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ANTIGENICITY OF CHLOROFORM-METHANOL-TREATED *COXIELLA BURNETII* PREPARATIONS

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Summary. — Phase I *Coxiella burnetii* (*C.b.*) cells untreated (*Cb I*) or treated with chloroform-methanol (CM) mixture (*Cb I-CM*) were compared as to their capacity to induce antibodies in laboratory animals and cattle, their ability to elicit delayed type hypersensitivity (DTH) reaction in mice and rabbits and protective effect in mice. In all animal species (mice, guinea pigs, rabbits, cattle) tested, the same doses of *Cb I-CM* cells induced lower levels of both phase I and phase II microagglutinating (MA) antibodies than *Cb I* cells at different intervals post-immunization (p.i.). Though for elicitation of DTH reaction in rabbits immunized with different *C.b.* preparations lower doses of *Cb I* than of *Cb I-C M*cells were necessary, *C.b.* cells caused inflammatory reaction at lower doses also in control rabbits. In mice immunized with *Cb I* and *Cb I-CM* cells, but not with trichloracetic acid extract (TCAE) from intact *Cb I* cells, DTH reaction was elicited by the same doses of *Cb I* and *Cb* I-*CM* cells. Higher immunizing doses of *Cb I-CM* than of *Cb* I cells were required, however, to induce DTH reaction (as tested by TCAE) as well as protection to phase I virulent challenge. TCAE from intact *Cb* I cells was protective in mice also at lower doses than TCAE from *Cb I-CM* cells (TCAE-CM). In humans who suffered from Q fever one year ago, higher proportion of positive skin test (ST) reactions and antibody recalls with higher mean geometric titres (MGT) of phase II MA antibodies was noticed following intradermal administration of TCAE than of TCAE-CM. When humans with no evidence of Q fever in past were vaccinated with TCAE or TCAE-CM, the former preparation not only caused higher proportion of both local and general post-vaccination reactions, but also of phase II MA antibody response and positive ST reactions as tested by TCAE 3 months post-vaccination in addition to higher proportion of phase II MA antibody recalls.

Key words: Coxiella burnetii; vaccines; antigenicity; chloroform-methanol treatment; laboratory animals; cattle; man

Introduction

In our accompanying paper (Kazár *et al.*, 1986), onset and duration of resistance to virulent challenge in mice and guinea pigs immunized with three different Q fever vaccine candidates, namely *Cb I* and *Cb-CM* cells and TCAE, was compared. In the present study, the effect of CM-treatment of formalin-killed *C.b.* cells on their antigenicity as determined by antibody--inducing capacity in laboratory animals and cattle, ability to induce and elicit DTH reaction in mice and rabbits and protection of mice from *C.b.* infection was investigated. Protective effects in mice were followed in parallel with TCAE obtained from intact whole *Cb I* cells and those pretreated with CM mixture (TCAE-CM). These two *C.b.* preparations were also studied in humans who overcame Q fever one year ago as to the possibility to elicit ST reaction and antibody recall upon their intradermal inoculation. In addition, humans with no evidence of Q fever in the past were immunized subcutaneously with TCAE or TCAE-CM to compare their reactogenicity and antigenicity as measured by occurrence of local and general post-vaccination reaction and by antibody response 3 months post-vaccination as well as by ability to induce DTH reaction determined by skin-testing with TCAE only.

Materials and Methods

C. burnetii strain Nine Mile serologically in phase I (the 3rd yolk sac passage) was used throughout. Seed pool prepared as 20 % suspension of heavy infected yolk sacs in brain heart infusion was stored at −60 °C. Its titre in log EID_{50}/ml values was determined in chick embryo yolk sacs. From the same pool inactivated by 1 % formalin, *Cb I* cells were purified by differential centrifugation and ether extraction. To prepare *Cb I-CM* cells, *Cb I* cells were treated with CM mixed in a 2 : 1 ratio as described (Kazár *et al.*, 1983). The soluble antigenic components were obtained by trichloroacetic acid extraction from intact *Cb I* cells (TCAE) or from *Cb I-CM* cells (TCAE-CM) according to the procedure previously described (Brezina and Úrvölgyi, 1962). All antigenic preparations were lyophilized so that their defined masses could be used for immunization and elicitation of DTH reaction in animals tested as well as for immunization or skin testing of humans.

