# Enzymic degradation of plasma arginine using arginine deiminase inhibits nitric oxide production and protects mice from the lethal effects of tumour necrosis factor α and endotoxin

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Septic shock is mediated in part by nitric oxide (NO) and tumour necrosis factor α (TNFα). NO is synthesized primarily from extracellular arginine. We tested the ability of an arginine-degrading enzyme to inhibit NO production in mice and to protect mice from the hypotension and lethality that occur after the administration of TNFα or endotoxin. Treatment of BALB/c mice with arginine deiminase (ADI) formulated with succinimidyl succinimide polyethylene glycol of $M_r$ 20000 (ADI-SS $PEG_{20000}$) eliminated all measurable plasma arginine (from normal levels of ~ 155 μM arginine to 2 μM). In addition, ADI-SS $PEG_{20000}$ also inhibited the production of NO, as quantified by plasma nitrate + nitrite. Treatment of mice with TNFα or endotoxin resulted in a dose-dependent increase in NO production and lethality. Pre-treatment of mice with ADI-SS $PEG_{20000}$ resulted in increased resistance to the lethal effects of TNFα and endotoxin. These observations are consistent with NO production resulting, to some extent, from the metabolism of extracellular arginine. The toxic effects of TNFα and endotoxin may be partially inhibited by enzymic degradation of plasma arginine by ADI-SS $PEG_{20000}$. Interestingly, pretreatment with ADI-SS $PEG_{20000}$ did not inhibit the anti-tumour activity of TNFα *in vitro* or *in vivo*. This treatment may allow greater amounts of TNFα, as well as other cytokines, to be administered while abrogating side effects such as hypotension and death.

Key words: cytokines, endotoxin shock, inflammation, rodent.

## INTRODUCTION

Septic shock is one of the leading causes of death in the U.S.A. [1,2]. The cytokine tumour necrosis factor α (TNFα) has been suggested to mediate septic shock. This is based on the observation that endotoxin causes TNFα to be synthesized by a variety of cells following treatment (for a review, see [3]). Furthermore, administration of TNFα has been shown to result in many shock-like symptoms in experimental animals (for reviews, see [3,4]).

Although TNFα may be an important mediator in septic shock, it was the anti-tumour activity of this molecule that stimulated much of the initial interest in it [3–6]. However, the early enthusiasm for TNFα as an effective cancer treatment was quickly dampened by the severe dose-dependent hypotension it caused in both humans and animals [3,5–7]. Thus considerable effort has been focused on trying to inhibit TNFα-induced hypotension, so that this cytokine may be utilized more effectively as an anti-cancer treatment.

The compromised vascular function that occurs following administration of endotoxin or TNFα is thought to be due, at least in part, to vasodilation and a decrease in vascular resistance caused by nitric oxide (NO) [8–13]. NO has been shown to be produced rapidly in response to TNFα and endotoxin treatment, and has potent vasodilatory activity [8,9,14,15]. Additional evidence for a role for NO in mediating septic shock has also been obtained from clinical studies, which demonstrated that inhibitors of NO production ameliorated many of the symptoms of shock, including hypotension, in mice, dogs and humans [1,2,15–22]. However, the inhibitors used were toxic.

NO is produced by at least three different mammalian NO synthase (NOS) isoenzymes. All NOS enzymes utilize arginine as substrate [8,9,23–26]. Although both intracellular and extracellular arginine may be metabolized into NO, it has been hypothesized that extracellular arginine is the major substrate for the NOS enzymes [8,9,27–30].

The enzyme arginine deiminase (ADI) metabolizes arginine into citrulline and ammonia. It has been demonstrated that injection of ADI into experimental animals results in a dramatic decrease in plasma arginine levels (F. Holtsberg, C. Ensor, J. Bomalaski and M. Clark, unpublished work; [31]). However, the efficacy of this enzyme in animals has been hampered by its short circulating half-life (~ 5 h in mice), and the safety of this treatment has been compromised by the fact that this enzyme is not found in mammals. As a result it must be derived from microbial sources and is therefore highly antigenic. We have formulated ADI by the covalent attachment of poly(ethylene glycol) (PEG) to produce the reagent termed ADI-SS $PEG_{20000}$ (defined as ADI formulated with succinimidyl succinimide PEG of $M_r$ 20000). This compound has a longer circulating half-life (~ 6 days in mice) and is less antigenic in both mice and rabbits than native ADI. Because some mammals, such as mice and humans, can synthesize arginine from citrulline using an intracellular pathway, injection of ADI-SS $PEG_{20000}$ and the subsequent elimination of arginine from the systemic circulation is very well tolerated, at least in mice. For example, we have shown that weekly injections for 12 weeks of ADI-SS $PEG_{20000}$ (5 i.u./mouse) decreased plasma arginine in mice from a basal level of ~ 155 μM to below the level of detection (< 2 μM) with no apparent adverse effects on the health of the animals (C.

