Comparison of Two Recombinant Major Outer Membrane Proteins of the Human Granulocytic Ehrlichiosis Agent for Use in an Enzyme-Linked Immunosorbent Assay

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Enzyme-linked immunosorbent assay (ELISA) for human granulocytic ehrlichiosis (HGE) using two different recombinant P44 proteins (rP44 and rP44-2hv) of the HGE agent as antigens was evaluated. Sera from a total of 72 healthy humans both from regions where HGE is nonendemic and regions where HGE is endemic were used as negative controls to determine the cutoff value for ELISA. Sera from a total of 14 patients (nine from whom the HGE agent was isolated and five who were HGE-PCR positive) were used as positive controls. One hundred nine sera from 72 patients in an area where HGE is endemic who were suspected of having HGE were examined by ELISA and indirect immunofluorescence assay (IFA). All IFA-negative sera were negative by both ELISAs. Of 39 sera that were IFA positive, 35 and 27 were positive by ELISA using rP44 and rP44-2hv, respectively, indicating that the use of rP44 is more sensitive. Western blot analysis of the four rP44-ELISA-negative IFA-positive sera using whole HGE agent as antigen suggests that these four sera were false IFA positive. There was no difference in results with or without the preabsorption of sera with Escherichia coli or with or without the cleavage of the fused protein derived from the vector. There was a significant positive correlation between IFA titers and optical densities of ELISAs. Four Ehrlichia chaffeensis-positive and 10 Borrelia burgdorferi-positive sera were negative by ELISA. However, two Babesia microti-positive sera showed strong cross-reactivity to the fused vector protein, which was eliminated after cleavage of the protein. Thus, ELISA using rP44 nonfusion protein would provide a simple, specific, and objective HGE serologic test which can be easily automated.

Human granulocytic ehrlichiosis (HGE) was first reported in 1994 from Wisconsin and Minnesota (2, 5) and subsequently from other areas of the United States (1, 9, 32, 36) and Europe (17, 25, 34). The etiologic agent of HGE is an obligate intracellular bacterium that belongs to the Ehrlichia equi-E. phagocytophila group on the basis of 16S rRNA gene sequence comparison (5) and serological cross-reactivity (8). Development of a specific, sensitive, and rapid diagnostic method to distinguish HGE from other tick-borne infections is desirable to ensure appropriate antimicrobial therapy, because early, but positive IFA results may not be definitive because of the subjective nature of evaluation and occasional cross-reactivity with other antigens. Comparison of sensitivity and specificity is, therefore, meaningful when compared among the same type of assay, such as serology, but not when compared between different types of tests. IFA using HGE agent- or E. equi-infected cells as antigen is currently the most widely used method for diagnosis of HGE (3, 6, 22). Although IFA is sensitive and simple, it has several problems. Since the HGE agent is an obligate intracellular bacterium, it is necessary to culture the HGE agent in eukaryotic host cells to prepare infected-cell antigen. Culturing is labor-intensive and produces batch-to-batch variation in antigens. Furthermore, the use of whole infected cells as antigen may increase the false-positive rate due to antigenic cross-reactivity. The visual evaluation of test results precludes rapid testing of a large number of samples, and the subjective evaluation of test results may cause variation in titers among different laboratories and technical personnel. Moreover, the cutoff titers for positive IFA reactions differ among laboratories, ranging from 20 to 80 (3, 4, 6, 21, 34, 37, 38).

Enzyme-linked immunosorbent assay (ELISA) is desirable for automated testing of large numbers of serum samples. Ravn et al. (26) reported that an ELISA using native HGE agent cultured in HL-60 cells as antigen is more sensitive than IFA. However, some samples are ELISA positive but Western blot negative. They recommended a two-test method of screening by ELISA and confirmation of specificity by Western blot analysis (26). Recently, an ELISA for HGE using a recombinant surface 44-kDa protein (HGE-44) of the HGE agent...
(N)H1 strain isolated from a patient in Massachusetts) fused with maltose binding protein was reported (total molecular mass of approximately 80 kDa) (12). We previously demonstrated that a dot blot assay using the recombinant major surface 44-kDa protein P44 (rP44) of the HGE agent (strain no. 13, isolated from a patient in New York) is useful for serodiagnosis of HGE (39). Further molecular analysis revealed that among approximately 20 P44 proteins of the multigenic family, P44-2 is most abundantly expressed by the HGE agent in HL-60 cell culture, and HGE patients' sera reacted with this band (20, 30, 40). In the present paper, we compare the usefulness of our rP44 and the recombinant P44-2 hypervariable region (rP44-2hv) as antigen with ELISAs using various sera, including B. microti antibody-positive sera, and investigate the need for either Escherichia coli preabsorption of sera or cleavage of fused vector peptide from the fusion protein.

