The Adjuvant Effect of a Single Dose of Interleukin-12 on Murine Immune Responses to Live or Killed *Brucella abortus* Strain Rb51

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ABSTRACT

This study was designed to determine if a single 0.5 μg administration of recombinant murine interleukin-12 (IL-12) would influence immune responses of mice vaccinated with live or killed *Brucella abortus* strain RB51 (SRB51). Mice were vaccinated intraperitoneally with 5×10^8 cfu of live or γ-irradiated SRB51 bacteria alone, or in combination with 0.5 μg of IL-12. Control mice received saline or 0.5 μg of IL-12. Serologic responses and spleen weights after vaccination were greater in mice vaccinated with live SRB51 when compared to mice receiving killed SRB51 or control treatments. Administration of a single dose of IL-12 as a vaccine adjuvant did not influence immune responses, clearance of live SRB51, or resistance against *B. abortus* strain 2308 (S2308) challenge. The results of this study suggest that a single administration of 0.5 μg of IL-12 at the time of vaccination does not have significant adjuvant effects on vaccine-induced immune responses against live or killed *Brucella*.

RÉSUMÉ

Afin de déterminer si l'administration unique de 0,5 μg d'interleukine-12 (IL-12) murine recombinante influencerait la réponse immunitaire de souris suite à une vaccination, des souris furent vaccinées par voie intrapéritonéale avec 5×10^8 unités formant des colonies de *Brucella abortus* souche RB51 (SRB51) vivantes ou tuées par irradiation, soit seules ou combinées à 0,5 μg d'IL-12. Des souris témoins ont reçu de la saline ou 0,5 μg d'IL-12. La réponse sérologique et le poids de la rate après vaccination étaient plus élevés chez les souris vaccinées avec les bactéries SRB51 vivantes comparativement aux souris ayant reçu les bactéries SRB51 tuées ou les souris témoins. L'administration d'IL-12 n'a pas influencé la réponse immunitaire, la capacité d'élimination des bactéries SRB51 vivantes ou la résistance à une inoculation défi avec la souche 2308 de *B. abortus*. Les résultats de l'étude suggèrent que l'administration unique de 0,5 μg d'IL-12 au moment de la vaccination n'a pas d'effet adjuvant significatif sur la réponse immunitaire suite à une vaccination avec une souche vivante ou tuée de *Brucella abortus*.

(Traduit par le docteur Serge Messier)

Interleukin-12 (IL-12) is a heterodimeric cytokine produced by macrophages, B cells and other accessory cells in response to bacteria, bacterial products, or parasites (1). Interleukin-12 has been reported to be required for generation of Th1 responses (2), which are important for induction of cellular immunity. While IL-12 and IFN-γ are key cytokines for the differentiation of Th1 cells, IL-12 also has inhibitory effects on cytokines which promote Th2 cell development (2). Due to its ability to preferentially induce Th1 cells, IL-12 has been considered as an adjuvant for vaccines against pathogens in which cellular immune responses and IFN-γ are important for protective immunity. Studies have demonstrated that 2 dosages of IL-12 have beneficial adjuvant effects on killed vaccines against *Listeria monocytogenes* (3) and *Leishmania major* (4).

Current vaccines used to protect cattle against brucellosis are composed of live bacteria and can be pathogenic in pregnant cattle or humans. In general, killed vaccines do not sufficiently induce cellular immune responses and are not as efficacious as live vaccines in preventing brucellosis in cattle and other animals (5). However, an efficacious vaccine for cattle that did not use live bacteria would be desirable as it would eliminate the pathogenic problems associated with live vaccines. Due to data suggesting an adjuvant effect for IL-12, we hypothesized that a single administration of exogenous IL-12 might have beneficial effects when administered with a live or killed brucellosis vaccine. This hypothesis was evaluated in a mouse model of brucellosis, to determine if a single administration of IL-12 with live or killed *Brucella* influenced clearance, immune responses, or protection against experimental *Brucella* challenge.

All cell culture experiments used RPMI 1640 medium (GIBCO Laboratories, Grand Island, New York, USA), containing L-glutamine, 25 mM HEPES (N-2-hydroxyethylpiperazine- N′-2-ethanesulfonic acid), 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah, USA), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 5×10^{-5} M 2-mercaptoethanol.

