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Rapid Sequential Changeover of Expressed *p44* Genes during the Acute Phase of *Anaplasma phagocytophilum* Infection in Horses

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Anaplasma phagocytophilum **immunodominant polymorphic major surface protein P44s have been hypothesized to go through antigenic variation, but the within-host dynamics of *p44* expression has not been demonstrated. In the present study we investigated the composition and changes of *p44* transcripts in the blood during the acute phase of well-defined laboratory *A. phagocytophilum* infections in naïve equine hosts. Three traveling waves of sequential population changeovers of the *p44* transcript species were observed within a single peak of rickettsemia of less than 1 month. During the logarithmic increase, the rapid switch-off of the initial dominant transcript *p44-18* occurred regardless of whether the bacterium was transmitted by ticks or by intravenous inoculation. Each of the subsequently dominant *p44* transcript species was phylogenetically dissimilar from *p44-18*. Development of antibody to the hypervariable region of P44-18 during the rickettsemia suggests the suppression of dominance of immuno-cross-reactive *p44* populations. When *A. phagocytophilum* was preincubated with plasma from the infected horse and then coincubated with HL-60 cells, the dominance of the *p44-18* transcript was rapidly suppressed in vitro and most of the newly emerged *p44* transcript species were previously undetected in this horse. This work provides experimental evidence of within-host *p44* antigenic variation. Results suggest that the rapid and synchronized switch of expression is an intrinsic property of *p44*s reinitiated after transmission to naïve mammalian hosts and shaped upon exposure to immune plasma.**

Anaplasma phagocytophilum is a tick-borne obligatory intracellular pathogen that causes persistent infections in various mammals (4). In humans, *A. phagocytophilum* causes an acute systemic and potentially fatal disease, human granulocytic anaplasmosis (2, 4). Antigenic variation belongs to the general survival strategy of the pathogen to enhance phenotypic variation within its hosts to prolong its duration of infection and the potential for transmission. Despite several mathematical models for the dynamics of antigenic variation (1, 8), the recent report of within-host dynamics of *var* gene expression by *Plasmodium falciparum* during the acute phase of human infection (16) underscores the importance and paucity of experimental data. In the bovine intraerythrocytic agent, *Anaplasma marginale*, researchers demonstrated antigenic variation of Msp2 major surface proteins between peak rickettsemia that occurred at 6- to 8-week intervals (6, 7). However, little is known about the dynamics of antigenic variation within each rickettsemic cycle. The *A. phagocytophilum* genome encodes a large number of immunodominant major surface protein P44s (homologs of Msp2). Expression of diverse *p44*s in patients, horses, mice, and ticks has been documented (5, 9, 15, 26). A unique *p44* expression locus (polymorphic *p44* expression locus) was discovered downstream of three tandem genes (*tr1*, *omp-1X*, and *omp-1N*), from which diverse *p44* genes have been proposed to be transcribed as a result of gene conversion (3, 14). Although *p44*s have been hypothesized to go through antigenic variation like *msp2*s of *A. marginale*, within-host dynamics of *p44* gene expression has not been demonstrated due in part to difficulty in monitoring switching behavior in a single infected human or in a laboratory mouse model of infection.

The horse is not only the natural host of *A. phagocytophilum*, but it also serves as a useful animal model for human granulocytic anaplasmosis (10, 26). The HZ strain, a human isolate of *A. phagocytophilum*, was well characterized in previous infection studies in horses, mice, ticks, and in cell culture (14, 23, 24, 26). This strain predominantly expresses *p44-18* in cell culture at 37°C and in the early stages of infection in the blood of horses and mice (26). Therefore, in the present study, in the horse model of infection with *A. phagocytophilum* strain HZ, within-host dynamics of *p44* expression and the P44-18 variant-specific humoral immune response were investigated. Furthermore, since *A. phagocytophilum* is readily cultivable using the human promyelocytic leukemia HL-60 cell line, in contrast to *A. marginale* (6, 7), it may provide the opportunity for in vitro investigation of antigenic variation. Therefore, we also determined the suppression of dominance of immuno-cross-reactive *p44* variants in cell culture by incubating host cell-free *A. phagocytophilum* with horse plasma followed by infection of HL-60 cells. Expressed *p44* compositions were determined through a newly developed variant-specific probe hybridization method. Our results suggest that the rapid and synchronized switch of expression is an intrinsic property of *p44*s, and the coordination necessary for this change might be provided by the host.

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TABLE 1. Sequences of oligonucleotides used in RT-PCR, PCR, colony hybridization, and cloning

Primer set no.	Primer name	Target	Purpose	Size (bp)	5′ primer	3′ primer
1	p3709-p4257	*p44* paralogs	C-PCR, RT-PCR	~550	GCTAAGGAGTTAGCTTATGA	AGAAGATCATAACAAGCATTG
2	p3709-pCompI	*p44* paralogs	Competitor construction	252	GCTAAGGAGTTAGCTTATGA	CTGGGATCCCACTACACTGCGACGTG[a]
3	pCompII-p4257	*p44* paralogs	Competitor construction	221	CGTGGATCCCGTAGATCGAGTAACAACAG[a]	AGAAGATCATAACAAGCATTG
4	pComp16SII-pComp16SI	*A. phagocytophilum* 16S rRNA	C-PCR	323	CGGGGGAAAGATTTATCGCTATTA	CGCTTGCCCCCTCCGTATTA
5	HB-ACTIN5-HB-ACTIN3	Horse β-actin	DNA PCR	628	CTGGCACCACACCTTCTACAACGAG	GTACAGGTCCTTACGGATGTCGACG
6	p5u37-p4257	*p44* upstream sequence (*p44* expression locus)	First-step DNA PCR	~1,230	CGTTATTTGTTCTAGAGAAAG	AGAAGATCATAACAAGCATTG
7	p18-5′-p18-3′	*p44-18* hv region	Colony hybridization	123	GTGATAGTAGCAATGCTG	CATCACTCAATGTGTTAGAA
8	*p44*con-p5247	*p44* paralog C terminus conserved region	Colony hybridization	102	AGTAGCAGGGTTACTAGC	AGAAGATCATAACAAGCATTG
9	18RECS-18RECA	*p44-18* hv region	rhvP44-18 cloning	271	TCCGAATTCGGAGACAAAGCGGA	CGCAAGCTTCTATACAGCTTTGGC

