INFECTION AND IMMUNITY, Oct. 2001, p. 6541–6544 Vol. 69, No. 10
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.10.6541–6544.2001

Mouse Cytokine Profiles Associated with *Brucella abortus* RB51 Vaccination or *B. abortus* 2308 Infection

PAOLO PASQUALI,[1]* ROSANNA ADONE,[1] LOUIS C. GASBARRE,[2] CLAUDIA PISTOIA,[1] AND FRANCO CIUCHINI[1]

Laboratory of Veterinary Medicine, Istituto Superiore di Sanità, 00161 Rome, Italy,[1] and Immunology and Disease Resistance Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20705[2]

Received 27 February 2001/Returned for modification 18 April 2001/Accepted 25 July 2001

This study indicated that mice immunized with *Brucella abortus* RB51 bacteria and subsequently challenged with *B. abortus* 2308 were protected from reinfection. After vaccination, both Th1 and Th2 cytokine patterns were observed. Of those, the early production of gamma interferon seems to have the prominent role in inducing an immunologically based protection.

An attenuated mutant of virulent *Brucella abortus* 2308, designated *B. abortus* RB51, is currently being evaluated as an alternative vaccine to *B. abortus* S19 because it does not induce antibodies to *Brucella* lipopolysaccharide (LPS) O antigens (12, 13, 15–17), and thus it is possible to discriminate between infected and vaccinated animals (1). Vaccination with *B. abortus* RB51 confers protection against a pathogenic challenge infection in both mice (8, 14, 16, 18) and cattle (2, 9, 10, 11, 15). In mice, the bacteria can persist for a short period of time in the spleens (14). Vaccination induces both antibody responses and proliferation in spleen cell populations. Protection induced by vaccination with *B. abortus* RB51 is based upon cell-mediated immunity and antibody plays a minor role in protection (8, 14–18). Because little information is available on cell-immune responses following *B. abortus* RB51 vaccination, the present study was performed to delineate cytokine induction after intraperitoneal (i.p.) administration of *B. abortus* RB51 and subsequent infection with the virulent strain *B. abortus* 2308.

Cell cultures were performed in RPMI 1640 medium containing 2 mM L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 10% fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol, 100 U of penicillin, and 100 μg of streptomycin per ml (RPMI).

Twelve- to 14-week-old BALB/c female mice (Charles River, Calco, Italy) were allocated to experimental groups consisting of five animals each. Mice were vaccinated i.p. with 0.2 ml of saline containing 2×10^8 CFU of *B. abortus* RB51. Unvaccinated control animals remained untreated throughout the experiment. At 42 days after vaccination, unvaccinated controls and i.p. vaccinated mice were challenged i.p. with 0.2 ml of saline containing 2×10^4 CFU of *B. abortus* 2308.

At 6, 18, and 42 days after vaccination and 3, 6, and 10 days after challenge, mice were euthanatized and spleens were aseptically removed. Approximately one-third of the spleen was weighed and homogenized in phosphate-buffered saline (PBS), and an aliquot of the resulting cell suspension was plated to determine the number of CFU. The remaining two-thirds of the spleens were weighed, minced, and used to prepare spleen cell suspensions. Cytokine expression was assayed in culture supernatants of splenocytes stimulated with 10^8 heat-inactivated *B. abortus* RB51 or 2308 bacteria per well. Spleen cells were maintained in duplicate in 24-well plates at 37°C in a 5% CO_2 atmosphere in RPMI at 2×10^6 cells/well in a total volume of 1 ml. Supernatants were harvested on day 2 for interleukin-12 (IL-12) p40 and p70 and on day 3 for gamma interferon (IFN-γ), IL-4, and IL-10 after bacterial stimulation. Cytokines were detected by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, Minneapolis, Minn.). Differences between groups were estimated by a one-way analysis of variance. Differences were considered significant with a P value of ≤ 0.05.

Vaccinated mice had higher spleen weights when compared to control animals, being (mean ± standard deviation [SD]) 168 ± 31, 358 ± 53, and 180 ± 6 mg at 6, 18, and 42 days, respectively, after vaccination (Table 1). Vaccination of mice with live *B. abortus* RB51 cells resulted in a pattern of bacterial growth in which there were $7.1 \times 10^5 \pm 3.6 \times 10^5$ CFU/spleen after 6 days, and peak numbers ($8.4 \times 10^6 \pm 2.8 \times 10^6$ CFU/spleen) were seen 18 days after vaccination, followed by a progressive decline. Bacteria were absent in the spleen at 42 days postvaccination. Counts on days 6 and 18 were significantly different ($P \leq 0.05$).

