Seroprevalence of Antibodies That React with *Anaplasma phagocytophila*, the Agent of Human Granulocytic Ehrlichiosis, in Different Populations in Westchester County, New York

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We determined the frequencies of antibodies to *Anaplasma phagocytophila*, the agent of human granulocytic ehrlichiosis (HGE), in different groups of adults and children from Westchester County, New York. The groups included 159 adult blood donors and 215 children who were seronegative for *Borrelia burgdorferi* antibodies, 118 adult patients and 57 children who were seropositive for *B. burgdorferi* antibodies, and 42 adult patients with culture-confirmed erythema migrans. Eighteen (11.3%) of the blood donors and 11 (5.1%) of the *B. burgdorferi*-seronegative children were found to have *A. phagocytophila* antibodies by indirect immunofluorescent-antibody assay (IFA). Nine of 42 (21.4%) patients with culture-confirmed erythema migrans tested at the baseline visit, 42 of 118 (35.6%) adults, and 3 of 57 (5.3%) children whose sera were reactive for *B. burgdorferi* antibodies also tested positive for *A. phagocytophila* antibodies. The geometric mean titer ranged from 219 to 315 for all groups, and the differences in titers among the groups were not statistically significant. Only one-third of the healthy blood donors reactive by IFA were confirmed to be positive by immunoblotting. We conclude that a significant proportion of adults and children without clinical evidence of HGE will test positive for *A. phagocytophila* antibodies when the conventional cutoff titer of 80 is used in the IFA. This information must be considered in interpretation of test results.

Human granulocytic ehrlichiosis (HGE) is an emerging tick-borne disease first described in 1994 in the midwestern United States (5, 7). The etiologic agent is an ehrlichial species closely related to *Ehrlichia phagocytophila*. It is often referred to as the HGE agent and was recently named *Anaplasma phagocytophila* (11). Since the original report, increasing numbers of cases of infection with this agent have been diagnosed in many of the same geographic areas where *Borrelia burgdorferi* infection is endemic (1, 5, 7, 8). Both infections are transmitted by the same *Ixodes* tick vectors (12).

Antibody testing by an indirect immunofluorescent-antibody assay (IFA) is the test most commonly used in the laboratory to confirm a diagnosis of HGE. We and others have shown that approximately 90% of acutely infected patients are seropositive by serologic testing during the acute or convalescent phase (≤ 30 days postinfection) (3, 7).

An antibody titer of ≥ 64 or ≥ 80 by IFA is considered a positive result (10). However, few studies exist on the rate of background seropositivity in areas of endemicity, and it is unclear if this cutoff is the most appropriate threshold for seropositivity. Indeed, evidence exists that this titer is too low. A study by Bakken et al. (6) of 475 individuals from Wisconsin without evidence of active tick-borne infection or a prior diagnosis of HGE found a seropositivity rate of 14.9%, and the patients usually had titers ≤ 320 . While such low titers occur at surprisingly high frequencies in individuals in the general pop-

ulation, in our experience patients acutely infected and confirmed to have HGE by recovery of the agent from blood have distinctly higher titers (greater than or equal to 640 in 95.2% of patients) (3). The purposes of the present study were to determine the background seropositivity rate in another area of endemicity and to attempt to determine at what age seropositivity occurs and to what extent any observed seropositivity represents subclinical or resolved *A. phagocytophila* infection versus antibody reactivity due to other causes.

Coded and uncoded sera frozen at -70°C were tested for antibodies to *A. phagocytophila*. The sera were obtained from five groups of individuals, as follows. (i) One hundred fifty-nine serum samples were collected from healthy blood donors from Westchester County, New York. These serum samples were randomly selected from the approximately 5,000 such serum samples collected during 1989 and 1990 that were known to be seronegative for antibodies to *B. burgdorferi*. (ii) A convenience sample consisted of sera from 118 adult patients (age, >19 years) whose sera were randomly selected from among those sera which tested positive for antibodies to *B. burgdorferi* by enzyme immunoassay and immunoblotting at the Westchester Medical Center from 1990 to 2000. The clinical histories of the donors of these samples were not available. (iii) A second convenience sample consisted of sera from 42 adult patients (age, >19 years) with erythema migrans who were diagnosed with *B. burgdorferi* infection at the Westchester Medical Center from 1991 to 2000 on the basis of recovery of the spirochete from culture of a skin biopsy sample. The sera tested were baseline samples. (iv) A third convenience sample consisted of sera from 57 pediatric patients (age, ≤ 19 years) who had tested positive for antibodies to *B. burgdorferi* at the Westchester

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TABLE 1. Seroprevalence of antibodies to *A. phagocytophila* by IFA in different populations from Westchester County, New York

