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HOST INTERACTIONS WITH INTESTINAL *LISTERIA*: EXCEPTION OR THE RULE?

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SUMMARY

Listeria monocytogenes normally infects the host by translocating from the intestinal lumen. Experiments were carried out to determine if, when and where tumour necrosis factor (TNF) and gamma interferon (IFN-γ) function in antibacterial resistance during enteric listeriosis. Groups of normal mice and severe combined immunodeficient (SCID) mice were injected with monoclonal antibodies (MAb) specific for either cytokine and then inoculated intragastrically with *L. monocytogenes*. The course of infection was monitored by enumerating listeriae in gut associated lymphoid tissues (GALT), livers and spleens. By the third day of infection, bacterial numbers in infected tissues and organs were greatly exacerbated in normal mice and SCID mice treated with anti-TNF Mab, whereas bacterial numbers in the organs of mice treated with anti-IFN-γ Mab did not differ from those present in the respective organs of control mice. However, by the fifth day of infection, bacterial numbers in the organs of anti-IFN-γ Mab-treated normal mice and SCID mice were much greater than in the corresponding organs of control mice. Experiments using immune mice revealed that TNF and IFN-γ are involved in the expression of anti-*Listeria* memory immunity, however, it was also found that the anti-IFN-γ Mab was relatively ineffective in inhibiting the expression of anti-*Listeria* immunity whereas, a monospecific polyclonal anti-IFN-γ was quite effective. This paradox between the ability of the anti-IFN-γ Mab to inhibit IFN-γ-mediated effects in innate antibacterial resistance and its inability to inhibit IFN-γ-mediated effects in anti-*Listeria* memory immunity suggest that the IFN-γ molecule possesses functional domains which mediate distinct activities.

INTRODUCTION

Carter and Pollard, Berg and Savage, Adrian Lee, and many others have shown that lactobacilli and other members of the mouse autochthonous flora do not induce an immune reaction when they colonise the intestinal tract. A close relative of members of the genus *Lactobacillus* is *Listeria monocytogenes*, an enteric organism which induces a somewhat unusual immune response. Mouse listeriosis is a widely used model for the study of host resistance mechanisms that are expressed against intracellular bacteria. Mackaness reported that

host cells, not humoral factors, effected anti-*Listeria* immunity in mice (*Mackaness*, 1962). Mackaness also established the importance of both lymphocytes and macrophages in the expression of anti-*Listeria* immunity (*Mackaness*, 1964, 1969). Newly generated and sensitised immune lymphocytes conferred the specific nature of immunity to listeriae, however, resolution of infection required the activation of macrophages which exhibit a heightened state of non-specific bactericidal activity. Later, *Lane* and *Unanue* (1972) and *North* (1973) established that T lymphocytes mediated the expression of specifically acquired anti-*Listeria* immunity. Since this time, considerable information concerning the importance and interactions of various host effector cells and cytokines in non-specific antibacterial resistance (innate immunity) and in specifically acquired T cell-mediated antibacterial immunity has come about using mouse models of listeriosis.

The identification of cytokines involved in host antimicrobial resistance has come largely from the use of mouse models of infectious diseases in which the action of a cytokine is inhibited. Inhibition of a cytokine-mediated effect has generally been accomplished by either blocking the action of an endogenously produced cytokine with specific anti-cytokine antibodies or using cytokine gene knockout mice which lack the capacity to synthesise functional cytokines or cytokine membrane receptors. Indeed, both approaches have been used to demonstrate the importance of IFN-γ and TNF in host anti-*Listeria* resistance in mice. *Buchmeier* and *Schreiber* (1985) showed that listeriosis was exacerbated in mice treated with monoclonal anti-IFN-γ Mab. Work carried out in our laboratory (*Havell*, 1987, 1989) and by others (*Nakane* et al., 1988) showed that anti-TNF antibody treatment of mice converted a normally immunising infection initiated by the intravenous injection of a sublethal *L. monocytogenes* inoculum into a lethal infection. Likewise, anti-TNF antibody treatment exacerbated listeriosis in immunoincompetent nude (*nu/nu*) mice (*Havell*, 1989; *Hauser* et al., 1990). Gene knockout mice lacking functional membrane receptors for either IFN-γ or TNF were found to be considerably more susceptible to listeriosis than the wild type mice (*Huang* et al., 1993; *Rothe* et al., 1993; *Pfeffer* et al., 1993). Similar experimental approaches have shown both that TNF and IFN-γ are also important components of host resistance against a variety of other microorganisms and that these cytokines are important both in innate antimicrobial resistance and in the generation and/or expression of specifically acquired antimicrobial immunity (*Nakane* et al., 1989; *Kindler* et al., 1989; *Chen* et al., 1992, 1993; *McCafferty* et al., 1994; *Aguirre* et al., 1995).

Most studies investigating host cells and cytokines in anti-*Listeria* resistance have used mice infected by parenteral routes of inoculation, however, *L. monocytogenes* normally infects the host by translocating from the intestinal lumen and then spreading to internal organs. *MacDonald* and *Carter* (1980) reported that listeriae present in the lumen of the gastrointestinal tract of mice were capable of infecting Peyer's patches. Peyer's patch-associated listeriae were shown capable of entering mesenteric lymph nodes, from where these bacteria can spread to other internal organs, including the liver and spleen. Like *L. monocytognes*, *Salmonella typhimurium* present in the intestinal lumen was also shown to invade the Peyer's patches In view of the reports that IFN-γ is found in S. *typhimurium*-infected Peyer's patches (*George*, 1996) and that intracellular IFN-γ is detected in intraepithelial lymphocytes in mice following the intragastric *Listeria* inoculation, experiments were carried out to deter-

mine whether IFN-γ and TNF play roles in innate resistance and specifically acquired antibacterial resistance in the GALT and other infected organs following the translocation of listeriae from the intestinal lumen of mice.

