Acta virol. **26**: 174—182, 1982

DIFFERENTIAL EFFECTS OF CYCLOPHOSPHAMIDE ON *COXIELLA BURNETII* INFECTION IN MICE

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Received November 30, 1981

Summary. — Treatment of mice with a single dose (200 mg/kg body weight) of cyclophosphamide (CPA) 3 days before intraperitoneal (i. p.) administration of *Coxiella burnetii* did not substantially affect either the yield of *C. burnetii* from mouse spleen or the splenomegaly induced by the infection. Administration of the same CPA dose 2 days after i. p. infection resulted in an increased yield of *C. burnetii* from the spleen and in a reduction of the spleen size as compared to CPA-untreated and CPA--pretreated infected mice. The yields of *C. burnetii* from the blood and peritoneum were similarly affected. The reduction of splenomegaly was accompanied by a marked decrease in the number of spleen lymphocytes especially in white pulp. Agglutinating antibody response to *C. burnetii* antigens was inhibited regardless whether CPA was given before of after infection. Whereas delayed hypersensitivity reaction to phase I soluble *C. burnetii* antigen was not influenced significantly by CPA pretreatment, it was markedly inhibited in mice treated with CPA after *C. burnetii* administration. The results stress the importance of cell-mediated immunity in *C. burnetii* infection.

Key words: *Coxiella burnetii*; *cell-mediated immunity*; *immunosuppression*; *cyclophosphamide*

Introduction

The cytotoxic drug cyclophosphamide (CPA) is an extensively used immunosuppressive agent. It also enhance experimental rickettsial infection (Kazár *et al.*, 1971; Tachibana and Kobayashi, 1975). These authors, however, have not taken into consideration the fact that effects of CPA treatment on humoral and cellular immune responses may vary depending on the dose and time of drug administration in respect to immunization (Kerckhaert *et al.*, 1977).

The purpose of the present study was to follow the splenomegaly as an indicator of *C. burnetii* infection (Franti *et al.*, 1974) and the multiplication of *C. burnetii* in the spleen, blood and peritoneum along with the humoral

(agglutinating antibody response) and cellular (delayed hypersensitivity-DH reaction) components of the immune response in mice given a single dose of CPA (200 mg per kg body weight) 3 days before or 2 days after *C. burnetii* administration. Histological changes induced in the spleen by CPA treatment and *C. burnetii* infection were compared.

Materials and Methods

C. burnetii strain Nine Mile in phase I (3rd chick embryo yolk sac passage) was partially purified by differential centrifugation, resuspended in brain-heart infusion (BHI) and stored at −30 °C. Titrations of this rickettsial pool and of 20% mouse organ suspensions were performed on 7 days old chick embryos inoculated into the yolk sacs. Results were expressed in median egg infectious doses (EID_{50}) calculated based on the reading Gimenez-stained impression smears on day 10—11 p.i.

Specific-pathogen-free outbred mice (VELAZ breed) weighing 25 ± 1 g were used throughout; they were infected i. p. with 10^4-10^6 EID_{50} of *C. burnetii* in 0.5 ml volumes. CPA-treated mice received a single dose of 200 mg/kg body weight of CPA (Rudolstadt, German Democratic Republic) in 0.3 ml of phosphate buffered saline (PBS) 3 days before or 2 days after inoculation (p. i.) of *C. burnetii*. In some experiments, control groups of CPA-treated uninfected or untreated infected mice were included. Groups of 4—6 mice each were examined at a given interval. On days 3, 7, 10 or 14 p. i.., the mice were aseptically bled from the heart. Pooled sera of each group were examined for the presence of phase I and phase II antibodies by the microagglutination (MA) test according to Fiset *et al.* (1969). From the pooled blood clots, 20% suspensions in BHI were prepared and stored. In some experiments, the peritoneal cavity of each mouse was washed with 3 ml of BHI; the washings were pooled and stored. In all groups of mice, the spleens were removed aseptically and weighed. Impression smears were made from each spleen, stained according to Gimenez and the average amount of rickettsiae seen in 20 fields of view was evaluated as described (Kazár *et al.*, 1973): − no rickettsiae; (+) single rickettsiae occasionally seen; + less than ten; + + tens; + + + hundreds; and + + + + uncountable number of rickettsiae per each field of view. The remaining sépleen pieces werre pooled; 20% suspensions in BHI were prepared from them, stored and titrated as mentioned above. For histological examination, spleens were fixed in 10% buffered formaldehyde and embedded into paraffin. Sections were stained with haematoxylin and erythrosin (HE) and according to Gimenez.

