materials were demonstrated to be massive cross-linked fibrin depositions by PTAH staining (Fig. 1d). Fibrin deposition was located along the basement membrane of the endothelial cells of the splenic sinuses and the interstitial space in the red pulp cord. Fibrin thrombi were also observed in the sinuses. In the splenic sinuses, enlarged mononuclear cells containing acidophilic inclusions in the cytoplasm were present (Fig. 1c).

In the kidneys, mild to moderate mononuclear cell infiltration was present around the arcuate veins. An increased number of circulating mononuclear cells was present in the renal vessel lumens. Many thrombi composed of acidophilic materials and small amounts of fibrin were present in the medulla of renal capillaries in some macaques. Six macaques (Nos. 2377, 2338, 2334, 2872, 2882 and 2878) showed hemorrhage in the renal tubules.

Mild to moderate perivascular infiltration of mononuclear cells was observed in the pulmonary parenchyma. Some macaque lungs showed evidence of fibrinous exudate and hemorrhage in the intra-alveolar area. Similar thrombi to those observed in the renal medulla were also present in the pulmonary venular.

Histopathologic findings in sacrificed macaques

The twelve sacrificed macaques showed more variable pathological changes than those that died of EBO-R infection (Table 1). Among them, 4 macaques (Nos. 2671, 2182, 2612 and 2669) showed no clinical signs, while 3 macaques (Nos. 956, 2939 and 2615) were ill at euthanasia (Table 1). Data on clinical manifestations were not available for the remaining 5 macaques.

Two macaques (Nos. 2671 and 2182) showed minimal fibrin deposition along the basement membrane of the splenic sinus. In addition, enlarged mononuclear cells in the sinuses of the liver and spleen occasionally contained acidophilic intracytoplasmic inclusions. However, inclusions in hepatocytes were not present in these 2 macaques.

On the other hand, two macaques (Nos. 2921 and 2728) showed minimal acidophilic intracytoplasmic inclusions in a few hepatocytes. Three macaques (Nos. 2739, 2644 and 2612) showed hepatic inclusions in many hepatocytes. No. 2644 showed massive fibrin deposition in the red pulp cords like most of the macaques that died of EBO-R infection while the other 4 macaques showed minimal deposition. None of these 5 macaques showed any lymphoid cell depletion in the white pulp of spleens. Hemorrhage and enlarged macrophages were present in the renal proximal tubules of No. 2644.

The other 5 macaques (Nos. 956, 2400, 2939, 2669 and 2615) showed pathological changes similar to those in the macaques that died of EBO-R infection in the liver and the spleen. Nos. 956 and 2939 showed hemorrhage in the renal proximal tubules, and Nos. 2669 and 2615 showed hemorrhage in the intra-alveolar area.

All twelve sacrificed macaques showed fibrin thrombi in the livers and the spleens. Five macaques (Nos. 2612, 956, 2400, 2939, and 2669) showed thrombi composed of acidophilic materials and small amounts of fibrin in the kidneys and/or the lungs.

The five uninfected macaques did not show any pathological changes in any of tissues examined.

EBO-R NP antigen distribution

The localization of EBO-R NP antigen was examined by immunohistochemistry, and the result is summarized in Table 1.

In all macaques that died of EBO-R infection, EBO-R NP antigens were detected in macrophages/monocytes, endothelial cells and fibroblasts in the livers (Fig. 1e), spleens (Fig. 1f), kidneys and lungs. Although the acidophilic inclusions were frequently observed in the hepatocytes by HE staining, EBO-R NP antigens were not frequently detected in these cells (Fig. 1e).

On the other hand, EBO-R NP antigen distribution among sacrificed macaques was variable compared to that of macaques that died of EBO-R infection. One sacrificed macaque (No. 2671) had the viral antigens only in the MPS cells of the spleen. Five sacrificed macaques (Nos. 2671, 2182, 2921, 2728, and 2739) had the viral antigens in circulating macrophages/monocytes in the livers, spleens, kidneys and lungs but not in the endothelium in these organs.

In addition, two macaques (Nos. 2644 and 2878) that had hemorrhage in the renal proximal tubules had EBO-R NP antigens in the epithelium of renal proximal tubles.

Increase in leukocyte antigen L1 Positive Cells

As shown above, an increased number of mononuclear cells was observed in the vessel lumens of livers and kidneys. These mononuclear cells were morphologically indistinguishable from macrophages and monocytes. Thus, we analyzed the number of blood-derived macrophages/monocytes in the livers, kidneys and lungs by counting the numbers of leukocyte antigen L1-positive

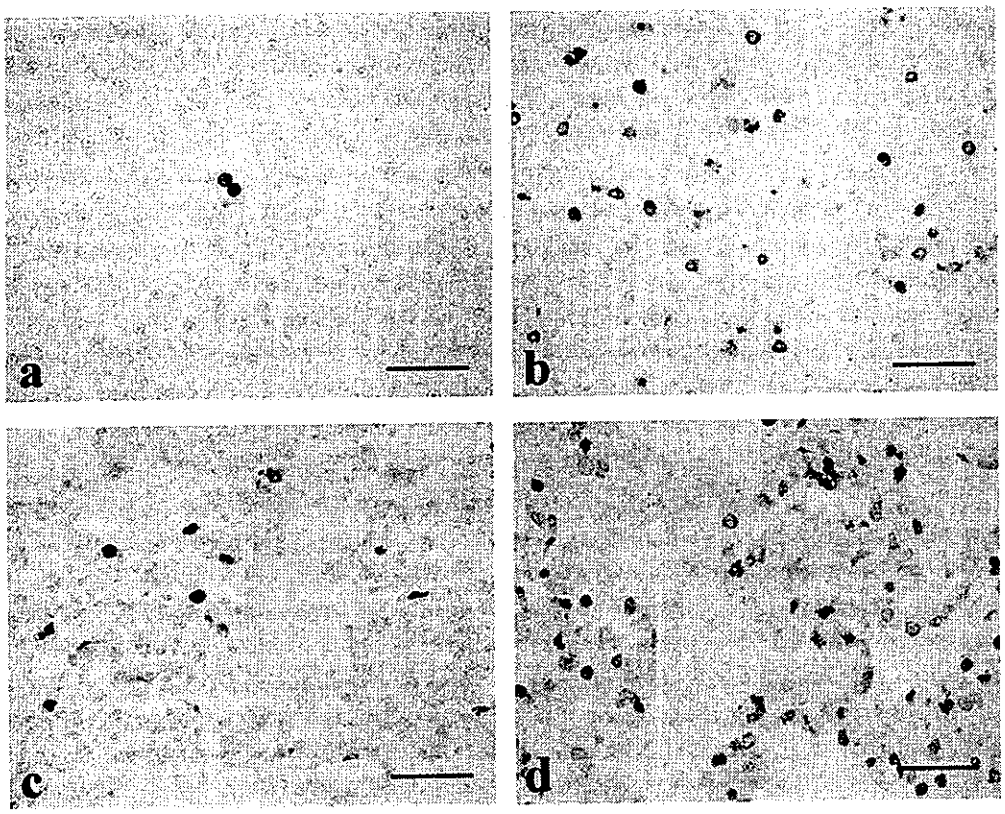

Fig. 2. An increase in leukocyte antigen L1 positive macrophages / monocytes in EBO-R infected macaques. Immunohistochemistry, Mayer's hematoxylin counterstain. Bar = 50 μm. (a) Liver, No. 2782. L1 antigen-positive cells in a non-infected macaque. (b) Liver, No. 2377. L1-positive cells are increased in the sinus in an EBO-R-infected macaque. (c) Lung, No. 2782. L1 antigen-positive cells in a normal macaque. (d) Lung, No. 2377. Increased number of L1-positive cells in an EBO-R-infected macaque.

cells. Since the fibrin was deposited in the red pulp cord of the spleen and the number of cells in the spleen were prominently decreased in severely affected macaques, we did not analyze the number of L1-positive cells in the spleen. The numbers of L1 antigen-positive cells in the livers, kidneys and lungs counted in 5 fields at 50X magnification are shown in Fig. 3. All sacrificed macaques and macaques that died through infection with EBO-R had increased numbers of L1-positive cells in the hepatic vasculature in comparison with the average of the 5 uninfected ones ($P<0.01$) (Figs. 2a, 2b, 3). Furthermore, 6 of 11 (55%) sacrificed and 10 of 11 (91%) dead macaques showed increased numbers of L1-positive cells in renal vessels, and 8 of 10 (80%) sacrificed macaques and all of the macaques that died of EBO-R infection showed increased numbers of L1-positive cells in the pulmonary venular ($P<0.01$) (Figs. 2c, 2d, 3).

