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Induction of Hyperreactivity to Endotoxin in Mice by *Coxiella burnetii*

S. SCHRAMEK,[1] J. KAZAR,[1] Z. SEKEYOVA,[1] M. A. FREUDENBERG,[2] AND C. GALANOS[2]*

Institute of Virology, Slovak Academy of Sciences, 817 03 Bratislava, Czechoslovakia,[1] and Max-Planck-Institut für Immunbiologie, D-7800 Freiburg im Breisgau, Federal Republic of Germany[2]

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Intraperitoneal inoculation of mice with live or killed *Coxiella burnetii* phase I or phase II cells induced a marked hyperreactivity to the lethal effect of bacterial endotoxin and was accompanied by a marked hepatosplenomegaly. The degree and duration of hyperreactivity depended on the dose of *C. burnetii* administered and were higher with phase I than with phase II cells. Sensitization to the lethal effects of endotoxin and induction of splenomegaly by phase I *C. burnetii* cells also proceeded in the endotoxin-resistant C3H/HeJ strain of mice. Preincubation of *C. burnetii* cells with the corresponding immune serum significantly diminished the ability of phase I but not phase II cells to induce hyperreactivity to endotoxin.

Endotoxins isolated from gram-negative bacteria induce a wide spectrum of biological changes that may lead to shock and death in different experimental animal species (8, 32). The normal sensitivity to endotoxin may be increased by pretreatment of the animals with various chemical agents and with certain microorganisms. Thus, treatment with actinomycin D (1), lead acetate (27), or D-galactosamine (6) was shown to increase the sensitivity of mice to the lethal effects of endotoxin several 1,000-fold. Similarly, pretreatment with bacteria, that possess properties generally designated as immunomodulatory, like BCG (29) or *Corynebacterium parvum* (8), was found to increase susceptibility to endotoxin lethality, often accompanied by splenomegaly and hepatomegaly (33). The bacterial components responsible for the above sensitization and the mechanisms involved have not yet been defined.

Coxiella burnetii, the causative agent of Q fever, was shown to elicit in experimental animals a number of biological activities, resembling those of the bacteria described above. In experimental models, *C. burnetii* induces resistance to bacterial infection (14, 17) and to blood parasites (3), stimulates interferon production (9), possesses antitumor activity (12, 15), and suppresses the normal response of spleen cells to mitogens (4, 16).

C. burnetii was also shown to lead to splenomegaly in mice (5, 11, 13, 34) and to increased sensitivity to the lethal effects of endotoxin in guinea pigs (20) after infection with live *C. burnetii* cells, and in mice (3) after treatment with killed *C. burnetii* phase I cells. These data were obtained in studies carried out independently and in different animal models, and, therefore, do not allow conclusions to be made on the relation between hyperreactivity to endotoxin and hyperplasia of the liver and spleen. Furthermore, the induction of the above phenomena was not studied in relation to the phase state of *C. burnetii* cells or in relation to viability (live or killed *C. burnetii* cells).

In the present communication, the effect of live and killed phase I and phase II *C. burnetii* cells on endotoxin sensitivity is studied in relation to splenomegaly and hepatomegaly in endotoxin-sensitive and endotoxin-resistant mice. Also, the effect of phase-specific antisera on the induction of the above activities is investigated.

MATERIALS AND METHODS

Cultivation and purification of rickettsial cells. *C. burnetii* Nine-Mile cells in phase I and in pure phase II (chicken embryo yolk sac passages 3 and 163, respectively) were propagated in chicken embryo yolk sacs. The strains were partially purified by differential centrifugation in phosphate-buffered saline (PBS), resuspended in brain-heart infusion, and stored at −20°C. The rickettsial cells were killed wth Formalin (0.5%) or aqueous phenol (1%) and purified as described previously (24). The purified cells were adjusted to a concentration of 2 mg/ml in PBS and stored at −20°C.

Antisera. Rabbits received two doses of 1 mg of phenol-killed *C. burnetii* cells administered intraperitoneally (i.p.) on days 1 and 21, and serum was collected 14 days later. Phase I antiserum was found to contain complement-fixing antibodies to both antigens 1 and 2 in titers of 64 and 256, respectively. Phase II antiserum contained antibodies directed to antigen 2 only, in a titer of 128 as measured by the complement-fixation test.