Abbreviations used: ADI, arginine deiminase; ADI-SS $PEG_{20000}$, arginine deiminase formulated with succinimidyl succinimide poly(ethylene glycol) of $M_r$ 20000; IL-2, interleukin-2; NOS, nitric oxide synthase; PEG, poly(ethylene glycol); TNBS, 2,4,6-trinitrobenzenesulphonic acid; TNFα, tumour necrosis factor α.

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Ensor, F. Holtsberg, J. Bomalaski and M. Clark, unpublished work). The purpose of the present experiments was to determine if NO synthesis could be inhibited by enzymic elimination of plasma arginine using ADI-SS $PEG_{20000}$. Also, we investigated whether this treatment resulted in an increase in resistance to the toxic effects of TNF$\alpha$ and endotoxin.

## MATERIALS AND METHODS

### Materials

*Salmonella abortus* endotoxin was obtained from Sigma (St. Louis, MO, U.S.A.). All chromatography resins were from Perseptive Biosystems (Boston, MA, U.S.A.). The succinimidyl succinimide PEG was from Sun Biowest (San Francisco, CA, U.S.A.). Endotoxin testing kits were from Cape Cod Associates (Woods Hole, MA, U.S.A.). All other reagents were obtained from Sigma.

### Animals

BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA, U.S.A.) and were 8–12 weeks of age at the time of use. All animals were allowed to acclimatize for at least 2 weeks after receipt prior to use. Plasma was collected as described previously [31]. The experimental protocols were approved by the Institutional Animal Care and Use Committee, The University of Kentucky.

### Meth A sarcoma

Meth A sarcoma tumour cells were obtained from the Memorial Sloan Kettering Cancer Center (New York, NY, U.S.A.), and were maintained as an ascites in BALB/c mice. Cells were collected from the mice and implanted subcutaneously on the backs of the animals, and were allowed to grow for 7 days prior to treatment, as described previously [4]. At the time of use, the tumours were 0.5–0.8 cm in diameter.

### Production of recombinant TNF$\alpha$

Human recombinant TNF$\alpha$ was produced as reported previously [32], with minor modifications. The resulting recombinant TNF$\alpha$ was $> 99\,\%$ pure as determined by SDS/PAGE, and contained less than 6 endotoxin units of endotoxin, as determined by *Limulus* amoebocyte assay. Recombinant TNF$\alpha$ inhibited L-929 cells [4] with an $LD_{50}$ of 50–70 pg/ml.

### Purification of recombinant ADI

Recombinant ADI was obtained by expression of the *Mycoplasma hominis* ADI gene in *Escherichia coli* using a modification of the method described previously for expression of ADI from *Mycoplasma arginini* [33]. Recombinant ADI was expressed ($10\,\%$ of total cell protein) as denatured inclusion bodies in *E. coli*. The *E. coli* cells that expressed recombinant ADI were collected in 10 mM sodium phosphate, pH 7.2, and disrupted with a Microfluidizer. The homogenate was then adjusted to $4\,\%$ (v/v) with respect to Triton X-100 and centrifuged (13000 ***g*** for 10 min). The resulting particulate fraction was solubilized in 1 litre of 5 M guanidine hydrochloride in 50 mM sodium phosphate, pH 7.2. The recombinant ADI was renatured by rapid dilution into 100 litres of 10 mM sodium phosphate buffer, pH 7.2. The renatured protein (which is $> 95\,\%$ pure at this step) was purified by anion-exchange chromatography using Poros HQ resin and a linear gradient of 0–1 M NaCl in 10 mM sodium phosphate, pH 7.2. ADI elutes as a sharp peak at approx. 200 mM NaCl. This method gives a yield of $\sim 4$ g of purified ADI, having a specific activity of 19–21 i.u./mg of protein, from a single 20-litre fermentation.

### Formulation of recombinant ADI with PEG

ADI was first diafiltered against 10 vol. of 10 mM sodium phosphate, pH 8.3. The protein concentration was then adjusted to 1 mg of protein/ml in the same buffer. A 4:1 molar ratio of succinimidyl succinimide $PEG_{20000}$ was then added to the ADI, and the PEG/protein mixture was stirred for 1 h at room temperature before being diafiltered against 20 vol. of 130 mM NaCl/20 mM sodium phosphate, pH 6.9, and concentrated to a final concentration of 10 mg of protein/ml. The filters used had a 100000-$M_r$ cut-off; because the ADI exists as a multimeric structure, typically less than $5\,\%$ was lost by this procedure.

