

Anaplasma phagocytophilum Antibodies in Humans, Japan, 2010–2011

To the Editor: Human granulocytic anaplasmosis (HGA) is an emerging tick-borne infectious disease caused by *Anaplasma phagocytophilum*, an obligatory intracellular bacterium (1). Recently, 2 cases of HGA were identified by a retrospective study in Japan (2). For serodiagnosis of HGA, *A. phagocytophilum* propagated in HL60 cells is usually used as an antigen, especially by indirect immunofluorescent assay (IFA) (3). However, the serum from these 2 patients in Japan reacted with antigens of *A. phagocytophilum* cultured in THP-1 cells rather than in HL60 cells in IFA (2). In *A. phagocytophilum*, a *p44/msp2* multigene family encoding multiple 44-kDa immunodominant major outer membrane protein species (so-called P44) exists on the genome, and these multigenes are similar, but not identical, to each other, and the bacterium generates antigenic variations because of gene conversion (4). The previous studies showed that *A. phagocytophilum* expresses predominantly 2 species of *p44/msp2* transcripts in THP-1 cells, but it produces

the variation of P44 protein species in HL60 cells (2,5). This finding strongly suggested that *A. phagocytophilum* grown in THP-1 cells differs serologically from that in HL60 cells. Our serologic analysis found 4 recent cases of HGA in Japan by using infected THP-1 and HL60 cells as antigens, and some P44 immunoreactive protein species of *A. phagocytophilum* that were associated with the respective cell line cultures, binding to antibodies from the 4 patients' serum, also were identified.

In 2010 and 2011, nine patients in Shizuoka Prefecture, Japan, who had rickettsiosis-like symptoms, were suspected to have Japanese spotted fever or scrub typhus, but they were serologically negative by IFA. Therefore, IFA for HGA was conducted. In 4 of the patients, antibodies to *A. phagocytophilum* were detected in serum by using *A. phagocytophilum* cultured in THP-1 and HL60 cells as antigens (Table). In IFA tests for HGA, IgM and/or IgG from the patients' serum samples reacted with *A. phagocytophilum* cultured in THP-1, HL60, or both, and the seroconversions were observed in convalescent-phase serum from all patients. The clinical manifestation and laboratory findings for the 4 patients are summarized in the online Technical Appendix Table, (wwwnc.cdc.gov/EID/article/20/3/13-1337-Techapp1.pdf). Western blot analysis

further confirmed the specific reaction to P44 protein antigens (P44s) of *A. phagocytophilum* cultured in THP-1 and HL60 and to recombinant P44–1 protein (rP44–1) in the serum samples (online Technical Appendix Figures 1 and 2), supporting the IFA results in the Table.

To identify P44 immunodominant protein species binding to antibodies from the patients' serum, we selected P44–47E and P44–60 proteins that are dominantly expressed by *A. phagocytophilum* propagated in THP-1 cells (2) and P44–18ES protein that frequently predominates by *A. phagocytophilum* cultured in HL60 cells (6) as representatives for the preparation of recombinant proteins. The central hypervariable regions of the respective P44 proteins (online Technical Appendix Figure 3) were produced as recombinant proteins in vitro by insect cell-free protein synthesis system (Transdirect Insect Cell Kit; Shimadzu Co., Kyoto, Japan) (7) to avoid the strong nonspecific reaction with human serum that occurs in the *Escherichia coli* expression system. In Western blot analyses using these 3 recombinant P44 proteins (rP44–60 and rP44–47E for THP-1 and rP44–18ES for HL60) as antigens, most of the serum from the patients was reactive with *A. phagocytophilum* cultured in THP-1 cells in IFA bound to either rP44–60 or rP44–47E, whereas the

Table. Immunofluorescence antibody titers to *Anaplasma phagocytophilum* in serum from 4 patients with human granulocytic anaplasmosis and reactive rP44 protein species, Japan, 2010–2011*

