JOURNAL OF CLINICAL MICROBIOLOGY, June 2000, p. 2398–2399 Vol. 38, No. 6
0095-1137/00/$04.00+0

Survival of the Human Granulocytic Ehrlichiosis Agent under Refrigeration Conditions

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Received 15 October 1999/Returned for modification 14 January 2000/Accepted 5 April 2000

The human granulocytic ehrlichiosis (HGE) agent in infected blood specimens remained viable during refrigeration at 4°C for up to 18 days. These findings suggest that blood specimens submitted for culture may withstand transportation to a remote laboratory. HGE should be added to the list of infections potentially transmitted by blood transfusion.

Human granulocytic ehrlichiosis (HGE) is an emerging tick-borne disease caused by a bacterium that infects leukocytes and that is closely related if not identical to *Ehrlichia equi*. Laboratory tests used to confirm the diagnosis are direct examination of the peripheral blood buffy coat smear for intragranulocytic inclusions; culture in HL-60 cells; a promyelocytic leukemia cell line; PCR; and serology (1, 2, 4, 9, 13). Culture is the most definitive method to confirm diagnosis, and in our experience it is a highly sensitive diagnostic tool during the acute phase of illness, comparable to PCR and better than serology (unpublished data). Since few laboratories have culture capabilities, we decided to investigate whether the HGE agent remains viable under refrigeration conditions, an important consideration if specimens are to be transported to remote laboratories. Since the HGE agent resides in leukocytes, this information is also of importance with regard to the potential for blood-borne transmission of the microorganism by transfusion of refrigerated blood products. Although transmission of the disease usually occurs through the bite of infected *Ixodes* ticks, transmission has also occurred perinatally (10) and perhaps by contact with blood of infected animals (3).

Patients. Five patients diagnosed with HGE between October 1997 and November 1998 provided the blood samples for this study. All patients had intragranulocytic inclusions on buffy coat smears and had not received prior antimicrobial treatment. They ranged in age from 35 to 78 years and included one female and four males. Other relevant parameters are shown in Table 1.

Specimens. Peripheral blood samples were collected in EDTA (15% K_3 EDTA solution, 8.55 mg) from four patients and in acidified citrate dextrose (ACD) (2.2% Na_3 citrate, 0.8% citric acid, 0.24% dextrose) from one patient. Collection tubes containing these anticoagulants are routinely used in clinical practice for blood cell counting. The blood specimens were transported refrigerated or maintained at 4°C for an average of 3.2 days (range, 1 to 5 days) prior to initial processing and culture. In addition, at the time of specimen processing, separate aliquots of the samples were stored at 4°C to be cultured after various periods of storage. The duration of refrigeration referred to in this study includes the transport time prior to initial specimen processing.

Culture. The human promyelocytic HL-60 cell line was used for culture as described previously (9). The HL-60 cells were cultured in RPMI 1640 supplemented with L-glutamine and 10% fetal bovine serum and incubated at 37°C in 5% CO_2. A 0.2-ml aliquot of EDTA- or ACD-anticoagulated blood was inoculated into 5 ml of the HL-60 cell culture at a cell density of approximately 2×10^5/ml. Culture aliquots were Wright stained and microscopically examined for infection every 3 to 4 days. Cultures were incubated for up to 30 days.

A total of 18 cultures were inoculated with infected blood from five patients. Three to five aliquots from each patient

TABLE 1. Selected clinical and laboratory parameters at time of blood collection and culture recovery

Patient no.	Duration of symptoms (days)	% Infected granulocytes	Absolute no. of infected granulocytes/μl	HGE antibody titer by IFA[a]	No. of days of storage before:	
					Last positive culture aliquot	First negative culture aliquot
1	6	6	225	<80	7	ND[b]
2	11	0.5	32	NA[c]	17	ND
3	4	0.8	34	<80	18	24
4	8	0.3	2.7	320	12	29
5	6	25	903	160	14	21

[a] IFA, indirect fluorescent-antibody assay.
[b] ND, not done.
[c] NA, not available.

