Immunization with Ehrlichia P28 Outer Membrane Proteins Confers Protection in a Mouse Model of Ehrlichiosis

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The obligately intracellular bacterium Ehrlichia chaffeensis that resides in mononuclear phagocytes is the etiologic agent of human monocytotropic ehrlichiosis (HME). HME is an emerging and often life-threatening, tick-transmitted infectious disease in the United States. Effective primary immune responses against Ehrlichia infection involve generation of Ehrlichia-specific gamma interferon (IFN-γ)-producing CD4+ T cells and cytotoxic CD8+ T cells, activation of macrophages by IFN-γ, and production of Ehrlichia-specific antibodies of the Th1 isotype. Currently, there are no vaccines available against HME. We evaluated the ability of 28-kDa outer membrane proteins (P28s) using a DNA prime-protein boost regimen and challenged with E. muris had significantly lower bacterial loads than the spleens of mock-vaccinated mice. Mice immunized with P28-9, P28-12, P28-19, or the mixture of these three P28 proteins (P28s) using a DNA prime-protein boost regimen and challenged with E. muris had significantly lower bacterial loads than the spleens of mock-vaccinated mice. Mice immunized with P28-9, P28-12, P28-19, or the mixture induced Ehrlichia-specific CD4+ Th1 cells. Interestingly, mice immunized with P28-14, orthologs of which in E. chaffeensis and E. canis are primarily expressed in tick cells, failed to lower the ehrlichial burden in the spleen. Immunization with the recombinant P28-19 protein alone also significantly decreased the bacterial load in the spleen and liver compared to those of the controls. Our study reports, for the first time, the protective roles of the Ehrlichia P28-9 and P28-12 proteins in addition to confirming previous reports of the protective ability of P28-19. Partial protection induced by immunization with P28-9, P28-12, and P28-19 against Ehrlichia was associated with the generation of Ehrlichia-specific cell-mediated and humoral immune responses.

Human monocytotropic ehrlichiosis (HME) is an important emerging tick-transmitted disease (24). HME in immunocompetent individuals can present as a flu-like illness that may develop into a severe disease with manifestations of toxic shock-like syndrome. Death occurs in 3% of cases and is preceded by lymphocytopenia, thrombocytopenia, and liver injury (8). HME is caused by Ehrlichia chaffeensis, one of the most prevalent life-threatening tick-borne pathogens in North America (22). This unusual pathogen, which infects monocytes and macrophages, is an obligately intracellular, Gram-negative bacterium that lacks the inflammatory pathogen-associated molecular pattern molecules, lipopolysaccharide and peptidoglycan, but contains several antigenic proteins (14, 27, 32). Lack of early diagnosis and treatment of HME and being immunocompromised are the main risk factors that lead to severe and fatal disease. Ehrlichia chaffeensis causes a transient subclinical infection with no reported pathology in immunocompetent mice and does not offer the best opportunity to study a model of disease resembling HME (35). However, murine models of systemic infection associated with the mildly virulent Ehrlichia muris or the highly virulent IOE (Ixodes ovatus Ehrlichia) in C57BL/6 mice have provided knowledge of immunological mechanisms involved in host defenses against ehrlichial infection (7, 11, 23, 29). Protective immunity against E. muris or IOE in the mouse models of mild or fatal ehrlichiosis, respectively, correlates with induction of strong cell-mediated CD4 and CD8 type 1 responses and humoral immunity (11). T cell-independent humoral immunity has also been reported to be sufficient for protection against fatal intracellular ehrlichial infection (2).

