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CUTANEOUS RESISTANCE AGAINST *FRANCISELLA TULARENSIS*

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To my father

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ABSTRACT

Francisella tularensis, the causative agent of tularemia, is a potent pathogen in humans and other mammals. The ulceroglandular form of the disease is the most common expression in humans with a clinical picture characterized by a skin ulcer, enlarged regional lymph nodes and fever. Despite being a preferred route of infection, the skin also affords an effective defense barrier against *F. tularensis*. Doses required to induce infection by intradermal inoculation are several logs higher than those needed for infection by other routes. In the present thesis, the requirements for the local and systemic host defense to intradermal infection with *F. tularensis* was studied in experimental mouse models. Naïve mice and mice immunized by previous infection were challenged, mostly with the live vaccine strain *F. tularensis* LVS but also with a clinical isolate of *F. tularensis*.

In naïve mice, intradermal inoculation of *F. tularensis* LVS resulted in a rapid increase of bacterial numbers during the first few days in the skin, lymph nodes, spleen and liver, followed by a decrease and eradication of the bacteria within two weeks of inoculation. Immune mice controlled the infection at the site of infection and very few bacteria spread to internal organs. When immunohistochemical staining of skin specimens was performed during the first 3 days, naïve mice showed a weak or barely discernible local expression of TNF-α, IL-12 and IFN-γ. In immune mice, the expression of all three cytokines was strongly enhanced, TNF-α and IL-12 within 24 h and IFN-γ within 72 h of inoculation.

To investigate the role of T cells in the defense against intradermal infection with *F. tularensis* LVS, naïve and immune T-cell knockout mice (e.g., αβ $TCR^{-/-}$, γδ $TCR^{-/-}$, αβγδ $TCR^{-/-}$) were used. Naïve mice lacking the αβ TCR had persistently high bacterial numbers in all organs and died at 4 weeks. Mice lacking the γδ TCR, on the other hand, controlled the infection as effectively as did wild-type mice. To enable αβ $TCR^{-/-}$ and αβγδ $TCR^{-/-}$ mice to survive, antibiotic treatment was given from day 10 to 20 of infection. When intradermally challenged 2 weeks later, these animals were found to control a secondary infection, resulting in decreasing viable counts in skin and lymph nodes and prevention of spread to liver and spleen. The results indicated the presence of a T-cell independent mechanism of resistance and analyses of serum showed high levels of *F. tularensis*-specific IgM, findings suggesting a role for antibodies in the protection against cutaneous tularemia.

To study the effect of *F. tularensis*-specific antibodies on host resistance, we adoptively transferred immune serum to B-cell-deficient mice. After receiving immune serum, both naïve and immunized mice became capable of surviving an otherwise lethal dose of *F. tularensis* LVS. Moreover, transfer of immune serum to wild type mice, afforded significant protection to a lethal dose of a wild-type strain of *F. tularensis* subsp. *holarctica*, as disclosed by reduced bacterial counts in spleen and liver. Finally, we studied the effect of immune serum on the local expression of proinflammatory cytokines and neutrophils in response to an intradermal injection of *F. tularensis* LVS. As compared to normal serum, transfer of immune serum resulted in increased expression of TNF-α, IL-12 and neutrophils. These findings afford a possible explanation for the effect of specific antibodies in the local host protection in the skin against tularemia.

Key words: tularemia, *Francisella tularensis,* skin, protection, cytokines, T-cells, B-cells, specific antibodies

ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Stenmark S, Sunnemark D, Bucht A, Sjöstedt A.
Rapid local expression of interleukin-12, tumor necrosis factor alpha, and gamma interferon after cutaneous *Francisella tularensis* infection in tularemia-immune mice. Infect Immun. 1999; 67: 1789-97.

II. Stenmark S, Lindgren H, Tärnvik A, Sjöstedt A.
T-cell-dependent and independent protection against *Francisella tularensis.* Manuscript

III. Stenmark S, Lindgren H, Tärnvik A, Sjöstedt A.
Specific antibodies contribute to the host protection against strains of *Francisella tularensis* subspecies *holarctica*. Microb Pathog. 2003; 35: 73-80.

IV. Stenmark S, Sjöstedt A.
Transfer of specific antibodies results in increased expression of TNF–α and IL-12 and recruitment of neutrophils to the site of a cutaneous *Francisella tularensis* infection. J Med Microbiol. 2004; 53: 501-4

SAMMANFATTNING

Bakterien *Francisella tularensis* är en potent patogen som orsakar sjukdomen tularemi (harpest) hos människor och djur. Den ulceroglandulära formen av sjukdomen är den vanligaste och har en klinisk bild som karaktäriseras av ett sår i huden, förstorade regionala lymfkörtlar och feber. Förutom att huden är den vanligast förekommande infektionsvägen så utgör den även en effektiv försvarsbarriär mot infektioner med *F. tularensis*. De doser som krävs för att orsaka infektion vid intradermal injektion är flera logaritmer högre än doserna som krävs via andra infektionsvägar. I den här avhandlingen har mekanismer för lokal och systemisk immunitet vid intradermal infektion med *F. tularensis* studerats i en musmodell. Naiva möss och möss som immuniserats genom tidigare infektion har injicerats med den levande vaccinstammen *F. tularensis* LVS, men även ett isolat från en human infektion med *F. tularensis* har använts. Intradermal injektion med *F. tularensis* i naiva möss leder under de första dagarna till en snabb ökning av bakterietalen i hud, lymfkörtlar, mjälten och levern, följt av sjunkande bakterietal med eradikering inom 2 veckor efter inokulationen. Immuniserade möss kontrollerar infektion i huden och mycket få bakterier sprids vidare till inre organ.

I immunohistokemiska färgningar, utförda på hudvävnad från de tre första dagarna efter infektionen, sågs i naiva möss ett svagt eller knappt synligt lokalt uttryck av TNF-α, IL-12 och IFN-γ. I immuniserade möss var däremot uttrycket av alla tre cytokinerna kraftigt förstärkt. TNF-α och IL-12 inom 24 h och IFN-γ inom 72 h efter inokulationen.

Naiva och immuniserade T-cell knockout möss (αβ $TCR^{-/-}$, γδ $TCR^{-/-}$, αβγδ $TCR^{-/-}$) användes för att undersöka T cellernas roll i försvaret mot intradermal infektion med *F. tularensis* LVS. Naiva möss med avsaknad av αβ TCR hade kontinuerligt höga bakterietal i alla undersökta organ och dog efter 4 veckor. Möss med avsaknad av γδ TCR, kontrollerade å andra sidan infektionen lika effektivt som möss av vildtyp. För att göra det möjligt för αβ $TCR^{-/-}$ och αβγδ $TCR^{-/-}$ möss att överleva en primär infektion gavs antibiotikabehandling mellan dag 10 och 20 under infektionen. Vid förnyad intradermal infektion 2 veckor senare visade sig dessa möss kunna kontrollera infektionen med sjunkande bakterietal i huden och lokala lymfkörtlar samtidigt som vidare spridning till lever och mjälte förhindrades. Dessa resultat indikerade en förekomst av en T cells-oberoende försvarsmekanism och serumanalyser visade höga nivåer av *F. tularensis*-specifikt IgM. Dessa fynd talar för att antikroppar kan spela en roll i försvaret mot tularemi i huden.

