



N^G -nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthesis, ameliorates interleukin 2-induced capillary leakage and reduces tumour growth in adenocarcinoma-bearing mice

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Summary We tested whether N^G -nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthesis, can prevent interleukin 2 (IL-2)-induced capillary leakage in tumour-bearing mice without compromising the therapeutic benefits of IL-2. C3H/HeJ female mice transplanted s.c. with 2.5×10^5 C3-L5 mammary carcinoma cells were treated with: nothing, IL-2 (ten injections of 15 000 Cetus units i.p. every 8 h), L-NAME (0.1, 0.5, or 1 mg ml⁻¹ drinking water), IL-2+L-NAME (0.1 or 0.5 or 1 mg ml⁻¹ drinking water). Therapies were given in one round (IL-2, days 10–13; L-NAME, days 9–13) or in two rounds (IL-2, days 10–13 and 20–23; L-NAME, days 9–13 and days 19–23) after tumour transplantation. Capillary leakage was measured from the water contents of the pleural cavities, lungs, spleen and kidneys. Effects of the therapies on the primary tumour size and the number of spontaneous lung metastases were also recorded. NO production was measured as the nitrite+nitrate levels in the serum and in the pleural effusion. After the first round of therapies, addition of L-NAME significantly reduced IL-2-induced pulmonary oedema and water retention in the spleen in a dose-dependent manner. It also significantly reduced the IL-2-induced rise in NO levels in the serum and pleural fluid, but did not affect IL-2-induced pleural effusion or water retention in the kidney. At later stages of tumour growth (day 23), tumours themselves induced significant fluid retention in the lungs and the kidney, which was not aggravated further with the second round of IL-2 therapy. At this time, L-NAME therapy alone ameliorated tumour-induced pulmonary oedema. During both rounds of therapy different doses of L-NAME alone caused a reduction of primary tumour growth as well as spontaneous lung metastases, which improved further with the addition of IL-2. The combination therapy was at least as effective as IL-2 therapy. In summary, L-NAME had anti-tumour effects *in vivo*, reduced the severity of IL-2-induced capillary leakage in some organs and did not compromise anti-tumour efficacy of IL-2 therapy. Thus, L-NAME could be a valuable adjunct to IL-2-based cancer therapy.

Keywords: capillary leak syndrome; interleukin 2 cancer immunotherapy; mammary adenocarcinoma; nitric oxide; N^G -nitro-L-arginine methyl ester

Interleukin 2 (IL-2) therapy, alone or in combination with *ex vivo*-generated lymphokine-activated killer (LAK) cells, can cause tumour regression in mice (Rosenberg *et al.*, 1985; Ettinghausen *et al.*, 1988) and in man (Rosenberg, 1989; Fisher *et al.*, 1988; Dutcher *et al.*, 1989; Parkinson *et al.*, 1990). Widespread clinical use of IL-2 has been limited by the low rate of complete remission and by severe toxicity (Hibbs *et al.*, 1992; Siegel and Puri, 1991). Capillary leak syndrome is a major side-effect of high-dose IL-2 therapy, characterised by retention of extravascular fluid and hypotension (Siegel and Puri, 1991). It has been observed in humans (Siegel and Puri, 1991; Margolin *et al.*, 1989; Rosenberg *et al.*, 1987) as well as in mice (Rosenstein *et al.*, 1986).

A number of *in vitro* as well as *in vivo* observations suggest that IL-2-induced capillary leakage may result from multiple mechanisms: damage to endothelial cells by LAK cells and natural killer (NK) cells (Amador *et al.*, 1991; Aronson *et al.*, 1988); a direct injury to endothelial cells inflicted by two cytokines induced by IL-2 therapy – interferon (IFN) γ (Montesano *et al.*, 1985) and tumour necrosis factor (TNF) α (Kahaleh *et al.*, 1988); and finally, vasodilation due to IL-2-induced production of nitric oxide (NO) (Hibbs *et al.*, 1992; Ochoa *et al.*, 1992), leading to systemic hypotension followed by pulmonary hypertension and oedema.

There have been several reports of therapies that combine IL-2 with agents that might ameliorate the capillary leakage. However, the added drugs also opposed the anti-tumour effects of IL-2. Corticosteroids (Rosenstein *et al.*, 1986; Faggioni *et al.*, 1994), which suppress inflammatory and immune responses as well as production of NO (Moncada and Higgs, 1993), and asialo-GM-1 antibody, which depletes

LAK cells (Ettinghausen *et al.*, 1988), both fall into this category. Puri *et al.* (1989) have reported that IL-1 α reduced IL-2-induced capillary leakage but did not improve animal survival. Welbourn *et al.* (1991) reported that certain cyclopeptides, e.g. antamanide and phalloidine, reduced IL-2-induced oedema in the rat, presumably by causing cytoskeletal changes in neutrophils, with consequent suppression of endothelial injury by thromboxane B₂. Interactions of these agents with the anti-tumour effect of IL-2 remain unknown. Further studies are therefore required to identify substances that can ameliorate capillary leakage without reducing the anti-tumour effects of IL-2.

