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J Clin Invest. 1997;100(12):3014-3018. <https://doi.org/10.1172/JCI119855>.

Research Article

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Immunization against the Agent of Human Granulocytic Ehrlichiosis in a Murine Model

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Abstract

The agent of human granulocytic ehrlichiosis (HGE) is a newly recognized tick-borne pathogen that resides within polymorphonuclear leukocytes. C3H/HeN mice can become infected with the agent of HGE (designated aoHGE) by syringe inoculation or tick-borne infection and develop transient neutropenia. They thereby partially mimic human disease and provide a model in which to study immunity to this microorganism. Mice vaccinated with lysates of purified aoHGE, or administered aoHGE antisera, were partially protected from both syringe- and tick-transmitted challenge with aoHGE. These data suggest that antibodies are sufficient to provide substantial, but not complete, immunity against aoHGE. (*J. Clin. Invest.* 1997. 100:3014–3018.) Key words: vaccine • *Ehrlichia* • antibody • ticks • intracellular pathogen

Introduction

Human granulocytic ehrlichiosis (HGE)¹ is an emerging tick-borne infectious disease, and the pathogen infects and survives within granulocytes (1–6). Patients may develop fever, myalgia, and neutropenia, among other symptoms. Severe infection can be fatal, usually due to secondary infections (3, 5, 7, 8). Horses, sheep, goats, and humans acquire granulocytic ehrlichiosis, and the respective agents, *Ehrlichia equi*, *Ehrlichia phagocytophila*, and the agent of HGE (designated aoHGE) are genetically similar (9–11). *Peromyscus leucopus*, the white-footed mouse, is an animal reservoir for aoHGE, and *Ixodes scapularis* (also known as *Ixodes dammini*) is an arthropod vector of aoHGE (6, 12, 13). Not surprisingly, HGE is prevalent in the upper midwest and northeast United States, areas

where other *I. scapularis*-borne microorganisms, including *Borrelia burgdorferi* and *Babesia microti*, are common (2, 5, 14). Laboratory mice have been infected with aoHGE (13). We now show that C3H/HeN mice are susceptible to aoHGE infection and also develop neutropenia, thereby partially resembling human infection. Moreover, when examined at 14 d after aoHGE challenge, mice vaccinated with aoHGE lysates are substantially protected from ehrlichiosis, and aoHGE-specific antibodies are sufficient to provide partial immunity from tick-borne aoHGE infection. These data provide a basis for beginning to understand protective immunity to this unique emerging pathogen.

Methods

Mice. 3-wk-old female C3H/HeNcr mice were obtained from the Frederick Cancer Research Center (Frederick, MD). C3H/HeJ mice, which are LPS unresponsive, have been shown to be susceptible to aoHGE infection (13). We chose C3H/HeNcr (C3H) mice because they are LPS responsive and therefore more capable of responding to bacterial cell wall products (15). Mice were housed in filter-frame cages and killed with CO₂. C3H mice were initially inoculated intraperitoneally with blood (50 μ l) from CD-1 mice that had been infected with the NCH-1 isolate (see purification of aoHGE and tick-borne aoHGE vaccination studies) 3 wk earlier. In subsequent studies, C3H mice were engorged upon by aoHGE-infected ticks.

Purification of aoHGE. aoHGE lysate was made from the NCH-1 isolate, which was recovered from the peripheral blood of a patient from Nantucket, MA (16). The NCH-1 isolate (passage 2) used for purification of aoHGE was the same organism used in subsequent needle- or tick-challenge studies. The NCH-1 isolate was cultured using the HL-60 (240-CCL; American Type Culture Collection, Rockville, MD) cell line in Iscove's modified Dulbecco's medium supplemented with 20% FCS, using previously described methodologies (17). Cultures were maintained at 37°C in 5% CO₂. Fresh cells and medium were added biweekly to maintain a density of $\sim 10^6$ cells/ml. To ensure that the HL-60 cells were infected with aoHGE, cell cultures were placed onto microscope slides, stained with DiffQuick (Baxter Healthcare Corp., Miami, FL), and examined by light microscopy.

