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# Expression of Members of the 28-Kilodalton Major Outer Membrane Protein Family of *Ehrlichia chaffeensis* during Persistent Infection

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The 28-kDa immunodominant outer membrane proteins (P28 OMPs) of *Ehrlichia chaffeensis* are encoded by a multigene family. As an indirect measure of the in vivo expression of the members of the *p28* multigene family of *E. chaffeensis*, sera from two beagle dogs experimentally infected with *E. chaffeensis* were evaluated for the presence of specific antibodies to P28 OMPs by enzyme-linked immunosorbent assay. Antigenic peptides unique to each of the P28s were identified within the first hypervariable region of each P28 OMP. Serological responses to peptides derived from all P28 OMPs were detected from day 30 postinoculation to day 468 and from day 46 until day 159 in the two beagles. Although antibody titers to the peptides fluctuated, the peak response to all of the peptides appeared simultaneously in each dog. The antibody responses to another outer membrane protein of *E. chaffeensis* (GP120) showed similar temporal and quantitative changes. These data suggest that the P28 OMPs are expressed concurrently during persistent *Ehrlichia* infection.

Human monocytic ehrlichiosis, an emerging infectious disease, is caused by *Ehrlichia chaffeensis*, an obligate intracellular gram-negative bacterium that resides in endosomes of host cells. *E. chaffeensis* is a member of the order *Anaplasmataceae*, which also includes the genera *Anaplasma*, *Neorickettsia*, and *Wolbachia* (9). The life cycle of *E. chaffeensis* involves a tick vector and a mammalian host. Mammals are infected with *E. chaffeensis* by infected ticks, and noninfected ticks acquire *E. chaffeensis* by a blood meal from infected animals. *E. chaffeensis* is not transovarially transmitted (20). Thus, mammalian hosts are essential for the persistence of *E. chaffeensis*. *Anaplasma* and *Ehrlichia* species can cause persistent infection in their natural mammalian hosts (1, 3, 6, 11, 14, 28, 29, 38). Persistent or prolonged ehrlichial infection in humans has been reported for both *E. chaffeensis* (10, 25) and *A. phagocytophilum* (8, 15).

Persistent ehrlichial infection required that the ehrlichiae evolve mechanisms to evade the host immune response. An evasion strategy utilized by many extracellular bacterial pathogens is variable expression of cell surface components. For example, the periodic cycling of acute febrile and afebrile episodes during relapsing fever caused by the spirochete *Borrelia hermsii* is associated with dramatic changes in the surface antigens of the spirochetes circulating in the blood (26). *Neisseria gonorrhoeae* also undergoes phenotypic variation of its surface proteins as a result of recombination among genes in a multigene family (13). The 28-kDa immunodominant outer membrane proteins (P28 OMPs) of *E. chaffeensis* are also encoded as a polymorphic multigene family (24, 36), which consists of 22 homologs (22, 36). Amino acid identity among the P28 OMPs ranges from 20 to 83%.

Three regions of the P28 OMPs are highly variable and have been designated hypervariable regions (HVRs). A previous study showed that mouse and human sera recognized an immunodominant epitope within the first HVR (HVR1) of one P28 (P28-19) (17), indicating that HVR1 is highly antigenic. Antigenic variability of the P28 OMPs has been reported for clinical isolates of *E. chaffeensis* (17, 22, 24, 36). Data from studies of *Anaplasma marginale* have suggested that antigenic variation in Msp-2 OMPs is responsible for bacterial persistence (2). The P28 OMPs share homology with the Msp-2 OMPs of *A. marginale*. However, a role for P28 OMPs in antigenic variation during ehrlichial infection has not been fully resolved.

A previous study indicated that there is no antigenic variation of P28 OMPs resulting from genetic recombination (35). One question remaining is whether the differential expression of *p28 omp* genes causes persistent infection. The transcription of the *E. chaffeensis p28* genes was investigated previously with reverse transcription-PCR (19, 34, 36). It was reported that transcripts of all the *p28 omp* genes were detected from an infected dog except for the *p28-2* gene (22). However, other studies have detected transcripts for fewer *p28* genes in cell culture (5, 19, 22). Differences in the detection of *p28 omp* gene transcripts by reverse transcription-PCR could be due to experimental variables, such as RNA template quantity and quality, or primer specificity. Alternatively, it is possible that mRNA and protein expression were not coincident due to posttranscriptional regulation.

An alternative approach to examine P28 OMP expression during persistent infection is to determine if antibodies are generated to individual P28 OMPs during persistent infection. This approach was possible because serological analyses of humans and animals have shown that the P28 OMPs are immunodominant (4, 31). Therefore, in this study we analyzed the host humoral response to the P28 OMPs as an indirect means of monitoring protein expression. Our findings suggest

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TABLE 1. Primers used for amplification of p28 genes