[a] BamHI site is underlined.

MATERIALS AND METHODS

***A. phagocytophilum* and horse infection.** The *A. phagocytophilum* HZ strain was cultured in HL-60 cells as previously described (18). Specimens from three infected horses were analyzed. Horse EQ005, a 5-year-old male, was infected by attaching 89 laboratory-reared *Ixodes scapularis* adult ticks infected with *A. phagocytophilum* as nymphs. A 12-year-old male horse (EQ006) was inoculated intravenously (i.v.) with 10^7 HL-60 cells infected with *A. phagocytophilum*. Horse EQ002 was infected by tick attachment in the previous study (26). All horses were confirmed to be seronegative for *A. phagocytophilum* by the indirect fluorescent antibody test (18) prior to tick placement or i.v. inoculation. On various days post-tick placement (p.t.) or post-i.v. inoculation (p.i.), blood samples (50 to 500 ml) were collected from the jugular vein into EDTA tubes or acid citrate dextrose anticoagulant bottles, and plasma and peripheral blood leukocytes (PBLs) were prepared as previously described (10).

C-PCR. Total DNA was extracted from the PBLs with a QIAamp blood kit (QIAGEN, Valencia, Calif.). To determine levels of *A. phagocytophilum* organisms in the horse blood, a sensitive *p44* competitive PCR (C-PCR) assay was developed to amplify *p44* paralogs using primer set 1 (Table 1) (24). A 463-bp competitor for *p44* homologs was prepared by ligating the two PCR products with primer sets 2 and 3 (Table 1), using *A. phagocytophilum* HZ chromosomal DNA as the template after digestion of BamHI sites included in primers pCompI and pCompII (Table 1). Primer set 4 (Table 1) was used to amplify the *A. phagocytophilum* 16S rRNA gene, resulting in a 361-bp product, whereas a competitor for the 16S rRNA gene (H. Niu and Y. Rikihisa, unpublished data) with the same primer pair yielded the 323-bp product. Densitometric analysis of PCR products was performed as previously described (12). To normalize the amount of PBL DNA across samples, PCR amplification for the horse β-actin gene was performed with primer set 5 in Table 1 over a linear range, as described previously (12). The number of PBL cells was estimated based on the predetermined ratio of cell number to the density of the β-actin gene PCR product.

Sequence analysis of *p44* cDNA and the polymorphic *p44* expression locus. The RNeasy kit (QIAGEN) was used to extract total RNA from PBLs of each horse and from *A. phagocytophilum*-infected HL-60 cells. The RNA was treated with DNase I (Invitrogen, Carlsbad, Calif.), and cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) with random hexamer (Invitrogen) as a primer, as described previously (15). The RNA sample without reverse transcriptase treatment served as a negative control. PCR was performed with primer set 1 (Table 1) to amplify cDNA encoding *p44* paralogs, and the resulting cDNA was cloned into the pCRII vector by using a TA cloning kit (Invitrogen) for sequencing the insert.

To determine the sequence of the polymorphic *p44* expression locus in the DNA specimen, PCR with primer set 6 (Table 1) (5′-upstream region sequence of the polymorphic *p44* expression locus and the 3′-end conserved region primers of *p44* paralogs [14, 24]) was performed, yielding a ~1,230-bp product. Amplified PCR products were cloned. Each insert was sequenced by the dideoxy termination method using the ABI Prism BigDye terminator v3.0 cycle sequencing reaction kit and an ABI 310 or 3730 sequencer. The deduced amino acid sequences were aligned using the CLUSTALV method in the MegAlign program (DNAStar, Madison, Wis.). Phylogenetic analysis was performed with the PHYLIP software package (version 3.6).

Cloning and expression of rhvP44-18. Primer set 9 (Table 1) was designed to clone the DNA fragment encoding the unique P44-18 hypervariable (hv) region. The resulting PCR products were digested with EcoRI and HindIII and ligated into the EcoRI- and HindIII-digested pET33b(+) vector (Novagen Inc., Madison, Wis.), and the plasmid was amplified in *Escherichia coli* Novablue cells (Novagen). *E. coli* BL21(DE3) cells (Novagen) were transformed with the recombinant plasmid and induced to express the recombinant P44-18 hypervariable region (rhvP44-18) with isopropyl-thio-β-D-galactoside. The recombinant protein was affinity purified with a His-Bind Quick column (Novagen).

Western blot analysis. *A. phagocytophilum* organisms purified as previously described (25) and rhvP44-18 were subjected to sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was incubated with either 5C11 (against pan-P44) or 3E65 (against hvP44-18) monoclonal antibodies (MAbs) (11) or with plasma samples from each horse that were preabsorbed with *E. coli* BL21(DE3) lysates and diluted to 1:50. The peroxidase-conjugated affinity-purified anti-mouse or anti-horse immunoglobulin G secondary antibodies (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.) were used at a dilution of 1:1,000. Finally, colorimetric reactions were developed with a 4-chloro-naphthol substrate solution (19).

