrosis in experimental murine tumours [5, 15], stimulate nitric oxide production *in vitro* and (as determined by increases in plasma nitrate) *in vivo* [13, 14, 16], and induce TNF-α [10, 15]. Both induce increases in plasma interferons [10, 17], and natural killer activity in the spleen [18, 19, unpublished].

The endotoxin-resistant C_3H/HeJ mouse provides an excellent model to investigate whether FAA acts on the endotoxin pathway. Macrophages from mice of the C_3H/HeJ mutant strain do not produce TNF-α in response to endotoxin [20], and stimulation of nitric oxide both *in vitro* and *in vivo* [16] is refractory to endotoxin in this strain. We have compared here the effect of FAA and XAA analogues and nitric oxide production, both *in vitro* by activated peritoneal macrophages from C_3H/HeN and C_3H/HeJ mice, and *in vivo* in C_3H/HeN and C_3H/HeJ mice. We have also investigated the relationship between nitric oxide production and the induction of haemorrhagic necrosis of subcutaneous M16/C tumours in C_3H/HeN and C_3H/HeJ mice.

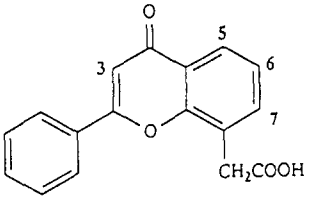
MATERIALS AND METHODS

Materials. FAA was supplied by Dr K. Paull, National Cancer Institute, U.S.A. XAA and its derivatives (Fig. 1) were synthesized in this laboratory by Drs W. A. Denny, G. J. Atwell and G. W. Rewcastle and were judged pure by TLC. Endotoxin (prepared by phenol extraction from *Escherichia coli* (055:B5) was from Sigma Chemical Co. (St Louis, MO, U.S.A.). Recombinant human TNF-α was kindly supplied by Professor J. D. Watson,

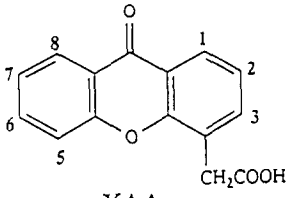
* Corresponding author: B. C. Baguley, Cancer Research Laboratory, University of Auckland School of Medicine, Private Bag, Auckland, New Zealand.

† Abbreviations: FAA, flavone-8-acetic acid; XAA, xanthenone-4-acetic acid; 5-MeXAA, 5-methyl xanthenone-4-acetic acid; 8-MeXAA, 8-methyl xanthenone-4-acetic acid; 5,6-MeXAA, 5,6-dimethyl xanthenone-4-acetic acid; TNF-α, tumour necrosis factor-α.

‡ Futami H, Eader L, Back TT, Gruys E, Young HA, Wiltrout RH and Baguley BC, manuscript submitted for publication.



FAA



XAA

Fig. 1. Structures of FAA and XAA, showing numbering system.

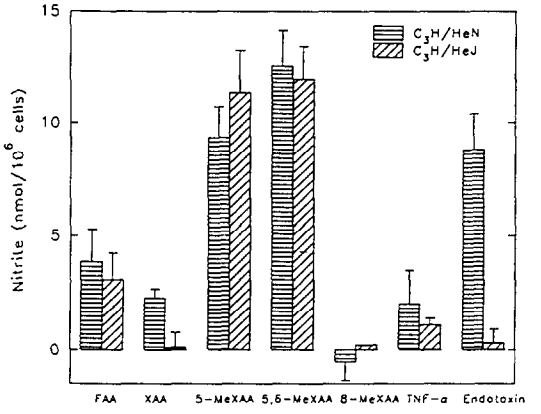


Fig. 2. *In vitro* nitrite production above control values (mean of 2–4 separate experiments ± SEM) by activated macrophages from C_3H/HeJ and C_3H/HeN mice stimulated with FAA (890 μM), XAA (1100 μM), 5-MeXAA (170 μM), 5,6-MeXAA (80 μM), 8-MeXAA (1380 μM), recombinant human TNF-α (500 ng/mL) or endotoxin (20 ng/mL). The data for C_3H/HeN mice, with the exception of those for TNF-α, has been published [13].