MATERIALS AND METHODS

Mice

Male BALB/c mice 8 to 12 wk of age were purchased from either Charles River Laboratories (Wilmington, MA) (BALB/c Crl) or Taconic Farms (Germantown, NY) (BALB/c Tac). C.B-17 SCID mice were purchased from Jackson Labs (Bar Harbor, ME). BALB/c mice were maintained under pathogen-free husbandry conditions while immunoincompetent SCID mice were maintained in autoclaved microisolator cages provided with sterile food and water.

Listeria monocytogenes

Listeria monocytogenes (strain EGD, serotype 1/2a) was grown overnight at 37°C in Trypticase soy broth (BBL Microbiology Systems, Becton Dickinson, Cockeysville, MD). The culture broth was centrifuged at 800 x g/20 minutes and the pelleted bacteria were re-suspended in Dulbecco's phosphate-buffered saline (DPBS) pH 7.4. The stock culture having a titre of 6.6 x 10^9 CFU/ml was aliquoted in tubes and stored at -70°C. Immediately before use, stock preparations were quick-thawed and diluted in DPBS (pH 7.4). The intravenous LD_{50} for *L. monocytogenes* in BALB/c Crl mice was determined to be 4 x 10^3 CFU. The standard intagastric (i.g.) inoculum was 2 x 10^8 CFU in 0.2 ml of DPBS. Mice were gavaged intragastrically with an 18 gauge feeding needle (Popper, Long Island City, NY).

Enumeration of organ-associated bacteria

Organ homogenates of livers, spleens, mesenteric lymph nodes and Peyer's Patches were prepared by grinding organs suspended in iced sterile saline (0.85%) with a Teflon motorised pestle. Enumeration of bacterial CFU in the organ homogenates were determined by plating serial 10-fold dilutions of liver, spleen, or mesentery/mesenteric lymph nodes homogenates on trypticase soy agar (TSA, BBL Microbiology Systems, Becton Dickinson, Cockeysville, D). Bacterial CFU in homogenates of the Peyer's patches were plated on *Listeri*a-selective phenylethanol (PEA) agar consisting of 1.5% Noble agar, 1.5% trypticase peptone, 0.5% phytone peptone, 0.5% NaCl, 1.0% Glycine, 0.05% LiCl, and 0.25% Phenylethanol (*MacDonald* and *Carter*, 1980). *Listeria monocytogenes* colonies on PEA agar were identified by their characteristic light blue colour when illuminated with oblique light. Tests for esculin, catalase, and/or motility were performed to insure that questionable colonies on PEA agar were indeed *L. monocytogenes* colonies.

Anti-cytokine antibodies

The R4-6A2 hybridoma (ATCC, HB170) which secretes a rat anti-murine IFN-γ Mab (IgG1) and the XT3.11 hybridoma (DNAX Research Institute, Palo Alto, CA) which secretes rat anti-murine TNF-α MAb (IgG1) were grown as ascites in the peritoneal cavities of pristane-primed $CB6F_1$ mice, according to our published procedures (*Havell*, 1986a; *Aguirre* et al., 1995). The R4-6A2 anti-IFN-γ MAb and the XT3.11 anti-TNF Mab were purified from ascitic fluids according to previously published procedures (*Havell*,

1986a). A rabbit anti-IFN-γ polyclonal IgG antibody was generated by immunising a New Zealand White female rabbit with pure recombinant mouse IFN-γ having a specific activity of 10^7 antiviral units/ml (u/ml) which was the kind gift of Genentech, Inc. (South San Francisco, CA). The rabbit anti-IFN-γ IgG or rabbit control IgG was purified from serum according to published procedures (*Havell* et al., 1988). The various purified antibody preparations were assayed for endotoxin concentrations by means of a quantitative chromogenic *Limulus* amoebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD).

The quantitation of the anti-IFN-γ antibody neutralising activity was performed as previously reported (*Spitalny* and *Havell*, 1984). Briefly, serial two-fold dilutions of sample (50 μl) were incubated with an equal volume of IFN-γ (20 antiviral units/ml) in wells of a 96-well flat bottom plate for 1 hour at 37°C. At the end of this time, $2x10^4$ L929B mouse fibroblasts in 100 μl were added to each well, and the plates were incubated at 37°C. Eighteen hours later, 10^3 PFU of vesicular stomatitis virus (VSV) were added to each well. The plates were incubated for 48 hours, after which viral cytopathic effect was scored. The neutralising titre (neutralising units/ml [NU/ml]) of the anti-IFN-γ Mab is defined as the reciprocal of the highest dilution of the sample that, when mixed with an equal volume of IFN-γ (concentration, 20 antiviral units/ml), neutralises 50% of the antiviral activity, as judged by the development of VSV cytopathic effect in the L929B cell monolayer.