Endotoxin. The preparation used was the lipopolysaccharide of *Salmonella abortus-equi* in sodium form. Details of its preparation were described elsewhere (7).

Animals. Outbred white mice weighing 16 to 20 g were obtained from the Dobra Voda breed; C57/BL6, C3H, and the endotoxin-resistant C3H/HeJ mice were obtained from the breeding stock of the Max-Planck-Institut für Immunbiologie, Freiburg im Breisgau, Federal Republic of Germany. All mice used were 6 to 8 weeks old.

Induction and measurement of hyperreactivity to endotoxin. Mice were inoculated i.p. with the given *C. burnetii* preparation in 0.5 ml of PBS. At different time intervals thereafter, the animals were injected i.p. with different amounts of *S. abortus-equi* lipopolysaccharide in 0.5 ml of PBS, and deaths occurrring within 24 h were recorded. (In all experiments all deaths of hypersensitive animals occurred within this time.) Mice inoculated with PBS only served as control. After 24 h, surviving animals were sacrificed. Spleens and livers were removed from all the mice and weighed. In some experiments, spleen or liver enlargement is expressed as a splenic or hepatic index (mean organ weight of tested group per that

* Corresponding author.

TABLE 1. Induction of hypersensitivity to endotoxin and hepatosplenomegaly in mice infected with phase I and phase II *C. burnetii* cells

Pretreatment	Endotoxin treatment on day	Endotoxin LD_{50} (μg)	Avg wt[a]	
			Spleen (mg)	Liver (g)
C. burnetii phase I cells	7	5	629 ± 166	3.05 ± 0.43
	14	3	975 ± 262	4.03 ± 1.02
C. burnetii phase II cells	7	53	397 ± 152	1.82 ± 0.39
	14	>100	289 ± 136	1.85 ± 0.23
None		>500	280 ± 126	1.80 ± 0.25

[a] Average for 18 mice with standard deviations.

of control group). The results given are representative of several experiments carried out at the Institute of Virology and at the Max-Planck-Institut.

Statistical analysis. Mean values and standard deviations of data obtained were calculated for all experiments. Differences between two mean values were assessed by Student's *t* test. Mean values of 50% egg infective doses (EID_{50}) of *C. burnetii* and mean values of 50% lethal doses (LD_{50}) of endotoxin were calculated by the method of Reed and Muench (21).

RESULTS

Sensitization of mice to the lethal effect of endotoxin by *C. burnetii* infection. Mice from the Dobra Voda breed were infected i.p. with 10^6 EID_{50} of phase I or phase II *C. burnetii* cells. On days 7 and 14 postinfection, groups of six animals received 100, 10, or 1 μg of endotoxin i.p., and deaths occurring within 24 h were recorded. Hypertrophy of the spleen and liver was investigated in dead and surviving animals. In mice infected with phase I *C. burnetii* cells, a marked increase in susceptibility to the lethal effect of endotoxin was observed, which was paralleled by splenomegaly and hepatomegaly (Table 1). In mice infected with phase II *C. burnetii* cells, the level of sensitization to endotoxin and the degree of spleen and liver hyperplasia were lower and only of short duration. It should be mentioned that after infection, the rate of multiplication of phase II cells in mice is known to be significantly lower than that of phase I cells. To see whether the low sensitization obtained with phase II infection was due to a lower rate of multiplication, the animals were treated with cyclophosphamide (CPA) which enhanced multiplication of phase I cells (11). Groups of six animals (outbred mice) were infected with 10^6 EID_{50} of *C. burnetii* phase II cells. On day 2, test groups received CPA (200 mg/kg) administered subcutaneously. Groups receiving PBS on day 2 served as controls. On days 7 and 14 after infection, CPA-treated and control groups received increasing amounts (1, 10, and 100 μg) of endotoxin, and lethality and liver and spleen weights were determined. In parallel, multiplication of phase II cells in the spleen was determined as described by Kazar et al. (10). CPA treatment enhanced the rate of multiplication of phase II cells in the spleen (Table 2). Susceptibility to endotoxin and spleen and liver weights markedly increased on days 7 and 14 and were comparable to those obtained with phase I infection (Table 1).

