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# BIOLOGICAL PROPERTIES OF CHLOROFORM/METHANOL EXTRACTS OF *COXIELLA BURNETII*

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*Summary.* – Biological properties of antigens of whole cells of *C. burnetii* in phase I (WCI), their residue (CMR) and extract (CME) obtained by extraction of *C. burnetii* with chloroform/methanol mixture were subjected to a comparative study. It was shown that CME is able to react specifically with sera containing antibodies against WCI. The inoculation of animals with CME, but not with WCI and CMR does not cause changes of humoral and cell immunity to *Coxiellae* and does not strengthen nonspecific antibacterial activity, but it can stimulate the formation of antibodies. Morphological changes of internal organs of mice were most expressed after the CMR inoculation and were practically absent after the CME inoculation.

*Key words: Coxiella burnetii; antigens, chloroform/methanol extraction; biological properties; antibodies; pathogenesis in mice*

## *Introduction*

In search of immunogenic but innocuous substances of *C. burnetii* that can serve as a basis for vaccine preparation special attention has been paid to the coxiellae extract obtained by chloroform/methanol procedure (Williams and Cantrell, 1982). In further investigations this extract was characterized as phospholipid which contained neither protein nor lipopolysaccharide (Lukáčová and Schramek, 1988). In response to the introduction of coxiellae antigens treated by chloroform/methanol specific agglutinins appeared in animals in either higher (Ruppaner *et al.*, 1985) or lower (Kazár *et al.*, 1987) titers than in the immunization with phase I whole cells coxiellae antigen containing phospholipid fraction.

However, the data concerning the role of this phospholipid in the formation of antibodies and the usage of inactivated coxiellae without phospholipid component as a vaccine are rather contradictory (Kazár and Řeháček, 1987). Thus the

aim of this work was a further study of immunomodulating features of *C. burnetii* phospholipid and morphological alterations which appeared in animal organism in response to the inoculation of this fraction.

## *Materials and Methods*

*Antigens.* The followig preparations were studied: the extract obtained by treatment of coxiellae with chloroform/methanol mixture (CME); coxiellae residue without phospholipid after chloroform/methanol treatment (CMR) and phase I whole cells *C. burnetii* antigens (WCI). All mentioned preparations were isolated from *Apodemus flavicollis* Luga and RSA strains of *C. burnetii* phase I (after 3–4 passages on embryonated chicken eggs). Besides that in some experiments the commercial antigen from *Rickettsia prowazekii* was used as a control.

Whole cells antigens (WCI) were isolated from coxiellae inactivated by 1 % formalin and purified by differential centrifugation and ether treatment. To obtain CME and CMR preparations WCI was treated twice by chloroform/methanol mixture (2:1) at 52 °C for 2 hr. For every extraction a freshly made mixture was used. After the extraction the mixture was filtrated through paper filter. The residue on the filter represented CMR. To obtain the phospholipid (CME), the filtrate were lyophilized that allowed to standardize the CME preparations. Sugars in CME were not detectable and proteins amounted to 2.4 %.

*Immunization.* Experimental animals were provided by the Rappolovo farm of Russian Academy of Medical Sciences. Outbred white mice (15 g and 22 g), C3HA line mice (15 g), guinea pigs (275 g) and rabbits (2750 g) were used.

The ability of CME to induce formation of antibodies to coxiellae was determined by intraperitoneal inoculation of guinea pigs with 200 μg of this preparation suspended in phosphate buffered saline pH 7.4 (PBS) (the first group of animals). Guinea pigs inoculated by the equal quantity of WCI (the second group) and CMR (the third one) served as controls. On the 12th and 21st days after the immunization the presence of antibodies to coxiellae in sera from every animal was tested.

The reactions of complement fixation (CFR), microagglutination (MAR), indirect immunofluorescence antibody analysis (IFA) and erythrocyte haemolysis were carried out by routine procedures. Enzyme-linked immunosorbent assay (ELISA) was realized with the help of immunoenzyme test system for detection of *C. burnetii* antigens (Gorbachev *et al.*, 1991). Immune active components of the test system were prepared on the basis of hyperimmune rabbit serum obtained by immunization with WCI of animals antigen of *Apodemus flavicollis* Luga strain.

