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# Characterization of *Bartonella henselae*-specific immunity in BALB/c mice

K. L. KAREM,*† K. A. DUBOIS,* S. L. MCGILL† & R. L. REGNERY† **Heska Corporation, Fort Collins, Colorado and* †*Viral and Rickettsial Zoonoses Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA*

## SUMMARY

BALB/c mice were inoculated with *Bartonella henselae* by both systemic and mucosal routes. Culture analysis of tissues from mice infected intraperitoneally with a high dose of *B. henselae* yielded positive results 24 hr after infection. However, culture analysis of blood taken between 6 hr and 7 days after infection from groups receiving live *B. henselae* were negative. Following intraperitoneal infection, *B. henselae* was detected by polymerase chain reaction in liver and mesenteric lymph nodes by 6 hr and up to 7 days after infection in liver, kidney and spleen tissue. Enzyme-linked immunosorbent assay (ELISA) of serum samples collected as early as 13 days after infection indicated humoral immune responses to *B. henselae*. Specific humoral responses remained through week 6. Analysis of faecal samples revealed induction of *B. henselae*-specific immunoglobulin A by day 28 after infection. In addition, *B. henselae*-specific cellular responses were indicated by a positive delayed-type hypersensitivity and a T helper 1 (Th1) ($CD4^+$ T cell)-type cytokine response following *in vitro* stimulation of splenocytes. The significance and implications of these data in relation to *B. henselae* infections are discussed.

## INTRODUCTION

*Bartonella henselae* is a fastidious, Gram-negative bacterium that has been associated with a variety of human diseases, including cutaneous bacillary angiomatosis, bacillary hepatic peliosis, bacteraemia, relapsing fever, central nervous system disorders and, more commonly, cat scratch disease (CSD).[1–9] CSD afflicts an estimated 24 000 persons in the United States annually,[10] and is characterized by a broad range of clinical symptoms, manifested in varying degrees of severity, largely dependent upon the immune status of the host. Although *B. henselae* has emerged as the cause of significant human disease, both in rate of infection as well as severity of disease, little is known regarding pathogenicity and immunity induced during infection.

Historically, human immune responses to CSD have been defined as regional lymphadenopathy in proximity to the inoculation papule (cat scratch or bite). In addition, skin testing based upon the Hanger–Rose diagnostic test indicates cellular immunity to the agent.[11] In more recent years, the identification and growth on artificial media have facilitated the use of serological clinical diagnosis. The immunofluorescence assay (IFA) developed by Regnery *et al.*,[12] has provided a routine detection assay for diagnosis of *Bartonella*-associated human disease. In addition, humoral analysis of human CSD patient sera provide insight into aspects of *B. henselae*-specific immunity.[13,14] However, other than serological analysis of *Bartonella* exposure, little is known about immunity following exposure to *B. henselae*. Likewise, serological tests have provided evidence of significant humoral immune responses in domestic cats.[15] However, detailed aspects of cat immunity remain elusive. Thus, limited immunological analysis in human or cat hosts has left a gap in our understanding of *B. henselae*-induced immunity. The characterization of immune responses generated by exposure to *Bartonella* species is crucial for the understanding of this organism's ability to cause disease. To advance our understanding of the immune response to *B. henselae*, a BALB/c mouse model of immunity for the fastidious bacterial pathogen *B. henselae* has been developed. BALB/c mice were found to be responsive to systemic and mucosal routes of infection as evidenced by the development of *B. henselae* humoral and cellular immune responses.

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Correspondence: Dr K. L. Karem, Mail Stop G-13, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA.

## MATERIALS AND METHODS

### *Bacterial strain*

*B. henselae* used in this study originated from the Houston-1 isolate (ATCC #49882). *B. henselae* stocks were grown on rabbit blood agar plates (BBL Microbiology systems, Cockeysville, MD) at 32° with 5% $CO_2$. Solid agar growth of this stock yields a mixed phenotype of rough and smooth colonies. No clonal selection for phenotype was performed in this study. Bacteria were harvested in a laminar flow hood by gently scraping colonies off the agar surface in brain heart infusion (BHI) broth media. The cells were then collected via

centrifugation and suspended in phosphate-buffered saline solution (PBS). Colony-forming units (CFU) of harvested *Bartonella* cultures were titred on blood agar plates prior to being either frozen for infection studies or inactivated by gamma irradiation ($5 \pm 10^5$ rads) and stored at $-70^\circ$ until used. A single freeze/thaw of stocks routinely results in less than a one log reduction in viability.