Quantitation of anti-TNF Mab neutralising activity was also performed as previously reported (*Havell* et al., 1988). Basically, the anti-TNF Mab assay procedure is the similar to that outlined above for anti-IFN-γ antibody assay procedure, except the titration of anti-TNF Mab activity measures the neutralisation of TNF cytotoxic activity on actinomycin D-treated L929B cell monolayers.

Anti-cytokine treatment of mice

Mice were injected intraperitoneally with a given antibody preparation 4 hours prior to the intragastric inoculation of bacteria. Mice were injected with 10^5 neutralising units of the R4-6A2 rat anti-IFN-γ Mab (sp act $1.8x10^5$ NU/mg) in PBS (pH 7.4). Mice injected with the XT3.11 rat anti-TNF Mab received $2x10^4$ NU (sp act 6 $x10^3$ NU/mg) in PBS (pH 7.4). The mice that were injected with the rabbit anti-IFN-γ IgG were given $2x10^4$ NU (sp act $2x10^3$ NU/mg) while the corresponding control mice were injected with an equivalent amount (mg) of control rabbit IgG.

At the time of sacrifice, antibody-treated mice were anaesthetised, bled by cardiac puncture, and the serum collected. The sera were assayed to determine the anti-cytokine antibody neutralising titres in order to insure that excess amounts of the anti-cytokine were present in the blood throughout the course of the experiments. In all cases, antibody titres exceeded 10^3 NU/ml of blood.

Statistical analysis

The experimental results were compared using Student's t-test, which requires that the populations be normally distributed and have equal variances. A significant difference between experimental groups was defined by a p value of <0.05. Experiments involving statistical comparisons were performed using 3 to 5 mice per group.

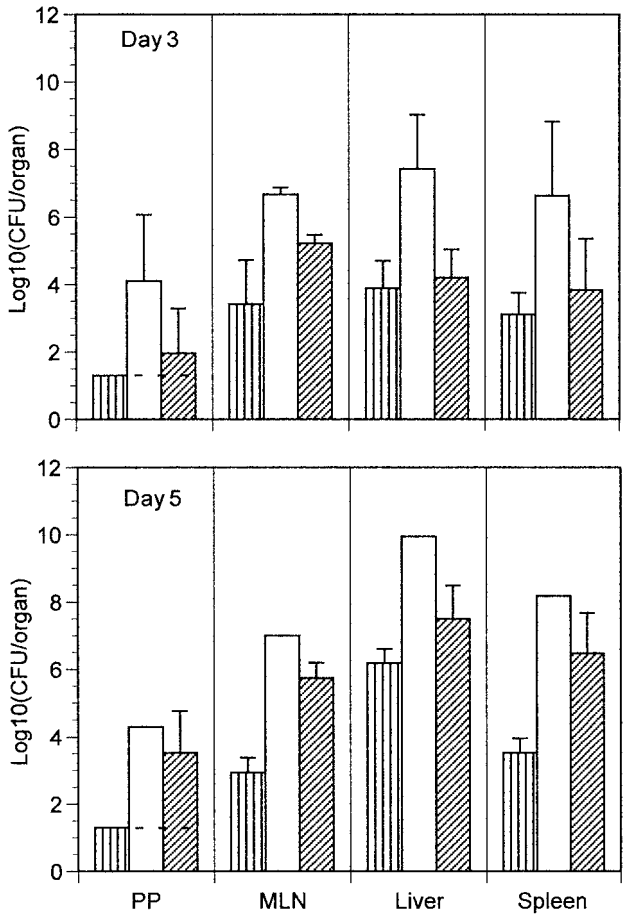

Figure 1: The effect of anti-TNF Mab or anti-IFN-γ Mab treatment on the course of enteric listeriosis. BALB/c mice were injected intraperitoneally with 10^5 NU of anti-IFN-γ Mab, $3x10^4$ NU of anti-TNF, or PBS and 4 hours later, all mice were inoculated intragastrically with $2x10^8$ CFU of *L. monocytogenes*. Organ *L. monocytogenes* CFU were determined on days 3 and 5 following the intragastic inoculation of bacteria. Data are presented as the means (bars) +/- standard deviations of organ CFU for an experimental group. Means lacking standard deviations indicate that either bacterial CFU were below detection limits in one or more organs from an experimental group, or insufficient numbers of mice survived treatment, as was the case on day 5 for the anti-TNF Mab-treated group (1 survivor). ▥: control; □: anti-TNF; ▨: anti-IFN. Dashed horizontal lines represent the detection limits of the assay.

RESULTS

The importance of TNF and IFN-γ in resistance to an immunising *Listeria* enteric infection

The inhibition of cytokine-mediated effects *in vivo* by administering specific antibodies has proven an effective means for establishing the importance of a cytokine in host resistance to infectious agents (*Buchmeier* and *Schreiber*, 1985; *Havell*, 1987, 1989; *Chen* et al., 1993). Experiments were carried out using specific anti-TNF or anti-IFN-γ Mab treated mice to determine if TNF and IFN-γ are involved in resistance to enteric listeriosis. Groups of BALB/c mice were treated intraperitoneally with