The number of PEG molecules attached to the primary amines of ADI was determined as follows. Briefly, serial dilutions of the ADI native enzyme and ADI–PEG were made in 100 mM sodium phosphate, pH 8.3. TNBS (2,4,6-trinitrobenzenesulphonic acid) reagent was then added (10 mg/ml), and the reactions were heated to 40 °C for 2 h. This quantifies the number of primary amines on the protein [34]; as PEG is attached to the primary amines, making them inaccessible to derivatization with TNBS, ADI–PEG has less absorbance than native ADI. The absorbance was determined ($A_{330}$) and plotted against the protein concentration, and the slope of the line was determined. Then:

$$\text{Number of PEG molecules} = 1 - \left(\frac{\text{slope of native ADI}}{\text{slope of ADI–PEG}}\right) \times 29$$

### Amino acid analyses of plasma samples

Plasma, collected in either EDTA or heparin, was mixed with an equal volume of Sera Prep (Pickering Laboratories, Mountain View, CA, U.S.A.). This acidic solution precipitates plasma proteins, which were then removed by centrifugation (13000 ***g*** for 10 min at room temperature). The plasma amino acids that remained in the supernatant were separated by cation-exchange chromatography, and quantified by post-column derivatization with *o*-phthaldehyde and *N*,*N*-dimethyl-2-mercaptoethylamine, using the reagents and methodologies suggested by the supplier (Pickering Laboratories). The HPLC system employed was from Hewlett Packard, and was equipped with a fluorescent detector and a post-column derivatization unit. The post-column derivatization unit, $Li^+$ ion-exchange column and all buffers used in the separation were obtained from Pickering Laboratories.

### Protein determinations

Protein concentrations were determined using Bradford Reagent (Bio-Rad, Richmond, CA, U.S.A.), as suggested by the manufacturer, using BSA (Sigma) as a standard.

### ADI enzyme activity

ADI enzyme activity was determined as described [31]. Briefly, the enzyme was incubated with 10 mM arginine and 20 mM sodium phosphate, pH 7.2, for 30 min at 37 °C. An equal volume of blood urea nitrogen reagent (BUN Reagent; Sigma) was then added, the samples were heated to 95 °C for 10 min and the $A_{540}$ was then determined. A standard curve was constructed with the same buffer (omitting enzyme) containing various concentrations of citrulline. For ADI, 1 i.u. of enzyme activity was defined as that amount of activity that converts 1 $\mu$mol of arginine into 1 $\mu$mol of citrulline in 1 min at 37 °C.

### Plasma nitrate + nitrite measurement

At the indicated times, ~ 0.04 ml of plasma was collected from the mice. Plasma nitrate + nitrite levels were determined as described previously [35].

### L-929 cytotoxicity assays

L-929 cells were obtained from the American Type Culture Collection (Bethesda MD, U.S.A.) and grown in Dulbecco's modified Minimal Essential Medium (GIBCO, Grand Island, NY, U.S.A.) supplemented with fetal calf serum (10 %, v/v) (Hyclone, Logan, UT, U.S.A.). Cells were then transferred to 96-well plates containing the same medium (3000 cells/well) and grown for 24 h. Next, the indicated concentrations of TNF$\alpha$ or ADI-SS $PEG_{20000}$ were added to the wells (four wells were used at each concentration), and the cells were grown for an additional 48 h. Then 0.20 ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (25 mg/ml in PBS, pH 7.4) was added to each well of the culture plate, followed by incubation of the cultures at 37 °C for 4 h. After that time, the culture supernatants were discarded and 150 $\mu$l of DMSO was added to each well. The absorbance of each well at 540 nm was determined using a microtitre plate reader.

### Measurement of systolic blood pressure in mice

Mice were conditioned by placing them in restrainers three times per day (15 min each time) for 4 days. On day 4, the animals were injected with either saline or 5 i.u. of ADI-SS $PEG_{20000}$ as indicated. The next day, animals were treated as indicated in the Figure legends and Table 2 with TNF$\alpha$ or saline, and blood pressure was measured at the indicated times. Systolic blood pressure was measured using a tail cuff and instrumentation obtained from Kemp Scientific (Boston, MA, U.S.A.) and used as suggested by the manufacturer. Briefly, the blood pressure cuff was placed at the base of the tail and the electronic pulse detector was placed distal to the cuff. Next, the cuff was inflated to a pressure sufficient to inhibit pulse detection, and then the pressure was released slowly until a pulse was again detected. A total of five separate blood pressure measurements were obtained at each of the indicated time points. Although this method produced reasonably reproducible measurements in normal mice, it was found that a lowering of blood pressure to values less than 50 % of normal was not easily determined by this methodology, as no pulse could be detected by the pulse detector.