Department of Molecular Medicine, University of Auckland Medical School. Anti-mouse TNF-α antibodies were from Endogen Inc. (Boston, MA, U.S.A.), *Bacillus Calmette Guerin* organisms (Institute Armand-Frappier, Canada) were prepared as a suspension (4.2×10^8 organisms/mL) in sterile water. α-Minimal essential culture medium (Gibco, Grand Island, NY, U.S.A.) was supplemented with fetal calf serum (10%; Gibco New Zealand Ltd), 2-mercaptoethanol (50 μM), penicillin (100 U/mL) and streptomycin sulphate (100 μg/mL).

Mice and tumours. C_3H/HeN and C_3H/HeJ mice were bred and housed in the laboratory animal facility under constant temperature and humidity with sterile bedding, water and food according to institutional ethical guidelines. Male and female mice 6–12 weeks of age were used for all experiments. M16/C mammary tumours were provided by Dr W. R. Leopold, Parke-Davis Division of Warner-Lambert, Ann Arbor, MI, U.S.A.

In vitro *detection of nitric oxide production.* Production of nitric oxide from activated macrophage cultures was tested according to the method previously described [13]. Mice were killed by cervical dislocation 14–15 days after i.p. injection of *Bacillus Calmette Guerin* organisms (10^8 organisms/mouse), and peritoneal exudate cells were collected. Adherent macrophages were prepared by plating 10^5 peritoneal exudate cells/150 μL culture medium in 96-well plates and incubating for 2 hr at 37° in 95% air/5% CO_2. Nonadherent cells and supernatant were then removed and the adherent cells were washed twice with phosphate-buffered saline. The macrophage monolayers were then covered with 200 μl culture medium containing FAA, XAA, 5-MeXAA, 5,6-MeXAA, 8-MeXAA, endotoxin or recombinant human TNF-α. Anti-mouse TNF-α antibodies (range; 250–2000 neutralizing U/mL) were added to some cultures immediately prior to adding 5,6-MeXAA (80 μM) or endotoxin (20 ng/mL). Control macrophages were prepared and cultured in medium without added agents.

In vivo *detection of nitric oxide production.* Production of nitric oxide was determined as previously described [14]. Experiments were carried out using groups of mice from each strain matched for weight. Mice were injected i.p. with maximal tolerated doses of FAA, 5,6-MeXAA or endotoxin (40 or 150 μg/mouse), or recombinant human TNF-α (16 μg/mouse). Blood, obtained by heart puncture under ether anaesthesia with heparinized syringes, was immediately centrifuged and the plasma removed and stored at −20°. Plasma nitrate concentrations were determined after preincubation of plasma proteins with 30% $ZnSO_4$, and reduction of nitrate in the supernatant to nitrate using acid-washed cadmium powder [21]. Nitrite concentrations in the supernatant were analysed using a microplate assay method [13] based on the Griess reaction [22].

Measurement of tumour haemorrhagic necrosis. Subcutaneous M16/C tumours were passaged in carrier mice by growing to a diameter of 10 mm, removing surgically and implanting 1 mm^3 fragments in recipient mice. Mice were treated i.p. with a single dose of FAA, 5,6-MeXAA or endotoxin when the tumours were 5–10 mm in diameter. Mice were killed 24 hr later and tumours were removed and fixed in formalin. Sections were assessed for haemorrhagic necrosis using a grid counting system as previously described [23].

Statistical analyses. Data points are mean values and error bars are SEM. Differences between means of nitrate concentrations from *in vitro* experiments, and means of plasma nitrate concentrations from experimental groups, were evaluated according to the Student's *t*-test. Values for $P < 0.05$ were considered significant.

