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CHANGING OF ANTIGENIC, IMUMNOGENIC AND CHEMICAL PROPERTIES OF A COXIELLA BURNETII STRAIN DURING CHICK EMBRYO YOLK SAC PASSAGING

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Summary. — *Coxiella burnetii* (*C.b.*) strain 48 after an increasing number of chick embryo yolk sac (CEYS) passages had lost at egg passages (EP) 15 — 20 its antigenic, immunogenic and chemical properties typical of phase I. The change refers namely to phase I antibody-inducing ability in mouse, the inability to react in microagglutination (MA) test with the phase II-specific serum, the loss of phase I antibody-binding capacity as detected by immunofluorescence (IF), the capacity to induce an increased number of peritoneal exudate cells (PEC) in the mouse, the resistance to nonspecific phagocytosis by guinea pig macrophages, the non-agglutinability by acriflavine and its behaviour in aqueous polymer two-phase system. At the same time the phase I antibody--inducing ability in rabbit and phase I antibody-binding capacity as detected by the MA and CF tests were preserved up to EP 45, while the phase II antibody-binding capacity in IF and complement-fixation (CF) tests was determined as early as in EP 10 and EP 15, respectively. The changes of other properties, such as splenomegaly and hepatomegaly-inducing abilities as well as immunogenicity by resistance to challenge with phase I virulent cells were more gradual.

Key words: Coxiella burnetii; phase variation; antigenicity; immunogenicity; physicochemical properties

Introduction

Our previous study on phase variation in *C.b.* stressed the complexity of this phenomenon (Kazár *et al.*, 1974). The present study was aimed at characterization of some antigenic, immunogenic and chemical properties of a *C.b.* strain after an increasing number of CEYS passages in order to determine the proper interval at which phase I to phase II conversion takes place. The study was also extended to the investigation of splenomegaly, hepatomegaly and PEC-inducing capacity of *C.b.* cells, their nonspecific phagocytosis and behaviour in aqueous polymer two-phase system as well as to the use of monophasic sera for determination of the antigenic properties.

Materials and Methods

C.b. strain 48 was isolated from *Haemaphysalis punctata* ticks collected in Central Slovakia (Řeháček *et al.*, 1970). It was originally passaged in mice, later in CEYS using 20% mouse spleen or CEYS suspensions at dilutions 1:10 — 1:100, so that also material which had never been grown in CEYS (EP 0) besides of the different EP (5—75) was available. As a control, pure phase II *C.b.* strain Nine Mile with an unknown number of CEYS which underwent 163 EP in our laboratory was employed (designated EP X). *C.b.* cells grown in CEYS or mouse spleen were killed with formalin and purified as described (Schramek *et al.*, 1978). All *C.b.* cell suspensions were adjusted to a concentration of 1 mg/ml in phosphate buffered saline (PBS), pH 7.2. Stock of live phase I Nine Mile strain was assayed in 6 to 7-day-old CEYS and its titre expressed in log EID_{50}/ml value, calculated by the method of Reed and Muench.

Animals were outbred mice (18—20 g) from the Dobrá Voda farm or the SPF mice from the VELAZ breed, and guinea pigs (250—300 g) and rabbits (2.5 kg) from the VELAZ breed. Mice and rabbits were used for testing phase I antibody-inducing ability of the *C.b.* strain 48. Mouse sera were collected 4 weeks after intraperitoneal (i.p.) inoculation of 100 μg of *C.b.* cells with a given EP. Rabbits were immunized intravenously (i.v.) on days 1, 4, 7 and 11 with 0.1. 0.3, 0.5 and 1.0 mg of *C.b.* cells, respectively, and tested on day 17. Rabbits were immunized by the schedule described (Schramek *et al.*, 1978) to prepare monophasic sera, i. e. sera containing antibodies against one of two main *C.b.* antigens only (phase I monophasic serum to antigen 1, phase II monophasic serum to antigen 2, respectively). SPF mice inoculated i.p. with 500 μg of different EP of strain 48 were searched after 3 weeks for development of splenomegaly and hepatomegaly and for the amount of PEC in two washings of peritoneal cavity with 3 ml of Eagle's basal essential medium (BEM). At the same time, a part of mice was challenged i.p. with 10^6 EID_{50} of phase I virulent strain Nine Mile (EP 3) and 6 days later the amount of *C.b.* in the mouse spleen was estimated by evaluation of the number of *C.b.* cells in spleen impression smears (in crosses) and by determining the yield of *C.b.* (in log EID_{50}/ml values) in the 20% spleen suspensions assayed in CEYS as described (Kazár *et al.*, 1977). Guinea pigs served as the source of macrophages used for determination of nonspecific phagocytosis (no serum added) of the strain 48 *C.b.* cells. The macrophages were collected and processed as described (Kazár *et al.*, 1975). After incubation of 10^6 macrophages with 100 μg of *C.b.* cells in 0.2 ml of BEM at 37 °C for 2 hr, macrophages were washed twice with BEM, air dried, stained according to Gimenez and the proportion of phagocytic cells containing *C.b.* corpuscles per 200 macrophages was calculated.