### Statistical analyses

The data were tested for statistical significance using Student's *t* test, with significance accepted at $P < 0.05$.

## RESULTS

Injection of endotoxin into mice caused hypoactivity, pyloerection, diarrhoea and ultimately death. Virtually all of the mice were dead within 24 h of treatment. Occasionally a few of the mice, which were morbid at 24 h, were later found dead within the next 24 h. Very few mice that survived until 48 h after treatment were observed to die within the next 30 days. Therefore in all experiments the number of mice surviving until 48 h after treatment was used in the calculation of all data shown. It was found that endotoxin caused dose-dependent death in mice, with all mice dying after exposure to a dose of 150 $\mu$g (Figure 1).

Endotoxic shock is mediated, at least in part, by NO synthesis, which results from the metabolism of extracellular arginine.

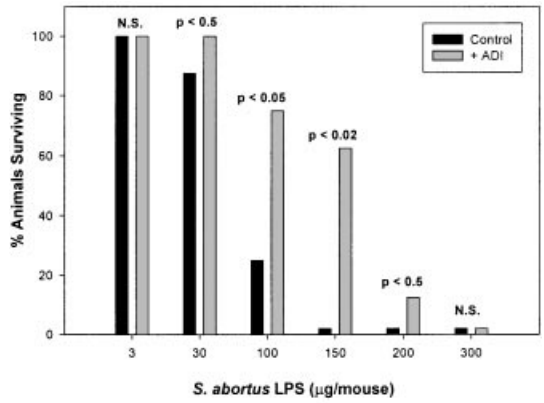


**Figure 1 Enzymic lowering of plasma arginine levels decreases endotoxin-mediated lethality in mice**

Female BALB/c mice were injected intraperitoneally with either saline or ADI-SS $PEG_{20000}$ (5 i.u./mouse). The next day the animals were injected intravenously with the indicated amounts of *Salmonella abortus* endotoxin. The survival of the animals following treatment was monitored. The number of animals that survived until 48 h after injection with endotoxin was noted as a percentage of the number of animals in each group. There were eight animals in each group (16 animals in total at each endotoxin concentration). The data were analysed by Student's *t* test; N.S., not significant.

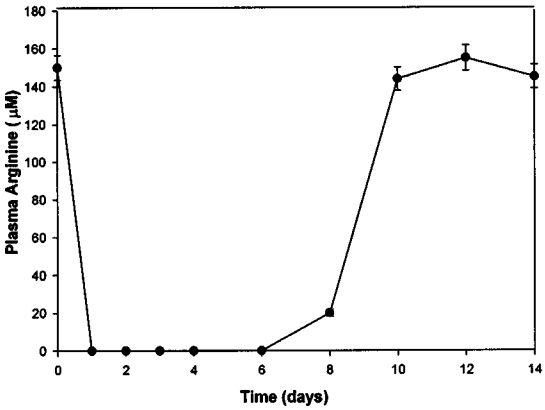


**Figure 2 Effect of a single injection of an arginine-degrading enzyme on plasma arginine levels in mice**

Five female BALB/c mice were injected intramuscularly with 5 i.u. of ADI-SS $PEG_{20000}$ on day 0. Day 0 represents plasma collection prior to ADI-SS $PEG_{20000}$ treatment. Plasma was then collected at the indicated times and the circulating levels of arginine were determined by HPLC amino acid analysis. The mice had basline levels of plasma arginine of 155 $\mu$M. The data shown represent the mean plasma concentration of arginine for five mice at each time point. The intra-assay variation was 6%, the inter-assay variation was 9% and the S.D. between the animals in each group was ~ 12%.

Therefore we utilized an arginine-degrading enzyme to decrease plasma arginine levels in order to determine the effect this may have on endotoxin-mediated lethality in mice. The effect of a single injection of ADI-SS $PEG_{20000}$ on the plasma levels of arginine in mice is shown in Figure 2. Normal mice have a plasma arginine concentration of ~ 155 $\mu$M. Within 24 h following a single injection of ADI-SS $PEG_{20000}$ (5 i.u./mouse, intramuscular), there was a lowering of plasma arginine to below the level of detection (< 2 $\mu$M) for up to 6 days (Figure 2). To test the effect of this enzyme on the survival of mice treated with endotoxin, animals were pretreated with a single injection of