Animals used were specific-pathogen-free outbred mice of the VELAZ breed weighing 18 to 20 g, guinea pigs and rabbits of the same breed weighing 250—300 g and 2,500—3,000 g, respectively. They were immunized intraperitoneally with different doses of *C.b.* preparation to determine their serological response at intervals post-immunization as well as the ability to respond in DTH reaction. In some experiments also subcutaneously immunized 3-month-old calves were employed.

Serological examination of pooled sera from 5—6 mice and of individual sera of guinea pigs, rabbits, calves and humans was carried out by MA test according to Fiset *et al.* (1969) with phase I and artificial (Schramek *et al.*, 1972) phase II *C.b.* antigen. Titres >2 were considered as positive; for calculation of mean geometric titre (MGT) of MA antibodies in guinea pigs, calves and humans, the values of negative sera were considered to be 1.

DTH reaction and protective effects of C.b. preparations in mice were assayed as described in accompanying paper (Kazár *et al.*, 1986).

Skin test (ST) of rabbits was performed with a slight modification according to Anacker *et al.* (1962). Animals inoculated with 100 μg of *Cb I* or *Cb I-CM* cells, TCAE and with PBS (4 in each group) were injected intradermally 6 weeks later along the shaved lateral surface of the back with 0.1 ml of PBS containing decreasing (from 30 to 0.003 μg) of *Cb I* and *Cb I-CM* cells and PBS as control. Rabbits were observed for 3 days post-inoculation and cutaneous lesions were evaluated in crosses as follows: + + + erythema with oedema and necrosis, + + erythema with edema, + erythema (at least 5 mm in diameter); — erythema of a lesser extent or absence

of any lesion was considered as negative reaction. As positive were considered cutaneous lesions occurring during 3 days observation period at least in 2 (50 %) rabbits in each group under study.

Immunization and ST of humans was carried out as previosly described (Kazár *et al.*, 1982). Humans with no evidence of Q fever in they history (as excluded also by serological examination in MA test and negative ST reaction) were immunized subcutaneously with 50 μg of TCAE or TCAE-CM. Their serological examination by MA test and skin testing with 0.5 μg of TCAE only was done 3 months post-vaccination. Two weeks later their sera were harvested again to determine eventual antibody recall, i.e. seroconversion and 4-fold or higher increase of phase II MA antibody titres. Another group of humans who overcame Q fever about one year ago (as determined by serological examination at the time of Q fever outbreak) was subjected to skin testing with 0.5 μg of TCAE or TCAE-CM. These were also examined serologically before skin testing and 2 weeks later to determine antibody recall as described in our previous paper (Kazár *et al.*, 1984).

Results

Antibody response in laboratory animals and cattle at different intervals post-immunization with Cb I and Cb I-CM cells

Mice, guinea pigs, rabbits and calves were immunized with different concentrations of *Cb I* and *Cb I-CM* cells and at different intervals post-immunization (Table 1) their sera were examined in MA test to determine the phase I and phase II antibody titres.

Table 1. Phase I and phase II MA antibody titres in laboratory animals and cattle at different intervals post-immunization with Cb I or Cb I-CM cells

Animal species	Preparation and dose used		Phase I and phase II MA antibody titres* at intervals post-immunization					
			1 week		4 weeks		4 months	
Mice	*Cb* I	100 μg	2	256	128	4096	64	1024
		10 μg			8	64	4	32
	Cb I-*CM*	100 μg	<2	4	8	32	<2	<2
		10 μg			<2	8	<2	<2
Guinea pigs	*Cb* I	100 μg			12.3	939.0	4.1	26.2
		10 μg			3.7	69.8	2.6	7.7
	Cb I-*CM*	100 μg			1.7	41.5	2.2	8.8
		10 μg			1.0	12.3	1.1	3.2
Rabbits	*Cb* I	1 mg	16	512	64	1024		
			16	256	128	4096		
	Cb I-*CM*	1 mg	4	128	16	256		
			8	128	16	256		
Cattle	*Cb* I	500 μg			2.0	85.7	10.0	57.4
		80 μg			1.3	26.9	6.7	16.7
	Cb I-*CM*	500 μg			1.5	5.4	1.8	3.0
		80 μg			1.0	4.0	1.0	2.8

* Titres in pooled sera from 5—6 mice, MGT calculated from 8 guinea pig and 20 cattle sera, respectively, in case of rabbits titres in sera examined in parallel.

