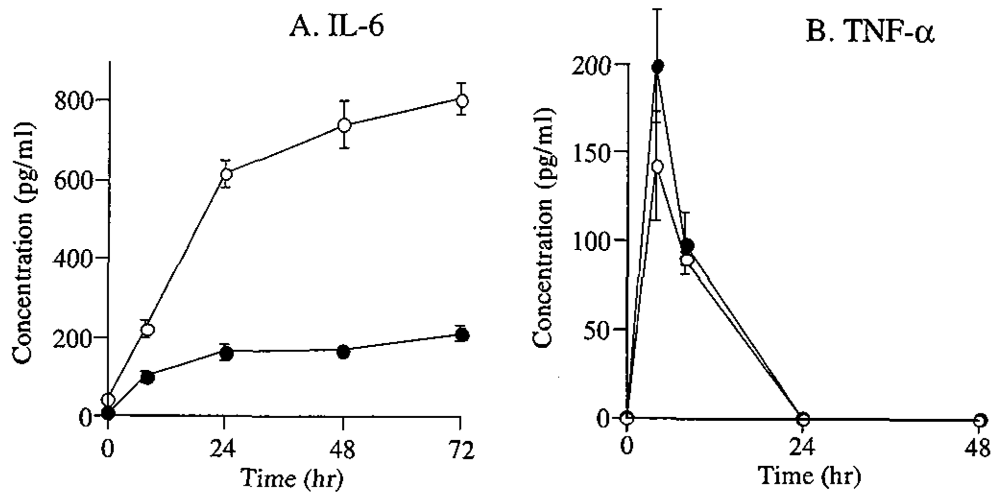

Fig. 5. Induction of high levels of IL-6 and absence of TNF-alpha induction by TNF-alpha. IL-6 (**A**) and TNF-alpha (**B**) concentrations in the supernatants of HTNV-infected (○) and uninfected (●) HUVEC were assayed at indicated hours after the addition of TNF-alpha (1 ng/ml). Time 0 indicates the concentrations before the addition of TNF-alpha. Each point represents the mean value of triplicate. Bars indicate SD

616 pg/ml. The concentration gradually increased further up to 807 pg/ml until 72 hr after the stimulation. On the other hand, in the uninfected cells, the concentration of IL-6 was slowly increased up to 163 pg/ml for the first 24 hr. During the next 48 hr, the concentration slightly increased and reached 210 pg/ml at 72 hr after the stimulation.

The HTNV infection of HUVEC itself did not induce TNF-alpha production (time 0 in Fig. 5B). The TNF-alpha concentration in the supernatants decreased rapidly after the addition of TNF-alpha, probably due to the degradation or absorbance to the cells, and completely disappeared at 24 hr after the addition. It was not detected thereafter until 96 hr (Fig. 5B and data not shown). At 4 hr after the stimulation, the concentration of TNF-alpha was marginally higher in the uninfected cell supernatant; however, the difference completely disappeared at 8 hr. Thus, the TNF-alpha stimulation did not induce significant level of TNF-alpha in either the HTNV-infected or uninfected HUVECs. IL-1beta was not produced above the detectable levels (10 pg/ml) by the TNF-alpha stimulation either in the HTNV-infected or uninfected HUVECs (data not shown).

We then investigated if the increased level of IL-6 was involved in the extension of hyper-permeability in HTNV-infected monolayers by adding the neutralizing antibody to IL-6 at the time of TNF-alpha stimulation. The antibody at concentrations of 50 or 500 ng/ml did not affect the time courses of hyper-permeability in either the HTNV-infected or uninfected monolayers (data not shown). Further, the uninfected monolayers treated with the mixtures of TNF-alpha and IL-6 did not show prolonged hyper-permeability (data not shown). These results suggest that IL-6 is not involved in the prolonged response of the HTNV-infected monolayers to TNF-alpha.

Discussion

We demonstrated that HTNV infection of HUVEC extended the duration of increased permeability of monolayers induced by low levels of TNF-alpha, while the magnitude of initial increase in permeability was comparable to the uninfected cells. In fact, this phenomenon was observed when 1 ng/ml of TNF-alpha was added to the culture medium, which is 10 times higher concentration than that detected in HFRS patients' sera [22]. This concentration is, at the same time, comparable to the reported concentration secreted by SNV-infected macrophages *in vitro* [17]. This is the first report demonstrating that the infection of hantavirus to endothelial cell monolayers modifies their barrier function. We speculate that the prolonged hyper-permeability of HTNV-infected endothelium induced by the TNF-alpha secreted from the infected monocytic cells may result in a gradual accumulation of hyper-permeable vascular endothelium (Fig. 6). In this hypothetical model, we assume the number of infected monocytic cells and the overall

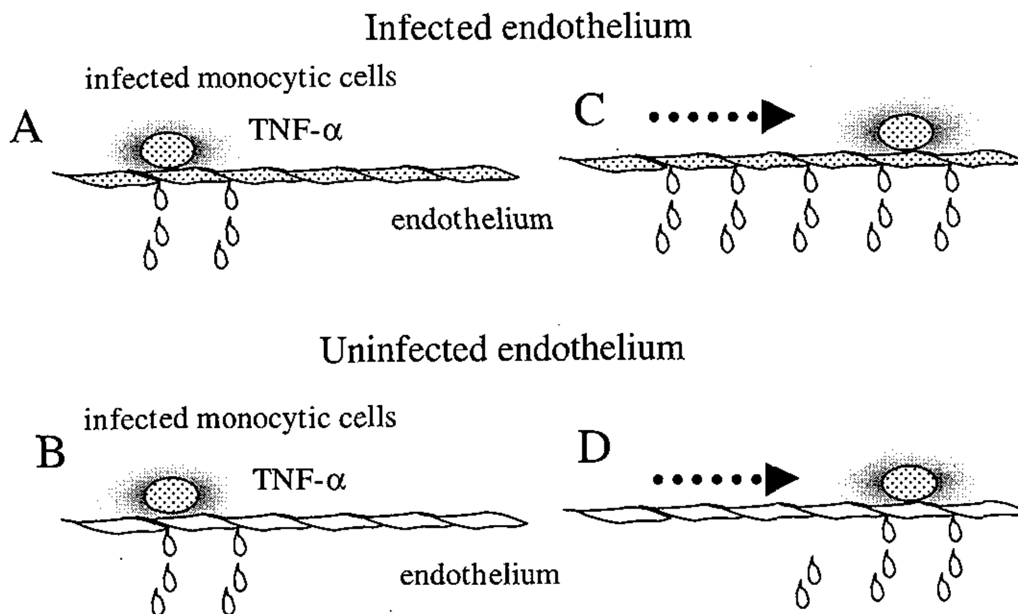

Fig. 6. A hypothetical scheme how the combination of the prolonged hyper-permeability of infected endothelium and the low-level TNF-alpha secretion from infected monocytic cells may contribute to a serious plasma leakage. In both infected and uninfected endothelium, the low-level TNF-alpha from the infected monocytic cells induces hyper-permeability only to the close proximity (**A** and **B**). When the infected monocytic cells move, plasma discharge continues from the infected endothelium which is no longer close proximity to the infected monocytic cells, as well as the leakage from the endothelium now becomes close proximity to them (**C**). Contrary, in the uninfected endothelium, only the endothelium newly becomes close proximity to the infected monocytic cells begins to leak, while the previously hyper-permeable endothelium recovers when the concentration of TNF-alpha is not high enough any more (**D**)

