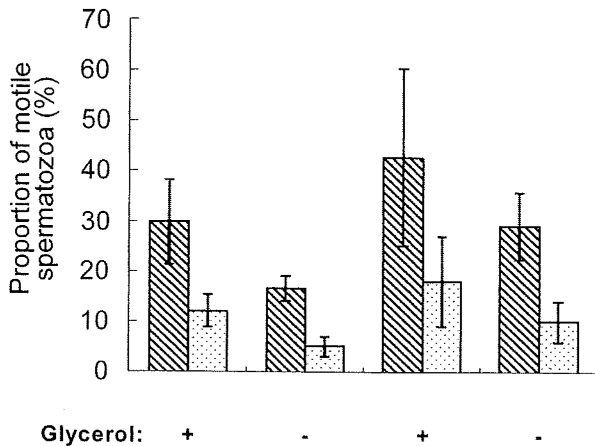

Fig. 2. Effect of semen exposure with or without glycerol on the proportion of total (slanted line bar) and progressively (stippled bar) motile spermatozoa after freezing and thawing. Experiments were replicated two or three times to examine the sperm motility using ejaculates of ICH or NAN. There was no significant difference between the groups with and without glycerol or cooling at 4 C for 1 and 3 h (P>0.05).

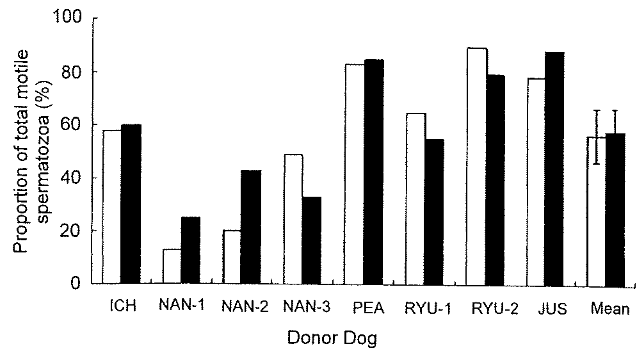

Fig. 3. The proportion of total motile spermatozoa frozen with the skim milk/glucose (SG)-based extender (black bar) and egg yolk (EY)-based extender (white bar).

freshly ejaculated semen from different dogs was performed to avoid failure of conception in one bitch. The bitch was inseminated with the cryopreserved and fresh semen on Days 5 and 6, respectively. Another bitch was inseminated with only the cryopreserved semen on Days 6, 7 and 8. Paternity for the delivered pups was examined using microsatellite markers, as described previously [8].

Statistical analysis

Data were compared using the *t*-test and the StatView software (Abacus Concepts, Berkeley, CA, USA). Differences were considered significant at a level of $P<0.05$.

Results

When 1-ml of the SG-based extender was cooled to 4 C, the temperature of the sample reached 4 C within 60 min. However, there was a tendency for the proportion of total motile spermatozoa in each of the samples exposed for 3 h in the case of the SG-based extender to be higher than that in the samples after 1 h, but the difference was not significant (Fig. 2). Addition of glycerol to the SG-based extender was remarkable in that in terms of the resulting motility of the cryopreserved canine semen. The results for the glycerol added groups were higher in both the proportion of total motile spermatozoa and progressive motility of spermatozoa compared with the no glycerol groups, although the difference was not significant (Fig. 2).

When 8 ejaculates from 5 dogs were frozen-thawed after 3 h exposure to the SG and EY extenders containing glycerol, as shown in Fig. 3, the proportion of total motile spermatozoa of the samples in the SG and EY extenders ranged from 25–89% (average: 58.8 ± 8.6) and 13–90% (average: 57.1 ± 10.1), respectively. Thus, the effect of the SG extender for cryopreservation of canine spermatozoa was similar to those obtained with the EY extender (Fig. 3). Other parameters of motility for frozen-thawed spermatozoa in the SG extender were similar to the corresponding parameters in the EY extender (Fig. 4).

Spermatozoa frozen in the SG-based extender containing glycerol after 3 h exposure were transcervically inseminated, resulting in the delivery of 6 pups from 2 recipient bitches (Table 1).

Discussion

Although egg yolk extender is the most commonly used extender for freezing canine sperm, the process for preparing the extender involves complicated procedures including storage for one to four days prior to use in addition to microbiological problems. Moreover, there are considerable individual differences in the fertilizing capacity of cryopreserved canine spermatozoa frozen in egg yolk-based extender. Thus, an improved system for cryopreservation of canine spermatozoa is required for successful breeding programs in companion and working dog colonies. Although skim milk is itself an extract from biological products (similar to egg yolk), skim milk is commercially available as a reagent and widely used as a cryoprotective additive in mouse [3] and goat [4] spermatozoa. In our preliminary experiment, in which the most suitable concentration of skim milk in terms of the effect on canine sperm motility was determined after freezing and thawing, 30 mg/ml was found to be the most effective concentration in terms of the kinematic parameters from among concentrations of 15, 30 and 60 mg/ml (data not shown). The results in Figs. 1 and 2 suggested that it may be necessary to expose the SG-based extender for 3 h at 4 C, although the temperature of the sample reached 4 C within 60 min. In addition, sufficient exposure time to the cryoprotectants may be a critical factor for the viability of frozen canine spermatozoa. Thus, we utilized 30 mg/ml of skim milk as a component of semen extender and 3 h as the exposure time for the SG extender. When 30 mg/ml of skim milk, 0.3 M glucose and 7% (v/v) of glycerol were provided as cryoprotectants, the motility and other related sperm viability parameters of canine spermatozoa after thawing were similar to those obtained with the EY-based extender (Figs. 3 and 4). Rota *et al.* [9] have similarly shown that

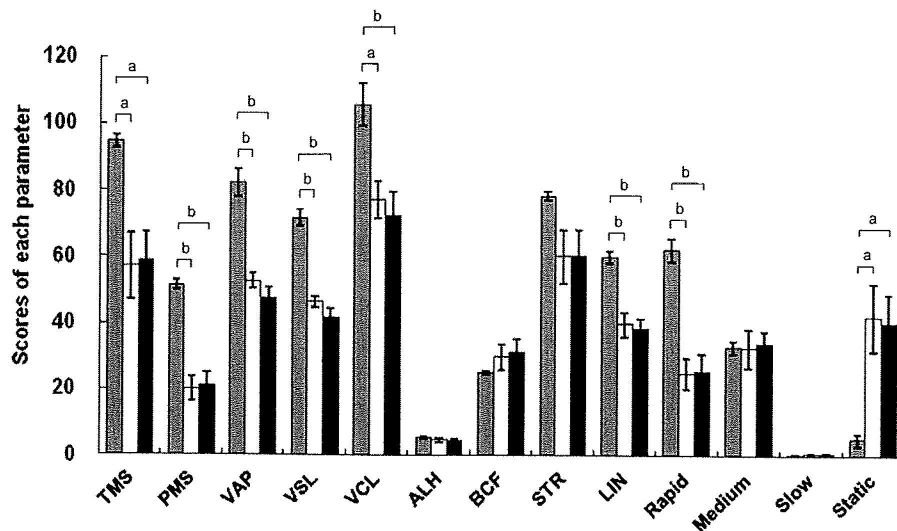

Fig. 4. Sperm motility parameters of fresh spermatozoa (gray bars) and spermatozoa frozen with skim milk/glucose-based (black bars) and egg yolk-based extender (white bars). TMS: The proportion of total motile spermatozoa. PMS: the proportion of progressively motile spermatozoa. VAP: velocity average pathway. VSL: velocity straight line. VCL: curvilinear velocity. ALH: amplitude lateral head. BCF: beat cross frequency. STR: straightness. LIN: linearity. The overall sperm population was subdivided into four categories: Rapid, ≥25 μm/sec; Medium, ≥5 μm/sec, <25 μm/sec; Slow, >0 μm/sec, <5 μm/sec; and Static, 0 μm/sec. Experiments were replicated four or eight times to examine the sperm motility in the ejaculates of two (ICH and NAN) or five (ICH, NAN, PEA, RYU and JUS) male Labrador Retrievers in the fresh or cryopreserved groups, respectively. Significantly different between the three groups at P<0.05 (a) and P<0.01 (b).

Table 1. Results of artificial insemination with canine spermatozoa frozen in a skim milk/glucose-based extender

Bitch no.	No. of pups Delivered	Treatment	
		Cryopreserved	Fresh
1*	5	1	4
2**	5	5	–

*Insemination with cryopreserved and fresh semen on Days 5 and 6, respectively. **Insemination with cryopreserved semen on Days 6, 7 and 8.

the use of skim milk in extenders for freezing canine semen results in sperm motility and viability after thawing comparable to that obtained using a Tris-based buffer with egg yolk, although they did not reportedly determine the fertilizing capacity of the cryopreserved spermatozoa by artificial insemination.

In the bull, it has been reported that egg yolk protects sperm function by preventing the binding of sperm to the major seminal plasma proteins, thereby preventing seminal plasma protein-mediated stimulation of lipid loss from the plasma membrane [10]. As in the case of egg yolk, skim milk prevents the binding of seminal plasma protein to bull sperm and reduces sperm lipid loss while also maintaining sperm motility and viability during storage at 4 C [11]. On the other hand, since it has been shown that the fertilizing lifespan of sperm stored in milk or milk-based extenders dose not exceed 12 h in the goat [12], further modification, such as antioxidant supplementation [13] or shortening of the exposure time in the extender before cryopreservation, might be required to prolong the survival and fertilizing ability of frozen canine spermatozoa.

In conclusion, the results presented here clearly demonstrate that an effective, simple extender composed of skim milk, glucose and glycerol is available for the cryopreservation of canine spermatozoa as an alternative to extenders containing egg yolk, and this may contribute to both efficient exchange of genetic materials and production of guide dogs for the blind.

Acknowledgements

This study was supported by Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Funds from the Ministry of Health, Labour and Welfare of Japan.