Discussion

In this study, we histologically examined formalin-fixed tissues from 24 EBO-R infected cynomolgus macaques, and compared the outstanding findings in sacrificed EBO-R infected macaques with those of macaques that died during the 1996 outbreak in the Philippines. Regrettably, the gross findings and sufficient clinical data were not available in this study. The light microscopic findings such as acidophilic inclusion bodies in the hepatocytes, fibrin deposition in the red pulp of the spleen, lymphoid cell necrosis in the white pulp of the spleen and fibrin thrombi formation were consistent with those reported in the 1989 American outbreak [11, 15, 19]. However, the histological changes in 7 (Nos. 2671, 2182, 2921, 2728, 2739, 2644 and 2612) of 12 sacrificed macaques were minimal or moderate as compared with

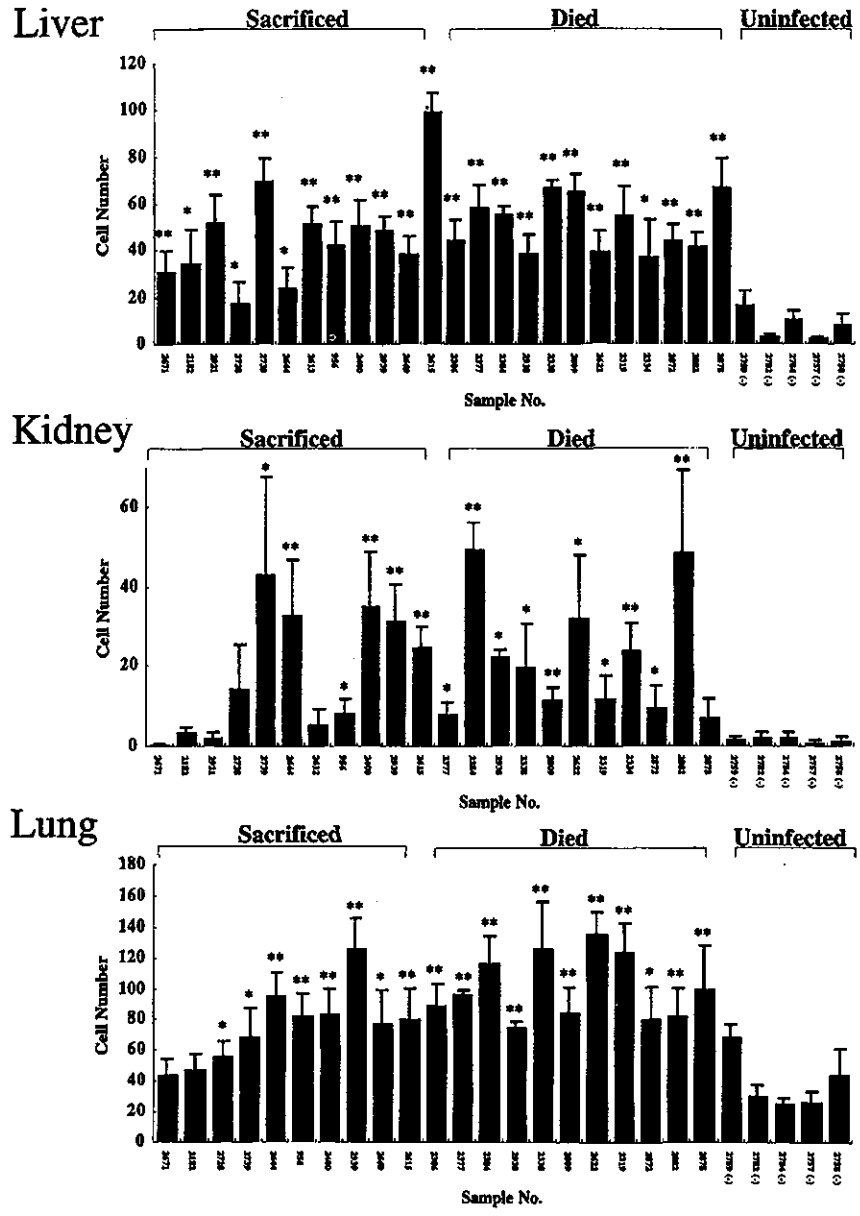

Fig. 3. The increase in leukocyte antigen L1 positive cells in the livers, kidneys and lungs of EBO-R infected macaques. Each column represents the total number of leukocyte antigen L1 positive cells in the fields of the livers, kidneys and lungs from the EBO-R-infected sacrificed macaques, EBO-R-infected dead macaques and non-infected macaques (5 fields at × 50, mean ± SD). *P<0.05, **P<0.01 versus the average of 5 negative controls.

those which died. These 7 sacrificed macaques showed no dramatic lymphoid cell depletion in the white pulp of the spleens which was evident in macaques that died. In addition, EBO-R NP antigens were not detected in the sera from 2 sacrificed macaques (Nos. 2182 and 2921) by antigen-capture ELISA, while EBO-R antigens were detected in the sera from 6 sacrificed macaques (Nos. 2728, 2739, 2644, 2612, 2669 and 2615) (data not shown) [24]. Therefore, the sacrificed macaques examined in this study were thought to be at various stages of the EBO-R infection.

The fibrin deposition in the red pulp of spleen was an

outstanding finding in EBO-R infected macaques in this study. Experimental EBO-R infection in cynomolgus macaques also revealed similar findings [13, 20]. It was reported that monkeys infected with SHFV showed similar fibrin deposition in the red pulp of the spleen [1]. SHFV was also isolated from cynomolgus macaques from the first outbreak of EBO-R in the Philippines [16]. We performed reverse transcription polymerase chain reaction (RT-PCR) to detect SHFV genomic RNA (p15 coding region) from frozen tissues (liver or spleen) of 7 EBO-R-infected monkeys during the same outbreak using primers p15F (5'-GTC CAG AGG AGG GAA TAG GCT-3') and p15R (5'-GCA GCA AAA TTG ATT CTC TGT CCG T-3'). We could not detect any evidence of SHFV infection (data not shown). However, the possibility of SHFV infection could not be excluded in all the EBO-R infected macaques in this study, because we could not examine all the monkey specimens with the RT-PCR. Experimentally induced endotoxemia also resulted in the fibrin deposition in the red pulp cord of the spleens of rhesus monkeys [3, 22]. Thus, fibrin deposition in the spleen may be a common change in macaques that suffer diseases with vessel disorders.

Considering the fibrin thrombi formation in the livers and spleens and fibrin deposition in the spleen in most of the EBO-R infected macaques, systemic coagulopathy seemed to occur in the EBO-R infected macaques. Increased levels of TNF-α, interleukin (IL)-2, IL-10, and interferon (IFN)-α were reported in fatally EBO-Z-infected human patients [2, 31]. Recently, EBO-R infected cynomolgus monkeys were also reported to have increased levels of TNF-α, IFN-γ, IL-2, IL-1β, and IL-6 in the blood [17]. Procoagulant activity in the endothelium was most likely enhanced by these cytokines produced in the process of EBO-R propagation, following tissue destruction and macrophage activation.

Thrombi composed of acidophilic materials and small amounts of fibrin in the kidneys and lungs were only observed in the macaques in which viral antigens were detected in the endothelium. Since these thrombi were immunohistochemically stained with a rabbit polyclonal antibody to CD62P (PharMingen, Co., Ltd. San Diego, USA) specific to platelet and endothelium (data not shown), the thrombi may contain platelets and/or the debris of endothelium as their ingredient.

We confirmed an increase in the number of leukocyte antigen L1 positive macrophages/monocytes in the liver, spleen, kidney and lung of the EBO-R infected macaques. A monoclonal antibody, MAC387, recognizes leukocyte antigen L1 (calprotectin) which is expressed in neutrophils and monocytes [6], and is a useful marker for newly blood-derived macrophages because monocytes gradually lose calprotectin after migration from blood into tissues [25, 27]. It was reported that MPS cells were the primary targets of Ebola virus [10, 28]. Granulomatous inflammation was demonstrated in the liver of guinea pigs experimentally inoculated with EBO-Z [29]. It is of interest to know whether the infected macrophages/monocytes work to gather other macrophages/monocytes by producing cytokine. The increase in the number of macrophages/monocytes in the vessels may also be advantageous to the rapid dissemination of EBO-R.

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Production and immunogenic efficacy of botulinum tetravalent (A, B, E, F) toxoid

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Abstract

A tetravalent (type A, B, E and F) toxoid was produced and its efficacy and safety were assessed. The toxoid preparation was inoculated from two to five times to 15 healthy adult volunteers participating in botulinum toxin research.

The serum samples taken from the toxoid recipients were titrated for the antitoxin potencies by enzyme-linked immunosorbent assay (ELISA) and the neutralization test. The neutralizing and ELISA titers were too low to correlate each other. The mean neutralization titer of four recipients in 9 months after three doses of toxoid was about 0.1 IU/ml for each of the four types, whereas, the one receiving five doses possessed a higher titer. Since the amount of the toxin handled in laboratory work is usually not so large, three or more doses of the present toxoid will bestow sufficient immunity on the workers participating in botulinum research. Nevertheless booster injections might be desirable to those at higher risk, handling the toxin of a high concentration. © 2002 Published by Elsevier Science Ltd.

Keywords: *Clostridium botulinum*; ELISA; Mouse bioassay

1. Introduction

Botulinum toxin, a neurotoxin that selectively attacks peripheral cholinergic nerve endings, is widely recognized as the most potent microbial toxin. Seven distinct serotypes of the toxin have been identified and designated from A through G [1]. Human botulism is classified into the following four types on the basis of the mode of intoxication, food-borne botulism, wound botulism, infant botulism and adult botulism from intestinal colonization [2,3]. In Japan, food-borne botulism outbreaks counted at 86 and patients 351 during the period from 1955 to 1998 [4].