To purify aoHGE lysates, 1,000 ml of aoHGE-infected HL-60 cells were centrifuged at 1,500 rpm for 10 min, the pellet was resuspended with PBS-glucose (0.02%), and centrifuged again using the same conditions. The resuspended cells were disrupted by shearing using 21-gauge needles and pelleted. The supernatant was then digested with DNase/RNase (50 μ g/ml) and subjected to density gradient ultracentrifugation using 42 and 30% discontinuous gradient renografin (Nycomed Princeton, NJ) (18). Centrifugation was performed at 22,000 rpm for 75 min. Ehrlichia bacteria were collected in a band at the 30 and 42% renografin interface.

Tick-borne aoHGE vaccination studies. *I. scapularis* larvae were allowed to feed to repletion on CD-1 mice that had been infected with the aoHGE NCH-1 isolate for 3 wk. C3H mice could also have been used for this purpose. However, both CD-1 and C3H mice can be infected with aoHGE, and CD-1 mice are less expensive and have been used for aoHGE tick infestation for > 1 yr by our group. The

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Received for publication 28 May 1997 and accepted in revised form 1 October 1997.

1. Abbreviations used in this paper: aoHGE, agent of human granulocytic ehrlichiosis; HGE, human granulocytic ehrlichiosis.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.
0021-9738/97/12/3014/05 \$2.00
Volume 100, Number 12, December 1997, 3014–3018
<http://www.jci.org>

goal of this portion of the protocol was to obtain aoHGE-infected ticks rather than examine ehrlichiosis in the mice. The engorged larvae were collected, and kept until they had molted to nymphs. A sample was then examined for aoHGE infection by visual inspection of the salivary glands using the Feulgen reaction (13). The ticks had an 85% aoHGE infection rate.

For the vaccination studies, three or four aoHGE-infected *I. scapularis* nymphs were then placed, and allowed to engorge to repletion, on individual C3H mice that were immunized with either aoHGE lysates in CFA or CFA alone (control). aoHGE lysate was dialyzed against PBS, and heat-treated at 56°C for 1 h, before immunization. Groups of three to five mice were immunized subcutaneously in the back with 15 µg of purified, heat-killed aoHGE lysates in CFA and boosted twice with the same preparation in incomplete Freund's adjuvant at bimonthly intervals.

14 d after the ticks had fallen from the immunized animals, the mice were killed with CO₂ and blood was obtained by cardiac exsanguination. To visualize morulae, blood smears were air-dried, and stained with DiffQuick. In each smear, 200 high power fields were examined for morulae. The presence of one or more definitive morulae was considered positive, and the percentage of aoHGE-infected neutrophils was calculated. At necropsy, 100 µl of anticoagulated blood from each mouse was inoculated into culture flasks containing 5 ml of 5 × 10⁵ to 1 × 10⁶ HL-60 cells/ml. aoHGE infection of the HL-60 cells was determined at weeks 2, 3, 4, and 6. Mice were examined for aoHGE by PCR as described (see PCR and quantitative PCR).

PCR. An aoHGE-specific 16S rDNA fragment was amplified by PCR from peripheral blood. 50 µl of blood was lysed in red cell lysis buffer (155 mM NH₄Cl; 10 mM KHCO₃; 1 mM EDTA) and then treated with 10 µg/µl proteinase K at 56°C for 1 h. 5 µl of the supernatant containing aoHGE was used as template. Oligonucleotide primers were 497–521 (5'-TGT AGG CGG TTC GGT AAG TTA AAG-3') and 747–727 (5'-GCA CTC ATC GTT TAC AGC GTG-3'), which amplify a region that distinguishes aoHGE from other ehrlichia (6). PCR was done for 35 cycles with the following denaturation, annealing, and extension conditions: 1 min at 94°C, 1 min at 54°C, and 2 min at 72°C. PCR fragments were separated in a 1% agarose gel and stained with ethidium bromide or then transferred to nitrocellulose for Southern blotting using an aoHGE 16 rDNA probe. In all of the PCR studies, blood from three to five individual naive (uninfected) mice was also used as a control template.