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FULL PAPER *Bacteriology*

Regulated upon Activation Normal T-Cell Expressed and Secreted (RANTES) Contributes to Abortion Caused by *Brucella abortus* Infection in Pregnant Mice

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(Received 17 December 2007/Accepted 25 March 2008)

ABSTRACT. *Brucella abortus (B. abortus)* is a facultative intracellular pathogen that can survive inside macrophages and trophoblast giant cells, and the causative agent of brucellosis. In the present study, we found that production of regulated upon activation normal T-cell expressed and secreted (RANTES) due to *B. abortus* infection contributes to abortion in pregnant mice. *B. abortus* infected pregnant interferon-γ (IFN-γ) knockout mice died within 15 days of infection, but non-pregnant IFN-γ knockout mice were still alive. With infection by wild type *B. abortus*, a large amount of RANTES production was observed in pregnant IFN-γ knockout mice, and induction of RANTES was also observed in normal pregnant mice infected with the wild type, but not in those infected with the intracellular replication-defective mutant. Production of RANTES and IFN-γ were inhibited in mice inoculated with the respective RANTES or IFN-γ antibody. Neutralization of RANTES, induced by *B. abortus* infection, served to prevent abortion. These results indicate that the production and function of RANTES are correlated with IFN-γ in pregnant mice infected with *B. abortus*.
KEY WORDS: abortion, *Brucella abortus*, IFN-γ, RANTES.

J. Vet. Med. Sci. 70(7): 681–686, 2008

Brucellosis is a serious debilitating disease in humans and results in abortion and sterility in domestic animals. The etiologic agents of brucellosis are *Brucella* spp., small gram-negative and facultative intracellular pathogens that can multiply within professional and non-professional phagocytes [8, 9]. In contrast to other intracellular pathogens, *Brucella* species do not produce exotoxins, antiphagocytic capsules or thick cell walls, resistant forms or fimbriae, and do not show antigenic variation [14]. A key aspect of the virulence of *Brucella* is its ability to proliferate within professional and non-professional phagocytic host cells, thereby successfully bypassing the bactericidal effects of phagocytes, and their virulence and chronic infections are thought to be due to their ability to avoid the killing mechanisms within host cells [20, 28]. Infection in humans is almost exclusively due to zoonosis, either through direct contact with infected animals or from contaminated dairy products [19]. The mouse model, particularly that using the non-pregnant mouse, has been used extensively to study some aspects of the pathogenesis of brucellosis [11]. While brucellosis is known to primarily affect the reproductive tract in the natural host and has been much studied, little is known regarding the cellular and molecular mechanisms of *Brucella* infection in the pregnant mouse [27]. The infectious abortion model using the pregnant mouse is a powerful tool for investigating the mechanisms of *Brucella* pathogenesis, and in our previous study we demonstrated that *B. abortus* causes abortion in pregnant mice by inoculating bacteria on day 4.5 of gestation [15]. We found that there was a higher degree of bacterial colonization in the placenta than in other organs, that there were many bacteria in trophoblast giant (TG) cells in the placenta and that an intracellular replication-defective mutant did not induce abortion. Transient interferon-γ (IFN-γ) production induced by infection with *B. abortus* also contributes to infectious abortion, and its neutralization served to prevent abortion.

It has been suggested that during normal pregnancy a shift from a Th1- to a Th2-polarized immune response allows the survival of the fetus [21]. Th2 cytokines such as TGF-β, IL-4 and IL-10 are not only able to prevent immunopathologic events, but also have beneficial effects in successful pregnancy [7]. It has also been suggested that cytokines and chemokines play a role in the pathogenesis of idiopathic recurrent spontaneous abortion [7], but their role in abortion induced by *B. abortus* infection is still unclear.

Regulated upon activation normal T-cell expressed and secreted (RANTES) is a chemokine that has recently been implicated in trophoblast and spermatozoa migration in view of its well-established chemoattractant properties [17, 26]. In the present study, we investigated the pathogenesis of *B. abortus*-induced abortion in pregnant IFN-γ knockout mice and noted that RANTES plays an important role in this process.

MATERIALS AND METHODS

Bacterial strains: All *B. abortus* derivatives were from 544 (ATCC23448) smooth virulent *B. abortus* biovar 1 strains. Ba598 (544Δ*virB4*) was also used in this study [30, 31]. *B. abortus* strains were maintained as frozen glycerol stocks and cultured on Brucella broth (Becton Dickinson, NJ, U.S.A.) or Brucella broth containing 1.5% agar.

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Mice: Six to ten-week-old BALB/c female mice were individually mated to 6- to 10-week-old BALB/c male mice. All of these mice were obtained from CLEA Japan (Tokyo, Japan). The normal gestational time for these mice is 19 days, and the vaginal plug was observed on day 0.5 of gestation. In addition, six to eight-week-old IFN-γ knockout mice on the BALB/c background were obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.). The animal experiments were permitted by Animal Research Committee of Obihiro University of Agriculture and Veterinary Medicine.

Virulence in pregnant mice: Groups of three or five pregnant mice were infected intraperitoneally with approximately 10^4 CFU of brucellae in 0.1 m*l* saline at the indicated days of gestation [15]. On day 18.5 of gestation or when mice died, their fetus, placenta, and spleen were removed and homogenized in saline. Tissue homogenates were serially diluted with PBS and plated on Brucella agar to count the number of CFU in each organ. Fetuses were determined to be alive if there was a heartbeat, and dead if there was no heartbeat.

Cytokine measurement: Serum levels of RANTES, IFN-γ and MCP-1 were measured for infected and uninfected virgin and pregnant mice. To achieve infection, groups of five mice were inoculated intraperitoneally with approximately 10^4 CFU of brucellae in 0.1 m*l* saline on day 4.5 of gestation, and blood was collected at 1, 3, 5 or 7 days after infection. Blood was collected at the same times for uninfected mice. On day 18.5 of gestation, uteri were removed, and a judgment was made as to whether mice were pregnant or not. Serum levels of RANTES, IFN-γ and MCP-1 were measured with an enzyme linked immunosorbent assay (ELISA) kit (PIERCE Endogen, Rockford, IL, U.S.A.) according to the instructions of the manufacturer. The cytokine antibody array used was obtained from RayBiotech (Norcross, GA, U.S.A.).

In vivo depletion of RANTES and IFN-γ: RANTES and IFN-γ were neutralized in the mice through the use of anti-mouse RANTES monoclonal antibodies (clone 53405) or IFN-γ monoclonal antibodies (clone HB170), administering 200 μg of the respective antibody in a volume of 0.1 m*l* intraperitoneally 24 hr before infection. As a control, mice were injected with 200 μg of normal rat IgG in 0.1 m*l* according to the same schedule as for the mice treated with anti-RANTES or anti-IFN-γ monoclonal antibodies. Bacterial infection was achieved as described above. Blood was collected at 1, 3, 5 or 7 days after infection, and serum levels of RANTES and IFN-γ were measured with an ELISA kit as described above. On day 18.5 of gestation, a judgment was made as to whether mice were pregnant or not as described above.

Statistical analysis: All statistical analysis was conducted using the Student's *t* test.

RESULTS

B. abortus infection in IFN-γ knockout mice: We previously reported that transient induction of IFN-γ production is a key event in abortion induced by *B. abortus* infection [15]. In order to clarify the contribution of IFN-γ to infectious abortion, pregnant IFN-γ knockout mice were infected with *B. abortus*, and abortion rates were examined. As reported previously, pregnant normal mice remained alive after *B. abortus* infection (Fig. 1A), but all *B. abortus* infected pregnant IFN-γ knockout mice died within 15 days of infection (Fig. 1A). Bacterial growth was examined in the spleens of infected pregnant IFN-γ knockout mice to determine if bacterial colonization was predominantly in the spleen. Colonization by *B. abortus* was much greater in the spleens of pregnant IFN-γ knockout mice than in those of pregnant normal mice (Fig. 1B). *B. abortus* induced splenomegaly as a consequence of the host inflammatory response in the pregnant normal mice, but splenomegaly was not induced in the pregnant IFN-γ knockout mice (Fig. 1C).

Transient increase in RANTES in pregnant IFN-γ knock-

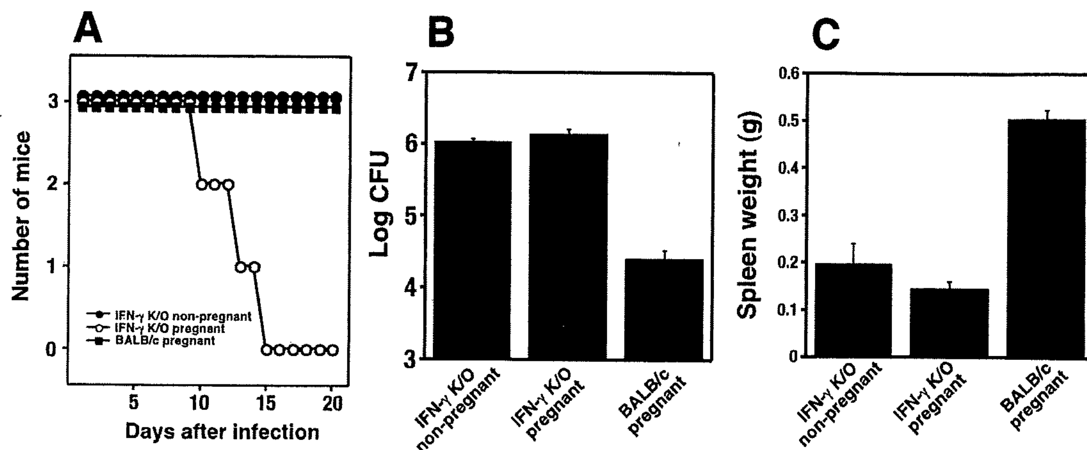

Fig. 1. *B.abortus* infection in pregnant IFN-γ knockout mice. Pregnant (IFN-γ K/O pregnant) and non-pregnant (IFN-γ K/O non-pregnant) IFN-γ knockout mice and pregnant normal mice (BALB/c pregnant) were infected with *B. abortus*. The figure shows number of mice (A), bacterial growth in spleen (B) and spleen weight (C).