As shown in Table 2, spleen weight in vaccinated and infected mice did not increase after challenge (158 ± 23, 180 ±

TABLE 1. Effect of vaccination with *B. abortus* RB51 on spleen weight[a]

Treatment	Spleen weight (mg)[b]		
	6 days	18 days	42 days
None	100 ± 10*	96 ± 5*	95 ± 7*
Vaccination	168 ± 31**	358 ± 53***	180 ± 6**

[a] BALB/c mice were treated as shown, and spleen weights were assessed at 6, 18, and 42 days after vaccination. Data are mean values of five animals ± SD.
[b] Groups with a different number of asterisks are significantly different ($P \leq 0.05$).

* Corresponding author. Mailing address: Laboratory of Veterinary Medicine, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. Phone: 39 (0)6 49902728. Fax: 39 (0)6 49387077. E-mail: pasquali@iss.it.

TABLE 2. Effect of challenge infection with *B. abortus* 2308 on spleen weight of vaccinated and unvaccinated mice[a]

Treatment	Spleen weight (mg)[b]		
	3 days	6 days	10 days
None + challenge	136 ± 13*	254 ± 34**	258 ± 68**
Vaccination + challenge	158 ± 23*	180 ± 42*	132 ± 22*

[a] BALB/c mice were treated as shown, and spleen weights were assessed at 3, 6, and 10 days after challenge infection. Data are mean values of five animals ± SD.
[b] Group with different number of asterisks are significantly different ($P \leq 0.05$).

42, and 132 ± 22 mg [mean ± SD] at 3, 6, and 10 days, respectively, after challenge infection) and were not significantly different from nonchallenged, vaccinated animals killed 42 days after vaccination (180 ± 6 mg). In contrast, spleen weights of unvaccinated and infected mice showed a significant enlargement as early as 6 days after infection. Vaccinated mice were almost refractory to a subsequent challenge infection with *B. abortus* 2308 cells (Table 3), exhibiting significantly lower levels of infection compared to unvaccinated infected animals 3, 6, and 10 days after infection.

Vaccination alone induced a number of significant effects upon cytokine expression (Table 4). Spleen cells from unvaccinated mice stimulated with heat-inactivated *B. abortus* RB51 cells did not produce measurable cytokines throughout the course of the experiment (data not shown). Similarly, spleen cells from vaccinated mice killed 6 and 18 days after vaccination did not produce measurable bio-active IL-12 (p70) when stimulated in vitro, but a low level of production was detectable in spleen cells from mice killed 42 days after vaccination. The non-bio-active form of IL-12 (p40) was detectable 6 days after vaccination and increased throughout the experiment, although the differences were not statistically significant. Spleen cells from animals killed 6 and 18 days after vaccination produced similar amounts of IFN-γ, and the level was noticeably increased in mice killed 42 days after vaccination ($P < 0.05$). Peak induction of IFN-γ in spleen cells from mice killed 42 days after vaccination corresponded to an observed reduction in spleen weight and bacterial counts in these animals. IL-4 was not detectable in any mice (data not shown). Finally, IL-10 production by spleen cells increased throughout the course of the experiment, but the levels were not significantly different over time.

Subsequent challenge infection of vaccinated and nonvaccinated mice induced even more marked increases in cytokine expression patterns. As shown in Table 5, IL-12 p40 was detectable in spleen cells from both vaccinated and unvaccinated mice after challenge infection, and there were no significant differences between vaccinated and unvaccinated mice. Similar results were seen throughout the course of the experiment for IL-12 p70 production, which was low at all times in all groups of mice. In contrast, IFN-γ production was detectable as early as 3 days after challenge infection in spleen cells from both vaccinated and unvaccinated mice, although levels were significantly higher in vaccinated mice. Levels of IFN-γ in spleen cells from vaccinated, challenged mice showed no differences throughout the experiment, in contrast to spleen cells from unvaccinated, challenged mice which showed increased IFN-γ production that reached levels seen in spleen cells from vaccinated, challenged mice 6 days after challenge. After challenge infection, low levels of IL-4 were detectable, reaching a peak in spleen cells 6 days after challenge. However, levels were not different between vaccinated and unvaccinated mice. IL-10 showed a pattern similar to that of IFN-γ. IL-10 was detectable in both vaccinated and unvaccinated mice after challenge infection. However, vaccinated and challenged animals did not show any differences at different times after challenge. Conversely, IL-10 production in unvaccinated, challenged mice was first detectable 6 days after challenge infection and reached a peak 10 days after challenge infection.