The development of effective vaccines for pathogens requiring cellular and humoral immunity has been impeded by lack of understanding of the factors required for generation of long-term effective and optimal memory responses. Understanding the factors required for the generation of vaccination-induced long-term memory immune responses that are sufficient to control an intracellular Ehrlichia infection is equally important as the identification of protective ehrlichial antigens is. The P28-19 (OMP-1g) outer membrane protein of E. chaffeensis has been identified as an effective target mediating clearance of the bacteria (14, 22). Recombinant P28-19 of IOE also elicited strong humoral and CD4 T cell responses in C57BL/6 mice and induced significant protection against lethal challenge (17). Additionally, a p28 gene-based naked-DNA vaccine (MAPI) was found to protect mice against challenge with a lethal dose of Ehrlichia ruminantium (19). Several vaccination strategies including regimens of pathogen DNA priming followed by administration of homologous recombinant
proteins have demonstrated enhanced immune responses compared with vaccines using DNA vaccination alone (6, 18). A significant constraint to vaccine development for *Ehrlichia* is the high antigenic diversity that is present in outer membrane protein genes among different isolates of a particular species including *E. chaffeensis*. In nature, there appear to be three stable variant *E. chaffeensis* lineages that can be identified based on their p28 alleles (3, 15). This antigenic diversity among strains of *E. chaffeensis* must be considered in the development of broadly effective vaccines. In this study, we examined whether DNA gene priming immunization followed by recombinant protein booster immunization would induce improved protection against *Ehrlichia*. We chose several P28 paralogs representative of different regions of the multigene p28 locus of *E. muris*, all of which are known to be transcriptionally active in mice (4), as experimental immunogens. P28-19 was also chosen because an epitope within the amino terminus of the first hypervariable region of the *E. chaffeensis* gene, which encodes a disulfide bond-forming protein (MAb) with high avidity (13). Predominant expression of P28-14 orthologs of *E. chaffeensis* and *Ehrlichia canis* in tick cells, but not in mammalian cells (27, 28, 33), suggests that it might be required for colonization and survival within the tick environment, and considering it as a potential vaccine in comparison with the other P28 paralogs was worth investigating. In this paper, we demonstrate that P28-9, P28-12, and P28-19, but not P28-14, confer partial protection against *Ehrlichia* in a mouse model by inducing T cell and antibody responses.

### MATERIALS AND METHODS

**Plasmid DNA constructs.** The open reading frames (ORFs) for *Ehrlichia muris* p28-9, p28-12, p28-14, and p28-19 (GenBank accession number DQ335244) were directionally cloned into pcDNA 3.1/CT-GFP-TOPO (GFP stands for green fluorescent protein) designed for high-level expression in mammalian hosts under the control of the cytomegalovirus (CMV) promoter and into pET102D-TOPO, which allows cloning the gene of interest as a fusion with His-Patch thioredoxin (Invitrogen, Carlsbad, CA) (Table 1). Sequence analysis using an ABI Prism 377 DNA sequencer (Perkin Elmer Applied Biosystems, Foster City, CA) was performed on all constructs to verify the proper orientation and frame of the insert. pWRG/mL-12 is a pBluescript plasmid that contains the two subunits of murine interleukin-12 (IL-12), p40 and p35. Both subunits are under separate CMV promoter regions and contain bovine growth hormone poly(A) signal. pWRG/mL-12 was kindly provided by Hua Yu, Moffitt Cancer Center, Tampa, FL. For vaccine purposes, the DNA was used after purifying using an endotoxin-free mega plasmid prep kit from Qiagen (Valencia, CA) following the manufacturer’s instructions. Plasmid DNA expressing *E. muris* p28-9, p28-12, p28-14, and p28-19 and control constructs used in the vaccine were dissolved in normal saline (0.9% NaCl) to a concentration of 2 μg/ml.

**Mice.** Six- to 8-week-old female C57BL/6 mice were used in all experiments. Mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed and cared for in the Animal Research Center at the University of Texas Medical Branch in accordance with the Institutional Animal Care and Use Committee guidelines under whose review and approval the experiments were conducted.

**Immunizations and *Ehrlichia muris* challenge.** C57BL/6 mice were divided into 6 groups with 8 mice per group. The mice in groups 1 to 4 were inoculated with 100 μg of p28-9, p28-12, p28-14, or p28-19 endotoxin-free plasmid DNA mixed with 100 μg of pWRG/mL-12 in saline. Group 5 was inoculated with a 100-μg mixture of all four p28 constructs (25 μg each), mixed with 100 μg of pWRG/mL-12, and group 6 was inoculated with 100 μg of pcDNA 3.1/CT-GFP vector only, which was also mixed with 100 μg of pWRG/mL-12. All DNA immunizations were administered intramuscularly in the tibialis anterior muscle. As stated above, all mice immunized with DNA received 100 μg of purified pWRG/mL-12 in saline to enhance a Th1-type cell-mediated immune response. A second DNA immunization identical to the first was administered 4 weeks later. Four weeks after the last DNA inoculation, 100 μg of purified p28-9, p28-12, P28-14, and P28-19 recombinant proteins or a mixture of the four proteins (25 μg each) in Freund’s complete adjuvant were administered subcutaneously to mice in groups 1 to 5, respectively. Similarly, mice in group 6 received His-Patch thioredoxin protein as a negative control. A booster immunization of His-Patch thioredoxin was given intramuscularly in the tibialis anterior muscle, 1 day prior to challenge, and the sera were collected. Seven days after challenge, the remaining mice were sacrificed, and their spleens and sera were harvested. Part of the spleen was used to determine the frequency of antigen-specific gamma interferon (IFN-γ)-producing CD4+ T cells, and the other part was used to determine the chorial load by real-time PCR. Sera were assayed for determination of antibody titers.