amount of secreted TNF-alpha from these cells are not great enough to induce systemic catastrophic change. On the other hand, the secretion of TNF-alpha from individual infected monocytic cell is high enough to induce prolonged hyper-permeability in the proximal infected endothelium. This might contribute to a local catastrophic plasma leakage after a certain period of time in specific organs where the infected endothelial cells and monocytic cells are close proximity. It should be noted that although HFRS is an acute disease, it takes order of weeks after infection before the critical condition appears [19]. A recent report showed that HTNV infection also induced dendritic cells to produce a low level of TNF-alpha [39]. Thus, the HTNV-infected monocytic cells can be a source of TNF-alpha *in vivo*, though TNF-alpha was supplied exogenously in our *in vitro* experiments. Of further interest, increased numbers of TNF-alpha producing cells in HPS patients' lungs were described and the involvement of local cytokine production in the HPS pathogenesis was suggested [32]. Furthermore, in dengue virus infection, which also causes hemorrhagic fever without serious damage on vascular endothelium, it was reported that TNF-alpha from the dengue virus-infected peripheral monocytes modulated endothelial cell protein expressions [1]. The mechanisms behind the hyper-permeability without disruption of the tight junction are not clear. One report suggested that the rearrangement of cytoskeleton by TNF-alpha changed the tension within the individual cells and resulted in hyper-permeability in endothelial monolayers without gap formation [2].

In HFRS and HPS patients' plasma, increased levels of NO were reported [9, 24]. In the former, the increased NO levels correlated to the TNF-alpha levels. However, our results suggested that NO was not involved in the prolonged hyper-permeability. The irrelevance of NO to the formation of pulmonary edema and alveolar flooding in a mice model system with lymphocytic choriomeningitis virus, which might mimic HPS, was recently reported [9]. The involvement of PG in the prolonged hyper-permeability was also not likely, while Cox-2 was one of the up-regulated genes by the HTNV infection in endothelial cells detected by the DNA array experiments [13].

The low level of TNF-alpha induced IL-6 above 700 pg/ml in the HTNV-infected HUVECs. This phenomenon might partly contribute to the elevated levels of IL-6 in HTNV-infected patients. It is noteworthy that a previous report showed no difference in IL-6 levels between the HTNV-infected and uninfected endothelial cells *in vitro*, as the pre-stimulation levels in our experiments [36]. The involvement of induced IL-6 in the pathogenesis of HTNV infection is not clear, though our data indicates that it is not involved in the prolonged hyper-permeability. In one report, IL-6 alone could induce hyper-permeability at higher concentrations above 20 ng/ml using normal bovine endothelial cell monolayers derived from carotid aorta [29]. In our study, HUVEC monolayers did not respond to IL-6 alone at concentrations up to 100 ng/ml (data not shown). Since endothelial cells derived from different organs or species may show different property [2, 26], it is not known whether this is due to the difference of source organs of the endothelial cells or to the species difference.

Difference in the sensitivity to cytokine stimulations in different species may even play a role in the pathogenesis of hantavirus infections. Hantaviruses are pathogenic to humans but not to the natural hosts, rodents, except for suckling animals [30, 34, 38, 44]. It is, therefore, tempting to postulate that the high sensitivity of human to pro-inflammatory cytokines [10] may be one of the possible explanations for the different pathogenesis between human and other animals, assuming that the combination of the low level of TNF-alpha and the endothelial cell infection plays a major role in the pathogenesis of human hantavirus infections.

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Short Communication

Possible Horizontal Transmission of Crimean-Congo Hemorrhagic Fever Virus from a Mother to Her Child

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SUMMARY: The case of a child with Crimean-Congo hemorrhagic fever (CCHF) presumably infected with CCHF virus from her 27-year-old mother is described. The mother with CCHF was treated with ribavirin and did not present with any symptoms of obvious hemorrhage. The child developed fever on the 5th day after the mother's onset. The partial virus genome was amplified by RT-PCR, and nested PCR from the child and the genome sequence were identical to that from the mother, indicating possible transmission of the virus from mother to child. This case indicates the importance of preventive measures for in-house outbreaks of CCHF.

Crimean-Congo hemorrhagic fever (CCHF) virus (CCHFV), a tick-borne virus distributed across Africa, Eastern Europe, the Middle East, and Asia, causes illness in humans and has a high fatality rate of up to 30% (1). Humans are usually infected with the virus through the bite of a tick (genus, *Hyalomma*) or by close contact with freshly slaughtered meat, or blood from viremic animals such as sheep, cattle, and goats (1). CCHF outbreaks have also occurred as nosocomial infections in several instances (2-5). In a review article by Hoogstraal (6), several cases of human-to-human infection of CCHF in households were described, indicating the importance of this infection route in CCHF outbreaks. However, the impact of human-to-human transmission of CCHFV in a household has not been studied with virological analysis, although in-house outbreaks of CCHF are considered to be relatively frequent, beyond expectations.

A 27-year-old female, who lived in a village in the Western part of the Xinjiang Uygur Autonomous Region, P. R. China, presented with fever, backache, headache, flushed face and general malaise without obvious hemorrhagic symptoms, and was transferred to a local hospital. She was diagnosed as having CCHF based on the epidemiology of CCHF in the area, and was hospitalized and treated with a 0.8 g/dose of ribavirin by drip infusion, twice daily for 7 days. Five days after the onset of the symptoms, her 4-year-old daughter also presented with high fever. She was clinically diagnosed as having CCHF, and was treated with intravenous administration of a 0.4 g/dose of ribavirin through drip infusion, twice a day for 7 days. No other proximate households showed any symptoms such as fever, arthralgia, and bleeding around that time. Both these patients lived in close contact with ticks, though neither recalled being bitten by one. Neither patient developed hemorrhagic manifestations. They recovered without any consequences.

Taking the day on which the fever first appeared as day 1, blood specimens were collected on days 3 and 11 from the mother and on days 3 and 8 from the daughter (Table 1). Serum samples were carefully separated under strict precautions, wearing a mask, protective glasses, double gloves, and a gown. RNA was extracted from serum samples using a High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. The reverse-transcription polymerase chain reaction (RT-PCR) and nested PCR was performed for amplification of a portion of the S-RNA segment according to the previous report (7) with some modifications (8). Serum samples were heat-inactivated at 56°C for 1 h for serological assays.

CCHFV immunoglobulin G (IgG) antibodies were detected by recombinant CCHFV nucleoprotein (CCHFV rNP)-based IgG enzyme-linked immunosorbent assay (ELISA) as described previously (9). CCHFV IgM antibodies were also detected by IgM-capture ELISA format using purified CCHFV rNP as an antigen (8). The cutoff optical density values for both ELISA tests were set at 0.200 (8,9).