Quantitative PCR. To determine the detection limits of the PCR assay, serial 10-fold dilutions of purified aoHGE DNA was subjected to amplification. 5 × 10⁻¹⁰ g (1:1,000 dilution of 500 ng) of purified aoHGE DNA was used as the initial template and an amplified product could be detected when up to 5 × 10⁻¹⁶ g of aoHGE DNA was used as the template. Pulse-field gel electrophoresis demonstrated that aoHGE has a chromosome that migrates at ~ 700 kb (not shown). We can estimate that 1 mol of aoHGE had a molecular mass of ~ 4.6 × 10⁸ g (7.0 × 10⁵ bp × 660 g/bp). 1 aoHGE therefore has an approximate molecular mass of 7.6 × 10⁻¹⁶ g [(4.6 × 10⁸ g/mol)/6.02 × 10²³ organisms/mol]. Thus, the limits of detection of the PCR assay is estimated at nearly one organism. In addition, the sensitivity of the PCR was unchanged when purified aoHGE DNA was added to 50 µl of murine blood and then subjected to red cell lysis buffer and proteinase K treatment (see PCR) before being used as the template, the same conditions as blood from experimental animals.

Passive antibody transfer studies. aoHGE antisera were obtained from C3H mice subcutaneously immunized with 15 µg of aoHGE lysate in CFA and boosted twice bimonthly with the same antigen in incomplete Freund's adjuvant. 2 wk after the final boost, blood was obtained by cardiac exsanguination. For the passive immunization studies, aoHGE antisera were diluted 1:5 in PBS and intradermally injected (200 µl) into naive mice. 1 d after immunization, the mice were inoculated intraperitoneally with blood (50 µl) from mice that had been infected with the NCH-1 isolate 2 wk earlier, or were engorged upon by three or four aoHGE-infected ticks. Mice were then administered the same amount of antisera (1:5 dilution in PBS, 200 µl)

on days 4, 8, and 12, and then killed on day 14, and examined for infection. In these studies, a 100-µl aliquot of anticoagulated blood was evaluated for neutropenia using a Coulter counter (Antech Diagnostics, Farmingdale, NY). For the assessment of splenomegaly, the whole spleen was removed from each animal at necropsy and immediately weighed.

Immunoblot. 500 ng of purified aoHGE lysate was separated using 15% SDS-PAGE and transferred onto nitrocellulose membranes. Membrane strips were incubated with sera (1:1,000, 1:10,000, and 1:50,000 dilutions) from aoHGE-immunized mice or control mice (immunized with CFA) at room temperature for 1 h, washed three times with PBS, and then incubated with alkaline phosphatase-conjugated anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). The color was developed using BCIP/NBT (Kirkegaard and Perry, Gaithersburg, MD) as the substrate.

Results and Discussion

We first show that all five C3H mice intraperitoneally inoculated with aoHGE developed transient neutropenia, with cytoplasmic inclusions known as morulae in 8–10% of the polymorphonuclear leukocytes at 2 wk. The aoHGE isolate (NCH-1) used in these studies was recovered previously from a woman with fever, neutropenia, and morulae in peripheral polymorphonuclear leukocytes (16). The average number of neutrophils in five aoHGE-infected mice at 14 d (814 cells/mm³, ±213 SD) was much lower than in five control animals (3,421 cells/mm³, ±426 SD) that were inoculated with normal mouse blood. aoHGE-infected mice also developed splenomegaly (0.32 g, ±0.05 SD) compared with controls (0.14 g, ±0.02 SD). Neutropenia resolved by 60 d and morulae were no longer readily detectable in peripheral smears at this time point. This experimental murine model partially resembles human infection and can therefore be used to study pathogenesis and immunity.