Delayed hypersensitivity (DH) was assayed by inoculation into the left hind footpad of 0.05 ml PBS containing 20 μg of lyophilized surface phase I *C. burnetii* antigen prepared by trichloroacetic acid extraction (Brezina and Úrvölgyi, 1962). The same mice inoculated into the right hind footpad with 0.05 ml of PBS served as controls. The DH reaction was read after 24 hr by the method of Kitamura (1980), by weighing legs of individual mice cut in the knee joint and calculating per cent of weight increase of the test (left) leg versus the control (right) leg in each mouse group.

The means of spleen and leg weights and their standard deviations were calculated and the differences obtained between the groups were evaluated by Student's t-test. Probability values of $p \leq 0.05$ were considered as significant.

Results

Multiplication of C. burnetii *in the spleen, spleen weight and agglutinating antibody response to* C. burnetii *in infected mice treated with CPA before or after infection*

Mice infected with different doses of *C. burnetii* (from 10^4 to 10^6 EID_{50}) were divided into three groups: A − untreated mice, B − mice treated with CPA 3 days before and C − mice treated with CPA 2 days after infection with *C. burnetii*. On day 7 p. i., the spleen weight, the amount of *C. burnetii* in spleen impression smears and the phase I and phase II MA antibody

Table 1. Spleen weight, multiplication of C. burnetii in the spleen and agglutinating antibodies on day 7 p. i.

Mouse group	Dose of *C. burnetii* (EID_{50})	Spleen weight (mean ± SD) mg	Amount of *C. burnetii* in the spleen	MA antibody titre	
				phase I	phase II
A	10^6	554 ± 34	++++	4	64
B		519 ± 49	+++	< 2	< 2
C		193 ± 45	++++	< 2	< 2
A	10^5	484 ± 51	+++	2	32
B		495 ± 45	+++	< 2	< 2
C		218 ± 25	++++	< 2	< 2
A	10^4	470 ± 33	++	< 2	32
B		480 ± 23	++	< 2	< 2
C		178 ± 34	++++	< 2	< 2
Uninfected CPA-treated	0	156 ± 21	−	< 2	< 2
Uninfected untreated	0	168 ± 16	−	< 2	< 2

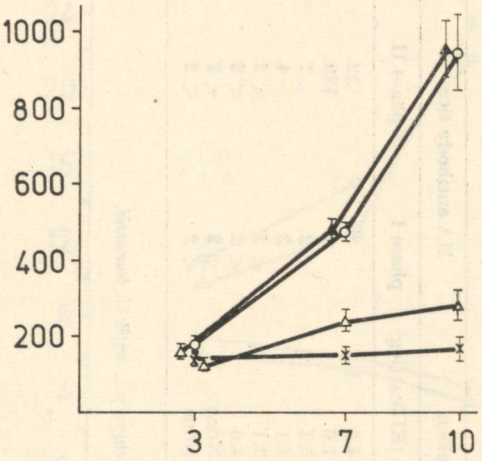

Fig. 1.
Effect of CPA treatment on the mouse spleen weight
Mice treated with CPA before (▲) or after (△) infection with 10^5 EID_{50} of *C. burnetii*
Controls: infected, untreated (○) and untreated, uninfected (×)
Abscissa: days p.i.; ordinate: mean spleen weight (mg)

response were determined. In parallel, control groups of CPA-treated and untreated unifected mice were similarly examined.