*Immunizations*

BALB/c mice (4–6 weeks old) were obtained from Harlan Sprague–Dawley (Indianapolis, IN). *B. henselae* was prepared as described above, and thawed on ice prior to immunization. Mice were anaesthetized with methoxyflurane (Metofane; Pitman-Moore Inc., Mundelein, IL) and administered live *B. henselae* at dose of $1 \pm 10^4$ or $3 \pm 10^6$ CFU/mouse by the routes indicated or $3 \pm 10^8$ CFU/mouse intraperitoneally (i.p.) as described below. For oral immunizations, mice were dosed by using a 22-gauge feeding needle attached to a 1 $cm^3$ syringe. For intranasal (i.n.) immunizations, mice were dosed with volumes of 10–20 µl containing $\approx 3 \pm 10^6$ CFU of *B. henselae* administered into the nostrils using a micropipette fitted with a 200-µl capacity tip. Intravenous (i.v.), i.p. and subcutaneous (s.c.) immunizations utilized a tuberculin syringe and needle. Tail vein injections were performed under anaesthesia and with the use of heat lamp to facilitate venous access. Following immunizations, mice were observed daily for signs of illness. Each immunized group contained at least five mice. None of the challenged mice showed any signs of overt illness during this study.

*Detection of* B. henselae in vivo

Detection of blood-borne organisms was attempted by using whole blood plating techniques as well as polymerase chain reaction (PCR). Bleeding was performed by using heparinized tubes at 6, 24 and 48 hr and on days 3, 7, 8 and 15 after immunizations. Whole blood was plated on rabbit blood agar plates (BBL) and incubated for up to 30 days at 32° with 5% $CO_2$. Spleen, liver, kidney and mesenteric lymph nodes (MLN) tissues from individual mice were harvested aseptically and homogenized together in 1 ml of BHI broth using disposable pellet pestles (Kontes, Vineland, NJ) before being plated (100 µl per plate) on rabbit blood agar plates. In addition, DNA was extracted from whole blood or tissues using Qiagen DNA extraction kits (Qiagen #29104 and #29306) and subsequently subjected to an ethanol precipitation prior to PCR analysis. Primers A1 (CAGATGATGATCCCAAGC CTTC) and A2 (GTTCTATTGAA-ATTGTGAAGAGAAG) designed by Minnick and Barbian[16] from the Intragenic spacer region of the 16 seconds and 23 seconds rRNA gene region of *Bartonella* were used. For primers A1 and A2, the thermal cycler programme format was as follows: 95° for 1 min, 58° or 1 min, 72° for 1 min for 39 cycles, then 95° for 1 min, 58° for 1 min and 72° for 2 min for one cycle. Spiking of naïve tissue and blood samples with *B. henselae* bacteria prior to DNA extraction was performed to confirm the detection and sensitivity of this assay. *B. henselae* DNA was detected down to 15 CFU/ml of whole blood and 3·3 CFU/mg of tissue.

*Enzyme-linked immunosorbent assay (ELISA)*

Serum and faecal samples were collected from preimmune (naïve) mice at day 0 and throughout the study at the days indicated post immunization. Faecal samples were prepared as previously described.[17] Microtitre plates (Immulon II) were coated overnight at 4° with a 1 : 50 dilution of stock whole cell inactivated (gamma-irradiated) *B. henselae* (titre $1 \pm 10^6$ CFU/ml). Plates were then blocked with 3% skim milk in $1 \pm$PBS for 1 hr at 37° and washed three times with PBST (0·05% Tween-20 in PBS). Samples were then added at either a 1 : 50 dilution (sera) or a 1 : 1 dilution (faecal extract). Samples were then doubly diluted in PBST and plates incubated for 2 hr at 37°. Plates were washed three times with PBST. Secondary conjugate antibodies were used according to the manufacturer's recommendations and include goat antimouse immunoglobulin G (IgG)–horseradish peroxidase (HRP) and goat antimouse IgA–HRP conjugates (Kirkegaard & Perry, Gaithersburg, MD). Secondary antibodies were incubated for 1 hr at 37°, washed three times and plates developed using 2,2 ± -azino-di-3-ethylbenzthiazoline sulphonic acid. Plates were allowed to develop for 30 min before absorbance values at 405 nm were determined.