In the experiments involving immunization with the recombinant P28-19 alone (see Fig. 4, 5, and 6), 50 μg of the protein was mixed with Freund’s complete adjuvant and administered by the i.p. route (3 animals per group). The second dose of immunization was administered on day 14 after the first immunization in Freund’s incomplete adjuvant. The mice were challenged 14 days after the second immunization with *E. muris* (~1 × 10<sup>9</sup> bacterial genomes) by the i.p. route.

**Assessment of chorial load in organs by quantitative real-time PCR.** The bacterial burdens in the organs were determined by quantitative real-time PCR. The *Ehrlichia*-specific *dab* gene, which encodes a disulfide bond-forming protein (GenBank accession numbers AY236484 and AY236485) was selected as the target gene for amplification of *E. muris* DNA. The sequences of the primers and probes and thermal cycle conditions were described previously (30). PCR analyses were considered negative for chorial DNA if the critical threshold values (*C<sub>T</sub>*) exceeded 40 cycles. The chorial load in organs was normalized relative to the total DNA. Each sample was run in triplicate.

### TABLE 1. Primers used for cloning and expressing P28 constructs of *E. muris*

| P28 paralog | Sequences of primer pairs for PCR and cloning into the following vector: | pET102D expression vector | pcDNA3.1CT/GFP vector |
|-------------|------------------------------------------------------------------------|--------------------------|------------------------|
| P28-9       | 5'-CACCATGGGAGGCAATACAG-3'                                               | 5'-GCCATGGGAGGCAATACAG-3' |
|             | 5'-AACCTGCGCACAAGATTCG-3'                                               | 5'-CTCTGGCACCAAGATCCG-3'  |
| P28-12      | 5'-CACCTGAGGATACCTAGGAGCA-3'                                            | 5'-GCCATGGAATATCATTAGGGCA-3' |
|             | 5'-ACCAAATGTTAATCACATAGG-3'                                             | 5'-CAAGATCCACACATCAAGTG-3'  |
| P28-14      | 5'-ACCGTAGGGAGGCATTACTATG-3'                                            | 5'-GCCATGTAAGGAGGCAGCGA-3'  |
|             | 5'-CTCAATGCTCCACCAAGATTCG-3'                                            | 5'-CTCAATGCTCCACCAAGATTCG-3'  |
| P28-19      | 5'-CACCGCTTCTACTTTGAGATTTTCGAC-3'                                       | 5'-GCCATGCGCTTCTACTTTGGAGATTTTCCGC-3' |
|             | 5'-CTCTTCTCAAGTCTATACCAAAATG-3'                                         | 5'-CTCTTCTCAAGTCTATACCAAAATG-3'  |
Antibody responses. One day before challenge and on day 7 after E. muris challenge, serum samples from vaccinated mice were collected for measurement of antibody titers against E. muris by indirect immunofluorescence assay (IFA) as described previously (see Fig. 3) (23). Antigen slides were prepared from E. muris-infected DH82 cells. Serum samples were diluted, and 10 μl was applied to each well on a slide and incubated for 30 min at 37°C in a humidified chamber. The antigen slides were washed and then incubated with 10 μl of fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at a 1:100 dilution. The slides were then washed, counterstained with Evans blue, and examined under a Nikon Labphoto UV microscope. Serological endpoint titers were expressed as the reciprocal of the highest dilution at which specific fluorescence was detected.