The CCHFV genome was successfully amplified from the samples taken from the mother on days 3 and 11 and from the daughter on day 3 (Table 1). The daughter's serum collected on day 8 showed a positive reaction in the IgM-capture ELISA. The serum sample collected from the mother on day 11 also showed a positive reaction in the IgM-capture ELISA. On the other hand, a significant IgG response was demonstrated in the daughter but not in the mother (Table 1). The 262-base viral genome fragments, which were amplified in the sera collected from the mother and the child, respectively, were sequenced using ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). The nucleotide sequences of these viral genomes were the same (Accession No. AB102852 and AB102853 in DNA Data Bank of Japan). Furthermore, the sequence was confirmed to be identical to

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Table 1. Results of RT-PCR, IgG ELISA, and IgM-capture ELISA

Virological tests	from	Serum samples collected			
		Mother		Daughter	
	on day	3	11	3	8
RT-PCR		+[1]	+	+	–[1]
IgG ELISA (OD_{405})		–(0.071[2])	–(0.059)	–(0.059)	+(0.310)
IgM-capture ELISA (OD_{405})		–(0.015[3])	+(0.216)	–(0.021)	+(1.433)

[1]: + and - indicate positive and negative results, respectively.
[2, 3]: The OD_{405} values in IgG ELISA and IgM-capture ELISA were measured at the dilution level of 1:400 and 1:100, respectively.

that of CCHFV Chinese strain 66019 (Accession No. AJ101648 in National Center for Biotechnology Information). Although the data are not shown here, the CCHF outbreak in the region of residency in 2002 was caused by multiple strains of CCHFV. The partial viral genomes amplified from three patients, including the mother and her daughter, out of 6 patients from whom CCHFV genomes were amplified, were identical. This result strongly suggests that the pair were infected with the same strain of CCHFV from the same source or that the daughter was infected by her mother.

The incubation period of CCHF is 4-7 days (1). The interval between the onset of the mother and that of the child was 5 days. If both of them were infected with CCHFV simultaneously from the same source on the same occasion, the expected incubation time for the mother and her daughter would be 4-7 and 9-12 days, respectively. The expected incubation time of 9-12 days in the daughter is too long, suggesting that she was not infected with CCHFV at the same time her mother was. Therefore, it is quite likely that she was infected by her mother. However, we must not exclude the possibility that they were infected with CCHFV from the same source but on different occasions.

The mother did not show any symptoms of bleeding; therefore, if the daughter was infected by her mother, the daughter was infected through close contact with visually non-bloody bodily fluids secreted from the mother such as saliva, respiratory secretions, and/or urine. It is also possible that traces of blood were present in the mother's bodily fluids. This case of possible mother-to-child horizontal transmission of CCHFV indicates the importance of preventive measures in a household, even in cases without any hemorrhagic manifestations. In order to prevent in-house outbreaks, it must be emphasized that the education of the residents in endemic areas concerning modes of CCHFV transmission, risk of infection, and preventive measures is essential. In addition, rapid and accurate diagnosis of CCHF is also necessary.

The present study indicates the necessity of preventive measures against transmission of CCHFV to caregivers such as family members and hospital staff. It must be stressed that not only blood but also other bodily fluids should be regarded as possible sources of human-to-human transmission.

These patients were treated with an intravenous administration of ribavirin with favorable outcomes, as reported previously (8). The efficacy of ribavirin should be studied as a treatment of CCHF in the future.

In summary, we reported a pediatric case of CCHF, confirmed by virological studies, in which a child was possibly infected by her mother.

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Epidemiological and Serological Survey of Brucellosis in Mongolia by ELISA Using Sarcosine Extracts

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Abstract: **Brucellosis is an important zoonosis, and serological surveillance is essential to its control. However, cross-reactions of attenuated live cells of *Brucella abortus* strain S-19 and *B. melitensis* strain Rev-1 with *Yersinia enterocolitica* O9 or vaccinated animal sera interfere with accurate serological diagnosis by the Rose Bengal test (RBT). Therefore, we used ELISA with sarcosine extracts from the virulent *B. abortus* strain 544 to eliminate false-positives among RBT positive-sera. A total of 697 serum samples were collected in Mongolia from humans and animals in 23 nomadic herds. The herds were classified into three groups as brucellosis-endemic (BE), brucellosis-suspected (BS), or *Brucella*-vaccinated (BV). The number of 295 animals (43.0%) was positive by RBT, but 206 (69.8%) of these were positive according to ELISA; therefore, 30.2% of the RBT-positive sera were found to be false positives. The false positive samples for RTB represent 4.1%, 27.4%, and 68.2% of the animals from the BE, BS, and BV herds, respectively. In addition, 32% of RBT-positive human sera were also false positives. Thus, our ELISA would be more specific than RTB and useful for epidemiological surveillance for brucellosis.**

Key words: **Brucellosis, Domestic animal, ELISA, Seroepidemiology, Mongolia**

Introduction

Brucellosis infects domestic and wild animals worldwide, as well as humans who have contact with infected animals or contaminated dairy products (5, 11). To control and eradicate brucellosis in Mongolia, cattle and sheep/goats have been vaccinated with the *B. abortus* S-19 and *B. melitensis* Rev-1 strains, respectively (19). *Brucella*-infected animals are generally slaughtered following conventional serological tests such as the Rose Bengal (RBT), tube agglutination, and complement fixation tests using inactivated whole bacterial cells or bacterial lipopolysaccharide (LPS) antigens (9, 15, 17, 20). However, a strong cross-reaction between *Brucella* spp. and *Yersinia enterocolitica* O9 in these tests has seriously complicated the diagnosis of animal brucellosis (1, 2, 6, 12–14, 16) because *Brucella* spp. has common antigenic determinants with *Y. enterocolitica* O9 in the smooth LPS region (3, 6, 16, 21–23). Furthermore, it is difficult to discriminate between infected and vaccinated animals because both animals have high titers to smooth LPS of *Brucella* antibody (4, 7, 8, 18).

In Mongolia, the economic loss caused by *Brucella* infection has been one of the most serious and important problems in nomadic animal breeding, prompting the government to vaccinate on all domestic animals except for camels (Fig. 1). After vaccination, an epidemiological survey is essential for tracing the occurrence of brucellosis. However, large numbers of vaccinated and healthy domestic animals could be diagnosed as positive for brucellosis by these tests, because of the cross-reac-

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide.

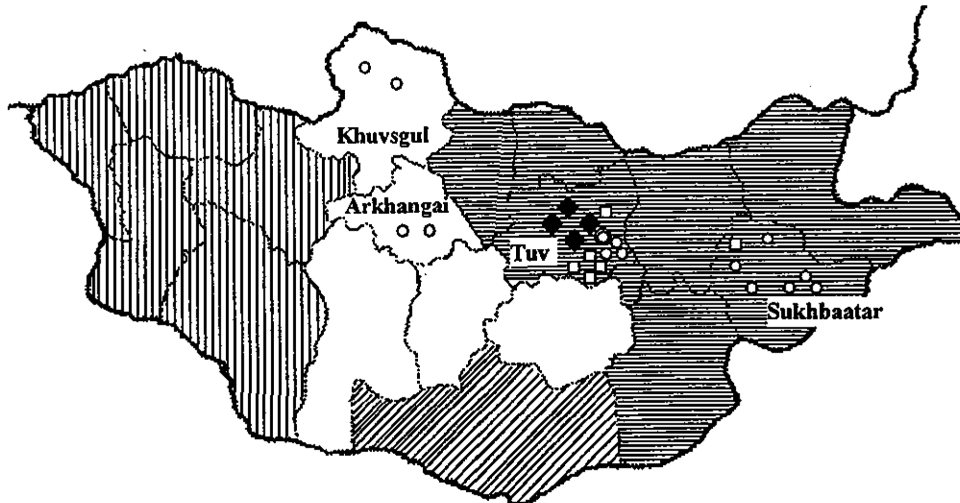

Fig. 1. Locations of herds used for serum sampling in Mongolia. A total of 23 herds was divided into three groups: ○, brucellosis-suspected herds; ◆, brucellosis-endemic herds; □, vaccinated herds. ◎, Ulaanbaatar. ▨, unvaccinated area. ▤, vaccinated in 2000. □, vaccinated in 2001. ▥, vaccinated in 2002.

tion with *Yersinia* or the high level of anti-smooth LPS of *Brucella* antibodies in vaccinated animals. Unfortunately, animals are culled if current serological tests show a false positive. Therefore, a simple diagnostic method to specifically detect authentic *Brucella*-infections must be established to avoid culling healthy animals.