Severe hypotension observed during IL-2 therapy has been recently attributed to the production of NO from L-arginine (Hibbs *et al.*, 1992; Ochoa *et al.*, 1992), indicating the potential value of NO synthase inhibitors in preventing IL-2-induced hypotension. This prompted our studies with two NO synthase inhibitors. We found that one such inhibitor, N^G -methyl-L-arginine (NMMA) failed to ameliorate IL-2-induced capillary leakage, but improved anti-tumour effect of IL-2 in mice (Orucevic and Lala, 1992, 1993). Subsequently, we discovered that N^G -nitro-L-arginine methyl ester (L-NAME; a more potent NO synthase inhibitor) given orally could effectively prevent capillary leakage induced by IL-2 in healthy mice (Orucevic and Lala, 1995a).

The objectives of the present study were to examine whether L-NAME can prevent IL-2-induced capillary leakage in tumour-bearing mice without compromising the therapeutic benefit of IL-2.

Materials and methods

Mice

C3H/HeJ female mice, 6–7 weeks old, were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). Animal

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care was in accord with guidelines of the Canadian Council on Animal Care. Mice were kept on a 12 h light/dark cycle, fed with a standard mouse chow and provided with water *ad libitum*.

Interleukin 2

Highly purified recombinant human IL-2 (lot LQP-046) was kindly provided by Chiron Corp (Emeryville, CA, USA). The sp. act. was 18×10^6 International units mg^{-1} or 3×10^6 Cetus units mg^{-1} IL-2. The lyophilised IL-2 (1.2 mg per vial) was first reconstituted with 1 ml of distilled water and further diluted with RPMI-1640 medium (ICN Biomedicals, Costa Mesa, CA, USA) in order to obtain the concentration of 15 000 Cetus units in 0.1 ml (volume per injection). The reconstituted IL-2 was stored at 4°C for up to 1 day.

N^G-nitro-L-arginine methyl ester

L-NAME (Sigma, St Louis, MO, USA) was added to the drinking water to provide concentrations of 0.1, 0.5 and 1 mg ml^{-1} of water. These doses were based on our studies conducted earlier to prevent IL-2-induced capillary leak syndrome in healthy mice (Orucevic and Lala, 1994, 1995a). In one additional experiment, we investigated the effects of the D-form of NAME (D-NAME; 1 mg ml^{-1} of water; obtained also from Sigma) in order to evaluate the specificity of L-NAME action, since D-NAME is incapable of inhibiting NO synthase (Ialenti *et al.*, 1993).

Tumour-cell line

A spontaneous mammary tumour in a C3H/HeJ mouse, which also exhibited lung metastases (Brodt *et al.*, 1985), was the source of a primary transplantable tumour, T58, from which the metastatic C3 line had been clonally derived (Lala *et al.*, 1986). Since the spontaneous lung metastatic ability of the C3 line declined after *in vitro* passages over the years (Lala *et al.*, 1986), a highly metastatic C3-L5 line was derived from the C3 line by five cycles of *in vivo* selection for spontaneous lung metastasis (Lala and Parhar, 1993) as follows. C3 cells were transplanted s.c. into C3H/HeJ mice and allowed to metastasise to the lungs. Cells from the lung micrometastases were then injected s.c. into fresh C3H/HeJ recipients, and again allowed to metastasise to the lungs. This cycle was repeated five times. The C3-L5 line has since maintained its strong metastatic phenotype both in C3H/HeN (Saarloos *et al.*, 1992) and C3H/HeJ (Lala and Parhar, 1993) strains of mice.

Tumour transplantation

C3-L5 mammary adenocarcinoma cells (2.5×10^5 in 0.1 ml of RPMI medium) were injected s.c. in the mammary line near the axilla. In addition to the formation of primary tumours, this procedure was expected to produce micrometastases in the lungs of C3H/HeJ mice within 10 days of transplantation (Lala and Parhar, 1993).

Protocols for immunotherapy

Tumour transplanted mice, randomly separated into eight groups ($n=10-20$), were treated with: nothing, IL-2 (ten injections of 15 000 Cetus U i.p. every 8 h), L-NAME (0.1 mg ml^{-1} drinking water), L-NAME (0.5 mg ml^{-1} drinking water), L-NAME (1 mg ml^{-1} drinking water), IL-2+0.1 mg ml^{-1} L-NAME, IL-2+0.5 mg ml^{-1} L-NAME, IL-2+1 mg ml^{-1} L-NAME. Therapies were given in one round (IL-2, days 10-13; L-NAME, days 9-13), or in two rounds (IL-2, days 10-13 and 20-23; L-NAME, days 9-13 and days 19-23) after tumour transplantation. In one additional experiment, mice ($n=5$) were treated with IL-2 or IL-2+1 mg ml^{-1} D-NAME (IL-2, days 10-13; D-NAME, days 9-13). Mice were killed after one or two rounds of treatments (1 h after last IL-2 injection) to measure capillary

leakage and anti-tumour effects of therapies. Some animals succumbed to IL-2 toxicity after the first round of IL-2, so that all animals did not survive to the beginning of the second round. Autopsy was performed on all dead animals and the quantity of pleural effusion was measured.