Table 2. Comparison of doses of Cb I and Cb I-CM cells needed to elicit DTH reaction in mice and rabbit immunized with different C.b. preparations

Preparation and dose used*		DTH reaction** in mice and rabbits 6 weeks post-inoculation with			
		Cb I	*Cb* I-*CM*	TCAE	PBS
Cb I	30 μg	<0.001 +++	<0.000 +++	<0.0005 +++	<0.05 ++
	3 μg	<0.005 +++	<0.005 ++	<0.0005 +++	>0.05 ++
	0.3 μg	<0.05 ++	<0.05 ++	<0.001 ++	>0.05 +
	0.03 μg	>0.05 +	>0.05 +	<0.005 +	>0.05 −
	0.003 μg	>0.05 −	>0.05 −	>0.05 −	>0.05 −
Cb I-CM	30 μg	<0.001 ++	<0.005 ++	<0.0005 ++	>0.05 ++
	3 μg	<0.005 ++	<0.005 ++	<0.001 ++	>0.05 +
	0.3 μg	>0.05 +	<0.01 +	<0.05 +	>0.05 −
	0.03 μg	>0.05 −	>0.05 −	>0.05 −	>0.05 −
PBS		>0.05 −	>0.05 −	>0.05 −	>0.05 −

* Used to elicit DTH reaction by foot-pad inoculation in mice and intradermal application in rabbits, respectively.

** P values in mice and ST positivity in rabbits (+++ necrosis, ++ edema, + erythema, − negative reaction) immunized with 100 μg of given *C.b.* preparation.

At each interval investigated and in all animal species tested, comparable doses of *Cb I* cells induced higher titres of both phase I and phase II antibodies. *Cb I-CM* cells were less immunogenic, namely as concerned phase I antibodies, which following immunization with lower doses of *Cb I-CM* cells (10 μg in mice and guinea pigs and 80 μg in calves, respectively) were either absent or detectable only occasionaly in very low titres. Differences between antibody-inducing capacity of *Cb I* and *Cb I-CM* cells were not so great in rabbits, which can be in part explained by higher immunizing dose used, since in rabbits 6 weeks post-immunization with 100 μg of either *C.b.* preparation, much lower antibody levels were induced by *Cb I* than by *Cb I-CM* cells (results not included in Table 1).

Comparison of the ability of Cb I and Cb I-CM cells to elicit DTH reaction in mice and rabbits immunized with different C.b. preparations

Mice and rabbits were immunized intraperitoneally with 100 μg *Cb I*, *Cb I-CM* cells and TCAE or inoculated with PBS only. Six weeks later they were bled to determine MA antibody response in pooled sera from 4—5 mice or individual sera from 4 rabbits of each group. After bleeding, mice were inoculated into hind foot-pad and rabbits intradermally with 30, 3, 0.3 and 0.003 μg of *Cb I* and *Cb I-CM* cells to compare their ability to elicit DTH reaction.

As shown in Table 2, similar doses of *Cb I* and *Cb I-CM* cells (0.3 μg) elicited DTH reaction in mice immunized with *Cb I* or *Cb I-CM* cells, but

Table 3. Antibody response, DTH reaction and protection to virulent challenge in mice 3 weeks post immunization with different concentrations of CM-treated and CM-untreated C.b. preparations

