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Amila Orucevic

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**Role of Nitric Oxide in Interleukin-2 Therapy
Induced Capillary Leak Syndrome**

by

Amila Orucevic

Department of Anatomy and Cell Biology

**Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy**

**Faculty of Graduate Studies
The University of Western Ontario**

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ABSTRACT

During the recent years, systemic interleukin-2 (IL-2) therapy has shown significant promise in certain types of cancer, but its practice has been limited by a major side effect known as "capillary leak syndrome", characterized by retention of extravascular fluid, severe hypotension, and multiple organ system dysfunction. The initial part of the present study tested the hypothesis that an overproduction of nitric oxide (NO) induced by IL-2 therapy was, at least in part, responsible for this syndrome. Studies were undertaken in healthy or C3-L5 mammary adenocarcinoma bearing C3H/HeJ mice to evaluate whether treatment with inhibitors of NO synthesis (N^G -Methyl-L-Arginine, NMMA and N^G -Nitro-L-Arginine methyl ester, L-NAME) can prevent IL-2 induced capillary leakage without compromising the therapeutic benefit of IL-2. Results revealed that intraperitoneal IL-2 therapy in both groups of mice caused substantial capillary leakage (pleural effusion, pulmonary edema, fluid retention in the kidney and the spleen), and a substantial rise in NO production *in vivo*. Addition of subcutaneous NMMA therapy in either mouse group, failed to ameliorate IL-2 induced capillary leakage, or NO overproduction, and did not compromise antitumor effects of IL-2. Subcutaneous NMMA therapy alone reduced primary tumor growth, spontaneous pulmonary metastasis and tumor-induced pulmonary edema. Chronic administration of NMMA by the oral route, however, succeeded in abrogating IL-2 induced NO overproduction as well as capillary leakage in healthy mice. Oral NMMA therapy was not tested in tumor-bearing mice, since L-NAME, a more potent and inexpensive inhibitor of NO was soon available.

L-NAME given chronically in the drinking water led to a parallel reduction in the

IL-2 therapy-induced NO overproduction and capillary leakage in healthy mice. Simultaneous morphological, immunohistochemical and biochemical analyses revealed that IL-2 therapy (1) caused structural damage to the lungs and its capillaries; (2) induced inducible type NO synthase (iNOS) expression in numerous tissues, including the endothelium, muscles of the anterior thoracic wall and splenic macrophages; (3) induced a high NOS activity in the lungs and the anterior thoracic wall of animals exhibiting pulmonary edema and pleural effusion. Addition of L-NAME therapy totally abolished NOS activity, but not necessarily iNOS expression, and significantly restored structural integrity of the lungs in conjunction with an amelioration of pulmonary edema and pleural effusion. Taken together, these results strongly suggested that local NO overproduction, resulting from an induction of iNOS enzyme by IL-2 therapy, was responsible for the structural damage and consequent fluid leakage from the capillaries.

In mammary adenocarcinoma bearing mice, oral L-NAME alone produced significant antitumor as well as antimetastatic effects, as was also shown with NMMA therapy. L-NAME in combination with IL-2 therapy in these mice succeeded in ameliorating IL-2 induced as well as tumor-induced capillary leakage and potentiated tumor reductive function of IL-2. An abrogation of IL-2 induced NO production by L-NAME treatment *in vivo* as well as *in vitro* markedly stimulated IL-2 induced generation of antitumor cytotoxicity of splenocytes of healthy as well as tumor-bearing mice. These results revealed that IL-2 induced increase in NO production interfered with optimal lymphokine activated killer (LAK) cell activation, which can be overcome by NO inhibition with L-NAME therapy.

The observed antitumor and antimetastatic effects of two NO inhibitors, NMMA

and L-NAME suggested that NO promoted tumor progression in the C3-L5 mammary tumor model. Tumor cells as well as endothelium of the tumor vasculature were identified as the source of NO within the tumor. Immunoreactive endothelial type NOS (eNOS) was detected in both cell classes within the primary as well as metastatic tumors. Furthermore, C3-L5 cells *in vitro* were positive for eNOS and expressed iNOS when incubated with LPS+IFN γ . At least one mechanism of NO-mediated promotion of metastasis was identified in this tumor model. Treatment with NO inhibitors; NMMA or L-NAME, reduced *in vitro* invasive ability of C3-L5 tumor cells, which was restored by addition of excess L-arginine. Treatment with IFN γ +LPS stimulated NO production by these cells and also stimulated their invasiveness. Thus, NO production by the tumor cells promoted tumor cell invasiveness.

Above results provided the first direct evidence that NO is instrumental in IL-2-induced capillary leakage and that a NO blocking agent such as L-NAME can mitigate this leakage without interfering with the beneficial antitumor effects of IL-2 therapy. NO blocking agent can, in fact, improve IL-2 induced antitumor effector cell activation, as well as tumor regression. NO blocking agents alone can reduce mouse mammary tumor growth and metastasis, at least in part, by mitigating the invasion-stimulating role of tumor-derived NO. Thus, NO blocking agents may have a place in tumor therapy and could be a valuable adjunct to IL-2 based immunotherapy of cancer and infectious diseases.

KEYWORDS

Interleukin-2, capillary leak syndrome, nitric oxide, nitric oxide synthase inhibitors, murine mammary adenocarcinoma

To my parents, Jasminka and Burhan Lomigoric, with love.

Thank you for your endless love, support and being always there for me.

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LIST OF ABBREVIATIONS

ABC - avidin biotin complex

ATP - adenosine triphosphate

BH₄ - tetrahydrobiopterin

BSA - bovine serum albumin

°C - degrees Celsius

Ca⁺⁺ - calcium

CO₂ - carbon dioxide

cGMP - cyclic guanosine monophosphate

⁵¹Cr - ⁵¹Chromium

d - day

DAB - diamino benzidine

dpm - disintegration per minute

DTT - DL-dithiothreitol

eNOS - endothelial nitric oxide synthase

EDTA - ethylenediamine tetracetic acid

EGTA - ethylenebis(oxyethylenitrilo)tetracetic acid

FITC - fluorescein isothiocyanate

g - gram

H₂O₂ - hydrogen peroxide

h - hour

[³H]Tdr - tritiated thymidine

IL - interleukin

IFN - interferon

iNOS - inducible nitric oxide synthase

i.p. - intraperitoneal

kg - kilogram

LAK - lymphokine-activated killer

L-NAME - N^G-Nitro-L-Arginine methyl ester

LPS - lipopolysaccharide

M - Molar

mCi - millicuries

mg - milligram

min - minute

ml - millilitre

mRNA - messenger ribonucleic acid

N - normal

NADPH - nicotinamide adenine dinucleotide phosphate, reduced form

NK - natural killer

NMMA - N^G-Methyl-L-Arginine

nNOS - neuronal nitric oxide synthase

NO - nitric oxide

NO₂⁻ - nitrite

NO₃⁻ - nitrate

NOS - nitric oxide synthase

PBS - phosphate buffered saline

s.c. - subcutaneous

TNF - tumor necrosis factor

U - Cetus Units

v/v - volume/volume

w/d - wet/dry

% - percentage

μg - microgram

μm - micrometer

μM - micro Molar

α - alpha

β - beta

τ - gamma

CHAPTER 1 - INTRODUCTION

Interleukin-2 (IL-2) is a very important lymphokine, produced primarily by the helper T cells in the body, and required for the functional activation of virtually all subsets of lymphocytes.

During the last decade, IL-2 therapy in various forms became a major focus of interest in cancer immunotherapy, because of IL-2 dependence of all antitumor effector cell lineages i.e. T cells (Robb *et al.* 1981), natural killer (NK) cells (Aribia *et al.* 1989) and macrophages (Holter *et al.* 1986; Parhar and Lala, 1988). High dose IL-2 therapy, initially tested as promising in the mouse model (Rosenberg *et al.* 1985) was applied to human trials either alone or in combination with ex-vivo generated lymphokine activated killer (LAK) cells (Rosenberg, 1989; Fisher *et al.* 1988; Dutcher *et al.* 1989; Parkinson *et al.* 1990; Bar *et al.* 1990) or other immunomodulators (Mertens *et al.* 1993; Mertens *et al.* 1993; Mertens *et al.* 1992) with modest and variable success in renal cell carcinomas and melanomas. Wide spread clinical use of IL-2 based therapy, has been limited by a major side effect known as "capillary leak syndrome". This syndrome is characterized by retention of extravascular fluid, severe hypotension, and multiple organ system dysfunction (Siegel and Puri, 1991; Oppenheim and Lotze, 1994), often requiring cessation of IL-2 therapy. Capillary leak syndrome has been documented in numerous species: humans (Siegel and Puri, 1991; Lotze *et al.* 1985; Rosenberg *et al.* 1985; Oppenheim and Lotze, 1994), mice (Ettinghausen *et al.* 1988; Rosenstein *et al.* 1986), sheep (Harms *et al.* 1989; Klausner *et al.* 1989; Jesmok and Gunther, 1989) and rats (Edwards *et al.* 1992). However, at this time, pathophysiological mechanisms underlying this syndrome remain poorly understood.

Possible mechanisms include a damage to endothelial cells by LAK cells (Kotasek *et al.* 1988; Amador *et al.* 1991) or by NK cells (Aronson *et al.* 1988) or certain IL-2 induced cytokines e.g. interferon (IFN) γ (Montesano *et al.* 1985) and tumor necrosis factor (TNF) α (Kahaleh *et al.* 1988). Injury to endothelial cells mediated by these two cytokines (Palmer *et al.* 1992; Estrada *et al.* 1992) has been recently linked with nitric oxide (NO) production, because it was prevented with dexamethasone and certain inhibitors of nitric oxide synthase (NOS) enzymes. Since IL-2 therapy induces production of LAK cells (Rosenberg *et al.* 1985; Rosenberg *et al.* 1987), IFN γ (Lotze *et al.* 1986) and TNF α (Lotze *et al.* 1985; Mier *et al.* 1988), it is reasonable to suggest that the observed increase in capillary permeability during IL-2 therapy may result from IL-2 induced cell-mediated injury to endothelial cells and/or cytokine-mediated effects on endothelial cells. It may also result from hemodynamic changes during IL-2 treatment. Severe hypotension observed during IL-2 therapy has also been recently attributed to NO production from L-arginine (Hibbs *et al.* 1992; Ochoa *et al.* 1992). Since NO is a potent vasodilator (Palmer *et al.* 1987), vasodilation and systemic hypotension due to NO production can indirectly cause pulmonary hypertension and thus increase pulmonary capillary pressure (Siegel and Puri, 1991) leading to fluid leakage in the lungs. Thus, NO may have a major role in the pathogenesis of IL-2 induced capillary leakage.

Several investigators have combined IL-2 with other agents in order to ameliorate the capillary leakage in rodents. However, the added drugs also countered the beneficial antitumor effects of IL-2. Corticosteroids (Rosenstein *et al.* 1986; Faggioni *et al.* 1994), which suppress inflammatory responses and induction of NO (Moncada and Higgs, 1993), and Asialo-GM-1 antibody, which depletes LAK cells in the mouse (Ettinghausen

et al. 1988), both fall in 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 edema in the rat, presumably by causing cytoskeletal changes in neutrophils, with consequent suppression of endothelial injury by neutrophil derived thromboxane B₂. Interactions of these agents with the antitumor effect of IL-2 remain unknown. Further studies were therefore required to identify substances that can ameliorate capillary leakage without reducing the antitumor effects of IL-2.

On the basis of the indirect evidence summarized earlier (Hibbs *et al.* 1992; Ochoa *et al.* 1992), it was hypothesized in the present study that overproduction of NO may underlie the pathogenesis of IL-2 induced capillary leak syndrome. This hypothesis was tested in normal as well as mammary adenocarcinoma bearing mice subjected to IL-2 therapy. The initial series of studies were designed to examine whether inhibitors of NO synthesis, N^o-Methyl-L-Arginine (NMMA) and N^o-Nitro-L-Arginine methyl ester (L-NAME) could prevent IL-2 induced capillary leakage (measured as fluid retention in the lungs, pleural cavities, kidneys and spleen) in both murine models without compromising the therapeutic benefit of IL-2; and whether these agents alone affected tumor growth or metastasis. Since NO inhibition *in vivo* reduced IL-2 induced capillary leakage and augmented antitumor effects of IL-2 therapy, further studies were undertaken to elucidate the mechanisms by which NO overproduction during IL-2 therapy led to the capillary leakage and compromised the antitumor effects of IL-2 therapy. Since NO inhibitors alone had antitumor as well as antimetastatic effects, studies were undertaken to investigate whether such effects could, at least in part, be explained by invasion

promoting role of NO.

CHAPTER 2 - HISTORICAL REVIEW, HYPOTHESIS AND OBJECTIVES

2.1 Nitric oxide

2.1.1 Origin and functions

Furchgott and Zawadski (1980) were the first to describe a potent vasodilator synthesized by endothelial cells and named it endothelium derived relaxing factor (EDRF). The real nature of this factor remained unknown until Palmer *et al.* (1987) reported that nitric oxide released from endothelial cells accounted for the biological activity of EDRF. Later, Palmer *et al.* (1988) found that amino acid L-arginine was the substrate from which vascular endothelial cells synthesized nitric oxide.

Nitric oxide is synthesized by many mammalian cells with the help of a family of enzymes named NO synthases (NOS)(Knowles and Moncada, 1994). Three NOS isoforms have been isolated and characterized so far, which vary in their amino acid sequence, subcellular location, regulation and functional roles (Knowles and Moncada, 1994; Morris and Billiar, 1994). In common, all three isoforms share following characteristics: they are homodimers; they use L-arginine as a substrate and NADPH, flavin adenine mono and dinucleotides, tetrahydrobiopterin and protoporphyrin IX heme as cofactors. However, only two of these enzymes are typically constitutively (always) expressed and were originally identified in endothelial cells (eNOS, or type 3 NOS) (Michel *et al.* 1993) and central and peripheral neurons (nNOS, or type 1 NOS) (Bredt *et al.* 1991). These enzymes have subsequently been identified in numerous other cells in the body. For example, nNOS is expressed in extraneuronal sites such as the kidney, the pancreas and skeletal muscles (Nakane *et al.* 1993; Nathan, 1992), and eNOS is found in endocardium, epithelium of the bronchial tree (Snyder, 1995) and even in some

neurons [pyramidal cells of the hippocampus (Snyder, 1995)]. Small amounts of basal NO are generated by these two types of enzymes, the activation of which is dependent on elevations in intracellular Ca^{++} , and is short lived. NO generated in this manner plays an active role in many physiological processes, including vasodilation and neurotransmission (Ignarro *et al.* 1987; Moncada *et al.* 1989). In contrast to the constitutive forms of NOS, the third isoform, the inducible NOS (iNOS, or type 2 NOS) is not typically expressed unless the cells have been exposed to certain microbes, microbial products or cytokines (Nathan and Hibbs, 1991; Nussler and Billiar, 1993). After induction, enzyme activation is long lived, independent of elevations in intracellular Ca^{++} , generating high levels of NO. NO produced in this manner can play roles in either cytostatic/cytotoxic or cytoprotective processes in mammalian tissues (Morris and Billiar, 1994). Examples of cells capable of iNOS expression, when appropriately stimulated, include macrophages, hepatocytes, endothelial cells, kidney cells, vascular smooth muscle cells, chondrocytes, cardiac myocytes and fibroblasts (Nathan and Hibbs, 1991; Billiar, 1995; Morris and Billiar, 1994).

2.1.2 Nitric oxide targets

NO is a small lipophilic molecule with one unpaired electron (Ignarro, 1991; Feldman *et al.* 1993), which freely diffuses through cell membranes in search for another molecule to accept or share this odd electron (Feldman *et al.* 1993). Oxygen, thiol groups, metals or other radicals are all potential target molecules for NO, so that in an environment abundant in these targets, NO has a short half life of a few seconds. Interaction of NO with oxygen results in the oxidation of NO to nitrite and nitrate and

inactivation of NO (Feldman *et al.* 1993). However, interaction of NO with thiols, other radicals or metals (eg. iron) is a mechanism by which NO is thought to exert its effects as cytotoxic or cytoprotective molecule in target cells (Feldman *et al.* 1993; Billiar, 1995). For example, interaction of NO with prosthetic iron groups or thiol groups in proteins can form complexes that activate or inactivate target enzymes (Billiar, 1995). Vascular smooth muscle relaxation (Ignarro *et al.* 1987), inhibition of platelet aggregation (Sinzinger *et al.* 1992), modulation of neurotransmission (Moncada *et al.* 1989) and other brain functions (Chapman *et al.* 1992; Venturini *et al.* 1991; Morley and Flood, 1991) are all mediated through the activation of soluble guanylate cyclase by NO. The activated soluble guanylate cyclase brings about an increase in cytoplasmic cGMP level (Craven and DeRubertis, 1978), which acts as a transduction signal in target cells and triggers the cellular responses within a localized environment (Ignarro, 1991). Antimicrobial and antitumor cytotoxicity of activated macrophages (Moncada and Higgs, 1993; Nathan and Hibbs, 1991; Moncada, 1992) have, at least in part, been linked to NO production and appears to be mediated through the binding of NO with iron-containing moieties present in key enzymes of DNA synthesis (Lepoivre *et al.* 1992; Nguyen *et al.* 1992) and of the respiratory cycle (Ignarro, 1991; Hibbs *et al.* 1987) in the target cells. Finally, a number of studies suggest that NO released at sites of inflammation can exert genotoxic effects (and thus have a carcinogenic role) due to the binding of NO or its reaction products (e.g. peroxynitrite) to the DNA molecule (Nguyen *et al.* 1992; Wink *et al.* 1991; Arroyo *et al.* 1992) or to an inhibition of a DNA repair enzyme Fpg (Wink and Laval, 1994).

2.1.3 Inhibitors of nitric oxide synthesis

L-arginine analogs block nitric oxide synthesis by substrate competition for the enzyme (Hibbs *et al.* 1987; Sacuma *et al.* 1988; Palmer *et al.* 1988; Kilbourn and Belloni, 1990; Kilbourn *et al.* 1990; Kilbourn *et al.* 1990; Billiar *et al.* 1990).

N^o-Methyl-L-Arginine (NMMA) has been used as a nitric oxide synthesis inhibitor in numerous studies *in vitro* (Palmer *et al.* 1988; Sacuma *et al.* 1988; Kilbourn and Belloni, 1990) and *in vivo* (Billiar *et al.* 1990; Kilbourn *et al.* 1990; Kilbourn *et al.* 1990). NMMA inhibited endothelium-dependent relaxation (Sacuma *et al.* 1988), raised the systemic blood pressure (Kilbourn *et al.* 1990; Kilbourn *et al.* 1990), and inhibited endothelium-dependent and acetylcholine-elicited cGMP formation in smooth muscle cells. These effects of NMMA provided confirmation of the role of endothelium-derived nitric oxide in regulating vascular tone (Fukito *et al.* 1990), and suggested that nitric oxide overproduction may be causal to endotoxin-mediated septic shock (Kilbourn *et al.* 1990). The role of nitric oxide in endotoxin-mediated septic shock still remains controversial. It appears that NMMA was able to protect animals from severe hypotension associated with experimental gram negative sepsis (Evans *et al.* 1994), by inhibiting NO production, but was unable to prevent death in these mice. In addition, Billiar *et al.* (1990) reported that the same inhibitor promoted hepatic damage in a murine model of endotoxic shock. These authors implied a protective role for nitric oxide in endotoxin-induced hepatic damage in mice. Nava *et al.* (Nava *et al.* 1992) have suggested that the degree of inhibition of NO synthesis is the critical factor determining whether NMMA has beneficial or adverse effects in the outcome of endotoxic shock.

Searching for new analogs of L-arginine, potent enough to inhibit endothelium

dependent relaxation, Fukito *et al.* (1990) described N^G-Amino-L-arginine, a nitric oxide synthesis inhibitor, 100 to 300-fold more potent than NMMA. This inhibitor could produce pharmacological responses not obtainable with NMMA (Fukito *et al.* 1990).

N^O-Nitro-L-Arginine (L-NNA) or its methyl ester (L-NAME) and N-iminoethyl-L-ornithine (L-NIO) can all act as competitive inhibitors of NO synthase (Rees *et al.* 1990; Joly *et al.* 1994; Gardiner *et al.* 1990; Gardiner *et al.* 1992).

Glucocorticoids do not act as competitive inhibitors of NOS, but rather inhibit the expression of the inducible enzyme. However, they can not block the activity of the inducible enzyme, once expressed (Moncada, 1992). They also do not act on the activity or expression of constitutive NOS enzyme (Moncada, 1992).

2.1.4 Nitric oxide in tumor biology

2.1.4.1 NO and tumor growth: NO levels have been reported to rise in the serum of cancer patients (Miles *et al.* 1994). A number of reports indicate a contributory role of NO to tumor progression. An abundant expression of NO synthase (Thomsen *et al.* 1994; Cobbs *et al.* 1995), as well as NOS activity (Thomsen *et al.* 1994) has been positively correlated with the degree of malignancy. The high NOS activity can be explained by the presence of constitutive form (eNOS) in both tumor cells (Thomsen *et al.* 1994; Cobbs *et al.* 1995) and tumor endothelial cells (Cobbs *et al.* 1995), and the inducible form (iNOS) in the tumor neovasculature (Buttery *et al.* 1993). Treatment with an inhibitor of NO synthesis (L-NAME) was reported to cause a reduction in NO production as well as regression of transplanted colon adenocarcinoma in the rat (Kennovin *et al.* 1993). The mechanism(s) by which NO may promote tumor growth remain undefined. The

possibilities are (a) a direct effect on tumor cell proliferation and/or invasiveness; (b) a promotion of tumor angiogenesis and/or blood flow in the tumor neovasculature; (c) a suppression of the host antitumor defence. Of these, some evidence exist on the last two possibilities.

2.1.4.2 Role of NO in angiogenesis and tumor blood flow: Regulatory roles of NO in angiogenesis remain highly controversial. Pipili-Synetos *et al.* (1994) suggested that NO is an endogenous inhibitor of angiogenesis. Using an angiogenesis assay which scores the number of blood vessels in a defined area of chick chorioallantoic membrane (CAM assay), as well as tube formation by endothelial cells on matrigel (artificially reconstituted basement membrane) they found that sodium nitroprusside (an NO-donor) reduced, and NO synthase inhibitors promoted basal angiogenesis. In contrast to these findings, Konturek *et al.* (1993) showed that inhibition of NO synthesis delayed healing of chronic gastric ulcers induced by acetic acid, by decreasing local blood flow as well as reducing angiogenesis at the ulcer edge. Similarly, using both an *in vivo* angiogenesis assay with rabbit cornea and an *in vitro* assay which measures the growth and migration of capillary endothelial cells, Ziche *et al.* (1994) showed that vasoactive substances such as substance P, or PGE₁ stimulated angiogenesis in an NO dependent manner, since it was blocked with NOS inhibitors NMMA, L-NNA and L-NAME; NO donors such as Na-nitroprusside and glycerol trinitrate also stimulated endothelial cell migration. Buttery *et al.* (1993) detected inducible NO synthase (iNOS) in the endothelial cells of neovasculature of experimental tumors and suggested that NO production was important both for an expansion of angiogenesis, as well as maintenance of tumor blood flow. It remains to be tested whether tumor-induced neo-angiogenesis is NO-dependent.

2.1.4.3 Nitric oxide in host immune responses: A number of reports suggest opposing roles of NO in tumor immunity. First, it has been shown that activated murine macrophages synthesize NO (Stuehr and Marletta, 1985), which may partly mediate their cytotoxic activity against tumor cells and bacteria (Nathan and Hibbs, 1991; Moncada, 1992; Lancaster and Hibbs, 1990). Mills *et al.* (Mills *et al.* 1992) reported that tumor growth in the mouse peritoneal cavity was associated with a decreased NO production by intratumor macrophages. It was not shown, however, whether this reduced the antitumor cytotoxicity of these macrophages. Similarly, it has been reported that *in vitro* tumoricidal function of IL-2 activated NK cells depend, at least in part, on their NO synthesizing ability, since deprivation of L-arginine in the medium or blocking of NO synthesis with NMMA reduced their killer function (Cifone *et al.* 1994). On the other hand, NO overproduction by rodent macrophages has been shown to down-regulate activation of T lymphocytes in response to antigen or mitogen (Hoffman *et al.* 1990; Albina *et al.* 1991), and thus may hinder antitumor immune responses of T cells. Indeed, excessive NO production has been implicated in tumor-induced immunosuppression in rats (Lejeune *et al.* 1994). In summary, NO appears to be an important bioactive component of the cytotoxic pathways of antitumor effector cells, however, excessive NO release in the microenvironment may be detrimental to their activation pathways.

2.1.5 NO induction during inflammation and infection

Pathological effects of excessive NO production induced by bacterial endotoxins and inflammation-associated cytokines have been well documented. Addition of endotoxin, interferon- γ (Palmer *et al.* 1992) and TNF α (Estrada *et al.* 1992) have been

shown to damage endothelial cells *in vitro* through the induction of NO synthase. Murine lung epithelial cells (Robbins *et al.* 1994) and fibroblasts (Willis *et al.* 1994) produce high levels of NO when cultured in the presence of IL-1, TNF α or IFN γ , and it is believed that NO contributes to inflammation-associated lung injury in the rat after immune complex deposition (Mulligan *et al.* 1991). High NO production followed by vasodilatation and hypotension has been implicated in the pathogenesis of septic and endotoxic shock (Petros *et al.* 1991; Kilbourn *et al.* 1990; Wright *et al.* 1992). NO has also been implicated in adjuvant induced arthritis (Ialenti *et al.* 1993) and immune complex-induced vasculitis (Mulligan *et al.* 1992). Protective effects of NO inhibitors (L-arginine analogues and/or glucocorticoids) against tissue injury have been reported in all of the above cases (Petros *et al.* 1991; Kilbourn *et al.* 1990; Palmer *et al.* 1992; Estrada *et al.* 1992; Robbins *et al.* 1994; Willis *et al.* 1994; Mulligan *et al.* 1991; Wright *et al.* 1992; Ialenti *et al.* 1993; Mulligan *et al.* 1992).

2.2 The lungs and the pleura: human vs. mouse

2.2.1 Anatomy of the lungs and the pleura

In contrast to the human lungs displaying three lobes on the right (superior, middle and inferior), and two lobes on the left (superior and inferior) (Gray, 1977), the murine right lung is divided into four lobes including lobus postcavalis, which has no human counterpart, and the left lung has only one lobe (Theiler, 1972).

Lung morphology in higher mammals has been broadly classified by McLaughlin *et al.* (1966) into three subtypes depending on the presence of interlobular septa dividing lung lobes into secondary lobules. Type I morphology is observed in sheep and pigs with

well-defined secondary lobules, type II is observed in the mouse having no secondary lobules, and type III morphology is noted in humans, showing secondary lobules only in peripheral but not in deeper parts of the lungs.

The lungs of all mammals are composed of an external serous membrane (visceral pleura), a subserous elastic layer, and pulmonary parenchyma. McLaughlin's subtypes of lung morphology are also used to characterize the visceral pleura in small and large mammals. Visceral pleura is thick in types I and III lungs, and thin in type II. In the human lung the different constitutive layers of the visceral pleura are mesothelium, submesothelial connective tissue, an external elastic layer with elastic fibres, a connective tissue layer with blood and lymph vessels and an internal elastic layer (Naigaishi, 1972). The mouse has a thin visceral pleura, and there is no internal elastic layer (Pinchon *et al.* 1980).

The parietal pleura (the serosal membrane underlining the thoracic wall) is a fibroelastic membrane. Its internal surface is covered by mesothelial cells, beneath which are, depending on the species, thicker or thinner connective tissue layers supplied with blood from the systemic circulation (Bernaudin and Fleury, 1985). In man there is a submesothelial connective tissue layer, two layers of fibroelastic tissue separated by connective tissue and a loose connective tissue layer beneath the second fibroelastic layer (Policard and Galy, 1942). Pinchor. *et al.* (1980) described a thick connective tissue layer separating the mesothelium from the muscle of the thoracic wall in the rat parietal pleura.

The pleural cavity is a potential space between the parietal and the visceral pleura, containing a very thin layer of fluid serving as a lubricant between the two layers. The turnover of this fluid as well as the lung interstitial fluid is tightly regulated by the blood

and lymphatic circulation of the pleura and the lungs under normal physiological conditions (Courtice and Simmonds, 1954; Staub, 1974). A breakdown in this regulation under pathological conditions can result in pleural effusion or pulmonary edema (Staub, 1974; Stewart, 1963).

2.2.2 Blood supply and lymphatic drainage of the lungs and the pleura

The blood supply and the lymphatic drainage of the interstitial space of the lungs and visceral pleura vary with the morphological type of the lungs (Mc Laughlin *et al.* 1966). This knowledge is relevant to understanding the pathophysiology of pleural effusion and pulmonary edema in various species. In type I and III lungs, the blood supply is largely from the systemic circulation (bronchial arteries), however according to von Hayek (1960) there is a small contribution from the pulmonary arteries. Details of the anatomical distributions of these two circulations, and the contribution of pulmonary blood supply to the visceral pleura are controversial (Mc Laughlin *et al.* 1966; von Hayek, 1960). In type II lung morphology (e.g. in the mouse), the blood supply is exclusively from the pulmonary vessels (Pinchon *et al.* 1980).

The pulmonary lymphatic network consists of a superficial or pleural plexus, localized in the subpleural connective tissue layer of the visceral pleura and a deep plexus located in the peribronchial, perivascular and interlobular connective tissue (Naigaishi, 1972). According to Leak and Jamuar (1983), the density of the lymphatic network is different in different types of lung morphology: extensive in the human visceral pleura and sparse in the mouse.

In contrast to the different densities of the lymphatic network of the visceral

pleura, there is no major variation among different mammals in the lymphatics of the parietal pleura. In rabbits, mice and humans, true anatomical connections, the stomata, have been found to connect the pleural space with the lumina of the lymphatic vessels in the parietal pleura (Wang, 1975). Pleural fluid is removed via the lymphatic vessels of the parietal pleura to the systemic venous circulation (Courtice and Simmonds, 1954).

2.3 Leakage of capillary vessels

Abnormal leakage of capillaries can be caused by numerous mechanisms: a loss of integrity in the endothelial lining, an increased hydrostatic pressure within the capillaries, or a reduced oncotic pressure of the plasma within the capillaries. Damage to the capillary endothelium increases permeability and permits transfer of fluid containing more protein than usual to the interstitial space, causing edema. Capillary injury can result from bacterial (Breider *et al.* 1991), chemical (Korpela, 1991), mechanical or thermal agents, or can be the consequence of a hypersensitivity reaction (Bacon, 1991; Schlag *et al.* 1991).

Increased capillary hydrostatic pressure resulting from local obstruction of venous drainage, congestive heart failure or increased blood volume, can also result in local or generalized edema (Aberle *et al.* 1988). Reduced oncotic pressure of the plasma caused by a reduction in the plasma protein levels resulting from malnutrition, liver disease or loss of protein into the urine (e.g. in kidney disease) or gastrointestinal tract can all lead to generalized edema (Braunwald, 1987).

In all cases, capillaries are "leaky". Endothelial permeability is increased, permitting an increase in the volume of the extravascular (interstitial) component of the

extracellular fluid. The symptoms of the capillary leakage are those of accumulation of excess fluid in tissue spaces. This may occur in the subcutaneous tissue (anasarca), the pleural cavity (hydrothorax), the peritoneal cavity (ascites) or the interstitial tissue of the lungs (pulmonary edema) (Braunwald, 1987). Accumulation of fluid in the lungs endangers life because of the quick development of the respiratory failure (Morgan and Goodman, 1991). Common consequences of generalized fluid accumulation are rapid weight gain, drop in urinary output, hypoproteinemia and hypotension (Braunwald, 1987).

2.4 Pulmonary edema

2.4.1 Pathogenesis of pulmonary edema

"Pulmonary edema, like edema in any organ, occurs when the net transmural pressure gradient in exchanging vessels is high enough in the direction of filtration that the fluid cannot be drained away adequately by lymphatics or because the nature of the filtering membrane is altered so that vessels leak excessively" (Brigham, 1978).

In both cases, filtered fluid accumulates in the interstitial tissue space despite further increases of lymphatic flow, producing symptoms that depend on the quantity of fluid. In early pulmonary edema, mild tachypnea is the only symptom. This increases with accumulation of fluid; gas exchange deteriorates, and radiographic changes appear. With further accumulation of interstitial fluid, the alveolar-capillary membrane becomes permeable to water. Tight junctions between alveolar cells are opened, and extracellular fluid floods the alveoli and airways. Without effective treatment of this stage, acidemia, hypoxia and respiratory arrest ensue (Ingram and Braunwald, 1987).

Pulmonary edema can result from a variety of etiological conditions:

- (a) Cardiogenic pulmonary edema is due to increased pulmonary venous pressure, resulting in elevation of pulmonary hydrostatic capillary pressure and more filtering of fluid into the interstitium (Luisada, 1970; Morgan and Goodman, 1991);**
- (b) Lymphatic blockade, secondary to fibrotic and inflammatory diseases can lead to poor drainage of the interstitial fluid (Ingram and Braunwald, 1987);**
- (c) Increased permeability of alveolar-capillary membranes may be brought about directly by inhaled toxic gases or fumes of acids (Szidon *et al.* 1972);**
- (d) Increased permeability of alveolar-capillary membranes in septicemia and/or endotoxemia is caused indirectly through activation and accumulation of neutrophilic granulocytes within pulmonary capillaries. Neutrophils undergo respiratory burst to inflict oxidant mediated injury and release numerous mediators of inflammation eg. leukotrienes, thromboxanes, prostaglandins. These mediators further increase the accumulation of interstitial fluid initiated with alveolar-capillary membrane injury because of their ability to increase pulmonary capillary pressure (Ingram, 1987; Schlag *et al.* 1991).**
- (e) High altitude pulmonary edema is probably caused by vasoconstriction of a large fraction of the pulmonary arterial network provoked with high altitude induced hypoxia (Hultgren and Flamm, 1969).**
- (f) Neurogenic pulmonary edema is related to massive sympathetic discharge from hypothalamic centres inducing pulmonary vasoconstriction which leads to increased intravascular pressure and more fluid escaping into the pulmonary interstitium (Moss *et al.* 1972; Ell, 1991).**

It can be noted from the above that an alteration of fluid filtration and clearance resulting in pulmonary edema can secondarily be followed by a disruption of both the structure and the function of the alveolar-capillary membrane, or that the alveolar capillary membrane damage may precede the increased vascular permeability (Ingram, 1987).

2.4.2 Experimental investigation of pulmonary edema

In order to prevent and treat pulmonary edema in humans, the early physiological and pathological changes must be recognized (Matthay, 1991). Lack of information about the lesion contributes to the 50-60 percent mortality rate associated with acute pulmonary edema (Ingram, 1987).

Experimental expectations have been hindered by the difficulties in designing a convenient experimental model of pulmonary edema in a common laboratory animal (Brigham, 1978). For example, hydrostatic pressure inside and outside exchanging vessels cannot be accurately measured (Gaar *et al.* 1967), although protein osmotic pressure outside and inside capillaries can be estimated from lymph and plasma protein concentrations (Garlick and Renkin, 1970).

Methods used for measuring the water content of the lungs include indicator dilution (Goresky *et al.* 1969) and postmortem desiccation (Pearce *et al.* 1965; Poulsen, 1954). According to Cunningham and Hurley (1972), quantitative measurements of pulmonary fluid content accompanied by microscopic evaluation of the lungs still remains as a valuable tool for gaining an insight into the pathobiology of pulmonary edema.

2.5 Pleural effusion

2.5.1 Pathogenesis of pleural effusion

Abnormal accumulation of liquid in pleural space is known as pleural effusion. Under physiological conditions, a thin layer of fluid serves as a lubricant in the pleural space. During the normal turnover process, it is drained from the parietal pleura, and possibly also from the visceral pleura. Parietal pleura is supplied with blood from the systemic circulation (Staub, 1974), so also is the visceral pleura in the thick type (sheep, pig and human) (McLaughlin *et al.* 1966), whereas the thin type (mouse) of the visceral pleura receives blood from the pulmonary circulation (Pinchon *et al.* 1980). A large fraction of the filtered water and electrolytes are immediately reabsorbed into the microvessels at their site of origin, and the remaining liquid with a higher protein content is reabsorbed through the stomata into the lymphatic vessels of the parietal pleura (Staub *et al.* 1985).

A pleural effusion may develop when there is a relative increase in hydrostatic pressure inside microvessels, e.g. in congestive heart failure, or when there is a relative decrease in oncotic pressure inside microvessels, eg. in hypoalbuminemia, resulting from a variety of pathological conditions such as in nephrosis or hepatic cirrhosis. In these cases, effusion is associated with a normal pleura. Effusions may also result from pleuritis (inflammation of the pleura) leading to increased permeability of the parietal pleura eg. in pulmonary tuberculosis. Neoplasia of the lungs and pleura can lead to effusion contaminated with blood (Ingram, 1987).

Effusions due to pleural disease are exudates and resemble plasma, whereas those occurring with a normal pleura are ultrafiltrates of the plasma known as transudates.

Sometimes it is impossible to distinguish between the types of effusion, because of a mixture of causes, but routine tests (measurement of the protein level and/or lactic dehydrogenase level in the pleural fluid, as compared to the levels in the serum, can generally differentiate a transudate from an exudate (Ingram, 1987).

2.6 Interleukin-2 (IL-2) based tumor immunotherapy

2.6.1 Interleukin-2 alone in tumor therapy

T cell growth factor (later named as interleukin-2) was initially identified in the supernatant of phytohemagglutinin-stimulated normal human lymphocytes, that supported the growth of T cells in cultures of normal human bone marrow (Morgan *et al.* 1976). IL-2 has since been characterized as a 133 amino-acid polypeptide of 15,500 daltons (Watson *et al.* 1983) and in humans it is encoded by a single gene (Shows *et al.* 1984) on chromosome 4. Recombinant IL-2 has been obtained by inserting the IL-2 gene from cultured lymphoma cells (Taniguchi *et al.* 1983) or from normal peripheral blood lymphocytes (Devos *et al.* 1983) in *E. coli*. This form of recombinant IL-2, although nonglycosylated, has biological activity *in vitro* and *in vivo*, identical to that of native IL-2.

The structure of IL-2 receptor consists of 3 peptide chains (α , β and γ) and the genes for encoding these components have been cloned and characterized (Hatakeyama *et al.* 1989). The γ chain, which has been recently found (Sagamura *et al.* 1990; Takeshita *et al.* 1992), is necessary for the formation of the intermediate and high-affinity receptors, which consists of either $\beta\gamma$ or $\alpha\beta$ heterodimers or $\alpha\beta\gamma$ heterotrimers (Taniguchi and Minami, 1993). IL-2 receptor expression has been variably found on T cells, NK

cells (Aribia *et al.* 1989), macrophages (Holter *et al.* 1986; Lotze *et al.* 1987), oligodendroglial cells (Bonveniste and Merrill, 1986), epidermal Langerhans' cells (Steiner *et al.* 1986) and B cells (Boyd *et al.* 1985). IL-2 receptor has also been located on the cell surface of squamous cell carcinoma cell lines derived from human head and neck tumors and also on some melanoma cells (Weidemann *et al.* 1992).

IL-2 is an augmentative factor in the generation of cytotoxic T lymphocytes as well as activation of natural killer cells (Kuribayashi *et al.* 1981; Domzig *et al.* 1983). The tumoricidal potential of immune effector cells including T cells (Robb *et al.* 1981), NK cells (Aribia *et al.* 1989), and macrophages (Holter *et al.* 1986; Parhar and Lala, 1988) can be stimulated with IL-2. Lymphocytes cultured in the presence of high dose IL-2 give rise to cytotoxic cells with a broad spectrum of antitumor cytotoxicity, known as lymphokine activated killer (LAK) cells capable of killing syngeneic as well as allogeneic tumor cells (Yron *et al.* 1981; Grimm *et al.* 1982; Rosenstein *et al.* 1984).

Rosenberg *et al.* (1985) showed that administration of IL-2 led to regression of pulmonary metastasis in mice by activation of LAK cells *in vivo*. These findings led to the application of IL-2 therapy in human cancers, revealing that it can cause a regression of melanomas and renal cell carcinomas in human (Rosenberg *et al.* 1985; Munn and Cheung, 1987; Sondel *et al.* 1988).

2.6.2 Interleukin-2 in combination with lymphokine activated killer (LAK) cells in tumor therapy

Rosenberg's group observed that a combination of IL-2 therapy with infusion of

LAK cells generated by IL-2 activation of lymphocytes *in vitro*, had significantly higher antitumor activity in mice than IL-2 therapy alone (Mule *et al.* 1984; La Freiniere and Rosenberg, 1985). IL-2 therapy in combination with LAK cells was then applied to treat human patients with solid tumors. Autologous lymphocytes were obtained from cancer patients by repeated leukophereses, incubated with IL-2 to generate LAK cells and then reinfused into the patients together with IL-2 (Rosenberg *et al.* 1985). This treatment resulted in regression of tumor in some patients for whom no other effective therapy was available (Rosenberg *et al.* 1987; Paciucci *et al.* 1989). However, it was soon apparent that the therapeutic benefit derived from this combination therapy was not better than that resulting from IL-2 therapy alone (Fisher *et al.* 1988).

2.6.3 Interleukin-2 in combination with tumor infiltrating lymphocytes (TIL) in tumor therapy

Lymphocyte-trafficking studies with radiolabeled LAK cells generated from blood or splenic lymphocytes showed that LAK cells did not localize at tumor metastatic sites but were trapped in the lungs and later in the liver (Maghazachi *et al.* 1990). Interestingly, lymphocytes retrieved from the tumor itself and expanded with IL-2 showed some selectivity for migration to the tumor metastatic site after infusion *in vivo* (Fisher *et al.* 1989). These "tumor infiltrating lymphocytes" (TIL), were expanded *in vitro* during 3-4 weeks after surgical excision of tumor (Topalian *et al.* 1988) for adoptive transfer. When TILs were given together with IL-2 to patients with melanoma or renal carcinoma, responses in tumor regression were reported to be better than with IL-2 treatment alone or IL-2 in combination with LAK cells (Kradin *et al.* 1987;

Topalian *et al.* 1988; Rosenberg *et al.* 1988).

2.6.4 Interleukin-2 in combination with chronic indomethacin therapy in tumor treatment

Lala *et al.* (1985) observed that natural killer cells were progressively inactivated with increasing tumor burden in the tumor-bearing host. This inactivation was largely caused by a high level of prostaglandin E₂ (PGE₂) produced by host macrophages (Parhar and Lala, 1985) in addition to the PGE₂ produced also by certain tumor cells (Young *et al.* 1985). PGE₂ has been shown to suppress lymphocyte proliferation (Goodwin and Weeb, 1980) and activation of all killer cell lineages (Parhar and Lala, 1988). These effects were held responsible for the promotion of metastatic ability of tumors by PGE₂ (Lala, 1989). The PGE₂-mediated inactivation of effector cells was attributed to inhibition of IL-2 production (Walker *et al.* 1983) and a down regulation of IL-2 receptors on the surface of all killer cell lineages (Lala *et al.* 1988).

Based on these findings, Lala's group devised an immunotherapy protocol combining IL-2 with chronic administration of indomethacin (Parhar and Lala, 1987), a drug that inhibits prostaglandin production (Goodwin and Weeb, 1980). Chronic indomethacin therapy alone was found to have antitumor and antimetastatic effect in murine tumor models (Fulton, 1984; Lala *et al.* 1986; Maca, 1988), and substantially restored natural killer cell function (Lala *et al.* 1986). However, this therapy alone was unable to eradicate advanced metastases (Parhar and Lala, 1987), possibly because of inadequate IL-2 production *in vivo*. Chronic indomethacin therapy (given in the drinking water) when combined with systemic injections of IL-2 resulted in lasting cure of B16F10

melanoma metastases in the lungs of large proportion of animals (Lala and Parhar, 1988). Reactivation of AGM-1+ and Thy-1+/- killer lymphocytes *in situ* accounted for the therapeutic benefit, since depletion of these cells *in vivo* abrogated the therapeutic effects. Similar eradication of metastases was also achieved with murine fibrosarcoma (Parhar *et al.* 1988), mammary adenocarcinomas (Lala and Parhar, 1993) as well as human melanomas grown in BALB/C nude mice (Lala *et al.* 1990). This combination therapy was then applied in a phase 2 human trial of advanced melanoma and renal cell carcinoma patients with promising results (Mertens *et al.* 1993; Mertens *et al.* 1993), comparable with the better success rates in other IL-2 based therapy trials (Lala *et al.* 1993).