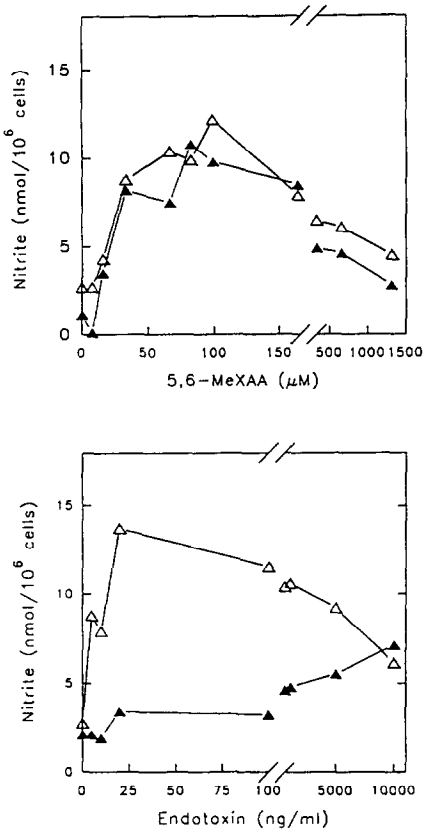


Fig. 3. Representative experiments showing *in vitro* stimulation of nitrite production by activated macrophages pooled from groups of C_3H/HeN (△) or C_3H/HeJ (▲) mice and cultured in the presence of increasing concentrations of 5,6-MeXAA or endotoxin. Each point is the mean value of triplicate cultures at each drug concentration.

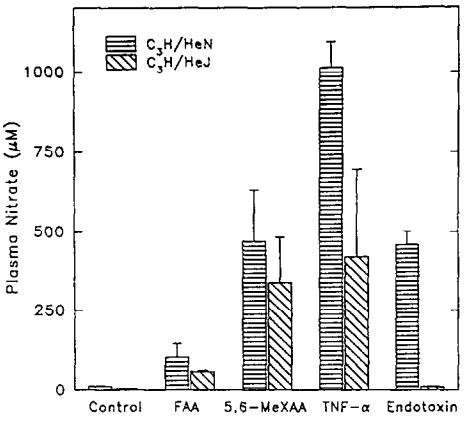


Fig. 4. Concentrations of nitrate in the plasma of C_3H/HeN or C_3H/HeJ mice 12 hr after administration of FAA (1180 μmol/kg), 5,6-MeXXA (100 μmol/kg), recombinant human TNF-α (16 μg/mouse), or endotoxin (40 μg/mouse). Control animals received no drug treatment. Values represent the mean of 3–5 mice for each treatment group. Bars are SEM.

RESULTS

In vitro *stimulation of nitrite production*

Nitrite production in control cultures of activated macrophages from both strains of mice was 2.5–5.6 and 1.8–2.9 nmol/10^6 cells for C_3H/HeN and C_3H/HeJ macrophages, respectively. The optimal concentrations for the maximal stimulation of *in vitro* nitrite production in macrophages from C_3H/HeJ mice were 890, 1100, 170 and 80 μM for FAA, XAA, 5-MeXAA and 5,6-MeXAA, respectively, the same as those found for C_3H/HeN mice [13]. The level of stimulation of nitrite production at these drug concentrations demonstrated that the ranking of activity (5,6-MeXAA > 5-MeXAA > FAA > XAA) was the same for C_3H/HeJ and C_3H/HeN mice (Fig. 2). 8-MeXAA did not stimulate nitrite production over the concentration range 10–1380 μM. The dose–response curves for stimulation of nitrite production by 5,6-MeXAA (8–1320 μM) were very similar for activated macrophage cultures from C_3H/HeN and C_3H/HeJ mice (Fig. 3).

Recombinant human TNF-α stimulated maximal *in vitro* nitrite production at concentrations above 500 ng/mL, and results for activated macrophage cultures from either C_3H/HeN and C_3H/HeJ mice were not significantly different (Fig. 2). For each murine strain, there was a highly significant difference between stimulation with recombinant human TNF-α and 5-MeXAA ($P < 0.05$) or 5,6-MeXAA ($P < 0.05$ for C_3H/HeN macrophages and $P < 0.02$ for C_3H/HeJ macrophages).

In contrast to the above results, endotoxin (20 ng/mL) stimulated nitrite production only from macrophages from C_3H/HeN mice (8.8 ± 1.6). The extent of stimulation was similar to that found with the most active antitumour agents, 5,6-MeXAA and 5-MeXAA (Fig. 2). Comparison of activated macrophage cultures from C_3H/HeN and C_3H/HeJ mice showed very different dose–response curves for stimulation of nitrite production by endotoxin (1–10,000 ng/mL). Stimulation of C_3H/HeN macrophages was observed over a wide range of endotoxin concentrations (5–10,000 ng/mL), while stimulation of C_3H/HeJ macrophages was observed only at very high endotoxin concentrations (500–10,000 ng/mL) (Fig. 3).