Preparation and dose used		Phase I and phase II MA antibody titres		DTH reaction (P)	Yields of *C.b.* from spleen*
Cb I	100 μg	128	1024	<0.001	2.6
	10 μg	8	32	<0.005	3.1
	1 μg	2	8	<0.01	4.6
	0.1 μg	<2	<2	>0.05	7.1
Cb I-CM	100 μg	8	64	<0.005	3.1
	10 μg	<2	8	<0.05	5.1
	1 μg	<2	<2	>0.05	6.6
	0.1 μg	<2	<2	>0.05	7.3
TCAE	100 μg	2	32	<0.01	3.6
	10 μg	<2	<2	<0.05	4.6
	1 μg	<2	<2	>0.05	6.8
TCAE-CM	100 μg	2	16	<0.05	4.8
	10 μg	<2	<2	>0.05	6.6
	1 μg	<2	<2	>0.05	7.3
Control mice		<2	<2	>0.05	7.1

* log EID_{50}/ml values from 20 % suspensions of 5—6 mouse spleens collected 5 days post-infection with 10^4 EID_{50} of phase I *C.b.* strain.
MA antibody titres were determined in pooled sera from 4—5 mice, P values of DTH reaction were calculated from 4—5 mice in each group tested.

not with TCAE, in which DTH reaction could be elicited also with the dose of 0.03 μg of *Cb* I cells. In *Cb I-CM* cells-immunized mice higher probability values were observed following application of *Cb I-CM* than of *Cb* cells. A dose of 30 μg of *Cb I*, but not *Cb I-CM* cells elicited non-specific DTH reaction also in control, PBS-inoculated mice. Phase I and phase II MA antibody titres were comparable, though slightly lower, to the values observed after immunization with 100 μg doses of each *C.b.* preparation as presented in Table 3.

Table 2 also present the results of ST of rabbits. Though *Cb I* cells elicited ST positive reaction in lower concentration (0.03 μg) than *Cb I-CM* cells (0.3 μg) in *Cb I*, *Cb I-CM* and TCAE-immunized rabbits, the least dose of *Cb* I cells producing lesions in control animals was also lower (o.3 μg) than that of *Cb* I-*CM* cells (3 μg), the extent of lesions caused by *Cb I* cells being always greater. Phase I MA antibody titres varied from 32 to 128, from 4 to 8 and were hardly detectable in rabbits immunized with *Cb I*, *Cb I-CM* cells and TCAE; those of phase II varied from 256 to 512, from 16 to 64 and from 4 to 8 (data not presented in Table 2).

Table 4. Comparison of antigenicity of TCAE and TCAE-CM preparations in humans

Group* and number of humans vaccinated or skin-tested with		Proportion (in %) of					MGT of phase II MA antibodies after ST	
		LR	GR	MA+	ST+	AR		
1 TCAE	52	61.5	5.8	52.8	74.3	61.8	7.7	
TCAE-CM	35	40.0	2.9	30.8	45.5	27.3	4.8	
2 TCAE	15			60.0	73.3	73.3	20.2	136.3
TCAE-CM	15			60.0	46.7	53.3	13.5	21.9

* Group 1 — humans with no previous evidence of Q fever were vaccinated with TCAE and TCAE-CM respectively, but skin-tested 3 months later with TCAE only;
* Group 2 — humans who suffered from Q fever one year ago as confirmed by serological examination at that time were skin-tested with TCAE or TCAE-CM.
LR = local reactions, GR = general reactions post-vaccination;
MA+ = phase II MA antibodies in sera collected 3 months post-vaccination or one year post-infection; ST+ = skin test positive following intradermal application of TCAE in vaccinated humans and TCAE or TCAE-CM in infected humans, respectively; AR = = antibody recalls (seroconversion and 4-fold or higher increase in phase II MA antibody titres).

Antibody response, DTH reaction and protection to virulent challenge in mice immunized with different concentrations of CM-treated and CM-untreated C.b. preparations

Mice immunized intraperitoneally with decreasing concentrations of *Cb I* and *Cb I-CM* cells (100, 10, 1 and 0.1 μg) or TCAE and TCAE-CM (100, 10, and 1 μg) were bled after 3 weeks to determine MA antibody titres, inoculated into foot-pad with 10 μg of TCAE to assay DTH reaction and challenged intraperitoneally with 10^4 EID_{50} of phase I *C.b.* virulent strain to compare protective potency of *C.b.* preparations tested.