Group	No. of serum samples positive/no. tested (%)	IFA titer		
		Range	Median	GMT ^a
Healthy blood donors	18/159 (11.3)	80–640	160	226
Pediatric <i>B. burgdorferi</i> -negative subjects	11/215 (5.1)	80–640	160	219
Pediatric <i>B. burgdorferi</i> -positive subjects	3/57 (5.3)	160–320	320	254
Adult patients with erythema migrans ^b	9/42 (21.4)	80–≥2,560	160	242
Adult <i>B. burgdorferi</i> -positive subjects	42/118 (35.6)	80–≥2,560	320	315

^a Differences in titer among groups were not statistically significant ($P = 0.695$ by Kruskal-Wallis analysis of variance on ranks).

^b Sera were collected at the baseline, prior to antimicrobial treatment.

Medical Center from 1991 to 1999. (v) The final sample consisted of sera from 215 pediatric patients from Westchester County collected from 1996 to 2000 for endocrinologic testing. These sera were selected in order to obtain representative samples from children in different age groups (<1 to 19 years).

Serologic testing. Antibodies to *B. burgdorferi* were measured by the recommended two-step approach. The sera were first assayed by an immunoglobulin G (IgG) and IgM enzyme-linked immunosorbent assay (ELISA; Wampole Laboratories, Cranbury, N.J.), and those that were reactive were subjected to separate IgG and IgM immunoblotting assays (MarDx Diagnostics, Inc., Carlsbad, Calif.) as described previously (2).

An IFA was used to detect antibodies to *A. phagocytophila* in assays with a local human isolate (isolate NY-13), as described previously (3). Briefly, slides were prepared for IFA when >90% of the HL-60 cells used in the tests were infected with *A. phagocytophila* NY-13, as detected by Wright staining. Suspensions of infected cells were applied to each well of 12-well Teflon-coated slides (Cell Line; Erie Scientific Co., Portsmouth, N.H.), air dried, and fixed in acetone. The sera were tested at an initial dilution of 1:80, and bound antibodies were detected after incubation with fluorescein isothiocyanate-labeled goat anti-human IgG, IgM, and IgA conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) at a dilution of 1:50 as a secondary antibody. Those sera that were reactive at the initial dilution of 1:80 were tested after serial dilution up to 1:2,560 to determine the titer. Sera with titers ≥2,560 were given a value of 2,560 for calculations of geometric mean titers (GMTs).

Antigen was purified from isolate NY-13 by a density gradient method and was used to prepare immunoblot strips for detection of antibodies to *A. phagocytophila*, as described previously (4). Sera were tested at 1:100 dilutions in separate assays for IgG and IgM antibodies. The criterion for positivity was the presence of a distinct band(s) in the area of 42 to 44 kDa.

Statistical methods. Categorical variables were compared by Fisher's two-tailed exact test. IFA titers were compared by the Kruskal-Wallis one-way analysis of variance on ranks. A P value of ≤0.05 was considered significant.

***A. phagocytophila* antibodies in sera from healthy blood donors.** Sera from 159 healthy blood donors who reside in an area of Westchester County where Lyme disease is endemic and which were previously tested and found to be nonreactive for antibodies to *B. burgdorferi* were retested. Eight serum samples (5.0%) were reactive for antibodies to *B. burgdorferi* by ELISA, but none of these eight serum samples was positive for borreliacidal antibodies by immunoblotting for IgM or IgG. Eighteen (11.3%) serum samples tested positive for antibodies to *A. phagocytophila* by IFA (Table 1). The titers of antibodies to the HGE agent ranged from 80 to 640, with a median of 160 and a GMT of 226. These 18 serum samples were also tested by separate immunoblotting assays for IgM and IgG antibodies to *A. phagocytophila*. On the basis of the reactivities to the 42- to 44-kDa immunodominant antigen, six serum samples (33.3%) were reactive: two serum samples were positive for only IgM antibodies, three serum samples were positive for only IgG antibodies, and one serum sample was positive for both IgM and IgG antibodies (Table 2). Seventeen additional serum samples randomly selected from blood donors that tested negative for antibodies to *A. phagocytophila* by IFA were similarly tested by immunoblotting; all were nonreactive ($P = 0.045$ for the comparison of 5 of 18 serum samples versus 0 of 17 serum samples).

Seroreactivity to *A. phagocytophila* in sera of children testing negative for *B. burgdorferi* antibodies. Among 215 children (114 males and 100 females plus 1 child whose gender was unknown) who were being tested for an endocrinologic disorder, 17 (7.9%) were reactive for antibodies to *B. burgdorferi* by ELISA, but none of these 17 tested positive for borreliacidal antibodies by immunoblotting for IgM or IgG. Eleven of the 215 (5.1%) samples tested positive for antibodies to *A. phagocytophila* by IFA.

