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Serology of Culture-Confirmed Cases of Human Granulocytic Ehrlichiosis

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We evaluated the antibody responses in the sera of 24 patients with culture-confirmed human granulocytic ehrlichiosis (HGE). Antibody titers were measured by an indirect immunofluorescent-antibody assay (IFA) by using a local human isolate as the source of antigen. All patients received appropriate antimicrobial treatment. One hundred five serum specimens collected at baseline and at periodic intervals for up to 14 months were included in the study. Seroconversion was observed in 21 of 23 patients (91.3%) from whom convalescent-phase sera were obtained. Antibodies were first detected at an average of 11.5 days after onset of symptoms. Peak titers (≥2,560 for 71.4% of patients and ≥640 for 95.2% of patients) were obtained an average of 14.7 days after onset of symptoms. Eleven of 13 patients (84.6%) from whom sera were collected between 6 and 10 months after onset of symptoms were still seropositive, and sera from 5 of 10 (50%) patients tested positive between 11 and 14 months after onset of symptoms. For a subset of 71 serum specimens from 17 patients with culture-confirmed HGE also tested by IFA by using either a human isolate from Wisconsin or an *Ehrlichia equi* isolate from a horse, there was qualitative agreement for 62 serum specimens (87.3%). Peak titers were higher, however, with the local human HGE isolate, but the difference was not statistically significant. In summary, most patients with culture-confirmed HGE develop antibodies within 2 weeks of onset of symptoms. Antibodies reach high titers during the first month and remain detectable in about one-half of patients at 1 year after onset of symptoms.

Human granulocytic ehrlichiosis (HGE) is an emerging vector-borne infectious disease transmitted through the bite of infected *Ixodes* ticks (14). Most cases to date have been reported from the Midwest and Northeast United States, where *Ixodes scapularis* ticks are highly prevalent (1, 3, 4, 16). Classically, patients with HGE present with high fever and constitutional signs and symptoms a few days following a tick bite (1, 3).

Routine laboratory tests show leukopenia and/or thrombocytopenia and elevation of liver enzyme levels (1, 3, 4). Specific tests used to confirm the diagnosis during the acute phase include microscopic detection of inclusions in peripheral blood granulocytes, PCR with whole blood, and culture of the agent from blood (1, 3, 4, 6).

Detection of antibodies has also been used to support the clinical diagnosis by using either human isolates or the closely related ehrlichial species *Ehrlichia equi* as the source of antigen (5, 11, 12). Results of most serologic studies, however, have been based on clinically defined patient populations or a small number of patients with culture-confirmed HGE (10, 12, 15). The present study reports on the serologic test results for 24 patients with culture-confirmed HGE, the largest cohort of patients with culture-confirmed HGE tested to date, obtained by using a local human isolate as the source of antigen in an indirect immunofluorescent-antibody assay (IFA). A total of 105 serum specimens collected at baseline and for up to 14 months after onset of symptoms were studied. A comparison of the serologic findings obtained with a Wisconsin human HGE isolate or *E. equi* as the antigen is also presented.

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MATERIALS AND METHODS

Patients. Twenty-four patients diagnosed with HGE by culture of the HGE agent from blood were included in the study. These patients were diagnosed with HGE at the Westchester Medical Center, Valhalla, N.Y., between 1995 and 1998. All patients were treated with doxycycline within 8 days of the initial visit. The clinical and laboratory features of 11 of these patients have been reported previously (7–9, 13).

Sera. A total of 105 serum specimens collected during the first visit and at different time intervals for up to 14 months after onset of symptoms were tested. Sera were frozen at −70°C if they were not tested within a few weeks of collection. All sera obtained from an individual patient were tested simultaneously.

IFA. A local HGE isolate designated NY-13, which was cultured in HL-60 cells as described previously (6), was the source of antigen. The isolation and identification of this organism were published previously (8). This isolate was chosen as the source of antigen because it was the first one to be maintained in continuous culture in our laboratory. Slides were prepared for IFA when >90% of the HL-60 cells were infected, as evidenced by the presence of intracellular inclusions after Wright staining. A suspension of approximately 5×10^3 infected cells was applied to each well of 12-well Teflon-coated slides (Cell Line; Erie Scientific Co., Portsmouth, N.H.), air dried, and fixed in acetone for 10 min. Prepared slides were stored at −70°C until they were used. To maintain the consistency of the antigen preparations, several hundred slides were prepared at the same time with low-passage-number (less than five passages) NY-13 isolates. Positive and negative controls were included on each slide. Slides with *E. equi*-infected horse neutrophils (MRK isolate cultured from a horse in California) or HL-60 cells infected with the Webster HGE isolate from Wisconsin were prepared as described previously (5) and were tested at the Johns Hopkins Medical Institutions by J.S.D. Serum specimens were tested in both laboratories (New York Medical College and Johns Hopkins) at an initial dilution of 1:80 in phosphate-buffered saline containing 0.5% of nonfat dry milk (PBSM). Bound antibodies were detected after incubation with fluorescein isothiocyanate-labeled goat anti-hu-