MATERIALS AND METHODS

Sera. A total of 109 sera were collected from 72 patients at Westchester Medical Center in New York State from June 1995 to September 1997 who were suspected of having HGE based on clinical signs and exposure to ticks. Sera were collected once from 45 patients, twice from 17 patients, and three times from 4 patients, with not yet been identified in Japan. Ten sera, which were demonstrated to be seropositive by ELISA and Western blotting analysis for B. burgdorferi (10), were also tested in the present study. Two sera from B. microti-infected patients were supplied by Lily I. Kong, MRL Diagnostic Laboratory (Cypress, Calif.). Five sera, positive for antibodies against Ehrlichia chaffeensis by IFA and Western immunoblot analysis as described later. ELISA was independently performed three times for each IFA-negative serum sample.

IFA. IFA was performed as previously described (27). Briefly, the HGE agent isolate no. 13, referred to as New York isolate (27) was cultured in the human promyelocytic leukemia cell line HL-60. Heavily infected (>80% infected) cultures of cells were suspended in RPMI 1640 medium and were dispensed onto 12-well slides at a concentration of 10^5 cells/well. A twofold serial dilution of test serum starting at 1:20 was prepared in 2× phosphate-buffered saline (PBS; 19 mM Na_2HPO_4, 1.2 mM Na_2HPO_4, 300 mM NaCl, pH 7.4). Ten microliters of each were also tested in the present study. Two sera from Westchester, New York, and sera from 53 healthy humans in Japan, kindly provided by Makoto Kawahara, Nagoya City Public Health Research Institute (Nagoya, Japan), were used as negative controls. HGE is endemic in Westchester but has not yet been identified in Japan. Ten sera, which were demonstrated to be seropositive by ELISA and Western blotting analysis for B. burgdorferi (10), were also tested in the present study. Two sera from B. microti-infected patients were supplied by Lily I. Kong, MRL Diagnostic Laboratory (Cypress, Calif.). Five sera, positive for antibodies against Ehrlichia chaffeensis by IFA and Western immunoblot analysis (33), were provided by MRL Diagnostic Laboratory.

Preabsorption of sera. E. coli BL21(DE3)/pLysS transformed with pET30a vector (39), was independently performed three times for each IFA-negative serum sample. ELISA was independently performed three times for each IFA-negative serum sample.

RESULTS

Determination of the cutoff value in ELISA. Sera from 20 healthy humans in the United States and 52 of 53 healthy humans in Japan were IgG antibody negative (<1:20) by HGE-IFA. One IFA-positive serum was further examined by Western immunoblot analysis as described later. ELISA was independently performed three times for each IFA-negative serum sample.

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ELISA using positive control sera. Convalescent-phase sera from 14 patients from whom HGE agent was isolated and/or who were HGE-PCR positive (11, 38) were used as positive controls in ELISA testing. The results from ELISA using EK-rP44 as an antigen are summarized in Table 1. The tests were independently repeated three times with ELISA plates coated with different batches of recombinant antigen; in every test all the samples were positive. The antibody titers were determined by IFA and ELISA were similar. Coefficients of variation were calculated by dividing the SDs of replicates by the means of replicates. A value of less than 20% indicates adequate reproducibility (15). In the present results, coefficients of variation of all samples were less than 20%, indicating the adequate precision of this system. The results of ELISA using rP44 or EK-rP44-2hv were similar (data not shown).

Reactivity of sera from patients positive for antibodies to E. chaffeensis, B. burgdorferi, or B. microti. To check cross-reactivity in ELISA, 4 E. chaffeensis, 10 B. burgdorferi-, and 2 B. microti-positive sera were tested. Sera from B. microti-infected patients reacted with rP44 antigen in ELISA, and OD$_{450}$ were 0.858 and 0.786, respectively. However, these reactivities disappeared after digestion of rP44 with EK, and the OD$_{450}$ reduced to 0.147 and 0.122, respectively. These sera from B. microti-infected patients did not react with EK-rP44-2hv antigen (OD$_{405}$ < 0.139). None of E. chaffeensis- or B. burgdorferi-infected sera reacted with rP44 antigen (OD$_{405}$ < 0.243), EK-rP44 antigen (OD$_{405}$ < 0.262), or EK-rP44-2hv antigen (OD$_{405}$ < 0.139).