| Gene | Forward primer | Reverse primer |
|---|---|---|
| p28-1′ | CACCGAAACAGCTGGGTTTTACGCA | GACTTCACCACCATAGTTGTCTGC |
| p28-1 | CACCCAGTACAAGCCAGCCAGACAACACC | TCCAATACTCCCACCGTAATATCC |
| p28-2 | CACCGGAGGACAATATAAACCAGG | ATTCCGATACTGCTAGTAAG |
| p28-3 | CACCATGCACCAAAACAACAATATAG | ACTTAATCCAATTTCACTGC |
| p28-4 | CACGCATTAATAGGCAATGTAGA | CCTTATTCCAATTTCACTTCC |
| p28-5 | CACCGGATTGTACATAAGTGGTCAAT | TCTCATCCCAACTTCACCACC |
| p28-6 | CACCGCTATAAATCATAATCATACA | TGTAAACTTTACTCCAGCTTCACC |
| p28-7 | CACCGGGCTATATGTCAGTGGACAAT | CCCAAATTCACTACCGAAATACTC |
| p28-8 | CACCAGTGGGCAATATAAACCAGGGA | GGCTACTGCGTATACAGGTCC |
| p28-9 | CACCACAGGAAATGTAAGTAACCATAC | TCTTACTCCAATTTCGCCACC |
| p28-10 | CACCGAGGTTACAAACAGCAG | TCTTACTCCAACTTCAGCGCCG |
| p28-11 | CACCGGAATCATAAATAACAATGC | TATAAACCTTACTCCAACTTCACCAC |
| p28-12 | CACCCAGTTTGGGTTATATGTTAG | TATAAGCCTTGCTCCAATTTCAC |
| p28-13 | CACCGAAACTATAAACAACAGTGC | TGTGAATCTTATTCCAACTTCGC |
| p28-14 | CACCGATCCTGTAACTTCAAATG | CTAGAAGGTGAACCTTACTCCAACT |
| p28-16 | CACCTCTGATCCAGTACAGGATG | GAAGTTAAACCTTCCTCCAAG |
| p28-17 | CACCGACAATATTAGTGGTAATTTC | TTAGAAGTTAAACCTTCCTCCAAG |
| p28-18 | CACCGTACGAACGACAATGT | TCCTCCAAGTTCCACTCCAAAG |
| p28-19 | CACCGAAGAAAAGAAATAACAACAG | TTAGAAAGCAAACCTTCCTCC |
| p28-20 | CACCGATGCAAACGTTCCTGAA | CTATAGTGTAAATCTCATACCAACT |
| p28-21 | CACCACATATAGGATAAATGGTG | CTATACAAATACCTTTATACCAAT |

that the P28 OMPs are expressed concurrently in persistently infected dogs. These data suggest that persistent *E. chaffeensis* infection is most likely not caused by antigenic variation of the P28 OMPs resulting from differential expression of the *p28 omp* genes.

## MATERIALS AND METHODS

**Dog sera.** The sera from two male beagle dogs (dog ACC and dog ADJ) experimentally infected with *E. chaffeensis* at 6 months of age were used in this study, and infection of the dogs was reported elsewhere previously (38). Briefly, the dogs were infected by subcutaneous inoculation of $10^6$ *E. chaffeensis* (Arkansas strain)-infected DH82 cells (7). *E. chaffeensis* was clonally purified at the beginning of the experiment by limiting dilution; 10 ml of blood was obtained from each dog prior to inoculation (day 0) and at 1-week intervals from day 8 to day 117 and at 2-week intervals from then until day 159 after inoculation. The blood was also drawn on days 248 and 462. The dogs were confirmed to be persistently infected with *E. chaffeensis* by reisolation of *E. chaffeensis* and detection of ehrlichial DNA from blood. Cell culture yielded ehrlichiae from the blood of dog ADJ collected from day 23 until day 81 after inoculation and yielded ehrlichiae from the blood of dog ACC collected from day 23 until day 102. *E. chaffeensis* DNA was detected from the dogs by PCR at the same time that culture yielded ehrlichiae, and 2 weeks after the cultures became negative.

Immune sera from a dog immunized with recombinant P28-1′ (dog AWF) was used to test the specificity of the synthetic peptides of P28 OMPs. The dog was immunized subcutaneously three times with 100 μg of recombinant p28-1′ protein each time. Blood samples were obtained from the dog prior to immunization and at 28 days after immunization.

**Cloning and expression of *E. chaffeensis* outer membrane proteins.** The primers listed in Table 1 were chosen manually from each gene to amplify all *p28* genes by PCR. The forward primers and the reverse primers were chosen as close as possible to the ends of each gene in order to express most of the coding sequences of the genes. The forward primer was downstream of the signal sequences, and the reverse primer was upstream of the stop codon. The primers were tested for formation of primer self-dimers, primer pair dimers, and self-loops with the PrimerSelect program (DNASTAR, Madison, Wis.). The specificity of the primers was predicted with the Editseq program (DNASTAR) by searching the *p28* locus sequences. Primers that were not predicted to amplify any of the *p28* genes except the gene from which they were derived were synthesized by Bio-synthesis (Lewisville, Tex.) and used for PCR.

The PCR products were directionally cloned into the pET102/D-TOPO vector (Invitrogen, Carlsbad, Calif.) in-frame with the thioredoxin gene on the 5′ end and V5 and His tag on the 3′ end. The positive clones were selected by PCR with a combination of one primer from the vector and one primer from the insert and

confirmed by DNA sequencing. The recombinant plasmids were transformed into *Escherichia coli* BLStar (DE3) (Invitrogen) to express the P28 proteins. The expression of the recombinant fusion proteins was confirmed with antibodies to V5 and thioredoxin by protein immunoblotting analysis. Recombinant P28 fusion proteins were purified with ProBond resin (Invitrogen), a nickel-charged Sepharose resin that binds to the His tag. The genes *p28-15* and *p28-19* were cloned into the PCR T7/CT TOPO vector as previously described (38). The genes for GP120 and P110, outer membrane proteins of *E. chaffeensis*, were cloned previously in the pGEX expression vector and expressed as GST fusion proteins in *E. coli* (32, 33). Glutathione *S*-transferase (GST) was used as a negative control for protein enzyme-linked immunosorbent assay (ELISA) and Western blot. The GST fusion proteins were purified with glutathione-Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, N.J.). Positope, a 53-kDa protein containing thioredoxin, His, and V5 epitopes, was also used as a negative control for protein ELISA and Western blot because most of the P28 recombinant proteins are fusion proteins containing thioredoxin, His, and V5 epitopes. The *E. coli*-expressed recombinant Positope protein was purchased from Invitrogen (Carlsbad, Calif.).