The effect of CME on antibody formation was studied in rabbits and C3HA line mice. This effect was compared to that obtained by commercial Freund's complete adjuvant. Rabbits were divided in to 3 groups: the first was inoculated with a suspension of sheep erythrocytes, the second with a suspension of sheep erythrocytes and 3 μg of CME and the third with a suspension of sheep erythrocytes and Freund's adjuvant. All the animals were immunized subcutaneously with sheep erythrocytes in equal dose ($10^8$ cells). On the 12th day animals' sera were analyzed in the reaction of erythrocyte haemolysis. C3HA line mice were subcutaneously immunized with sheep erythrocytes ($5 \times 10^6$ cells) with different doses of CME (0.5–1.75 μg per 1 g of mouse weight) suspended in 0.5 ml PBS. The control animals were inoculated with sheep erythrocyte suspension in the same dose but without the coxiellae extract. After 5 days the animals were killed with chloroform, spleen was extracted and direct antibody (IgM) generating cells were detected.

Immune response of mice to sheep erythrocytes was evaluated by the detection of direct (IgM) antibody generating cells (AGC) in mouse spleen according to Jerne and Nordin method. The AGC quantity was calculated by $10^6$ splenocytes.

The lymphocyte sensibilization rate (SR) of mice immunized by WCI, CMR and CME was determined in the reaction of leukocyte adhesion inhibition (LAIR) according to the formula:

$$SR = (1 - o/K) \times 100\ \%$$

where **o** – quantity of cells adhered to the bottom of plate wells after their incubation with *C. burnetii* test antigen, **K** – the same but without antigen (Tokarevich, 1985).

*Antibacterial activity of peritoneal cells* was tested by infecting mice with a virulent strain of *Salmonella typhimurium*. These mice were 6 hr, 2, 4, 7 and 15 days before infection inoculated by WCI, CMR and CME. Each experimental series the abdominal cavity of intact and inoculated animals was washed with lactoalbumin hydrolysate solution straight after the infection and two hours later. Peritoneal washing fluid was seeded on the selective media. The identification of colonies was carried out by standard methods. According to the number of typical colonies grown up we calculated the change of bacteria concentrations in abdominal cavity 2 hr after the inoculation of *S. typhimurium* in control ($\log C_0 - \log C_2$) and experimental ($\log E_0 - \log E_2$) mice, where $C_0$ – bacteria concentration in abdominal cavity of control animals straight after the infection, $C_2$ – the same 2 hr later, $E_0$ – bacteria concentration in experimental animals straight after the infection, $E_2$ – 2 hr later.

To evaluate morphological alterations elicited by WCI, CMR and CME mice (15 g) were intraperitoneally injected with mentioned preparations in a dose of 500 $\mu$g per individual resuspended in 0.5 ml PBS. Control animals were injected with PBS. Mouse liver and spleen were fixed in Carnoy's fluid and embedded in paraffin for histological studies. Paraffin sections (up to 5 $\mu$m thick) were stained with eosin-methylene blue according to Leishman or May-Grünwald and additionally with the Romanowsky-Giemsa stain.

All obtained data were statistically evaluated with the help of Student's test ($p \leqq 0.05$).

## *Results*

*The ability of CME to interact specifically with antibodies against C. burnetii*

The interaction of WCI and CME with rabbit blood serum containing antibodies to *Apodemus flavicollis* Luga strain antigen was investigated inCFR (chess variant) and ELISA. As a control these preparations were studied in CFR with rabbit blood serum containing antibodies against *Rickettsia prowazekii* and blood serum from intact rabbits.

We found that *C. burnetii* WCI and CME did not react in CFR with sera of intact rabbit and rabbit with antibodies to *Rickettsia prowazekii*. However, CFR for these preparations was positive with serum of a rabbit immunized with *C. burnetii*. The observed concentrations of WCI and CME differed essentially.