TABLE 2. Immunoblotting results for sera from healthy blood donors and children being tested for endocrinologic disorders positive for *A. phagocytophila* by IFA

Population	No. of serum samples IFA positive	No. of serum samples reactive for <i>A. phagocytophila</i> by immunoblotting			
		IgM positive, IgG negative	IgM positive, IgG positive	IgM negative, IgG positive	IgM negative, IgG negative
Adult blood donors	18	2	1	3	12
Children	11	4	0	4	3

^a Both groups were seronegative for antibodies to *B. burgdorferi* by two-stage testing.

TABLE 3. Frequency of *A. phagocytophila* antibodies in children by age and presence of *B. burgdorferi* antibodies

Age group (yr)	No. of serum samples positive/no. tested (%)	
	<i>B. burgdorferi</i> negative ^a	<i>B. burgdorferi</i> positive
<4	1/160 (1.7)	1/2 (50.0)
5–9	6/48 (12.5)	0/16 (0)
10–14	3/65 (4.6)	1/31 (3.2)
15–19	1/42 (2.4)	1/8 (12.5)
Total	11/215 (5.1)	3/57 (5.3)

^a Sera were from children evaluated for endocrinologic disorders.

phila by IFA (Table 1); the 11 samples were from 4 of the 114 (3.5%) males and 7 of the 100 (7%) females ($P = 0.35$). Of these 11 samples, 8 (72.7%) also tested positive for HGE by immunoblotting (Table 2). The titers of those serum samples that tested positive by IFA ranged from 160 to 640, with a median of 160 and a GMT of 219. The difference in the rates of seroprevalence of *A. phagocytophila* antibodies determined by IFA between the healthy adult blood donors and this group of pediatric patients was significant ($P = 0.03$). The rate of antibody reactivity to *A. phagocytophila* was greatest for the group of children ages 5 to 9 years (12.5%) and was significantly higher for that age group than for the group of children ages <1 to 4 years (1.7%) ($P = 0.04$) (Table 3).

***A. phagocytophila* antibodies in sera of children and adults seropositive for antibodies to *B. burgdorferi* and in sera of adults with culture-confirmed erythema migrans.** Significantly higher rates of seropositivity for *A. phagocytophila*, however, were observed among adults (42 of 118 [35.6%]) versus 18 of 159 [11.3%]; $P < 0.001$) but not among children (3 of 57 [5.3%]) versus 11 of 215 [5.1%]; $P = 1.0$) seropositive for *B. burgdorferi* by two-stage testing (Table 1). The IFA titers of the adults positive for *B. burgdorferi* ranged between 80 and $\geq 2,560$, with a median titer of 320 and a GMT of 315 (Table 1). Comparison of the titers of the positive samples from blood donors and the *B. burgdorferi*-positive groups was, however, not significant ($P = 0.695$). In addition, among the 42 baseline serum samples collected between 1991 and 2000 from adults with culture-confirmed erythema migrans, 9 (21.4%) were seropositive for antibody to *A. phagocytophila* (Table 1), including 5 of 22 (22.7%) patients diagnosed with erythema migrans in 1991 and 4 of 20 (20.0%) patients diagnosed with erythema migrans between 1996 and 2000 ($P = 1.0$). Among the groups of sera from children and adults reactive for antibodies to *B. burgdorferi*, the rate of IFA reactivity to *A. phagocytophila* was slightly higher in the groups of sera containing IgM antibodies to *B. burgdorferi* (23 of 85; 27.1%) than in those testing positive for IgG only (22 of 90; 24.4%) ($P = 0.73$) (Table 4).

The results presented here extend and confirm the report of Bakken et al. (6), who found an *A. phagocytophila* seropositivity rate of 14.9% among 475 individuals without evidence of HGE or a prior clinical history of HGE when tested by IFA by using a cutoff titer for seropositivity of 80.

