INTERACTION OF *RICKETTSIA PROWAZEKII* STRAINS OF DIFFERENT VIRULENCE WITH WHITE RAT MACROPHAGES

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Summary. – The growth of mildly pathogenic strain E, its virulent revertant EVir, and prototype virulent strain Breinl of *Rickettsia prowazekii* in peritoneal macrophage cultures of outbread white rats (WR) was evaluated by light microscopy and bioassay in chick embryos (CE). Macrophage cultures infected with strain E were characteristic by limited number of infected cells, poor or moderate accumulation of rickettsiae in individual cells, poor or nil spread of infectious process during first 7 days of infection, and the death of rickettsiae in cultures as determined by the bioassay in CE. Moreover, rickettsiae were not determined in 20.7% of infected macrophage cultures by either microscopic or bioassay methods. In contrast, the growth of virulent strains EVir and Breinl was characteristic by higher proportion of infected cells, considerable accumulation of rickettsiae, and intensive spread of infectious process within 5–7 days post infection (p.i.). However, the intensity of infectious process in macrophage cultures was less expressed with strain EVir than with strain Breinl.

Key words: *Rickettsia prowazekii*; mild and virulent strains; macrophages; white rats

Introduction

As peritoneal macrophages of different animals, human monocytes and macrophage-like cells are widely used for differentiation of R. prowazekii strains of different virulence (Gambrill and Wisseman, 1973; Turco and Winkler, 1982, 1991; Winkler and Dougherty, 1983; Winkler and Turco, 1988; Turco and Winkler, 1994), and play an important role in typhus fever pathogenesis (Beaman and Wisseman, 1976a,b; Kekcheeva *et al.*, 1981; Osterman, 1985; Popov *et al.*, 1987a,b; Vovk *et al.*, 1980; Walker *et al.*, 1991; Winkler and Turco, 1988), we have conducted a comparative study of interactions of isogenic strains, mild pathogenic E, its virulent revertant EVir and prototype virulent strain Breinl with WR macrophages. The main purpose of this study was to find out in what way the reversion of the virulence in strain EVir of *R. prowazekii* affected its ability to grow in cells, namely, in WR macrophages. This system was chosen because the different seroimmunological answers of WR depend on the virulence of *R. prowazekii* strain used for infection (Ignatovich and Rybkina, 1963; Zdrodovski and Golinevich, 1960).

Materials and Methods

Rickettsial strains used in this study were described previously (Ignatovich *et al.*, 1990). Rickettsial materials were characterized by their ID_{50} titers for CE ($CEID_{50}$), white mice ($WMID_{50}$) and WR ($WRID_{50}$), and by their haemolytic activity (HA). The values of $CEID_{50}$/ml ($3.0 \times 10^7 - 2.0 \times 10^8$) and HA units/ml ($9.6 \times 10^8 - 8.5 \times 10^9$) were identical for all the three strains. The pathogenic strains Breinl and EVir were characteristic by high values of $WMID_{50}$/ml ($0.5 \times 10^6 - 1.8 \times 10^8$) and $WRID_{50}$/ml ($1.0 \times 10^6 - 1.8 \times 10^8$). The mildly pathogenic strain E had low values of $WMID_{50}$/ml ($3.2 \times 10^1 - 3.2 \times 10^2$) and $WRID_{50}$/ml ($1.8 \times 10^2 - 4.0 \times 10^3$). Egg cultures of rickettsiae were partially purified by differential centrifugation. Each culture was standardized by $CEID_{50}$/ml and/or HA units/ml (Walker and Winkler, 1979), and stored at -60 °C before use.

Macrophage cultures. Activated peritoneal macrophages from outbreed male WR (70 – 80 g) were collected at the day 3 after interperitoneal injection of 10 ml thioglycollate broth. The peritoneal fluid was added to medium 199 with 10 U/ml heparin. After counting the cells were resuspended in medium 199 with 10%

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Abbreviations: CE = chick embryo; HA = haemolytic activity; p.i. = post infection; WM = white mouse; WR = white rat

bovine serum without antibiotics to concentration of 5 – 6 x 10^5 cells/ml and added into a tube with glass coverslip.