The spleen weights of group B mice were comparable with those of group A mice ($p > 0.05$), in contrast to spleen weights of group C mice; the latter were significantly lower ($p < 0.01$), not exceeding the values in uninfected controls (Table 1). The amounts of *C. burnetii* in spleen impression smears were similar and depended on the size of *C. burnetii* inoculum in mouse groups A and B. On the other hand, in the mouse group C, great amounts of *C. burnetii* were present in the spleen regardless the infectious dose used.

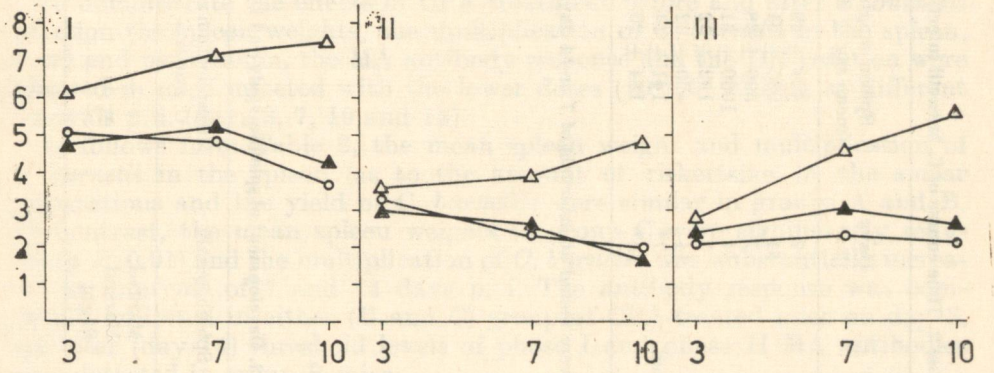

Fig. 2.
Effect of CPA treatment on the yield of *C. burnetii* in mouse spleen (I), blood (II) and peritoneum (III)
Mice treated with CPA before (▲) or after (△) infection with 10^5 EID_{50} of *C. burnetii*
Controls: infected, untreated (○) mice
Abcsissa: days p. i.; ordinate: yield of *C. burnetii* (log EID_{50}/ml)

Table 2. Time course of C. burnetii infection in mice treated with CPA before or after infection with 10^4 EID_{50}

Mouse group	Days p.i.	Spleen weight (mean mg ± SD)	*C. burnetii* in mouse spleen		MA antibody titre	
			Amount of *C. burnetii*	Yield (EID_{50}) (log)	phase I	phase II
A	7	424 ± 49	++	4.3	2	32
	14	985 ± 81	(+)	1.6	4	128
B	7	539 ± 28	++	5.1	< 2	< 2
	14	1050 ± 61	(+)	1.1	2	4
C	7	182 ± 32	++++	7.1	< 2	< 2
	14	420 ± 32	+++	4.6	< 2	< 2
Control, uninfected, untreated	7*	141 ± 14	−	None	< 2	< 2
	14*	184 ± 23	−	None	< 2	< 2

* Control mice were tested at intervals of 7 or 14 days, corresponding to the respective days p.i. with *C. burnetii*.

Table 3. Development of splenomegaly and DH reaction as related to the multiplication of C. burnetii in mouse spleen

Mouse	Splenomegaly	Multiplication of *C. burnetii*	MA antibody response		DH reaction	Death of mice
			phase I	phase II		
A	Marked	++	Low	High	High	Absent
B	Marked	++	None	None	High	Absent
C	Slight	++++	None	None	None	Frequent