Studies were performed to determine whether mice could be protected against aoHGE. Indeed, infection of a horse with aoHGE has been shown to confer resistance to challenge with *E. equi* (19). Groups of mice were actively immunized with purified aoHGE lysates. 2 wk after the final boost, mice developed antibodies to aoHGE, detectable at a dilution of up to 1:10,000 on immunoblot. Antisera reacted predominantly against 40-, 44-, 65-, and 80-kD aoHGE antigens, among others (Fig. 1). Control mice, immunized with adjuvant in an identical fashion, did not develop aoHGE antibodies.

aoHGE-vaccinated mice were challenged with aoHGE, killed at 2 wk, and examined for infection (Table I). To simu-

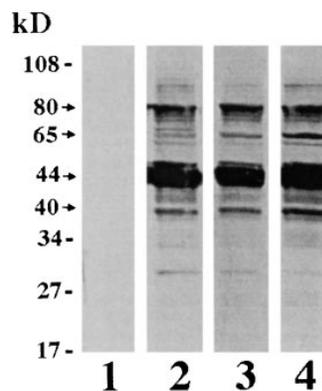


Figure 1. Mice immunized with aoHGE lysates developed aoHGE antibodies. aoHGE lysates were probed in immunoblot with sera (1:1,000) from controls (lane 1), and mice immunized with aoHGE lysates (lanes 2–4).

Table I. Mice Immunized with aoHGE Lysates Were Protected from Tick-borne Infection with aoHGE at 14 d after Challenge

Immunogen	Mouse	Culture	Morulae	PCR
Experiment 1				
CFA, control	1	+	+	+
	2	+	-	+
	3	+	+	+
	4	+	+	+
aoHGE lysates	5	-	-	-
	6	-	-	+
	7	-	-	+
	8	-	-	-
Experiment 2				
CFA, control	1	+	+	+
	2	+	+	+
	3	-	-	+
	4	-	-	+
	5	-	-	+
aoHGE lysates	6	-	-	+
	7	-	-	+
	8	-	-	-
	9	-	-	+
	10	-	-	-

Peripheral smears were examined (200 granulocytes per smear) for aoHGE morulae within neutrophils. The presence of one or more definitive morulae was considered positive for aoHGE infection. For cultivation of aoHGE, 100 μ l of murine blood was inoculated into a culture of HL-60 cells. The cells were maintained for 6 wk and examined for aoHGE infection. PCR was performed using 16S rDNA primers and 5 μ l of blood as the template.

late the natural mode of transmission, mice were engorged upon by nymphal ticks that had been infected with the NCH-1 isolate (Table I). Mice were assessed for infection at 14 d after tick feeding by examination of peripheral blood smears for morulae, culture, and by 16S rDNA PCR of blood. Five of nine control animals had morulae in peripheral polymorphonuclear leukocytes, six of nine mice were culture positive, and aoHGE-specific DNA was detected in blood of all control mice (Table I). In contrast, morulae were not detected in blood smears of aoHGE-vaccinated mice (χ^2 , $P < 0.01$, compared with control mice) and aoHGE was not cultured from any of the nine mice (χ^2 , $P < 0.005$, compared with control mice), but five of nine animals were PCR positive (χ^2 , $P < 0.01$, compared with control mice). An amplified DNA product was not detected in samples from naive, uninfected mice, validating the specificity of the PCR assays. PCR reactions in which distilled water was used as the template were also uniformly negative. In addition, a DNA product was not obtained when blood from a mouse that had been immunized with aoHGE lysates in CFA (obtained 14 d after immunization) was used as the template, indicating that DNA from the vaccination procedure does not result in false-positive PCR reactivity.