TABLE 1. Characteristics of the serology of 24 patients with culture-confirmed HGE

Patient no.	Age (yr)/ gender[a]	Duration of symptoms at baseline (days)	Baseline serology titer[b]	First positive titer	Days after onset of symptoms for seroconversion	Possible time of seroconversion after onset of symptoms (range [days])[c]	Peak titer	Time after onset of symptoms for peak titer
1	47/M	17	640	640	17	0–17	2,560	26 days
2	20/M	3	<80	NA[d]				
3	37/M	5	<80	640	16	5–16	640	16 days
4	56/F	4	<80	1,280	12	4–12	1,280	12 days
5	71/F	8	<80	2,560	36	16–36	2,560	36 days
6	45/M	32	<80	None[e]				
7	64/F	2	80	80	2	0–2	2,560	7 days
8	1 wk/F	1	NA[f]	320	10	0–10	320	10 days
9	35/F	6	<80	2,560	13	6–13	2,560	13 days
10	60/F	4	<80	2,560	25	12–25	2,560	25 days
11	65/M	1	NA	160	18	12–18	2,560	4 mo
12	54/M	6	<80	640	13	6–13	2,560	30 days
13	50/F	3	<80	2,560	8	3–8	2,560	8 days
14	30/F	3	<80	1,280	11	3–11	1,280	11 days
15	35/M	3	<80	2,560	11	3–11	2,560	11 days
16	48/M	3	160	160	3	0–3	1,280	10 days
17	47/M	11	NA	2,560	16	0–16	2,560	16 days
18	40/F	1	320	320	1	0–1	2,560	7 days
19	72/M	4	<80	2,560	11	4–11	2,560	11 days
20	60/M	8	640	640	8	0–8	640	8 days
21	80/F	6	160	160	6	0–6	2,560	11 days
22	45/M	8	320	320	8	0–8	2,560	15 days
23	74/M	1	<80	None				
24	74/M	8	2,560	2,560	8	0–8	2,560	8 days

[a] M, male; F, female.
[b] Titer for serum obtained during the first visit.
[c] Range in days after onset of symptoms when detectable antibodies may have appeared.
[d] NA, no convalescent-phase serum was available.
[e] None, no seroconversion was observed.
[f] NA, not available.

man immunoglobulin G (IgG), IgM, and IgA (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted 1/50 in PBSM. Specimens showing fluorescent intracellular inclusions at a dilution of 1:80 were considered reactive and were serially diluted up to 1:2,560 and tested by IFA to determine the titer (5, 17). Specimens with titers of ≥2,560 were considered to have a titer of 2,560 for the purpose of analysis. For calculation of geometric mean titers (GMTs), titers of <80 were given an arbitrary value of 40.

RESULTS

Patients. Selected characteristics of the 24 patients included in this study are shown in Table 1. There were 14 males with ages ranging from 20 to 74 years (median age, 47.5 years) and 10 females ranging in age from 1 week to 80 years (median age, 53 years). The patients presented at an average of 6.2 days after onset of symptoms. Five of these patients had concomitant Lyme borreliosis with erythema migrans, with the Lyme borreliosis in four patients confirmed by isolation of *Borrelia burgdorferi* from the skin lesion. Data for three of the coinfected patients were reported previously (7, 8, 13).

Acute-phase serology. Eight of 21 evaluable patients (38%) tested positive for antibodies to the HGE agent at the first visit. The mean and median durations of disease prior to treatment for those with positive baseline serologies were 6.6 and 7 days, respectively, whereas they were 6.3 and 4 days, respectively, for the group of patients who tested negative at this visit ($P = 0.53$ for the comparison of medians; Mann-Whitney rank sum test). One patient who tested positive at baseline (patient 7, Table 1) had a prior episode of HGE that had been confirmed by PCR and serology 2 years earlier. Data for this patient were reported previously (8).

Serology in relation to disease duration. To assess the evolution of the antibody response, serum specimens obtained at baseline and thereafter were arbitrarily categorized into the following time intervals after onset of symptoms: <7 days, 7 to 14 days, 15 days to 1 month, 2 to 5 months, 6 to 10 months, and 11 to 14 months. For all but one patient at least one specimen was collected during the first month after onset of symptoms. For 5 of 23 patients (21.7%) two specimens were collected, and for 13 of 23 patients (56.5%) three specimens were collected within the first month after onset of symptoms. Twenty-one of 23 patients (91.3%) who provided specimens after they provided the baseline specimen developed antibodies (seroconverted), as determined by IFA. One of the patients who did not seroconvert was treated within 1 day of onset of symptoms (patient 23). The other patient who did not seroconvert presented 32 days after onset of symptoms and also tested negative by PCR and microscopy during the acute phase (patient 6).