Serological examination. Mice and rabbit sera were examined for the presence of phase I and phase II antibodies by the MA test according to Fiset *et al.* (1969) using unstained antigenic preparation. This test and micromodifications of CF and indirect IF tests were used for determination of antibody-binding capacity of *C.b.* cells. As positive titres were considered ≥ 4 in MA and CF tests, and ≥ 8 in IF test.

Chemical procedures. Agglutination of *C.b.* cells with 0.1% neutral acriflavine solution was performed as described (Schramek *et al.*, 1972). For separation of the cells in aqueous polymer two-phase system, a mixture of 4.4% (w/w) polyethylenglycol (PEG) 6 000 and 6.2% (w/w, dextran (DEX) T 500 (Pharmacia, Uppsala) in 0.03 mol/l Tris-HCl buffered solution was used (Albertson, 1960). Into tubes containing 2 ml of the lower (DEX-rich) and 2 ml or the upper (PEG-rich) phases were added 0.2 ml of *C.b.* cells resuspended in 0.03 mol/l Tris-HCl buffer to final concentration of 2 mg/ml; finally, 0.1 ml of negative (no antibodies to *C.b.*) rabbit serum was added. After thorough mixing and 1 hr incubation at room temperature, 1 ml samples from each tube were carefully collected and their extinction was measured at 450 nm. From the extinction values obtained a proportional representation (in %) of *C.b.* cells in upper and lower phase was calculated.

Results

Antibody-binding capacity of strain 48 C.b. cells with an increasing number of EP

Purified, formalin-killed *C.b.* cells with different number of EP were examined in MA, CF and IF tests with two-fold dilutions of phase I or phase II

Table 1. Antibody-binding capacity of C.b. cells strain 48 with an increasing number of EP

Number of EP	Antibody titre in monophasic rabbit serum phase I			phase II		
	MA	CF	IF	MA	CF	IF
0	256	64	128	<4	<4	<8
5	256	128	128	<4	<4	<8
10	256	128	128	<4	<4	16
15	256	64	32	<4	8	16
20	256	32	<8	16	32	32
25	512	16	<8	16	64	64
30	512	32	<8	32	64	256
45	512	32	<8	32	32	256
75	<4	<4	<8	128	64	256
X	<4	<4	<8	128	64	256

* Reciprocals of serum dilutions in which *C.b.* cells still reacted in a given serological test.

monophasic rabbit serum. As shown in Table 1, *C.b.* cells reacted with phase I serum in the MA and CF tests up to EP 45, but in the IF test only up to EP 15. On the other hand, their reactivity with phase II antibodies in the MA test appeared in EP 20, but in the IF and CF tests as early as in EP 10 and EP 15, respectively.

Similar results were obtained also by cross-titration in the CF test using two-fold dilutions of *C.b.* cells with a given EP and rabbit monophasic sera (Fig. 1). Again, reactivity with phase I monophasic serum preserved up to EP 45 and that with phase II monophasic serum was noted from EP 15.

Phase I antibody-inducing ability of strain 48 C.b. cells with an increasing number of EP

Groups of 6 mice and 2 rabbits were bled 4 weeks after i.p. inoculation of 100 μg of *C.b.* cells obtained from different EP and their sera were examined individually by MA and CF tests for the presence of phase I antibody response. Though different levels of antibodies were detectable by tests used, the CF antibody titres lower in all cases in rabbit as well as in mouse sera, both MA and CF test gave consistent results, i.e. they revealed phase I antibodies in rabbit sera up to EP 45 and in mouse sera up to EP 15, respectively (Table 2).