2.7 Capillary leak syndrome due to interleukin-2

There was a belief that efficacy of IL-2 in the therapy of cancer increased with the IL-2 dose (Sondel *et al.* 1988; Paciucci *et al.* 1989). Although true for animal models, this association was very weak in a recent study at the National Institutes of Health on renal carcinoma patients, receiving high or low-dose of intravenous IL-2 (Oppenheim and Lotze, 1994) and not detectable in a local study at the London Regional Cancer Centre, using indomethacin in combination with IL-2 (Mertens *et al.* 1993; Mertens *et al.* 1993; Mertens *et al.* 1992). However, dose-related toxicity was observed in most trials and still remains a major obstacle to IL-2 based therapy. Capillary leak syndrome is a major side effect of high doses of IL-2 observed in many species, including mice (Rosenstein *et al.* 1986) rat (Edwards *et al.* 1992), sheep (Klausner *et al.* 1989; Jesmok and Gunther, 1989), dog (Kilbourn *et al.* 1994) and humans (Lotze *et al.*

1986). There is an increase in vascular permeability causing marked retention of extravascular fluid in all organ systems and hypotension, often requiring treatment with intravenous fluids and vasopressor agents (Margolin *et al.* 1989). Retention of extravascular fluid results in a rapid weight gain of up to 20%, manifested as peripheral edema, pleural effusion and ascites (Siegel and Puri, 1991; Oppenheim and Lotze, 1994). Occasionally, life threatening respiratory or cardiac failure or neurological abnormalities resulting in coma can develop during IL-2 therapy, requiring cessation of the therapy (Oppenheim and Lotze, 1994; Siegel and Puri, 1991). Interestingly, symptoms of capillary leakage begin to reverse within 24 h of cessation of IL-2 therapy and usually completely disappear within a few days (Oppenheim and Lotze, 1994). Capillary leak syndrome has been observed with IL-2 therapy alone and IL-2 therapy in combination with LAK cells or TIL.

2.7.1 Possible mechanisms of the capillary leak syndrome

At least one of two conditions have to be satisfied to have capillary leak syndrome. The capillary endothelium must be damaged or the capillary hydrostatic pressure increased. Five mechanisms have been proposed by which IL-2 therapy can induce the capillary leak syndrome.

- (a)** IL-2 induces LAK cells to adhere to and later damage the endothelial cells.
- (b)** IL-2 induces natural killer cells to adhere to and damage endothelial cells.
- (c)** Endothelial cells are damaged by tumor necrosis factor α (TNF α) produced by IL-2 activated leucocytes.
- (d)** Changes in endothelial cell architecture are brought about by interferon τ (INF τ),

produced by IL-2 activated leucocytes.

(e) Systemic hypotension is due to production of a vasodilator nitric oxide, induced by IL-2 therapy, which indirectly causes pulmonary hypertension resulting in an increase in pulmonary capillary pressure.

2.7.1.1 Lymphokine activated killer cells and capillary leak syndrome

Lymphokine activated killer cells have been shown to adhere to endothelial cells and lyse them *in vitro* (Kotasek *et al.* 1988; Damle *et al.* 1987; Amador *et al.* 1991). Kotasek *et al.* (1988) proposed that the dense granules secreted by LAK cells, which contain serine esterase I (an enzyme with high proteolytic and cytolytic activity), were responsible for causing breaches in the endothelial cell membranes. Observations on cultured endothelial cells led Savion *et al.* (Savion *et al.* 1984) to propose that LAK cells migrated through and broke endothelial cell tight junctions. Once they have reached the level of the basement membrane and subendothelial matrix, LAK cells would secrete enzymes to degrade the matrix underlying the endothelial cells. These events with or without the lysis of endothelial cells, would cause capillaries to leak. This hypothesis of LAK cell mediated capillary injury is substantiated by the findings that LAK cell depletion *in vivo* by treatment with asialo-GM-1 antibody in mice ameliorated IL-2 therapy induced capillary leakage (Ettinghausen *et al.* 1988).

2.7.1.2 Natural killer cells and capillary leak syndrome

Aronson *et al.* (1988) showed that IL-2 can induce natural killer cells to adhere to human endothelial cells in culture. These authors implied that vascular leakage induced

by IL-2 resulted from NK cell mediated endothelial cell injury. However, there has been no direct evidence of NK cells causing endothelial cell damage *in vivo*, in the absence of activation into LAK cells.

2.7.1.3 Tumor necrosis factor α and capillary leak syndrome

Interleukin-2 therapy activates leucocytes (monocyte-macrophages in particular) to produce $\text{TNF}\alpha$ (Lotze *et al.* 1985; Mier *et al.* 1988). Several authors have reported controversial findings about the ability of $\text{TNF}\alpha$ to damage endothelial cells. Collins *et al.* (1986) reported that $\text{TNF}\alpha$ activated human endothelial cells to express class 1 HLA antigen, suggesting that $\text{TNF}\alpha$ would make them prone to cytolytic T lymphocyte mediated injury. Kahaleh *et al.* (1988) showed that $\text{TNF}\alpha$ inhibited endothelial cell growth in culture. When they were applied in high concentrations they induced endothelial cell lysis.

In 1990, Doukas and Pober reported that $\text{TNF}\alpha$ alone led to endothelial cell "activation", which was enhanced by $\text{IFN}\gamma$. "Activation" was indicated by appearance of new morphologic, antigenic and functional characteristics of endothelial cells. Increases in specific endothelial cell surface molecules like ELAM-1 (endothelial leucocyte adhesion molecule 1) or ICAM (intercellular cell adhesion molecule) were observed by these authors after stimulation by $\text{TNF}\alpha$ and $\text{IFN}\gamma$. Endothelial cell activation resulting in increased endothelial adhesiveness for leucocytes may play a role in increased capillary permeability.

On the other hand, Mier *et al.* (1989) reported that $\text{TNF}\alpha$ and $\text{IFN}\gamma$ could activate endothelial cells and increase binding of CD16^+ lymphocytes to endothelial cells in

culture, but that the lymphocyte binding was not responsible for increased capillary permeability. In fact, these authors reported that $\text{TNF}\alpha$ and $\text{IFN}\gamma$ protected endothelial cells from LAK cell-mediated injury.

2.7.1.4 Changes of endothelial architecture induced by interferon γ

Interferon γ is a lymphokine that appears in the blood of cancer patients as early as 6 hours after administration of IL-2 (Lotze *et al.* 1985). Cytotoxic activity of $\text{IFN}\gamma$ is well known, but its possible role in IL-2 induced capillary leak syndrome is still obscure.

Montesano *et al.* (1985) showed that certain lymphokines could change human endothelial cell architecture *in vitro*. IL-2 had no effect, and $\text{IFN}\gamma$ had only a slight effect, but IL-1 and $\text{IFN}\gamma$ in combination completely changed the appearance of endothelial cells. They became elongated, with many "dendrite like" processes, and there were changes in cytoskeletal structure. Similarity between changes of endothelial cells induced by these lymphokines *in vitro* and in the capillary leak syndrome remain to be established. In fact, Puri *et al.* (1989) reported that administration of recombinant interleukin-1 *in vivo* reduced IL-2 induced vascular leakage in the lungs of mice. These authors could not explain the failure of IL-1 to increase survival of mice treated with IL-2 or with IL-2 and $\text{IFN}\gamma$.

2.7.1.5 Nitric oxide and the capillary leak syndrome

As discussed earlier, severe hypotension is a major component of IL-2 induced capillary leak syndrome in experimental animals and humans. Nitric oxide production,

induced by IL-2-induced cytokines, can potentially contribute both to fluid leakage as well as hypotension. Damage to endothelial cells by IL-2 induced cytokines IFN γ and TNF α (Palmer *et al.* 1992; Estrada *et al.* 1992) has been at least partially attributed to NO production. Similarly, NO production induced by these cytokines can lead to systemic vasodilatation with consequent hypotension. This, in turn, can indirectly cause pulmonary hypertension, owing to hypoxemia induced constriction of the pulmonary artery, followed by pulmonary edema, as discussed below.

Based on the findings that nitric oxide can be produced by activated macrophages after treatment with endotoxin, IFN γ or certain other cytokines, (Hibbs *et al.* 1988; Stuehr and Nathan, 1989; Marletta *et al.* 1988), Kilbourn and Belloni (1990) investigated the effects of INF γ , TNF, IL-1, IL-2 and endotoxin on the production of nitric oxide by endothelial cells. They showed that culture of murine brain endothelial cells produced nitric oxide in response to various combinations of cytokines. They, then speculated that endothelium- derived nitric oxide played a role in the development of hypotension in patients treated with IL-2 or TNF α . In support of this hypothesis, Kilbourn *et al.* (1990) showed that N^o-Methyl-L-Arginine (inhibitor of nitric oxide synthesis) inhibited TNF α and endotoxin-induced hypotension in dogs. However, in a study on murine brain endothelial cells (Kilbourn and Belloni, 1990) IL-2 alone or in combination with IFN γ did not increase nitric oxide production.

Subsequently, increased levels of final metabolites of NO (nitrates and nitrites; Moncada and Higgs, 1993; Kelm *et al.* 1992) have been reported in human cancer patients receiving IL-2 therapy (Hibbs *et al.* 1992; Ochoa *et al.* 1992), indicating the potential role of NO in the pathogenesis of severe hypotension that complicates such

therapy. Since NO is a potent vasodilator (Palmer *et al.* 1987), vasodilation and systemic hypotension due to NO production can indirectly cause pulmonary hypertension and thus increase pulmonary capillary pressure (Siegel and Puri, 1991) leading to fluid leakage in the lungs. In addition, injury to endothelial cells mediated by IFN γ and TNF α (Palmer *et al.* 1992; Estrada *et al.* 1992) has been linked with NO production, because it was prevented with dexamethasone and inhibitors of NOS. Thus, NO may have a major role in the pathogenesis of IL-2 induced capillary leakage.

2.8 Proposed mechanisms of IL-2 induced capillary leak syndrome

In view of the literature reviewed earlier, it is reasonable to suggest that increase in capillary permeability is caused by multiple factors initiated by high dose of IL-2. These factors operate by causing damage to capillary endothelial cells and/or by increasing capillary pressure. Capillary leak syndrome may result from numerous simultaneous or sequential events: 1) IL-2 induces LAK cell activation *in vivo* and promotes their adhesion to and later damage of endothelial cells; 2) IL-2 induces high level of IFN γ which can change cytoarchitecture of endothelial cells, making the endothelial lining more prone to leakage; 3) high level of TNF α produced by IL-2 activated leucocytes induces endothelial cell activation and adhesivity for leucocytes and may play a role in increased capillary permeability; 4) TNF α and IFN γ may also cause damage of endothelial cells through induction of NO. 5) Finally, NO as a potent vasodilator induces systemic hypotension which may indirectly lead to pulmonary hypertension and increased pulmonary capillary pressure.

2.9 Hypothesis

IL-2 induced overproduction of NO contributes to the capillary leak syndrome resulting from high dose IL-2 therapy.

This hypothesis was tested by documenting the effects of treating healthy or tumor-bearing mice with two inhibitors of NO synthesis, N^G-Methyl-L-Arginine (NMMA) or N^G-Nitro-L-Arginine methyl ester (L-NAME) on capillary leakage resulting from IL-2 therapy. Simultaneously, the effects of these inhibitors on IL-2 induced NO production *in vivo* as well as antitumor responses to IL-2 therapy were evaluated. In addition, the effects of treatment with inhibitors alone on tumor growth and metastasis was documented. Since inhibition of NO production *in vivo* reduced IL-2 induced capillary leakage, further histological analysis were undertaken to test whether an induction of NO synthase was related to capillary leakage. Furthermore, since NO inhibition had some potentiating influence on the tumor regression caused by IL-2 therapy, effects of these inhibitors was evaluated on the IL-2 induced LAK cell generation *in vitro* as well as *in vivo*.

2.10 Objectives

1. To examine the effects of treating healthy mice with NMMA on IL-2 induced capillary leakage (pulmonary edema, pleural effusion, fluid retention in the spleen and the kidney) and NO production *in vivo* (Chapter 4).

2. To examine the effects of treating mammary adenocarcinoma bearing mice with NMMA on IL-2 therapy induced capillary leakage, NO production and antitumor responses to IL-2 (Chapter 4).

3. To examine the effects of treating mammary adenocarcinoma bearing mice with NMMA alone on tumor growth and metastasis (Chapter 4).

4. To examine the effects of treating healthy mice with L-NAME on IL-2 induced capillary leakage (pulmonary edema, pleural effusion, fluid retention in the spleen and the kidney), NO production and mortality (Chapter 5).

5. To examine the effects of treating mammary adenocarcinoma bearing mice with L-NAME on IL-2 therapy induced capillary leakage, NO production *in vivo* and antitumor responses to IL-2 (Chapter 6).

6. To examine the effects of treating mammary adenocarcinoma bearing mice with L-NAME alone on tumor growth and metastasis (Chapter 6).

7. To examine the effects of L-NAME on IL-2 induced LAK cell generation *in vitro* with splenocytes from healthy and tumor bearing mice (Chapter 7).

8. To examine the effects of L-NAME therapy on IL-2 induced antitumor cytotoxicity of splenocytes *in vivo* in healthy and tumor bearing mice (Chapter 7).

9. To examine the effects of L-NAME therapy on IL-2 induced expression of NO synthase protein and activity in the lungs and the pleura, and their relationship to histological evidence of capillary leakage (Chapter 8).

10. To examine the effects of NO production by C3-L5 mammary adenocarcinoma cells on tumor cell invasiveness (Chapter 9).

CHAPTER 3 - MATERIALS AND METHODS

3.1 Mice.

Six to nine week old C3H/HeJ female mice were obtained from the Jackson Laboratories (Bar Harbour, ME). This strain of mice was chosen for two reasons: 1) C3H/HeJ female mouse was a host of a spontaneous mammary tumor, from which C3-L5 mammary adenocarcinoma cell line was later derived (as described below - section 3.5.) and C3-L5 cells were used for subcutaneous transplantation of primary tumors; 2) C3H/HeJ mice were susceptible to IL-2 induced toxicity (Lala *et al.* unpublished). Animals were fed with standard mouse chow, provided with water ad libitum, and kept on a 12 h light/dark cycle in the animal quarters. Animal care was in accord with the guidelines set out by the Canadian Council on Animal Care.

3.2 Interleukin-2.

Recombinant human IL-2, highly purified (lot LSP-805, or lot LQP-046, endotoxin content 8×10^{-3} ng/mg), was initially provided by the Cetus Corporation (currently Chiron Corporation), and later by Chiron Corporation, Emeryville, CA. The specific activity was 3×10^6 Cetus Units or 18×10^6 International Units / mg of IL-2. The lyophilized IL-2 (1.2 mg/vial) was first reconstituted with 1 ml of distilled water. RPMI 1640 medium (ICN Biomedicals Inc., Costa Mesa, CA) was used to dilute it further for injections in order to obtain the required dose in 0.1 ml volume per injection. The reconstituted IL-2 was stored at 4°C up to 2 days.

3.3 N^G-Methyl-L-Arginine.

NMMA acetate obtained from Sigma Chemical Company (St. Louis, MO), was reconstituted with phosphate-buffered saline, pH 7.4 to achieve doses of 5-20 mg/kg/inj.

A volume of 0.1 ml was used per injection in healthy or tumor-bearing mice. These doses were based on *in vivo* studies reported earlier to block nitric oxide synthesis (Kilbourn *et al.* 1990). The reconstituted material was stored at 4°C up to 2 days. In one additional experiment, NMMA was given in the drinking water in a dose of 1 mg/ml, to test whether this route of chronic administration influenced IL-2 induced capillary leakage in healthy mice. This dosage was chosen on the basis of pilot studies, showing that it was nontoxic, had no antidipsogenic effects and blocked IL-2 induced NO increase in the serum.

3.4 N^G-Nitro-L-Arginine Methyl Ester.

L-NAME obtained from Sigma Chemical Company (St.Louis, MO) was added to the drinking water to provide concentrations of 0.01, 0.1, 0.5 and 1 mg/ml. These doses were based on studies reported by Moncada's group earlier to inhibit endothelial nitric oxide synthase activity (Rees *et al.* 1990) and increase mean systemic arterial blood pressure in anaesthetized and conscious rats (Gardiner *et al.* 1990; Gardiner *et al.* 1992).

3.5 Tumor-cell line.

C3-L5 mammary adenocarcinoma cell line was selected in Dr. Lala's laboratory from its parent C3 line, which had been clonally derived from a primary transplantable tumor T58. T58 was grown from a spontaneous mammary tumor in a C3H/HeJ mouse, which also exhibited lung metastases (Brodt *et al.* 1985). C3 line initially showed a strong ability for spontaneous metastasis to the lungs when transplanted s.c., but with *in vitro* passages for a number of years, this ability declined (Lala *et al.* 1986). C3-L5 line was derived from C3 line by 5 cycles of *in vivo* selections for spontaneous lung micrometastases following s.c. transplantation in C3H/HeJ mice (Lala and Parhar, 1993).

This line has since maintained a strong ability to metastasize spontaneously to the lungs, in both C3H/HeJ and C3H/HeN strains of mice.

3.6 Tumor transplantation.

C3-L5 mammary adenocarcinoma cells were injected subcutaneously (s.c., 5×10^5 cells/0.1 ml of RPMI medium in NMMA studies, and 2.5×10^5 cells/0.1 ml in L-NAME studies) in the mammary line near the axilla of C3H/HeJ mice (one injection / mouse). The resultant primary tumor usually produces lung micrometastases within two weeks.

3.7 Measurement of capillary leakage.

For measurement of water content, the left lung, the spleen and the left kidney were recovered from animals, and their wet weight was recorded. The organs were frozen at -80°C and then freeze-dried to constant weight in a freeze-drying system (Labconco Corporation, Kansas City, MO). Dry weights were measured, and wet/dry weight ratio of the organs was calculated.

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 exposed and effusion collected with a syringe. This procedure allowed collection of blood free aspirates. On rare occasions of blood contamination, the samples were excluded from the study.

3.8 Measurement of nitric oxide.

The samples of serum and pleural effusion were collected at the end of the therapies to measure the production of NO. Endogenously synthesized NO in mammals

is quickly oxidized to the stable inorganic NO_2^- (Moncada and Higgs, 1993), which reacts rapidly with oxyhemoglobin from blood giving NO_3^- (Kelm *et al.* 1992). Thus, basal concentrations of NO_2^- in the blood are low, while those of NO_3^- are nearly 100 times higher (Kelm *et al.* 1992). In our samples, cadmium filings were used for conversion of NO_3^- to NO_2^- (Davison and Woof, 1978) and Griess reaction for measurement of NO_2^- (Green *et al.* 1982). The basal concentrations of NO_2^- were not detectable and measurable amounts of NO_2^- from our samples were obtained by reducing the NO_3^- to NO_2^- .

The samples of pleural effusion or serum were first diluted 5 times with deionized distilled water, and proteins were precipitated with 30% ZnSO_4 (0.05 ml/ ml of sample). After centrifugation (8000 x g for 5 min), 1 ml of supernatant mixed with 300 μl of 0.5 M ammonium chloride, 100 μl of 0.06 M sodium borate and 50 mg of freshly prepared cadmium filings (Davison and Woof, 1978) was incubated at room temperature for 30 minutes with constant shaking to reduce NO_3^- to NO_2^- . After further centrifugation (400 x g, 7 min), 1 ml of supernatant and 1 ml of Griess reagent were mixed and incubated for 10 minutes. Griess reagent is a mixture of equal parts of 1% sulphanilic acid and 0.1% naphthylethylenediamine in 2% phosphoric acid. The absorbance was read at 543 nm in a DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). The final concentration of nitrite in pleural effusion and serum was calculated from a sodium nitrite standard curve, which was linear from 0 to 90 μM nitrite.

3.9 Measurement of anti-tumor effects of various therapeutic protocols

Effects of therapies were evaluated on the growth of primary tumors as well as on the incidence of spontaneous lung metastases.

The size of the primary tumor was measured with callipers, and the maximum and

minimum diameters were recorded. The average of the maximum and minimum diameters or tumor volumes were then calculated and the size represented in mm or mm³, respectively.

Isolated right lungs (at the end of therapeutic treatment) were fixed with Bouin's fixative and the number of visible lung metastatic nodules was scored using a dissecting microscope.

3.10 Statistical evaluation.

Microstat Statistics Package (Ecosoft, Inc., Indianapolis, IN) was used to perform the analysis of variance test. One-way ANOVA test was used for normal distributions and Kruskal-Wallis test for skewed distributions. Newman-Keuls multiple range test or nonparametric multiple range test was used further to determine which means or sums of ranks were different from one another (Zar, 1974). $P < 0.05$ was considered significant.

CHAPTER 4 - EFFECTS OF N^G-METHYL-L-ARGININE ON INTERLEUKIN-2 INDUCED CAPILLARY LEAKAGE AND ANTITUMOR RESPONSES IN HEALTHY AND TUMOR-BEARING MICE

4.1 Introduction

It has been demonstrated that IL-2 based immunotherapy (Rosenberg *et al.* 1985) can cause regression of metastases in mice. IL-2 therapy alone or in combination with *ex vivo* generated lymphokine activated killer (LAK) cells (Rosenberg, 1989; Fisher *et al.* 1988; Dutcher *et al.* 1989) has been utilized with some success in treating human patients with certain forms of cancer, particularly melanomas and renal carcinomas. Although high dose IL-2 therapy has been shown as promising, the major obstacle for its widespread clinical use is a serious side effect known as capillary leak syndrome, characterized by retention of extravascular fluid and severe hemodynamic instability inclusive of hypotension (Lotze *et al.* 1985). This syndrome was observed during IL-2 therapy in mice (Rosenstein *et al.* 1986), sheep (Klausner *et al.* 1989; Jesmok and Gunther, 1989), rat (Edwards *et al.* 1992), as well as in humans (Lotze *et al.* 1985; Siegel and Puri, 1991; Oppenheim and Lotze, 1994), or when IL-2 therapy was combined with LAK cells (Rosenberg *et al.* 1985). The pathophysiology of IL-2 induced capillary leakage still remains poorly understood.

Capillaries leak if their endothelial lining is damaged and/or if the capillary pressure is increased. *In vitro* studies have shown that LAK cells can adhere to and then damage endothelial cells (Kotasek *et al.* 1988). Indeed treatment with asialo-GM-1 antibody, which depletes LAK cells in mice, has been reported to ameliorate capillary

leak syndrome (Ettinghausen *et al.* 1988), however it also removes the main therapeutic effect of IL-2. Increased capillary permeability resulting from IL-2 induced inflammatory mediators may underlie capillary leakage, since treatment with glucocorticoids can reduce it (Rosenstein *et al.* 1986; Faggioni *et al.* 1994). However such treatment also abrogates the therapeutic benefit of IL-2. IL-1 α treatment although reported to reduce capillary leak syndrome (Puri *et al.* 1989), failed to prevent IL-2 induced mortality. Treatment with phalloidine and antamanide has also been reported to abrogate capillary leakage (Welbourn *et al.* 1991), but it remains unknown whether these agents compromise antitumor effect of IL-2.

Lala's group observed that overproduction of PGE₂ by tumor cells or host macrophages interfered with the optimal activation of antitumor effector cells by IL-2. Thus addition of chronic indomethacin therapy (CIT) to IL-2 therapy was found to improve the therapeutic efficacy of IL-2 in numerous models of murine metastatic cancer by enhancing the activation of tumoricidal cells in situ (Lala and Parhar, 1988; Lala and Parhar, 1993). They subsequently tested this combination therapy in a phase 2 human trial of advanced kidney cancer and melanomas (Mertens *et al.* 1992; Mertens *et al.* 1993), with promising results. Since PGE₂ is a potent vasodilator, which can also be induced by IL-2 therapy, Orucevic and Lala (1992; 1993) hypothesized that PGE₂ production may contribute to systemic hypotension, and in turn pulmonary hypertension, which may underlie IL-2 therapy-induced pulmonary edema, a significant component of capillary leakage. Unfortunately, however, we found that CIT, in spite of its added antitumor effects, was inadequate in ameliorating IL-2 induced capillary leak syndrome in healthy or tumor bearing mice (Orucevic and Lala, 1992; Orucevic and Lala, 1993).

It is possible that capillary leak syndrome is an end result of multiple mechanisms: endothelial injury caused by LAK cells as mentioned earlier (Kotasek *et al.* 1988), as well as other cytokines induced *in vivo* with IL-2, eg. interferon (IFN) γ and tumor necrosis factor (TNF) α (Lotze *et al.* 1986; Mier *et al.* 1988). IFN γ has been reported to change endothelial architecture *in vitro* (Montesano *et al.* 1985), and TNF α to cause endothelial cell injury *in vivo* (Kahaleh *et al.* 1988), effects which can be mediated by the induced production of nitric oxide (NO). TNF α can induce NO production by endothelial cells as well as macrophages (Kilbourn *et al.* 1990). Indeed, a rise in the NO level (as reflected by the levels of nitrites and nitrates) in the body fluids has been reported after human tumor immunotherapy with IL-2 (Hibbs *et al.* 1992; Ochoa *et al.* 1992). NO can possibly mediate capillary leak syndrome in two ways: first, cytokine induced NO production can damage endothelial cells (Palmer *et al.* 1992). Second, NO is a potent vasodilator (Palmer *et al.* 1987), and thus may underlie severe systemic hypotension observed during IL-2 therapy. This, in turn, can indirectly cause pulmonary hypertension, leading to fluid leakage in the lungs. Thus, pulmonary edema encountered during IL-2 therapy could also result from hemodynamic changes in addition to endothelial damage.

The objectives of the present study were to examine the possible role(s) of NO production *in vivo* in the pathogenesis of capillary leak syndrome during IL-2 therapy in healthy and tumor-bearing mice, as well as its influence on the antitumor responses to IL-2 therapy. N^G-Methyl-L-Arginine (NMMA), a potent inhibitor of nitric oxide synthesis (Kilbourn *et al.* 1990) has been tested in therapeutic settings to prevent IL-2 induced hypotension in a dog model (Kilbourn *et al.* 1994), as well as in human cancer

patients treated with IL-2 (Fonseca *et al.* 1994; Kilbourn *et al.* 1995). However, the effects of NMMA on IL-2 induced capillary leakage or *in vivo* antitumor responses were not evaluated in those studies. Experiments were therefore designed to test whether inhibition of NO synthesis with NMMA in healthy or tumor-bearing mice could reduce pulmonary edema or pleural effusion caused by high doses of IL-2. These are the most severe side effects which can lead to cardiovascular failure (Siegel and Puri, 1991), requiring an interruption of IL-2 therapy. The influence of NMMA treatment alone on tumor growth, and the addition of NMMA to IL-2 on antitumor effects of IL-2 therapy were also examined.

4.2 Materials and Methods

4.2.1 Mice.

Seven to nine week old C3H/HeJ female mice were obtained from the Jackson Laboratories (Bar Harbour, ME). Animal care was previously described in Chapter 3.

4.2.2 Interleukin-2.

Recombinant human IL-2, highly purified (lot LSP-805, endotoxin content 8×10^3 ng/mg), was kindly provided by the Cetus Corporation (currently Chiron Corporation, Emeryville, CA). Specific activity, methods of reconstitution and storage were the same as described earlier (Chapter 3).

4.2.3 N^G-Methyl-L-Arginine.

NMMA acetate obtained from Sigma Chemical Company (St. Louis, MO), was reconstituted with phosphate-buffered saline, to achieve doses of 5-20 mg/kg/inj in a volume of 0.1 ml as described in Chapter 3. In one additional experiment, NMMA was

given in the drinking water in a dose of 1 mg/ml, to test whether this route of chronic administration influenced IL-2 induced capillary leakage in healthy mice. This dosage was chosen on the basis of pilot studies, showing that it was nontoxic, had no antidipsogenic effects and blocked IL-2 induced NO increase in the serum.

4.2.4 Tumor-cell line.

C3-L5 mammary adenocarcinoma cell line was used in this part of the study, the origin of which was described in Chapter 3.

4.2.5 Tumor transplantation.

C3-L5 mammary adenocarcinoma cells (5×10^5) were injected s.c. in the mammary line near the axilla of C3H/HeJ mice. The resultant primary tumors usually produce lung micrometastases within two weeks.

4.2.6 Experimental design.

Capillary leak syndrome during IL-2 therapy was studied in healthy or tumor bearing mice in five series of experiments ($n = 5-14$ mice per group, 10 mice in most experiments with healthy mice; $n=10-25$ for tumor transplanted mice).

In the first series of experiments (series A), the dose response of capillary leakage to IL-2 \pm NMMA therapy was explored. Fluid content of the lungs and pleural cavities was measured in groups of healthy mice receiving vehicles for IL-2 and NMMA; or 7,500, 15,000 or 35,000 Cetus U of IL-2 given i.p. every 8h for 10 injections; or NMMA at 5, 10 and 20 mg/kg s.c. every 8h for 10 injections; or a combination of IL-2 (7,500 or 35,000 U/injection) and NMMA (5, 10 and 20 mg/kg/inj) at the same schedule.

In the second series of experiments (series B), the middle dose (15,000 U/inj) of

IL-2 and the highest dose (20 mg/kg/inj) of NMMA were chosen to measure the effects of NMMA on IL-2 induced capillary leakage. Healthy mice received one of the following therapies, given as one or two rounds: nothing; IL-2; IL-2 + NMMA. IL-2 was injected i.p. at a dose of 15,000 Cetus U every 8 h, for a total of 10 injections / round. NMMA was injected s.c. (20 mg/kg every 8 h, for a total of 10 injections per round, together with IL-2). The second round of treatments followed 5 days after the first round, to mimic therapeutic settings of tumor treatments (Lala and Parhar, 1988; Lala and Parhar, 1993).

In a third series of experiments (series C), treatment was given in one round as in series B, except for the fact that NMMA was given in the drinking water (1 mg/ml of water, starting 1 d before IL-2 therapy).

In all of the above series (A,B,C) mice were killed 1 h after the last IL-2 injection to measure the water content of the lungs and the pleural cavities. The NO_2^- and NO_3^- levels (the final product of NO metabolism) (Kelm *et al.* 1992) were also measured in the serum, pleural effusion or daily (24h) urine collections retrieved from mice housed in plastic metabolic cages ($n = 5/\text{cage}$, Nalgene Company, Rochester, NY).

The fourth series of experiments (series D) was conducted in tumor bearing mice in order to measure the effects of NMMA on IL-2 induced capillary leakage and antitumor responses. Tumor-transplanted mice, randomly separated into 6 groups ($n = 10-25$), were treated with : nothing, IL-2 (10 inj. 15,000 Cetus U i.p. every 8h), NMMA (10 inj. 20 mg/kg/inj every 8h), IL-2+NMMA. IL-2 and NMMA were given in one round on d 10-13 or in two rounds on d 10-13 and d 19-22 after tumor transplantation. Animals were killed 1h after the last IL-2 or NMMA inj. to measure water content of

the lungs and pleural cavities, and the effects of therapies on the growth of the primary tumor and the number of spontaneous lung metastasis. This series of experiments were duplicated in series E (6 groups of mice, n = 10-15 / group).

4.2.7 Measurement of capillary leakage.

For measurement of water content, the left lung was recovered from animals, and the wet/dry weight ratio of the lungs was calculated as described in Chapter 3.

The volume of pleural effusion was measured as described in Chapter 3.

4.2.8 Measurement of nitric oxide.

Nitrite levels from body fluids were measured by reducing NO_3^- to NO_2^- with cadmium filings (Davison and Woof, 1978), and then NO_2^- concentrations were measured using the Griess reagent (Green *et al.* 1982) as described in Chapter 3.

4.2.9 Measurement of antitumor effects

The size of the primary tumor was measured with callipers on day (d) 10, 13 and 22 after tumor transplantation, and recorded as the average of the maximum and minimum diameters.

Isolated right lungs (d 22 of tumor growth) were fixed with Bouin's fixative and the number of visible lung metastatic nodules was scored using a dissecting microscope.

4.2.10 Statistical evaluation.

Methods of statistical evaluation were the same as described in Chapter 3.

4.3 Results

4.3.1 Experimental series A: This series tested the effects of various doses of IL-2, or NMMA or IL-2+NMMA on water content of the lungs and pleural cavities.

4.3.1.1 IL-2 dose response: Vehicles for IL-2 and NMMA did not influence the water content in the lungs, nor did they induce any pleural effusion (data not shown). Water content in the lungs increased with all doses of IL-2 (7,500; 15,000 or 35,000 U/inj). However, no significant difference was noticed amongst different IL-2 doses. On the other hand, significantly ($p < 0.05$) higher amount of pleural effusion resulted from 15,000 or 35,000 U than with 7,500 U of IL-2 (Figure 4.1.).

4.3.1.2 NMMA dose response: Different doses of NMMA (5, 10 and 20 mg/kg/inj.) did not influence IL-2 (35,000 U/inj.) induced pulmonary edema or pleural effusion in the dose response experiment (Table 4.1). Repeated administration (10 injections) of different doses of NMMA alone (5, 10 or 20 mg/kg/inj.) did not induce any pulmonary edema or pleural effusion ($n = 10/\text{group}$) (data not shown).

4.3.1.3 NMMA on IL-2 dose response: NMMA (20 mg/kg/inj.) therapy again did not influence pulmonary edema or pleural effusion induced by the lowest or the highest doses (7,500 or 35,000 U/inj) of IL-2 (data not presented).

4.3.2 Experimental series B and

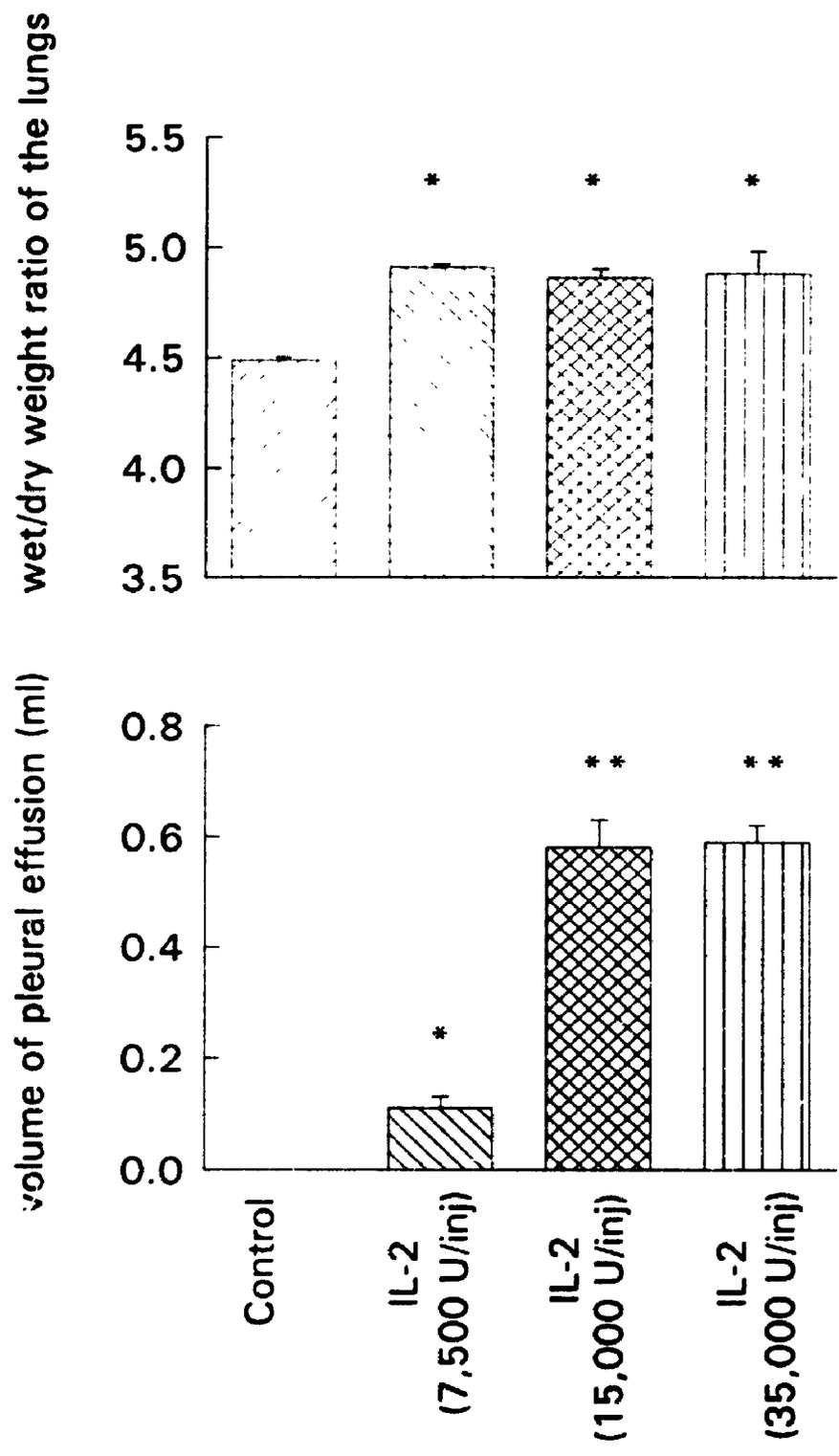
4.3.3 Experimental series C: This series tested the effects of NMMA (subcutaneous vs. oral administration) on IL-2 induced capillary leakage in healthy mice. In these experiments, the middle IL-2 dose (15,000 U/inj) was used, since capillary leakage reached a maximum at this dosage (as observed in experimental series A); the highest NMMA dose (20 mg/kg/inj) was used, since it was found to be nontoxic and expected to block NO production (Kilbourn *et al.* 1990; Kilbourn *et al.* 1990).

Subcutaneous administration of NMMA (20 mg/kg/inj) did not influence IL-2

Figure 4.1 Change in the water content of the lungs (top) and pleural cavities (bottom) after different doses of IL-2 therapy in healthy mice. Data represent mean \pm SE (n = 10)

* All IL-2 doses induced significant ($p < 0.05$) increase in the water content in the lungs and pleural cavities.

** 15,000 or 35,000 U/inj of IL-2 induced significantly ($p < 0.05$) higher accumulation of pleural fluid than 7,500 U/inj of IL-2.



**Table 4.1 Influence of different doses of NMMA on
IL-2^a induced pulmonary edema and pleural effusion**

(n=10/group)	wet/dry weight of the lungs ^b	pleural effusion ^c
Control	4.41 ± 0.03	no effusion
NMMA (20 mg/kg/inj)	4.41 ± 0.01	no effusion
IL-2	4.55 ± 0.04	0.79 ± 0.03
IL-2 + NMMA ^d (5 mg/kg/inj)	4.7 ± 0.09	0.80 ± 0.08
IL-2 + NMMA ^d (10 mg/kg/inj)	4.62 ± 0.02	0.98 ± 0.08
IL-2 + NMMA ^d (20 mg/kg/inj)	4.6 ± 0.04	0.97 ± 0.05

^a Animals received a single round of IL-2 (35,000 U i.p. every 8h, 10 times) or single round of different doses of NMMA (5, 10 and 20 mg/kg/inj s.c., every 8h, 10 times) together with IL-2. NMMA alone (20 mg/kg/inj, s.c.) followed IL-2 schedule.

^b Showing a relative measure of the water content in the lungs, expressed as the mean ± SE.

^c Mean ± SE of the quantity of liquid in pleural cavities in ml.

^d NMMA in any dose did not change pulmonary edema or pleural effusion induced by IL-2.

(15,000 U/inj) induced capillary leakage (indicated by the water content of the lungs and pleural cavities and measured at the end of the first round of therapies) (Figure 4.2). However, oral administration of NMMA (1 mg/ml of drinking water) significantly reduced IL-2 induced increase in the water content of the lungs (measured at the end of the first round of therapies), while the reduction of IL-2 induced pleural effusion was not significant (Figure 4.2).

At the end of the second round of IL-2 therapy (15,000 U/inj), healthy mice exhibited pulmonary edema, but no pleural effusion. Addition of NMMA (20 mg/kg/inj; s.c.) did not influence these results (Table 4.2.).

4.3.4 NO production during IL-2 and NMMA therapy (series A, B, C; single round of therapies):

Basal levels of NO_2^- in the samples of serum and pleural effusion were not detectable, and all measurable amounts of NO_2^- were obtained by reducing NO_3^- to NO_2^- . A single round of IL-2 administration (7,500, 15,000 or 35,000 U/inj; experimental series A) caused a dose dependent increase in NO generation, as was reflected by the nitrite + nitrate levels in the serum and pleural effusion (Figure 4.3).

In an independent experiment (series B) addition of NMMA therapy (s.c.; 20 mg/kg/inj) to a single round of IL-2 therapy slightly reduced nitrite + nitrate levels only in the urine, but not in the serum or pleural effusion (data not presented).

Oral administration of NMMA (experimental series C) significantly reduced IL-2 induced increase in NO levels both in the serum and pleural effusion (Figure 4.4.).

It may be noted that in spite of differences in the control levels of $\text{NO}_2^- + \text{NO}_3^-$ in the serum in different experiments, the relative increase in $\text{NO}_2^- + \text{NO}_3^-$ levels induced

Figure 4.2 Influence of the addition of NMMA therapy by the subcutaneous (A) or the oral route (B) to IL-2 therapy on the water content of the lungs (top) or pleural cavities (bottom). Data represent mean \pm SE (n = 5).

* Oral administration of NMMA (1 mg/ml of drinking water) significantly ($P < 0.05$) reduced IL-2 (15,000 U/inj) induced pulmonary edema. The reduction of pleural effusion was not significant ($p=0.07$).

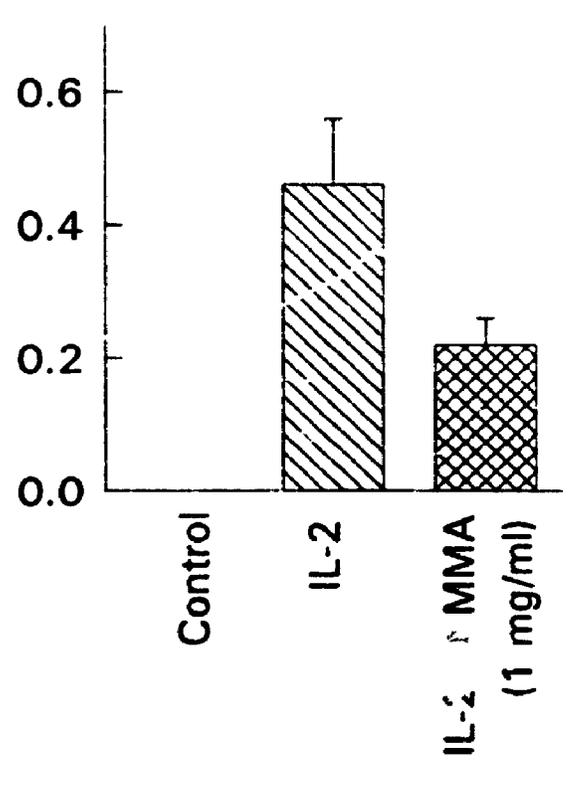
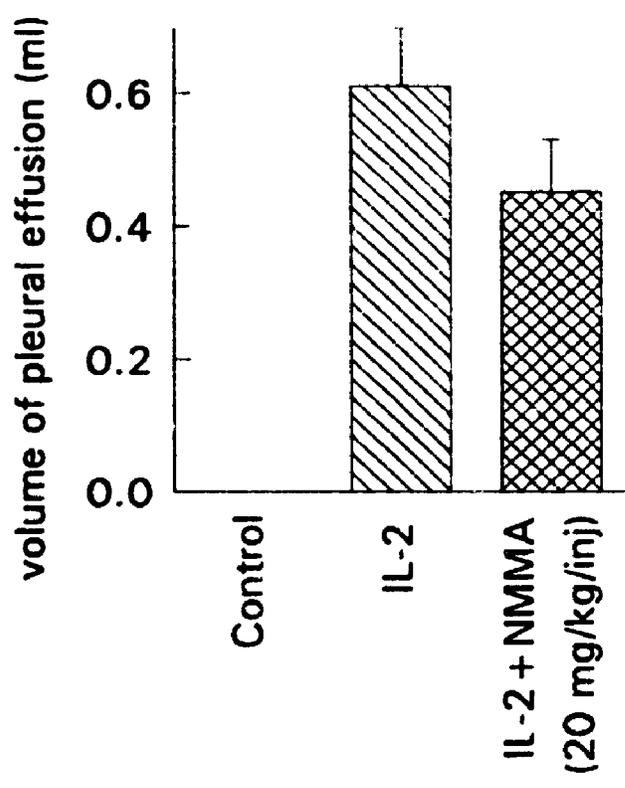
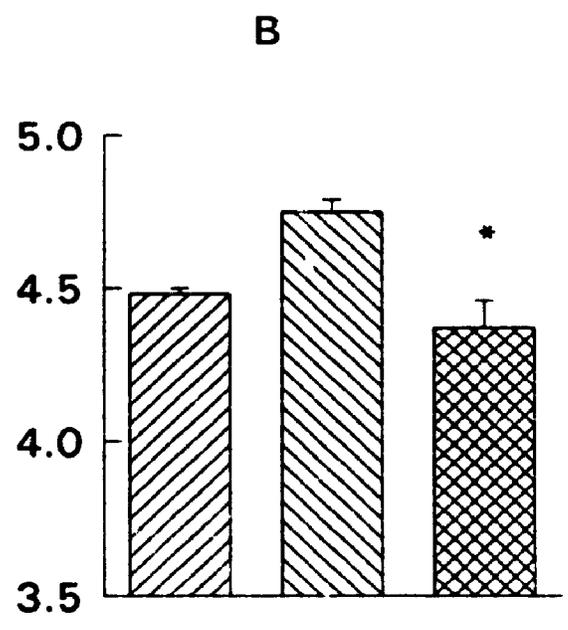
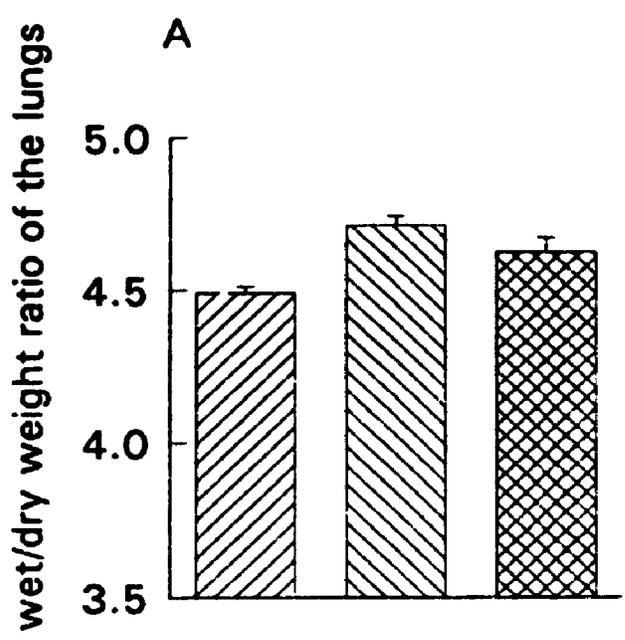


Table 4.2 Influence of NMMA on IL-2 induced pulmonary edema after the second round of therapies^d

	Number of mice	water content in the lungs ^a
Control	7	4.34 ± 0.04
NMMA	6	4.48 ± 0.03
IL-2 ^b	9	4.5 ± 0.03
IL-2 + NMMA ^{b,c}	7	4.71 ± 0.06

^a Water content in the lungs is expressed as the mean ± SE

of the wet/dry lung weight ratios.

