Acta virol. 33: 474—481, 1989

CELL-MEDIATED IMMUNE RESPONSE TO *COXIELLA BURNETII* ANTIGENS IN Q FEVER CONVALESCENTS AND VACCINEES

E. GAJDOŠOVÁ, R. BREZINA

Institute of Virology, Slovak Academy of Sciences, 817 03 Bratislava, Czechoslovakia

Received June 10, 1988

Summary. — Cellular immunity against phase I and II *Coxiella burnetii* antigens was evaluated by the lymphocyte blast transformation test in a) Q fever convalescents; b) persons immunized with a chemovaccine against Q fever; and c) control persons. The first two groups consisted of workers in a ricketsiology department. Of the 9 convalescents, all (100 %) were positive against phase I antigen and 4 (44.4 %) against phase II antigen. Of the 22 vaccinees, 77.3 % were positive against phase I antigen and all were negative against phase II antigen. All 10 controls were negative against both antigens.

Key words: *Coxiella burnetii*; *cellular immunity*; *Q fever vaccine*; *skin test*; *lymphocyte proliferation*

Introduction

Vaccination of men against Q fever is indicated especially in workers with a professional contact with *Coxiella burnetii*. This applies mainly to animal keepers on farms, workers in food and dairy industries and laboratory personnel.

Vaccination of the latter in the department of rickettsiae of our institute has been used since 1970. The vaccine has been prepared from highly purified phase I *C. burnetii* cells by extraction with trichloroacetic acid (Brezina and Úrvölgyi, 1961, 1962). The antigenic, immunogenic and protective properties of the chemovaccine were described by Brezina (1977). The skin test proved to be a good indicator of pre- and post-vaccination immunity (Kazár *et al.*, 1982, 1984; Ascher *et al.*, 1983; Marmion *et al.*, 1984). Several hundred persons professionally exposed to Q fever were successfully immunized with the chemovaccine in Czechoslovakia (Brezina *et al.*, 1981; Kazár *et al.*, 1982). Because the antibody response after immunization is insufficient and the vaccine offers long-term protection against Q fever (Brezina *et al.*, 1974) we became interested in the cellular component of immunity. We evaluated it by lymphocyte blast transformation test.

Materials and Methods

Sources of lymphocytes. Convalescents, vaccinees and control donors. Thirty-one workers in the Dept. of rickettsiae of our institute who have been in professional contact with *C. burnetii* were examined. Three of nine convalescents had overcome an inapparent infection. Twenty-two

persons were immunized with the chemovaccine in the period from 1970 to 1988. The third group consisted of blood donors who had no contact with *C. burnetii*.

Antigens. C. burnetii strain Nine Mile was propagated in chick embryo yolk sacs. Phase I (EP 3) was kindly supplied by Dr. R. A. Ormsbee (Rocky Mountain Laboratory, Hamilton, Montana, U.S.A.); phase II had undergone 163 egg passages at our Institute. Formalin-killed cell suspensions were purified with ether and adjusted to a final concentration of 1 mg/ml according to Fiset *et al.* (1969). These antigens were used in lymphocyte transformation tests in concentrations of 25, 50, 75 and 100 μg/ml. *Rickettsia prowazekii* strain Breinl, propagated in chick embryo yolk sacs and purified according to Ormsbee (1962) was used as nonspecific antigen in concentrations of 50 and 100 μg/ml.

Mitogens. Proliferation ability of lymphocytes was tested with the use of nonspecific mitogens, lectins. Concanavalin A (Con A; Sigma) was dissolved in sterile phosphate buffered saline (PBS) and used in a concentration of 2 μg per well. Leukoagglutinin (PHA-L; Flow Laboratories) was reconstituted in sterile distilled water and used in concentrations of 5.5 and 55 μg per well. These solutions were stored at −20 °C and thawn immediately before use.

Vaccine and skin test. The chemovaccine was prepared by extraction with trichloroacetic acid from phase I antigenic component of *C. burnetii* (Brezina and Úrvölgyi, 1961). This vaccine was characterized as a lipopolysaccharide — protein complex (Schramek, 1978). One vaccination dose contained soluble material extracted from 1 mg dry rickettsial cells, dissolved in 1 ml PBS. One day before imunization, the vaccinees were bled for serological assays (microagglutination and complement fixation reactions) and subjected to a skin test. In the latter, 0.1 ml of the vaccine was injected into the arm to detect any previous contact with *C. burnetii* or any hypersensitivity (Kazár *et al.*, 1984). The results were read after 24 hr and the skin test was considered positive if an erythema of $\geq$ 5 mm diameter with induration had developed (Brezina *et al.*, 1974).