ELISA of patient sera using EK-rP44 or rP44. One hundred nine sera from 72 patients suspected of having HGE were examined by ELISA testing. All IFA-negative sera were negative by ELISA using EK-rP44 or rP44. Of 39 IFA-positive (>1:20) sera, 35 from 21 patients were positive by ELISA using EK-rP44 or rP44. To verify that an ELISA-positive reaction was not against E. coli proteins that may be present in the affinity-purified rP44 antigen preparation, all patient sera were preabsorbed with E. coli transformed with pET-30a expression vector in order to remove antibodies which might be present and potentially react with E. coli proteins, and the ELISA was repeated. The same results as obtained without preabsorption of sera were obtained (data not shown). We, therefore, conclude that the ELISA reactivity was against rP44, not against E. coli proteins, and that preabsorption of sera with E. coli is not required for this ELISA.

Reactivities of four IFA-positive but ELISA-negative patient sera were examined by Western blot analysis using the purified whole HGE agent antigen. One additional serum from a healthy Japanese individual (N34), which was IFA positive (1:320) but ELISA negative, was also included in the analysis. The results are shown in Fig. 1 and Table 2. Two sera (serum ID.B66 and A19) from patient P63 and one serum (serum ID.J14) from healthy human N34 reacted only with an approximately 70-kDa protein of the purified HGE agent in Western blotting analysis. One serum (serum ID.B20) from patient P24 reacted only with a single band of 44 kDa of the HGE agent even at the serum dilution of 1:100. None of these four sera reacted with rP44 or uninfected HL-60 cells in Western blotting analysis.

### Table 1. IFA and reproducibility of ELISA in sera from seven culture and/or HGE-PCR-positive patients

| Serum ID | HGE isolation | HGE-PCR | IFA titer$^a$ | ELISA titer$^a$ | OD$_{450}$ in ELISA$^b$ (n = 3) | CV$^c$ |
|----------|---------------|---------|---------------|----------------|-------------------------------|-------|
| A12      | +             | 5.120   | 2.560         | 1.028 ± 0.101  | 0.098                         |
| A30      | NT$^d$        | 5.120   | 2.560         | 0.960 ± 0.043  | 0.045                         |
| B35      | +             | 2.560   | NT            | 0.917 ± 0.032  | 0.035                         |
| B58      | +             | 40      | 80            | 0.301 ± 0.025  | 0.083                         |
| C11      | NT            | 640     | 320           | 0.856 ± 0.056  | 0.065                         |
| C12      | NT            | 160     | 40            | 0.521 ± 0.049  | 0.094                         |
| C14      | NT            | 160     | 80            | 0.306 ± 0.044  | 0.144                         |
| C15      | NT            | 640     | 320           | 0.734 ± 0.063  | 0.086                         |
| D03      | +             | 80      | 320           | 0.767 ± 0.033  | 0.043                         |
| D11      | +             | 1,280   | 640           | 0.808 ± 0.045  | 0.056                         |
| D17      | +             | 160     | 640           | 0.834 ± 0.028  | 0.034                         |
| D46      | +             | 80      | 160           | 0.581 ± 0.039  | 0.067                         |
| D50      | +             | 320     | 640           | 0.844 ± 0.020  | 0.024                         |
| D74      | +             | 320     | 160           | 0.657 ± 0.052  | 0.049                         |

$^a$ Reciprocal of the highest serum dilution.
$^b$ ELISA using EK-rP44. Mean ± standard deviation.
$^c$ CV, coefficients of variation (standard deviation of OD$_{450}$/mean of OD$_{450}$).
$^d$ NT, not tested.