Results of this study indicated that mice vaccinated with *B. abortus* RB51 cells were protected as early as 3 days after a challenge infection with virulent *B. abortus* 2308 cells. Vaccination induced high levels of IFN-γ that were found to be positively correlated with clearance of the infection. Moreover, in vitro-stimulated spleen cells from vaccinated and challenged mice produced higher levels of IFN-γ than did unvaccinated, challenged mice during the early phase of infection. This high level of early IFN-γ production coupled with the lower bacterial persistence in the spleens of vaccinated mice argues that IFN-γ could be crucial during the early phase of infection, but once infection is established it plays a minor role in resistance. This finding may explain why virulent strains of *B. abortus* induce chronic infection in spite of the fact that they stimulate IFN-γ production (19–22). Our findings further suggest that temporal relationships between brucellae, macrophages, and IFN-γ may determine the outcome of the infection.

In this study, the induction patterns of other cytokines that have been implicated in resistance to infectious agents was also investigated. Both IL-12 p70 and IL-12 p40 levels from spleen cells were assessed to determine their role in the development of protection. The level of IL-12 p70 was very low in vaccinated mice and did not change after challenge infection, suggesting that *Brucella* is not a potent inducer of IL-12 p70 (4, 7). One possible explanation is that in brucellosis, IL-12 may not act in a soluble form but rather as a membrane-bound protein on macrophages (3). Conversely, the production of IL-12 p40 was

TABLE 3. Persistence of *B. abortus* 2308 in spleens of vaccinated and unvaccinated mice[a]

Treatment	CFU of *B. abortus* 2308 cells in spleen[b]		
	3 days	6 days	10 days
None + challenge	$9.3 \times 10^4 \pm 6.7 \times 10^4$**	$2 \times 10^6 \pm 1 \times 10^6$***	$4.7 \times 10^6 \pm 1 \times 10^6$***
Vaccination + challenge	$7.3 \times 10^3 \pm 5.4 \times 10^3$*	$1.2 \times 10^4 \pm 2.1 \times 10^4$*	$1.9 \times 10^4 \pm 2.2 \times 10^4$*

[a] BALB/c mice were treated as shown, and bacteria present in spleen were assessed at 3, 6, and 10 days after vaccination. Data are mean values of five animals ± SD.
[b] Group with different number of asterisks are significantly different ($P \leq 0.05$).

TABLE 4. Production of cytokines in stimulated spleen cells from mice after *B. abortus* RB51 vaccination[a]

Cytokine	Cytokine production (pg/ml)		
	6 days	18 days	42 days
IFN-γ	566 ± 188	903 ± 278	2,568 ± 413
IL-12 p70	0	0	9 ± 6
IL-12 p40	269 ± 86	400 ± 69	379 ± 61
IL-10	154 ± 63	98 ± 28	302 ± 115

[a] BALB/c mice were vaccinated and cytokine productions in stimulated spleen cells were assessed at 6, 18, and 42 days after vaccination. Data are mean values of five animals ± SD.

detected early. Significant differences between vaccinated and unvaccinated mice were observed only in mice killed 6 days after challenge infection. The basis of this observation remains to be investigated. Interestingly, *B. abortus* RB51 and 2308 seem to induce different patterns of IL-12 p40 production. IL-12 p40 production was higher in mice vaccinated with *B. abortus* RB51 cells and killed 6 days later than in unvaccinated mice challenged with *B. abortus* 2308 cells and killed 3 ($P = 0.06$) and 6 ($P < 0.05$) days after infection. This suggests that chronic infection with virulent *B. abortus* 2308 cells is not the result of the secretion of the nonimmunoactive form of the cytokine which then can interfere with the establishment of immunity (4).

Finally, the kinetic pattern of Th2 cytokines after vaccination and challenge was investigated. IL-4 was never detected in *B. abortus* RB51-vaccinated mice. However, after challenge infection with *B. abortus* 2308 cells, a very low level of IL-4 was detectable. This level did not differ between vaccinated and unvaccinated mice, indicating little involvement of IL-4 in the immune response to *Brucella* infection. The kinetics of IL-10 production of *B. abortus* 2308-stimulated spleen cells from immunized and infected mice showed an enhanced production of IL-10 in vaccinated mice compared to that in control mice. In vaccinated mice, challenge infection resulted in high levels of IL-10 production at all times tested. In contrast, in unvaccinated mice, IL-10 production reached levels comparable to that in the vaccinated mice beginning 6 days after infection. Previous studies indicate that both IL-10 and IFN-γ are produced following a *B. abortus* infection and that IL-10 induction does not down-regulate IFN-γ production. This implies that in brucellosis, the effect of IL-10 on the immune response is to limit the consequences of an exaggerated proinflammatory response more than to counterbalance the production of Th1 cytokines. These findings are in agreement with others which measured IL-10 production following *Brucella* infection (6, 19). Although there is evidence that IL-10 may be detrimental in brucellosis (5), it is reasonable to assume that optimal development and maintenance of a protective response against the infection relies on a finely regulated balance of cytokines rather than upon the level of a single cytokine.