Resistance to phase I virulent challenge in mice immunized by C.b. cells with an increasing nuber of EP

Mice immunized i.p. with 500 μg of *C.b.* cells with a different number of EP and control, nonimmunized mice, were infected after 3 weeks i.p. with 10^6 EID_{50} of virulent phase I *C.b.* strain Nine Mile (EP 3). On day 6 p.i., the amount of *C.b.* in spleen impression smears was estimated and the yield of *C.b.* from 20% pooled spleen suspensions was determined by their titra-

tion in CEYS. As follows from Table 3, a gradual decrease in immunogenicity of strain 48 *C.b.* cells with an increasing number of EP was observed. High degree of resistance was induced in mice immunized with *C.b.* cells from EP 0 to EP 15, lower degree by those from EP 20 to EP 45, whereas *C.b.* cells from EP 75 induced very low degree of resistance comparable with that provided by pure phase II Nine Mile strain (EP X).

Ability of C.b. cells with an increasing number of EP to induce splenomegaly, hepatomegaly and PEC in mice

In mice previously inoculated by i.p. route with 500 μg of *C.b.* cells differing in the number of EP, the amount of PEC was calculated in peritoneal washings (10^6 values per mouse), and the weights of spleen and liver (mean values ± S.D.) were determined. Whereas with an increasing number of EP the weight of both spleen and liver gradually decreased, the amount of PEC increased, the most marked differences being found between EP 15, and EP 45 and EP 75, respectively (Table 4).

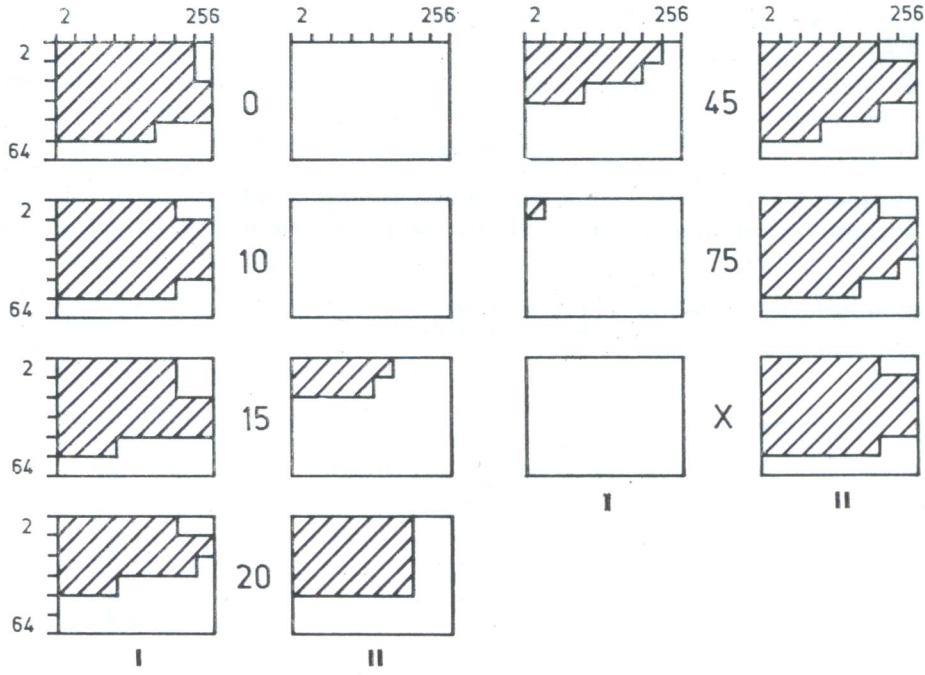

Fig. 1.
Cross titration in CF test of rabbit monophasic sera with *C.b.* cells strain 48 at increasing number of EP
Abscissae: serum dilutions; ordinates: dilutions of *C.b.* cells.
Left (I) and right (II) columns correspond to phase I and phase II monophasic sera, respectively.
Numbers in the middle indicate the number of EP.