CME was characterized by a considerably smaller zone of positive reactions than WCI. CME was revealed also in ELISA provided much higher concentra-

**Table 1. Reactivity of WCI and CME with sera containing antibodies against C. burnetii**

| *C. burnetii* strains | Tested preparation | Minimal concentration of the preparation reacting with serum (ng/ml)* | |
|---|---|---|---|
| | | CFR | ELISA |
| *Apodemus flavicollis* – Luga | WCI | $2.2 \times 10^4$ | $5.8 \times 10^1$ |
| | CME | $7.5 \times 10^5$ | $1.0 \times 10^5$ |
| RSA | WCI | $3.0 \times 10^4$ | $13.3 \times 10^1$ |
| | CME | $6.3 \times 10^5$ | $6.2 \times 10^4$ |

* Average values from two series of three experiments

tion as compared to WCI was used (Table 1). The results of tests with WCI and CME preparations obtained from different *C. burnetii* strains were quite similar.

*CME antigenicity studies*

CMR was shown to be less immunogenic than WCI. Antibodies to *C. burnetii* was found in not a single animal inoculated by CME (Table 2).

In the experiments for detection of animal sensibilization to coxiellae using LAIR we established that CME did not challenge it while the immunization by WCI and CMR caused pronounced animal sensibilization (Fig. 1). LAIR was negative in control animals immunized by *Rickettsia prowazekii* antigen.

*The effect of CME on antibody formation*

The concentration of antibodies to sheep erythrocytes in all animals from the second group was shown to be higher than in animals from the 1st and 3d groups (Fig. 2). The optimal dose of CME increasing formation of antibodies was determined in experiments with mice. It was established that in experimental animals the number of direct (IgM) AGC depended on the CME dose. The optimal dose was 1–1.25 $\mu$g per 1 g of weight. The AGC number was $12.9 \pm 1.8$ and $14.0 \pm 2.1$ as compared to $5.2 \pm 1.2$ of control. Further increase of CME dose led to lowering of the effect.

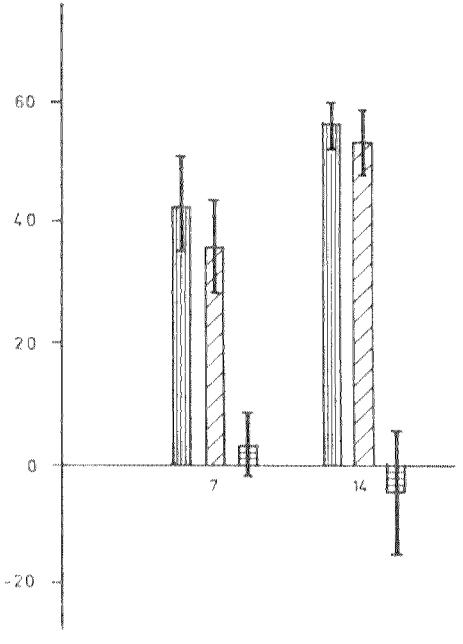


Fig. 1
LAIR test indices in mice inoculated with WCI, CMR and CME
Abscissa: days after inoculation; ordinate: level of the sensibilization of animals inoculated with WCI - ▥, CMR - ▨, CME - ▤
Vertical segments: confidential intervals at $P = 0.05$

**Table 2. Antigenic properties of the CME, WCI and CMR**

| Preparation inoculated | Days after inoculation | Titers of antibodies to *C. burnetii* | | | | |
|---|---|---|---|---|---|---|
| | | CFR | | MAR | IFAR | |
| | | phase I | phase II | phase II | phase I | phase II |
| CME | 12 | < 3.3 | < 3.3 | < 3.3 | < 3.3 | < 3.3 |
| | 21 | < 3.3 | < 3.3 | < 3.3 | < 3.3 | < 3.3 |
| WCI | 12 | < 3.3 | 6.3 | 10.0 | 5.6 | 12.0 |
| | 21 | 4.9 | 9.3 | 11.0 | 6.3 | 11.3 |
| CMR | 12 | 3.3 | 4.9 | 7.6 | 3.6 | 9.6 |
| | 21 | 3.6 | 7.6 | 9.3 | 4.6 | 8.3 |

Antigens were obtained from phase I *C. burnetii Apodemus flavicollis* - Luga strain. Titers of antibodies are $\log_2$ values of geometrical averages.