Infection of macrophage cultures and measurement of rickettsial growth. At the day 2 the macrophage cultures were infected with rickettsiae with doses 10^6 $CEID_{50}$ and/or 10^8 HA units. After adsorption for 2 hrs at 35 °C the inoculum was removed, tubes were twice washed and medium 199 with 10% bovine serum was added. Cell cultures were kept at 34 °C.

Infected macrophage cultures were examined at the days 1, 2, 5, and 7 p.i. Glass coverslips were removed, washed, fixed in ethanol and stained by the Giemsa method. In each stained coverslip 100 – 400 cells were investigated by determining the percentage of infected cells and the number of rickettsiae in individual cells. The extent of rickettsial accumulation per cell was grouped in the following way: (a) up to 10, (b) up to 50, and (c) over 50 rickettsiae per cell. The intensity of rickettsial accumulation was expressed as percentage of each category of such infected macrophages. After removing glass coverslips the tubes with the rest of cells and medium were stored at -65°C until the next use. To detect viable rickettsiae in the macrophages on coverslips the cells were removed from the glass surface with a rubber policeman, suspended in the medium and injected into 3 – 4 CE. Infected CE were examined immediately after death and viable CE at the day 12 p.i. On the whole, 210 samples of 61 infected macrophage cultures from 30 WR were investigated.

Statistics. The data were analyzed by the Student's test.

Results

Characteristics of the interaction of *R. prowazekii* strains of different virulence with peritoneal macrophages of WR *in vitro* were obtained by analyzing the dynamics of spread of infectious process in cultures, accumulation of rickettsiae in an individual macrophage, and determination of viable rickettsiae in infected macrophage cultures.

It can be seen that the ability of each rickettsial strain to infected cells and to spread in WR macrophage cultures had its peculiarity (Table 1). The infectious process in macrophage cultures infected with mild strain E differed markedly from those infected with virulent strains EVir or Breinl. The proportion of infected cells with E strain was small and increased slowly. On the whole, the mean percentage of infected macrophages were 1.2 ± 2.1%, 2.0 ± 2.1%, 1.9 ± 2.1% and 2.3 ± 1.9% at days 1, 2, 5 and 7 p.i., respectively. Six of 29 (20.7%) examined cultures were negative. The negative results of microscopic test were confirmed by bioassay in CE (see below, Table 3).

Table 1. Kinetics of spread of different strains of *R. prowazekii* in macrophage cultures

Strains	Positive/ total cultures	Infected macrophages in %* Days p.i.			
		1	2	5	7
E	23/29	1.2 ± 2.1	2.0 ± 2.1	1.9 ± 2.1	2.3 ± 1.9
EVir	15/15	4.1 ± 2.1	5.1 ± 4.0	17.7 ± 9.7	19.1 ± 15.9
Breinl	17/17	15.8 ± 6.5	12.1 ± 5.3	38.4 ± 14.8	35.2 ± 21.4

*Each value represents the mean ± SE from 7 experiments. Differences between the strain E, EVir and Breinl are significant ($p < 0.05$) for all days except the difference between the strains EVir and Breinl on day 7 ($p > 0.05$).

In contrast, the virulent strains EVir and Breinl grew well in WR macrophages with the intensive spread of infectious process in cultures. The percentage values of infected macrophages were significantly higher ($p < 0.05$) than those for the strain E at all days tested. At the same time the kinetics of infectious process in macrophage cultures infected with EVir or Breinl strains were different. The differences were significant ($p < 0.05$) at all days except the day 7. So the mean statistic figures of the percentage of EVir-infected macrophages were 4.1 ± 2.1%, 5.1 ± 4.0%, 17.7 ± 9.7% and 19.1 ± 15.9% at days 1, 2, 5 and 7 p.i., respectively. The strain Breinl-infected macrophage cultures were characteristic by figures 15.8 ± 6.5%, 12.1 ± 5.3%, 38.4 ± 14.8%, and 35.2 ± 21.4% at the same days, respectively.