TABLE 5. Production of cytokines in stimulated spleen cells from vaccinated or unvaccinated mice challenged with *B. abortus* 2308 and killed 3, 6, and 10 days after challenge[a]

Cytokine	Treatment	Cytokine production (pg/ml)[b]		
		3 days	6 days	10 days
IFN-γ	None + challenge	807 ± 465	1,676 ± 122	1,518 ± 249
	Vaccination + challenge	1,665 ± 409*	1,919 ± 204	1,404 ± 677
IL-12 p70	None + challenge	15 ± 13	7 ± 11	2 ± 3
	Vaccination + challenge	22 ± 12	0	0
IL-12 p40	None + challenge	168 ± 72	77 ± 47	417 ± 290
	Vaccination + challenge	184 ± 56	252 ± 192*	223 ± 133
IL-10	None + challenge	0	18 ± 19	71 ± 55
	Vaccination + challenge	35 ± 29	35 ± 52	91 ± 79
IL-4	None + challenge	9 ± 5	32 ± 16	10 ± 12
	Vaccination + challenge	9 ± 5	31 ± 26	9 ± 4

[a] BALB/c mice were treated as shown, and cytokine productions in stimulated spleen cells were assessed at 3, 6, and 10 days after challenge. Data are mean values of five animals ± SD.

[b] Groups with different number of asterisks are statistically different compared to unavaccinated controls ($P \leq 0.05$).

In conclusion, our results confirm and extend knowledge regarding the immune response to *Brucella* infection. *B. abortus* RB51 confers a solid immunity that protects mice against a challenge infection with virulent *B. abortus* 2308. After vaccination, both Th1 and Th2 cytokine patterns were observed. Although the definitive confirmation of our findings would require the use of IFN-γ-depleted or IFN-γ knockout mice, it is conceivable that *B. abortus* RB51-induced protection is the result of a precocious production of IFN-γ that may prime macrophages, resulting in the inhibition of the establishment of persistent infections after challenge infection. Thus, protection may be more dependent upon the timing of the cytokine response rather than on the absolute level of cytokine expression.

We are grateful to Giacomo Marcon and Antonella Romeo for their excellent technical assistance.

REFERENCES

1. **Adone, R., and F. Ciuchini.** 1999. Complement fixation test to assess humoral immunity in cattle and sheep vaccinated with *Brucella abortus* RB51. Clin. Diagn. Lab. Immunol. **6:**787–790.
2. **Elzer, P. H., F. M. Enright, L. Colby, S. D. Hagius, J. V. Walker, M. B. Fatemi, J. D. Kopec, V. C. Beal, and G. G. Schurig.** 1998. Protection against infection and abortion induced by virulent challenge exposure after oral vaccination of cattle with *Brucella abortus* strain RB51. Am. J. Vet. Res. **59:** 1575–1578.
3. **Fan, X., V. Sibalic, E. Niederer, and R. P. Wüthrich.** 1996. The proinflammatory cytokine interleukin-12 occurs as a cell membrane-bound form on macrophages. Biochem. Biophys. Res. Commun. **225:**1063–1067.
4. **Fernandez-Lago, L., E. Rodriguez-Tarazona, and N. Vizcaino.** 1999. Differential secretion of interleukin-12 (IL-12) subunits and heterodimeric IL-12 p70 protein by CD-1 mice and murine macrophages in response to intracellular infection by *Brucella abortus*. J. Med. Microbiol. **48:**1065–1073.
5. **Fernandes, D. M., and C. L. Baldwin.** 1995. Interleukin-10 downregulates protective immunity to *Brucella abortus*. Infect. Immun. **63:**1130–1133.
6. **Hoover, D. L., R. M. Crawford, L. L. Van De Verg, M. J. Izadjoo, A. K. Bhattacharjee, C. M. Paranavitana, R. L. Warren, M. P. Nikolich, and T. L. Hadfield.** 1999. Protection of mice against brucellosis by vaccination with *Brucella melitensis* WR201 (16MΔ*purEK*). Infect. Immun. **67:**5877–5884.
7. **Huang, L.-Y, A. M. Krieg, N. Eller, and D. E. Scott.** 1999. Induction of regulation of Th1-inducing cytokines by bacterial DNA, lipopolysaccharide, and heat-inactivated bacteria. Infect. Immun. **67:**6257–6263.
8. **Jimenez De Bagues, M. P., P. H. Elzer, S. M. Jones, J. M. Blasco, F. M. Enright, G. G. Schurig, and A. J. Winter.** 1994. Vaccination with *Brucella abortus* rough mutant RB51 protects BALB/c mice against virulent strains of *Brucella abortus, Brucella melitensis*, and *Brucella ovis*. Infect. Immun. **62:** 4990–4996.
9. **Lord, V. R., G. G. Schurig, J. W. Cherwonogrodzky, M. J. Marcano, and G. E. Melendez.** 1998. Field study of vaccination of cattle with *Brucella*