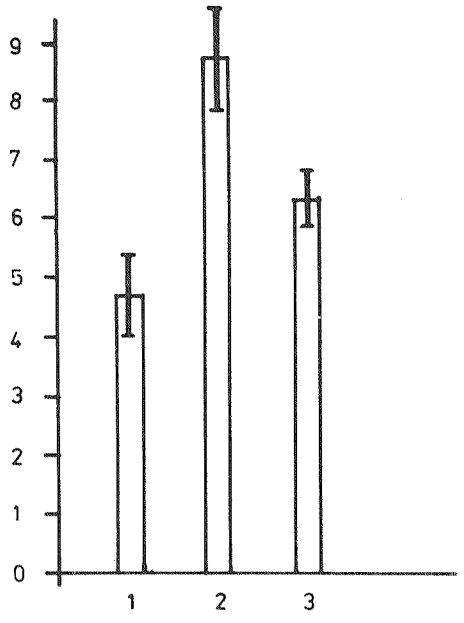


**Fig. 2**
Results of sera titration in the reaction of erythrocyte haemolysis
Abscissa: rabbits inoculated with sheep erythrocytes (1), sheep erythrocytes plus CME (2) and sheep erythrocytes plus complete Freund's adjuvant (3); ordinate: negative $\log_2$ of the average geometrical titer of antibodies in rabbits.

*The effect of CME on nonspecific antibacterial activity of mouse peritoneal cells*

After the infection of intact white mice by *S. typhimurium* we observed rather pronounced elimination of these bacteria from abdominal cavity ($\log C_0 - \log C_2 = 1.49 \pm 0.15$) during 2 hr. This fact is considered to be linked to the natural antibacterial activity of peritoneal cells of these animals. Preliminary injection of 100 $\mu$g of CME did not lead to any noticeable increase of antibacterial activity of mouse peritoneal cells whereas the same dose of WCI or CMR caused 2 peaks of cell clearance activity rise - on the 2nd and 7th - 15th day (Fig. 3).

*Morphological changes in mouse spleen*

During the first hours (6-24 hr) after intraperitoneal CME inoculation pycnosis, lymphocyte migration from white to red pulp, lymphocyte blasttransformation and leukocytic reaction were weakly expressed and practically did not differ from these indices in control animals. On the other hand, the inoculation of WCI and in a lesser degree CMR quite pronounced pycnosis and lymphocyte degradation in T-dependent zone of spleen white pulp were observed.