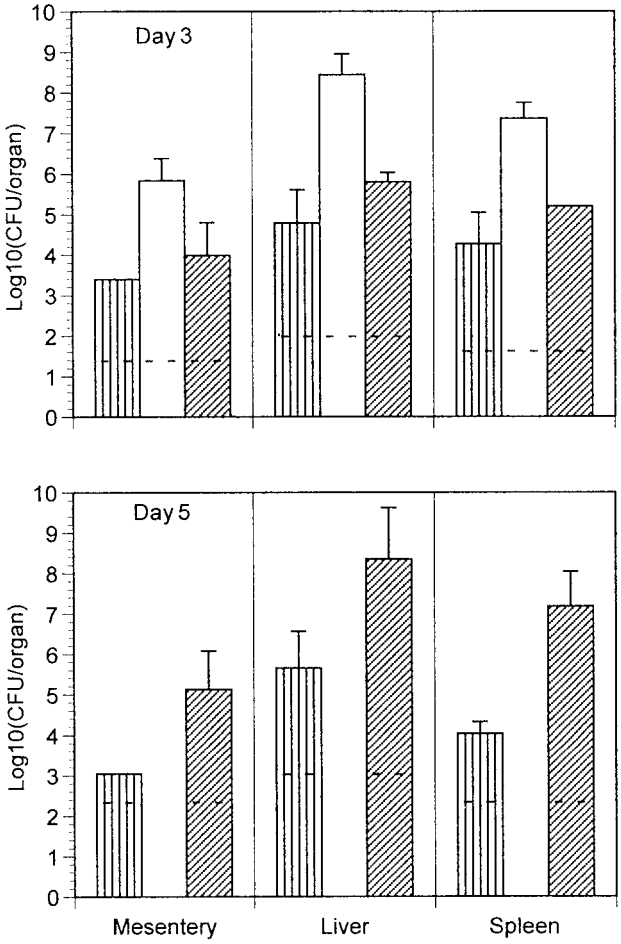

Figure 2: The effect of anti-TNF Mab or anti-IFN-γ Mab treatment on the course of enteric listeriosis in immunoincompetent SCID mice. C.B-17 SCID mice were injected intraperitoneally with 10^5 NU of anti-IFN-γ Mab, $3x10^4$ NU of anti-TNF, or PBS and 4 hr later, all mice were inoculated intragastrically with $2x10^8$ CFU of *L. monocytogenes*. Organ *L. monocytogenes* CFU were determined on days 3 and 5 following the intragastric inoculation of bacteria. All anti-TNF Mab-treated mice were dead by day 5 of infection. Data are presented as the mean (bars) +/- standard deviations of organ CFU. Means lacking standard deviations indicate that bacterial CFU were below detection limits in one or more organs from an experimental group of mice. ▥: control; □: anti-TNF; ▨: anti-IFN. Dashed horizontal lines represent the detection limits of the assay.

anti-TNF Mab or anti-IFN-γ Mab and 4 hr later, inoculated intragastrically with $2x10^8$ CFU of *L. monocytogenes*. The course of enteric listeriosis was then monitored at progressive times by enumerating listerial CFU in the PP, MLN, livers and spleens of the treated mice and control mice. It was found that by the end of the first day of infection, no significant differences existed in numbers of listeriae present in the corresponding organs (MLN, livers, and spleens) of mice in the different experimental groups of mice (results not presented). Moreover, based on the limits of detection of the assay, listeriae were absent from the PP of control mice groups (results not presented). However, by day 3 of infection (Figure 1) the numbers of listeriae in the organs of

the anti-TNF Mab-treated mice were greatly exacerbated, whereas numbers of listeriae were elevated only in the MLN of the anti-IFN-γ-treated mice. Also, at this time of infection, anti-TNF Mab-treated mice were lethargic and hypothermic, and most died by day 5 of infection. Enumeration of listeriae in the organs of the one remaining anti-TNF Mab-treated mouse on day 5 of infection revealed overwhelming numbers of listeriae in the organs (Figure 1). On day 5 of infection, the infected organs of the anti-IFN-γ Mab-treated mice had greater numbers of listeriae than did the corresponding organs of the control mice. These results establish that TNF has an effect that is important in antilisterial resistance during the first 3 days of enteric listeriosis, whereas IFN-γ mediates an important effect in resistance following this time.

The importance of TNF and IFN-γ in innate immunity to enteric listeriosis

Results from the foregoing experiment established that an IFN-γ-mediated effect is expressed in *Listeria*-infected organs of mice after the time (day 3) when the host normally begins to generate a T cell-mediated anti-*Listeria* immune response that is capable of effecting the resolution of infection (39-41). In an attempt to dissociate IFN-γ- or TNF-mediated effects in innate antibacterial immunity from possible effects which could be important in the generation and/or expression of specifically acquired anti-*Listeria*-immunity, immunoincompetent SCID mice were used in an experiment to determine the importance of these cytokines in innate antibacterial immunity to enteric listeriosis. Groups of C.B-17 SCID mice were inoculated intraperitoneally with anti-TNF Mab or anti-IFN-γ Mab and 4 hr later, inoculated intragastrically with $2x10^8$ CFU of *L. monocytogenes*. Since SCID mice lack discernible PP and MLN, listeriae were enumerated in the mesentery, livers and spleens of groups of the Mab-treated SCID mice and control SCID mice on days 3 and 5 of listeriosis. It can be seen in Figure 2 that anti-TNF Mab treatment, but not anti-IFN-γ Mab treatment of SCID mice greatly enhanced numbers of listeriae in the mesentery, liver, and spleen on day 3 of infection. By day 5 of infection, all anti-TNF Mab-treated SCID mice had succumbed to overwhelming infection whereas, infected SCID mice treated with anti-IFN-γ Mab were only beginning to show signs of morbidity associated with overwhelming bacterial infection. Bacterial numbers present in the mesentery, liver and spleens of the anti-IFN-γ Mab-treated SCID mice and control SCID mice on day 5 of listeriosis are also presented in Figure 2, where it can be seen that the numbers of listeriae in the organs of the anti-IFN-γ-treated mice are much greater than in the respective organs of control mice. Thus, the collective results presented in Figure 1 and Figure 2 establish that the magnitude and temporal manifestation of TNF- and IFN-γ-mediated antibacterial effects are, respectively, similar in the organs of both immunocompetent mice and immunoincompetent mice during enteric listeriosis.