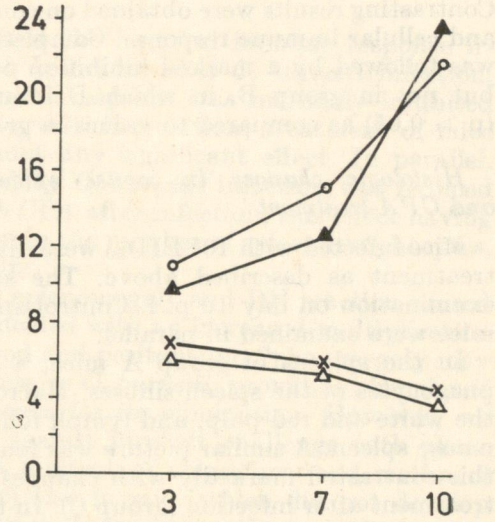

Fig. 3.
Effect of CPA treatment on the DH reaction
Mice treated with CPA before (▲) or after (△) infection with 10_5 EID_{50} of *C. burnetii*
Controls: infected, untreated (○) and uninfected (×) untreated mice
Abscissa: days p. i.; ordinate: log weight increase (in per cent)

Some mice infected with higher doses died, obviously due to increased multiplication of *C. burnetii*. Phase I and phase II MA antibody response was completely inhibited in all groups of CPA-treated mice, irrespective of whether CPA was given before or after *C. burnetii* infection.

Time dependence of changes in mice by C. burnetii *infection and treatment with CPA before or after infection*

To demonstrate the effects of CPA-treatment before and after *C. burnetii* infection the spleen weights, the multiplication of *C. burnetii* in the spleen, blood and peritoneum, the MA antibody response and the DH reaction were followed in mice infected with the lower doses ($10^4/10^5$ EID_{50}) at different intervals p. i. (days 3, 7, 10 and 14).

As follows from Table 2, the mean spleen weight and multiplication of *C. burnetii* in the spleen (as to the amount of riskettsiae in the smear preparations and the yield of *C. burnetii*) were similar in groups A and B. By contrast, the mean spleen weights in group C were significantly reduced ($p < 0{,}01$) and the multiplication of *C. burnetii* was substantially increased at intervals of 7 and 14 days p. i. The antibody response was completely inhibited in either (B and C) group of CPA-treated mice on day 7, but later (day 14) threshold levels of phase I and phase II MA antibodies were detected in group B mice.

Reduction of the spleen weight, substantially greater at later intervals, was observed only in mice of group C (Fig. 1). In these mice, in addition to an increased yield of *C. burnetii* from the spleen, *C. burnetii* multiplied in the blood and peritoneum, as evidenced by an increased yield at later intervals that contrasted with a decreased yield in mice of groups A and B (Fig. 2).

Contrasting results were obtained on comparing the effects of CPA on humoral and cellular immune response. Complete inhibition of MA antibody response was followed by a marked inhibition of DH reaction ($p < 0.01$) in group C but not in group B, in which DH reaction was not significantly affected ($p > 0.05$) as compared to values in group A mice (Fig. 3).

Histologic changes in mouse spleens induced by C. burnetii *infection and CPA-treatment*

Mice infected with 10^5 EID_{50} were divided into 3 groups, according to CPA treatment as described above. The spleens were removed for histological examination on day 10 p. i. Control spleens from untreated and uninfected mice were examined in parallel.

In the spleens of group A mice, a few *C. burnetii* were found in fixed phagocytes of the spleen sinuses. Macrophages were more abundant in both the white and red pulp, and lymph follicles were greater than in the control mouse spleen. A similar picture was found in the spleens of group B (Fig. 4); this contrasted markedly with changes in the mouse spleen caused by CPA treatment after infection (group C). In the latter, lymph follicles were clearly reduced (Fig. 5). Marked depletion of lymphocytes was seen in white pulp namely in the T zone around *a. centralis* accompanied by a relative increase in number of free macrophages (Fig. 6). In the red pulp, the increase in the number of free macrophages and swelling of the cytoplasm of fixed macrophages lining the sinuses was abundant. Numerous rickettsiae (stained according to Gimenez) were seen in fixed macrophages filling up the cytoplasm and pushing the nuclei to the cell margin (Fig. 7).

