INFECTION AND IMMUNITY, Apr. 1996, p. 1197–1202 Vol. 64, No. 4
0019-9567/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Acquired Resistance against a Secondary Infection with *Listeria monocytogenes* in Mice Is Not Dependent on Reactive Nitrogen Intermediates

JANNEKE N. SAMSOM, JAN A. M. LANGERMANS,* PAUL H. P. GROENEVELD, AND RALPH VAN FURTH

Department of Infectious Diseases, University Hospital, 2300 RC Leiden, The Netherlands

Received 2 October 1995/Returned for modification 22 November 1995/Accepted 12 January 1996

During an infection, inflammatory mediators can induce the production of nitric oxide, a reactive nitrogen intermediate (RNI) which plays a role in antimicrobial activity against a wide variety of pathogens. In vitro experiments have shown that release of RNI by macrophages is mediated by tumor necrosis factor alpha (TNF). Since TNF is essential for acquired resistance during a secondary *Listeria monocytogenes* infection in mice, the aim of the present study was to determine whether RNI are also involved in the course of such an infection. Mice which had recovered from a sublethal primary infection with 0.1 50% lethal dose of (LD_{50}) *L. monocytogenes* were infected intravenously with 10 LD_{50} of *L. monocytogenes*. During a primary infection, the number of bacteria in the liver and spleen, as well as the concentration of RNI in plasma, increased. During a secondary infection, the number of bacteria in the liver and spleen decreased whereas no significant increase in the concentration of RNI in plasma was observed. Neutralization of endogenously produced TNF and gamma interferon by subcutaneous injection of alginate-encapsulated monoclonal antibody-forming cells during a secondary infection resulted in an increase in the number of bacteria in the liver and spleen and an increase in the concentration of RNI in plasma. When the production of RNI was inhibited by treatment of mice with the competitive NO-synthase inhibitor N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) and an L-arginine-deficient diet during a secondary infection, the proliferation of *L. monocytogenes* in the liver and spleen was not affected whereas the concentration of RNI in plasma of these mice was significantly reduced. Our findings that inhibition of RNI formation during a secondary infection does not affect the proliferation of *L. monocytogenes* in the liver and spleen and that enhanced elimination of bacteria from these organs is not accompanied by an increase in the concentration of RNI in plasma led to the conclusion that resistance against a secondary infection with *L. monocytogenes* is not dependent on RNI.

Nitric oxide is a reactive nitrogen intermediate (RNI) which is formed in small amounts by the constitutive form of NO synthase (cNOS) (26, 27). Inflammatory mediators can increase the production of NO by induction of another isoform of this enzyme, inducible NO synthase (iNOS), which converts L-arginine to L-citrulline in a calcium-independent fashion and simultaneously releases NO as a short-lived intermediate (28). NO is rapidly converted to the stable metabolites nitrate and nitrite (17). In vitro experiments with competitive inhibitors of NOS demonstrated that RNI are involved in the antimicrobial activity of activated mouse macrophages against a variety of intracellular microorganisms, e.g., *Toxoplasma gondii* (2, 18), *Leishmania major* (21), herpes simplex virus type 1 (9), *Mycobacterium tuberculosis* (7), and *Listeria monocytogenes* (3). In vivo studies with competitive inhibitors of NOS have confirmed the involvement of RNI in resistance against infection with *L. major* (22) and *M. tuberculosis* (6) but yield conflicting results on the role of RNI during an infection with *L. monocytogenes*. Inhibition of RNI production during a primary infection in mice has been shown to increase (3, 4) but also to decrease (13) *Listeria* proliferation in the liver and spleen. Recently, it was reported that mice deficient in iNOS were not able to eliminate listeriae as efficiently as did control mice during a primary infection (24).

During the course of a secondary *Listeria* infection, the bacteria are eliminated from the organs faster than during a primary infection (23). We reported earlier that TNF is essential for this enhanced elimination of *L. monocytogenes* (30), and in vitro studies have shown that this cytokine stimulates the release of RNI by activated macrophages (11, 19). The aim of the present study was to determine whether RNI are involved in the decrease in the number of bacteria in the liver and spleen during a secondary *L. monocytogenes* infection in mice.

MATERIALS AND METHODS

Mice. Female, specific-pathogen-free CBA/J mice, aged 6 to 8 weeks, were purchased from IFFA Credo, Saint Germaine-sur-l'Abersle, France, and given dry food (Hope Farms, Woerden, The Netherlands) and tap water. In one experiment, the mice were kept on a well-defined L-arginine-deficient amino acid rodent diet (Hope Farms) consisting of small particles and deionized distilled water without nitrite or nitrate, starting 8 days prior to infection. Control mice received a regular diet (Hope Farms) which was ground into particles of equal size to those in the deficient diet and deionized distilled water. The concentration of creatinine in plasma for mice under study was determined by a routine laboratory procedure.