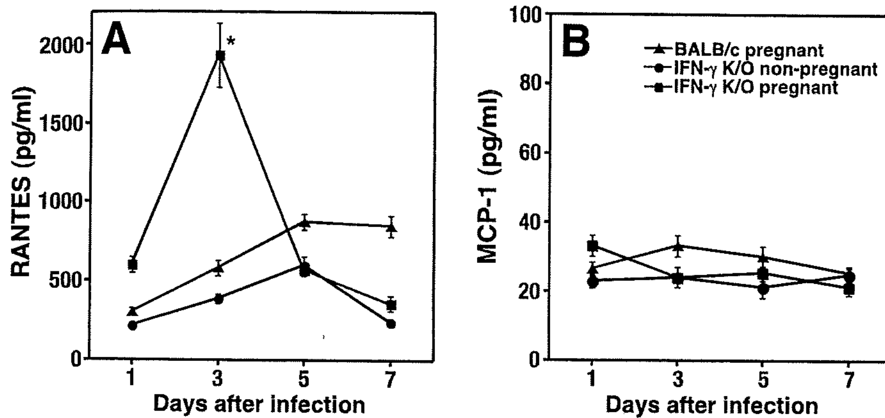

Fig. 2. Induction of RANTES in pregnant IFN-γ knockout mice by *B. abortus* infection. RANTES (A), and MCP-1 (B) serum levels were measured in each mouse by ELISA at the indicated numbers of days after infection. The means and SE for groups of 5 mice are shown. Statistically significant differences between pregnant and non-pregnant mice are indicated by asterisks (*, P<0.01).

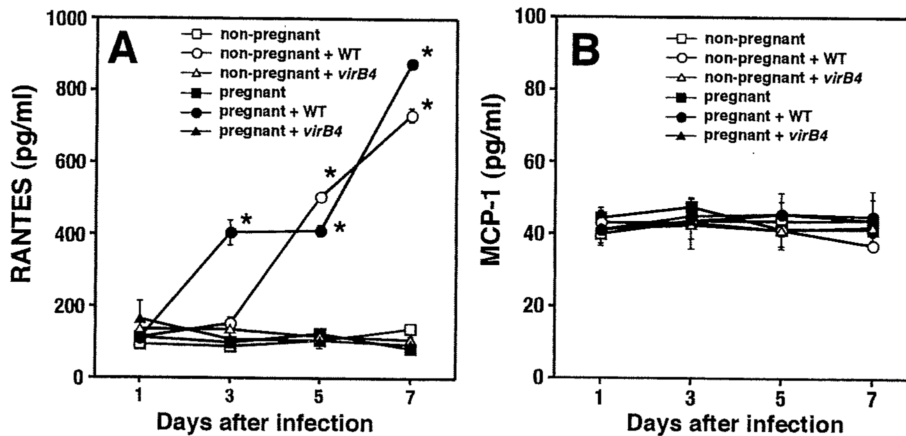

Fig. 3. Transient increase in RANTES in normal mice induced by *B. abortus* infection. Pregnant and non-pregnant normal mice were infected with wild type (WT) or *virB4* mutant (*virB4*). RANTES (A), and MCP-1 (B) serum levels were measured in each mouse by ELISA at the indicated numbers of days after infection. The means and SE for groups of five mice are shown. Statistically significant differences between mice infected with and without wild type *B. abortus* are indicated by asterisks (*, P<0.01).

out and normal mice induced by B. abortus infection: To examine cytokine patterns in *B. abortus* infected pregnant IFN-γ knockout mice, we conducted rough screening using a cytokine antibody array. As this showed that RANTES was greatly induced, we decided to determine if RANTES contributes to induction of abortion by *B. abortus* infection by using ELISA to measure the production of RANTES in pregnant mice. As a result, we observed a large amount of induced RANTES production in pregnant IFN-γ knockout mice at 3 days after infection with *B. abortus*, but afterwards RANTES production decreased rapidly (Fig. 2A). No significant MCP-1 production was observed in infected pregnant IFN-γ knockout mice or pregnant normal mice (Fig. 2B).

As these results imply that RANTES contributes to abortion due to *B. abortus* infection, we next examined RANTES production in normal mice with *B. abortus* infection in detail. For wild type *B. abortus* infection, RANTES was induced within 3 days of infection in pregnant mice, but the start of induction was 2 days later in non-pregnant mice. There was no induction of RANTES in pregnant and non-pregnant mice infected with *virB4* mutant, which does not have the ability of intracellular replication (Fig. 3A). No significant MCP-1 production was observed in pregnant or non-pregnant mice infected with the wild type or *virB4* mutant (Fig. 3B). These results suggest that the type IV secretion system contributes to RANTES production.

Preventing abortion by neutralizing RANTES: To determine if abortion is prevented by neutralizing the RANTES produced as a result of bacterial infection, pregnant mice

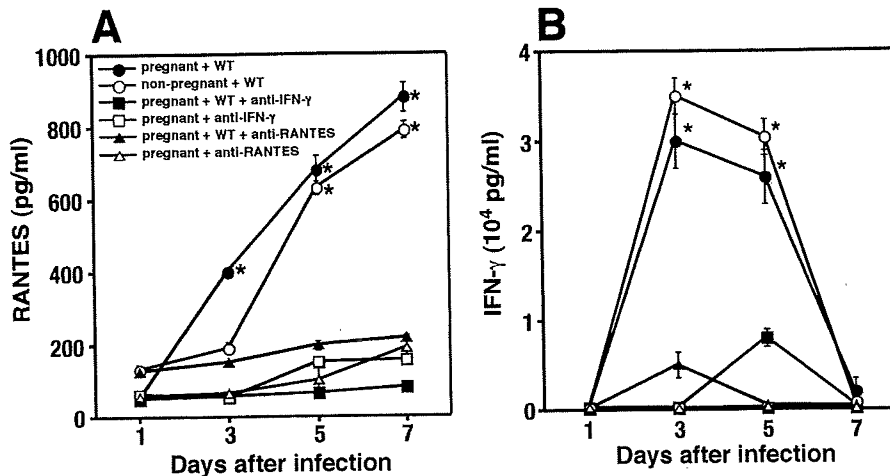

Fig. 4. Neutralization of RANTES and IFN-γ using monoclonal antibodies. RANTES and IFN-γ were neutralized in the mice by administering anti-mouse RANTES or IFN-γ monoclonal antibodies, respectively, and then the mice were infected with wild type *B. abortus* (WT). RANTES (A) and IFN-γ (B) were measured by ELISA. The means and SE for groups of 5 mice are shown. Statistically significant differences between the untreated control and antibody treated mice are indicated by asterisks (*, $P<0.01$).

were inoculated with monoclonal anti-RANTES antibodies or anti-IFN-γ antibodies 1 day before infection with *B. abortus*. Thereafter, serum levels of RANTES and IFN-γ, and abortion rates were recorded. Induction of RANTES and IFN-γ production was observed within 3 days of infection in pregnant mice, and this was inhibited by inoculation with RANTES or IFN-γ antibodies, respectively (Fig. 4A and B). Prior inoculation with anti-RANTES antibodies was also observed to inhibit abortion as compared to non-inoculated mice (Fig. 5A). However, there was no significant difference between pregnant mice inoculated with anti-RANTES antibodies and non-inoculated pregnant mice as regards bacterial growth in the spleen (Fig. 5B).

DISCUSSION

In the present study, we found that RANTES production contributes to abortion due to *B. abortus* infection in pregnant mice. We also noted that the production of RANTES and IFN-γ are influenced by each other in pregnant mice. Our previous study showed that a transient increase in IFN-γ due to *Brucella* infection causes abortion in pregnant mice [15]. For many other intracellular bacterial and protozoan pathogens, it has been shown that IFN-γ is an important component of Th1 immune responses and contributes to control through its ability to activate macrophages to enhance microbial killing. The role of IFN-γ in the control of *B. abortus* infections has been demonstrated by administering recombinant IFN-γ to BALB/c mice. This treatment resulted in a 10-fold decrease in the number of bacteria at 1 week after infection [24]. Similarly, the neutralization of endogenous IFN-γ through the administration of anti-IFN-γ monoclonal antibodies resulted in a decrease in control of *B. abortus* infection [35]. IFN-γ is important for control of *B. abortus* infection in BALB/c mice during the first week of infection, since an increase in bacteria has been measured in IFN-γ knockout mice at 1 week post infection [12, 13]. However, by 3 weeks post infection, the number of bacteria recovered was equivalent regardless of whether IFN-γ was present or not, and BALB/c mice survived for 10 weeks when IFN-γ was knocked out [18].

In the present study, pregnant IFN-γ knockout mice died within 15 days of *B. abortus* infection. Pregnancy leads to a generalized suppression of the adaptive immune system, typified by significantly decreased cell-mediated immunity and reduced Th1 responsiveness [21, 33, 34], and this immunosuppressed state prevents maternal rejection of the fetus but has the unfortunate consequence of increasing maternal susceptibility to certain infectious agents [16, 23]. This is considered to be the reason that pregnant IFN-γ knockout mice died more rapidly due to *B. abortus* infection than non-pregnant IFN-γ knockout mice.

Immune cells and their secretory products have been recognized as important pathophysiological mediators of recurrent spontaneous abortions and endometriosis in humans [7, 25, 32]. On the other hand, chemokines are involved in T-cell trafficking during normal processes and also in pathological events such as inflammation and endothelium damage [29]. RANTES is a chemokine that has recently been implicated in trophoblast and spermatozoa migration in view of its well-established chemoattractant properties [17, 26]. The specific ability of RANTES to downregulate T-cell responses suggests it might be relevant for fetal tolerance induction, but other than this no role of RANTES in pregnancy has been established [22]. A recent study reported that the secretion of RANTES, macrophage inflam-

