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THE EFFECTS OF REFRIGERATION AND FREEZING (WITH GLYCEROLIZATION) OF PACKED RED BLOOD CELLS ON THE RECOVERY AND VIABILITY OF *ORIENTIA TSUTSUGAMUSHI*

Brian G. Casleton

A Thesis

Submitted to the Graduate College of Bowling Green State University in partial fulfillment of the requirements for the degree of

MASTER OF BIOLOGY

August 1996

Committee:

Stan L. Smith, Chair

Daryl J. Kelly, (Research Advisor)

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ABSTRACT

Daryl J. Kelly, Research Advisor

Scrub typhus, caused by infection with *Orientia tsutsugamushi*, accounts for up to 23% of all fevers in endemic areas of the Asia-Pacific region. Patients often become rickettsemic approximately 1 to 3 days before symptoms of the disease are evident. Infection is common in rural areas and the clinical syndrome can vary from asymptomatic to a fatal illness. In an effort to determine if *O. tsutsugamushi* could survive in units of stored blood and present a potential threat to the blood supply, we infected human mononuclear cells isolated from whole blood by density gradient centrifugation and subsequently inoculated them with *O. tsutsugamushi*, Karp strain. Infection of the mononuclear cells was determined by Giemsa stain, direct fluorescent antibody (DFA) staining, and polymerase chain reaction (PCR). Infected autologous mononuclear cells were returned to aliquots of packed red blood cells (PRBC) which were then refrigerated at 4°C or glycerolized and frozen at -70°C. Mice were inoculated with 0.5 ml aliquots of the human blood at 24 hours, 10 days, and 30 days after PRBC refrigeration or 45 days after frozen glycerolized storage. *O. tsutsugamushi* caused illness in the mice after inoculation with the 24 hour and 10 day refrigerated aliquots, and the 45 day deglycerolized aliquot, but not the 30 day refrigerated blood. Infection was confirmed by DFA staining, ELISA, and PCR. The data indicate that *O. tsutsugamushi* can survive within human mononuclear cells in blood products stored in accordance with standard blood bank methods for extended

periods of time. Scrub typhus and perhaps other intracellular bacterial infections could present a risk in blood collected from donors in endemic areas.

To my wonderful family (especially my wife Deanna and my children Matthew and Alex) without whose patience and understanding this project could not have been completed. To my parents Ronald and Harriet whose love and encouragement have provided an important source of motivation and inspiration, and to my friends for their continuous support.

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INTRODUCTION

One of the most serious adverse effects of blood and blood component transfusion is the transmission of disease. This, combined with the public's demand for a safer blood supply, has resulted in intense investigation into infectious disease detection in blood and blood products over the past two decades. Research intensified in the early 1980's after the discovery of human immunodeficiency virus (HIV), the etiologic agent of AIDS, a fatal blood-borne infection transmitted through transfusion.[1] As a result, the number of infectious disease tests performed on donated blood has increased since that time and now includes tests for hepatitis B and C, HIV 1 and 2, human T-lymphotropic virus (HTLV) I and II, and syphilis. The implementation of improved testing and stringent donor selection procedures has reduced the risk of infection via blood transfusion.[2] Nonetheless, many other viruses, parasites, and bacteria can and have been transmitted through blood or blood component transfusion and must not be overlooked or forgotten (Table 1).[3] The possibility of transfusion transmitted disease remains a constant concern for all blood recipients and transfusion facilities.

Table 1. Potential Transfusion Transmitted Diseases.*

Hepatitis A	Hepatitis B
Hepatitis C	Hepatitis D
Cytomegalovirus	Epstein-Barr virus
HIV	HTLV
Babesiosis	Lyme disease
Chagas disease	Creutzfeldt-Jakob disease
Syphilis	Malaria
Toxoplasmosis	Parvovirus B19
Leishmaniasis	Yersinia enterocolitica

* Summary of diseases documented as transfusion transmitted and/or shown to survive the normal preparation of blood and blood components.[3]

Any microorganism that can survive the normal preparation and storage environment of blood and blood components has the potential of being transmitted through transfusion. For example, investigators have reported that the causative agent of Lyme disease, *Borrelia burgdorferi*, can survive the normal processing of whole blood and has the potential to be transfusion transmitted.[4] However, since hematogenous spread of the extracellular spirochete occurs early in the illness leading to rapid development of symptoms (fever and headache), potential blood donors would most likely be deferred during physical examination under current Food and Drug Administration (FDA) regulations, and American Association of Blood Banks (AABB) standards.[5] Microorganisms of the family Rickettsiaceae may present a similar threat with some notable differences: studies have shown that certain members of the Rickettsiaceae, notably *Rickettsia prowazekii,* can become latent after initial infection and reactivate when the host becomes immunocompromised resulting in Brill-Zinsser disease or recrudescent typhus.[6] Researchers have also demonstrated that *Rickettsia rickettsii* and *Orientia* (formerly *Rickettsia*) *tsutsugamushi* are harbored in the tissues of humans and laboratory rodents for at least a year after primary infection.[7,8,9,10] Hayashi et al., were able to induce a rickettsemia in a patient who had recovered from *O. tsutsugamushi* infection using intravenous injection of typhoid fever vaccine 4 to 8 weeks after fever subsided.[11,12] Additionally, cases of asymptomatic rickettsial disease have been documented; thus, leading to the possibility of people donating potentially infectious blood.[13,14,15]

Rickettsiaceae are small pleomorphic gram-negative coccobacilli that are obligate intracellular parasites and are usually transmitted to humans by arthropod vectors. They are

a group of organisms that infect wild animals and are passed to humans by arthropod vectors: ticks, mites, lice, and fleas. The organisms multiply by binary fission within cells which are eventually lysed, releasing mature rickettsiae into the blood stream. At least four species (*R. rickettsii*, *R. conorii*, *O. tsutsugamushi*, *R. akari*) are transmitted transovarially (generation to generation) in the arthropod, which serves as both vector and reservoir.[16] Rickettsiaceae are classified on the basis of clinical features, epidemiological aspects, and immunologic characteristics into clinically distinct groups (Table 2).[17,18] Most organisms of the family Rickettsiaceae infect humans following the bite from an infected arthropod vector. After being deposited directly into the blood stream through the bite of an ixodid tick or mite (chigger), or by self inoculation of the infected feces of the louse or flea, the organisms are engulfed by the host cells, and quickly escape the vacuole to become free in the cytoplasm; thus, evading destruction by host cell defenses. Damage to cells results in vascular changes and an influx of inflammatory cells (neutrophils and monocytes) that serve to magnify the host's immune response and disseminate the disease.[19]

Rickettsioses are emerging as important causes of imported fevers in non-endemic areas and continue to be prevalent in various geographic locations throughout the world. In Switzerland, rickettsioses are estimated to be the third most frequent cause of imported acute febrile disease behind malaria and enteric fever.[20] In Germany, investigators have reported 22 cases of imported rickettsiosis from returning travelers during a five year period: 18 with boutonneuse fever (*Rickettsia conorii*) and 3 with scrub typhus (*O. tsutsugamushi*).[21] In the United Kingdom, boutonneuse fever has been reported in military personnel and returning travelers.[22] In the United States, only 500 cases of Rocky Mountain

Table 2. Selected Rickettsial Disease of Humans.*

Disease	Etiological Agent	Geographic Area of Prevalence	Arthropod Vector	Mammalian Reservoir
Typhus group				
Epidemic typhus	*Rickettsia prowazekii*	South America, Africa, Asia	Louse	Humans
Murine typhus	*R. typhi*	Worldwide	Flea	Rodents
Scrub typhus	*Orientia tsutsugamushi* †	Southeast Asia, Pacific region	Mite §	Rodents
Spotted Fever Group				
Rocky Mountain spotted fever	*R. rickettsii*	Western Hemisphere	Tick §	Rodents, dogs
Boutonneuse fever	*R. conorii*	Africa, India, Mediterranean	Tick §	Rodents, dogs
Kenya tick typhus South African tick typhus	*R. africae*			
Indian tick typhus Japanese spotted fever	*R. japonica*	Japan	Unknown	Rodents
Queensland tick typhus	*R. australis*	Australia	Tick §	Rodents
Rickettsialpox	*R. akari*	USA, Korea, Russia	Mite §	Mice
Siberian tick typhus	*R. sibrica*	Siberia, Mongolia	Tick §	Rodents
Flinders Island spotted fever	*R. honei*	Australia	Unknown	Rodents
Other				
Q fever	*Coxiella burnetii*	Worldwide	Tick, aerosol	Cattle, Sheep
Ehrlichiosis	*Ehrlichia* sp.	Worldwide	Tick	Rodents
Trench fever ‡	*Bartonella quintana*	Worldwide	Louse	Humans

* This chart shows the common antigenic groups and lists the worldwide locations of some of the common rickettsial diseases with their arthropod vectors and animal reservoirs.

† Recently proposed to change the name of *Rickettia tsutsugamushi* to *Orientia tsutsugamushi*.[12]

§ Arthropod also serves as a reservoir, by maintaining the rickettsiae through transovarian transmission.

‡ Recently proposed to combine the genera *Bartonella* and *Rochalimaea* and to remove the family Bartonellaceae from the order Rickettsiales.[23]

spotted fever (RMSF) (*Rickettsia rickettsii*) were reported annually before 1960; since then the incidence has more than doubled.[24] In 1990, a prospective, seroepidemiologic study conducted on infections caused by spotted fever group rickettsiae (SFGR) and *Ehrlichia* species among 1094 US military personnel at Fort Chaffee, Arkansas estimated that at least 700 new infections occur annually in this region with only 200 being symptomatic. In addition, the first isolation of the human rickettsial pathogen, *Ehrlichia chaffeensis*, was a product of that study.[14] Serological studies of 325 blood donors from southern France demonstrated that 18% percent had antibodies to *R. conorii* with the highest prevalence occurring in urban areas.[25] In Tunisia, 500 sera collected from healthy blood donors were tested and 41% had antibodies to various rickettsiae, confirming endemicity in the region.[26] Serological surveys in China show that up to 34% of healthy adults have antibodies to rickettsiae.[27] Additionally, sixty-nine cases of scrub typhus (*O. tsutsugamushi*) were detected in Chinese military personnel stationed in the Pescadore Islands between May and November 1975.[28] In Malaysia, scrub typhus was found to cause up to 23% of febrile illness in certain areas and the prevalence of antibody was found to be 64% in rural populations.[29] Risk to U.S. military continued to be a problem as documented by a 1975 seroepidemiological study conducted on 507 US Marines stationed at Okinawa, Japan as a result of deployment to the Mount Fuji region of Japan. Investigators found that *O. tsutsugamushi* accounted for six infections in this group.[15] These statistics affirm that there is a significant worldwide prevalence of rickettsial disease. The current socioeconomic climate in underdeveloped and war-torn countries favors the emergence of dangerous rickettsial disease due, in part to the urbanization of endemic areas, as well as man's

intrusion into the normally stable ecosystem of vectors and animal hosts as a result of recreational activities, military exercises, and travel.[22,30]

In light of the worldwide prevalence of rickettsial infections, accurate and early diagnostic measures are needed to prevent serious illness. Individuals engaged in military operations encounter the same types of habitats as rural residents and often disrupt the normally stable ecosystem of arthropod vectors and animal hosts; thus, increasing the chance of exposure to arthropod-borne disease. Military blood banks rely heavily and, at times, solely on the military population to supply blood needs. With the prevalence of *R. rickettsii* and *Erhlichia* species and their vectors in the United States, *R. conorii* in Europe, Africa and the Mediterranean littoral, and *O. tsutsugamushi* in Asia-Pacific region, non-immune American troops could experience significant disease in certain areas. For example, scrub typhus was the most serious rickettsial disease among allied forces in the Southwest Pacific Theater during World War II with over 18,000 cases diagnosed.[31] During the Vietnam war, scrub typhus was the cause of 11% of all serologically diagnosed fever cases in American military personnel and accounted for up to 23% of all fever in endemic areas of the Asia-Pacific region.[32] During the spring of 1989, 86 military members participated in a two-week training mission in Arkansas and Virginia. Of those, 38% who trained in Arkansas demonstrated seropositivity and symptoms suggestive of tick-borne illness (rickettsiosis). Twenty percent of those had elevated titers and extensive tick bite exposures, yet were asymptomatic.[33] In 1992, 196 US Army soldiers participated in a 10 day training exercise in Botswana, Africa. Upon return to their home station, 30% sought medical attention for African tick bite fever (*R. africae*).[34] Similarly, *O.*

tsutsugamushi has continued to cause morbidity in troops conducting military operations in Vietnam, New Guinea, Malaysia, and Taiwan.[32,35,36,37] Those military members who donate blood and remain asymptomatic, yet carry rickettsiae, may transmit rickettsioses to blood recipients through transfusion.