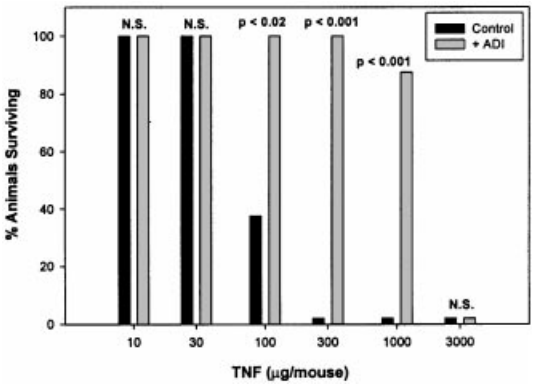


**Figure 3 Enzymic lowering of plasma arginine levels decreases TNFα-mediated lethality in mice**

Female BALB/c mice were injected intraperitoneally with either saline or ADI-SS $PEG_{20000}$ (5 i.u./mouse). The next day the animals were injected intravenously with the indicated amounts of recombinant TNFα. The number of animals that survived until 48 h after injection with TNFα was noted as a percentage of the number of animals in each group. There were eight animals in each group (16 animals in total at each TNFα concentration). The data were analysed by Student's *t* test; N.S., not significant.

**Table 1 Effects of the enzymic degradation of plasma arginine on TNFα-mediated hypotension in mice**

Mice (four female BALB/c mice in each group) were injected intraperitoneally with saline alone or saline containing 5 i.u. of ADI-SS $PEG_{20000}$. The next day the animals were injected intravenously with saline or TNFα (10 or 100 μg/mouse). Systolic blood pressure was determined by taking five separate consecutive measurements (over a 5–10 min time period) 2 h after administration of TNFα. A limitation of this method for determining blood pressure was that when the blood pressure decreased below about 50% of the resting value, the electronic pulse detector could not detect a pulse and therefore it was difficult to determine blood pressures accurately at the lower values. Thus, at the highest dose of TNFα, blood pressure in the control animals is given as no pulse detectable (ND), although the animals were alive. Data are means ± S.D.

| | Blood pressure (mmHg) | |
|---|---|---|
| Treatment | Saline control | ADI-SS $PEG_{20000}$ |
| Control | 88 ± 7 | 83 ± 8 |
| TNFα (10 μg) | 54 ± 9 | 86 ± 5 |
| TNFα (100 μg) | ND | 85 ± 10 |

ADI-SS $PEG_{20000}$ (5 i.u./mouse, intraperitoneal), and the animals were challenged with endotoxin 24 h later. It was found that mice pretreated with ADI-SS $PEG_{20000}$ were more resistant to endotoxin than untreated animals (Figure 1).

Recombinant TNFα also induced dose-dependent lethality in mice, with most animals dying within 24 h and a few surviving an additional 24 h. Therefore survival was again noted at 48 h after treatment (Figure 3). To determine if ADI-SS $PEG_{20000}$ was able to protect mice from the lethal effect of TNFα, animals were first injected with ADI-SS $PEG_{20000}$ (5 i.u./mouse) 24 h prior to testing. Then the animals were challenged with the indicated amounts of recombinant TNFα. After 48 h, survival of the animals was noted (Figure 3). Mice pretreated with ADI-SS $PEG_{20000}$ were more resistant to TNFα than the untreated animals, as was observed for endotoxin (Figures 1 and 3). When the systolic blood pressure of the mice was measured (Table 1), it was found that ADI pretreatment prevented the decrease in blood pressure caused by TNFα [similar data (not shown) were obtained with endotoxin].

**Table 2 Effects of enzymic depletion of plasma arginine on NO production in mice**

Systemic NO production [as quantified by total plasma nitrate + nitrite (μM)] in response to treatment with TNFα or *Salmonella abortus* endotoxin is shown. Animals were pretreated with either saline or ADI-SS $PEG_{20000}$ (5 i.u./mouse), and then 24 h later challenged with the indicated amounts of either endotoxin or TNFα. The amount of NO produced was determined by quantifying the total amount of nitrate + nitrite in the plasma 12 h later. The lower limit of detection by this method was ~ 1 μM. Data are means ± S.D. for four mice in each group. Significance of differences compared with saline control: $^{*}P < 0.02$, $^{**}P < 0.005$.

| | Nitrate + nitrite (μM) | |
|---|---|---|
| Treatment | Saline control | ADI-SS $PEG_{20000}$ |
| Control | 358 ± 27 | 188 ± 18* |
| *S. abortus* endotoxin (3 μg) | 603 ± 18 | 396 ± 9** |
| *S. abortus* endotoxin (30 μg) | 2732 ± 44 | 735 ± 9** |
| TNFα (10 μg) | 948 ± 27 | 509 ± 27** |
| TNFα (100 μg) | 4962 ± 89 | 559 ± 9** |

