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*Article*

# Recombinant Protein-44-Based Class-Specific Enzyme-Linked Immunosorbent Assays for Serologic Diagnosis of Human Granulocytic Ehrlichiosis

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**Abstract** Recombinant protein 44, expressed and purified as a maltose-binding protein fusion peptide of the human granulocytic ehrlichiosis (HGE) agent (*Ehrlichia phagocytophila* genogroup), was used as antigen in enzyme-linked immunosorbent assays (ELISAs) to detect total antibodies, immunoglobulin (Ig) M antibodies, and IgG antibodies. Of the 67 human sera obtained from 64 HGE patients 3–5 weeks after the onset of illness and confirmed as having total immunoglobulins to whole-cell antigen by indirect fluorescent antibody analyses, 63 were positive in a polyvalent ELISA. Fifty-six and 61 sera had IgM or IgG antibodies, respectively. Fifty sera had both IgM and IgG antibodies. In specificity tests of 110 sera, one serum sample from a patient who had Lyme borreliosis reacted to the protein 44 antigen in the analysis for IgM antibody (specificity, 99%). There were no false-positive results in an ELISA for IgG antibodies. With their high sensitivity and specificity, class-specific ELISAs can be used in conjunction with indirect fluorescent antibody analyses or immunoblotting methods to help diagnose human granulocytic ehrlichiosis.

## Introduction

The human granulocytic ehrlichiosis (HGE) agent is found in California, northeastern and upper midwestern USA, Europe, and Asia, where *Ixodes pacificus*, *Ixodes persulcatus*, *Ixodes scapularis*, or *Ixodes ricinus* ticks are abundant [1–8]. Considered an emerging disease, HGE occurs in areas where Lyme borreliosis is reported. Patients exposed to more than one tickborne agent may have concurrent HGE and Lyme borreliosis [1]. Clinical signs of HGE usually include leukopenia and/or thrombocytopenia, often accompanied by headache and fever and, less frequently, associated with moderate increases in concentrations of serum hepatic transaminase.

Closely related bacteria in the *Ehrlichia phagocytophila* genogroup cause infections in humans [1, 2, 7], white-tailed deer (*Odocoileus virginianus*) [3], horses [4], and other mammals [5, 6]. Nucleotide sequence analysis of the 16S rRNA gene has shown that the HGE agent, *Ehrlichia equi*, and *Ehrlichia phagocytophila* are nearly identical, with 99.8% homology [9]. Past or current infection is indicated by the presence of ehrlichial inclusion bodies (i.e. morulae) in granulocytes, the detection of ehrlichial DNA in blood, a positive culture of the pathogen, or a positive serologic test result for antibodies. Usually, however, laboratory diagnosis relies on antibody detection assays.

Although indirect fluorescent antibody (IFA) and Western blot analyses are suitable for detecting serum

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antibodies to the HGE agent, enzyme-linked immunosorbent assays (ELISAs) are preferred because of automation and ease of standardization. A newly developed polyvalent ELISA [10] containing a purified recombinant fusion peptide, designated as HGE-44 or protein (p) 44, has aided the laboratory diagnosis of HGE. High sensitivity and specificity were noted in analyses for total immunoglobulins. Further development of class-specific ELISAs, however, is needed to measure concentrations of immunoglobulin (Ig) M and IgG antibodies. Detection of specific IgM antibody may help identify recent ehrlichial infections. The purpose of the present study was to evaluate class-specific ELISAs for diagnosis of HGE by using the p44 recombinant antigen.

## Materials and Methods

*Test Sera.* Sixty-seven serum samples from 64 persons, diagnosed by physicians as having HGE, were obtained 3–5 weeks after the onset of illness and were positive by polyvalent IFA analysis (the reference method) or immunoblotting [10, 11] methods for antibodies to whole-cell ehrlichial antigen (NCH-1 strain). The former method has been relied on heavily to diagnose HGE. All blood samples were obtained during the period 1996–1999 from patients in Connecticut, USA, who had leukopenia and/or thrombocytopenia associated with acute febrile illnesses, headache, and malaise. Further evidence of HGE infections included the identification of morulae in peripheral blood smears or detection of ehrlichial DNA by polymerase chain reaction methods. An additional four sera from patients with human monocytic ehrlichiosis (HME), whose homologous IFA titers to *Ehrlichia chaffeensis* ranged from 1:128 to 1:2,048, were selected from archived collections at the Connecticut Agricultural Experiment Station to assess specificity along with 59 sera from patients who had Lyme borreliosis ($n=25$), babesiosis ($n=9$), syphilis ($n=18$), or rheumatoid arthritis ($n=7$). Sera from subjects who had Lyme borreliosis had homologous antibody concentrations of 1:160 to 1:20,480 but lacked antibodies to the HGE agent, as determined by IFA, polyvalent ELISA, or immunoblotting methods. In addition, 47 sera from healthy persons were included as negative controls. Details on the sources of sera and prior serologic test results have been reported [10–12].