### Table 2. Results of IFA, ELISA, and Western blotting in sera from three patients and one healthy human who were IFA positive but ELISA negative

| Patient ID | Serum ID | IFA titer$^a$ | OD$_{450}$ in ELISA$^b$ (n = 3) | Molecular mass of reacting protein (kDa)$^c$ |
|------------|----------|---------------|-------------------------------|---------------------------------------------|
| P24        | B20      | 320           | 0.189 ± 0.051                 | 44                                          |
| P32        | B14      | 80            | 0.129 ± 0.009                 | No band                                     |
| P63        | A19      | 80            | 0.162 ± 0.037                 | 70                                          |
| P63        | B66      | 1,280         | 0.157 ± 0.025                 | 70                                          |
| N34        | J14      | 320           | 0.106 ± 0.015                 | 70                                          |

$^a$ Reciprocal of the highest serum dilution.
$^b$ ELISA using EK-rP44. Mean ± standard deviation.
$^c$ Western blotting using purified HGE agent.
All IFA-negative sera were negative by ELISA using rP44-2hv. EK-rP44 ELISA, indicating a positive correlation between IFA titer and OD of correlation is 0.740 and is statistically significant (P < 0.001) (n = 181).

The results of IFA and ELISA using EK-rP44 are compared in Table 3. The relative diagnostic sensitivity and specificity of IFA relative to ELISA, calculated by this manner, were 100 and 97%, respectively. These results indicate that ELISA using EK-rP44 is as sensitive as IFA and more specific than IFA. The correlation of titer and OD of rP44 ELISA was as specific and sensitive as IFA (39). Furthermore, dot immunoblot assay using a synthetic oligopeptide specific to the hypervariable region, P44-2hv of one of P44 proteins, P44-2, and convalescent sera from patients with HGE demonstrated that antibody specific to P44-2hv was developed in these patients (40). However, Western blot analysis and dot immunoblot assay are not convenient for automated testing of a large number of clinical samples. We developed an ELISA system using a recombinant P44 as antigen because ELISA is easily adapted to automation, allowing rapid testing of a large number of patient samples at a relatively low cost. There was significant positive correlation between IFA titer and OD of rP44 ELISA.

Comparison of results of IFA and ELISA using EK-rP44. The results of IFA and ELISA using sera from patients and healthy individuals were compared (Table 3). Usually, a new assay is evaluated by comparison with another serological assay or combination of assays, and relative sensitivity and specificity are calculated by making the previous assay result the standard. In the present study, however, false-positive cases in IFA were observed and ELISA was more specific than IFA. In such a case, the relative diagnostic sensitivity and specificity can be calculated by making the new method the standard of comparison (15). The relative diagnostic sensitivity and specificity of IFA, calculated by this manner, were 100 and 97%, respectively. These results indicate that ELISA using EK-rP44 is as sensitive as IFA and more specific than IFA. The correlation of titers and ODs of ELISA using EK-rP44 is shown in Fig. 2. The rho value calculated by Spearman’s rank correlation was 0.740 (P < 0.001), indicating a positive correlation between IFA titer and OD of EK-rP44 ELISA.

The 38- to 49-kDa proteins of the HGE agent have been shown to be immunodominant antigens in human infection (13, 26, 38). IgG antibodies against 44-kDa protein were detected in all culture-positive and PCR-positive patients’ sera (39, 39) and in seven of nine acute-phase patients (1 week after onset of symptoms) and in 10 day sera of mice exposed to Ehrlichia-infected (NCH-1 strain) ticks (13). These proteins are encoded by the p44 multigene family (40). Recently, Zhi et al. cloned several genes belong to this family (39, 40). Zhi et al. demonstrated that Western blot analysis and dot immunoblot assay using rP44 as antigen were as specific and sensitive as IFA (39). Furthermore, dot immunoblot assay using a synthetic oligopeptide specific to the hypervariable region, P44-2hv of one of P44 proteins, P44-2, and convalescent sera from patients with HGE demonstrated that antibody specific to P44-2hv was developed in these patients (40). However, Western blotting analysis and dot immunoblot assay are not convenient for automated testing of a large number of clinical samples. We developed an ELISA system using a recombinant P44 as antigen because ELISA is easily adapted to automation, allowing rapid testing of a large number of patient samples at a relatively low cost. There was significant positive correlation between IFA titer and OD of rP44 ELISA. Since another report of ELISA using recombinant HGE-44 does not describe
ODs (12), it is difficult to compare that data with our data. Although this was not examined in another ELISA study (12), it is difficult to compare that data with our data. Thus, it is difficult to interpret the high background OD of that ELISA. This variation may be due to the difference of HGE agent strains or 44-kDa protein genes cloned or expression vectors used. Amino acid identities between P44 and HGE-44 and between P44-2 and HGE-44 are 75.3 and 80.2%, respectively (40).