**Protein immunoblotting.** Proteins were separated on a two-dimensional NuPAGE 4 to 12% Bis-Tris gel (Invitrogen) and electroblotted onto nitrocellulose membranes. Each membrane was incubated with dog sera diluted at 1:100 in a Mini-Protean II multiscreen system (Bio-Rad Laboratories, Hercules, Calif.). Alkaline phosphatase-labeled anti-canine immunoglobulin G (heavy and light chains) purchased from Kirkegaard & Perry Laboratories (Gaithersburg, Md.) was used at 1:7,000 dilution.

**Epitope mapping the P28-19 protein.** Overlapping DNA fragments representing the entire *p28-19* gene were PCR amplified and subcloned into pET32-LIC as thioredoxin fusion proteins in a previous study (17). The recombinant peptides expressed in *E. coli* BL21(DE3) were used in ELISA to map the portion of P28-19 which stimulated the production of antibodies.

**Synthetic peptides of P28 protein.** The amino acid sequences of all 22 proteins of the P28 family were aligned with Clustal W (DNASTAR). Peptides of 12 to 20 amino acids were designed from the unique sequence of the first hypervariable region of each P28 OMP. The peptides were synthesized chemically by Biosynthesis (Lewisville, Tex.). The sequences of the peptides and their alignment are listed in Fig. 1. To obtain a baseline of dog sera reacting with synthetic peptides, we used three nonrelevant peptides derived from OmpB of *Rickettsia conorii* as negative controls. The nonrelevant peptides were OmpB 4 (TVGG QQGNK), OmpB 7 (LENGTTVKF), and OmpB 19 (ITVTLNKQA) (18).

**ELISA.** ELISA was used to determine the reactivity of dog sera with synthetic peptides and recombinant proteins. The synthetic peptides and recombinant proteins were diluted at a concentration of 1.2 μg/ml in distilled water. Peptides P28-9 to P28-12, P28-13, and P28-14 were dissolved in 50% dimethyl sulfoxide because they were insoluble in water. Fifty microliters of the peptide or protein solution was used to coat each well of 96-well plates at 4°C overnight. Each well

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Majority YISGQYKPSVSHFSNFSVKETN-----NTTVELFG-LKKDIDSIAIS------ISTNAN--FN-----I--PYSPKFQNN-FLGFSGAIGY-SMDGPRIE
p28-1    .VGI....ARQ.L.KLLI..S-----AAN...V...-...LLNDLLT-----G.KD.T.---.-------.--K.N.YYE..-R.....IF..YYNKNF...    113
p28-1'   .A..H.NIQFPR..PI...YKT----DEN.EKELTLFTLKESTDTPT------FKQKSE--..-----DKKG...IYNR.-YT.........-.GG.L.V.    135
p28-2    ..G.....G.PR.NR.L.TNN.IRELMSSDE.CRSTIPHMVQ.V.QGTLPPEALEEL.KGLLHGGYLFFTL..N.TYKK.-L..AG.V...-.TTHF.V.     134
p28-3    .VTIK.QLTTP..K..YI...D-----FD.Q.PI..A.ITANTKFDT-----LKE.FS--.SP---LHQTDSYKSY..D-L..IGLSV.L-FVKSF...      114
p28-4    .V.S.....IF..R...IQ.SHP---KKSSE.FKK-I.ANLNN.LK---------S.------------A.NLQ..D.-TTS...T...-FSK.L.L.       116
p28-5    .........IP..K....E.-.-----DKV.D.I.-.TT.VTY.TEH-----ILRD.TK--.-.-----T--H.IA..K..-.IN..S....Y.GQ...L.    117
p28-6    ..T...R.G.................-----VD.IQ.V.-.Y..SAS..DPN---------.YS.---.Q-----G-...TVT..D.-AAS.......SYPESL.L.    116
p28-7    .V......T........L...Y-----TD.K..L..A...K..TD-------.T..KK--.-----.---.NT...D.-AVS..A.V..I.Q.S..V.         118
p28-8    ........GI....K.....IY-----.DNIQ.I.-.RHNAI.TST-------LNI.TD--.-----.---.KVT....-ITS.......SDPT.A.F.          114
p28-9    ......R.G.....K..........-----YN.TQ.V.-.....SV.GN..-N-----.T.YT.---.-----F-...IAE..D.-AIS.......LYSENF...    119
p28-10   ...........V..S..I...........-----TI.KN.IA-.....N.LEVNADASQG..HPG.---.T-----.---.IAA.ED.-AFN.N.....-ITE.L...    121
p28-11   ...........V................-----F..QQ.VA-.......VD..TNADSG.NNPQ.---.T-----.---.I....D.-AAS....L.FFYAR.L.L.    122
p28-12   .V.........I................-----FP.KY.AA-.....N.VEFDDSVTAG..YPL.---.S-----T---.IAV..D.-ISN.N....TFVE.....    123
p28-13   ...........V...............-----VP.KQ..N.-.....N.V.VGSNATTG..NPG.---.T-----.---.TAE..D.-VAN.N..V..SFP.SL...    127
p28-14   ...VK.N...I...RK..AE.APINGNTSI.KKV..-....G.-..Q.------------.--.------R-TDPALE....LIS....S...-A........    121
p28-15   ....K.M..A....GV..A..E-----K.P..A.Y...Q.WNGVSA.------SHAD.D--..-----N-KG..F.YE..P...A......-..G....    116
p28-16   ....K.M..A....GV..A..E-----R....GV..-IEQ.W.RCV..R-----TTLSDI--.T-----V-PN..F.YE..L.S..A.....-........    117
p28-17   .V..K.M..A....GM..A..E-----K.P..A.Y...Q.WEG.SS.-----SHNDNH--.------N-KG..F.YE..P....A......-...G...V.    116
p28-18   ....K.V.......GV..A.QE-----R...TGV..-..Q.W.GST..K-----N.PENT--.-----V-PN..F.YE..P...A...V..-.L.N....    117
p28-19   ....K.M..A....GV..A..E-----R....GV...-.QNW.GS...N-----S.P.DV--.T-----V-SN..F.YE..P....A.....-........    116
p28-20   .VGT...VG.PN.....AE.-----LPGL.KSI.A-.GL.KS..SDH----------.G--.T-------QA.N.TYAS.-.A..G.V...-YVNDF.V.    110
p28-21   FLGIS..L.APL..S...IG..YRI-NGVK.DRVV.-...S..LLD.DK-----AMKDFN.---.------FSEE.V..YD..-IF.L.FIF..SF-RNL.V.    121
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FIG. 1. Alignment of the amino acid sequences of HVR1 and adjacent sequences of *E. chaffeensis* P28 OMPs. The corresponding sequences of the synthetic peptides for each P28 OMP are underlined. Dots represent residues that were identical in the majority of sequences. Dashes indicate gaps that were introduced to facilitate alignment.