Rickettsial disease normally presents as an acute illness. However, studies indicate that the initial incubation can be 5-14 days with rickettsemia occurring 1-3 days before the onset of symptoms. Some infections can be completely asymptomatic.[38] RMSF is normally described as a febrile illness of sudden onset accompanied by headache and a rash beginning on the extremities and spreading to the trunk. The characteristic rash is seen in only 50-60 percent of serologically confirmed cases of RMSF.[14] Therefore, the possibility exists that early signs of the infection can go unnoticed. Human monocytic ehrlichiosis (HME), caused by *Ehrlichia chaffeensis*, also presents as a febrile illness clinically similar to RMSF, but in 60-70 percent of these cases there is no rash.[14] Due to cross-reactivity between *R. rickettsii* and other non-pathogenic SFGR, investigators are unsure if asymptomatic cases of *R. rickettsii* occur.[14] Asymptomatic infections with *E. chaffeensis,* and *R. conorii* have been documented.[39,13] Like certain SFGR, infection with *O. tsutsugamushi* can range from asymptomatic to fatal, but often presents as a febrile illness with eschar formation, regional lymphadenopathy, severe headache and fatigue.[40] Rickettsial diseases, in general, remain difficult to diagnose upon presentation of illness and are often confused with other febrile illnesses.[22]

Laboratory diagnosis of rickettsial disease currently include cell culture, Weil-Felix agglutination, direct and indirect fluorescent antibody tests, direct Giemsa staining,

polymerase chain reactions, enzyme linked immunosorbent assay (ELISA), and flow cytometry.[22,41] If undiagnosed, rickettsial disease can lead to severe morbidity and mortality. However, in some individuals, exposure has only been demonstrated by seroconversion.[14] To date, no study has been conducted to determine the potential risk of transfusion transmitted rickettsial disease or the survival of organisms from the family Rickettsiaceae in blood products collected from symptomatic or asymptomatic donors and stored in accordance with AABB, and FDA guidelines. This study was designed to determine if *O. tsutsugamushi* and potentially other rickettsiae are viable after storage in refrigerated and/or frozen deglycerolized packed red blood cells (PRBC); and therefore, potentially transfusion transmissible. Viability of *O. tsutsugamushi* was assessed using an established mouse model. The laboratory mouse has been used extensively for the study of *O. tsutsugamushi* to determine its virulence/pathogenesis, the efficacy of antibiotics, and to characterize its antigenic variations.[42,43] One or two organisms can kill or immunize a mouse; thus, providing adequate sensitivity for this experiment.[44]

MATERIALS AND METHODS

The organism selected for this study was *O. tsutsugamushi*, Karp strain (ATCC VR-150: L11V29/30 8 Feb 95 Semi-purified, Naval Medical Research Institute). It is the most typical and pathogenic strain; therefore, it has received the most study. All work with this organism was conducted at the Naval Medical Research Institute in accordance with Center for Disease Control guidelines for biosafety level 3 facilities.[45] Human mononuclear cells were isolated from units of whole blood and infected with *O. tsutsugamushi* at or above levels which correspond to a rickettsemic asymptomatic individual (Figure 1).[38] Mononuclear cells were selected because Rickettsiaceae are obligate intracellular organisms and reports have shown that these cells (primarily monocytes) play a role in the eradication and dissemination of the microorganisms.[46] Mouse fibroblasts (L-929 cells, ATCC, Cat. No. CCL-1, Rockville, MD) were also used in the preliminary experiments in the event the isolated mononuclear cells were not easily infected (Figure 2). The units of blood were split into 5 aliquots of approximately 50 ml each. The infected cells were returned to the aliquots of blood and stored as refrigerated (PRBC) or frozen glycerolized red cells. Samples of the stored PRBC were injected into the intraperitoneal cavity of mice following 24 hours, 10 days, 30 days of refrigerated storage, and 45 days after storage as frozen glycerolized red cells. The animal experiments reported herein, were conducted according to the principles set forth by the Institute of Laboratory Animal Resources.[47]

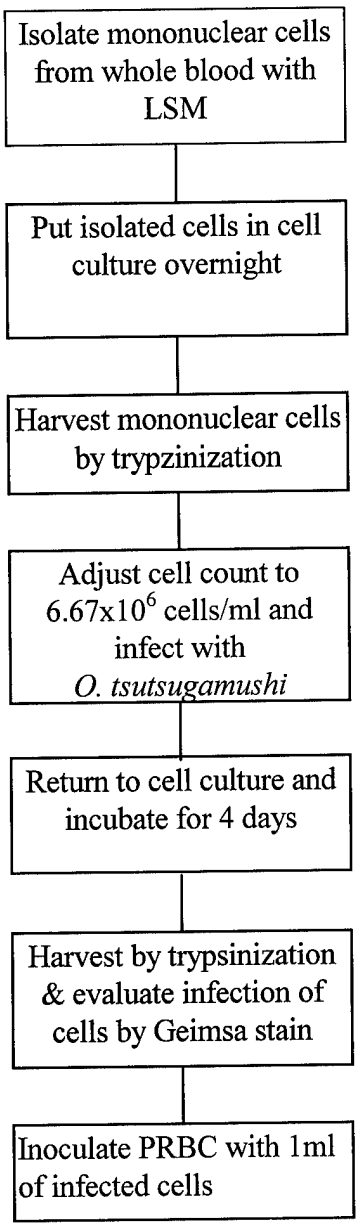

Figure 1. Preparation of autologous mononuclear cells for inoculation into PRBC. Infected autologous mononuclear cells were infected in-vitro with *O. tsutsugamushi*, Karp strain then returned to the aliquots of PRBC.

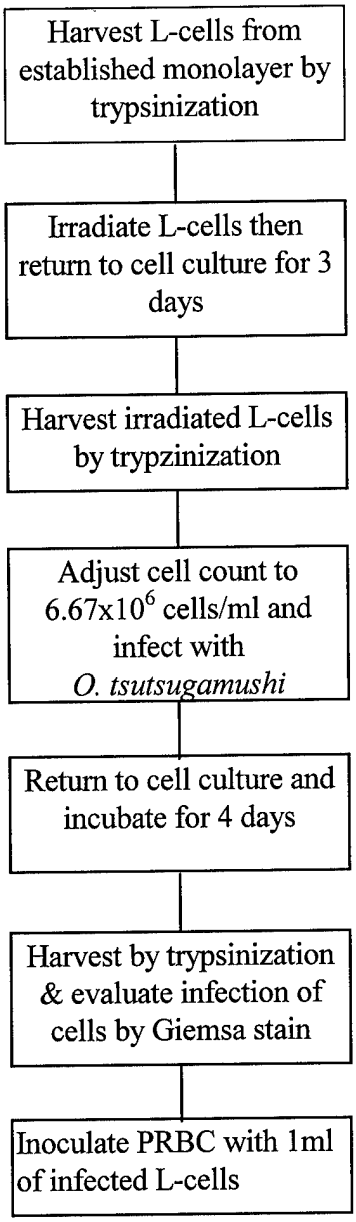

Figure 2. Outline of L-929 cell preparation. Infected mouse L-929 cells were used in the preliminary experiments.

Processing the whole blood unit into packed red blood cells

Units of whole blood were obtained from donations given at the Walter Reed Army Medical Center Blood Donor Center. Sixty grams of whole blood were aseptically transferred into one of the attached satellite bags for mononuclear cell isolation. The tubing between the bags was sealed in two places and the satellite bag removed. The remainder of the unit was processed into PRBC. The cellular components were separated from the plasma in the unit of whole blood by centrifugation (Sorvall Model RC-3B, Dupont Co. Wilmington, DE) at 5000*g* for 5 minutes at 4°C.[48] The plasma was expressed into one of the satellite bags and saved. The remaining PRBC were separated into 5 equal aliquots (approximately 50 ml each) using the quadruple transfer container (Baxter Fenwal Cat. No. 4R2958, Deerfield, IL). The amount of each aliquot was determined by weight on a OHAUS GT 8000 digital scale tared with an empty satellite bag and then converted to milliliters using the specific gravity of PRBC (1.060).[48]

Isolation of mononuclear cells from whole blood

A sampling site coupler (Baxter Fenwal Cat. No. 4C2405, McGaw Park, IL) was inserted into one of the ports on the satellite bag. The whole blood was diluted with 60 ml of sterile 0.9% sodium chloride (Baxter Fenwal Cat. No. J5A275). Six 20 ml aliquots were aseptically removed with a 16 gauge needle and syringe and dispensed into each of six 50 ml sterile Falcon Blue Max conical tubes (Becton Dickinson Labware; Cat. No. 14432-24, Lincoln Park, NJ).

Mononuclear cells were isolated by underlaying the diluted whole blood with 10 ml lymphocyte separation medium (LSM) density of 1.077-1.080 (Organon Teknika Cat. No. 50494, Durham NC). The mixture was separated at 400*g* in a Sorvall RT6000D centrifuge (Dupont Co. Wilmington, DE) at room temperature (20-24°C) (RT) for 30 minutes. The top layer of clear plasma was aspirated to within 3 mm above the mononuclear cell layer and discarded using a sterile 10 ml pipet (Fisher Scientific Cat. No. 13678-11E, Pittsburgh, PA). The mononuclear cell layer and half of the LSM layer were aspirated using a 10 ml pipet and placed into a clean 50 ml conical tube. The cells were washed twice with sterile phosphate buffered saline (PBS) pH 7.4 (Sigma Chemical Co. Cat. No. P3813, St. Louis, MO) using centrifugal separation at 200*g* for 10 minutes at RT.[49] After the second wash, the mononuclear cells were resuspended with 5 ml growth medium (GM) (Medium E-199, Quality Biologicals Cat. No. 112-022-100, Gaithersburg MD; 5% fetal calf serum, Grand Island Biological Co. Cat. No. 200-6140, Grand Island NY; 5% Tryptose Phosphate Broth, Quality Biologicals Cat. No. 40-108-5; and 1% L-Glutamate, Quality Biologicals Cat. No. 118-084-060). One drop of the recovered cells was placed onto a glass slide for a differential count.