Antibodies were first detected an average of 11.5 days (range, 1 to 36 days) following onset of symptoms with a GMT of 780 (Table 1). Peak titers of ≥640 were obtained for 20 of 21 (95.2%) patients who developed antibodies in the convalescent phase, and 15 of 21 (71.4%) had peak titers of ≥2,560. Peak titers were first obtained an average of 14.7 days after onset of symptoms (range, 7 days to 4 months).

Duration of antibodies. Twenty of 22 (90.9%) specimens collected between 2 and 5 months after onset of illness tested positive by IFA. Antibodies to the HGE agent were detected by IFA (titers ≥80) by 6 to 10 months for 11 of 13 patients (84.6%) and by 11 to 14 months for 5 of 10 patients (50%) (Table 2). The GMT increased to 964 between 2 and 4 weeks after onset of symptoms and gradually declined over the next few months. By 11 to 14 months after antimicrobial therapy the GMT was 130, with titers ranging from <80 to 2,560.

Comparison of different antigen preparations for detection of antibodies to HGE agent by IFA. Seventy-one serum spec-

TABLE 2. Titers of antibody to the HGE agent for 24 patients with culture-confirmed HGE at selected time intervals after onset of symptoms

Time interval	GMT	Median titer	No. of positive patients/ no. tested (%)
<7 days	61	<80	4/13 (30.7)
7–14 days	666	1,280	14/17 (82.3)
15 days–1 mo	964	1,280	21/22 (95.4)
2–5 mo	599	640	20/21 (95.2)
6–10 mo	356	320	11/13 (84.6)
11–14 mo	130	80	5/10 (50)

imens from 17 patients with culture-confirmed HGE were also tested by IFA with *E. equi* MRK-infected neutrophils or the Webster HGE isolate. Forty-four of the 71 specimens obtained from eight patients were tested with *E. equi*-infected neutrophils, and 27 specimens from nine patients were tested with the Webster HGE isolate. Concordant positive or negative results were obtained for 62 of 71 specimens (87.3%). Discrepant results were obtained for three baseline and six convalescent-phase specimens (Table 3). Two of the baseline specimens were found to be positive only with the Webster isolate, and one of the baseline specimens was found to be positive only with the NY-13 isolate. When the IFA results obtained with the NY-13 isolate were compared with the results obtained with the MRK isolate, titers of ≥2,560 were obtained for six of seven patients (85.7%) who seroconverted when the NY-13 isolate was used and for four of seven patients (57.1%) when the MRK isolate was used. Similarly, when the results obtained with the NY-13 and the Webster isolates were compared, peak titers of ≥2,560 were obtained for four of six patients (66.6%) in the convalescent phase when the NY-13 isolate was used and for three of six patients in the convalescent phase (50%) when the Webster isolate was used. The GMT of the peak titers was higher for tests with the NY-13 isolate than for tests with the MRK isolate (2,317 versus 1,279; $P = 0.172$, paired t test) or the Webster isolate (2,031 versus 1,140; $P = 0.317$, paired t test).

DISCUSSION

The antibody response to the HGE agent was evaluated by IFA for a cohort of 24 treated patients with culture-confirmed HGE. Twenty-one of 23 patients (91.2%) developed detectable antibodies (titers ≥80). Antibodies were first detected an average of 11.5 days after onset of symptoms (range, 1 to 36 days), with peak titers of ≥640 achieved within 4 weeks after onset of symptoms. Ravyn et al. (15) published a report on the use of IFA with culture-derived human isolates from Minnesota for five patients with culture-confirmed HGE. In that study they tested a few convalescent-phase specimens by separate IgG- and IgM-specific IFA assays and obtained titers lower than those that we found in our study (15). For two of their five patients the highest IgG or IgM titer was 160. Unfortunately, due to technical variables inherent to IFA, it is difficult to standardize titer cutoff values. Differences in antigen sources and preparations, use of polyvalent or monovalent fluorescent conjugates, and variability in reading of the reactivity under a fluorescent microscope may account for titer differences among laboratories (17).

