Inducible nitric-oxide synthase plays a minimal role in lymphocytic choriomeningitis virus-induced, T cell-mediated protective immunity and immunopathology

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By using mice with a targeted disruption in the gene encoding inducible nitric-oxide synthase (iNOS), we have studied the role of nitric oxide (NO) in lymphocytic choriomeningitis virus (LCMV)-induced, T cell-mediated protective immunity and immunopathology. The afferent phase of the T cell-mediated immune response was found to be unaltered in iNOS-deficient mice compared with wild-type C57BL/6 mice, and LCMV-induced general immunosuppression was equally pronounced in both strains. In vivo analysis revealed identical kinetics of virus clearance, as well as unaltered clinical severity of systemic LCMV infection in both strains. Concerning the outcome of intracerebral infection, no significant differences were found between iNOS-deficient and wild-type mice in the number or composition of mononuclear cells found in the cerebrospinal fluid on day 6 post-infection. Likewise, NO did not influence the up-regulation of proinflammatory cytokine/chemokine genes significantly, nor did it influence the development of fatal meningitis. However, a reduced virus-specific delayed-type hypersensitivity reaction was observed in iNOS-deficient mice compared with both IFN-γ-deficient and wild-type mice. This might suggest a role of NO in regulating vascular reactivity in the context of T cell-mediated inflammation. In conclusion, these findings indicate a minimal role for iNOS/NO in the host response to LCMV. Except for a reduced local oedema in the knockout mice, iNOS/NO seems to be redundant in controlling both the afferent and efferent phases of the T cell-mediated immune response to LCMV infection.

Introduction

Nitric oxide (NO) synthesized by the inducible nitric-oxide synthase (iNOS) is a multifunctional mediator in immune defences. Thus, NO has been reported to exert protective functions against a number of viruses, many intracellular bacteria and parasites and has been implicated in the control of cancers (Karupiah et al., 1993; Xie et al., 1996; Bogdan, 1997; MacMicking et al., 1997; Fang, 1997). On the other hand, disease-mediating effects, reflecting the role of NO as an effector molecule in inflammation, have been seen in several rodent autoimmunity models as well as in some infections (Bogdan, 1998). Thus, progressive insulitis and depletion of pancreatic islet cells in non-obese diabetic mice is correlated with increased expression of iNOS and is prevented by iNOS inhibitors (Corbett et al., 1993; Green et al., 1994). Similarly, iNOS/NO has been shown to contribute to the severity of influenza virus- and herpes simplex virus type 1 (HSV-1)-induced pneumonia (Akaike et al., 1996; Adler et al., 1997; Karupiah et al., 1998b). Finally, an immune-regulatory role for iNOS-derived NO has been proposed (Kolb & Kolb-Bachofen, 1998), and at certain concentrations NO markedly inhibits T cell proliferation (Hoffman et al., 1990; Albina et al., 1991), in particular the response of Th1 cells (Taylor-Robinson et al., 1994; Wei et al., 1995), thus serving as a potential co-regulator of the Th1/Th2 balance (MacLean et al., 1998; Karupiah et al., 1998b).

Induction of iNOS expression has been found not to be restricted solely to the T cell-dependent late phase of infection, but up-regulation of iNOS may also be an important component of the innate host response. Thus, iNOS-knockout (iNOS−/−) mice are highly susceptible to ectromelia virus, yet show no impairment of specific antiviral immune responses.
(Karupiah et al., 1998a). Moreover, in leishmania-infected mice, where iNOS is focally induced by IFN-γ/β within the first 24 h of infection, lack of iNOS leads to a reduction in cytotoxic activity of natural killer (NK) cells, decreased expression of IL-12 mRNA and the complete absence of up-regulation of IFN-γ (Diefenbach et al., 1998).