was blocked with 1% bovine serum albumin for 1 h at 37°C and incubated with dog sera at 1:200 dilution. The plates were washed five times with BluePhos washing solution (Kirkegaard & Perry Laboratories, Gaithersburg, Md.); 50 μl of a 1:1,000 dilution of alkaline phosphatase-labeled anti-canine immunoglobulin G (heavy and light chains) was added to each well and incubated at room temperature for 30 min. After washing, color was developed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), and the optical density (OD) of the samples was read at a wavelength of 595 nm with the VERSAmax tunable microplate reader with Pro 4.0 software (Softmax, Sunnyvale, Calif.). The data were analyzed with Microsoft Excel. The ELISA data were normalized to determine the relative antibody titers of the sera obtained after inoculation of *E. chaffeensis* (infected serum) compared with that of the serum obtained prior to inoculation (preinfection serum) with the following equation: relative antibody titer of an infected serum = OD of the infected serum/OD of the noninfected serum − 1. The change of each sample was subtracted from 1 to set the baseline for preinfection serum at 0.

## RESULTS

**Antibody recognition of recombinant P28 OMPs by infected dogs.** To develop immunoassays for serological detection, 14 P28 OMPs were cloned and expressed in *E. coli*, and the recombinant P28 OMPs were used as antigens in Western blotting for detection of antibodies to *E. chaffeensis*. Twelve *p28* genes were cloned into the pET102/D-TOPO expression vector as part of this study, and *p28-15* and *p28-19* were cloned into pCRT7/CT TOPO previously (38).

Dog sera collected from day 0 to day 462 post-*E. chaffeensis* infection were used in Western blotting with 10 recombinant P28 OMPs (P28-8 to P28-19). All of the recombinant P28 OMPs were recognized by dog ACC sera starting from day 30 and continuing until day 462 and by dog ADJ sera starting from day 46 and continuing until day 462 (data not shown). These data suggested that all sera after day 30 in dog ACC and after day 53 in dog ADJ might react with other P28 OMPs. Thus, we tested antibody reaction with the remaining four recombinant proteins (P28-1′, -4, -5, and -7) with the serum collected on day 88 postinfection from each dog. The day 88 serum of dog ACC (Fig. 2) and dog ADJ (data not shown) reacted with all four recombinant P28 OMPs. Dog sera obtained prior to inoculation of *E. chaffeensis* did not react with

any recombinant P28 OMPs (data not shown), which suggested that the antibody reaction was specific to *E. chaffeensis* P28 OMPs. These data suggested either that all the P28 proteins were expressed during infection or that cross-reaction occurred among the homologous P28 OMPs. Therefore, the antibody response to the P28 OMPs was analyzed further with synthetic peptides specific to each P28 OMP.

**Epitope mapping of the P28-19 proteins.** To identify antibody epitopes in P28 OMPs, we performed epitope analysis with dog sera that recognized P28-19. P28-19 was selected for epitope mapping because a previous study showed that mouse and human sera recognized an immunodominant epitope within HVR1 of P28-19 (17) and recombinant P28-19 truncation variants were available. Truncation Δ3 included amino acids 26 to 172 and the first two hypervariable regions; truncation Δ2/3 contained amino acids 26 to 100, which included the first hypervariable region; truncation Δ1/2/3 consisted of amino acids 26 to 70, which were amino terminal to the first hypervariable region; and truncation Δ1 contained amino acids 101 to the carboxy terminus and included the second and third hypervariable regions (Fig. 3A).

Dog sera collected from days 8 to 95 postinoculation as well as day 0 serum were used to detect the P28-19 truncation variants by protein immunoblotting. Dog ACC (day 38 to day 95) and dog ADJ (day 53 to day 95) postinoculation sera reacted with variants Δ3 and Δ2/3, but none of the sera reacted with truncation Δ1/2/3. Dog ADJ sera collected between days 67 and 95 reacted weakly with truncation Δ1, but none of the dog ACC sera reacted with the truncation Δ1. Sera collected on day 0 did not react with any of the recombinant antigens (Fig. 3). Variants Δ1/2/3 and Δ3 but not Δ2/3 lacked HVR1, which suggested that HVR1 contained epitopes that were immunodominant, as has been demonstrated previously in mice (17). The data suggested that HVR1 peptides from the highly divergent P28 OMPs could be used to assay dog sera.