The importance of TNF and IFN-γ in the expression of anti-*Listeria* memory immunity in the intestine

The results of the preceding experiments do not allow conclusions to be made as to whether TNF and IFN-γ function in the expression of anti-*Listeria* immunity because of the similar results which were obtained with normal mice and SCID mice undergoing a primary *Listeria* enteric infection. To determine whether TNF or IFN-γ plays a role in anti-*Listeria* immunity in the intestine, *Listeria*-immune mice, immunised by an intragastric inoculation of

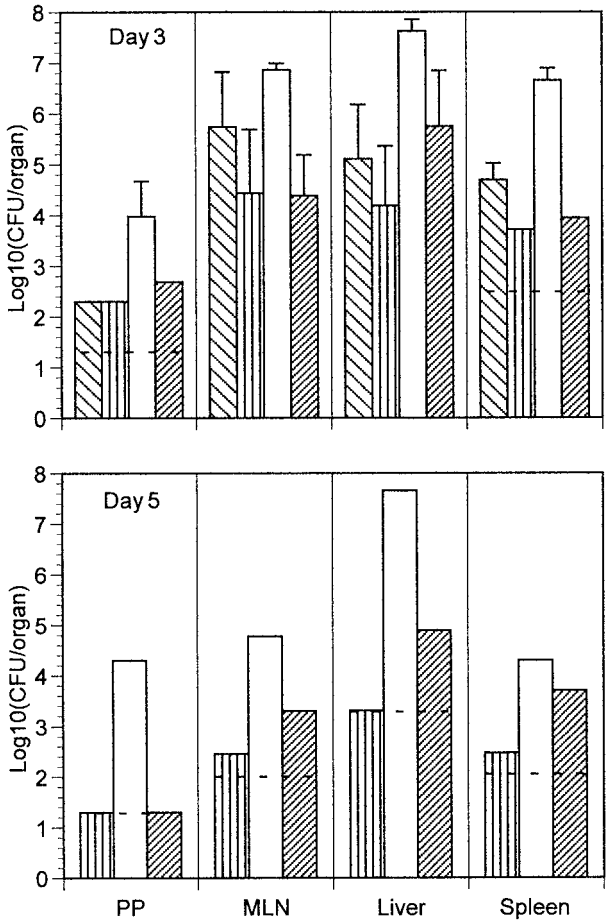

Figure 3: The effect of anti-TNF Mab or anti-IFN-γ Mab treatment on the expression of memory anti-*Listeria* immunity. BALB/c mice rendered *Listeria* immune by intragastric inoculation of $2x10^8$ *L. monocytogenes* 28 days earlier were injected intraperitoneally with 105 NU of anti-IFN-γ Mab, $3x10^4$ NU of anti-TNF, or PBS. Four hours later, all treated *Listeria* immune mice and a group of non immune mice were challenged with an intragastric inoculum of $6x10^9$ CFU of *L. monocytogenes*. On days 3 and 5 following rechallenge, organ CFU were enumerated. Data are presented as means (bars) and +/- standard deviation. Means lacking standard deviations indicate that bacterial CFU were below detection limits in one or more organs from an experimental group of mice, or insufficient numbers of mice survived treatment, as was the case on day 5 for the anti-TNF Mab-treated group (1 survivor). ▧: non-immune controls; ▥: immune controls; □: anti-TNF; ▨: anti-IFN. Dashed horizontal lines represent the detection limits of the assay.

$2x10^8$ CFU of *L. monocytogenes* 28 days earlier, were injected intraperitoneally with either anti-TNF Mab or anti-IFN-γ Mab and, 4 hr later, challenged with an intragastric dose of $6x10^9$ CFU of *L. monocytogenes*. In Figure 3 are presented the CFU present in the PP, MLN, livers and spleens of the Mab-treated *Listeria*-immune mice on days 3 and 5 of a secondary challenge. It can be seen that on day 3 of infection listerial numbers were elevated in the organs of the anti-TNF Mab-treated immune hosts relative to the listerial numbers in the corresponding organs of immune control mice ($p<0.01$). At this time of infection, listerial numbers in the respective organs of non-