Recently, we developed an enzyme-linked immunosorbent assay (ELISA) using a soluble antigen extracted from *B. abortus* 544 by *n*-lauroylsarcosine (sarcosine extracts) (10). The ELISA shows a highly specific reaction to authentic brucellosis and differentiates between vaccinated and *Y. enterocolitica* O9-infected animals. In this study, a serological survey of brucellosis was performed to evaluate the ELISA in nomadic animal husbandry in Mongolia.

Materials and Methods

Serum samples. A total of 697 serum samples were collected from 23 herds and 47 humans in 4 prefectures in Mongolia from July to September 2001. Of 47 human samples, 36 were obtained from healthy individuals who had been involved in domestic animal breeding in 4 different prefectures and 11 from the patients with brucellosis in a hospital in Mongolia. The animal samples consisted of 222 sheep, 219 cattle, 155 goats, 17 camels, 17 yaks, and 20 reindeer. The 23 herds were divided into three groups: brucellosis-endemic (BE), brucellosis-suspected (BS), and *Brucella*-vaccinated (BV) (Fig. 1 and Table 1). BE herds were identified on the basis of clinical evidence such as abortion, placenta retention, and previous results of serological tests. In the BS herds, clinical symptoms for brucellosis were not observed, but conventional serological tests and information from the local veterinary service suggested the likelihood of brucellosis. In the BE and BS herds, it was confirmed that animals examined were not vaccinated before the serological survey according to the information of the local veterinary service. In the BV herds, all the animals were vaccinated by vaccine strains of *B. abortus* S-19 or *B. melitensis* Rev-1 9 to 11 months before the samples were taken.

Preparation of soluble Brucella *antigen (sarcosine extracts).* The antigen for ELISA was prepared as previously described (10). Briefly, *B. abortus* 544 cells were cultured in *Brucella*-broth (Becton Dickinson, Sparks, Md., U.S.A.) to AD_{600}=3.0, collected by centrifugation at 3,500 rpm for 15 min at 4 C, and washed once with distilled water (DW). The culture was resuspended in the original volume of DW containing *n*-lauroylsarcosine at a final concentration of 0.5%. After incubation at room temperature for 30 min with gentle shaking, the solubilized suspension was filtered to remove bacterial residues. The filtrate was kept at −20 C and used directly for the ELISA.

Serological tests. The RBT was performed using internationally standardized *Brucella* whole-cell antigen (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) (17). The ELISA was performed as described previously (10). Briefly, 50 μl of sarcosine extracts (4 μg/ml) was coated onto a 96-well immuno plate (Nunc, Rochester, N.Y., U.S.A.) at 4 C for 20 hr. Each well was then blocked with PBS containing 0.5%

Table 1. Total number of tested samples

Animals	Prefectures from which samples were collected																	
	Arkhangai				Khuvsgul				Tuv				Sukhbaatar				Ulaanbaatar	Total
	T	BE	BS	BV	T	BE	BS	BV	T	BE	BS	BV	T	BE	BS	BV	BP	
Cattle	24	—	24	—	3	—	3	—	163	61	—	102	29	—	29	—	—	219
Sheep	40	—	40	—	8	—	8	—	117	58	37	22	57	—	50	7	—	222
Goat	36	—	36	—	5	—	5	—	63	8	40	15	51	—	47	4	—	155
Camel	—	—	—	—	—	—	—	—	—	—	—	—	17	—	17	—	—	17
Yak	13	—	13	—	4	—	4	—	—	—	—	—	—	—	—	—	—	17
Reindeer	—	—	—	—	20	—	20	—	—	—	—	—	—	—	—	—	—	20
Human	9	—	9	—	7	—	7	—	6	6	—	—	14	—	14	—	11	47
Total	122	—	122	—	47	—	47	—	349	133	77	139	168	—	157	11	11	697

T, Total number; BE, brucellosis-endemic herd; BS, brucellosis-suspected herd; BV, *Brucella*-vaccinated herd; BP, patients with brucellosis who had been under the treatment in the hospital.

Table 2. Number of serum samples showing positive results with the RBT and ELISA

Animals	No. of sera examined	No. (%) of RBT-positive sera	No. (%) of ELISA-positive among the RBT-positive
Cattle	219	118 (53.9)	85 (72.0)
Sheep	222	95 (42.8)	67 (70.5)
Goat	155	51 (32.9)	33 (64.7)
Camel	17	4 (23.5)	4 (100)
Yak	17	10 (58.8)	8 (80.0)
Reindeer	20	3 (15.0)	3 (100)
Human	47	25 (53.2)	17 (68)
Total	697	306 (43.9)	217 (70.9)

BSA at room temperature (RT) for 30 min. The test sera were diluted 200-fold with PBS and applied to the wells. After incubation at 37 C for 1 hr, the wells were washed three times with PBS containing 0.01% Tween-20 (PBS-T) for 5 min, then 1,000-fold diluted protein-G conjugated with horseradish peroxidase was added and the wells were incubated at 37 C for 1 hr. After washing five times with PBS-T for 5 min, the substrate O-phenylenediamine was added to the wells. The absorbance was measured at 492 nm using an ELISA reader model 450 (Bio-Rad, Hercules, Calif., U.S.A.). When sera of OD_{492} was greater than 0.5, the samples were judged to be positive, as previously described (10).

Statistical analysis. Statistical analysis was done by Student's *t*-test.

Results

Screening by the Conventional Serological Tests

To determine whether the ELISA system is effective for epidemiological surveillance or not, all the sera were screened by the RBT and the ELISA was then performed using the RBT-positive sera. Of 697 serum samples examined, 306 (44.5%) were judged positive for brucellosis by the RBT (Table 2). These positive samples included 118 (53.9%) of 219 cattle, 10 (58.8%) of 17 yaks, 95 (42.8%) of 222 sheep, 51 (32.9%) of 155 goats, 4 (23.5%) of 17 camels, 3 (15.0%) of 20 reindeer, and 25 (53.2%) of 47 humans (Table 2). The ELISA results indicated that 217 (70.9%) of RBT-positive samples reflected authentic brucellosis (Table 2).

ELISAs of the BE, BS, and BV Herd Samples

The results of the RBT and ELISA in the BE herds are summarized in Table 3. Of 127 samples, including 61 cattle, 58 sheep, and 8 goats, 98 (77.2%) were positive according to RBT. Of the RBT-positive sera, 94 (95.9%) were also positive according to ELISA. These were diagnosed as true brucellosis. In contrast, 4 RBT-positive sera (4.1% of RBT-positive sera) from cattle and sheep were eliminated by the ELISA as false positives, suggesting that these animals might be exposed to *Y. enterocolitica* O9 or other Gram-negative bacteria that have a cross-reaction with *Brucella* spp.