Determination of the number of mononuclear cells

Cells were counted using a Neubauer hemocytometer (American Optical, Buffalo, NY). The mononuclear cell suspension was diluted 1:10 in PBS. Of this dilution, 10 µl was pipetted onto the hemocytometer and the cells allowed to settle for 10 minutes at RT.[50] The cells in the 5 large squares on each side of the chamber were counted. The number of cells per microliter was determined using the following formula:

$$\text{Formula: } \frac{\#\ of\ Cells\ Counted \times Dilution\ Factor}{\#\ of\ Squares\ Counted \times 0.1\ mm} = \text{Cells}/\mu l$$

The mononuclear cells were then placed into a 75 cm^2 culture flask (Costar Cat. No. 3075, Cambridge, MA) with 10 ml of GM and incubated (Forma Scientific Model 3326, Marietta, OH) at 35°C with 5% CO_2 for 24 h. A sterility check was performed by inoculating 0.01 ml of the suspension onto a sheep blood agar plate (Remel Cat. No. 1200, Lenexa, KS).

Trypsinization of the cells for infection with Orientia tsutsugamushi

The medium containing the suspended cells was transferred from the culture flask into a sterile 15 ml conical tube from Corning (Fisher Scientific Co. Cat. No. 05538-51A). Enough 0.1% trypsin versene (Biofluids Inc. Cat. No. 301, Rockville, MD) and 0.1% disodium-ethylenediamine-tetraacetic acid (EDTA) (Sigma Chemical Co. Cat. No. E-5134) in Hank's balanced salt solution (Quality Biologicals Cat. No. 14-102-5) was dispensed into the flask to completely cover the monolayer of adherent cells. The flask was placed into the 35°C incubator for 5 minutes. The cells were dislodged by firmly hitting the bottom of the flask. The trypsinized suspension was added to the 15 ml tube and the cells were separated by centrifugation (IEC model PR-J) at 200*g* for 5 minutes at RT. The medium was decanted and the cell pellet was resuspended with PBS and separated by centrifugation at 200*g* for 5 minutes. This step was repeated and the cells were adjusted to a cell count of 6.67×10^6 cells per ml in GM (For example: If count yields 7.60×10^6 cells/ml in 3 ml GM, then 7.60/6.67 x 3 ml = 3.42 ml, therefore 0.42 ml of GM needs to be added to the 3 ml suspension to yield a final count of 6.67×10^6 cells/ml).[51]

Inoculation of cellular suspensions

The inoculation method used was adapted to mononuclear cells from the method described by Strickman for infection of mouse L-929 cells.[51] For inoculation, 0.6 ml of the mononuclear cell suspension ($6.67x10^6$ cells/ml) and 0.3 ml of the rickettsial suspension were added to a 2 ml biofreeze vial (Costar Cat. No. 2228, Pittsburgh, PA). The tightly capped vials were rotated 360° at 10 rpm for 60 minutes on a Scientific Industries model 151 rotator (CMS Cat. No. 205-435, Houston, TX) at 34°C. Rotation ensures even distribution of the rickettsiae throughout the mononuclear cell suspension. Two 25 cm^2 culture flasks (Costar Cat. No. 3050) were prepared with 4.0 ml GM, and a third with 4.0 ml GM containing 10% human plasma from the unit of whole blood (bioassay to determine if the human plasma inhibited the growth of the *O. tsutsugamushi*). Each flask was inoculated with the contents of one vial of infected mononuclear cells. The mixture was incubated at 35°C in an atmosphere of 5% CO_2 for 4 days.[51] A fourth flask was prepared containing only non-infected mononuclear cells and incubated with the other flasks.

Preparation of mouse L-929 fibroblasts for inoculation

Monolayers of L-929 cells grown in 75 cm^2 flasks were harvested by trypsinization.[41] The suspended cells were concentrated by centrifugation and resuspended in GM.[51] The suspension was irradiated 5 minutes at 3000 Gy with ^{137}Cesium. The cells were replated into a 75 cm^2 culture flasks and incubated at 35°C in 5% CO_2 for 2 days to permit the cells to cease replicating. The cells were trypsinized again, and cells shown to be viable by trypan blue staining (Sigma Chemical Co. Cat. No. T 8154, St. Louis MO) were counted in a hemocytometer to determine the number of viable cells in the suspension.[50]

For infection, the number of viable cells was adjusted to 6.67 X 10^6 cells per ml, and 0.6 ml of this suspension was added to a 2 ml biofreeze vial. The cells were inoculated with *O. tsutsugamushi* by the method described above for mononuclear cells.[51]

Harvesting of infected cells for inoculation into PRBC aliquots

After 4 days incubation, the mononuclear and L-929 cells were harvested by the previously described trypsinization method with the following alteration: after the final wash, the cells were resuspended in 2 ml PBS. The resulting suspension was used to inoculate the PRBC. A manual cell count was performed to determine the number of cells in the inoculum.

Preparation of slides to evaluate infection of cells

After harvesting the cells, slides were prepared to evaluate the infection within the cells. Two slides from each suspension (infected cells, infected cells with serum control, and uninfected cells) were prepared using the Shandon Cytospin model 2 (Shandon Lipshaw, Pittsburgh, PA). From each suspension, 100 µl was pipetted into the chambers and the cells were concentrated onto the slides by centrifugation for 5 minutes at 500 rpm.[51] After air drying, one of the slides from each suspension was fixed for a least 5 minutes in absolute methanol (Mallinckrodt Specialty Chemicals Co. Cat. No. 3412, Chesterfield, MO) for staining with Giemsa stain (EM Science Cat. No. GX 0085-3, Gibbstown, NJ), the remaining slide was fixed for 10 minutes in cold acetone (Mallinckrodt Cat. No. 2440, Chesterfield, MO) for direct fluorescent antibody (DFA) staining.

Giemsa staining

The Giemsa stain was diluted 1:20 with phosphate buffer pH 6.8. The buffer was prepared by dissolving 1.16 g of sodium phosphate dibasic heptahydrate (Na_2HPO_4) (Mallinckrodt Specialty Chemical Co. Cat. No. 7914, Paris KY) and 0.71 g of potassiuim phosphate (KH_2PO_4) (Sigma Chemical Co. Cat. No. P5379, St. Louis, MO) in 1,000 ml of distilled water. The pH was adjusted with 1 N hydrochloric acid (HCL) (Sigma Chemical Co., Cat. No. 920-1) using a pH meter (Orion Research Inc. Ionalyzer model 601A, Cambridge, MA). The slides were stained for 7 minutes, then rinsed with distilled water, air dried, and examined using an Olympus BH-2 microscope (Olympus America Inc., Melville, NY) equipped for light microscopy at 1000x magnification for the presence of deep blue staining inclusion bodies in the cytoplasm (perinuclear space) of the cells.[52]

Evaluation of slides to determine concentration of rickettsiae per ml PRBC

The percentage of infected cells was determined by classifying 200 cells as either infected or uninfected. The rickettsiae in 50 infected cells were counted to determine the average number or organisms per infected cell.[51] Using the formula:

# of cells/ml inoculum x percent infected cells = # infected cells per ml
Total rickettsiae counted / 50 cells counted = average # rickettsiae per infected cell
 # infected per ml x average # rickettsia per cell = # rickettsiae in 1 ml

the estimated number of rickettsiae per ml of inoculum was calculated. Only 1 ml of each inoculum was used to inoculate the PRBC aliquots. Therefore, the calculated number of rickettsiae per ml inoculum was adjusted for the addition of 1 ml of inoculum into the volume of PRBC in each aliquot (i.e., 1 ml inoculum + 49 ml PRBC = 50 ml total volume; the # of rickettsiae/ml inoculum divided by 50 ml = # of rickettsiae/ml PRBC).

Inoculation of the aliquots of PRBC

In the preliminary study, 2 aliquots of PRBC were inoculated with infected autologous mononuclear cells, and 2 were inoculated with infected L-929 cells. The remaining un-inoculated aliquot served as a PRBC control.

In subsequent units, only infected autologous mononuclear cells were used and 10-fold serial dilutions (1:10, 1:100, 1:1000) of the undiluted inoculum were prepared in PBS prior to inoculation into the PRBC aliquots (Figure 3). Of the 4 PRBC aliquots, each received 1 ml of inoculum, either undiluted, 1:10, 1:100, or 1:1000. The remaining uninoculated aliquot served as a PRBC control. Inoculation was performed using a 18 gauge needle, syringe, and inserted sample site coupler. After removal of 2 ml PRBC from each aliquot for glycerolization and freezing, the PRBC aliquots were stored at 4°C. The number of rickettsiae per ml PRBC in the aliquots which received 10-fold serial dilutions of inoculum was estimated by dividing the number of rickettsiae per ml PRBC from the aliquot which received undiluted inoculum by the inverse of each dilution (i.e., if 35,000 rickettsia/ml PRBC in aliquot which received undiluted inoculum, then the aliquot which received a 1:100 dilution of this inoculum would have approximately 350 rickettsiae per ml PRBC: 35,000/100=350).