As follows from Table 3, all criteria studied, i.e. phase I and phase II antibody titres, the value of DTH reaction as well as the yield of *C.b.* from the mouse spleen reflecting the protection of mice against *C.b.* infection depended on both the *C.b.* preparation used and on its immunization dose. When comparing the same doses of individual *C.b.* preparations, of highest immunogenicity were *Cb* I cells, higher values of parameters tested being observed with CM-untreated than with CM-treated *C.b.* preparations. We also found that the least dose exerting protective effect was 1 μg in the case of *Cb I* cells, but 10 μg in the case of *Cb I-CM* cells and TCAE, and in case of TCAE-CM even 100 μg were necessary to produce significant protection.

Comparison of antigenicity of TCAE and TCAE-CM preparations in humans

Though TCAE soluble vaccine was used for prevention of humans against Q fever (Cracea *et al.*, 1973; Brezina *et al.*, 1974; Kazár *et al.*, 1982), nothing is known about the effects of TCAE-CM in Q fever convalescents or vaccinees.

For this reason TCAE and TCAE-CM preparations were compared as to their reactogenicity and immunogenicity and ability to prepare for elicitation of ST and antibody recall in humans with no previous evidence of Q fever and for ability to elicit ST and antibody recall in humans with Q fever in their history.

In humans with no previous evidence of Q fever, TCAE-CM vaccine caused less proportion of both local (erythema of diameter higher than 10 mm and edema and induration, respectively) and general (elevation of body temperature over 37.0 °C and influenza-like symptoms) post-vaccination reactions (Table 1). At the same time, TCAE-CM was less immunogenic than TCAE as to the proportion of sera containing phase II MA antibodies 3 months post-vaccination (30.8 versus 52.8 %), but namely as to the proportions of subjects reacting positively to skin testing with TCAE (45.5 versus 74.3 %) and those displaying antibody recall (27.3 versus 61.8 %). MGT of post-vaccination phase II MA antibodies did not differed markedly in humans vaccinated with TCAE or TCAE-CM preparation.

In the group of humans who overcame Q fever one year ago, higher proportion of positive ST reactions (73.3 versus 46.7 %) and antibody recalls (73.3 versus 53.3 %) was noticed following intradermal application of TCAE than TCAE-CM preparation. Though at the time of ST same proportion of sera (60.0 %) contained phase II MA antibodies with comparable MGT (20.2 and 13.5 in subjects to be tested with TCAE or TCAE-CM), TCAE elicited higher antibody response than TCAE-CM, MGT 2 weeks later being 136.3 and 21.9, respectively.

Discussion

The finding that CM treatment of formalin-killed *Cb I* cells abrogated their pathological effects in mice, namely liver necrosis and hepatosplenomegaly but preserved their immunological properties (Williams and Cantrell, 1982), was later confirmed in our study (Kazár *et al.*, 1983) suggesting the possibility to prepare a Q fever vaccine devoid of untoward side effects eliminating post-vaccination reactions, and at the same time of high immunogenic potency.

Favourable influence of CM treatment on *Cb I* cells was found also by Ascher *et al.* (1983) who observed the loss of potential of *Cb I-CM* cells to cause dermal granulomatous reaction in guinea pigs immunized with *Cb I* cells. Absence of deleterious effects on the mouse liver and spleen upon intraperitoneal administration of *Cb I-CM* but not of *Cb I* cells was also observed in our histological and ultrastructural studies (Jakubovský *et al.*, 1985; Kokorin *et al.*, 1985). Treatment of *Cb I* cells with CM mixture markedly reduced their ability to sensitize mice to lethal effects of bacterial endotoxin (Schramek *et al.*, 1984) and rickettsial toxin (Kazár and Schramek, 1984*a*). When comparing effects of *Cb I* and *Cb I-CM* cells on non-specific resistance in mice, similar levels of interferon induction and NK cells stimulation were observed, but activation of macrophages and degree of resistance

to tumours and some microorganisms induced by *Cb I-CM* cells was lower (Kazár and Schramek, 1984*b*; Macela *et al.*, 1985).