*Delayed-type hypersensitivity*

Immunized mice were subjected to intradermal skin testing to monitor delayed swelling reactions as an indication of specific cellular immune induction. Mice were boosted on day 100 after primary immunization with $1{\cdot}5 \pm 10^6$ CFU using the same route of immunization. One week following the boost, each mouse was challenged in one ear pinna with gamma-irradiated *B. henselae* ($10^6$ CFU) and in the other with $1 \pm$PBS as a control. For each mouse, the antigen-injected ear pinna was monitored at 24, 48 and 72 hr for swelling responses and compared to the control ear pinnae as an internal negative control. Swelling was determined from three readings using a 'General' #142, 6 inch Dial Caliper (General Hardware, Mfg. Co. Inc., New York, NY). Average swelling differences of antigen-injected versus control ears for each group of five to 10 mice were then compared to naïve controls using a one-tailed, two-sample unequal variance *t*-test. *P*-values $\pm 0{\cdot}05$ are considered statistically significant.

In vitro *stimulation of splenocytes*

For each of two experiments, spleens were harvested from two mice from each group immunized either s.c., orally or i.p. with live *B. henselae* or killed *B. henselae* at $3 \pm 10^6$ CFU s.c. Single cell suspensions were made to $1 \pm 10^6$ cells/ml in RPMI medium plus 10% fetal calf serum (FCS). Splenocytes were plated into 96-well microtitre plates at a concentration of $1 \pm 10^5$ cells/well. Cells were then stimulated with either killed total *B. henselae* antigen at $10^5$ CFU/ml per well, RPMI medium plus 10% FCS alone as a negative control or concanavalin A at a final concentration of 1 µg/ml as a positive control. Cells were incubated with antigen at 37° plus 5% $CO_2$ for 5 days. Cell cultures were then harvested and filtered through 0·22 µm filters, diluted and screened for interferon-γ (IFN-γ) and interleukin-4 (IL-4) using ELISA kits as recommended by the manufacturer (Endogen, Woburn, MA).

## RESULTS

### Experimental infection with *B. henselae*

Tissues harvested at hours 6, 24, 48 and 72 and at day 7 after i.p. infection with $10^6$ CFU yielded positive PCR results for

*B. henselae* (Table 1). Positive PCR results were observed in tissues as early as 6 hr (liver and MLN) and remained positive up to 7 days (spleen, liver, kidney) after i.p. infection. Despite positive results in tissues, PCR failed to detect *B. henselae* in whole blood of mice receiving $10^4$ or $10^6$ CFU of live *B. henselae*. In addition, attempts to culture *B. henselae* from blood or tissues following i.v., s.c., i.p. or oral challenge with $10^4$ or $10^6$ CFU failed (data not shown). Subsequently, in a further attempt to recover viable *B. henselae* from challenged mice, $3 \pm 10^8$ CFU of live *B. henselae* was administered i.p. Culture analysis of tissue homogenates from this group indicated positive cultures within a 24-h harvest period, although blood cultures remained negative (data not shown). However, by 48 hr post challenge with $3 \pm 10^8$ CFU, cultures from tissue homogenates were negative.

### Humoral immunity

Mice dosed i.v., s.c. or orally with $10^4$ CFU of live *B. henselae* were monitored for serum IgG responses by ELISA. Serum *B. henselae*-specific IgG was detected in all groups by day 13 after infection with titres increasing through day 42 (Fig. 1a,b,c). The highest titres were observed at day 70 in the i.v. and s.c. groups following a second dose of $10^4$ CFU on day 50 (Fig. 1a,b), while an oral boost failed to enhance titres at day 70 (Fig. 1c). An additional study using a single dose of $3 \pm 10^6$ CFU per mouse dosed either s.c., orally or i.n. with live *B. henselae* or s.c. with killed *B. henselae* was performed and *B. henselae*-specific IgG levels determined. Titres were apparent by day 21 in all four groups, with peak responses occurring at day 42 after immunization. Levels of IgG began to drop by day 52 falling to those of the day 0 (naïve) control by day 81 (Fig. 2a). In addition, faecal samples collected at day 28 after immunization indicated *B. henselae*-specific IgA induction in mice receiving live *B. henselae* orally, i.n. or s.c. and killed *B. henselae* s.c. when compared to naïve animals (Fig. 2b).