Table 2. Phase I antibody-inducing ability of C.b. cells strain 48 with an increasing number of EP

Number of EP	Antibody titres in rabbit sera MA	CF	Antibody titres* in mouse sera MA	CF
0	2048, 1024	256, 256	84	11
5	2048, 2048	256, 256	147	7
10	2048, 2048	256, 128	338	11
15	2048, 1024	256, 128	195	7
20	512, 256	64, 32	<4	<4
25	256, 256	32, 32	<4	<4
30	128, 64	16, 16	<4	<4
45	128, 32	16, 8	<4	<4
75	<4, <4	<4, <4	<4	<4
X	<4, <4	<4, <4	<4	<4

* Mean geometric antibody titre calculated from 4—6 mice.

The change of some surface properties of C.b. cells with an increasing number of EP

To determine whether *C.b.* cells with a different number of EP differ also in some of their surface properties, nonspecific phagocytosis of *C.b.* cells, their agglutinability by acriflavine and behaviour in aqueous polymer two-phase system was followed out. As shown in Table 5, appearance of agglutinability by acriflavine in EP 20 was accompanied by a marked increase in nonspecific phagocytosis and the disappearance of *C.b.* cells from the

Table 3. Resistance to challenge with virulent phase I C.b. cells in mice immunized with C.b. at increasing number of EP

Number of EP	Multiplication of *C.b.* in the mouse spleen (4—5 mice) of EP	
	Amount in spleen smears*	Yield in CEYS**
0	(+)	3.1
5	(5)	2.6
10	(+)	3.1
15	(+)	3.3
20	+	4.4
25	++	4.8
30	++	4.6
45	++	4.6
75	+++	6.3
X	+++	6.6
Control	++++	7.3

* Estimated in 10 different fields of view at a magnification of x1000 and scored as follows: (+) single *C.b.* occasionally seen, + less than ten, ++ two, +++ hunder and ++++ uncountable number of *C.b.* per each field of view.
** Expressed in log EID_{50} units per ml of 20% spleen suspension.

Table 4. Splenomegaly, hepatomegaly and PEC-inducing ability of C.b. cells in mice with an increasing number of EP

Number of EP	Ability of *C.b.* to induce in mice× splenomegaly M± SD (g)	hepatomegaly M± SD (g)	PEC (10^6/mouse)
5	1.07 ± 0.34	4.9 ± 1.1	1.8
10	0.96 ± 0.26	4.7 ± 1.2	2.6
15	0.88 ± 0.32	4.8 ± 1.0	3.3
20	0.96 ± 0.29	4.9 ± 1.3	7.5
25	0.67 ± 0.23	4.2 ± 0.9	9.0
30	0.71 ± 0.27	4.5 ± 1.3	9.8
45	0.52 ± 0.18	4.0 ± 1.0	12.5
75	0.43 ± 0.12	4.3 ± 1.1	18.5
X	0.38 ± 0.15	3.7 ± 1.9	17.8
Control	0.12 ± 0.09	2.0 ± 0.3	2.2

Values calculated from 5—6 mice per each group.

upper phase of aqueous polymer two-phase system, indicating the sudden change of investigated surface properties of *C.b.* cells between EP 15 and EP 20.

Discussion

Phase variation of *C.b.* described originally as a serological phenomenon (Stoker and Fiset, 1956) has later turned out more complex reflecting not only antigenic changes, but also alterations in other properties of *C.b.* The accumulated data stressed the need for a more precise characterization of the phase state of *C.b.* populations and cells, respectively, and led to recognition of the so-called intermediate phase II of *C.b.* differing as from phase I

Table 5. Changes of surface properties of C.b. cells with increasing number of EP

Number of EP	Nonspecific phagocytosis (% of cells containing *C.b.*)	Agglutinability by acriflavine	% of *C.b.* cells in upper phase of two-phase system
0	7	none	91
5	5	none	89
10	15	none	83
15	32	none	91
20	74	yes	7
25	88	yes	11
30	84	yes	11
45	82	yes	11
75	86	yes	7
X	82	yes	6

so from pure phase II cells (Pautov and Igumnov, 1968; Kazár *et al.*, 1974; Brezina, 1978).