Immunserum överfördes till B cells-defekta möss för att kunna studera effekten på värdförsvaret av *F. tularensis*-specifika antikroppar. Efter tillförsel av immunserum erhöll både naiva och immuniserade möss förmågan att överleva en normalt sett letal dos med *F. tularensis* LVS. Immunserum överfört till möss av vildtyp ledde utöver detta till signifikant förstärkt försvar mot en letal dos med en vildtyp stam av *F. tularensis* subsp. *holarctica*, genom sänkta bakterietal i mjälte och lever.

Slutligen studerades effekten av immunserum på det lokala uttrycket av proinflammatoriska cytokiner och neutrofiler som svar på en intradermal injektion med *F. tularensis* LVS. I jämförelse med normalserum resulterade överfört immunserum i ett ökat uttryck av TNF-α, IL-12 och neutrofiler. Dessa fynd utgör en möjlig förklaring till specifika antikroppars effekt i det lokala värdsvaret i huden vid infektion med tularemi.

ABBREVATIONS

αβ	alfa beta
CFU	colony forming units
DETC	dendritic epidermal T cells
ELISA	enzyme-linked immunosorbent assay
FDA	federal drug agency
γδ	gamma delta
i.d.	intradermal
i.n.	intranasal
i.p.	intraperitoneal
i.v.	intravenous
LC	Langerhans cells
LD_{50}	lethal dose for 50%
LVS	live vaccine strain
MHC	major histocompability complex
NK cells	natural killer cells
MRNA	messanger RNA
PCR	polymerase chain reaction
RT-PCR	reversed transcriptase PCR
Subsp.	subspecies
SCID	severe combined immunideficiency
TCR	T cell receptor
Th1/ Th2	T helper cells 1/ T helper cells 2

INTRODUCTION

The intracellular pathogen *Francisella tularensis* is the causative agent of tularemia, a zoonotic disease spread to humans by direct contact with infected animals, bites from arthropod vectors, inhalation of contaminated dust, or intake of contaminated food or water. The most common clinical presentation of the disease is the ulcero-glandular form, characterized by a skin ulcer, enlarged regional lymph nodes, and fever. Although in a majority of human cases, the infection is acquired via the skin, there is little knowledge on mechanisms behind local immunity to *F. tularensis*. The present thesis is focused on local and systemic protection to cutaneous *F. tularensis* infection. For the purpose, a mouse model of tularemia was used.

BACKGROUND

Francisella tularensis and Tularemia

History

F. tularensis was first isolated by G.W. McCoy in 1906 when he was sampling ground squirrels and rats in Tulare county in California. He named it *Bacterium tularense* (McCoy, 1911; McCoy and Chapin, 1912). In human disease, the agent was first isolated from a patient with conjunctivitis and regional lymphadenopathy (Wherry and Lamb, 1914). In the beginning of the 1920's, Edward Francis was able to isolate *B. tularense* from blood samples of severely ill patients and named the disease tularemia (Francis, 1921; Francis et al., 1922). In the honor of his extensive research on diagnostic methods and pathological changes in animals and humans, the bacterium was later renamed *Francisella tularensis* (Rockwood, 1983).

The pathogen

F. tularensis is a small gram-negative, facultative intracellular bacterium (Tärnvik, 1989; Tärnvik and Berglund, 2003). It is strictly aerobic and grows relatively slowly on culture media enriched with L-cystein and glucose (Sjöstedt, 2000). The species *F. tularensis* includes two predominant subspecies, *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B) (Jellison, 1974). The former subspecies is found only in North America, where most strains have been isolated from ticks and rabbits. Isolates of *F. tularensis* subsp. *tularensis* are highly virulent with an LD_{50} in rabbits less than 10 bacteria. *F. tularensis* subsp. *holarctica* is spread throughout the Northern hemisphere and is the only subspecies isolated in Europe. It is less virulent than type A, with an LD_{50} for rabbits of more than one million bacteria. However, *F. tularensis* subsp. *holarctica* causes invasive disease, often associated with complications such as lymph node suppuration and a prolonged period of convalescence.

Vaccination and the live vaccin strain, *F. tularensis* LVS

Vaccine research on tularemia was initiated during the 1930's. Preparations based on killed *F. tularensis* were found to induce only a marginal protection toward infections with virulent strains (Saslaw et al., 1961) From a natural isolate in the former Soviet Union, an attenuated strain of *F. tularensis* subsp. *holarctica* was developed in the 1940's and a safe and effective live vaccine was introduced for mass vaccination in 1946 (Pollitzer, 1967; Tigertt, 1962). In 1956, in the middle of the cold war, a batch of viable vaccine bacteria was sent to the US. One of two bacterial colony variants of this batch was successively tested for safety and efficacy in humans and named *F. tularensis* Live Vaccine Strain, LVS.

Since 1960, LVS has been used at the US Army Research Institute of Infectious Diseases, Fort Detrick, Md for vaccination of at-risk personnel by skin scarification. The attenuation of the vaccine is still not defined and, therefore, it is not approved by FDA for public use. Vaccination protects against respiratory tularemia and mitigates the course of ulceroglandular disease. After the introduction of LVS at Fort Detrick, the incidence of respiratory tularemia fell from 5.7 to 0.27 cases per 1,000 at-risk employees (Burke, 1977).

Although *F. tularensis* LVS is avirulent in humans it is still virulent in mice and causes a disease with histopathological changes similar to those seen in human tularemia (Elkins et al., 2003; Tärnvik and Berglund, 2003) In mice, a sublethal inoculum of *F. tularensis* LVS develops a long-lasting immunity and allows survival after reinfection with up to 100 LD_{50} doses (Sjöstedt et al., 1994; Sjöstedt et al., 1996; Stenmark et al., 2003).

For several decades, *F. tularensis* LVS has been used extensively to characterize mechanisms of anti-*Francisella* resistance (Anthony et al., 1989; Anthony and Kongshavn, 1987; Conlan and North, 1992; Eigelsbach and Downs, 1961; Eigelsbach et al., 1975; Elkins et al., 2003; Elkins et al., 1993; Fortier et al., 1994; Fortier et al., 1991; Leiby et al., 1992)

Epidemiology

Tularemia occurs endemically in most countries of the Northern Hemisphere. In Sweden, the endemic area is found in the northern part of the country. *F. tularensis* has a complex ecology and a broader host distribution than any other zoonotic disease and the reservoir is still unknown. The disease is endemic in rodents and lagomorphs and for *F. tularensis* subsp. *holarctica,* there is an epidemiological association to natural water *i.e.*, streams, lakes, ponds and rivers (Hopla, 1974; Hopla and Hopla, 1994). In Sweden, transmission by mosquitoes, direct contact with infected animals and inhalation of bacteria-containing dust are the most common modes of acquisition of tularemia in humans (Eliasson et al., 2002; Olin, 1942).

Although the ulceroglandular form predominates, a few larger outbreaks of respiratory tularemia have been described among farmers (Tärnvik and Berglund, 2003). From 1931 to 2001, more than 6,000 cases of tularemia have been reported in Sweden.