Measurement of capillary leakage and NO metabolites

The left lung, the spleen and the left kidney were recovered for measurement of their water content. The wet weights were recorded, then the organs were frozen (-80°) and freeze dried to constant weight. Water content of each organ was expressed as wet-dry weight ratio.

The volume of liquid from both pleural cavities was measured directly by complete aspiration with a 1 ml syringe as follows. After removing the skin, the anterior thoracic wall was cut at three levels: inferior to the sternum, and at both anterior axillary lines, so that it could be lifted without severing major blood vessels of the thoracic wall or the mediastinum. Both costodiaphragmatic recesses were then accessed with a syringe. This procedure allowed collection of blood-free aspirates. On rare occasions of blood contamination, the samples were excluded from the study.

Samples of serum and pleural effusion were collected after the first or second round of treatments to measure NO_2^- and NO_3^- , the principal metabolites of NO (Moncada and Higgs, 1993; Kelm *et al.*, 1992). Griess reagent (Green *et al.*, 1982) was used for measurement of NO_2^- and cadmium filings for conversion of NO_3^- to NO_2^- (Davison and Woof, 1978).

Measurement of anti-tumour effects

The size of the primary tumours was measured with callipers on days 9, 14, 19 and 23, by recording the maximum and minimum diameters. Tumour volumes were then calculated as $0.52a^2b$, where a and b are the minimum and maximum diameters (Baguley *et al.*, 1989), and the tumour volume represented in mm^3 .

The right lung was isolated and fixed with Bouin's fixative. The number of metastatic nodules in the lungs was scored using a dissecting microscope.

Statistical analysis

The Microstat statistics package (Ecosoft, Indianapolis, IN, USA) was used in the analysis of collected data. One-way ANOVA was used for normal distributions and the Kruskal-Wallis test for non-normal distributions. Newman - Keuls test and non-parametric multiple range test were respectively used to determine which means or sums of ranks differed significantly ($P<0.05$) from one another (Zar, 1974).

Results

Effects of L-NAME on IL-2-induced capillary leakage after one or two rounds of therapies

A single round of IL-2 therapy induced a significant amount of pleural effusion. This was 0.38 ± 0.05 ml in animals that were killed immediately after the first round of therapy, and 0.92 ± 0.07 ml in animals that died within 5 days after the first round of therapy. The reduction of IL-2-induced pleural effusion noted in either group was not significant with the addition of different doses (0.1, 0.5 and 1 mg ml^{-1}) of L-NAME (data not presented). There was no detectable pleural effusion after the second round of the therapies inclusive of IL-2. L-NAME therapy alone did not induce pleural effusion in any of the animals (data not shown).

Addition of various doses of L-NAME significantly ($P<0.05$) reduced IL-2-induced pulmonary oedema after the first round of therapies (Figure 1a). At the time of completion of the second round of therapy (day 23), untreated tumour-bearing mice showed a higher water content in the lungs as compared with healthy animals. This was not aggravated further by IL-2 therapy. The

tumour-induced water retention in the lungs was significantly ameliorated only by L-NAME therapy alone ($P < 0.01$) (Figure 1b).

IL-2-induced water retention in the spleen decreased significantly ($P < 0.001$) with addition of L-NAME in a dose-dependent manner after the first round of therapies (Figure 2a). After the second round of therapies, different doses of L-NAME did not significantly influence IL-2-induced water retention in the spleen (Figure 2b). L-NAME therapy alone did not have any effect on the water content in the spleen after the first or the second round of therapy (Figure 2).

