Transfer of specific antibodies results in increased expression of TNF-α and IL12 and recruitment of neutrophils to the site of a cutaneous Francisella tularensis infection

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This study demonstrates that passive transfer of Francisella tularensis-specific antibodies before experimental cutaneous infection with the live vaccine strain of F. tularensis has profound effects. Recipient mice showed stronger staining for TNF-α and IL12, and larger numbers of neutrophils in skin samples after infection than control mice.

INTRODUCTION

The skin comprises an important first line of defence against infection. Besides providing an important physical barrier against invading pathogens, it has become obvious that it also constitutes a sophisticated immunological defence system. Infection with Leishmania donovani results in the generation of an effective local and systemic host response after cutaneous infection, whereas intravenous inoculation leads to a progressive visceral infection (Melby et al., 1998). This example, and examples from other infection models (Chong-Cerrillo et al., 2001; Nizet et al., 2001; Ramaswamy et al., 1997), indicate that specific memory immunity is generated in the skin and, upon reinfection, local rapid recruitment of inflammatory cells and prominent expression of Th1 cytokines result. Our previous studies have shown that the cutaneous mouse model of tularaemia, a zoonotic infection caused by the facultative intracellular bacterium Francisella tularensis, is an interesting model for studies of cutaneous host-protective mechanisms (Stenmark et al., 1999, 2003).

We have observed that immune mice, in contrast to naïve mice, respond to cutaneous infection with rapid local expression of IL12 and TNF-α, cytokines known to be crucial for host protection against tularaemia (Stenmark et al., 1999). Concomitantly, although bacteria were not eradicated immediately in the skin, there was an almost absolute containment and no bacteria were isolated in the spleen and liver. More recently, we have demonstrated that a similar containment of F. tularensis occurs in the skin of mice after they have been administered immune serum (Stenmark et al., 2003). To this end, we wanted to characterize more specifically how these antibody-mediated effects may be mediated.

METHODS

We have previously demonstrated that immune serum affords protection in a dose-dependent manner (Stenmark et al., 2003). Transfer to naïve mice 24 h before challenge with F. tularensis live vaccine strain (LVS) afforded significant protection, since there were significantly fewer bacteria in liver and spleen (Stenmark et al., 2003). Following the protocol of the previous study, 0.5 ml immune or control serum was administered intraperitoneally to C57BL/6 mice. Immune serum had been obtained by collecting sera from mice 4 weeks after they had been given a sublethal intradermal inoculum of F. tularensis LVS. Sera were found to be sterile before use. All sera were screened by ELISA for the presence of various cytokines. The levels of IL1β, IL4, IL6, IL10, TNF-α and IFN-γ were below the detection limit of 30 pg ml−1 in sera from immune and naïve mice. Levels of IFN-α and IFN-β in both types of sera were also below the immunoassay detection limit of 12 IU ml−1 (Eloranta et al., 1996). Levels of IL12p40 were approximately 310 pg ml−1 in non-immune serum and 640 pg ml−1 in immune serum. Levels of F. tularensis lipopolysaccharide (LPS) were determined by an ELISA based on mAbs. In both naïve and immune sera, levels were below the detection limit of the assay, the equivalent of 103 bacteria ml−1 (Grunow et al., 2000).

Mice were 8–14 weeks of age at the start of an experiment. Control mice were age- and sex-matched. The mice were bred and housed at the Animal Facility, Umeå University, Umeå, Sweden and found to be specific-pathogen free upon repeated testing. The research conducted in this study was approved by the Animal Care Committee, Umeå.

An area of approximately 3 cm² of skin on the upper thorax was shaved 1 day before inoculation. Mice were challenged intradermally with 5 × 103 c.f.u. of F. tularensis LVS 24 h after administration of serum and killed by cervical dislocation. The dose was approximately 0–1 LD50. Immunohistochemical staining for expression of TNF-α, IL12 and IFN-γ in infectious foci was performed as previously described (Stenmark et al., 1999). One to three days after bacterial challenge, 1 cm² of the skin at the site of inoculation was excised and snap-frozen in liquid propane. Tissues were placed in OCT compound (Tissue Tek) and samples stored at −70 °C until sectioned. Rat anti-mouse IL12 (10 μg ml−1), TNF-α (15 μg ml−1), IFN-γ (5 μg ml−1) and anti-granulocyte Gr-1 (0.5 μg ml−1) antibodies were all from Pharmingen. Secondary antibody was biotinylated rabbit anti-rat IgG (adsorbed against mouse antiserum) from Vector Lab. Primary antibodies were visualized using a peroxidase-labelled antibody. Using a Sony camera and a Leica DMLB 100T microscope, five to nine visual fields from two separate sections

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per skin biopsy were analysed. The areas of peroxidase-stained cells in the epidermis and dermis were calculated as a percentage of the total area in each visual field by use of the analysis program LEICA QWin. For statistical evaluation, Student’s one-tailed t test in SPSS (version 11.0) was used; a P value of < 0.05 was considered significant.

RESULTS AND DISCUSSION

Preliminary studies indicated that staining for TNF-α in sections of infected skin from mice given immune serum increased up to 3 days after infection. At this time point, no increased staining was observed in samples from the infected mice versus the non-infected mice (P > 0.30), whereas mice given immune serum before infection showed a strong increase in staining (P < 0.01 versus the infected control group; Fig. 1a). A representative illustration of the staining for TNF-α is shown in Fig. 2. No changes in staining for IFN-γ were observed, whereas IL12 staining was significantly increased on day 3 (P < 0.01; Fig. 1a). Thus, the increase in both IL12 and TNF-α observed in immune mice (Stenmark et al., 1999) may, at least in part, be dependent on the presence of specific antibodies.