Natural route of infection and clinical presentation

In man, tularemia presents in different ways, depending on the route of entry (Tärnvik, 1989; Tärnvik and Berglund, 2003). Ulceroglandular tularemia, the most common clinical form, appears after direct contact with an infected animal or after a bite from a blood-feeding arthropod. A pustule or a small dry skin ulcer is accompanied by an inflamed regional lymph node and high fever. The patient seeks medical attention because of fever and an enlarged and tender lymph node, whereas the ulcer is usually of no concern. Glandular tularemia shows the same clinical picture, although with no ulcer. In oropharyngeal tularemia, ulcerative exsudative stomatitis and pharyngitis develop after ingestion of food or water containing *F. tularensis.* Inhalation of infected dust leads to the respiratory form of the disease. Besides fever, the respiratory form presents with general symptoms as headache, myalgia, and arthralgia. Dry cough and other respiratory symptoms occur in about one-half of the cases and radiographical signs of pneumonia in <50% of the patients (Syrjälä et al., 1985). Pneumonia as a hematogenic spread secondary complication to other forms of tularemia was diagnosed in only one of 400 cases during a Swedish outbreak (Christenson, 1984).

The most severe cases of the disease occur in the United States, where the highly virulent subspecies *F. tularensis* subsp. *tularensis* is prevalent. Before the use of antibiotics, the fatality rate in the pneumonic form was up to 30% (Dienst, 1963; Stuart and Pullen, 1945). Several outbreaks of respiratory tularemia caused by *F. tularensis* subsp. *holartica* have been described also in the Nordic countries, although with no mortality.

Antibiotic treatment

Streptomycin early became established as the drug of choice for treatment of tularemia (Foshay, 1946). Similar to other aminoglycosides, the drug is bactericidal *in vitro* and highly efficacious against *F. tularensis*. Due to side effects, streptomycin has been replaced by other aminoglycosides and in tularemia, feasible alternatives are gentamicin or netilmicin (Enderlin et al., 1994). The main drawbacks are their toxicity and the necessity for parenteral administration. Tetracyclines, including doxycycline, are currently the alternative for oral therapy of tularemia, and has been the drug of choice in most cases of type B tularemia. To minimize the risk for relapse using this bacteriostatic drug, the treatment duration is extended to 3 weeks. During the last years, quinolones have offered new treatment options, due to their low minimal inhibitory concentration (MIC) values (Johansson et al., 2002). In contrast to tetracyclines, quinolones are bactericidal and can be used for treatment both in children and adults. In the few clinical reports so far published, ciprofloxacin has shown high efficacy in treatment of type B tularemia (Johansson et al., 2000; Johansson et al., 2001; Limaye and Hooper, 1999; Perez-Castrillon et al., 2001).

***F. tularensis* as an intracellular pathogen**

In primates and other mammals, *F. tularensis* induces tissue changes typical to those of intracellular bacterial diseases. After intracutaneous or respiratory exposure of monkeys to *F. tularensis*, the bacteria disseminate systemically to regional lymph nodes and the liver and spleen. Macrophages infiltrate diffusely and form granulomas (Hall et al., 1973; McGravan et al., 1962; Schricker et al., 1972).

F. tularensis is capable of intracellular replication in mononuclear phagocytes. Early studies suggested that the bacteria reside in a vacuolar compartment of macrophages (Anthony et al., 1991). The demonstration of an inhibitory effect on intracellular replication

by agents interfering with endosome acidification suggested that the bacteria need the acid environment of vacuoles for their acquisition of iron (Fortier et al., 1995). However, more recent data have questioned the capability of *F. tularensis* to multiply in a phagolysosomal compartment. By use of confocal microscopy and transmission electron microscopy, the bacterium has be shown to escape from the phagosome into the cytoplasm within a few hours of uptake by the mononuclear phagocyte (Clemens et al., 2004; Golovliov et al., 2003).

The adhesion to and uptake of intracellular bacteria in mononuclear phagocytes leads to the mobilization of a wide range of antimicrobial mechanisms inherent in the cells. In turn, the intracellular bacteria developed various mechanisms for evasion of the antimicrobial host response. These mechanisms vary widely from one obligate or facultative intracellular pathogen to another. Mechanisms for adaptation of *F. tularensis* to an intracellular lifestyle have been described. *F. tularensis* withstands relatively high levels of hydrogen peroxide, an antimicrobial reactant produced by macrophages in response to bacteria (Löfgren et al., 1984). Moreover, new evidence indicates that *F. tularensis* may interfere with transcription pathways of the macrophages, thereby abrogating their TNF-alpha and IL-1 response to microbial cell wall components (Telepnev et al., 2003). This abrogation of the cytokine response may at least partly explain the extreme potency of virulence of *F. tularensis*. The bacterium also induces apoptosis in the macrophages (Lai et al., 2001; Lai and Sjöstedt, 2003), an event that may facilitate bacterial survival and spread to new target cells.

An early T-cell independent host resistance to *F. tularensis*

Irrespective of these potentially strong virulence mechanisms inherent in *F. tularensis*, the infected mammalian host may control the infection. At an early stage, T cell-independent host resistance is induced and later, T cell-dependent mechanisms develop and may mediate both complete eradication and a long-term memory cell-dependent protection to reinfection.

Experience from work on murine models of tularemia show that already during the first few days of infection, a T-cell independent host resistance to *F. tularensis* is induced. When congenitally athymic *nu*/*nu* mice were immunized by intradermal inoculation of a sublethal dose of LVS, they acquired resistance to a lethal intraperitoneal dose of the bacteria (Elkins et al., 1993; Elkins et al., 1992). Remarkably, the time for development of this resistance was only 2 days. By administration of neutralizing specific antibodies against TNF-α or IFN-γ, both cytokines were shown to be indispensible for induction of the early host resistance (Anthony et al., 1989; Elkins et al., 1996; Leiby et al., 1992; Sjöstedt et al., 1996). Possible candidate cells responsible for the cytokine response are NK cells, dendritic cells and keratinocytes, cells that are present in the skin and may be rapidly activated upon intradermal immunization (Elkins et al., 1993). A role of antibodies seemed less likely, due to the short interval needed for development of resistance.

In experimental tularemia, neutrophils are the first phagocytic cell to be recruited to the site of infection. During the first few days of a primary infection, neutrophils play a critical role. Depletion of neutrophils or prevention of their recruitment causes a dramatic exacerbation of the infection (Conlan and North, 1992; Conlan and North, 1994). Irrespective of whether the bacteria were intradermally or intravenously inoculated, the LVS bacteria grew unrestrictedly in liver, spleen, and lung of the neutrophil-deficient animals, leading to death within 8 days. The neutrophil-dependent antimicrobial activity was believed to involve killing of defenseless infected hepatocytes rather than a direct phagocytic killing of the bacteria (Conlan and North, 1992). Neutrophils may be of vital importance also in human tularemia, as suggested by a case report of fatal infection caused by *F. tularensis* in a neutropenic patient (Sarria et al., 2003).