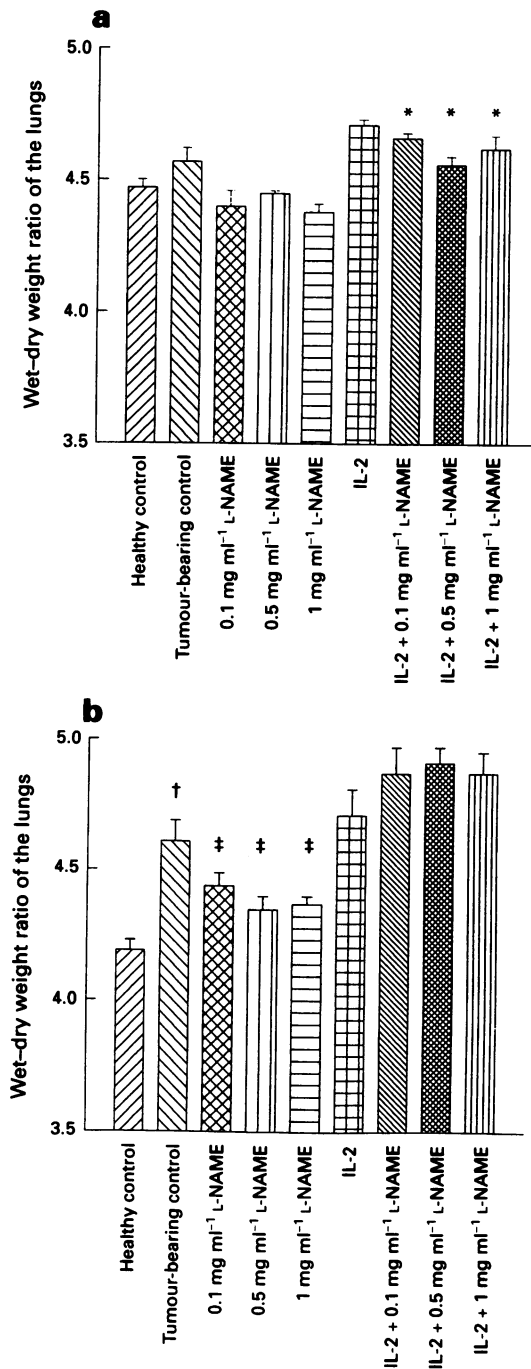


Figure 1 Water content in the lungs after one (a) or two (b) rounds of IL-2 and L-NAME therapy. Data represent mean \pm s.e. (a) $n = 10$. (b) $n = 9-12$. *Addition of L-NAME significantly ($P < 0.05$) reduced IL-2-induced pulmonary oedema after the first round of therapies. †Tumour bearing by itself at a time coinciding with the end of the second round of therapies, induced a significant ($P < 0.05$) increase in the water content in the lungs. ‡L-NAME therapy significantly ($P < 0.01$) reduced tumour-induced water retention in the lungs.

After the first or the second round of therapy, IL-2-induced fluid retention in the kidney remained unaffected with addition of any of the L-NAME doses (Figure 3). Tumour-bearing by itself induced a significant increase in the water content in the kidney (Figure 3), which was significantly ($P < 0.05$) reduced by L-NAME therapy alone, but only after the first round of therapy (Figure 3).

Effects of therapies on animal morbidity

After the first round of treatments, some tumour-transplanted animals died from IL-2-induced toxicity in all IL-2 treated groups. There was no death due to tumour-transplantation at this time. The reduction of IL-2-induced mortality by the addition of various doses of L-NAME

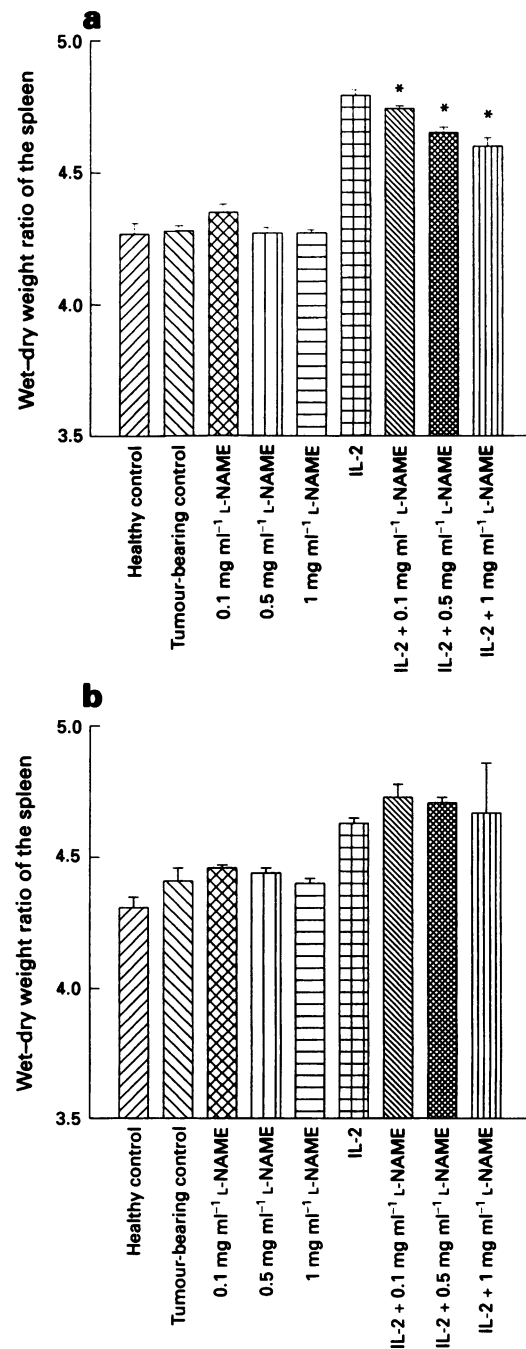


Figure 2 Water content in the spleen after one (a) or two (b) rounds of IL-2 and L-NAME therapy. Data represent mean \pm s.e. (a) $n = 10$. (b) $n = 9-12$. *Addition of L-NAME significantly ($P < 0.001$) decreased IL-2-induced water retention in the spleen in a dose-dependent manner after the first round of therapies.