Patient no.	Time after illness onset, d	Antigen			
		<i>A. phagocytophilum</i> propagated in THP-1 cells (rP44 species)		<i>A. phagocytophilum</i> propagated in HL60 cells (rP44 species)	
		IgM	IgG	IgM	IgG
1	1	80 (r60)	<20	80 (r18ES)	<20
	15	160 (r60)	<20	160 (r18ES)	<20
	30	320 (r60)	20 (r60)	320 (r18ES)	<20
2	13	40	40 (r47E)	<20	20
3	3	40	80 (r60)	<20	20 (r18ES)
	7	40	80 (r60)	<20	20 (r18ES)
	24	80 (r60)	160 (r60)	<20	40 (r18ES)
4	4	160 (r47E)	40	<20	<20
	15	160 (r47E)	80	<20	<20

*Three recombinant P44 (rP44) protein species (r18ES, r47E, r60) were prepared and either one bound to antibodies in each serum sample from 4 patients in Western blot analyses (online Technical Appendix Figure 4, wwwnc.cdc.gov/EID/article/20/3/13-1337-Techapp1.pdf). r18ES represents rP44–18ES immunoreactive outer membrane protein that is known to predominate in *A. phagocytophilum* cultured in HL60 cells (6). r47E and r60 show rP44–47E and rP44–60 proteins, respectively, that are dominantly transcribed in *A. phagocytophilum* propagated in THP-1 cells (2).

patients' serum reactive with *A. phagocytophilum* cultured in HL60 cells in IFA bound to rP44–18ES (online Technical Appendix Figure 4; Table). This finding strongly supports the results of IFA and Western blot analyses with the infected THP-1 and HL60 cells.

In Japan, rickettsioses such as Japanese spotted fever and scrub typhus, caused by *Rickettsia japonica* and *Orientia tsutsugamushi*, respectively, occur frequently. However, fever of unknown cause and rickettsiosis-like symptoms still occur in some patients. Detection of *A. phagocytophilum* in ticks was first reported in 2005 in central Japan (8). Since then, DNA of *A. phagocytophilum* has been detected in ticks inhabiting several places of Japan (9,10). However, little was known about human infection with *A. phagocytophilum* for many years, probably because of the poor selection of the culture cell line used as infected cell antigens for serodiagnosis. Our previous study first documented HGA in Japan and recommended that *A. phagocytophilum* propagated in THP-1 and in HL60 cells be used as antigens to avoid misdiagnosing cases of HGA. Our current study demonstrates the presence of specific antibodies against the central hyper-variable regions of P44–47E, P44–60, or P44–18ES proteins that predominate in infected THP-1 or HL60 cells, probably being suitable as protein antigens for serodiagnosis of HGA. The rP44–1 protein whose recombinant plasmid had previously been constructed for *E. coli* expression system may be available as well. Thus, our study provides substantial information about the usefulness of suitable P44 immunoreactive protein species of *A. phagocytophilum* as antigens for serodiagnosis of HGA.

This work was supported in part by a grant for Research on Emerging and Re-emerging Infectious Diseases from The Association for Preventive Medicine of Japan; grants for Research on Emerging and Re-emerging Infectious Diseases from the Japanese Ministry of Health, Labour and Welfare (H18-Shinkou-Ippan-14)

and (H21-Shinkou-Ippan-014); a grant for Global Center of Excellence Program from Japanese Ministry of Education, Culture, Sports, Science and Technology; and a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (no. 23590514) for N.O.