Lymphocyte transformation (LT) tests. Human peripheral blood mononucleate cells (PBMC) were isolated from 10 ml heparinized venous blood by centrifugation in a Ficoll-Verografin gradient by a modification of the method of Boyum (1968). Interphase cells were washed three times with isotonic saline (Infundibile natrii chlorati isotonici, Imuna, Czechoslovakia) and resuspended in RPMI 1640 medium (Gibco) supplemented with 2 mmol/l L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, 2 mmol/l HEPES, 7 % $NaHCO_3$ and 10 % heat-inactivated human serum (AB group, Rh factor positive, pool from at least five male donors). The lymphocyte concentration was adjusted to 10^6 cells per ml medium. Cell viability, estimated with 0.4 % trypan blue (Gibco) solution, was always higher than 95 %.

Wells of multi-well plastic plates (type P, KOH-I-NOOR, Czechoslovakia) were seeded with 10^5 PMBC in 100 μl complete medium and 50 μl mitogen or antigen. All experiments were done in triplicate and included control cultures without mitogen or antigen. The cultures were incubated for 5 days at 37 °C in humid atmosphere containing 5 % CO_2. Eighteen hr before the end of incubation, 80 kBq (methyl-^{3}H) thymidine (ÚVVVR, Prague; spec. activity 1.4−2.2 GiBq/mmol) were added into each well in 20 μl volumes. After the end of incubation, the cells were harvested on Whatman glass microfibre filters 934-AH with the aid of a semiautomatic cell collector (Automash II, Dynatech). After drying, the filters were transferred into 4 ml of scintillation liquid (SLT-31, Spolana, Czechoslovakia). Incorporation of (methyl-^{3}H) thymidine was measured in a beta scintillation counter (LKB 1217 Rackbeta) and expressed in counts/min.

The results were treated as follows: mean ± standard deviation (SD) was calculated from the triplicate sample and the relative value was determined by adding 3 SD values to the mean. The stimulation index (SI) was calculated from these relative values as the ratio (test sample − background) to (corresponding autologous control − background). SI $\geq$ 2 was considered positive (Sabbe *et al.*, 1983.) Significance was estimated by Student's t test.

Results

The results of LT tests on cellular immunity of persons vaccinated in 1986−87 are illustrated in Fig. 1. Of the 11 persons 3 were vaccinated in 1986 (17−21 months before testing) and 8 in 1987 (2−11 months before testing). Mean SI ± SD in relation to the dose of *C. burnetii* phase I antigen

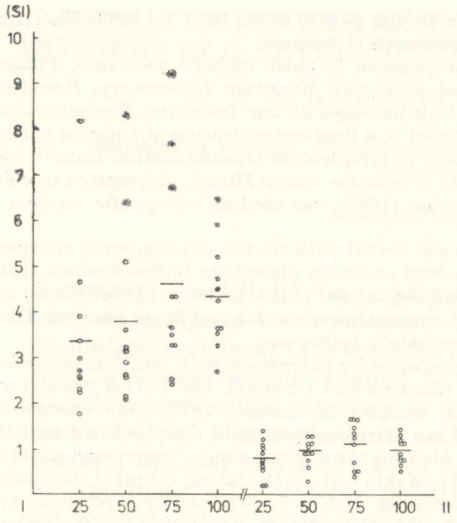


Fig. 1.

Lymphocyte proliferation responses after stimulation with antigens of phase I (left) and phase II (right) *C. burnetii* in men immuni ed with a chemovaccine in the years 1986—87

The results were expressed as mean stimulation indices (SI) from triplicate samples. Bars represent standard deviation (SD). Proliferation index of PBMC coming the vaccinees was significantly different from that of controls when stimulated with phase I antigen ($p < 0.01$) but not with phase II antigen ($p > 0.5$) (compare Fig. 2).

(25, 50, 75 and 100 μg/ml) varied from 3.36 ± 0.67 to 4.62 ± 2.13; SI was the highest with the antigen concentration of 75 μg/ml.

Positive responses were recorded as early as two months after immunization: the SI varied from 2.54 to 5.20. With phase II antigen the SI values were < 2 (from 0.78 ± 0.36 to 1.11 ± 0.48).

