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INDUCTION OF SPLENOMEGALY IN MICE BY KILLED *COXIELLA BURNETII* CELLS

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Summary. — Splenomegaly induced in mice inoculated intraperitoneally (i.p.) with purified formalin-killed phase I and phase II *Coxiella burnetii* (*C.b.*) cells was dose-dependent. The phase I cells induced higher splenomegaly than phase II cells. The splenomegaly-inducing ability of phase I cells was reduced upon incubation with phase I but not with phase II antiserum, whereas the phase II cells preincubated with phase I or phase II immune sera induced higher splenomegaly than the phase II cells alone. Phase I cells caused lower splenomegaly in mice previously immunized with *C.b.* The splenomegaly-inducing ability of phase I cells was abolished by mild acid hydrolysis, by treatment either with phenol-chloroform-petroleum ether (PCP) or with a chloroform-methanol (CM) mixture. However, either the CM or the PCP-treated phase I cells retained their capacity to protect mice challenged with virulent phase I *C.b.*

Key words: *C. burnetii*; *splenomegaly*; *mice*; *immunity*

Introduction

Splenomegaly is a common feature of i.p. *C.b.* infection in mice (Franti *et al.*, 1974). In our studies splenomegaly was observed not only in *C.b.*-infected mice (Kazár *et al.*, 1982), but also in mice inoculated i.p. with antigenic preparations of *C.b.* The purpose of this study was to compare the splenomegaly inducing ability of i.p. inoculated purified killed phase I and phase II *C.b.* cells, after their preincubation with phase I and phase II antisera, after treatment with chemicals attacking the components of the bacterial cell-wall and after administration of phase I cells into mice previously immunized with *C.b.* The splenomegaly was evaluated 3 weeks after i.p. inoculation of *C.b.* cells, i.e. at the time when a fair antibody response had developed and protection against challenge with virulent phase I *C.b.* could be demonstrated (Kazár *et al.*, 1977).

Materials and Methods

C.b. strains and their treatments. Coxiella burnetii strain Nine Mile (phase I) in the 3rd chick embryo yolk sac passage (EP_3) and the same phase II strain passaged in our laboratory (EP_{162}) were partially purified by differential centrifugation, resuspended in brain-heart infusion (BHI)

and stored at —20 °C. Formalin-killed phase I and phase II cells of this strain purified by differential centrifugation and ether treatment were adjusted to the concentration of 2 mg/ml in phosphate buffered saline (PBS) and stored at —20 °C. Phase I cells were subjected to mild acid hydrolysis (0.1 mol/l HCl for 30 min at 100 °C) or were treated with PCP mixture (5 min at room temperature) according to Galanos *et al.* (1969) or with the CM mixture (2:1 v/v, 4 hr, 37 °C). Then the cells were sedimented, washed with ethanol and acetone, and air-dried. For immunization, in addition to live and formalin-killed phase I *C.b.*, also the lipopolysaccharide (LPS)-protein complex was extracted from phase I cells with trichloracetic acid as described (Brezina and Ürvölgyi, 1961).

Experimental animals. Specific pathogen-free outbred mice from the breed VELAZ weighing 25 ± 1 g were used throughout. Mice inoculated i.p. with a given *C.b.* preparation in 0.5 ml of PBS were bled 3 weeks after inoculation and their spleens were individually weighed. Means of spleen weights with standard deviations were calculated and differences obtained between the groups were evaluated by the Student's t-test. Probability values < 0.05 were considered significant. Each group consisted of 8—10 mice. In some experiments, mice were challenged with 10^6 EID_{50} (egg infectious dose) of phase I virulent *C.b.* Six days after challenge the mouse spleens were aseptically removed, their impression smears were tested microscopically for the presence of rickettsiae and evaluated in crosses as described (Kazár *et al.*, 1973). Twenty per cent spleen suspensions in BHI were titrated on 7-day-old chick embryos inoculated into yolk sacs by reading Gimenez-stained impression smears on day 10—11 p.i. The yields of *C.b.* from the spleen as well as the titre of phase I virulent strain used for the challenge were calculated according to Reed and Muench and expressed in log EID_{50} units.