**Gaowa, Yuko Yoshikawa,
Norio Ohashi, Dongxing Wu,
Fumihiko Kawamori,
Asaka Ikegaya,
Takuya Watanabe,
Kazuhito Saitoh,
Daisuke Takechi,
Yoichi Murakami,
Daisuke Shichi, Katsumi Aso,¹
and Shuji Ando**

Author affiliations: University of Shizuoka and Global Center of Excellence Program, Shizuoka City, Japan (Gaowa, Y. Yoshikawa, N. Ohashi, D. Wu.); Shizuoka Institute of Environment and Hygiene, Shizuoka City (F. Kawamori, A. Ikegaya); Seirei Hamamatsu General Hospital, Shizuoka (T. Watanabe, K. Saitoh, D. Takechi); Seirei Mikatagahara General Hospital, Shizuoka (Y. Murakami, D. Shichi); Seirei Numazu Hospital, Shizuoka (K. Aso); and National Institute of Infectious Diseases, Tokyo, Japan (S. Ando)

DOI: <http://dx.doi.org/10.3201/eid2003.131337>

References

- Bakken JS, Dumler S. Human granulocytic anaplasmosis. *Infect Dis Clin North Am.* 2008;22:433–48. <http://dx.doi.org/10.1016/j.idc.2008.03.011>
- Ohashi N, Gaowa, Wurutu, Kawamori F, Wu D, Yoshikawa Y, et al. Human granulocytic anaplasmosis, Japan. *Emerg Infect Dis.* 2013;19:289–92. <http://dx.doi.org/10.3201/eid1902.120855>
- Walls JJ, Agüero-Rosenfeld M, Bakken JS, Goodman JL, Hossain D, Johnson RC, et al. Inter- and intralaboratory comparison of *Ehrlichia equi* and human granulocytic ehrlichiosis (HGE) agent strains for serodiagnosis of HGE by the immunofluorescent-antibody test. *J Clin Microbiol.* 1999;37:2968–73.
- Dunning Hotopp JC, Lin M, Madupu R, Crabtree J, Angiuoli SV, Eisen JA, et al. Comparative genomics of emerging human ehrlichiosis agents. *PLoS Genet.* 2006;2:e21.
- Lin M, Kikuchi T, Brewer HM, Norbeck AD, Rikihisa Y. Global proteomic analysis of two tick-borne emerging zoonotic agents: *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*. *Front Microbiol.* 2011;2:24. <http://dx.doi.org/10.3389/fmicb.2011.00024>
- Sarkar M, Troese MJ, Kearns SA, Yang T, Reneer DV, Carlyon JA. *Anaplasma phagocytophilum* MSP2 (P44)-18 predominates and is modified into multiple isoforms in human myeloid cells. *Infect Immun.* 2008;76:2090–8. <http://dx.doi.org/10.1128/IAI.01594-07>
- Ezure T, Suzuki T, Shikata M, Ito M, Ando E. A cell-free protein synthesis from insect cells. *Methods Mol Biol.* 2010;607:31–42. http://dx.doi.org/10.1007/978-1-60327-331-2_4
- Ohashi N, Inayoshi M, Kitamura K, Kawamori F, Kawaguchi D, Nishimura Y, et al. *Anaplasma phagocytophilum*-infected ticks, Japan. *Emerg Infect Dis.* 2005;11:1780–3. <http://dx.doi.org/10.3201/eid1111.050407>
- Gaowa, Ohashi N, Aochi M, Wurutu, Wu D, Yoshikawa Y, et al. Rickettsiae in ticks, Japan, 2007–2011. *Emerg Infect Dis.* 2013;19:338–40.
- Gaowa, Wurutu, Wu D, Yoshikawa Y, Ohashi N, Kawamori F, et al. Detection and characterization of *p44/msp2* transcript variants of *Anaplasma phagocytophilum* from naturally infected ticks and wild deer in Japan. *Jpn J Infect Dis.* 2012;65:79–83.

Address for correspondence: Norio Ohashi, Laboratory of Microbiology, Department of Food and Nutritional Sciences, School of Food and Nutritional Sciences, Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan; email: ohashi@u-shizuoka-ken.ac.jp

Find emerging
infectious disease
information on

facebook

<http://www.facebook.com>

¹Current affiliation: Aso Clinic, Numazu, Japan.