The complex investigation of antigenic, immunogenic and chemical properties of *C.b.* strain 48 during its passaging in CEYS justifies the need to distinguish between the phase states of *C.b.* Dissociation in altered individual properties of *C.b.* strain 48 depending on its CEYS passage history made it possible to include the *C.b.* population from particular EP to one of the above mentioned phase states, namely the phase I up to EP 15, the transition phase II from EP 20 to EP 45, and the pure phase II from EP 75.

Investigation of antigenic properties of *C.b.* cells showed that the change of their antibody-binding capacity depended not only on the EP but also on the serological test used, i.e. *C.b.* cells behaved as phase II based on the reactivity with phase II monophasic serum in IF test as early as in EP 10, but in CF and MA tests from EP 15 and EP 20, respectively. However, up to EP 45 they should be considered as intermediate phase II only, because of their preserved ability to react with phase I monophasic serum as detected by MA and CF tests, but not by IF test. Similar results were obtained when testing the antibody-inducing capacity of *C.b.* cells with an increasing number of CEYS passages. In mice a single administration of *C.b.* cells induced phase I antibodies up to EP 15, whereas hyperimmunization of rabbits resulted in phase I antibody response up to EP 45 probably due to the increased possibility of expression of traces of phase I antigenic determinants (which still may be present in transition phase II *C.b.* cells) after repeated antigenic stimulation, though the animal species must be also taken into consideration. Thus, determination of the phase state of *C.b.* based on the antigenic properties of the agent, can be influenced by the serological test, the animal species employed, the immunization schedule, etc. Nevertheless, the transition from phase I to intermediate phase II was reflected best by serological examination in the MA test as evidenced by investigation of the surface properties of *C.b.* cells, namely by their nonpecific phagocytosis, agglutinability by acriflavine and behaviour by two phase aqueous system, which all revealed clear-cut different properties of *C.b.* cells just between EP 15 and EP 20.

The existence of three phase states of *C.b.* was confirmed also by different degree of resistance to phase I *C.b.* virulent challenge afforded by *C.b.* cells with different CEYS passage history. The highest degree of resistance was observed in mice immunized with *C.b.* strain 48 from EP 0 to EP 15 corresponding to phase I state, less resistance in mice immunized with *C.b.* cells from EP 20 to EP 45 corresponding to transition phase II, and in mice immunized with *C.b.* cells in EP 75 the lowest degree of resistance corresponded to that found after administration of pure phase II *C.b.* cells with EP X.

On the other hand, nonsignificant difference in splenomegaly, hepatomegaly and PEC-inducing ability of *C.b.* cells from different EP reflecting the change of phase state were found, but a decrease in the spleen and liver weights or increase in the amount of PEC was gradual accompanying the increasing number of CEYS passage.

Based on the results obtained and previous observations from our labora-

tory (Kazár *et al.*, 1974; Brezina, 1978; Kováčová and Brezina, 1978; Schramek and Brezina, 1983), the mechanism of change taking place during serial passages in CEYS can be suggested. The first change occuring on the surface of phase I *C.b.* cells after several CEYS passages (from EP 10 to EP 20, depending on *C.b.* strain, the mode of its pasaging in CEYS and the serological test used) results in unmasking the binding sites of phase II antigenic determinants. This is probably due to the partial loss of ability of *C.b.* cells to polymerize saccharide o-chains of phase I antigen, which is of lipopolysaccharide (LPS) nature (Schramek and Mayer, 1982), and which may mask by steric hindrance the phase II antigenic determinants. *C.b.* cells from these EPs behave as intermediate phase II reacting in serological tests with phase II sera, but they still possesss phase I antibody-binding and antibody--inducing capacities and can be converted to phase I upon passaging in laboratory animals.

Further passaging in CEYS leads to the complete, irreversible loss of ability of *C.b.* cells to synthesize o-specific chains in LPS molecules, resulting in the pure phase II state of *C.b.* and reaching the point of no return to phase I upon passaging in laboratory animals. The change from phase I to intermediate phase II is realized obviously on the level of repression and derepression of regulatory systems of synthesis or rather polymerization of polysacchaide o-chains in the LPS molecule, though a complete genetic information for their synthesis is still preserved. The transition from intermediate to pure phase II cell is determined, however, by an irreversible change in the genetic information (mutation?) coding for synthesis of o-specific chains making a reversed conversion to intermediate phase II or phase I improbable even under altered evnironmental conditions.

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