^b significant increase in pulmonary edema compared to control ($P < 0.05$).

^c Values are not significantly different from IL-2 alone.

^d Because of non-normal distribution, all data were analysed by Kruskal Wallis test.

Figure 4.3 Nitrate + nitrite levels in the serum (top) and pleural effusion (bottom) after different doses of IL-2 therapy in healthy mice. Data represent mean \pm SE (n = 3-5, each done in duplicate).

* IL-2 at doses of 15,000 and 35,000 U/inj induced an increase in NO production in the serum and pleural effusion in a dose dependent manner, whereas a dose of 7,500 U/inj did not have a significant effect on the serum NO level.

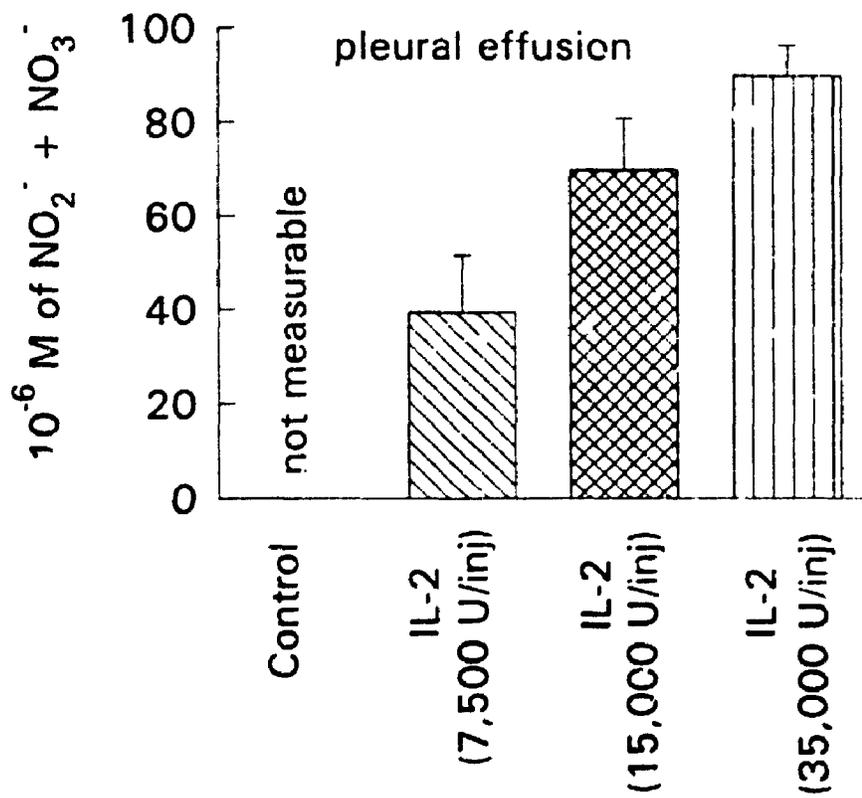
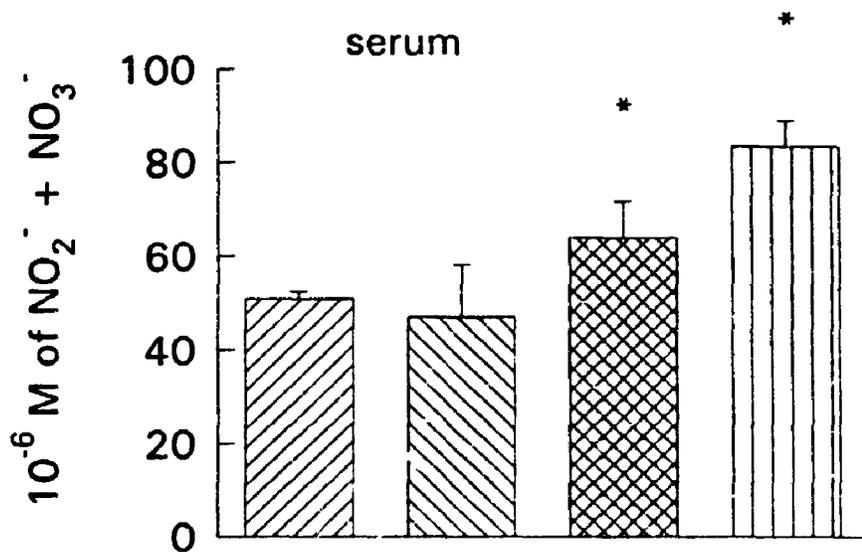
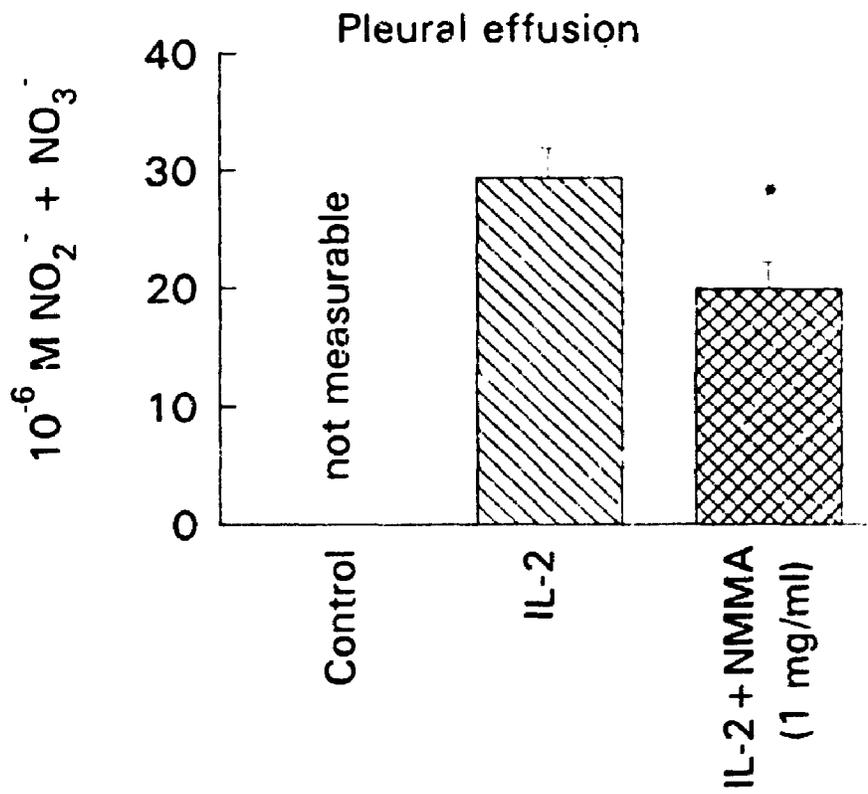
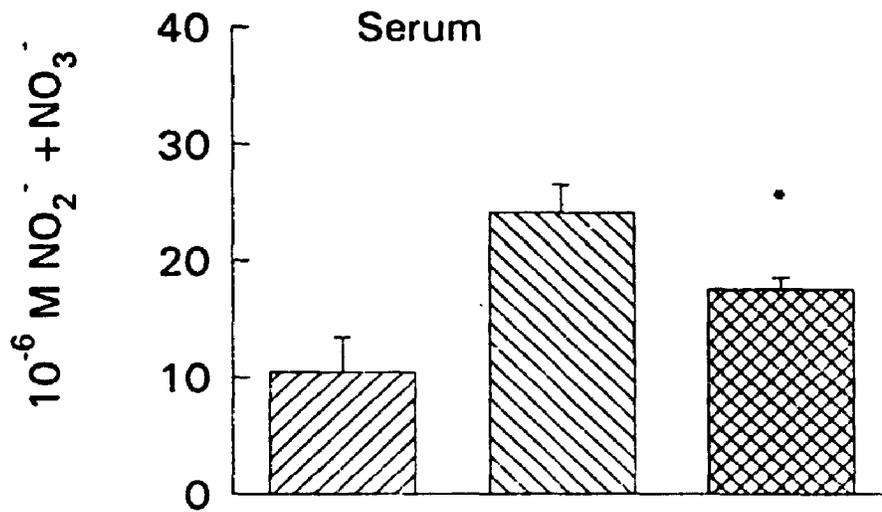


Figure 4.4 Nitrate + nitrite levels in the serum (top) and pleural effusion (bottom) after IL-2 (15,000 U/inj) and IL-2 + NMMA (1 mg/ml of drinking water) therapy of healthy mice. Data represent mean \pm SE (n = 3-5, each done in duplicate).

* IL-2-induced increase in NO production, measured from the nitrite + nitrate levels in the serum and pleural effusion, was significantly ($p < 0.05$) reduced with addition of oral NMMA therapy.



by a single round of IL-2 (15,000 U/inj) was of the similar range (1.5 - 2.5 times control levels) (Figures 4.3, 4.4, and unrepresented data).

4.3.5 Experimental series D: This series tested the effects of NMMA (s.c.; 20 mg/kg/inj) on IL-2 (15,000 U/inj) induced capillary leakage and antitumor responses to IL-2 immunotherapy in tumor bearing mice.

4.3.5.1 Effects of NMMA on IL-2 induced capillary leakage in tumor-bearing mice: The quantity of liquid in the pleural cavities induced by IL-2 was not significantly ($p < 0.05$) influenced with addition of NMMA after the first round of therapies in tumor bearing mice (Table 4.3.).

Pleural effusion appeared to be the main cause of mortality in animals treated with IL-2 as monotherapy or in combination therapy. At necropsy, these animals had 0.9-1.2 ml of pleural effusion irrespective of the treatment applied. Interestingly, there was no pleural effusion after the second round of therapies in those animals which survived the first round. These results (data not presented) in tumor-bearing mice were similar to those noted in healthy animals subjected to the same therapies, as described earlier.

NMMA or IL-2 therapy alone or IL-2 in combination with NMMA did not change the water content in the lungs of tumor-bearing animals after the first round of therapies (Table 4.3).

4.3.5.2 Effects of tumor-bearing on water content of the lungs: Tumor-bearing by itself induced a significant ($p < 0.05$) increase in the water content in the lungs at the time coinciding with the end of the second round of therapy (d 22). The second round of all therapies (NMMA, IL-2, IL-2+NMMA) resulted in a significant ($p < 0.05$) amelioration of tumor induced fluid leakage (Figure 4.5.). However, there was no significant

Table 4.3 Influence of NMMA on IL-2^a induced pulmonary edema and pleural effusion in tumor bearing mice after first round of therapy

(n= 10/group)	wet/dry weight of the lungs^b	pleural effusion^c
Tumor control	4.47±0.09	no effusion
NMMA (20 mg/kg/inj)	4.49±0.04	no effusion
IL-2	4.58±0.04	0.38±0.03
IL-2+NMMA (20 mg/kg/inj)	4.69±0.04	0.32±0.05

^a Animals received a single round of IL-2 (15,000 U i.p. every 8h, 10 times, from d 10-13 after tumor transplantation) or single round of NMMA (20 mg/kg/inj s.c., every 8h, 10 times) together with IL-2. NMMA alone (20 mg/kg/inj, s.c.) followed IL-2 schedule.

^b Showing a relative measure of the water content in the lungs, expressed as the mean ± SE.

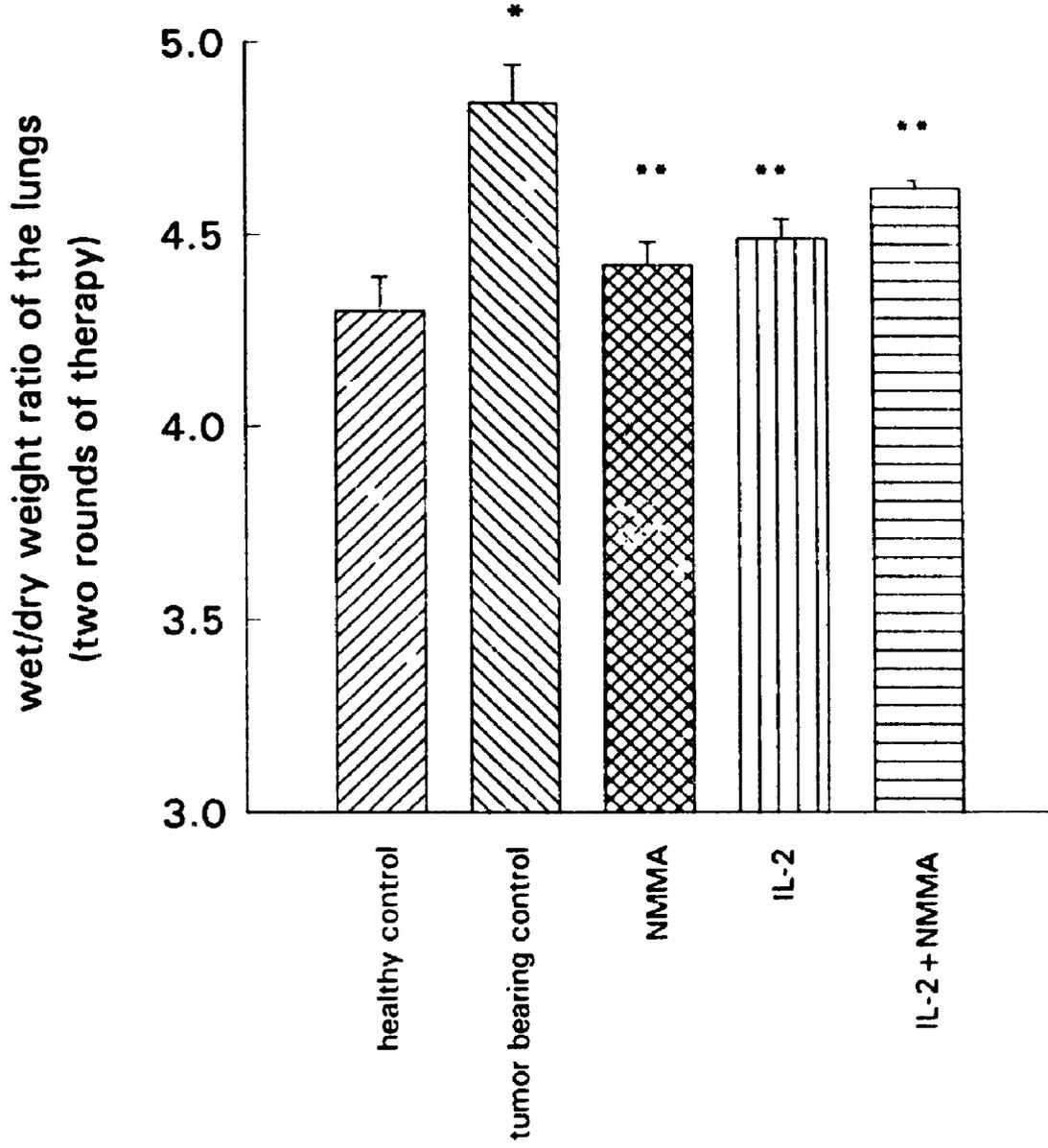
^c Mean ± SE of the quantity of liquid in pleural cavities in ml.

Figure 4.5 Changes in the water content of the lungs after two rounds of IL-2 and NMMA therapy in tumor-bearing animals. Data represent mean \pm SE (n=7-9).

* indicates significant ($p < 0.05$) difference from the healthy control.

** indicates significant difference from tumor-bearing control ($p < 0.05$).

All therapies caused significant reduction of tumor induced increase in the water content in the lungs after the second round of therapies.



difference between different treatment groups.

4.3.5.3 Antitumor effects of NMMA and IL-2 therapy: All therapies (NMMA, IL-2, IL-2+NMMA) caused a significant ($p < 0.05$) reduction of the primary tumor mass only at the end of the first round (d13) of therapies (Figure 4.6.).

Although the primary tumor mass was not significantly influenced by any of the therapies at the end of the second round (d 22), the number of lung metastatic nodules was significantly ($p < 0.05$) reduced by all therapies (NMMA, IL-2, IL-2+NMMA) at this time (Figure 4.7).

In an independent series of experiments (series E), using tumor bearing animals ($n = 10-15$ /group), the effects of NMMA on tumor growth / metastasis as well as on IL-2 induced capillary leakage and reduction of tumor/metastasis burden were essentially similar (data not presented).

4.4 Discussion

In the present study, the influence of treatment with NMMA, an inhibitor of nitric oxide synthesis, on capillary leak syndrome and antitumor responses during IL-2 therapy was examined in healthy and tumor-bearing C3H/HeJ mice. The results showed that subcutaneously administered NMMA during IL-2 therapy was not adequate in mitigating IL-2 induced capillary leakage in healthy or tumor bearing mice. However, in latter animals, subcutaneous NMMA therapy alone reduced the growth of primary tumors, formation of spontaneous lung metastasis, as well as tumor induced pulmonary edema. In combination with IL-2 therapy, it did not compromise antitumor effects of IL-2. Chronic oral administration of NMMA, however, succeeded in reducing the severity of

Figure 4.6 Growth of the primary tumor during IL-2, NMMA and IL-2 + NMMA therapy. Data represent tumor diameter, mean \pm SE (d 10: n=17-25; d 13: n=10-17; d 22: n =5-14).

All therapies caused a significant reduction ($p < 0.05$) of the primary tumor size at the end of the first round of therapies (d 13), which was not sustained to the end of the second round (d 22) of therapy.

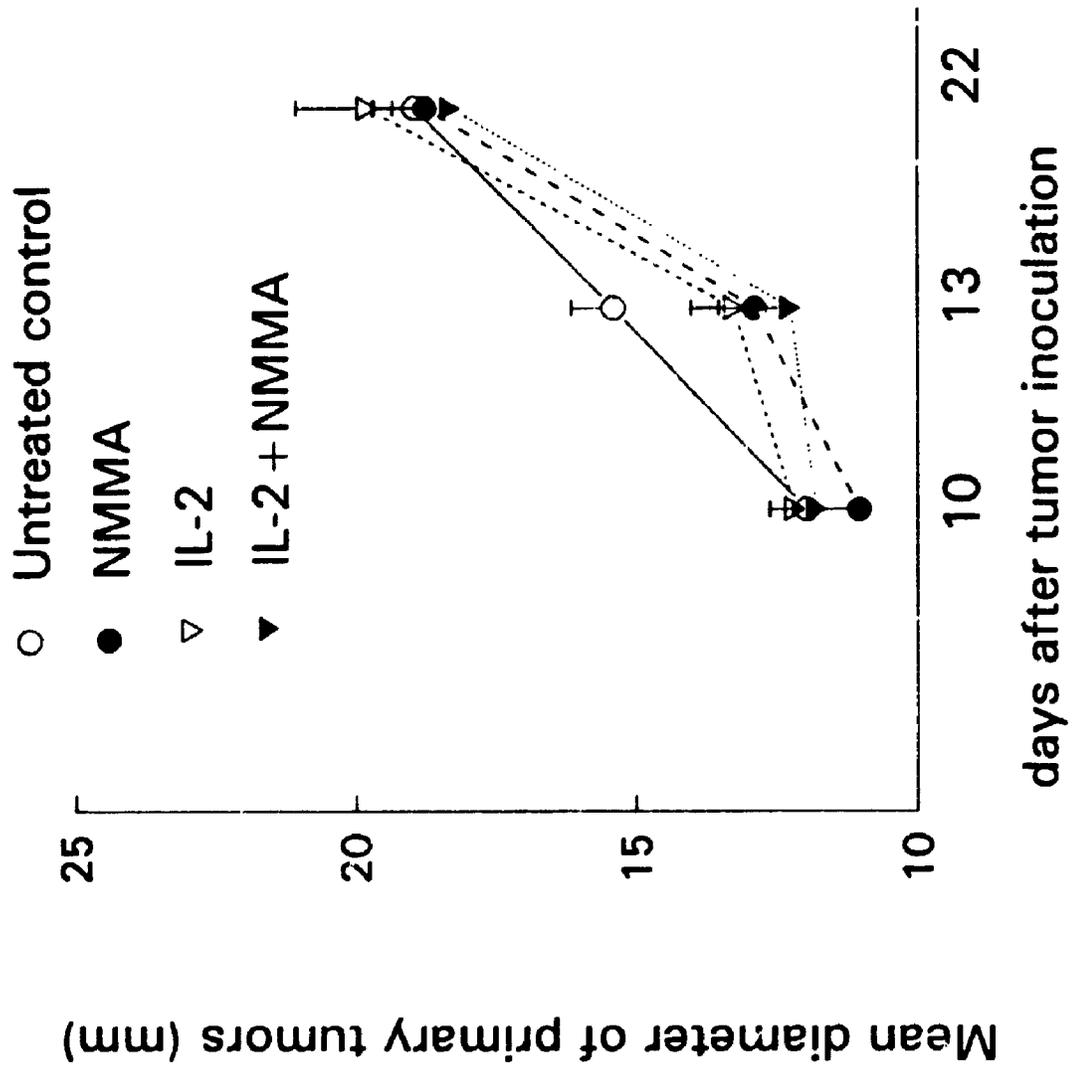
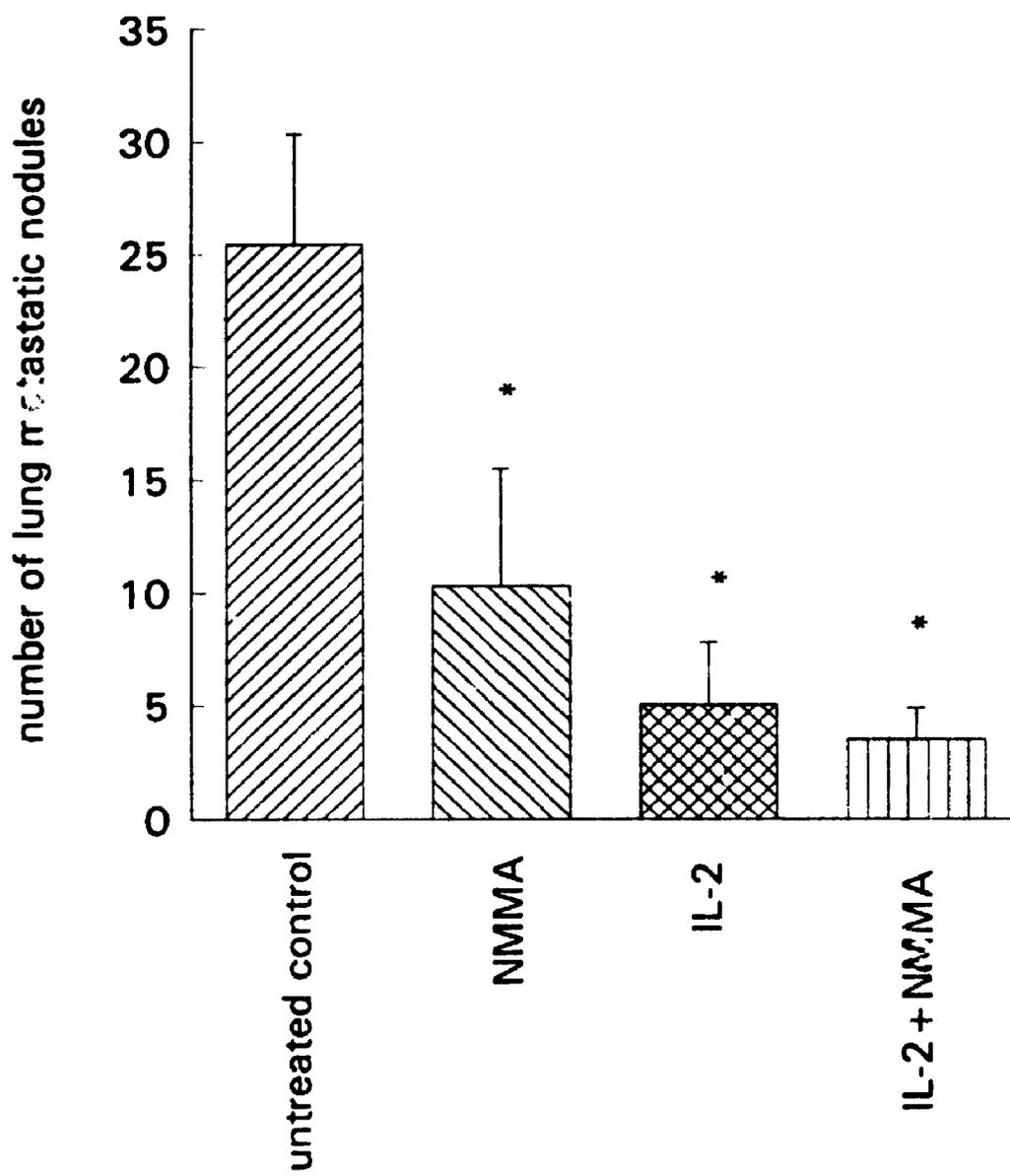


Figure 4.7 Changes in the number of metastatic nodules in the lungs (scored on d 22 of tumor growth) after NMMA, IL-2 and IL-2 + NMMA therapy. Data represent mean \pm SE (n = 7-9).

* indicates significant difference from tumor-bearing control ($p < 0.05$).

All therapies significantly ($p < 0.05$) reduced the number of spontaneous lung metastatic nodules scored at the end of the second round of therapies.



IL-2 induced capillary leakage, as well as the IL-2 induced rise in NO production in healthy mice.

The demonstration of the development of pulmonary edema and pleural effusion with numerous doses of single round of IL-2 in C3H/HeJ mice is in accord with observations by Rosenstein *et al.* in C57BL/6 mice (Rosenstein *et al.* 1986) and Faggioni *et al.*, in C3H/HeJ mice (Faggioni *et al.* 1994), respectively. The resistance of the surviving mice to pleural effusion during the second round of IL-2 in normal as well as tumor bearing mice is a novel finding. One would suggest that pleural endothelium is quicker to recover from the IL-2 induced vascular injury than pulmonary endothelium. Acquisition of resistance to IL-2 induced toxicity has also been noticed in some human patients during repeated rounds of IL-2 therapy (Mertens *et al.* 1993).

Increased water content of the lungs noted in tumor-bearing mice (tumor-induced pulmonary edema) possibly results from the presence of lung metastasis, since tumor microvasculature is often leakier than normal vessels. A lack of aggravation of this edema following the second round of IL-2 therapy in tumor-bearing mice can be explained by antimetastatic effects of IL-2 therapy. A reduction of the metastasis burden would also reduce tumor-induced pulmonary edema and thus mask IL-2 induced pulmonary edema.

Nitric oxide production has been reported to increase during IL-2 therapy of human cancer patients (Hibbs *et al.* 1992; Ochoa *et al.* 1992). In this study, it was shown that IL-2 induced rise in NO production (measured in the serum and pleural effusion) was IL-2 dose dependent. Since protective effects of agents blocking NO synthesis against IL-2 induced hypotension have been reported (Kilbourn *et al.* 1994; Fonseca *et al.* 1994;

Kilbourn *et al.* 1995), it was reasonable to expect that NMMA should prevent fluid leakage, another toxic side effect of high dose IL-2. The present study fulfilled this expectation only when NMMA was given orally, but not subcutaneously, most likely because a continuous administration of the drug via the oral route was effective in blocking the rise in the serum NO level induced by IL-2 therapy (Figure 4.4), whereas the subcutaneous administration, in spite of repeated delivery, was inadequate in fully blocking NO production. These results strongly indicate that NO had a major role in capillary leakage induced by IL-2. It is plausible that the route of administration, as well as scheduling are important determinants of therapeutic efficacy of NO inhibitors in the mitigation of IL-2 induced capillary leakage. Scheduling has also been shown to be an important variable in influencing the outcome of NMMA therapy during septic shock (Petros *et al.* 1994).

Antitumor effects of IL-2 were neither augmented significantly, nor compromised by addition of NMMA. Interestingly, NMMA therapy alone had significant antitumor and antimetastatic effects. These findings are similar to the observations of Kennovin's group with another NOS inhibitor L-NAME (Kennovin *et al.* 1994). They reported growth retarding effects of L-NAME in a rat colonic adenocarcinoma model. These findings indicate that NO had a promoting role on tumor progression in these tumor models. Indeed, high NOS activity has been positively correlated to the progression of a number of human tumors eg. human gynaecological cancer (Thomsen *et al.* 1994), central nervous system tumors (Cobbs *et al.* 1995) and breast cancer (Thomsen *et al.* 1995). The high NOS activity has been explained by the presence of constitutive form(s) in tumor cells (Thomsen *et al.* 1994; Cobbs *et al.* 1995) and / or tumor endothelial cells (Cobbs

et al. 1995), and the inducible form in the tumor endothelial cells (Cobbs *et al.* 1995) and/or tumor associated macrophages (Thomsen *et al.* 1995). Expression of iNOS in the tumor neovasculature has also been reported in experimental tumors (Buttery *et al.* 1993). It is obvious from aforementioned studies that the source of NO within the tumor can be tumor cells themselves, endothelial cells or macrophages within the tumor. In concurrence with these observations, the source of NO in the C3-L5 tumors was investigated and the results are presented in chapter 9.

The relationship of NO to tumor progression still remains controversial. In apparent contradiction to the above data, high NOS activity has been inversely correlated to tumor growth and metastasis in a murine melanoma model, in which an engineered overexpression of iNOS made the cells nontumorigenic, because of NO mediated apoptosis of tumor cells (Xie *et al.* 1995). Similarly, NADPH diaphorase activity (a common correlate of NOS activity) has been reported to have an inverse relationship to human colonic tumor progression (Chhatwal *et al.* 1994). In contrast, numerous human colon cancer cell lines are found to express NOS activity (Jenkins *et al.* 1994), and engineered overexpression of iNOS in a human colonic adenocarcinoma line was found to increase tumor growth when transplanted in nude mice (Jenkins *et al.* 1995). These reported discrepancies may possibly be explained by dual role of NO on tumor growth. Whereas very high NO producing tumor cell clones may delete themselves by apoptosis, NO may facilitate growth of surviving clones *in vivo* by numerous mechanisms, eg. promotion of neoangiogenesis (Buttery *et al.* 1993) and/or tumor blood flow (Andrade *et al.* 1992).

Finally, NO can also have a dual action on host antitumor effector cells. For

example, macrophage mediated cytotoxicity may, in part be NO dependent (Lancaster and Hibbs, 1990). On the other hand, high NO levels in the microenvironment can suppress lymphocyte proliferation (Albina *et al.* 1991) required for the early activation phase. The role of inhibition of IL-2 induced NO production on LAK cell generation *in vivo* and *in vitro* was investigated in this animal model and results are presented in chapter 6.

Findings from this study raised following questions:

1. Can another more potent inhibitor of NO synthesis (L-NAME) given orally prevent IL-2 induced capillary leakage in tumor-bearing mice without compromising the therapeutic benefit of IL-2?
2. Are the route of administration as well as scheduling of NO inhibitors important determinants of their therapeutic efficacy in the mitigation of IL-2 induced capillary leakage?
3. What was the source of NO in C3-L5 mammary adenocarcinoma model?
4. What are the mechanism responsible for antitumor effects of NO inhibition in this animal model?

CHAPTER 5 - N^G-NITRO-L-ARGININE METHYL ESTER AMELIORATES INTERLEUKIN-2-INDUCED CAPILLARY LEAK SYNDROME IN HEALTHY MICE

5.1 Introduction

It has been proposed that NO synthase inhibitors might be useful in the control of hypertension during IL-2 therapy (Hibbs *et al.* 1992; Ochoa *et al.* 1992; Moncada, 1992). However, IL-2-induced capillary leakage in healthy or tumor-bearing mice was not ameliorated by combining subcutaneously administered L-NMMA with systemic IL-2 therapy, although L-NMMA was found to have antitumor effects (Chapter 4). Oral administration of this inhibitor reduced IL-2 induced rise in NO production and the severity of capillary leakage in healthy mice. Because of the prohibitive cost of this inhibitor, it was not feasible to further explore effects of oral NMMA therapy.

N^G-Nitro-L-Arginine methyl ester (L-NAME), is an NO inhibitor shown to be ten times more potent than NMMA (Gardiner *et al.* 1990), because of higher affinity for the NO synthase. L-NAME also exhibits a prolonged ability to inactivate NO synthase (Mayer *et al.* 1993), because of a slower rate of dissociation, and can be given in the drinking water (Gardiner *et al.* 1990; Gardiner *et al.* 1992) at a low cost. For these reasons, the objective of the present study was to test the efficacy of oral L-NAME therapy in reducing capillary leakage induced by IL-2 therapy in healthy mice. The effects of this inhibition upon the development of pulmonary edema, pleural effusion and fluid retention in the spleen and kidney were examined in healthy mice treated with three doses of IL-2. The results were correlated with the degree of inhibition of NO production

by L-NAME.

5.2 Materials and methods

5.2.1 Mice.

C3H/HeJ female mice (7-8 weeks old) were obtained from the Jackson Laboratories. Animal care was described in chapter 3.

5.2.2 Interleukin-2.

Recombinant, highly purified human IL-2 (lot LQP-046) was kindly provided by the Chiron Corporation (Emeryville, CA). The specific activity and the methods of reconstitution were described in chapter 3. In this study, 7,500; 15,000 or 35,000 Cetus Units were the doses used in 0.1 ml per injection. These doses were found previously to induce capillary leakage (Chapter 4), whereas a dose of 750 U/inj did not induce capillary leakage (unpresented data). The reconstituted material was stored at 4°C up to 1 day.

5.2.3 N^G-Nitro-L-Arginine Methyl Ester.

L-NAME obtained from Sigma Chemical Company (St. Louis, MO) was added to the drinking water to provide concentrations of 0.01, 0.1, 0.5 and 1 mg/ml. These doses were based on earlier studies reported in the rat by Moncada's group (Rees *et al.* 1990; Gardiner *et al.* 1992; Gardiner *et al.* 1990).

5.2.4 Experimental protocols.

5.2.4.1 Effects of inhibition of NO synthesis on IL-2-induced capillary leak syndrome.

Healthy mice (n = 5/group) received one of the following treatments: nothing; 7,500, 15,000 or 35,000 Cetus U of IL-2 alone given i.p. every 8 h for 10 injections;

or L-NAME alone at 0.01, 0.1, 0.5 and 1 mg/ml of drinking water starting one day before beginning IL-2 therapy; or a combination of the above doses of IL-2 and L-NAME at the same schedule. Mice were killed 1 h after the last IL-2 injection to measure the water content in the lungs, pleural cavities, spleen and kidney as well as NO_3^- levels in the serum and pleural effusion. Control mice received no treatment, since pilot experiments established that injections with vehicles alone did not influence water content in any of the above organs.

5.2.4.2 Effects of inhibition of NO synthesis on IL-2 induced mortality.

Healthy mice (n = 15/group) received the same treatments as above in order to observe the influence of different therapies on their survival. Autopsy was performed on every dead animal, and the volume of pleural effusion was measured. This experiment was repeated in groups (n=15/group) of mice treated with IL-2 (15,000 U/inj) \pm L-NAME (1 mg/ml of water).

5.2.5 Measurement of capillary leakage.

The left lung, spleen and the left kidney were recovered for measurement of water content, and the water content of each organ was calculated as described in chapter 3.

Liquid was collected from both pleural cavities using 1 ml syringe and the volume of pleural effusion was recorded in an unbiased manner for animals coded by ear-tags.

5.2.6 Measurement of NO production.

The samples of serum and pleural effusion were collected at the end of the therapies to measure the production of NO, the methods were described in Chapter 3.

5.2.7 Statistical analysis.

Data were subjected to analysis of variance using Microstat Statistics Package

(Ecosoft, Inc., Indianapolis, IN) as described earlier (Chapter 3).

5.3 Results

5.3.1 Effects of L-NAME on IL-2 induced pleural effusion.

While all L-NAME doses reduced pleural effusion induced by high dose of IL-2 (35,000 U/inj, $p < 0.05$) only high L-NAME dose (1mg/ml) significantly reduced pleural effusion induced by lower IL-2 dose (15,000 U/inj or 7,500 U/inj, $p < 0.05$) (Figure 5.1). There was no pleural effusion in L-NAME alone treated mice (data not shown).

5.3.2 Effects of L-NAME on IL-2 induced pulmonary edema

L-NAME (0.1 mg/ml or more) significantly reduced IL-2 (35,000 U/inj or 15,000 U/inj) induced pulmonary edema in a dose dependent manner, the edema being abolished at a dose of 1 mg/ml (Figure 5.2). The low dose of IL-2 (7,500 U/inj) also induced pulmonary edema, which was not affected by addition of L-NAME (at any of the doses tested). L-NAME therapy alone did not have any effect on the water content in the lungs (Table 5.1.).

5.3.3 Effects of L-NAME on IL-2 induced water retention in the spleen

L-NAME at higher doses significantly reduced IL-2 induced water retention in the spleen, but not to the control level (Figure 5.3). The reduction was significant at 1 mg/ml of L-NAME for all IL-2 doses and at 0.5 mg/ml for the lowest IL-2 dose. L-NAME therapy alone resulted in a small but significant ($p < 0.05$) decline in the water content of the spleen (Table 5.1.).

5.3.4 Effects of L-NAME on IL-2 induced fluid retention in the kidney

IL-2 therapy at all three doses induced significant fluid retention in the kidney. However, addition of L-NAME did not have any effect on this IL-2 induced fluid

Figure 5.1 Pleural effusion after IL-2 and L-NAME therapy.

Data represent mean \pm SE (n=5).

* indicates significant difference from IL-2 treatment ($p < 0.05$).

L-NAME (0.01, 0.1 or 1 mg/ml of drinking water) significantly ($p < 0.05$) reduced IL-2 (35,000 U/inj) induced pleural effusion. Significant reduction ($p < 0.05$) of pleural effusion induced by lower IL-2 dose (15,000 U/inj or 7,500 U/inj) was noticed only with high L-NAME dose (1 mg/ml of drinking water). Neither control (untreated) nor L-NAME alone treated mice showed any pleural effusion (data not shown).

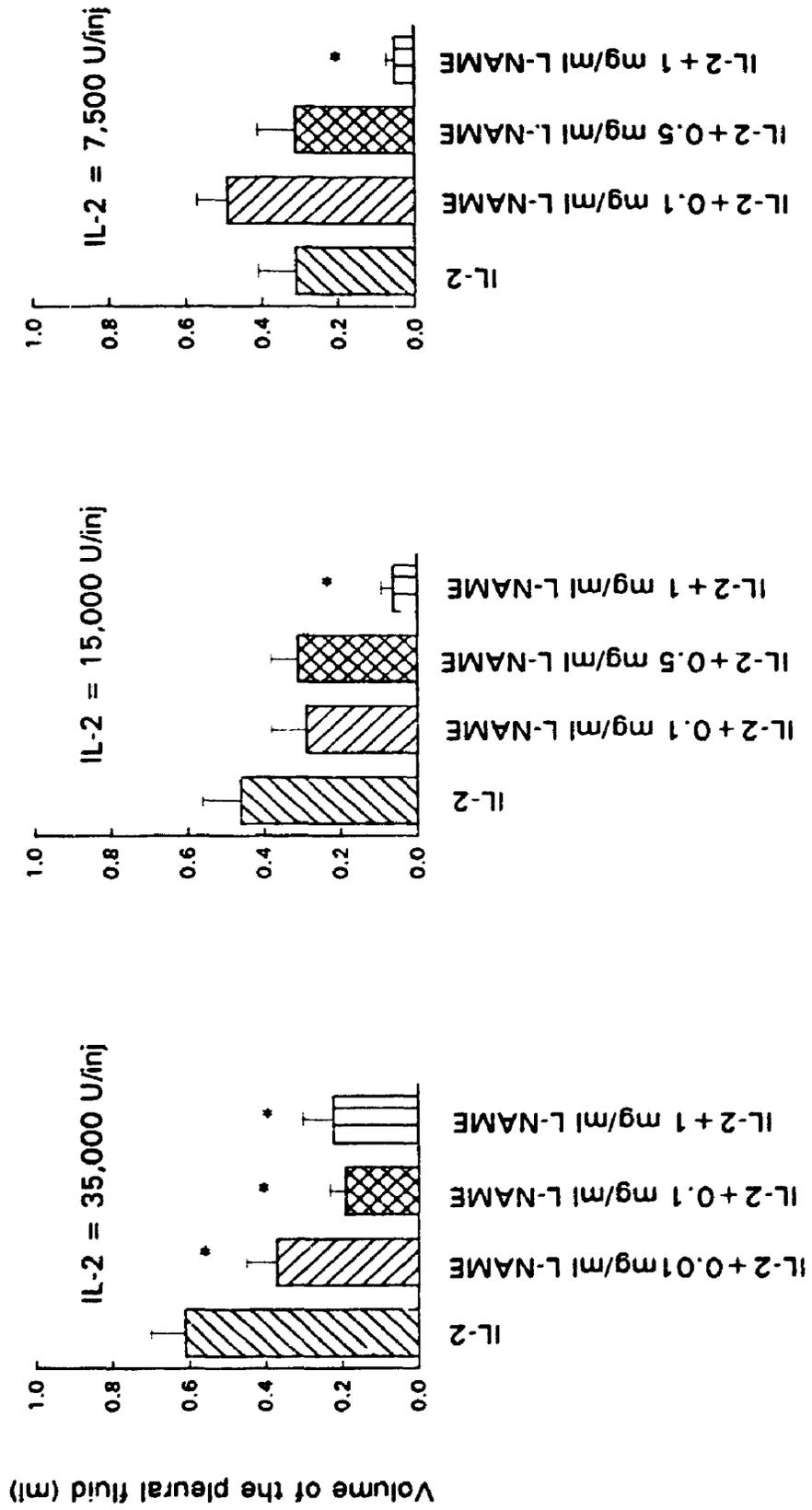


Figure 5.2 Water content of the lungs after IL-2 and L-NAME therapy.

Data represent mean \pm SE (n = 5).

* indicates significant difference from control ($p < 0.05$).

** indicates significant difference from IL-2 treatment ($p < 0.05$).

IL-2 (35,000 U/inj or 15,000 U/inj) induced pulmonary edema was significantly ($p < 0.05$) reduced with addition of L-NAME (0.1 mg/ml or more) in a dose dependent manner, being abolished at a dose of 1 mg/ml. Low dose of IL-2 (7,500 U/inj) also induced pulmonary edema, but addition of L-NAME did not have any significant effect.