As to the value of accumulation of rickettsiae in an individual macrophage, relative indices were used because of impossibility to count exactly rickettsiae in a cell which contained more than 50 of them (Table 2). At the days 1 and 2 p.i. with strain E, the macrophages containing not more than 1 – 10 rickettsiae predominanted. The proportion of such macrophages was 81.0% and 61.0%, respectively. At the later time intervals, 5 and 7 days p.i., the proportion of poorly infected macrophages (1 – 10 rickettsiae) decreased to 44.0 – 40.0%, and that with more than 10 to 50 rickettsiae/cell increased to 56.0 – 60.0%, respectively. As it is seen from these results the number of strain E rickettsiae per infected cell was not above 50. Unlike the poor or moderate accumulation of strain E rickettsiae in WR macrophages, the growth of the virulent strains in these cells was characteristic with higher intensity. The percentage of macrophages with low number of strain EVir rickettsiae fell from 73.2% at the day 1 to 16.1% at the day 7 p.i. The percentage of macrophages with low number of strain Breinl rickettsiae fell from 53.8% at the day 1, 23.7% at the day 7 p.i. At the same periods the percentage of cells with considerable number of rickettsiae (above 50) increased greatly, namely from 2.4% to 35.0% (strain EVir), and from 1.3% to 30.2% (strain Breinl). The accumulation of two virulent strains in an individual cell had practically similar characteristic.

To determine viable rickettsiae in infected macrophage cultures, the latter were investigated by the bioassay at the days 1, 2, and 5 p.i. The data are summarized according to the percentage of positive probes, the percentage of CE with rickettsiae in probes, and the mean day of CE death (Table 3). The lower number of positive results of the bioassay

Table 2. Accumulation of different strains of *R. prowazekii* in infected macrophages

Strains	Percentage of macrophages with different amount of rickettsiae per infected cell* Days p.i.											
	1			2			5			7		
	a	b	c	a	b	c	a	b	c	a	b	c
E	81.0	19.0	0	61.0	39.0	0	44.0	56.0	0	40.0	60.0	0
EVir	73.2	24.4	2.4	43.2	49.0	7.8	17.0	61.0	22.0	16.1	48.1	35.8
Breinl	53.8	44.9	1.3	44.6	48.0	7.4	19.5	46.9	33.6	23.7	46.1	30.2

*a – up to 10 rickettsiae/cell.
b – up to 50 rickettsiae/cell.
c – over 50 rickettsiae/cell.

was observed in the E strain-infected cultures. Twenty-three cultures which were microscopically positive gave positivity only in 50.0%, 85.7% and 81.3% at the days 1, 2, and 5 p.i., respectively, by the bioassay. The percentage of CE with rickettsiae in these probes was 31.7%, 46.3% and 52.0%, respectively. The mean time of the infected CE death was 12.8 ± 0.83 days p.i. that corresponded to minimum of live rickettsiae in the probes. Six negative macrophage cultures tested microscopically turned out to be negative also in the bioassay. Positive results were obtained with EVir strain-infected macrophage cultures in 75.0, 93.8, and 95.5% of samples in comparison with the 100% positive microscopic figures (Table 1). The proportion of CE with rickettsiae in these probes was 55.3, 70.0, and 75.3%, respectively. The mean day of infected CE death was close to that obtained for strain E-infected cultures. Most positive results were obtained by the examination of strain Breinl-infected macrophage cultures by the bioassay. As positive were determined 92.9, 100.0, and 100.0% of probes, respectively. The percentage of CE with rickettsiae in all probes varied from 80.9% to 87.3%. The mean time of infected CE death in probes of strain Breinl-infected cultures (10.3 ± 1.93 days p.i.) was shorter and that for strains E ($p < 0.2$) and EVir ($p < 0.5$). These results showed that there are differences in the quantity of viable rickettsiae in macrophage cultures infected with E, EVir and Breinl strains of *R. prowazekii*.