TABLE 3. Time course and duration of hyperreactivity to endotoxin induced by different infective doses of phase I *C. burnetii* cells in mice[a]

C. burnetii EID_{50}	Lethality (dead/total) with 100 μg of endotoxin on day:				
	1	3	7	21	90
10^7	1/6	3/6	6/6	6/6	3/6
10^5	ND[b]	0/6	6/6	6/6	1/6
10^3	ND	ND	6/6	6/6	0/6
10^1	ND	ND	5/6	0/6	0/6

[a] In normal mice no lethality occurred with doses of up to 500 μg of endotoxin.
[b] ND, Not determined.

To determine the effect of different doses of phase I *C. burnetii* cells on the degree and duration of sensitization, outbred mice were infected i.p. with graded doses of phase I cells. On days 1, 3, 7, 21, and 90 postinfection, groups of six mice were administered 100 μg of endotoxin i.p., i.e., a nonlethal dose for normal mice, and lethality was recorded. The results indicate that duration of hyperreactivity to endotoxin was dependent on the infective dose of *C. burnetii* cells (Table 3). With 10^7 EID_{50} of cells, hyperreactivity was still detectable 90 days later. Hepatosplenomegaly in this experiment was followed only up to day 21 postinfection (Table 4). It increased with increasing infective dose, reaching the maximum with 10^5 EID_{50} of cells.

The duration of hepatosplenomegaly induced by *C. burnetii* phase I infection was investigated in mice over a period of 120 days, after inoculation with 10^6 EID_{50} of live cells. Figure 1 shows the index of hepatomegaly and splenomegaly over the above period of observation. After infection, both organs increased in weight by day 5, and reached maximum weights on days 15 to 35. Thereafter, mean weight values decreased slowly. Hepatomegaly was detectable up to about day 70, whereas splenomegaly persisted up to the end of the observation time (120 days). At the height of hepatosplenomegaly, mean weights for the liver and spleen were 3.25 and 9.4 times higher, respectively, than in the controls.

Effect of killed *C. burnetti* cells on endotoxin sensitivity and

TABLE 2. Influence of CPA treatment of mice on their hyperreactivity to endotoxin induced by infection with 10^6 EID_{50} of phase II *C. burnetii* cells

Mice	Day of *C. burnetii* infection and endotoxin administration	Degree of *C. burnetii* multiplication in mouse spleen[a]	Endotoxin LD_{50} (μg)	Avg wt	
				Spleen (mg)	Liver (g)
CPA untreated	7	+	85	301 ± 144	1.74 ± 0.49
CPA treated[b]	7	+++	3	337 ± 170	1.46 ± 0.35
CPA untreated	14	+	>100	252 ± 62	1.85 ± 0.46
CPA treated[b]	14	++	7	579 ± 175	2.46 ± 0.43

[a] +, Less than 10; ++, 10s; and +++, 100s of rickettsiae per each field of view from 10 different fields of view at a magnification of ×1,000 after staining the spleen impression smears by the method of Gimenez.
[b] 200 mg of CPA per kg of body weight given subcutaneously 2 days after infection with 10^6 EID_{50} of *C. burnetii* cells.

TABLE 4. Hepatosplenomegaly in mice on day 21 postinfection with different doses of *C. burnetii* phase I cells

C. burnetii phase I cell EID_{50}	Mean wt on day 21[a]	
	Spleen (mg)	Liver (g)
10^7	1,493 ± 329	4.76 ± 0.90
10^5	1,858 ± 302	4.51 ± 0.71
10^3	817 ± 234	2.68 ± 0.31
10^1	301 ± 104	2.31 ± 0.17
None	242 ± 79	1.97 ± 0.19

[a] Mean weights for groups of six outbred mice with standard deviations.

on liver and spleen in mice. Outbred mice received 1, 0.1, or 0.01 mg of killed phase I cells or 1 mg of killed phase II cells i.p. After 14 days, groups of six animals were injected with various amounts (1, 10, and 100 μg) of endotoxin, and the resulting lethality was recorded. After 24 h, spleen and liver weights were determined for dying and surviving animals. A high susceptibility to the lethal effects of endotoxin was obtained with the highest dose (1 mg) of phase I cells (LD_{50} = 2.3 μg) (Table 5). A lower degree of sensitization was obtained with 0.1 mg of phase I cells (LD_{50} = 32 μg), whereas 0.01 mg had virtually no effect (LD_{50} < 100 μg).