Wet/dry weight ratios of the lungs

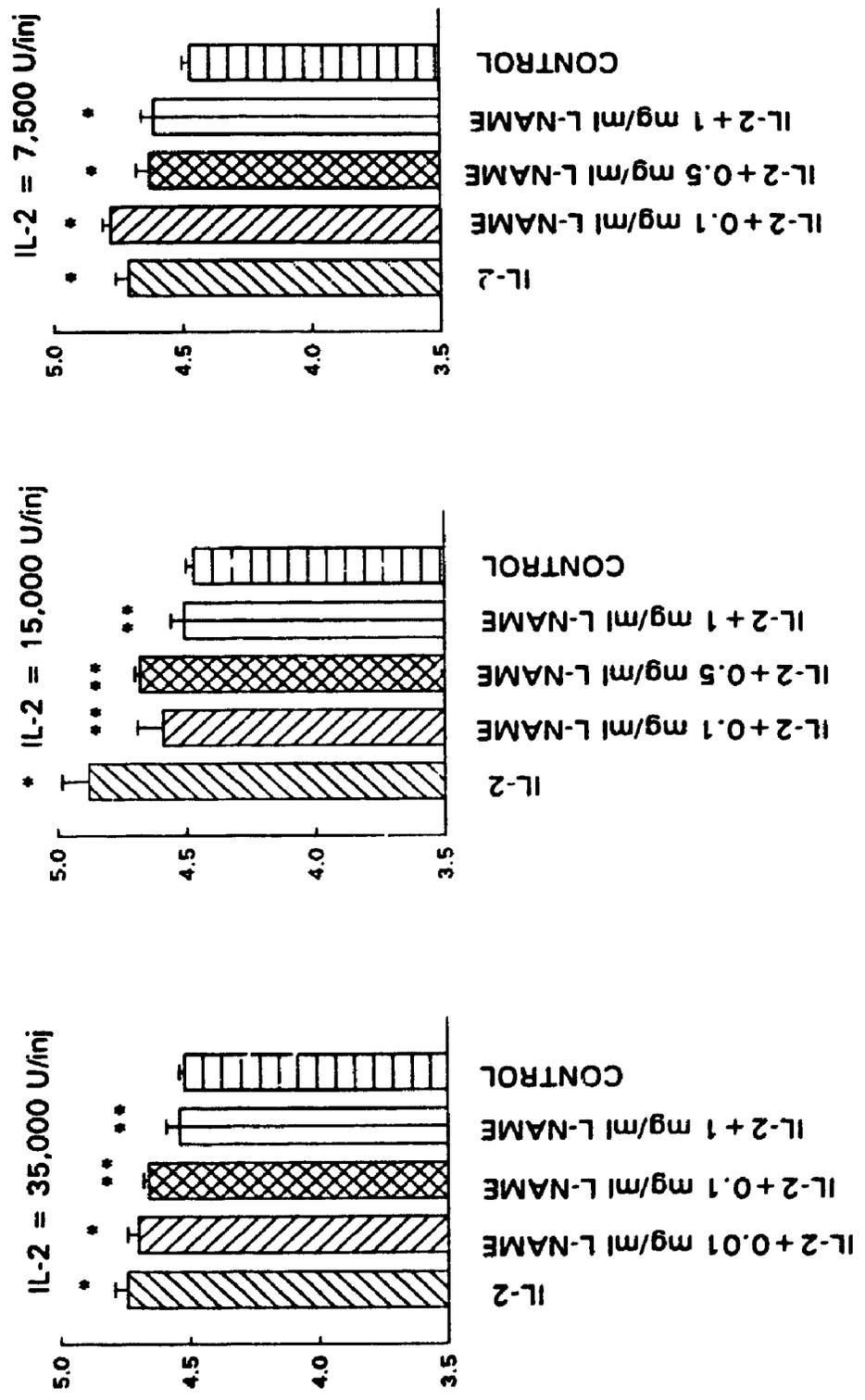


Table 5.1 Influence of L-NAME on the water content of the lungs, spleen and kidneys as indicated by the wet/dry weight ratio (mean \pm SE)^a

	lung	spleen	kidney
Control	4.19 \pm 0.04	4.31 \pm 0.04	3.78 \pm 0.02
0.1 mg/ml L-NAME ^b	4.16 \pm 0.01	4.15 \pm 0.02 ^c	3.71 \pm 0.03
0.5 mg/ml L-NAME ^b	4.19 \pm 0.03	4.20 \pm 0.02 ^c	3.73 \pm 0.04
1 mg/ml L-NAME ^b	4.12 \pm 0.05	4.13 \pm 0.01 ^c	3.67 \pm 0.02

^a n = 5 in each group.

^b Animals were given 0.1, 0.5 or 1 mg/ml of L-NAME in drinking water for 4 days.

Controls drank tap water.

^c Significantly lower than the control value ($p < 0.05$).

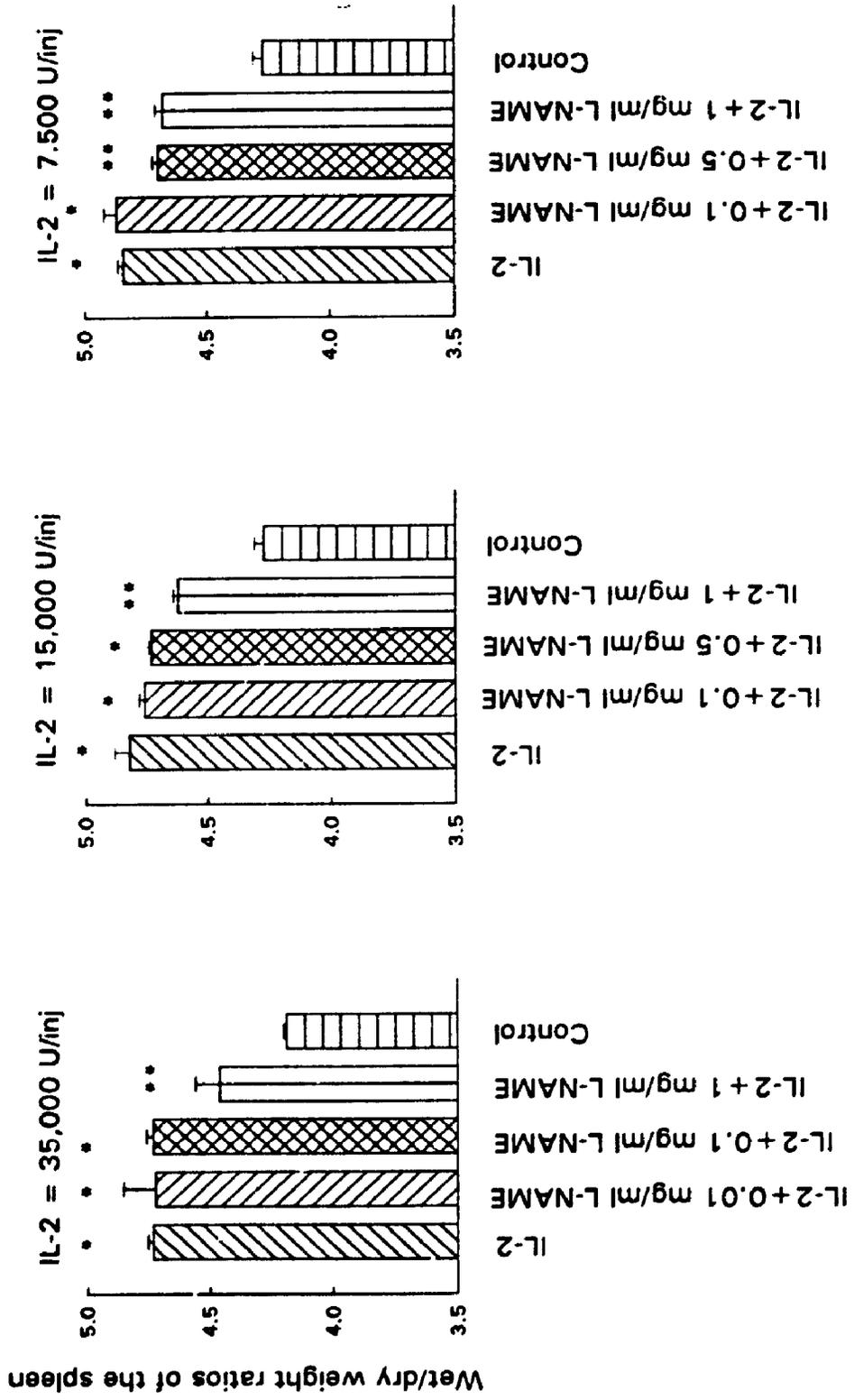
Figure 5.3 Water content of the spleen after IL-2 and L-NAME therapy.

Data represent mean \pm SE (n = 5).

* indicates significant difference from control ($p < 0.05$).

** indicates significant difference from IL-2 treatment ($p < 0.05$).

IL-2 (35,000 U/inj, 15,000 U/inj or 7,500 U/inj) - induced water retention in the spleen was significantly ($p < 0.05$) decreased with addition of 1 mg/ml of L-NAME to drinking water; 0.5 mg/ml of L-NAME was only effective against the lowest IL-2 dose.



retention (Figure 5.4). Water content in the kidney remained unaffected with L-NAME therapy alone (Table 5.1.).

5.3.5 Effects of L-NAME on IL-2 induced NO production in the serum and pleural effusion

Higher doses of IL-2 (35,000 U/inj or 15,000 U/inj) induced an increase in NO production (measured as NO_3^- levels) in the serum. This increase was ameliorated by L-NAME (0.1 mg/ml or more) in a dose dependent manner, reducing it to the control levels at a dose of 1 mg/ml, when combined with the higher IL-2 dose (35,000 U/inj) (Figure 5.5.). NO_3^- levels in the serum after therapy with low dose of IL-2 (7,500 U/inj) were not significantly different from the control levels. Addition of different doses of L-NAME did not change these levels further. Experiments were done at different times (treatment with 35,000 and 7,500 U of IL-2 at the same time, but 15,000 U separately), so the results on the effects of L-NAME on NO_3^- levels should be compared only within the particular experiment. In a separate IL-2 dose response experiment, IL-2 in above doses induced a dose dependent rise in NO production in the serum (data presented in Chapter 4, Figure 4.3.). Treatment with different doses of L-NAME alone did not have any effect on NO_3^- levels in the serum (data not shown).

Addition of L-NAME (0.1 mg/ml or more) significantly reduced IL-2 (35,000 U/inj or 15,000 U/inj) induced increase in NO production (measured as NO_3^- levels) in the pleural effusion (Figure 5.6.). L-NAME did not significantly influence NO_3^- levels in the pleural effusion induced by low IL-2 dose (7,500 U/inj). These experiments were done at the same time, and can be compared with one another.

Figure 5.4 Water content of the kidney after IL-2 and L-NAME therapy.

Data represent mean \pm SE (n = 5).

* indicates significant difference from control ($p < 0.05$).

IL-2 (35,000 U/inj, 15,000 U/inj or 7,500 U/inj) induced significant increase in water content of the kidney, which was not significantly influenced by the addition of any of the L-NAME doses.

Wet/dry weight ratios of the kidneys

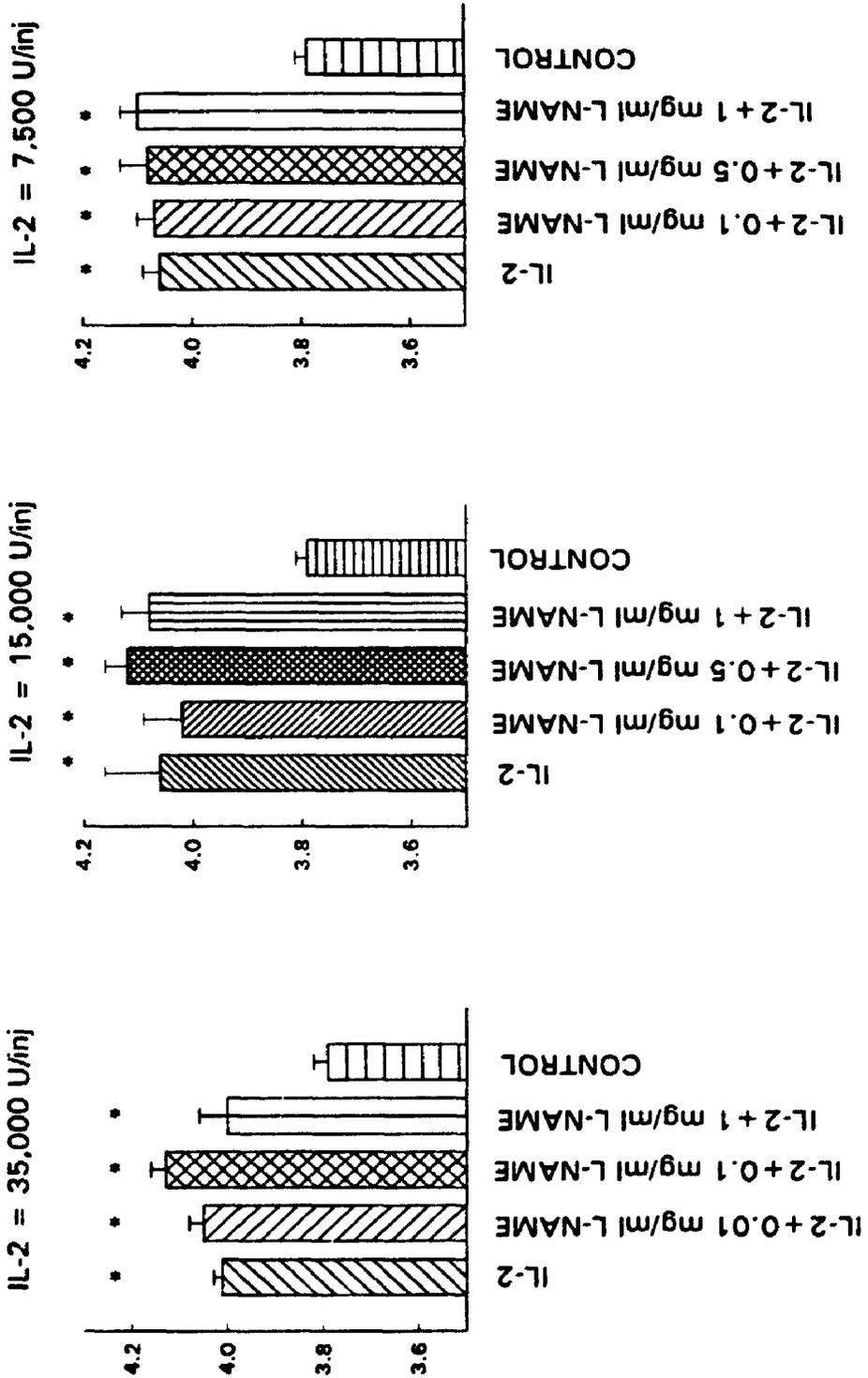


Figure 5.5 $\text{NO}_2^- + \text{NO}_3^-$ levels in the serum after IL-2 and L-NAME therapy ($10^{-6} \times \text{M} = \mu\text{M}$).

Data represent mean \pm SE (n = 3-5, each done in duplicate).

Experiments A and B were not conducted at the same time.

* indicates significant difference from control ($p < 0.05$).

** indicates significant difference from IL-2 treatment ($p < 0.05$).

Higher doses of IL-2 treatment (15,000 U/inj and 35,000 U/inj) induced an increase in NO production (measured as $\text{NO}_2^- + \text{NO}_3^-$ levels in the serum). Addition of L-NAME (0.1 mg/ml or more) significantly reduced it in a dose dependent manner, reducing it to the control level at a dose of 1 mg/ml, when added to higher IL-2 dose (35,000 U/inj). Low dose of IL-2 (7,500 U/inj) as well as L-NAME therapy alone (data not shown) did not significantly influence $\text{NO}_2^- + \text{NO}_3^-$ levels in the serum.

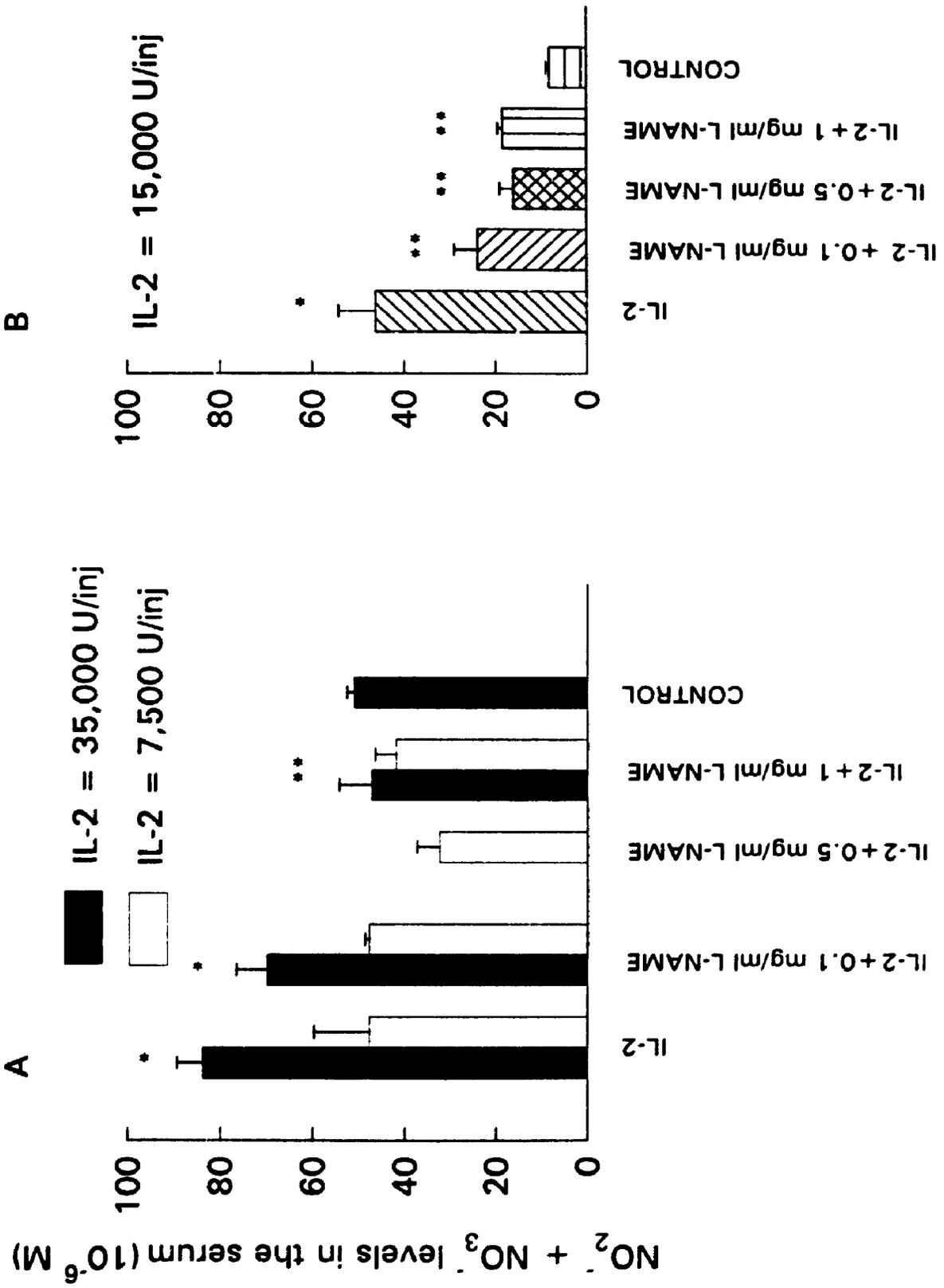
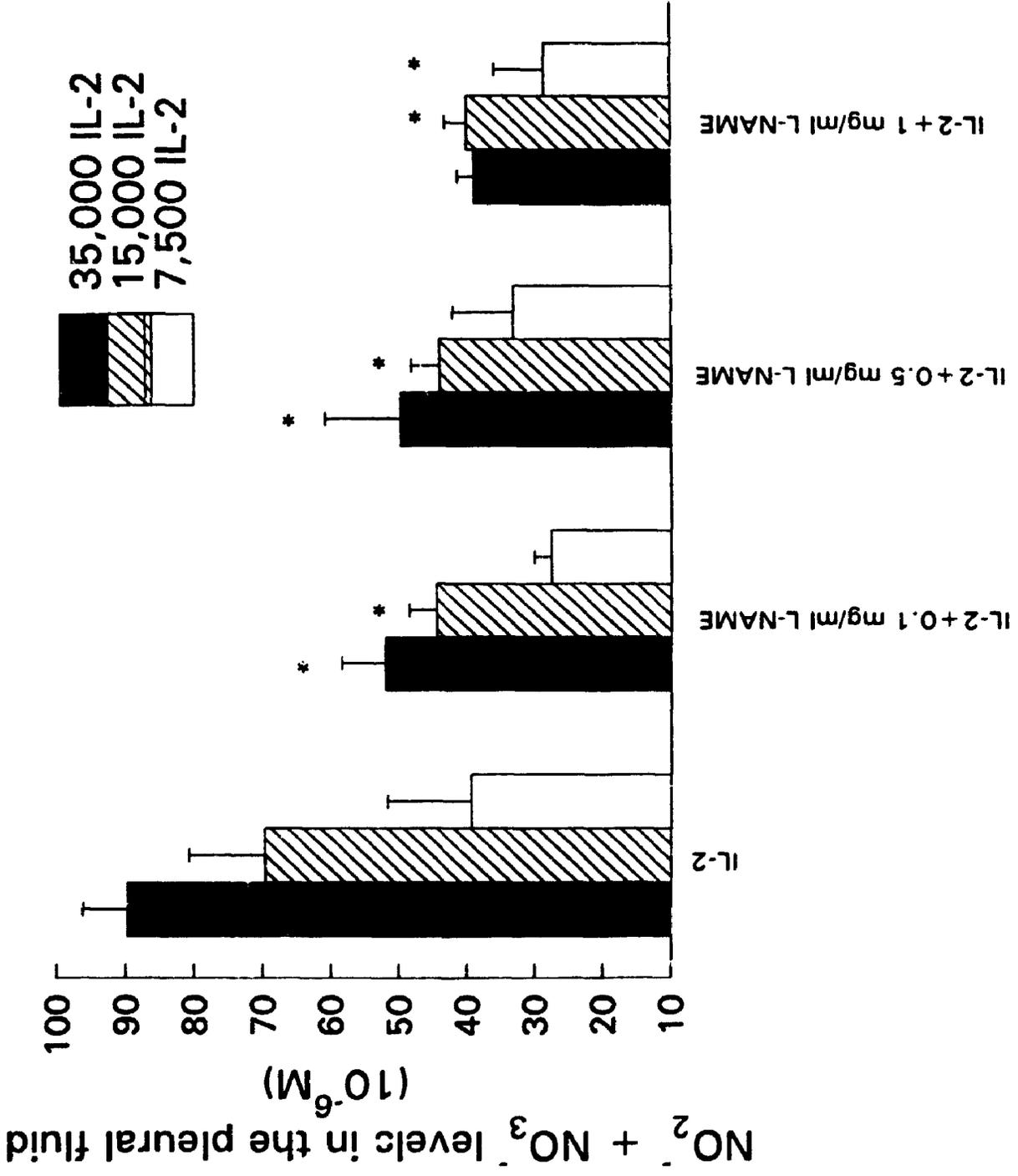


Figure 5.6 $\text{NO}_2^- + \text{NO}_3^-$ levels in the pleural effusion after IL-2 and L-NAME therapy ($10^{-6} \times \text{M} = \mu\text{M}$).

Data represent mean \pm SE (n = 3-5, each done in duplicate).

* indicates significant difference from IL-2 treatment ($p < 0.05$).

IL-2 (15,000 U/inj or 35,000 U/inj) - induced dose dependent increases in $\text{NO}_2^- + \text{NO}_3^-$ levels in the pleural effusion were significantly ($p < 0.05$) reduced with addition of L-NAME (0.1 mg/ml or more). L-NAME did not have any effects on the $\text{NO}_2^- + \text{NO}_3^-$ levels induced by low IL-2 dose (7,500 U/inj).



5.3.6 Effects of L-NAME on mortality of animals treated with IL-2

IL-2 induced mortality with the present regimens was: 53% with the highest (35,000 U/inj), 50% with the intermediate (15,000 U/inj) and 13% with the lowest (7,500 U/inj) IL-2 dose. This was not significantly influenced by addition of L-NAME (at any dosage) to the highest or the lowest IL-2 dose. A significant reduction in mortality (by 46%; $p=0.035$) was noted only by addition of the highest L-NAME (1 mg/ml) dose to the intermediate (15,000 U/inj) IL-2 dose. Animals died within 4 days after completion of the therapies. Treatment with L-NAME alone at any dosage caused no morbidity or mortality in mice (data not shown).

Quantity of IL-2 induced pleural effusion noted post-mortem was significantly reduced with the addition of L-NAME (Figure 5.7), and thus pleural effusion did not appear to be the primary cause of death in animals subjected to the combination therapies. Haemorrhages in the mesentery were consistently noted in these mice at necropsy, thus may have accounted for the mortality in animals subjected to the combination therapy. There was no such haemorrhage observed at necropsy of mice treated with IL-2 alone, so that the primary cause of death in these animals appeared to be pleural effusion, confirming earlier findings in IL-2 treated healthy mice (Chapter 4).

5.4 Discussion

The present study clearly reveals that oral treatment with L-NAME, a potent and inexpensive inhibitor of NO synthesis, can ameliorate IL-2-induced capillary leakage in healthy mice.

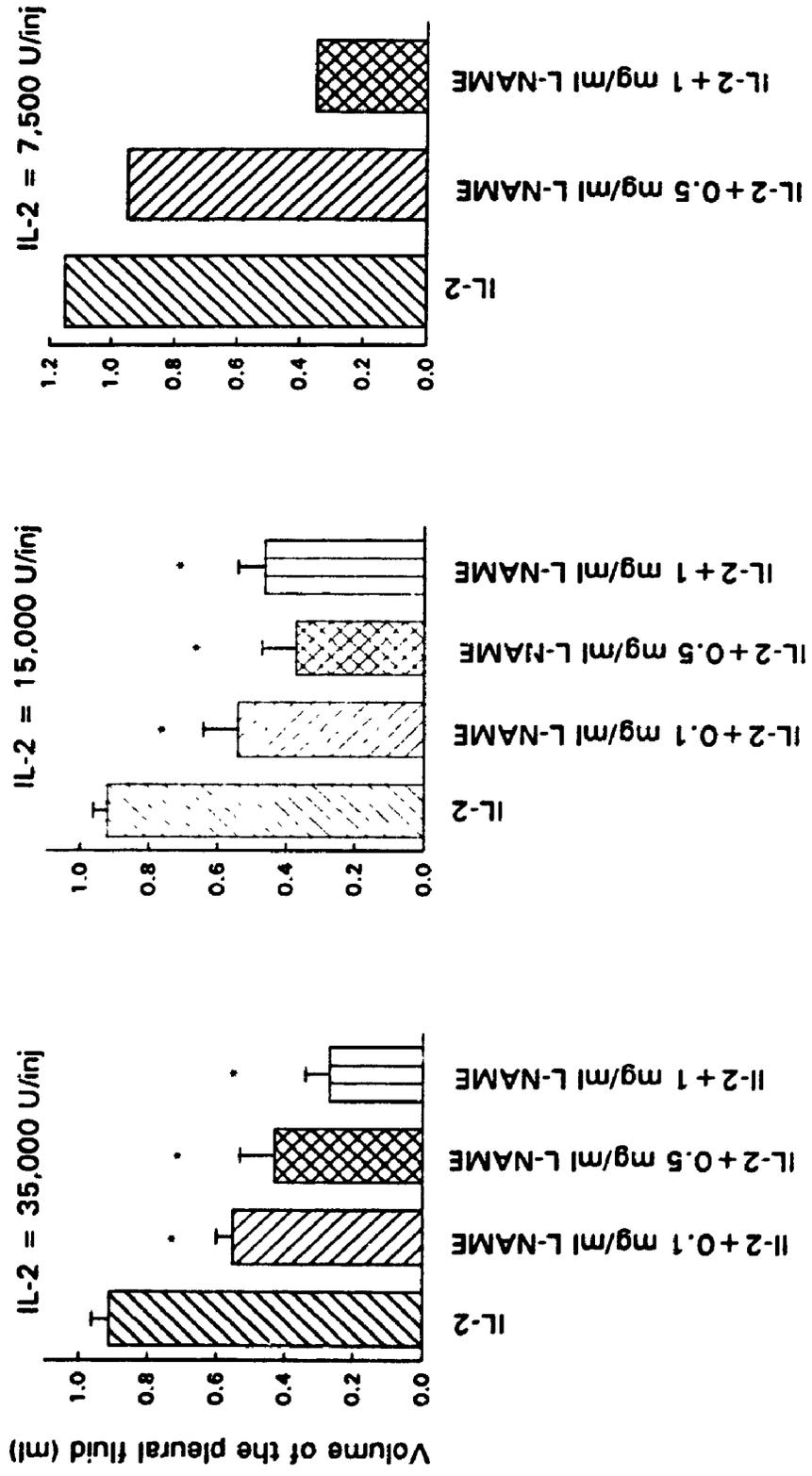
Excessive NO production has been implicated in septic and endotoxic shock

Figure 5.7 Pleural effusion in mice that died after IL-2 and L-NAME therapy.

Data represent mean \pm SE when $n \geq 3$, or mean when $n \leq 3$ (IL-2 = 7,500 U/inj).

* indicates significant difference from IL-2 treatment ($p < 0.05$).

IL-2-induced pleural effusion noted at post-mortem was significantly ($p < 0.05$) reduced with the addition of L-NAME.



(Petros *et al.* 1991; Kilbourn *et al.* 1990; Wright *et al.* 1992) and reported in human cancer patients receiving IL-2 therapy (Hibbs *et al.* 1992; Ochoa *et al.* 1992). It has also been shown that damage of endothelial cells by *in vitro* exposure to IFN γ and TNF α (both cytokines induced by IL-2 therapy) is due to excessive NO production (Palmer *et al.* 1992; Estrada *et al.* 1992). These findings, taken together, suggested the potential value of NO synthase inhibitors for controlling the toxic effects of IL-2 therapy. Indeed, Fonseca and colleagues (1994) reported that L-NMMA prevented hypotension in cancer patients treated with IL-2. However, no effect on capillary leakage was evaluated in that study. The results from previous chapter (chapter 4) showed that repeated subcutaneous injections of NMMA did not succeed in controlling IL-2-induced capillary leakage, but once administered orally, NMMA succeeded in reduction of pulmonary edema. Therefore L-NAME, more potent inhibitor of NO synthase (Gardiner *et al.* 1990; Gardiner *et al.* 1992) was tested in the present animal model.

All doses of IL-2 employed in the present study induced capillary leak syndrome as indicated by pleural effusion and fluid retention in the lungs, spleen and kidneys. Furthermore, NO $_3^-$ levels in the pleural effusion were directly related to IL-2 dose. L-NAME therapy significantly mitigated all the manifestations of IL-2 induced capillary leakage, except fluid retention in the kidney. A decrease in the water retention in the lungs was accompanied by a significant reduction in the thickness of the interalveolar connective tissue space (as measured by computerized histomorphometry; data presented in chapter 8) indicated that there was a true reduction in the IL-2 induced pulmonary edema. There was a concomitant reduction of IL-2 induced NO production in the serum and pleural effusion. These results, combined with previous findings of mitigation of IL-2

induced capillary leakage with oral NMMA, strongly indicated that NO had a major role in capillary leakage induced by high dose IL-2. It is therefore possible that earlier reported protective effects of glucocorticoids from IL-2 induced capillary leakage (Rosenstein *et al.* 1986; Faggioni *et al.* 1994) were most likely due to inhibition of inducible NO synthase (Moncada and Higgs, 1993; Knowles and Moncada, 1994), in addition to a general reduction of inflammation (Rosenstein *et al.* 1986) or an inhibition of TNF production (Faggioni *et al.* 1994).

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 (antidipsogenic effect): (a) it was noted that higher doses of L-NAME (eg. 1 mg/ml), as well as the present doses of IL-2 led to a significant reduction in water consumption (45% to 60% of pre-treatment values at the end of the treatments). However, the water consumption in IL-2 treated mice remained unaffected by the addition of L-NAME. Indeed, if the water consumption in IL-2 and L-NAME treated mice had remained normal, the therapeutic effects of L-NAME in reducing capillary leakage would have likely been more pronounced. (b) In spite of antidipsogenic effects, L-NAME therapy alone did not influence the water content of the lungs and kidneys.

IL-2-induced fluid retention in the kidney was not mitigated by L-NAME therapy in the present study. IL-2 therapy can lead to renal dysfunctions as indicated by low sodium excretion, elevated plasma renin activity and in severe cases, azotemia and oliguria (Textor *et al.* 1987). Reduced sodium excretion and glomerular damage have also been reported during chronic inhibition of NO synthesis (Baylis *et al.* 1992). This

may account for the negative results with additional L-NAME therapy. It was also noticed in the present study that treatment with L-NAME (0.5 and 1 mg/ml) mildly aggravated IL-2-induced oliguria (data not shown), indicating that kidney function should be closely monitored in humans receiving L-NAME.

The combination of L-NAME to IL-2 therapy reduced IL-2 induced mortality only at the intermediate IL-2 dose level. This was not the case at the high IL-2 dosage in spite of a reduction of IL-2 induced capillary leakage by L-NAME therapy. Haemorrhages in the mesentery appeared to be the main cause of death in these animals receiving the combination of IL-2 and L-NAME therapy, whereas pleural effusion was the main cause of death in animals treated with IL-2 alone. Since gastrointestinal pathology was not observed with L-NAME alone, or with IL-2 alone, it is reasonable to ascribe these changes to the combination therapy. Similar pathology have been reported earlier in rat intestine following LPS treatment (Laszlo *et al.* 1994). In that study, initial suppression of the constitutive NO synthase by L-NAME aggravated endotoxin-induced vascular injury in rat intestine, but delayed administration of NO synthase inhibitors provided protection against the consequent damage to the intestinal vasculature. In a preliminary study (data not presented), a delayed administration of 0.1 mg/ml of L-NAME reduced only IL-2 induced pleural effusion, and had no effect on other parameters of capillary leakage. It remains to be established whether a delayed administration of L-NAME can prevent mortality induced by high dose IL-2 or avoid presently observed intestinal damage.

Route of administration was important for this inhibitor as well, because in a pilot study, subcutaneously administered L-NAME did not ameliorate IL-2 induced capillary

leakage (data not presented).

In summary, L-NAME therapy given orally significantly mitigated the rise in NO level in the body fluids as well as the capillary leakage induced by IL-2 therapy in healthy mice. The results from this study led to the new sets of experiments which were designed to test whether L-NAME can prevent IL-2 induced capillary leak syndrome in tumor-bearing mice without compromising the therapeutic effects of IL-2 therapy.

CHAPTER 6 - N^G-NITRO-L-ARGININE METHYL ESTER AMELIORATES INTERLEUKIN-2 TOXICITY AND AUGMENTS ANTITUMOR EFFECTS OF INTERLEUKIN-2 THERAPY IN TUMOR BEARING MICE

6.1 Introduction

There have been several reports of therapies that combined IL-2 with other agents in order to ameliorate the capillary leakage. However, the added drugs also opposed the antitumor effects of IL-2. Corticosteroids (Rosenstein *et al.* 1986; Faggioni *et al.* 1994), which suppress inflammatory responses and production of NO (Moncada and Higgs, 1993), and Asialo-GM-1 antibody, which depletes LAK cells (Ettinghausen *et al.* 1988), both fall in this category. Puri *et al.* (1989) 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 edema in the rat, presumably by causing cytoskeletal changes in neutrophils, with consequent suppression of endothelial injury by neutrophil-derived thromboxane B₂. Interactions of these agents with the antitumor effect of IL-2 remain unknown. Further studies were therefore called for identification of substances that can ameliorate capillary leakage without reducing the antitumor effects of IL-2.

It was demonstrated so far, that NMMA (an inhibitor of NO synthesis) given subcutaneously did not ameliorate IL-2 induced capillary leakage in tumor-bearing mice, but it improved antitumor effect of IL-2 in mice (chapter 4). It was also found that L-NAME (another NO synthase inhibitor, more potent than NMMA) given orally could effectively prevent capillary leakage induced by IL-2 in healthy mice (chapter 5).

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

6.2 Materials and methods

6.2.1 Mice.

C3H/HeJ female mice, 6-7 weeks old, were obtained from the Jackson Laboratories. Animal care was described in chapter 3.

6.2.2 Interleukin-2.

Highly purified recombinant human IL-2 (lot LQP-046) was kindly provided by the Chiron Corporation (Emeryville, CA), the activity and reconstitution of which were described in chapter 3. In this particular study, 15,000 Cetus Units in 0.1 ml (volume per injection) was used. The reconstituted IL-2 was stored at 4°C up to 1 day.

6.2.3 N^G-Nitro-L-Arginine Methyl Ester.

L-NAME (Sigma Chemical Company, St. Louis, MO) was added to the drinking water to provide concentrations of 0.1, 0.5 and 1 mg/ml of water. These doses were based on the studies conducted earlier to prevent IL-2 induced capillary leak syndrome in healthy mice (chapter 5). In one additional experiment, D-form of NAME (1 mg/ml of water; obtained also from Sigma) was used in order to demonstrate the specific effects of L-NAME as an inhibitor of NO synthase.

6.2.4 Tumor-cell line.

C3-L5 mammary adenocarcinoma line, originated and maintained in Dr. Lala's laboratory was used in this study (Lala and Parhar, 1993; Brodt *et al.* 1985; see also

description in chapter 3).

6.2.5 Tumor 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 tumors, this procedure was expected to produce micrometastases in the lungs of C3H/HeJ mice within 2 weeks after transplantation.

6.2.6 Protocols for immunotherapy.

Tumor transplanted mice, randomly separated into 8 groups ($n = 10-20$), were treated with: nothing, IL-2 (10 inj. 15,000 Cetus U i.p. every 8h), L-NAME (0.1 mg/ml of drinking water), L-NAME (0.5 mg/ml of drinking water), L-NAME (1 mg/ml of drinking water), IL-2 + 0.1 mg/ml L-NAME, IL-2 + 0.5 mg/ml L-NAME, IL-2 + 1 mg/ml L-NAME. Therapies were given in one round (IL-2: d 10-13; L-NAME: d 9-13), or in two rounds (IL-2: d 10-13 and 20-23; L-NAME: d 9-13 and d 19-23) after tumor transplantation. In one additional experiment, mice ($n=5$) were treated with IL-2 or IL-2+1mg/ml D-NAME (IL-2: d 10-13; D-NAME: d 9-13). Mice were killed after one or two rounds of treatments (1h after last IL-2 inj.) to measure capillary leakage and antitumor effects of therapies. Some of the animals that were subjected to the first round of therapy did not survive to the beginning of the second round, because of IL-2 induced toxicity. Necropsy was performed on all dead animals and the quantity of pleural effusion was measured.

6.2.7 Measurement of capillary leakage and NO metabolites.

The left lung, the spleen and the left kidney were recovered for measurement of their water content. Water content of each organ was expressed as wet/dry weight ratio

(calculations described in chapter 3).

The volume of liquid from both pleural cavities was measured directly by taking it all out with a 1 ml syringe.

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).

6.2.8 Measurement of antitumor effects.

The size of the primary tumors was measured with callipers on d 9, 14, 19 and 23, by recording the maximum and minimum diameters. Tumor volumes were then calculated as $0.52a^2b$, where a and b are the minimum and maximum diameters (Baguley *et al.* 1989), and the tumor 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.

6.2.9 Statistical analysis.

The Microstat statistics package was used in the analysis of collected data, as described in chapter 3.

6.3 Results

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

IL-2-induced pleural effusion was not significantly changed with the addition of different doses of L-NAME, measured either immediately after the end of the first round

of therapies, or in animals which died after the first round of therapies (data not presented). There was a trend in the reduction of IL-2 induced pleural effusion with addition of different doses of L-NAME in the latter case, which was not significant. There was no pleural effusion after the second round of therapies. L-NAME therapy alone did not induce pleural effusion (data not shown).

Addition of L-NAME (0.1 mg/ml or more) significantly ($p < 0.05$) reduced IL-2-induced pulmonary edema after the first round of therapies (Figure 6.1 - left). After the second round of therapies, tumor-bearing by itself induced a significant increase in the water content in the lungs which was significantly ameliorated only by L-NAME therapy alone ($p < 0.01$)(Figure 6.1 - right).

IL-2-induced water retention in the spleen was significantly ($p < 0.001$) decreased with addition of L-NAME in a dose dependent manner after the first round of therapies (Figure 6.2 - left). After the second round of therapies, different doses of L-NAME did not significantly influence IL-2-induced water retention in the spleen (Figure 6.2 - right). 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 6.2).

After the first or second round of therapies, IL-2 induced fluid retention in the kidney remained unaffected with addition of any of the L-NAME doses (Figure 6.3.- left and right). Tumor-bearing by itself induced significant increase in the water content in the kidney (Figure 6.3 - left and right), which was significantly ($p < 0.05$) reduced by L-NAME therapy alone, but only after the first round of therapy (Figure 6.3 - left).

6.3.2 Effects of L-NAME on animal morbidity due to IL-2 toxicity

After the first round of treatments, some animals died from IL-2 induced toxicity

Figure 6.1 Water content of the lungs after IL-2 and L-NAME therapy (left: one round of therapies, right: two rounds of therapies).

Data represent mean \pm SE (left: n = 10; right: n = 9-12)

* Addition of L-NAME significantly ($p < 0.05$) reduced IL-2 induced pulmonary edema after the first round of therapies.

** Tumor-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 tumor induced water retention in the lungs.

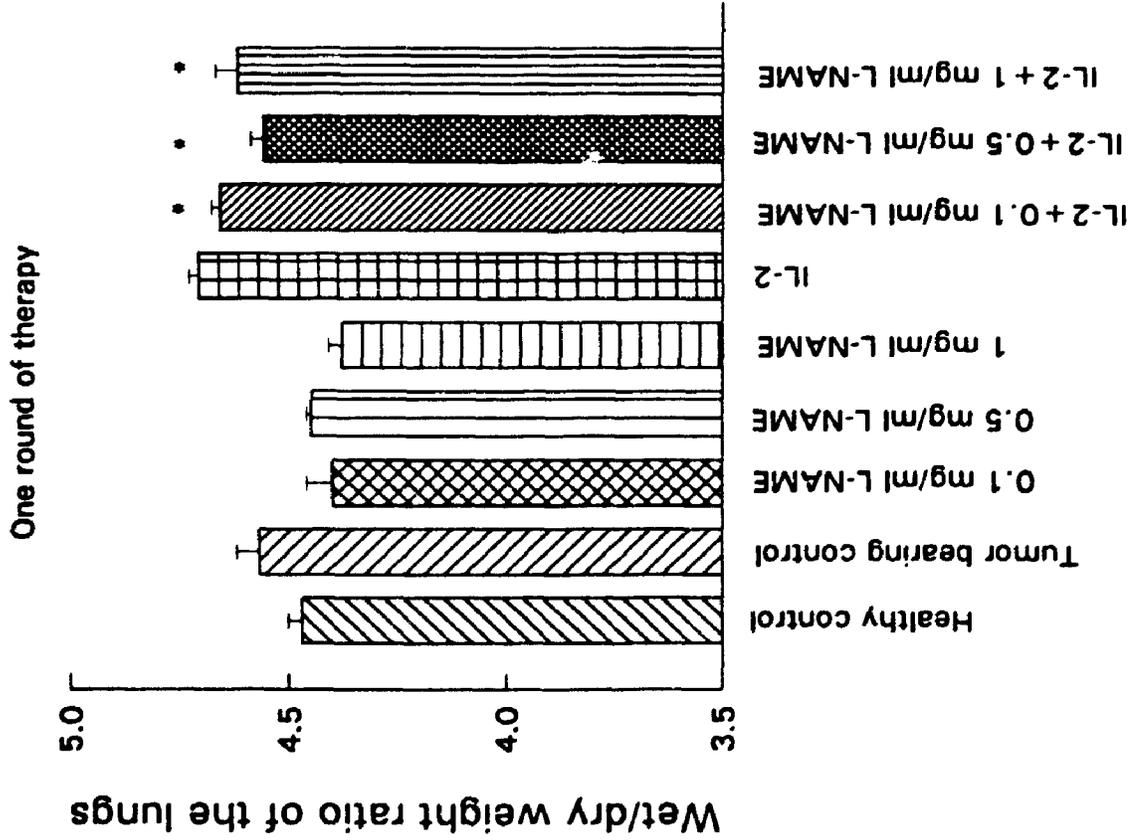
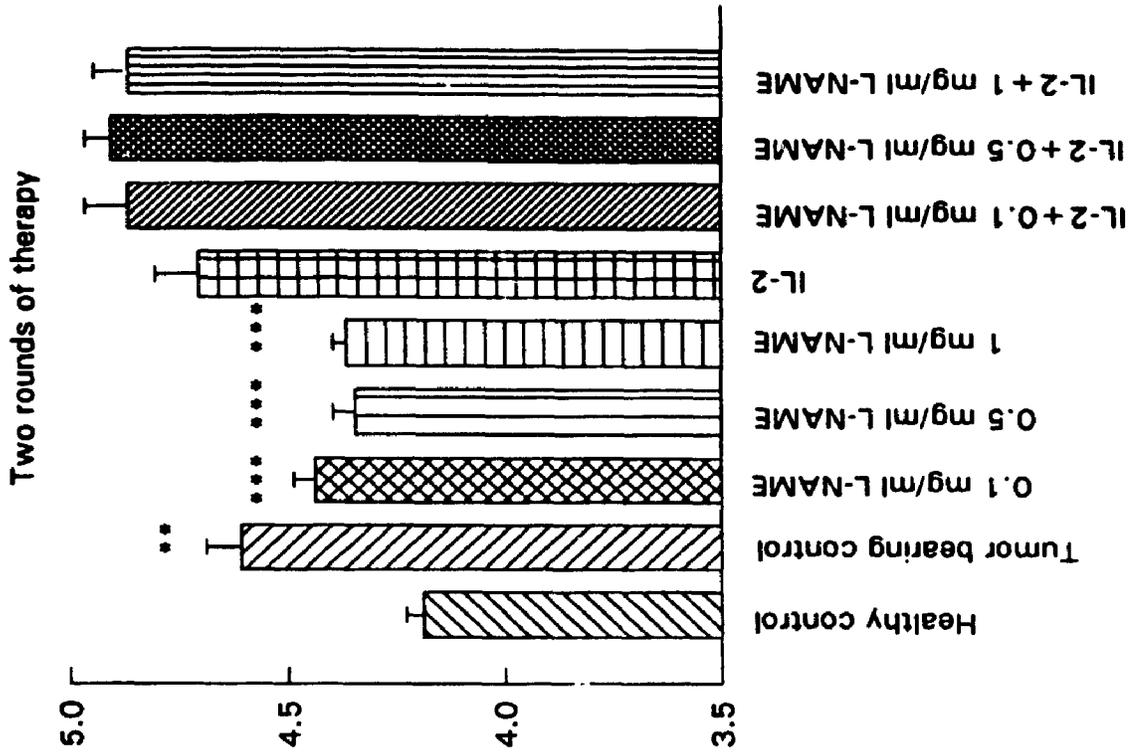


Figure 6.2 Water content of the spleen after IL-2 and L-NAME therapy (left: one round of therapies; right: two rounds of therapies).