Preparation of glycerolized packed red blood cells for freezing

PRBC were prepared for freezing by slowly adding 4 ml of 6.2 M glycerolizing solution (Cytosol Laboratories Inc., Glysol-500, Braintree MA) to 2 ml of PRBC in a 15 ml sterile conical tube. The contents were mixed by inversion to ensure even distribution of the glycerol with the red blood cells. The mixture was allowed to equilibrate for 10 minutes

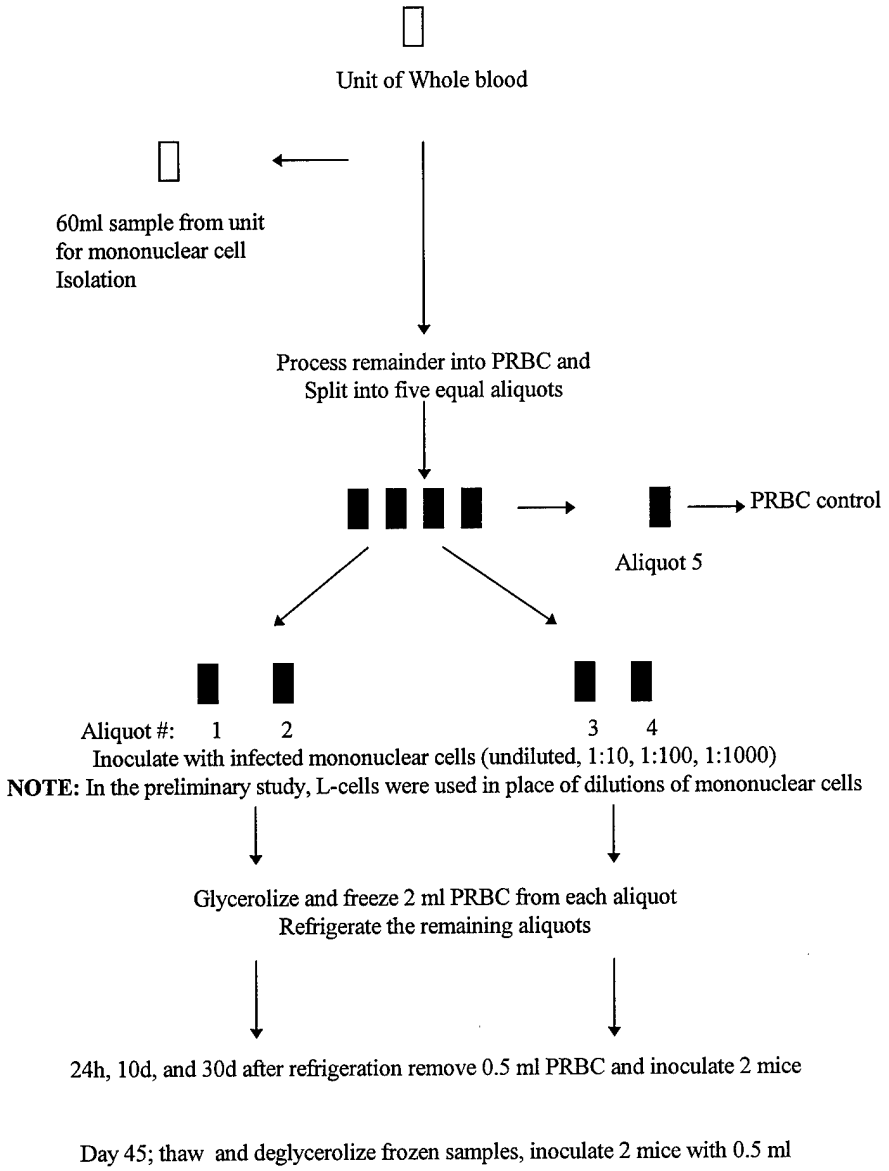

Figure 3. Flow chart of preparation, inoculation of PRBC, and inoculation of mice. Units of whole blood were processed into PRBC then separated into 5 equal aliquots. In-vitro infected autologous mononuclear cells or infected L-929 cells were inoculated into the aliquots then the blood was refrigerated or glycerolized and frozen. Samples of PRBC were injected into the intraperitoneal cavity of 2 mice for each aliquot at each sampling time for each unit.

at RT.[53] A 4 ml aliquot was transferred to a 5 ml cryovial (Nagle, Fisher Scientific Co., Cat. No. 5000-0050) and stored -70°C for 45 days.

Sampling of units

A sample of each refrigerated PRBC aliquot was removed at 24 hours, 10 days, and 30 days. The frozen glycerolized red cells were thawed and deglycerolized on day 45 after storage. Two outbred CD-1 mice (Charles River Laboratories) were inoculated with 0.5 ml PRBC at each sampling point for each aliquot of PRBC. Additionally, 25 µl of PRBC from each aliquot was pipetted onto a 1 cm^2 filter paper square, air dried, and stored in the refrigerator at 4°C for later PCR.

Deglycerolization of frozen glycerolized infected PRBC

The frozen blood was thawed at RT for 30 minutes then mixed by inversion. The thawed blood was transferred to a clean 15 ml conical tube. A volume of 12.0% sodium chloride (Baxter Fenwal Cat. No. 4B7874) equal to one-half the volume of thawed blood was added slowly to the tube with constant mixing. The mixture was allowed to equilibrate for 3 minutes at RT. The tube was filled with 1.6% sodium chloride (Baxter Fenwal Cat. No. 4B7870), mixed by inversion, and the mixture separated by centrifugation at 200*g* for 1 minute. The supernatant was discarded, the tube filled with 1.6% sodium chloride, and again separated by centrifugation at 200*g* for 1 minute. The supernatant was discarded and the cells were washed two more times with 0.9% sodium chloride (McGaw Inc., Cat No. R5200-01, Irvine, CA) or until supernatant was hemoglobin free by visual inspection.[53]

Inoculation of mice

Using a tuberculin syringe (26 gauge needle), 0.5 ml of the rickettsiae inoculated PRBC was injected into the intraperitoneal cavity (IP) of two outbred CD-1 mice. Two additional mice were inoculated from each PRBC sampling point (24 hours, 10 days, 30 days after refrigeration and 45 days after frozen glycerolized storage). Each mouse was restrained and positioned head pointing downward and abdomen exposed for the inoculation. The area was cleansed with isopropyl alcohol (J.T. Baker Chemical Co. Cat. No. 9080-01-BC, Phillipsburg, NJ). The needle was inserted into the abdomen at a 30-45 degree angle, caudal to the umbilicus and lateral to the midline. Mice were visually evaluated daily for signs of infection. If no signs were apparent by 9-11 days post inoculation then a 25 µl retro-orbital blood sample was collected for PCR using a 50 µl capillary tube (25µl blood on a 2 cm^2 filter paper square, air dried, and stored at 4°C). The mice were anesthetized with methoxyflurane (metophane) (Pitman-Moore Cat. No. 55685, Mundelein, IL) for this procedure. If the mice were symptomatic, then one was sacrificed. Two impression smears were prepared from peritoneal exudate of sacrificed mice, one fixed in methanol for Giemsa and one fixed in cold acetone for DFA staining. If the mice remained asymptomatic or recovered, then blood was collected at 30 days post-inoculation for ELISA.[42] Data were compiled as the number of units causing a positive result (i.e., illness, lethality, *O. tsutsugamushi* DNA amplification, antibody detection, or direct detection) rather than as individual mouse data.

Direct fluorescent antibody stain

Direct fluorescent antibody staining was used to confirm the presence of *O. tsutsugamushi* in the inocula and in infected mice. Slides from the infected inocula (prepared by cytospin) and impression smears were fixed in cold acetone at -20°C for at least 10 minutes, allowed to air dry, then placed into a slide box and stored at -70°C until stained. Prior to staining the slides were warmed to RT. The primary antibody was fluorescein isothiocyanate (FITC)-labeled rabbit anti-Karp immunoglobulin G (Conjugate #302 USAMRIID/PAI 12/90, Ft. Detrick, MD). FITC-labeled normal rabbit immunoglobulin G was used as a control antibody. A 50 µl drop of each conjugate was applied to separate areas of the slides which were then placed in a 37°C incubator for 30 minutes. The slides were removed, then rinsed twice for 5 minutes using PBS, air dried, and then coverslips were applied using Vectashield mounting medium for fluorescence (Vector Laboratories Inc. Cat. No. H-1000, Burlingame, CA). The slides were examined under the Olympus BH-2 microscope (Olympus America Inc., Melville, NY) equipped for fluorescence at 400x magnification. DFA staining was used to confirm the presence of *O. tsutsugamushi*.[54]

Analysis of inoculum by flow cytometry

A 200 µl sample of the initial inoculum (used in the inoculation of the PRBC) and the uninfected cells were concentrated by centrifugation at 500 x *g* and the supernatant discarded. The pellets were resuspended in 500 µl 4% paraformaldehyde (Fisher Scientific Co. Cat. No. T-353, Fairlawn, NJ) and incubated for 10 minutes at RT. The fixed cells were washed three times in PBS containing 1% bovine serum albumin (BSA) (Sigma

Chemical Co. Cat. No. A3803). After the last wash, the cells were resuspended in 0.5 ml of PBS with BSA. The cells were then treated with 0.5 ml 0.2% saponin (J.T. Baker Chemical Co. Cat. No. 3388) and 20% inactivated human serum in PBS for one hour at 4°C. The infected cells and the uninfected cells were separated equally into two tubes (0.5 ml each tube). FITC-labeled rabbit anti-Karp IgG (50 µl) was added to one tube from each set. FITC-labeled normal rabbit IgG (50µl) was added to the other tube in each set. The tubes were incubated for 60 minutes at 4°C in the dark. After two washes with PBS containing 0.1% saponin, the cells were fixed with 1% paraformaldehyde in PBS (0.5 ml) and stored at 4°C in the dark until analyzed (within 1-3 h). Cell suspensions were analyzed by flow cytometry using a FACScan™ flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15 mW argon laser. A total of 5,000 cells were counted. Data acquisition was performed using Lysis II software version 1.1 (February 1992) (Becton Dickinson, San Jose, CA). Gates were appropriately set on both dotplots and histograms to capture fluorescence intensity of the cells of interest.[55]

Analysis of serum from mice by enzyme linked immunosorbent assay

Antibody titers were determined using the ELISA described by Dasch et.al., 1979.[56] A casein blocking buffer was prepared by dissolving 2.5 g of 0.5% casein (Calbiochem Cat. No. 21859, San Diego, CA) in 50 ml 0.1N NaOH (Mallinckrodt Cat. No. 7708, Paris, KY), and adding 450 ml of PBS. The pH was adjusted to 7.4 with 1 N HCL and 0.01 ml of phenol red (Flow Laboratories Cat. No. 16-900-49, McLean, VA) was added. The PBS coating buffer was prepared by dissolving 1 package of Sigma Diagnostics prepackaged PBS in 1 L distilled water. The wash buffer was prepared by dissolving 5 packages of

Sigma Diagnostics prepackaged PBS in 5 L distilled water with 5 ml polyoxyethylene-sorbitan monolaurate (tween-20) (Sigma Chemical Co. Cat. No. P1379). Each well of a 96 well microtiter plate (ICN Biomedical Cat. No. 76-311-05, Costa Mesa, CA) was coated with 100 µl of diluted Karp antigen (Lot#-A007; RTSU, 100 µl/ml diluted 1:1000 with PBS coating buffer) and stored overnight at 4°C. The plates were washed 5 times with the PBS wash buffer and blotted dry. Residual binding sites in the plates were blocked by pipetting 100 µl casein blocking buffer into each well and incubating for 1 hour at RT. The microtiter plates were again washed 5 times with the PBS wash buffer and blotted dry. The samples were diluted 1:50 in the first row of the plate by pipetting 196 µl of PBS coating buffer into each well, then adding 4 µl of each sample to be tested. The remaining rows received 100 µl of the PBS coating buffer in each well. Serial dilutions were made by pipetting 100 µl from the first row of wells into the second row and continued until the end of the plate, then 100 µl was discarded from the last row. The plates were incubated for 1 hour at RT. Following incubation, the plate was washed 5 times with PBS wash buffer and blotted dry, then 100 µl of horse radish peroxidase labeled goat anti-mouse IgG(fc) antibody (Accurate Chemical & Scientific Corp., Cat. No. JGM035008, Westbury, NY) diluted 1:5000 with PBS was dispensed into each well and incubated for 1 hour at RT. The plate was washed 5 times with PBS wash buffer and blotted dry, then 100 µl of 2.2′-azino-di[3-ethyl-benzthiazoline sulfonate (6)] (ABTS) substrate (Kirkegaard & Perry Laboratories Inc. Cat. No. 50-62-01, Gaithersburg, MD) was added into each well and incubated 15 minutes at RT. The reaction was stopped by pipetting 100 µl of 1% sodium dodecyl sulfate (SDS) (BioRad Laboratories Cat. No. 161-0302, Richmond, CA) into each well. Color

development was detected at a wavelength of 405-410 nm using a spectrophotometric mircroplate reader (Molecular Devices Corp., Model V-max, Menlo Park, CA).

