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Transfer of Immunity against Lethal Murine *Francisella* Infection by Specific Antibody Depends on Host Gamma Interferon and T Cells

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Both serum and spleen cells from mice immune to *Francisella tularensis* transfer protection to naive recipients. Here we characterize the mechanism of protection induced by transfer of immune mouse serum (IMS). IMS obtained 4 weeks after intradermal infection with 10^3 bacteria of the live vaccine strain (LVS) contained high levels of immunoglobulin G2 (IgG2a) and IgM (end point titers, 1:16,600 and 1:7,200, respectively) and little IgG1, IgG2b, or IgG3. LVS-specific antibodies were detected 5 days after intradermal infection, and reached peak levels by 2 weeks postinfection. Only sera obtained 10 days or more after sublethal infection, when IgG titers peaked, transferred protection against a challenge of 100 50% lethal doses (LD_{50}s). Purified high-titer IgG anti-LVS antibody but not IgM anti-LVS antibody was responsible for transfer of protection against an intraperitoneal challenge of up to 3,000 LD_{50}s. IMS had no direct toxic effects on LVS and did not affect uptake or growth of bacteria in association with peritoneal cells. One day after LVS infection, liver, spleen, and lung tissue from mice treated with IMS contained 1 to 2 log units fewer bacteria than did tissue from mice treated with normal mouse serum or phosphate-buffered saline. Between 2 and 4 days after infection, however, bacterial growth rates in tissues were similar in both serum-protected mice and unprotected mice. Bacterial burdens in IMS-treated, LVS-infected mice declined in infected tissues after day 5, whereas control animals died. This lag phase suggested that development of a host response was involved in complete bacterial clearance. In fact, transfer of IMS into normal recipients that were simultaneously treated with anti-gamma interferon and challenged with LVS did not protect mice from death. Further, transfer of IMS into athymic *nu/nu* mice did not protect against LVS challenge; protection was, however, reconstituted by transfer of normal T cells into *nu/nu* mice. Thus, "passive" transfer of protection against LVS with specific antibody is not passive but depends on a host T-cell response to promote clearance of systemic infection and protection against lethal disease.

We have previously developed a model for human tularemia by using the attenuated *Francisella tularensis* live vaccine strain (LVS) inoculated intraperitoneally (i.p.) or intradermally (i.d.) in mice (9, 10, 13). Although this strain has met with only variable success as a human vaccine, it is pathogenic for mice, and the infection mimics human disease (8, 33); a single *F. tularensis* LVS bacterium given to mice i.p. is lethal, whereas mice survive as much as 10^4 LVS given i.d. or subcutaneously (9, 10, 13). Furthermore, surviving mice are protected against lethal i.p. challenge (12, 15). Using this mouse model, we have described specific immunity, both humoral and cellular, that develops after i.d. infection with sublethal doses of LVS. Although *F. tularensis* is generally considered to be an intracellular pathogen, transfer of either immune cells or serum containing LVS-specific antibody was able to protect against lethal LVS infection (13).

Passive-transfer systems have been studied both to elucidate the role of antibody in infection and to investigate their potential therapeutic utility. Such studies have suggested that specific antibody has little, if any, role in protection against infection with intracellular bacteria such as *Listeria* spp. (25) and *Mycobacterium* spp. (17); transfer of serum may not be efficient or consistent in protection against experimental *Francisella* infection (32, 33), particularly when animals are challenged with fully virulent *Francisella* strains such as Schu 4 (1, 33). On the other hand, passive immunotherapy was used at one time to modulate the course of disease in human tularemia (14, 15).

The LVS model may be a particularly appropriate system with which to reexamine the potential contribution of specific antibody to both natural infection and potential immunotherapies for intracellular bacteria, since neither cells nor sera from normal inbred mice cross-react with LVS (32) and since lethal infection with similarities to human disease is easily established (8, 10, 13, 32). To further address questions on the role of antibodies in protection against intracellular bacteria, we characterize here the polyclonal humoral response to LVS in mice and analyze its contribution to overall protection against lethal *Francisella* infection. Most importantly, we begin to study the mechanism(s) by which LVS-specific antibody exerts its protective effects.

Relatively little information on the mechanism by which "passive" transfer of antibody mediates protection is available, and the need for further understanding of the mechanism of action of such products as intravenous immunoglobulin (IVIg) has been noted (24). It is generally assumed that antibody

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transfer serves to replace missing specificities or subclasses of antibody and that antibody functions largely through direct effects such as complement-dependent lysis or opsonization for phagocytosis. The opsonic capacity of serum does not always correlate with in vivo models of protection, however (16). Using the LVS model system, we demonstrate a novel mechanism for antibody-mediated protection: passive transfer of protection by specific anti-LVS antibody is not at all passive but is dependent on a T-cell immune response and gamma interferon (IFN-γ) production by the recipients that results in systemic clearance of the bacteria.

MATERIALS AND METHODS

Animals. Specific-pathogen-free male C3H/HeNHSD mice (Harlan Sprague Dawley, Frederick, Md.) were used at 5 to 7 weeks of age. Male BALB/c *nu*/+ and *nu*/*nu* mice, 4 to 5 weeks of age, were purchased from the Biological Resources Branch, Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, Md. Mice were housed in a barrier environment at the Walter Reed Army Institute of Research and routinely tested for common murine pathogens by a diagnostic service provided by the Division of Veterinary Medicine, Walter Reed Army Institute of Research. In conducting the research described in this report, we adhered to the Guide for Laboratory Animal Facilities and Care as promulgated by the Committee of the Guide for Laboratory Animals and Care of the Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences.