Live cultures of a standard virulent strain of *B. abortus*, strain 2308 (S2308), and a strain currently used as a vaccine in the United States, *B. abortus* strain RB51 (SRB51), were prepared as previously described (6). Killed cultures of SRB51 and S2308 were prepared by γ-irradiation with 1.4×10^6 rads. Recombinant murine IL-12 was obtained from R & D Systems in Minneapolis, Minnesota, USA.

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A total of 180 (n = 30/treatment) female, 10-week-old BALB/c AnNHsD mice (Harlan Sprague Dawley, Indianapolis, Indiana, USA) were used in the experiments. Mice were vaccinated intraperitoneally (IP) with 0.2 mL of saline containing 5×10^8 colony forming units (cfu) of live or γ-irradiated SRB51 alone (live SRB51 and killed SRB51), or combined with 0.5 μg of murine recombinant IL-12 (live SRB51+IL-12 and killed SRB51+IL-12). Other mice were injected IP with 0.2 mL of saline alone (saline control) or saline containing 0.5 μg of IL-12 (IL-12 control). Mice (n = 10/treatment) were challenged IP with 2×10^4 cfu of S2308 at 12 wk after vaccination. The time of challenge was based on data from previous studies which demonstrated that a dosage of 5×10^8 cfu of SRB51 is cleared from Balb/c mice by 12 wk after IP vaccination (6).

Blood samples and spleens were obtained from mice in all treatments after CO_2/O_2 euthanasia at 2, 4, 8, and 12 wk after vaccination (n = 5/treatment/time). Following CO_2/O_2 euthanasia at 2 wk after S2308 challenge, blood and spleens were obtained from mice in all treatments (n = 10/treatment). Spleens were weighed and approximately one-third of each spleen was excised and weighed. The excised portion of the spleen was used for bacterial culture and the remaining portion was used to prepare spleen cell suspensions.

Serum samples were measured for antibody to SRB51 or S2308 by a dot enzyme-linked immunosorbent assay (ELISA) with a goat anti-mouse immunoglobulin G (heavy- and light-chain specific)-horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) as described previously (6,7). Results from the dot ELISA were expressed as mean $\log_{10}$ titer ± standard error of the mean (SEM).

The excised portion of the spleen was processed using a tissue grinder to form a cell lysate and the cfu of *B. abortus* in the lysate were determined. Results were then evaluated as mean $\log_{10}$ cfu/g spleen ± SEM, or as mean $\log_{10}$ cfu/total spleen (cfu/g × total spleen weight).

The remaining portion of the spleen was placed on a sterile, 60-mesh stainless steel screen, minced with scissors, and processed to form a spleen cell suspension as described previously (6). Cells from 5 mice from each treatment were evaluated separately at 2, 4, 8, and 12 wk after vaccination. Following S2308 challenge, spleen cell suspensions from 10 mice from each treatment were combined into 5 separate suspensions (2 mice/suspension, 5 suspensions/ treatment) for evaluation of lymphocyte proliferative responses. Briefly, 50 μl of RPMI containing 3×10^5 spleen cells was added to each of 2 separate flat-bottom wells of a 96-well microtiter plate that contained 100 μl of γ-irradiated S2308 (10^8, 10^7, 10^6, 10^5, and 0 bacteria/well), alone, or with 10, 1, 0.1, 0 ng/mL of exogenous IL-12. Cell cultures were incubated for 5 d at 37°C in 5% CO_2 and pulsed for an additional 18 h with 1.0 μCi of [^{3}H] thymidine per well. Cells were then harvested (Harvester 96, Tomtec Inc., Orange, Connecticut, USA) and measured for radioactivity in a liquid scintillation counter (1450 Microbeta, Wallace Inc., Gaithersburg, Maryland, USA). Cell proliferation results were expressed as stimulation indices (counts/min in presence of antigen divided by counts/min in medium only).

Statistical differences between treatments were determined by a 2-way analysis of variance procedure, and significant differences were reported when $P \leq 0.05$. Means for each treatment were compared by use of a least significant difference. Means are reported as least square means ± SEM.