Scrub typhus polymerase chain reaction (PCR)

DNA was extracted from the blood spots on the filter paper by boiling in 300 µl 5% Chelex®-100 resin (BioRad Laboratories Cat No. 143-2832, Richmond, CA) in sterile distilled water for 10 minutes.[57] The suspension was then separated by centrifugation at 12,000*g* in an Eppendorf Model 5414 centrifuge (Brinkman Instruments Inc., Westbury, NY) for 10 minutes. The extract was used as the DNA template. DNA was amplified using a nested PCR designed to amplify a 1,412-base pair region of the gene encoding the scrub typhus 58-kilodalton antigen (Sta58) of *O. tsutsugamushi* (Figure 4).[58,59] The first amplification was performed using 6 µl each of two specific (526 forward (F), 2371 reverse (R)) oligonucleotide primers (Figure 5) (Midland Corp., 11/58KD-526F & 11/58KD-2371R, Midland, TX) and 0.25 µl Taq DNA polymerase (Geneamp DNA Amplification Reagent Kit, Perkin-Elmer Cetus, Norwalk, CT, Cat. No. N801-0055) in a total 50 µl volume (1 µl extracted DNA template, 5 µl reaction buffer, 8 µl of 200 µM dATP, dCTP, dGTP, dTTP mix, 23.75 µl filtered distilled water). The reaction buffer, nucleotides, primers, and distilled water were combined in 0.5 ml reaction tubes (Perkin Elmer Cetus, Cat. No. N801-0180) and then overlayed with 50 µl of mineral oil (Sigma Chemical Co., Cat. No. 400-5). Using the 'hot start' method described by Chou et al., the PCR mixture was heated to 80°C for 10 minutes followed by the addition of the DNA sample and the Taq polymerase.[60] The reaction mixture was heated to 94°C for 4 minutes, followed by 35 cycles of 94°C for 1 minute (denaturation), 50°C for 2 minutes (annealing), and 70°C for 2

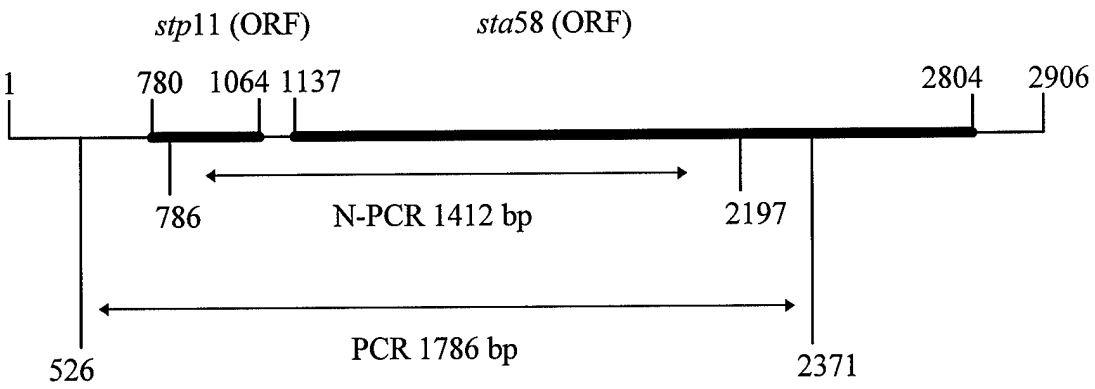

Figure 4. Visual depiction of the *O. tsutsugamushi* Sta 58 major antigen gene. This shows the regions being amplified by the nested PCR reactions where N-PCR is the second amplification and the region of interest. This gene has been shown to be homologous to the *Escherichia coli* groESL operon.[59]

11/58KD-526F	20 mer	5′-TGA-TAG-GCT-ATA-AGC-AAT-GA-3′
11/58KD-2371R	20 mer	5′-TCT-TCA-ACA-GCT-GCT-CTA-GT-3′
11/58KD-786F	20 mer	5′-TAC-CAA-CAA-CTG-TAT-GAT-CG-3′
11/58KD-2197R	20 mer	5′-TCA-CGG-ATC-TGT-TCA-CAA-CG-3′

Figure 5. Sizes and sequences of oligonucleotide primers. Depiction of the forward (F) and reverse (R) primers used for the nested polymerase chain reaction (PCR) amplification of *O. tsutsugamushi* DNA.[59]

minutes (extension) using a DNA thermal cycler (model PTC-100, MJ Research Inc, Watertown, MA). Samples were then held at 4°C overnight. The second amplification was performed using 6 µl each of two specific (786F, 2197R) oligonucleotide primers (Figure 5) internal to the first pair (Cruachem, 11/58KD-786F & 11/58KD-2197, Sterling, VA) and 1 µl of the primary PCR product in place of extracted DNA template. The reaction mixture and amplification conditions were also the same for the primary amplification except that a 57°C annealing step was used in place of 50°C. Amplification of the extracted DNA was determined by visual detection of the ethidium bromide (Sigma Chemical Co., Cat. No. E-4391) stained bands of the expected size after agarose gel electrophoresis of 5 µl of the PCR product in a 14-well 2.0% agarose minigel (FMC Bioproducts, SeaKem LE Cat. No. 50004, Rockland, ME) submerged in tris-borate buffer (TBE) using the Horizon 58 apparatus set on low 100 (Gibco BRL, Life Technologies Inc., Cat. No. 1060 with Model 200 power supply Cat No. 1061, Gaithersburg, MD). Stock TBE pH 8.3 was prepared by dissolving 108 g tris-hydroxymethyl-aminomethane (Calbiochem, Cat. No. 648311), 55 g boric acid (Mallinkrodt, Cat. No. 2549), and 5.44 g Na_2EDTA (Sigma Chemical Co. Cat. No. E-5134) in 1,000 ml of distilled water. This was diluted 1:10 in distilled water for use. All gels were stained 10-15 minutes with 0.5 µg/ml ethidium bromide solution and photographed with Type 55 or Type 57 Polaroid film on a UV transilluminator (Hoefer Scientific Instruments, San Francisco, CA).[46,61,62]

Restriction enzyme digestion

A 6 µl sample of each PCR product was digested in a 20 µl volume with 1 unit of the restriction enzymes Nla IV (Gibco BRL, Cat. No. 71714), and Hinf I (Gibco BRL, Cat. No. 1B12245). Buffers were provided by the manufacturer. After 2 hours of digestion at 37°C, digests were held at RT overnight before electrophoresis. Separation and visualization of digested DNA bands was performed using the previously described agarose gel electrophoresis using 2.0% agarose gel.

RESULTS

Separation of mononuclear cells from whole blood

A clear band of separated cells was produced in between the lymphocyte separation medium and the diluted plasma in 3 of 4 attempts. The 3 successful separations gave a mononuclear cell concentration adequate to provide 3 x 10^6 cells per ml after dilution with cell culture medium. The unsuccessful separation occurred on a unit of blood received from a polycythemia vera patient. No clear bands were produced and streaks of red blood cells contaminated the ficoll concentration gradient. No further testing was performed on the blood from this unit. The cellular differential performed on the mononuclear cells after isolation from each unit of CPDA-1 whole blood revealed a predominance of lymphocytes (Table 3).

Table 3. Differential analysis of isolated mononuclear cells*

Unit #	% Neutrophils	% Lymphocytes	% Monocytes
1	ND†	96	4
2	ND	97	3
3	ND	96	4

* Cellular differential was performed on 200 Giemsa stained cells after isolation of mononuclear cells with lymphocyte separation medium.

† ND = none detected

Recovery of infected cells after incubation and trypsinization

The number of recovered cells after incubation and trypsinization ranged from 1.0 x 10^6 to 1.7 x 10^6 per ml for the human mononuclear cells. The recovery for the L-929 cells used in the initial experiment was 8.3 x 10^4 cells per ml (Table 4).

Table 4. Recovery and degree of intracellular infection of cells used as inoculum

Inoculum #*	# of cells/ml	% cells Infected	Avg. # of Rickettsiae/cell	# of Rickettsiae/ml of Inoculum†
1 (MNC)	1.7×10^6	18	11	3.4×10^6
1 (L-Cell)	8.3×10^4	60	18	9.0×10^5
2 (MNC)	1.8×10^6	12	8	1.7×10^6
3 (MNC)	1.0×10^6	19	7	1.3×10^6

* Includes mononuclear cells (MNC) and the L -929 cells (L-cells) used in the initial unit. The numbers 1-3 correspond to the units of whole blood.

† Calculation: *Total cells/ml of inoculum x % infected x avg number of rickettsiae/cell = # rickettsiae/ml inoculum*

After examination of the Giemsa stained cytospin smears (Figure 6), the percent of infected cells ranged from 12 to 19 percent for the human mononuclear cells and was 60 percent for the mouse L-929 cells. The average number of rickettsiae per infected cell ranged from 7 to 18 with mouse L-929 exhibiting the highest average per cell. In the mononuclear cell inocula, monocytes were the predominately infected cell. Some infection of lymphocytes was noted: the number of rickettsiae per infected lymphocyte ranged from 1-3. The bioassay for each collected unit (i.e., autologous infected cells incubated with 10% plasma from each unit) demonstrated growth equivalent to the cells incubated without plasma added. This confirmed that the plasma from the units had no inhibitory effect on the growth of *O. tsutsugamushi*. The presence of *O. tsutsugamushi* within the cells was confirmed by DFA staining (Figure 7).