T-cell mediated immunity

Thus, experimental evidence suggests the presence of innate cytokine-dependent host mechanisms allowing an infected mammal to survive the initial phase of tularemia. The cytokines mediate host resistance by activating the macrophages, *i.e*., the target cells of infection. To eventually eradicate the infection and afford an enhanced host resistance against reinfection, an adaptive cell-mediated immune response is required. In particular, αβ T cells need to be activated. Along with antigenic stimulation, αβ T cells are differentiated into effector cells of the Th1 phenotype, *i.e*., cells producing IFN-γ. This differentiation is initiated with the aid of the first macrophages that becomes infected. Upon infection, the macrophages secrete TNF-α and IL-12 that in turn stimulate NK cells to produce IFN-γ (Golovliov et al., 1995; Tripp et al., 1993). These cytokines in concert seem to form the prerequisites for directing a T-cell response to bacterial peptides into the Th1 phenotype. Similar to premises for the early host resistance, an important function of αβ T cells of the Th1 type is their capability of activating macrophages by cytokine production, and by production of IFN-γ in particular.

A crucial role of T cells for control and eradication of *F. tularensis* has been demonstrated in several experimental systems (Conlan et al., 1994; Elkins et al., 1993; Yee et al., 1996). Congenitally athymic nude mice, SCID mice, or T-cell-depleted mice do not die within the first weeks of infection with *F. tularensis* (Elkins et al., 1996) but instead develop a protracted infection to which they eventually succumb. Mice with αβ TCR-gene disruption died after five weeks of infection. The importance of αβ T cells is not exclusively attributed to either CD4 or CD8 T cells, in as much as knockout mice lacking only one of these subsets of αβ T cells remained capable of eradicating an *F. tularensis* infection.

In humans recovering from tularemia, a long-lasting αβ T-cell mediated immunity is developed, as evidenced by the presence of a T-cell memory response to *F. tularensis* up to 25 year after the infection (Ericsson et al., 2001; Ericsson et al., 1994).

Humoral immunity

In the 1930's, a series of attempts of treating human tularemia by injection of specific antiserum was performed (Foshay, 1934). A significant increase in the survival time of mice injected with immune serum over that of control mice had been demonstrated. However, attempts to treat humans with antitularemic serum from horses, sheep, rabbits, or from a human tularemia convalescent were unsuccessful. Moreover, killed tularemia vaccines induced antibodies in humans but no evident protection to *F. tularensis* (Burke, 1977). Taken together, the data strongly indicated an absence of valuable protection by antibodies alone. However, none of the studies answered the question of whether, together with cell-mediated immunity, antibodies might contribute to host resistance to virulent strains of *F. tularensis*.

In the 1960's, extensive work on murine tularemia showed a significant protective effect of antibodies against attenuated strains but not against fully virulent strains of *F. tularensis* subsp. *tularensis* (Allen, 1962; Pannell and Cordle, 1962; Thorpe and Marcus, 1965).

Skin-associated immunity

Mechanisms of host protection in the skin are part of a first line of defense. Besides providing an important physical barrier against invading pathogens, the skin also constitutes a sophisticated immunological defense system. In 1978, Strelein introduced the concept of skin-associated lymphoid tissue or "SALT" (Streilein, 1989) and in 1986, Bos summarized the

complexity of humoral and cellular immunity in the normal skin under the term “SIS”, skin immune system (Bos and Kapsenberg, 1993)

The skin consists of two distinct layers, a thin outer layer, the epidermis, and a thicker layer, the dermis. The epidermis contains several layers of tightly packed epithelial cells joined by tight junctions, including and the outer epidermal layer of dead cells filled with keratin, a waterproofing protein. The normal bacterial flora of the epidermis defends against invading pathogens by secreting antibacterial substances and by competing for nutrients. Besides keratinocytes, Langerhans cells (LC) and T cells are important for the control of infections in the epidermis.

The dermis is composed of connective tissue, blood vessels, hair follicles, sebaceous glands, and sweet glands. The sebaceous glands produce an oily secretion consisting of fatty acids and lactic acid. A low pH of the skin, contributes to inhibit bacterial growth. Besides affording a physical barrier against pathogenic bacteria (Bjerke, 2002), the skin can be considered as a highly specialized immune organ. Once bacteria penetrate through the outer layer of the epidermis, various types of cells of the normal skin are called into action. Among these are the cells in the epidermis, T cells, macrophages, dendritic cells, neutrophils and NK cells.

The keratinocytes in the epidermis have several important immunological functions. In the presence of IFN-γ, they are induced to express class II MCH and thereby acquire a capability of presenting antigenic peptides to $CD4^+$ T cells (Nickoloff et al., 1995). Keratinocytes also produce a wide spectrum of cytokines (IL-1, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, TNF-α, TGF-β) (Bjerke, 2002; Schmitt, 1999; Takashima and Bergstresser, 1996) and thereby contribute to the local immune response. A number of antimicrobial peptides, so called defensins, are produced by keratinocytes, neutrophils and lymphocytes in the skin (Oppenheim et al., 2003). Some are produced constitutively and some are induced by

microbial products and proinflammatory cytokines. The defensins are a part of the innate immunity but also amplify the adaptive immune defense.

The LC represent about 2% of the epidermal cells (Bjerke, 2002) and are leukocytes of myeloid origin, belonging to the dendritic cell system (Peters et al., 1996; Romani et al., 2003). LC express HLA class II molecules and receptors for C3b and Fc on their surface (Bjerke, 2002). In the presence of IL-1 and TNF-α, the expression of class II MHC molecules is increased on LCs (Salmon et al., 1994).

LC are professional antigen-presenting cells and serve as initial gatekeepers by transporting antigens to regional lymph nodes. Proinflammatory cytokines, like IL-1β and TNF-α, induce maturation of LC and transportation of the cells to lymphoid tissue, where they interact with lymphocytes. After activation in lymph nodes, lymphocytes are exported to the skin to act as memory and effector cells. LC express surface markers that allow them to home to the skin and to localize in the epidermis. Locally produced TGF-β induces expression of E-cadherin on the LC surface. E-cadherin anchors the LC to keratinocytes, thereby keeping them in the epidermis (Jakob et al., 2001).

In the epidermis, LC contribute to local inflammatory responses by producing IL-1β (Salmon et al., 1994), IL-6, IL-8, TNF-α, MIP-1α (Takashima and Bergstresser, 1996) and may contribute to micobicidal activity by expressing iNOS and producing NO (Qureshi et al., 1996)

In the skin, macrophages are located perivascularly in papillary dermis. Like in other organs they are important for phagocytosis, antigen presentation and cytokine production (Bjerke, 2002)

Mice have a special subset of T cells in the epidermis, the dendritic epidermal T cells (DETC). These cells express the γδ TCR and are Thy-1^+, class II MHC^-. $CD3^+$, $CD45^+$, $CD5^-$, $CD4^-$ and most $CD8^-$ (Salmon et al., 1994). DETC are activated by IL-7, IL-8, IL-15 and

TNF-α produced by keratinocytes, LC and fibroblasts. IL-2 produced by DETC can in a autocrine way promote growth and cytotoxicity. Besides IL-2 DETC can produce IL-3, IL-4 IFN-γ and GM-CSF (Takashima and Bergstresser, 1996). There is no exact equivalent in humans but there are subsets of nondendritic γ/δ T cells and double negative ($CD4^-$, $CD8^-$) α/β T cells (Salmon et al., 1994)

Besides being a natural route of infection and the inoculation site for vaccination, the skin has been a common inoculation site for experimental tularemia. The LD_{50} values for *F. tularensis* infection in mice are many $\log_{10}$ higher by intradermal or subcutaneous inoculation, as compared to intravenous or intraperitoneal injection (Elkins et al., 1992). The underlying mechanisms for the efficient resistance to tularemia in the skin have not been systematically investigated. This lack of knowledge was the underlying motif for the studies of this thesis.