Studies were then performed to determine whether antibodies were sufficient for protection. In three separate experiments, groups of three to five mice were passively immunized with aoHGE antisera, then challenged by either ticks or by direct inoculation of whole blood from an aoHGE-infected

mouse (Table II). Regardless of the mode of aoHGE challenge, passive immunization of mice with aoHGE antisera was sufficient to afford substantial protection as determined by the lack of visualization of morulae in polymorphonuclear leukocytes on peripheral blood smears (7 of 11 control animals, 1 of 12 immunized mice: χ^2 , $P < 0.005$), lack of recovery of aoHGE by culture (7 of 11 control animals, 1 of 12 immunized mice: χ^2 , $P < 0.005$), and the incomplete ability to amplify aoHGE DNA from blood by PCR (10 of 11 control animals, 4 of 12 immunized mice: χ^2 , $P < 0.01$). Furthermore, neutropenia was evident in aoHGE-infected control mice (462 cells/mm³, ± 280 SD) compared with uninfected animals (3,240 cells/mm³, $\pm 1,340$ SD). Splenomegaly was also observed in aoHGE-infected control animals (0.27 g, ± 0.05 SD) but not in experimental mice (0.12 g, ± 0.03 SD).

Moreover, serial dilution PCR analysis indicated that the amount of aoHGE DNA was much lower in the PCR-positive, immunized mice compared with PCR-positive, control animals. Serial 10-fold dilutions of an aliquot of purified aoHGE DNA estimated that PCR could detect as little as one organism, indicating that the assay is sensitive. A product could be identified in PCR-positive, control mice at a serum dilution of 10⁵-10⁸, whereas amplified DNA was discernible in the PCR-positive, vaccinated mice at a dilution of 10⁰-10³ (Fig. 2, as an example of one experiment). This suggested two possibilities.

Table II. Mice Passively Administered aoHGE Antisera Were Protected from either Tick-transmitted or Syringe-mediated Infection with aoHGE at 14 d after Challenge

Immunogen (aoHGE challenge)	Mouse	Culture	Morulae	PCR	
Experiment 1 (tick-borne)					
NMS, control	1	-	-	-	
	2	+	+	+	
	3	+	+	+	
	aoHGE antisera	4	-	-	-
		5	-	-	-
		6	-	-	-
Experiment 2 (tick-borne)					
NMS, control	1	+	+	+	
	2	+	+	+	
	3	-	-	+	
	4	-	-	+	
	5	-	-	+	
aoHGE antisera	6	-	-	-	
	7	-	-	-	
	8	+	+	+	
	9	-	-	+	
	10	-	-	+	
Experiment 3 (syringe-inocula)					
NMS, control	1	+	+	+	
	2	+	+	+	
	3	+	+	+	
aoHGE antisera	4	-	-	-	
	5	C	-	-	
	6	C	-	-	
	7	-	-	+	

Peripheral smears, culture, and PCR were assessed as in Table I. NMS, Normal mouse sera; C, contaminated.