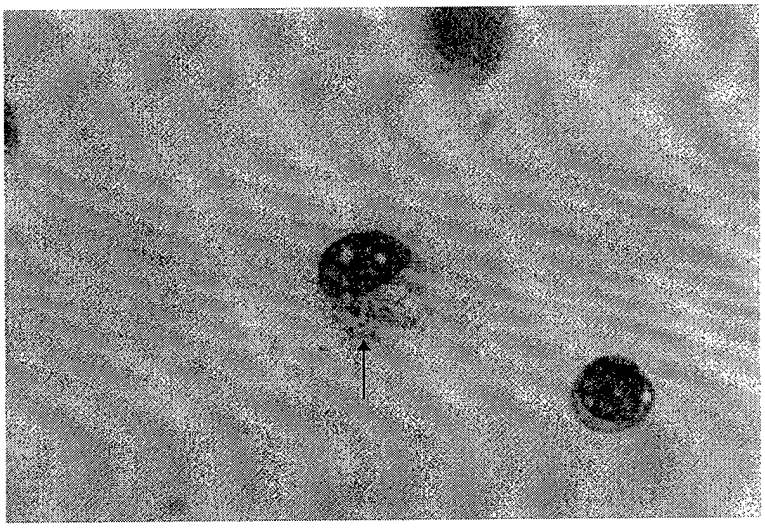

Figure 6. Giemsa stained cytospin smear. This shows a human monocyte with deep blue staining inclusions consistent with rickettsiae in the cytoplasm as indicated by the arrow. (Original magnification 1,000x)

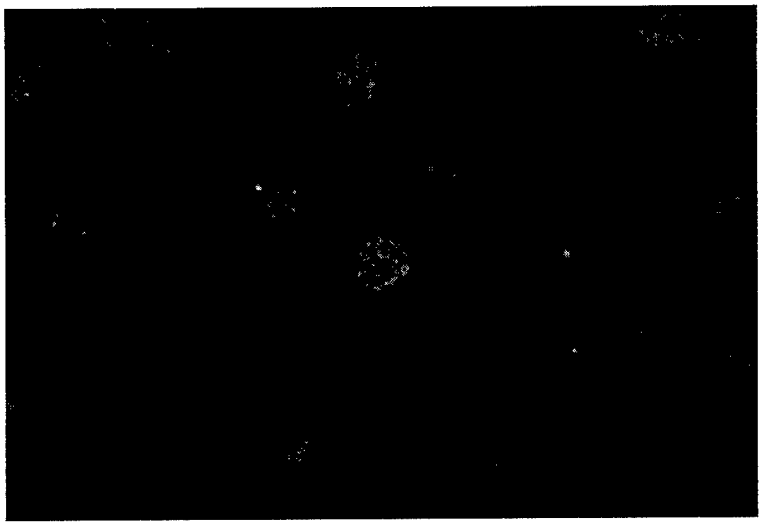

Figure 7. Direct fluorescent antibody stained cytospin smear. Shows brightly stained inclusions consistent with *O. tsutsugamushi.* (Original magnification 400x)

Inoculation of packed red blood cell aliquots

Using the rickettsiae per ml in each inoculum (Table 4), the number of rickettsiae per ml PRBC in each of the aliquots was calculated. The number of rickettsiae per ml PRBC was approximated to be 67,000 in unit 1 (MNC), 30,000 in unit 1 (L-cell), 35,000 in unit 2, and 27,000 per ml in unit 3 (Table 5).

Table 5. Estimated number of rickettsiae per ml PRBC

Unit #	# of Rickettsiae/ml of Inoculum*	Volume PRBC/ aliquot (ml)†	Dilution Factor‡	# of Rickettsiae/ml PRBC§
1 (MNC)	3.4×10^6	49	50	67,000
1 (L-cell)	9.0×10^5	29	30	30,000
2 (MNC)	1.7×10^6	47	49	35,000
3 (MNC)	1.3×10^6	47	49	27,000

* Estimated number of rickettsiae per ml inoculum derived in Table 4. Only 1 ml of each inoculum was added to each aliquot of PRBC.

† Determined by weight then converted to ml by dividing by the approximate specific gravity of PRBC (1.060).

‡ The dilution factor was used to calculate the number of rickettsiae/ml PRBC after the addition of 1 ml of inoculum into the volume of PRBC.

§ *# rickettsiae/ml inoculum ÷ dilution factor = # rickettsiae/ml PRBC.* Numbers were rounded to the nearest thousand.

Based on the results of the calculated number of rickettsiae per ml PRBC (Table 5), the number of rickettsiae per ml PRBC in the aliquots which received 10-fold serial dilutions (1:10, 1:100, 1:1000) of inoculum was estimated (Table 6). For unit #2, the number of rickettsiae per ml PRBC in each aliquot after dilution of the inoculum was 3,500, 350, and 35, respectively. For unit #3, the estimated numbers were 2,700, 270, and 27, respectively.

Table 6. Estimated number of rickettsiae per ml of PRBC after serial dilution of inoculum*

Aliquot #	Dilution	Unit 1 # rickettsiae/ml	Unit 2 # rickettsiae/ml	Unit 3 # rickettsiae/ml
1	undiluted	67,000	35,000	27,000
2	1:10	NT†	3,500	2,700
3	1:100	NT	350	270
4	1:1000	NT	35	27

* For each unit, the initially prepared infected autologous mononulcear cell inoculum was 10-fold serially diluted (1:10, 1:100, 1:1000). Each dilution was returned to one of the three remaining aliquots of PRBC (#2-#4). The estimated number of rickettsiae/ml in the aliquots which received diluted inoculum was determined by dividing the number of rickettsiae/ml PRBC (aliquot #1) by the inverse of the dilution for each unit. Only aliquots from units #2 and #3 received diluted inoculum, unit #1 received L929 cells instead.

† NT = not tested; mouse L-929 cells were used in place of infected mononuclear cell dilutions.

Mouse Inoculations

Mononuclear cells infected with *O. tsutsugamushi* demonstrated prolonged infectivity after refrigerated and frozen storage in PRBC (Table 7). Infection was confirmed by DFA staining of impression smears from the intraperitoneal cavity (Figure 8), and PCR on blood collected from the retro-orbital sinus at the first sign of illness (Figure 9). Infection was evident in mice inoculated with PRBC's from all three aliquots which received undiluted inoculum and both aliquots which received a 1:10 dilution of infected inoculum after 24 h and 10 days of refrigerated storage. Mice became ill on day 8-10 after inoculation as evidenced by ruffled fur, ascites, and diminished activity. All mice from these inoculations died by day 17.

Both PRBC aliquots which received a 1:100 dilution of inoculum caused infection in mice after 24 h refrigerated storage (Table 7). Mice inoculated with blood from unit #2, aliquot #3 (Table 6), became sick on day 12 and died by day 16. One mouse inoculated

Table 7. Infectivity data: Mouse inoculation results (MNC).*

Sample†	PRBC Aliquot ‡	Sick	Death	PCR	ELISA	DFA
24 h	Undiluted	3	3	3	nd¶	3
	1:10	2	2	2	nd	nd
	1:100	2	2	2	0	nd
	1:1000	1	1	0	0	nd
10 d	Undiluted	3	3	3	nd	3
	1:10	2	2	2	nd	nd
	1:100	1	1	0	1§	nd
	1:1000	1	1	0	0	nd
30 d	Undiluted	0	0	0	1‖	nd
	1:10	0	0	0	0	nd
	1:100	0	0	0	0	nd
	1:1000	0	0	0	0	nd
45 d (DG)**	Undiluted	3	3	3	nd	3
	1:10	2	2	1	1§	nd
	1:100	0	0	0	0	nd
	1:1000	0	0	0	0	nd

* Data are presented as the number of aliquots giving a positive result (n=3 for the undiluted aliquots; n=2 for each of the dilutions). A positive result was illness in mice, lethality, amplification of DNA by PCR, detection by direct fluorescent antibody staining, and/or presence of antibody by ELISA.

† Storage time after rickettsiae were seeded into human PRBC

‡ Dilutions are of infected cells inoculated into PRBC prior to storage

§ One mouse inoculated with PRBC from unit #2 became sick and later recovered

‖ Mice inoculated with PRBC from unit #1 revealed no visible signs of infection, but did develop antibody

¶ nd = not determined

** DG = deglycerolized

Figure 8. Direct fluorescent antibody stained intraperitoneal cavity impression smear. Shows both intracellular and extracellular organisms consistent with *O. tsutsugamushi.* (Original magnificaton was 400x)

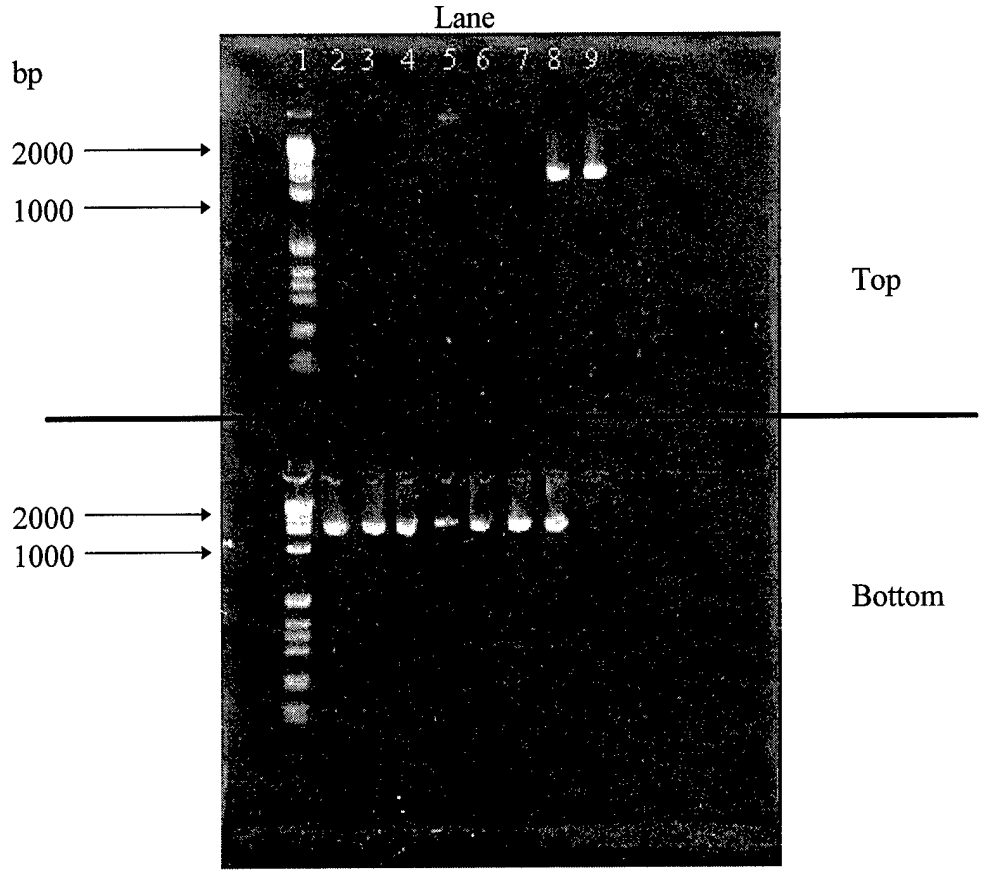

Figure 9. PCR results. The amplified region of the *O. tsutsugamushi* Sta58 major antigen protein used in this experiment is 1412 base pairs (bp). The top half of gel is PCR amplified DNA from *Escherichia coli*, *Mycoplasma pneumoniae*, and various Rickettsiales. This served as a specificity control for the PCR. (**Lane 1**) 1-kilobase (kb) DNA marker ladder. (**Lane 2**) *E. coli*; (**lane 3**) *Bartonella quintana*; (**lane 4**) *R. rickettsii*; (**lane 5**) *R. typhi*; (**lane 6**) *Ehrlichia risticii*; (**lane 7**) *Mycoplasma pneumoniae*; (**lane 8**) *O. tsutsugamushi,* Kato; (**lane 9**) *O. tsutsugamushi*, Gilliam. The bottom half of the gel contains extracted mouse retro-orbital blood samples after inoculation with PRBC aliquots at the first sign of illness. (**Lane 1**) 1-kilobase (kb) DNA marker ladder. (**Lane 2**) Control, *O. tsutsugamushi*, Karp; (**lane 3**) mouse, 24 h undiluted; (**lane 4**) mouse, 10 day undiluted ; (**lane 5**) mouse, 24 hr 1:10 dilution; (**lane 6**) mouse, 10 day 1:10 dilution; (**lane 7**) mouse, 24 hr 1:100 dilution; (**lane 8**) mouse, 45 day deglycerolized undiluted; (**lane 9**) uninfected mouse blood control. Lanes 8, 9 (top), and 2 (bottom) show amplification of *O. tsutsugamushi* DNA from strains Kato, Gilliam, and Karp, respectively. Lanes 3-8 on the bottom show amplification of *O. tsutsugamushi* DNA in the blood collected from the infected mice.

blood from unit #3, aliquot #3, became sick on day 19 and died on day 20, the remaining mouse had no signs of infection and no antibody was detected as determined by ELISA. This was still considered a positive result for unit #3, aliquot #3. At 10 days after refrigerated storage, only blood from unit #2, aliquot #3 caused illness in mice. Mice from this inoculation became sick on day 17. One died on day 18, the other recovered and produced antibody to *O. tsutsugamushi* as determined by a positive ELISA. The aliquot with approximately 270 rickettsiae/ml (unit #3, aliquot #3; Table 6) failed to cause infection.