**Specificity of dog sera reacting with P28 synthetic peptides.** The background levels of nonspecific reactions of dog ACC and dog ADJ sera with synthetic peptides were determined
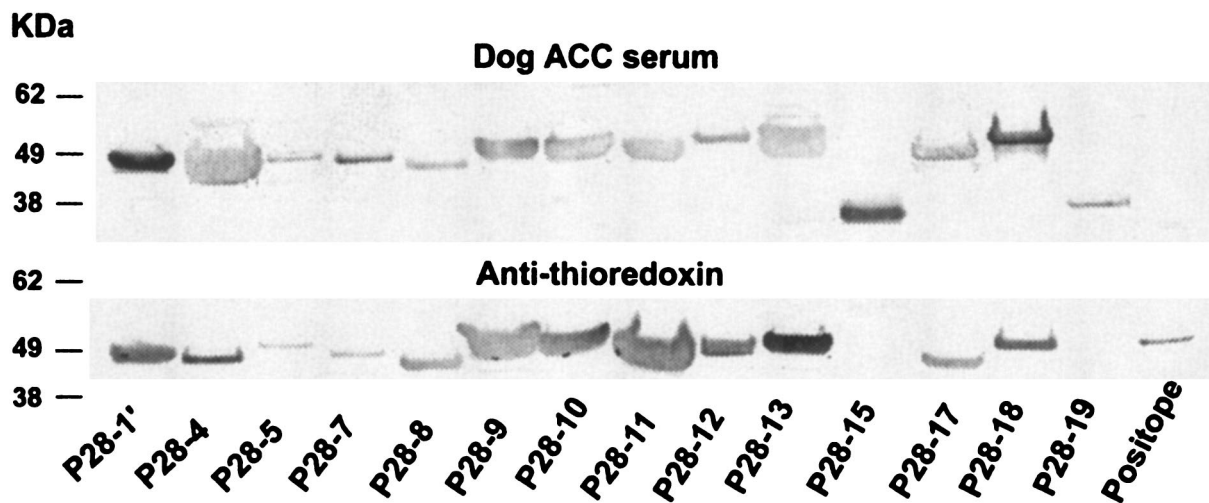
FIG. 2. Protein immunoblotting of recombinant P28 proteins reacting with dog ACC serum obtained on day 88 postinoculation of *E. chaffeensis* diluted at 1:200 (top). The dog sera did not react with the Positope protein that was used as a negative control. Positive controls for each recombinant P28 protein reacting with antithioredoxin antibodies are shown on the bottom. The antithioredoxin did not react with P28-15 and P28-19 because these proteins were not thioredoxin fusion proteins.

with nonrelevant peptides OmpB4, OmpB7, and OmpB19, which were derived from *R. conorii* OmpB (18). There was no significant increase in the ODs of the sera from both dogs postinoculation of *E. chaffeensis* (from day 8 to day 462) reacting with all P28 synthetic peptides compared to the OD of the preimmunization serum from each dog reacting with the same peptides (data not shown). The highest OD increase of post-inoculation sera above the OD of day 0 serum of dog ACC was 0.15-fold for day 88 serum reacting with the OmpB19 peptide. The highest OD increase of postinoculation sera above the OD of day 0 serum of dog ADJ was 0.16-fold for day 74 serum reacting with the OmpB19 peptide.

The cross-reaction between the synthetic peptides was tested with hyperimmune sera (dog AWF) that were immunized with recombinant P28-1′ protein. The antiserum to p28-1′ protein reacted strongly with the homologous synthetic peptides but very weakly with other P28 synthetic peptides (Fig. 4). The OD of the dog immune serum reacting with P28-1′ was at least fourfold higher than that of the serum reacting with other P28 synthetic peptides. Thus, the reaction between the P28 synthetic peptides and the antibodies to the P28s was specific.

**Reactivity of dog sera with synthetic peptides derived from HVR1 of P28 OMPs.** To detect antibodies that uniquely recognized each P28 OMP, we tested the reaction of sera of two persistently infected dogs with synthetic peptides representing each P28 HVR1 by ELISA. Synthetic peptides 12 to 20 amino acids in length derived from unique sequences of HVR1 of
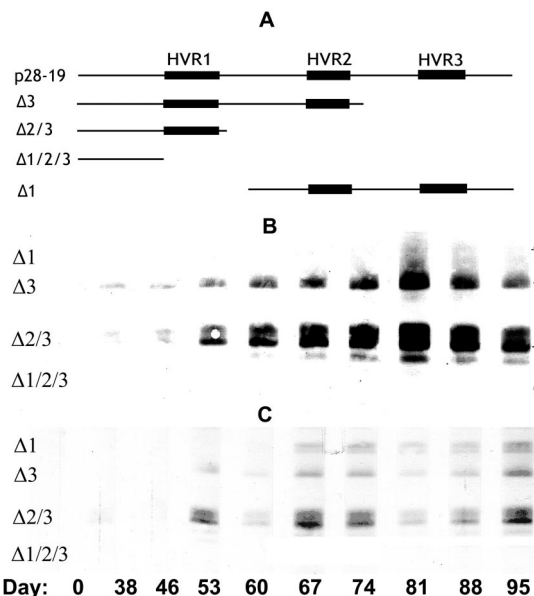


FIG. 3. Structures of truncated P28-19 proteins and their reactivity with dog sera. (A) Schematic diagram of the truncated P28-19 proteins illustrating three hypervariable regions (HVR1, HVR2, and HVR3) and their flanking sequences. Protein immunoblotting of sera from dog ACC (B) and dog ADJ (C) reacted with overlapping truncated recombinant proteins of P28-19: Δ1, Δ3, Δ2/3, and Δ1/2/3. The days postinoculation of *E. chaffeensis* when the sera were obtained are shown below the blots. The same amount of each protein was used in the Western blots for both dog sera.
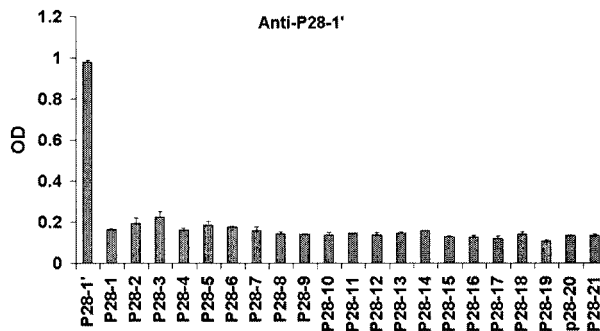