AIMS

- To study local and systemic immunity to a dermal infection with the intracellular pathogen *F. tularensis* in a murine model.

- To characterize the bacterial growth in the skin and internal organs and the expression of cytokines in naïve and immune mice

- To study the role of αβ- and γδ-T-cells in the development of immunity against a dermal infection with *F. tularensis.*

- To study the role for *F. tularensis* specific antibodies in the immunity to a dermal infection with *F. tularensis.*

- To identify factors of importance for the antibody-mediated local immunity in the skin.

METHODOLOGICAL ASPECTS

Arguments and rationale for using a mouse model in studies on the host response to *F. tularensis*

Experimental tularemia in the mouse is closely mimicking natural infection in man and is generally held to be an appropriate model for understanding the host-parasite interaction of human infection. Mice that survive and clear a sublethal infection with *F. tularensis* develop a long-lasting immunity and survive high challenge inocula that are otherwise lethal. Thus, studies of *F. tularensis* infection in the mouse may provide important information regarding the protective mechanisms against infections caused by facultative intracellular bacteria. Work on the murine model of tularemia has disclosed mechanisms of host resistance that are similar to those relevant for intracellular bacteria in general.

Mice

Wild type mice of the strains BALB/cJBom, C57BL/6 and C57BL/10 were used. Mice were 8-14 weeks of age at the start of an experiment and control mice were age- and sex matched.

T cell-knockout mice with defects in the TCR genes were used to study the role of T cells in primary and secondary infection. αβ TCR-gene deficient (αβ $TCR^{-/-}$; C57BL/6J-*Tcrb* ^{tm1Mom}), γδ TCR-gene deficient (γδ $TCR^{-/-}$; C57BL/6J-*Tcrd* ^{tm1Mom}), and αβ- and γδ-TCR-deficient (αβγδ-$TCR^{-/-}$, C57BL/6J-*Tcrb* ^{tm1Mom} *Tcrd* ^{tm1Mom}) (Itohara et al., 1993; Mombaerts et al., 1993; Mombaerts et al., 1992) were used.

The development of B-cell-deficient ($Ig\mu^{-/-}$) mice has offered new possibilities to investigate the role of antibodies. These animals have a disruption of one of the membrane exons of the μ-chain gene (Kitamura et al., 1991). They have no detectable B cells or

circulating antibodies but show normal development of the T-cell compartment (Epstein et al., 1995). Moreover, the B-cell-deficient mice have a normal antigen-presenting function for priming of T cells (Baird and Parker, 1996).

In vivo infection in mice

An area of approximately 3 cm^2 of the skin of the upper thorax was shaved one day before intradermal inoculation of 3 to 5 $\log_{10}$ *F. tularensis* LVS organisms. Mice were killed at various intervals by cervical dislocation and samples from skin (~1cm^2), axillar lymph nodes, liver, spleen and serum were taken under sterile conditions for further analyses. In experiments using transfer of serum, normal or immune serum was injected intraperitoneally to recipient mice 24 h before challenge with *F. tularensis.*

Immunohistochemistry

Skin biopsy specimens excised from the site of inoculation were prepared for immunocytochemical staining by snap freezing in liquid propane. Tissues were placed in OCT compound (Tissue Tek), and samples were stored at -70°C until sectioned. Immunohistochemistry for intracellular cytokine expression in infectious foci was performed as previously described (Sunnemark et al., 1996). Rat anti-mouse IL-12, TNF-α , IFN-γ, and anti-granulocyte GR-1 were used as primary antibodies. As a secondary antibody, biotinylated rabbit anti-rat IgG was used. The primary antibody was visualized by use of a peroxidase-labeled antibody. Microscopy was performed using a Leitz DRMBE or Leica DMLB 100T microscope. No staining was visualized after incubation with isotype-matched irrelevant antibodies. To determine the frequency of cytokine-expressing cells, the number of peroxidase-stained epidermal cells per tissue section was determined. In the experiments of

paper I, the slides were scored in a blinded fashion by two observers. The score was recorded as 1+ if 1 to 5 cells were stained in each visual field, 2+ for 5 to 10 cells, and 3+ for >10 cells. For each organ, the average score of 15 to 30 visual fields was calculated. In experiments described in paper IV, using a Sony camera connected to the microscope, five to nine visual fields from two separate sections per skin biopsy were analyzed. 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.

RESULTS AND DISCUSSION

Paper I. *F. tularensis*-immunized mice respond to a secondary intradermal challenge by enhanced local expression of TNF-α, IFN-γ, and IL-12 and by rapid control of infection

In the mammalian host defense against *F. tularensis*, the skin seems to constitute an extraordinarily well-equipped barrier. In BALB/c mice, the lethal intradermal dose of infection with *F. tularensis* LVS is ~ 10^6 organisms, as compared to one or at most a few hundred bacteria when injected intraperitoneally or intravenously or when administrated orally or by inhalation, *i.e.*, even when the bacteria need to pass through intact mucous membranes. The present studies aimed at understanding mechanisms involved in the barrier. In the studies reported in paper I, using BALB/c mice and *F. tularensis* LVS, we monitored the bacterial replication at the infectious skin site and in internal organs in relation to the host expression of IL-12, TNF-α and IFN-γ, cytokines known to mediate host resistance to intracellular bacteria. The host response of naïve mice was compared to that of mice immunized 5 weeks previously by skin infection with *F. tularensis* LVS. The primary infection was cleared within 2 weeks.

When intradermally inoculated with 3.8 x 10^5 CFU of *F. tularensis* LVS, naïve mice showed a 1 $\log_{10}$ increase of bacterial numbers in the skin, with a peak value at 3 days of ~ 10^8 CFU per sample followed by a slow decrease during the next few days into non-detectable numbers at 2 weeks. Also in draining lymph nodes, spleen and liver, significant bacterial numbers were found, peak values at 3-5 days being 10^5 to 10^6/bacteria per organ, followed by control of the infection within 2 weeks. Previously immunized mice seemed to immediately control the infection. Already within 3 days, bacterial numbers decreased more than 3 $\log_{10}$ and at 8 days, no bacteria were found in the skin samples. In draining lymph nodes, a few

bacteria were found during the first few days whereas in spleen and liver, no bacteria were isolated through the whole 2-week period of monitoring. Altogether, intradermal inoculation of naïve mice with *F. tularensis* LVS resulted in a transient bacterial replication in skin and invasion of spleen and liver, and eradication of infection within 2 weeks of infection. Previously immunized mice seemed not to allow bacterial replication and contained the infection much more rapidly.

To appraise the duration of protective immunity induced by a primary infection, mice were challenged intradermally with the homologous organism, either 1 or 3 months after the infection. At 3 days after challenge, bacterial numbers at the local site of infection were 2.3 ± 0.1 $\log_{10}$ CFU per skin sample in the former and 3.3 ± 0.5 $\log_{10}$ CFU in the latter group ($P < 0.05$). In naïve animals, 7.8 ± 0.1 $\log_{10}$ CFU bacteria were recovered. Thus a long-term protective immunity was induced by a primary infection, although possibly with a tendency to wane within 6 months.