Regarding neuroimmunological functions, iNOS expression is indicated to be one of the factors contributing to the expansion of the brain damage that occurs following an ischaemic insult (Iadecola et al., 1995, 1997). In contrast, iNOS appears to play a predominantly protective role in the development of experimental allergic encephalitis (Bogdan, 1998). Local iNOS expression has also been suggested to influence the outcome of lymphocytic choriomeningitis (LCM) by mediating protective functions (Campbell, 1996). The development of fatal LCM in mice occurs as a response to intracerebral (i.c.) infection with lymphocytic choriomeningitis virus (LCMV) and is characterized at the pathological level by marked recruitment and extravasation of immunoinflammatory cells to the sites of virus replication in the meninges and ependyma (Doherty et al., 1990). This results eventually in death, 6–8 days post-infection (p.i.). Experiments have shown that CD8+ MHC class I-restricted cytotoxic T lymphocytes (Tc cells), but not CD4+ T lymphocytes, are pivotal in the development of LCM (Doherty et al., 1990; Kagi et al., 1994), and the temporal and spatial expression of iNOS in the infected brain is clearly associated with the development of the virus-induced inflammatory infiltrate (Campbell et al., 1994b). High levels of IFN-γ are present in the cerebrospinal fluid (CSF) (Frei et al., 1988; Nansen et al., 1998), but the outcome of this infection in iNOS−/− mice has not been investigated previously. In addition, iNOS-dependent NO production may be one important pathway through which IFN-γ mediates its antiviral action (Croen, 1993; Karupiah et al., 1993), and previous studies by our group have revealed a pivotal role of IFN-γ in controlling both NO production in vitro and virus clearance after systemic infection with viscerotropic strains of LCMV. A consequence of the latter is severely exacerbated Tc-mediated immunopathology in IFN-γ−/− mice. In fact, lack of IFN-γ results in a fatal outcome of intravenous (i.v.) infection in the majority (~ 85%) of mice infected with LCMV 1 (unpublished results).

Therefore, in the present study, we have investigated the role of iNOS/NO in LCMV-induced meningitis, as well as in the T cell-mediated immune response to LCMV in general. This also included an analysis of LCMV-induced immune suppression, which is known to reflect an increased propensity of T lymphocytes to undergo apoptosis upon T-cell receptor (TCR) stimulation (activation-induced cell death; AICD) (Razvi & Welsh, 1993), a phenomenon that has recently been found to be dependent partly on IFN-γ (Lohman & Welsh, 1998).

By using mice lacking iNOS (Wei et al., 1995; MacMicking et al., 1995; Laubach et al., 1995), we found a minimal role for iNOS/NO in the host response to LCMV. Thus, besides a reduced local oedema observed in the knockout mice, iNOS seems to be redundant in controlling both the afferent and efferent phases of the T cell-mediated immune response to LCMV infection.

**Methods**

**Mice.** C57BL/6 (B6) wild-type mice were obtained from Bomholtgaard Ltd (Ry, Denmark). C57BL/6 iNOS−/− and IFN-γ−/− mice were the progeny of breeding pairs obtained from Jackson Laboratory (Bar Harbour, ME, USA). Mice were used at 7–10 weeks old in all experiments and animals were always allowed to acclimatize to the local environment for at least 1 week before use. All animals were housed under specific-pathogen-free conditions as validated by testing of sentinel for unwanted infections, according to Federation of European Laboratory Animal Science Association guidelines.

**Virus.** LCMV of the Taub strain, produced and stored as described previously, was used in most experiments (Marker & Volkert, 1973). Mice to be infected received a dose of 10^6 LD_{50} in an i.v. injection of 0.3 ml. Infection by this route is followed by transient immunizing infection (Marker & Volkert, 1973; Thomsen & Marker, 1989). LCMV of the Armstrong clone 53b strain was kindly provided by M.B.A. Oldstone ( Scripps Clinic and Research Foundation, La Jolla, CA, USA) and used for i.c. infection. Mice were infected with 200 p.f.u. in an i.c. injection of 0.3 ml. This inoculation induces a fatal, T cell-mediated meningitis, to which the animals succumb days 6–8 p.i. (Campbell et al., 1994a).