Bacteria. *L. monocytogenes* EGD was kept virulent by repeated passage through CBA/J mice and stored on blood agar plates at 4°C. The bacteria were cultured in tryptose phosphate broth for 18 h at 37°C, collected by centrifugation (10 min at 900 × *g*), washed in phosphate-buffered saline (pH 7.4), and resuspended in pyrogen-free saline (saline).

Induction of primary or secondary *L. monocytogenes* infection in mice. A primary *L. monocytogenes* infection was induced in mice by injecting 5×10^4 *L. monocytogenes* organisms (10 50% lethal doses [LD_{50}]) intravenously (i.v.)

* Corresponding author. Mailing address: Department of Infectious Diseases, University Hospital, Building 1, C5-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands. Phone: 31-71-5262613. Fax: 31-71-5266758.

(35). A secondary infection was induced in mice by first injecting 5×10^2 *L. monocytogenes* organisms (0.1 LD_{50}) i.v. and 3 weeks later injecting 10 LD_{50} of *L. monocytogenes* i.v. On fixed days of a primary or secondary infection, the liver and spleen were removed and homogenized with a tissue homogenizer (type X-1020; Ystral GmbH, Döttingen, Germany), blood samples were taken by heart puncture and collected in a heparinized tube, and peritoneal macrophages were collected by peritoneal lavage. Serial 10-fold dilutions of the organ suspensions were plated onto blood agar plates and incubated for 24 h at 37°C; the number of colonies was used to calculate the number of viable *L. monocytogenes* per organ. The blood samples were stored as plasma at −20°C until use. Peritoneal macrophages were cultured without additional stimulation for 24 h at 10^6 macrophages per ml.

Neutralization of endogenous cytokine production during infection. Hybridoma cell lines forming either rat anti-mouse gamma interferon (IFN-γ) monoclonal antibodies (MAb) (XMG1.2; immunoglobulin G1) (8) or rat anti-mouse tumor necrosis factor alpha (TNF) MAb (XT22; immunoglobulin G1) (1) were a gift from DNAX Research Institute, Palo Alto, Calif. The cells were encapsulated in alginate entrapment medium (FMC Bioproducts-Europe, Vallensbaek Strand, Denmark) by a method described previously (30, 32). Four days before a primary or secondary infection, the mice were given a subcutaneous injection in the nuchal region with 2×10^6 encapsulated anti-IFN-γ antibody-forming cells (anti-IFN-γ-FC), 10^6 encapsulated anti-TNF antibody-forming cells (anti-TNF-FC), or a combination of 2×10^6 anti-IFN-γ-FC and 1×10^6 anti-TNF-FC in a volume of 1 ml. Control mice received 2×10^6 anti-β-galactosidase antibody-forming cells (anti-β-gal-FC) (GL113; immunoglobulin G1) (DNAX) (31) in a volume of 1 ml. Endotoxin levels in the solution of alginate-encapsulated antibody-forming cells were below 10 pg/ml, as determined by a *Limulus* assay.

Measurement of RNI. The concentrations of NO_3^- and NO_2^- in plasma and the amount of NO_2^- in culture supernatant, each considered a measure of the production of NO, are referred to as RNI. A plasma sample was diluted twofold with deionized H_2O and deproteinized with 30% zinc sulfate. NO_3^- in the sample was reduced to NO_2^- by incubation with spongy cadmium (14), and the concentration of NO_2^- was determined with Griess reagent (29). The amount of NO_2^- released by peritoneal macrophages was determined in the culture supernatant with Griess reagent.

In vivo inhibition of NOS. N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma, St. Louis, Mo.) was given i.v. once a day at a dose of 200 mg/kg. Control mice received N_ω-nitro-D-arginine methyl ester (D-NAME) (Sigma). Aminoguanidine hemisulfate salt (Sigma) was given twice a day in a dose of 50 mg/kg intraperitoneally as described by others (3). Treatment was started 24 h before the secondary infection, as indicated. Endotoxin levels in the L-NAME, D-NAME, and aminoguanidine solutions were below 40 pg/ml.

Statistical analysis. Results are expressed as the mean ± standard deviation for at least five mice per time point, unless otherwise indicated. Differences between various groups were assessed by means of the Mann-Whitney two-sample test or a one-way analysis of variance with a Fisher's least-significant-difference comparison report. For all analyses, the level of significance was set at 0.05. Pearson correlation was used to determine the possible relationship between two variables, and Bartlett chi-square analysis was used to determine whether the correlation was significant. The correlation coefficient (r) denotes the Pearson product moment correlation coefficient.

