The requirement of tumour necrosis factor- α and interferon- γ for the expression of protective immunity to secondary murine tularaemia depends on the size of the challenge inoculum

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The present study was conducted to determine the extent to which the cytokines tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) are required to protect against primary or secondary murine tularaemia caused by the live vaccine strain of the facultative intracellular bacterium *Francisella tularensis*. It is shown that non-immune mice treated with neutralizing monoclonal antibodies (mAbs) against TNF- α and IFN- γ are rendered defenceless against otherwise sublethal intravenous inocula of the bacterium. Treatment with either of the anti-cytokine mAbs resulted in even a very small inoculum of 500 c.f.u. of the pathogen multiplying unrestrictedly in the livers, spleens and lungs of non-immune mice to rapidly reach lethal numbers. By contrast, *Francisella*-immune mice treated with either of the mAbs remained capable of resolving secondary infection with 50-fold larger inocula. However, the need for TNF- α and IFN- γ for controlling secondary tularaemia became critical when challenge inocula exceeded 10^6 c.f.u. Overall, the results imply that different defence mechanisms operate to control primary versus secondary murine tularaemia. Additionally, they show that the need for TNF- α and IFN- γ to combat secondary infection depends on the size of the challenge inoculum.

Keywords: *Francisella tularensis*, tumour necrosis factor- α , interferon- γ

INTRODUCTION

The Gram-negative facultative intracellular bacterium *Francisella tularensis* causes tularaemia, a potentially severe and fatal infection of humans and other mammals (reviewed by Tärnvik, 1989). In humans, resolution of naturally acquired primary tularaemia or vaccination with an attenuated live vaccine strain of *F. tularensis* (strain LVS) can confer long-lasting specific protective immunity to reinfection (Burke, 1977). Because *F. tularensis* can invade and multiply inside host cells, including macrophages (Anthony *et al.*, 1991) and hepatocytes (Conlan & North, 1992), specific immunity to it is considered to be primarily T-cell-mediated rather than humoral. This implies that to resolve tularaemia, *Francisella*-specific T cells generated during infection must secrete cytokines

that activate macrophages to kill the francisellae they ingest. Experimentally, this is supported by findings (Fortier *et al.*, 1992) showing that tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) can activate murine macrophages to kill phagocytosed *F. tularensis* strain LVS cells that remain highly virulent for mice despite being attenuated for humans (Eigelsbach & Downs, 1961). Furthermore, these cytokines were shown to be critical for controlling primary infection of mice by *F. tularensis* strain LVS (Leiby *et al.*, 1992).

However, more recent findings using mice infected with *F. tularensis* strain LVS suggest that host resistance to tularaemia is a highly complex process requiring more than the participation of T cells. Studies with this model clearly show that innate defences as well as specific T-cell-mediated and humoral immunity can participate in anti-*Francisella* resistance. For instance, a recent publication from this laboratory (Sjöstedt *et al.*, 1994) shows that host defence against primary *F. tularensis* strain LVS infection

Abbreviations: IFN- γ , interferon- γ ; i.v., intravenous; TNF- α , tumour necrosis factor- α .

is critically dependent on the actions of neutrophils, a class of phagocytes previously considered irrelevant for defence against intracellular bacterial pathogens. In contrast, nude mice (Elkins *et al.*, 1993) and mice depleted of CD4⁺ or CD8⁺ or both T-cell subsets (Conlan *et al.*, 1994) remain remarkably capable of controlling the early progressive phase of primary *Francisella* infection. However, unlike immunocompetent mice, T-cell-deficient mice fail to resolve primary infection, but instead develop a chronic infection that can become overwhelming with the passage of time. This indicates that the crucial early anti-*Francisella* defences mediated by TNF- α and IFN- γ are distinct from those mediated by classical $\alpha\beta$ ⁺ T cells.

In contrast to host resistance to primary infection, immunity of mice to secondary tularaemia appears to require the operation of different host defences. Thus, although depleting immune mice of neutrophils results in exacerbated secondary infection, these mice can go on to resolve infection initiated by inocula of *F. tularensis* strain LVS that are lethal for non-immune neutropenic mice (Sjöstedt *et al.*, 1994). Similarly, immune mice treated with anti-IFN- γ or anti-TNF- α monoclonal antibodies (mAbs) do not develop lethal infections following challenge with doses of *F. tularensis* strain LVS that kill immunocompetent non-immune mice (Leiby *et al.*, 1992; Rhinehart-Jones *et al.*, 1994). The latter results, particularly, are surprising, given that in adoptive immunity studies, the expression of transferred specific anti-*Francisella* T-cell-mediated and humoral immunity reportedly requires the participation of TNF- α or IFN- γ or both cytokines (Leiby *et al.*, 1992; Rhinehart-Jones *et al.*, 1994).