Data represent mean \pm SE (left: n = 10; right: 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.

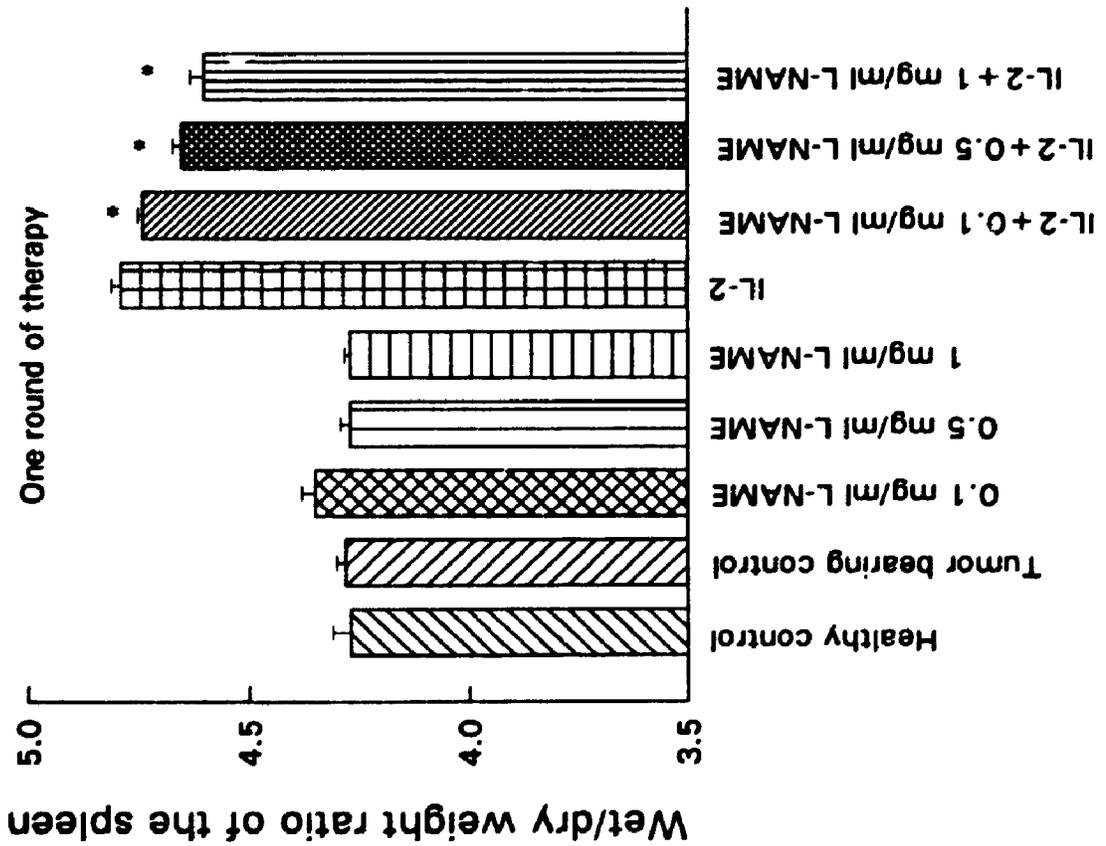
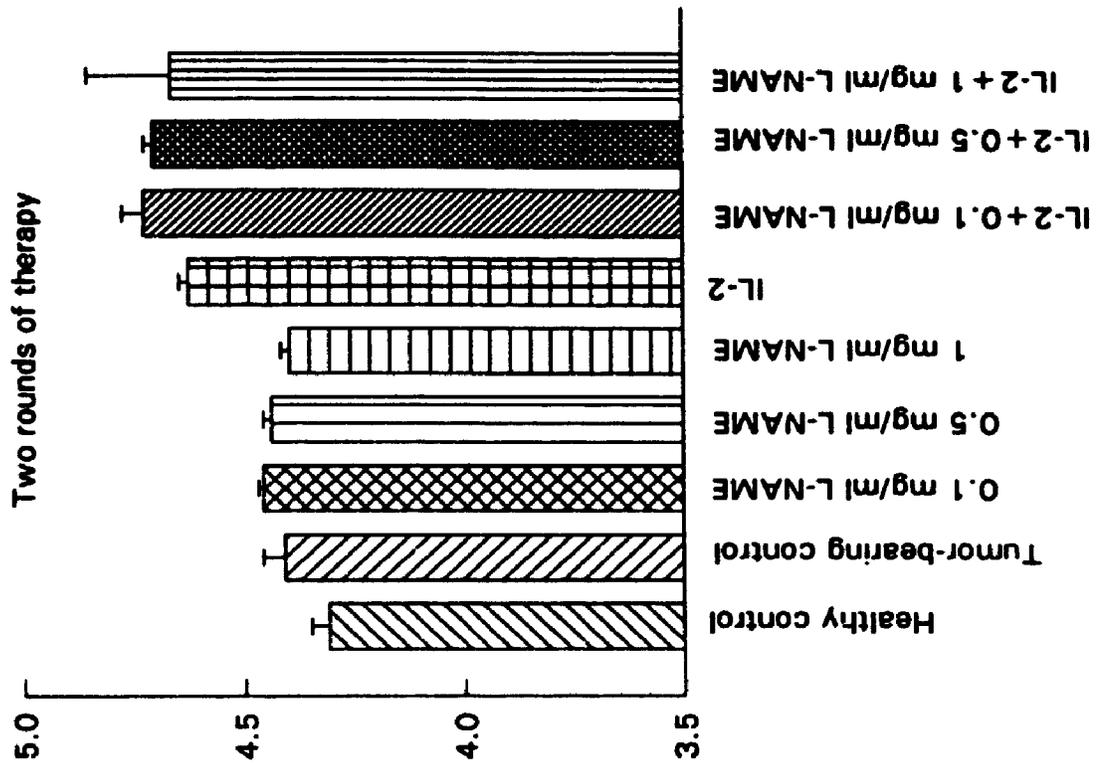
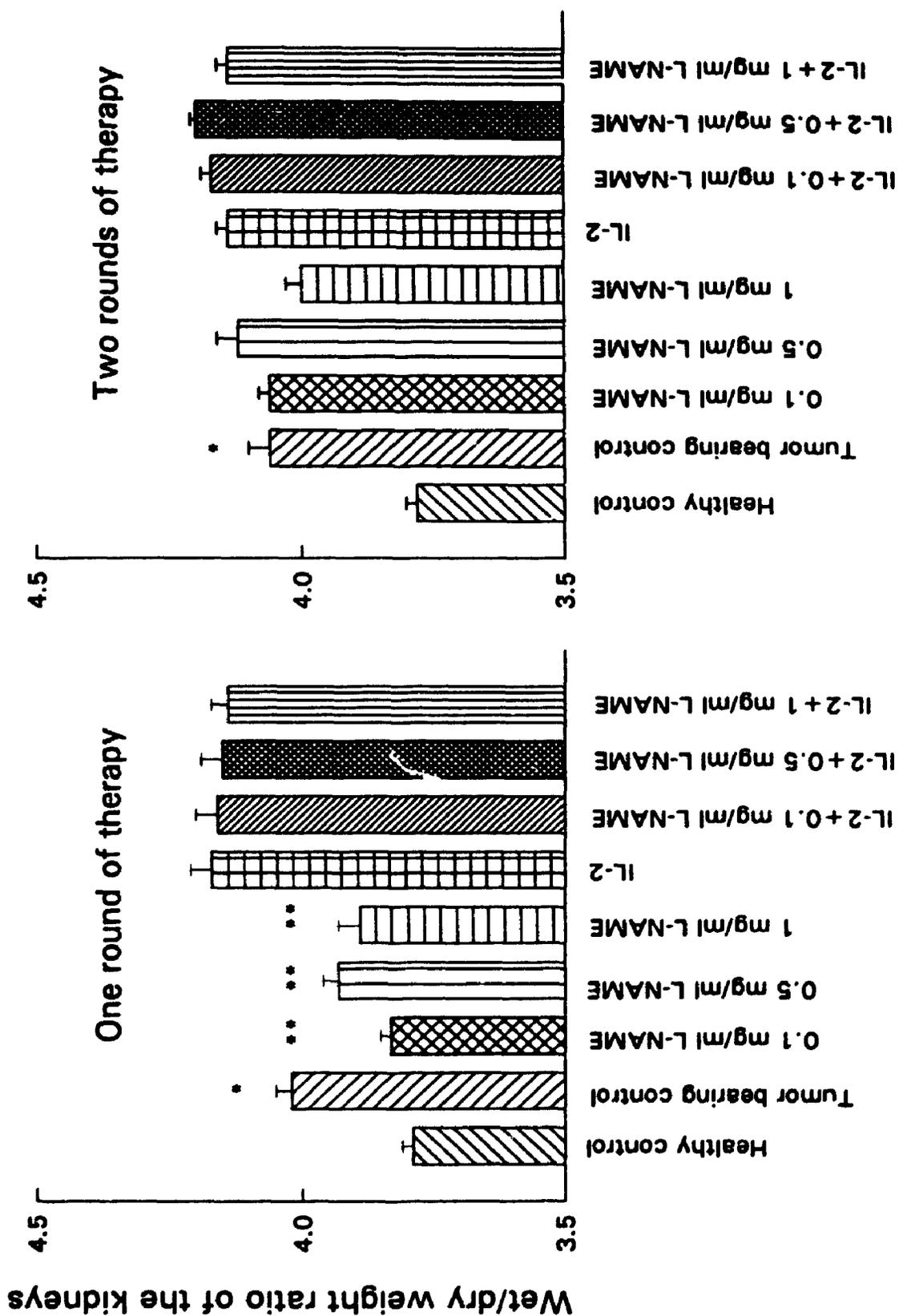


Figure 6.3 Water content of the kidney after IL-2 and L-NAME therapy (left: one round of therapies; right: two rounds of therapies)

Data represent mean \pm SE (left: n = 10; right: n = 9-12)

* Tumor bearing by itself induced an early (d 13 - top) and sustained increase (d 22 - bottom) in the water content in the kidney ($p < 0.05$).

** L-NAME therapy alone significantly ($p < 0.05$) decreased tumor induced fluid retention in the kidney after the first round of therapy.



in all IL-2 treated groups. There was a trend in the reduction of IL-2 induced mortality with addition of L-NAME, which was not significant (Table 6.1).

6.3.3 Effects of L-NAME and IL-2 therapy on NO₃⁻ levels in the serum and pleural effusion

Different doses of L-NAME significantly reduced IL-2 induced increases in NO₃⁻ levels in the serum and pleural effusion in tumor-bearing mice after the first round of therapies (Figure 6.4 - left and right). The basal concentrations of NO₂⁻ in the samples were not detectable. NO₂⁻ levels shown in figure 6.4 were obtained by reducing NO₃⁻ to NO₂⁻ with cadmium filings and thus represent NO₃⁻ levels. After the second round, IL-2 therapy did not influence NO₃⁻ levels in the serum of these animals, while addition of 1 mg/ml L-NAME significantly increased these levels (Figure 6.4 - middle). L-NAME therapy alone did not have any effect on NO₃⁻ level in the serum measured after the first or second round (Figure 6.4 - left and middle).

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

IL-2 therapy alone or in combination with any dose of L-NAME therapy significantly ($p < 0.05$) reduced the growth of the primary tumor measured after the first as well as after the second round of therapies (Figure 6.5.). L-NAME alone (0.5 mg/ml or 1 mg/ml) also significantly ($p < 0.05$) reduced the growth of the primary tumor, measured at any given time point. Addition of increasing doses of L-NAME to IL-2 therapy, significantly improved IL-2 induced retardation of primary tumor growth in a dose dependent manner after the first round of therapies only.

All therapies, except 1 mg/ml of L-NAME alone, significantly reduced the Table

6.1 Animal mortality after IL-2 and L-NAME therapy*

n = 20 / group	number of dead animals	%
IL-2	11	55
IL-2 + 0.1 mg/ml L-NAME	9	45
IL-2 + 0.5 mg/ml L-NAME	8	40
IL-2 + 1 mg/ml L-NAME	10	50

* Animal mortality was followed after the first round of IL-2 (10 inj. 15,000 U i.p. every 8 h on d 10-13) and L-NAME therapy (0.1, 0.5 or 1 mg of L-NAME per ml of drinking water on d 9-13). Animals died within 5 days (between d14 and 18). There was no mortality in animals receiving L-NAME alone at any dosage.

Figure 6.4 NO_3^- levels in the serum (left: one round of therapy; middle: two rounds of therapy) and pleural effusion (right: after one round of therapy) in tumor bearing mice. Data represent mean \pm SE (n = 3-5, each done in duplicate)

* Dose dependent trend in reduction of NO_3^- 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 of L-NAME caused significant ($p < 0.05$) abrogation of IL-2 induced rise in NO_3^- levels in the serum after the first round of therapies.

*** Addition of 1 mg/ml L-NAME to IL-2 significantly ($p < 0.05$) increased NO_3^- levels in the serum after the second round of therapies.

**** IL-2 induced production of NO, measured as NO_3^- levels in pleural effusion after the first round of therapy, was significantly ($p < 0.05$) reduced with addition of L-NAME.

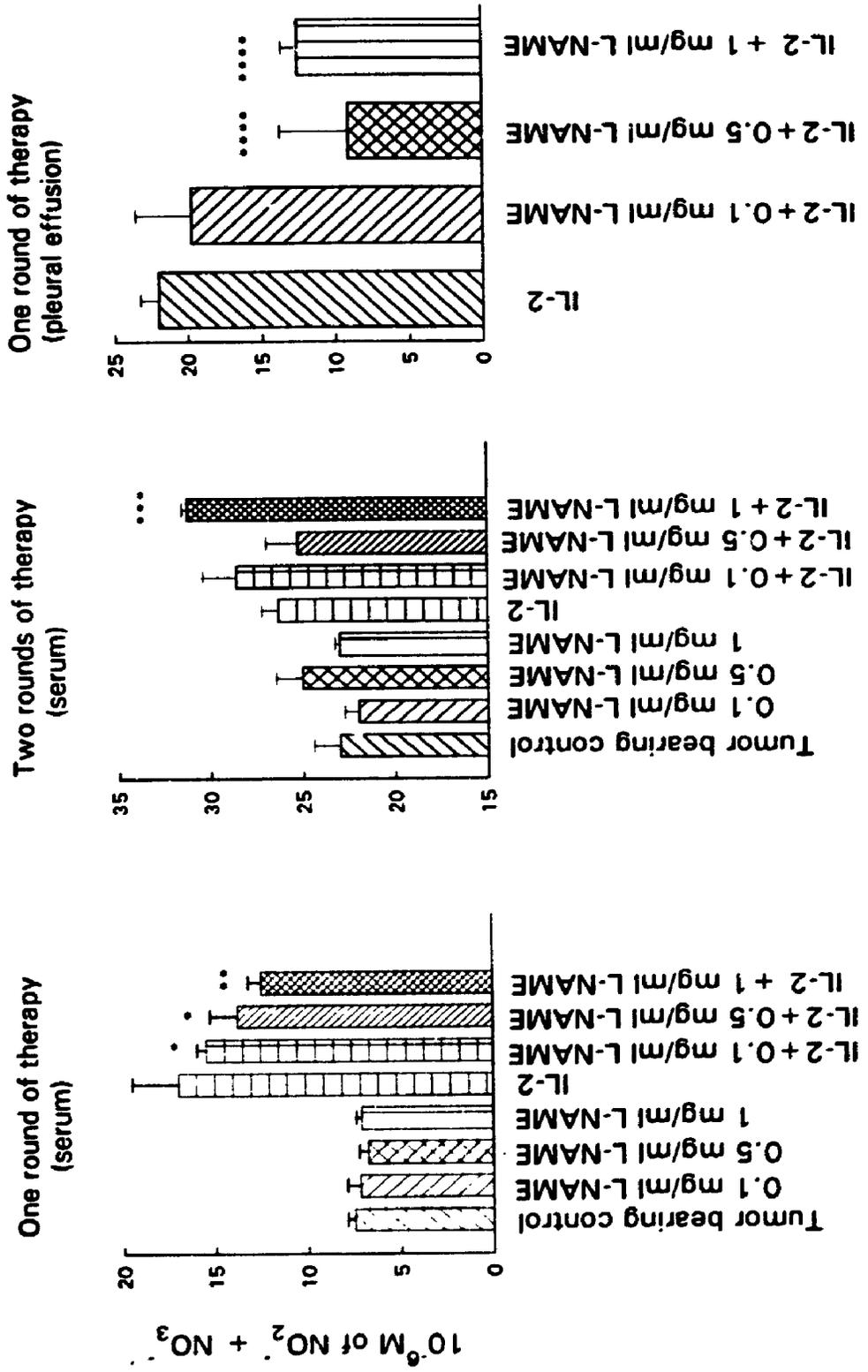


Figure 6.5 Growth of the primary tumor during IL-2 and L-NAME therapy, as given by the mean tumor diameter on days 9, 14, 19 and 23.

Data represent mean \pm SE (d 9 and 14: n = 10-20; d 19 and d 24: n = 9-12)

After the first (d 14) or the second round (d 23) of therapies, IL-2 alone, or in combination with any dose of L-NAME, or L-NAME alone (0.5 or 1 mg/ml) significantly ($p < 0.05$) reduced the growth of the primary tumor.

IL-2 induced reduction of the primary tumor was significantly ($p < 0.05$) enhanced in a dose dependent manner with addition of increasing doses of L-NAME (d 13).

number of lung metastatic nodules. Reduction with IL-2 alone or in combination with L-NAME was greater than with L-NAME alone (Figure 6.6).

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

N^o-Nitro-D-Arginine methyl ester (D-NAME)(1 mg/ml) significantly reduced IL-2 induced pulmonary edema, but had no significant effect on IL-2 induced pleural effusion, water content in the spleen and kidney, as well as on the growth of the primary tumor after the first round of therapies (data not presented). D-NAME did not reduce IL-2 induced rise in the levels of NO₃⁻ in the serum and pleural effusion of tumor bearing mice after one round of therapies (data not presented). D-NAME alone (1 mg/ml) also did not have any beneficial effect on the growth of the primary tumor. (data not shown).

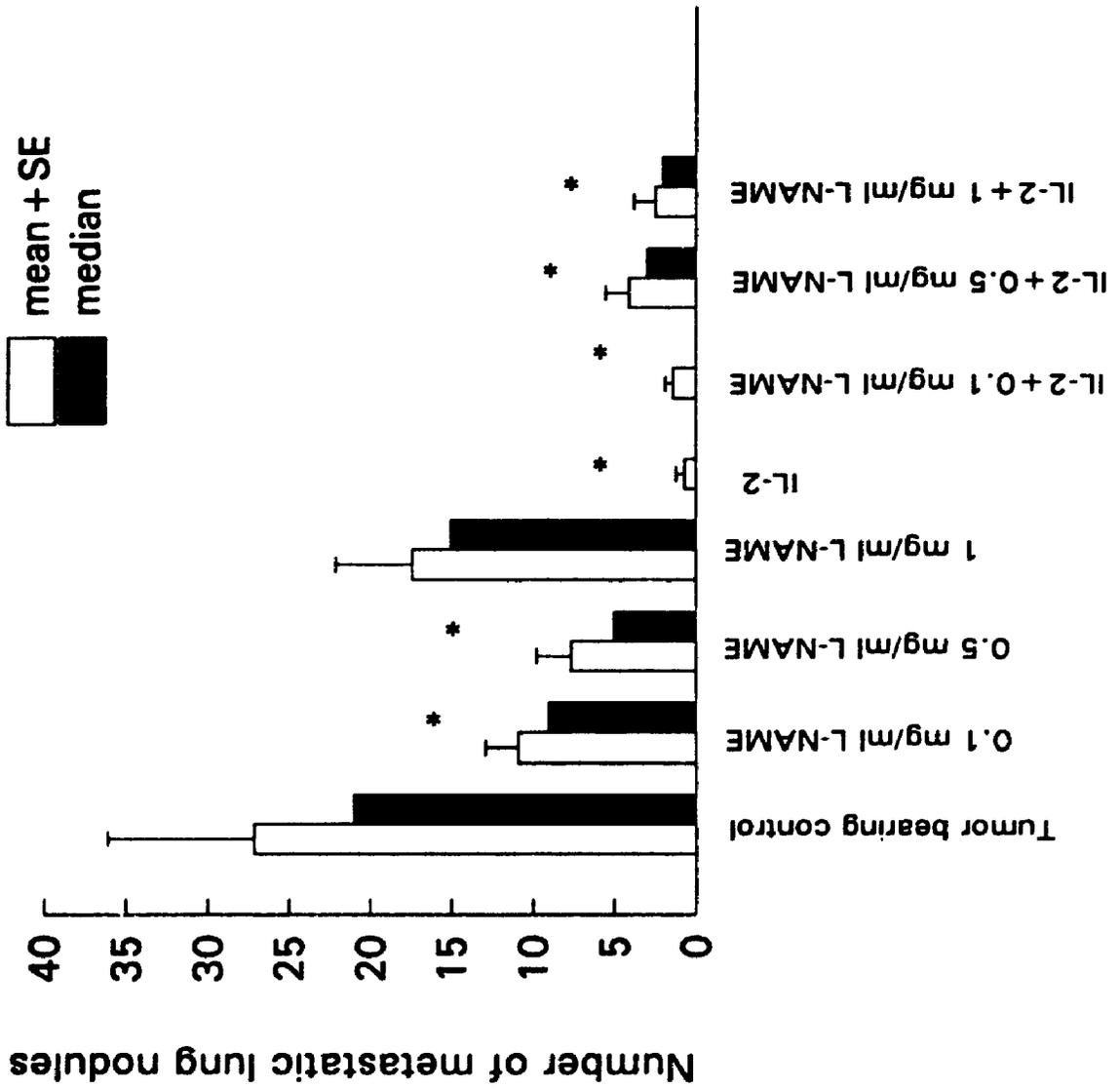
6.4 Discussion

The results of this study showed that oral administration of L-NAME, a potent inhibitor of NO synthesis, significantly ameliorated IL-2-induced capillary leakage in tumor-bearing mice without compromising the therapeutic benefits of IL-2. Furthermore, L-NAME therapy alone had antitumor effects.

This study demonstrated that IL-2 induced pulmonary edema, water retention in the spleen and rise in NO₃⁻ levels (in the serum and pleural effusion) after the first round of therapy were all significantly reduced with addition of L-NAME. However, this inhibition of NO synthesis did not affect either the volume of IL-2-induced pleural effusion or the water content of the kidney after one round of therapy. The results from this study are in general accord with those previously reported in healthy animals

Figure 6.6 Number of metastatic nodules in the lungs after IL-2 and L-NAME therapy (n = 9-12).

* Significant reduction ($p < 0.05$) of the number of metastatic nodules in the lungs scored at the end of the second round.



(chapter 5), in which L-NAME also prevented IL-2 induced capillary leakage. Our results reveal that tumor bearing animals were more susceptible to IL-2 induced capillary leakage and morbidity and that L-NAME therapy succeeded in only a partial abrogation of the IL-2 toxicity.

At later stages of tumor growth (d 23), tumors 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 increased fluid retention in the lungs was a direct result of pulmonary metastasis and that its amelioration with L-NAME was due to its antimetastatic effects.

Surprisingly, no significant change in NO production was noted in any of the groups of mice receiving a second round of treatments, except an increase in animals given IL-2 in combination with the highest dosage of L-NAME. This paradox remains to be investigated further.

The findings of the absence of pleural effusion following the second round of IL-2 therapy was similar to that reported in chapter 4 in healthy or tumor-bearing animals. It is not known at this moment which cells are mostly responsible for production of NO induced by IL-2 in these animals. The expression of inducible NO synthase is known to vary from tissue to tissue and within a given tissue under different circumstances (Nussler and Billiar, 1993). So, it is possible that the second round of IL-2 or the combination therapy did not induce NO synthase 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 Hibbs *et al.*

(Hibbs *et al.* 1992) might be similarly explained.

The findings of augmentation of antitumor effects of IL-2 by L-NAME and of the antitumor effect of L-NAME therapy alone were similar to previous observations with another NO synthase inhibitor, NMMA (chapter 4). Although L-NAME alone did not significantly change serum levels of NO_3^- , it was demonstrated that L-NAME mediated reduction of tumor size was related to NO pathway, since D-NAME (an amino acid not involved in an inhibition of NO generation) (Ialenti *et al.* 1993) did not have any effect on tumor size (data not shown). The mechanisms responsible for the antitumor effects of L-NAME treatment in C3-L5 tumor model as well as the cellular source of NO within the tumor were raised again and are the objectives of next two chapters.

In summary, L-NAME strongly augmented antitumor effects of IL-2, and had antitumor effect when given alone. IL-2-induced capillary leakage was also significantly ameliorated with the addition of L-NAME.

CHAPTER 7 - EFFECTS OF N^G-NITRO-L-ARGININE METHYL ESTER ON IL-2 INDUCED LAK CELL GENERATION IN VIVO AND IN VITRO IN HEALTHY AND TUMOR-BEARING MICE

7.1 Introduction

Interleukin-2 (IL-2) is an important lymphokine which potentiates the functional responses of all cells of the immune system including T cells (Zarling and Bach, 1979), B cells (Emmrich *et al.* 1985) and natural killer cells (Domzig *et al.* 1983). Lymphocytes cultured in the presence of IL-2 give rise to lymphokine activated killer (LAK) cells, by recruitment of primarily NK cells and to a minor extent T lymphocytes (Phillips and Lanier, 1986). LAK cells are capable of killing NK-resistant tumor cells in the absence of prior exposure to tumor antigens and without major histocompatibility locus restriction (Grimm *et al.* 1982; Perussia, 1991). Functional activation of LAK cells mediating antitumor effects *in vivo* has been demonstrated with IL-2 therapy in numerous species including mice (Lafreniere and Rosenberg, 1985; Lala and Parhar, 1988) and humans (Rosenberg *et al.* 1985). Cytolytic function of LAK cells has been shown to be associated with numerous lytic molecules eg. perforin and granule associated enzymes (eg. serine esterase) (Reynolds and Ortaldo, 1987).

There is growing evidence to suggest that full therapeutic potential of IL-2 in cancer therapy has not been achieved because of the appearance of suppressor molecules, which can interfere with LAK cell activation. For example, it has been shown in murine models that production of prostaglandin E₂ (PGE₂) in the tumor bearing host by tumor cells or host cells (eg. macrophages)(Parhar and Lala, 1988; Elkashab and Lala, 1991),

can suppress LAK cell generation by downregulating IL-2 receptors on effector cells (Lala *et al.* 1988). Thus, a combination of chronic indomethacin therapy (CIT) with IL-2 markedly improved the antitumor effects of IL-2 therapy, by improving killer cell activation in situ (Lala and Parhar, 1988; Lala and Parhar, 1993; Khoo *et al.* 1992; Saarloos *et al.* 1993). The possibility also remains, that IL-2 therapy itself can induce the production of short range molecules capable of interfering with LAK cell activation. Nitric oxide (NO) remains a strong candidate.

Nitric oxide (NO), a molecule with a short half life, has been identified as a potent biological mediator (Palmer *et al.* 1987). The role of NO in tumor immunity remains controversial. First, it has been shown that activated murine macrophages synthesize NO (Stuehr and Marletta, 1985), which may partly mediate their cytotoxic activity against tumor cells and bacteria (Nathan and Hibbs, 1991; Lancaster and Hibbs, 1990; Mills *et al.* 1992). Mills *et al.* (1992) reported that tumor growth in the mouse peritoneal cavity was associated with a decreased NO production by intratumor macrophages. Similarly, it has been reported that *in vitro* tumoricidal function of IL-2 activated NK cells depend, at least in part, on their NO synthesizing ability, since deprivation of L-arginine in the medium or blocking of NO synthesis with NMMA reduced their killer function (Cifone *et al.* 1994). In contrast, NO overproduction by rodent macrophages has been shown to suppress activation of T lymphocytes in response to antigen or mitogen (Hoffman *et al.* 1990; Albina *et al.* 1993), and thus may hinder antitumor immune responses of T cells. Indeed, excessive NO production has been implicated in tumor-induced immunosuppression in rats (Lejeune *et al.* 1994). Thus NO can play a dual role in antitumor defense. While it appears to be an important bioactive

component of the cytotoxic pathways of antitumor effector cells, excessive NO release in the microenvironment may be detrimental to their activation pathways.

The contributory role of NO in "capillary leak syndrome" during IL-2 therapy has been demonstrated in previous chapters. Treatment with N^G-Nitro-L-Arginine methyl ester (L-NAME), a potent inhibitor of NO synthesis, ameliorated IL-2 induced capillary leakage in normal (chapter 5), as well as tumor-bearing mice (chapter 6), whereas treatment with N^G-Methyl-L-Arginine (NMMA), another NO inhibitor, was not effective when given subcutaneously (chapter 4). Both agents, however, were found to augment the effects of IL-2 therapy in reducing the tumor burden. One explanation of the latter finding was that NO induction by IL-2 interferes with the optimal activation of antitumor effector cells.

The present study was therefore designed to test whether inhibition of NO synthesis with L-NAME could influence IL-2 induced generation of antitumor cytotoxicity *in vivo* or *in vitro* in splenocytes of healthy and C3-L5 mammary adenocarcinoma bearing C3H/HeJ mice. In addition, the contributory role of macrophages on IL-2 induced NO production *in vitro* was explored by using macrophage depleted splenocytes.

7.2. Materials and methods

7.2.1 Mice

C3H/HeJ female mice, 6-7 weeks old, were obtained from the Jackson Laboratories (Bar Harbor, ME). Animal care was described in chapter 3.

7.2.2 Interleukin-2.

Highly purified recombinant human IL-2 (lot LQP-046) was kindly provided by the Chiron Corporation (Emeryville, CA). The specific activity was 18×10^6 International Units / mg or 3×10^6 Cetus Units / mg of IL-2. The lyophilized IL-2 (1.2 mg/vial) was first reconstituted with 1 ml of distilled water and for *in vivo* studies further diluted with RPMI 1640 medium 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 up to 1 day. For *in vitro* studies, IL-2 was further diluted with complete medium in order to obtain the concentration of 1,000 Cetus Units / ml / 4×10^6 cells. Complete medium was made of RPMI 1640 medium, 10% (v/v) heat inactivated fetal calf serum and 1% (v/v) antibiotics. Fetal calf serum was obtained from Gibco BRL, Burlington, ON, Canada, and antibiotics from Mediatech, Washington, DC. Antibiotics contained 5,000 I.U./ml of Penicillin and 5,000 mcg/ml of Streptomycin.

7.2.3 N^G-Nitro-L-Arginine Methyl Ester.

L-NAME (Sigma Chemical Company, St. Louis, MO) was added to the drinking water to provide concentrations of 0.1, 0.5 and 1 mg/ml of water for *in vivo* experiments. These doses were based on the studies conducted earlier to prevent IL-2 induced capillary leak syndrome in healthy (chapter 5) and tumor-bearing mice (chapter 6). For *in vitro* experiments, L-NAME was added in concentration of 0.1, 0.5, and 1 mg/ml of complete medium/ 4×10^6 cells, based on the experience with other *in vitro* studies, which showed a dose dependent reduction in NO production (Orucevic and Lala, unpublished). 1 mg/ml is equivalent of 3 mM of L-NAME.

7.2.4 Tumor-cell line

C3-L5 mammary adenocarcinoma was used in this study (see chapter 3).

7.2.5 Tumor 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 tumors, this procedure was expected to produce micrometastases in the lungs of C3H/HeJ mice within 2 weeks after transplantation.

7.2.6 Protocols for immunotherapy

Healthy or tumor-bearing mice ($n = 5$ / group) were randomly separated into 5 groups and treated with: nothing, IL-2 (10 inj. 15,000 Cetus U i.p. every 8h), L-NAME (1 mg/ml of drinking water), IL-2 + 0.1 mg/ml L-NAME, IL-2 + 0.5 mg/ml L-NAME, or IL-2 + 1 mg/ml L-NAME. Therapies were given in one round. Healthy mice started with L-NAME in drinking water 1 day before IL-2 therapy. Tumor-bearing mice received IL-2 from d 10-13 and L-NAME from d 9-13 after tumor transplantation. Animals were killed 1h after last IL-2 inj. to measure splenocytes antitumor cytotoxicity and nitrite+nitrate levels in the serum.

7.2.7 Preparation of single cell suspension of spleen cells

Spleens from healthy or tumor-bearing mice subjected to immunotherapy were pooled (5 spleens per each treatment group) and used to isolate effector splenocytes for measurement of LAK cell generation *in vivo*. For *in vitro* LAK cell generation, 30 spleens from healthy or 30 spleens from tumor-bearing mice (14 d after tumor transplantation) were used to isolate effector splenocytes. Single cell suspension was prepared by homogenization using glass homogenizer. Pooled cells were resuspended in

RPMI 1640, and density gradient centrifugation with sterile modified Ficoll-Paque (1.5 g Ficoll 400 / 100 ml Ficoll-Paque; Pharmacia, Dorval, PQ, Canada) was used to remove red blood cells and dead cells. Mononuclear cells from the medium-Ficoll-Paque interface were collected, washed, counted and tested for viability.

7.2.8 Generation of LAK cells in vitro

Unfractionated or splenocytes depleted of plastic adherent macrophages (Elkashab and Lala, 1991) from healthy or tumor bearing mice ($n=30$), were plated in 6-well plates (Corning Glass Works, Corning, NY). Cell input was 20×10^6 cells in 5 ml of complete medium alone or complete medium containing 1,000 Cetus Units of IL-2 / ml of medium, or a combination of IL-2 and different concentrations of L-NAME (0.1, 0.5, or 1 mg/ml of medium). Cells were cultured in duplicate for each experimental condition for 4 days, and were used as effector cells to measure tumoricidal activity. In some experiments, cells were cultured in the same conditions as above, but for 3 days only. Cells were then washed and resuspended in complete medium alone or medium containing IL-2 only, without addition of L-NAME, and incubated for following 24 h. This protocol was intended to dissociate the possible requirement of NO production by effector cells during their later phase of activation for their acquisition of killer activity, from the possible suppressive role of NO during the early activation phase of lymphocyte proliferation. Culture medium was collected and kept frozen at -20°C for measurement of nitrite levels [final metabolic product of NO production in the culture conditions - (Kilbourn and Belloni, 1990; Moncada and Higgs, 1993)].

7.2.9 Measurement of splenocyte antitumor cytotoxicity

Cytotoxic activity of *in vivo* or *in vitro* IL-2 activated splenocytes against a NK

sensitive murine tumor line (YAC-1 lymphoma, American Type Culture Collection, Rockville, MD) and a NK resistant murine tumor line (C3-L5 adenocarcinoma) was measured by ^{51}Cr release assay (Lala and Parhar, 1988). Various effector : target ratios (25, 50 and 100 : 1) were used in triplicates or quadruplicate employing tumor targets labelled for 1 h with 0.2 mCi of sodium chromate (Amersham Canada Limited, Oakville, ONT.) per 10^7 target cells. Percentage of cytotoxicity was calculated using the formula (experimental release - spontaneous release) \div (maximum - spontaneous release) x 100.

7.2.10 Measurement of NO production

Samples of serum were collected after the end of treatments to measure NO_2^- and NO_3^- , the principal metabolites of NO (Moncada and Higgs, 1993; Kelm *et al.* 1992). Culture media from wells used for *in vitro* generation of LAK cells were collected at the end of the incubation period, and kept frozen at -20°C , until assayed for NO_2^- levels. Griess reagent (Green *et al.* 1982) was used for measurement of NO_2^- and cadmium filings for conversion of N to NO_2^- (Davison and Woof, 1978), as described in chapter 3.

7.2.11 Measurement of the sizes of the primary tumors

The size of the primary tumor was measured with callipers on d 9 and d 13, by recording the maximum and minimum diameters. Tumor volumes were then calculated as $0.52a^2b$, where a and b are the minimum and maximum diameters (Baguley *et al.* 1989). The tumor volume was represented in mm^3 .

7.2.12 Statistical analysis

The Microstat statistics package was used in the analysis of collected data, as described in chapter 3.

While comparing antitumor cytotoxicity of effector cells (*in vivo* or *in vitro* experiments) under different conditions, the results obtained from triplicate or quadruplicate determinations at all effector : target ratios were combined for each group, so that significant differences in overall cytotoxicity could be identified between groups.

7.3 Results

7.3.1 Generation of antitumor cytotoxicity *in vivo* in splenocytes of healthy mice

Splenocyte cytotoxicity against both NK sensitive and NK resistant targets was markedly ($p < 0.05$) increased after IL-2 treatment of healthy mice. Addition of L-NAME (via drinking water) significantly ($p < 0.05$) enhanced this cytotoxicity further (Figure 7.1.). The effect was more pronounced in the case of C3-L5 (NK resistant) target. L-NAME therapy alone did not have any effect on NK activity (data not presented). At the same time, IL-2 induced increase ($p < 0.05$) in NO production measured in the serum was significantly ($p < 0.05$) abrogated by addition of L-NAME therapy (Figure 7.2.).

7.3.2 Generation of antitumor cytotoxicity *in vivo* in splenocytes of tumor-bearing mice

IL-2 treatment of tumor-bearing mice significantly ($p < 0.05$) increased splenocyte cytotoxicity against both NK sensitive and resistant targets. Increasing doses of orally administered L-NAME significantly ($p < 0.05$) enhanced this cytotoxicity further, reaching a plateau with a dose of 0.5 mg/ml of drinking water (Figure 7.3.). L-NAME alone did not have any effect on NK activity. A significant abrogation ($p < 0.05$) of IL-2 induced rise in $\text{NO}_2^- + \text{NO}_3^-$ levels in the serum were noticed again with addition of L-NAME (data not presented), as reported in chapter 6.

Figure 7.1 In vivo killer cell generation in healthy mice. Data represent mean \pm SE (every effector : target ratio done in triplicate).

* IL-2 therapy significantly ($p < 0.05$) improved splenocyte cytotoxicity (all three effector : target ratios combined) against NK sensitive and NK resistant targets).

** Addition of L-NAME therapy significantly ($p < 0.05$) enhanced IL-2 induced splenocyte cytotoxicity (all three effector: target ratios combined) against NK sensitive and NK resistant targets.

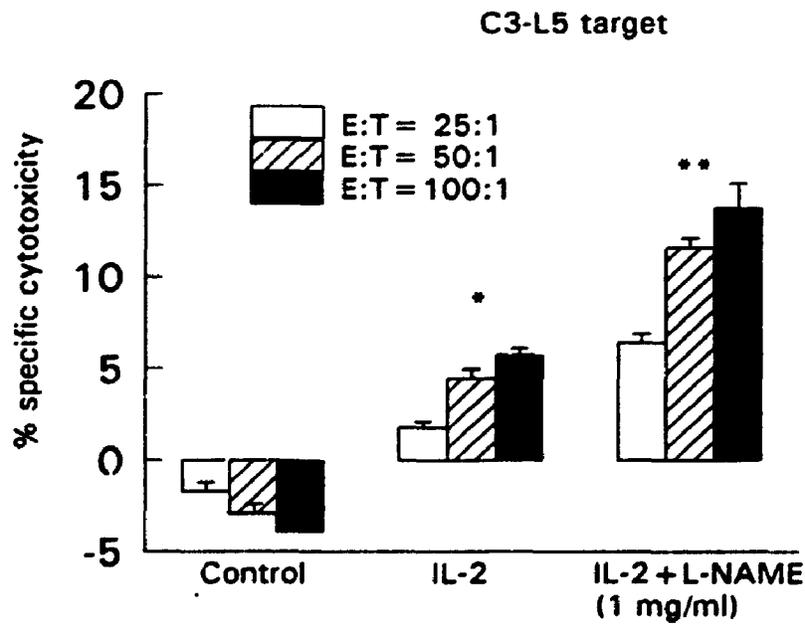
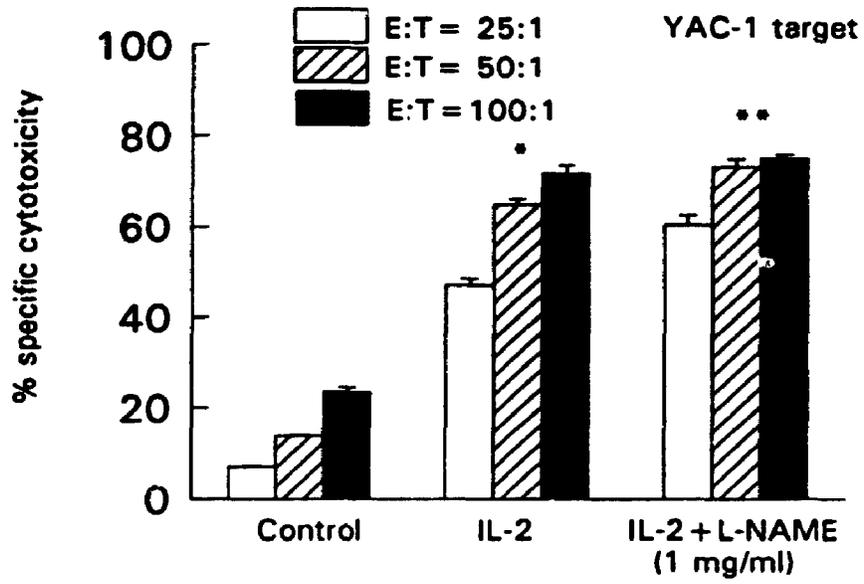


Figure 7.2 NO_3^- levels in the serum of healthy mice, measured as NO_2^- after reduction.

Data represent mean \pm SE (n = 3-5, each done in duplicate).

* IL-2 therapy significantly ($p < 0.05$) increased $\text{NO}_2^- + \text{NO}_3^-$ levels in the serum, measured at the end of the treatment.

** Addition of 1 mg/ml of L-NAME in drinking water significantly ($p < 0.05$) abrogated IL-2 therapy induced rise in NO_3^- levels in the serum measured at the end of the treatment.