Our findings showed that 11.3% of 159 healthy adult blood donors from Westchester County who were seronegative for antibodies to *B. burgdorferi* were seropositive for *A. phagocytophila* (antibody titers, ≥ 80) (Table 1). The explanation for

TABLE 4. *A. phagocytophila* IFA reactivity in sera of patients with serologic evidence of prior *B. burgdorferi* infection

<i>B. burgdorferi</i> antibody reactivity by immunoblotting	No. of serum samples positive for <i>A. phagocytophila</i> antibodies by IFA/no. tested (%)	
	Adults	Children
IgM positive, IgG negative	9/16 (56.3)	0/15 (0)
IgM positive, IgG positive	11/30 (36.7)	3/24 (12.5)
IgM negative, IgG positive	22/72 (30.6)	0/18 (0)

this seropositivity is unclear. Unexplained seropositivity for *A. phagocytophila* was also found in 11 of 215 (5.1%) pediatric patients whose sera had been submitted for endocrinologic testing, but at a significantly lower rate ($P = 0.03$). Bakken et al. (6) tested sera from only eight children, but they also demonstrated that the donors whose sera were positive were significantly older than those whose sera were negative. In our study, among adults who were seropositive for antibodies to *B. burgdorferi*, 35.6% were seropositive for antibodies to *A. phagocytophila* (Table 1). The difference in the rate of seropositivity between this group and the healthy blood donors was significant ($P < 0.001$). A similar seroprevalence (21.4%) was observed among adults with culture-confirmed erythema migrans whose sera were obtained at the time of diagnosis (before antibiotic therapy). In contrast, the rate of *A. phagocytophila* seropositivity (5.3%) among 57 children who tested seropositive for antibodies to *B. burgdorferi* was almost identical (5.1%) to the rate among children without borrelial seropositivity. Similar to the findings of De Martino et al. (S. J. De Martino, J. A. Carlyon, and E. Fikrig, Letter, N. Engl. J. Med. **345**:150–151, 2001), adults with IgM antibodies to *B. burgdorferi* had a higher frequency of antibody reactivity to *A. phagocytophila* (20 of 46; 43.5%) compared with the frequency of antibody reactivity for those with only IgG reactivity to *B. burgdorferi* (22 of 72; 30.6%), but this difference was not significant ($P = 0.17$).

We are aware of only one prior study that assessed seropositivity for *A. phagocytophila* by an IFA in a pediatric population. In that study, conducted in Slovenia, children were tested at the time of their routine medical examinations. When reactivity was considered a titer of ≥ 64 , 12 of 95 (12.6%) children tested positive, including 2 of 22 (9.1%) children 5 to 9 years of age, 7 of 31 (22.6%) children 10 to 14 years of age, and 3 of 42 (7.1%) children 15 to 19 years of age (9).

The high rates of background seropositivity for antibodies to *A. phagocytophila* found in both New York State and Wisconsin suggest that a single low-positive titer should be interpreted with caution in patients suspected of having recent HGE. Raising the threshold titer to 640 is supported by our findings. If this titer were considered the breakpoint, the seroprevalence rates would fall to 3.1% for our healthy blood donors and to 2.9% for the Wisconsin cohort. In contrast, in our laboratory, among 24 patients with culture-confirmed HGE, 95.2% would have still been considered seropositive if this cutoff had been used with an acute- or convalescent-phase serum sample (3).

Although it has been speculated that the high background rate of seropositivity for antibodies to *A. phagocytophila* indicates that a substantial proportion of the population has had subclinical HGE, this seems to be an unlikely explanation for

several reasons. First, testing of sera from our seropositive healthy blood donors by immunoblotting demonstrated a pattern suggestive of prior *A. phagocytophila* infection in only 6 of 18 (33.3%) samples (Table 2). It should be emphasized, however, that the accuracy of immunoblotting in distinguishing true-positive from false-positive serologic reactivity to *A. phagocytophila* remains to be established. Second, the seroprevalence rate was substantial among the serum samples from the pediatric population tested, yet unlike adults, children with seroreactivity to *B. burgdorferi* were not more likely to be seropositive for antibodies to *A. phagocytophila* than those without reactivity to *B. burgdorferi*. In addition, the pattern of reactivity did not show an increase with the age of the child. Both observations would be surprising if subclinical infection were the only explanation for seropositivity.

The background seropositivity might represent cross-reactivity to common bacterial antigens or autoantibodies reactive with the HL-60 cells in which *A. phagocytophila* is cultivated, but no data are available to substantiate these hypotheses.

In summary, by using a cutoff titer of 80 for IFA, approximately 11 to 15% of healthy adults from Wisconsin or Westchester County and 5% of children from Westchester County will test positive for antibodies to *A. phagocytophila*. This seroreactivity likely does not indicate the occurrence of a prior subclinical case of HGE for some, if not most, of these individuals. Regardless of whether this seroreactivity represents the occurrence of a prior subclinical case of HGE, it will certainly negatively affect the positive predictive values of test results for patients undergoing serologic evaluation because of clinical symptoms. Individual laboratories performing IFA for antibodies to *A. phagocytophila* need to determine the most suitable cutoff titer for positivity by testing well-characterized serum samples from both healthy individuals residing in areas where HGE is endemic and patients with well-established acute HGE.

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