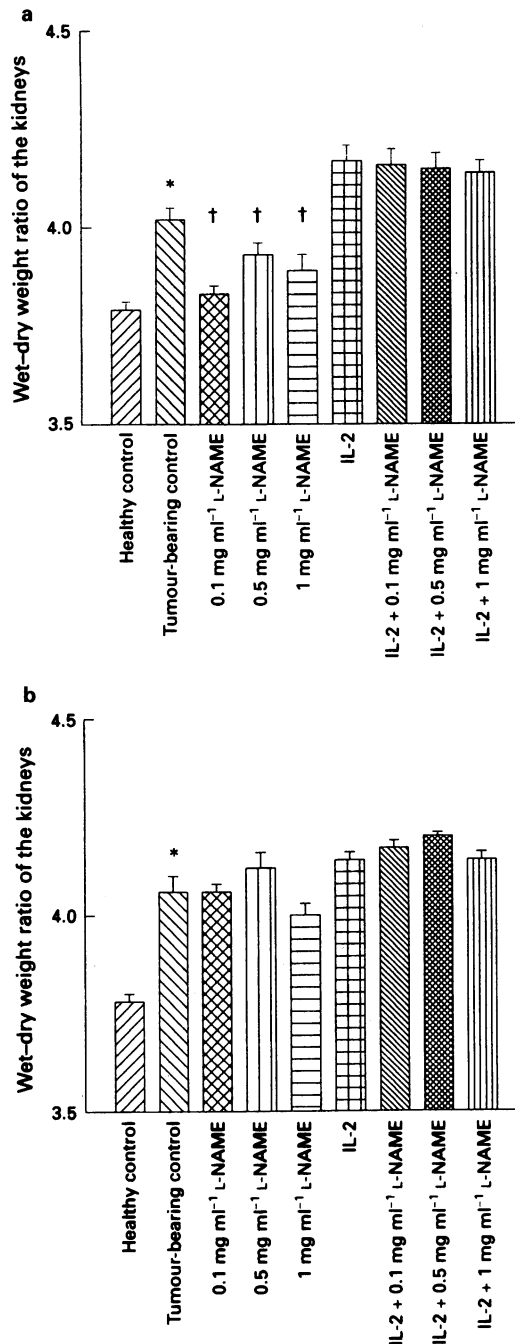


Figure 3 Water content in the kidney after one (a) or two (b) rounds of IL-2 and L-NAME therapy. Data represent mean \pm s.e. (a) $n=10$. (b) $n=9-12$. *Tumour-bearing by itself induced an early, day 13 (a) and sustained, day 23 (b), increase in the water content in the kidney ($P<0.05$). †L-NAME therapy alone significantly ($P<0.05$) decreased tumour-induced fluid retention in the kidney after the first round of therapy.

therapy was not significant. L-NAME therapy alone at any dose level did not cause any mortality in healthy or tumour-transplanted mice (data not presented).

Effects of L-NAME and IL-2 therapy on $\text{NO}_2^- + \text{NO}_3^-$ levels in the serum and pleural effusion

The basal concentrations of NO_2^- in the serum and pleural effusion were not detectable, since they were below the sensitivity of the assay ($<1 \mu\text{M}$). NO_2^- levels shown in Figure 4 were obtained by reducing NO_3^- to NO_2^- with cadmium filings and thus represent $\text{NO}_2^- + \text{NO}_3^-$ levels. Different doses of L-NAME significantly reduced IL-2-induced increases in $\text{NO}_2^- + \text{NO}_3^-$ levels in the serum and

pleural effusion in tumour-bearing mice after the first round of therapies (Figure 4). After the second round, IL-2 therapy did not influence $\text{NO}_2^- + \text{NO}_3^-$ levels in the serum of these animals, while addition of 1 mg ml^{-1} L-NAME to IL-2 significantly increased these levels (Figure 4b). L-NAME therapy alone did not have any effect on $\text{NO}_2^- + \text{NO}_3^-$ levels in the serum measured after the first or the second round (Figure 4a and b).

Effects of L-NAME and IL-2 therapy on the primary tumour size and number of lung metastatic nodules

All forms of therapy (L-NAME alone at various doses, IL-2 alone or IL-2+L-NAME) significantly ($P<0.05$) reduced the growth of primary tumours. The best responses were seen with L-NAME alone at 1 mg ml^{-1} , IL-2 alone or IL-2+L-NAME (at various doses) (Figure 5). Addition of L-NAME to IL-2 therapy had only a transient (noted on day 14, at the end of the first round) beneficial effect on primary tumour growth (Figure 5), which was reproduced in another experiment (data not shown).

All therapies, except 1 mg ml^{-1} L-NAME alone, significantly reduced the number of lung metastatic nodules. Reduction with IL-2 alone or IL-2 in combination with L-NAME was greater than with L-NAME alone (Figure 6).