By the 7th day leukocytic reaction in animals immunized with WCI or CMI

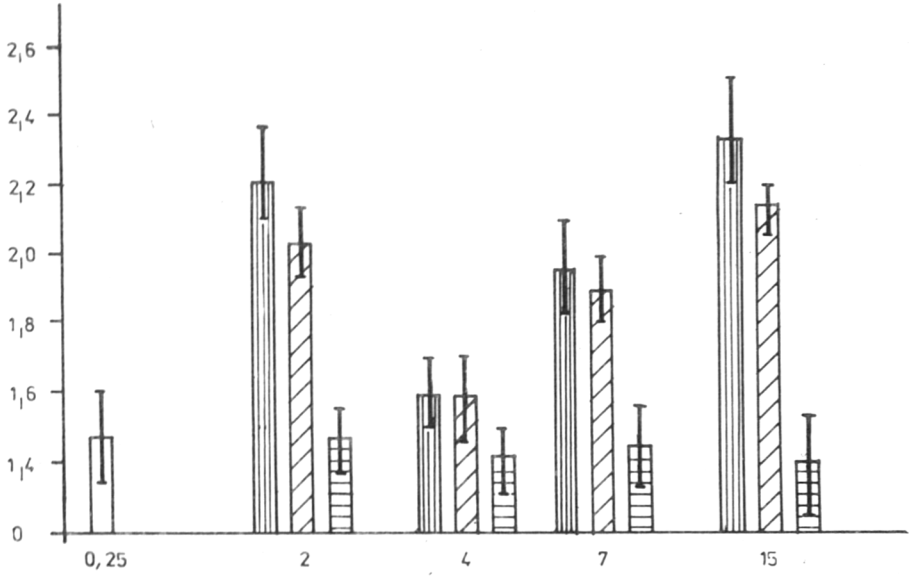


Fig. 3

Antibacterial activity of peritoneal cells in mice inoculated with WCI, CMR and CME
Abscissa: days after inoculaton; ordinate: log difference of bacteria concentration in mice: ☐ - intact, ▥ - inoculated with WCI, ▨ CMR and ▤ CME.
Vertical segments: confidential intervals at P = 0.05.

almost disappeared and the lymphocyte blasttransformation and the migration of lymphocytes to the red pulp increased. From this moment a pronounced hyperplasia of reticulum cells and macrophages with a relative decrease of follicles and white pulp periarterial lymphoid muffs were noted (Fig. 4). The spleen weight and its ratio to the weight of the animal markedly increased. The number of blasts and mitoses in the red pulp reached its maximum 14 days after WCI inoculation (Fig. 5A). Increased number of macrophages, blasts and mitoses was also observed in T-dependent zones of white pulp, marginal zones of follicles and periarterial lymphoid muffs (Fig. 5C, D). At the same time the number of blasts and mitoses in B-dependent zones of white pulp and follicles was also higher after the WCI inoculation (Fig. 5B) but hyperplasia of reticulum stroma was not noted there. About 15 % of reticulum cells macrophages in T-dependent white pulp zones contained vacuoles filled by watery fluid with isolated coxiellae particles. The same picture was observed in the red pulp.

After the CMR inoculation reticulum cells and macrophages hyperplasia as well as spleen weight increase were considerably less pronounced than after the WCI inoculation. In other aspects the picture was similar. After the CME inoculation a hyperplasia of reticulum cells and macrophages or an increase of blast forms in marginal follicular zone were almost absent, namely they were 2–

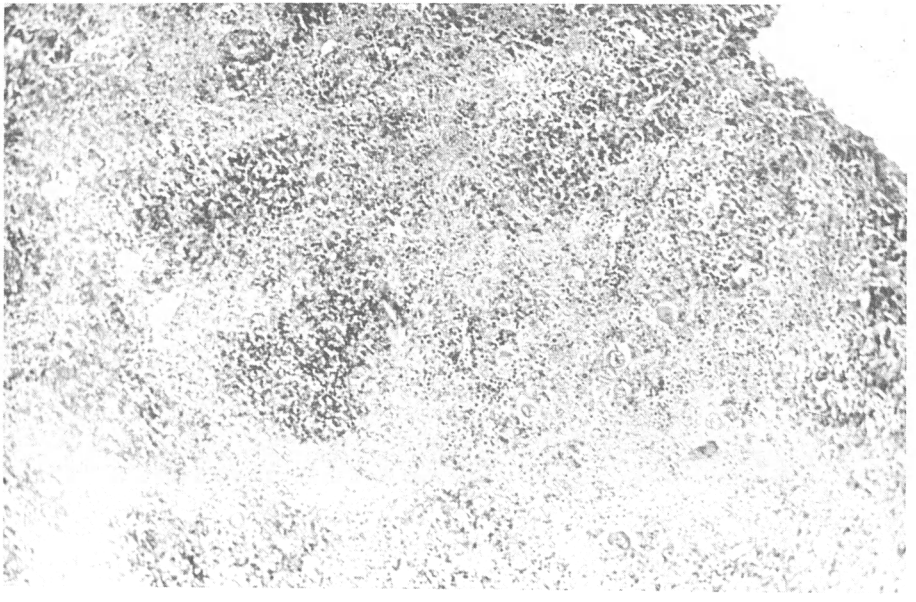

Fig. 4
Hyperplasia of the spleen red pulp and small rare lymphatic follicles after WCI of CMR inoculation Leishman-azure-eosin stain. Magn. x 100.