There is no current consensus on the appropriate use of serology for the diagnosis of HGE. Screening serum dilutions range from 1:40 to 1:80, and many assays detect IgG selectively. When serum specimens collected early in convalescence are tested, it seems appropriate to use conjugates that detect both IgG and IgM antibodies. The findings of Ravyn et al. (15), as well as those of IJdo et al. (10) and Walls et al. (17) and our own findings (unpublished data), seem to indicate that IgM reactivity develops early in the antibody response to the HGE agent.

On the basis of our results, if only a single blood specimen is available, it would seem appropriate to consider a titer of 640 rather than a titer of 80 by a polyvalent IFA that detects both IgG and IgM antibodies to support the diagnosis of a recent infection with the HGE agent. Whether a titer of 80 is an appropriate cutoff for seropositivity will require further study of control groups without evidence of HGE. As for other infectious diseases, patients who develop high titers of antibodies in the acute phase may remain seropositive with gradually declining titers for long periods of time. Such patients may have titers to HGE of 640 without indicating a recent infection. In our cohort, 6 of 13 patients (46%) and 2 of 10 patients (20%) had titers of ≥640 by 6 months and 1 year after onset of illness, respectively. Furthermore, approximately one-half of our patients still had antibody reactivity (titer ≥80) 1 year after treatment for HGE infection; this is similar to the observations of Bakken et al. (4) on the basis of testing for antibody to *E. equi* in patients from the Midwest (11 of 24 patients had antibodies at 1 year after onset of symptoms).

In this study we also studied geographically unrelated isolates as a source of antigens, such as the Webster isolate of HGE from Wisconsin and the MRK isolate of *E. equi* cultured in California. Discrepant results were found for 9 of 71 (12.7%) serum specimens. Earlier detection of antibodies was found for two serum specimens when the Webster isolate was used and for one serum sample when the NY-13 isolate was used. Persistence of seropositivity was most frequent when the NY-13 isolate was used as the source of antigen. These differences might be due to technical factors. However, antigenic differences among HGE isolates do exist and might also account for variations in serologic assay results (2, 12, 17, 18). Preliminary studies in our laboratory have shown that the NY-13 isolate contains an immunodominant antigen of about 44 kDa which is also exhibited by other isolates of HGE (J. Knowles, K. Barber, B. Menefee, A. Markovits, G. Wormser, and M. Aguero-Rosenfeld, Abstr. VIII Int. Conf. Lyme Bor-

TABLE 3. Comparison of HGE serology by IFA with three antigen sources

Isolates compared and patient no.	Discrepant specimens (time after onset of symptoms)[a]	Titer to the following antigen:		
		MRK	Webster	NY-13
NY-13 vs MRK[b]				
3	Convalescent phase (4 mo)	<80		640
8	Convalescent phase (11 mo)	160		<80
9	Convalescent phase (4 mo)	<80		2,560
9	Convalescent phase (7 mo)	<80		640
NY-13 vs Webster[c]				
1	Convalescent phase (14 mo)		320	<80
7	Baseline		640	<80
11	Baseline		80	<80
11	Convalescent phase (8 days)		<80	2,560
15	Baseline		<80	320

[a] Discrepant indicates qualitative disagreement (positive or negative).
[b] Forty-four specimens were tested. Results were concordant (qualitative agreement [positive or negative]) for 40 specimens (90.9%).
[c] Twenty-seven specimens were tested. Results were concordant for 22 specimens (81.4%).

reliosis and Other Emerging Tick-Borne Diseases, abstr. P255, p. 69, 1999). When choosing a particular isolate as a source of antigen, it would be prudent to validate its reactivity with sera from different geographic areas.

Although most patients in our series seroconverted, two did not develop detectable antibodies. One of these patients (patient 23) received antimicrobial treatment within 1 day of onset of symptoms. Prompt treatment of this patient may have aborted the development of antibodies. Two other patients who received antimicrobial therapy within 1 day of onset of symptoms, however, did develop antibodies. It is unlikely that the reason for the lack of detectable antibodies was the antigen source since those patients were also found to be negative by use of the other isolates of *Ehrlichia* used in this study. Furthermore, they were also found to be negative when their sera were tested against several other local human isolates (data not shown).

Enzyme immunoassays such as enzyme-linked immunosorbent assays or immunoblot assays are feasible alternatives to IFA for detection of antibodies to the HGE agent (15). Technical difficulties in reproducing the IFA and reading of the IFA results will stimulate the development of assays that use purified antigens. Zhi et al. (19) recently published a report on the cloning and expression of the 44-kDa immunodominant outer membrane protein of the HGE agent. Whether this cloned antigen is broadly cross-reactive among patients infected with different strains of HGE is unknown. In the meantime, IFA of an acute-phase serum sample plus a convalescent-phase serum sample collected 2 to 4 weeks after onset of symptoms is a sensitive test for confirmation of the diagnosis of HGE.

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