Antibody determination. Individual mouse sera in each group were examined for the presence of antibodies directed to antigen 1 or antigen 2 of *C.b.* (Schramek *et al.*, 1978) by microagglutination (MA) test according to Fiset *et al.* (1969) and the mean geometric titres were calculated. This test was used also for determination of antibodies in hyperimmune Q fever sera obtained 10 days after i.p. challenge (one month interval between infecting and reinfecting *C.b.* dose) of the rabbits with 10^7 EID_{50} of phase I or phase II *C.b.* cells.

Results

Comparison of splenomegaly inducing ability of phase I and phase II C.burnetii cells

Splenomegaly inducing ability of phase I and phase II *C.b.* cells was compared by i.p. inoculation of 1 mg, 100 μg, of 10 μg of either cells. As follows from Table 1, significantly higher spelnomegaly was induced by both 1 mg and 100 μg of phase I than by phase II cells ($p < 0.05$). In mice inoculated with 10 μg of phase I or phase II cells the spleen weights did not significantly differ ($p > 0.05$) from values found in control mice.

Table 1. Comparison of splenomegaly-inducing ability of phase I and phase II C. burnetii cells

Mice immunized with	Amount of C. burnetii cells		
	1 mg	100 μg	10 μg
phase I cells	1256 ± 336	561 ± 380	149 ± 56
phase II cells	651 ± 269	256 ± 89	173 ± 39
control mice	134 ± 27		

Values calculated from two experiments represent mean spleen weight (in mg) and standdar deviations (SD); for details see Materials and Methods.

Table 2. Splenomegaly and antibody production in mice inoculated with phase I or phase II C. burnetii cells preincubated with phase I or phase II serum

Mice inoculated with	Spleen weight mg ± SD	Mean geometric titre of MA antibodies to	
		antigen 1	antigen 2
phase I cells alone	1271 ± 374	256	1260
phase I cells + phase I serum	305 ± 180	10	104
phase I cells + phase II serum	1206 ± 337	315	1260
phase II cells alone	307 ± 171	<2	223
phase II cells + phase I serum	558 ± 344	<2	315
phase II cells + phase II serum	833 ± 489	<2	315
control mice	117 ± 29	<2	2

Values calculated from two experiments (for details see Materials and Methods).

Effect of preincubation of phase I or phase II antisera on the splenomegaly- and antibody-inducing abilities of phase I and phase II cells.

Three ml volumes of phase I or phase II cells (concentration of 2 μg/ml) were incubated for one hr at 37 °C with the same volumes of either phase I immune serum (MA antibody titres 128 and 1024 to antigen 1 and 2, respectively) or phase II immune serum (antibodies only to antigen 2 in a titre 2048) diluted 1 : 4 or with the same volume of PBS only. Mice were inoculated i.p. with 0.5 ml volumes of the mixtures, so that each mouse received 500 μg of either phase I or phase II cells. In parallel, mice received i.p. the immune sera only, which did not induce any splenomegaly. In the absence of immune sera higher splenomegaly was induced by phase I than by phase II cells ($p < 0.05$). Splenomegaly inducing ability of phase I cells was greatly reduced ($p < 0.05$) after preincubation with phase I immune serum. Reduction of splenomegaly was accompanied by a decrease in the mean geometric titres

Table 3. Induction of splenomegaly by killed phase I cells in mice previously immunized with C. burnetii

Mice immunized with	Mice		MA antibody titres[b] to	
	inoculated*	non-inoculated	antigen 1	antigen2
phase I C.b. (live 10^6 EID_{50})	1078 ± 434[a]	800 ± 312[a]	128	1024
phase I C.b. (killed 500 μg)	1081 ± 399	664 ± 269	64	128
LPS-protein complex (100 μg)	627 ± 238	257 ± 104	4	16
non-immunized (control) mice	1765 ± 642	245 ± 56	<2	<2

* Three weeks after i.p. administration of 1 mg of killed phase I cells to previously (3 months before) immunized mice.