Effects of D-NAME on IL-2-induced capillary leakage, NO production and anti-tumour effects

N^G -nitro-D-arginine methyl ester (D-NAME) (1 mg ml^{-1}) had no significant effect on IL-2-induced pleural effusion, rise in water content in the spleen and kidney, or the reduction of the primary tumour size after the first round of therapies ($n=5$ per group, data not presented). Furthermore, D-NAME had no influence on IL-2-induced rise in the levels of $\text{NO}_2^- + \text{NO}_3^-$ in the serum and pleural effusion of the tumour-bearing mice after one round of therapies (data not presented). However, there was a minor reduction in IL-2-induced pulmonary oedema caused by addition of D-NAME. D-NAME alone (1 mg ml^{-1}) had no effect on the growth of the primary tumour (data not shown).

Discussion

The results of the present study showed that oral administration of L-NAME, a potent inhibitor of NO synthesis, reduced the severity of IL-2-induced capillary leakage in some organs of tumour-bearing mice, without compromising the therapeutic benefits of IL-2. Furthermore, L-NAME therapy alone had significant anti-tumour effects.

We demonstrated that IL-2-induced pulmonary oedema, water retention in the spleen and rise in nitrite + nitrate levels (in the serum and pleural effusion) after the first round of therapy were all significantly reduced with addition of L-NAME. However, this addition did not affect the level of IL-2-induced pleural effusion or water retention in the kidney after one round of therapy. These results in tumour-transplanted mice are in general accord with those previously reported by us in healthy mice (Orucevic and Lala, 1995a). In both of these studies, two lines of evidence excluded the possibility that the therapeutic effects of L-NAME in reducing the water content of certain organs in IL-2-treated mice were due to L-NAME-induced reduction in water consumption (antidypsogenic effect): (1) we noted that higher doses of L-NAME (eg. 1 mg ml^{-1}), as well as the present dose of IL-2 led to a significant reduction in water consumption (up to 60% of pretreatment values at the end of the first round of therapy). However, the water consumption in IL-2-treated mice remained unaffected by the addition of L-NAME; (2) In spite of antidypsogenic effects, L-NAME therapy alone did not influence the water content of any organ in healthy mice. Furthermore, L-NAME is well recognised for its vasoconstrictive effects in healthy animals (Gross *et al.*, 1990). Since this effect alone did not influence

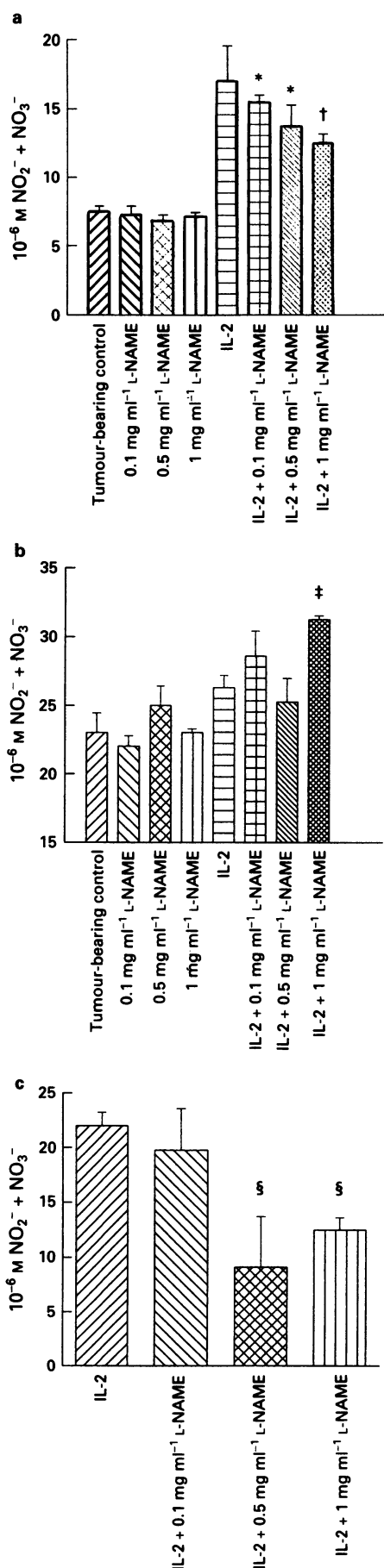


Figure 4 NO₃⁻ levels in the serum (a) after one round of therapy (b) after two rounds of therapy; and (c) pleural effusion after one round of therapy, measured as NO₂⁻ after reduction. Data represent mean ± s.e. (n=3-5, each done in duplicate). *Dose-dependent trend in reduction of NO₃⁻ levels in the serum with addition of different doses of L-NAME to IL-2 after the first round of therapy. †Only the dose of 1 mg ml⁻¹ L-NAME caused