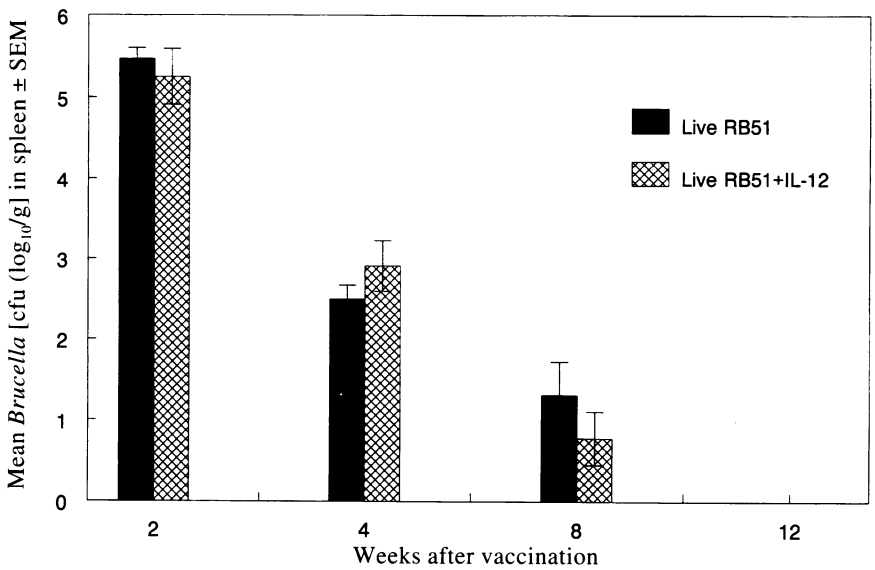

Figure 1. Colonization of splenic tissues (cfu/g) at 2, 4, 8, and 12 wk after vaccination with 5×10^8 cfu of *B. abortus* strain RB51 alone, or combined with 0.5 μg of murine recombinant IL-12 (n = 5 mice/treatment).

TABLE I. Serologic responses in mice vaccinated with SRB51

	Log_{10} antibody titer to SRB51 at time (wk) after vaccination[a]			
SRB51 Vaccine (n = 5)[b]	2	4	8	12
Live	2.54 ± 0.11[a]	3.41 ± 0.16[a]	3.41 ± 0.22[a]	2.77 ± 0.04[a]
Live + IL-12	1.93 ± 0.23[b]	3.22 ± 0.19[a]	3.26 ± 0.28[a]	2.81 ± 0.07[a]
Killed	1.72 ± 0.07[b]	2.24 ± 0.12[c]	2.99 ± 0.27[ab]	2.24 ± 0.29[b]
Killed + IL-12	1.87 ± 0.15[b]	2.66 ± 0.14[b]	2.81 ± 0.11[b]	2.43 ± 0.10[ab]
IL-12	0[c]	0[d]	0[c]	0[c]
Saline	0[c]	0[d]	0[c]	0[c]

[a] Mice were given 5×10^8 cfu of live or killed vaccine alone, or combined with 0.5 μg of recombinant murine IL-12. Control mice received 0.2 μl of saline alone (Saline) or combined with 0.5 μg of IL-12 (IL-12)

[b] Dot ELISA antibody titers to SRB51 were measured in vaccinated mice at 2, 4, 8, or 12 wk after vaccination

Results are expressed as mean ± SEM. Means with different superscripts within a sampling time are statistically different ($P \leq 0.05$)

TABLE II. Mean stimulation indices of spleen cells from SRB51-vaccinated mice to 10^8 cfu of irradiated S2308 in the presence or absence of added recombinant murine IL-12.