[a]) Spleen weights (mg ± SD).

[b]) Determined at the time of secondary administration of the phase I cells.

Table 4. Effects of the chemical treatment on the splenomegaly-inducing ability and immunogenic properties of phase I C. burnetii cells

Phase I cells	Spleen weight[a] mg ± SD	MA antibodies to		Protection to challenge	
		antigen 1	antigen 2	C.b. in spleen smears[b]	yield of C.b. from the spleen[c]
untreated	1045 ± 457	111*	1444*	(+)	1.6
HCl-treated	118 ± 28	n.t.	n.t.	n.t.	n.t.
PCP-treated	116 ± 27	26	294	++	3.3
CM-treated	128 ± 25	14	209	+	2.1
control mice	117 ± 27	2	2	++++	5.8

[a]) Mice inoculated with 1 mg of either treated or untreated phase I cells.
[b]) The amount of C. burnetii in splen smears was estimated in 10 fields of view at a magnification, of × 1000 and scored as follows: — no rickettsiae, (+) single rickettsiae occasionally seen + less than ten, ++ tens, +++ hundreds, and ++++ uncountable.
[c]) The yield of C.b. expressed in log EID_{50}/ml (tested in chick embryo yolk sacs).
* Geometric mean titre.

of MA antibodies directed to both, antigen 1 and 2. By contrast, preincubation of phase II cells with phase I as well as with phase II antiserum resulted in an increase of splenomegaly and the levels of MA antibodies directed to the antigen 2 were not affected (Table 2).

Induction of splenomegaly by killed phase I cells in mice previously immunized with C. burnetii

Mice i.p. immunized with 10^6 EID_{50} of live phase I C.b., with 500 μg of killed phase I *C.b.* cells or with 100 μg of the LPS-protein complex, as well as control (non-immunized) mice were inoculated 3 months later with 1 mg of the formalin-killed phase I *C.b.* cells. At this time the spleen weights were assumed to return to normal. Contemporarily the levels of serum MA antibodies were determined. The splenomegaly was evaluated 3 weeks later and compared with mice which did not receive the second dose of the killed phase I cells.

As follows from Table 3, a lower but still significant splenomegaly ($p < 0.05$) persisted in mice for 3 months after immunization with live or killed phase I *C.b.* cells, but not in those which received the LPS-protein complex. Administration of the killed phase I cells resulted in a significant ($p < 0.05$) increase of the spleen weight of control mice and in a substantially less but still significant ($p < 0.05$), increase in spleen weight of mice immunized with the LPS-protein complex, respectively. The increase of splenomegaly in mice immunized with live or killed phase I *C.b.* cells was non-significant ($p > 0.05$). Refracterness of these groups of mice to develop a significant splenomegaly correlated with the higher level of MA antibodies directed to antigen 1. The higher was a residual splenomegaly, the lower was an increase in the spleen weights following the secondary administration of killed phase I cells.

Effect of chemical treatment on splenomegaly inducing ability and immunogenicity of phase I C. burnetii cells

The splenomegaly was followed in mice inoculated with 1 mg of phase I cells which were untreated, treated with PCP or CM mixture or subjected to mild acid hydrolysis. In mice inoculated with PCP- and CM-treated phase I cells also MA antibody response and immunity to challenge with 10^6 EID_{50} of phase I virulent *C.b.* strain were tested. As shown in Table 4, all treatments led to complete reduction of splenomegaly inducing ability of phase I *C.b.* cells. Both PCP and CM treatment reduced the levels of MA antibodies to antigen 1 and 2. The degree of protection of mice against phase I *C.b.* virulent challenge by PCP-treated cells was lower than and by CM treated cells similar to that of untreated phase I *C.b.* cells.