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TABLE 2. Effect of duration of refrigeration of blood at 4°C on recovery of the HGE agent in culture

Mean no. of days (range) of refrigeration at 4°C	No. of cultures[a]	Mean no. of days to positive culture[b]
3.3 (1–5)	8	3.3
9.0 (7–13)	4	6.5
16.3 (14–18)	3	12.0
24.7 (21–29)	3	No growth

[a] A total of 18 refrigerated blood aliquots from five patients were cultured in an HL-60 cell line after different periods of storage at 4°C.
[b] Cultures were examined for infection every 3 to 4 days.

were cultured after different periods of refrigeration, as shown in Table 2. All 16 cultures of blood refrigerated for up to 18 days were positive for the HGE agent. Three cultures inoculated with blood refrigerated for 21 to 29 days did not yield growth. The incubation time needed to detect growth was directly related to the number of days of refrigeration ($r = 0.893$, $P < 0.001$).

No correlation was found between the number of infected granulocytes and recovery of the HGE agent in culture ($r = 0.104$, $P = 0.712$) (Table 3). Similarly, the duration of symptoms prior to blood collection and the presence of antibodies at the time of obtaining the blood did not affect recovery of the HGE agent. These results, however, must be interpreted cautiously due to the small number of patient samples included.

Other factors that may be involved in recovery in culture that were not considered in this study are the number of microorganisms per cell and biological differences among strains. Intragranulocytic HGE inclusions may contain from few organisms to hundreds or perhaps thousands of bacterial cells. Moreover, our experience has suggested that there are biological differences among different HGE isolates under in vitro culture conditions. While some strains invade the HL-60 cell line quite rapidly, others reach a high degree of infection only after prolonged incubation (data not shown).

Our findings suggest that HGE should be added to the list of potential infections transmitted by blood transfusion. A recent preliminary report provided presumptive evidence for this mode of transmission in a single patient (6). It could not be proven in that case, however, that the blood product was the source of the infection. When considering the implications for blood transfusion, it is important to note that volumes of infected blood much larger than those used in this study will be infused. Theoretically, in these circumstances blood stored for longer times could be still be infectious. Although *Babesia microti* has been found to survive for up to 21 days under in vitro conditions (7), it has been reported that transfusion-transmitted cases have occurred with blood refrigerated for up to 35 days (12).

TABLE 3. Effects of number of infected granulocytes and refrigeration time at 4°C on days to detection of the HGE agent in culture

No. of infected granulocytes/μl	Avg no. of days to detection of growth with the indicated no. of days of refrigeration			
	1–5	7–13	14–18	21–29
903	5	ND[a]	7	NG[b]
225	2.5	3	ND	ND
34	3	7	17	NG
32	2.3	6	12	ND
2.7	7	10	ND	NG

[a] ND, not done.
[b] NG, no growth.

It should be also emphasized that our experiments were conducted using infected blood showing inclusions on buffy coat smears. Similar studies have not been conducted with smear-negative HGE patients or with patients with subclinical HGE infection. It is currently unknown whether HGE organisms are found in blood of infected individuals during incubation of the infection or how long ehrlichemia persists in individuals with subclinical, untreated illness. *Ehrlichia phagocytophila*, the agent of granulocytic ehrlichiosis in sheep, may persist in blood from infected animals for up to 2 years following an acute infection (8). Based on PCR evidence, *B. microti* persisted for at least 82 days in the blood of untreated infected individuals who did not have specific symptoms (11).

Other *Rickettsia* spp. have been reported to survive under refrigeration conditions. *E. phagocytophila* has been reported to remain viable in infected sheep blood stored refrigerated at 4°C for up to 13 days (8). *Orientia tsutsugamushi*, the cause of scrub typhus, has been reported to remain infectious in experimentally infected blood stored at 4°C for up to 10 days and for up to 45 days in frozen packed cells (5).

An important practical application of our findings is that storage of infected blood specimens at 4°C for a few days does not prevent recovery of the HGE agent in culture. This is of particular importance for blood specimens that require transport to a remote laboratory for culture.

This work was supported by grants HLT-27018 and HLT-27017 from the Westchester County Department of Health to M.E.A.-R.

We thank Philip Kurpiel for providing blood specimens from one patient and the assistance of Louis Rosenfeld and Daniel Byrne.

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