Histological examination of the infected skin sections indicated that an increase in the number of neutrophils occurred. To this end, we quantified infiltrating neutrophils using the granulocyte-specific antibody Gr-1. The histological examination indicated that virtually all granulocytes were neutrophils (data not shown). Infection per se triggered recruitment and a threefold increase was observed in infected mice versus control mice (Fig. 1b). A further increase was observed in infected mice given immune serum, since a fivefold increase was observed in this group (P < 0.05 versus the infected control group). The localization of the infiltrating neutrophils was largely in the dermis and subcutis and the pattern of staining resembled that seen with the TNF-α-specific antibody (Fig. 3).

F. tularensis causes an ulceroglandular form of tularemia after its introduction in the skin by arthropod bites. Although this is the most common form of the disease, previous studies have focused on characterization of systemic host-protective mechanisms. In a previous study, we demonstrated that the cutaneous immune response plays a crucial role in controlling the infection, since local bacterial numbers in the skin decrease within 24 h and no spread to liver and spleen occurs (Stenmark et al., 1999). Concomitantly, TNF-α and IL12 are expressed locally. Transfer of specific antibodies confers recipient mice with an ability to contain a cutaneous F. tularensis infection effectively (Stenmark et al., 2003). We now demonstrate that one mechanism whereby pathogen-specific antibodies contribute to this control of infection may be via the triggering of cutaneous TNF-α and IL12 expression. Thus, our data confirm previous findings that specific antibodies play an important role in the acquired immune response to this prototypic intracellular pathogen (Conlan et al., 2002; Drabick et al., 1994; Fulop et al., 2001; Stenmark et al., 2003).

Work on other models of intracellular infections, e.g. murine listeriosis, the prototypic model of intracellular bacterial infection, has revealed a critical requirement for TNF-α, IL12 and IFN-γ in innate and acquired host resistance (Unanue, 1997). A similar requirement exists in the experimental model of murine tularemia, since neutralization of IFN-γ or TNF-α leads to lethal exacerbation of infection (Anthony et al., 1989; Elkins et al., 1996; Leiby et al., 1992; Sjöstedt et al., 1996). Thus, the presence of TNF-α and IL12 during the early phase of infection will be most likely to benefit the host. The mechanisms for cytokine-mediated control of tularemia remain unknown, but a number of possibilities have been suggested in other studies. For example, previous studies on F. tularensis have suggested that TNF-α and IFN-γ may endow monocytes with an enhanced capability to kill the pathogen (Fortier et al., 1992; Polsinelli et al., 1994). TNF-α is also known to play a critical role in dermal immune mechanisms by upregulating MHC class II antigen on Langerhans’ cells, and to increase the trafficking of these cells from the epidermis to the regional lymph nodes (Jakob et al., 2001).

In a previous communication, we have shown that neutrophil depletion renders mice defenceless against an intra-
dermal infection with *F. tularensis* (Sjöstedt et al., 1994). It was suggested that the neutrophil-mediated effect was not due to direct killing of *F. tularensis*. The present finding implies that rapid triggering of cytokine expression may be one effect mediated by neutrophils infiltrating the cutaneous site of infection. Our results do not afford direct evidence to link TNF-α secretion to neutrophils, since other cells, such as keratinocytes or dendritic cells, may be a source of TNF-α. However, the appearance of TNF-α secretion and neutrophil recruitment occur with similar kinetics and this coordination may be important for protection.

For many decades, it was generally assumed that antibodies played only a minor, or no, role in protection against facultative and obligate intracellular bacteria and parasites. However, it has become obvious that this view has to be modified, since a number of studies have demonstrated that antibodies can mediate critically required functions for control of intracellular infections. Our findings provide direct evidence that the presence of pathogen-specific antibodies will benefit the host during infection with a prototypic intracellular pathogen. It should be noted that protective antibody-mediated mechanisms seem to exert minimal or no effect against infections caused by the highly virulent North American type A strains, *F. tularensis* subspecies *tularensis* (Conlan et al., 2002; Fulop et al., 2001). We have shown, however, that significant protection is conferred by transfer of immune serum against a clinical isolate from Europe belonging to *F. tularensis* subspecies *holarctica* (Stenmark et al., 2003). Since most infections in North America and all European infections are caused by the latter subspecies, and the LVS used in the present study also belonged to this

**Fig. 2.** Representative immunohistochemical staining for TNF-α after 3 days of infection. No staining was visualized after incubation with isotype-matched irrelevant antibodies. FT, Samples from mice infected with *F. tularensis* LVS; FT + IS, samples from mice given immune serum and infected 24 h later with *F. tularensis* LVS.

**Fig. 3.** Representative immunohistochemical staining for neutrophils with the granulocyte-specific antibody Gr-1, after 3 days of infection. No staining was visualized after incubation with isotype-matched irrelevant antibodies. FT, Samples from mice infected with *F. tularensis* LVS; FT + IS, samples from mice given immune serum and infected 24 h later with *F. tularensis* LVS.
subspecies, our findings advance our understanding of host immunity to \textit{F. tularensis} and may be a basis for strategies to develop future \textit{F. tularensis} vaccines.

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**REFERENCES**


Conlan, J. W., Shen, H., Webb, A. & Perry, M. B. (2002). Mice vaccinated with the \(O\)-antigen of \textit{Francisella tularensis} LVS lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or aerosol challenge with virulent type A and type B strains of the pathogen. Vaccine 20, 3465–3471.


Grunow, R., Splettstoesser, W., McDonald, S. & 7 other authors (2000). Detection of \textit{Francisella tularensis} in biological specimens using a capture enzyme-linked immunosorbent assay, an immunochromatographic handheld assay, and a PCR. Clin Diagn Lab Immunol 7, 86–90.