### Delayed-type hypersensitivity

To assess the induction of *Bartonella*-specific cellular immune responses, the presence of delayed-type hypersensitivity (DTH) was tested for in mice receiving $10^6$ CFU of live *B. henselae* by the s.c., i.n. or oral routes of immunization or $10^6$ CFU of killed *B. henselae* s.c. Inactivated *B. henselae* was injected into one ear pinna of immunized mice and ear swelling measured 48 hr later. The only statistically significant DTH response occurred in the s.c. immunized mice receiving live *B. henselae* (Table 2). In addition, although below statistically significant levels, orally and i.n. immunized mice receiving live *B. henselae* and mice receiving gamma-irradiated *B. henselae* s.c. developed some degree of ear swelling in response to *B. henselae* compared to non-immune animals (Table 2).

**Table 1.** Detection of *B. henselae* by PCR from tissues following live intraperitoneal immunization

| | Time after immunization | | | | |
|---|---|---|---|---|---|
| Tissue* | 6 hr | 24 hr | 48 hr | 72 hr | 7 days |
| Spleen | – | – | – | f+ | f+ |
| Liver | + | – | + | + | + |
| Kidney | – | – | – | f+ | f+ |
| MLN† | + | + | + | f+ | – |
| Blood | – | – | – | – | – |
| Bone | – | – | – | – | – |
| Brain | ND | ND | ND | ND | – |

*Tissues were extracted following i.p. inoculation with 106 CFU of *B. henselae*.
†MLN = mesenteric lymph node.
f+ = faint positive band.
ND = not determined.

### Induction of cellular cytokine expression

In addition to DTH as an indicator of cellular immune induction, *in vitro* stimulation of splenocytes with *B. henselae* antigen was performed to assess the production of IFN-γ and IL-4 in response to antigen stimulation. Splenocytes were harvested from mice previously immunized with live *B. henselae* via the s.c., i.p. and oral routes or s.c. with killed *B. henselae*. The results shown in Table 3 indicate induction of both IL-4 and IFN-γ expression in splenocytes from immunized animals following *in vitro* antigen stimulation. In fact, IFN-γ expression in immunized as well as non immunized groups exceeded levels obtained with ConA positive control stimulation. In contrast, levels of IL-4 production increased only in the group immunized with live *B. henselae* subcutaneously while remaining below or near the detectable limits (<15 pg/ml) in all other groups (Table 3).

## DISCUSSION

This report provides novel information regarding the immunity induced by *B. henselae*, the aetiological agent of cat scratch disease. In an attempt to provide data that would advance our understanding of *B. henselae*-induced immunity, BALB/c mice were infected with *B. henselae* and assessed for the development of bacteraemia, organ dissemination and the induction of *B. henselae*-specific immunity. By establishing a murine model of immunity, a basis for future immune studies of this organism is provided.

*B. henselae* was cultured from organ homogenates 24 hr following a $10^8$ CFU dose. However, attempts at recovery of viable bacteria from blood and tissues failed in mice receiving $10^4$ or $10^6$ CFU, and by 48 hr, no organisms were recovered from animals receiving $10^8$ CFU. Thus, it is likely that recovery of viable bacteria at 24 hr after high dose challenge ($10^8$ CFU) is representative of residual organisms from the original inoculum rather than *in vivo* growth. Despite the absence of viable organisms in blood and tissues by culture techniqes, *B. henselae* DNA was detected by PCR in mouse tissues from 6 hr up to 7 days after i.p. infection (Table 1). Thus, it appears that *B. henselae* is incapable of maintaining viability for extended periods in BALB/c mice although organ distribution is evidenced by PCR analysis.