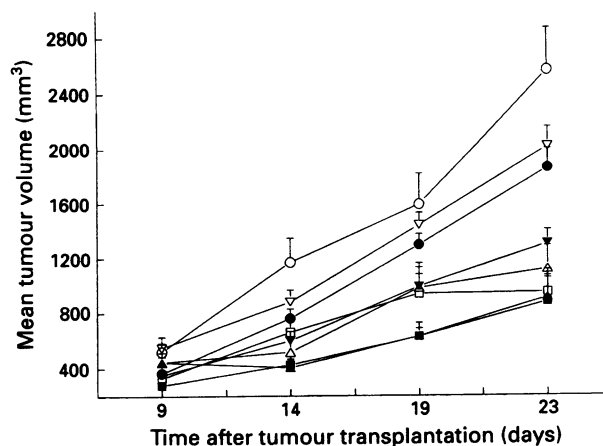


Figure 5 Growth of the primary tumour during IL-2 and L-NAME therapy, as given by the mean tumour volume on days 9, 14, 19 and 23. Data represent mean ± s.e. (days 9 and 14: n=10-20; day 19 and day 23: n=9-12). After the first (day 14) or the second round (day 23) of therapies, IL-2 alone, or in combination with any dose of L-NAME, or L-NAME alone significantly (P<0.05) reduced the growth of the primary tumour. ○, Tumour control; ●, 0.1 mg ml⁻¹ L-NAME; ▽, 0.5 mg ml⁻¹ L-NAME; ▼, 1 mg ml⁻¹ L-NAME; □, IL-2; ■, IL-2 + 0.1 mg ml⁻¹ L-NAME; △, IL-2 + 0.5 mg ml⁻¹ L-NAME; ▲, IL-2 + 1 mg ml⁻¹ L-NAME.

the water content of any organ in healthy mice treated with L-NAME alone, the observed L-NAME-induced reduction of water content of the lungs and the spleen in IL-2-treated mice cannot be explained solely on the basis of generalised or selective vasoconstriction of normal vascular beds.

We suggest that the beneficial effects of L-NAME against IL-2-induced capillary leakage resulted from an abrogation of IL-2-induced NO overproduction (measured in the serum and pleural fluid) documented here, as well as in healthy tumour-free mice (Orucevic and Lala, 1995a). This abrogation occurred in spite of reported higher selectivity of L-NAME for binding and inactivation of the constitutive isoforms of NO synthase (NOS) enzymes in comparison with the inducible isoform (i-NOS) (Gross *et al.*, 1990; Dwyer *et al.*, 1991; Furfine *et al.*, 1993). For these reasons, further investigation is needed to identify the cellular source of NO as well as the NOS isoform(s) responsible for increased NO production after IL-2 therapy. This information may also indicate the relative contribution of haemodynamic (owing to eNOS) and other effects (owing to iNOS) of NO towards IL-2-induced capillary leakage.

At later stages of tumour growth (day 23), tumours themselves induced significant fluid retention in the lungs and the kidney, and the former was reduced with L-NAME therapy alone. It is likely that the increased fluid retention in the lungs was a direct result of pulmonary metastases due to either an increased leakiness of the capillaries supplying the metastatic foci, or an increased leakiness of the lung vasculature in the presence of metastasis. Thus a reduction in tumour-induced pulmonary oedema can be explained by the anti-metastatic effects of L-NAME (documented here) and/or a selective reduction in tumour blood flow (Andrade *et al.*, 1992) within the metastatic foci. IL-2 therapy alone, in spite of strong anti-metastatic effects, failed to reduce the

significant (P<0.05) abrogation of IL-2-induced rise in NO₃⁻ levels in the serum after the first round of therapies. ‡Addition of 1 mg ml⁻¹ L-NAME to IL-2 significantly (P<0.05) increased NO₃⁻ levels in the serum after the second round of therapies. § IL-2-induced NO production in pleural effusion after the first round of therapy was significantly (P<0.05) reduced with addition of L-NAME.

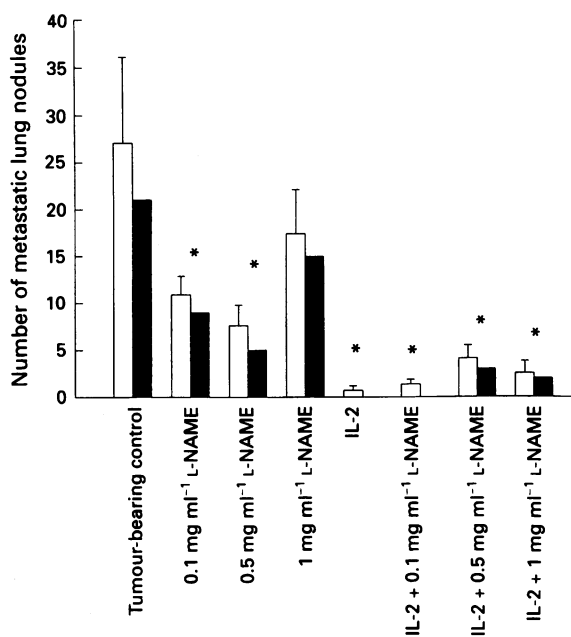


Figure 6 Number of metastatic nodules in the lungs scored on day 23 after two rounds of IL-2 and L-NAME therapy ($n=9-12$). *Significant reduction ($P<0.05$) was noted with all therapies except with 1 mg ml^{-1} L-NAME. □, mean \pm s.e.; ■, median.

water content of the lungs, probably because of additional IL-2-induced pulmonary oedema.