Measurement of P28-19-specific antibody responses. An enzyme-linked immunosorbent assay (ELISA) was performed to measure the concentration of E. muris-specific IgG subclasses antibodies as described previously (11, 16). Briefly, each well in a 96-well ELISA plate was coated with 100 μl of a 22-amino-acid synthetic peptide corresponding to a previously identified dominant B cell epitope in the amino terminus of the first hypervariable region of the P28-19 protein (see Fig. 5 and 6) (13) at a concentration of 4 μg/ml in 50 mM sodium bicarbonate buffer, pH 9.6. Serum samples were diluted 1:100, and 100 μl of each sample was added to each antigen-coated well and incubated at 24°C for 2 h. Alkaline phosphatase-conjugated goat anti-mouse IgG or IgG1, IgG2a, IgG2b, IgG3, and IgM antibodies (Southern Biotech, Birmingham, AL) were added at a dilution of 1:1,000, and the plates were developed using Blue Pharos phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Optical densities were measured using an ELISA plate reader (Molecular Devices, Sunnyvale, CA) at 650 nm after 30 min of incubation at room temperature. The wells (on microtiter plates) coated with an unrelated control 22-amino-acid peptide were used to determine the background level for the ELISA. All assays were performed in duplicate wells, and the average values were calculated for analysis after subtraction of the background absorbance.

Splenocyte cultures and in vitro recall cellular immune responses. The frequencies of antigen-specific IFN-γ-producing T cells in the spleens were determined by flow cytometric analysis. Splenocytes, either pooled from 3 mice/group (on microtiter plates) coated with an unrelated control 22-amino-acid peptide were used to determine the background level for the ELISA. All assays were performed in duplicate wells, and the average values were calculated for analysis after subtraction of the background absorbance.

RESULTS

Immunization with the outer membrane proteins P28-9, P28-12, and P28-19 confers partial protection against E. muris infection. The protective capacity of the memory immune response induced by different genes of the E. muris P28 family was investigated in C57BL/6 mice. To this end, C57BL/6 mice were immunized with recombinant DNA plasmids carrying the p28-9, p28-12, p28-14, and p28-19 genes or a mixture of all four in combination with IL-12 DNA on days 0 and 28 followed by two homologous recombinant protein booster immunizations on days 56 and 84. Mock-immunized mice were immunized similarly with empty vector/unrelated protein (His-Path thioredoxin) and served as controls. Immunized mice were challenged intraperitoneally with a high dose (~1 x 10^6 bacterial genomes) of E. muris 28 days after the last booster immunization, and the bacterial burden in the spleen was determined on day 7 after challenge. A high bacterial burden was detected in the spleens of mice immunized with empty vector/protein (mock-vaccinated mice) (Fig. 1). In contrast, the spleens of mice vaccinated with P28-9, P28-12, P28-19, and the P28 mixture exhibited significantly reduced bacterial loads on day 7 postinfection than those of the mock-vaccinated control group (Fig. 1). Interestingly, our data indicated that immunization of mice with P28-14, orthologs of which in E. canis are primarily expressed in tick cells, failed to lower the E. muris burden in the spleen.

 Outer membrane proteins P28-9, P28-12, and P28-19 induce strong antigen-specific type 1 immune responses. Previous studies have shown that infection of C57BL/6 mice with E. muris, a mildly virulent Ehrlichia species, elicits a potent effect- tor CD4^+ and CD8^+ type 1 T cell response in the spleen (11, 17). In addition, recently we showed that heterologous protec-
tion against lethal ehrlichial infection following immunization with live *E. muris* organisms is associated with expansion of effector memory CD4^+^ and CD8^+^ T cells producing IFN-γ as well as a substantial antibody response (31). To determine the induction of memory T cells in mice vaccinated with different P28 proteins (P28s), we measured *Ehrlichia*-specific CD4^+^ and CD8^+^ T cell responses in mice before and after *E. muris* challenge. Compared to the vector/protein control group, the animals vaccinated with P28-9, P28-12, and P28-19 and those immunized with the mixture had higher frequencies of *Ehrlichia*-specific IFN-γ-producing CD4^+^ Th1 cells 7 days after *E. muris* challenge (Fig. 2). We did not detect higher frequencies of *Ehrlichia*-specific CD4^+^ Th1 cells after *in vitro* antigenic stimulation in the spleens of mice vaccinated with P28-14, which failed to induce protective immunity, compared to the other P28-vaccinated groups and the vector/protein controls. No difference in the number of antigen-specific IFN-γ-producing CD8^+^ memory T cells in the vaccinated groups compared to vector controls was observed (data not shown). These results indicate that vaccination with P28-9, P28-12, or P28-19 effectively increased the magnitude of protective antigen-specific CD4^+^ Th1 memory cells, which correlated with lower bacterial burdens in the vaccinated mice.