Of the mice receiving blood from the aliquots which received a 1:1,000 dilution of inoculum, only blood from unit #2, aliquot #4 (Table 6) caused infection in mice at 24 h and 10 days after storage. Mice from the 24 h inoculation became sick on day 12 and died by day 21. One mouse from the 10 day inoculation became sick on day 14 and died on day 21. The remaining mouse from this inoculation displayed no signs of infection and developed no antibody as determined by ELISA. The aliquots which originated from unit #3, aliquot #4 did not cause illness in mice at this dilution and none produced antibody to *O. tsutsugamushi.*

For all aliquots tested, mice which were inoculated with PRBC following 30 days of refrigerated storage did not develop visible signs of infection. However, mice inoculated with PRBC from unit #1, aliquot #1 (approximated 67,000 rickettsiae per ml PRBC) did develop antibodies to *O. tsutsugamushi* as determined by ELISA.

Infection was also observed in mice inoculated with the deglycerolized blood 45 days after frozen storage for all three aliquots which received undiluted inoculum and both

that received the 1:10 dilution of the mononuclear cell inoculum. Mice from these inoculations became sick on day 12 after inoculation and died by day 19. Those PRBC aliquots which received the 1:100 and 1:1000 dilution of inoculum caused no illness in mice and no antibody was produced.

L-cells used in the preliminary study also demonstrated prolonged infectivity following refrigerated storage in PRBC (Table 8). Infection was evident in mice inoculated with infected L-cell seeded PRBC after 24 h and 16 days of refrigerated storage. Mice from the 24 h inoculation became sick on day 15, and one died on day 16. The other recovered and produced antibody to *O. tsutsugamushi* as determined by a positive ELISA. No mice from the day 16 inoculation became sick; however, one did produce antibody to *O. tsutsugamushi* as determined by a positive ELISA. Mice inoculated with 45 day deglycerolized red cells developed signs of illness on day 11 and died on day 16.

Table 8. Infectivity data: Mouse inoculation results (L-cell).

Sample*	Sick	Death	PCR	ELISA	DFA
24 h	1‡	1	1	1§	1
16 d	0	0	0	1‖	0
45 d †	1	1	1	nd	1

* Storage time after rickettsiae infected L-cells seeded into human PRBC.
† Time after frozen glycerolized storage
‡ Aliquot giving a positive result (n=1). A positive result was illness in mice, lethality, amplification of DNA by PCR, detection by direct fluorescent antibody staining, and/or presence of antibody by ELISA.
§ One mouse was sick, but recovered from infection
‖ No signs of illness, but antibody produced in mice
nd = not determined

Evaluation of infected inoculum by flow cytometry

As indicated by the complete overlapping of the uninfected cells and infected cells stained with FITC labeled anti-Karp IgG (Figure 10, panel A), *O. tsutsugamushi* infection of human mononuclear cells was not detected by flow cytometry. An attempt was made to enhance the fluorescence of infected cells by selecting only the monocytic region of the scan with the LYSIS II™ software (Figure 10, panel B). Even with this, no difference was detected. As a control, uninfected cells stained with FITC-labeled normal rabbit IgG were overlayed with uninfected cells stained with FITC-labeled anti-Karp IgG. This demonstrated that the human mononuclear cells had a slightly greater non-specific uptake of the FITC-labeled anti-Karp IgG, as indicated by the right-shift in the peak for the anti-Karp stained cells (Figure 10, panels C and D). For this reason, uninfected human mononuclear cells stained with FITC-labeled anti-Karp IgG were used to evaluate the infected cells.

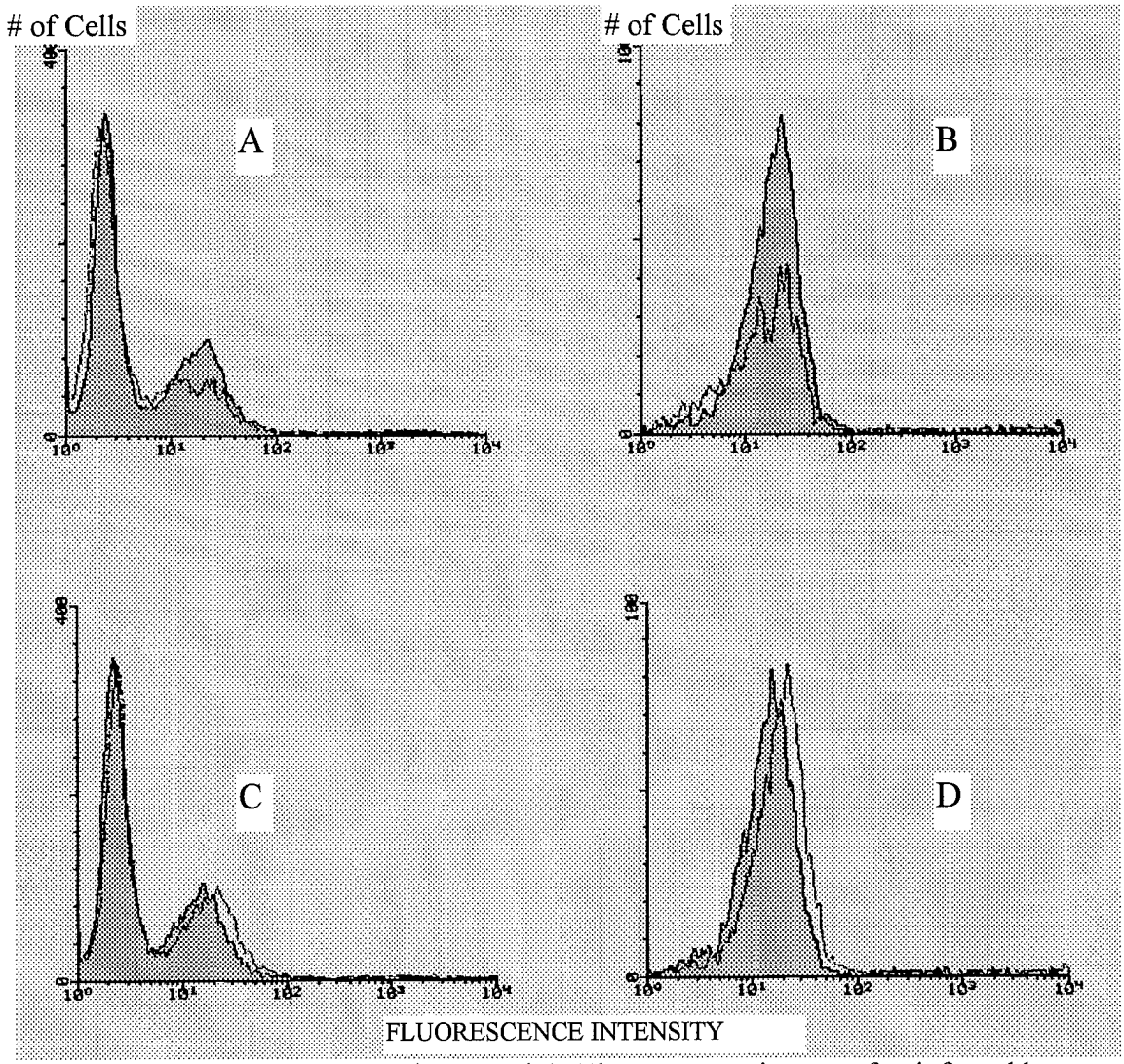

Figure 10. Flow cytometry results. Panel A: Flow cytometric scan of uninfected human mononuclear cells (shaded area), and cells 96 h post infection with *O. tsutsugamushi*, Karp strain, stained with fluorescein isothiocyanate (FITC)-labeled anti-Karp IgG. Panel B: Same as panel A with only the monocyte region selected. Panel C: Flow cytometric scan of uninfected mononuclear cells stained with FITC-labeled normal rabbit IgG (shaded area) and FITC-labeled anti-Karp IgG. Panel D: Same as panel C with only the monocyte region selected. As a control, uninfected cells stained with FITC-labeled normal rabbit IgG (shaded area) were overlayed with infected cells stained with FITC-labeled anti-Karp IgG (panels C and D). Uninfected cells had a slightly greater non-specific uptake of FITC-labeled anti-Karp IgG as indicated by the slight shift in the peaks. For this reason, infected cells stained with FITC-labeled anti-Karp IgG were compared to uninfected cells stained with FITC-labeled anti-Karp IgG. Infection of mononuclear cells was not detected by flow cytometry as indicated by the complete overlapping of the uninfected cells (shaded area) and infected cells stained with FITC-labeled anti-Karp IgG (panels A and B).

Restriction enzyme analysis

Restriction enzyme analysis of the positive PCR products generated restriction enzyme-cleaved fragments of identical molecular sizes to a known *O. tsutsugamushi*, Karp strain for all but one of the PCR products tested (Figure 11); thus, confirming the similarity of the PCR products. Two restriction enzymes were used (Hinf I, and Nla IV), but only the results of Hinf I are displayed. The PCR product from the mouse inoculated with PRBC containing a 1:100 dilution of inoculum did not show the expected bands when digested with Hinf I. Nla IV produced faintly staining bands of similar size for all PCR products digested.