Though the chemical composition of the component(s) extractable by CM is not precisely known, phospholipids are supposed to be of importance (Schramek *et al.*, to be published). These components may play some adjuvant role in increasing both non-specific host resistance and at least the humoral part of specific immune response. As a matter of fact, in all animal species (mice, guinea pigs, rabbits, cattle) tested at different intervals post-immunization, higher levels of both phase I and phase II MA antibodies were induced by *Cb I* than by *Cb I-CM* cells. This was partly in accord with results of Williams and Cantrell (1982) in mice, at least as phase I antibodies are concerned, but contradictory with those of Ruppaner *et al.* (1985) who found much higher levels of MA antibodies in sera of lambs inoculated with *Cb I-CM* cells than with *C.b.* cells, though different sources of these two types of formalin-killed *C.b.* vaccines should be also taken into consideration.

Of higher importance, however, are the effects of CM treatment of *Cb I* cells on induction of specific cellular immune responses and on protection against *C.b.* infection. Intraperitoneal injection of *Cb I* cells into endotoxin-resistant mice resulted in marked and persistent suppression of the proliferative response of spleen cells to mitogens (Damrow *et al.*, 1985). In contrast to *Cb I* cells, in experiments in guinea pigs *Cb I-CM* cells elicited strong DTH reaction without subsequent granuloma formation and were equivalent to *Cb I* cells in ability to elicit and immunize for specific lymphocyte proliferative response (Ascher *et al.*, 1983; 1984). In our experiments the same doses of *Cb I*-CM and *Cb I* cells were able to elicit DTH reaction in mice immunized with *Cb I* and *Cb I-CM* cells, but for elicitation of DTH reaction in mice immunized with TCAE higher doses of *Cb* I-CM cells were necessary. On the other hand, DTH reaction with TCAE could be elicited in mice immunized with lower doses of *Cb I* than *Cb I-CM* cells. Although to elicit DTH reaction in rabbits immunized with different *C.b.* preparations lower doses of *Cb I* than *Cb I-CM* cells were required, intradermal application of *Cb* I cells led to non-specific inflammatory reactions in lower doses than *Cb I-CM* cells also in control non-immunized rabbits. The doses of *Cb I* cells producing cutaneous lesions in both sensitized and normal rabbits were similar to those observed by Anacker *et al.* (1962). Similar results were obtained in humans with Q fever in their history, in which higher proportion of ST positivity and antibody recall was registered after intradermal application of TCAE than of TCAE-CM preparation. Immunization with TCAE rather than with TCAE-CM induced higher proportion of positive serological post-vaccination response and in a greater extent senzitized for elicitation of ST reaction, though it should be considered that for skin testing only TCAE was used. Based on these results, we are of an impression that CM treatment of *Cb I* cells may lead to the decrease of not only humoral but also cellular immune response. It does mean, however, that *Cb I-CM* cells must differ from *Cb I* cells in ability to elicit cellular immune responses. As

a matter of fact, for in vitro lymphocyte stimulation *Cb I-CM* cells were recommended and successfully used (Ascher *et al.*, 1983; Koster *et al.*, 1985*a*, 1985*b*).

Though 4 weeks post-immunization the degree of mouse protection to virulent phase I *C.b.* challenge provided by *Cb I* and *Cb I-CM* cells was similar (Kazár and Schramek, 1985) to that found in our recent study (Kazár *et al.*, 1986), it was lower at later intervals and of shorter duration. The same can be applied to TCAE-CM preparation which was of lower protective potency in guinea pigs than the TCAE vaccine (Votruba *et al.*, 1985). As follows from the results of our study, higher doses of CM-treated *C.b.* preparations were necessary to induce both DTH reaction and protection from *C.b.* infection. Thus, although there is no doubt about reduced reactivity and side effects of CM-treated *C.b.* preparations, whether their immunogenic potential as Q fever vaccine candidates was sufficient enough remains questionable.

In the meantime paper by Williams *et al.* (1986) appeared demonstrating higher antigenicity of *Cb I-CM* than *Cb I* cells as measured by responsiveness to mitogens and specific recall antigens and resistance to lethal intraperitoneal challenge in C57 Bl/10 ScN endotoxin-nonresponder mice. The differences observed in this and our study can be explained by animal species and line used.

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