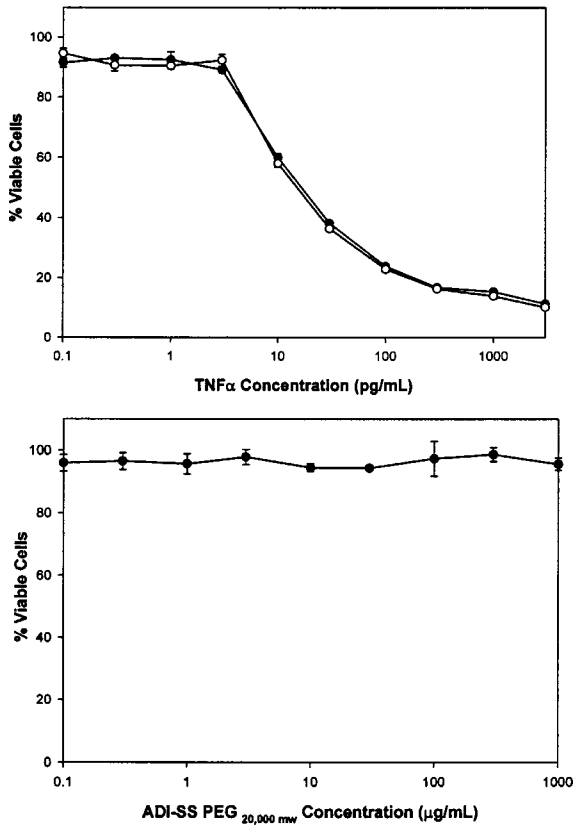


**Figure 4 Effect of arginine-degrading enzyme on TNFα-mediated cytotoxicity towards L-929 cells *in vitro***

L-929 cells were grown in 96-well plates for 24 h. Then the indicated concentrations of TNFα (upper panel; ●) or ADI-SS $PEG_{20000}$ (lower panel) was added to each well for 48 h (all treatments were performed in quadruplicate wells) and the viability of the cells was determined. The effect of TNFα on L-929 cell cytotoxicity was also tested in the presence of 100 μg/ml ADI-SS $PEG_{20000}$ (upper panel; ○). In this experiment, ADI-SS $PEG_{20000}$ and TNFα were added at the same time. The means ± S.E.M. of the four wells are shown.

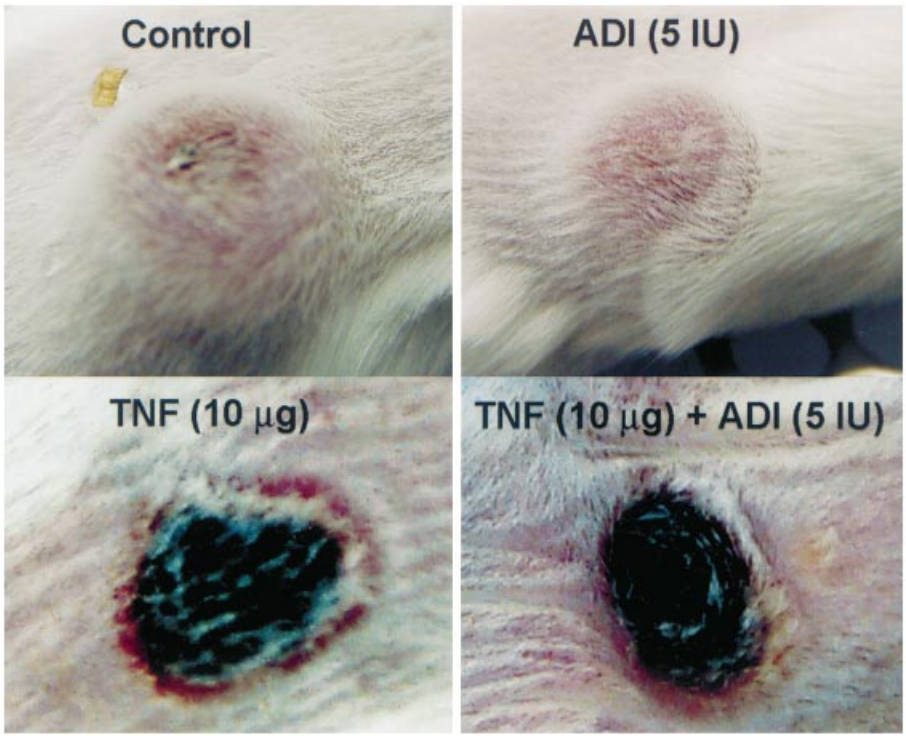


**Figure 5 Effects of treatment with arginine-degrading enzyme on TNFα-mediated anti-tumour activity**

BALB/c mice were implanted with Meth A sarcomas, which were allowed to grow for 7 days until they were ~ 0.5–0.8 cm in diameter. The animals were then pretreated with either saline or ADI-SS $PEG_{20000}$. After 24 h, the animals were given a single injection of 10 μg of recombinant TNFα, and the effects on the tumours were noted after an additional 24 h.