abortus strain RB51 and 19 under high and low disease prevalence. Am. J. Vet. Res. **59:**1016–1020.
10. **Olsen, S. C., B. Bricker, M. V. Palmer, and A. E. Jensen.** 1999. Response of cattle to two dosages of *Brucella abortus* RB51: serology clearance and efficacy. Res. Vet. Sci. **66:**101–106.
11. **Palmer, M. V., S. C. Olsen, and N. F. Cheville.** 1997. Safety and immunogenicity of *Brucella abortus* strain RB51 vaccine in pregnant cattle. Am. J. Vet. Res. **58:**472–477.
12. **Schurig, G. G., R. M. Roop II, T. Bagchi, S. Boyle, D. Buhrman, and N. Sriranganathan.** 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet. Microbiol. **28:**171–188.
13. **Stevens, M. G., S. G. Hennager, S. C. Olsen, and N. F. Cheville.** 1994. Serologic responses in diagnostic tests for brucellosis in cattle vaccinated with *Brucella abortus* 19 or RB51. J. Clin. Microbiol. **32:**1065–1066.
14. **Stevens, M. G., S. C. Olsen, G. W Pugh, Jr., and M. V. Palmer.** 1994. Immune and pathologic response in mice infected with *Brucella abortus* 19, RB51, or 2308. Infect. Immun. **62:**3206–3212.
15. **Stevens, M. G., S. C. Olsen, and N. F. Cheville.** 1995. Comparative analysis of immune responses in cattle vaccinated with *Brucella abortus* strain 19 or RB51. Vet. Immunol. Immunopathol. **44:**223–235.
16. **Stevens, M. G., S. C. Olsen, and G. W Pugh, Jr.** 1995. Comparison of spleen cell proliferation in response to *Brucella abortus* 2308 lipopolysaccharide or proteins in mice vaccinated with strain 19 or RB51. Infect. Immun. **63:**3199–3205.
17. **Stevens, M. G., S. C. Olsen, M. V. Palmer, G. W Pugh, Jr., and D. Brees.** 1995. Comparison of immune responses and resistance to brucellosis in mice vaccinated with *Brucella abortus* 19 or RB51. Infect. Immun. **63:**264–270.
18. **Stevens, M. G., S. C. Olsen, M. V. Palmer, and G. W Pugh, Jr.** 1996. Immune responses and resistance to brucellosis in mice vaccinated orally with *Brucella abortus* RB51. Infect. Immun. **64:**4534–4541.
19. **Svetic, A., Y. C. Jian, P. Lu, F. D. Finkelmann, and W. C. Gause.** 1993. *Brucella abortus* induces a novel cytokine gene expression pattern characterized by elevated IL-10 and IFN-γ in CD4+ T cells. Int. Immunol. **5:**877–883.
20. **Zaitseva, M. B., H. Golding, M. Betts, A. Yamauchi, E. T. Bloom, L. E. Butler, L. Stevan, and B. Golding.** 1995. Human peripheral blood CD4+ and CD8+ T cells express Th1-like cytokine mRNA and proteins following in vitro stimulation with heat-inactivated *Brucella abortus*. Infect. Immun. **63:**2720–2728.
21. **Zaitseva, M., H. Golding, J. Manischewitz, D. Webb, and B. Golding.** 1996. *Brucella abortus* as a potential vaccine candidate: induction of IL-12 secretion and enhanced B7.1 and B7.2 and intercellular adhesion molecule 1 surface expression in elutriated human monocytes stimulated by heat-inactivated *B. abortus*. Infect. Immun. **64:**3109–3117.
22. **Zhan, Y., A. Kelso, and C. Cheers.** 1993. Cytokine production in the murine response to *Brucella* infection or immunization with antigenic extracts. Immunology **80:**458–464.

Editor: R. N. Moore