Analysis of *p44* species expressed in cell culture after incubation with horse

plasma. All horse plasma and sera were filtered through 0.22-μm mixed cellulose ester membrane filters (Fisher Scientific, Pittsburgh, Pa.) and inactivated by incubation at 56°C for 30 min. Host cell-free *A. phagocytophilum* was freshly prepared from 2×10^6 *A. phagocytophilum*-infected HL-60 cells (>90% cells infected) per treatment, as previously described (22). The organisms were suspended in 1 ml of culture medium and incubated at 4°C for 30 min with 0.5 ml of plasma collected from horse EQ005 either on day 0 (before tick placement) or day 22 or 31 p.t. Control samples were incubated with 0.5 ml of heat-inactivated fetal bovine serum (FBS) or RPMI 1640 medium. Each of the mixtures was added to 10^6 uninfected HL-60 cells in 0.5 ml of RPMI 1640 medium supplemented with 5% FBS and 2 mM L-glutamine (final concentration, 5×10^5 cells/ml) and incubated at 37°C in 95% air–5% CO_2. After 12 h, the medium was replaced with fresh 5% FBS–RPMI medium, and the medium was replaced every 2 days thereafter. About 10^6 infected cells were harvested from each sample 2.5, 6, or 12 days postculture (p.c.) and immediately preserved in RNAlater (QIAGEN). *p44* transcripts were amplified by reverse transcription-PCR (RT-PCR), and the polymorphic *p44* expression locus was amplified by DNA PCR as described above and cloned.

Colony hybridization to detect *p44-18* and total *p44* cDNA and DNA clones. *E. coli* colonies harboring pCRII vector with *p44* homolog inserts were randomly picked up and plated onto two nitrocellulose membranes placed on Luria-Bertani plates. After incubation at 37°C overnight, the membranes were peeled off and treated with denaturing solution and UV cross-linked at 1,200 μJ for 50 s with a Stratalinker UV cross-linker (Stratagene Cloning Systems, La Jolla, Calif.) (19). The *p44-18* hv region probe (*p44-18*hv; 123 bp) was prepared by PCR using primer set 7 (Table 1) and digoxigenin labeled with a DIG DNA labeling and detection kit (Roche Molecular Biochemicals, Mannheim, Germany). The pan-*p44*-specific probe was prepared by amplification of a C-terminal fragment from the conserved *p44* region (102 bp) with primer set 8 (Table 1) and digoxigenin labeled. The pan-*p44* and *p44-18*hv probes were hybridized to each of the duplicate membranes, and hybridization was detected according to the manufacturer's instructions.

Complexity analysis. To quantify complexity, the Shannon entropy (H) calculation (20, 21) was applied. Shannon entropy incorporates both the number of *p44* species and the number of cloned cDNAs in each *p44* species. It is defined as $H = -\Sigma_i = 1 \text{ to } {}_N P(i) \ln[P(i)]$, where N is the total number of *p44* species and $P(i)$ is the number of clones represented in each *p44* species. A normalized value of H, denoted H', was defined as $H/\ln(N)$. This resulted in a range of possible values for H' from 0 to 1, representing 1 to N distinct *p44* species, respectively. H' values of specimens incubated in immune horse plasma and preimmune horse plasma were compared with a paired Student's t test. A P value of <0.05 was considered significant.

Nucleotide sequence accession numbers. GenBank accession numbers of sequences newly identified in the present study are as follows: *p44-43*, AY147269; *p44-46*, AY147263; *p44-47*, AY147264; *p44-48*, AY147267; *p44-49*, AY147268; *p44-58*, AY279320; *p44*-59, AY279321; *p44-60*, AY279319. The remaining *p44* sequences and GenBank numbers are described elsewhere (5, 15, 26).

RESULTS

Logarithmic rise and decline in rickettsemia in horses. In order to determine the pattern of initial rickettsemia in naïve hosts, we newly developed a *p44* C-PCR assay to detect low levels of infection. We chose C-PCR over other PCR methods since in this method the competitor coexists with the target DNA in the same reaction tube and serves as an internal control for variation of each PCR. The *p44* gene is composed of a central hv region of approximately 280 bp flanked by conserved sequences ranging from 100 to 500 bp in length (14, 15, 24). Primer set 1 (Table 1), which hybridizes to the conserved 5′ and 3′ regions, was used to amplify a group of *p44* paralogs in the genome of *A. phagocytophilum*. In order to estimate the number of organisms detected by *p44* C-PCR, we also developed C-PCRs specific for the 16S rRNA gene, which is a single copy within the *A. phagocytophilum* genome (www.tigr.org). Both *p44* paralog and 16S rRNA gene C-PCRs were performed with the same amount of *A. phagocytophilum* chromosomal DNA to determine the ratio of *p44* paralogs to the 16S rRNA gene. The finding from C-PCR indicated that this ratio was 48 under the assay conditions used. Thus, the number of *p44* paralogs detected by *p44* C-PCR divided by 48 represents the approximate *A. phagocytophilum* genome equivalent. The amount of horse PBLs in each specimen was normalized based on the horse β-actin gene, as described previously (12). Figure 1 shows similar levels and a similar monotopic, but not oscillating, pattern of rickettsemia: an initial logarithmic increase followed by a precipitous decline during 1 month p.t. in EQ005 and p.i. in EQ006.