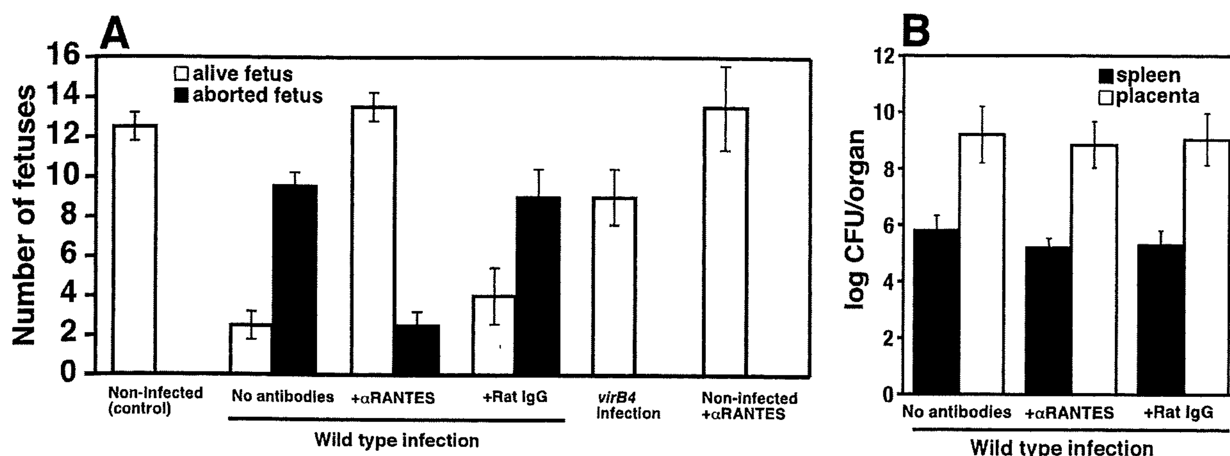

Fig. 5. Preventing abortion by neutralizing RANTES. RANTES was neutralized in the mice by administering anti-mouse RANTES monoclonal antibodies, and then they were infected with wild type (WT) or *virB4* mutant (*virB4*). The figure shows number of fetuses (A), and bacterial growth in the spleen and placenta (B).

matory protein (MIP)-1α, MIP-1β, and activated-induced, T cell-derived, and chemokine-related cytokine (ATAC) in polyclonally activated NK and T cells was closely associated with the secretion of IFN-γ [10]. RANTES is not only co-secreted to a great extent with IFN-γ at the single-cell level but also synergize functionally with IFN-γ on a common target population [10]. In this sense, the production and function of RANTES correlate with IFN-γ in *B. abortus* infected pregnant mice. Further, RANTES may act as a component of Th1 immune responses with IFN-γ in pregnant mice.

NK cells are large granular lymphocytes found in peripheral blood and also in the maternal decidua during pregnancy. The actions of NK cells on trophoblast lineage cells are likely mediated by NK cell secretory products, including cytokines, and may be direct or indirect. Uterine NK cells produce several cytokines and are the primary source of IFN-γ in the metrial grand [1, 2]. IFN-γ has been implicated as a major mediator of uterine NK cell function during pregnancy [3, 4]. Trophoblast cells are among a variety of different IFN-γ targets, and *in vitro* trophoblast cell differentiation, survival, and outgrowth are affected by IFN-γ [1, 5]. However, the function of IFN-γ is more diverse than the induction of bactericidal function and includes the stimulation of antigen presentation through class I and class II MHC molecules, the orchestration of leukocyte-endothelium interactions, the effects on cell proliferation and apoptosis, as well as stimulation and repression of a variety of genes whose functional significance remains obscure [6]. NK cells may play a role in controlling brucellosis in pregnant mice and shift from Th2- to Th1-polarized immune response may induce the abortion by *B. abortus* infection.

ACKNOWLEDGEMENTS. We thank Dr. Alexander Cox for his critical reading of the manuscript and Dr. Yoshitaka Omata for his valuable suggestions. This work was supported, in part, by grants from Program for Promotion of Basic Research Activities for Innovative Bioscience, and from Scientific Research (19041006 and 18790298), Japan Society for the Promotion of Science.

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Heat shock cognate protein 70 contributes to *Brucella* invasion into trophoblast giant cells that cause infectious abortion

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Published: 5 December 2008

BMC Microbiology 2008, **8**:212 doi:10.1186/1471-2180-8-212

Received: 21 April 2008

Accepted: 5 December 2008

This article is available from: http://www.biomedcentral.com/1471-2180/8/212

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Abstract

Background: The cell tropism of *Brucella abortus*, a causative agent of brucellosis and facultative intracellular pathogen, in the placenta is thought to be a key event of infectious abortion, although the molecular mechanism for this is largely unknown. There is a higher degree of bacterial colonization in the placenta than in other organs and many bacteria are detected in trophoblast giant (TG) cells in the placenta. In the present study, we investigated mechanism of *B. abortus* invasion into TG cells.

Results: We observed internalization and intracellular growth of *B. abortus* in cultured TG cells. A monoclonal antibody that inhibits bacterial internalization was isolated and this reacted with heat shock cognate protein 70 (Hsc70). Depletion and over expression of Hsc70 in TG cells inhibited and promoted bacterial internalization, respectively. IFN-γ receptor was expressed in TG cells and IFN-γ treatment enhanced the uptake of bacteria by TG cells. Administering the anti-Hsc70 antibody to pregnant mice served to prevent infectious abortion.

Conclusion: *B. abortus* infection of TG cells in placenta is mediated by Hsc70, and that such infection leads to infectious abortion.

Background

Brucellosis is a widespread and economically important infectious disease of animals and humans caused by members of the genus *Brucella*. *Brucella* spp. are small gram-negative, facultative intracellular pathogens that cause abortion, retained placenta and infertility in numerous domestic and wild mammals, and a disease known as undulant fever in humans [1-3]. Transmission of *Brucella* spp. from infected animals to humans may be either direct or indirect. Direct transmission involves the respiratory, conjunctival and cutaneous routes, and is more important in people in close contact with infected animals. Indirect

transmission is through the consumption of contaminated dairy products [3]. *Brucella* spp. occasionally causes spontaneous abortion in pregnant women [4].

There have been several histological studies on the placentas of *Brucella* infected animals [5]. Further, it has been found that *Brucella* internalizes into the caprine erythrophagocytic trophoblastic epithelial cells from the maternal circulation [6] and that the internalized bacteria replicate within the rough endoplasmic reticulum, resulting in secondary infection of adjacent trophoblastic epithelial cells [6,7]. Researches have also shown that after necrosis of infected trophoblasts, large numbers of brucellae are released, and proximity of the fetal capillaries in the ulcerated placenta to the lumenal bacteria has been proposed as the source of the fetal bacteremia and further placental infection [6,8]. However, the molecular mechanism of abortion induced by *Brucella* spp. remains unknown.

The mouse model, particularly that using the unpregnant mouse, has been used extensively to study some aspects of the pathogenesis of brucellosis [2]. While brucellosis is known to primarily affect the reproductive tract in the natural host, little is known regarding the cellular and molecular mechanisms of *Brucella* infection in the pregnant mouse [9]. Although the structure of bovine placenta is completely different from mouse placenta, the infectious abortion model using the pregnant mouse is a powerful tool for investigating the mechanisms of *Brucella* pathogenesis. In our previous study, we demonstrated that *B. abortus* causes abortion in pregnant mice by inoculating bacteria on day 4.5 of gestation [10]. We found that there was a higher degree of bacterial colonization in the placenta than in other organs, that there were many bacteria in trophoblast giant (TG) cells in the placenta and that an intracellular replication-defective mutant did not induce abortion. These findings suggest that bacterial infection of TG cells plays a key role in abortion induced by *B. abortus* infection.

Pregnancy leads to a generalized suppression of the adaptive immune system, typified by significantly decreased cell-mediated immunity and reduced T helper cell (Th) 1 responsiveness [11-13]. This immunosuppressed state prevents maternal rejection of the fetus but has the unfortunate consequence of increasing maternal susceptibility to certain infectious agents [14,15]. Our previous study showed that a transient increase in interferon (IFN)-γ due to *Brucella* infection contributes to abortion in pregnant mice [10]. In addition to examining the balance of inflammatory and regulatory cytokines in bacteria infected pregnant mice, analysis of bacterial internalization into the TG cells, a specific host cells in placenta, will help to advance our knowledge regarding the control of *Brucella*-induced abortion.

In the present study, we investigated the internalization of *B. abortus* into TG cells and identified heat shock cognate protein 70 (Hsc70) as a candidate receptor against *Brucella* or bacterial uptake-associated molecule. We noted that IFN-γ enhances bacterial internalization into TG cells.

Methods

Bacterial strains

All *B. abortus* derivatives were from 544 (ATCC23448) smooth virulent *B. abortus* biovar 1 strains. GFP expressed 544 strain was used in this study [16,17]. *B. abortus* strains were maintained as frozen glycerol stocks and cultured on Brucella broth (Becton Dickinson) or Brucella broth containing 1.5% agar.

Mice

Six to ten-week-old ICR female mice were individually mated to 6- to 10-week-old ICR male mice. The parent mice were obtained from CLEA Japan. Day 0.5 of gestation was the day the vaginal plug was observed. The normal gestational time for these mice is 19 days.

Virulence in pregnant mice

Groups of five pregnant mice were infected intraperitoneally with approximately 10^4 colony forming unit (CFU) of brucellae in 0.1 ml saline on day 4.5 of gestation [10]. On day 18.5 of gestation, placenta and spleen were removed and homogenized in phosphate buffered saline (PBS). Tissue homogenates were serially diluted with PBS and plated on Brucella agar to count the number of CFU in each organ. Fetuses were determined to be alive if there was a heartbeat, and dead if there was no heartbeat. The animal experiments were permitted by Animal Research Committee of Obihiro University of Agriculture and Veterinary Medicine.

Cell culture

Trophoblast stem (TS) cells were cultured in TS medium in the presence of FGF4, heparin and mouse embryonic fibroblast (MEFs)-conditioned medium as described previously [18]. The TS medium was prepared by adding 20% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, and 2 mM L-glutamine to RPMI 1640. To induce differentiation to trophoblast giant (TG) cells, the cells were cultured in the only TS medium alone for 3 days at 37°C in CO_2 incubator. The TG cells were seeded ($1-2 \times 10^5$ per well) in 48 well tissue culture plates for all assays.

Efficiency of bacterial internalization and replication within cultured cells

Bacterial infection and intracellular survival assays were performed according to a modified version of the method of Kim *et al* [19]. *B. abortus* strains were deposited onto TS or TG cells at a multiplicity of infection (MOI) of 100 which had been grown on 48-well microtiter plates containing TS medium but no antibiotics by centrifugation at 150 × g for 10 min at room temperature. To measure bacterial internalization efficiency after 30 min of incubation at 37°C, the cells were washed once with TS medium and then incubated with TS medium containing gentamicin (30 μg/ml) for 30 min. Next, cells were washed three times with PBS and lysed with cold distilled water. CFU values were determined by serial dilution on Brucella plates. To measure intracellular replication efficiency, infected cells were incubated at 37°C for 30 min, washed once with TS medium and then incubated with TS medium containing gentamicin (30 μg/ml) for 2, 24, 48 and 72 h. The cell washing, lysis and plating procedures were the same as for the bacterial internalization efficiency assay. Percentage protection was determined by dividing the number of bacteria surviving by the number in the infectious inoculum. The purified the R2–25 antibody or recombinant IFN-γ (Cedarlane Laboratories) was added to the TS medium at the indicated concentrations 2 or 12 h before infection.