Bacteria and growth conditions. *F. tularensis* LVS (ATCC 29684; American Type Culture Collection, Rockville, Md.) was cultured on modified Mueller-Hinton agar (MHA) plates for 3 to 4 days at 37°C under 5% CO_2 and 95% humidity (4, 12). Colonies were selected for growth in modified Mueller-Hinton broth (MHB; Difco Laboratories, Detroit, Mich.) supplemented with ferric PP_i and IsoVitaleX (Becton Dickinson, Cockeysville, Md.) (4). Inoculated broth cultures were incubated for 24 to 36 h until the bacterial density reached 10^8 to 10^9 CFU/ml. Aliquoted bacteria were then frozen in broth alone at −70°C. Aliquots (1 ml) were periodically thawed, and viable bacteria were quantified by plating serial dilutions in 0.1% fetal bovine serum (FBS)–saline on MHA plates. The number of CFU after thawing varied less than 5% over a 4-month period.

Neisseria gonorrhea was a gift from Carolyn Deal, Walter Reed Army Institute of Research and was cultured on MHA for 2 days at 37°C under 5% CO_2 and 95% humidity.

Animal inoculations. To determine an i.p. lethal dose for 50% of the mice (LD_{50}) of LVS, we gave mice 0.5 ml of a 1:5 dilution of immune mouse serum (IMS) i.p. and challenged them i.p. 2 h later with various doses of LVS. The LD_{50} was calculated for this route by the method of Reed and Muench as discussed by Lennette (19). Both LD_{50} and transfer experiments used groups of five or six mice, as indicated.

For serum analyses by enzyme-linked immunosorbent assay (ELISA) and determinations of anti-LVS titers, mice were individually marked, prebled, and sublethally infected with 10^3 LVS bacteria i.d., and sera were obtained via tail bleeds throughout 14 weeks after infection. To obtain serum for normal mouse serum (NMS) and IMS pools, we obtained prebleed serum via the lateral tail vein and then inoculated mice i.d. with 10^3 LVS bacteria. At 4 weeks after infection, groups of mice were sacrificed and bled by cardiac puncture. Pooled blood was clotted, and serum was collected and frozen at −20°C until needed for experimental use.

Characterization of antibody response. The reactivity of the immune serum was analyzed by LVS ELISA with whole live bacteria from frozen LVS stock (9). *F. tularensis* LVS was diluted to a concentration of 5×10^7 bacteria per ml in bicarbonate buffer (pH 9.0). Immulon I plates were coated with 5×10^6 bacteria per well and incubated for 2 h at 37°C and then overnight at 4°C. The following day, plates were washed three times (this and all subsequent steps) with a saline-Tween solution (0.05% [vol/vol] Tween 20). Plates were blocked with 200 μl of 10% calf serum in phosphate-buffered saline per well (PBS) for 30 min at 37°C and washed. Serum samples were serially diluted with saline-Tween-serum solution (saline-Tween solution, 10% serum) onto LVS-coated and blocked plates and incubated for 90 min at 37°C. After washing, enzyme-labeled antibodies (goat anti-mouse immunoglobulin [Ig] or subclass specific antibodies against IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA) directly conjugated to horseradish peroxidase (Southern Biotech, Birmingham, Ala.) were added for detection, and plates were incubated at 37°C for another 90 min and then washed. Optimal concentrations for specificity and sensitivity of the various lots of enzyme-labeled antibodies were determined in separate experiments. For color development, a 1:1 mixture of ABTS Peroxidase Substrate and Peroxidase Solution B (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was added. Maximal color developed by 20 min, and the reaction was stopped by addition of 1.5% KF (in double-distilled H_2O). Plates were read on a kinetic microplate reader (Molecular Devices, Menlo Park, Calif.) at 410 nm (reference, 490 nm). The end point titer was defined as the lowest dilution of serum that gave an optical density at 405 nm (OD_{405}) that was twofold greater than the value of the matched dilution of normal, prebleed mouse serum and also was greater than 0.050. End point titers obtained from individual assays of groups of three to five mice were averaged by geometric means. Standard errors are omitted for clarity of presentation but were less than 20%. To ensure that antibody responses in IMS samples were specific to *F. tularensis* LVS, we also assayed sera on uncoated Immulon I plates (without any bacteria) as well as on plates coated with an unrelated bacterium, *Salmonella typhimurium*; we further compared these with the binding of germfree mouse serum (GFMS) to LVS-coated plates. Very low ODs (<0.1) were observed and no OD of IMS above that of NMS or GFMS was observed when using either uncoated or *S. typhimurium*-coated plates. Since this assay uses plates coated with live, intact *F. tularensis* LVS grown to stationary phase and then diluted in coating buffer, it measures primarily antibody responses to bacterial cell surface antigens rather than secreted antigens.

Isolation and purification of isotype by column separation. To isolate the IgM fraction of IMS (obtained from mice infected 4 weeks earlier with a sublethal i.d. dose of LVS [10^3 bacteria]), a 2-ml column of anti-mouse IgM agarose (Sigma, St. Louis, Mo.) was prepared and equilibrated in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.5 M sodium chloride. Pooled IMS (5 ml) was applied slowly to the column, followed by a thorough wash with phosphate buffer. The eluted fraction, containing only IgM antibody, was obtained by washing the column with 0.1 M glycine–0.15 M sodium chloride (pH 2.4). The IgM fraction was immediately brought to neutral pH and stored at −20°C until needed.

To isolate the IgG subfraction of IMS, a 4-ml column of protein A-Sepharose (Pharmacia, Piscataway, N.J.) was prepared and equilibrated in PBS (pH 8.0). IMS (5 ml) was applied slowly to the column and was followed by a wash with PBS. The eluate, all IgG, was eluted from the column with 0.1 M glycine (pH 3.0) or 3.5 M magnesium chloride, and the fraction was neutralized immediately and stored at −20°C.

Direct complement lysis. *Neisseria gonorrhoeae* and LVS organisms, grown on MHA plates at 37°C under 5% CO_2, were diluted in Hanks buffered saline solution to an OD_{600} of 0.1 (approximately 10^8 CFU/ml). The bacteria were mixed with 1 ml of either Hank's buffered saline solution for controls, heat-inactivated (56°C for 30 min) antiserum, or heat-inactivated antiserum containing 10% exogenous source of complement (rabbit complement; Pel Freez Biologicals, Rogers, Ark.). The reaction mixtures were rotated on a shaker for 1 h at 37°C. After incubation, mixtures were serially diluted and plated to assess the number of CFU per milliliter of reaction mixture.