Nowadays, horse immune serum preparations are being used for the treatment of botulism patients. No prophylactics have been commercialized for botulism. In some countries, toxoid has been produced for the military personnel [5–7]. Type E toxoid was produced experimentally for human use and its immunogenic efficacy proved in Japan [8]. We anew produced a tetravalent toxoid (types A, B, E and F) for researchers, and its efficacy and safety were assessed. The serum samples taken periodically from the recipients were titrated by enzyme-linked immunosorbent assay (ELISA) and the neutralization test in mice to assess the antigenicity of the toxoid preparation.

2. Materials and methods

2.1. Production of a tetravalent botulinum toxoid

Clostridium botulinum type A 97, type B Okra (proteolytic), type E German sprats and type F Langeland (proteolytic) were used. The toxins were each purified by the methods described previously [3]. L-lysine hydrochloride was added to 0.05 M and formalin in 0.2% every 4 days to a final concentration of 0.6% to each purified toxin (0.2 mg/ml, type A, B, E and F toxin were containing 3.5, 2.2 (upon tryptic activation), 3.6 (upon tryptic activation), and 0.68×10^6 LD_{50}/ml), detoxification was proceeded at

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30 °C. The formalized toxin was sampled at intervals for mouse inoculation until it became completely nontoxic. The samples were inoculated intraperitoneally in a dose of 0.5 ml into each of two mice, and they were obserbed 7 days. The formalized toxin confirmed nontoxin that all animals do not die due to intoxication, or show any of such specific symptoms of intoxication as spasms, stiffening, or any other abnormal sign during the observation period. The folmalized toxin became completely nontoxic in 24 days. The toxoid was kept at 4 °C for further 21 days, dialyzed against PBS (pH 6.0), and the toxicity reversion test was performed in mice. A sample of each toxoid was kept for 20 days at 37 °C before injecting intraperitoneally into guinea pigs, which were observed for 50 days. No aberration was found, and all toxoid preparations were regarded as passing the test. The toxoid preparations of the four types were mixed together, which was adsorbed to aluminum gel, and thimerosal was added to it. Since there was no standard for botulinum toxoid, that standard for absorbed tetanus toxoid was applied to this preparation [9]. The following tests were conducted, pH, aluminum content, thimerosal content, formaldehyde content, sterility, freedom from abnormal toxicity in guinea pigs, detoxification in guinea pigs, and potency in mice and guinea pigs. Test for freedom from abnormal toxicity was carried out in two guinea pigs. They were injected intraperitoneally with 5 ml of a sample product. This examination conformed when it was observed for 7 days and there was not significant weight decrease of the guinea pig. The detoxification test was conducted on the test sample and that kept standing at 37 °C for 20 days. The sample was injected subcutaneously at a dose of 5 ml into three guinea pigs, and they were observed for 21 days. This examination conformed that all animals did not show their remarkable weight decrease, the toxic symptoms and other abnormal sign during the observation period. The potency test was carried out by antitoxin titration and toxin challenge. The undiluted toxoid and its twofold and 4-fold dilutions were each injected subcutaneousely in a dose of 0.4 ml into each of five guinea pigs twice at a 2 week interval. Blood samples were taken from the guinea pigs 5 weeks after the first injection. The antitoxin titers higher than 0.1 IU/ml were regarded as passing the test. In the toxin challenge test, the immunized guinea pigs were injected with each toxin of 10 LD_{50} 3 days after blood collection and observed for 3 weeks. If no aberration was found, the toxoid was regarded as passing the test. The toxoid met all the requirements, proving its effectiveness and safety.

2.2. Collecting blood samples from toxoid recipients

The toxoid was inoculated to 15 healthy adult volunteers, who were participating in botulinum toxin research. All of them gave written informed consents and received physical examinations. The toxoid was injected intramuscularly in 0.5 ml doses to healthy individuals only. The immunization schedule was based on the current dosing schedule for adsorbed tetanus toxoid, the inoculation of three times at a 4–8 week interval. The serum antitoxin was titrated by ELISA and the toxin neutralization test in mice.

The immunization schedules were grouped into three based on the circumstances of the volunteers (Tables 3 and 4). Four volunteers (a–d) of group A were immunized three times in 0, 4–6, and 20 weeks. Blood samples were collected 2–12 weeks after the second and 2 weeks after the third injections. The samples taken after the second injection were titrated by ELISA, and those after the third injection by the neutralization test in mice. Ten volunteers (e–n) of group B were inoculated two to four times at 4–5 week intervals. Serum samples were collected 3 weeks after the last injection. The single volunteer (o) of group C was injected five times. The volunteer was immunized in 0, 4, 6 weeks, 79 and 90 months. The sample taken 2 weeks after the fourth inoculation was titrated by ELISA, and 2 weeks after the fifth inoculation by the neutralization test. In addition, blood sample were taken 9 and 12 months after the last inoculation from all volunteers of groups A and C to find the periodical change of the antibody titer by the neutralization test.

2.3. Antitoxin titration by ELISA and the neutralization test

ELISA was performed in 96-well microtiter plates with purified botulinum type A, B, E and F toxins as the capture antigen. Each well received 100 μl of purified toxin diluted to 1 μg/ml in carbonate buffer. The plates were incubated for 2 h at 37 °C and washed five times with PBS containing 0.05% Tween 20. Toxin-unbound wells were each blocked by addition of 200 μl of 3% bovine serum albumin in phosphate-buffered saline, pH 6.0 (PBS). After overnight incubation at 4 °C, the plates were washed five times with PBS containing 0.05% Tween 20. Human serum samples were diluted twofold serially and 100 μl of each dilution was added to a well. After incubation for 2 h at room temperature, 100 μl per well of 20,000-fold diluted goat anti-human IgG conjugated with horseradish peroxidase was added. After incubation for 2 h at room temperature, *o*-phenylendiamine was added as a chromogenic substrate. The plates were incubated at room temperature for 45 min and the absorbance at 450 nm was measured with a microplate reader. The ELISA titer was expressed in the highest dilution factor showing an absorbance higher than double of negative control serum.

Neutralizing antibodies to botulinum type A, B, E and F toxins were titrated by mouse bioassay with each standard antitoxin [9]. The standard antitoxins were diluted in gelatin PBS (pH 6.0) so as to contain 0.032, 0.04, 0.05, 0.063, and 0.08 units per 0.25 ml. Each human serum sample was diluted in the same manner as to contain approximately the same units per 0.25 ml. The test toxin corresponding to each standard antitoxin was diluted so as to contain one test dose (a dose of the toxin, when mixed with 0.05 IU of antitoxin, killing half of the mice weighing about 16 g in 72 h when injected intraperitoneally) per 0.25 ml. One IU of corresponding type of antitoxin neutralizes 10,000 mouse

intraperitoneally LD_{50} of toxin types A, B, F or 1000 mouse intraperitoneally LD_{50} of toxin type E [2]. Equal quantities of each dilution of standard antitoxin or a test serum sample and the test toxin dilution were mixed well. The mixtures were allowed to stand for 1 h and 0.5 ml doses were injected intraperitoneally into two mice weighing about 16 g. The injected mice were observed for 3 days. The titer was expressed in relation to that of the standard antitoxin.

3. Result

3.1. Toxoid production

C. botulinum types A, B, E, and F were each cultured and their toxins were purified [3]. The purified toxins comprised L and LL toxin for type A, L toxin only for type B, and M toxin only for types E and F.

The toxoid preparation passed all the tests performed in accordance with the minimum requirements, and its properties are shown in Tables 1 and 2. The toxoid preparation contained 0.1 mg of the toxoid of the four types, 0.23 mg of aluminum, 0.011% thimerosal, and 0.0006% formalin per ml.

3.2. Titration for antitoxin by ELISA and the neutralization test

The toxoid was inoculated two to five times to 15 volunteers. The serum samples taken from them were titrated for the antitoxin by ELISA and the neutralization test.

Table 1
The properties of the toxoid preparation

Protein (mg/ml)	0.10
Formaldehyde (%)	0.0006
Thimerosal (%)	0.011
pH	5.67
Aluminium	0.23
Sterility test	Passed
Test for freedom from abnormal toxicity	Passed
Detoxification test	Passed

Table 2
Potency test by antitoxin neutralization

Antitoxin type	IU/ml[a]			Final decision
	1	2	4	
Type A	16.0	13.6	4.8	Passed
Type B	5.6	1.4	1.4	Passed
Type E	3.4	1.4	0.4	Passed
Type F	4.0	2.0	1.2	Passed
Toxin challenge test[b]	Passed	Passed	Passed	Passed

[a] All dilutions are passed the challenge test.
[b] Antitoxin titer higher than 01 IU/ml is regarded as passed.

For ELISA, each purified botulinum toxin was used as the capture antigen (Table 3). No difference was recognized in the ELISA titer due to the immunization schedule. The six volunteers (f–k) in group B receiving three injections gave ELISA titers of 11–15 (average 12.7) for type A. The antitoxin titers for types B, E and F were 10–14 (average 12.2), 10–12 (average 10.8), and 10-14 (average 12.5), respectively. The seven volunteers immunized twice, four (a–d) in group A and three (l–n) in group B, showed somewhat lower titers for type E than for type F.