RESULTS

Number of bacteria in liver and spleen and concentration of RNI in plasma from mice during the course of a primary or secondary *Listeria* infection. During the course of a primary infection, the number of listeriae cells recovered from the liver and spleen increased significantly (Fig. 1A and B). Simultaneously, the concentration of RNI in plasma of mice with a primary infection increased from a mean of 22 μM at 24 h to 330 μM at 72 h (Fig. 1C). During the course of a secondary infection, the number of listeriae cells recovered from the liver and spleen of mice decreased significantly and was smaller than that found for mice with a primary infection (Fig. 1A and B). The concentration of RNI in plasma during a secondary *Listeria* infection increased initially from a mean of 23 μM at 24 h to a maximum of 76 μM at 48 h and then decreased (Fig. 1C). After 72 h of infection, the concentration of RNI in plasma of mice with a secondary infection was sevenfold lower than that determined for mice with a primary infection (Fig. 1C). The concentration of RNI in plasma of naive mice amounted to 32 ± 9 μM. Together, these results show that enhanced elimination of bacteria from the liver and spleen during a secondary infection is not accompanied by high concentrations of RNI in plasma.

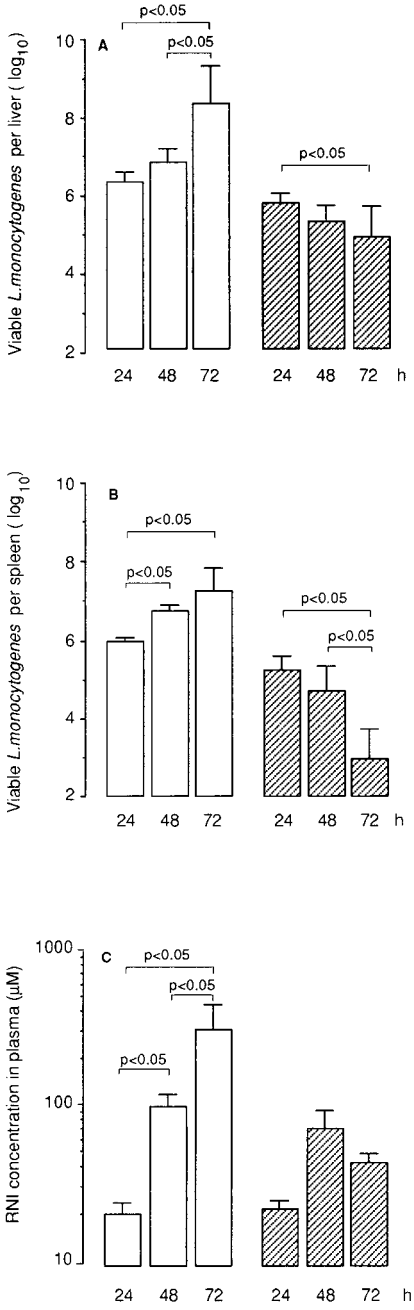


FIG. 1. Number of *L. monocytogenes* in the liver and spleen and concentration of RNI in plasma of mice with a primary or a secondary infection. Naive mice (□) or mice immunized with 0.1 LD_{50} of *L. monocytogenes* 3 weeks earlier (▨) were infected with 10 LD_{50} of *L. monocytogenes*. After 24, 48, and 72 h of infection, the number of listeriae cells in the liver (A) and spleen (B) and the concentration of RNI in plasma (C) were assessed. Values are means ± standard deviations of results for five mice.

Number of bacteria in the liver and spleen and concentration of RNI in plasma from mice treated with anti-cytokine-forming cells during a secondary infection. Since TNF and, to a lesser extent, IFN-γ are involved in enhanced resistance during a secondary infection with *L. monocytogenes* (30), we studied the effects of neutralization of these cytokines on RNI