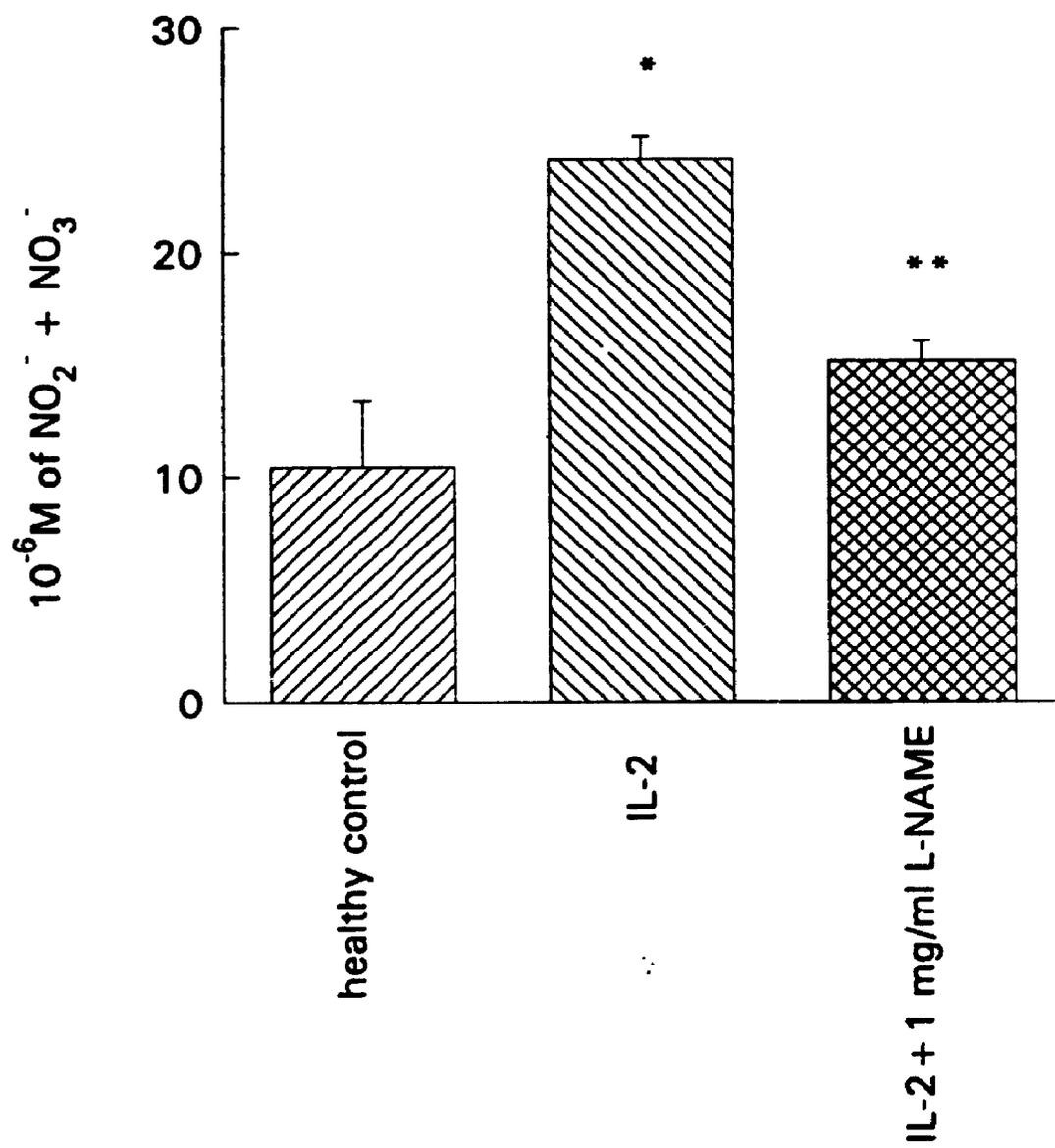
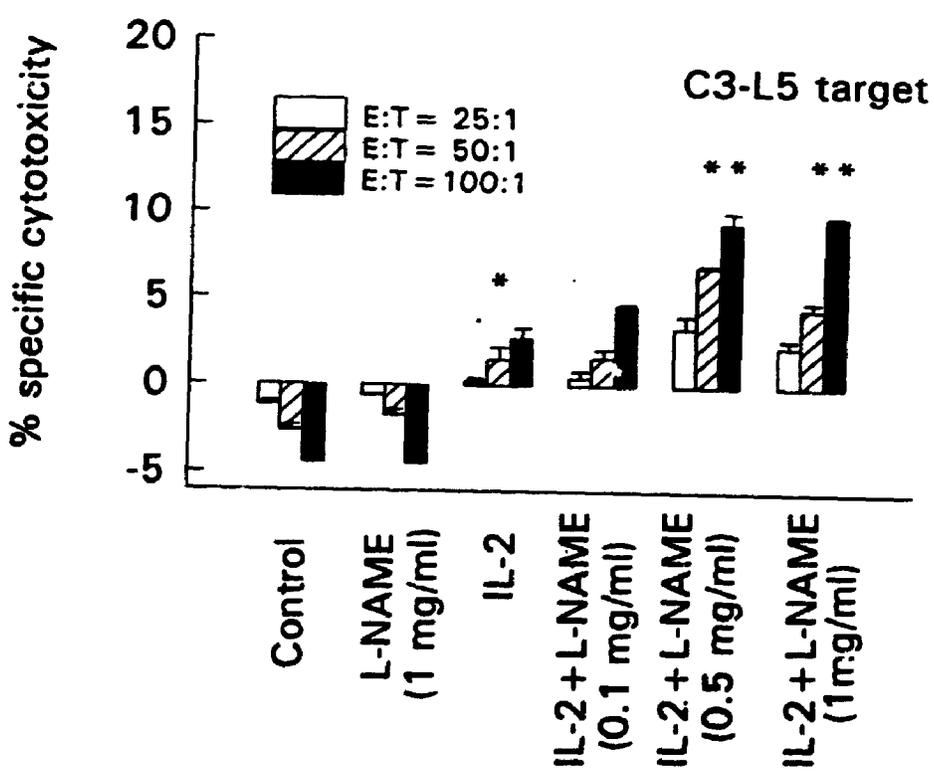
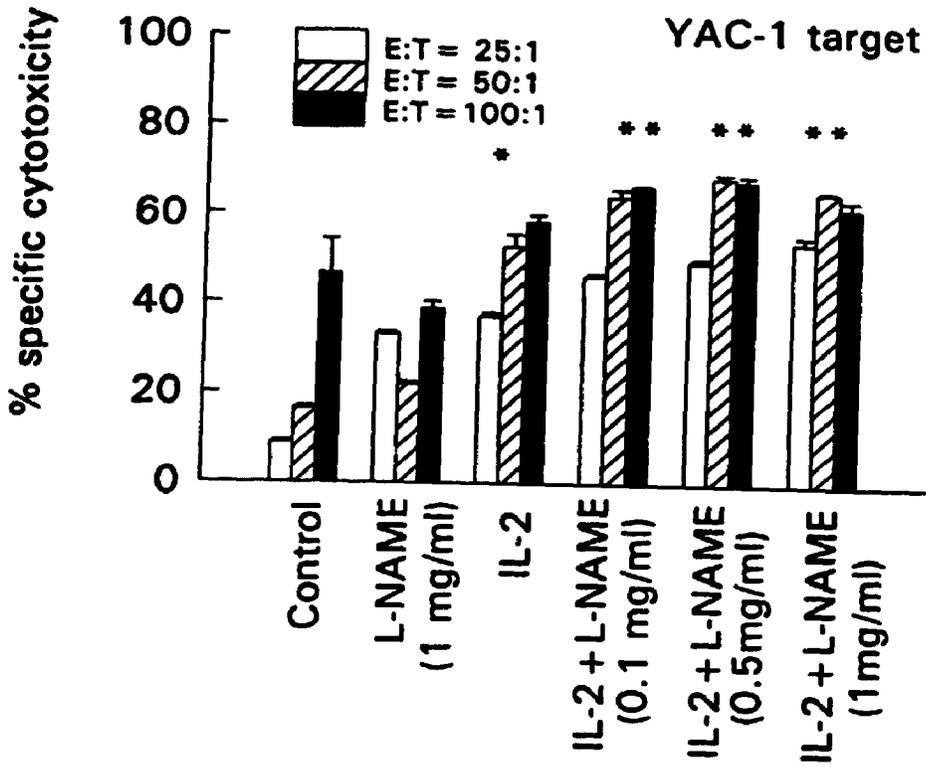


Figure 7.3 In vivo killer cell generation in tumor-bearing mice. Data represent mean \pm SE (every effector : target ratio done in triplicate).

* IL-2 therapy alone significantly ($p < 0.05$) improved antitumor cytotoxicity of splenocytes (all three effector : target ratios combined), both against NK sensitive and NK resistant targets.

** Addition of 0.5 or 1 mg/ml of L-NAME significantly ($p < 0.05$) enhanced IL-2 induced splenocyte cytotoxicity (all effector : target ratios combined) against NK sensitive and NK resistant targets. This was also seen with addition of 0.1 mg/ml L-NAME in the case of NK sensitive target.



7.3.3 Tumor growth during IL-2 and L-NAME therapy

All therapies significantly ($p < 0.05$) reduced the growth rate of the primary tumors as given by the changes in tumor volume between days 9 and 13. The best responses were seen with IL-2+L-NAME (at various doses) (Figure 7.4.), confirming the data reported earlier that addition of L-NAME had beneficial effect on IL-2 mediated reduction of primary tumor growth (chapter 6).

7.3.4 Generation of antitumor cytotoxicity *in vitro* in splenocytes of healthy mice

L-NAME addition *in vitro* significantly ($p < 0.05$) increased IL-2 induced cytotoxicity of unfractionated splenocytes against NK resistant C3-L5 target (Figure 7.5 - top) and did not have any influence on IL-2 induced cytotoxicity against NK sensitive YAC-1 target (data not presented), in the 4 d incubation experiment. Macrophage depletion from the splenocytes caused a minor but significant ($p < 0.05$) improvement of LAK cell activity with IL-2 alone (Figure 7.5 bottom vs. top). Addition of L-NAME (0.1 or 1 mg/ml of medium) still led to a minor but significant ($p < 0.05$) improvement of LAK cell activity (Figure 7.5 - bottom).

When L-NAME was excluded for the last 24 h from the incubation medium, there was no significant difference between the cytotoxicity generated with IL-2 alone and the cytotoxicity generated with IL-2+L-NAME both for unfractionated and macrophage depleted splenocytes (data not presented).

7.3.5 Generation of antitumor cytotoxicity *in vitro* in splenocytes of tumor-bearing mice

Exposure to IL-2 caused a significant stimulation of antitumor cytotoxicity *in vitro* in splenocytes of tumor bearing mice. Addition of L-NAME (4 d incubation) *in vitro* did not, however, significantly influence IL-2 induced cytotoxicity of unfractionated or

Figure 7.4 Growth of the primary tumor during IL-2 and L-NAME therapy, as given by the mean tumor volume on days 9 and 13. Data represent mean \pm SE (n = 14-24). L-NAME alone, IL-2 alone or in combination with any dose of L-NAME significantly ($p < 0.05$) reduced the growth of the primary tumor. Reduction of tumor growth with IL-2+L-NAME (at any of the dosage presented) was significantly ($p < 0.05$) greater than with IL-2 alone.

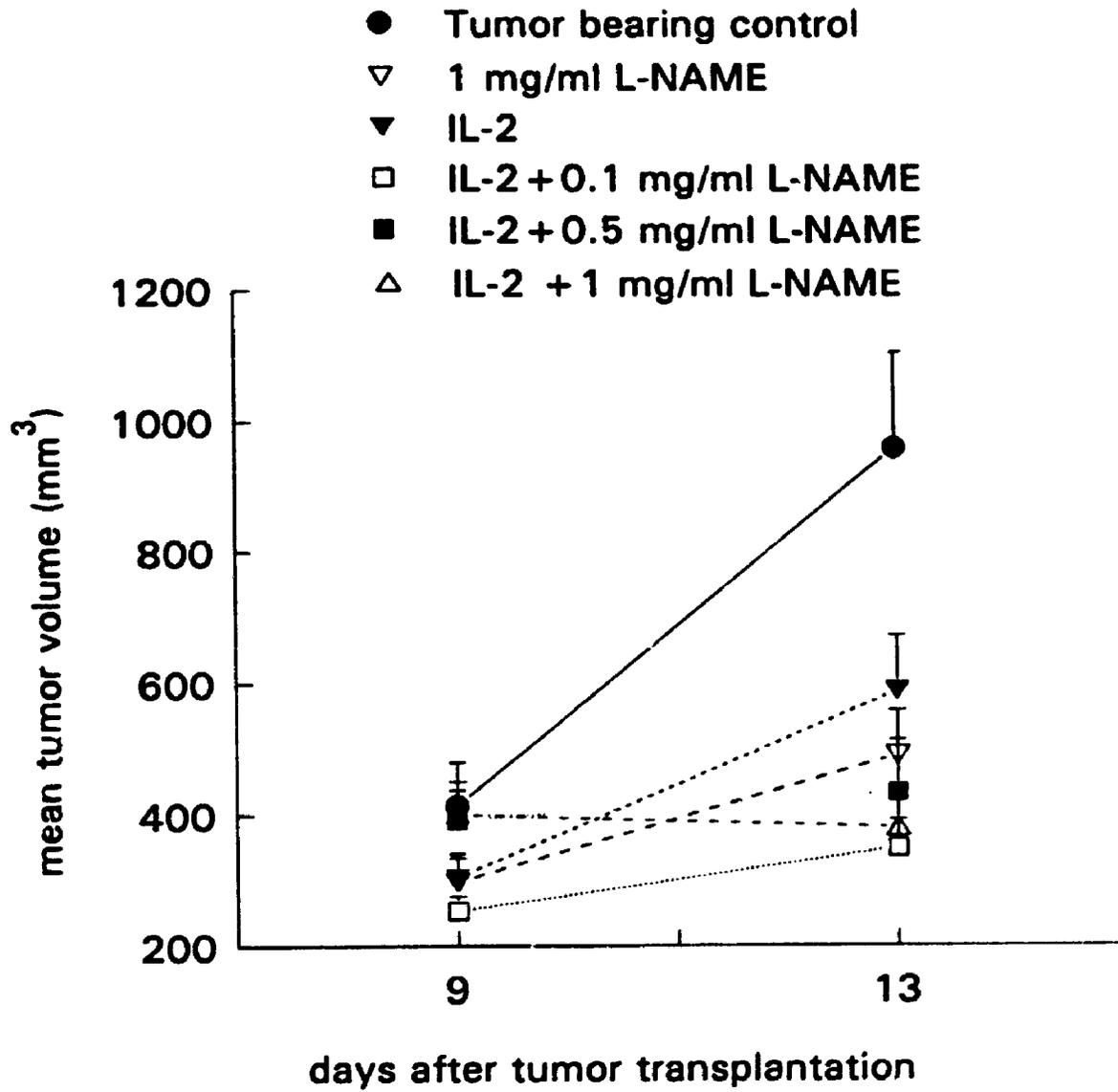
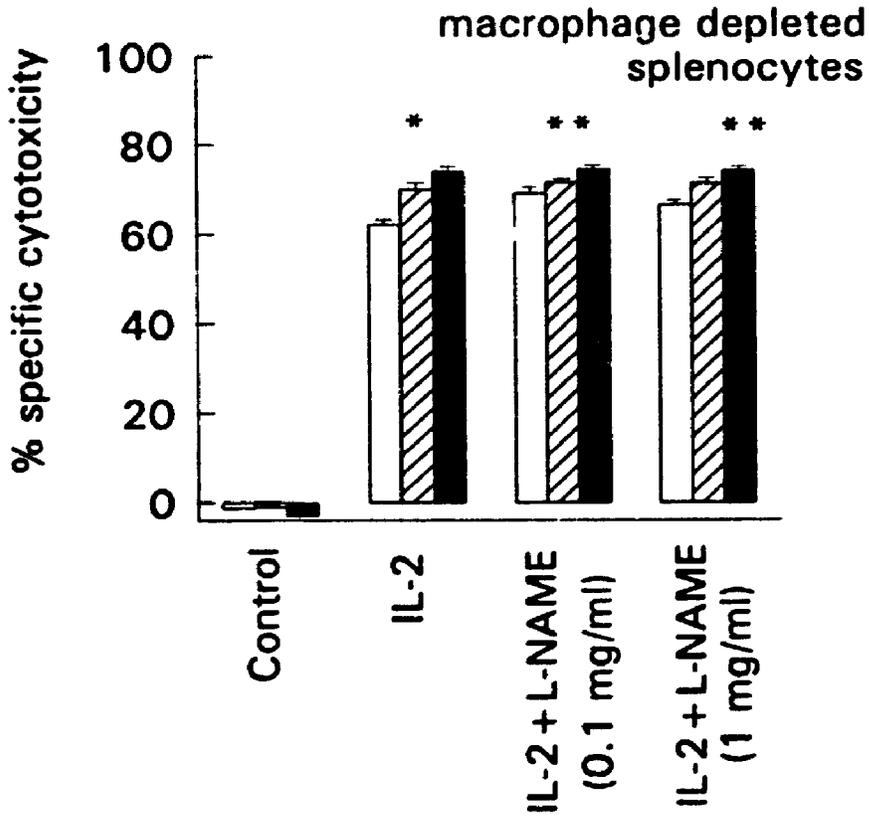
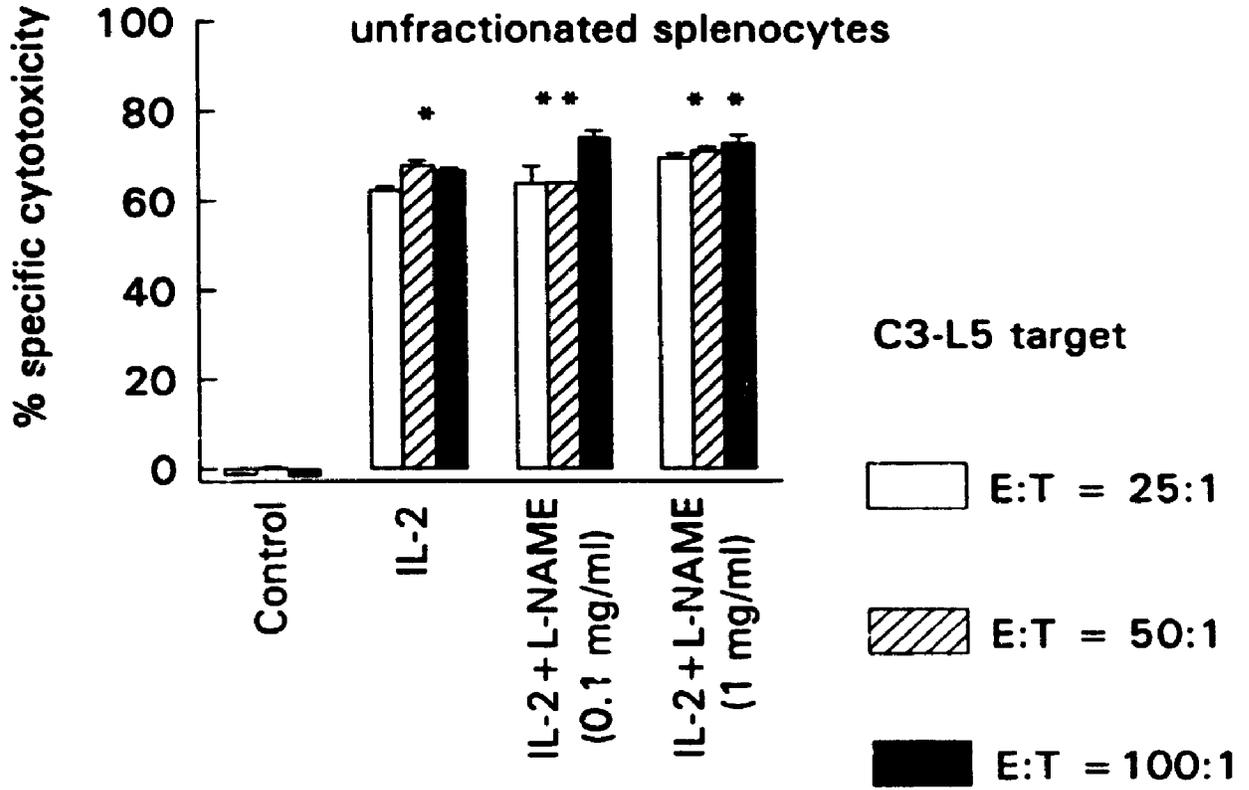


Figure 7.5 In vitro killer cell generation of unfractionated or macrophage depleted splenocytes from healthy mice against NK resistant C3-L5 target. Data represent mean \pm SE (each effector : target ratio done in quadruplicate).

* Addition of IL-2 significantly ($p < 10^{-7}$) induced LAK cell activity in unfractionated (top) or macrophage depleted (bottom) splenocytes.

** Addition of L-NAME (0.1 or 1 mg/ml) significantly ($p < 0.05$) increased LAK cell activity (all effector : target ratios combined) of unfractionated (top) or macrophage depleted splenocytes (bottom).



macrophage depleted splenocytes (data not presented).

7.3.6 NO production *in vitro* by cultured splenocytes from healthy or tumor-bearing mice

NO production (as given by the nitrite levels) during 4d culture of splenocytes alone from healthy or tumor bearing mice was low. NO production was significantly ($p < 0.05$) increased when splenocytes from healthy mice or tumor-bearing mice were incubated with IL-2. However, IL-2 induced increase in NO production in splenocytes from tumor-bearing mice was markedly ($p < 0.05$) higher than in splenocytes from healthy mice (Figure 7.6 - top vs. bottom). Addition of L-NAME significantly ($p < 0.05$) reduced the NO levels in both cases, reducing it to the control level at the dose of 1 mg/ml (Figure 7.6 - top and bottom).

Macrophage depletion significantly ($p < 0.05$) reduced the nitrite levels in cultures incubated with IL-2 and addition of L-NAME reduced them to the control levels, but only in splenocytes from healthy mice (Figure 7.6 - top).

7.4 Discussion

The present study revealed that inhibition of NO synthesis with L-NAME significantly enhanced IL-2 induced activation of antitumor cytotoxicity of splenocytes *in vivo* in healthy as well as tumor-bearing mice, and *in vitro* in healthy mice.

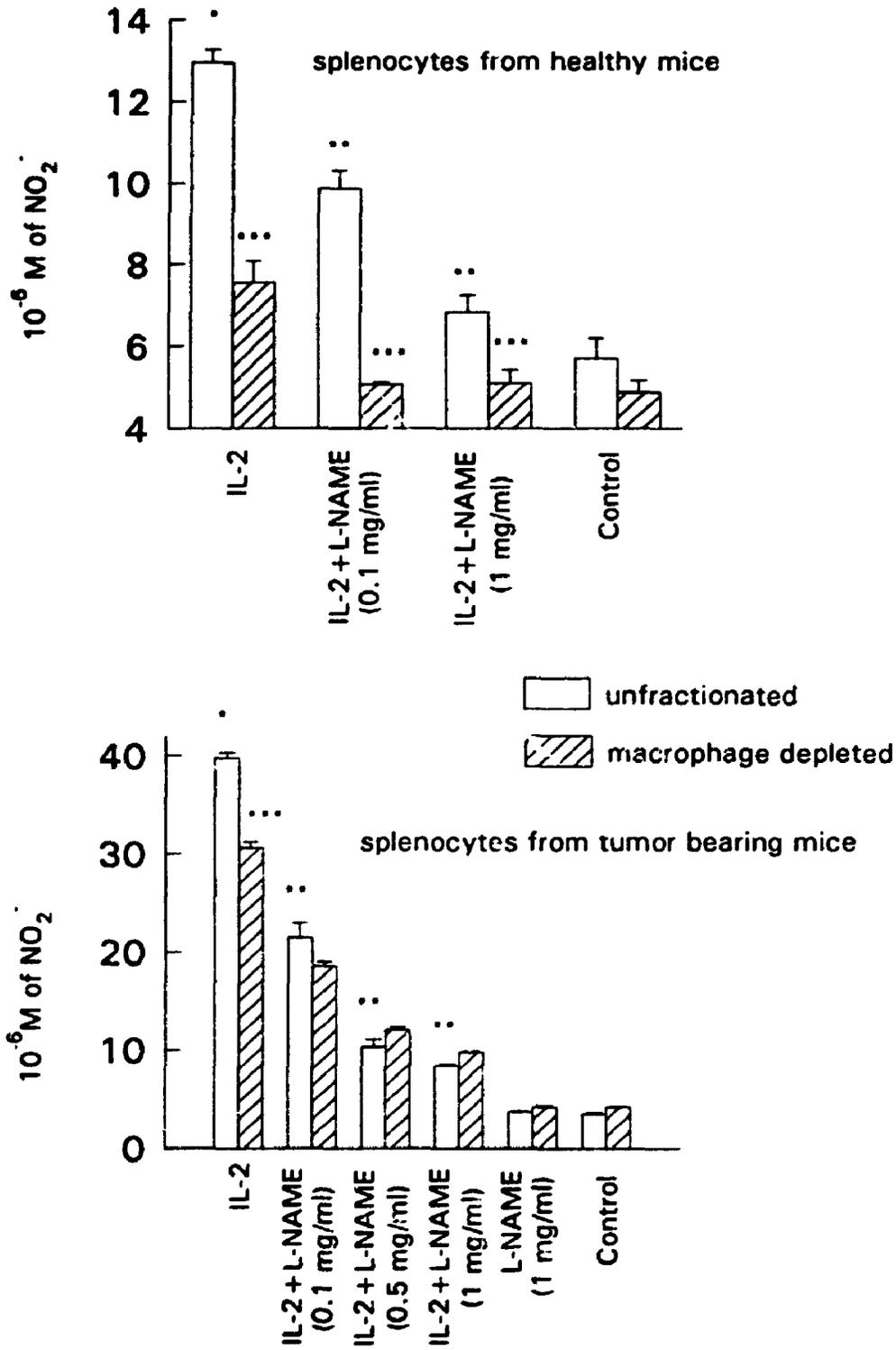
The immunopotentiating effect of L-NAME on IL-2 induced LAK cell generation *in vivo* in healthy as well as in tumor bearing mice is a novel finding. It was demonstrated earlier that IL-2 induced rise in NO production was directly related to the severity of IL-2 induced capillary leak syndrome (toxic side effect of IL-2 therapy) in healthy mice (chapter 4 and 5). Inhibition of NO synthesis with L-NAME reduced the

Figure 7.6 Nitrite production during 4 d culture of splenocytes. Data represent mean \pm SE (n = 4).

* NO production was significantly ($p < 0.05$) increased when splenocytes from healthy (top) or tumor bearing mice (bottom) were incubated with IL-2.

** Addition of L-NAME significantly ($p < 0.05$) reduced IL-2 induced rise in NO production from splenocytes of healthy or tumor bearing mice.

*** Macrophage depletion significantly ($p < 0.05$) reduced the nitrite levels in cultures incubated with IL-2 and addition of L-NAME reduced them to the control levels, but only in splenocytes from healthy mice (top).



severity of IL-2 induced capillary leakage in healthy and tumor bearing mice. Furthermore, L-NAME therapy alone reduced tumor growth and metastases, and in combination with IL-2 augmented early antitumor effects of IL-2, as reported before in chapter 6 and confirmed here. Thus, addition of L-NAME to IL-2 therapy provides the dual benefit of reducing IL-2 toxicity and increasing its therapeutic efficacy, both of which were likely due to a reduction of IL-2 induced NO production. Present results, in tumor-free or tumor-bearing mice, reveal that enhanced NO production during IL-2 therapy, irrespective of the presence of the tumor, interferes with optimal LAK cell activation *in vivo*. L-NAME, by blocking NO production, removes this interference leading to an improvement of antitumor effects of IL-2 therapy.

In vitro results in the present study were in accord with the *in vivo* results in healthy mice. L-NAME significantly increased IL-2 induced LAK cell activity of unfractionated or macrophage-depleted splenocytes. Similar to the results seen *in vivo*, it was also shown that L-NAME blocked IL-2 induced NO production in the medium, in a dose-dependent manner. Thus, it is evident that IL-2 induced NO production was responsible for suboptimal LAK cell activation with IL-2. Macrophages were shown to be the major (but not exclusive) source of IL-2 induced NO production in the splenocyte culture, since macrophage depletion also reduced NO levels in the medium. This finding is in agreement with the reports that macrophages after appropriate induction are the major iNOS expressing cell population in the body (Nathan and Hibbs, 1991). Small, but discernable L-NAME effects even after macrophage depletion may have been due to the residual macrophage contamination or some NO production by lymphoid cells after exposure to IL-2.

In the case of tumor bearing mice, addition of L-NAME did not further stimulate LAK cell activation of IL-2 treated splenocytes *in vitro*, in spite of the fact that NO production in the medium was reduced in the presence of L-NAME, and that L-NAME improved IL-2 induced LAK cell generation *in vivo*. The differences between *in vivo* and *in vitro* results may be due to the presence of additional cells in tumor bearing mice, which were stimulated to produce NO by IL-2 therapy. Indeed, endothelial cells of tumor vasculature (Cobbs *et al.* 1995), as well as macrophages within the tumor have been reported to express iNOS (Thomsen *et al.* 1995). Furthermore, one can not exclude the possibility that L-NAME effects would have been discernable when added to a lower concentration of IL-2 than employed in the present study for LAK cell generation.

Previous reports on the role of NO on IL-2 induced LAK cell generation *in vitro* from healthy subjects remain conflicting. Kilbourn *et al.* (1994) showed that IL-2 induced LAK cell proliferation and tumoricidal activity of canine lymphocytes toward a canine glioblastoma target was unaffected when NO production was blocked with L-NMMA (another NO inhibitor). Juretic *et al.* (1994) reported that L-NMMA suppressed IL-2 induced LAK cell generation from rat or murine splenic cells only when added in culture together with IL-2. The effect was lost when L-NMMA was added a day after IL-2, suggesting that NO might be important in early activation of LAK cells. This group also found that the observed L-NMMA action was species specific, since L-NMMA addition did not affect generation of LAK cells from human peripheral blood or spleen mononuclear cells. Cifone *et al.* (1994) showed that tumoricidal activity of IL-2 induced LAK cells from rat splenocytes was reduced when cytotoxicity assays were performed in the presence of L-NMMA or in arginine-free medium, implying that NO might be

important for the effector phase of LAK cell killing. In the present study, the effector cells were isolated from healthy or tumor bearing C3H/HeJ female mice, incubated for 4 days in media containing IL-2 or IL-2+L-NAME, and cytotoxicity assays were performed in media free from IL-2 and L-NAME. Removal of L-NAME for the last 24 h of culture did not influence the results. It is plausible that culture conditions as well as species differences may account for the differences in the *in vitro* results among previous (Kilbourn *et al.* 1994; Juretic *et al.* 1994; Cifone *et al.* 1994) and present studies with healthy animals; that *in vivo* results from the present study, which were similar in healthy and tumor-bearing mice, offer more reliable information on the role of NO on LAK cell activation.

In summary, it has been shown that IL-2 induced increase in NO production *in vivo* interferes with LAK cell activation, which can be overcome with L-NAME therapy. Augmentation of early antitumor effects of IL-2 with addition of L-NAME therapy are partly due to this L-NAME action. This provides at least one reason for the beneficial effects of adding L-NAME to IL-2 therapy in reducing the tumor burden (chapter 6). These findings, combined with the observation that L-NAME can also mitigate IL-2 induced capillary leakage in healthy (chapter 5), as well as in tumor-bearing mice (chapter 6) suggest that L-NAME could be a valuable adjunct to IL-2 therapy of cancer and infectious diseases.

CHAPTER 8 - TISSUE ANALYSIS OF NITRIC OXIDE SYNTHASE ACTIVITY AND PROTEIN EXPRESSION IN MICE TREATED WITH IL-2 ALONE OR IN COMBINATION WITH N^G-NITRO-L-ARGININE METHYL ESTER.

8.1 Introduction

"Capillary leak syndrome" (fluid leakage into tissue spaces) remains a major road block to IL-2 based immunotherapy of cancer and infectious diseases (Siegel and Puri, 1991; Oppenheim and Lotze, 1994). Excessive NO production has been implicated in the pathogenesis of septic and endotoxic shock (Petros *et al.* 1991; Kilbourn *et al.* 1990; Wright *et al.* 1992). High NO levels reported in human cancer patients receiving IL-2 therapy (Hibbs *et al.* 1992; Ochoa *et al.* 1992; Miles *et al.* 1994) appear to be responsible for severe hypotension, a common side effect of high dose IL-2 therapy, since it is controllable with NO synthase inhibitors (Kilbourn *et al.* 1995; Fonseca *et al.* 1994). It was previously demonstrated (chapter 4, 5 and 6) that moderate to high doses of IL-2 therapy in healthy or mammary adenocarcinoma bearing C3H/HeJ mice, resulted in capillary leakage, as indicated by pleural effusion and fluid retention in the lungs, spleen and kidneys. It was also shown that stable metabolic products of NO measured in the pleural effusion and the serum of these animals, were directly related to the IL-2 dose. L-NAME therapy significantly mitigated all the manifestations of IL-2 induced capillary leakage, except fluid retention in the kidney. There was a concomitant reduction of IL-2 induced NO production in the serum and pleural effusion. Above results, combined with the previous findings of mitigation of IL-2 induced capillary leakage with oral NMMA (chapter 4), strongly indicated that NO plays a major role in the

pathogenesis of capillary leakage induced by high dose IL-2.

We hypothesized that IL-2 therapy directly or indirectly induced iNOS protein and iNOS activity in specific cells, leading to the local rise of NO production in tissues. This led to the capillary leak syndrome by the dual mechanism of NO-induced damage to endothelial cells (Palmer *et al.* 1992; Estrada *et al.* 1992) as well as vasodilation (Palmer *et al.* 1987). The former caused a direct leakage of the capillaries. The latter, in addition, resulted in systemic hypotension, which in turn, indirectly caused pulmonary hypertension, thus further precipitating the pulmonary edema. Both mechanisms may also underlie the IL-2 induced pleural effusion in the mouse, since the blood in the murine pleura is partially derived from the pulmonary arteries (Pinchon *et al.* 1980). The present study was therefore designed in healthy C3H/HeJ mice: (1) to identify the tissue source of NOS activity and NOS protein induced by IL-2 therapy, (2) to identify histologically the nature of structural damage to the lungs during IL-2 therapy induced pulmonary edema and (3) to test whether addition of L-NAME therapy abrogated the increase in NOS activity and IL-2 induced structural damage to the lungs. NOS activity of the lungs and the anterior thoracic wall, iNOS expression in the lungs, anterior thoracic wall and spleen, as well as morphological changes in the lungs were evaluated in mice subjected to systemic IL-2 therapy \pm oral L-NAME therapy.

8.2 Materials and methods

8.2.1 Mice.

C3H/HeJ female mice (7-8 weeks old) were obtained from the Jackson Laboratories. Animal care was described in chapter 3.

8.2.2 Interleukin-2.

Recombinant, highly purified human IL-2 (lot LQP-046) was kindly provided by the Chiron Corporation (Emeryville, CA). The specific activity and the methods of reconstitution were described in chapter 3. In this study, 15,000 Cetus Units was used in 0.1 ml per injection. This dose was found previously to induce capillary leakage (Chapter 4, and 5). The reconstituted material was stored at 4°C up to 1 day.

8.2.3 N^G-Nitro-L-Arginine Methyl Ester.

L-NAME obtained from Sigma Chemical Company (St.Louis, MO) was added to the drinking water to provide concentrations of 1 mg/ml. This dose was found previously to significantly reduce IL-2 induced capillary leakage, as well as IL-2 induced rise in NO production in healthy mice (Chapter 5).

8.2.3 Experimental design.

Healthy mice (n = 20/group) received one of the following treatments: nothing; 15,000 Cetus U of IL-2 alone given i.p. every 8 h for 10 injections; or IL-2+L-NAME (1 mg/ml of drinking water starting one day before IL-2 therapy). Mice (n = 8/group) were killed 1 h after the last IL-2 injection to measure nitric oxide synthase activity in the lungs and anterior thoracic wall, as well as tissue distribution of iNOS enzyme in the lung, intercostal muscles and spleen. Water content in the pleural cavities (shown as a reliable marker of IL-2 induced capillary leak syndrome in mice - chapter 4, 5, and 6), as well as NO₃⁻ levels in the serum and pleural effusion were also measured. Structural changes of the lungs during IL-2 therapy were examined using light and electron microscope. All of the above parameters were examined in order to establish the relationship between nitric oxide synthase activity and protein expression within the

tissues and the degree of IL-2 induced capillary leak syndrome.

The rest of the mice ($n = 12/\text{group}$) were left for treatment with the second round of IL-2, which started 6 days after the first, with the same schedule, doses and route of administration, as the first round. L-NAME was also given in drinking water (1 mg/ml, starting 1 day before second round of IL-2). Mice were killed at the end of the second round of IL-2 (1 h after the last IL-2 injection) to measure the same parameters as after the first round. The differences in nitric oxide synthase activity or iNOS protein expression between one or two rounds of IL-2 therapy were examined.

8.2.6 Measurement of pleural effusion and NO production

Liquid was collected from both pleural cavities using 1 ml syringe as described in chapter 3.

Samples of serum and pleural effusion were collected after the end of treatments to measure NO_2^- and NO_3^- , the principal metabolites of NO (Moncada and Higgs, 1993; Kelm *et al.* 1992), as described in chapter 3.

8.2.6 Light and electron microscopy of the lungs

Lungs were fixed in 2.5 % glutaraldehyde in 0.1M/l of sodium cacodylate buffer by immersion, postfixed in 1% osmium tetroxide in 0.1M/l cacodylate buffer, infiltrated and embedded in plastic (epoxy resin). Semi-thin sections (0.5 μm) were stained with toluidine blue for light microscopic analysis. Thin sections (90 nm) were stained with lead citrate and uranyl acetate (Hunter, 1984) and examined with electron microscope. Light microscopic image analysis (Jandel Scientific Mocha image software) established the degree of pulmonary edema (induced by IL-2 therapy and its abrogation by L-NAME therapy), calculating the percent of area occupied by the connective tissue of the lungs

vs the percent of area occupied by the air spaces of the lungs. Ultrastructural changes in the lung morphology (interalveolar septa, as well as blood-air barrier) were analyzed with electron microscopy. Alterations in endothelial and epithelial cell morphology, endothelial continuity, thickness of the endothelial and epithelial basement membrane and the characteristics of migratory cells in the interalveolar septa were analyzed.

8.2.7 Measurement of nitric oxide synthase activity

The lung and anterior thoracic wall (without the skin) tissues were collected from mice receiving nothing or one or two rounds of IL-2 \pm L-NAME, snap frozen in liquid nitrogen and stored at -70°C until assayed for NOS activity. Assay of NO synthase was performed as described by Thomsen *et al.* (1995). All reagents were obtained from Sigma, if not otherwise stated.

Frozen tissue ($n = 5/\text{treatment group}$) was homogenised (with a polytron) in 5 volumes of a buffer containing 20 mM HEPES, 0.1 mM EDTA, 0.2 mM sucrose, 5 mM DL-dithiothreitol (DTT) (Boehringer Mannheim, Laval, Quebec), 10 $\mu\text{g}/\text{ml}$ of each leupeptin (Boehringer Mannheim, Laval, Quebec) and soybean trypsin inhibitor and 1 $\mu\text{g}/\text{ml}$ pepstatin. The homogenates were centrifuged at 10,000 g at 4°C for 30 min. Endogenous arginine from obtained supernatants (cytosol plus microsomes) was removed by addition of 1:5 ratio of cation exchange resin (DOWEX 50x8-400), followed by short centrifugation (1 min, 10,000 g). Nitric oxide synthase in the supernatants was measured by conversion of L-[U- ^{14}C] arginine (Amersham Life Science, Canada) to [U- ^{14}C]citrulline at 37°C for 10 min as described by Salter *et al.*, 1991. In brief, 100 μl of substrate ("uninhibited substrate") containing 10 μM tetrahydrobiopterin (BH_4), 2.5 mM DTT, 4000 U/ml Calmodulin, 250 μM CaCl_2 , 0.5 mg/ml bovine serum albumin, 125 μM

NADPH, 1500 pmol arginine ("cold + hot") and 100 μ M of L-Citrulline in HEPES buffer was incubated with 50 μ l of "enzyme" (supernatant) in the water bath, set at 37°C for 10 min. The reaction was stopped with addition of 500 μ l of DOWEX and 1000 μ l of distilled water. 20-30 min. later, when DOWEX settled at the bottom of the tube, and all 14 C-L-Arginine adhered to DOWEX, 975 μ l of clear upper phase containing only 14 C-L-Citrulline was mixed with 3 ml of scintillation cocktail (Amersham Canada Ltd.), and counted on scintillation counter.

NOS activity in the citrulline assay was calculated from the difference of counts/min between a substrate and an appropriate blank. Blank in these incubations was achieved by addition of a competitive NOS inhibitor, while Ca^{++} chelator, ethylenebis(oxyethylenitrilo)tetracetic acid (EGTA) served to block Ca^{++} dependent NOS activity. Thus, the activity of a calcium dependent enzyme was calculated from the difference (Δ) between the [14 C]citrulline generated from "uninhibited samples" and samples containing 1mM EGTA. The activity of the calcium-independent enzyme was calculated from the difference (Δ) between samples containing 1 mM EGTA and samples containing both 1mM EGTA and 1mM NMMA. The following formula was used:

$$\Delta \text{dpm}/50 \mu\text{l of "enzyme"} : \text{specific activity} : 10 \text{ min.} = \text{pmol/ min/ } 50\mu\text{l of "enzyme"}.$$

Specific activity is calculated as difference (Δ) dpm between total counts (substrate only) and background counts (substrate + resin, no "enzyme") corrected for volume added to scintulant and concentration of arginine in 100 μ l of substrate. Total protein content of tissue supernatant was determined spectrophotometrically (Bio-Rad assay), and final NOS activity was expressed as pmol of citrulline / min / mg of protein.

8.2.8 Immunocytochemical localization of iNOS protein in tissues

Unfixed samples of lungs, intercostal muscles and spleen were frozen in cryopreservative (OCT) in liquid nitrogen-cooled isopentane, sectioned on a cryostat (20 μm), and melted directly onto glass slides. Sections were fixed in 10% buffered formalin, endogenous peroxidase activity was blocked with 3% H_2O_2 in absolute methanol, and tissue permeabilized with 0.25% Triton X - 100 in phosphate-buffered-saline (PBS). After washing (3x5 min PBS), 10% normal horse serum in 0.2% bovine serum albumen (BSA) was added onto the sections as blocking serum. This was followed by the addition of a complex of a primary (anti-mac NOS, mouse monoclonal antibody against mouse macrophage iNOS, Transduction Laboratories, Lexington, KY) and a secondary horse anti mouse biotinylated antibody (Dimension Laboratories, Mississauga, ON) (1:50 and 1:200 dilution, respectively). The complex was made by shaking primary and secondary antibody overnight at 4°C, followed by addition of heat inactivated normal mouse serum to final concentration of 0.2% (v/v), 2 h before addition of the complex to the sections. This procedure was described by Hierck *et al.* (1994), and proven to significantly reduce the high background usually resulting with mouse monoclonal antibodies, applied to a mouse tissues. High background is due to nonspecific binding of secondary anti mouse Ab to mouse immunoglobulins normally present in the mouse tissues. In the above mentioned procedure, a complex between primary and secondary antibody is made in a test tube, and normal mouse serum added to bind to all unbound secondary Ab, so when added, theoretically, only specific binding of a complex is to iNOS antigen present in the tissues. As a negative control, anti-iNOS Ab from a complex was replaced with a mouse monoclonal antibody against *Aspergillus niger* glucose oxidase (Dako, Dimension

Laboratories, Mississauga, Canada), an enzyme which is neither present nor inducible in mammalian tissues. Sections were incubated with a complex for 4 h, washed and ABC substrate was added, followed by diaminobenzidine (DAB) chromogen treatment. Sections were counterstained with hematoxylin, mounted with aqua-mount and analyzed for a presence of iNOS protein. Image analysis was performed using a computer package (Northern Exposure, version 2.3) and an IBM-compatible computer. Photomicrographs were prepared in a digital darkroom by using Adobe Photoshop and Corel Draw software programs. Prints were generated with a Tektronix Phaser 440 printer.

8.2.9 Statistical analysis

Data were subjected to analysis of variance using Microstat Statistics Package (Ecosoft, Inc., Indianapolis, IN) as described earlier (Chapter 3).

8.3 Results

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

L-NAME significantly reduced pleural effusion induced by IL-2 after the first round of therapy (Figure 8.1.), as was also previously reported (Chapter 5, Fig. 5.1.). IL-2 therapy-induced rise in $\text{NO}_2^- + \text{NO}_3^-$ levels in the serum and pleural effusion were significantly prevented by addition of L-NAME (data presented in chapter 7, Figure 7.2.), similarly to previously described results (Chapter 5, Figure 5.5 and 5.6.).

8.3.2 Effects of L-NAME on IL-2 induced morphological changes of the lungs after first round of therapy

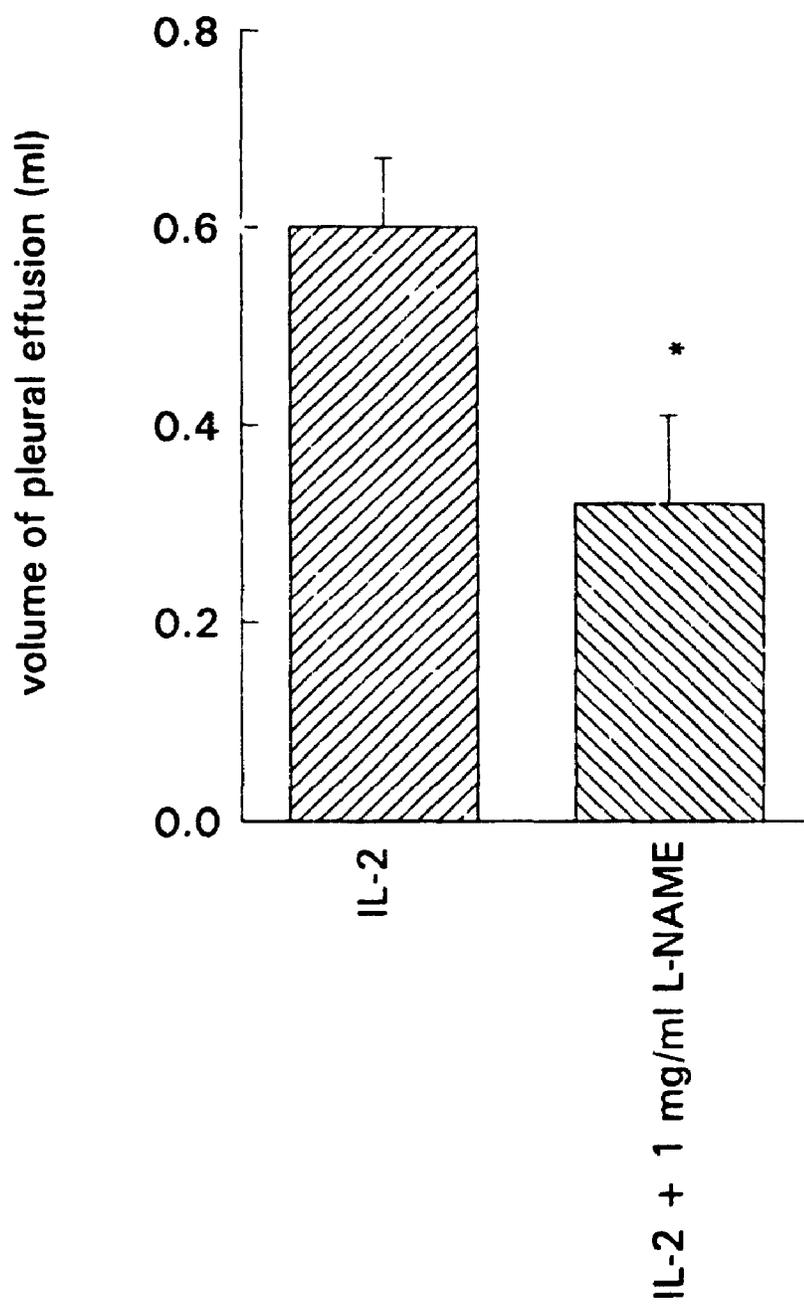
Light microscopic image analysis (Jandel Scientific Mocha image software) of

Figure 8.1 Pleural effusion after IL-2 and L-NAME therapy.