Serum samples of 10 persons injected three times, and one person each injected two, four times, or five times were subjected to titration by the neutralization test (Table 4). The antibody titers of the one injected twice were 0.4 IU for both types A and B. The sample volumes of group B were too small to titrate for type E and F antitoxins. The type A titers of those immunized three times (a–d, f–k) were 0.12–8.9 IU (average 1.6 IU). The type B titers of six persons (a–d, f–g) were 0.07–0.8 IU (average 0.5 IU), those of the other four (h–k) were below the minimum detective level. The type E and F titers of four persons (a–d) were 0.4–3.2 IU (average 1.4 IU) and 0.8–1.4 IU (average 1.1 IU), respectively. The type A, B, E and F antibody titers of the one inoculated four times (e) were 0.7, 0.2, 0.4, and 0.3 IU, respectively. The antibody titers of the one immunized five times (o) were 4, 2, 16, and 5.6 IU, respectively (Table 4).

A scattergram was depicted to correlate the neutralization and ELISA titers (Fig. 1). No correlation can be seen between these titers ($r = 0.03$).

Then, to follow the trend of neutralization titers after immunization with the toxoid, serum samples were collected from five recipients of groups A and C, 2 weeks, 9 months and 12 months after the final injection and titrated for the neutralizing antitoxin (Table 5). Type A antitoxin titers were higher than those of the other types and such higher titers persisted. The neutralization titers for type B were lower than those for the other types. The one inoculated toxoid five times maintained a high antibody titer throughout the experimental period.

The decreasing rates of the neutralization titers were compared. In the four persons inoculated three times, the antitoxin titers of all types decreased by 90% from 2 weeks to 9 months after the last injection. The mean titer 9 months after the toxoid inoculation became to about 0.1 IU/ml.

4. Discussion

Recently, studies on botulinum toxin, which is utilized for treating dystonia, have been made in many laboratories and advanced a great deal [10]. Practical use and laboratory handling of botulinum toxin have become customary, and avoidance of biohazard has become important. Especially, adequate treatment is required in case of emergency. Serum therapy has successfully been applied to botulism patients [2]. However, the effect of antitoxic serum is lessened if

Table 3
ELISA titers of serum of human volunteers vaccinated with botulinum tetravalent toxoid

Group	Frequency of injection	Volunteers	ELISA titers (log 2)			
			Type A	Type B	Type E	Type F
A	2	a	11	10	10	12
		b	11	9	<6	13
		c	8	9	<6	11
		d	<6	<6	<6	7
B	4	e	15	14	12	15
	3	f	15	13	11	14
		g	11	11	12	12
		h	15	14	11	14
		i	12	10	11	13
		j	12	12	10	12
		k	11	13	10	10
	2	l	13	12	11	13
		m	9	11	9	11
		n	8	9	12	14
C	4	o	8	11	13	15

administration is delayed. Vaccination with botulinum toxoid has been carried and the effectiveness proved in some foreign countries [5–7,11,12]. Type E toxoid has experimentally been produced in Japan [8]. Although the effects of polyvalent toxoid have not been assessed in Japan, tetravalent toxin was produced this time for protecting botulinum toxin research workers and its effectiveness and safety were demonstrated. Since no standard for botulinum toxoid is available in Japan, the minimum requirements for tetanus toxoid were followed in producing botulinum toxoid. It was confirmed that the toxoid met all the requirements. When the toxoid was injected into humans, some recipients developed slight local reddening and swelling, but no other untoward reactions developed.

According to the previous report, it did not have the correlation of ELISA and neutralization titers in the condition of the low titer [13–15]. We could confirm that it did not have their correlation in low titer like this experiment, too. This seemed to happen, since it has the property in which botulinum toxin of low concentrate is difficult to combine with antitoxin of low titer. This property is seen remarkably in ELISA. However, the ELISA titers were correlated to the neutralizing titers with serum samples of higher potencies [6,7,12]. It seem that ELISA is suitable for the detection of the high concentration, and detection is possible for neutralization test at all concentration. Since it was considered that the neutralization titer was more reliable than the ELISA titers with serum samples of low antitoxin potencies, subsequent titrations were conducted by the neutralization test.

The Centers for Disease Control reported that type F antibody titers over 0.02 IU/ml may assure sufficient protection and recommended a titer higher than 0.25 IU/ml

Table 4
Neutralization titers of serum of human volunteers vaccinated with botulinum tetravalent toxoid

Group	Frequency of injection	Volunteers	Neutralization titers (IU/ml)			
			Type A	Type B	Type E	Type F
A	3	a	1.6	0.6	0.4	1.6
		b	8.9	0.8	3.2	0.8
		c	2.3	0.6	1.6	1.1
		d	1.1	0.8	0.4	0.8
B	4	e	0.7	0.2	0.4	0.3
	3	f	0.4	0.4	ND[a]	ND
		g	0.12	0.07	ND	ND
		h	0.4	<0.1	ND	ND
		I	0.2	<0.05	ND	ND
		j	0.2	<0.2	ND	ND
		k	0.34	<0.1	ND	ND
	2	l	0.4	0.4	ND	ND
C	5	o	4	2	16	5.6

[a] ND: No data.

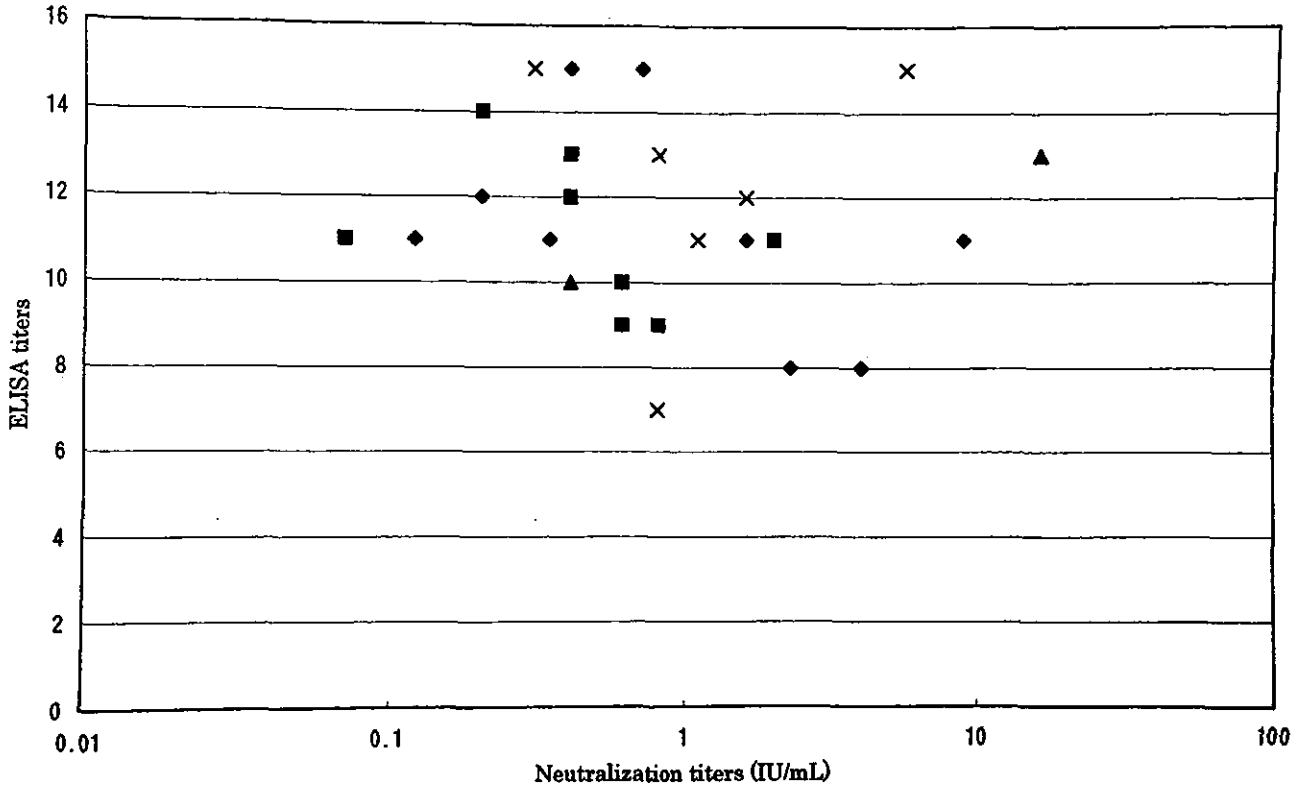

Fig. 1. Relationship between neutralization and ELISA titer of serum samples of volunteers vaccinated with botulinum tetravalent toxoid. Symbols: (●) type A, (■) type B, (△) type E, (×) type F. Relation was not recognized in two titers ($r = 0.03$).

Table 5
Time course of neutralization titer (IU/ml) after the last immunization

Toxin	Group	Frequency of injection	Volunteers	In second weeks	In nineth months	In twelth months
Type A	A	3	a	1.6	0.1	0.07
		3	b	8.9	0.7	0.56
		3	c	2.3	0.125	0.05
		3	d	1.1	0.1	0.07
	C	5	o	4.0	5.7	5.6
Type B	A	3	a	0.6	0.05	<0.05
		3	b	0.8	0.1	0.14
		3	c	0.6	0.05	0.07
		3	d	0.8	<0.03	<0.05
	C	5	o	2.0	1.0	0.7
Type E	A	3	a	0.4	0.05	<0.03
		3	b	3.2	0.4	0.2
		3	c	1.6	0.2	0.1
		3	d	0.4	0.05	<0.03
	C	5	o	16	16	8.0
Type F	A	3	a	1.6	0.05	0.05
		3	b	0.8	0.07	0.07
		3	c	1.1	0.07	0.04
		3	d	0.8	<0.02	<0.02
	C	5	o	5.6	2.8	2.0

for those at high risk [16]. The mean titer of four volunteers inoculated three times and titrated 9 months after the last injection was about 0.1 IU/ml. The volunteer inoculated five times maintained higher neutralization titers. When the amount of the toxin handled at a time by the laboratory workers is not so large, this antitoxin titer might be sufficient to protect them from botulism even in an emergency. It was found that the more frequently the toxoid is inoculated, the higher and the longer the immunity will be. We recommend continual frequent inoculations of a toxoid preparation like the present one and periodic titration of antitoxin potencies for those who work in laboratories handling *C. botulinum* toxin and organisms of high concentration.