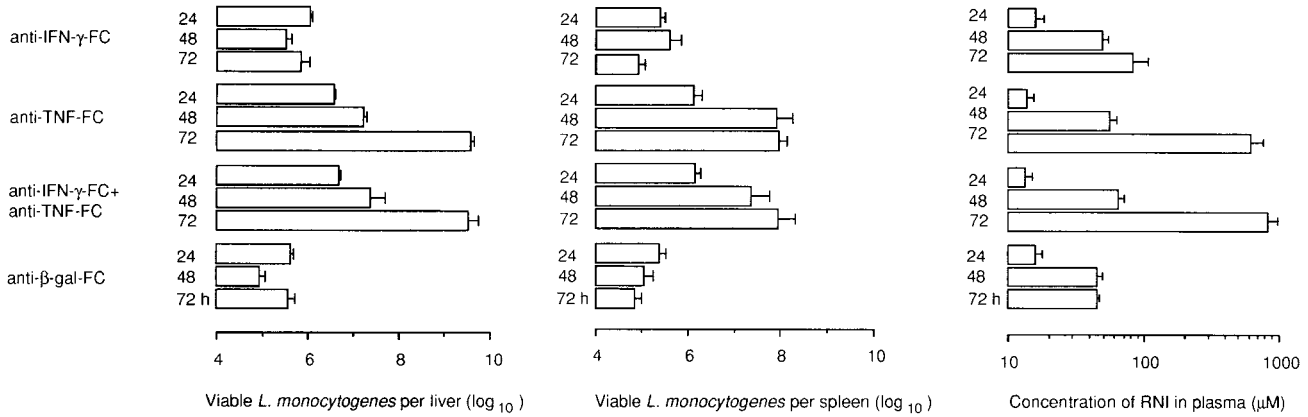


FIG. 2. Number of *L. monocytogenes* in the liver and spleen and concentration of RNI in plasma of mice treated with anti-cytokine-FC during a secondary *L. monocytogenes* infection. Naive mice were immunized with 0.1 LD_{50} of *L. monocytogenes*; 16 days later, they received either 2×10^6 anti-IFN-γ-FC, 1×10^6 anti-TNF-FC, a combination of 2×10^6 anti-IFN-γ-FC and 1×10^6 anti-TNF-FC, or 2×10^6 anti-β-gal-FC as a control. Five days later, these mice were infected with 10 LD_{50} of *L. monocytogenes*. After 24, 48, and 72 h of infection, the number of listeriae in the liver and spleen and the concentration of RNI in plasma were determined. Values are means ± standard errors of the means for eight mice. For *P* values, see the text.

formation. To obtain a constant level of anti-TNF MAb and anti-IFN-γ MAb in the circulation, alginate-encapsulated cells which produce these MAb were injected subcutaneously, as described previously (30, 32). The number of *L. monocytogenes* in the liver and spleen of mice treated with anti-TNF-FC or a combination of anti-IFN-γ-FC and anti-TNF-FC during a secondary infection increased significantly compared with that found for mice treated with anti-β-gal-FC, whereas treatment with anti-IFN-γ-FC resulted in only a slight increase, as reported previously (30) (Fig. 2). After 72 h of infection, the concentration of RNI in plasma of mice treated with anti-IFN-γ-FC was 2-fold higher than that in mice treated with anti-β-gal-FC, whereas treatment with anti-TNF-FC or a combination of anti-IFN-γ-FC and anti-TNF-FC during the secondary infection resulted in a 10-fold increase in the concentration of RNI after 72 h of infection compared with anti-β-gal-FC-treated mice (Fig. 2). Thus, neutralization of TNF during secondary infection does not lead to a decrease in the concentration of RNI in plasma. Together, these results show that a large number of *L. monocytogenes* in the liver and spleen is accompanied by a high concentration of RNI in plasma.

RNI release by peritoneal macrophages from mice during a primary or a secondary *Listeria* infection. To demonstrate that macrophages release RNI during a secondary infection, we used peritoneal macrophages, since it is difficult to obtain macrophages from the liver and spleen. The release of RNI by peritoneal macrophages from mice with a primary or secondary *Listeria* infection reached a maximum at 24 h of infection and then decreased (Fig. 3). At all times, the amount of RNI released by peritoneal macrophages from mice with a secondary infection was slightly but not significantly smaller than that released by peritoneal macrophages from mice with a secondary infection was slightly but not significantly smaller than that released by peritoneal macrophages from mice with a primary infection (Fig. 3). Treatment of mice with anti-TNF-FC during a secondary infection resulted in a decrease in the amount of RNI released by the peritoneal macrophages (data not shown). Peritoneal macrophages from naive mice did not release RNI. In summary, these results show that macrophages from mice with a secondary infection release RNI but that this can be inhibited by neutralization of endogenously produced TNF.

Inhibition of RNI formation during a secondary infection. To further clarify the role of RNI in the elimination of bacteria from the organs during a secondary infection, RNI production was inhibited by treating mice with a combination of L-NAME and an L-arginine-deficient diet. Preliminary experiments showed that L-NAME inhibited RNI formation by peritoneal macrophages activated in vitro with IFN-γ and lipopolysaccharide whereas D-NAME could not. However, in vivo treatment of mice with L-NAME alone did not effectively decrease the concentration of RNI in plasma (data not shown). When mice were treated with L-NAME in combination with an L-arginine-deficient diet during infection, the concentration of RNI in plasma was significantly lower than that in mice treated with D-NAME (Table 1). At all times, the number of *L. monocytogenes* in the liver and spleen of mice treated with L-NAME and an L-arginine-free diet was slightly but not significantly smaller than that in control mice treated with D-NAME during a sec-