Anaplasma phagocytophilum Antibodies in Humans, Japan, 2010–2011

Technical Appendix

Technical Appendix Table. Clinical manifestations and laboratory findings of 4 patients with HGA, Japan, 2010–2011*

Patient	Age, y/sex	Occupation	Hospital	Days with fever		Symptom onset	Fever	Rash	Additional symptom/sign	Underlying disorder	Laboratory findings (reference values)†	Treatment
				after possible tick bite								
1	87/F	Farmer	A	5 d after working on farm		2010 May	39.7°C	Yes	Malaise	Hypertension, hyperlipidemia	WBC, 8.7×10^9 cells/L (3.5–9.2 $\times 10^9$ cells/L); Plt, 196×10^9 cells/L (155–365 $\times 10^9$ cells/L); AST, 145 U/L (<38 U/L); ALT, 95 U/L (<36 U/L); LDH, 318 U/L (125–237 U/L); CRP, 16.6 mg/dL (0.3 mg/dL)	Minocycline (200 mg/d) by oral administration for 24 d
2	49/M	Electronics worker	B	5 d after construction in forest		2010 May	39°C	Yes	Anorexia, diarrhea, acute renal failure	Diabetes	WBC, 13.1×10^9 cells/L (3.5–9.2 $\times 10^9$ cells/L); Plt, 190×10^9 cells/L (155–365 $\times 10^9$ cells/L); AST, 69 U/L (<38 U/L); ALT, 49	Minocycline (200 mg/d) by oral administration for 21 d

Patient	Age, y/sex	Occupation	Hospital	Days with fever		Symptom onset	Fever	Rash	Additional symptom/sign	Underlying disorder	Laboratory findings (reference values)†	Treatment
				after possible tick bite								
											U/L (<36 U/L); LDH, 236 U/L (125–237 U/L); CRP, 6.6 mg/dL (0.3 mg/dL); BUN, 54 mg/dL (9– 21 mg/dL); Cre, 5.62 mg/dL (0.6– 1.2 mg/dL)	
3	83/M	Retired	A	3 d after traveling to mountains	2011 Jun	38.2°C	No	None	Diabetes, hypertension, arteriosclerosis obliterans	WBC, 6.3×10^9 cells/L (3.5–9.2 \times 10^9 cells/L); Plt, 88×10^9 cells/L (155–365 $\times 10^9$ cells/L); AST, 34 U/L (<38 U/L); ALT, 27 U/L (<36 U/L); LDH, 266 U/L (125–237 U/L); CRP, 10.9 mg/dL (0.3 mg/dL)	Minocycline (200 mg/d) by intravenous administration for 6 d and then oral administration for 9 d	
4	38/M	Industry worker	C	18 d after working in depository of industry	2011 Jun	39°C	Yes	Chills, arthralgia, headache	None	WBC, 2.1×10^9 cells/L (3.5–9.2 \times 10^9 cells/L); Plt, 126×10^9 cells/L (155–365 $\times 10^9$ cells/L); AST, 31 U/L (<38 U/L); ALT, 53 U/L (<36 U/L); LDH, 277 U/L (125–237 U/L); CRP, 0.7 mg/dL (0.3 mg/dL)	Minocycline (200 mg/d) by oral administration for 10 d	

Patient	Age, y/sex	Occupation	Hospital	Days with fever		Symptom			Additional symptom/sign	Underlying disorder	Laboratory findings (reference values)†	Treatment
				after possible tick bite	onset	Fever	Rash					

*Three hospitals were located in Shizuoka Prefecture, Japan. Three patients excluding case-patient 4 were hospitalized for treatment of HGA. HGA, human granulocytic anaplasmosis; WBC, white blood cells (leukocytes); Plt, platelets (thrombocytes); AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CRP, C-reactive protein; BUN, blood urea nitrogen; Cre, creatinine.

†Laboratory value at time of hospital admission.