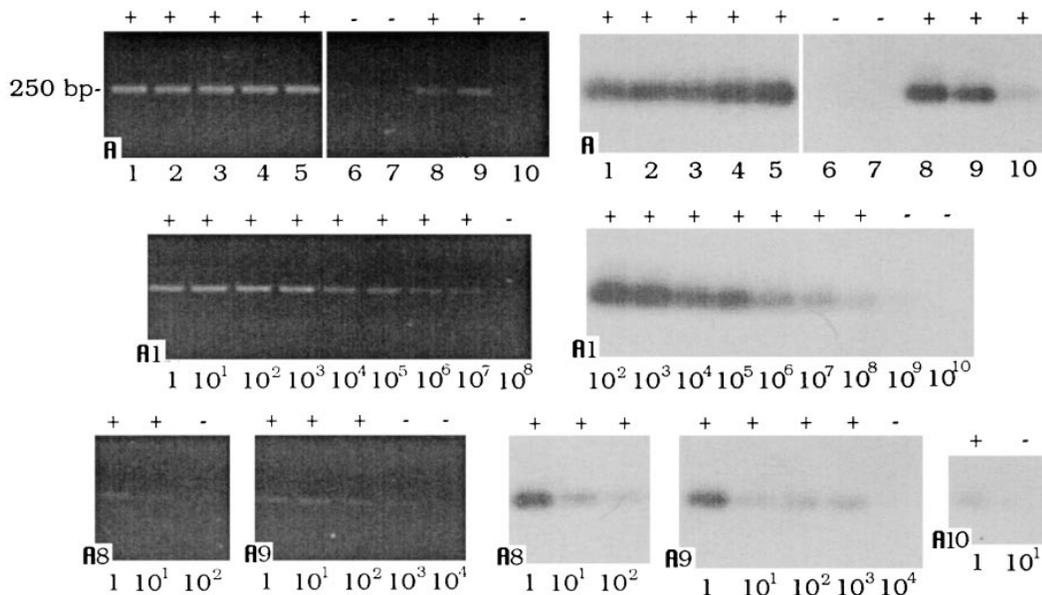


Figure 2. aoHGE-specific DNA amplified from mice challenged with aoHGE (data in Table II, experiment 2). Ethidium bromide staining (left) or Southern blot using a 16S rDNA probe (right). (A) Mice administered control (lanes 1–5) or aoHGE antisera (lanes 6–10). (A1, A8, A9, and A10) Serial dilution PCR to quantitate aoHGE. 10-fold dilutions of blood from an aoHGE-infected, control mouse (A, lane 1) or aoHGE-infected, experimental mice (A, lanes 8–10). 5 μ l of blood, prepared as described in Methods, was used as the initial aliquot. For example, A1 (10^8) represents a 10^8 dilution of 5 μ l of blood from the mouse depicted in A, lane 1.

In cases where aoHGE immunization did not fully prevent infection (based on PCR), the number of aoHGE organisms was markedly reduced when compared with infected, control mice, due to the effect of aoHGE-specific antibodies. Conversely, the presence of residual aoHGE DNA in the host, from aoHGE that had been injected into the host during tick-borne transmission, resulted in PCR positivity. In either case, morulae visualization, culture, and PCR data indicate that aoHGE vaccination affords substantial protection against tick-transmitted challenge, and that PCR is the most sensitive method of detection.

Ehrlichia are obligate intracellular pathogens related to *Rickettsia*, *Coxiella*, and *Chlamydia* (20). In general, cellular immune responses are necessary for effective immunity to these groups of microbes, and for that matter, against gram-negative intracellular bacteria such as *Listeria* (21–27). These data show that it is possible to elicit partial immunity against aoHGE in an experimental murine model. Furthermore, the protective capacity of vaccination with aoHGE lysates extended to tick-borne infection, the natural mode of disease transmission, and passive administration of aoHGE antisera were sufficient for substantial protection. Effective humoral immunity against aoHGE challenge suggests that aoHGE may reside, albeit transiently, in the extracellular environment during part of its life cycle: perhaps in the bloodstream as the pathogen moves among neutrophils, or as the microbe migrates from the tick to the mammalian host. Antibodies could potentially interact directly with aoHGE to facilitate complement-mediated lysis or opsonization at either of these intervals. Now that it is possible to induce immunity against tick-transmitted aoHGE in a model system, the specific antigens that elicit protective antibodies can be identified, the duration of long-term immunity studied, and the mechanisms by which

humoral responses afford substantial protection can be elucidated.

Acknowledgments

This study was supported by National Institutes of Health grant A141440. W. Sun is a postdoctoral fellow of the American Heart Association. J.W. IJdo is a Daland Fellow of the American Philosophical Society and a postdoctoral fellow of the Markey Foundation.

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