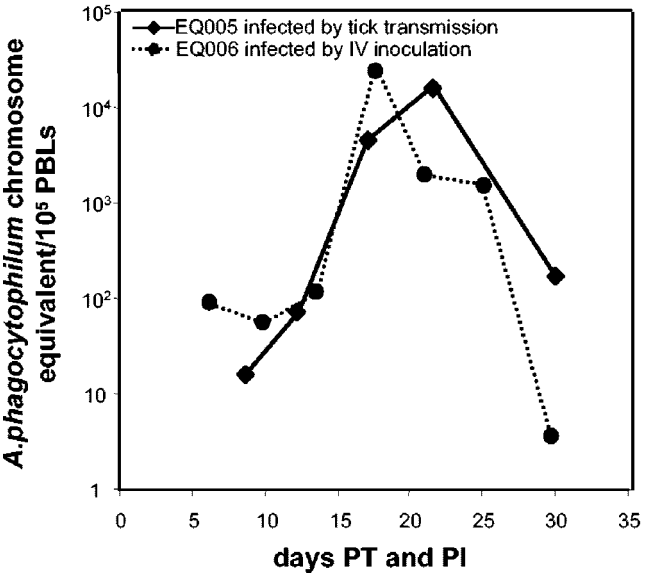

FIG. 1. Levels of *A. phagocytophilum* organisms in the blood of two horses during the course of infection, as determined by *p44* C-PCR. EQ005 was infected by tick attachment, and EQ006 was inoculated i.v.

Sequential appearance of dominant *p44* transcript species. To determine the within-host dynamics of *p44* transcript species, RNA specimens from the PBLs from three experimentally infected horses were subjected to *p44*-specific RT-PCR followed by sequencing of the ~550-bp amplicon as previously described (26). Two horses, EQ005 and EQ002, were infected by tick transmission of the HZ strain. One horse, EQ006, was infected by i.v. inoculation of the cultured HZ strain. No amplicon was detected in any of the samples that lacked reverse transcriptase, indicating that the fragments were not due to amplification of contaminating DNA (data not shown). A total of 249 *p44* cDNA clones (20 to 35 clones for each horse at each time point) were randomly selected for sequencing after cloning of RT-PCR products. *p44* species can be identified based on the sequences of the central hv regions (5, 15, 26). A previous study showed that the HZ strain predominantly expresses *p44-18* in cell culture and day 8 postinfection in the blood of both tick- and i.v.-infected horses (26). *p44* transcripts could not be detected in the blood of tick-infected EQ005 on day 8 p.t., perhaps due to the very low level of *A. phagocytophilum* infection (1.6 rickettsiae/10^4 PBLs, based on *p44* C-PCR) (Fig. 1). On day 12 p.t., all *p44* transcripts detected were *p44-18*. On day 17 p.t., *p44-30, p44-2*, and other new transcript species emerged. On day 22 p.t., the dominance of the *p44-18* transcript was replaced with new dominant *p44* transcript spe-

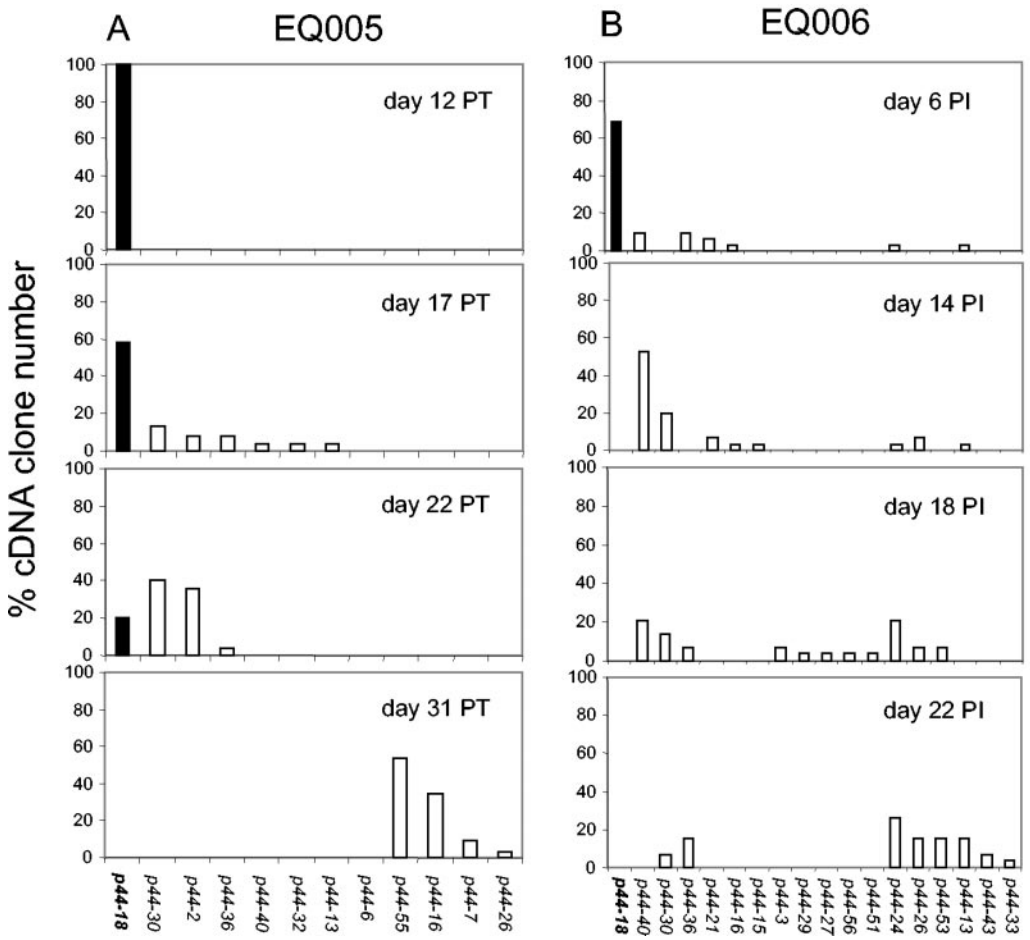

FIG. 2. Relative proportion of each of the *p44* transcript species in the blood of horses EQ005 (A) and EQ006 (B). The vertical axes show the percentage of cDNA clones of each *p44* species at each time point. The horizontal axes show the various *p44* cDNA clone species detected.

