Host Cell–Specific Expression of a p44 Epitope by the Human Granulocytic Ehrlichiosis Agent

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The human granulocytic ehrlichiosis agent (HGEA) survives extreme differences between ticks and humans, possibly by use of differential expression of specific antigens for survival in different hosts. The role of the immunodominant p44 antigens is unknown. In this study, HGEA cultured in human or tick cells was probed with human, mouse, and hamster serum and with monoclonal antibodies (MAbs). p44 antigens were strongly expressed in human HL-60 cells but were strikingly reduced in tick cells. In HGEA alternately grown in HL-60 or tick cells, a p44 epitope recognized by MAb RSE4 was expressed in human but not tick cells. This was not a temperature effect, because incubation of infected tick cells at 37°C did not induce expression of the p44 epitope. The p44 antigen predominates in human but not tick cells and may be involved in regulatory changes that mediate survival of the HGEA by immune modulation after tick transmission.

The mammalian host is generally in homeostasis, but pathogens must overcome its powerful immune response. By contrast, ticks undergo severe fluctuations in temperature and metabolism but lack a highly specific immune system [9], and pathogens are not subject to immune selection. Replication and activities such as cyclic expression of variant surface antigens cease [8, 10–13]. Attachment to a new host, increased temperature, and influx of nutrients initiate changes in many pathogens that mediate infection of the vertebrate. For example, Rickettsia rickettsii [14, 15] and Borrelia burgdorferi [16, 17] regain infectivity only after activation by elevated temperature or the blood meal. These genetically unrelated pathogens have developed similar strategies to ensure survival in their hosts, a phenomenon referred to as convergent evolution [18]. We assumed that the HGEA would respond likewise to changing conditions. We focused on the p44 antigens because they are immunodominant in humans and animals. We also predicted that they would be down-regulated in ticks (i.e., differentially expressed in their hosts). To test this hypothesis, we used immune serum and monoclonal antibodies (MAbs) to probe proteins expressed by the HGEA cultured alternately in tick and human cells.

Materials and Methods

Host cell lines, HGEA isolate, and culture conditions. Tick cell line ISE6 from Ixodes scapularis and the human promyelocytic cell line HL-60 were grown at 34°C or 37°C, respectively, in closed flasks [4, 19, 20] with L15B cell culture medium with 5% fetal bovine serum (Harlan), 10% tryptose phosphate broth (Difco), and 0.1% lipoprotein (ICN) and were buffered to pH 7.5 with 0.25% NaHCO3 (Sigma), 25 mM HEPES (Sigma), and 1 N NaOH [19] (ehrlichia medium). HL-60 cell suspensions were diluted 10-fold with ehrlichia medium once a week. The HGE2 isolate [4] was

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The human granulocytic ehrlichiosis agent (HGEA) is an emerging, obligate, intracellular pathogen closely related to Ehrlichia equi and E. phagocytophila [1]. It is transmitted by Ixodes ticks [2, 3]. Infection with HGEA causes a potentially life-threatening acute febrile illness characterized by leukopenia, thrombocytopenia, and elevated serum transaminase levels [1, 4]. Antibodies are often not detectable until after onset of illness [5] and strongly react with the dominant p44 antigens. This suggests that their expression is initiated after infection and that the HGEA may activate differential expression of specific antigens (DESA) to facilitate survival after tick transmission.

p44 of the HGEA and major surface protein 2 (MSP2) of the closely related cattle pathogen Anaplasma marginale are homologous and are encoded by multigene families [6, 7]. Although the role of MSP2 in immune evasion by antigenic variation is well documented [7, 8], the role of p44 is not understood.
maintained in parallel in HL-60 and ISE6 tick cells. Infection was monitored by phase-contrast microscopy and examination of Giemsa-stained cells.

**Serum and MAbs.** Human serum (titer 1: 1280, immunofluorescence assay [IFA]) was obtained from a patient with HGE. Female mice (C3H/HeJ, 6 weeks old; Jackson Laboratories) and male hamsters (outbred Syrian, 3–4 weeks old, Dept. of Entomology, University of Minnesota) were injected intraperitoneally with 0.2 mL of an HGE1-infected ISE6 culture [4], and serum specimens were obtained 6 weeks later. Serum titers for mice and hamsters by IFA were 1:2560 and 1:640, respectively. Mouse MAbs [21, 22] were raised against HGEA grown in either HL-60 cells (R5E4, anti-p44) or ISE6 cells (3C9 and 15D4, anti-p60).