The addition of anti-mouse TNF-α antibodies was also investigated. Antibody (≤2000 neutralizing U/mL) had no effect on nitrite production from macrophages stimulated with 5,6-MeXAA (80 μM) or endotoxin (20 ng/mL).

In vivo *stimulation of nitrate production*

Plasma nitrate concentrations measured 12 hr after *in vivo* administration of maximum tolerated doses of drugs, were 100 ± 40 μM and 60 ± 5 μM for FAA (1180 μmol/kg), 470 ± 160 μM and 340 ± 140 μM for 5,6-MeXAA (100 μmol/kg), for C_3H/HeN and C_3H/HeJ mice, respectively (Fig. 4). These results were not significantly different between the two strains of mice for each drug treatment. Analysis of plasma

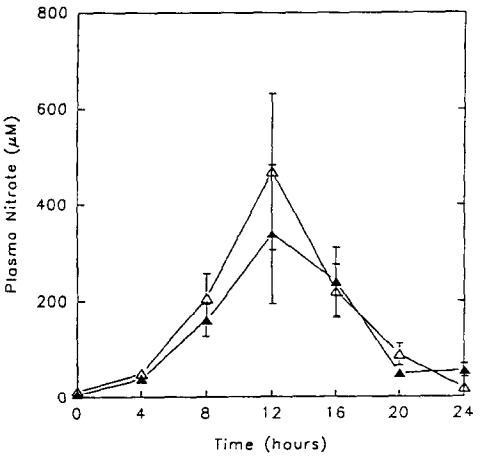


Fig. 5. Concentration of nitrate in the plasma of C_3H/HeN (△) or C_3H/HeJ (▲) mice over the 24 hr time period after administration of 5,6-MeXAA (100 μmol/kg). Points are the mean of 4–6 mice per time increment. Bars are SEM.

taken from mice at 4-hourly intervals after administration of 5,6-MeXAA (100 μmol/kg) showed peak plasma nitrate concentrations at 12 hr for both C_3H/HeN and C_3H/HeJ mice (Fig. 5).

Recombinant human TNF-α induced maximal plasma nitrate levels in C_3H/HeN mice after *in vivo* administration of ≥4 μg/mouse [14]. Plasma nitrate levels were 1010 ± 160 μM for C_3H/HeN mice and 420 ± 280 μM for C_3H/HeJ mice 12 hr after administration of recombinant human TNF-α (16 μg/mouse) (Fig. 4), and were not significantly different. In contrast, in response to endotoxin, a highly significant difference was observed between the responses of C_3H/HeN and C_3H/HeJ mice ($P < 0.01$). Endotoxin (40 μg/mouse) administered to C_3H/HeN mice induced increased plasma nitrate levels (460 ± 44 μM) which were similar to those induced by 5,6-MeXAA, but failed to induce increased plasma nitrate levels (12 ± 1 μM) in C_3H/HeJ mice substantially above basal levels (6 ± 1 μM) (Fig. 4). Even when a much higher dose (150 μg/mouse) was administered to the endotoxin-resistant strain, plasma nitrate concentrations (17 ± 2 μM) were not substantially elevated above basal levels.

Induction of tumour haemorrhagic necrosis

Results are summarized in Table 1. A small amount of spontaneous tumour necrosis was observed with all tumours and with both strains of mice. The percentage of necrotic cells scored in histological sections of tumours treated with both FAA and 5,6-MeXAA increased to a similar extent in both strains of mice. Endotoxin (100 μg/mouse) induced a high percentage of tumour necrosis in tumours growing in C_3H/HeN mice but a significantly lower ($P < 0.05$) degree of necrosis in tumours in C_3H/HeJ mice. The latter were not significantly different from untreated mice. Endotoxin at a dose which was toxic to C_3H/HeN mice (600 μg/mouse) induced a moderate degree of tumour necrosis in C_3H/HeJ mice.