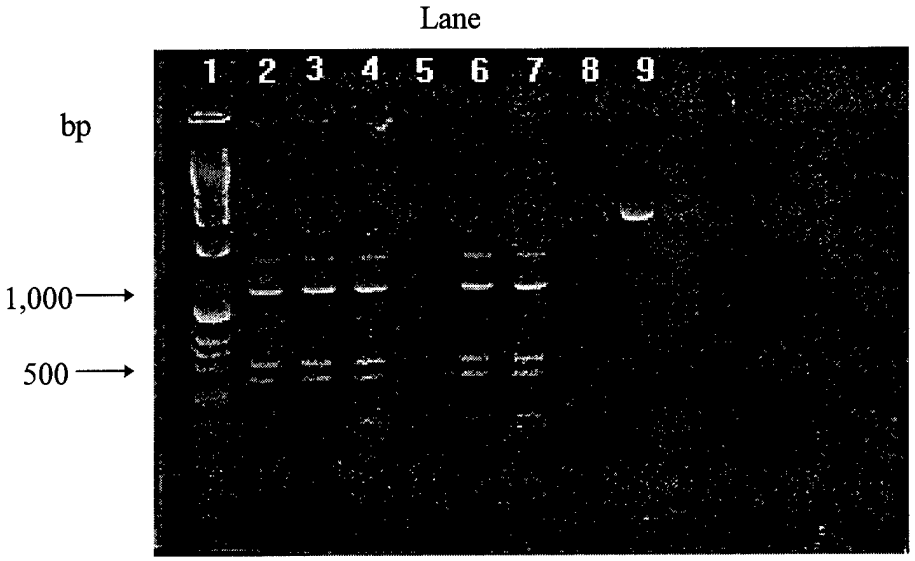

Figure 11. Restriction enzyme results. The 1412 bp region of the *O. tsutsugamushi* Sta58 major protein antigen amplified by PCR was digested with the Hinf I restriction enzyme. (**Lane 1**) 1-kilobase (kb) DNA marker ladder. (**Lane 2**) Digested PCR product from *O. tsutsugamushi* Karp strain; (**Lane 3**) mouse with 24h undiluted inoculum; (**Lane 4**) mouse with 24h 1:10 dilution. (**Lane 5**) mouse with 24h 1:100 dilution; (**Lane 6**) mouse with 10d undiluted inoculation; (**Lane 7**) mouse with 45d deglycerolized undiluted inoculum; (**Lane 8**) restriction enzyme control, no template added; (**Lane 9**) Karp strain, undigested. Fragments of identical molecular sizes to known *O. tsutsugamushi* (**Lane 2**) for all but one PCR product tested were generated confirming similarity of the PCR products.

DISCUSSION

This study was designed to examine whether *O. tsutsugamushi* could retain viability in blood products stored in accordance with standard blood banking practices. The findings demonstrate that *O. tsutsugamushi* can survive the normal blood processing procedures employed in the United States. This is the first time that data have been presented on the survival of *O. tsutsugamushi* in blood processed for transfusion. Survival of *O. tsutsugamushi*, and potentially other members of the family Rickettsiaceae, in stored blood products may justify an increased awareness in geographical regions where organisms and vectors are endemic. This study presents results from the investigation of *O. tsutsugamushi*, Karp strain, only one of many strains. Viability of *O. tsutsugamushi* in human blood during refrigerated and frozen storage may vary among strains, as well as, with other members of the family Rickettsieaceae.

An interesting, but expected observation in this experiment was the apparent decrease in infectivity of the thawed deglycerolized blood as compared to the refrigerated aliquots at 24h (i.e. only caused illness through the 1:10 dilution of inoculum, Table 7). The deglycerolization process is known to remove 95-99 percent of white blood cells (2 $\log_{10}$ reduction).[63,64] Since rickettsiae are obligate intracellular organisms, this would account for the reduction in infectivity in the deglycerolized PRBC aliquots. It has been shown that residual white blood cells in cellular blood components cause many complications in transfusion recipients, including, but not limited to, febrile nonhemolytic transfusion reactions, alloimmunization, and transmission of cytomegalovirus (CMV). For this reason, there is currently a push for pre-storage leukodepeletion of blood products in the blood

banking community. Using the model described in this study, it would be relatively easy to assess filtration as a means to reduce the potential infectivity of rickettsiae in stored blood products by removal of leukocytes.

Kelly et al. were able to detect the presence of *O. tsutsugamushi* infected L-929 cells by flow cytometric analysis.[41] In that study, the best correlation between flow cytometry and cell counts was achieved when the intensity of infection was high (i.e., 40% of cells infected with $\geq$ 50 rickettsiae) and the percent infected exceeded 83%.[41] In this study, infection of human mononuclear cells was assessed. As indicated, no difference was detected between infected and un-infected human mononuclear cells. This was attributed, in part, to the fact that no cells were maximally infected and that on average only 11 percent of the total cells were infected. The majority of cells were lymphocytes which were not infected. To overcome this problem, monocytes (the predominantly infected cell in this experiment) were electronically isolated through software gating after the cellular data were collected, but no difference was evident. Future studies should be conducted with collection gates set on the monocyte region. In this study, a total of 5,000 cells were counted and analyzed. Since the majority of the cells were lymphocytes, recovery of infected cells was low. Using collection gates, it would be possible to count more monocytes. Thus, focusing on the monocyte region could potentially demonstrate infected cells versus un-infected cells using flow cytometry. Another approach would be to attempt to initially collect and infect only monocytes rather than lymphocytes and monocytes as was the case with the separation technique used in this study.

Shirai et al., demonstrated that individuals become rickettsemic approximately 4-7 days post chigger attachment and 1-3 days prior to becoming symptomatic using mice and sampling at 3 day intervals. They were able to show a level of 15-50 rickettsiae per ml whole blood using mouse titration studies prior to the development of clinical signs and symptoms.[38] Rickettsemia at that level would correspond to 6,750 to 22,500 rickettsiae in a unit of donated PRBC representing a significant infectious load when one considers that a single organism is capable of causing disease and that blood recipients are often immunocompromised. In the present study, we approximated that transfusion load in the mice inoculations through dilution of the inoculum used to infect the PRBC aliquots and were able to demonstrate infectivity with as low as 35 rickettsiae per ml of PRBC. However, infectivity was erratic in the units of blood which were separated into aliquots and inoculated with dilutions of infected inoculum. Blood from one unit (unit #2) consistently caused infection at all dilutions, while blood from the other unit (unit #3) only caused infection through the 1:10 dilution. Part of this disparity was attributed to sampling and mixing error, but there was no way to assess the reason for the apparent lack of infectivity of unit #3 at dilutions of 1:100 and 1:1000. Based on the estimated numbers of rickettsiae per ml estimated for unit #2 (Table 6), unit #3 should have caused infection in mice at least through the 1:100 dilution. Another postulated explanation for the disparity between the infectivity of the two units was that since outbred mice were used and they were inoculated with human blood, there could have been a cellular response to remove the human cells. Thus, the rickettsiae could have been eliminated as innocent by-standers when low numbers of rickettsiae were present.

While most rickettsial diseases are currently under control, it seems unlikely that they will ever be eradicated because of the prevalence of their hosts, vectors, and their ability to exist transovarially within their vectors. In endemic tropical and subtropical areas, rickettsial diseases can lead to significant morbidity with more than 4,000 infections reported annually from 34 countries.[21] Scrub typhus was a significant medical problem in the Pacific Theatre during WWII and in the Vietnamese conflict.[31,32] Scrub typhus is readily diagnosed when patients in endemic regions present with the characteristic signs and symptoms.[65] Unfortunately, these signs are often not present, and individuals can be rickettsemic before symptoms are evident.[38] Epidemiological data indicate that *O. tsutsugamushi* is unlikely to pose a transfusion risk in this country. However, since it is highly endemic in Asia and the Southwest Pacific it may pose a significant risk to military personnel assigned to that region. The United States military has a significant presence throughout those regions with bases in Japan, Okinawa, and Korea. Additionally, training missions are frequently conducted in Malaysia, Thailand, Australia, Korea, and Japan. For the military, the potential threat becomes real in situations when blood is collected for emergency situations within endemic areas. Should a war of international scope occur or even the continuance of regional conflicts like operations in Bosnia, Somalia, and Liberia, the potential exists for rickettsial diseases to cause morbidity in non-immune residents and military troops as sanitary conditions worsen and normally stable ecosystems are disrupted.

This study focused on *O. tsutsugamushi*, but it does raise questions about the viability of other organisms from the family Rickettsiaceae under similar conditions. In the United States, cases of two emerging diseases, human monocytic ehrlichiosis (HME) and

human granulocytic ehrlichiosis (HGE) are on the rise.[66,67] More than 400 cases of HME and several dozen cases of HGE have been reported since 1986.[66] A study, in a golf oriented retirement community in Tennessee, suggested that 330 cases per 100,000 occur annually.[67] Illness ranges from subclinical to fatal with a nonspecific presentation of disease.[68] Many human infections with *E. chaffeensis* are entirely asymptomatic or have symptoms that may not prompt medical consultation, treatment or serologic testing.[69,70] Considering this, the possibility exists that patients infected with these agents may well be rickettsemic prior to presentation of symptoms. Studies should be conducted to assess this potential.

Many diseases are transmitted by arthropods and some may co-exist within vectors of certain geographical areas. For example, Magnarelli et al. demonstrated antibodies to *Borrelia burgdorferi*, *Babesia microti*, and *Ehrlichia chaffeensis* in ten patients in Connecticut.[71] Patients diagnosed with babesiosis are permanently deferred from blood donation, but no test is currently conducted for the detection of this or any other arthropod borne disease in blood donors. A study in New York suggested that the majority of infections with *B. microti* are asymptomatic and that 23% of patients with babesiosis had concurrent Lyme disease.[72] Since babesiosis is a permanent deferral from blood donation, has been transfusion transmitted, and is endemic in the North Eastern United States, Boustani et al. have suggested that in areas where babesiosis is endemic, blood donors who have had a febrile illness within 2 months of donation (between May and September) or donors with a recent history of a tick bite should be temporarily deferred.[73] Such precautions for rickettsial disease do not seem necessary at this time, but additional studies

should be conducted to determine the prevalence of rickettsial disease in endemic geographic areas.

With the apparent increase in cases of ehrlichiosis in this country and the worldwide prevalence of other rickettsial disease, further studies on the risk of transfusion acquired rickettsial disease may be indicated. Serological testing of donors for rickettsial disease does not seem feasible at this time since testing may not differentiate active from past infections. Because the sensitivity of such tests is low in the early stages of illness, persons most likely to transmit the infection would be missed. At the present time, no confirmed cases of transfusion-associated rickettsial disease have been reported to the Centers for Disease Control. Until the possibility of transmission of rickettsial disease through transfusion is demonstrated by epidemiological studies, blood centers in endemic areas should consider taking simple precautions against this potential problem. Such precautions would include deferring potential blood donors with symptoms consistent with infection, and/or fever of unexplained origin within the previous two weeks if in an area where rickettsial diseases are endemic. Aside from exclusion of donors, it may be possible to prevent the transmission of rickettsial disease with leukoreduction of blood products. In this study, as previously discussed, leukoreduction by deglycerolization appeared to reduce the infectivity of PRBC aliquots inoculated with intracellular rickettsiae. Other studies have shown that removal of white blood cells from blood components by filtration prevents the transmission of CMV infection (transmitted by leukocytes).[74] With recent advances in leukocyte filters, greater than 99.9% of white blood cells can be removed from blood components. Since rickettsiae are obligate intracellular organisms, pre-storage filtration of

blood components may be a useful procedure to prevent transfusion transmitted rickettsial disease.

Over the years, the technology of storing and collecting blood products has improved. For the military, the ability to collect blood and products such as platelets closer to the front lines in a field setting increases the chance of spreading rickettsial and other novel diseases through blood product transfusion. Although this is by no means the norm, in some instances blood products are needed in emergency situations. Military commanders, medical providers, and medical personnel planning operations in areas where scrub typhus and other rickettsial disease are endemic must be aware of the threat these diseases pose and of the potential to transmit rickettsial disease through transfusion.[31]

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