**IFA.** Serum specimens and MAbs were screened for specificity and reactivity by IFA, using HGEA-infected HL-60 or ISE6 cells [5] (authors’ unpublished data). Antibodies were diluted as follows: MAb 15D4, 1:10; MAb 3C9, 1:500 (both hybridoma culture supernatants); MAb R5E4, 1:50,000 (mouse ascites fluid); human serum, 1:500; rodent serum, 1:200. To distinguish human from tick cells, we used fluorescein isothiocyanate (FITC)–labeled MAb CSLEX-1 (Beckman Coulter) specific for sialylated Lewis-x (CD15s), which is not present on invertebrate cells but is abundant on HL-60 cells [23]. In combination with rhodamine-labeled secondary antibodies, the FITC-labeled CSLEX-1 allowed distinction of infected HL-60 and ISE6 cells.

**Alternating host cell cultures.** The HGEA isolate HGE2 was passaged in HL-60 for 6 months [4] and then was alternated between human and tick cells. All cultures were duplicated. To start, 10^5 infected (84%) HL-60 cells were mixed with 10^7 ISE6 cells. All cultures were duplicated. To passaged in HL-60 for 6 months [4] and then was alternated. In a secondary antibody, the FITC-labeled CSLEX-1 allowed distinction of infected HL-60 and ISE6 cells.

In **Alternating host cell cultures**, the HGEA isolate HGE2 was passaged in HL-60 for 6 months [4] and then was alternated between human and tick cells. All cultures were duplicated. To start, 10^5 infected (84%) HL-60 cells were mixed with 10^7 ISE6 cells and were incubated at 34°C. The first subculture to new tick cells was made after 3 weeks by repeated passage of the cells through a 27-gauge needle to release the HGEA. Cells and large debris were removed by centrifugation at 275 g for 10 min, and ehrlichiae were spun with 10^7 uninfected ISE6 cells at 2000 g for 15 min. The pellet was resuspended in 5 mL of ehrlichia medium and was cultured at 34°C. Five weeks later, another passage with host cell–free ehrlichiae was made. Subsequently, 0.25 mL of an infected (80%–90%) cell suspension was transferred to fresh ISE6 cultures twice monthly. At each passage, samples were taken for Giemsa staining, IFA, and Western blotting. After 3.5 months and 4 tick cell–to–tick cell passages, the HGEA was returned to HL-60 culture by mixing 10^7 HL-60 cells with 1 mL of infected (>90%) ISE6 cell suspension (2 x 10^6 cells). After 2 weeks, 5% of the culture was transferred to fresh HL-60 cells, and this was done once weekly thereafter. Tick cells did not survive incubation at 37°C for >2 weeks.

**Influence of temperature on protein expression by HGEA in ISE6 tick cells.** We used HGE2 passaged 5 times in HL-60 cells and then 20 times in ISE6 cells. A 5-mL ISE6/HGE2 culture (90% infected) was mixed with 40 mL of uninfected ISE6 cells (1.5 x 10^8 cells/mL) in ehrlichia medium at a 1:8 ratio of infected to uninfected cells. The suspension was aliquoted into 8 25-cm² flasks and was incubated overnight at 34°C to allow invasion by the HGEA [20]. Duplicate cultures were then transferred to 25°C, 31°C, and 37°C; 2 remained at 34°C. On days 4 and 10, samples were taken for Giemsa staining and IFA [5]. Cultures were extracted for Western blot analysis upon termination on day 10. The experiment was repeated twice.

**Antigen preparation and Western blotting.** At each passage, infected cells were frozen at −70°C. HGEA was separated from rapidly thawed cultures as was done for cell-free ehrlichia suspensions, which was concentrated at 13,000 g for 15 min, and was solubilized in 5 times the pellet volume of buffer [24]. For temperature experiments in ISE6 cells, 10 μL of ehrlichia extract per well was separated by SDS-PAGE [24] through a 1-mm 10% minigel at 200 V for 50 min. For comparison, HGEA extracted from continuous HL-60 culture at 37°C was run in the same gel. Duplicate gels were run together. For experiments involving alternating host cells, 100-μL samples were loaded onto preparative gels. Pre-stained molecular weight markers (SeeBlue and Multimark [Novex/InVitroGen]) were electrophoresed in the same gels. Proteins and peptides were transferred to Immobilon P membranes (Millipore). Membranes were blocked, and those from 10-well gels were incubated with human patient serum. Strips cut from preparative membranes were used to test reactions of hamster, mouse, or human serum or of MAbs against p60 (3C9 and 15D4) or p44 (R5E4). R5E4 was diluted 1:1000, 3C9 1:200, and 15D4 1:5 in PBS with 3% bovine serum albumin (Sigmac). Polyclonal serum specimens were diluted 1:100. Blots were incubated with horseradish peroxidase–labeled secondary antibodies (Pierce) and were developed by using the ICN membrane peroxidase system (Kirkegaard & Perry Laboratories).