In comparison, phase II cells were less effective in inducing hyperreactivity to endotoxin. The sensitization (LD_{50} = 36 μg) obtained with 1 mg of cells was comparable to that obtained with 0.1 mg of phase I cells. In all cases, increased sensitivity to endotoxin correlated with liver and spleen enlargement.

A degree of sensitization to endotoxin and of hepatosplenomegaly comparable to the one observed in Dobra Voda mice was also obtained with the corresponding *C. burnetii* phase in C57/BL6 and C3H mice (results not shown).

The duration of sensitization to endotoxin by killed *C. burnetii* cells is shown in Table 6. Mice received 1 mg of cells of either phase i.p. At different times, up to 75 days thereafter, groups of four animals were challenged with 100 μg of endotoxin. Deaths occurring within 24 h were recorded, and spleen and liver weights of all animals were determined. After treatment with phase I cells, hypersensitivity persisted for a long time (up to 8 weeks) and correlated with a high increase in liver and spleen weight (Table 6). With phase II cells, the duration of hypersensitivity was shorter, practically disappearing by week 5.

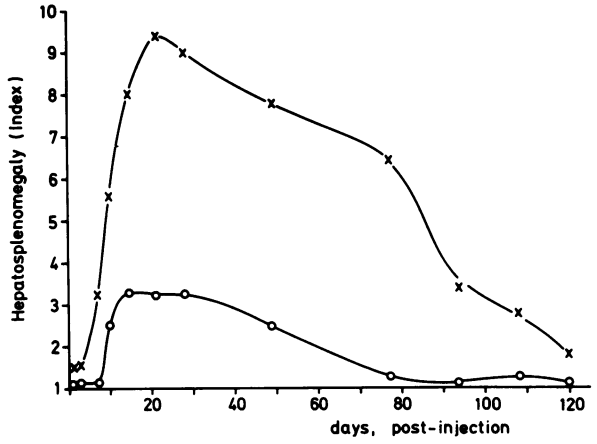

FIG. 1. Duration of hepatosplenomegaly in mice infected with *C. burnetii* phase I cells. Mice were inoculated i.p. with 10^6 EID_{50} of live, phase I cells in 0.5 ml of PBS. Control animals received PBS alone. At different times thereafter groups of six infected and six control animals were sacrificed, and liver and spleen weights were determined. Splenomegaly or hepatomegaly is expressed as an index. Index = mean organ weight in infected mice per mean organ weight in controls. Symbols: ×, Spleen; ○, liver.

TABLE 5. Induction of hyperreactivity to endotoxin and hepatosplenomegaly in mice by killed *C. burnetii* cells

C. burnetii phase	Dose of *C. burnetii* cells (mg)	Endotoxin LD_{50} on day 14 (μg)	Mean wt[a]	
			Spleen (mg)	Liver (g)
I	1	2.3	1,055 ± 336	5.46 ± 1.27
	0.1	32	595 ± 232	3.83 ± 1.07
	0.01	>100	174 ± 69	2.14 ± 0.34
II	1	36	400 ± 168	3.03 ± 0.95
	0.1	>100	198 ± 94	1.97 ± 0.21

[a] Mean weights of 18 animals with standard deviations.

Induction of hyperreactivity to endotoxin by killed *C. burnetii* phase I cells in endotoxin-resistant mice. Female mice of the endotoxin-resistant strain C3H/HeJ received 500 or 100 μg of killed *C. burnetii* phase I cells i.p. Animals receiving PBS served as control. On day 10, groups of six animals received 5, 50, or 100 μg of endotoxin administered intravenously, and deaths occurring within 24 h were recorded. Treatment with *C. burnetii* phase I cells rendered the above mice sensitive to endotoxin, the sensitization being dependent on the dose of cells used (Table 7). Here also, the sensitization correlated with splenomegaly (liver weights not determined); the weight of this organ increased up to sevenfold. Results very similar to those in Table 7 were also obtained in several experiments in which male C3H/HeJ mice were used.