A second round of IL-2 therapy caused a milder degree of capillary leakage (fluid retention in the spleen, but not the lungs, kidney or the pleural cavities), which was not ameliorated by addition of L-NAME. In parallel, there was no significant change in NO production in most groups receiving IL-2 or the combination therapy. While this association may reinforce the role of NO in IL-2-induced capillary leakage, the reasons for a resistance of healthy mice (Orucevic and Lala, 1995b) as well as tumour-bearing mice (this study) to the induction of pleural effusion by the second round of IL-2 remains unclear. Identification and cellular localisation of NOS isoforms in the treated mice may resolve this issue. The expression of iNOS is known to vary from tissue to tissue and within a given tissue under different circumstances (Nussler and Billiar, 1993). Thus, it is possible that our second round of IL-2 did not induce iNOS in the pleural vessels, which consequently remained resistant to leakage. The so called 'tolerance' to IL-2 toxicity after the initial round reported by our laboratory in the human (Mertens *et al.*, 1993) and a milder fall in blood pressure after the second course of IL-2 reported by another laboratory (Hibbs *et al.*, 1992) might be similarly explained.

We found that anti-tumour effects of IL-2 were not compromised with addition of L-NAME and that L-NAME therapy alone had anti-tumour effects. These findings were similar to our previous observations with another NOS inhibitor, NMMA (Orucevic and Lala, 1993). The observed anti-tumour effects of NOS inhibitors in our tumour model are in accord with those documented in a rat model (Kennovin *et al.*, 1993, 1994).

Mechanisms responsible for the anti-tumour effects of NOS inhibitors in the present as well as other studies remain to be fully investigated. They may include a reduction in the tumour blood flow, tumour angiogenesis, tumour invasiveness and an abrogation of NO-induced immunosuppression in the host. Evidence exists for some of the above. Kennovin *et al.* (1993, 1994) observed that the anti-tumour effects were not sustained after withdrawal of chronic L-NAME therapy of tumour-bearing rats. For this and other reasons, they postulated that the therapeutic effects were probably due to a reduction in tumour blood flow, caused by a selective constriction of the tumour vasculature owing to an

inhibition of iNOS activity. Indeed, NO production by experiment tumours in mice has been implicated in maintaining tumour blood flow in the neovasculature (Buttery *et al.*, 1993), and its blockade with L-NAME has been shown to selectively reduce flow in tumour-associated neovasculature (Andrade *et al.*, 1992) and to cause a severe tumour hypoxia (Wood *et al.*, 1993).

There is a growing number of reports on the opposing roles of NO on the immune system that may influence tumour growth. While NO may be required for tumoricidal function of certain effector cells, excessive NO production can also suppress lymphocyte activation. It has been shown that activated murine macrophages synthesise NO from L-arginine (Stuehr and Marletta, 1985), which may partly mediate the cytotoxic activity of these cells against tumour cells and bacteria (Lancaster and Hibbs, 1990). Mills *et al.* (1992) reported that tumour growth in the peritoneal cavity of mice was associated with a marked decline in the production of NO by intra-tumour macrophages. On the other hand, murine macrophages have been shown to down-regulate lymphocyte activation by an NO-dependent mechanism (Hoffman *et al.*, 1990; Albina *et al.*, 1991), which may compromise the tumoricidal function of lymphocytes. Indeed, NO has been implicated in the tumour-induced immunosuppression in rats (Lejeune *et al.*, 1994) and high NO synthase activity has been correlated with the degree of malignancy (Thomsen *et al.*, 1994).

In a preliminary study (A Orucevic and PK Lala, unpublished), we found an improvement in IL-2-induced LAK cell function *in vivo* with addition of L-NAME in tumour-bearing mice after the first round of therapy. This may explain our findings of a transient improvement of IL-2-induced retardation of tumour growth by addition of L-NAME after the first round of therapy (present study). Although L-NAME alone did not significantly change serum levels of nitrite+nitrate, we demonstrated that L-NAME-mediated reduction of tumour size was related to NO pathway, since treatment of mice with D-NAME, an amino acid incapable of inhibition of NO generation (Ialenti *et al.*, 1993), failed to influence tumour growth rate (data not shown). We are currently investigating the identity of the NOS isoforms and the cellular source of NO in our tumour model.

In summary, L-NAME therapy had an anti-tumour effect when given alone. In combination with IL-2, L-NAME reduced the severity of IL-2-induced capillary leakage in some organs after the first round of therapy and did not compromise anti-tumour effects of IL-2 therapy. Thus, L-NAME could be a valuable adjunct to IL-2-based immunotherapy of cancer.

Abbreviations

IL-2, interleukin 2; LAK, lymphokine-activated killer; NK, natural killer; IFN, interferon; TNF, tumour necrosis factor; NO, nitric oxide; NMMA, N^G -methyl-L-arginine; L-NAME, N^G -nitro-L-arginine methyl ester; NO_2^- , nitrite; NO_3^- , nitrate; U, Cetus units; NOS, nitric oxide synthase.

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