In view of these apparently conflicting findings, the present study was undertaken to examine in greater detail the requirement of TNF- α and IFN- γ for the expression of protective immunity to secondary tularaemia. To this end, the effect of treating immune mice with neutralizing mAbs specific for TNF- α or IFN- γ on the subsequent course of secondary tularaemia with different doses of *F. tularensis* strain LVS was monitored. The results show that the need for TNF- α or IFN- γ for combating secondary infection varies depending on the size of the challenge inoculum.

METHODS

Mice. Female B6D2F1 (C57BL/6 $\times$ DBA/2) mice were used in all experiments. Mice were obtained from the Trudeau Institute Animal Breeding Facility, Saranac Lake, NY, USA, and were free of common viral pathogens, according to the results of routine screening by the Research Animal Diagnostic Laboratory, University of Missouri, Columbia, USA. Five mice per group per time-point were used throughout. Mice were between 7 and 9 weeks of age at the start of each experiment.

Bacteria and infection of mice. *F. tularensis* strain LVS (ATCC 29684) was grown in modified Mueller-Hinton broth to exponential phase (2.5×10^8 c.f.u. ml⁻¹), aliquotted, and frozen at -70 °C in the presence of 10% (w/v) sucrose. *Listeria monocytogenes* (strain EGD) was grown to exponential phase in trypticase soy broth at 37 °C with shaking, dispensed into 1 ml

volumes (approximately 2×10^8 c.f.u. ml⁻¹), and frozen at -70 °C. Primary *Francisella* infection was initiated by intravenous (i.v.) inoculation of approximately 5×10^2 or 5×10^3 c.f.u. *F. tularensis* strain LVS. The LD₅₀ of *F. tularensis* strain LVS for naïve B6D2 mice is approximately 5.2 log₁₀ c.f.u. (Sjöstedt *et al.*, 1994). Immune mice were used in reinfection experiments 5 weeks after receiving an i.v. inoculum of 5×10^3 c.f.u. strain LVS. The i.v. LD₅₀ for immune mice is approximately 7.5 log₁₀ c.f.u. strain LVS (Sjöstedt *et al.*, 1994). *F. tularensis* cells in the livers, spleens and lungs of infected naïve and immune mice were enumerated by plating serial 10-fold dilutions of organ homogenates on cystine heart agar supplemented with 1% (v/v) haemoglobin (Conlan & North, 1992). Livers and spleens were homogenized to a final volume of 10 ml, and the lungs to a final volume of 2 ml. *Francisella* colonies were counted following incubation at 37 °C for 48–72 h. Bacterial burdens are reported as c.f.u. per organ in all experiments. To determine whether non-specific antibacterial defences were enhanced during primary tularaemia, mice were challenged intravenously with 5×10^3 c.f.u. *L. monocytogenes* on various days following initiation of *Francisella* infection. *L. monocytogenes* cells in the livers and spleens were enumerated 2 d later as previously described by Conlan & North (1992). Non-specific resistance mechanisms were considered to be activated if fewer listeriae were recovered from the organs of *Francisella*-infected mice than from the organs of naïve mice by day 2 following *Listeria* challenge.

mAb treatments. Anti-TNF- α mAb MP6-XT3.11, produced by a hybridoma that was a kind gift from Dr J. Abrams (DNAX, Palo Alto, CA, USA), anti-IFN- γ mAb R46A2 (Spitalny & Havell, 1984) and control isotype-matched (IgG1) mAb 91b directed against horseradish peroxidase (Dean *et al.*, 1984) were purified from ascitic fluid as described elsewhere by Sjöstedt *et al.* (1994). Anti-TNF- α mAb and anti-IFN- γ mAb had neutralizing activities of 10^4 and 1.4×10^5 neutralizing units mg⁻¹, respectively, as measured by bioassays previously described by others (see Havell, 1989). mAbs were administered intraperitoneally in a dose of 0.5 mg to non-immune mice 12 h before infection. They were given by the same route in a dose of 1.0 mg to immune mice 12 h before reinfection.