FIG. 4. Reaction of dog immune serum to P28-1′ proteins with synthetic peptides. The 22 synthetic peptides are shown on the *x* axis, and the OD values of the immune serum reacting with each peptide are shown on the *y* axis.
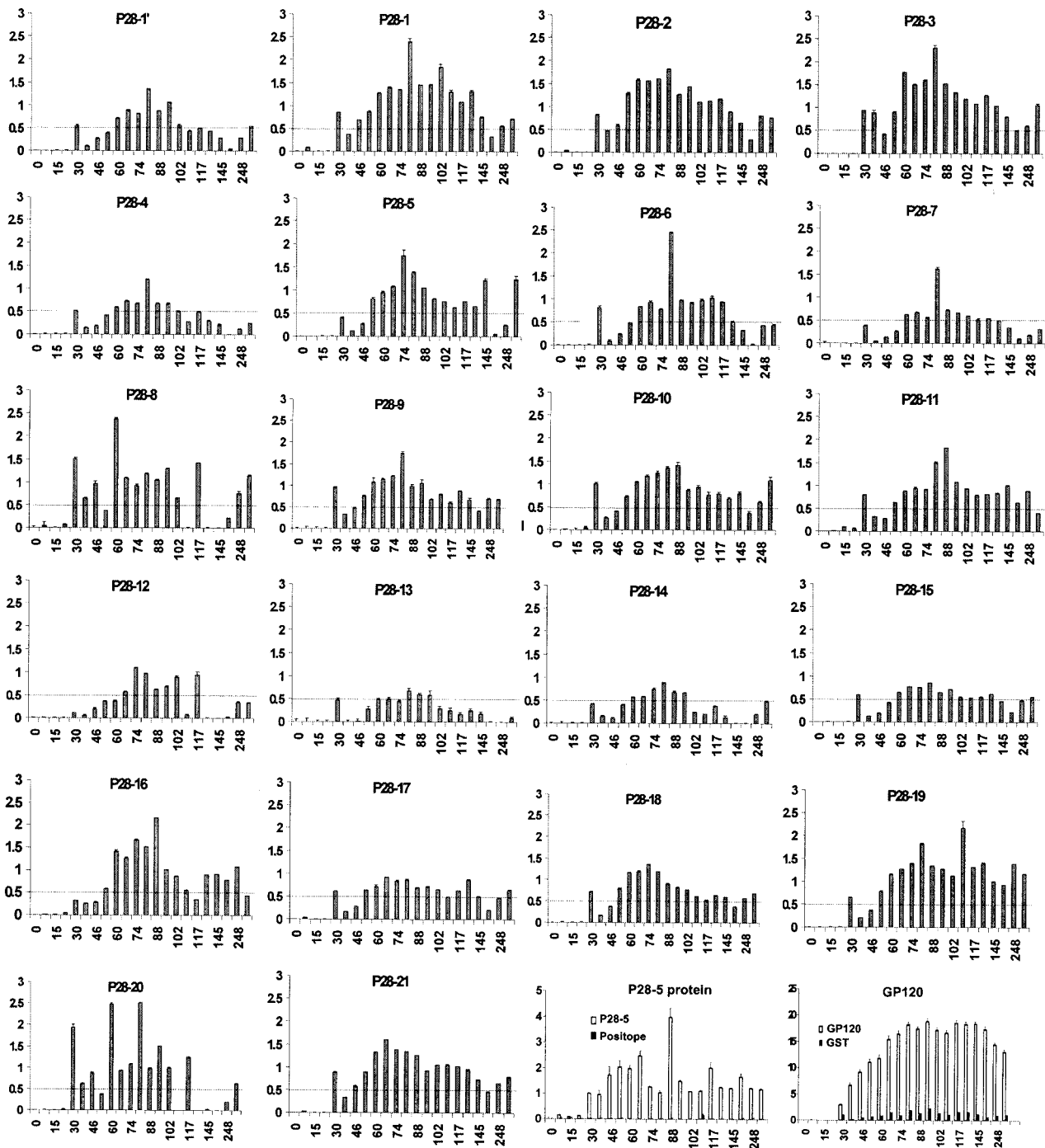
FIG. 5. Reactivity of all 22 synthetic peptides derived from HVR1 of P28 OMPs with the sera of dog ACC. The 22 time points representing dog sera obtained on days 0 (prior to inoculation of *E. chaffeensis*), 8, 15, 23, 30, 38, 46, 53, 60, 67, 74, 81, 88, 95, 102, 110, 117, 131, 145, 159, 248, and 462 postinoculation of *E. chaffeensis* are shown on the *x* axis. The height of the bars represents the increase in OD of an infected dog serum reacted with a P28 peptide over the OD of the day 0 serum from the same dog reacting with the same peptide shown on the *y* axis. Each bar represents the reaction of a peptide with a serum collected at one time point. The reactions of the dog sera with recombinant proteins P28-5 and GP120 were included for comparison with the synthetic peptides and recombinant outer membrane proteins. The dashed lines indicated the 0.5-fold cutoff for a positive ELISA reaction.