The results of the RBT and ELISA in the BS herds are given in Table 4. Of 373 serum samples from 56 cattle, 135 sheep, 128 goats, 17 camels, 17 yaks, and 20 reindeer, 117 (31.4%) were positive according to RBT. Of these samples, 85 (72.6%) were positive according to ELISA and were diagnosed as true brucellosis. As a result, 32 RBT-positive sera (27.4% of RBT-positive

Table 3. Serological reactions in brucellosis-endemic (BE) herds

Animals	No. of sera examined	No. (%) of RBT-positive	No. (%) of ELISA-positive among the RBT- positive	No. (%) of animals suspected [a]
Cattle	61	52 (85.2)	50 (96.2)	2 (3.8)
Sheep	58	38 (65.5)	36 (94.7)	2 (5.3)
Goat	8	8 (100)	8 (100)	0 (0)
Total	127	98 (77.2)	94 (95.9)	4 (4.1)

[a] The percentage of animals eliminated from suspicion of brucellosis was determined as the percentage of sera that were RBT-positive and ELISA-negative.

Table 4. Serological reactions in brucellosis-suspected (BS) herds

Animals	No. of sera examined	No. (%) of RBT-positive sera	No. (%) of ELISA-positive among the RBT-positive	No. (%) of animals suspected[a]
Cattle	56	21 (37.5)	17 (80.9)	4 (19.1)
Sheep	135	45 (33.3)	29 (64.4)	16 (35.6)
Goat	128	34 (26.5)	24 (70.6)	10 (29.4)
Camel	17	4 (23.5)	4 (100)	0 (0)
Yak	17	10 (58.8)	8 (80.0)	2 (20.0)
Reindeer	20	3 (15.0)	3 (100)	0 (0)
Total	373	117 (31.4)	85 (72.6)	32 (27.4)

[a] The percentage of animals eliminated from suspicion of brucellosis was determined as the percentage of sera that were RBT-positive and ELISA-negative.

Table 5. Serological reactions in vaccinated animals by RBT and ELISA

Animals	No. of sera examined	No. (%) of RBT-positive sera	No. (%) of ELISA-positive among RBT-positive	No. (%) of animals suspected[a]
Sheep	29	12 (41.4)	2 (16.7)	10 (83.3)
Goat	19	9 (47.4)	1 (11.1)	8 (88.9)
Cattle	71	19 (26.8)	0 (0)	19 (100)
Cattle[b]	31	26 (83.9)	18 (69.2)	8 (30.8)
Total	150	66 (44.0)	21 (31.8)	45 (68.2)

[a] The percentage of animals eliminated from suspicion of brucellosis was determined as the percentage of sera that were RBT-positive and ELISA-negative.
[b] Thirty-one cattle were from one BS herd.

Table 6. Serological reactions of human samples

Place of sampling	No. of sera examined	No. (%) positive by	
		RBT	ELISA
Arkhangai	9	4	0
Khubsgul	7	3	2
Tuv	6	2	1
Sukhbaatar	14	5	3
Hospital	11	11	11
Total	47	25	17

sera) from cattle, sheep, goats, and yaks were eliminated by the ELISA as false positives. However, all RBT-positive camels and reindeer were confirmed as true brucellosis by the ELISA.

The results of the RBT and ELISA in the BV herds are summarized in Table 5. Of 150 sera from 102 cattle, 29 sheep, and 19 goats, 66 (44.0%) were declared positive by RBT. Of these, 21 (31.8%) were judged positive by ELISA and diagnosed as true brucellosis. Thus, 45 RBT-positive sera (68.2% of RBT-positive sera) from cattle, sheep, goats, and yaks were eliminated by ELISA as false positives, suggesting that the RBT might have detected antibodies raised against the vaccine strains or that these animals might have been exposed to *Y. enterocolitica* O9 or another Gram-negative bacterium. Of 48 serum samples from vaccinated sheep and goats in one BV herd (Table 5), 21 (43.8%) sera were positive according to RBT, but only 3 (14.3%) were positive according to ELISA, meaning that 85.7% of the RBT-positive animals did not carry brucellosis. Though RBT-positive cattle were found in 26 (83.9%) of 31 samples from BS herds, 18 (69.2%) of RBT-positive animals were judged positive by ELISA, suggesting that these ELISA-positive animals might have had brucellosis before vaccination.

The serological reactions of 47 human sera are summarized in Table 6. The results showed that 36 healthy humans (38.9%) were RBT-positive, but 8 (57.1%) of

these were eliminated as false positives by the ELISA. However, all 11 sera of patients, who had been under treatment for brucellosis, were judged positive by both RBT and ELISA.

Discussion

To control and eradicate animal brucellosis, more sensitive and specific serological tests are needed for the diagnosis. However, this also means that the results of false positives could be a complicating problem (24). In fact, the RBT is very sensitive for the diagnosis of brucellosis, but has a problem of cross-reaction with *Y. enterocolitica* O9-infected animals, *Brucella*-vaccinated animals, and animals infected with other Gram-negative bacteria (3, 17). Actually, *Y. enterocolitica* O9-infected animals, which were diagnosed by RBT to have brucellosis, were reported in France (14). Therefore, to confirm brucellosis, we previously proposed applying the ELISA system with sarcosine extracts from *B. abortus* 544 after screening by RBT; although 200-fold-diluted sera from vaccinated and *Y. enterocolitica* O9-infected animals reacted positively with the RBT, they did not exceed an OD_{492} of 0.5, but sera from animals with brucellosis had an OD_{492} of more than 0.5 in the ELISA (10). These findings might be explained by the low concentration of LPS in the sarcosine extracts (10).

In this study, we examined the practical usefulness of the ELISA with sarcosine extracts in Mongolian nomadic husbandry. We found 95.9% of RBT-positive sera from BE herds were also positive for brucellosis according to our ELISA. This is significantly higher than the 72.6% positivity we found in the BS herds ($P<0.001$) (Tables 3 and 4). In addition, both percentages were significantly greater than the positivity (31.8%) in the BV herds ($P<0.001$) (Tables 3, 4 and 5). Before the laboratory diagnosis of brucellosis, it was confirmed by the local veterinary service in Mongolia that none of the animals were vaccinated and typical clinical symptoms like abortion had occurred in the BE herds, that none of the animals were vaccinated but they showed no clinical symptoms in the BS herds, and that all the animals were vaccinated and showed no clinical symptoms in the BV herds. The results demonstrate that the RBT was highly sensitive as a screening test, but its specificity was much lower than that of the ELISA, and the ELISA could be used to identify the infected animals among the RBT-positive ones.