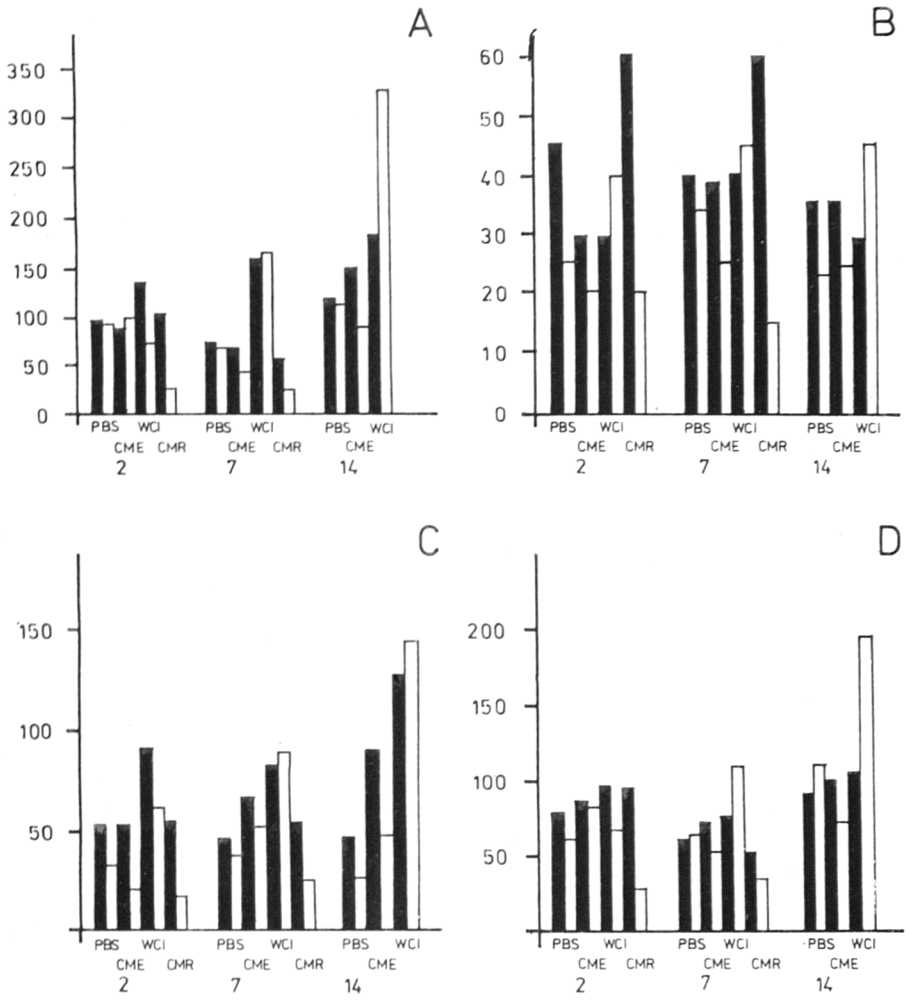


Fig. 5

Number of blasts and mitoses in spleen section after WCI inoculation

A – red pulp, B – B-dependent zone of a white pulp (follicles), C – and D – T-dependent zone of the white pulp: C – marginal zone of follicles, D – periarterial lymphoid muffs.

Abscissa: time of antigen inoculation (days) and its character; ordinate: number of cells

3 times lower than after the WCI inoculation. The number of mitoses in these animals unlike in the WCI immunized mice remained on the control level or even lower. In accord with this observation the spleen weight increase was not observed in animals after the CME inoculation.

*Morphological changes in mouse liver*

6 hr after the WCI inoculation and in a lesser degree after the inoculation of CMR and CME separate accumulations of neutrophiles, monocytes and lymphocytes were observed in periportal zones and more seldom in centers of lobes around central veins. By 24 hr the number of cells in such clusters increased as well as the number of infiltrations in animals immunized with WCI and slightly less after the CMR inoculation. In experiments with CME this infiltration did not increase and even it decreased.