RESULTS AND DISCUSSION

Treating mice with anti-TNF- α or anti-IFN- γ mAbs exacerbates primary infection by *F. tularensis* strain LVS

According to Leiby *et al.* (1992), TNF- α and IFN- γ are critical components of host defence against primary murine tularaemia caused by intradermal administration of *F. tularensis* strain LVS. The results of the present study indicate that this is also the case for infection initiated by i.v. inoculation of strain LVS. Fig. 1 shows the course of primary infection in the livers, spleens and lungs of mice treated with anti-TNF- α mAb, anti-IFN- γ mAb or control mAb, and inoculated intravenously with 500 c.f.u. *F. tularensis* strain LVS. This dose is at least 300-fold smaller than the i.v. LD₅₀ for control mice, yet still allows early detection of *F. tularensis* cells in target organs. By day 1 of infection, all three groups of mice were similar with respect to the numbers of bacteria isolated from infected organs. Mice treated with irrelevant control mAb began to control hepatic, splenic and pulmonary infection between days 2 and 5 of infection and resolved infection between days 5 and 10. By contrast, mice treated with

Fig. 1. Growth curves of *F. tularensis* strain LVS in non-immune control mice and mice treated with anti-cytokine mAbs. Mice were treated with 0.5 mg of a control mAb (▽), anti-TNF- α mAb (●) or anti-IFN- γ mAb (○) 12 h prior to initiation of infection by i.v. inoculation of 500 c.f.u. *F. tularensis* strain LVS. Bacterial growth was monitored in the livers, spleens and lungs for up to 8 d. Means of five mice per group ($\pm$ SEM) are shown. The arrow indicates the size of the inoculum, asterisks indicate the deaths of individual mice, and the broken line indicates the detection limit.

either of the anti-cytokine mAbs were unable to restrict *F. tularensis* from multiplying to lethal numbers.

Transient expression of enhanced non-specific antibacterial resistance during primary tularaemia

During bacterial infection, host defences are often activated to kill challenge inocula of heterologous bacteria more efficiently (Mackanness, 1964). Enhanced non-specific resistance usually rapidly wanes after infection with the primary organism has resolved. Experiments were performed to ensure that this would be the case for studies of specific acquired immunity to tularaemia conducted here. To this end, mice were monitored on various days of primary *Francisella* infection for enhanced non-specific resistance to an i.v. challenge with 5×10^3 c.f.u. *L. monocytogenes* strain EGD. The decreased recovery of *L. monocytogenes* from the livers and spleens of *Francisella*-infected versus control mice following *Listeria* challenge was used as an indicator of acquired non-specific resistance in the former mice. The results of this study, presented in Table 1, show that *Francisella*-infected mice exhibited enhanced non-specific resistance to *L. monocytogenes* during the first 12 d of primary tularaemia, but not thereafter.

Effect of anti-TNF- α and anti-IFN- γ mAbs on the course of secondary tularaemia varies depending on the size of the reinfection inoculum

Others have shown (Leiby *et al.*, 1992) that *Francisella*-immune mice treated with anti-TNF- α or anti-IFN- γ mAbs retain the capacity to resist reinfection with a dose

Table 1. Expression of enhanced non-specific resistance to *L. monocytogenes* by *Francisella*-infected mice

Mice received an i.v. inoculum of 5×10^8 c.f.u. *F. tularensis* strain LVS on day 0. Thereafter, at indicated time-points, mice were challenged with an i.v. inoculum of 5×10^3 c.f.u. *L. monocytogenes* strain EGD. *Listeria* burdens in the livers and spleens of *Francisella*-infected mice were determined 2 d post-challenge with *L. monocytogenes*. The means $\pm$ SD of five mice per group are shown; BDL, below detection limit of 100 c.f.u. per organ.

Time of <i>Listeria</i> challenge in <i>Francisella</i> -infected mice (d)	log ₁₀ c.f.u. <i>L. monocytogenes</i>	
	Spleen	Liver
Non-infected	5.41 $\pm$ 0.09	4.50 $\pm$ 0.05
1	5.22 $\pm$ 0.05	4.34 $\pm$ 0.10
4	BDL	BDL
6	BDL	2.70 $\pm$ 0.35
12	5.26 $\pm$ 0.06	2.75 $\pm$ 0.42
20	5.26 $\pm$ 0.13	4.35 $\pm$ 0.34
28	5.56 $\pm$ 0.06	4.56 $\pm$ 0.10

of *F. tularensis* that is lethal for naïve mice. However, the aforementioned study measured resistance in terms of ability of mice to survive the challenge doses of *Francisella* employed. It did not monitor the growth of *Francisella* in the organs of immune mice treated with anti-cytokine mAbs. Therefore, it is not known whether infection in