An obvious question was whether the rapid control of a secondary infection in the skin would have an association with the expression of cytokines in the skin. By immunohistochemistry, we studied the expression of TNF-α, IFN-γ, and IL-12 at the site of infection at 24, 48 and 72 hours after intradermal inoculation of *F. tularensis* LVS. Naïve mice showed a weak expression of all three cytokines at all time intervals assayed. By comparison, previously immunized mice showed a markedly increased expression of all three cytokines, although with different kinetics. The expression of TNF-α and IL-12 peaked already at 24 h and declined thereafter, whereas the expression of IFN-γ was markedly increased only after 72 h of inoculation.

Qualitative RT-PCR analyses and quantitative assay of cytokine mRNA on skin samples partially conformed to the histochemistry data. In competitive PCR analysis of samples obtained at 24 and 48 h after intradermal inoculation of *F. tularensis* LVS, both naïve and

immune mice showed expression of TNF-α and IFN-γ mRNA. As compared to naïve mice, immune mice showed, at 24 h after inoculation, a several-fold higher expression of both cytokines, whereas at 48 h they showed a several-fold lower expression of the cytokines. This result indicated the presence of a more rapid cytokine response in immune animals, and also a capability of these animals to rapidly down-regulate the expression in relation to control of infection. For IL-12, results from the qualitative and quantitative mRNA assays were mutually less consistent and therefore less readily interpreted.

To summarize the results of paper I, immunization of BALB/c mice by intradermal inoculation of a sublethal dose of *F. tularensis* LVS induced an enhanced capability to control a secondary intradermal infection and an enhanced early expression of IL-12, TNF-α and IFN-γ at the local site of infection.

When the present studies were initiated, an importance of the skin as a barrier to *F. tularensis* infection had been suggested by the demonstration of a wide difference in infective dose due to the route of administration of the bacteria (Elkins et al., 1992; Fortier et al., 1991). Using a dose of 3.0 $\log_{10}$ *F. tularensis* LVS, Balb/c mice were given an intraperitoneal, intradermal, intranasal, or intravenous injection. For comparison, Balb/c mice resisted an intradermal dose of > 10^7 bacteria whereas all mice died after injection of 10^2 bacteria intraperitoneally. The authors asked the question of whether this difference was due only to a physical barrier retarding spread, or whether an innate immune mechanism resided in the skin. The results favored an immune mechanism because on day 5 of infection, bacterial numbers in the spleen were the same irrespective of intradermal or intranasal administration, although the intradermal but not the intranasal infection was controlled. The presence of an innate immune mechanism in the skin was favored also by a control of bacterial growth in the lungs restricted to intradermal injection. Based on these results the authors suggested that “murine

skin contains cells that are very efficient in responding to bacterial infection with cytokine production, which ultimately results in limiting bacterial replication and effective clearance".

The suggestion was corroborated by the results of paper I. According to our results, these are cells in the murine skin capable of responding with a rapid cytokine production to *F. tularensis* and this capability is enhanced in animals previously immunized with the agent.

Our results on primary infection gave some support in favor of the assumption that the skin may afford innate immunity to *F. tularensis*. TNF-α and IFN-γ mRNA was detected in the skin within 24 h of intradermal infection. The strength of this evidence was limited, since the expression of protein was weak. Nevertheless, the results of our studies on rechallenge strongly indicate the presence in the skin of cells ready to produce TNF-α and IL-12 within 24 hours of exposure to *F. tularensis*.

The early presence of IL-12, TNF-α and IFN-γ, although weak, may represent prerequisites to direct the T-cell response into a Th1 phenotype after intradermal infection. In line with the development of a Th1 response, no IL-4 mRNA has been detected in the skin during the first days after primary infection (unpublished data). Due to the extreme virulence of *F. tularensis* LVS when administered in Balb/c mice by routes other than the skin, this is less easily studied.

In a murine model of leishmaniasis, cutaneous infection with *Leishmania donovani* resulted in the development of a prominent local and systemic Th1 immune response and no detectable visceral parasitism, whereas intravenous inoculation resulted in a delayed Th1 immune response, as evidenced by minimal IL-12 mRNA expression and a progressive visceral parasite burden (Melby et al., 1998). In experimental tularemia, effective protection results regardless of the route of immunization, and control of infection occurs with similar kinetics, regardless of whether inoculation is cutaneous or parenteral. Thus, in contrast to *Leishmania* infection, the control of *F. tularensis* infection seems not to be dependent on the

route of immunization. This result is probably related to the fact that *F. tularensis*, without known exceptions, induces a Th1 immune response, whereas *Leishmania* infection and other parasitic diseases may under certain circumstances, result in the expansion of Th2 T cells and thereby disease progression.

Paper II. *F. tularensis* LVS induces a partial host protection locally in the skin of mice lacking αβ T cells.

Previous studies on murine tularemia have shown that the survival of a primary infection is dependent on IFN-γ and T cells, with special emphasis on the αβ T cell. By using naïve and immunized T-cell knockout mice (e.g., αβ $TCR^{-/-}$, γδ $TCR^{-/-}$, αβγδ $TCR^{-/-}$) we investigated the role of T cells in the defense against an intradermal infection.

First we studied the course of infection in naïve mice. When intradermally inoculated with *F. tularensis* LVS, γδ $TCR^{-/-}$ mice were found to control the infection as effectively as did normal mice. In skin samples obtained from the site of inoculation, bacterial numbers continuously decreased to reach eradication within 7 days. In liver and spleen, bacteria were eradicated within 14 and 23, days respectively. In αβ $TCR^{-/-}$ and αβγδ $TCR^{-/-}$ mice, on the other hand, bacterial numbers in skin, liver and spleen were persistently high during the whole 23-day period of observation. When *F. tularensis* LVS was intradermally inoculated at a dose sublethal to normal mice, αβ $TCR^{-/-}$ mice died within a mean period of 30.4 ± 3.4 days and αβγδ $TCR^{-/-}$ mice at 27.8 ± 2.8 days, whereas γδ $TCR^{-/-}$ cleared the infection within 3 weeks. In conclusion, αβ T cells were essential to control the infection, locally in the skin as well as in liver and spleen, whereas γδ T cells seemed dispensable.

Macrophages are effector cells crucial in the host defense against *F. tularensis* and other intracellular bacteria. Since, in order to be able to kill the bacteria, they need to be activated by IFN-γ, we asked whether the lack of control of a primary infection in αβ $TCR^{-/-}$ mice might be due to an impaired production of IFN-γ. At various time intervals after intradermal inoculation of *F. tularensis* LVS, we analyzed serum levels of IFN-γ. In wild-type and γδ $TCR^{-/-}$ mice, an initial high serum level was followed by a continuous decline down to baseline at day 10. By contrast, αβ $TCR^{-/-}$ mice had persistent high serum levels of IFN-γ from day 4 until the end of the experiment at day 24. These high levels of IFN-γ paralleled the presence of high bacterial numbers in the organs. Thus, an extensive production of IFN-γ occurred in αβ $TCR^{-/-}$ mice which was, apparently insufficient for bacterial killing and control of the infection.