By the 7th day the cell infiltration reached maximum in experiments with WCI and was less expressed after the CMR inoculation. Moreover the content of neutrophiles substantially decreased and the infiltrations looked like a lymphoid-macrophagal granuloma. At the same time the pronounced hyperplasia of Kupffer's cells and numbers of macrophages and lymphocytes in sinus areas

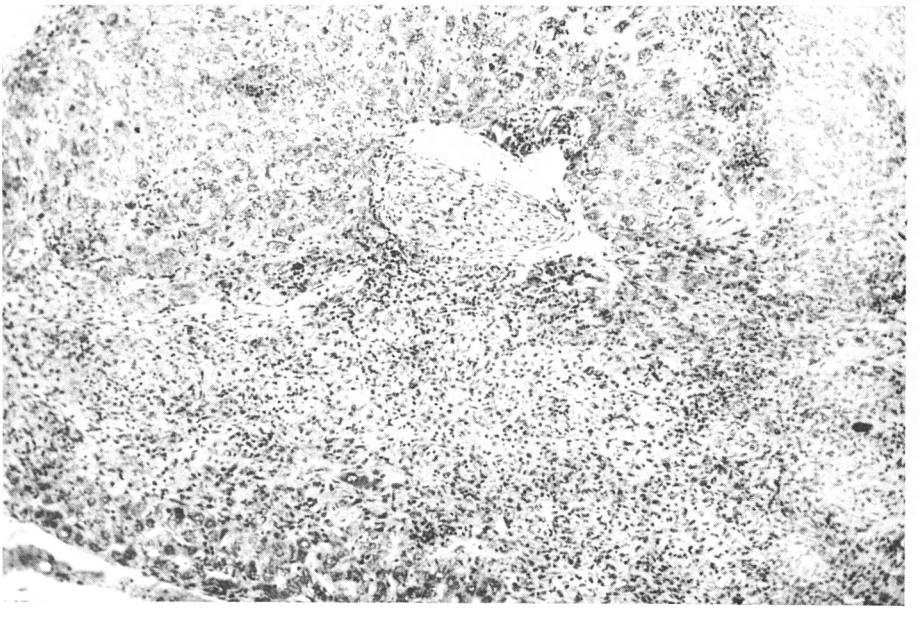

**Fig. 6**

Organized thrombosis of the portal vein branch 14 days after WCI inoculation Leishman-azure-eosin stain. Magn. x 100.

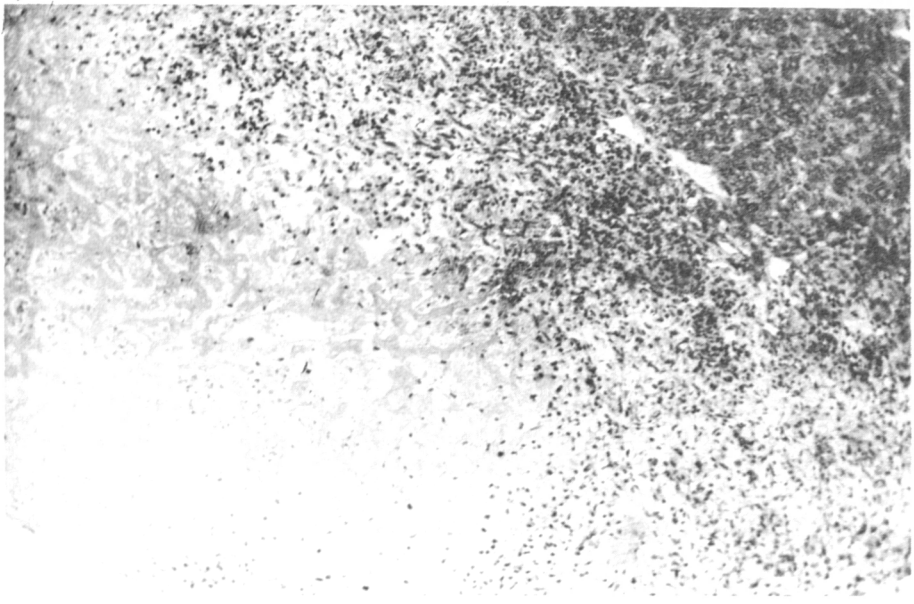

Fig. 7
Ischemic necrosis of the liver lobule 14 days after WCI inoculation
Leishman-azure-eosin stan. Magn. x 100.

increased. As it was shown in experiments with mice perivascular granulomas developed after the WCI inoculation almost in all portal zones (92.8 %) and around a half of central veins (50.7 %). Considerably more seldom and less pronounced granulomas were observed after the CMR inoculation (65.3 and 25.3 %) and especially after the CME inoculation (43.1 and 10.0 % respectively). In experiments with the inoculation of WCI and CMR apparently unchanged particles were often found in cytoplasm of granuloma's macrophages and Kupffer's cells.