**Virus titration.** Virus titrations were carried out by i.c. inoculation of 10-fold dilutions of a 10% organ suspension into young adult Swiss mice. Titration end-points were calculated by the Kärber method and expressed as LD_{50}.

**Severity of LCMV-induced meningitis.** Mortality and CSF cell numbers were used to evaluate the clinical severity of acute LCMV-induced meningitis (Doherty et al., 1990; Marker et al., 1995). Mice were checked twice daily for a period of up to 14 days after i.c. inoculation.

**Clinical severity of systemic LCMV infection.** Mice were monitored daily for a period of 4 weeks. Weight loss was measured as the percentage difference between initial weight and weight on the indicated day.

**Assay of LCMV-specific delayed-type hypersensitivity (DTH).** Mice were primed by i.v. injection of LCMV Armstrong (200 p.f.u.) and, in addition, were inoculated in their right hind footpad with 30 µl peptide (200 p.f.u.). Footpad thickness was measured with a dial calliper (Mitutoyo 7309) daily from day 6 p.i. until termination of the experiment and virus-specific swelling was determined as the difference in thickness of the infected right and the uninjected left feet (Thomsen & Marker, 1989). LCMV-specific DTH was also evaluated by footpad challenge 8 days after an i.v. infection with an immunodominant class I-restricted viral peptide (LCMV GP 33–41) (Nansen et al., 1998). Mice were injected with 30 µl of a solution containing 50 µg/ml peptide.

**Cell preparations.** Spleen single-cell suspensions were obtained by pressing the organ through a fine steel mesh and erythrocytes were lysed by treatment with 0.83% NH_4Cl (Gey’s solution). CSF cells were obtained from the fourth ventricle of mice that had been etheranaesthetized and exsanguinated; background levels in uninfected mice are < 100 cells/µl (Marker et al., 1995).

**Cytotoxicity assays.** Virus-specific Tc cell activity was assayed in a standard ^{31}Cr-release assay by using EL-4 cells incubated for 1 h at
37 °C with either of two different immunodominant class I-restricted viral peptides (LCMV GP 33–41 and NP 396–404); EL-4 cells incubated without peptide served as control targets. NK cell activity was assayed in a standard 51Cr-release assay by using NK-sensitive YAC-1 cells; spleen cells from uninfected mice were used as controls (Thomsen & Marker, 1989). The time of assay was 4 and 5 h for NK cell activity and Tc cell activity, respectively, and percentage specific release was calculated as described previously (Marker & Volkert, 1973).

T cell proliferation. Splenocytes from individual mice were plated at 2 × 10^6, 1 × 10^6 and 0.5 × 10^6 cells per well in 90-well flat-bottomed microtitre plates. IL-2 responsiveness was evaluated as proliferation after 48 h incubation in the presence of 1000 IU/ml murine recombinant IL-2 (R&D Systems) (Andersson et al., 1995), anti-CD3 responsiveness as proliferation after 48 h incubation in the presence of 1 µg/ml anti-CD3 (PharMingen) and ConA responsiveness as proliferation after 72 h incubation in the presence of 2.5 µg/ml ConA (Pharmacia). Cultures were labelled by adding 1 µCi[^3]H]tdR per well (specific activity 2 Ci/mmol) during the last 6 h of incubation.

MAB for flow cytometry. The following MABs were purchased from PharMingen as rat anti-mouse antibodies: FITC-conjugated anti-CD49d (common α-chain of LPAM-1 and VLA-4), PE-conjugated anti-CD8a, biotin-conjugated anti-CD62L (L-selectin (L-sel), MEL-14), PE-conjugated anti-IL-5 and PE-conjugated anti-IFN-γ.