Effect of anti-phase I and -phase II antisera on the ability of killed *C. burnetii* to sensitize mice toward endotoxin. An equal volume of the corresponding antiserum was added to a suspension (2 mg/ml of PBS) of phase I- or phase II-killed *C. burnetii* cells and incubated for 1 h at 37°C. Similar incubations of cells of both phases with normal rabbit serum,

TABLE 6. Duration of hyperreactivity to endotoxin and of hepatosplenomegaly in mice induced by killed *C. burnetii* cells

Day of endotoxin administration after inoculation with *C. burnetii* (1 mg)	Lethality (dead/total) with 100 μg of endotoxin	Mean wt[a]	
		Spleen (mg)	Liver (g)
Phase I			
3	0/4	ND	ND
7	3/4	551 ± 82	ND
14	3/4	1,040 ± 340	4.79 ± 1.04
21	4/4	1,286 ± 250	
28	4/4	1,970 ± 392	8.05 ± 3.10
35	4/4	1,299 ± 576	4.07 ± 1.07
52	6/6	1,041 ± 720	3.18 ± 0.70
75	1/5	625 ± 291	2.73 ± 0.19
Phase II			
14	3/4	480 ± 169	3.05 ± 0.95
21	4/4	533 ± 286	3.25 ± 1.08
35	1/4	512 ± 294	2.85 ± 0.38
42	0/4	377 ± 199	2.41 ± 0.92

[a] Mean weights of groups of four mice with standard deviations. ND, Not determined.

TABLE 7. Induction of sensitization of C3H/HeJ mice to the lethal effect of endotoxin and spleen enlargement by killed *C. burnetii* phase I cells

Killed *C. burnetii* phase I cells (μg)	Lethality (dead/total) on day 10 with endotoxin (μg):			Mean spleen wt (mg)[a]
	5	50	100	
100	0/6	0/6	6/6	485 ± 87
500	0/6	5/6	6/6	756 ± 78
None	0/6 with 5 mg			107 ± 11

[a] Mean values for six mice with standard deviations.

devoid of detectable antibodies to the two antigens, served as controls. An additional control of each antiserum alone (without cells) diluted with an equal volume of PBS was incubated alongside. From each incubation mixture, 0.5 ml (either 500 μg of cells, 0.25 ml of serum, or both) was administered to mice i.p. On day 21, the animals received different doses (1 to 100 μg) of endotoxin i.p.; groups of six mice received each dose. Deaths occurring within 24 h were recorded, and LD_{50} values were calculated.

The ability of phase I cells to sensitize to endotoxin and to induce hepatosplenomegaly was completely abolished after incubation with anti-phase I antiserum (Table 8). In contrast, anti-phase II antiserum had no effect on the sensitizing properties of phase II cells, nor on liver and spleen enlargement. In the controls, normal rabbit serum had no effect, on either phase I or phase II cells. Similarly neither immune serum alone had any influence on the normal sensitivity of the mice toward endotoxin.

DISCUSSION

C. burnetii cells in phase I or phase II administered i.p. rendered mice more susceptible to the lethal effects of endotoxin by a factor of up to 100. Sensitization to endotoxin was always paralleled by splenomegaly and hepatomegaly, in which up to an eightfold increase in organ weight was seen. These properties were expressed by live and phenol-killed cells. Lethality in the sensitized mice occurred faster than in normal mice, commencing within 1 to 3 h of endotoxin injection and being completed 10 to 15 h later. In this respect, lethality in *C. burnetii*-sensitized animals is similar to that seen in other models of sensitization to endotoxin (BCG [29], *Corynebacterium parvum* [8], adrenalectomy [2], galactosamine [6], etc.).