*Enzyme-Linked Immunosorbent Assays.* The materials and methods used to detect total and class-specific antibodies to the p44 antigen were essentially the same as those described earlier for serodiagnosis of Lyme borreliosis and HGE [10, 12] for either whole-cell or recombinant antigens. The HGE recombinant antigen, fused with maltose-binding protein (MBP), was the same reagent evaluated before [10] in a polyvalent ELISA. Methods of antigen preparation, purification, and the use of MBP controls likewise have been reported [10]. Use of MBP as a fusion partner over glutathione transferase improved the solubility of the recombinant protein. The concentration of the p44 antigen used to coat polystyrene plates, however, was increased to 3 μg/ml for optimal reactivity in both class-specific ELISAs. The working dilutions of commercially prepared (Kirkegaard & Perry Laboratories, USA), affinity-purified horseradish peroxidase-labeled goat antihuman antibodies in IgM (μ-chain specific) or IgG (γ-chain specific) analyses were 1:8,000 and 1:10,000, respectively. Human sera were diluted to 1:80 in phosphate-buffered saline solution (PBSS, pH=7.2) for initial testing; if positive, samples were re-analyzed to determine titration endpoints.

Forty-seven or 45 negative control sera were diluted in PBSS to 1:80, 1:160, and 1:320 and tested with p44 antigen and conjugates to calculate net absorbance values to define critical regions for IgM- and IgG-positive results, respectively. Statistical analyses (3 standard deviations plus the mean) of net optical density (OD) values were used to establish cut-off values for a positive reaction. In tests for IgM antibodies, a net OD value of 0.13 was considered positive for sera that reacted at dilutions of 1:80 or greater. Net OD values of 0.24, 0.18, and 0.16 marked the critical regions for IgG antibodies at serum dilutions of 1:80, 1:160, and ≥1:320, respectively. All polystyrene plates contained the same positive and negative control sera as well as checks for PBSS, antigen, MBP, and peroxidase-labeled antibodies. Groups of 10 and 7 positive sera were re-tested along with 3 negative sera on different days to assess reproducibility of IgM and IgG assay results, respectively.

*Immunoblotting.* Western blot analysis of 35 human sera, also positive for antibodies to the HGE agent by IFA analyses, was used to verify reactivity to the p44 antigen of whole cells. The immunoblotting procedures for measuring total immunoglobulins have been described previously [10, 12].

*Statistical Analyses.* A z-test determined significant differences in percentages of positive results. The Yates correction was applied to calculations as a part of the statistical software program (SigmaStat; SPSS, USA).

## Results

Of the 67 sera positive for antibodies to the HGE agent in IFA analyses, 63 were positive for total immunoglobulins in an ELISA with the p44 antigen. When compared to the expected rate of 100% for IFA analyses, the percentage value (94) was statistically insignificant ($z=1.529$, $P=0.126$). One of four sera containing homologous antibodies (titer=1:1,280) to *Ehrlichia chaffeensis*, the etiologic agent of HME, also had antibodies to the p44 antigen at a serum dilution of 1:160 in a polyvalent ELISA. This sample was negative by IFA analysis for HGE. All other sera in specificity testing plus the 47 normal sera from healthy subjects were negative by IFA staining methods and a polyvalent ELISA for HGE.

Seropositivity rates for IgM or IgG antibodies (84% and 91%, respectively) were statistically insignificant ($z=0.964$, $P=0.335$) (Table 1). Fifty of 67 sera had both IgM and IgG antibodies. Sera from all 64 subjects in the study group were positive in either or both class-specific ELISAs. Geometric mean antibody titers for ELISAs designed to detect IgG antibodies (mean=1,251) or total immunoglobulins (mean=951) were similar to results obtained for IgM antibody (mean=1,050). In each case, a maximal antibody titer of 1:20,480 was noted for sera in all three ELISAs. The geometric mean antibody titer (232) and maximal antibody titer (1:2,560) for sera tested by IFA staining methods were lower. Results for 13 of 17 positive sera re-tested on different days indicated changes of twofold or less for either group of class-specific antibodies. Titration endpoints for the remaining four positive sera differed by fourfold. Findings for three negative sera were identical in the second trials. In tests of specificity,

**Table 1** Reactivity of human sera to the recombinant p44 antigen of the human granulocytic ehrlichiosis agent by polyvalent or class-specific enzyme-linked immunosorbent assays

| Study group | Total sera tested | No. (%) of sera positive for antibodies[a] | | |
|---|---|---|---|---|
| | | Total immunoglobulin | IgM | IgG |
| HGE patients | 67 | 63 (94)[b] | 56 (84)[b] | 61 (91) |
| HME patients | 4 | 1 (25) | 0 | 0 |
| Babesiosis patients | 9 | 0 | 0 | 0 |
| LB patients[c] | 25 | 0 | 1 (4) | 0 |
| Syphilis patients | 18 | 0 | 0 | 0 |
| Arthritis patients | 7 | 0 | 0 | 0 |