Despite failure to maintain viability *in vivo*, induction of specific humoral immunity following administration of live or killed *B. henselae* was observed. *B. henselae*-specific systemic humoral IgG responses were observed by day 21 after administration of live *B. henselae* via the s.c., i.v. oral and i.n. routes or killed *B. henselae* s.c. Thus, *B. henselae* is capable of

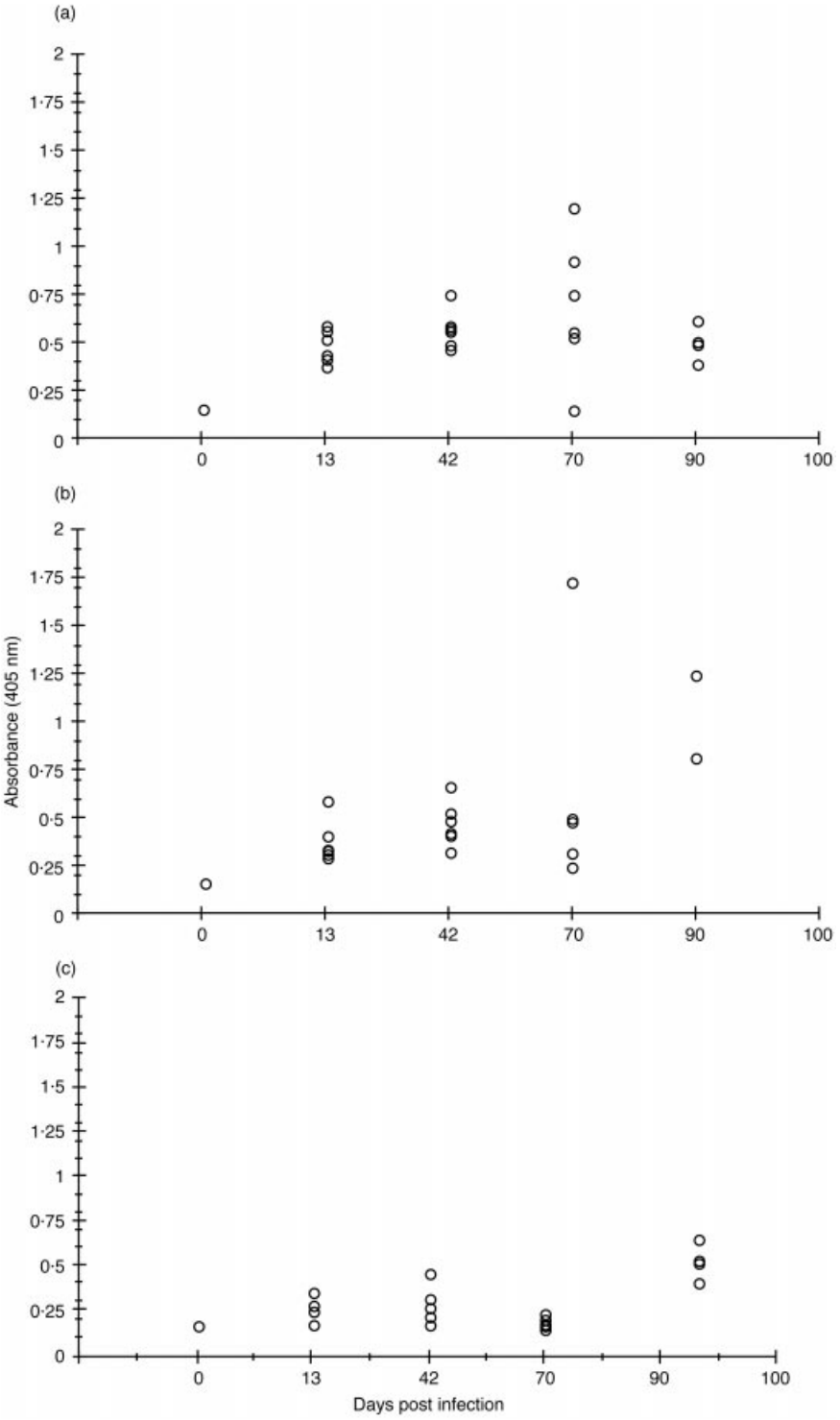


**Figure 1.** ELISA analysis of *B. henselae-specific IgG in serum samples of mice receiving* $10^4$ CFU of live *B. henselae either* (a) intravenously (b) subcutaneously or (c) orally. Data represent the absorbance (405 nm) reading of the 1 : 100 dilution of each serum tested.

inducing systemic immunity following a variety of infection routes including mucosal delivery. The development of systemic antibody following mucosal delivery of *B. henselae* suggests that the organisms may reach the gut-associated lymphoid tissue, where inductive immune responses are induced. Furthermore, the presence of *B. henselae*-specific faecal IgA following both systemic and mucosal challenge may be indicative of a mucosal component of *B. henselae* infection.