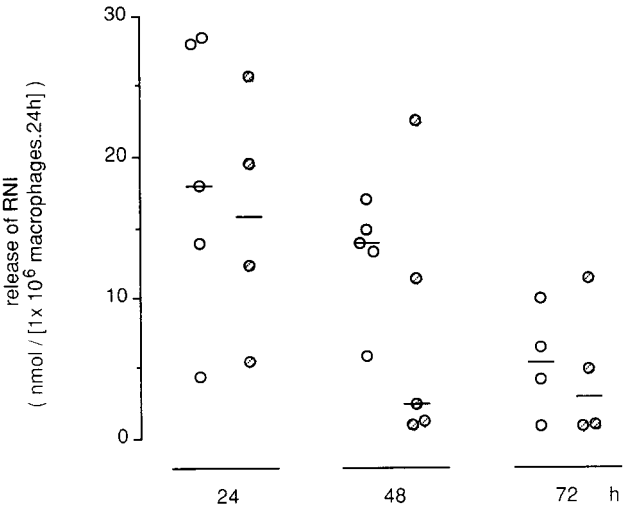


FIG. 3. Release of RNI by peritoneal macrophages from mice with a primary or secondary *L. monocytogenes* infection. Naive mice (○) or mice immunized with 0.1 LD_{50} of *L. monocytogenes* 3 weeks earlier (⊘) were infected with 10 LD_{50} of *L. monocytogenes*. After 24, 48, and 72 h of infection, peritoneal macrophages were collected and cultured for 24 h; the RNI release was determined in the supernatant. Release of RNI by macrophages from uninfected mice was not detectable. Dots represent individual mice, and horizontal bars represent median values. The differences were not significant.

TABLE 1. Number of *L. monocytogenes* in the livers and spleens of mice treated with an L-arginine-free diet and an NOS inhibitor during a secondary infection[a]

Treatment		Duration of infection (h)	Concn of RNI in plasma (μM)[b]	Log_{10} no. of viable *L. monocytogenes*[c] cells in:	
Inhibitor	Diet			Liver	Spleen
L-NAME	L-Arginine free	24	3.7 ± 2.2*	6.0 ± 0.3	5.9 ± 0.1
D-NAME	Control	24	21.6 ± 12.5	6.1 ± 0.2	6.0 ± 0.1
L-NAME	L-Arginine free	72	51.7 ± 24.6*	7.1 ± 1.5	5.8 ± 0.5
D-NAME	Control	72	194.9 ± 64.2	7.9 ± 0.9	6.7 ± 0.7

[a] Mice were immunized with 0.1 LD_{50} of *L. monocytogenes* and received a challenge with 10 LD_{50} of *L. monocytogenes* 3 weeks later. The mice were adapted to an L-arginine-deficient or control diet for 8 days before challenge and were treated once a day with 200 mg of L-NAME or D-NAME (control) per kg i.v. starting 1 day before the challenge.

[b] *, $P < 0.05$ versus mice treated with D-NAME.

[c] Values are means ± standard deviations for four mice.

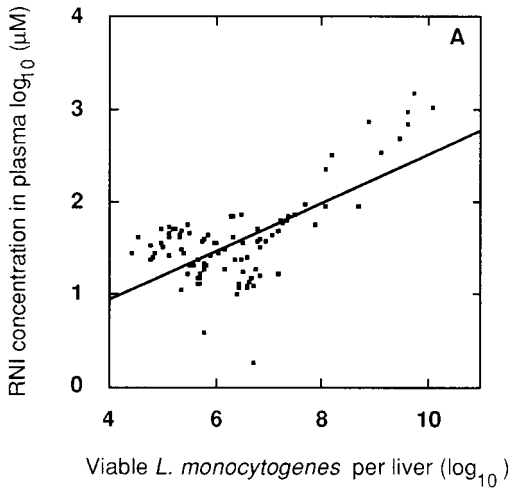
ondary *Listeria* infection (Table 1). Treatment of mice with aminoguanidine during a secondary infection did not significantly affect the proliferation of *L. monocytogenes* in the organs. At 72 h of a secondary infection, the number of *L. monocytogenes* recovered from the livers of mice treated with aminoguanidine (mean $\log_{10}$ 6.42 ± 0.60) did not significantly differ from that recovered from the livers of mice treated with saline (mean $\log_{10}$ 6.31 ± 0.60) and the number of *L. monocytogenes* in the spleens of mice treated with aminoguanidine (mean $\log_{10}$ 5.16 ± 1.00) did not differ significantly from that recovered from the spleens of mice treated with saline (mean $\log_{10}$ 5.00 ± 0.87). Together, these results demonstrate that inhibition of RNI formation does not result in an increase in the number of bacteria in the liver and spleen during a secondary infection.

Correlation between the RNI concentration in plasma and the number of bacteria per organ. Since the results of the experiments described above indicated that a large number of bacteria in the liver and spleen is associated with a high concentration of RNI in plasma, the correlation between the concentration of RNI in plasma and the number of bacteria in the liver and spleen was assessed by using all the data obtained so far. Statistical analysis based on the actual number of bacteria revealed a significant and positive correlation between the RNI concentration in plasma and the number of listeriae in the liver ($r = 0.83$; $P < 0.01$) (Fig. 4A) and spleen ($r = 0.50$; $P < 0.01$) (Fig. 4B). The concentration of RNI in plasma did not correlate ($r = 0.003$; $P = 0.8$) with the concentration of creatinine in plasma, indicating that high concentrations of RNI in plasma during infection are not due to renal failure.