each of the P28 OMPs were used in ELISA (Fig. 1). Most peptides reacted with dog ACC sera from day 30 to day 462 postinoculation when a 0.5-fold increase was used as the cutoff at a minimal titer of 1:200 (Fig. 5). The antibodies to peptides P28-5, P28-12, P-28-14, and P28-16 appeared late in the dog sera, from day 53 to day 67 postinoculation. The antibodies to P28-4, P-12, P28-13, and P28-14 lasted a short period in dog ACC sera. Most peptides were recognized by antibodies in the
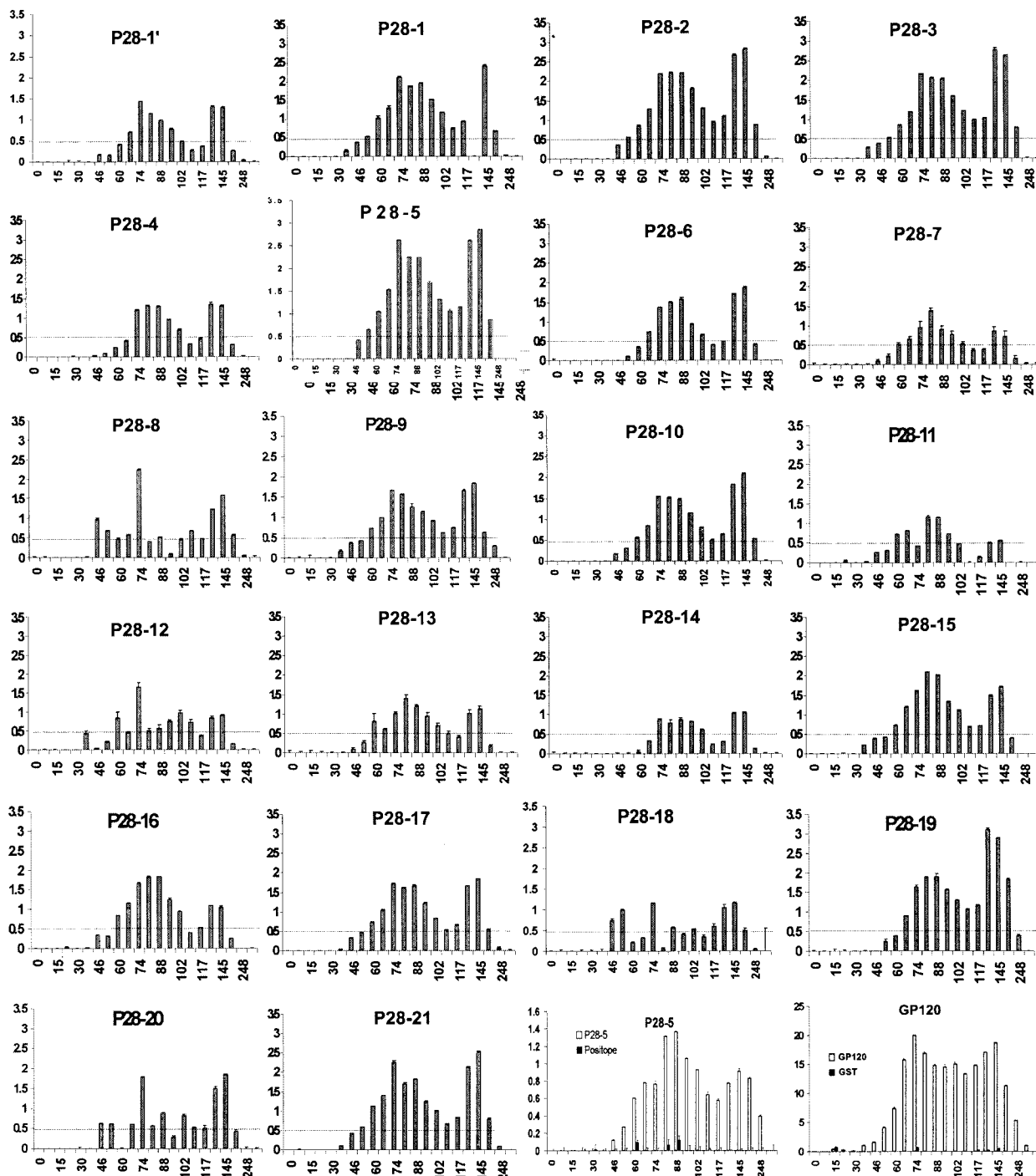
FIG. 6. The reactivity of 22 HVR1 synthetic peptides with the sera from dog ADJ. The 22 time points representing dog sera obtained on days 0 (prior to inoculation of *E. chaffeensis*), 8, 15, 23, 30, 38, 46, 53, 60, 67, 74, 81, 88, 95, 102, 110, 117, 131, 145, 159, 248, and 462 postinoculation of *E. chaffeensis* are shown on the *x* axis. Each bar represents the reaction of a peptide with one serum collected at one time point. The height of the bars represents the increase in OD of an infected dog serum reacted with a P28 peptide over the OD of the day 0 serum from the same dog reacting with the same peptide. The reactions of the dog sera with recombinant proteins P28-5 and GP120 were included for comparison with the antibody reaction to the synthetic peptides and recombinant outer membrane proteins. The dashed lines indicate the 0.5-fold cutoff for a positive ELISA reaction.

sera of dog ADJ between day 53 to day 67, except two peptides (P28-8 and P28-18), which were recognized from day 46 by the antibodies, and one peptide (P28-14) was recognized much later, at day 74 by the sera (Fig. 6).

The antibodies in ADJ sera to all peptides were no longer

detectable from day 145 to day 159 postinoculation (Fig. 6). The antibody titers against the P28 peptides changed during infection (Fig. 5 and 6) and the changes appeared at similar time in each dog. These data suggested that the antibodies specific to all of the P28 OMPs were generated during infec-

tion, which in turn suggested that all of the P28 OMPs were expressed in vivo.