DISCUSSION

These investigations indicate that C_3H/HeN (endotoxin-responsive) and C_3H/HeJ (endotoxin-resistant) mice respond equally to stimulation of L-arginine-dependent nitric oxide production by FAA or XAA analogues. *In vitro* nitrite production from activated macrophage cultures, and *in vivo* plasma nitrate levels, were not significantly different for both strains of mice following treatment with each of these antitumour agents. Furthermore, the responses of subcutaneous M16/C tumours, as assessed histologically by necrosis induction, were similar for both strains of mice. These results contrast strikingly with the divergent nitric oxide and antitumour responses of C_3H/HeN and C_3H/HeJ mice to endotoxin. Whereas normal responses were obtained in C_3H/HeN mice, production of nitrite by activated macrophage cultures from C_3H/HeJ mice was observed only at very high concentrations of endotoxin and no elevation of plasma nitrate levels following endotoxin treatment was observed. Induction of haemorrhagic necrosis of tumours in C_3H/HeJ mice was observed only at a dose of endotoxin which was toxic to C_3H/HeN mice.

One possible explanation of the results is that FAA and XAA analogues do not act on the endotoxin pathway. However, in view of the numerous similarities between these agents including the induction of cytokines (see introduction), it is more likely that FAA and XAA act at some point in the endotoxin-responsive pathway downstream from the primary endotoxin membrane receptor. The biochemical basis of the mutation which renders C_3H/HeJ mice resistant to the toxic and immunostimulatory effects of endotoxin is not known and attempts to identify an endotoxin receptor deficiency have been unsuccessful [24]. In particular, investigations of a specific endotoxin receptor have identified an 80-kDa endotoxin-binding protein which appears to be indistinguishable when murine macrophages and T and B lymphocytes from endotoxin-responsive and endotoxin-resistant mice are compared [24]. With no compelling evidence of missing endotoxin binding sites or receptors, it may be that defective transmembrane or intracellular signalling is the cause of the C_3H/HeJ defect. Additional evidence, including reestablishment of endotoxin mitogenic activity in endotoxin-resistant mice by the addition of trypsin to cultures of endotoxin-stimulated C_3H/HeJ splenocytes [25], favours a defect in transmembrane triggering responses as the cause of this genetic abnormality.

Recently, Flebbe *et al.* [26] have reported responsiveness of C_3H/HeJ macrophages to endotoxin extracted from rough mutant strain bacteria. They suggest that manifestation of activity may depend on the physicochemical properties of endotoxin preparations rather than biochemical differences in cell activation. Thus, while FAA and XAA analogues are lipophilic low molecular mass compounds which are expected to enter cells rapidly,

Table 1. Induction of haemorrhagic necrosis of M16/C tumours in C_3HHeN and C_3H/HeJ mice

Treatment	Haemorrhagic necrosis	
	C_3H/HeN mice	C_3H/HeJ mice
No treatment	12 ± 4	18 ± 2
FAA (1180 μmol/kg)	81 ± 11	78 ± 9
5,6-MeXAA (100 μmol/kg)	50 ± 14	58 ± 18
Endotoxin (10 μg/mouse)	63 ± 24	8 ± 4
Endotoxin (100 μg/mouse)	95 ± 3	27 ± 22*
Endotoxin (600 μg/mouse)		50 ± 0

* $P < 0.05$ compared with C_3H/HeN mice treated with endotoxin (100 μg/mouse).

the possibility that they may interact at the cell surface cannot be excluded.

In vitro nitrite production or plasma nitrate concentrations stimulated by recombinant human TNF-α were not significantly different for C_3H/HeN and C_3H/HeJ mice, suggesting that the nitric oxide synthesis pathway in both strains is apparently equally responsive to TNF-α. However, wide variations in plasma nitrate levels were induced in individual C_3H/HeJ mice in response to administration of recombinant human TNF-α (16 μg/mouse) (Fig. 4). This may reflect a subtle difference between the two strains, in that an *in vivo* dose of 16 μg/mouse may be optimal for the induction of maximal plasma nitrate levels in C_3H/HeN mice, but may be suboptimal for C_3H/HeJ mice.

Endotoxin [20], FAA [10] and XAA analogues* induce TNF-α. TNF-α induces nitric oxide production by activated murine macrophage cultures in the absence of endotoxin (Fig. 2; [27]). However, at optimal *in vitro* concentrations, nitrite production by activated macrophage cultures from both C_3H/HeN and C_3H/HeJ mice stimulated with recombinant human TNF-α was very low compared with the most active XAA analogues, 5,6-MeXAA and 5-MeXAA ($P < 0.05$). Furthermore, results from this study and from others [27, 28] show that the antibody to TNF-α does not or only partially suppresses nitrite synthesis by activated murine macrophage cultures. Although other explanations are possible, these results suggest that there may be two pathways involved in the stimulation of macrophages by endotoxin, FAA or XAA analogues to produce nitric oxide. The first functions via the production of TNF-α as an intermediate, which in turn induces nitric oxide synthesis, while the second functions independently of TNF-α.