DISCUSSION

From the present results, it can be concluded that resistance against a secondary *L. monocytogenes* infection in mice is not dependent on RNI. Support for this conclusion is provided by the following observations. Inhibition of RNI formation during a secondary infection had no effect on the proliferation of *L. monocytogenes* in the liver and spleen. Furthermore, the enhanced elimination of bacteria during a secondary infection was not accompanied by a high concentration of RNI in plasma. When the secondary immune response was abrogated by treatment with anti-cytokine-forming cells the number of listeriae in the liver and spleen, as well as the concentration of RNI in plasma, increased.

Our finding that treatment of mice with L-NAME and an L-arginine-deficient diet resulted in a marked reduction in the concentration of RNI in plasma but did not affect the proliferation of *L. monocytogenes* in their organs during a secondary infection is supported by the results of our studies with aminoguanidine. Moreover, Maybodi et al. (25) and Mielke et al. (26a) observed no effect of treatment with N^G-monomethyl-L-arginine on the proliferation of *L. monocytogenes* during a secondary infection in mice. Furthermore, we reported earlier that the enhanced killing of *L. monocytogenes* by activated peritoneal macrophages in vitro is only partly mediated by RNI (33), and others have shown that reactive oxygen radicals play a more important role than RNI in the intracellular killing of *L. monocytogenes* by cells of a macrophage-like cell line (16). Recently, an antimicrobial protein has been identified in the cytosol of IFN-γ-stimulated macrophages with activity against *L. monocytogenes* (15). Possibly, a combination of oxygen-de-



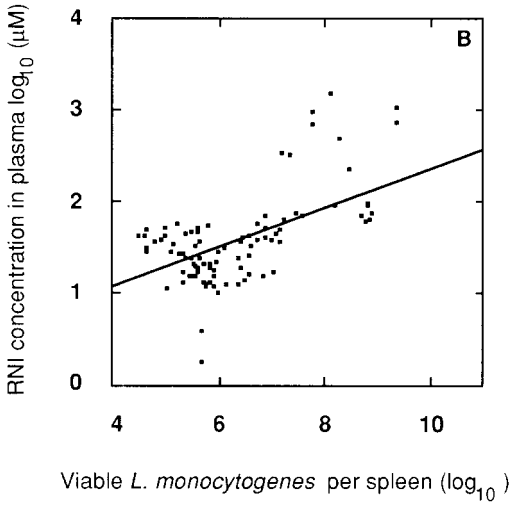


FIG. 4. Correlation between the concentration of RNI in plasma and the number of bacteria per organ. The relationship between the concentration of RNI and the number of bacteria in the liver (A) or spleen (B) after 24, 48, and 72 h of a primary infection, a secondary infection, and anti-cytokine-FC treatment during a secondary infection was assessed ($n = 96$). The analysis was performed with the actual bacterial numbers; for the purpose of illustration, logarithmic transformation of the data was performed.

pendent and oxygen-independent mechanisms is involved in the elimination of *L. monocytogenes* during a secondary infection in mice.

During the course of a secondary *L. monocytogenes* infection, the concentration of RNI in plasma remains low while the bacteria are efficiently eliminated from the organs. Our findings do not exclude the possibility that despite a low concentration of RNI in the plasma, large amounts of RNI are produced in the tissues. However, others have reported that in adoptively immunized mice with an *L. monocytogenes* infection, the urinary nitrate excretion was low and was accompanied by a low level of iNOS mRNA expression in the tissues, whereas in nonimmunized mice, the urinary nitrate excretion was high and was accompanied by a high iNOS mRNA expression in the tissues (25). These data suggest that during an infection, the concentration of RNI in plasma correlates with the production of RNI in the tissues. In our study, the concentration of RNI in plasma correlated positively with the number of *L. monocytogenes* in the liver and spleen, which suggests that the concentration of RNI in plasma can be considered to be a parameter for the severity of infection. This is supported by another study showing that an increase in the infecting dose of listeriae results in an increase in the concentration of RNI in plasma (13). In our study, neutralization of TNF during a secondary infection led to an increase in the number of listeriae in the liver and spleen, confirming an earlier report (30). The finding that in vivo neutralization of TNF led to a decrease in RNI release by peritoneal macrophages is consistent with earlier reports (11, 19). However, neutralization of TNF led to an increase in the concentration of RNI in plasma. These results could suggest that cells other than macrophages are responsible for the increase in the concentration of RNI in plasma. Others have shown that hepatocytes can release RNI in response to products of activated Kupffer cells (10); also, endothelial cells from the livers of mice injected with lipopolysaccharide release RNI (20).

During the course of a primary as well as a secondary *L. monocytogenes* infection, the bacteria are rapidly ingested by Kupffer cells and eliminated, but some bacteria will evade elimination by Kupffer cells and invade hepatocytes (5, 12). During a primary infection, the bacteria can replicate in the hepatocytes, whereas during a secondary infection, the hepatocytes are capable of eliminating listeriae very rapidly (12). It has been shown that Kupffer cells and hepatocytes derived from mice with an *L. monocytogenes* infection produce RNI but that the in vitro killing of listeriae by these cells occurs in an RNI-independent fashion (13). It has also been reported recently that killing of *L. monocytogenes* by cells of an activated hepatocyte cell line occurs independently of RNI (34). Together, these studies (12, 13, 34) support our finding that during a secondary infection with *L. monocytogenes*, elimination of the bacteria is not dependent on RNI.