Technical Appendix Figure 1. Western blot analyses of serum from 4 case-patients, performed by using recombinant P44-1 protein (rP44-1) and *Anaplasma phagocytophilum*-infected THP-1 cells as antigens, Japan, 2010–2011. The recombinant *Escherichia coli*-producing rP44-1 was kindly provided by Yasuko Rikihisa at The Ohio State University (Columbus, OH, USA). The preparation of purified rP44-1 protein and the rabbit hyperimmune serum (positive serum control) has been described (2). A human serum sample (negative control) is shown. The primary human serum samples tested were 250-fold diluted, and the rabbit serum as positive control was 10,000-fold diluted. The goat antihuman IgG and IgM alkaline phosphatase conjugates (Life Technologies, Grand Island, NY, USA) were used as secondary antibodies. rP, recombinant P44-1 protein (rP44-1); In; infected THP-1; Un, uninfected THP-1; M, marker in size.

Technical Appendix Figure 2. Western blot analyses of serum from 4 case-patients, performed by using recombinant P44-1 protein (rP44-1) and *Anaplasma phagocytophilum*-infected HL60 cells as antigens, Japan, 2010–2011. The rabbit hyperimmune anti-rP44-1 serum and a human serum sample were used as positive and negative controls, respectively. rP, recombinant P44-1 protein (rP44-1); In, infected HL60; Un, uninfected HL60.

Technical Appendix Figure 3. The amino acid sequence comparison of the hypervariable regions of 3 recombinant P44 protein species (rP44-47E, rP44-60, and rP44-18ES). The rP44-47E protein encodes 651 bp in the 460–1,110-bp position of open reading frame corresponding to 217 amino acids. The rP44-60 protein encodes 633 bp in the 40–672-bp position of the truncated P44-60 corresponding to 211 amino acids. Both P44-47E and P44-60 are dominantly transcribed in *Anaplasma phagocytophilum* cultured in THP-1 cells (2). The rP44-18ES protein encodes 624 bp in the 460–1,083-bp position of P44-18E open reading frame corresponding to 208 aa. The P44-18ES protein is known to predominate in *A. phagocytophilum* propagated in HL60 cells (6). The hypervariable regions of 3 recombinant P44 protein species produced have 74.9% identity between rP44-47E and rP44-60, 70.2% between rP44-47E and rP44-18ES, and 70.2% between rP44-60 and rP44-18ES. For

preparation of these 3 rP44 protein species (rP44–47E, rP44–60, and rP44–18ES), the DNA including a central hypervariable region of each P44 protein species was artificially synthesized in consideration of codon use for insect. After synthesis, the DNA region was cloned into a pUC57 plasmid. Then, the insert region with in-fusion tags in both 5' and 3' ends was amplified, and the PCR product was cloned into pTD1 expression vector by using in-fusion cloning kit (Life Technologies, Grand Island, NY, USA). The mRNA of respective rP44 protein species was transcribed in vitro from the constructed plasmids by T7 RiboMAX express large-scale RNA production system (Promega Co, Madison, WI, USA). Then, the respective recombinant proteins were produced from the transcribed mRNA in vitro by insect cell-free protein synthesis system (Transdirect Insect Cell Kit; Shimadzu Co., Kyoto, Japan) (7) and used as antigens for Western blot analysis (see Technical Appendix Figure 4).

Technical Appendix Figure 4. Western blot identification of P44 protein species (r18ES, r47E, or r60) binding to antibodies in 4 case-patients. r18ES represents rP44-18ES protein antigen that is known to predominate in *Anaplasma phagocytophilum* cultured in HL60 cells (6). r47E and r60 show rP44–47E and rP44–60 proteins, respectively, that are dominantly transcribed in *A. phagocytophilum* propagated in THP-1 cells (2). The hypervariable regions of 3 recombinant proteins with 23–25 kDa (r47E, r60, and r18ES, see Technical Appendix Figure 3) were all detectable by the rabbit hyperimmune anti-rP44-1 serum as a positive serum control previously prepared (2) as shown in the top left panel.