It has previously been shown that FAA stimulates *in vitro* tumouricidal activity of resident peritoneal macrophages from C_3H/HeN mice but not C_3H/HeJ mice [12]. However, FAA stimulates the *in vitro* tumouricidal activity of activated macrophages from both C_3H/HeJ and C_3H/HeN mice.† Resident peritoneal macrophages do not produce nitric oxide [13] suggesting that more than one cytotoxic pathway responds to FAA and XAA analogues. Alternatively, cytotoxicity may reflect the response of a subpopulation of resident macrophages which arises as a consequence of chronic low-level stimulation of macrophages by endogenously produced endotoxin in C_3H/HeN mice.

In conclusion, the results on nitric oxide production both *in vitro* and *in vivo* parallel those of the antitumour responses of tumour-bearing C_3H/HeN and C_3H/HeJ mice to treatment with FAA, 5,6-MeXAA or endotoxin (Table 1). Taken together with previous data [13, 14], the results strengthen the relationship between the stimulation of nitric oxide production and antitumour activity.

Acknowledgements—This work was supported by the Ruth Spencer Medical Research Fellowship Trust, the Auckland Division of the Cancer Society of New Zealand, the Health Research Council of New Zealand and a Warner-Lambert Fellowship. The authors are grateful to Dr Li Zhuang for histological assessment of the tumour sections.

REFERENCES

1. O'Dwyer PJ, Shoemaker D, Zaharko S, Grieshaber C, Plowman J, Corbett T, Valeriote F, King SA, Cradock J, Hoth F and Leyland-Jones B, Flavone acetic acid (LM 975, NSC 347512), a novel antitumor agent. *Cancer Chemother Pharmacol* **19**: 6–10, 1987.
2. Kerr DJ and Kaye SB, Flavone acetic acid—preclinical and clinical activity. *Eur J Cancer Clin Oncol* **25**: 1271–1272, 1989.
3. Rewcastle GW, Atwell GJ, Baguley BC, Calveley SB and Denny WA, Potential antitumor agents. 58. Synthesis and structure-activity relationships of substituted xanthenone-4-acetic acids active against the Colon 38 tumor *in vivo*. *J Med Chem* **32**: 793–799, 1989.
4. Rewcastle GW, Atwell GJ, Zhuang L, Baguley BC and Denny WA, Potential antitumor agents. 61. Structure-activity relationships for *in vivo* colon 38 activity among disubstituted 9-oxo-9H-xanthene-4-acetic acids. *J Med Chem* **34**: 217–222, 1990.
5. Smith GP, Calveley SB, Smith MJ and Baguley BC, Flavone acetic acid (NSC 347512) induces haemorrhagic necrosis of mouse colon 26 and 38 tumours. *Eur J Cancer Clin Oncol* **23**: 1209–1212, 1987.
6. Evelhoch JL, Bissery M-C, Chabot GG, Simpson NE,

* Futami H, Eader L, Back TT, Gruys E, Young HA, Wiltrout RH and Baguley BC, manuscript submitted for publication.

† Ching L-M *et al.*, manuscript in preparation.

McCoy CL, Heilbrun LK and Corbett TH, Flavone acetic acid (NSC 347512)-induced modulation of murine tumor physiology monitored by *in vivo* nuclear magnetic resonance spectroscopy. *Cancer Res* **48**: 4749–4755, 1988.