Although the development of specific immunity following exposure to *B. henselae* indicates successful immune induction, determination of protective immunity remains elusive. A cellular component of immunity occurs during human CSD,[14] and

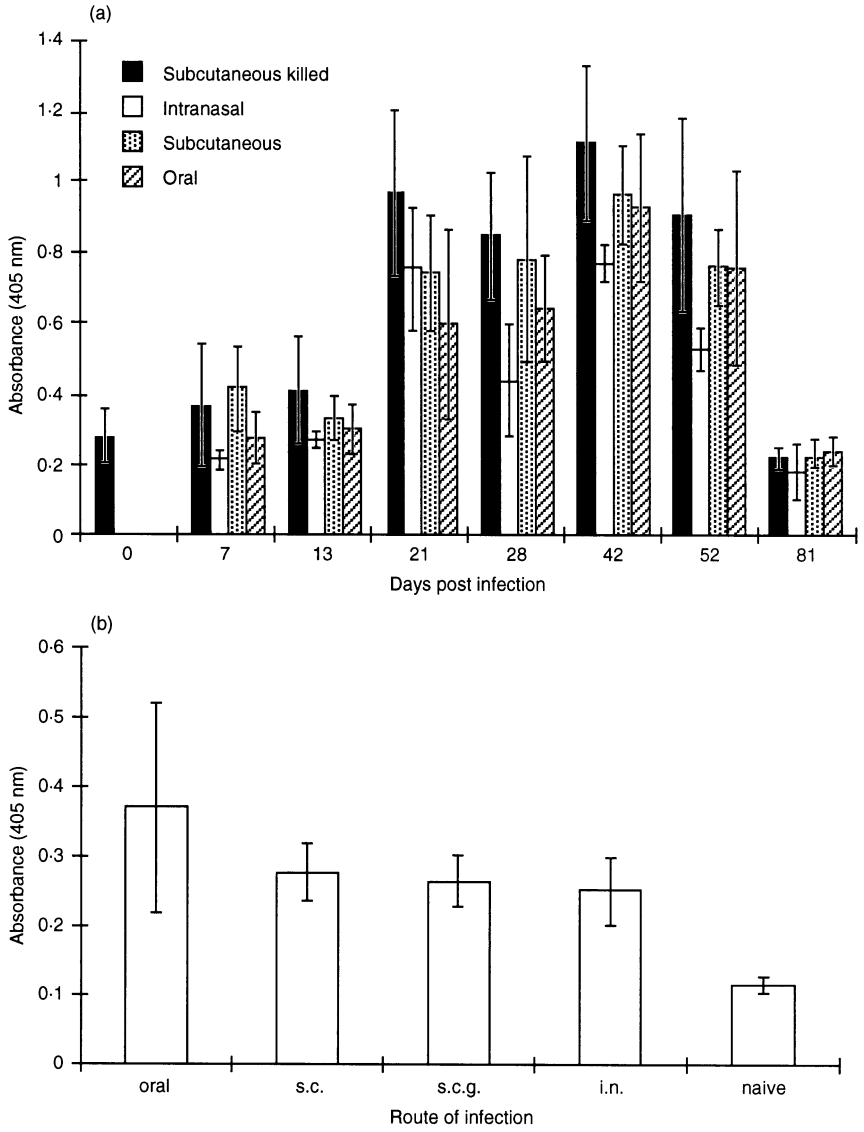


**Figure 2.** ELISA analysis of *B. henselae-specific* (a) serum IgG or (b) faecal IgA in mice receiving $10^6$ CFU of live *B. henselae* either subcutaneously, orally or intranasally or mice receiving $10^6$ CFU of killed *B. hensleae* subcutaneously. Data represent the absorbance (405 nm) reading of a 1:100 dilution of each serum and 1:2 dilution of faecal samples tested.

**Table 2.** Measurement of DTH responses in mice following administration of *B. henselae*

| Route of administration | Ear pinnae thickness* | *P*-value† |
|---|---|---|
| Subcutaneous | 1·35±0·72 | 0·011 |
| Killed subcutaneous | 0·75±0·65 | 0·105 |
| Orally | 0·85±0·71 | 0·07 |
| Intranasally | 1·2 ±0·91 | 0·056 |
| Non-immunized | 0·17±0·28 | |

*Results are expressed as an average of the ear swelling of the antigen ear minus the swelling of the control ear in units of $10^{-2}$ inches for each group.