Results obtained in persons vaccinated in 1980—85 (2—7 years before testing) are presented in Fig. 2. SI with phase I antigen varied from 2.37 ±

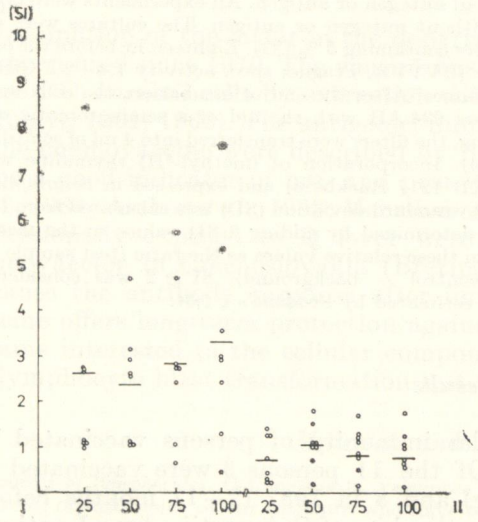


Fig. 2.

Lymphocyte proliferation responses after stimulation with antigens of phase I (left) and phase II (right) *C. burnetii* in men immunized with a chemovaccine in the years 1980—1985

For further explanations see legend to Fig. 1.

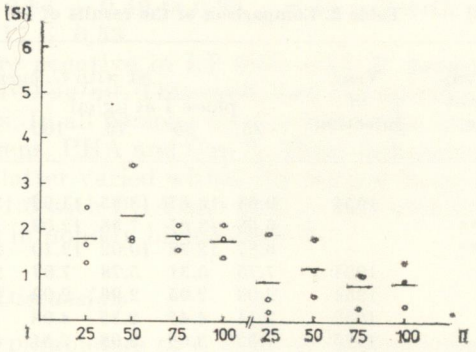
Fig 3.

Lymphocyte proliferation responses after stimulation with antigens of phase I (left) and phase II (right) *C. burnetii* in men immunized with a chemovaccine in the years 1970—1972

Proliferation index of PBMC from vaccinees in group C (1970—1972) was not significantly different from controls when stimulated with both *C. burnetii* antigens ($p > 0.05$), except for the results with phase I antigen in a concentration of 50 μg/ml ($p < 0.01$).

Abscissa: I — in the left: phase I antigen
II — in the right: phase II antigen

Ordinate: stimulation index (SI)



1.20 to 3.31 ± 2.13; the highest values were recorded with an antigen concentration of 100 μg/ml. Three persons were negative in these LT tests. Tests done with phase II antigen were negative throughout (SI < 2; from 0.72 ± 0.50 to 1.40 ± 0.48).

Fig. 3 illustrates the results of LT tests in three persons vaccinated in 1970—72 (15—17 years before testing). The SI values with phase I antigen were low as compared with the preceding two groups (from 1.77 ± 0.29 to 2.33 ± 0.77). One person immunized in 1972 was positive (SI from 2.10 to 3.42) and two persons immunized in 1970 were negative (SI from 1.25 to 1.82). With phase II antigen, the results were negative in all three persons (SI < 2; from 0.79 ± 0.34 to 1.17 ± 0.51).

Figs. 1—3 clearly show that in 22 persons vaccinated with a Q fever chemovaccine the cellular immune response assayed by the LT test with *C. burnetii* phase I antigen gradually decreassed with time. At an antigen

Table 1. Results of LT and skin tests on persons vaccinated with a Q fever chemovaccine

Vaccinee No.	Year of vaccination	SI with *C. burnetii* antigen (mean ± SD)		Skin test Erythema diam. (mm)/ degree of induration
		phase I	phase II	
1	1986—87	3.95 ± 0.52	1.33 ± 0.27	No reaction
2		5.13 ± 1.76	0.80 ± 0.21	No reaction
3		3.79 ± 0.55	0.45 ± 0.13	2.0/II
4	1980—85	2.91 ± 0.17	1.04 ± 0.34	2.5/III
5		1.81 ± 0.76	1.61 ± 0.22	2.2/I
6		3.85 ± 1.21	1.03 ± 0.22	0.5/necrosis
7		6.61 ± 1.46	1.36 ± 0.26	No reaction
8		2.88 ± 0.41	0.62 ± 0.32	No reaction
9	1970—72	1.75 ± 0.06	0.35 ± 0.13	No reaction
10		1.50 ± 0.21	1.42 ± 0.45	No reaction
11		2.52 ± 0.53	0.98 ± 0.30	No reaction