F-actin staining

GFP-expressing bacteria were deposited onto the cultured cells by centrifugation and the incubation was conducted at 37°C for 30 min. The infected cells were incubated with TS medium containing gentamicin (30 μg/ml) at 37°C for 30 min to kill extracellular bacteria and were then fixed in 4% paraformaldehyde for 30 min at room temperature. Next, samples were permeabilized in 0.2% Triton X-100, washed three times with PBS and incubated with Alexa Fluor 594-phalloidin (Molecular Probes) at 20 μg/ml for 30 min at 37°C. After three washes with PBS, samples were placed in mounting medium (90% glycerol containing 1 mg/ml phenylenediamene in PBS, pH 9.0) and visualized by fluorescence microscopy.

Isolation of monoclonal antibodies

Hybridomas producing monoclonal antibodies that inhibit bacterial internalization into TG cells were obtained from fusions of BALB/c P3-X63-Ag8.653 (8-azaguanine-resistant and non-producer cell line) myeloma cells with spleen cells from Wister rats that had been immunized with TG cells. The screening of hybridoma supernatants for inhibiting antibodies was performed by adding antibodies to the TS medium in a bacterial internalization assay. Monoclonal antibodies obtained from hybridoma supernatants were purified using a protein G column (GE Healthcare Life Science) and the class and subclass of the purified monoclonal antibodies were determined using an Immunogloblin Typing Kit (WAKO Pure Chemical). The R2–25 monoclonal antibody used in this study was typed as IgG1.

Subcellular fractionation of TS and TG cells

TS and TG cells (3×10^5/ml) were seeded into each well of a 6-well plate. Protein isolation for the cytoskeleton, nuclear, membrane, and cytosol fraction was performed using a ProteoExtract Subcellular Proteome Extraction Kit as described by the manufacturer (Calbiochem).

Immunoblotting

The cell lysates (500 μg/ml) and fractionated proteins (50 μg/ml) were separated on 10% polyacrylamide gels and transferred to a PVDF membrane, which was incubated for 1 h at room temperature with primary antibody (0.5 μg/ml) in 5% skim milk. It was then washed three times in Tris buffered saline (TBS) with 0.02% Tween 20, incubated for 30 min with a horseradish peroxidase (HRP)-conjugated secondary antibody at 0.01 μg/ml and then washed again. Immunoreactions were visualized by ECL (GE Healthcare Life Science). Antibodies for β-actin, β-tublin and histone H1 were purchased from SIGMA or Abcam. Anti-IFN-γ receptor rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology.

Mass spectrometry analysis

Identification of proteins reacting with monoclonal antibodies that inhibited bacterial internalization into TG cells was conducted by means of nano LC-MS/MS analysis and a search of MASCOT database (APRO life Science Institute, Japan).

RNA isolation and RT-PCR

The total RNA of TG cells was isolated using an RNA Purification Kit (Qiagen) and purified RNA samples were stored at -30°C until use. The RNA was quantified by absorption at 260 nm using a SmartSpec3000 spectrophotometer (Bio-Rad). RT-PCR was carried out using a Sperscript II Kit (Invitrogen). The primers used for mouse Hsc70 or β-actin amplification had the following sequence 5'-GCAGCTGGGCCTACACACAAG-3' and 5'-CCCTGTGGAACAAAGCTACAC-3', or 5'-CGTGACATTAAGGAGAAGCTGTGC-3' and 5'-CTCAGGAGGAGCAATGATCTTGAT.

Expression and purification of recombinant proteins

Mouse Hsc70 cDNA (GenBank Accession No. BC066191) was amplified from RNA isolated from TG cells by means of RT-PCR with the pair of primers described above. The product was cloned into the pCR2.1-TOPO vector (Invitrogen) (pCR-Hsc70). To achieve expression of recombinant Hsc70 protein, amplified DNA encoding Hsc70 from pCR-Hsc70 in PCR was cloned into pCold TF vector

(Takara Bio Inc.). The His-tagged Hsc70 was expressed in the *E. coli* strain DH5α, and its purification and cleavage of His-tagged by HRV 3C protease were performed as described by the manufacturer (Novagen). Bovine Hsc70 and the rat anti-Hsc70 monoclonal antibody (SPA-815) were obtained from Stressgen for use as control materials.

To achieve expression of Hsc70 in TG cells, amplified DNA encoding Hsc70 from pCR-Hsc70 in PCR was cloned into the pcDNA4/TO vector in the T-Rex System (Invitrogen). pcDNA4/TO-Hsc70 was transfected into TG cells using the FuGENE 6 Transfection Reagent (Roche) with a final concentration of 1.2 μg/ml.

siRNA experiment

The siRNA duplexes used for silencing mouse Hsc70 (target sequence: AACAAGTAACATGGAATAATA), and β-actin (target sequence: CACTGACTTGAGACCAATAAA) and AllStars Negative Control siRNA were purchased from QIGEN. TG cells were transiently transfected using oligofectamine (Invitrogen) with or without a final concentration of 10 nM for siRNAs.

Immunofluorescence microscopy

Samples grown on coverslips were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, and permeabilized with or without 0.2% Triton X-100 in PBS for 20 min at room temperature. After blocking with 5% BSA in PBS, the cells were incubated with primary antibody (25 μg/ml) for 1 h at 37°C, and detection was conducted with TRITC-labeled goat anti-rat IgG (0.01 μg/ml) (Chemicon). Fluorescent images were taken using an Olympus BX51 microscope and a cooled CCD camera Olympus DP70.

In vivo *depletion of Hsc70*

Hsc70 was neutralized in the mice by administering an anti-mouse Hsc70 monoclonal antibody (clone R2-25)*in vivo* using 100 or 200 μg of antibody in a volume of 0.3 or 0.6 ml intraperitoneally 24 h before infection. Control mice were given 100 μg of normal rat IgG in a volume of 0.1 ml according to the same injection schedule as used for the anti-Hsc70 monoclonal antibody treated groups. Bacterial infection was conducted as described previously. On day 18.5 of gestation, fetuses were removed from the mice and a judgment made as to whether they were pregnant or not. Fetuses were determined to be alive if there was a heartbeat, and dead if there was no heartbeat.

Statistical analysis

All statistical analysis was conducted using the Student *t* test.

Results

B. abortus *internalizes and replicates in trophoblast giant cells*

We previously reported that there were many bacteria in trophoblast giant (TG) cells in the placenta by inoculation of pregnant mice with *B. abortus* [10]. To examine this bacterial infection into TG cells further, we used *in vitro* cell culture system of trophoblast stem (TS) cells and TG cells differentiated from TS cells. The *B. abortus* internalized into TG cells more efficiently than TS cells (Fig. 1A). We also investigated the intracellular replication of *B. abortus* in TS and TG cells. The bacteria replicated more efficiently in TG cells than TS cells (Fig. 1B).

Several intracellular pathogens attached to the host plasma membrane induce actin polymerization around the site of bacterial attachment and the process is essential for bacterial entry [20]. We therefore examined actin polymerization by means of fluorescence microscopy after 30 min and 48 h of incubation of TS and TG cells infected with *B. abortus*. It has been noted that differentiated TG cells dramatically rearrange their actin cytoskeleton into thick bundles of stress fibers [21]. There was no apparent actin polymerization around the site of the bacterial entry after 30 min incubation on TG cells or 48 h of incubation of infected TG cells (Fig. 1C).

Isolation of monoclonal antibodies that inhibit bacterial internalization into TG cells

To identify a receptor against *B. abortus* on TG cells, rats were immunized with TG cells, and monoclonal antibodies that inhibit bacterial internalization into TG cells were isolated. Seventy-five clones of bacterial internalization inhibiting antibodies (3.9%) were isolated from 1,920 hybridoma supernatants. From among the monoclonal antibodies, we selected R2-25, since it significantly inhibited internalization and showed clear reactions with protein in immunoblotting. The purified R2-25 antibody significantly inhibited bacterial internalization concentration dependently, but there was no inhibition with rat IgG (negative control) (Fig. 2A). The R2-25 antibody reacted with protein of around 70 kDa which was localized in membrane and cytosol, with the protein amount in the membrane fraction being especially large (Fig. 2C). On examining the distribution of protein reacting with R2-25 on the surface of TG cells by immunofluorescence microscopy, we observed an islet-like distribution on the surface of TG cells which was no permeabilized cells (Fig. 2B). It was difficult to detect the protein reacting with the R2-25 antibody on the surface of permeabilized cells.