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An essential virulence protein of *Brucella abortus*, VirB4, requires an intact nucleoside-triphosphate-binding domain

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***Brucella abortus* is a facultative intracellular bacterium capable of surviving inside macrophages. The VirB complex, which is highly similar to conjugative DNA transfer apparatuses, is required for intracellular replication. A conserved *NTP-binding domain* in VirB4 suggests that one or both proteins couple energy by NTP hydrolysis to transport of putative effector molecule(s). Here it is shown that a mutant strain of *B. abortus* that contains an in-frame deletion in *virB4* is unable to replicate in macrophages and survives in mice. Intracellular replication and virulence in mice are fully restored by expressing *virB4 in trans*, indicating that VirB4 is essential for intracellular replication and virulence in mice. An alteration within the NTP-binding region of VirB4 by site-directed mutagenesis abolished complementation of a *virB4* mutant, demonstrating that an intact NTP-binding domain is critical for VirB4 function. Intracellular replication was inhibited in wild-type *B. abortus* after introducing a plasmid expressing a mutant VirB4 altered in the NTP-binding region. The dominant negative phenotype suggests that VirB4 either functions as a multimer or interacts with some other component(s) necessary for intracellular replication. Wild-type *B. abortus*-containing phagosomes lack the glycoprotein LAMP-1, which is an indicator of the normal endocytic pathway. Mutant strains were found in phagosomes that co-localized with LAMP-1, indicating that VirB4 containing the intact NTP-binding region is essential for evasion of fusion with lysosomes.**

Keywords: type IV secretion, macrophage

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 (Acha & Szylres, 1980). The bacterium is endemic in many underdeveloped countries and is responsible for large economic losses and chronic infections in human beings (Zavala *et al.*, 1994). *Brucella* spp. are facultative intracellular pathogens that survive in a variety of cells, including macrophages, and their virulence and ability to cause chronic infections are thought to be due to their ability to avoid the killing mechanisms within macrophages (Baldwin & Winter, 1994; Sangari & Aguero, 1996). The molecular mechanisms of their virulence and infection are incompletely understood. Studies with HeLa cells have confirmed the observations that *Brucella* inhibits phagosome–lysosome fusion and transits through an intracellular compartment that resembles an autophagosome. Bacteria replicate in a different compartment, containing protein markers normally associated with the endoplasmic reticulum, as shown by confocal microscopy and immunogold electron microscopy (Comerci *et al.*, 2001; Pizarro-Cerda *et al.*, 1998a, b).

Genetic loci encoding export mechanisms specializing in transferring a variety of multimolecular complexes across the bacterial membrane to the extracellular space or into other cells have been described. These complexes, named type IV secretion systems, have been reported in organisms such as *Agrobacterium tumefaciens* (*virB* genes) (Kuldau *et al.*, 1990; Stachel & Nester, 1986), *Bordetella pertussis* (*ptl* genes) (Kotob *et al.*, 1995; Weiss *et al.*, 1993), *Escherichia coli* (*tra* genes) (Pohlman *et al.*, 1994; Winans & Walker, 1985), *Legionella pneumophila* (*dot/icm* genes) (Segal *et al.*, 1998, Vogel *et al.*, 1998) and *Helicobacter pylori* (*cag* genes) (Covacci *et al.*,

1999). Recently, the *virB* operon of *Brucella* has been identified (O'Callaghan *et al.*, 1999; Sieira *et al.*, 2000). This operon comprises 13 ORFs, designated *virB1* to *virB11*, *orf12* and *orf13*, that share homology with components of other bacterial type IV secretion systems involved in the intracellular trafficking of pathogens. Polar mutations introduced in the first gene of the operon, *virB1*, abolish the ability of *Brucella* to replicate intracellularly, indicating that this system is essential for the intracellular lifestyle of this pathogen. Mice infected with polar and non-polar mutations in *virB10* demonstrated that the *virB* operon is a major determinant of *Brucella* virulence (Sieira *et al.*, 2000). Thus, *Brucella abortus* VirB proteins are thought to be constituent elements of the secretion apparatus, but their specific molecular functions are unknown.

Two *virB* proteins, VirB4 and VirB11, contain the putative NTP-binding site (Sieira *et al.*, 2000) first described by Walker *et al.* (1982). Nucleotide-binding proteins might have several roles in the secretion process, including providing energy for transport or signalling the opening of a gate or channel by a kinase activity. In this study, we examined one of these putative nucleotide-binding proteins, VirB4, to determine its importance in intracellular replication and the role that the putative NTP-binding site might play in the *Brucella* virulence.

METHODS

Bacterial strains and media. All *Bru. abortus* derivatives are from strain 544, which is a smooth virulent *Bru. abortus* biovar 1 strain (Table 1). *Bru. abortus* strains were maintained as frozen glycerol stocks and were cultured on Brucella broth (BBL) or Brucella broth containing 1·5% agar.

Initial isolation of replication-proficient plasmids was from *E. coli* strain DH5α. For the propagation of suicide plasmids requiring R6K π protein, *E. coli* strain DH5α (λ*pir*) (Kolter *et al.*, 1978) was used. Kanamycin was used at 40 μg ml^{-1}.

Construction of in-frame deletion mutant of *virB4*. pMAW14 (Δ*virB4*) was constructed by cloning two PCR fragments into *Sal*I/*Sac*I-cleaved pSR47s (Andrews *et al.*, 1998). Fragment 1 was a 1640 bp *Sal*I–*Bam*HI fragment spanning a site located 1620 nt upstream of the 5′ end of *virB4* to 20 nt downstream from the 5′ end and was amplified by PCR using primers 5′-GTCGACCAAGTCAACAGGTACAATCTG-3′ (*Sal*I site underlined) and 5′-GGATCCGCCCATTATGATTCTCTT-TTG-3′ (*Bam*HI site underlined) (nucleotide positions 690 and 2290 in GenBank accession no. AF226278, respectively; Sieira *et al.*, 2000). Fragment 2 was a 1617 bp *Bam*HI–*Sac*I fragment spanning the region starting 6 nt upstream of the 3′ end of *virB4* to a position 1611 nt downstream from the 3′ end and was amplified using primers 5′-GGATCCAGGTGAC-ACTATGAAGAAGAT-3′ (*Bam*HI site underlined) and 5′-GAGCTCGCGGCTGAAGATTGCACTCGA-3′ (*Sac*I site underlined) (nucleotide positions 4785 and 6385 in AF226278, respectively; Sieira *et al.*, 2000).

pMAW14 (Δ*virB4*) was introduced into DH5α (λ*pir*), and subsequently the plasmid was transferred into *Bru. abortus* 544 by using electroporation (Gene Pulser; Bio-Rad). Cells were spread onto Brucella agar containing 5% sucrose and incubated at 37 °C to isolate recombinants in which the entire plasmid integrated into the appropriate chromosomal site. The resulting sucrose-resistant colonies were tested for kanamycin sensitivity which indicates loss of the suicide vector. The kanamycin-sensitive colonies thus selected were analysed to confirm that in-frame deletion had occurred in the *virB4* gene by PCR amplification.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and relevant characteristic(s)	Source or reference
Bru. abortus		
544	Biovar 1 virulent strain	ATCC 23448
Ba598	544 Δ*virB4*	This study
Ba603	Ba598(pMAW15)	This study
Ba615	544(pMAW18)	This study
Ba616	Ba598(pMAW18)	This study
Ba620	544(pMAW15)	This study
E. coli		
DH5α	*supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Woodcock *et al.* (1989)
DH5α λ*pir*	DH5α (λ*pir*) *tet*::Mu *recA*	Kolter *et al.* (1978)
Plasmids		
pSR47s	*ori*TRP4 *ori*R6K *kan sacB*	Andrews *et al.* (1998)
pMAW14	pSR47s Δ*virB4*	This study
pBBR1MCS-2	Broad-host-range cloning vector	Kovach *et al.* (1995)
pMAW15	pBBR1MCS-2 *virB4*$^+$	This study
pKF19k	Vector for site-directed mutagenesis	TAKARA
pMAW16	pKF19k *virB4*$^+$	This study
pMAW17	pKF19k *virB4* K463R	This study
pMAW18	pBBR1MCS-2 *virB4* K463R	This study

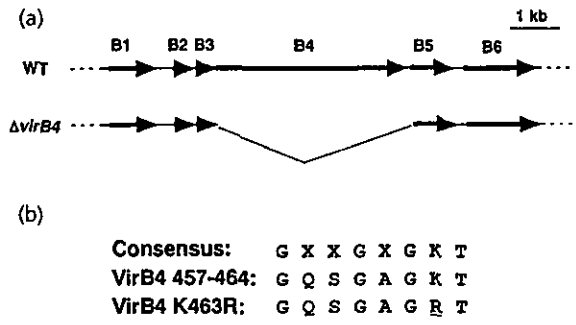

Fig. 1. Genetic map of the *virB4* flanking region and alteration of the putative nucleoside-binding region of VirB4. (a) Construction of an in-frame deletion mutation is shown for Δ*virB4*. Nucleotides are numbered as described previously by Sieira *et al.* (2000). (b) The consensus Walker box A region is shown with the corresponding region from VirB4 (aa 457–464). The K463R alteration made by site-directed mutagenesis is underlined.

pMAW15 (*virB4*$^+$) was constructed by cloning a PCR fragment into *Eco*RI/*Bam*HI-cleaved pBBR1MCS-2 (Kovach *et al.*, 1995). The 2786 bp *Eco*RI–*Bam*HI PCR fragment spanned a site located 270 nt upstream of the 5′ end of *virB4* to a position 20 nt downstream from the 3′ end (Sieira *et al.*, 2000) and was amplified using the primers 5′-GAATTCAGCCATG-TTGTTTGGGGTTCC-3′ (*Eco*RI site underlined) and 5′-GGATCCAGAATTATCTTCTTCATAGTG-3′ (*Bam*HI site underlined).