cies (*p44-30* and *p44-2*). On day 31 p.t., *p44-30* and *p44-2* were replaced with the new dominant *p44* transcript species (*p44-55* and *p44-16*) (Fig. 2). In horse EQ002, which was also infected by tick transmission, *p44-18* was the major transcript species detected on day 8 p.t. (26). When we analyzed RNA specimens from this horse that were preserved in the −80°C freezer, we found that on day 16 p.t. the dominance of the *p44-18* transcript was replaced with a new transcript species, *p44-1* (25 of 25 cDNA clones). In i.v.-inoculated horse EQ006, results for days 6, 14, 18, and 22 p.i. are shown (Fig. 2B), since *p44* transcript species changeover was faster in this horse than in EQ005 and on days 30 and 37 p.i. *p44* transcripts could not be detected in the blood, perhaps due to the very low level of *A. phagocytophilum* infection (below 3.5 rickettsiae/10^5 PBLs, based on *p44* C-PCR) (Fig. 1). Nonetheless, in EQ006 a similar changeover pattern of the initially dominant *p44-18* transcript species to that of tick-transmitted EQ005 was observed, i.e., traveling waves of sequential population changeovers of the *p44* transcript species within a single peak of rickettsemia.

To determine the similarity among these sequentially dominant *p44* transcript species, phylogenetic analysis was performed by defining the dominant *p44* transcript species as those that represented greater than 20% of the total number of cDNA clones at each time point for each horse sample. Investigators from our laboratory previously showed that hv region sequences of *p44* paralogs are clustered into three major groups (α, β, and γ) (15). *p44-18*, which is located in the α cluster, was the dominant transcript initially detected in all six horses infected with *A. phagocytophilum* HZ, including EQ001 to EQ004 in the previous study (26), regardless of whether inoculation was i.v. or through ticks. All dominant *p44* transcript species in horses EQ005 and EQ006 that subsequently appeared within the initial rickettsemia period belonged to the β or γ clusters. The *p44-1* transcript detected in horse EQ002 on day 16 p.t. also belonged to the β cluster. These results suggest suppression of dominance of the α cluster *p44* transcript species during subsequent *p44* transcript species changeovers by immunologic cross-reactivity.

To investigate whether the sequential appearance of dominant *p44* transcript species is due to sequential change of the bacterial genetic population having corresponding *p44* species in the polymorphic *p44* expression locus, *p44* genes in the locus were cloned by DNA PCR. DNA fragments (~1,230 bp) were amplified with primer set 6 (Table 1) from EQ005 PBL specimens obtained on days 17 and 22 p.t. PBL specimens obtained on days 12 and 31 p.t. did not yield significant products due to the insufficient levels of *A. phagocytophilum* DNA present in these specimens. The resulting PCR products were cloned, and