We also studied the capacity of TCR-deficient mice to control a secondary intradermal infection with *F. tularensis* LVS. These experiments were complicated by the inability of αβ TCR-deficient mice to survive the primary infection. For this reason we created a protocol, including 10 days of netilmicin treatment starting 10 days after immunization, followed by a secondary intradermal inoculation of the homologous agent 4 weeks later. Although netilmicin treatment enabled αβ TCR-deficient mice to survive the primary infection, they did not eradicate the bacteria. To be able to identify and quantify bacteria from the secondary infection in the presence of bacteria remaining from the primary infection, we used a strain (*F. tularensis* pKK214) with a tetracycline resistance gene enabling selection during cultivation.

After intradermal challenge of previously immunized mice, using 6.7×10^4 tetracycline-resistant *F. tularensis* LVS, bacterial numbers were monitored in various organs for 5 days. As might be expected, γδ $TCR^{-/-}$ mice controlled a secondary infection quite well. Actually, no bacteria were found in the skin, draining lymph nodes, liver or spleen of the γδ $TCR^{-/-}$ mice

on day 5. However, αβ TCR$^{-/-}$ and αβγδ TCR$^{-/-}$ mice also showed some degree of resistance against a secondary infection with *F. tularensis*. In these mice, skin samples and lymph nodes obtained 5 days after challenge contained significantly lower numbers of organisms than did samples from naïve immunocompetent mice. In spleen and liver of αβ TCR$^{-/-}$ or αβγδ TCR$^{-/-}$ mice, no bacteria were detected, as compared to 3-4 log organisms per organ of naïve normal mice. Thus, mice deficient in γδ T cells showed an intact capacity to evoke immune protection by intradermal inoculation with *F. tularensis* LVS. Less expected was our finding of a partial host resistance after intradermal infection of animals lacking αβ T cells, suggesting the presence of an αβ T-cell independent mechanism of protection.

Asking the question of whether in αβ TCR$^{-/-}$ mice, the *F. tularensis* LVS induced host resistance might depend on antibodies, we analyzed serum for *F. tularensis*-specific IgG and IgM antibodies. Five weeks after infection, serum from wild-type mice and γδ TCR$^{-/-}$ mice contained high levels of both *F. tularensis*-specific IgG and IgM. αβ TCR$^{-/-}$- and αβγδ TCR$^{-/-}$ mice, on the other hand, had high levels of IgM, whereas specific IgG levels were as low as those of naïve wild-type mice. Thus, our results raised the possibility that *F. tularensis*-specific IgM antibodies might play a role in the intradermal immune defense against cutaneous tularemia, besides the well-established role of T cells.

The results of paper II conform well to a previous study by Yee et al. on *F. tularensis* LVS (Yee et al., 1996). When 10^5 bacteria were given intradermally to αβ-TCR-gene-deficient, or αβ- and γδ-TCR-deficient mice, death occurred within 5 weeks. On the contrary, all γδ-TCR-gene-deficient survived the same dose. Our results confirm these results on primary infection and we extended the investigation by studying αβ TCR-gene deficient mice that had been manipulated so to survive a primary infection by antibiotic treatment.

By taking this novel experimental approach, we disclosed an adaptive immunity in αβ TCR-gene deficient mice. A non-T cell-dependent protective immunity was found to be

acquired during the course of the primary infection. As far as I know, no similar observation has been described in infection models with other intracellular bacteria. Nor is there any data in human infection indicating an importance of non-T cell-mediated immunity in tularemia. The fact that humoral immunity afforded by immunization with killed bacteria or by adoptive transfer of immune serum lacks, per se, a protective effect does not exclude the possibility of a role for humoral immunity in the presence of T cells.

Dendritic epidermal T cells, DETC, in mice have a γδ TCR. Although DETC are shown to be of importance for the immune response in the skin, they seem not to be of critical importance in infection with *F. tularensis* LVS since γδ $TCR^{-/-}$ mice cleared the infection as effectively as wild type mice.

The results of this paper are of interest for treatment of tularemia in humans. Using therapeutically relevant doses of netilmicin, ciprofloxaxin or moxifloxacin, complete eradication of the bacteria was not achieved in spite of very low MIC-values. This indicates that in the absence of cell-mediated immunity, bactericidal antibiotics will not be sufficient to clear the infection.

Paper III. *F. tularensis*-specific antibodies afford protection against intradermal challenge of mice with *F. tularensis* LVS as well as a wild-type isolate of *F. tularensis* subsp. *holarctica*

All since immunotherapy by means of adoptive transfer of immune serum was used to treat tularemia in the 1930s, the role of antibodies in the mammalian host resistance against *F. tularensis* has been disputed. A contributory effect in the defense against less virulent strains such as LVS has been incontrovertibly demonstrated, whereas a role in host protection against wild strains has remained uncertain (Allen, 1962; Pannell and Cordle, 1962; Thorpe and

Marcus, 1965). When interpreting studies on adoptive immunity afforded by transfer of immune serum, a problem is to find a true negative control. Since an antibody response will be rapidly induced by the challenge itself, the immune state of control animals becomes blurred. To more reliably estimate the contribution of transferred *F. tularensis*-specific antibodies on primary infection, and to enable tests on secondary infection, we here used B-cell-deficient mice. Immune or normal serum was transferred by intraperitoneal injection, after confirming that the sera were free from bacteria and bacterial lipopolysaccharide. Moreover, screening of serum samples for various cytokines showed values at or below detection limits.

We first studied the effect of immune serum on lethal tularemia. One day after intraperitoneal injection of 0.5 ml normal or immune serum, mice were intradermally challenged with a lethal dose of *F. tularensis* LVS, *i.e.*, 6×10^7 bacteria for naïve mice and 3×10^8 bacteria for previously immunized mice. When given normal serum, time to death was 7.9 ± 1.5 days (mean ± SEM) in naïve and 5.3 ± 2.6 days in previously immunized mice. In both groups of mice, immune serum afforded full protection to lethality during a 28-day observation period and no bacteria were found in spleens at the end of the period.

We also studied the effect of *F. tularensis*-specific antibodies on the course of non-lethal tularemia. One day after intraperitoneal injection of 0.7 ml normal or immune serum in B-cell-deficient or wild-type mice, 10^4 LVS organisms were intradermally inoculated and three days after the inoculation, animals were killed for determination of bacterial counts. In naïve mice, including both B-cell-deficient and wild-type animals, immune serum caused a significant decrease of bacterial counts in liver and spleen. Only in the former mice, a reduction occurred also in bacterial counts in skin. Already one day after challenge, immune serum was found to effectively prevent bacterial spread to spleen and liver but not to reduce the number of bacteria in skin.

As compared to these results on naïve mice, data on immune animals were less easily interpreted. In immunized B-cell deficient mice, immune serum caused a reduction of bacterial counts in spleen but not in liver and skin. Undoubtedly, a possible effect of antibodies on the host resistance may have been masked by a T cell-dependent immune response induced by immunization. Notably, we demonstrated a strong recall T cell response to *F. tularensis* antigen in previously immunized B-cell-deficient and wild-type mice. In summary, adoptive transfer of immune serum conferred on B-cell-deficient mice a capacity to control *F. tularensis* LVS infection. The protective effect of immune serum was disclosed as a retarded spread of infection to internal organs rather than enhanced killing of the bacteria in the skin.