† *P*-values calculated using average difference in ear thickness of each group compared to the naïve group. *P*-values $\leq 0{\cdot}05$ are statistically significant

experimentation supports a role for cellular responses in feline protective immunity against *B. henselae* (our unpublished observation). However, until now the only animal infection model for *B. henselae* reported to date is the domestic cat,[15,18] a model for which immunological reagents are limiting. Thus, analysis of immunity in a murine model provides a model to access the strength of induced immunity. The development of DTH in animals receiving live *B. henselae* s.c. indicates induction of $CD4^+$ T-cell activity against *B. henselae* antigen.[19] However, mucosal delivery of live or parenteral delivery of killed *B. henselae* fail to induce statistically significant DTH reactions. In addition, although elevated levels of IFN-γ were observed following *in vitro* stimulation of naïve as well as all immunized groups, elevated levels of IL-4 along with of IFN-γ induction were detected only following parenteral exposure to live *B. henselae*. Thus, prior parenteral exposure to live organisms facilitates both T helper 1 (Th1)- and Th2-type immune parameters while only Th1-type cytokine responses are

**Table 3.** Cytokine expression from splenocytes following *in vitro* stimulation with whole cell *B. henselae* antigen

| Route of immunization* | *In vitro* stimulation† | Cytokine measured by ELISA (pg/ml) | |
|---|---|---|---|
| | | Interleukin-4 | Interferon-γ |
| Naïve | Media alone | <15 | <37 |
| Naïve | Con A | 156±50·9 | 117 800±350 |
| Naïve | *B. henselae* | <15 | 685 000±21 500 |
| Subcutaneous | Media alone | <15 | <37 |
| Subcutaneous | Con A | 187·5±10·6 | 80 500±2121·3 |
| Subcutaneous | *B. henselae* | 52±19 | 1 697 500±31819·5 |
| Intraperitoneal | Media alone | <15 | 53±ND |
| Intraperitoneal | Con A | 195±120 | 87 500±2121 |
| Intraperitoneal | *B. henselae* | 18·5±0·7 | 532 500±24748·7 |
| Oral | *B. henselae* | <15 | 232 333·33±19655·4 |
| Subcutaneous killed | *B. henselae* | <15 | 331 500±2121·3 |

*All mice received $10^6$ CFU *B. henselae* via the route indicated.
†$10^4$ splenocytes were stimulated *in vitro* with either $10^4$ CFU of killed *B. henselae*, 1 μg of Con A or media alone.

detected following live mucosal or killed parenteral immunizations. The analysis of DTH and cytokine profiles provide evidence of cellular immune induction following *B. henselae* exposure both parenterally and mucosally. However, it suggests that live parenteral exposure is superior in inducing both aspects of Th1 and Th2 immune responses.

Aside from the analysis of cellular and humoral immunity, detection of bacterial DNA suggests details regarding the fate of *B. henselae* following parenteral infection. The presence of *B. henselae* in lymph and liver tissue by PCR 6 hr following i.p. injection suggests the uptake of this organism by professional phagocytes, which then migrate to these organs. In fact, rapid uptake by murine phagocytic cells could explain the absence of *B. henselae*-induced bacteraemia and viable counts in tissues for extended periods post challenge. The induction of IFN-γ supports a role for phagocyte activity as well as enhanced class I- and class II-mediated immunity, which includes both cellular and humoral components.[20] With this in mind, *B. henselae* may be highly susceptible to immune effectors such as INF-γ and $CD4^+$ T-cell activity. In concordance with this supposition, *B. henselae*-induced bacteraemia during human infection is typically found only in immunocompromised patients.[1,12] Thus, the reduction in $CD4^+$ T-cell activity and IFN-γ may play a role in the development of bacteraemia in these patients. Therefore, although human and murine immune responses to *B. henselae* may include T-cell activity, IFN-γ and subsequent macrophage-killing activity, the immuno-status of the host may dictate disease progression. By defining immune parameters in a murine model, perhaps more details of *B. henselae* immunopathogenesis will be unveiled.

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