Construction of a point mutation in the NTP-binding domain of VirB4. A point mutation was made in the coding sequence (AAA; 1387–1389) for the invariant Lys_{463} residue (K463) within the Walker-type NTP-binding motif of VirB4 (Fig. 1). pMAW16 was constructed by cloning a 2786 bp *Eco*RI–*Bam*HI fragment from pMAW15 into *Eco*RI/*Bam*HI-cleaved pKF19k for site-directed mutagenesis (TAKARA). The coding sequence for Lys_{463} (AAA) was mutated to encode Arg (AGA) using the site-directed mutagenesis system Mutan-Express Km (TAKARA) and mutagenic oligonucleotide 5′-CAGGACAG-TTCTACCAGCGCC-3′ which includes the codon that alters Lys_{463} to Arg (K463R). The point mutation within this region of *virB4* was confirmed by DNA sequence analysis and the resultant plasmid was designated pMAW17. pMAW18 (*virB4* K463R) was constructed by cloning a 2786 bp *Eco*RI–*Bam*HI fragment from pMAW17 into *Eco*RI/*Bam*HI-cleaved pBBR1MCS-2.

Cell culture. Bone-marrow-derived macrophages from female BALB/*c* mice were prepared as described by Watarai *et al.* (2001). After culturing in L-cell conditioned medium, the macrophages were replated for use by lifting cells in PBS on ice for 5 to 10 min, harvesting by centrifugation and resuspending in RPMI 1640 containing 10% fetal bovine serum. The mouse macrophage-like cell line J774 was maintained in RPMI 1640 containing 10% fetal bovine serum. The macrophages were seeded (2×10^5–3×10^5 per well) in 24-well tissue culture plates for all assays.

Determination of efficiency of intracellular growth of bacteria. Bacteria were deposited onto macrophages at an m.o.i. of 20 by centrifugation at 150 *g* for 5 min at room temperature and were then incubated at 37 °C in 5% CO_2 for 1 h. The macrophages were then washed once with medium and incubated with 30 μg gentamicin ml^{-1}. At different time points, cells were washed and lysed with 1% Triton X-100 and the number of bacteria was counted in plates of a suitable dilution.

Detection of bacteria in macrophages. Mouse bone-marrow-derived macrophages were infected with *Bru. abortus* as described above. Infected monolayers were washed extensively with fresh medium to remove non-cell-associated *Bru. abortus*. Infected cells were fixed in periodate/lysine/paraformaldehyde (PLP) (McLean & Nakane, 1974) containing 5% sucrose for 30 min at 37 °C, washed three times in PBS and permeabilized in 0·1% Triton X-100 for 30 min at room temperature. Samples were then washed three times in blocking buffer (2%, v/v, goat serum in PBS) for 5 min and stained with anti-*Bru. abortus* rabbit serum (diluted 1:1000) in blocking buffer for 1 h at 37 °C. To visualize antibodies, samples were washed three times in blocking buffer, incubated with FITC-conjugated goat anti-rabbit IgG (Zymed) diluted 1:500 in blocking buffer for 1 h at 37 °C and visualized in mounting medium (90% glycerol containing 1 mg phenylenediamine ml^{-1} in PBS, pH 9·0).

LAMP-1 staining. Infected macrophages were fixed in PLP-sucrose for 1 h at 37 °C. Samples were washed three times in PBS and wells were successively incubated three times for 5 min in blocking buffer (2% goat serum in PBS) at room temperature. All antibody-probing steps were carried out for 1 h at 37 °C in a humidified incubator. After blocking, samples were stained with anti-*Bru. abortus* polyclonal rabbit serum diluted 1:1000 in blocking buffer to identify extracellular bacteria. Samples were washed three times for 5 min with blocking buffer, stained with Cascade-Blue-conjugated goat anti-rabbit IgG diluted 1:500 in blocking buffer and incubated as above. Samples were washed three times in PBS for 5 min and then permeabilized in −20 °C methanol for 10 s. After incubating three times for 5 min with blocking buffer, samples were stained with anti-LAMP-1 rat mAb ID4B (obtained from Developmental Studies Hybridoma Bank of the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA, and the Department of Biology, University of Iowa, Iowa City, IA, USA) diluted 1:100 in blocking buffer (Swanson & Isberg, 1996). After washing three times for 5 min in blocking buffer, samples were stained simultaneously with TRITC-conjugated goat anti-rat IgG (Molecular Probes). Samples were placed in mounting medium and visualized by fluorescence microscopy.

Fluorescence microscopy. The specimens were analysed using an Olympus IX70 inverted phase microscope and the images were collected using a cooled CCD camera (CoolSNAP; Roper Scientific) and processed using Openlab software (Improvision) on a Power Macintosh G4 computer.

Virulence in mice. Virulence was determined by quantifying the survival of the strains in the spleen after 10 days. Six-week-old female BALB/c mice were injected intraperitoneally with approximately 10^4 c.f.u. brucellae in 0·1 ml saline. Groups of five mice were injected with each strain. At 10 days post-infection the mice were sacrificed by decapitation and their spleens were removed, weighed and homogenized in saline. Tissue homogenates were serially diluted with PBS and plated on Brucella agar to count the number of c.f.u. in each spleen.

RESULTS

Effect of alteration of the putative NTP-binding region of VirB4 on intracellular replication

To investigate the role of the *virB4* gene in intracellular replication in macrophages, an in-frame deletion mutation was constructed in the *virB4* gene. This deletion mutation was designated Δ*virB4* and the mutant was designated Ba598 (Fig. 1a). As survival and multipli-

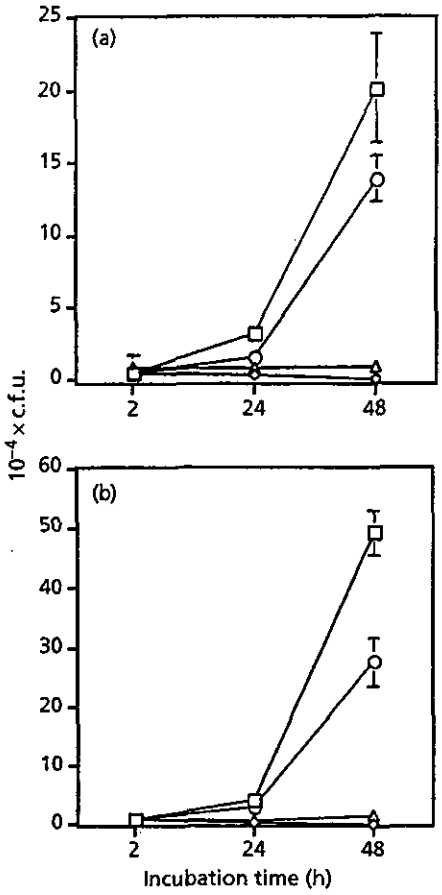

Fig. 2. Intracellular replication of *Bru. abortus* strains in J774 cells and mouse bone-marrow-derived macrophages. J774 cells (a) and mouse bone-marrow-derived macrophages (b) were infected with 544 (wild-type, □), Ba598 (Δ*virB4*, ◇), Ba603 (*virB4*$^+$, ○) or Ba616 (*virB4* K463R, △) for the indicated time. Data points and error bars represent the mean c.f.u. of triplicate samples from a typical experiment (performed at least four times) and their standard deviation.

cation in macrophages is an important virulence attribute of *Brucella*, we examined the intracellular replication of *Brucella* strains in two mouse macrophage models, using macrophage-like cell line J774 and bone-marrow-derived macrophages. J774 or mouse bone-marrow-derived macrophages were infected for various periods at an m.o.i. of 20. The results show that Ba598 (Δ*virB4*) failed to replicate in both J774 and bone-marrow-derived macrophages (Figs 2 and 3). The mutation was complemented *in trans* by introducing plasmid pMAW15, which carries a wild-type copy of *virB4* (Ba603). Introduction of the complementing plasmid into Ba598 (Δ*virB4*) restored replication to 544 (wild-type) levels (Fig. 2).