Discussion

A bulk of evidence has accumulated recently indicating the role of cell-mediated immunity in *C. burnetii* infection. Cellular immune responses to *C. burnetii* antigens were demonstrated in Q-fever convalescents (Jerrells *et al.*, 1975), in the course of infection of guinea pigs with *C. burnetii* (Kishimoto and Burger, 1977) as well as in animals immunized with inactivated *C burnetii* phase I vaccines or purified phase I antigenic preparations (Heggers *et al.*, 1974; Kishimoto *et al.*, 1978*a*). Lymphokines were found to increase macrophage resistance to *C. burnetii* infection (Hinrichs and Jerrells, 1976) and macrophages obtained from immune animals were shown to destroy *C. burnetii* more rapidly (Kishimoto *et al.*, 1977). Spleen cells from immune donors conferred protection against *C. burnetii* in recipient mice (Kazár *et al.*, 1977) and nude mice responded with a prolonged illness to *C. burnetii* infection (Kishimoto *et al.*, 1978*b*).

The importance of cell-mediated immunity in *C. burnetii* infection is also stressed by the present results. Differential effects of CPA on the B- and T-cell compartments of the mouse immune system (Stockman *et al.*, 1973) were emphasized when the effects of CPA-treatment before or after antigen administration were compared (Robinett and Rank, 1979). In our case, a single

large dose of CPA completely inhibited the humoral immune response irrespective of whether CPA was given before or after *C. burnetii* infection. On the other hand, the cellular immune response was markedly inhibited only when CPA was given after *C. burnetii* inoculation; treatment of mice with CPA before infection was without any significant effect. In parallel, development of splenomegaly induced by *C. burnetii* infection was reduced only in the group of mice treated with CPA after infection, this effect having been manifested mostly by a reduction in the number of spleen lymphocytes, namely in the T zone of the white pulp.

As follows from Table 3, absence of splenomegaly and DH reaction caused by CPA treatment after infection coincided with an increased multiplication of *C. burnetii* in the mouse spleen, blood and peritoneum. Splenomegaly may represent a part of the immune response to *C. burnetii*, providing stimulated lymphocytes and activated macrophages for its elimination. However, we observed a fair degree of protection against virulent challenge with phase I *C. burnetii* vaccines (treated with a mixture of chloroform and methanol or phenol-chloroform-petroleum ether) also in mice which did not develop splenomegaly after vaccination (Kazár *et al.*, in preparation).

In the meantime, Asher *et al.* (1980) described the preservation and even an increase of the protective immunogenicity of phase I particulate *C. burnetii* vaccine by CPA pretreatment of guinea pigs. This effect was accompanied by inhibition of the humoral immune response, but the cellular immune response fDH reaction and lymphocyte transformation) was enhanced. Elimination of a suppressive B-cell response by CPA pretreatment was thought to be responsible. Our results are in accordance with those of Ascher *et al.* (1980) except that we found no enhancement of DH reaction in CPA-pretreated mice. This is not surprising when taking into consideration the fact that the regulative effect of CPA-sensitive T cells on the reaction may depend on the dose of antigen and on the level of immune response to it obtained in control animals (Schwartz *et al.*, 1978). The DH skin reaction represents an in vivo marker of the cell-mediated immune response. It remains to be elucidated to which extent it reflects the actual state of resistance to *C. burnetii*.

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Explanation of Micrographs (Plate XVIII):

Fig. 4. Spleen of a mouse treated with CPA 3 days before infection (group B) examined on day 10 p.i. A large lymph follicle containing lymphocytes and lymphoblasts. × 120, HE.

Fig. 5. Spleen of a mouse treated with CPA 2 days after infection (group C) examined on day 10 p.i. Two lymph follicles appear smaller than those shown in Fig. 4. A relative increase in the number of macrophages in the red pulp and at the margin of the white pulp. × 120, HE.

Fig. 6. The white pulp of the same spleen as shown in Fig. 5. A marked reduction of the number of lymphocytes in the T zone around *a. centralis*. × 400, HE.

Fig. 7. The red pulp of the same spleen. Fixed macrophages in the shape of a sealing ring (arrows) are filled up with rickettsiae pushing the nucleus to the margin of the cell. × 960, Gimenez staining.