In Mongolia, a national mass vaccination has been carried out to control brucellosis of cattle, sheep, and goats starting in 2000 (Fig. 1). The vaccination program was completed in 10 prefectures in 2000 (Fig. 1). In addition, vaccination was performed in 10 other prefectures in 2001 and 2002 after our samples were taken in September 2001 (Fig. 1). Vaccinated animals had a high titer of antibodies against whole-cell *Brucella* antigens, mainly against the O-chain of the smooth polysaccharide (16–18, 21, 22), but our ELISA could clearly discriminate these animals from the infected ones (Table 5). In addition, the ELISA-positive ratio in BV herds was lower than in the BS and BE herds. Therefore, we believe that vaccinated animals have protective immunity against brucellosis, and the vaccination program in Mongolia is largely successful although not all animals were vaccinated because of their owners' nomadic life. Moreover, although of 31 cattle sera from one BS herd, 18 were positive for brucellosis by ELISA and the value was higher than in the other BV herds (Table 5). Since the vaccination status of these cattle was confirmed by the local veterinary service, they had been infected with *Brucella* before the vaccination. If this herd was eliminated from the results of Table 5, only 3 of 40 RBT-positive animals had brucellosis, meaning that the ELISA might be helpful in understanding the history of the infection in each animal and that the vaccination program was successful in reducing brucellosis. Therefore, it is important to monitor the antibody titer by ELISA after vaccination.

Although cattle, sheep, and goats are the domestic animals that are most frequently vaccinated against brucellosis, some breeding herds of yak, reindeer, and camel are also kept as livestock. We showed that 17 sera from these animals were RBT-positive and 15 (88.2%) of these were positive according to the ELISA for the *Brucella*-specific antibody. The reason for the high infection rate (88.2%) can probably be attributed to the fact that the animals had not been vaccinated, suggesting that vaccination of these animals is essential to control brucellosis in Mongolia.

Human brucellosis has increased recently in Mongolia, especially among certain occupational groups, including herdsmen, veterinarians, and animal product processing employees. These people also have a high risk of exposure to other Gram-negative bacteria, like *Y. enterocolitica* O9, *Francisella tularensis*, and *E. coli* O157, which are known to cross-react with antisera against *Brucella* (6, 9, 12–14, 16, 23). Since we had no systematic diagnostic methods for human brucellosis, the RBT had been used as a first screening in the clinical field, even though the RBT has false positives at a high rate. Fourteen (38.9%) of 36 human sera involved in domestic animal breeding in rural area were RBT-positive (Table 6) and were also positive in complement-fixation tests. The test had been used as the final diagnostic method for brucellosis in animals; therefore, these

14 individuals have been diagnosed as suffering from brucellosis (data not shown). However, we showed that only 6 (42.9%) of the RTB-positive sera were positive in our ELISA system, and 8 (53.1%) were negative (Table 6), meaning that these 8 sera were false positives. On the other hand, all 11 patients with clinical symptoms of brucellosis were confirmed as true brucellosis victims (Table 6). Therefore, we believe that our ELISA would be a useful tool for identifying brucellosis not only in animal but also in human cases.

In addition to the diagnostic effectiveness, our ELISA was convenient for practical applications, because the preparation of the antigen and the method are easier than those of complement fixation tests (20, 24). Therefore, our ELISA with sarcosine extracts would be useful as a confirmatory test for the control and eradication program for brucellosis in Mongolia in combination with the RBT.

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Lipid raft microdomains mediate class A scavenger receptor-dependent infection of *Brucella abortus*

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Abstract

Brucella abortus is a facultative intracellular bacterium that can survive inside macrophages. Intracellular replication of *B. abortus* requires the VirB complex, which is highly similar to the conjugative DNA transfer system. In this study, we showed that a class A scavenger receptor (SR-A) of macrophages is required to internalize *B. abortus* and contributes to the establishment of bacterial infection in mice. Macrophages from SR-A-deficient mice inhibited internalization and intracellular replication of both wild type strain and the *virB4* mutant, and that bacterial proliferation was inhibited in SR-A-deficient mice. Adding lipopolysaccharide from *B. abortus* and *Salmonella enterica* serovar Typhimurium, but not from *Escherichia coli*, to macrophages inhibited bacterial internalization. VirB-dependent bacterial internalization induced localization of SR-A into detergent-resistant membrane lipid rafts. These results indicate that *B. abortus* internalizes into macrophages by using SR-A as a receptor and that the VirB type IV secretion system of *B. abortus* regulates signal transduction dependent on SR-A to form replicative phagosomes, and which is mediated by lipid rafts.
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1. Introduction

Brucella spp. are Gram-negative bacteria that cause abortion and infertility in numerous domestic and wild mammals, and a disease known as undulant fever in humans [1]. The bacterium is endemic in many underdeveloped countries and is responsible for large economic losses and chronic infections in human beings [2]. *Brucella* species are facultative intracellular pathogens that survive in a variety of cells, including macrophages, and their virulence and chronic infections are thought to be due to their ability to avoid the killing mechanisms within macrophages [3,4]. The molecular mechanisms of their virulence and chronic infections are incompletely understood.

Recent studies with non-professional phagocyte HeLa cells have confirmed these observations, showing that *Brucella* inhibits phagosome–lysosome fusion and transits through an intracellular compartment that resembles autophagosomes. Bacteria replicates in a different compartment, containing protein markers normally associated with the endoplasmic reticulum, as shown by confocal microscopy and immunogold electron microscopy [5,6].

Brucella internalizes into macrophages by swimming on the cell surface with generalized membrane ruffling for several minutes, and then the bacteria are enclosed by macropinosomes [7]. In this period, the phagosomal membrane continues to be dynamic. Lipid raft-associated molecules, such as glycosylphosphatidylinositol (GPI)-anchored proteins, GM1 gangliosides and cholesterol, are selectively incorporated into macropinosomes containing

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Brucella abortus. In contrast, the late endosomal marker lysosomal-associated membrane protein (LAMP)-1 and host cell transmembrane proteins are excluded from macropinosomes. The disruption of lipid rafts on macrophages markedly inhibits VirB-dependent macropinocytosis and intracellular replication [7,8]. These results indicate that the entry route of *B. abortus* into macrophages decides the intracellular fate of the bacteria that are modulated by lipid rafts [7,8].

The operon coding for export mechanisms specializing in transferring a variety of multimolecular complexes across the bacterial membrane to the extracellular space or into other cells has been described [9]. These complexes, named type IV secretion systems, are also found in *B. abortus* (*virB* genes) [10–12]. This operon comprises 13 open reading frames that share a homology with other bacterial type IV secretion systems participating in the intracellular trafficking of pathogens. Type IV secretion systems export three types of substrates: (i) DNA conjugation intermediates; (ii) the multisubunit pertussis toxin; (iii) monomeric proteins including primase, RecA, the *Agrobacterium tumefaciens* VirE2 and VirF proteins, and the *Helicobactor pylori* CagA protein [9]. However, the substrates of the VirB secretion system of *B. abortus* and the target of the effector in host cells remain undefined.

In this study, we investigated a receptor on the macrophage surface against *B. abortus* and showed that *B. abortus* internalized into mouse bone marrow-derived macrophages through a class A scavenger receptor (SR-A). Bacterial internalization dependent on SR-A does not associate with the type IV secretion system, and lipopolysaccharide (LPS) has a critical role in the interaction between *B. abortus* and SR-A. However, the type IV secretion system of *B. abortus* has an important role downstream of the signal transduction from SR-A, which is mediated by lipid rafts.