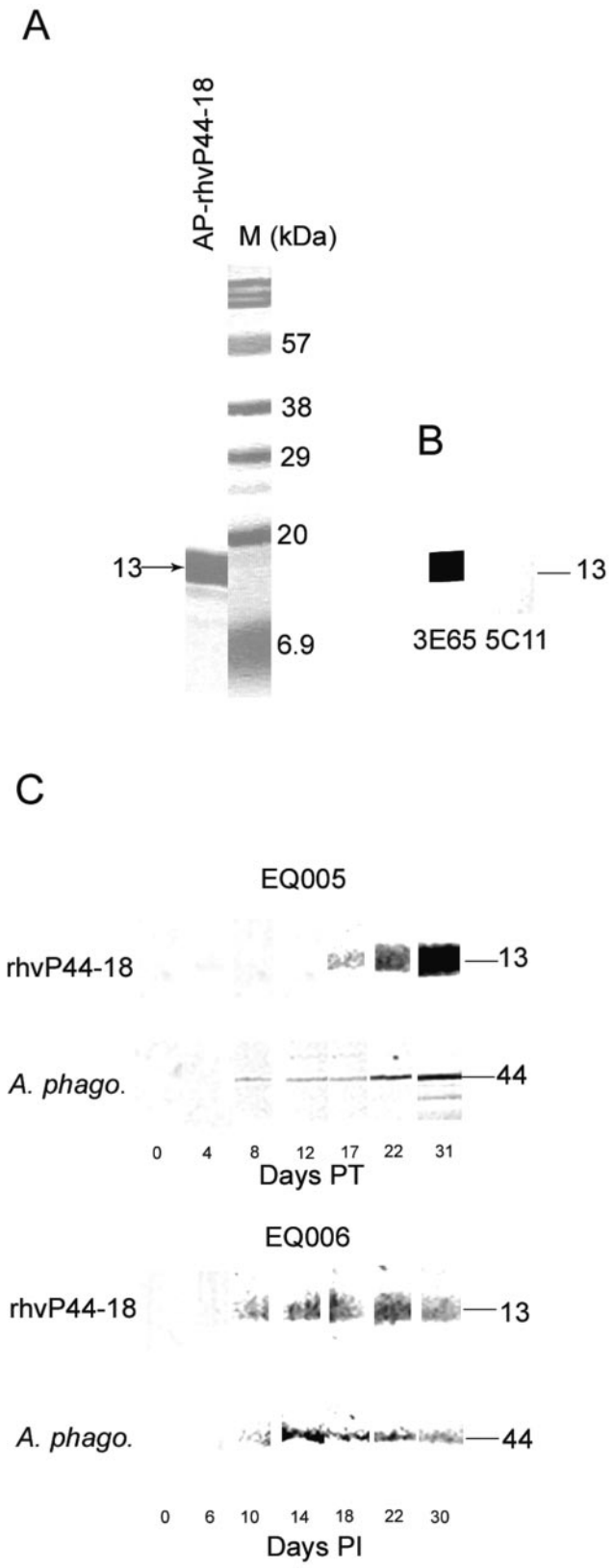

FIG. 3. Development of anti-hvP44-18 antibody in the plasma of *A. phagocytophilum*-infected horses. (A) Three micrograms of affinity-purified rhvP44-18 (AP-rhvP44-18) was subjected to SDS-PAGE followed by Coomassie blue staining. M, molecular size marker. The recombinant hvP44-18 is indicated by the arrow. (B) Purified rhvP44-18 (2.5 μg/lane) was separated by SDS-PAGE and blotted to the membrane. The membrane was incubated with the hvP44-18-specific MAb (3E65) or pan-P44-specific MAb (5C11). (C) Purified rhvP44-18 (2.5 μg/lane) and *A. phagocytophilum* (3 μg/lane) were separated by SDS-PAGE and blotted to the membrane. The membrane was incubated with horse sera collected at the indicated days p.t. or p.i. Numbers on the right indicate molecular masses (in kilodaltons) based on the broad-range prestained standards (Bio-Rad).

13 and 12 clones from days 17 and 22 p.t., respectively, were randomly selected for DNA sequencing. The DNA clones in the PBL specimen from day 17 p.t. were *p44-18* (11 clones), *p44-30* (1 clone), and *p44-2* (1 clone). The major DNA clones in the PBL specimen from day 22 p.t. were *p44-2* (6 clones), *p44-30* (5 clones), and *p44-18* (1 clone). The major *p44* cDNA species roughly correlated with the *p44* species present within the *p44* polymorphic expression locus, suggesting that sequential appearance of dominant *p44* transcript species is due to sequential recombination at the *p44* polymorphic expression locus and growth of the newly recombined population.

Development of P44 variant-specific antibodies during the course of *A. phagocytophilum* infection. To explore the possibility that the suppression of dominance of the α cluster *p44* transcript species is associated with immunologic cross-reactivity, we determined the kinetics of P44 variant-specific antibody. Since P44-18 is an initially dominant P44 variant, the DNA fragment encoding the P44-18 unique hv region (83 amino acids), based on the alignment of all *p44* paralogs, was cloned into the pET33b(+) vector. *E. coli* transformed with the plasmid expressed a 124-amino-acid (13,096-Da) fusion protein, including His_6 residues at its N terminus. The expressed rhvP44-18 was purified through Ni-affinity chromatography, and a single band of approximately 13 kDa was detected on SDS-PAGE (Fig. 3A). Western blot analysis revealed that rhvP44-18 specifically reacted with MAb 3E65, which binds only to the 44-kDa protein expressed by strain HZ in HL-60 cell culture and not to 44-kDa proteins expressed by the other five different *A. phagocytophilum* strains in HL-60 cells (12). rhvP44-18 did not react with MAb 5C11, which is specific to the invariable N-terminal regions of P44s (11, 26) (Fig. 3B). Immunoreactivity to native P44s was first detectable in the plasma at day 8 p.t. in EQ005 and day 10 p.i. in EQ006. Anti-rhvP44-18 immunoglobulin G became detectable in the plasma of EQ005 and EQ006 starting on day 17 p.t. and day 10 p.i., respectively (Fig. 3). This was roughly the time point when the *p44-18* transcript population declined in each horse. The result also confirmed the expression of the P44-18 protein by *A. phagocytophilum* in horses and recognition of the hv region by the horse immune system.

Infected horse plasma reduced dominance of the *p44-18* variant in cell culture. To test whether the infected horse plasma was involved in the suppression of *p44-18* transcript dominance, host cell-free *A. phagocytophilum* was preincubated with the plasma from infected horses and then the mixture was coincubated with HL-60 cells. Since there is no efficient and reliable method to determine a specific *p44* transcript population in a large number of specimens, we developed the colony hybridization method. In this method, we verified 100 *p44* cDNA clones using the pan-*p44* probe and determined the