Data represent mean+SE (n=5)

* indicates significant difference from IL-2 therapy ($p < 0.05$).

L-NAME therapy (1 mg/ml of drinking water) significantly ($p < 0.05$) reduced IL-2 (15,000 U/inj) induced pleural effusion. Control (untreated) mice did not show any pleural effusion.



semithin sections of the lungs showed that IL-2 therapy led to a significant increase in the relative area occupied by the connective tissue of the lung and a significant reduction in the relative area occupied by air spaces compared to the control lungs (Figure 8.2.), due to IL-2 induced pulmonary edema (Chapter 5, Figure 5.2.). Addition of L-NAME therapy significantly reduced IL-2 induced pulmonary edema (Chapter 5, Figure 5.2.) and significantly restored the balance between the area occupied by the connective tissue and air spaces close to the control levels (Figure 8.2.). Percentage of area occupied by the connective tissue in the lungs during different treatments, expressed as mean \pm SE (n=5), was as follows: IL-2 = 69 ± 2.5 (p < 0.05 compared to control); IL-2 + L-NAME = 62.4 ± 1.3 (p < 0.05, comparing to IL-2 alone) and control = 55.2 ± 3.1 .

Ultrastructural analysis of the lungs revealed that IL-2 therapy led to major distortions in the capillary ultrastructure of the lungs. The changes included endothelial cell and type I pneumocyte swelling or damage, thickening of the basement membrane of the thin portion of the capillaries, or herniation of the thin segment of endothelial cell into the vessel lumen because of accumulation of fluid between the plasma membrane and the basement membrane, and the presence of cellular debris in the "blebs" in endothelial cells or type I pneumocytes, indicating cellular damage (Figure 8.3.b. and 8.4.b). Addition of L-NAME therapy, in general, diminished the ultrastructural damage induced by IL-2. Although some swelling of endothelial cells was observed, there was no noticeable damage of endothelial cell membranes nor thickening of the basement membrane of the thin portion of the capillaries (Figure 8.3.c. and 8.4.c.).

Figure 8.2 Effects of IL-2 and L-NAME therapy on histology of the lungs.

IL-2 therapy caused a significant increase in the relative area occupied by the connective tissue of the lung and significant reduction in the relative area occupied by air spaces as compared to the control lungs (lower panel, pie charts), due to IL-2 induced pulmonary edema and significant interstitial mononuclear cell infiltration (upper panel, semithin section of the lungs, toluidine blue staining, x40). Addition of L-NAME therapy significantly reduced IL-2 induced pulmonary edema and mononuclear cell infiltration, and restored the balance between the area occupied by the connective tissue and air spaces (semithin section of the lungs and pie charts).

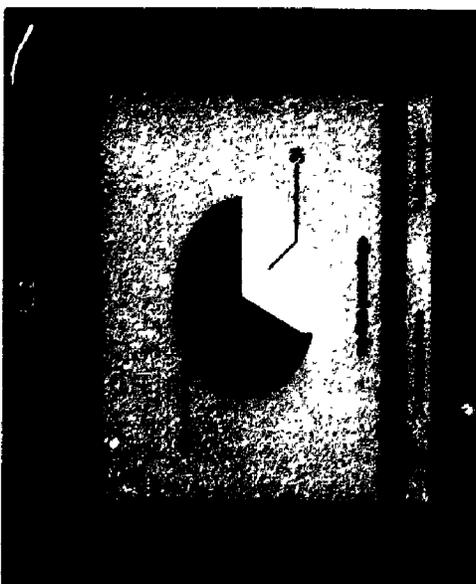
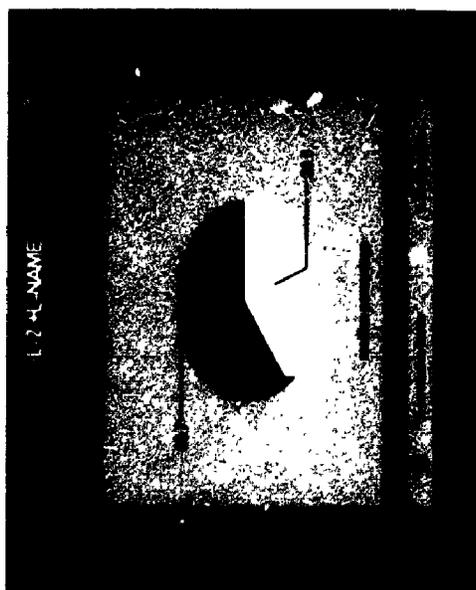
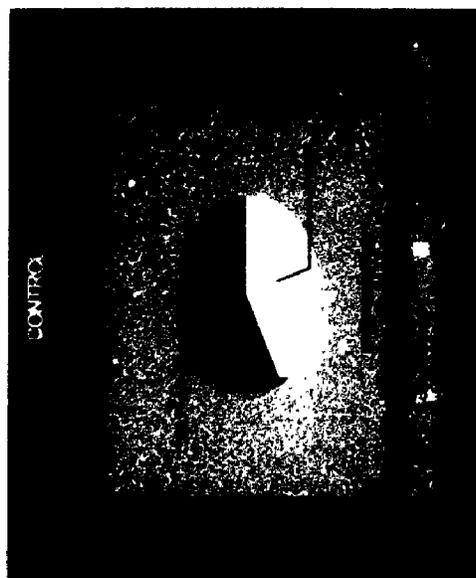
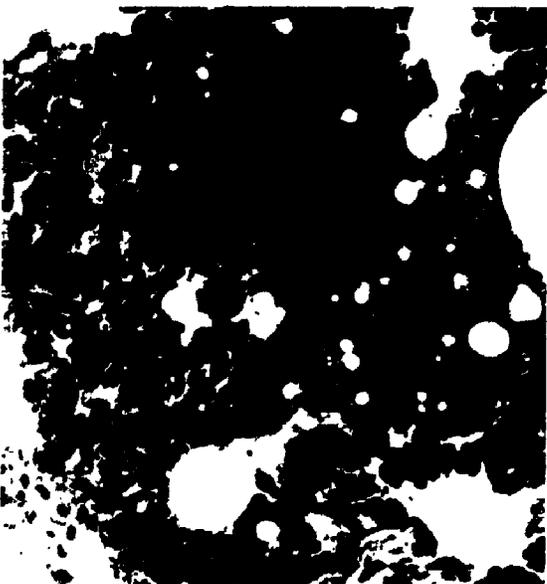
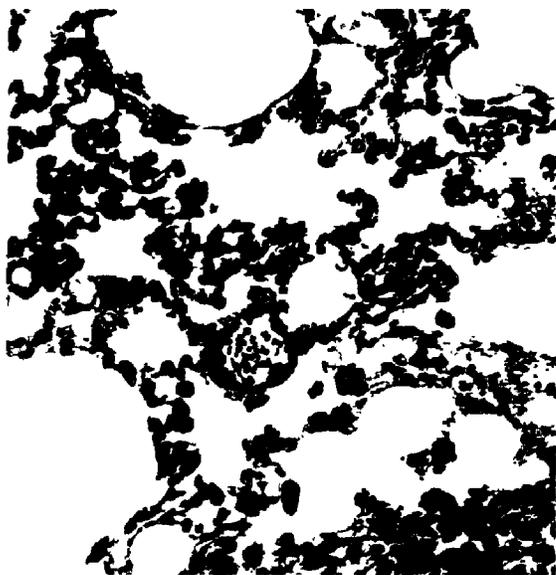


Figure 8.3 Ultrastructure of the lungs of mice given IL-2 or IL-2+L-NAME therapy.

A = control; B = IL-2; C = IL-2+L-NAME

Low-power view showing part of several capillaries and distortion in the capillary architecture of the lungs of IL-2 treated animals. The changes included endothelial cell and type I pneumocyte swelling and thickening of the basement membrane of the thin portion of the capillaries.

> shows thin part of the blood-air barrier, magnification x 3,000.

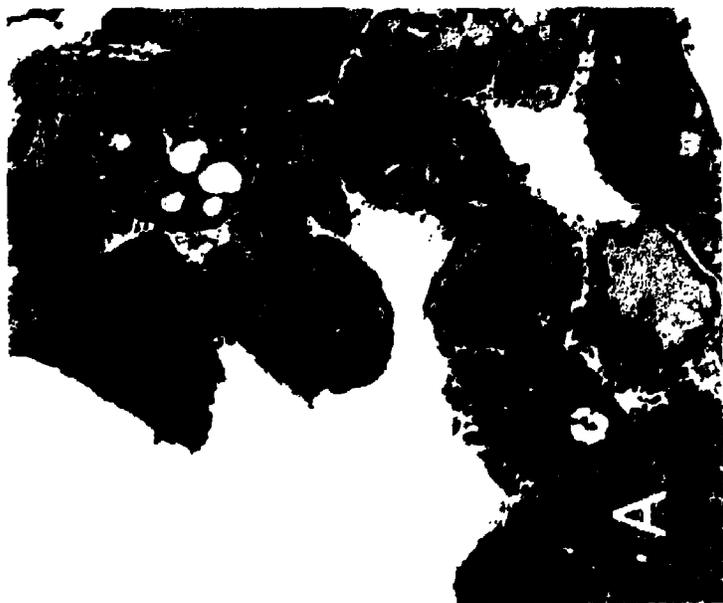
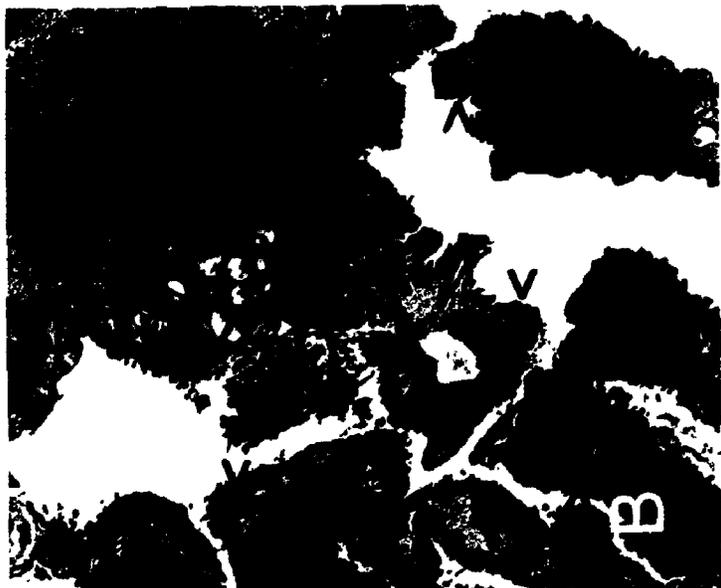


Figure 8.4 Ultrastructure of the lungs of mice given IL-2 or IL-2+L-NAME therapy.

A = control; B = IL-2; C = IL-2+L-NAME; magnification x 7000.

Basement membrane is discontinuous in IL-2 treated mice. Endothelial as well as pneumocyte type I cells are severely damaged. There is also swelling of endothelial as well as pneumocyte type I cells. > indicates an area of blood-air barrier showing such damage.

Basement membrane is continuous and thin at the thin part of the capillary (*) in IL-2 + L-NAME treated animal. Endothelial cells, although showing occasional swelling, are never detached from their basement membrane.

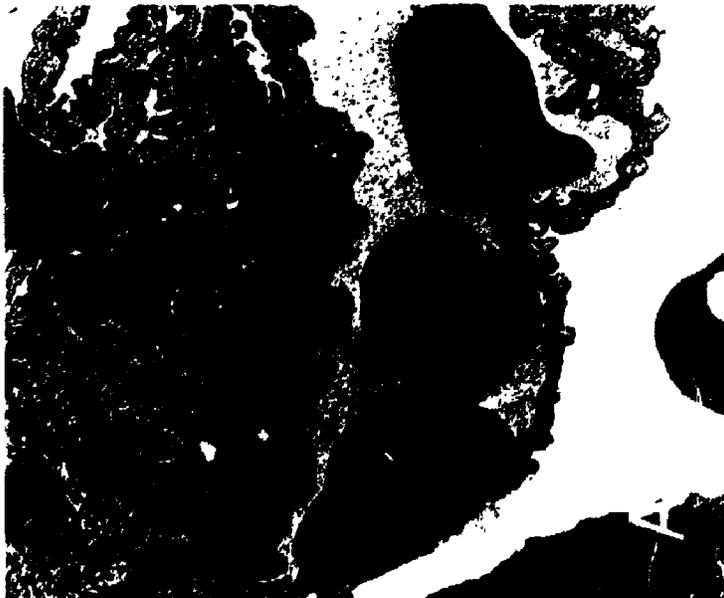
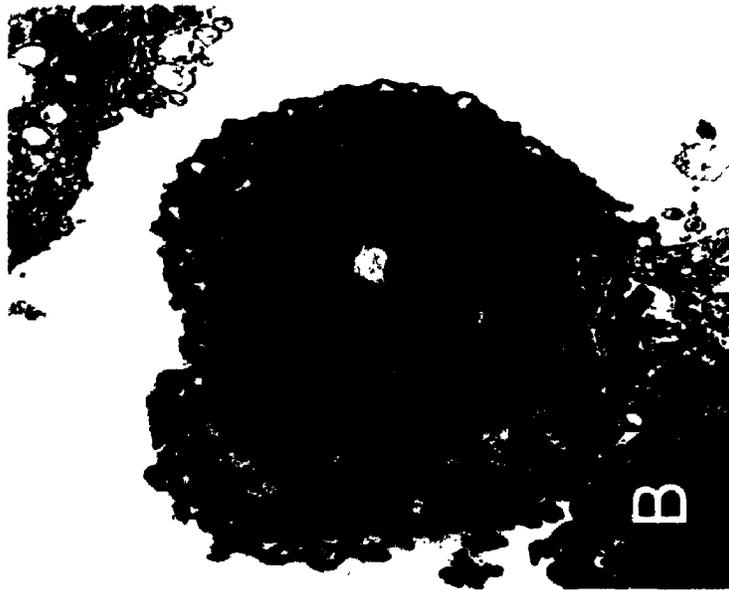
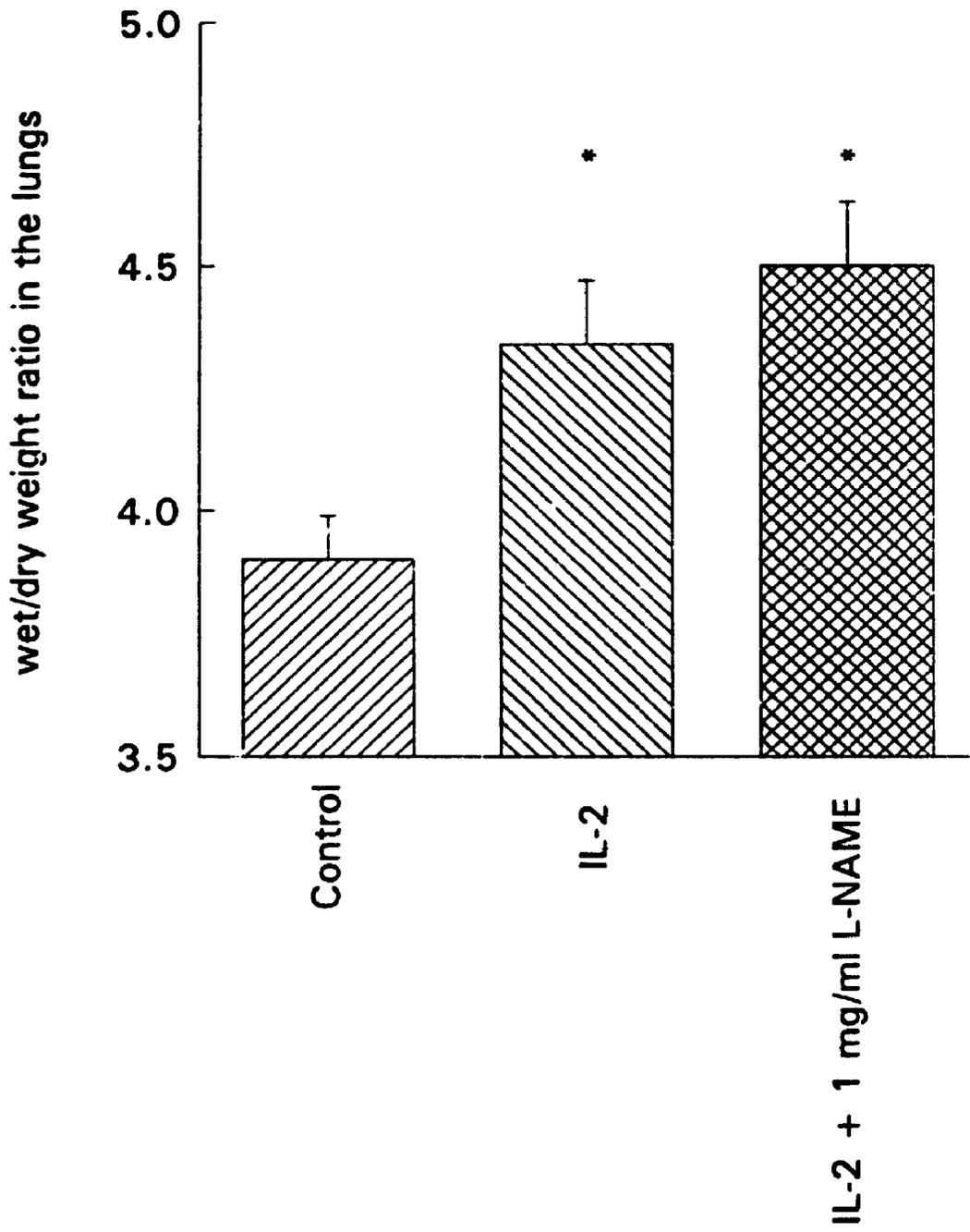


Figure 8.5 Water content of the lungs after second round of IL-2 \pm L-NAME therapy.

Data represent mean \pm SE (n=5)

Addition of L-NAME therapy did not influence IL-2 induced pulmonary edema after second round of therapy.



8.3.3 Effects of L-NAME on IL-2 induced capillary leakage and NO production after two rounds of therapy.

IL-2 therapy caused an increase in the water content in the lungs after second round of therapy, which was not significantly affected by the addition of L-NAME therapy (Figure 8.5.), although $\text{NO}_2^- + \text{NO}_3^-$ levels in the serum were significantly reduced with addition of L-NAME (data not presented). There was no pleural effusion at this time in animals treated either with IL-2 alone or IL-2 + L-NAME, as also documented earlier (Chapter 4, 6).

8.3.4 Nitric oxide synthase activity in the lungs and anterior thoracic wall after one or two rounds of IL-2 and L-NAME therapy

One round of IL-2 therapy significantly increased Ca^{++} independent (primarily explained by inducible) nitric oxide synthase activity in the anterior thoracic wall (Figure 8.6.), whereas the increase of Ca^{++} independent NOS activity in the lungs was not significant (Figure 8.7.). However, second round of IL-2 therapy increased Ca^{++} independent NOS activity in the lungs, but did not induce any Ca^{++} independent NOS activity in the thoracic wall (Figure 8.6 and 8.7.). Addition of L-NAME therapy during the first or the second round of IL-2 therapy abolished any nitric oxide synthase activity in the lungs or in the anterior thoracic wall (Figure 8.6 and 8.7).

8.3.5 Tissue distribution of immunoreactive iNOS protein during IL-2 ± L-NAME therapy

Thoracic wall: Immunocytochemical staining for iNOS enzyme revealed that one round of IL-2 therapy induced iNOS expression in endothelial cells of capillaries surrounding the fibres of intercostal muscles of the anterior thoracic wall (Figure 8.8.c.). Addition of L-NAME therapy, although abolishing all nitric oxide synthase activity from

Figure 8.6 Nitric oxide synthase activity in the thoracic wall after one or two rounds of IL-2 ± L-NAME therapy.

* One round of IL-2 therapy significantly ($p < 0.05$) increased Ca^{++} independent NOS (iNOS) activity in the anterior thoracic wall.

** Addition of L-NAME therapy abolished any NOS activity, either after one or two rounds of therapy.

+ There was no significant iNOS activity induced by the second round of IL-2 therapy.

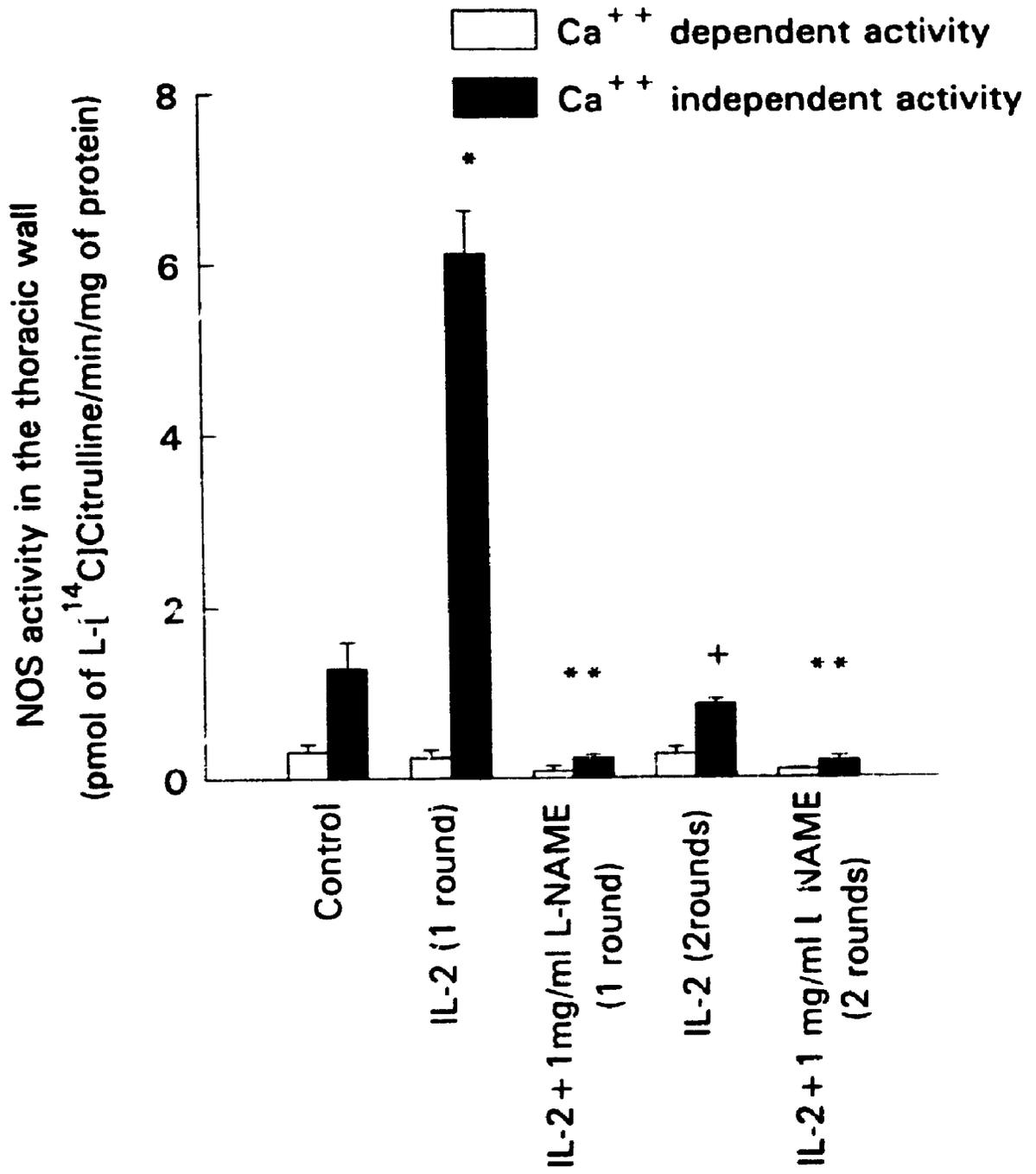


Figure 8.7 Ca⁺⁺ independent (inducible) NOS activity in the lungs after one or two rounds of IL-2 ± L-NAME therapy.

* IL-2 therapy induced significant iNOS activity in the lungs after the second round of therapy.

** L-NAME therapy abolished all iNOS activity in the lungs either after one or two rounds of therapy.

Ca^{++} independent NOS activity in the lungs
(pmol of L^{14}C]Citruilline/min/mg of protein)

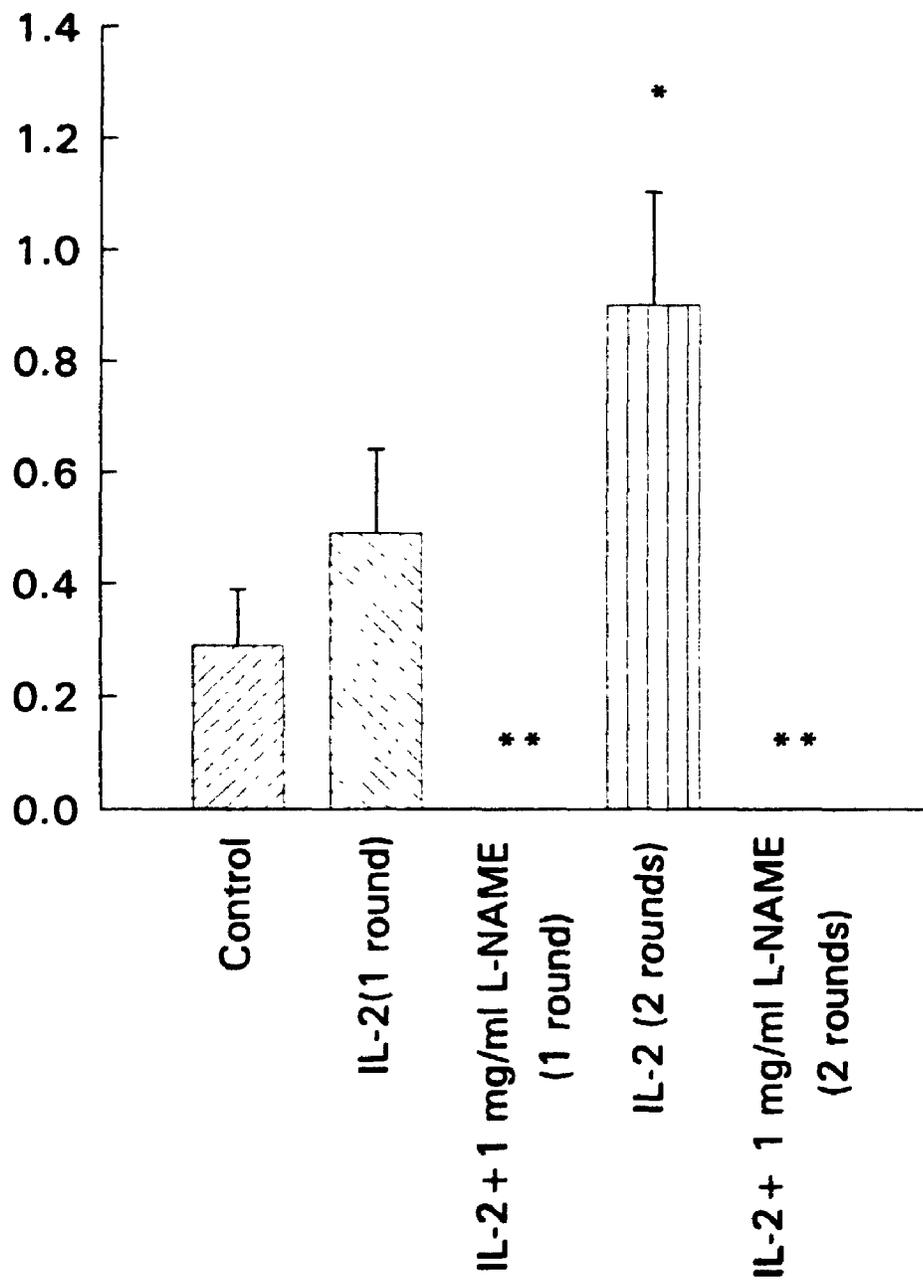
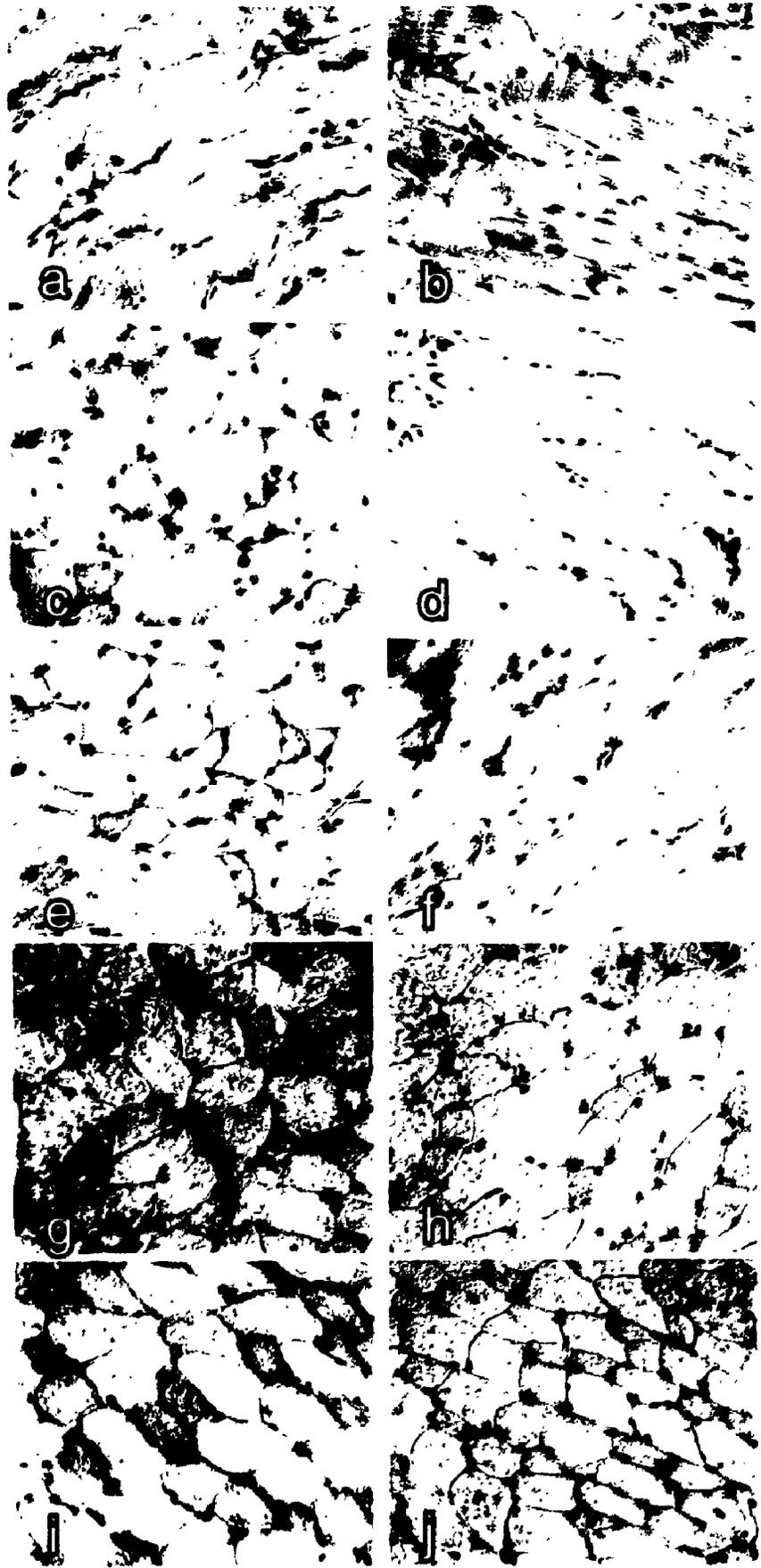


Figure 8.8 Immunostaining with a mouse monoclonal antibody against iNOS (a,c,e,g,i) and a negative control antibody of same isotype (b,d,f,h,j) in the anterior thoracic wall after one or two rounds of IL-2 ± L-NAME therapy, lightly stained with hematoxylin (x 40).

a&b control mice; c&d IL-2, 1 round; e&f IL-2 + L-NAME, 1 round; g&h IL-2, 2 rounds; i&j IL-2+L-NAME, 2 rounds.

Strong endothelial cell immunostaining for iNOS is present in all IL-2 treated groups. Staining of endothelial cells appears stronger after two rounds of therapy, when some staining is also noted in some muscle fibres.



anterior thoracic wall, did not influence expression nor distribution of iNOS enzyme. Two rounds of IL-2 ± L-NAME was followed by stronger staining for iNOS enzyme present in the endothelial cells of capillaries surrounding the fibres of intercostal muscles, as well as a punctate staining of some muscle fibres (Figure 8.8.g. and 8.8.i.).

Lungs: Although nitric oxide synthase activity in the lungs was significantly induced by two rounds of IL-2 therapy, there was no significant difference in iNOS staining between the lungs of control and treated mice (data not presented).

Spleen: iNOS positive macrophages were scanty in the spleen of normal mice, and abundant in the spleen of IL-2 or IL-2 + L-NAME treated animals, either after one or two rounds of therapies (Figure 8.9.). After one round of therapies, iNOS positive macrophages were mostly present in the red pulp of the spleen of IL-2 treated mice, whereas in mice subjected to IL-2+L-NAME, they also accumulated around the periphery of the white pulp. After two rounds of therapies, iNOS positive macrophages accumulated within the white pulp of IL-2 treated animals, and were most numerous at the periphery of the white pulp of IL-2+L-NAME treated animals.

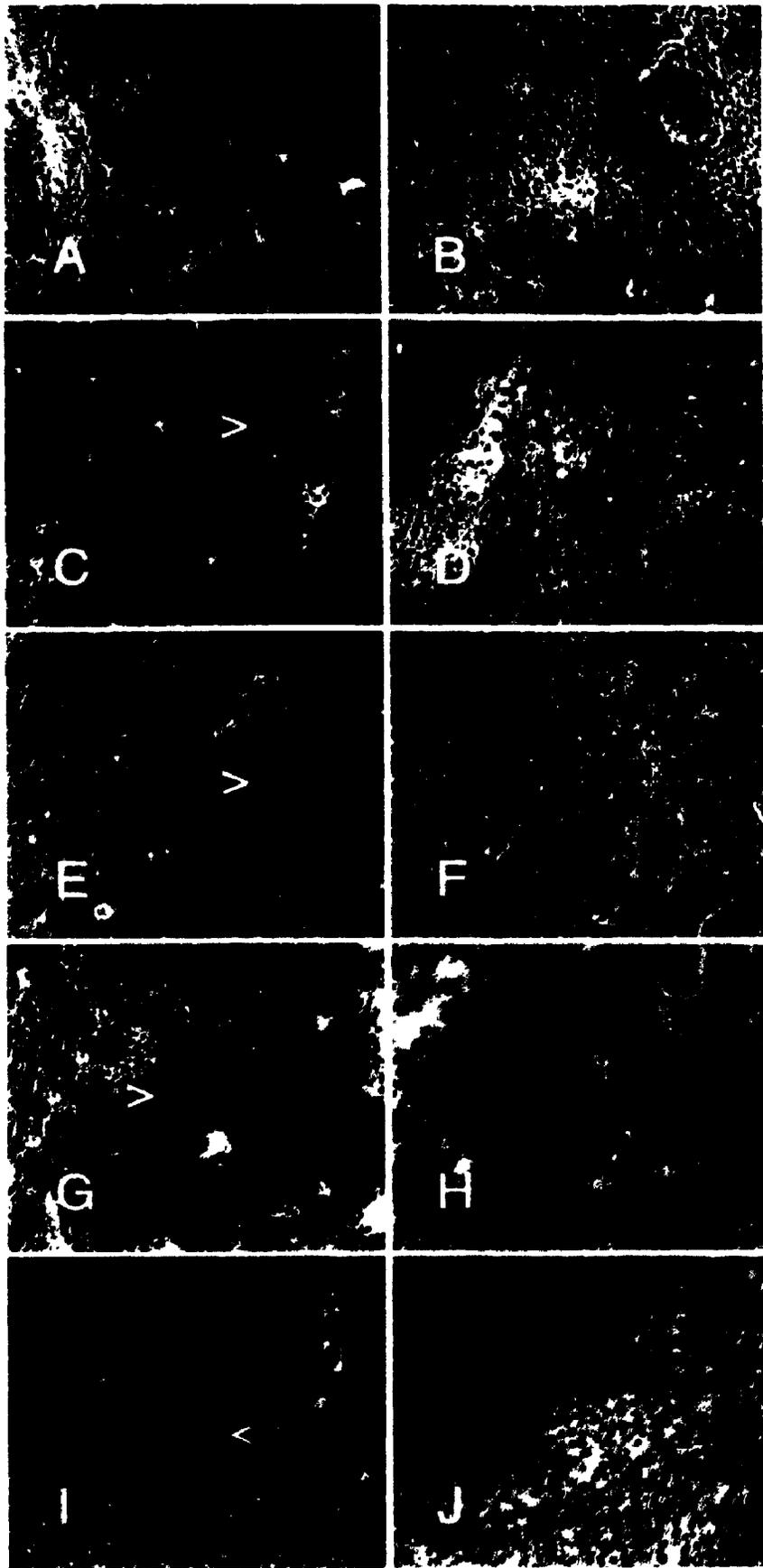
8.4 Discussion

Present study revealed that IL-2 therapy induced significant Ca^{++} independent (i.e. inducible) NOS activity in the lungs and the thoracic wall, which possibly played a central role in mediating IL-2 induced pulmonary edema and pleural effusion. An amelioration of these most severe symptoms of IL-2 induced capillary leakage with addition of L-NAME was associated with an abrogation of NOS activity in these tissues.

Pleural effusion following one round of IL-2 therapy was accompanied by an

Figure 8.9 Immunostaining with a mouse monoclonal antibody against iNOS (a,c,e,g,i) and a negative control antibody of the same isotype (b,d,f,h,j) in the spleen after one or two rounds of IL-2 ± L-NAME therapy, lightly counterstained with hematoxylin (x 40). A&B control; C&D IL-2, 1 round; E&F IL-2 + L-NAME, 1 round; G&H IL-2, 2 rounds; I&J IL-2+L-NAME, 2 rounds.

> Immunostaining for iNOS is present in a substantial cell population (identified subsequently as macrophages at higher magnification) in the spleens of all IL-2 treated groups.



induction of highly active NOS enzyme in the anterior thoracic wall. The enzyme was mostly localized in the endothelium of the capillaries surrounding the intercostal muscle fibres. Since the parietal pleura is supplied by the vessels which also supply the thoracic wall, this local source of NO may have likely contributed to the high NO level in the pleural fluid. There was no pleural effusion in mice following two rounds of IL-2 therapy, confirming previously described findings (chapter 4, 6). Interestingly, there was also a lack of significant induction of iNOS activity in the thoracic wall at this time, again confirming the association of pleural effusion with local NOS activity. This can best be interpreted on the basis of a feedback inhibition of NOS activity by high NO levels, as reported by Moncada laboratory for a macrophage cell line *in vitro* (Assreuy *et al.* 1993). Subsequently, it was shown by Billiar's group (Luss *et al.* 1994) that NO not only exerted feedback inhibition of NOS activity, but also decreased the level of iNOS protein expression, most likely by inhibiting translation of the iNOS protein. Surprisingly, however, an abundance of immunoreactive iNOS protein (both after the second round of IL-2 or IL-2+L-NAME) was still detected in the anterior thoracic wall in the present study, and seemed to be localized to endothelium of capillaries surrounding the intercostal muscle fibres and to some muscle fibres after IL-2 + L-NAME. Endothelial cell staining by the anti iNOS antibody was not due to a cross reactivity to eNOS or nNOS, based on the following findings: (1) There was no staining of endothelial cells or muscle cells in the tissues from untreated control animals; (2) C3-L5 cells, which constitutively express eNOS (Chapter 9), but not iNOS, were never found to exhibit positive immunoreactivity to iNOS antibody under these conditions.

Currently, it is not possible to give an explanation for the detection of iNOS

protein in the absence of iNOS activity after the second round of IL-2. This paradox remains to be resolved by further quantitative studies, e.g. Western analysis of the iNOS protein.

IL-2 induced pulmonary edema was present after either round of IL-2 therapy and accompanied with a significant NOS activity in the lungs. Addition of L-NAME therapy during either round abolished NOS activity, but reduced pulmonary edema only after first round of IL-2 therapy. Thus, one would assume that NO induction as well as other mechanism(s) may be responsible for the presence of pulmonary edema after the second round of IL-2 therapy, so that inhibition of NO synthesis with L-NAME was not enough to prevent IL-2 induced pulmonary edema. It is likely that a direct LAK cell mediated injury to the pulmonary endothelium was higher after the second round of IL-2.

A significant increase in the thickness of the interalveolar connective space (as measured by computerized histomorphometry) accompanied IL-2 induced pulmonary edema. Histological analysis revealed marked lymphocyte infiltration similar to those showed by Parhar and Lala (1987) and Dubinett *et al.* (1994). Ultrastructural analysis revealed that IL-2 induced pulmonary edema was due to major distortion in the capillary architecture of the lungs. The changes included endothelial cell and type I pneumocyte swelling or damage, thickening of the basement membrane of the thin portion of the capillaries or herniation of the thin segment of endothelial cell into the vessel lumen because of the accumulation of fluid between the plasma membrane and the basement membrane. Similar findings were described in the lungs of rats and rabbits treated with IL-2 (Renzi *et al.* 1991; Goldblum *et al.* 1990). Dubinett *et al.*, (1994), suggested that IL-2 induced TNF α played a central role in mediating the pulmonary vascular leakage,

since IL-2 upregulated the *in situ* expression of both the TNF α mRNA the protein in the lungs, whereas administration of a soluble TNF α receptor significantly reduced IL-2 induced pulmonary edema. Present study revealed that NO inhibition with L-NAME reduced IL-2 induced pulmonary edema, concomitant with a significant reduction of the thickness of interalveolar spaces. This was associated with ultrastructural evidence of significant restoration of capillary architecture in the lungs. Since TNF α induced endothelial cell damage *in vitro* through production of NO (Palmer *et al.* 1992; Estrada *et al.* 1992), it is reasonable to postulate that IL-2 induced TNF α production *in vivo* may have been instrumental in high NO production, leading to pulmonary edema.

Macrophages immunoreactive for the iNOS protein became abundant in the spleen of IL-2 or IL-2 + L-NAME treated animals, either after one or two rounds of therapies. It appeared that there were more macrophages stained in the spleens of mice treated with IL-2 + L-NAME group, than in IL-2 treated group. Luss *et al.*, (1994) reported that chronic inhibition of NO production can result in an increase in iNOS mRNA level and protein in iNOS expressing cells, although such iNOS may remain inactive due to the presence of L-NAME.

It was earlier shown (Chapter 7) that *in vivo* LAK cell activation in splenocytes of IL-2+L-NAME treated mice was significantly higher than that in IL-2 alone treated animals, suggesting that IL-2 induced increase in NOS activity followed by increased NO production within the spleen interfered with optimal LAK cell activation. Whether a cessation of L-NAME therapy would lead to a rebound in NO production in the presence of high iNOS protein and how would this influence splenocyte cytotoxicity, remains to be investigated.

In summary, IL-2 therapy in mice induced iNOS expression in numerous tissues including the endothelium and muscles of the anterior thoracic wall and splenic macrophages. High NOS activity in the lungs and the thoracic wall was accompanied with the presence of pulmonary edema, pleural effusion and structural damage to the lungs and its capillaries. Addition of NOS inhibitor, L-NAME, totally abolished NOS activity, but not necessarily iNOS expression. It also reduced IL-2 induced pulmonary edema and pleural effusion and significantly restored structural integrity of the lungs. Thus, high tissue activity of iNOS enzyme plays a crucial role in the pathogenesis of IL-2 induced capillary leak syndrome.