Flow cytometric analysis. Cells (1 × 10^6) were stained with directly labelled MAB in staining buffer (1% rat serum, 0.1% BSA, 0.1% NaN₃ in PBS) for 20 min in the dark at 4 °C and then washed. Biotin-conjugated antibodies were additionally incubated with streptavidin–Tricolor (Caltag Laboratories), washed and fixed with 1% paraformaldehyde (Christensen et al., 1996). To detect intracellular IFN-γ, splenocytes were cultured at 37 °C in 96-well round-bottomed plates at a concentration of 1 × 10⁶ cells per well in 0.2 ml complete RPMI medium supplemented with 10% FCS and 3 µM monensin (Sigma), either with or without 0.1 µg/ml LCMV GP 33–41 peptide. After 5 h incubation, cells were washed once in staining buffer (1% BSA, 0.1% NaN₃ and 3 µM monensin) and subsequently incubated in the dark for 30 min at 4 °C with relevant antibodies to cell-surface antigens. Cells were washed twice in staining buffer and resuspended in the dark for 20 min at 4 °C in fixation buffer (1% paraformaldehyde in PBS). Cells were then washed in staining buffer, resuspended in permeabilization buffer (1% rat serum, 1% BSA, 0.1% NaN₃ and 0.5% saponin in PBS) and incubated for 30 min at 4 °C in the dark with the relevant cytokine-specific antibodies (Christensen et al., 1996). Finally, cells were washed in permeabilization buffer and resuspended in staining buffer.

Samples were analysed with a Becton Dickinson FACSCalibur, and 1–2×10⁵ viable mononuclear cells were gated by using a combination of forward angle and side scatter to exclude dead cells and debris. Data analysis was carried out using the PC-LYSIS program and results are presented as dot plots.

Detection of cytokine and chemokine gene expression by RNase protection assays. Mice were exsanguinated under ether anaesthesia and brains were removed and immediately frozen in liquid nitrogen. Total RNA was isolated and gene expression was evaluated by RNase protection assays (Campbell et al., 1994a). Total RNA was extracted from homogenized organs by use of the RNeasy Midi kit (Qiagen). Detection of cytokine and chemokine mRNA was done by using the following kits obtained from PharMingen: RibonQuant in vitro Transcription kit, mouse Multi-Probe Template sets mCK-3 and mCK-5 and the RiboQuant ribonuclease protection assay kit. All analyses were carried out according to manufacturer’s instructions.

Results

LCMV-induced NK cell activation and Tc cell activity in iNOS−/− mice

In order to investigate the importance of iNOS in regulating the afferent phase of the antiviral immune response, functional evaluation of NK cell and Tc cell activity was performed. LCMV-induced NK cell activity, measured as cytotoxicity against NK-sensitive YAC-1 target cells 4 days after virus inoculation, tended to be slightly higher in iNOS−/− mice compared with wild-type animals, but the difference was not statistically significant (Fig. 1a). LCMV-specific cytotoxicity was assayed on day 8 p.i. and was found to be of similar magnitude in both mouse strains (Fig. 1b–e). Comparable results were obtained by flow cytometry. Thus, the frequency of NK1.1+ cells was found to be around 5% of splenocytes in infected animals of both strains (data not shown) and the frequencies of CD8+ T cells with an activated phenotype (VLA-4high L-sellow) were identical (Fig. 2). Direct visualization of virus-specific CD8+ T cells, detected through intracellular staining for cytokines, gave the same result: similar numbers of CD8+ IFN-γ+ IL-5− Tc1 (Christensen et al., 1996) cells were detected in both strains.