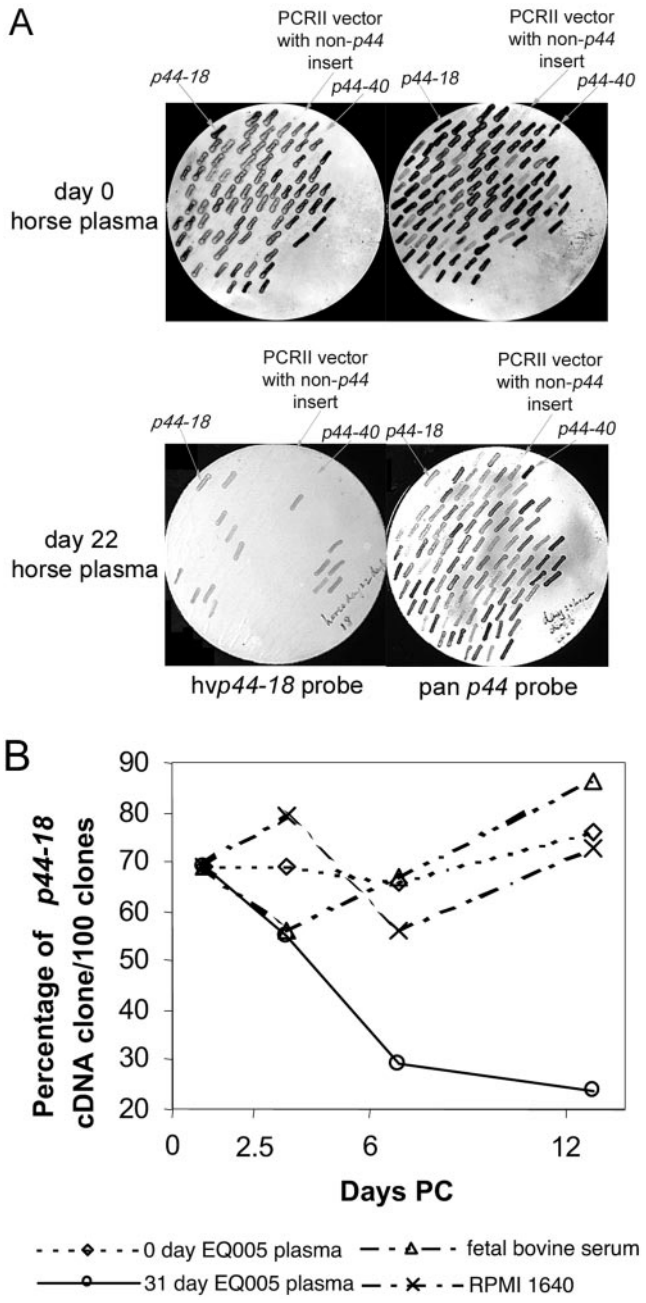

FIG. 4. Infected horse plasma reduced dominance of the *p44-18* transcript in cell culture. (A) Colony hybridization of *p44* cDNA clones from *A. phagocytophilum* HZ incubated with horse (EQ005) day zero plasma (pre-tick attachment) and day 22 plasma at 6 days p.c. hv*p44-18* and pan-*p44* probes each were hybridized to one of the duplicated membranes each with 100 *p44* cDNA clones. Two cDNA clones with *p44-18* and *p44-40* inserts were used as a positive and a negative control, respectively. A pCRII plasmid with a non-*p44* insert was also used as negative control. Approximately 14% of *p44* cDNA clones had the *p44-18* insert with *A. phagocytophilum* incubated with day 22 p.t. plasma, and approximately 80% of *p44* cDNA clones had the *p44-18* insert with those incubated with day zero plasma. (B) Temporal changes in the *p44-18* transcript population in *A. phagocytophilum* incubated with plasma obtained on day zero (preinfection) and day 31 p.t. from EQ005, FBS, or RPMI 1640 medium. Colony hybridization analysis was performed on 100 *p44* cDNA clones in each specimen at each day p.c. to determine the percentage of cDNA clones with *p44-18* inserts.

TABLE 2. Characteristics of *p44* cDNA clone type distribution after incubation of *A. phagocytophilum* with horse EQ005 day 31 and day 0 plasma

Days p.c.	Horse plasma	*p44* clone type distribution[b]	No. of clone types[c]	Entropy[d]
2.5	day 31	14, 3, 2, 2, 1, 1, 1	7	0.71
	day 0	18, 1, 1, 1	4	0.41
6	day 31	10, 3, 2, 1, 1, 1, 1, 1, 1, 1, 1, 1	13	0.82
	day 0	19, 2, 1, 1, 1, 1, 1, 1, 1	9	0.58
12	day 31	10, 4, 3, 2, 1, 1, 1, 1, 1, 1	10	0.82
	day 0	20, 2, 1, 1, 1	5	0.48
12[a]	day 31	6, 4, 3, 3, 3, 3, 2, 2, 1, 1, 1	11	0.94
	day 0	18, 1, 1, 1, 1, 1, 1	7	0.52

[a] Independent experiment.
[b] Each series shows the number of clones comprising each clone type for the given sample.
[c] Number of sequence indistinguishable cloned cDNAs.
[d] Normalized Shannon entropy (H′), a calculated value that incorporated the number and distribution of clone types. A paired *t* test was used to compare the difference in entropy between samples of day 31 and day 0 horse plasma; $P < 0.003$.

percentage of cDNA clones which had the *p44-18* insert among *p44* cDNA clones by using the *p44-18* species-specific probe (Fig. 4A). Two cDNA clones with *p44-18* and *p44-40* inserts (confirmed by sequencing) were used as positive and negative controls, respectively, in each hybridization reaction. PCRII vector plasmid with a non-*p44* insert was also used as a negative control. An advantage of this method over real-time PCR, for example, is that it is not influenced by primer sensitivity, plasmid stability, or variation of PCR. The variation in this assay in duplicate specimens was less than 10%. Using this method it was found that when incubated with infected horse plasma (EQ005 day 22 or 31 p.t.) the dominance of the *p44-18* transcript rapidly declined in vitro (Fig. 4). The inhibitory effect was specific to infected horse plasma, since incubation with EQ005 day 0 (pre-tick attachment) plasma, FBS, or RPMI medium did not reduce the dominance of the *p44-18* transcript (Fig. 4).