TABLE 8. Effect of immune serum on the ability of killed phase I and phase II *C. burnetii* cells to induce hyperreactivity to endotoxin and hepatosplenomegaly

Mice inoculated with	Endotoxin LD_{50} (μg) on day 21	Avg wt[a]	
		Spleen (mg)	Liver (g)
Phase I + normal serum	1	1,588 ± 476	5.85 ± 1.15
Phase I + phase I immune serum	>100	359 ± 258	2.85 ± 0.33
Phase II + normal serum	54	490 ± 263	3.21 ± 0.83
Phase II + phase II immune serum	29	564 ± 316	3.43 ± 1.04
Phase I immune serum alone	>100	127 ± 27	1.47 ± 0.36
Phase II immune serum alone	>100	141 ± 21	1.62 ± 0.22

[a] Average of 18 mice with standard deviations.

The sensitization to endotoxin obtained by phase I-killed cells, as well as the induction of hepatosplenomegaly, could be suppressed by anti-phase I immune serum. In contrast, a similar effect on phase II cells was not observed with anti-phase II serum.

Generally, *C. burnetii* phase I cells were more potent in inducing the above changes than were phase II cells. In the case of infection with live cells, the lower activity to phase II cells is explained by the low rate of multiplication of the cells in the infected animals. This is suggested by the results obtained after CPA treatment which increased the rate of multiplication of phase II cells in the spleen and enhanced splenomegaly, hepatomegaly, and sensitization to endotoxin to a degree comparable to that obtained with phase I cells. Although a lower rate of multiplication of phase II cells may contribute to a lower degree of sensitization, still it does not explain the difference in the sensitizing properties seen with killed cells, of which, on a weight basis, phase I cells were at least 10 times more active than phase II cells. Therefore, intrinsic qualitative differences between the two phases must account for the higher activity of phase I cells. The phase variation in *C. burnetii* is a complex phenomenon, and the change of phase state is accompanied by a change of most of the biological properties of this agent. *C. burnetii* is classified today as a gram-negative microorganism. In this respect, an analogy may be drawn to smooth and rough forms of other gram-negative bacteria, phase I cells being comparable to the virulent smooth forms, and phase II cells to the less virulent or apathogenic rough-form bacteria (26).

The induction of hyperreactivity to endotoxin by *C. burnetii* phase I cells in C3H/HeJ mice is of particular interest. This endotoxin-resistant mouse strain (27, 28) is refractory to the effects of other known endotoxin-sensitizing treatments, such as adrenalectomy or galactosamine (M. A. Freudenberg and C. Galanos, unpublished data). To our knowledge, enhancement of endotoxin sensitivity in C3H/HeJ mice has only been demonstrated so far after treatment with BCG (29), a gram-positive microorganism. *C. burnetii* is, therefore, the first example of a gram-negative agent rendering C3H/HeJ mice highly susceptible to the lethal effects of endotoxin.

As with BCG, *Corynebacterium parvum*, and most other sensitizing agents, it is not known which components in *C. burnetii* are responsible for endotoxin sensitization or hepatosplenomegaly. It was shown earlier (13, 34) that treatment of *C. burnetii* with a mixture of chloroform and methanol (2:1 [vol/vol]) to remove lipids also abolished induction of splenomegaly and hepatomegaly. In agreement with the above studies, a similar treatment in the present study (data not presented) of phase I or phase II cells with chloroform-methanol abolished their ability to induce splenomegaly, hepatomegaly, and sensitization to endotoxin. The above activities were thereby lost and could not be detected in the chloroform-methanol extract. Although these findings are indicative of lipidic components being responsible for the above activities, definite conclusions at this stage would be premature.

Equally unknown are the mechanisms by which treated mice acquire the state of hyperreactivity to endotoxin or those leading to hepatosplenomegaly. It should be mentioned that enlargement of the liver and spleen involves a considerable increase in the number of lymphocytes and macrophages (S. Schramek, et al., unpublished data). These cells that are potentially capable of releasing pharmacologically active substances (18, 19, 22, 23) are strong candidates for being mediators of endotoxic activities.

According to the present data, *C. burnetii* is an additional example of a microorganism with the ability to enhance natural sensitivity toward the lethal effects of endotoxin. *C. burnetii* is an infectious gram-negative agent, also carrying endotoxically active lipopolysaccharide in its cell wall (25). During infection, any sensitization to endotoxin would proceed in the concomitant presence of lipopolysaccharide, whereby a role for endotoxin as an important factor of pathogenicity in rickettsial infection may be envisaged.

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