Peritoneal cells. Resident peritoneal cells were collected by i.p. injection of 10 ml of culture medium consisting of Dulbecco-modified Eagle medium (DMEM; GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated FBS (Sterile Systems, Inc., Logan, Utah). Normal resident peritoneal cells were collected from untreated mice by withdrawing peritoneal fluid through the abdominal wall with a 19-gauge needle. Fluids from 3 to 10 mice were pooled, samples were removed for differential and total cell counts, and the remaining fluid was centrifuged to collect cells at 250 × *g* for 10 min at 4°C. Differential cell counts were made on Wright-stained cell smears (Diff-Quick; Dade Diagnostics, Aquado, P.R.) prepared by cytocentrifugation (Cytospin centrifuge; Shandon Southern Instruments, Camberley, England). Washed peritoneal cell suspensions were adjusted to 10^6 macrophages per ml in culture medium and incubated as unfractionated cell cultures (including lymphocytes) in polypropylene tubes (no. 2063; Falcon Plastics, Oxnard, Calif.) under 5% CO_2 at 37°C before exposure to LVS or antibody-treated LVS.

Opsonization. *F. tularensis* LVS, starting at a concentration of 5×10^8/ml, was diluted 1:10 directly into 1 ml of NMS, IMS, or PBS to obtain a concentration of 5×10^7/ml. This ratio of serum to bacteria is sufficient to cause visible bacterial agglutination in IMS. The bacteria-serum mixtures were incubated on ice, or under 5% CO_2 at 37°C, for 1 h and then vortexed to disrupt agglutination. After this initial incubation, 10 μl (or 10^5 bacteria) of each mixture was added to 10^6 cultured peritoneal cells (60% macrophages). Cell-bacteria mixtures were incubated under 5% CO_2 at 37°C for 2 h and washed twice with culture medium (DMEM supplemented with 10% FBS) by low-speed centrifugation (70 × *g* for 8 min) to minimize uningested bacteria; cell pellets were resuspended in fresh culture medium. To assess the short-term opsonic ability of serum, infected-cell suspensions were lysed with 0.05% sodium dodecyl sulfate (SDS) after a 2-h infection, and supernatants were serially diluted in PBS and plated on MHA to quantify bacteria. To assess the long-term capacity of IMS to block or enhance survival and replication of bacteria in PC, infected-cell suspensions were lysed and plated at 24, 48, and 72 h after infection. Only results for 72 h are shown (Table 2). Previous results demonstrate that LVS does not replicate in tissue culture medium but only in association with cells (12); thus, lysis of the washed cell suspension with SDS measures total cell-associated bacteria but cannot distinguish between intracellular bacteria and those adhering to the cell surface.

Detection of IFN in serum and in vivo depletion of IFN. The level of IFN in serum was quantitated by ELISA (22). Briefly, this ELISA is a double-sandwich procedure in which hamster monoclonal antibody (H22; a gift from R. D. Schreiber, Washington University, St. Louis, Mo.) is used to coat Immulon II plates. After blocking, standards and unknowns were serially diluted onto plates and incubated for 1 h at 25°C. Polyclonal rabbit anti-IFN antibody (a gift from R. D. Schreiber) was added, and plates were incubated for another 1 h. Horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad, Richmond, Calif.) was then added, and color was developed with ABTS (Kirkegaard and Perry). The concentration of IFN (in units per milliliter) in serum samples was estimated from a standard curve generated with a standard preparation of recombinant IFN (Genentech, South San Francisco, Calif.). IFN was depleted in vivo by i.p. inoculation of 200 μg of H22 anti-IFN monoclonal antibody (18); polyclonal hamster IgG (HIgG; Organon Teknika, Rockville, Md.) was used as a control.

Preparation and transfer of mouse T cells. For reconstitution of athymic *nu/nu* mice with enriched T cells, spleens were obtained from normal, age-matched BALB/c mice. A single-cell suspension was prepared, and erythrocytes were lysed with ammonium chloride buffer. Spleen cells were then depleted of B cells by treatment with monoclonal antibodies RA3-3A1 (a rat IgM anti-B220 antibody) and 331.12 (a rat IgG2b anti-mouse IgM antibody) (both obtained from American Type Culture Collection). Antibodies were diluted in DMEM plus 5% FBS to a final concentration of 10 μg/ml, and cells were incubated for 30 min on ice with this solution. Following incubation, the cells were collected by centrifugation and the incubated with 1:15 rabbit complement (H-2; Pel Freeze) for 30 min at 37°C. Both steps were repeated, and the resulting cells were washed extensively after the final incubation before analysis by flow cytometry (FACScan; Becton Dickinson, San Francisco, Calif.) with a panel of monoclonal antibodies to murine cell surface antigens (Pharmingen, San Diego, Calif.). Optimal staining concentrations of antibodies were determined in separate experiments with normal spleen cells. Cells were inoculated at the indicated numbers both i.p. and intravenously (i.v.) via the lateral tail vein in a volume of 200 μl.

Enumeration of bacteria in tissues. For the clearance studies, organs were aseptically removed from infected mice and placed in sterile sampling bags (Fisher Scientific, Pittsburgh, Pa.) containing DMEM with 10% heat-inactivated FBS (Hyclone). Organs were homogenized (in a Stomacher 80 Laboratory Blender [Seward Medical, London, England]), serially diluted, and plated on MHA plates. Colonies were counted after 3 days of incubation at 37°C under 5% CO_2.