NO has a very short circulating half-life; therefore it is most often quantified *in vitro* by measurement of nitrite (a stable metabolite of NO) using the Griess assay [35–37]. However, since nitrate is the predominant metabolite of NO in the blood, plasma nitrates were reduced with nitrate reductase to nitrites and then analysed by the Griess assay as described previously [35]. Plasma samples were taken 12 h after treatment with TNFα or endotoxin. Both TNFα and endotoxin caused a time- and dose-dependent increase in NO production in mice (Table 2). Pretreatment of mice with ADI-SS $PEG_{20000}$ not only increased the survival of the animals (Figures 1 and 3), but also appeared to lower NO production significantly. In addition, data from the control animals indicated that elimination of plasma arginine lowered NO production not only from the TNFα-induced NOS, but also from the constitutive enzyme.

We next set out to determine if inhibition of NO production using ADI-SS $PEG_{20000}$ affected the ability of TNFα to inhibit tumour cell growth *in vitro* and *in vivo*. L-929 cells have long been used as a model system for demonstrating the cytotoxic activity of TNFα *in vitro* [4], although these cells do not produce NO in response to TNFα treatment. Therefore this cell line was used to determine if ADI-SS $PEG_{20000}$ affected the cytotoxicity of TNFα *in vitro*. TNFα caused a dose-dependent inhibition of L-929 cells *in vitro*, with an $LD_{50}$ of ~ 50–70 pg/ml (Figure 4). L-929 cells were not inhibited by the addition of ADI-SS $PEG_{20000}$ to the culture medium (Figure 4). Moreover, addition of 100 μg/ml ADI-SS $PEG_{20000}$ had no effect on TNFα cytotoxic activity *in vitro*.

Meth A sarcomas are not sensitive to the cytotoxic actions of TNFα *in vitro*, but are sensitive *in vivo* [4]. Therefore we used this model system to determine the effect of pretreatment with ADI-SS $PEG_{20000}$ on the anti-tumour activity of TNFα *in vivo*. The results from four representative animals are shown in Figure 5. These data suggest that, although pretreatment with ADI-SS $PEG_{20000}$ decreases NO production and improves animal resistance to the lethality of high doses of TNFα, it does not inhibit the anti-tumour activity of TNFα *in vivo*.

## DISCUSSION

The initial enthusiasm for using TNFα as an anti-cancer treatment was quickly dampened when it was found by several different groups that this molecule caused a precipitous fall in blood pressure which resulted in shock and death ([3]; for a review, see [5]). This was observed in nearly all of the 19 clinical trials performed with systemically administered TNFα in humans [5]. At the same time, it has been determined, by performing isolated limb perfusion with TNFα, that a very high response rate can be obtained with this cytokine if its hypotensive effects can be curtailed. Thus a great deal of research has focused on the mechanisms by which TNFα causes hypotension, and several different laboratories have identified NO as being a possible mediator of the adverse effects of TNFα [11,12,15,22,38].

Other cytokines, such as interleukin-2 (IL-2), also cause hypotension, which limits the therapeutic utility of these agents as well. For example, IL-2 has also been shown to induce NO. Inhibitors of NO synthesis have been demonstrated to be effective in the maintenance of normal vascular function following treatment with IL-2 in humans [15,39]. Thus inhibition of NO

synthesis has been suggested as a way to allow safer usage of these cytokines, as has been recommended with TNF$\alpha$.

NO may be an important signalling molecule that has numerous effects, including regulation of vascular tone, immunity, glucose levels and neurotransmission [2,8,9,40,41]. Overproduction of NO has been associated with numerous pathological conditions, including septic shock, stroke, diabetes mellitus, and demyelinating disorders such as multiple sclerosis. Thus considerable interest lies in inhibiting the overproduction of this potentially harmful molecule.

Several different strategies have been explored as a means of inhibiting NO production. One of these approaches is the use of arginine analogues, which act as competitive inhibitors of NOS [15]. Several of these inhibitors have proven effective in blocking NO production. Moreover, these inhibitors appear to be effective in both experimental animals and humans, and can significantly inhibit TNF$\alpha$-, IL-2- and endotoxin-induced NO production. Although these inhibitors were also found to be toxic, results obtained from their use suggest the utility of NOS inhibitors for blocking hypotension in both humans and experimental animals.

Another approach is to limit the substrate (arginine) for the NOS enzymes through dietary restriction. This was deemed feasible, as human adults [42] and infants [43] do not require arginine in the diet. However, it was found that elimination of arginine from the diet resulted in only about a 30 % decrease in plasma arginine, and had little effect on NO production [44]. One possible explanation for these results may be that, because normal levels of arginine in human plasma are $\sim 155\ \mu$M and the $K_m$ of the NOS enzymes is $\sim 1$–$5\ \mu$M [23], a 50 % decrease in plasma arginine would not decrease substrate levels sufficiently below the $K_m$ of the enzyme such that it would inhibit its catalysis.