Fig. 1. NK cell activity and Tc cell activity in iNOS−/− and wild-type mice. iNOS−/− ( ●) and wild-type ( □) mice were infected i.v. with 10⁶ LD₅₀ LCMV Txub. E/T, Effector:target. (a) Splenic NK activity was assayed on day 4 p.i. by using YAC-1 target cells; spleen cells from uninfected mice were included for comparison (△). (b)–(d) LCMV-specific Tc cell activity was assayed on day 8 p.i. by using EL-4 cells pulsed with either GP 33–41 (b) or NP 396–404 (c); unpulsed EL-4 cells were used as negative controls (□). Results from individual mice are depicted and one of two similar experiments is shown.
Fig. 2. (a)–(b) Identical frequency of CD8⁺ splenic T cells with an activated phenotype (VLA₄⁺, L-sel⁻⁺⁺) in iNOS⁻/⁻ and wild-type mice. Cells were stained with anti-CD8–PE, anti-VLA-4–FITC and biotinylated anti-L-sel (plus streptavidin–Tri-color). (c)–(d) Similar numbers of virus-specific Tc1 cells (IFN-γ⁺-producing CD8⁺ T cells) in iNOS⁻/⁻ and wild-type mice. Cells were stained with anti-CD8–PE and anti-IFN-γ–PE. (e)–(f) Cells stained with anti-IL-5 instead of anti-IFN-γ were used as isotype (negative) control. All mice were infected i.v. 8 days previously with 10⁷ LD₅₀ LCMV Traub. Numbers represent medians from three mice. Numbers in parentheses refer to the mean sizes of the same subpopulations in uninfected mice.

Fig. 3. Proliferation of splenocytes stimulated with ConA, anti-CD3 (α-CD3) or IL-2. Spleen cells from virus-infected (day 8 p.i.) mice (○, wild-type; ●, iNOS⁻/⁻) and uninfected controls (△, wild-type; ▲, iNOS⁻/⁻) were stimulated with ConA, soluble anti-CD3 or IL-2 and proliferation was assayed at 48 (anti-CD3 and IL-2) or 72 (Con A) h after initiation of culture. Curves represent individual mice; one of two identical experiments is depicted.

iNOS plays no role in LCMV-induced inhibition of T cell proliferation

High concentrations of NO have been found in vitro to inhibit the proliferation of and to induce apoptosis of T lymphocytes (Albina et al., 1991; Hoffman et al., 1990; Fehsel et al., 1995; Okuda et al., 1996). Moreover, it has been observed recently that IFN-γ⁻/⁻ mice are partly resistant to LCMV-induced AICD (Lohman & Welsh, 1998). As AICD is assumed to be the underlying cause of LCMV-induced immune deficiency (Razvi & Welsh, 1993), we found it pertinent to test whether iNOS played a role in this regard. iNOS⁻/⁻ and wild-type mice were infected with LCMV, and at the nadir of responsiveness towards ConA and soluble anti-CD3 antibody in wild-type mice (day 7–8 p.i.), we evaluated the induced proliferative response in both strains. Irrespective of mouse strain, a marked depression of ConA- and anti-CD3-induced T cell proliferation was observed (Fig. 3). In contrast, cells from infected animals of both strains responded to IL-2, confirming that the unresponsiveness was restricted to agents inducing T
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Fig. 4. Organ virus titres in iNOS−/− mice infected with LCMV Traub. iNOS−/− (●) and wild-type (○) mice were infected i.v. with 10^3 LD_{50} LCMV Traub and organs were harvested and assayed for virus content on the days indicated. Points represent individual mice. On day 10 p.i., results from similarly infected IFN-γ−/− mice (⋆) are included for comparison.

Unimpaired virus clearance in iNOS−/− mice

The most relevant measure of the capacity of CD8+ effector T cells to function in vivo is virus clearance in infected animals. In a previous study, we found that IFN-γ is important for the T cell-mediated clearance of LCMV Traub, but plays little if any role in controlling virus replication prior to the development of the antiviral T cell response (unpublished results). To evaluate the possible role of iNOS/NO in this context, the kinetics of virus clearance were studied in iNOS−/− mice and compared with those from wild-type controls. Mice were infected i.v. with 10^4 LD_{50} LCMV Traub, groups of three or four mice were sacrificed at different times after infection (4, 8, 10 and 28 days p.i.) and their spleens and livers were removed for determination of virus content. From the results shown in Fig. 4, it is seen that iNOS−/− mice are able to control the infection just as efficiently as wild-type C57BL/6 mice. This is in marked contrast to the pattern in IFN-γ−/− mice, and therefore up-regulation of iNOS does not seem to be the effector pathway through which this cytokine participates in the clearance of LCMV.