Table 2. Comparison of the results of LT and skin tests in convalescents

Convalescent No.	Year of infection	SI with *C. burnetii* antigen								Skin test*
		phase I at μg/ml				phase II at μg/ml				
		25	50	75	100	25	50	75	100	
1	1954	9.64	16.67	13.95	11.92	3.60	3.60	3.46	4.12	1.2/II
2**		6.58	13.57	7.96	12.09	1.01	1.78	2.67	3.30	3/II
3**		6.87	12.32	10.02	12.10	2.23	2.57	2.92	3.45	2/II
4	1954	7.75	5.31	5.78	7.97	2.18	2.72	2.76	4.50	6/IV
5	1964	2.03	2.05	2.96	2.99	1.81	0.69	1.25	1.59	4/III
6	1962	2.11	4.45	2.35	4.98	1.09	1.22	1.10	1.85	5/III
7	1976	1.32	3.08	2.98	2.51	0.59	0.37	0.98	0.62	1/II
8**		2.33	2.69	2.67	2.19	1.96	1.42	1.02	0.44	1.5/II
9	1959	1.57	2.70	3.11	3.37	1.51	0.76	1.49	0.85	No reaction

* Erythema diameter (mm)/degree of induration
** Inapparent infection

concentration of 25 μg/ml the decrease reached 22.33 % (Fig. 2) and 46.73 % (Fig. 3) as compared with the first group of vaccinees (Fig. 1).

At an antigen concentration of 50 μg/ml the decrease reached 37.8 % (Fig. 2) and 38.8 % (Fig. 3). At 75 μg/ml, the decrease reached 38.5 % (Fig. 2) and 60.6 % (Fig. 3). At 100 μg/ml, the decrease was to 23.9 % (Fig. 2) and 59.3 % (Fig. 3).

Eleven of the vaccinees were also subjected to the skin test and its results were compared w th those of the LT tests (Table 1). Only 4 of 11 vaccinees were positive in the skin test as compared with 7 in the LT test (36.3 % to 63.6 %). One person (No. 5) negative in the LT test was positive in the skin test. On the other hand, higher positivity in the LT test was not related to positivity in the skin test (Nos. 1, 2, 7, 8). Other vaccinees examined by the LT test (Figs. 1—2) were not subjected to the skin test.

Table 2 presents the results of LT and skin tests on 9 convalescents, 6 of whom had undergone laboratory Q fever infections 12—28 years ago and 3 of whom had overcome an inapparent infection with *C. burnetii*.

Convalescents Nos. 1—4 had high SI with phase I antigen (from 5.31 to 16.67). They were also positive with phase II antigen (SI from 2.18 to 4.50), with the exception of convalescent No. 2, who was negative with phase II antigen concentrations of 25 and 50 μg/ml, but positive with its concentrations of 75 and 100 μg/ml. Skin tests were positive in all these convalescents. Other five convalescents were positive in the LT test with phase I antigen (SI from 2.03 to 4.98) with the exception of the concentration of 25 μg/ml, at which two persons were negative. These five convalescents were negative with phase II antigen (SI from 0.37 to 1.96). Skin tests were positive in four of them (total of 8 positive out of 9 tested).

In the control group consisting of 10 blood donors, the results of LT tests were negative with both phase I and II antigens of *C. burnetii*. SI values

ranged with phase I antigen from 0.84 ± 0.40 to 0.97 ± 0.46 and with phase II antigen from 0.45 ± 0.19 to 1.17 ± 0.53.

All 41 persons under study were negative in LT tests with *R. prowazekii* antigen concentrations of 50 and 100 μg/ml. This confirmed the specificity of phase I and II *C. burnetii* antigens. In all persons we also tested the lymphocyte response to nonspecific mitogens, PHA and Con A. These tests served as a control of mitotic activity. The latter varied within the normal range since the mean SI values were 45.8 ± 21.5 with PHA and 23.6 ± 11.7 with Con A. The immunoregulation index was in nobody higher than 3.

Discussion

We studied the immunological parameters of a Q fever chemovaccine, in particular the cellular component of immunity and evaluated it by the skin test and LT test.