**Reaction of the dog sera with recombinant proteins of P28-5 and GP120 by ELISA.** To investigate whether the antibody responses of dog sera to synthetic P28 peptides were similar to the antibody responses to *E. chaffeensis* outer membrane proteins, the reactivity of dog sera to P28-5 and P28-7 as well as to two other unrelated surface antigens (GP120 and P110) was analyzed. Serological responses against recombinant P28-5 (Fig. 5 and 6) and P28-7 (data not shown) were very similar to those of the synthetic peptides. Serological responses against recombinant GP120 (Fig. 5 and 6) and P110 (data not shown) had similar temporal patterns compared to those of recombinant P28-5 and p28-7. The recombinant proteins reacted with sera of dog ACC starting from day 30 and continuing through day 462 after *E. chaffeensis* infection. These data suggested that the serological response against the synthetic P28 peptides was representative of those directed against the corresponding P28 OMPs and that antibodies to P28 OMPs, GP120, and p110 appeared in the dog sera concurrently. There was no significant difference between the preimmunization serum and postimmunization sera when they were reacted with the GST and positope proteins (Fig. 5 and 6).

## DISCUSSION

We used synthetic peptides derived from HVR1 of each P28 to detect canine antibody response to P28 OMPs as an indirect means of monitoring the expression of P28 OMPs in infected dogs. HVR1 is divergent within each P28, and the synthetic peptides used in the study were unique to each of the P28 OMPs. Thus, it is highly likely that the serological responses against the synthetic peptides derived from the HVR1s were specific. This was supported by the observation that serological responses to the recombinant P28-5 and P28-7 antigens were similar to those to the corresponding synthetic peptides. Our data indicate that antibodies to all of the P28 OMPs were detected in dogs persistently infected with *E. chaffeensis* and in turn suggest that all of the P28 OMPs were expressed in persistently infected dogs.

A positive cutoff is essential for interpretation of the ELISA results. A higher cutoff would avoid false-positive results, but the test might become insensitive. We used a 0.5-fold increase in OD value as a cutoff for a positive P28 peptide ELISA. The criterion was threefold higher than the maximum OD increase when the sera were reacted with the nonrelevant peptides. Western blotting indicated that P28-15 was recognized by dog ACC sera from day 23 and by dog ADJ sera from day 38 postinoculation (38). Our ELISA results showed that the dog sera recognized the P28 peptides at much later time points, when a 0.5-fold increase in OD value was used as a criterion for a positive result. Thus, we believe that a 0.5-fold increase was a stringent criterion, and our ELISA results showed the specific reaction of the antibodies to the P28 proteins with the P28 synthetic peptides.

Antibodies to most P28 OMPs appeared in each dog at a similar time, around day 30 postinoculation in one dog and day 53 in the other dog, indicating that the P28 OMPs were expressed simultaneously. The antibodies to a few peptides appeared late in the dog sera and/or disappeared early, which

may be an artifact of the higher cutoff for positive reactions, because most of these results would be positive if the cutoff was decreased to a 0.2-fold increase. The simultaneous expression of the P28 proteins may have resulted from infection by homogeneous populations of *E. chaffeensis*, in which each bacterium expressed all of the P28s, or from a heterogeneous population of organisms, in which each organism expressed one or a few different P28 OMPs. The *E. chaffeensis* organisms used in this study were clonally purified by limiting dilution and passaged three times in cell culture prior to inoculation of dogs, which suggested that the initial inoculum contained a homogeneous population or at lest a limited number of populations if the mutation rate was very high. Regardless of whether a homogeneous or heterogeneous population of *E. chaffeensis* existed in the initial inocula, concurrent expression of all P28 OMPs in each persistently infected dog does not support the hypothesis that sequential expression of the P28 OMPs is important for persistent infection. If the sequential expression of P28 OMPs was an essential feature of persistent infection, one would expect that *E. chaffeensis* would have been cleared soon after inoculation of a mixed population owing to the host humoral immune response to all P28 OMPs simultaneously.

Our current data and previous results (34) suggest that the mechanism of immune evasion by *E. chaffeensis* may be fundamentally different from those used by *A. marginale*. Antigenic variation of surface proteins is a likely mechanism whereby *A. marginale* avoids the humoral immune response. In contrast, antigenic variation of the P28 OMPs may not play an important role in immune evasion by *E. chaffeensis*, because in our study serological responses were observed against all of the P28 OMPs simultaneously. Although antibodies to the P28 proteins have been demonstrated to protect mice from *E. chaffeensis* infection (16, 17), it may be that cellular responses to specific T-cell epitopes in P28 are more critical in host immune response to *E. chaffeensis*. Our results suggest that *E. chaffeensis* may utilize other mechanisms to evade the host immune response. Downregulation or avoidance of stimulation of production of cytokines such as interleukin-12, interleukin-15, and interleukin-18, that are critical for innate immune and adaptive immune response, by *E. chaffeensis* suggests that persistent *E. chaffeensis* infection may be caused by modulation of cytokine production (37).

The different structures of P28 OMPs and Msp-2 may explain their different roles in antigenic variation. Although the *p28* genes of *E. chaffeensis* and the *msp-2* genes of *A. marginale* share amino acid homology, they are quite different in structure. The *msp-2* gene family contains pseudogenes, and only one gene is expressed in each bacterium (2). The *p28* gene family contains no pseudogenes, and all genes are expressed in vivo (this study). All *msp-2* genes contain a central variable region (2, 23). However, the *p28* genes are highly divergent over their entire sequence, with homology as low as 20%. The hypervariable regions have been defined in only a few core members of the *p28* genes (36).

Orthologues of the *p28* multigene family have been found in other *Ehrlichia* species, such as *E. canis* (the *p30* multigene family) (21, 24, 30), *E. ewingii* (12), *E. ruminantium* (Map multigene family) (27), and *E. muris* (*p28* multigene family) (34). The conservation of the *p28* multigene family in the genus *Ehrlichia* suggests an important biological function for these

proteins. However, our data suggest that the P28 OMPs are not required for immune evasion at the population level because persistent infection occurred in the presence of antibody responses against the entire family of P28 OMPs.

## ACKNOWLEDGMENTS

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