Other investigators have attempted to lower arginine levels using various arginine-degrading enzymes. The first of these to be used was arginase, a mammalian enzyme most often isolated from the liver [8]. Arginase metabolizes arginine into ornithine. However, administration of this enzyme into animals is largely ineffective in lowering plasma arginine (and inhibiting NO production), for at least four reasons. First of all, arginase has an alkaline pH optimum (pH 9.3) and has little enzymic activity at physiological pH. Secondly, arginase has a very low affinity for arginine ($K_m$ of $\sim 2$ mM), and even large amounts of enzyme result in only an $\sim 50$ % decrease in plasma arginine levels. Thirdly, this enzyme has a short circulating half-life ($< 4$ h). Finally, arginase metabolizes arginine into ornithine, a metabolite that can only be reconverted back into arginine in the liver, and thus high levels of this enzyme can cause toxicity to extra-hepatic tissues by inhibiting protein synthesis (for a review, see [45]).

ADI is another arginine-degrading enzyme, which was first utilized by Takaku et al. [31] as a means of lowering plasma arginine levels in mice. ADI isolated from *Mycoplasma* has a pH optimum of 6.8 and retains 60–80 % of its activity at physiological pH. ADI isolated from *M. hominis* also has a high affinity for arginine ($K_m$ of 20 $\mu$M). Moreover, ADI converts arginine into citrulline. Most normal cells and tissues are able to take up citrulline from the circulation and, in two steps of the Krebs (urea) cycle catalysed by the intracellular enzymes argininosuccinate synthetase and argininosuccinate lyase, reconvert citrulline back into arginine for protein synthesis. By formulating ADI with PEG to produce a drug we have termed ADI-SS $PEG_{20000}$, we have been able to greatly increase its circulating half-life to $\sim 6$ days, and a single injection of 5 i.u. into a mouse will deplete circulating arginine levels from 155 $\mu$M to $< 2\ \mu$M (F. Holtsberg, C. Ensor, J. Bomalaski and M. Clark, unpublished work). This enzyme also appears to be relatively non-toxic, as we have been able to administer weekly injections to eliminate all detectable arginine from the circulation of mice for up to 3 months with no observable adverse effects in the mice. However, the species requirement for arginine varies considerably. For example rats, dogs and cats all have an absolute requirement for arginine, and elimination of plasma arginine in these species would be expected to be fatal [46].

ADI-SS $PEG_{20000}$ was originally developed as a means of inhibiting the growth of arginine auxotrophic tumours (C. Ensor, F. Holtsberg, J. Bomalaski and M. Clark, unpublished work). However, because the arginine that is metabolized into NO is extracellular, we hypothesized that ADI-SS $PEG_{20000}$ may be useful in inhibiting NO production, and thus may facilitate the use of TNF$\alpha$ as an anti-cancer therapy. This enzyme was used to pretreat normal BALB/c mice before challenge with TNF$\alpha$ or endotoxin. By lowering levels of L-arginine substrate, we were able to prevent the increases in NO production (Table 2) and the concomitant lethality normally associated with TNF$\alpha$ and endotoxin (Figures 1 and 3). However, even though the $LD_{50}$ was shifted by ADI-SS $PEG_{20000}$, all mice given sufficient amounts of either TNF$\alpha$ or endotoxin were killed in spite of a decrease in NO synthesis. Since death due to severe hypotension is an early event in TNF$\alpha$- and LPS-mediated shock, other lethal pathways must exist. Thus our observations are very similar to those made by others using knockout mice lacking inducible NOS [46]. It has been suggested, for example, that hepatotoxicity and neutrophil-mediated endothelial injury may also play important roles in mediating the lethal effects of TNF$\alpha$ and endotoxin [3,47]. In addition, it has been shown that NO inhibits complex IV in the mitochondrion respiratory chain and thus may contribute to cell death [48].

The effect of prolonged inhibition of NO in humans is unclear. Specific inhibitors of NOS have been shown to be toxic [15]. Blocking all NO synthesis for extended periods may lead to deleterious side effects. However, results from knockout mice lacking inducible NOS suggest that they develop normally and can reproduce [47]. Also, mice have been treated for several months will ADI-SS $PEG_{20000}$ and have demonstrated no harmful effects (results not shown).

Taken together, the present data indicate that the enzymic degradation of plasma arginine by ADI-SS $PEG_{20000}$ not only may be beneficial in preventing some of the adverse events associated with septic shock, but may also be of use in overcoming some of the toxicity associated with the use of TNF$\alpha$ as an anti-cancer agent.

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