[a] All sera were tested for total antibodies to the NCH-1 strain of the HGE agent or to the p44 recombinant antigen of the NCH-1 strain by polyvalent or class-specific ELISAs, respectively.
[b] Differences in percentages are statistically insignificant ($z=1.574$, $P=0.116$)
[c] Negative by immunofluorescence assay for total immunoglobulins to the HGE and syphilis (secondary or latent) agents
HGE, human granulocytic ehrlichiosis; HME, human monocytic ehrlichiosis; LB, Lyme borreliosis

there was one false-positive reaction. A serum sample from a Lyme borreliosis patient, negative by IFA analysis with whole-cell NCH-1 antigen, reacted to the p44 antigen of the HGE agent in tests for IgM antibody (specificity, 99%). The antibody titer (1:320) for this serum was less than the homologous titer of 1:1,280 to *Borrelia burgdorferi*, as determined by a polyvalent ELISA with whole-cell spirochetes. All remaining sera, MBP, PBSS, and other controls were negative.

Results of IFA analyses, immunoblotting, and ELISAs were compared for a subset of sera to further assess ELISA sensitivities. Of the 35 sera positive by IFA and immunoblotting procedures, 34 were positive by a polyvalent ELISA. Results of ELISAs with p44 antigen for IgM (80%) and IgG antibodies (91%) were statistically insignificant ($z=0.967$, $P=0.333$).

## Discussion

Class-specific ELISAs with the p44 recombinant antigen of the HGE agent are suitable for detecting antibodies to organisms belonging to the *Ehrlichia phagocytophila* genogroup. By performing both class-specific ELISAs, we were able to confirm HGE infections in all 64 subjects in the study group. Like the polyvalent ELISA developed earlier and shown to have similar sensitivity as IFA analysis [10], both class-specific ELISAs can be used as adjunct tests along with IFA or immunoblotting methods. However, false-positive reactions can sometimes occur in IFA analysis [13, 14], while banding patterns can be weak and inconclusive in blots. Results for IgM or IgG antibody titers in class-specific ELISAs were highly reproducible and specific. Therefore, the use of class-specific ELISAs as supportive diagnostic tests provides another option for verifying or clarifying serologic results determined by IFA or immunoblotting tests that contain whole-cell antigens. With automation, large numbers of serum samples can be analyzed by ELISA, which is the preferred initial screening method.

Assay sensitivities for polyvalent and class-specific ELISAs were statistically insignificant. There were 19 sera, however, judged as negative in one or more ELISAs, 11 of which were nonreactive in tests for IgM antibodies. Humoral responses in patients vary during early HGE infection [15, 16], and IgM antibody titers tend to be lower than IgG antibody concentrations. In analyses of sera from culture-confirmed HGE infections, most patients normally developed antibodies within 2 weeks after the onset of illness, but antibody titers continued to increase during the first month of illness [15]. Therefore, the time when blood samples are collected relative to the onset of illness is an important factor in determining seropositivity rates. When using class-specific ELISAs, it is suggested that sequential serum samples be tested.

The majority (75%) of our samples contained both IgM and IgG antibodies and were collected during summer and fall, times when *Ixodes scapularis* nymphs or adults are abundant. We suspect, like others [17], that nymphs and female ticks transmit the HGE agent over extended periods during the warmer months. Obtaining blood samples about 3–5 weeks after the onset of illness seems to be suitable for detecting adequate concentrations of IgM and IgG antibodies and was helpful in verifying early infections. In other work, antibodies were detected 11–14 months after illness onset [15].

Minor cross reactivity was noted in the ELISAs. One serum sample from a Lyme borreliosis patient reacted to the p44 antigen in an ELISA for IgM antibody, while another serum sample from an HME patient reacted weakly in a polyvalent ELISA for total immunoglobulins to p44. Both persons may have had prior exposure to the HGE agent. The geographic distributions of these diseases overlap in eastern USA. Alternatively, both reactions could be false-positive reactions caused by broadly reacting, nonspecific IgM antibody. In IFA analysis with whole-cell HGE antigen, false-positive results were likewise noted when sera from persons

who had Lyme borreliosis or HME infections were tested [13]. These results, albeit infrequent, have been attributed to the reactivity of nonspecific antibodies produced to heat-shock proteins or other peptides shared among unrelated whole-cell bacteria [13, 14]. The potential for false-positive results in IFA analysis with whole-cell antigen underscores the importance of developing highly specific ELISAs that can be used as alternative tests. It is particularly important to have such tests available because the number of HGE cases is expected to rise over the next several years [18]. Moreover, in the absence of culturing, it is also important to consider the results of polymerase chain reaction, the presence of morulae in neutrophils, or the results of blood cell counts whenever possible. Laboratory diagnosis of HGE should be based on data from different sources.

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