SRB51 Vaccine	Time (wk) after vaccination				
	4		8		12
(n = 10)[a]	No IL-12	Added IL-12	No IL-12	Added IL-12	No IL-12
Live	0.34 ± 0.69	32.48 ± 2.13[b]	3.82 ± 0.84	35.03 ± 18.89[bc]	6.27 ± 1.84[b]
Live + IL-12	0.95 ± 0.78	28.39 ± 7.44[b]	4.88 ± 2.48	40.18 ± 7.26[c]	3.46 ± 0.70[c]
Killed	2.16 ± 1.07	2.98 ± 1.57[c]	0.51 ± 0.16	32.16 ± 5.39[b]	2.00 ± 0.78[c]
Killed + IL-12	1.35 ± 0.75	3.72 ± 1.19[c]	0.31 ± 0.07	18.01 ± 4.63[d]	2.39 ± 1.40[c]
IL-12	1.16 ± 0.59	4.35 ± 1.88[c]	0.91 ± 0.69	5.97 ± 1.19[d]	1.81 ± 0.68[c]
Saline	0.46 ± 0.69	7.13 ± 2.13[c]	1.07 ± 0.23	3.27 ± 0.50[e]	3.63 ± 0.29[c]

[a] Mice were given 5 × 10^8 cfu of live or killed vaccine alone, or combined with 0.5 μg of recombinant murine IL-12. Control mice received 0.2 μl of saline alone (Saline) or combined with 0.5 μg of IL-12 (IL-12)

Five spleen cell suspensions per vaccine treatment, (2 mice/suspension) were prepared; 3 × 10^5 cells per suspension were incubated with 10^8 cfu of γ-irradiated S2308, with or without 0.1 ng/mL of exogenous IL-12, for 5 d at 37°C in 5% CO_2. Cells were then pulsed for 18 h with 1.0 μCi of [^{3}H] thymidine per well

Data are presented as mean stimulation indices ± SEM

Column means with different superscripts differ significantly ($P < 0.05$)

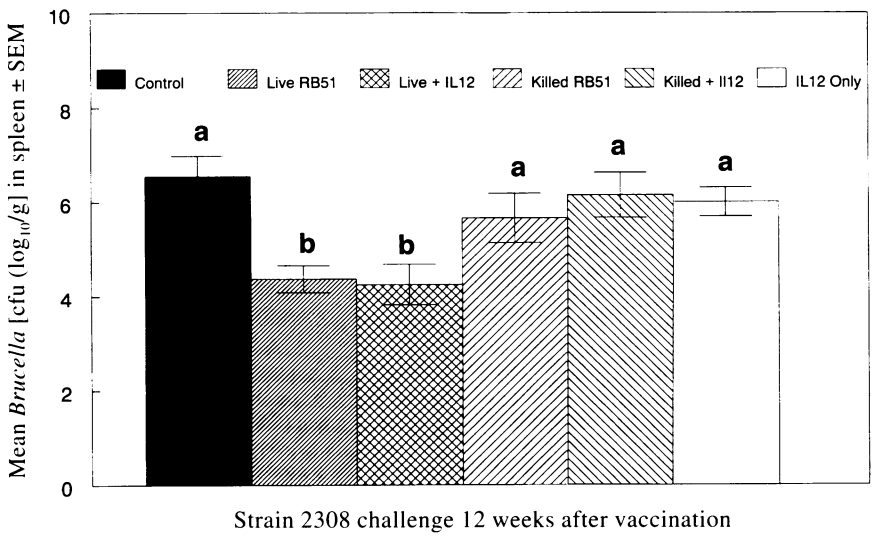

Figure 2. Colonization of splenic tissues (mean log cfu/g ± SEM) 2 wk following intraperitoneal challenge with 2 × 10^4 cfu of S2308. Twelve weeks prior to S2308 challenge, mice were vaccinated with saline, 0.5 μg of recombinant murine IL-12 alone, or 5 × 10^8 cfu of live or γ-irradiated *B. abortus* strain RB51 alone, or combined with 0.5 μg of murine recombinant IL-12 (n = 5 mice/treatment). Means with different superscripts differ significantly ($P < 0.05$).

The clearance of SRB51 from spleens of mice vaccinated with live SRB51 alone, or in combination with IL-12, did not differ at 2, 4, 8, or 12 wk after vaccination (Fig. 1). At 12 wk after vaccination, spleens of mice vaccinated with either live SRB51 or live SRB51+IL-12 were culture negative for SRB51. IL-12 did not influence the clearance of strain RB51 from splenic tissue. Spleen weights of mice vaccinated with live SRB51 or live SRB51+IL-12 were greater ($P \leq 0.01$) at 2, 4, 8, or 12 wk after vaccination when compared to saline controls, IL-12 controls, or mice receiving either vaccination treatment using killed SRB51 (data not shown).