In our ELISA systems using EK-rP44 and EK-rP44-2, no sera from the limited number of patients infected with B. burgdorferi, B. microti, or E. chaffeensis had positive reactions. Although B. microti-infected patients’ sera reacted with rP44 without EK treatment, the reactivity disappeared after treatment with EK or rP44. The exact reason for this cross-reactivity is unknown. The rP44 used in the present experiments was cloned in the pET-30a expression vector. This vector encodes some affinity tags which are useful for assaying expression levels and purifying proteins. EK treatment is able to separate the affinity tags from the recombinant protein. B. microti-infected patients’ sera also reacted with recombinant rP30 of Ehrlichia canis (data not shown), which was prepared by using the same vector system (23). These results suggest that the false reactivity might be directed to the affinity tag region. Another report of an ELISA using rHGE-44 fused with maltose binding protein (12) did not examine whether human babesial infection sera cross-react with the fusion protein. B. burgdorferi, B. microti, and the HGE agent are carried by the same tick vector, Ixodes scapularis (28, 35). Simultaneous infections of patients with these agents have been reported (7, 21). Antibiotics effective for these microorganisms are different and it is important to distinguish among these diseases. Treatment of rP44 by EK had no effect on the results of HGE-ELISA. Therefore, an ELISA system using the EK-rP44 described here may be able to distinguish HGE from human monocytic ehrlichiosis, babesiosis, and Lyme borreliosis.

The five sera, which were positive by IFA testing but negative by ELISAs using EK-rP44 and EK-rP44-2h, were further examined by Western blotting analysis. Three sera from one patient and one healthy individual reacted only with an approximately 70-kDa protein of the HGE agent. Previously, Ildo et al. reported that heat shock protein 70 (HSP70) of the HGE agent (an 80-kDa protein by their description) was cross-reactive with B. burgdorferi HSP70 (14). HSP70s of many microorganisms such as E. coli, Mycobacterium tuberculosis, and Plasmodium falciparum share common antigenicity. It has been identified as an immunodominant antigen in these infections (30). Although we did not determine whether the 70-kDa protein that reacted with the sera was HSP70 of the HGE agent or not, it is possible that the protein is HSP70. ELISA using EK-rP44 or EK-rP44-2h could eliminate false-positive reactions due to cross-reactions caused by common antigens including HSP70 present in many microorganisms. One serum reacted with a single 44-kDa band of the HGE agent but not with rP44 in Western blot analysis. The rP44 used in the present study lacks one-third of of P44 C terminus (39). Because the HGE agent expresses multiple P44 homologous proteins encoded by a polymorphic multigene family (40), it is possible that this serum reacted with one or more of these P44 homologous proteins distinct from rP44. However, since P44 homologous genes have highly conserved N-terminal regions (40), mouse polyclonal antibody against rP44 strongly recognizes multiple 44- to 42-kDa proteins in six HGE isolates (39), and sera from patients infected with the HGE agent reacted with not only multiple P44s but also with other proteins in Western blotting analysis using the purified HGE agent (13, 26, 38). Thus, it is unlikely that individuals infected with the HGE agent develop an antibody against only a single band of the HGE agent. Lastly, one serum collected more than 3 months after the first sampling, at a time when the IFA titer was significantly decreased, did not react with any proteins of the native HGE agent or rP44 in Western blotting. The disappearance of antibody against the HGE agent after recovery from disease has been reported (11, 26). We speculate that the antibody against the HGE agent had disappeared in this case and that the reactivity in IFA was considered false positive.

The rP44 used in this study is coded by the N-terminal conserved region and a part of the hypervariable region of p44 homologous genes (39, 40). As one of the membrane proteins, this protein is hydrophobic, thus relatively difficult to handle (39). In contrast, rP44-2h is encoded by hypervariable region of the p44-2 gene and is hydrophilic (40), thus easy to handle. However, the sensitivity of rP44-2 in ELISA was lower than that of rP44. This means that the protein coded by conserved regions of the p44 gene may be required for sensitive detection of anti-HGE antibodies in patients. Alternatively, since three patients were repeatedly positive with rP44 but negative with rP44-2h antigen, they may be infected with different strains of the HGE agent which lack or do not express p44-2. Because of the excellent specificity, objectiveness, and ease of the assay, this ELISA system using EK-rP44 as antigen is expected to improve serodiagnosis of HGE.

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