**Ehrlichia**-specific IgG antibody production is associated with protection in vaccinated mice. To address the roles of antibodies in the control of *Ehrlichia* infection in vaccinated mice, we determined the titers of anti-*E. muris* antibodies in the sera of all vaccinated and control mice 28 days after the last booster immunization and on day 7 after challenge infection. A higher concentration of *E. muris*-specific IgG antibodies was detected in the sera of all vaccinated animals, including P28-14-immunized mice, compared to mice vaccinated with the vector/protein control (Fig. 3). Furthermore, the infection boosted the antibody response as indicated by an increase in antibody titers in the sera from vaccinated and subsequently challenged mice (Fig. 3).

**Recombinant P28-19 protein induces a strong antibody response and confers protection.** Previous studies suggested a role for the orthologs of P28-19 of *E. chaffeensis* and IOE in protection (12, 17, 21). In the present study, the P28-19 DNA/protein immunization was found to reduce the ehrlichial burden significantly. Therefore, we further evaluated recombinant P28-19 (rP28-19) as a vaccine candidate. To this end, we immunized mice with two doses of rP28-19 (15 days apart) and challenged them with *E. muris* (≈1 × 10^8^ bacterial genomes) 15 days after the last dose of immunization. We assayed the bacterial burden in the spleens and livers of mice vaccinated with the P28-19 protein and unvaccinated mice harvested on days 7 and 14 after *E. muris* challenge by quantitative real-time PCR. The spleens of rP28-19-vaccinated mice had significantly lower bacterial loads on day 7 than the spleens of unvaccinated mice (Fig. 4A). Furthermore, on day 14, there were no detectable bacteria by PCR in either the spleens or livers of the rP28-19-vaccinated mice (Fig. 4A and B).

Analysis of P28-19-specific antibody responses by ELISA on days 7, 14, and 21 after *E. muris* challenge demonstrated that the IgG response in vaccinated mice challenged with *E. muris* was highest on day 14 compared to day 7 or 21 (Fig. 5). We further analyzed the isotypes of P28-19-specific antibodies in sera from mice. Both vaccinated and unvaccinated mice challenged with *E. muris* had higher concentrations of P28-19-specific IgM and IgG2b antibodies on day 7 after the challenge (Fig. 6A). The rP28-19-vaccinated mice challenged with *E.
were transcriptionally active and were associated with the development of antigen-specific IgG responses. P28-19-specific antibody responses were measured by an ELISA using a 22-amino-acid synthetic P28-19 peptide as the antigen. Mice immunized with the recombinant E. muris P28-19 protein had higher concentrations of P28-19-specific IgG antibodies on day 14 after E. muris challenge than mice in the unvaccinated control group. Antibody responses in mice immunized with the recombinant P28-19 alone are presented for comparison. The data were expressed as means plus standard deviations, and three mice from each group were used for this analysis. OD650nm, optical density at 650 nm.

**FIG. 5.** Protection induced by the recombinant P28-19 protein was associated with the development of antigen-specific IgG responses. P28-19-specific antibody responses were measured by an ELISA using a 22-amino-acid synthetic P28-19 peptide as the antigen. Mice immunized with the recombinant E. muris P28-19 protein had higher concentrations of P28-19-specific IgG antibodies on day 14 after E. muris challenge than mice in the unvaccinated control group. Antibody responses in mice immunized with the recombinant P28-19 alone are presented for comparison. The data were expressed as means plus standard deviations, and three mice from each group were used for this analysis. OD650nm, optical density at 650 nm.