Mice vaccinated with live SRB51 or live SRB51+IL-12 had greater serum antibody titers against SRB51 at 2, 4, 8, and 12 wk after vaccination when compared to all other treatments (Table I). When combined with live or killed SRB51, IL-12 as an adjuvant did not influence serologic responses at 2, 4, 8, or 12 wk after vaccination.

In the absence of IL-12 as an adjuvant, spleen cells obtained at 12 wk after IP vaccination with live SRB51 had greater ($P \leq 0.05$) stimulation indexes against γ-irradiated S2308 when compared to responses of spleen cells from other treatments (Table II). Administration of IL-12 as an adjuvant with live or killed SRB51 did not enhance lymphocyte proliferative responses to S2308.

Addition of exogenous IL-12 to culture supernatants of spleen cells obtained at 4 and 8 wk, but not at 2 or 12 wk after vaccination, enhanced lymphocyte proliferative responses of all vaccine treatments (Table II). Maximal enhancement of lymphocyte responses was associated with the addition of 0.1 ng/mL of exogenous IL-12 to cell cultures.

Mice vaccinated with live SRB51 (5 × 10^8 cfu) had lower ($P \leq 0.01$) spleen weights (data not shown), total spleen cfu (data not shown), and spleen cfu/g (Fig. 2) following challenge at 12 wk after vaccination when compared to mice receiving saline control, IL-12 control or killed SRB51 treatments. Administration of IL-12 as an adjuvant with live or killed SRB51 did not enhance resistance against experimental challenge with S2308.

The current study was designed to determine if a single administration of 0.5 μg of IL-12 in conjunction with vaccination with live or killed bacteria would modify immune responses or enhance protective immune responses against *Brucella*. This cytokine has been demonstrated to play a role in murine resistance to *B. abortus* (8) and could be beneficial as an adjuvant for vaccines against organisms for which cell-mediated immunity is required for protection. As single administration vaccines are more readily accepted for routine field use in domestic livestock, we were interested in determining if a single administration of IL-12 would have beneficial adjuvant activities on brucellosis vaccines. Two administrations (Days 0 and 5, or 0 and 10) of the dosage evaluated in this study (0.5 μg/mouse) had been successfully used previously in vaccination regimens to augment protective immunity against *Listeria* (3) and *Leishmania* (4).

Data from our study did not suggest that the addition of IL-12 (0.5 μg) to a vaccine containing live or killed SRB51 enhanced protective immunity against *Brucella*. Our data also did not suggest that single administration of 0.5 μg of IL-12 augmented cellular

immune responses to live or killed *Brucella* as clearance of the vaccine strain, lymphocyte proliferative responses, and protection against experimental challenge were not enhanced in mice receiving IL-12 as an adjuvant. The IL-12 used in this study had biological activity in mice as, in accordance with other reports, in vitro proliferative responses were enhanced by exogenous IL-12. The possibility that dosages above 0.5 μg, or multiple IL-12 dosages, might have beneficial adjuvant activities on *Brucella* vaccines cannot be eliminated. Based on reports which demonstrated that *B. abortus* or its lipopolysaccharide provide a strong stimulus for induction of IL-12 synthesis in human monocytes (9), an alternate hypothesis may be that live or γ-irradiated SRB51 can maximally stimulate IL-12 synthesis in BALB/c mice. If this hypothesis was true, it may imply that other cytokines or signals are required for protective immunity against *Brucella* which are not induced by γ-irradiated SRB51 alone, or in combination with 0.5 μg of IL-12.

Due to the potential for human infection following accidental exposure to live *Brucella* vaccines, it would be of benefit to develop a *Brucella* vaccine using killed bacteria or *Brucella* antigens. At the present time, non-living *Brucella* vaccines have not been identified which are acceptable alternatives to the live vaccines currently used in livestock in the United States. The present study cannot eliminate the possibility that use of IL-12 in other vaccination regimens could have beneficial adjuvant properties on live or killed brucellosis vaccines. However, our data would suggest that 0.5 μg of IL-12 at the time of vaccination does not influence immune responses to live or killed *Brucella* vaccines.

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