CHAPTER 9 - NITRIC OXIDE PRODUCTION BY C3-L5 MURINE MAMMARY ADENOCARCINOMA CELLS PROMOTES TUMOR CELL INVASIVENESS

9.1 Introduction

The role of NO in tumor biology remains controversial and still poorly understood. High serum NO levels have been reported in some cancer patients (Miles *et al.* 1994). A number of reports indicate a contributory role of NO to tumor progression. The level of NOS protein (Thomsen *et al.* 1994; Cobbs *et al.* 1995), as well as NOS activity (Thomsen *et al.* 1994) has been positively correlated with the degree of malignancy in a number of human cancers, including human gynaecological (ovarian, uterine) cancers (Thomsen *et al.* 1994), central nervous system tumors (Cobbs *et al.* 1995) as well as breast cancer (Thomsen *et al.* 1995). The high NOS activity has been explained by the presence of constitutive form(s) in tumor cells (Thomsen *et al.* 1994; Cobbs *et al.* 1995) and / or tumor endothelial cells (Cobbs *et al.* 1995), and the inducible form in the tumor endothelial cells (Cobbs *et al.* 1995) and/or tumor associated macrophages (Thomsen *et al.* 1995). Expression of iNOS in the tumor neovasculature has also been reported in experimental tumors (Buttery *et al.* 1993). Furthermore, two lines of direct evidence exist for a facilitatory role of NO on tumor progression: (a) Numerous human colon cancer cell lines are found to express NOS activity (Jenkins *et al.* 1994), and engineered overexpression of iNOS in a human colonic adenocarcinoma line was found to increase tumor growth when transplanted in nude mice (Jenkins *et al.* 1995). (b) In a rat colonic adenocarcinoma model, treatment with L-NAME, an NO synthase inhibitor, was shown to reduce NO production as well as tumor growth (Kennov'n *et al.*

1994). Similarly, antitumor as well as antimetastatic effects of two inhibitors of NO, NMMMA and L-NAME, have been observed in the present study (Chapter 4, 6). In apparent contradiction to the above reports, a histochemical localization of NAD(P)H diaphorase staining (believed to parallel NOS activity) appeared to be inversely related to colonic tumor progression (Chhatwal *et al.* 1994). Similarly, high NOS activity has been inversely correlated to tumor growth and metastasis in a murine melanoma model, in which an engineered overexpression of iNOS made the cells nontumorigenic, because of NO mediated apoptosis of tumor cells (Xie *et al.* 1995). These reported discrepancies may possibly be explained by dual role of NO on tumor growth. Whereas very high NO producing tumor cell clones may delete themselves by apoptosis, NO may facilitate growth of surviving clones *in vivo* by numerous mechanisms, eg. promotion of neoangiogenesis, tumor blood flow or invasiveness. While some evidence exist for contributory role of NO in promotion of neoangiogenesis (Buttery *et al.* 1993) and/or tumor blood flow (Andrade *et al.* 1992), possible role of tumor derived NO on tumor cell invasiveness has not been explored.

The mechanisms by which NO may have promoted tumor growth or metastasis remained undefined in C3H/HeJ mammary adenocarcinoma model. The objectives of the present study were to identify the source of NO in the spontaneously metastasizing C3-L5 mammary adenocarcinoma model and the possible role of tumor-derived NO on tumor cell invasiveness.

9.2 Materials and methods

9.2.1 Tumor cell line

C3-L5 mammary adenocarcinoma cell line was selected and maintained in Dr.

Malala's laboratory as described in chapter 3. After thawing, cells were grown in RPMI 1640 medium (Gibco BRL, Burlington, ON) with 1% antibiotics (Mediatech, Washington, DC), supplemented with 10% fetal calf serum (Gibco BRL, Burlington, ON) in 75 cm² flasks (Becton Dickinson and Co., Lincoln Park, NJ) until confluent. Antibiotics contained 5,000 I.U./ml of Penicillin and 5,000 mcg/ml of Streptomycin. All experiments were done with cells passaged 3 - 5 times after thawing.

9.2.2 N^G-Nitro-L-Arginine Methyl Ester and N^G-Methyl-L-Arginine.

L-NAME and NMMA (Sigma Chemical Company, St. Louis, MO) were added to the medium in concentrations of 0.01 - 1 mM, and L-NAME was added in some experiments in concentration of 1 mg/ml of medium (equivalent of 3 mM).

9.2.3 Interferon τ (IFN τ)

Murine recombinant IFN τ (lot FC2B11) was obtained from Gibco (Burlington, ON), reconstituted with sterile water in aliquots of 10,000 U/100 μ l and stored at -70°C until used for assays.

9.2.4 Lipopolysaccharide (LPS)

LPS powder was obtained from Sigma (St. Louis, MO), stored at 4°C, dissolved with complete medium on the day of assay and sterilized by filtration (0.2 μ m filter pore size, Nalgene syringe filters, Nalgene Co., Rochester, New York).

9.2.5 Experimental procedures

9.2.5.1 Immunocytochemical localization of NOS enzymes in C3-L5 cells and primary tumor tissue

C3-L5 cells were grown for 24 h on chamber slides, either in complete medium alone or in medium containing 1000 U/ml of IFN τ or 100 ng/ml of LPS or combination

of IFN γ + LPS in a humidified incubator (37°C, 5% CO₂ atmosphere). Slides were briefly washed with phosphate buffered saline (PBS), fixed in 10% buffered formalin, and permeabilized with 0.25% Triton X-100 in PBS. After washing (3x5 min PBS), 10% normal goat serum was added to the slides as blocking serum. Mouse monoclonal antibody against macrophage iNOS (Transduction Laboratories, Lexington, KY, 1:50 dilution) was used for identification of inducible NOS expression in C3-L5 cells, and rabbit polyclonal antibody against eNOS (Affinity Bioreagents, Neshanic Station, NJ, 1:200 dilution) was used for constitutive endothelial NOS expression in C3-L5 cells. Secondary goat anti-mouse or goat anti-rabbit fluorescein conjugated antibody (1:150 dilution, Vector Laboratories, Burlingame, Canada) was added after primary antibody, and positive immunostaining was identified under fluorescent microscope. Incubation time for both primary and secondary antibodies was 1h each. Negative controls were provided by omission of primary antibodies.

Conventional histological sections of primary tumors grown in C3H/HeJ mice for 24 days (Chapter 6), following subcutaneous transplantation of 250,000 C3-L5 cells were fixed in 10% buffered formalin, paraffin embedded, and cut at 4 μ m thick sections. After deparaffinization, and blocking of endogenous peroxidase activity (3% H₂O₂ in absolute methanol), sections were permeabilized with 0.25% Triton X-100 in PBS. 10% normal horse serum or 10% normal goat serum was added to the slides as blocking sera, followed by mouse monoclonal or rabbit polyclonal primary antibodies, respectively, incubated overnight. Mouse monoclonal antibody against macrophage iNOS was again used for identification of iNOS expression in tumor tissue, and rabbit polyclonal antibody against eNOS for eNOS expression in tumors. Secondary horse anti-mouse biotinylated

antibody was added after primary monoclonal antibody, and goat anti-rabbit biotinylated antibody after primary polyclonal antibody. Sections with secondary antibodies (Dimension Laboratories, Mississauga, ON, 1:200 dilution) were incubated 30 min, and followed with ABC complex (1h) and DAB chromogen. Sections were counterstained with hematoxylin, and NOS expression identified by positive brown staining from DAB chromogen under light microscope. Negative controls were provided by omission of primary antibodies.

9.2.5.2 Matrigel invasion assay by C3-L5 cells

An *in vitro* transwell matrigel invasion assay (Graham *et al.* 1993; Graham *et al.* 1994) was used to test invasiveness of C3-L5 cells in the presence or absence of 0.01, 0.1, and 3 mM NO blocking agent L-NAME or NMMA or iNOS inducers, IFN γ (500-1000 U/ml) + LPS (50-100 ng/ml), in the presence or absence of L-NAME. Some wells contained L-arginine (5 mM, specificity control, which was supposed to abrogate the effects of NO inhibitors). In this assay, tumor cells were prelabelled with ³HTdR for 24 h, and then added to the invasion chamber of the transwell containing a matrigel (reconstituted basement membrane, Collaborative Research, Inc.)-coated millipore membrane. Percentage of labelled cells penetrating the matrigel-millipore membrane were scored as percent radioactivity appearing in the lower well and bottom of the millipore membrane, as a function of time (1-3 days). All assays were done in triplicate.

9.2.5.3 Measurement of NO production in the media from C3-L5 cells

C3-L5 cells were grown in 24 well plates (10⁶ cells/800 μ l media/well) either in the medium alone, L-NAME alone (1mg/ml) or in the presence of IFN γ (500 - 1000 U/ml) + LPS (50 - 100 ng/ml) + L-NAME. Culture media from wells were

collected after 24h of the incubation period, and kept frozen at -20°C , until assayed for NO_2^- levels. 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), as described in chapter 3.

9.3 Results

9.3.1 Expression of immunoreactive eNOS and iNOS protein by C3-L5 cells *in vitro*

Immunocytochemical staining for eNOS enzyme revealed that C3-L5 mammary carcinoma cells constitutively expressed high level of eNOS protein *in vitro* (Figure 9.1.A). They also acquired iNOS protein after induction with combination of $\text{IFN}\gamma$ +LPS (Figure 9.1.B). $\text{IFN}\gamma$ alone or LPS alone did not induce iNOS in C3-L5 cells (data not presented). None of the negative controls in the above experiments expressed any labelling (data not shown).

Subcutaneous tumors grown in C3H/HeJ mice for 24 d after transplantation of 250,000 C3-L5 cells, as well as their spontaneous metastatic counterparts (lungs) expressed eNOS protein (Figure 9.2 a and c), but not iNOS protein (data not shown). eNOS protein was present in majority of tumor cells at the primary tumor site, and in approximately half of the tumor cells in the lung metastatic nodules.

9.3.2 Invasiveness of C3-L5 cells *in vitro*

C3-L5 cells exhibited significant invasiveness in 3d matrigel invasion assay, as indicated by an invasion index of $50 \pm 5\%$. This value is normalized to 100% in fig. 9.3 to test the effects of NMMA. NMMA at 0.25-1 mM doses reduced this invasiveness of C3-L5 cells. Addition of excess L-arginine abrogated the NMMA effects, indicating

Figure 9.1 Immunofluorescent staining of C3-L5 cells in culture.

A - eNOS labelling is present in 90-95% of cells (x 16).

B - iNOS labelling is present in 15-20% of cells after induction with IFN γ +LPS (x 40).

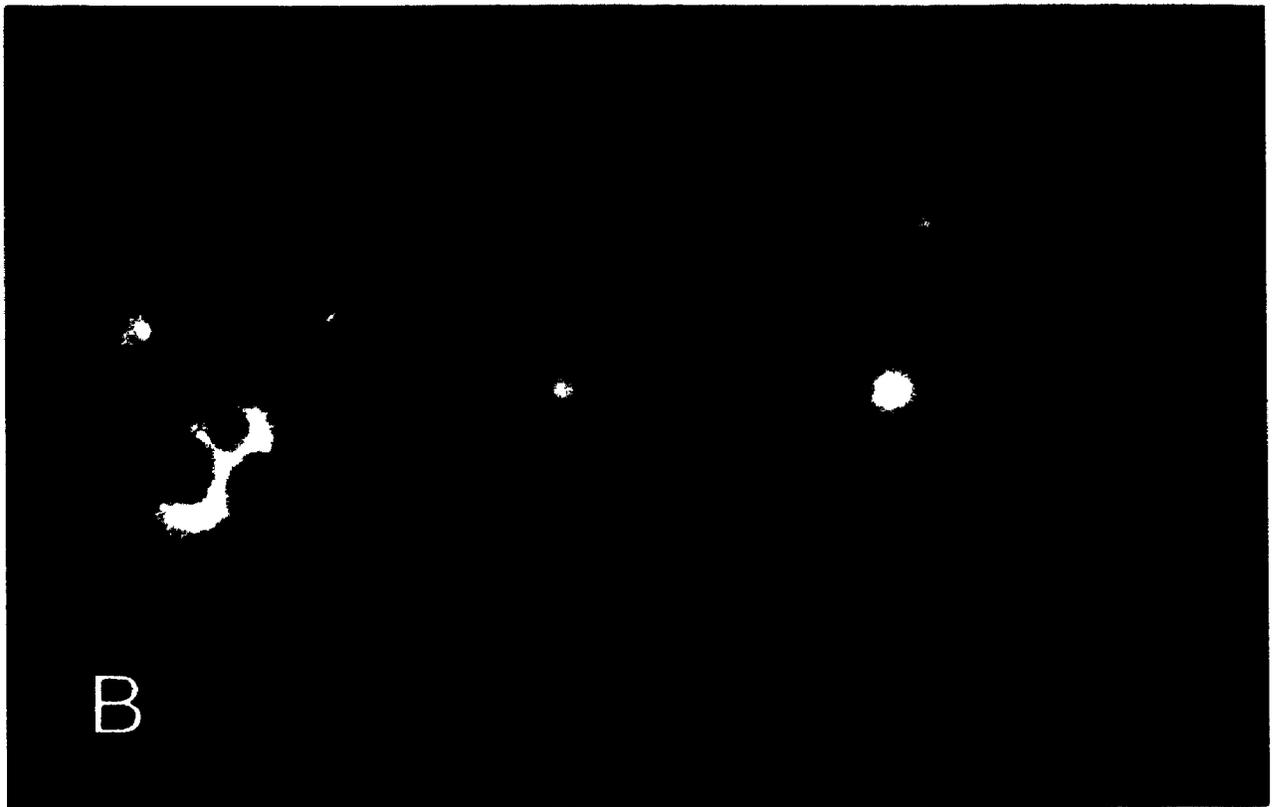
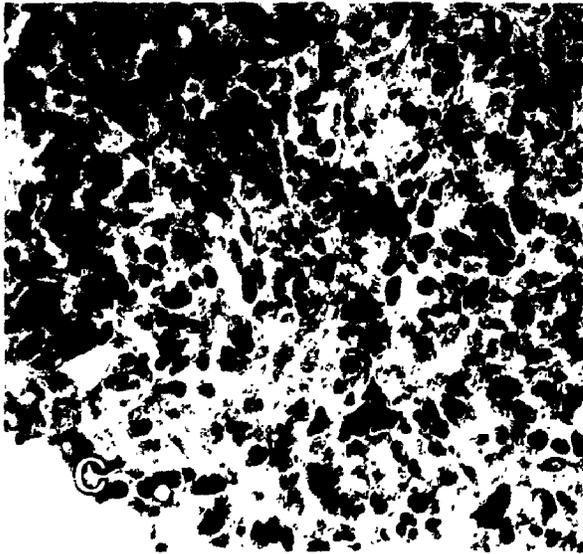
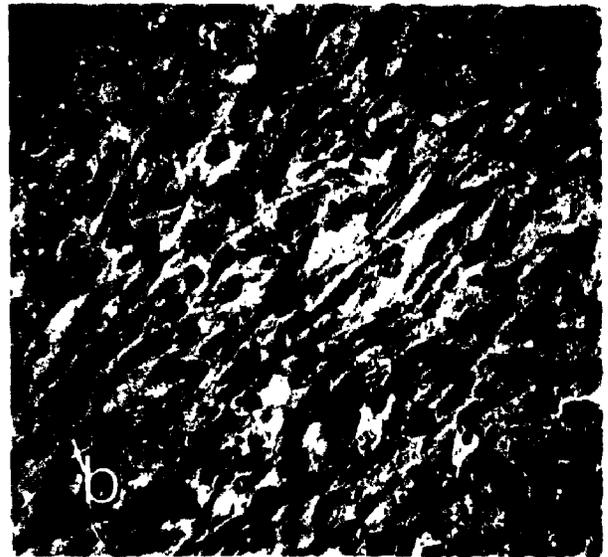
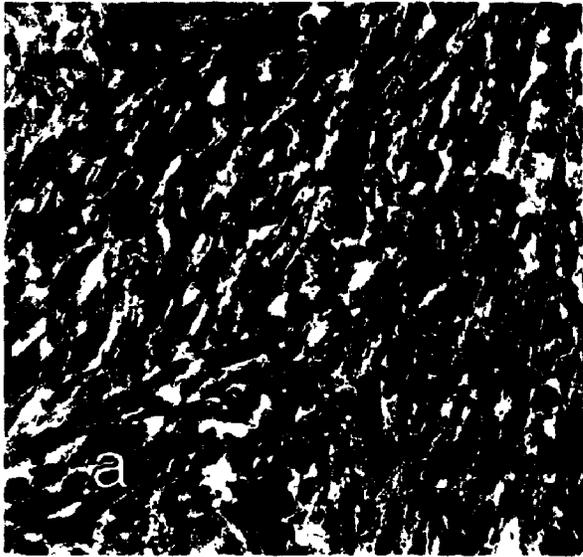


Figure 9.2 Immunocytochemical localization of eNOS in primary tumors and their metastatic lung nodules, 24 days after sc.transplantation of C3-L5 cells into C3H/HeJ mice (x 40).

eNOS is present in approximately 80 % of tumor cells at the primary site (a) and approximately 50% of tumor cells in a lung metastasis (c). (b) and (d) are corresponding negative control (omission of primary antibody). Sections were counterstained with hematoxylin.



the specificity of NMMA action (Figure 9.3.).

In another experiment, L-NAME at doses of 0.01 - 1 mM significantly ($p < 0.05$) reduced invasiveness of C3-L5 cells in a 3 d matrigel invasion assay (Figure 9.4).

Combination of IFN γ and LPS (500 U/ml and 50 ng/ml, respectively) significantly ($P < 0.05$) stimulated invasiveness of C3-L5 cells in 3 d matrigel invasion assay (Figure 9.5.). Reduction of IFN γ +LPS induced invasiveness with addition of L-NAME (1 mg/ml) was not, however, significant in this experiment (Figure 9.5.). A combination of IFN γ and LPS at higher concentrations (1000U/ml and 100 ng/ml, respectively), also significantly stimulated invasiveness of C3-L5 cells in both 1 d and 3 d matrigel invasion assays in separate experiments (data not presented).

9.3.3 NO production in the medium of C3-L5 cells treated with IFN γ + LPS \pm L-NAME

Nitrate + nitrite levels in the medium were significantly ($p < 0.05$) increased after 24 h stimulation of C3-L5 cells with IFN γ +LPS (500 U/ml and 50 ng/ml, respectively). Reduction of IFN γ +LPS induced NO production in the medium with addition of 1 mg/ml of L-NAME was not significant (Figure 9.6.). These data relate to the same experiment, as presented in Fig. 9.5.

9.4 Discussion

Results from the present study revealed that *in vitro* propagated C3-L5 mammary adenocarcinoma cells expressed eNOS protein and in addition, were stimulated to express iNOS protein, when grown in the presence of IFN γ +LPS. Tumor cells grown *in vivo* expressed eNOS, but not iNOS protein, both at the primary site, as well as the sites of spontaneous lung metastasis. These cells exhibited a strong ability to invade matrigel, and

Figure 9.3 Matrigel invasion by C3-L5 cells treated with different doses of NMMA
(± L-arginine)

NMMA at 0.25 - 1 mM doses reduced ($p < 0.05$) tumor cell invasiveness. Excess of L-arginine abrogated the NMMA effects indicating the specificity of NMMA action.

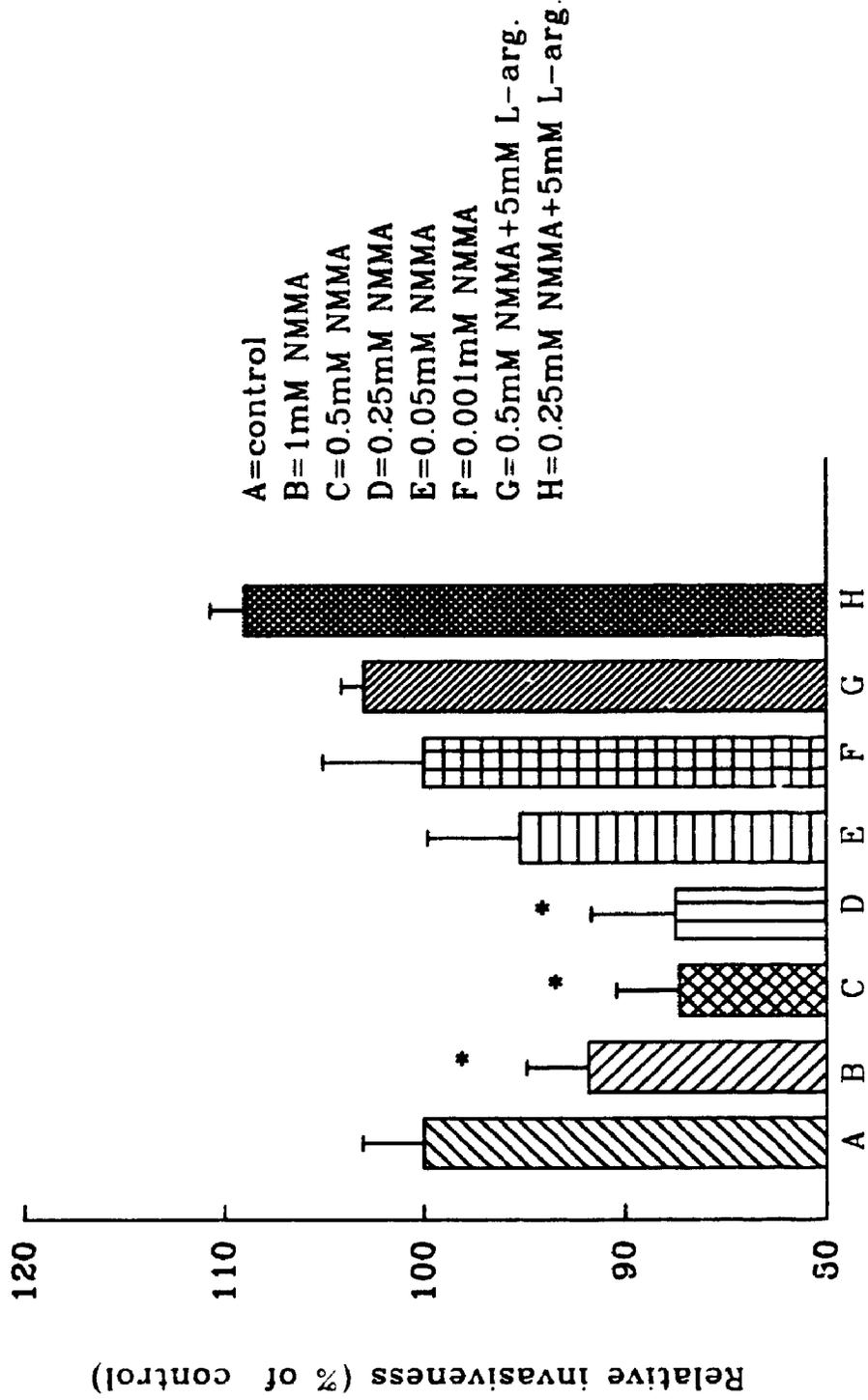


Figure 9.4 Matrigel invasion by C3-L5 cells treated with different doses of NMMA and L-NAME.

Both agents at all doses (0.01 to 1 mM) reduced ($p < 0.05$) tumor cell invasiveness.

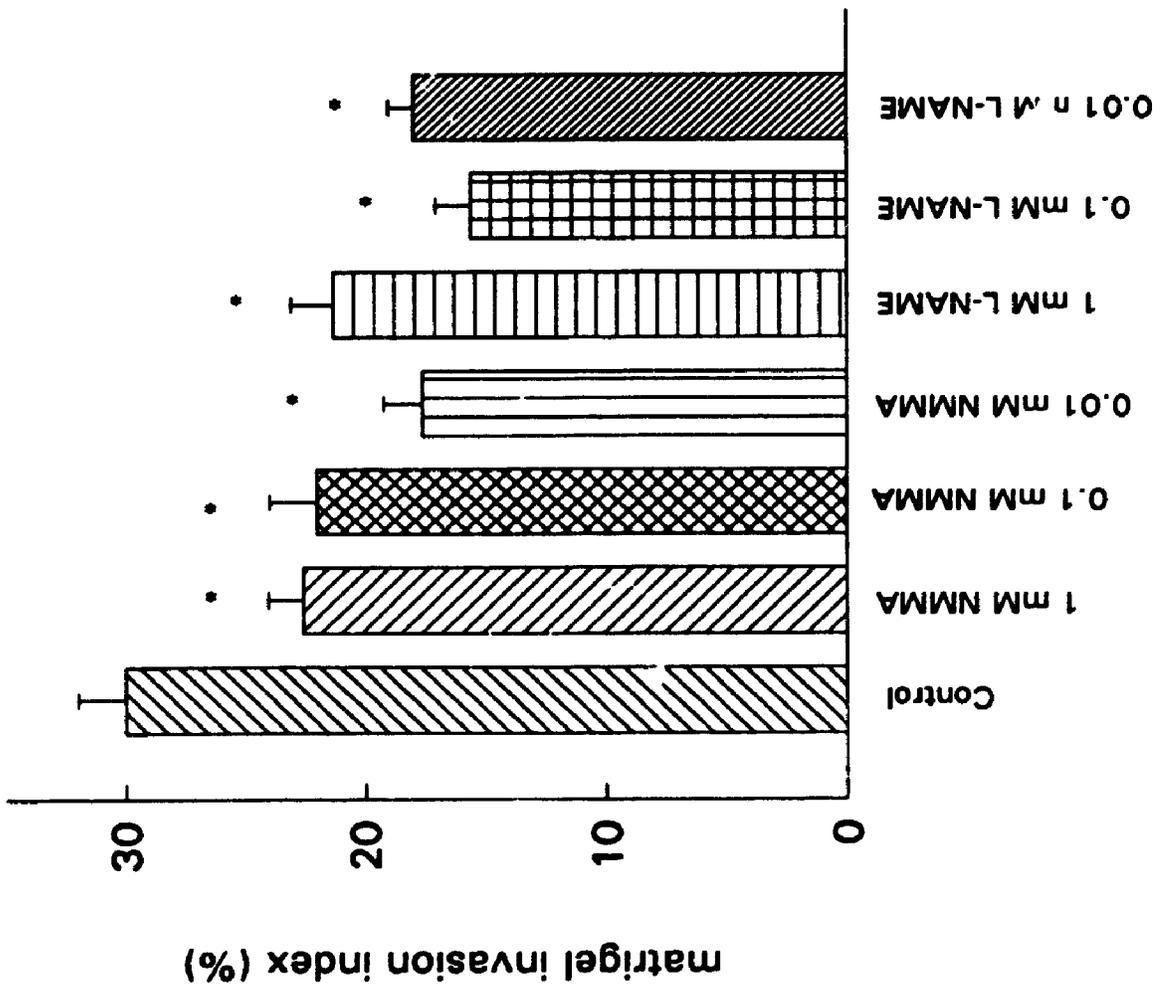


Figure 9.5 Matrigel invasion by C3-L5 cells treated with IFN γ (500 U/ml) + LPS (50 ng/ml).

* significantly ($p < 0.05$) different from control.

IFN γ + LPS significantly ($p < 0.05$) stimulated invasiveness of C3-L5 cells in 3 d matrigel invasion assay. Reduction of IFN γ +LPS induced invasiveness with addition of L-NAME (1 mg/ml) was not significant.

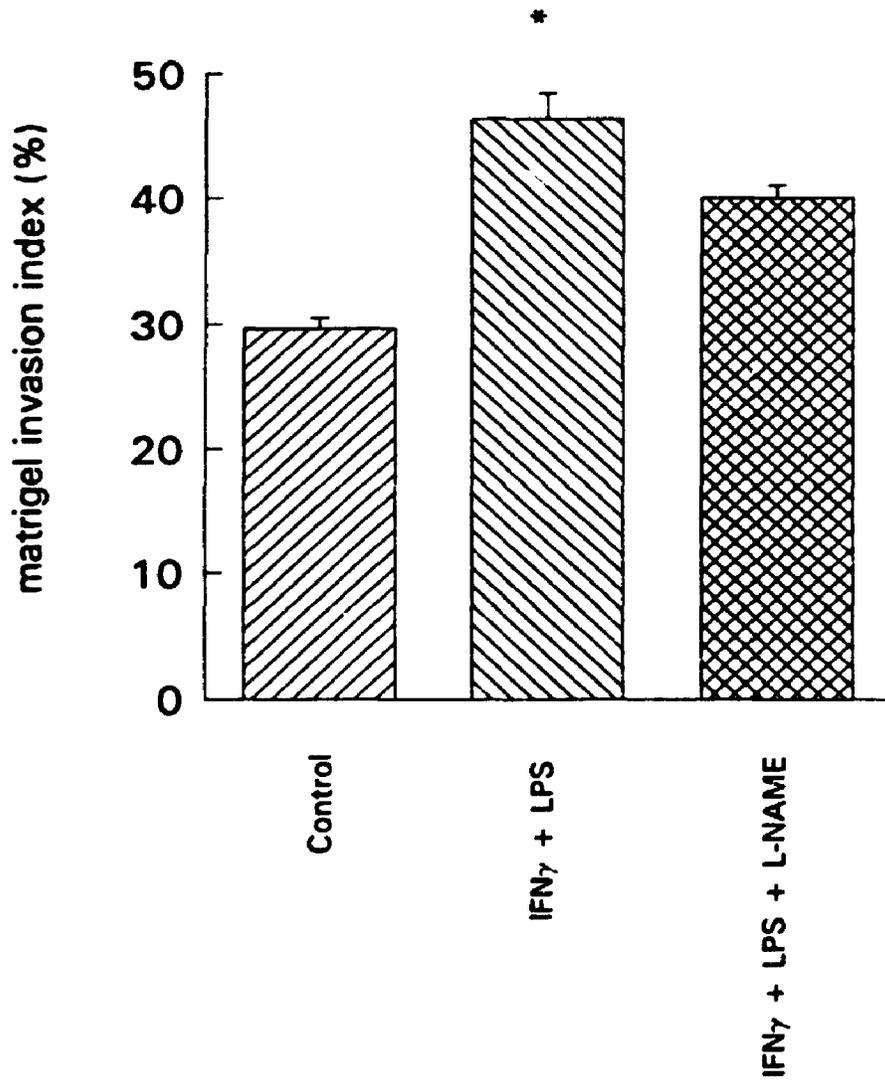
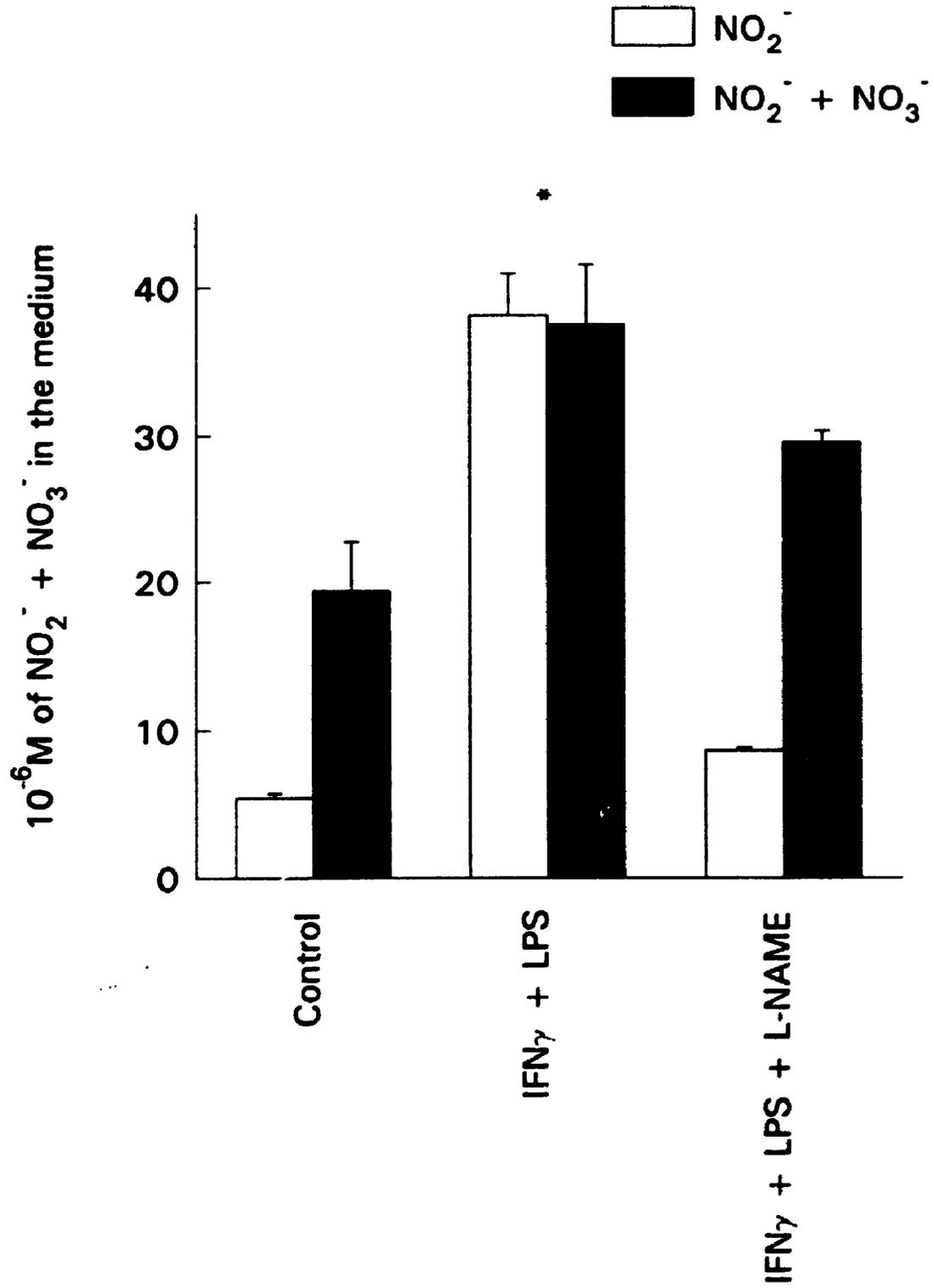


Figure 9.6 One day accumulation of nitrate+nitrite in the medium of C3-L5 cells treated with IFN γ (500 U/inj), LPS (50 ng/ml) and L-NAME (1 mg/ml).

Nitrate+nitrite levels in the medium of C3-L5 cells were significantly ($p < 0.05$) increased after 24 h stimulation with IFN γ +LPS. Reduction of IFN γ +LPS induced nitrate+nitrite production with addition of L-NAME (1 mg/ml) was not significant.



their invasiveness was reduced in the presence of NO blocking agents (NMMA and L-NAME). The anti-invasive effects were abrogated in the presence of excess L-arginine, attesting to the fact that the effects were due to an inhibition of NO synthesis. Finally, invasiveness of C3-L5 cells was stimulated in the presence of iNOS inducing agents IFN γ +LPS, with a concomitant increase in NO production *in vitro*. In this case, addition of L-NAME failed to abrogate the invasiveness significantly, apparently due to a failure of significant reduction in NO production. Taken together, these results demonstrated that NO production by C3-L5 cells promoted tumor cell invasiveness.

It was previously reported (chapter 4 and 6) that treatment of C3H/HeJ mice bearing C3-L5 mammary adenocarcinoma transplants, with NOS inhibitors, NMMA and L-NAME had significant antitumor and antimetastatic effects. Growth retarding effects of L-NAME were also observed in a rat colonic adenocarcinoma model (Kennovin *et al.* 1994), indicating that NO had a promoting role on tumor progression in these tumor models. Indeed, high NOS activity has been positively correlated to the progression of a number of human tumors eg. human gynecological cancer (Thomsen *et al.* 1994), central nervous system tumors (Cobbs *et al.* 1995) and breast cancer (Thomsen *et al.* 1995). In concurrence with these observations, C3-L5 mammary carcinoma cells, used in the present study, are found to express high levels of eNOS protein *in vitro*, as well as *in vivo* in primary and metastatic tumors. They also expressed iNOS protein following culture with IFN γ and LPS. These findings attest to the NO producing ability of these cells under constitutive conditions, which may be enhanced under inductive circumstances.

Multiple mechanisms may be postulated for the ability of NO produced by tumor

cells or host-derived cells in the tumor to promote tumor growth or metastases. Because of its vasodilatory function (Palmer *et al.* 1987), NO may promote the blood flow through the tumor vasculature and thus indirectly promote tumor cell nourishment. This hypothesis was supported by the temporal relationship of the reduction in tumor growth to L-NAME therapy in a rat adenocarcinoma model (Kennovin *et al.* 1994). NO has been shown to have a stimulatory effect on angiogenesis *in vitro* (Ziche *et al.* 1994), as well as *in vivo* studies with a rabbit cornea model (Ziche *et al.* 1994), or a model of healing gastric ulcer (Konturek *et al.* 1993). Angiogenesis-promoting role of NO was supported in a tumor model by the demonstration of a high vascularity of transplants of human colonic adenocarcinoma cells in nude mice, when these cells were engineered to over-express mouse iNOS gene (Jenkins *et al.* 1995). The preliminary findings of a reduction in the number of microvessels per unit area of primary C3-L5 mammary tumors in C3H/HeJ mice, when the mice were subjected to NMMA therapy (Orucevic and Lala, unpublished), also support this hypothesis. Another possible mechanism, a direct NO-mediated stimulation of tumor cell proliferation, has been excluded in the C3-L5 tumor model. Treatment of C3-L5 cells *in vitro* with NMMA had no effect on ³HTdR uptake by these cells (Orucevic and Lala, unpublished). Finally, as demonstrated in this study, NO production by C3-L5 cells promoted tumor cell invasiveness, and this mechanism may explain, at least in part, the observed reduction of primary tumor growth, as well as spontaneous lung metastasis following NMMA or L-NAME therapy (Chapter 4, 6).

The invasion promoting mechanisms by the presence of NO in the cellular microenvironment remain to be investigated. They include (a) a promotion of cellular migratory ability and (b) a promotion of cellular matrix degrading ability either because

of an upregulation of matrix degrading enzymes, e.g. matrix metalloproteinases (MMP's) or urokinase plasminogen activator (uPA) or a down-regulation of their inhibitors, e.g. tissue inhibitor of metalloproteinases (TIMP's) and plasminogen activator inhibitors (PAI's). Studies are in progress to identify these mechanisms.

In summary, C3-L5 cells express high level of eNOS protein *in vitro*, as well as *in vivo* in primary and metastatic tumors. They also express iNOS protein following culture with iNOS inducers. Since NO produced by C3-L5 cells promoted tumor cell invasiveness, one could suggest that the observed antitumor and antimetastatic effects of NMMA and L-NAME *in vivo* in the C3-L5 tumor model were at least partially due to inhibition of NO mediated tumor cell invasiveness.

CHAPTER 10. - SUMMARY AND CONCLUSIONS

The initial parts of this study were designed to test the hypothesis that an overproduction of NO was instrumental in the pathogenesis of "capillary leak syndrome", a major side effect of systemic IL-2 therapy, which has recently been recognized as a valuable mode of immunotherapy of cancer and infectious diseases. Studies were conducted in healthy as well as mammary adenocarcinoma-bearing C3H/HeJ mice to measure IL-2 therapy induced capillary leakage (pleural effusion, pulmonary edema and water content of the spleen and kidneys), NO production *in vivo* and the influence of additional therapy with two nitric oxide inhibitors, NMMA and L-NAME, on these parameters. Influence of these two inhibitors on IL-2 therapy-induced regression of the primary tumors and their lung metastases was also examined. Finally, the effects of these NO inhibitors alone on mammary tumor growth and metastases were also evaluated. Since NO inhibitors alone exhibited antitumor and antimetastatic effects, further experiments were designed to identify the source of NO production by the tumor, and whether tumor-derived NO promoted tumor cell invasiveness. Since L-NAME therapy also potentiated tumor-reductive effects of IL-2 therapy when there was a reduction of IL-2 induced rise in NO production *in vivo*, experiments were designed to test whether L-NAME treatment *in vivo* or *in vitro* had a potentiating effect on IL-2 induced antitumor cytotoxicity of splenocytes of healthy or tumor-bearing mice.

Results revealed that intraperitoneal IL-2 therapy caused substantial capillary leakage, both in healthy and tumor-bearing mice, as well as substantial rise in NO production *in vivo* (measured in the serum and pleural effusion) in an IL-2 dose dependent manner. Subcutaneously administered NMMA, when combined with IL-2

therapy, failed to ameliorate IL-2 induced capillary leakage in both groups of mice, and was inadequate in reducing IL-2 induced rise in NO production *in vivo*. It did not compromise antitumor effects of IL-2 therapy. In mammary adenocarcinoma bearing mice, s.c. NMMA therapy alone reduced tumor growth, spontaneous pulmonary metastasis and tumor induced pulmonary edema. Continuous oral administration of NMMA, when added to IL-2 therapy was, however, effective in reducing NO production as well as capillary leakage induced by IL-2 therapy in healthy mice. Because of a prohibitive cost of NMMA, oral NMMA therapy was not tested in tumor-bearing mice, since L-NAME, a more potent and inexpensive NO inhibitor was soon available.

It was shown that L-NAME given chronically in the drinking water was effective in preventing capillary leakage induced by IL-2 therapy in healthy mice. It also reduced IL-2 induced mortality when IL-2 dose was not too high. NO production appeared to be a strong determinant of the severity of this syndrome, because L-NAME treatment had a parallel effect in ameliorating the IL-2 induced capillary leakage and rise in NO production. Morphological studies revealed that IL-2 therapy induced the expression of iNOS enzyme in numerous tissues, including the endothelium, muscles of the anterior thoracic wall and splenic macrophages. Biochemical studies revealed that high NOS activity in the lungs and the anterior thoracic wall in IL-2 treated mice was associated with pulmonary edema, pleural effusion and structural damage to the lungs and its capillaries. Addition of L-NAME totally abolished NOS activity, but not necessarily iNOS expression. It also reduced IL-2 induced pulmonary edema and pleural effusion and significantly restored structural integrity of the lungs, identified both by light and electron microscopy. Thus, high tissue activity of IL-2 induced iNOS enzyme leading to NO

overproduction played a crucial role in the pathogenesis of IL-2 induced capillary leak syndrome.

In mammary adenocarcinoma bearing mice, oral L-NAME therapy alone produced significant antitumor as well as antimetastatic effects, noted also with NMMA therapy. L-NAME in combination with IL-2 therapy succeeded in ameliorating IL-2 induced as well as tumor-induced capillary leakage in tumor-bearing mice and potentiated the tumor-reductive function of IL-2. The latter effect was explained, at least in part, by a potentiation of LAK cell activation. It was found that L-NAME treatment *in vivo* as well as *in vitro* markedly stimulated IL-2 induced generation of antitumor cytotoxicity in splenocytes of healthy as well as mammary adenocarcinoma bearing mice, concomitant with a drop in the IL-2 induced NO production *in vivo* and *in vitro*. These results revealed that IL-2 induced increase in NO production had a compromising effect on optimal LAK cell activation, which can be overcome by NO inhibition with L-NAME therapy.

The antitumor and antimetastatic effects of both of NOS inhibitors (NMMA and L-NAME) when given alone to C3-L5 mammary adenocarcinoma bearing mice suggested that tumor-derived NO promoted tumor progression in this tumor model. The cellular source of NO in this tumor model was identified as tumor cells and endothelial cells of the tumor vasculature. Both cell types expressed high levels of eNOS protein *in vivo* in primary and metastatic tumors. C3-L5 cells *in vitro* expressed iNOS protein following culture with iNOS inducers (LPS+IFN γ). The mechanism of NO mediated tumor-progression was explored by testing whether tumor-derived NO promoted tumor cell invasiveness *in vitro* in a matrigel invasion assay. NO inhibitors, NMMA and L-NAME

reduced tumor cell invasiveness. Furthermore, an induction of high NO production by C3-L5 cells after exposure to LPS+IFN γ stimulated cellular invasiveness. Thus, NO produced by C3-L5 cells had an autocrine invasion promoting role. It may be suggested that the observed antitumor and antimetastatic effects of NMMA and L-NAME *in vivo* in the C3-L5 tumor model were at least partially due to inhibition of NO mediated tumor cell invasiveness. Other beneficial effects of NO inhibition on tumor regression, e.g. an abrogation of NO mediated promotion of tumor blood flow and angiogenesis remain as additional possibilities, which should be investigated in the future.

Above results provide the first direct evidence that NO is instrumental in IL-2-induced capillary leakage and that a NO blocking agent such as L-NAME can mitigate this leakage without interfering with the beneficial antitumor effects of IL-2 therapy; that NO blocking agents alone can reduce mammary tumor growth and metastasis and when combined with IL-2, can improve IL-2 induced antitumor cytotoxicity, as well as tumor regression. Therefore, NO blocking agents may have a place in treating NO producing tumors and could be valuable adjuncts to IL-2 based therapy of cancer and infectious diseases.

ORIGINAL CONTRIBUTIONS

1. IL-2 therapy in mice induces active iNOS enzyme in different tissues leading to an overproduction of NO. This NO, in turn, is instrumental in the pathogenesis of IL-2-induced capillary leakage in healthy and C3-L5 mammary adenocarcinoma bearing mice.
2. Oral administration of an NO blocking agent such as L-NAME can mitigate IL-2 therapy induced capillary leakage without interfering with the beneficial antitumor effects of IL-2 therapy.
3. NO blocking agents alone can reduce mammary tumor growth and metastasis and when combined with IL-2, can improve IL-2 induced antitumor cytotoxicity of effector cells, as well as tumor regression.
4. NO produced by tumor cells can stimulate tumor cell invasiveness. This may, in part explain the observed antitumor and antimetastatic effects of NMMA and L-NAME *in vivo* in the C3-L5 tumor model.

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