Fig. 2. Effect of anti-cytokine mAb treatment on the course of secondary tularemia. Immune mice that had resolved a sublethal primary infection 5 weeks earlier were treated with 1.0 mg of a control mAb (▽), anti-TNF- α mAb (●) or anti-IFN- γ mAb (○) 12 h before initiation of reinfection by i.v. inoculation of 9×10^3 c.f.u. (experiment 1), 2×10^6 c.f.u. (experiment 2) or 6×10^6 c.f.u. (experiment 3) of *F. tularensis* strain LVS. The means of five mice per group ($\pm$ SEM) are shown, except in experiment 3, where organs of only two mice from the anti-TNF- α -treated group were plated on day 3. Arrows indicate inoculum sizes, asterisks denote the time of death of individual mice, and the broken line indicates the detection limit.

anti-cytokine-treated immune mice was non-lethally exacerbated. In this regard, recent studies from this laboratory (Sjöstedt *et al.*, 1994) showed that, whereas immune mice depleted of neutrophils or T cells survived reinfection with certain i.v. inocula of *F. tularensis* strain LVS that were lethal for similarly depleted non-immune mice, they nevertheless succumbed to reinfection with

very high inocula of strain LVS that immunocompetent immune mice cleared.

In view of these findings, experiments were performed to monitor the course of secondary tularemia in immune mice treated with anti-cytokine mAbs or control mAb 12 h before reinfection with graded doses of *F. tularensis*

strain LVS. These experiments used mice infected 5 weeks earlier with 5×10^3 c.f.u. strain LVS. According to the results presented in Table 1, enhanced non-specific antibacterial resistance in these mice would have completely waned. Therefore, any enhanced resistance to *F. tularensis* reinfection displayed by these mice would probably represent specific immunity.

In the first of a series of experiments, immune mice treated with one or the other anti-cytokine mAb or control mAb were reinfected with an i.v. inoculum of 9×10^3 c.f.u. *F. tularensis* strain LVS. This inoculum is uniformly lethal for non-immune mice treated with anti-cytokine mAbs (Fig. 1). In contrast, experiment 1 (Fig. 2) shows that immune mice treated with anti-TNF- α or anti-IFN- γ mAbs were able to control and resolve secondary infection initiated with this dose of strain LVS. Thus, different defences appear to operate to control progressive primary versus secondary murine tularaemia. However, experiment 2 (Fig. 2) shows that immune mice treated with anti-TNF- α mAb, but not anti-IFN- γ mAb, died from secondary infection initiated by an i.v. inoculum of 2×10^6 c.f.u. *F. tularensis*. Finally, experiment 3 (Fig. 2) shows that unlike control immune mice, immune mice deprived of either TNF- α or IFN- γ rapidly succumbed when the reinfection inoculum was increased to 6×10^6 c.f.u.

In the experiments shown in Fig. 2, substantial amounts of neutralizing anti-TNF- α and anti-IFN- γ mAbs were found in the blood of surviving mice on days 4 and 8 of infection. For instance, in mice from experiment 2, the amounts of neutralizing anti-TNF- α antibodies ranged from 410 to 820 neutralizing units ml⁻¹ in blood drawn at day 4. In the group that had received anti-IFN- γ mAbs, the levels of antibodies varied between 6550 and 26200 neutralizing units ml⁻¹ in blood drawn at day 4 and between 3270 and 6550 units at day 8. Similar levels of neutralizing anti-TNF- α and anti-IFN- γ mAbs were detected in blood from mice included in experiment 1. Therefore, it is unlikely that the failure of immune mice treated with anti-cytokine mAbs to resist reinfection with large inocula of *F. tularensis* strain LVS is due to exhaustion of these antibodies. Instead, the results suggest that host defences can operate at several levels to resist secondary tularaemia. Firstly, immune mice can clear a relatively small challenge of *F. tularensis* strain LVS by a TNF- α - and IFN- γ -independent mechanism. However, as the challenge inoculum exceeds this threshold, the need for TNF- α and IFN- γ becomes critical.

Previous studies from this laboratory (Conlan *et al.*, 1994) and elsewhere (Fortier *et al.*, 1991; Rhinehart-Jones *et al.*, 1994) documented the existence of specific protective humoral and T-cell-mediated acquired immunity to *F. tularensis* strain LVS. Additionally, antibody- and T-cell-independent specific protective immunity to *F. tularensis* strain LVS has been described (Conlan *et al.*, 1994; Elkins *et al.*, 1992, 1993). It is possible that the varying need for TNF- α and IFN- γ in defence against secondary tularaemia is determined by which of the aforementioned defences are elicited by the particular challenge dose of *Francisella*.

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