7. Zwi LJ, Baguley BC, Gavin JB and Wilson WR, Blood flow failure as a major determinant in the antitumor action of flavone acetic acid (NSC 347512). *J Natl Cancer Inst* **81**: 1005–1013, 1989.
8. Bibby MC, Double JA and Loadman PM, Reduction of tumor blood flow by flavone acetic acid: a possible component of therapy. *J Natl Cancer Inst* **81**: 216–220, 1989.
9. Mahadevan V, Malik STA, Meager A, Fiers W, Lewis GP and Hart IR, Role of tumor necrosis factor in flavone acetic acid-induced tumor vasculature shutdown. *Cancer Res* **50**: 5537–5542, 1990.
10. Mace KF, Hornung RL, Wiltrout RH and Young HA, Correlation between *in vivo* induction of cytokine gene expression by flavone acetic acid and strict dose dependency and therapeutic efficacy against murine renal cancer. *Cancer Res* **50**: 1742–1747, 1990.
11. Ching L-M and Baguley BC, Enhancement of *in vitro* toxicity of mouse peritoneal exudate cells by flavone acetic acid (NSC 347512). *Eur J Cancer Clin Oncol* **24**: 1521–1525, 1988.
12. Ching L-M and Baguley BC, Hyporesponsiveness of macrophages from C_3H/HeJ (endotoxin-resistant) mice to the antitumour drug flavone acetic acid (NCS 347512). *Eur J Cancer Clin Oncol* **25**: 1513–1515, 1989.
13. Thomsen LL, Ching LM and Baguley BC, Evidence for the production of nitric oxide by activated macrophages treated with the antitumour agents flavone-8-acetic acid and xanthenone-4-acetic acid. *Cancer Res* **50**: 6966–6970, 1990.
14. Thomsen LL, Ching LM, Zhuang L, Gavin JB and Baguley BC, Tumor-dependent increased plasma nitrate concentrations as an indication of the antitumor effect of flavone-8-acetic acid and analogues in mice. *Cancer Res* **51**: 77–81, 1991.
15. Palladino MA, Are there more tumor necrosis factors? *J Natl Cancer Inst* **81**: 474–475, 1989.
16. Stuehr DJ and Marletta MA, Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipolysaccharide. *Proc Natl Acad Sci USA* **82**: 7738–7742, 1985.
17. Vogel SN, Moore RN, Sipe JD and Rosenstreich DL, BCG-induced enhancement of endotoxin sensitivity in C3H/HeJ mice. I. *In vivo* studies. *J Immunol* **124**: 2004–2009, 1980.
18. Ching L-M and Baguley BC, Induction of natural killer cell activity by the antitumour compound flavone acetic acid (NSC 347512). *Eur J Cancer Clin Oncol* **23**: 1047–1050, 1987.
19. Ching L-M, Joseph WR, Zhuang L, Atwell GJ, Rewcastle GR, Denny WA and Baguley BC, Induction of natural killer activity by xanthenone analogues of flavone acetic acid: relation with antitumour activity. *Eur J Cancer* **27**: 79–83, 1991.
20. Beutler B and Cerami A, Cachectin and tumour necrosis factor as two sides of the same biological coin. *Nature* **32**: 584–587, 1986.
21. Davison W and Woof C, Comparison of different forms of cadmium as reducing agents for the batch determination of nitrate. *Analyst* **103**: 403–405, 1978.
22. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS and Tannenbaum SR, Analysis of nitrate, nitrite, and (^{15}N)nitrate in biological fluids. *Anal Biochem* **126**: 131–138, 1982.
23. Baguley BC, Calveley SB, Crowe KK, Fray LM, O'Rourke SA and Smith GP, Comparison of the effects of flavone acetic acid, fostriecin, homoharringtonine and tumour necrosis factor α on Colon 38 tumors in mice. *Eur J Cancer Clin Oncol* **25**: 263–269, 1989.
24. Lei M-G and Morrison DC, Specific endotoxin lipopolysaccharide-binding proteins on murine splenocytes. I. Detection of lipopolysaccharide-binding sites on splenocytes and splenocyte subpopulations. *J Immunol* **141**: 996–1005, 1988.
25. Kuus K, Johnston AR and Ulevitch RJ, Enhancement of C_3H/HeJ B-cell responsiveness to lipopolysaccharide (LPS) with trypsin. *Fed Proc* **44**: 974, 1985.
26. Flebbe LM, Chapes SK and Morrison DC, Activation of C3H/HeJ macrophage tumoricidal activity and cytokine release by R-chemotype lipopolysaccharide preparations. *J Immunol* **145**: 1505–1511, 1990.
27. Drapier J-C, Wietzerbin J and Hibbs JB, Interferon-gamma and tumor necrosis factor induce the L-arginine-dependent cytotoxic effector mechanism in murine macrophages. *Eur J Immunol* **18**: 1587–1592, 1988.
28. Ding AH, Nathan CF and Stuehr DJ, Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. *J Immunol* **141**: 2407–2412, 1988.