RESULTS

Characterization of the anti-LVS response in serum. At 4 weeks after sublethal LVS challenge in mice, serum contains anti-LVS antibody that transfers specific immunity to naive animals (13). To determine the time course of the antibody response, serum taken from mice inoculated i.d. with 10^3 LVS bacteria at various time points was analyzed for anti-LVS antibody content by ELISA (Fig. 1). Using polyclonal anti-Ig antibody that reacts equally well with all antibody isotypes, we detected LVS-specific antibody in the serum by day 5 after i.d. infection. This response peaked at 14 days, and anti-LVS titers remained high up to 98 days after infection. Characterization of the immune serum by using isotype-specific antibody showed that the IgM LVS-specific response was detected at day 3, peaked with a titer of 1:7,200 by day 14, and declined gradually thereafter. The IgG2a titer reached 1:16,600 by day 14 but remained high through 98 days (Fig. 1). The IgG2a isotype was predominant, with small amounts of IgG1 (1:800), IgG2b (1:400), and IgG3 (1:800) observed and virtually no IgA (<1:20) detected at day 30 after i.d. infection. Therefore, substantial amounts of antibody to *F. tularensis* can readily be detected in the serum of sublethally infected mice; most of the antibody is IgM and IgG2a. Peak amounts of antibodies are present from day 14 through approximately day 30.

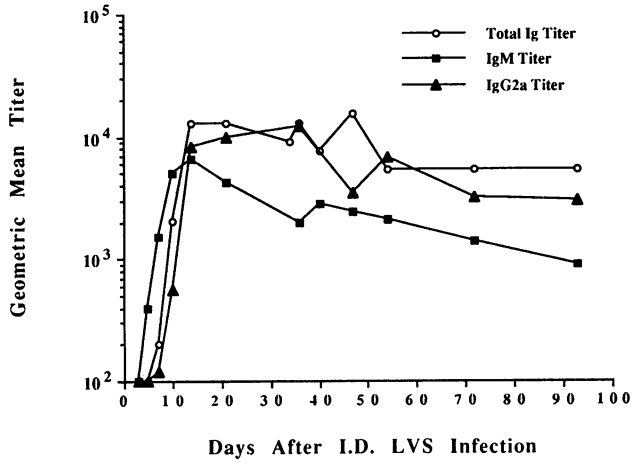

FIG. 1. Time course and isotype pattern of anti-LVS antibody production in mice. C3H/HeN mice were individually marked, prebled, and inoculated i.d. with a sublethal dose of 10^3 LVS bacteria, and serum was obtained from days 3 to 96 after inoculation. Serum from different time points was characterized for antibody response to LVS by ELISA and end point titers were determined as described in Materials and Methods. Error bars are omitted for clarity but were less than 20%. This experiment is representative of three experiments of similar design.

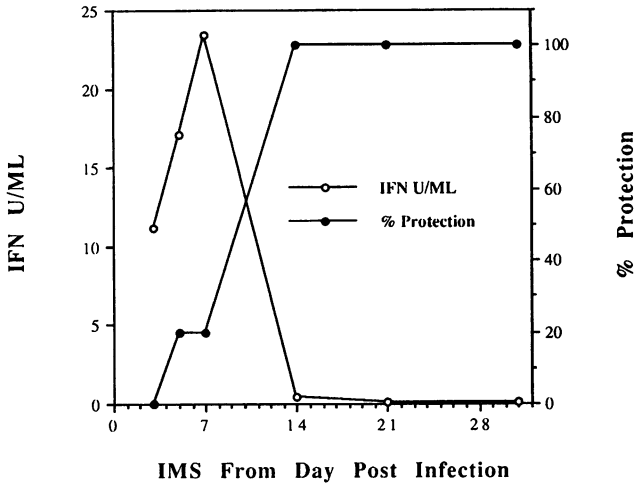

FIG. 2. Time course of development of protective capacity and IFN content of IMS. Groups of five C3H/HeN mice were prebled and infected i.d. with 10^3 LVS bacteria, and serum was collected via cardiac puncture at various time points thereafter (from 3 to 31 days after inoculation). Serum samples were assessed by ELISA for IFN content and ability to transfer protection to groups of five recipient C3H/HeN mice; mice were inoculated i.p. with 0.5 ml of either NMS or IMS at different dilutions as indicated and then challenged i.p. with a lethal dose of 10^3 LVS bacteria. Survival was observed daily for 21 days. Data are shown as units of IFN per milliliter of serum on the left y axis and percent protection (number of animals surviving/total number of animals inoculated) on the right y axis. This experiment is representative of three experiments of similar design.

Characterization of protection by immune serum. In previous transfer studies, 1 ml of undiluted immune serum (from mice inoculated 4 weeks previously with a sublethal i.d. dose of LVS) protected naive mice from 10^3 LVS bacteria given i.p. but not from higher doses (15). This dose is 3 log units greater than the i.p. LD_{50} of LVS (10, 13). Here, further analysis of the protective capacity of this high-titer antibody indicated that relatively large amounts of antibody are needed to effect protection. IMS obtained 4 weeks after i.d. infection protected 100% of mice (five of five) against 10^3 LVS bacteria given i.p. when diluted 1:5 but only 20% (one of five) when diluted 1:10 and not at all (none of five) when diluted 1:100. The magnitude of protection afforded by IMS was determined by calculating the LD_{50} for mice treated with 0.5 ml of NMS compared with that for mice treated with 0.5 ml of a 1:5 dilution of IMS. Treatment of mice with a 1:5 dilution of NMS results in an i.p. LD_{50} of 3, whereas treatment of naive mice with a 1:5 dilution of IMS results in an i.p. LD_{50} of 3.6×10^3, about 1,000 LD_{50}s for NMS. Although IMS clearly protects against substantial lethal infection, its capacity is notably lower than protection seen in the intact immune animal: immune mice can withstand a challenge of over 10^6 LVS bacteria inoculated i.p. (8, 10, 13).