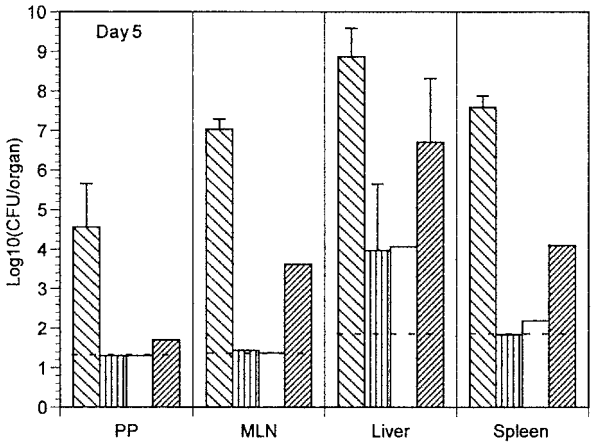

Figure 4: A comparison of the abilities of the R4-6A2 anti-IFN-γ Mab and a rabbit anti-IFN-γ IgG preparation to inhibit the expression of memory anti-*Listeria* immunity. BALB/c mice rendered *Listeria* immune by intragastric inoculation of $2x10^8$ *L. monocytogenes* 30 days earlier were injected intraperitoneally with 10^5 NU of anti-IFN-γ Mab, $2.5x10^4$ NU of a rabbit polyclonal anti-IFN-γ IgG, or control rabbit IgG. Four hours later, all treated *Listeria* immune mice and group of non immune mice were challenged with an intragastric inoculum of $7.2x10^8$ CFU of *L. monocytogenes*. Five days later, organ CFU (mean +/- standard deviation) were enumerated. Means lacking standard deviations indicate that bacterial CFU were below detection limits in one or more organs from an experimental group of mice. ▨: non-immune controls; ▥: immune controls; □: Mab-anti-TNF; ▨: Pab-anti-IFN. Dashed horizontal lines represent the detection limits of the assay.

immune control mice, *Listeria*-immune control mice, and the anti-IFN-γ Mab-treated and *Listeria*-immune mice were similar. However, by day 5 of infection listeriae were not detected in the organs of the control *Listeria*-immune mice whereas, substantial numbers of listeriae were present in the corresponding organs of the non-immune control mice which indicates that memory anti-*Listeria* immunity is not expressed until after the first 3 days of infection. All anti-TNF Mab-treated *Listeria*-immune mice were dead by day 5 of infection. On day 5, the bacterial numbers in the organs of the anti-IFN-γ Mab-treated and *Listeria*-immune mice were only marginally higher than those of the *Listeria*-immune control mice, but were not as high as the bacterial numbers in the organs of the non-immune control mice. Moreover, the anti-IFN-γ Mab treatment of immune mice did not completely prevent the expression of immunity, since these mice survived the secondary challenge. This was not due to a lack of serum anti-IFN-γ Mab neutralising activity during the course of the experiment, for it was found that substantial quantities of anti-IFN-γ Mab were present in the peripheral circulation of treated mice on day 5 of infection (results not presented).

Comparative analysis of the ability of different anti-IFN-γ antibody preparations to block the expression of anti-Listeria memory immunity

The failure of the anti-IFN-γ Mab treatment of *Listeria*-immune mice to completely subvert the expression of immunity (Figure 3) conflicts with previous reports showing that anti-IFN-γ Mab treatment of *Listeria*-immune mice converted what would be normally a sublethal infection initiated by an extravascular challenge into a lethal one

(*Tripp* et al., 1995). One possible explanation for the apparent discrepancy between the results reported in this publication and those reported elsewhere, is that anti-IFN-γ antibody preparations may have specificities for different molecular domains mediating distinct IFN-γ activities (*Schreiber* et al., 1985; *Caruso* et al., 1993). In view of such a possibility, a comparison was made as to abilities of the anti-IFN-γ Mab used in the preceding experiments and a monospecific rabbit anti-IFN-γ polyclonal antibody (Pab) to inhibit the expression of memory immunity in *Listeria*-immune mice. The results presented in Figure 4 show *Listeria* CFU in the PP, MLN, livers, and spleens of immune mice treated with anti-INF-γ Mab, rabbit anti-IFN-γ Pab, or control rabbit IgG on day 5 following the intragastric inoculation of listeriae. The *Listeria* CFU in the organs from the immune host treated with the anti-IFN-γ Mab did not differ from the immune control mice whereas, immunised mice treated with the anti-IFN-γ Pab preparation possessed greatly enhanced numbers of bacteria in the organs as compared to listerial numbers in the corresponding organs of either the immune control mice or anti-IFN-γ Mab-treated immune mice. This indicates that the anti-IFN-γ Pab is more effective than the anti- IFN-γ Mab in inhibiting the expression of anti-*Listeria* immunity in memory immune hosts.