By the 14th day of WCI immunization thrombi in portal vein branches (Fig. 6) appeared and ischemic nidus of necrosis in liver lobules (Fig. 7) were frequently observed. These processes were not marked in animals injected with CMR or CME.

## *Discussion*

WCI and CME obtained from different strains of *C. burnetii* were found to react specifically with serum obtained from animals immunized with antigens of

coxiellae strain *Apodemus flavicollis* Luga.

This study shows that in different immunological reactions lower concentrations of WCI as compared to CME are revealed without any differences using preparations from different strains. At the same time the CME inoculation of animals does not lead to the formation of antibodies to this microorganism as well as it does not cause lymphocyte sensibilization in response coxiellae. Moreover it should be noted that the dose of CME used by us for immunization was 200 times higher than that used by Kazár *et al.* (1987) for intraperitoneal immunization of mice with coxiellae phase I whole cells antigen when 1 $\mu$g of it was enough for the formation of antibodies. Besides that we have to underline that in order to detect them the highly sensitive method of IFA (Brezina, 1985) has been used.

Obtained data confirm the fact mentioned earlier (Williams, 1986) that the inoculation of CME does not induce change of humoral immunity indices in animals. They also suggest that neither the cell immunity response not the nonspecific antibacterial activity are strengthened by the inoculation of CME unlike WCI and CMR. Besides that the isolation of this component from coxiellae cell wall causes a decrease of its antigenic activity.

CME was shown to stimulate antibody formation in dependence on a dose of the preparation. The revealing of the effect of the CME's adjuvant allows to explain higher antibody level in animals immunized with WCI as compared to CMR.

Comparative analysis of morphological changes in mouse organism showed that the leukocytic reaction which appeared after the intraperitoneal inoculation of WCI, CMR and CME was stronger with WCI, weaker with CMR and minimal with CME. The most remarkable changes in spleen in experiments with WCI were the following: the strongly pronounced hyperplasia of reticulum cells and macrophages in red pulp and T-dependent zone of white pulp; the strengthening of their mitotic activity leading to splenomegaly.

The changes in liver appeared to be rather informative either for the estimation of development of T-cell immunity (characteristic lymphoid-macrophage granulomas') or for the estimation of harmlessness. Leukocytic reaction increased and disappeared at the same time as in spleen and it was most pronounced after the WCI inoculation. From the 2nd day it was already replaced by lymphoid-macrophage infiltration which reached its maximum by the 14th day. The number of plasma cells increased in the same infiltrate and reached maximum in 7–14 days. During this period there appeared most frequently thromboses of portal vein/branches and necrotic foci in liver lobules in mice inoculated by WCI only. They were obviously connected with the destruction of endothelium and simultaneous increase of blood coagulability characteristic for the lesion of vessels caused by circulating immune complexes.

It is quite possible that cytokines, e.g. tumour necrosis factor (TNF) and interleukin I play certain role in the development of pathogenic processes so far as WCI was shown to be a stronger inductor of these cytokines than CMR. The

latter does not lead to thromboses and necroses (Tokarevich, unpublished results).

Thus the described results confirm previous data (Williams and Cantrell, 1982; Kokorin *et al.*, 1985) concerning the WCI detoxication after phospholipid extraction and limited ability of CMR to stimulate immunomorphological changes characteristic to the development of cell immunity. It was shown that CME does not cause the pronounced toxic effect even in high doses. The toxic effect of WCI is suppose to be connected with the particular loci of CMR and CME on the coxiellae cell membrane.

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