Time course of development of protection, and role of IFN-γ in serum in passive transfer. We (18) and others (2) have shown that IFN is essential for development of immunity after i.d. infection with LVS. To examine the possibility that IFN present in serum samples contributed to protection, we quantitated IFN protein levels in serum by IFN ELISA (23). IFN was present in serum of i.d. infected mice, but peak levels were observed at 7 to 10 days; levels were below limits of detection by 14 days. However, serum obtained from mice at days 3, 5, 7, and 10 had little or no protective capacity. Complete protection was observed only when mice were treated with serum obtained 14 days or more after sublethal i.d. infection (Fig. 2). Thus, the protective capacity of serum does not correlate with IFN levels in the serum itself (Fig. 2) but correlates well with the development of IgG levels (Fig. 1).

Characterization of isotype responsible for passive transfer of protection. To directly test whether antibody was responsible for protection, purified IgM and IgG fractions of IMS were isolated from an anti-mouse IgM agarose column and a protein A-Sepharose column, respectively, and inoculated i.p. Amounts of purified IgM and IgG were adjusted to be approximately the amounts of IgM- or IgG-specific antibody in IMS, as determined by measurement of end point titer. Mice receiving 0.5 ml of the IgM fraction (with an end point IgM titer of 1:2,560 and no detectable IgG) were not protected against a lethal dose of LVS and died within 5 days, as did controls that received NMS and LVS i.p. (Table 1). On the other hand, mice receiving 0.5 ml of the purified IgG fraction (with an end point

TABLE 1. Purified IgG LVS-specific antibody protects mice against i.p. LVS infection[a]

Serum transferred	Titer		No. dead/ total no.
	IgM	IgG	
NMS (1:5)	<1:10	<1:10	5/5
IMS (1:5)	1:5,120	1:10,240	0/5
Protein A	1:20	1:5,120	0/5
Anti-IgM	1:2,560	<1:10	5/5

[a] Pools of NMS and IMS from C3H/HeN mice were tested for titers of LVS-specific IgM and IgG antibodies, and then portions of the IMS pool were purified by using a protein A affinity column or an anti-IgM affinity column as described in Materials and Methods. Resulting fractions were again tested for titers of LVS-specific IgM and IgG antibodies, and 0.5 ml of either a 1:5 dilution (for NMS or IMS) or undiluted antibodies (for protein A or anti-IgM fractions) was inoculated i.p. into groups of five naive C3H/HeN mice. All mice were challenged immediately thereafter with 10^3 LVS i.p., and survival was monitored for 21 days. This experiment is representative of three experiments of similar design.

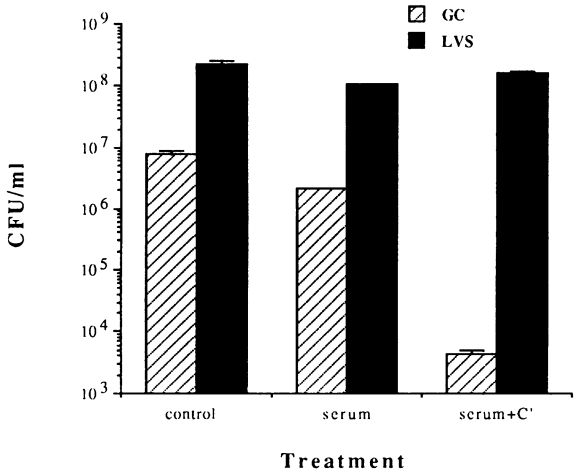

FIG. 3. Direct effects of IMS and complement on in vitro growth of bacteria. *N. gonorrhoeae* (GC) and LVS were grown on gonococcal agar and MHA plates, respectively, at 37°C under 5% CO_2 and were diluted in Hanks buffered saline solution to an OD_{600} of 0.1. The bacteria were mixed with either Hanks buffered saline solution, heat-inactivated (56°C for 30 min) antiserum, or an exogenous source of complement. Triplicate reaction mixtures were rotated for 1 h at 37°C. After incubation, mixtures were serially diluted and plated on appropriate plates to determine viable CFU per milliliter; percent survival was calculated by comparison with CFU remaining after control PBS treatment. Error bars represent one standard error of the mean. This experiment is representative of three experiments of similar design.

IgG titer of 1:5,120 and 1:20 IgM) i.p. were protected against a lethal i.p. challenge by LVS. Thus, purified LVS-specific IgG, most which is IgG2a (Fig. 1), was primarily responsible for the protective capacity of IMS.

Mechanisms of action. (i) Direct effects. Many intracellular pathogens, such as *N. gonorrhoeae*, are susceptible to either the neutralizing effects of antibody or direct toxic effects of serum mediated by complement activation. LVS susceptibility to direct effects of IMS in the presence or absence of exogenous complement was compared with that of *N. gonorrhoeae* (Fig. 3). *N. gonorrhoeae* and LVS were mixed with either Hanks buffered saline solution for controls, heat-inactivated antiserum alone, or heat-inactivated antiserum with an exogenous source of complement. Both LVS IMS (not cross-reactive with *N. gonorrhoeae*) (Fig. 3) and NMS (data not shown) inhibited *N. gonorrhoeae* growth, since 68% fewer bacteria grew in serum-treated cultures than in untreated cultures. *N. gonorrhoeae* is also acutely susceptible to lysis in the presence of a serum factor and complement (34), and 99% of these organisms are killed in the presence of IMS and complement (Fig. 3). In contrast, IMS, with or without exogenous complement, had no effect on the growth of LVS in vitro. Thus, although the preparation of IMS studied clearly contained factors that were toxic to *N. gonorrhoeae* and the effect of these factors was enhanced by the addition of exogenous complement (35), IMS was apparently not directly cytotoxic toward LVS itself.