2. Results

2.1. SR-A promotes internalization and intracellular replication of Brucella

To investigate the role of SR-A in *Brucella* infection, several phenotypes of *B. abortus* virulence were tested by using bone marrow-derived macrophages from SR-A deficient mice [13]. The differences in the rate of phagocytosis for parent or SR-A-deficient mice were quantitated at various times of incubation. Macrophages from parent mice supported internalization of the wild type *B. abortus* strain, but macrophages from SR-A-deficient mice inhibited the internalization (Fig. 1A). Macrophages from SR-A-deficient mice also showed inhibition of internalization of the *virB4* mutant (Fig. 1B), suggesting that the type IV secretion system does not contribute to bacterial internalization dependent on SR-A. SR-A deficiency did not affect internalization of opsonized bacteria and polystyrene beads (Fig. 1C and D).

We next investigated if SR-A contributes to the intracellular replication of *B. abortus*. In the infection of the wild type *B. abortus* strain and complemented strain, macrophages from parent mice supported intracellular replication, but not macrophages from SR-A-deficient mice until 24 h after infection. The wild type *B. abortus* strain replicated slightly in macrophages from SR-A-deficient mice between 24 and 48 h after infection, but the intracellular growth rate was still lower than those macrophages from parent mice (Fig. 2A and C).

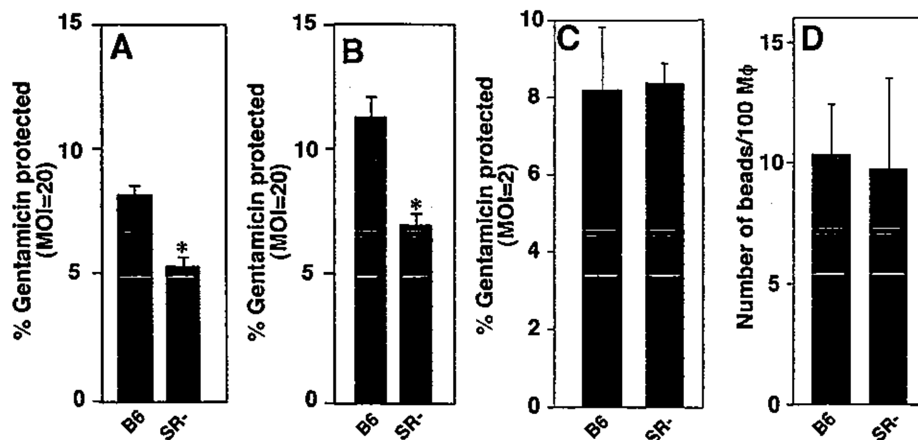

Fig. 1. Inhibition of *B. abortus* internalization into SR-A-deficient mouse macrophages. Wild type (A), *virB4* mutant (B), opsonized wild type (C) or polystyrene beads (D) were deposited onto macrophages from normal (B6) or SR-A-deficient mice (SR-), and then were incubated for 30 min. The efficiency of internalization into macrophages was measured by the protection of internalized bacteria from gentamicin killing. One hundred macrophages were examined per coverslips to determine FITC-labeled polystyrene beads by fluorescence microscopy. Data are the average of triplicate samples from three identical experiments, and error bars represent the standard deviation. The significance of the data were evaluated by Student's *t*-test. Statistically significant differences between the bacterial internalization into macrophages from normal mice and that from SR-A-deficient mice are indicated by asterisks (* $P < 0.01$).

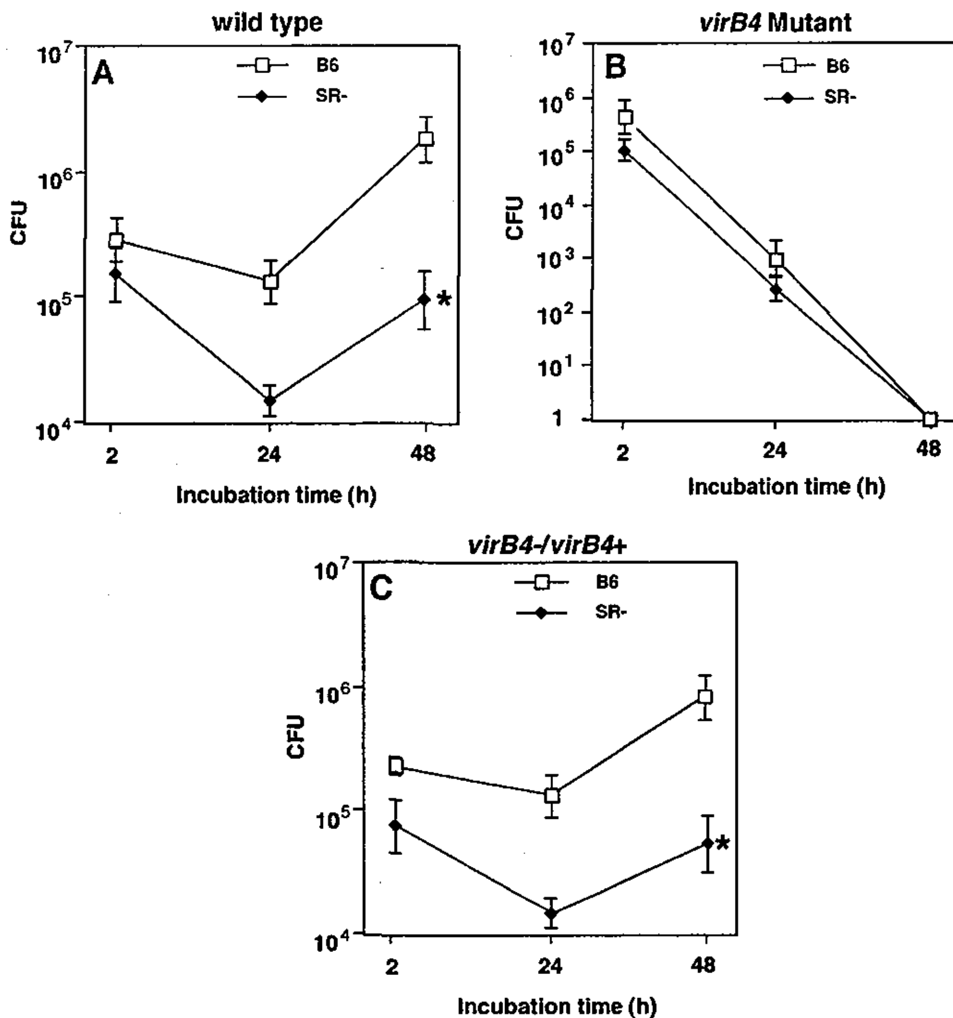

Fig. 2. Inhibition of intracellular replication of *B. abortus* in SR-A-deficient mouse macrophages. Macrophages from normal or SR-A-deficient mice were infected with the wild type (A), the *virB4* mutant (B) or complemented strain (C). Data points and error bars represent the mean CFU of triplicate samples from a typical experiment (performed at least six times) and their standard deviation, respectively. Statistically significant differences between the bacterial replication in macrophages from normal mice and that from SR-A-deficient mice are indicated by asterisks (* $P < 0.01$).

Macrophages from parent mice and SR-A-deficient mice were not significantly different in the intracellular replication of *virB4* mutant (Fig. 2B).