DISCUSSION

Listeria monocytogenes is capable of infecting man and animals following its ingestion in great numbers (*Burn*, 1936; *Osebold* and *Inouye*, 1954). Immunocompromised individuals, women in the first trimester of pregnancy, and neonates are at the greatest risk for infection. Following ingestion, this facultative intracellular bacterium moves rapidly through the intestinal tract and normally does not become a permanent component of the microflora (*Zachar* and *Savage*, 1979). To establish enteric infections in mice, great numbers of listeriae have to be deposited intragastrically, even in mouse strains (e.g., BALB/c) which are highly susceptible to infections initiated by para-enteral routes of inoculation. Peyer's patches are known to be a translocation route of for listeriae present in the intestinal lumen (*MacDonald* and *Carter*, 1980). However, results presented in this paper show that SCID mice become infected following intragastric inoculation of listeriae (Figure 2). This suggests the possibility of an alternate route of translocation route for listeriae, since immunoincompetent SCID mice lack PP. With regards to such a possibility, *Racz* et al. (1972) reported the presence of listeriae in mucosal epithelial cells following the intragastric inoculation of bacteria into guinea pigs. Moreover, the results of unpublished experiments carried out in this laboratory have shown that listeriae are capable of entering, proliferating and destroying the mouse Mode K small intestinal epithelial cell line (*Vidal* et al., 1993). Collectively, these observations and the *in vivo* observations of *Racz* et al. (1972) suggest the possibility that invasion of intestinal epithelial cells may constitute the first step in a PP-independent translocation route. Listeriae present in epithelial cells are capable of multiplying, transiting through the cytoplasm by polymerising actin, and penetrating into neighbouring cells (*Havell*, 1986b; *Dabiri* et al., 1990). Ultimately, the parasitised host cells are destroyed and internalised listeriae are released. Such a sequence of events in enterocytes could result in lis-

teriae entering the intestinal lamina propria and spreading to draining lymph nodes, from where listeriae are free to access the peripheral circulation and spread to other tissues and organs.

The results of experiments presented in this paper clearly establish roles for TNF and IFN-γ in anti-*Listeria* resistance mechanisms that are brought into play during primary and secondary infections caused by listeriae translocating from the gut lumen. TNF and IFN-γ are detected in organs within hours of listerial implantation (*Ehlers* et al., 1992; *Poston* and *Kurlander*, 1992). Previously, we reported that TNF is important in anti-*Listeria* resistance during the first 3 days of infection following the intravenous inoculation of *L. monocytogenes* into normal mice, immunodeficient mice, and *Listeria*-immune mice (*Havell*, 1989). Similarly, the results presented in this paper also establish that TNF is important in anti-*Listeria* resistance mechanisms in these same hosts during the first 3 days of an infection caused by bacteria translocating from the intestine. *Buchmeier* and *Schreiber* (1985) established the importance of IFN-γ in resistance to listeriosis by showing that anti-IFN-γ Mab treatment of mice infected by intraperitoneal inoculation caused an exacerbation of infection and the death of the host. The results presented in this paper also show that anti-IFN-γ Mab treatment exacerbated listeriosis in normal mice and immunoincompetent SCID mice. Moreover, the results of these experiments establish not only the importance of IFN-γ in anti-*Listeria* resistance during an enteric infection in either immunocompetent or immunoincompetent hosts, but also reveal that the IFN-γ-mediated effect occurs in the infected organs following the time when the TNF-mediated effect is expressed. In addition, TNF- and IFN-γ-mediated effects in antibacterial resistance occurred, respectively, at corresponding times during both a primary infection in naive mice and a secondary infection in *Listeria*-immune mice.

Evidence presented in a earlier publication from this laboratory suggested the importance of TNF in focusing host cells having antibacterial function at infectious sites (*Havell*, 1989). A histological examination of infected livers of mice given anti-TNF IgG revealed great numbers of listeriae in hepatocytes and a paucity of both neutrophils and macrophages at infectious foci. Indeed, TNF has activities that would be important in directing the migration of neutrophils, monocytes and lymphocytes to sites of inflammation in infected organs. For example, TNF causes the upregulation of ICAM-1 on endothelial cells (*Gamble* et al., 1985) and such an effect would result in adherence of neutrophils and other leukocytes to the vascular endothelium of infected organs and the subsequent extravasation of these cells into infectious foci. The work of Conlan and North (*Conlan* et al., 1993; *Conlan* and *North*, 1991) showed that neutrophils play an important role in antilisterial resistance by destroying *Listeria*-infected hepatocytes. TNF is known to cause the activation and degranulation of neutrophils (*Ferrante* et al., 1993) and such an effect in the vicinity of *Listeria*-infected non-professional cells could ultimately lead to the destruction of the infected host cells. This effect could serve the host by terminating the intracellular infection in cells incapable of coping with intracellular bacteria, thus allowing host cells having antibacterial function access to the previously internalised bacteria. Monocytes newly recruited from the peripheral circulation begin to supplant neutrophils at infectious foci following the first day of listeriosis (*Mackaness*, 1962). TNF has been shown to be important in granuloma formation, granuloma maintenance, and in triggering antimicrobial actions of macrophages (*Kindler* et al., 1989;

Oswald et al., 1992). Indeed, these effects of TNF early in listeriosis would be important in the expression of antibacterial resistance mechanisms in infected organs and tissues in either immunocompetent or immunoincompetent hosts.

TNF and IFN-γ regulate the synthesis of one another. TNF induces IL-12 which elicits the secretion of IFN-γ from natural killer cells and T lymphocytes (*D'Andrea* et al., 1993). In turn, IFN-γ is capable of greatly augmenting the host's potential for TNF production (*Havell*, 1993) . Natural killer cells produce IFN-γ during the first 24 hr of listeriosis in mice (*Dunn* and *North*, 1991). However, based on the results of experiments presented in this paper using anti-IFN-γ Mab to neutralise IFN-γ in *Listeria*-infected immunocompetent or immunoincompetent hosts, the anti-*Listeria* effect mediated by this cytokine is not evident until after the third day on infection. This observation raises the question as to whether IFN-γ is involved in the implementation and/or expression of the anti-*Listeria* resistance mechanism(s). Since the IFN-γ mediated effect occurs when macrophages populate infectious foci, it seems reasonable to assume that these phagocytes are involved in the IFN-γ-mediated antilisterial effect. On the one hand, it is possible that IFN-γ functions in events that result in the recruitment of monocytes/macrophages at these cells at sites of inflammation. With regards to such a possibility, IFN-γ alone, or in combination with other cytokines, induces the expression of certain beta chemokines (C-C) which can function to focus monocytes at sites of infection (*Proost* et al., 1996; *Cassatella* et al., 1997). On the other hand, IFN-γ is believed to prime macrophages for enhanced listericidal activity (*Buchmeier* and *Schreiber*, 1985; *Denis* and *Gregg*, 1990). Indeed, such a function may result in maintaining the chronic infection that characterises listeriosis in immunoincompetent mice (*Emmerling* et al., 1975).