Antibody inhibiting bacterial internalization reacts with heat shock cognate protein 70

We performed mass spectrometry analysis to identify the protein reacting with the R2-25 antibody. Proteins of the

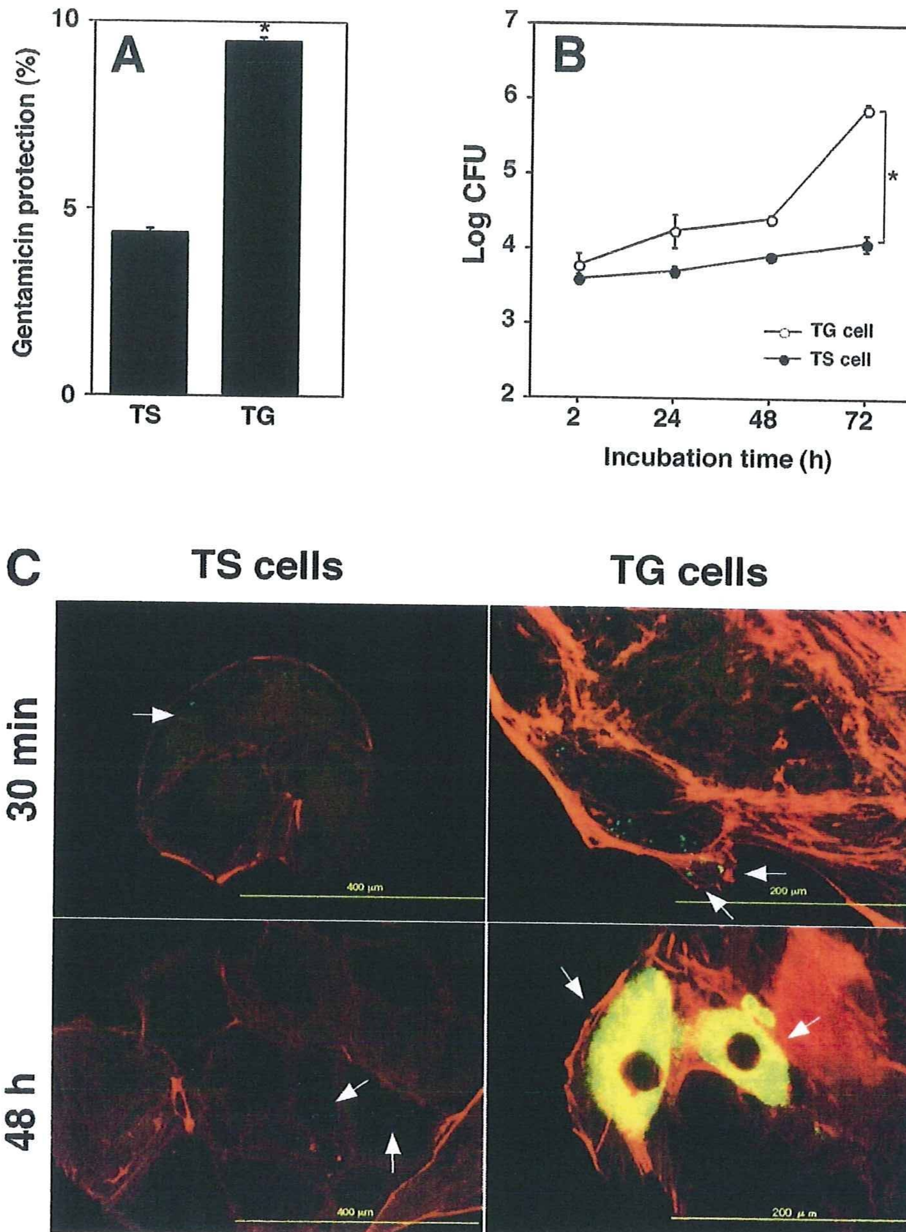

Figure 1

***B. abortus* infection in trophoblast giant cells**. (A) Bacterial internalization into trophoblast stem (TS) and giant (TG) cells. Data are the averages of triplicate samples from three identical experiments, and the error bars represent the standard deviations. Statistically significant difference between the bacterial internalization into TS and TG cells is indicated by asterisk (*, $P < 0.01$). (B) Intracellular replication of *B. abortus* in TS and TG cells. Datum points and error bars represent the mean of CFU of triplicate samples from a typical experiment (performed at least four times) and their standard deviations. Statistically significant difference between bacterial replication of *B. abortus* in TS and TG cells after 72 h of inoculation is indicated by asterisk (*, $P < 0.01$). (C) F-actin staining of bacteria infected cells. The figure shows GFP (bacteria) and Alexa Fluor 594 (actin filaments) channel merged images. Arrows indicate TS and TG cells containing bacteria.

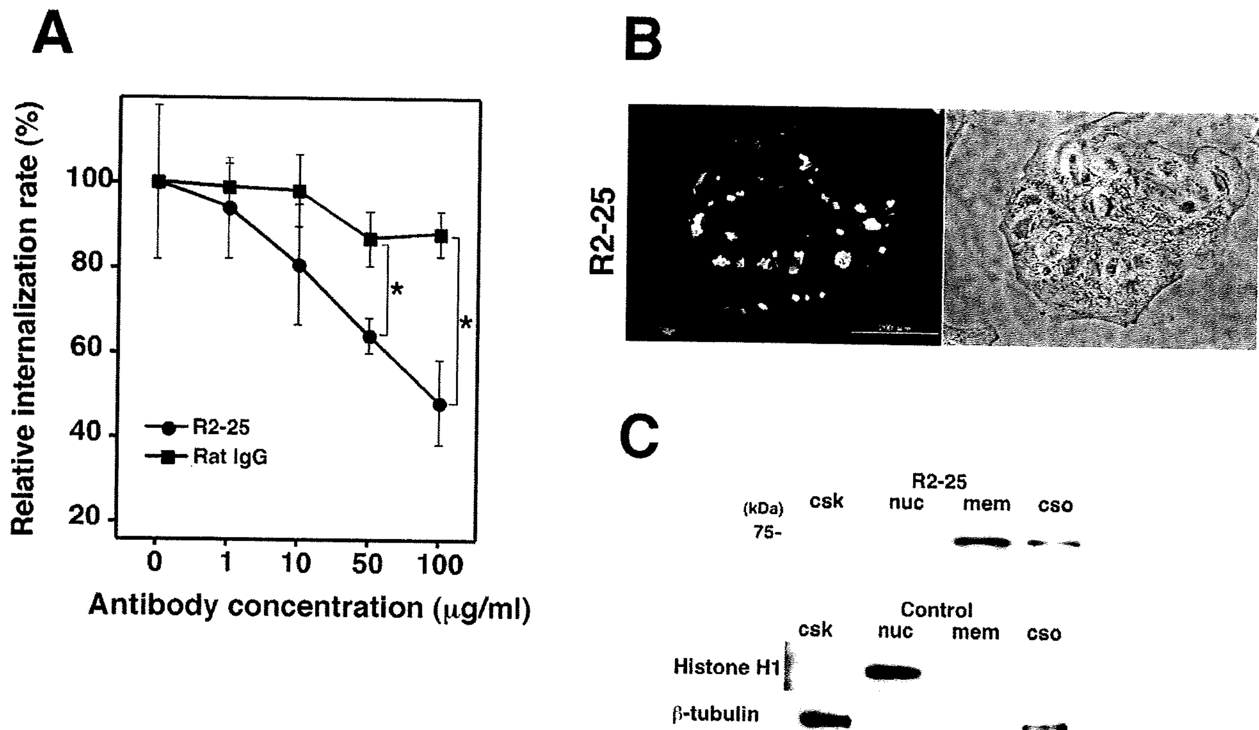

Figure 2
Characterization of the monoclonal antibody that inhibits bacterial internalization into TG cells. (A) Inhibition of bacterial internalization by the R2–25 antibody treatment. Data are the averages of triplicate samples from three identical experiments, and the error bars represent the standard deviations. Statistically significant differences between bacterial internalization into TG cells treated with the R2–25 antibody and those treated with rat IgG are indicated by asterisks (*, $P < 0.01$). (B) Distribution of protein reacting with monoclonal antibodies in TG cells. The left panels show fluorescence microscopy of the antibody stained TG cells and the right panels phase contrast microscopy of the corresponding microscopic fields. (C) Immunoblot analysis was performed on TG cell subcellular fractions with the monoclonal antibodies R2–25. Cells were fractionated to cytoskeleton (csk), nucleus (nuc), membrane (mem) and cytosol (cso). The anti-histone H1 and anti-β-tubulin antibody were used for fraction control for the nucleus and cytoskeleton.

membrane fraction were separated by SDS-PAGE and transferred to a PVDF membrane (Fig. 3A). The protein reacting with R2-25 was extracted from the PVDF membrane and the subjected to LC-MS/MS analysis. Through a search of the MASCOT database, the protein reacting with R2-25 was determined to be heat shock cognate protein 70 (Hsc70) (Fig. 3D). The anti-Hsc70 antibody also reacted with this protein (Fig. 3A). To confirm that the R2-25 antibody reacted with Hsc70, we tested its reactivity with mouse and bovine recombinant Hsc70. As expected, the R2-25 and anti-Hsc70 antibodies reacted with both types of recombinant Hsc70 (Fig. 3B and 3C). The mouse recombinant Hsc70 had a slightly greater molecular weight than the bovine recombinant Hsc70 because 25 amino acid residues had been added to the former after HRV 3C protease cleavage. These results indicate that the protein reacting with R2-25 was Hsc70.

Hsc70 contributes to bacterial internalization into TG cells

To examine the effect of Hsc70 on bacterial internalization into TG cells further, we reduced the amount of endogenous Hsc70 by transfecting Hsc70-specific small interfering RNA (siRNA) duplexes into the TG cells. After 48 h of transfection with Hsc70-specific siRNA, the expression level of Hsc70 was no longer detectable, but was not affected by transfection with β-actin or the control siRNA (Fig. 4A and 4D). Thus, the internalization efficiency of *B. abortus* into TG cells was significantly reduced by transfection with Hsc70-specific siRNA (Fig. 4C). Next, excessive production of endogenous Hsc70 was induced