2.2. Roles of LPS in the SR-A dependent internalization of Brucella

LPS is a major cell surface component of Gram-negative bacteria and a ligand of SR-A [14], and is an important virulence factor of *Brucella* spp. [15]. Therefore, LPS was the best candidate of a bacterial ligand for the specific binding to SR-A on macrophages. To examine this possibility, macrophages were infected with *B. abortus* in presence or absence of LPS from several bacterial species and the differences in rates of phagocytosis were quantitated at 30 min after infection. LPS from smooth and rough *B. abortus* inhibited internalization of *B. abortus*, but not LPS from *Escherichia coli*. Interestingly, LPS from *Salmonella enterica* serovar Typhimutium also inhibited internalization of *B. abortus* into macrophages (Fig. 3A), suggesting that soluble LPS from *B. abortus* and *S. enterica* serovar Typhimurium was competing with LPS on the surface of *B. abortus* for receptors on the macrophage plasma membrane. In addition to LPS, internalization of *B. abortus* was inhibited by another SR-A ligand, polyinosinic acid, whereas a control reagent, polycytidylic acid that does not block the SR-A receptor had no effect (Fig. 3A). Furthermore, 2F8, a monoclonal antibody that specifically recognizes mouse SR-A [16] and that blocks phagocytosis of apoptotic thymocytes mediated by SR-A [17], inhibited internalization of *B. abortus* (Fig. 3A). These inhibitors did not affect internalization of opsonized bacteria (Fig. 3B). These results suggest that LPS on the surface of *B. abortus* interacts directly with SR-A, and that this interaction is an essential first step in initiating internalization of *B. abortus*.

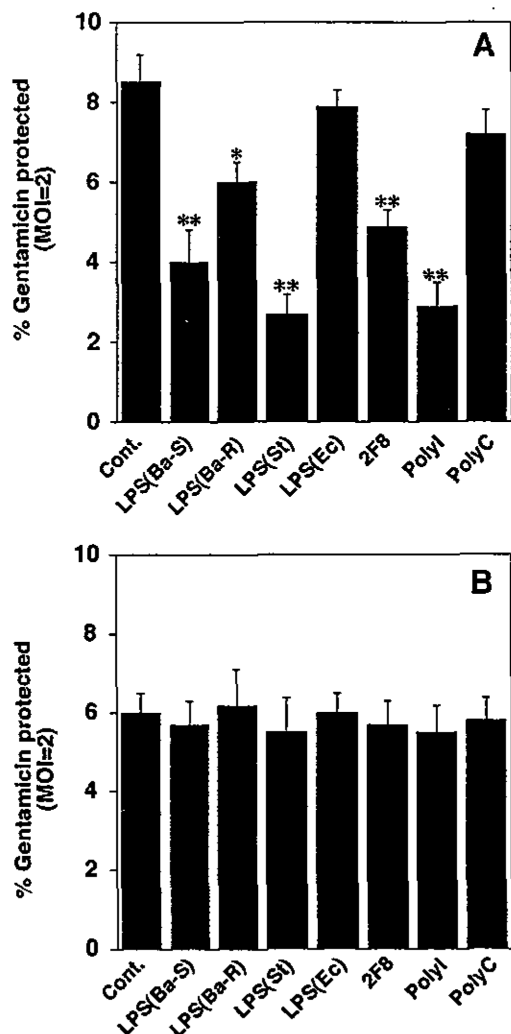

Fig. 3. Internalization of *B. abortus* into macrophages interfered by LPS and SR-A ligands. Macrophages from BALB/c mice in the presence or absence (Cont.) of various SR-A ligands were infected with the unopsonized (A) or opsonized (B) wild type strain. Macrophages were treated with LPS from the smooth *B. abortus* wild type strain (LPS (Ba-S)), rough *B. abortus* strain (LPS (Ba-R)), *S. enterica* serovar Typhimurium (LPS (St)), *E. coli* O111:B4 (LPS (Ec)), anti-SR-A antibody 2F8 (2F8), polyinosinic acid (polyI), and polycytidylic acid (polyC). Wild type strain were deposited onto macrophages and then were incubated for 30 min. Statistically significant differences between the bacterial internalization into macrophages and between the presence and absence of various SR-A ligands are indicated by asterisks (* $P < 0.05$; ** $P < 0.01$).

2.3. Decreased proliferation of Brucella in SR-A deficient mice

To find if the defect in internalization and intracellular replication of *B. abortus* correlate with an inability to establish infection in the host, we experimentally infected parent and SR-A-deficient mice with *B. abortus*. Many bacteria were recovered from the spleen of parent mice infected with the wild type *B. abortus* strain at 10 and 20 days after infection, but markedly fewer bacteria were recovered from SR-A-deficient mice, judged from the number of CFU in each spleen (Fig. 4). These results

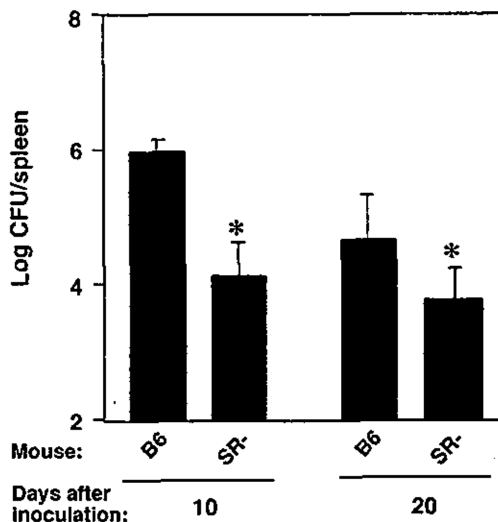

Fig. 4. Inhibition of *B. abortus* proliferation in SR-A-deficient mice. Normal (B6) or SR-A-deficent (SR-) mice were infected with wild type *B. abortus*. Recovery of viable bacteria from the spleen at 10 or 20 days postinfection are shown. Error bars indicate standard deviation. Statistically significant differences between the bacterial proliferation in the spleen from normal mice and from SR-A-deficient mice are indicated by asterisks (* $P < 0.01$).

indicated that bacterial internalization mediated by SR-A contributed to *B. abortus* proliferation in mice.

2.4. Association between SR-A and lipid rafts during bacterial internalization

Our previous studies showed that lipid raft-associated molecules were selectively incorporated into macropinosomes containing *B. abortus* to induce signal transduction into macrophages and to form replicative phagosomes [7,18]. If SR-A has an important role in the signal transduction into host cells, it may be contained in lipid rafts on the macrophage plasma membrane infected with *B. abortus*. To ensure that SR-A localizes in lipid rafts, we prepared detergent-resistant membranes (DRMs) fraction from macrophages infected with wild type *B. abortus* strain or *virB4* mutant. Lipid rafts can be isolated as DRMs from cells [19,20]. To isolate DRMs from infected macrophages, we extracted them in cold 1% Triton X-100 lysis buffer, and analyzed them on sucrose gradients for DRMs (Section 4) that float in light membrane fractions away from bulk cellular proteins. SR-A was contained in detergent soluble fractions isolated from the uninfected control or macrophages infected with the *virB4* mutant and it was not detected in detergent insoluble fractions, which were fractions 1–3 of the gradient (15–30% sucrose). In macrophages infected with the wild type strain, in contrast, most SR-A was contained in detergent insoluble fractions (Fig. 5A). These results suggest that SR-A shifted into detergent insoluble fractions, lipid rafts, from detergent soluble fractions by bacterial internalization dependent on the type IV secretion system and that SR-A may participate