During a sublethal immunising infection in mice, *Listeria monocytogenes* is rapidly eradicated following the appearance of specifically sensitised T cells that are capable of adoptively transferring anti-*Listeria* immunity to naive mice. The numbers of these sensitised T cells increase and decrease in concordance with the host's capacity to produce IFN-γ during listeriosis (*Havell* et al., 1982). In addition to having effects in innate antibacterial resistance, IFN-γ has actions that could be important in the generation and expression of specifically acquired T cell-mediated antibacterial immunity. With regards to effects in the generation of T cell-mediated immunity, IFN-γ causes the upregulation of MHC class II antigen expression on antigen presenting cells (*Stein* et al., 1984; *Inaba* et al., 1986). This cytokine is also capable of regulating the induction of Th1 helper T cells which regulate T cell immunity (*Belosevic* et al., 1989; *Swain* et al., 1991). As to possible roles for IFN-γ in the expression of T cell-mediated immunity, this cytokine is capable of augmenting the activity of specifically sensitised $CD8^+$ cytolytic T cells either directly, by enhancing the activity of these cells (*Blanchard* et al., 1988) or indirectly, by increasing the expression of MHC class I expression on infected target cells (*Halloran* et al., 1992). However, it is also important to mention both that *Harty* and *Bevan* (1995) have reported that $CD8^+$ T cells capable of adoptively transferring anti-*Listeria* immunity are generated during *Listeria* infection in IFN-γ gene knockout mice, and *Harty* et al. (1992) also found that anti-*Listeria* $CD8^+$ T cells can protect the host in an IFN-γ-independent manner. These findings would seem to suggest that IFN-γ may not be important in the mediation of anti-*Listeria* resistance by $CD8^+$ T cells, however,

this does not exclude the possibility that IFN-γ may be important in the mediation of anti-*Listeria* resistance by other phenotypically distinct T cells which have also been reported to be protective against this intracellular pathogen (*Kaufmann* et al., 1987, 1988; *Rakhmilevich*, 1994). Following the generation of a primary anti-*Listeria* immune response the numbers of T cells capable of adoptively transferring immunity rapidly decline, however, a state of long lived-state of memory immunity ensues. The T cells that are responsible for immunological memory are both physiologically and phenotypically distinct from those that mediate resistance during a primary *Listeria* infection (*Orme*, 1989; *North* and *Deissler*, 1975).

In order to establish the importance of IFN-γ in anti-*Listeria* T cell-mediated memory immunity, *Listeria* immune mice were treated with an anti-IFN-γ Mab preparation and challenged with an intragastric dose of *L. monocytogenes*. It was found that treatment with an anti-IFN-γ Mab had little, or no effect on the expression of *Listeria* memory immunity (Figures 3,4). Both in view of this finding and the knowledge that the monoclonal anti-IFN-γ Mab exacerbated a primary *Listeria* infection in either immunocompetent mice (Figure 1) or immunoincompetent SCID mice (Figure 2), it seems reasonable to assume that IFN-γ does not function in the expression of anti-*Listeria* memory immunity. However, the finding that listeriosis was exacerbated during a secondary infection in immune mice treated with a monospecific anti-IFN-γ Pab establishes the importance of this cytokine in the expression of memory immunity. Of interest was the finding that while the anti-IFN-γ Pab treatment caused an increase in listerial CFU in all organs examined, however, the extent of the increase was not as great as the increase in CFU in the organs of anti-IFN-γ Mab-treated mice during a primary infection (Figure 1). These findings indicate that both IFN-γ-dependent and IFN-γ-independent resistance mechanisms serve to resolve the secondary infection. This conclusion is similar to that reached by *Samsom* et al. (1995) who concluded that IFN-γ played only a minor role in the expression of anti-*Listeria* immunity against a secondary infection initiated by intravenous inoculation of bacteria. This conclusion was based on results showing that in anti-IFN-γ Mab-treated memory immune mice the liver bacterial CFU were only ~1 $\log_{10}$ higher than in control memory immune mice.

The apparent contradiction between the capacities of the anti-IFN-γ Mab and anti-IFN-γ Pab to interfere with the expression anti-*Listeria* memory immunity may be explained by different specificities of the two antibody preparations for distinct molecular domains on the IFN-γ molecule. Indeed, anti-IFN-γ Mab preparations have been shown to differ in abilities to neutralise certain IFN-γ activities, which indicates the presence of different molecular domains involved in signal transduction, which could account for the multiple activities of IFN-γ (*Schreiber* et al., 1985; *Caruso* et al., 1993).

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