To determine whether the putative NTP-binding region of VirB4 plays a role in intracellular replication, we used site-directed mutagenesis to specifically alter *virB4* such that the encoded Lys_{463} located in the Walker box A region of the protein is changed to an Arg residue (Fig. 1b). We introduced a plasmid, pMAW18, containing full-length *virB4* with that specific mutation into Ba598 (Δ*virB4*) and the transformant was designated Ba616 (*virB4* K463R). Ba616 failed to restore replication to 544 (wild-type) levels (Fig. 2). These results implied that the putative NTP-binding region of VirB4 is necessary for intracellular replication of *Brucella*.

The VirB proteins have been proposed to interact with other VirB proteins to form a multicomponent membrane apparatus which can mediate export of putative substrate(s) (Christie, 2001). If VirB4 constitutes one of the transporter subunits, or if the protein functions as a multimer, then a merodiploid expressing both the wild-type and mutant allele may differ phenotypically from 544 (wild-type). To test this possibility, we introduced pMAW18 (*virB4* K463R) into the wild-type strain (Ba615) and then examined the ability of Ba615 to replicate in macrophages. Bone-marrow-derived macrophages were infected for 48 h at an m.o.i. of 20. Introducing a control plasmid containing either no *virB4* or a wild-type copy of *virB4* did not affect intracellular replication ($5{\cdot}1 \times 10^5 \pm 0{\cdot}5 \times 10^5$ or $5{\cdot}4 \times 10^5 \pm 0{\cdot}8 \times 10^5$ c.f.u., respectively). In contrast, introducing a plasmid containing the mutated *virB4* into 544 (wild-type) resulted in greatly reduced intracellular replication ($7{\cdot}6 \times 10^4 \pm 0{\cdot}7 \times 10^4$ c.f.u.). These data indicated that the *virB4* K463R mutant exhibits a dominant negative phenotype.

The putative NTP-binding region of VirB4 is required for inhibition of phagosome–lysosome fusion by *Bru. abortus* early in infection

Phagosomes containing virulent *Bru. abortus* are reluctant to fuse with lysosomes, whereas dead *Bru. abortus* phagosomes co-localize with endocytic compartments in the early stage of infection in macrophages (Arenas *et al.*, 2000). To test the ability of *Bru. abortus* to target properly within bone-marrow-derived macrophages early in infection, interaction of the mutants with the endocytic pathway was quantified by immunofluorescence localization of LAMP-1, a membrane protein of late endosomes and lysosomes (Chen *et al.*, 1988; Harter & Mellman, 1992).

As expected, most phagosomes containing 544 (wild-type) did not co-localize with the late endosomal and lysosomal marker: $14{\cdot}3 \pm 3{\cdot}2$% of the wild-type phagosomes were LAMP-1-positive (Fig. 4). In contrast, phagosomes containing Ba598 (Δ*virB4*) with severe intracellular growth defects were frequently stained brightly by an antibody specific for LAMP-1 ($74{\cdot}6 \pm 5{\cdot}3$% LAMP-1-positive) (Fig. 4). Introduction of the complementing plasmid into Ba598 (Δ*virB4*) restored evasion of the endocytic pathway to 544 (wild-type) levels ($17{\cdot}0 \pm 2{\cdot}9$% LAMP-1-positive). To determine whether the putative NTP-binding region of VirB4 plays a role in intracellular trafficking, interaction of Ba616 (*virB4* K463R) with the endocytic pathway was quantified. Similar results were obtained for the *virB4* mutant: $76{\cdot}1 \pm 2{\cdot}9$% of the Ba616 (*virB4* K463R) cells resided in

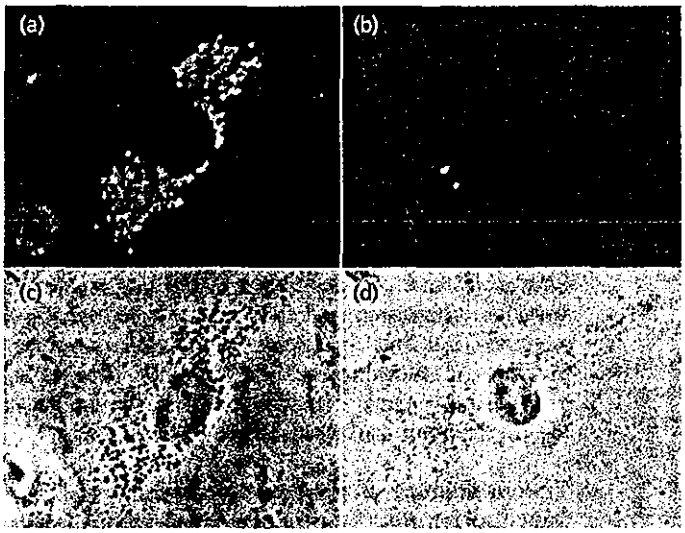

Fig. 3. Immunofluorescence micrograph of intracellular replicated *Bru. abortus*. Mouse bone-marrow-derived macrophages were infected with 544 (*wild-type*; a, c) or Ba598 (Δ*virB4*; b, d) for 48 h, as described in Methods. (a, b) FITC-labelled intracellular bacteria; (c, d) phase-contrast images.

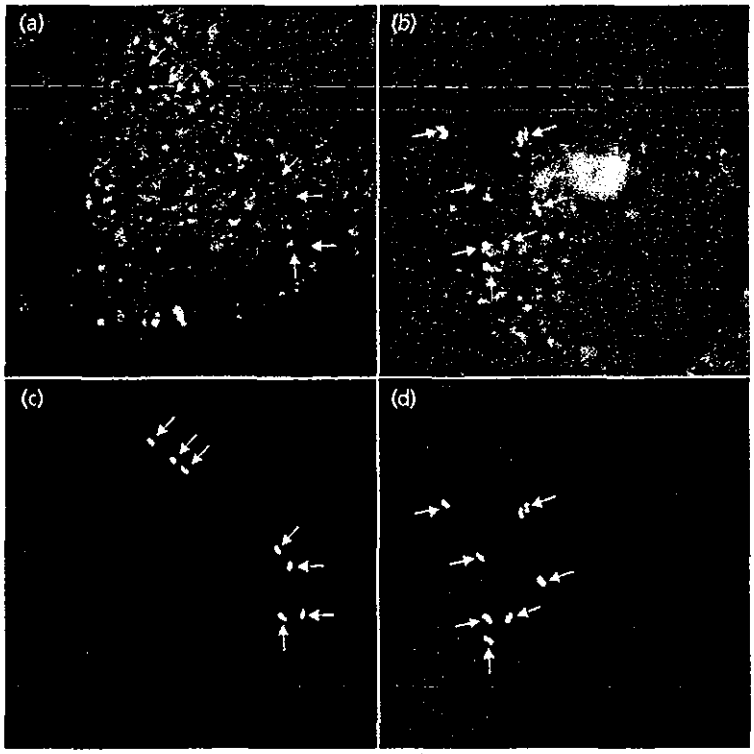

Fig. 4. Co-localization of *virB4 mutant* with late endosomal and lysosomal marker LAMP-1 in mouse bone-marrow-derived macrophages by immunofluorescence microscopy. Macrophages were infected with 544 (wild-type; a, c) or Ba598 (Δ*virB4*; b, d) for 1 h, fixed and stained for LAMP-1 co-localization (a, b) and intracellular bacteria (c, d).

a LAMP-1-positive compartment. Introducing a control plasmid containing either no *virB4* or a wild-type copy of *virB4* did not affect LAMP-1 staining (12·5±2·7 or 13·5±2·1 %, respectively). In contrast, introducing a plasmid containing the mutated *virB4* into 544 (wild-type) resulted in 73·9±2·3 % LAMP-1-positive phagosomes.

The putative NTP-binding region of VirB4 is essential for virulence in mice

Groups of five mice were injected intraperitoneally with 10^4 c.f.u. of *Bru. abortus* 544 (wild-type), Ba598 (Δ*virB4*), Ba603 (*virB4*$^+$) or Ba616 (*virB4* K463R). At 10 days post-inoculation, mice were sacrificed and their spleens were weighed and examined for *Brucella* proliferation. The number of viable bacteria recovered from the spleens of mice injected with 544 (wild-type) and Ba603 (*virB4*$^+$) were $3{\cdot}8\times10^7\pm1{\cdot}4\times10^7$ and $8{\cdot}6\times10^7\pm0{\cdot}7\times10^6$ c.f.u. per spleen, respectively. On the other hand, no viable bacteria were recovered from mice injected with Ba598 (Δ*virB4*) and Ba616 (*virB4* K463R), based on counting the number of c.f.u. in each spleen. The weights of the spleens of mice injected with Ba598 (Δ*virB4*) (58·3±5·2 mg) and Ba616 (*virB4* K463R) (57·2±4·9 mg) were markedly lower than those of mice injected with 544 (wild-type) (271·0±18 mg) and Ba603 (*virB4*$^+$) (216·6±11 mg). These results indicated that the

putative NTP-binding region is involved directly or indirectly in the splenomegaly typically associated with infection by wild-type *Bru. abortus*.