Table 3. Results of determination of rickettsiae in macrophage cultures by bioassay in CE

Indices	Strains	Days p.i.			Mean ± SE
		1	2	5	
Positive results of bioassay(%)	E[a]	50.0	85.7	81.3	–
	EVir	75.0	93.8	95.5	
	Breinl	92.9	100.0	100.0	
CE with rickettsiae in probes examined(%)	E[a]	31.7	46.3	52.0	–
	EVir	55.3	70.0	75.3	
	Breinl	80.9	87.3	81.7	
Average day of CE death	E[a]	12.8	13.0	12.4	12.8 ± 0.83
	EVir	12.0	12.0	11.6	12.2 ± 1.65
	Breinl	10.7	10.9	9.4	10.3 ± 1.93

[a] Without 6 negative cultures determined by microscopic and bioassay methods.

Discussion

The characteristics of the growth of genetically related mildly pathogenic strain E and its virulent mutant strain EVir as well as of the prototype virulent strain Breinl of *R. prowazekii* in peritoneal macrophages of WR were obtained. The typical outcome of interaction of strain E rickettsiae with macrophages was the limited infectious process, i.e. the small percentage of infected macrophages in culture and the poor growth of rickettsiae in an individual cell. Besides 6 of 29 infected macrophage cultures were completely free of rickettsiae.

The interaction of two virulent *R. prowazekii* strains with WR macrophages differed greatly from that of E strain with these cells. It was characteristic by intensive growth of rickettsiae in macrophages, intensive dynamics of infectious process and considerable amount of viable rickettsiae in macrophage cultures. However, the parameters of the growth characteristic of EVir and Breinl strains were not the same. The lower proportion of infected cells and slower spread of infectious process were observed for strain Evir in comparison with strain Breinl. During the first 5 days p.i. the

difference was significant and decreased to the day 7. The results of the bioassay of infected macrophage cultures showed that there were less viable rickettsiae for strain EVir than for strain Breinl. It is possible to assume a higher stability of the strain Breinl rickettsiae in WR macrophages as compared to the strain EVir.

On the whole, the determined peculiarities of the interaction of E and Breinl strains of *R. prowazekii* with peritoneal WR macrophages correspond to the previously described characteristics of the growth of strains E and Breinl in macrophages of man (Gambrill and Wisseman, 1973), macrophage-like cells (Turco and Winkler, 1982, 1991; Winkler and Dougherty, 1983) and some non-macrophage cell lines (Gudima, 1979; Ignatovich, 1976; Ignatovich *et al.*, 1990; Winkler and Dougherty, 1983).

Certain differences in the growth of virulent strains EVir and Breinl rickettsiae in peritoneal WR macrophage were determined. The lower ability of strain EVir rickettsiae to grow in FL cell cultures in comparison with strain Breinl rickettsiae was determined previously (Ignatovich *et al.*, 1990). It is necessary to emphasize that EVir and Breinl strains are highly virulent for experimental animals (guinea pigs, cotton rats, WR) grow intensively in CE, lungs of white mice (Balayeva, 1969; Ignatovich *et al.*, 1990) and also in macrophage-like RAW 264.7 cells (Turco and Winkler, 1994). It seems reasonable to note that not all phenotypic signs of virulence coincide in virulent *R. prowazekii* strains. So there are differences in virulent properties for WM, WR and guinea pigs (Ignatovich and Rybkina, 1963; Kazár *et al.*, 1973; Ormsbee *et al.*, 1978). Besides it was found that the virulent strains EVir and Breinl differ in sensitivity and resistance to some cytokines (Turco and Winkler, 1994).

The data received by the use of the system of peritoneal WR macrophage cultures demonstrated that the differences between the mildly pathogenic strain E and the virulent strains EVir and Breinl of *R. prowazekii* correlate with those in the reaction of WR infected with these strains. Moreover, this system allows to determine some differences between the two virulent strains which are not detectable in infected experimental animals by conventional tests (temperature reaction, antibody titers, $CEID_{50}$, $WRID_{50}$, $WMID_{50}$).

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