**DISCUSSION**

P28 family members are the most studied E. chaffeensis outer membrane proteins (OMPs). They have multiple predicted transmembrane β strands and are encoded by an antigenically variant multigene family composed of 22 paralogous genes clustered in a 27-kb gene locus of the E. chaffeensis genome (20, 21). Although these proteins seem to play a critical role in host-microbe interaction, they have not yet been explored as potential vaccine candidates against monocytotropic Ehrlichia infection. Several studies have shown that these P28 proteins are highly conserved among Ehrlichia species (4, 21, 26, 34), and therefore, we examined whether different P28 paralogs of E. muris are appropriate candidates for vaccines against Ehrlichia. In this study, we analyzed the ability of recombinant DNA vaccines expressing different genes of the E. muris P28 family and the homologous recombinant proteins given in a prime-boost regimen to stimulate long-term protective memory T and B cell responses and protect mice against Ehrlichia infection. Our study indicated, for the first time, the protective roles of P28-9 and P28-12 in addition to confirming the previous reports of the protective role of P28-19 against monocytotropic Ehrlichia (13, 17, 21). Immunization of mice with P28-9, P28-12, and P28-19 provided protection against E. muris as evidenced by reduction in bacterial burden (Fig. 1). A previous study showed that mice immunized with the recombinant E. chaffeensis P28-19 (rP28) enhanced the spontaneous clearance of the infection in BALB/c mice (21). A study by J. S. Li et al. indicated that MAb directed against the E. chaffeensis P28-19 (OMP-1g) reduced the bacterial burden in SCID mice (13). Further, the P28-19 (rOMP-19) of IOE was demonstrated to provide protection against fatal disease caused by IOE in C57BL/6 mice (17).

Our study indicated that mice immunized with P28-14 failed to develop protective immunity against challenge with E. muris cultured in mammalian cells (DH82 cells) administered i.p., which could be due to low levels of expression of P28-14 in the vertebrate host. Although it is not known whether P28 proteins of E. muris are differentially expressed in the vertebrate and tick hosts, our previous study indicated that all 21 p28 genes of E. muris were transcriptionally active in vivo on day 9 postinfection in mice (4). Previous reports suggested that E. chaffeensis and E. canis predominantly express orthologs of P28-19 and P28-20 in mammalian cell lines, compared with expression of orthologs of P28-14 in tick cell lines (27, 28, 33). Further studies indicated that mice infected with E. chaffeensis cultured in tick cells developed prolonged infection with higher bacterial burden than mice infected with E. chaffeensis grown in....
macrophages (9). Although proteomic analysis indicated the predominant expression of the P28-19 and P28-20 proteins of *E. chaffeensis* and their orthologs of *E. canis* in mammalian cells, reverse transcription-PCR (RT-PCR) analysis provided evidence for transcriptional activity of the majority of the genes within the p28 family (3, 15, 20). In addition, Zhang et al. provided indirect evidence for expression of all 22 p28 genes in dogs persistently infected with *E. chaffeensis* by demonstrating the presence of antibodies to the specific region of the individual P28s (36). These studies not only suggest different levels of expression of P28 proteins in mammalian cells but also reflect differences in the sensitivities of the methods employed. It is interesting to note that *Borrelia burgdorferi* OspA and OspC, which are primarily expressed in tick and mammalian cells, respectively, induce protective immunity against tick transmission of *Borrelia* (5, 10). However, unlike OspC, the protective role of antibodies to OspA, which is primarily expressed in tick cells, is restricted to killing of spirochetes in the midgut of engorging ticks (5). Although the *Ehrlichia* P28-14 protein did not induce protection against i.p. challenge with *E. muris* cultured in mammalian cells in the present study, it remains to be determined whether P28-14 is effective in blocking tick transmission of *Ehrlichia* or is effective against i.p. challenge with the bacteria cultured in tick cells.

Our study indicated that the protection induced by P28-9, P28-12, and P28-19 was associated with the induction of antigen-specific IFN-γ-producing CD4+ T cells. Furthermore, we provided evidence of the induction of P28-19-specific CD4+ Th1 cells in mice infected with *E. muris*, which suggests that P28-19 targets CD4+ T cells during the infection (Fig. 7). Previous studies from our laboratory and others have indicated that CD4+ T cells and IFN-γ are required for protection against *Ehrlichia* (1, 7). Moreover, the cross-protection induced by *E. muris* against fatal infection with IOE in mice was associated with the induction of Th1 responses (11, 31). These findings are consistent with the increased severity of *E. chaffeensis* infection observed in HIV-infected patients (25). We did not find higher frequencies of IFN-γ-positive CD4+ T cells in mice immunized with P28-14, which could be due to low levels of P28-14 expression in *E. muris* cultured in DH82 cells used for *in vitro* stimulation of T cells.