DISCUSSION

In this study, we have examined the importance of VirB4 for *Bru. abortus* intracellular replication and the functional significance of NTP binding or hydrolysis by this protein. VirB4 is a component of the VirB secretion apparatus that has a high sequence similarity to members of the type IV secretion system (Sieira *et al.*, 2000). In *A. tumefaciens*, a type IV secretion system participates in delivering oncogenic T-DNA from the bacterium to the plant cell, whereas in *Bor. pertussis* it participates in the secretion of pertussis toxin (Christie, 2001; Christie & Vogel, 2000). Recently, O'Callaghan *et al.* (1999) and Sieira *et al.* (2000) have described the presence of a *virB* region in *Brucella suis* and *Bru. abortus* which is involved in intracellular growth in macrophages. We found that VirB4 is a critical component of the VirB secretion apparatus, as an in-frame deletion mutant of *virB4* exhibited greatly impaired intracellular replication in the macrophage-like cell line J774 and mouse bone-marrow-derived macrophages. The fact that this mutation could be complemented by introducing a plasmid containing a wild-type copy of *virB4* confirms that this defect was due to a mutation in *virB4* rather than to a polar effect of the mutation.

We have also demonstrated the importance of the putative NTP-binding motif of *virB4* by specifically altering the region of *virB4* that encodes a Walker box A motif. To date, two other organisms, *A. tumefaciens* and *Bor. pertussis*, have been shown to have a functionally important NTP-binding domain. The intact NTP-binding domain of VirB4 is essential for *A. tumefaciens* virulence (Berger & Christie, 1993) and the NTP-binding domain of PtlC, a member of a set of proteins necessary for the secretion of pertussis toxin from *Bor. pertussis*, is essential for transport of pertussis toxin across bacterial membranes (Cook *et al.*, 1999). We have shown that alteration of Lys_{463} to Arg in *Bru. abortus* VirB4 protein had a marked effect on intracellular replication in macrophages and virulence in mice. As this mutation is in the putative NTP-binding region of VirB4, our results suggest that this region is critical for VirB4 function and support the idea that NTP binding is also an important aspect of VirB4 function. Three families of putative ATPases are associated with type IV transfer systems: (1) the TraG family of coupling proteins, (2) homologues of *A. tumefaciens* VirB4 protein, and (3) homologues of the RP4 TrbB and of *A. tumefaciens* VirB11 proteins (Christie, 2001). These proteins are ubiquitous among the type IV systems and are sometimes present in two or more copies. Further studies have provided evidence for transmembrane topology, self-association and a structural contribution to channel formation that is independent of VirB4 ATPase activity. Based on these properties, this family of ATPases might transduce information, possibly in the form of ATP-induced conformational changes, across the cytoplasmic membrane to extracytoplasmic subunits (Dang *et al.*, 1999). Additional work needs to be done to determine the function of VirB4, since introduction of a plasmid containing an NTP-binding domain mutation into a wild-type strain of *Bru. abortus* resulted in drastically reduced intracellular replication. Our finding of a dominant negative phenotype for this mutation suggests that the altered protein may interfere with the action of the wild-type protein. Our study also indicates that VirB4 might interact with another component of the putative secretion system, possibly another molecule of VirB4, another VirB protein or unidentified substrate(s). These findings provide important information towards understanding the mechanism underpinning VirB function.

The mechanism of virulence of *Brucella* spp. is not yet fully understood. *Brucella* spp. infect their hosts through mucosae and wounds and initially enter into professional phagocytes where they survive and reproduce (Liautard *et al.*, 1996). It is known that intracellular pathogens have developed a series of strategies to survive inside cells (Sinai & Joiner, 1997). Alteration of the normal process of phagosome maturation has been described for several micro-organisms, such as *Mycobacterium*, *Legionella* and *Chlamydia* (Sinai & Joiner, 1997). The intracellular survival of *Brucella* spp. has been documented for several cell types. *Bru. abortus* shows a different intracellular trafficking pattern between professional and non-professional phagocytes. Multiple observations have been reported that *Bru. abortus* is incorporated into phagosomes and remains in membrane-bound compartments until the host cell dies (Comerci *et al.*, 2001; Pizarro-Cerda *et al.*, 1998a, b). In non-professional phagocytes, *Brucella* is located in structures that resemble the endoplasmic reticulum (Pizarro-Cerda *et al.*, 1998a, b). Other evidence has indicated that *Brucella* is transported through the autophagic pathway before accumulating in the endoplasmic reticulum (Comerci *et al.*, 2001; Pizarro-Cerda *et al.*, 1998a, b). Macrophages are particularly important for the survival and spreading of *Brucella* during infection (Liautard *et al.*, 1996) and these autophagosomes are not observed in macrophages. Arenas *et al.* (2000) monitored the intracellular transport of *Bru. abortus* in macrophages and observed the kinetics of the fusion of phagosomes with preformed lysosomes labelled with colloidal gold particles by electron microscopy. In that study, phagosomes containing live *Bru. abortus* delayed fusion with lysosomes and newly endocytosed material was not incorporated into these phagosomes (Arenas *et al.*, 2000). In this study, we tested the ability of a wild-type strain, a Δ*virB4* mutant and a mutant of the putative NTP-binding motif of *virB4* to target properly within bone-marrow-derived macrophages early in infection. Bacterial phagosomes were scored for acquisition of the lysosomal glycoprotein LAMP-1, an abundant transmembrane protein found predominantly in late endosomes and lysosomes (Chen *et al.*, 1988; Harter & Mellman, 1992). Our results, together with previous

evidence, indicate that *Bru. abortus* prevents phagosome–lysosome fusion after uptake by macrophages.

This report shows that *Bru. abortus* VirB4 plays a critical role in intracellular growth and virulence in mice. Moreover, VirB4 may be a nucleotide-binding protein that interacts with other members of the VirB secretion apparatus to facilitate transport of unidentified substrate(s) across the bacterial membrane. Future investigations directed toward the clarification of the nature of the effector molecules may shed light on the molecular mechanism underlying the infection process of *Bru. abortus*.

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Modulation of *Brucella*-induced macropinocytosis by lipid rafts mediates intracellular replication

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Summary

Intracellular replication of *Brucella* requires the VirB complex, which is highly similar to conjugative DNA transfer systems. In this study, we show that *Brucella* internalizes into macrophages by swimming on the cell surface with generalized membrane ruffling for several minutes, after which the bacteria are enclosed by macropinosomes. Lipid raft-associated molecules such as glycosylphosphatidylinositol (GPI)-anchored proteins, GM1 gangliosides and cholesterol were selectively incorporated into macropinosomes containing *Brucella*. In contrast, lysosomal glycoprotein LAMP-1 and host cell transmembrane protein CD44 were excluded from the macropinosomes. Removing GPI-anchored proteins from the macrophage surface and cholesterol sequestration markedly inhibited the VirB-dependent macropinocytosis and intracellular replication. Our results suggest that the entry route of *Brucella* into the macrophage determines the intracellular fate of the bacteria that is modulated by lipid raft microdomains.

Introduction

Phagocytosis by 'professional' phagocytes, macrophages and neutrophils constitutes the initial step for the degradation of dying cells, inert particles and live infectious agents. It plays a critical part in essential biological functions such as inflammation, immunity and development (Lopes *et al.*, 2000). By confining the mechanisms of microbial killing and digestion to distinct intracellular compartments of specialized cells, host cells and tissues are subjected to less damage by the process of killing offending microbes (Emst, 2000). After uptake by professional phagocytes, inert particles are found in a membrane-derived phagosome, which undergoes a maturation process into a hydrolase-rich phagolysosome. In contrast, intracellular pathogens control the membrane-derived phagosomes, circumventing host defences and further degradation, thus transforming their compartments into replicative phagosomes (Méresse *et al.*, 1999).

Brucella spp. are Gram-negative bacteria that cause abortion and infertility in numerous domestic and wild mammals, as well as undulant fever in humans (Acha and Szylres, 1980). The bacterium is endemic in many underdeveloped countries and responsible for large economic losses and chronic infections in humans (Zavala *et al.*, 1994). *Brucella* species are facultative intracellular pathogens that survive within a variety of cells, including macrophages, and the virulence of these species and the establishment of chronic infection by them are thought to be essentially due to their ability to avoid the killing mechanisms within macrophages (Baldwin and Winter, 1994; Sangari and Aguero, 1996). The molecular mechanisms accounting for these properties are not understood completely. Recent studies with non-professional phagocyte HeLa cells have shown that *Brucella* inhibits phagosome–lysosome fusion and transits through an intracellular compartment that resembles autophagosomes. Bacterial replication occurs in another novel compartment, which has been shown by confocal microscopy and immunogold electron microscopy to contain protein markers that are normally associated with the endoplasmic reticulum (Pizarro-Cerda *et al.*, 1998a, b; Comerci *et al.*, 2001).

The operons coding for export mechanisms that specialize in the transfer of a variety of multimolecular complexes across the bacterial membrane, to the extracellular space or into other cells, have been described. These complexes, named type IV secretion systems, are present in *Agrobacterium tumefaciens* (*virB* genes) (Stachel and Nester, 1986; Kuldau *et al.*, 1990), *Bordetella pertussis* (*ptl* genes) (Weiss *et al.*, 1993; Kotob *et al.*, 1995), *Escherichia coli* (*tra* genes) (Winans and Walker, 1985; Pohlman *et al.*, 1994), *Legionella pneumophila* (*dot/icm* genes) (Segal *et al.*, 1998; Vogel *et al.*, 1998), and *Helicobacter pylori* (*cag* genes) (Covacci *et al.*, 1999). Recently, the *virB* operon of *Brucella* has been identified

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