ACKNOWLEDGMENTS

We express our gratitude to Herman Mattie and Annelies Stevenhagen for assistance with the statistical analysis and to Akke Annema for technical assistance.

REFERENCES

1. **Abrams, J. S., M. G. Roncarolo, H. Yssel, U. Andersson, G. J. Gleich, and J. E. Silver.** 1992. Strategies of anti-cytokine monoclonal antibody development: immunoassays of IL-10 and IL-5 in clinical samples. Immunol. Rev. **127:**5–24.
2. **Adams, L. B., J. B. Hibbs, Jr., R. R. Taintor, and J. L. Krahenbuhl.** 1990. Microbistatic effect of murine activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. J. Immunol. **144:**2725–2729.
3. **Beckerman, K. P., H. W. Rogers, J. A. Corbett, R. D. Schreiber, M. L. McDaniel, and E. R. Unanue.** 1993. Release of nitric oxide during the T-cell-independent pathway of macrophage activation. J. Immunol. **150:** 888–895.
4. **Boockvar, K. S., D. L. Granger, R. M. Poston, M. Maybodi, M. K. Washington, J. B. Hibbs, Jr., and R. L. Kurlander.** 1994. Nitric oxide produced during murine listeriosis is protective. Infect. Immun. **62:**1089–1100.
5. **Chakraborty, T., and W. Goebel.** 1988. Recent developments in the study of virulence in *Listeria monocytogenes*. Curr. Top. Microbiol. Immunol. **138:** 41–58.
6. **Chan, J., K. Tanaka, D. Carrol, J. Flynn, and B. R. Bloom.** 1995. Effects of nitric oxide synthase inhibitors on murine infection with *Mycobacterium tuberculosis*. Infect. Immun. **63:**736–740.
7. **Chan, J., Y. Xing, R. S. Magliozzo, and B. R. Bloom.** 1992. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. J. Exp. Med. **175:**1111–1122.
8. **Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. M. Mosmann.** 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J. Exp. Med. **166:**1229–1244.
9. **Croen, K. D.** 1993. Evidence for an antiviral effect of nitric oxide. Inhibition of herpes simplex virus type I replication. J. Clin. Invest. **91:**2446–2452.
10. **Curran, R. D., T. R. Billiar, D. J. Stuehr, K. Hofmann, and R. L. Simmons.** 1989. Hepatocytes produce nitrogen oxides from L-arginine in response to inflammatory products of Kupffer cells. J. Exp. Med. **170:**1767–1774.
11. **Green, S. J., R. M. Crawford, J. T. Hockmeyer, M. S. Meltzer, and C. Nacy.** 1990. Leishmania major amastigotes initiate the L-arginine-dependent killing mechanism in IFN-γ stimulated macrophages by induction of tumor necrosis factor-α. J. Immunol. **145:**4290–4297.
12. **Gregory, S. H., L. K. Barczynski, and E. J. Wing.** 1992. Effector function of hepatocytes and Kupffer cells in the resolution of systemic bacterial infections. J. Leukocyte Biol. **51:**421–424.
13. **Gregory, S. H., E. J. Wing, R. A. Hoffman, and R. L. Simmons.** 1993. Reactive nitrogen intermediates suppress the primary immunologic response to Listeria. J. Immunol. **150:**2901–2909.
14. **Groeneveld, P. H. P., P. Colson, K. M. C. Kwappenberg, and J. Clement.** 1995. Increased production of nitric oxide in patients infected with the European variant of hantavirus. Scand. J. Infect. Dis. **27:**453–456.
15. **Hiemstra, P. S., P. B. Eisenhauer, S. S. L. Harwig, M. T. van den Barselaar, R. van Furth, and R. I. Lehrer.** 1993. Antimicrobial proteins of murine macrophages. Infect. Immun. **61:**3038–3046.
16. **Inoue, S., S. Itagaki, and F. Amano.** 1995. Intracellular killing of *Listeria monocytogenes* in the J774.1 macrophage-like cell line and the lipopolysaccharide (LPS)-resistant mutant LPS1916 cell line defective in the generation of reactive oxygen intermediates after LPS treatment. Infect. Immun. **63:** 1876–1886.
17. **Iyengar, R., D. J. Stuehr, and M. A. Marletta.** 1987. Macrophage synthesis of nitrite, nitrate and N-nitrosamines: precursors and role of the respiratory burst. Proc. Natl. Acad. Sci. USA **84:**6369–6373.
18. **Langermans, J. A. M., M. E. B. van der Hulst, P. H. Nibbering, P. S. Hiemstra, L. Fransen, and R. van Furth.** 1992. Interferon-γ-induced L-arginine-dependent toxoplasmastatic activity in murine peritoneal macrophages is mediated by endogenous TNF-α. J. Immunol. **148:**568–574.
19. **Langermans, J. A. M., M. E. B. van der Hulst, P. H. Nibbering, and R. van Furth.** 1992. Endogenous TNF-α is required for enhanced antimicrobial activity against *Toxoplasma gondii* and *Listeria monocytogenes* in rIFN-γ-treated mice. Infect Immun. **60:**5107–5112.
20. **Laskin, D. L., D. E. Heck, C. R. Gardner, L. S. Feder, and J. D. Laskin.** 1994. Distinct patterns of nitric oxide production in hepatic macrophages and endothelial cells following acute exposure of rats to endotoxin. J. Leukocyte Biol. **56:**751–758.
21. **Liew, F. Y., Y. Li, and S. Millot.** 1990. Tumor necrosis factor-α synergizes with IFN-γ in mediating killing of Leishmania major through the induction of nitric oxide. J. Immunol. **145:**4306–4310.
22. **Liew, F. Y., S. Millot, C. Parkinson, R. M. J. Palmer, and S. Moncada.** 1990. Macrophage killing of Leishmania parasite in vivo is mediated by nitric oxide from L-arginine. J. Immunol. **144:**4794–4797.
23. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116:** 381–406.
24. **MacMicking, J. D., C. Nathan, G. Hom, N. Chartrain, D. S. Fletcher, M. Trumbauer, K. Stevens, Q. Xie, K. Sokol, N. Hutchinson, H. Chen, and J. S. Mudgett.** 1995. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell **81:**641–650.
25. **Maybodi, M., R. M. Poston, R. J. Kurlander, and D. L. Granger.** 1993. The nitric oxide surge observed during primary listeriosis is absent in adoptively immunized mice, abstr. 28. *In* Abstracts of the 31st Annual Meeting of the Infectious Diseases Society of America.
26. **Mayer, B., K. Schmidt, R. Humbert, and E. Bohm.** 1989. Biosynthesis of endothelium derived relaxing factor: a cytosolic enzyme in porcine aortic-endothelial cells Ca^{2+} dependently converts L-arginine into an activator of soluble guanylyl cyclase. Biochem. Biophys. Res. Commun. **164:**678–685.