Discussion

Administration of formalin-killed *C.b.* cells leads to a marked splenomegaly. It is difficult to say whether splenomegaly is a toxic reaction caused by *C.b.* or whether it represents some kind of immune or immunopathologic reaction. Reduction of splenomegaly by treatment of mice with cyclophosphamide after *C.b.* i.p. infection was accompanied by an increased multiplication of *C.b.* in the mouse spleen and other viscera (Kazár *et al.*, 1982) indicating that splenomegaly could be in some extent connected with immunity. In contrast, however, immunity to the challenge with virulent *C.b.* had been induced by CM-treated phase I cells in the absence of splenomegaly. Similar results were obtained by Cantrell and Williams (cited by Williams *et al.*, 1981), who observed that killed *C.b.* cells were immunogenic and toxic (inducing liver necrosis, splenomegaly, and hepatomegaly), whereas CM--extracted cells induced immunity but were not toxic. An observation of prolonged splenomegaly accompanied by rickettsaemia in athymic but not euthymic mice infected with *C.b.* aerosol (Kishimoto *et al.*, 1978) suggests that in mice with defective immunity the splenomegaly corresponds rather to pathologic reaction than to immune response.

In mice immunized previously with live or killed phase I *C.b.* (or with the LPS-protein complex which we also found to induce splenomegaly in mice but within 3 days after intravenous administration), phase I cells induced significantly lower splenomegaly. This could be due to the presence of phase I antibodies which reduced splenomegaly inducing ability of phase I cells (Table 2) or to the refractorness of cells involved in the development of splenomegaly not responding to secondary administration of the killed phase I *C.b.* cells.

Higher splenomegaly-inducing ability of phase I than phase II cells and reduction of splenomegaly inducing ability of phase I cells by mild acid hydrolysis, by preincubation with phase I immune serum, and in mice immunized previously with *C.b.*, suggests the importance of substance(s) connected with the phase I state of *C.b.* in induction of a marked splenomegaly. Nevertheless, further studies are necessary to find out components of *C.b.*

cells responsible for development of splenomegaly and to elucidate the significance of splenomegaly as the part of immune response or its relation to pathogenicity of *C.b.* Similarly, it remains to be determined which cells contribute to splenomegaly, though as follows from our study with live *C.b.* (Kazár *et al.*, 1982), both macrophages and lymphocytes might be involved.

References

Brezina, R., and Úrvölgyi, J. (1961): Extraction of *Coxiella burnetii* phase I antigen by means of trichloracetic acid. *Acta virol.* **5**, 193.

Fiset, P., Ormsbee, R. A., Silberman, R., Peacock, M., and Spielman, S. H. (1969): A microagglutination technique for detection and measurement of rickettsial antibodies. *Acta virol.* **13**, 60—66.

Franti, C. E., Behymer, D. E., Goggin, J. E., and Wright, M. E. (1974): Splenomegaly, sex, and other characteristics of laboratory animals used for primary isolations of *Coxiella burnetii* Lab. *Animal Sci.* **24**, 656—665.

Galanos, C., Lüderitz, O., and Westphal, O. (1969): A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* **9**, 245—249.

Kazár, J., Rajčáni, J., and Schramek, Š. (1982): Differential effects of cyclophsphamide on *Coxiella burnetii* infection in mice. *Acta virol.* **26**, 174—182.

Kazár, J., Brezina, R., Kováčová, E., and Úrvölgyi, J. (1973): Testing in various systems of the neutralizing capacity of Q fever immune sera. *Acta virol.* **17**, 79—89.

Kazár, J., E-Najdawi, E., Brezina, R., and Schramek, Š. (1977): Search for correlates of resistence to virulent challenge in mice immunized with *Coxiella burnetii*. *Acta virol.* **21**, 422—430.

Kishimoto, R. A., Rozmiarek, H., and Larson, E. W. (1978): Experimental Q fever infection in congenitally athymic nude mice. *Infect. Immun.* **22**, 69—71.

Schramek, Š., Brezina, R., Kazár, J. (1978): Influence of mild acid hydrolysis on the antigenic properties of phase *I Coxiella burnetii*. *Acta virol.* **22**, 302—308.

Williams, J. C., Peacock, M. G., and McCaul, T. F. (1981): Immunological and biological characterization of *Coxiella burnetii*, phase I and II, separated from host components. *Infect. Immun.* **32**, 840—851.