**Results.**

**DESA.** All polyclonal anti-HGE serum specimens, whether human (1:1480 titer), mouse (1:2560 titer), or hamster (1:640 titer), showed specific immunofluorescence reactivity only with the HGEA—not with host cells. Polyclonal serum specimens reacted equally well with organisms from human and tick cell culture, as did anti-p60 MAbs 3C9 and 15D4. In contrast, the anti-p44 MAb R5E4 reacted only with organisms propagated in HL-60 cells and not with those from ISE6 cells. Likewise, only infected HL-60 cells bound both CSLEX-1 and R5E4, whereas infected tick cells sampled 3 weeks after inoculation with infected HL-60 cells bound neither. Blots probed with polyclonal serum or MAbs revealed striking differences between antigen patterns in ehrlichiae grown in HL-60 (figure 1, top left) versus those grown in ISE6 cell culture (figure 1, top right). In HL-60–grown HGEA extracts, p44 showed strong reactivity with MAb R5E4, whereas 3C9 and 15D4 bound to p60 antigen expressed by these organisms. Patterns from ISE6 cultures were more complex than those from HL-60 cells, and reactivity with p44 was much reduced, both in intensity and in number of bands. MAb R5E4 did not label p44 in ISE6-HGE blots, whereas MAbs 3C9 and 15D4 did bind to p60.

**Host cell–dependent cyclic expression of p44 epitope.** To examine the kinetics of antigen expression by the HGEA grown under different temperature conditions and in 2 different host cells, the HGEA was transferred from HL-60 culture at 37°C to ISE6 cells at 34°C and then back to HL-60 at 37°C. At first, we...
used host cell–free HGEA, but infectivity for the alternate host cell proved to be very low. Therefore, we used whole infected cells as the source of infection, except as noted. Ehrlichiae readily transferred from infected HL-60 to ISE6 cells, and within 3–4 days, 10%–12% of ISE6 cells contained ehrlichial inclusions. However, subsequent growth was slow. The first passage was done after 3 weeks, when only ~28% of the ISE6 cells were infected, and the second was done 5 weeks later, with 43% infected cultures. Two passages from ISE6 to ISE6 cells (after initial transfer from HL-60 to ISE6) were made with host cell–free ehrlichiae, to ensure that HL-60 cells were eliminated. Expression of the p44 epitope recognized by R5E4 was lost within 3 weeks (figure 1, bottom right). At the same time, cultures of the HGEA continuously grown in HL-60 cells abundantly expressed the R5E4 p44 epitope (figure 1, top left), but organisms continuously grown in ISE6 did not (figure 1, top right). All ehrlichiae, regardless of host cell, bound polyclonal serum and anti-p60 MAbs 3C9 and 15D4. Additional samples taken 1.5–4 months later yielded the same results.

Five months after the initial HL-60 to ISE6 transfer, the HGEA were returned to HL-60 cells, resulting in 95% infection within 6 weeks. Examination of Western blots from these cultures (figure 1, bottom left) revealed a pattern similar to that of HGEA grown continuously in HL-60 cells (figure 1, top left). Of note, p44 antigens were again numerous and strongly expressed, and MAb R5E4 avidly reacted with an epitope of

Figure 1. Effect of alternating host cell type on antigen expression in human granulocytic ehrlichiosis agent. Western blot of pathogen continuously grown in human (HL-60; top left, H-H) or Ixodes scapularis tick cells (ISE6; top right, i-i) or transferred from HL-60 to ISE6 (bottom right, H-i) and then back to HL-60 (bottom left, H-i-H). Blot strips were probed with antisera or antibodies indicated at bottom of each panel. Arrows, location of 44- and 60-kDa antigens (p44 and p60). Molecular weight markers are from 2 standards (not shown), SeeBlue and Mutimark, run in each gel and blotted to membrane with the antigens.
that molecular weight. Tick cells did not survive incubation at 37°C, and none were seen in IFA slides or Giemsa-stained smears.