(ii) Opsonic abilities. Antibody not only mediates complement activation and neutralization but also can opsonize particles for uptake by cells. To study the effects of serum on interactions with host cells, we used LVS treated with either IMS or NMS to infect resident peritoneal cells (Table 2). Treatment of LVS with IMS at either 4°C (results not shown) or 37°C to promote complement deposition (Table 2) did not

TABLE 2. IMS has no effect on uptake and growth of LVS in peritoneal cells

LVS treatment[a]	LVS counts (CFU) after[b]	
	2 h	72 h
PBS	5.0×10^4	1.4×10^8
NMS	6.3×10^4	5.8×10^8
IMS	6.1×10^4	2.5×10^8

[a] LVS (10^5 bacteria) and PBS, NMS, or IMS were mixed, incubated at 37°C, and used to infect mouse peritoneal cells.

[b] Infected cells were washed at the indicated times after infection and sampled to assess total bacterial CFU; standard errors for groups of three cultures were less than 10%. This experiment is representative of three experiments of similar design.

opsonize the bacteria for increased uptake by resident peritoneal cells. Further, none of these treatments altered the replication of intracellular bacteria in peritoneal cells after infection (Table 2, 72 h). Therefore, pretreatment of LVS with control NMS or with anti-LVS IMS has no significant effect on either entry into cells or growth within cells, at least in vitro.

(iii) Clearance from tissues. Infection foci in mice inoculated i.p. with LVS are localized in the spleen, liver, and lungs (13). To determine the effect of serum on localization of bacteria in organs, we obtained organs from NMS-treated and IMS-treated mice at various days after LVS inoculation and assessed them for the number of LVS CFU per organ (Fig. 4). As early as 1 day after NMS treatment and i.p. inoculation of a lethal dose of LVS, spleen tissues from infected mice had 10^3 to 10^5 bacteria per organ, while cultures of spleens taken from IMS-treated LVS-infected mice at day 1 had 1 to 2 log units fewer bacteria. Bacteria replicated in the organs of both groups of mice, however, between 2 and 4 days after infection, and achieved substantial burdens even in spleens of IMS-treated mice (Fig. 4a); previous results indicate that burdens of this magnitude were observed in lethally infected mice (10, 13). Growth rates of bacteria in tissues from NMS-treated and IMS-treated LVS-infected mice were similar in spleens from 2 to 4 days after infection (Fig. 4a). By 5 days the majority of NMS-treated LVS infected mice died but IMS-treated mice survived. After 5 days there was a precipitous drop in bacterial counts from all organs of IMS-treated mice, and clearance of bacteria was complete by 14 to 21 days (Fig. 4b). Very similar trends were observed in growth and clearance of bacteria from livers and lungs, although growth rates were somewhat slower in lung tissue (data not shown).

(iv) Requirement for recipient IFN production and T-cell function. The above time course of bacterial replication in host tissues, coupled with the negative in vitro data, suggested that IMS (in addition to affecting the uptake of bacteria by infected organs) provided time for development of a contributing host response. To directly test the requirement for a contribution by the host immune system, we treated immunocompromised athymic *nu/nu* mice, which lack mature functional T cells, with either NMS or IMS and challenged them with LVS i.p. Both normal and T-cell-deficient *nu/nu* mice died within 6 days after treatment with NMS and LVS (Table 3). Although normal *nu/+* control mice were protected from lethal LVS infection by passive transfer, *nu/nu* mice were not. Treatment of *nu/nu* mice with IMS extended their mean time to death from subsequent LVS infection by about 3 days, but the mice died by day 10. Further, *nu/+* mice treated with a monoclonal antibody to IFN at the time of IMS transfer and LVS challenge did not survive the infection (Table 3). T-cell-deficient *nu/nu* mice treated with anti-IFN at the time of IMS transfer and LVS challenge

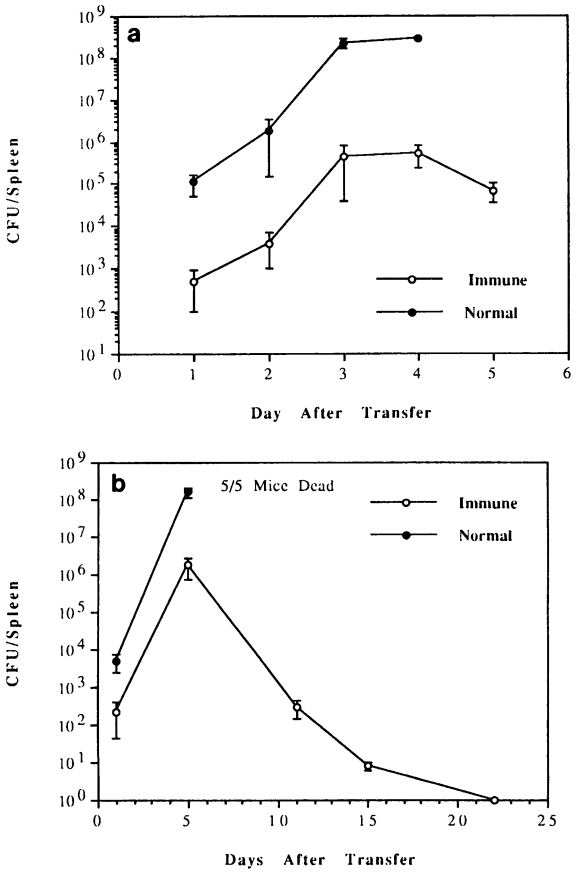

FIG. 4. Bacterial burdens in organs of serum-treated, LVS infected mice. Groups of two (a) or five (b) C3H/HeN mice were inoculated i.p. with 0.5 ml of 1:5 IMS or NMS and then challenged with a lethal i.p. dose of 10^3 LVS bacteria. Mice from each group were sacrificed on days 1 to 5 (a) or days 1, 5, 11, 15, and 22 (b) after inoculation, and the spleens, livers, and lungs were removed and homogenized in DMEM plus 10% heat-inactivated FBS. Tissue homogenates were serially diluted in DMEM plus 10% heat-inactivated FBS and plated on duplicate MHA plates, and the number of CFU per organ was calculated. Only results for spleen homogenates are shown. No detectable bacteria were found in any sample at day 22. Error bars represent one standard error of the mean. These experiments are representative of four experiments of similar design.

also died, with a mean time to death of 6 days (Table 3). The IMS itself contained no detectable IFN (Fig. 2). Thus, transfer of protection by IMS is not effective in T-cell-deficient *nu/nu* mice and requires host IFN production even in normal mice.