The group of 22 persons covered a long time interval after vaccination (from 2 months to 17 years). We found that 77.3 % of the vaccinees were positive in the LT test with *C. burnetii* phase I antigen, all being negative with phase II antigen (Fig. 1). An exception were three persons immunized 2, 6 and 7 years ago and two persons immunized 17 years ago. Two months after immunization we recorded increased SI against phase I *C. burnetii*. Eleven of the vaccinees were also subjected to a skin test which was positive only in four of them (36.6 %).

Jerrels *et al.* (1975) examined Q fever convalescents by the LT test and found that this *in vitro* lymphocyte response develops soon after recovery and persists for a long time (over 8 years). Also persons working with live *C. burnetii* under laboratory conditions were positive in the LT test. They used antigens obtained by extraction of phase I and II *C. burnetii* with trichloroacetic acid. Their results showed that phase I contains a component capable of stimulating immune lymphocytes while phase II is devoid of this ability. This is in partial agreement with our results of LT tests on five Q fever convalescents positive with phase I antigen and negative with phase II antigen (Table 2) like vaccinated persons (Figs. 1—3). An exception were four persons who had overcome Q fever in the past and were highly positive with *C. burnetii* phase I antigen and also positive with phase II antigen (Table 2). Of these nine convalescents eight were positive in the skin test (88.8 %). It must be stressed that our group of persons is in continuous contact with *C. burnetii* so that a continuous antigenic stimulus could be involved.

Kazár *et al.* (1983) showed that the cell-mediated immune response is of decisive importance in immunity against Q fever. The skin test acts as a repeated antigenic stimulus and the chemovaccine prevents the development of an infectious process for at least four years following vaccination. According to our results, cellular immunity as evaluated by the LT test persists for up to 15 years after vaccination. The LT test can be used not only for the detection of previous contact with *C. burnetii* antigens but also as an indicator of a need for revaccination.

Kazár *et al.* (1984) evaluated the time course (from 3 weeks to 12 months) of cellular and humoral immunity in persons vaccinated against Q fever. There was a good correlation between the results of LT and skin tests, but antibody formation (evaluated by the microagglutination reaction) was low.

In a group of 32 vaccinees only 44 % had antibodies (detected by complement fixation and immunofluorescence) against phase I and II of *C. burnetii*, while 94 % had increased SI values. The persons were examined later than 40 months after vaccination (Marmion *et al.*, personal communication 1986).

Ascher *et al.* (1983) studied cellular and humoral immune responses in 74 persons vaccinated with formalin-killed *C. burnetii* in phase I (strain Henzerling). LT tests on the vaccinees were positive with both phase I and II antigens, the SI with phase II having been higher. Blast transformation was in good correlation with skin test diameters.

Similarly, cell-mediated immune response evaluated by the LT test in 21 persons showed a higher positivity with phase II antigen than with phase I *C. burnetii* antigen (Marmion *et al.*, personal communication 1986). But in a group of another 12 persons, previously working in a slaughterhouse, the SI values after vaccination were higher than with phase II antigen. The vaccinees were fully protected from natural infection in the slaughterhouse, although antibody formation was demonstrated only in 60 % of them (Marmion *et al.*, personal communication 1986).

We obtained similar results on four Q fever convalescents positive with phase I antigen (SI from 5.31 to 16.67) as well as with phase II antigen (SI from 2.18 to 4.50, see Table 2). The results of the study by Benenson (1959) in which volunteers vaccinated with killed phase I *C. burnetii* antigen had antibodies in 50 % only but all were resistant to aerosol challenge with virulent *C. burnetii* strains also support the view that cell-mediated immune response is independent on antibody formation.

The decisive role of cellular immunity in *C. burnetii* infection was also demonstrated by the fact that in endocarditis patients lymphocyte proliferation specific of *C. burnetii* is lowered. This lowered response of T cells represents an important factor of persistent infection so that the LT test may serve as a good diagnostic tool for distinguishing endocarditis cases (Koster *et al.*, 1985).

Cell-mediated immunity was studied also in other ricketsioses. Coonrod and Shepard (1971) reported that lymphocytes from men vaccinated or infected with rickettsiae of the spotted fever and typhus groups were positive in the LT test. Similarly, human lymphocytes were stimulated by soluble and membrane fractions of *R. prowazekii* and *R. mooseri*, purified by renografin. The cell-mediated immunity was fully independent of antibody formation (Bourgeois *et al.*, 1980).

We examined a group of vaccinees and convalescents who have been in professional contact with live *C. burnetii*. In the future we intend to broaden our study to include immunized persons or convalescents who are no more in contact with *C. burnetii*.

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