26a. **Mielke, M. E. A., et al.** Unpublished data.
27. **Moncada, S., and R. M. J. Palmer.** 1990. The L-arginine:nitric oxide pathway in the vessel wall, p. 19–33. *In* S. Moncada and E. A. Higgs (ed.), Nitric oxide from L-arginine: a bioregulatory system. Elsevier Biomedical Press, Amsterdam.
28. **Mulsch, A., E. Bassenge, and R. Busse.** 1989. Nitric oxide synthesis in endothelial cytosol: evidence for a calcium-dependent and a calcium-independent mechanism. Naunyn-Schmiedeberg's Arch. Pharmacol. **340:**767–770.
29. **Nibbering, P. H., J. A. M. Langermans, J. S. van de Gevel, M. E. B. van der Hulst, and R. van Furth.** 1991. Nitrite production by activated murine macrophages correlates with their toxoplasmastatic activity, Ia antigen expression, and production of H_2O_2. Immunobiology **184:**93–105.
30. **Samsom, J. N., J. A. M. Langermans, H. F. J. Savelkoul, and R. van Furth.** 1995. Tumour necrosis factor, but not interferon-γ, is essential for acquired resistance to Listeria monocytogenes during a secondary infection in mice. Immunology **86:**256–262.
31. **Savelkoul, H. F. J., B. W. P. Seymour, L. Sullivan, and R. L. Coffman.** 1991. IL-4 can correct defective IgE production in SJA/9 mice. J. Immunol. **146:** 1801–1805.
32. **Savelkoul, H. F. J., R. van Ommen, A. C. T. M. Vossen, E. G. Breedveld, R. L. Coffman, and A. van Oudenaren.** 1994. Modulation of systemic cytokine levels by implantation of alginate encapsulated cells. J. Immunol. Methods **170:**185–196.
33. **Stokvis, H., J. A. M. Langermans, E. de Backer-Vledder, M. E. B. van der Hulst, and R. van Furth.** 1992. Hydrocortisone treatment of BCG-infected mice impairs the activation and enhancement of antimicrobial activity of peritoneal macrophages. Scand. J. Immunol. **36:**299–305.
34. **Szalay, G., J. Hess, and S. H. E. Kaufmann.** 1995. Restricted replication of *Listeria monocytogenes* in a gamma interferon-activated murine hepatocyte line. Infect. Immun. **63:**3187–3195.
35. **Van Dissel, J. T., J. J. M. Stikkelbroeck, M. T. van den Barselaar, W. Sluiter, P. C. J. Leijh, and R. van Furth.** 1987. Divergent changes in antimicrobial activity after immunologic activation of mouse peritoneal macrophages. J. Immunol. **139:**1665–1672.

Editor: S. H. E. Kaufmann