To directly test the role of T cells in transfer of protection by IMS, we reconstituted *nu/nu* mice with T cells from normal mice. Cells enriched for normal T cells were prepared by treatment of normal spleen cells with anti-B-cell antibodies and complement; analysis of the enriched cells by flow cytometry indicated that the cells were 63% $CD4^+$, 23% $CD8^+$, 89% $CD3^+$, and less than 3% $B220^+$ (with control staining of 2%). The T cells were then transferred both i.p. and i.v. into immunocompromised *nu/nu* mice. One day later, anti-LVS serum was transferred and mice were challenged with LVS, with and without anti-IFN treatment. Athymic *nu/nu* mice given IMS died from LVS challenge in about 10 days, and anti-IFN treatment further reduced the mean time to death to 5 days (Table 4). T-cell-reconstituted mice, however, survived

TABLE 3. Protection by transfer of LVS-specific antibody requires IFN and is not effective in athymic mice

Mouse strain	Serum transferred[a]	Antibody treatment[b]	No. dead/ total no.	Mean time (days) to death[c]
BALB/c *nu/+*	NMS	HIgG	5/5	6.0
	IMS	HIgG	0/5	
	IMS	Anti-IFN	5/5	6.0
BALB/c *nu/nu*	NMS	HIgG	5/5	6.5
	IMS	HIgG	5/5	9.5
	IMS	Anti-IFN	5/5	6.0

[a] Groups of five normal BALB/c *nu/+* mice or athymic, T-cell-deficient *nu/nu* mice were given 0.5 ml of a 1:5 dilution of NMS or IMS i.p., as indicated.

[b] Immediately after serum treatment, mice were treated i.p. with either 200 μg of control HIgG or 200 μg of the H22 monoclonal antibody to IFN-γ (Anti-IFN). Immediately thereafter, all mice were challenged i.p. with 10^3 LVS bacteria. Survival was monitored for 21 days. This experiment is representative of three experiments of similar design.

[c] The standard error of the mean was less than 10%.

LVS infection following IMS transfer, unless they were also treated with anti-IFN (Table 4). Thus, transfer of protection by IMS requires active participation of mature T cells in the recipient host animal, and at least one function of host T cells may involve IFN production.

DISCUSSION

Although previous studies have suggested that antibodies play only a small role in protective immune responses to intracellular bacteria, including *Francisella* species (1, 33, 34), the studies described here clearly show that IgG LVS-specific antibodies mediate protection in this transfer model (Table 1). Transfer protects against a challenge of over 1,000 LD_{50}s (see text), but this is noticeably less than the challenge that an animal with an intact immune system is able to withstand (10, 13). Thus we believe that the LVS-specific antibody response is a useful component of the protective immune response to lethal *Francisella* infection. Most interestingly, characterization of the passive transfer of protection by antibodies in the LVS model reveals that protection is not at all passive but is dependent on IFN production and the active participation of mature T cells in the recipient to effect clearance of bacteria from host tissues (Tables 3 and 4; Fig. 4). This, in turn, suggests that one function of the antibody response in infections such as these is to promote cytokine production and activation of specific T cells, which then function in a complex series of events leading to clearance of bacteria.

Outside of the recently developed *scid* mouse system, it has been difficult to establish a mouse model that permits study of infection and immunity in the absence of B cells and B-cell products (i.e., antibody); passive-transfer systems have been widely used to assess the role of antibodies both in secondary immunity and as immunotherapeutic agents. However, little direct evidence on the mechanism of protection afforded to patients by products such as IVIg is actually available (24). As shown here (Fig. 3) and previously (20), neither IMS nor IMS with an exogenous source of complement is directly toxic to the bacteria in vitro, nor does serum affect the in vitro uptake or growth of LVS in mouse peritoneal cells (Table 2). Other results further suggest that neither NMS nor IMS affects uptake or killing by polymorphonuclear leukocytes (data not shown) (26, 27). Nonetheless, on day 1 after infection with a lethal dose of LVS, NMS-treated mice had higher bacterial burdens in organs than did IMS-treated mice. This difference could be due to agglutination and/or alteration by antibodies of

TABLE 4. Protection by transfer of LVS-specific antibody requires IFN and T cells

Mouse strain	Medium or T cells transferred[a]	Serum transferred[b]	Antibody treatment[c]	No. dead/ total no.	Mean time (days) to death[d]
BALB/c *nu/+*	Medium	NMS	HIgG	5/5	4.8
	Medium	IMS	HIgG	0/5	
	Medium	IMS	Anti-IFN	5/5	
BALB/c *nu/nu*	Medium	NMS	HIgG	5/5	4.8
	Medium	IMS	HIgG	5/5	9.4
	Medium	IMS	Anti-IFN	5/5	5.0
	T cells	IMS	HIgG	0/5	
	T cells	IMS	Anti-IFN	5/5	6.0

[a] On day 0, groups of five normal BALB/c *nu/+* mice or athymic T-cell-deficient *nu/nu* mice were given either medium or both 3×10^6 enriched T cells i.p. and 1.5×10^7 enriched T cells i.v., as indicated.

[b] On day 1, mice were given 0.5 ml of a 1:5 dilution of NMS or IMS i.p., as indicated.

[c] Immediately after serum treatment, mice were treated i.p. with either 200 μg of control HIgG or 200 μg of the H22 monoclonal antibody to IFN-γ (Anti-IFN). Immediately after treatment, all mice were challenged i.p. with 10^3 LVS bacteria. Survival was monitored for 21 days. This experiment is representative of two experiments of similar design.