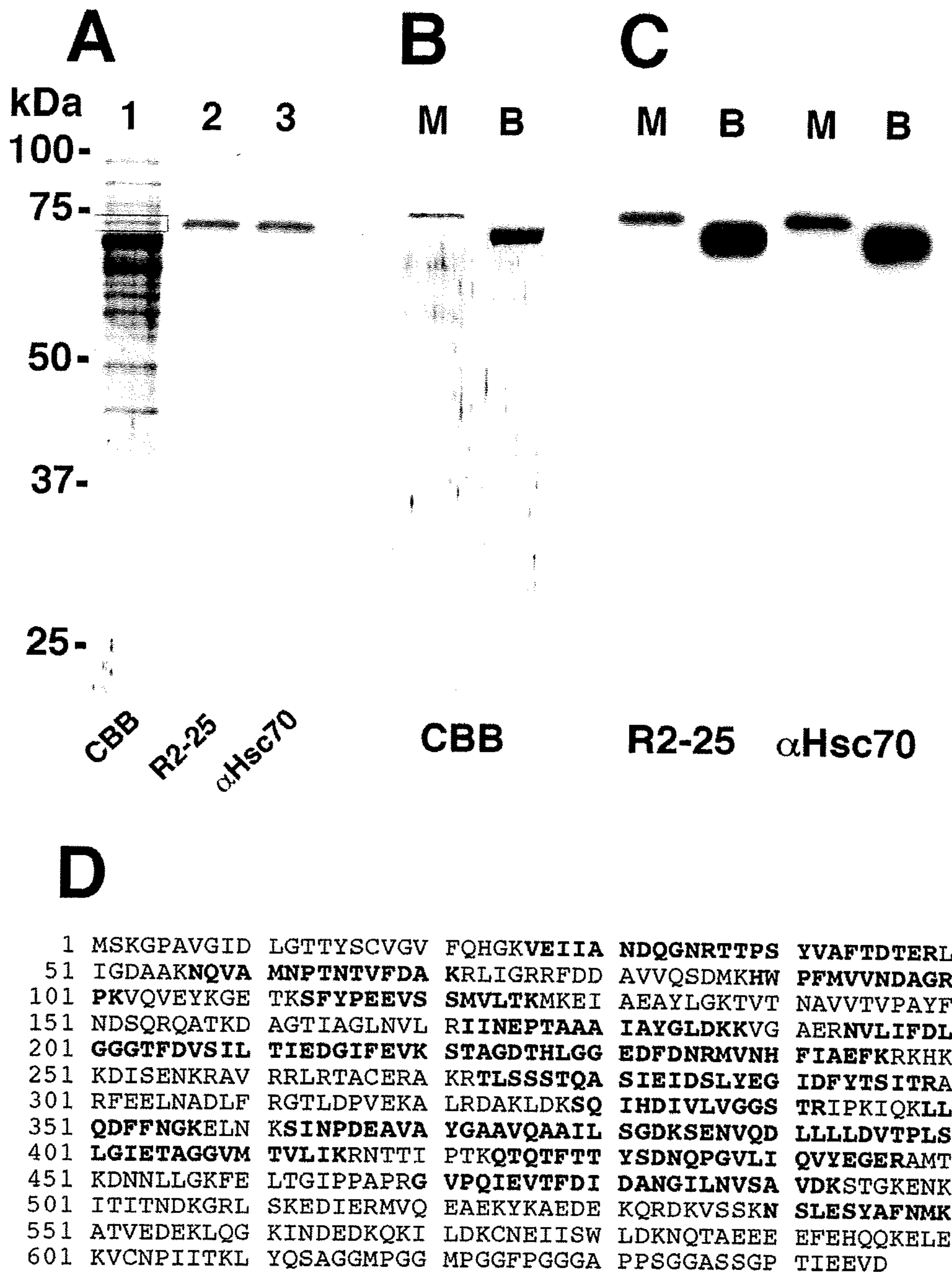

Figure 3

Bacterial internalization inhibiting antibodies react with Hsc70. (A) A membrane to which TG cell membrane fraction proteins had been transferred was stained with Coomassie brilliant blue (CBB) (lane 1), immunoblotted with the R2–25 antibody (lane 2) and the anti-Hsc70 antibody (clone SPA-815) (lane 3). (B) CBB staining of membrane to which mouse (M) or bovine (B) recombinant Hsc70 had been transferred. (C) Immunoblot analysis of mouse (M) or bovine (B) recombinant Hsc70 with R2–25 antibody and anti-Hsc70 antibody (clone SPA-815). (D) Amino acid sequences of Hsc70. The protein reacting with R2–25 was extracted from the PVDF membrane (square in lane 1 of panel A) and was used for LC-MS/MS analysis. The amino acids detected for Hsc70 in the analysis are shown in bold.

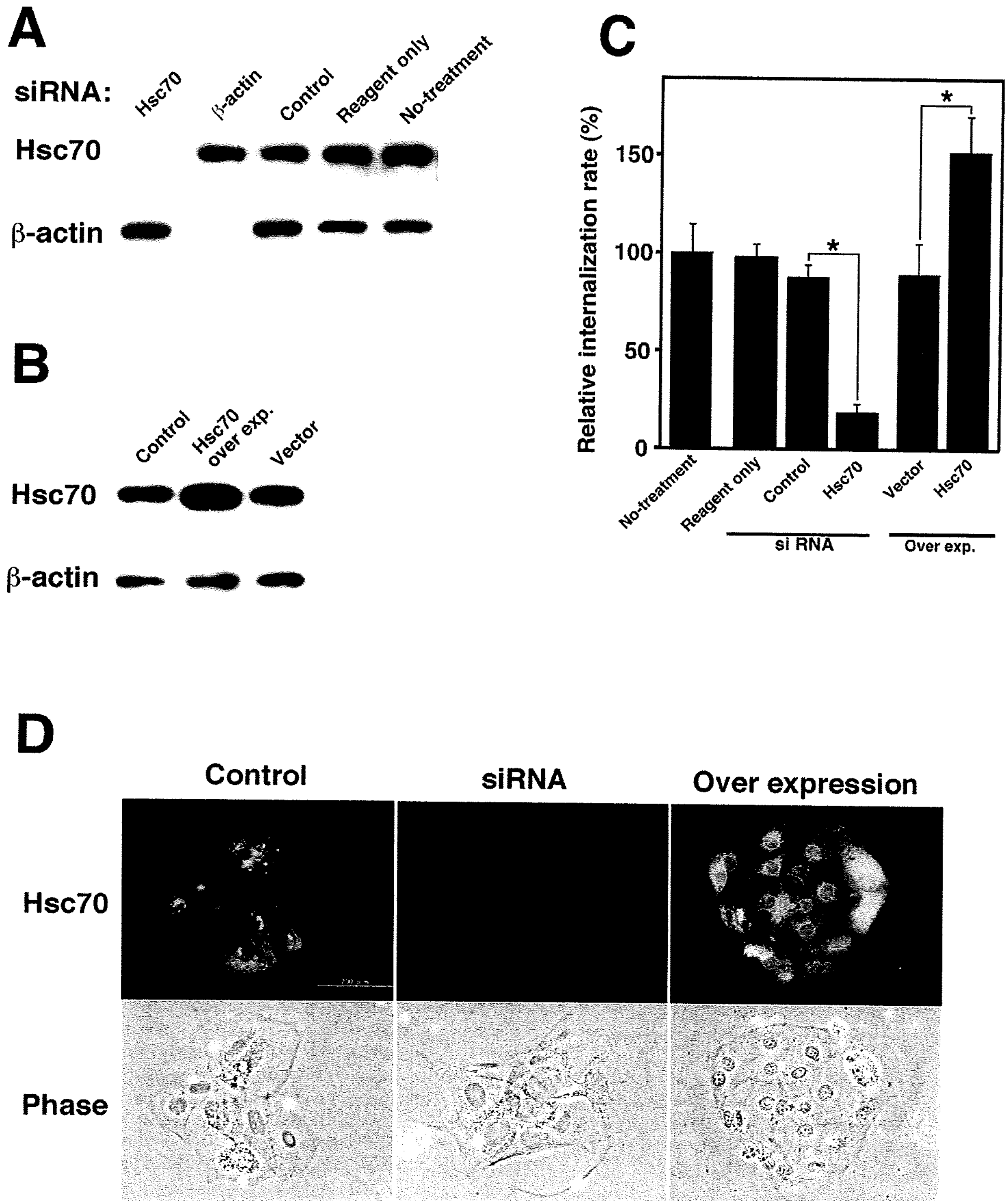

Figure 4 *(see legend on next page)*

Figure 4 *(see previous page)*
Effect of depletion and over expression of Hsc70 in TG cells on bacterial internalization. (A) Depletion of Hsc70. TG cells were treated for 48 h with siRNA targeting Hsc70 or without it (reagent only or no treatment), or β-actin or the control (QIAGEN AllStars Negative Control). Expression of the indicated proteins was monitored by immunoblotting. β-actin was used as an internal control. (B) Over expression of Hsc70. TG cells were transfected with or without (control) pcDNA4/TO-Hsc70 or vector only. (C) Bacterial internalization into Hsc70 depleted (siRNA) or over expressed (over exp.) TG cells was studied in a bacterial internalization assay. Lanes correspond to panels A and B. Data are the averages of triplicate samples from three identical experiments, and the error bars represent the standard deviations. Statistically significant differences between bacterial internalization into TG cells with (Hsc70) and without siRNA (control), and over expression and the control (vector) are indicated by asterisks (*, P < 0.01). (D) Distribution of Hsc70 in non-treated (control), Hsc70 depleted (siRNA), or over expressed (over expression) TG cells. Fluorescence microscopy of stained TG cells with the R2–25 antibody (upper panels) and phase contrast microscopy of the corresponding microscopic fields (lower panels) are shown.

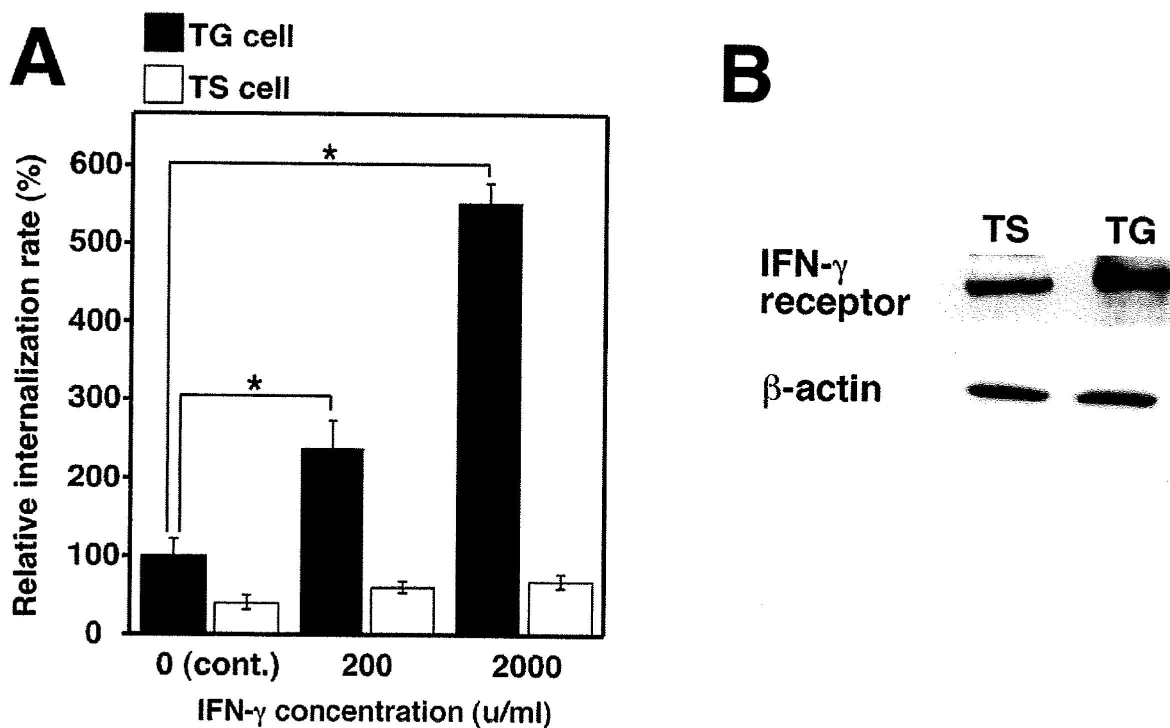

Figure 5
IFN-γ promotes bacterial internalization into TG cells. (A) Bacterial internalization into IFN-γ treated TS or TG cells. *B. abortus* was deposited onto TS and TG cells which were treated with or without (cont.) IFN-γ at the indicated concentrations. Data are the averages of triplicate samples from three identical experiments, and the error bars represent the standard deviations. Statistically significant differences between bacterial internalization in TG cells with and without IFN-γ treatment are indicated by asterisks (*, P < 0.01). (B) Expression of IFN-γ receptor in TS and TG cells. Immunoblot analysis was performed with anti-IFN-γ receptor and anti-β-actin rabbit polyclonal antibody.