The colony hybridization method was confirmed by sequencing a total of 226 *p44* cDNA clones (21 to 29 clones each per culture at each time point for two independent experiments) at 2.5, 6, and 12 days p.c. (Table 2). At 2.5 days p.c. with day 31 p.t. horse plasma samples, *p44-18* represented 58% of the total clones and *p44-13* (3 clones), *p44-47* (2 clones), *p44-29* (2 clones), *p44-15* (2 clones), *p44-32* (1 clone), and *p44-49* (1 clone) were also detected in 24 cDNA clones. Whereas, when incubated with day zero plasma *p44-18* represented 86% of all clones and only *p44-2* (1 clone), *p44-16* (1 clones), and *p44-32* (1 clone) were also detected in 21 cDNA clones. These four P44 variants were detected in horse blood by day 31 p.t. However, none of the cDNA clones were detected after incubation with day 31 plasma, except *p44-13*, which was a minor transcript at one time point (1 of 24 cDNA clones on day 17 p.t.) in the horse blood. We applied Shannon entropy analysis (20, 21) to measure *p44* species population complexity. With immune plasma the entropy value was significantly greater than that with preimmune plasma (Table 2).

Day 31 p.t. plasma from horse EQ005 also reduced the percentage of the *A. phagocytophilum* genetic population that contained *p44-18* at the polymorphic *p44* expression locus: relative to the culture incubated with day zero plasma from horse EQ005, the average percentage of the population with *p44-18* at the polymorphic *p44* expression locus was 20% (the average of 10 and 30%, obtained in two independent experiments). These results suggest that reduction of *p44-18* transcript frequencies in vitro is also accompanied by genetic population change at this locus.

DISCUSSION

This study is the first, to our knowledge, to report the within-host dynamics of transcribed *p44* genes during the acute phase of *A. phagocytophilum* infections. During the logarithmic increase of rickettsiae in the blood, the rapid switch-off of the initial dominant transcript *p44-18* occurred regardless of whether the bacterium was transmitted by ticks or by i.v. inoculation. *p44-18* was not expressed by *A. phagocytophilum* strain HZ in the salivary gland of ticks that transmitted *A. phagocytophilum* to naïve horses (26). Thus, this result suggests timed or programmed *p44-18* expression after transmission to mammalian hosts and reinitiation of rapid switch-off of *p44-18* expression. This is similar to the *var* gene expression by *P. falciparum* reported during the acute phase in two human volunteer infections (16). Although in the *P. falciparum* study subsequent changes in the same individual were not investigated, in our study two subsequent population changeovers in the *p44* transcripts were observed in each horse: the second and third synchronized waves of dominant *p44* transcripts appeared during peak rickettsemia and the declining phase of rickettsemia, respectively, suggesting that the switch is not dependent on rickettsial density. It is unclear how the entire population of *A. phagocytophilum* within the host can synchronize *p44* expression. These changeovers do not resemble the simple clearance of the previous *p44* variant population by the immune system and the rapid growth of new *p44* escape variants, since the characteristic oscillating pattern of rickettsemia was not evident during these changeovers.

Each of the subsequently dominant *p44* transcripts was phylogenetically dissimilar from *p44-18*. Concomitant development of an immune response to the hv region of P44-18 suggests that antibodies against the *p44* hv regions in the previously expressed *p44*s prevent dominance of similar *p44* transcripts, and thus shape the next *p44* transcript population in infected horses. Our work suggests that immune responses to multiple *p44* genes with diverse antigenic properties also shape the *A. phagocytophilum* genetic population, since the changeover of *p44* transcript species was accompanied by a corresponding changeover of *p44* species at the polymorphic *p44* expression locus.

The present study is the first to demonstrate that immune plasma contributes to changes in the phenotype and the genotype of the *A. phagocytophilum* population in cell culture. This supports the hypothesis that antibodies specific to the P44 hv region are responsible for preventing the reemergence of *A. phagocytophilum* cells that have a similar *p44* hv region at the polymorphic expression locus. Furthermore, the emergence of more diverse *p44* transcript species in the presence of immune plasma suggests that the immune plasma not only changes transcription levels but also promotes *p44* recombination. The data also showed that a 12-h treatment with heat-inactivated immune horse plasma is sufficient to cause a continuous decline of the *p44-18* transcript population for up to 12 days (corresponding to approximately 40 bacterial fission events, at an estimated doubling time of 7 h). Thus, this *p44* transcript changeover appears to be an intrinsic property of *p44*s which is rapidly reinitiated and sustained when introduced into mammals or exposed to immune plasma, but not by some chance event. The in vitro system developed in this study would provide a useful and simple model for studying mechanisms of *p44* antigenic variation, as an alternative to the use of experimental animals.

The present and previous studies showed that low levels of bacteria persisted in the blood of horses that were infected with *A. phagocytophilum* HZ by either tick attachment or by the i.v. route for more than 1 month. *A. phagocytophilum* can cause persistent infections in ruminants and horses (17). This bacterium was detected by PCR in the serum of an untreated human 30 days after the onset of illness (13). The persistence of *A. phagocytophilum* in a reservoir rodent host would be an important adaptation which allows greater access for uninfected tick populations to an infectious blood meal. The present study suggests that dynamic interactions with the host innate and adaptive immune systems orchestrate a prolonged *A. phagocytophilum* infection by spreading the expansion of different *p44* antigenic variants over time to avoid rapidly exhausting the *p44* repertoire.

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