Partial protection induced by P28-9, P28-12, and P28-19 was correlated with the development of *Ehrlichia*-specific IgG antibodies. Studies from our laboratory and others have previously demonstrated the importance of antibodies in protection against *Ehrlichia*. Passive transfer of polyclonal immune sera or MAbs conferred protection in SCID mice against *E. muris* and *E. chaffeensis*, respectively (7, 12, 13). Mice immunized with the recombinant P28-19 protein alone had higher concentrations of antigen-specific IgG1, IgG2b, and IgG3 on day 14 after *E. muris* infection (Fig. 6B), which coincided with the reduction of bacteria to undetectable levels in the spleens and livers of these mice. In contrast, unimmunized mice challenged with *E. muris* developed P28-19-specific IgG2c, IgG2b, and IgG3 antibodies by day 21 postchallenge. The data were expressed as means plus standard deviations, and three mice from each group were used for this analysis.

FIG. 6. Induction of *Ehrlichia*-specific IgG1, IgG2b, and IgG3 antibody isotypes was associated with protection in mice immunized with P28-19 protein. P28-19-specific antibody responses in mice immunized with the P28-19 protein on days 7, 14, and 21 after *E. muris* challenge were measured by ELISAs. (A) Comparable concentrations of P28-19-specific IgM and IgG2b antibodies were found in sera from mice immunized with P28-19 protein and unimmunized mice on day 7 after *E. muris* challenge. (B) Mice immunized with the recombinant P28-19 protein had higher concentrations of P28-19-specific IgG1, IgG2b, and IgG3 antibodies on day 14 after *E. muris* challenge than the controls. (C) In contrast, unimmunized mice challenged with *E. muris* developed P28-19-specific IgG2c, IgG2b, and IgG3 antibodies by day 21 postchallenge. The data were expressed as means plus standard deviations, and three mice from each group were used for this analysis.
grown in DH82 cells as discussed above. The induction of P28-14 in the antigen preparations obtained from specific antibodies as determined by IFA of sera from mice infected with E. muris developed during P28-19-specific IFN-γ-producing CD4⁺ T cells in uninfected mice. The frequencies of E. muris-infection-induced immune responses. The presence of CD4⁺ T cells in spleens of mice infected with E. muris by flow cytometry. The spleens of mice infected with E. muris had higher frequencies of P28-19-specific IFN-γ-producing CD4⁺ T cells on day 45 after infection than the spleens of naive uninfected mice. The frequencies of E. muris-specific IFN-γ-producing CD4⁺ T cells in the spleens of the same mice detected following in vitro stimulation with the E. muris whole-cell lysate were shown for comparison. In the graph at the bottom of the figure, each symbol represents the value for an individual mouse, and the short horizontal lines represent the mean for the group.

Similar to these findings, J. S. Li et al. reported that the majority of highly effective P28-19-specific MAbs recovered from E. chaffeensis-infected C57BL/6 mice were of the IgG2c isotype, but not IgG1 (13). The same group reported the production of all IgG isotypes (IgG1, IgG2c, IgG2b, and IgG3) in C57BL/6 mice immunized with the recombinant OPE P28-19 (OMP-19) (17). These studies suggested that the quality of antibody responses elicited by immunization, which usually involves use of adjuvants, is fundamentally different from infection-induced immune responses. The presence of Ehrlichia-specific antibodies as determined by IFA of sera from mice immunized with P28-14 suggests either possible cross-reactivity with the other P28s or the presence of a low concentration of P28-14 in the antigen preparations obtained from E. muris grown in DH82 cells as discussed above. The induction of antibody responses in mice immunized with P28-14 also suggests that the lack of protection observed in these mice is not due to the failure of the development of immune responses.

In conclusion, our study showed that the Ehrlichia P28 outer membrane proteins exhibit differential roles in protective immunity against Ehrlichia. Our data also indicate for the first time the protective roles of P28-9 and P28-12 in addition to confirming previous findings of the protective role of P28-19 against Ehrlichia. The partial protection induced by the P28 outer membrane proteins was associated with the generation of CD4⁺ Th1 and Ehrlichia-specific IgG responses. The lack of protection against Ehrlichia by i.p. challenge observed in mice immunized with P28-14 possibly suggests the importance of using a challenge model involving tick transmission of Ehrlichia to identify transmission blocking protective candidate antigens.

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