The p44 epitope is not temperature inducible in tick cells. Next, we tested whether expression of the p44 epitope could be induced in HGEA–tick cell culture by elevating the temperature to 37°C. The influence of lower temperatures (31°C and 25°C) also was studied alongside control cultures incubated at 34°C. Uninfected ISE6 cells mixed with infected cells at a ratio of 1:8 were 22% infected after incubation overnight at 34°C. Cultures subsequently transferred to 25°C and 31°C showed no further increase in the percentage of infected cells but maintained ehrlichiae during the following 10 days of the experiment. At 34°C and 37°C, the HGEA spread and had infected >90% of cells by the end of the experiment. By week 1, there was noticeable cytopathic effect and detachment, progressing to nearly total detachment and pronounced cell lysis with release of ehrlichiae on day 10. Tick cells did not replicate at 37°C, but the HGEA continued to multiply at a rate similar (92% infected cells) to that of HGEA incubated at 34°C (94%).

Western blots of HGEA from ISE6 grown at various temperatures. Ehrlichiae liberated from host cells were dissolved in sample buffer at the same ratio for all samples, so that extracts contained equivalent antigen concentrations (figure 2, left panel). However, the yield of ehrlichia extract from 31°C and 25°C cultures was ~25% that of 34°C and 37°C cultures. Western blots indicated little difference in the profile of antigens recognized by human patient serum (figure 2, left panel), regardless of temperature. Proteins with molecular masses of 38–50 kDa, including a single distinct 44-kDa band, were of comparable intensity at all temperatures. Above those migrated lesser antigens of 55, 58, 64 and 80 kDa. By comparison, the pattern obtained from HL-60–grown HGEA showed ≥2 bands in the 44-kDa region, and their staining intensity was much greater than that in ISE6-grown HGEA (figure 2, left panel, right lane). HL-60–grown ehrlichiae shared 38-, 55- and 80-kDa antigens plus a 48-kDa antigen of very low reactivity with organisms from tick cell culture but lacked 50- and 58-kDa bands, whereas minor bands of 100 and 130 kDa were present only in HL-60 cultures (figure 2, left panel). In lanes loaded with uninfected host cell protein extracts, the serum did not recognize any bands (data not shown). A duplicate blot developed by using MAb R5E4 showed reactivity with a single p44 antigen (figure 2, right panel) only in the lane loaded with ehrlichia extract from HL-60 cells.

Discussion

The HGEA is a remarkable organism capable of multiplying in neutrophil granulocytes, highly efficient phagocytes of mammals. Presentation of recombinant p44 to peripheral blood leukocytes induces proinflammatory cytokines in monocytes (interleukin [IL]–1β, IL-6, and tumor necrosis factor–α) and in granulocytes (IL-1β only [25]). Also, HL-60 and primary human bone marrow cells respond to ehrlichiae by production of myelosuppressive chemokines. It is tempting to speculate that bone marrow precursors could respond similarly in humans,
which would help to explain some of the major features of this disease, including pancytopenia [26].

The invertebrate immune system is less sophisticated than that of vertebrates. Defense reactions are much less specific and involve small antimicrobial peptides and hemocytes that phagocytize or encapsulate invading organisms. This system efficiently eliminates common bacteria, whereas arthropodborne disease agents appear to be tolerated unless they are not in their natural vector [9, 27, 28]. Therefore, hyperexpression of proteins that protect or hide the microbe from the mammalian immune system is not useful in the invertebrate, in which these proteins are subsequently down-regulated [11, 15, 16]. Once the tick attaches to a new host, transcription of specific antigens is activated to prepare the pathogen for successful invasion [29, 30]. DESA is one of many evasive strategies that pathogens have developed to aid transmission and to avoid being eliminated via host immune mechanisms.

Reduced expression of dominant immunogens by the invasive tick-derived forms of HGEA may serve to head off and delay the immune response by the mammalian host until the pathogen has established itself. The cyclical expression of p44 antigens in tick versus human cells may serve this function in the HGEA. The related pathogen _A. marginale_ expresses successive MSP2 antigenic variants that follow immune clearance of previous variants [7]. This process continues throughout the life of infected cattle and seems to stop only when the pathogen is ingested by ticks [8, 12]. Thus, MSP2 antigens are not down-regulated in the tick; rather, antigenic variation comes to a halt. Earlier evidence suggests a role of ticks in selection of variants expressed in salivary glands [12], but _A. marginale_ in ticks apparently express those variants ingested with the blood meal [8, 13]. p44 has not been shown to undergo antigenic variation in a manner similar to that of MSP2, and our data suggest that, like OspC of _B. burgdorferi_ [16], it is down-regulated in a tick environment and up-regulated in a mammalian system. The strong expression of p44 proteins in HL-60 culture, together with its immunodominance, indicates a possible role in adaptation of the HGEA to its host cell, the neutrophil granulocyte. It seems unlikely that it would function like MSP2 of _A. marginale_, which exclusively parasitize erythrocytes that are not immune effector cells in mammals.

**References**