We also studied the effect of immune serum on host resistance to a virulent strain of *F. tularensis* subsp. *holarctica*, *F. tularensis* FSC171, isolated from a patient with ulceroglandular tularemia. On day 5 after challenge of 1.3×10^3 bacteria intradermally, viable counts in liver and spleen were estimated. As compared to transfer of normal serum, the transfer of immune serum caused a reduction of bacterial numbers from 9.2 ± 0.1 to 4.5 ± 0.9 $\log_{10}$ in livers and 9.2 ± 0.1 to 5.1 ± 0.2 in spleens, indicating a highly efficient protection against primary intradermal infection with a virulent *F. tularensis* subsp. *holarctica* strain.

In mice, immune serum affects the distribution of *F. tularensis* LVS after intravenous injection (Anthony and Kongshavn, 1987). When in that study, mice were pre-treated with immune serum, and killed 15 min after intravenous injection, only 0.2% of injected bacteria were found in the blood, and almost 40% in the liver. In control mice, receiving normal serum, 70% of injected bacteria were recovered from blood and 5% from the liver. Notably, the total number of bacteria recovered from immune serum-treated animals was only half the number recovered from control animals. Obviously immune serum might have opzonized the bacteria and directed them to the liver and other organs for killing by phagocytes.

Also in humans, antibodies afford opsonization and killing of *F. tularensis.* There is, however, a difference between LVS and a wild strain of *F. tularensis* subsp. *holarctica* in susceptibility to opsonin-dependent intracellular killing by human polymorphonuclear leukocytes (Löfgren et al., 1983). In the presence of immune serum, both LVS and the wild strain were efficiently phagocytosed in vitro and within an hour, 40% of bacteria of the wild strain and 98% of LVS bacteria were killed intracellularly (Löfgren et al., 1983). In the presence of nonimmune serum, no phagocytosis occurred. Considering the susceptibility to antibody-mediated phagocytosis of *F. tularensis* subsp. *holarctica* demonstrated in the latter experiments and the complete resistance of *F. tularensis* subsp. *tularensis* to the effects of adoptively transferred antibodies in vivo repeatedly demonstrated by others (Allen, 1962; Fulop et al., 2001; Thorpe and Marcus, 1965), it seems reasonable to anticipate that the subspecies *tularensis* may be more resistant to antibody-mediated phagocytic killing.

Paper IV. *F. tularensis*-specific antibodies induce a local expression of TNF-α and IL-12 and recruitment of neutrophils early upon intradermal infection with *F. tularensis* LVS.

So far, our results had suggested a role for Th1 cytokines, *i.e.*, TNF-α, IFN-γ and IL-12, in the resistance to an intradermal *F. tularensis* infection (paper I) and also a role in the resistance for immunospecific antibodies (paper III). Since upon secondary challenge, an enhanced local cytokine response was found in the skin, an obvious question was whether the antibodies might be involved in the induction. As reported in paper IV, we applied immunohistochemistry to study the effect of immune serum on the local cytokine response to *F. tularensis* LVS. Since during the study, an accumulation of neutrophils was observed in the skin upon intradermal *F. tularensis* LVS infection, we also included a granulocyte-specific antibody GR-1 in the assay.

Immune serum or normal serum (0.5 ml) was intraperitoneally injected, mice were challenged by intradermal injection of *F. tularensis* LVS and on day 3, animals were killed for preparation of skin specimens from the site of infection. As compared to normal serum, administration of immune serum resulted in increased expression of TNF-α and IL-12p40, but not IFN-γ. In parallel, there was a significant recruitment of neutrophils in animals given immune serum. These observations afford a possible explanation for the effect of antibodies in the local host defense in the skin against tularemia, *i.e.*, a capacity to induce cytokines important to mediate resistance.

In a recent study, Prina et al. investigated the role of opsonins in parasite uptake and dendritic cell maturation in infection with *Leishmania amazonensis* (Prina et al., 2004). Dendritic cells from Balb/c mice were used in an in vitro system. Internalization of antibody-opzonized parasites induced dendritic cells to mature rapidly, as shown by overexpression of co-stimulatory, adhesion and MHC class II molecules. Furthermore, uninfected cells were transactivated as a consequence of the stimulation of the infective cells, suggesting production of soluble activating molecules. In summary, the presence of antibodies may lead to activation of immune cells.

In studies on mechanisms of phagocytosis, comparing uptake via the Fc receptor and complement receptor 3, important differences in the activation of macrophages have been found. Fc receptor ligation with antibody-opsonized bacteria is accompanied by the activation of the respiratory burst and cytokine production, such as TNF-α. Complement receptor 3 mediated uptake, on the other hand, may occur in the absence of proinflammatory signals (Caron and Hall, 1998). In a study of murine tularemia, transfer of immune serum to normal mice afforded protection from death due to intraperitoneal injection of LVS, an effect that was neutralized by simultaneous injection of anti-gamma interferon (Rhinehart-Jones et al., 1994). Our data are well in line with those results, which suggest the existence of antibody-induced

activation of immune cells including cytokine production. Transfer of immune serum increased the production of IL-12 and TNF-α locally in the skin after infection with *F. tularensis* LVS.

Intracellular bacteria that survive and multiply in professional phagocytes may interfere with the maturation of the phagosome into a phago-lysosome, escape from the phagosome or adapt to the hostile milieu (Amer and Swanson, 2002). Activated macrophages enhance phagocytosis by increasing the expression of Fc receptors on the cell surface. In infections with *Listeria* it has been shown that phagocytosis through the Fc receptor is associated with prevention of bacterial escape from the phagosome (Edelson et al., 1999). In infection with *Rickettsia conorii*, Fc-dependent antibodies inhibit phagosomal escape (Feng et al., 2004). If this is true also for *F. tularensis*, the presence of specific antibodies leading to enhanced Fc-mediated phagocytosis, might interfere with the escape from the phagosome, an event described by Golovliov *et. al.* (Golovliov et al., 2003), and subsequently result in enhanced bacterial killing.

CONCLUSIONS

- Immunization of BALB/c mice by intradermal inoculation of a sublethal dose of *F. tularensis* LVS induces an enhanced capability to control a secondary intradermal infection and an enhanced early expression of IL-12, TNF-α and IFN-γ at the local site of infection in the skin.
- αβ T cells are essential to control the infection, locally in the skin as well as in liver and spleen, whereas γδ T cells seem dispensable.
- A T cell-independent protection is acquired during the course of the primary infection.
- Adoptive transfer of immune serum confers on B cell-deficient mice a capacity to control *F. tularensis* LVS infection.
- The protective effect of immune serum is disclosed as a retarded spread of infection to internal organs rather than enhanced killing of the bacteria in the skin.
- Transfer of immune serum causes a reduction of bacterial numbers in spleen and liver, indicating a highly efficient protection against primary intradermal infection with a virulent *F. tularensis* subsp. *holarctica* strain.
- Transfer of immune serum results in increased expression of TNF-α and IL-12p40 at the local site of infection in the skin. In parallel, there is a significant recruitment of neutrophils.
- Immune serum confers a capacity to induce cytokines of importance to mediate resistance in the local host defense in the skin against tularemia.

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