by transfecting the Hsc70 expression vector into TG cells. After 48 h, expression levels of Hsc70 were significantly higher than the control levels (Fig. 4B and 4D). The internalization efficiency of *B. abortus* into TG cells in which Hsc70 was over-expressed was significantly higher than the control levels (Fig. 4C).

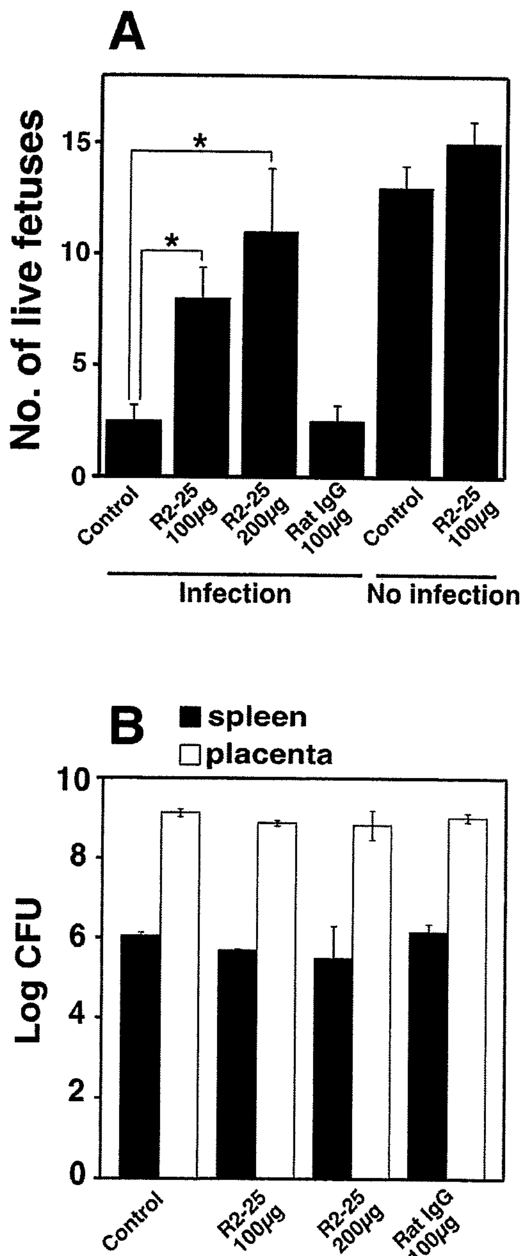

Figure 6

Figure 6
Preventing abortion by inoculating with anti-Hsc70 antibody. (A) Number of live fetuses. Hsc70 was neutralized in the mice by administering with or without (control) an anti-mouse Hsc70 monoclonal antibody (clone R2–25)*in vivo* using 100 or 200 μg of the antibody. The control mice were given 100 μg of normal rat IgG. Statistically significant differences between the untreated control and antibody treated mice are indicated by asterisks (*, $P < 0.01$). (B) Bacterial numbers in spleen and placenta. On day 18.5 of gestation, the placenta and spleen were removed and homogenized in PBS. Tissue homogenates were serially diluted with PBS and plated on Brucella agar in order to count the number of CFU in each organ.

IFN-γ enhances bacterial uptake by TG cells

Since a transient increase in IFN-γ brought about by *Brucella* infection promotes abortion in pregnant mice [10], we investigated the effect of IFN-γ treatment on bacterial internalization and Hsc70 expression in TG cells. IFN-γ treatment significantly increased the internalization efficiency of *B. abortus* into TG cells as their concentration, but had no effect in TS cells (Fig. 5A). To determine whether the enhancement of bacterial internalization by IFN-γ treatment was due to up-regulate Hsc70 expression or not, RNA was isolated from IFN-γ treated TG cells and subjected to RT-PCR. This showed that IFN-γ treatment did not affect Hsc70 expression (data not shown). IFN-γ receptor was expressed in TS and TG cells (Fig. 5B).

Preventing abortion by inoculating pregnant mice with anti-Hsc70 antibody

To determine if abortion is prevented by neutralizing the Hsc70 expressed on TG cells in the mouse placenta, pregnant mice were inoculated with the R2–25 antibody 24 h before infection with *B. abortus*, which was done on day 4.5 of gestation. While there was no change in the number of abortions observed in the non-inoculated mice, there was a significant increase in number of live fetuses in the inoculated mice (Fig. 6A). Inoculation of uninfected pregnant mice with the R2–25 antibody did not affect on pregnancy (Fig. 6A). Upon examining bacterial numbers in the spleen and placenta of infected pregnant mice, it was found that bacterial numbers were similar in both mice inoculated with the R2–25 antibody and those not inoculated with it (Fig. 6B).

Discussion

Previous mouse model studies have shown that *Brucella abortus* specifically replicates in trophoblast giant (TG) cells in the placenta [9,10]. TG cells are polyploid cells that play a crucial role in implantation, in remodeling of the embryonic cavity, and preventing maternal blood

flow to the implantation site [22]. Since *B. abortus* internalizes into TG cells and replicates in them, cell functions are not exhibited completely, which leads to abortion since implantation and placental development are inhibited. Therefore, it is thought that bacterial infection of TG cells is a key event in inducing abortion. To analyze the molecular mechanisms of *B. abortus* infection of TG cells *in vitro*, we used trophoblast stem (TS) cells and TG cells differentiated from TS cells for the infection assay in this study. Although TG cell differentiation is fairly well understood at the morphological and molecular level [23], the role of immune responses in fighting against pathogens of TG cells is poorly understood and in this regard a model of host-pathogen interaction using TG cells would be useful for obtaining new information of the effect of TG cell functions on pregnancy.

Hsc70 has been reported to be present on the surface of several types of cells [24]. In this regard, though Hsc70 congregates on the surface of TG cells, it is present to a much lesser extent on the surface of TS cells (data not shown). This may be a reason that the internalization of *B. abortus* into TG cells was greater than that into TS cells. As Hsc70 and many other factors will be present on TG cells differentiated from TS cells, there is a possibility that other receptors or bacterial uptake-associated molecules may contribute to *B. abortus* infection of TG cells. Little is known about how Hsc70, a protein with no signal sequence for secretion, exits cells by mechanisms other than escape from cells undergoing necrotic lysis. In previous studies, Hsc70 has seen to be released from a late endsomal lysosomal location where it participates in protein degradation [25,26]. Further, the secretion of the Hsp70 family and its association with lipid rafts have also been observed in epithelial cells under normal conditions, and a lipid raft-based mechanism has been suggested for the membrane delivery and release of Hsp70 family [27]. Although receptors for the extracellular Hsp70 family have still not been fully defined, several cell surface receptors have been suggested, such as CD14, CD40, CD91 and scavenger receptor Lox-1 [28-31]. Since it has also been noted that class A scavenger receptor (SR-A) contributes to *B. abortus* infection in macrophages [32], SR-A may be receptors for Hsc70, and the mechanism for *B. abortus* internalization into TG cells may be the same pathway as that for Hsc70 uptake by TG cells. Hsc70 may have a function that is catching antigens and anti-Hsc70 would inhibit binding between Hsc70 and antigens. IFN-γ treatment enhanced bacterial internalization into TG cells and these observations agreed with results obtained in pregnant mice model [10], and thus expression of unidentified receptors against Hsc70 may be upregulated by IFN-γ treatment. IFN-γ should therefore promote internalization of *B. abortus* into TG cells *in vivo* and this would be one of ways in which infectious abortion is induced.

Conclusion

The finding of this study that the anti-Hsc70 antibody prevents abortion caused by *B. abortus* infection is expected to be applied in the development of methods of preventing abortion. Since intracellular bacteria such as *Brucella* replicate in host cells, it is difficult to completely eliminate them from the host through treatment with antibiotics and develop effective vaccines against them. An alternative strategy in treating infection due to *Brucella* would be inhibition of bacterial internalization into TG cells and this could be an effective means of protecting against abortion due to brucellosis. Recently, Carvalho Neta *et al.* reported that *B. abortus* modulates innate immune response by bovine trophoblastic cells [33]. Although the structure of bovine placenta is completely different from mouse placenta, bovine and mouse trophoblastic cells may have similar function in the immune system. However, it is not known whether the mechanism of host-pathogen interaction observed in this study could be used to develop protective methods against other abortion-inducing pathogen infections, and thus further analysis of TG cell function in the immune system will be needed to clarify host defense mechanisms in the placenta and those contributing to the success of pregnancy.

Authors' contributions

MW conceived the study. MW, HS and KW designed the experiments, interpreted the results and worked on the manuscript. KW and MT carried out most of the experimental work. ST, HF and MH participated in cell culture and pathological experiments. HS and MW participated in animal experiments. All authors read and approved the final manuscript.

Acknowledgements

We thank Dr. Alexander Cox for critical reading of the manuscript, and Dr. Suk Kim for valuable discussion. This work was supported, in part, by grants from Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), and grants from the Naito Fundation, and Institute for Fermentation, Osaka.

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