[d] The standard error of the mean was less than 10%.

the dissemination of bacteria from the peritoneum, but further study is necessary to determine the precise cause of this difference. The initial effects observed may also contribute to the slight delay in time to death observed when *nu/nu* mice were treated with IMS and challenged (Table 4). More importantly, all bacterial burdens increased between days 2 and 4 (Fig. 4a), and previous results indicate that these bacterial burdens in the spleen following i.p. infection are uniformly lethal (9, 10, 13). After 5 days, however, bacterial counts decreased in IMS-treated mice, and complete resolution of systemic *Francisella* infection was observed by day 21. The time course of clearance suggested an active host contribution: some initial effects were observed, but the most apparent effects, i.e., decline of bacterial burdens in IMS-treated mice, occurred very late after infection. Very similar findings were reported almost 20 years ago by Collins (7). These studies demonstrated that mice treated with anti-*Salmonella enteritidis* IMS and then challenged with virulent *S. enteritidis* had 1 log unit fewer bacteria in livers and spleens initially; however, growth rates in these organs paralled growth rates in NMS-treated mice until day 7 after infection but declined thereafter. Collins concluded that an emerging cellular response, rather than remaining transferred antibody, was responsible for the eventual clearance of bacteria (7). Here we find a similar pattern of response in the LVS model, and for the first time we extend these observations to directly demonstrate a requirement for recipient IFN production and participation of mature host T cells (Tables 3 and 4). Further study is necessary to determine whether the protective effects of antibodies are due to simple delay of dissemination of bacteria during the infection, permitting time for T-cell responses to the bacteria. Alternatively, antibodies themselves may be involved in direct activation of T cells in an antibody-dependent cellular cytotoxicity-like mechanism, such as was previously observed in immunity to *Pseudomonas aeruginosa* (21). In either case, these results suggest that evaluation of the direct protective capacity of antibodies in passive-transfer models must be interpreted with caution.

It is most likely that the T-cell requirement reflects a need for specific cell-mediated recognition and/or cytokine production. T-cell products such as IFN play a role in determining the character of humoral responses, and our previous results (18) suggest a major role for IFN in survival of i.d. LVS infection. IFN is detectable in the serum of infected mice (but not healthy mice) soon after i.d. infection (Fig. 2), and its presence may result in the preference for production of the IgG2a isotype after i.d. infection (11, 31). Time course (Fig. 1) and purification (Table 1) studies demonstrate that IFN itself is not responsible for the transfer of protection, however. Rather, IFN must be produced by transfer recipients for successful protection (Tables 3 and 4). IFN production by host NK cells probably also contributes to the small delay in mean time to death of *nu/nu* mice given IMS and challenged (Tables 3 and 4). Future studies will examine whether T cells are responsible for the majority of IFN production or whether IFN production followed by other T-cell functions is part of a sequence of events leading to protection and will also examine the T-cell subpopulation(s) involved.

Clearly, not all successful transfer of protection by antibody requires T cells; for example, antibodies to *Streptococcus pneumoniae*, which can be produced independently of helper T-cell activity (5), protect athymic *nu/nu* mice from experimental *S. pneumoniae* infection (35). Monoclonal antibodies to the outer surface protein A of *Borrelia burgdorferi* protect *scid* mice from symptomatic infection, and protection correlates with antibody-mediated activation of an oxygen burst by bone marrow-derived macrophages (28, 30). On the other hand, other studies also suggested an interesting interaction between T cells and specific antibody to another extracellular bacterium: successful transfer of protection by *Pseudomonas*-specific T cells was dependent on adsorption of cytophilic IgG3 *Pseudomonas*-specific antibody (21). The mechanisms by which specific antibodies participate in protection to these extracellular pathogens are probably quite different from those for intracellular pathogens. However, the similarities between our results and the observations made by Collins with the *Salmonella* model (7) suggest to us that this T-cell-dependent antibody-mediated protection may not be not limited to *Francisella* species but is most likely to be operative with other intracellular bacteria also. We therefore believe that the requirement for active T-cell participation in protection against disease by transfer of specific antibodies deserves further study and may explain a variety of ambiguous results on the role of specific antibody in protection against infection.

These results may also have implications for the use of commercial IVIg preparations in treatment of infectious diseases and the development of high-titer antisera specific for individual pathogens. Currently, IVIg products, prepared from

pooled serum of normal donors and containing relatively low-affinity antibodies, are used primarily as replacement therapy for individuals with primary and secondary immunodeficiencies (6); these products are expected to be effective mainly against environmental (primarily extracellular) pathogens when phagocytic cell function is intact (6, 24). Although the effectiveness of IVIg is well established for immunodeficient patients (6), its efficacy in prevention of nosocomial infections in low-birth-weight infants has been equivocal (3, 29); the immaturity of the neonatal immune system could be a contributing factor. Interestingly, delay in time to serious bacterial infection was observed in asymptomatic human immunodeficiency virus-infected children treated with IVIg only when their $CD4^+$ T-cell counts were $>2 \times 10^9$ cells per liter at the time of treatment (23). The results presented here suggest that lack of T-cell function may contribute to these observations, and they emphasize the need for understanding the mechanism by which transferred antibody exerts its protective effects (24).

Thus, immunotherapy with specific antibodies may first permit a delay in an infectious process, giving the recipient an important head start for induction of cytokine production and specific T-cell-mediated protective immunity against some lethal pathogens. Similarly, specific antibody present in the immunocompetent animal at the time of secondary challenge may alter the time course and/or stimulation of LVS-specific T-cell memory responses such that cell-mediated immunity is ultimately able to effectively combat the bacteria. Adjunctive therapies, such as the combined use of antibiotic and antibody treatment, should be evaluated in this context: antibiotic treatment may lower the bacterial burden but at the expense of generating strong secondary immunity from active infection. The means by which specific antibodies, cytokines, and T cells interact in effecting protection against LVS is therefore an area for further study.

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