In addition to impaired virus clearance in IFN-γ−/− mice, we have also found that most of these mice developed a severe wasting disease and subsequently died after systemic infection with LCMV Traub. The clinical severity of systemic LCMV infection in mice deficient of NO production was therefore determined by daily evaluation of weight loss and mortality. The clinical status of iNOS−/− mice after LCMV infection was identical to that of wild-type mice. Thus, only a transient and minor weight loss was observed around day 7 p.i. in both strains (Fig. 5), and all mice survived.

iNOS deficiency does not influence the outcome of i.c. infection with LCMV

We wanted next to investigate the role of NO in the pathogenesis of i.c. infection with LCMV Armstrong. iNOS−/− (●) and wild-type (○) mice were inoculated i.c. with 200 p.f.u. LCMV Armstrong and mortality was registered. The number of mononuclear cells per µl CSF at day 6 p.i. is given as the medians from four mice; ranges are given in parentheses. Flow-cytometric analysis of CSF cells revealed an essentially similar composition with regard to CD8+ and Mac-1+ cells (not shown).
Fig. 7. Up-regulation of proinflammatory cytokine genes in the brain after an i.c. infection with LCMV Armstrong in iNOS−/− (ko) mice compared with wild-type (wt) mice. Mice were infected with 200 p.f.u. LCMV Armstrong and, on the days indicated, total RNA from brains was analysed for the levels of expression of proinflammatory cytokine genes in an RNase protection assay. Lanes represent individual mice. One of two identical experiments is presented.

Reduced virus-specific DTH reactivity in iNOS−/− mice

In order to obtain a more quantitative evaluation of the LCMV-induced inflammatory reaction, four to six mice from each strain were primed by i.v. injection of 200 p.f.u. LCMV Armstrong, followed immediately by inoculation of the same virus dose in the right hind footpad; virus-induced inflammation was measured as footpad swelling on days 6–11 p.i. As seen in Fig. 8(a), the increase in footpad thickness was reduced in iNOS−/− mice on day 7 p.i., which was also the day of peak reactivity. Since some of the variation between individuals found in this assay may reflect small variations in local virus replication, we also measured the response of i.v.-primed mice to local challenge (day 8 p.i.) with an immunodominant class I-restricted viral peptide (Nansen et al., 1998). Significantly reduced footpad swelling was observed for the first 24 h after peptide injection in iNOS−/− mice, but not in IFN-γ−/− mice, compared with the response of wild-type animals (Fig. 8b).

Discussion

It was the aim of this study to investigate the role of iNOS/NO in the pathogenesis of LCMV infection, particularly in the virus-induced meningitis that results from the antiviral T cell-mediated inflammatory response in i.c.-infected mice. On the basis of a number of previous studies, it was suspected that iNOS might be important in (i) regulating the host response to LCMV, (ii) LCMV-induced immunodeficiency and (iii) the T cell-mediated effector phase, which involves inflammation and virus clearance. We therefore found it pertinent to investigate the role of iNOS as a regulatory molecule in the afferent and efferent phases of the LCMV-induced, T cell-mediated immune response.
We analysed the role of NO during LCMV infection by using mice deficient in iNOS (Laubach et al., 1995), and compared the experimental results with those of wild-type mice. We found that LCMV-induced NK cell activation was comparable in iNOS$^{-/-}$ and wild-type mice, and FACS analysis revealed equal numbers of NK cells 4 days after i.v. infection with LCMV. Thus, in contrast to the situation in leishmania-infected mice (Diefenbach et al., 1998), iNOS-derived NO appears not to be involved in the up-regulation of NK cell activity following virus infection (similar results were obtained in mice infected with vesicular stomatitis virus; unpublished observations). Regarding the generation of effector T cells, our results clearly demonstrate that the generation of LCMV-specific Tc1 cells is unaffected by the absence of iNOS. No difference was observed in either clonal expansion or effector cell differentiation, as evaluated both functionally and by flow cytometry, and similar results were obtained by flow-cytometric evaluation of Tc1 cells induced by i.v. infection with vesicular stomatitis virus (data not shown). Thus, NO does not seem to exert the same marked feedback inhibition on Tc1 cells as has recently found for antiviral Th1 cells in influenza virus- and HSV-1-infected mice (Karupiah et al., 1998b; MacLean et al., 1998).

We found no involvement of iNOS in the immunodeficiency induced by systemic LCMV infection, despite the recent finding that IFN-γ plays an important role (Lohman & Welsh, 1998). Thus, our findings confirm and extend previous results obtain by treatment with N-monomethyl-L-arginine (Butz et al., 1994).

Analysis in vivo revealed identical kinetics of virus clearance (which is an indirect measure of Tc1 effector capacity; Kagi et al., 1994), and no clinical signs of illness, measured in terms of weight loss and survival rate, were observed in either strain. Since impaired virus clearance and severe wasting is observed in similarly infected IFN-γ$^{-/-}$ mice, it may be concluded that the protective effect of IFN-γ is not mediated through the up-regulation of iNOS. Quantitative and qualitative analyses of CSF exudate cells indicated that cell migration into the inflammatory site can proceed to the same extent in the absence of NO; this is in keeping with analogous findings in IFN-γ$^{-/-}$ mice, in which up-regulation of iNOS cannot be detected (Nansen et al., 1998). Likewise, the up-regulation of proinflammatory cytokine and chemokine genes in the brain after i.c. infection with LCMV seems to be regulated independently of NO. This result may seem to conflict with data from mice treated with amino-guanidine (Campbell, 1996), a NOS inhibitor with relative selectivity for iNOS, which indicated a role for NO in the up-regulation of TNF-α, IL-1α and IL-1β in the brain 3 days after i.c. LCMV infection. Furthermore, time to death was slightly shortened in mice treated in this way (Campbell, 1996). In the same study, however, ruffled fur and diarrhea were noted in drug-treated control animals, suggesting that toxic levels of amino-guanidine had been administered. Therefore, our results with iNOS$^{-/-}$ mice probably provide a more valid indication of the role of iNOS in LCMV-induced immunity.

By using another model system of LCMV-induced, T cell-mediated inflammation, virus-induced footpad swelling, we found a significant role for NO. Thus, footpad swelling was significantly reduced in iNOS$^{-/-}$ and wild-type mice. This agrees well with the results of a study on the role of NO in contact hypersensitivity reactions, which suggested that epidermal cell-derived NO contributed to the ear-swelling reaction (Ross et al., 1998). As the swelling reaction is more a measure of the induced oedema than of cellular infiltration per se, and assuming that our findings on cell infiltration and expression of cytokine/chemokine genes can be extrapolated to apply also to the footpad, the reduced reaction in iNOS$^{-/-}$ mice would suggest that NO may be involved in regulating secondary effects of the inflammatory response; vascular reactivity is a strong possibility (Wei et al., 1995; Peng et al., 1998). The apparent redundancy of IFN-γ in the swelling reaction (Nansen et al., 1998) is puzzling if NO indeed plays a role. However, this could be the result of a compensatory phenomenon. Thus, we find a higher antigenic load and more extensive virus dissemination in IFN-γ$^{-/-}$ mice (Nansen et al., 1998), which may accelerate and augment the specific T cell response in these mice compared with matched wild-type mice. Indeed, we have previously found some indications that this might be the case (Nansen et al., 1998), and the observed redundancy of IFN-γ in the swelling reaction should therefore be interpreted with some care.

In summary, this study discloses a minor role for iNOS/NO in LCMV-induced, T cell-mediated protective immunity and immunopathology. Our results indicate that NO plays a role in regulating the magnitude of the inflammatory reaction but is otherwise redundant in LCMV infection.

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