MICROBIAL PATHOGENICITY

The interaction of *Yersinia pestis* with erythrocytes

V. A. FEODOROVA and Z. L. DEVDARIANI

Laboratory of Hybridomas, Russia State Antiplague Research Institute 'Microbe', Saratov, Russia

Human and murine erythrocytes (RBC) were invaded by *Yersinia pestis* *in vivo* and *in vitro* during a short period and were probably used as an essential source of iron and porphyrin for survival, effective gross multiplication and rapid spread of these bacteria in the bloodstream of mammals. Both iron and porphyrin were extracted by *Y. pestis* from the RBC through oxidase-catalase activity which produced oxidation of the RBC glucose with generation of H₂O₂ in large concentration leading to oxidative transformation of haemoglobin into haemin. Furthermore, some mainly chromosomally encoded effector proteins were implicated in this process because all were synthesised by *Y. pestis* grown in media simulating the intracellular conditions of mammalian RBC. Damaged RBC lost the ability to transport O₂ in the mammalian organism. As a result, significant oxygen deficiency developed in host tissues providing specific clinical disease features of plague which are similar to characteristic symptoms of poisoning with haemotoxic substances when the transformation of the haemoglobin Fe²⁺ to Fe³⁺ occurs.

Introduction

Several reports have demonstrated that *Yersinia pestis* is able to survive and grow *in vitro* within mouse peritoneal macrophages which are considered to be the host target cells for these bacteria [1–5]. However, after a short period (2–3 h) *in vivo*, as well as under *in vitro* conditions imitating those *in vivo*, virulent *Y. pestis* organisms acquired the ability to resist phagocytosis by mouse polymorphonuclear leukocytes [6, 7]. Moreover, *Y. pestis* bacteria are not recognised by human phagocytes *in vitro* or *in vivo* [7] and extensive multiplication of bacteria *in vivo* occurs mainly outside the macrophage, i.e., at the site of infection, in regional lymph nodes and also in bloodstream resulting in septicaemia [1, 4, 8]. Therefore, it seems possible that blood cell(s) other than macrophages are invaded and used by *Y. pestis* for its normal life cycle.

The current study examined the invasion by *Y. pestis* of mammalian erythrocytes (RBC) but not phagocytes, both *in vivo* and *in vitro*, and the use of RBC as nutrient sources. Subsequent damage to the RBC is essential for development of specific clinical features of plague as a result of progressive oxygen deficiency in host tissues.

Materials and methods

Bacterial strains and culture conditions

The *Y. pestis* strains used in this study are listed in Table 1. [1, 5, 9, 10]. *Y. pestis* strains EV-76, 231, U-2422, U-2377 and PKR-133 were cultured in RPMI-1640 medium, pH 7.2, containing 2 mM glutamine, 2.2 mM sodium bicarbonate, 20 mM HEPES, 2.5 mM CaCl₂ and fetal calf serum 10% v/v together with human erythrocytes (HRBC) at 37°C for 24 h or 48 h.

Y. pestis strain EV-76 and its isogenic derivatives were grown in Hottinger broth, pH 7.2, supplemented with 1 mM ϵ -aminocaproic acid (ϵ -aca) only or in combination with 5.5 mM glucose or 20 mM MgSO₄ together with ϵ -aca and glucose at the same concentrations at 28°C for 24 h and then incubated at 37°C for 24 h. The same strains grown in Hottinger broth, pH 7.2, without any supplements were used as a control.

After cultivation, the bacteria were harvested and washed twice with phosphate-buffered saline (PBS), pH 7.2. Viable cell yields were determined by turbidity measurements and by standard plating techniques. Viable cells were counted and, after killing with sodium merthiolate at a concentration of 1 in 10 000, used for assays.

Y. pestis strain 231 cultivated on Hottinger agar, pH 7.2–7.4, at 28°C for 24 h was used for acute challenge of mice.

Received 25 July 2001; accepted 9 Aug. 2001.

Corresponding author: Dr V. A. Feodorova (e-mail: microbe@san.ru).

Table 1. The *Y. pestis* strains studied

Strain no.	Plasmid profile	Pgm*	Reference or source
EV-76	pFra ⁺ ;pCad ⁺ ;pPst ⁺	Pgm ⁻	5, 9, 10
KM-217	pFra ⁻ ;pCad ⁺ ;pPst ⁻	Pgm ⁻	5, 10
KM-218	pFra ⁻ ;pCad ⁻ ;pPst ⁻	Pgm ⁻	5, 10
U-2377	pFra ⁺ ;pCad ⁻ ;pPst ⁺	Pgm ⁺	Institute 'Microbe' Collection
U-2422	pFra ⁻ ;pCad ⁺ ;pPst ⁺	Pgm ⁺	Institute 'Microbe' Collection
C-534	pFra ⁺ ;pCad ⁺ ;pPst ⁻	Pgm ⁺	Institute 'Microbe' Collection
PKR-133	pFra ⁻ ;pCad ⁻ ;pPst ⁻	Pgm ⁺	Institute 'Microbe' Collection
231	pFra ⁺ ;pCad ⁺ ;pPst ⁺	Pgm ⁺	Institute 'Microbe' Collection

*Pgm⁺ phenotype means the capability of the relevant *Y. pestis* strain to form pigmented colonies on the media containing exogenous haemin [1].

Infection of the RBC

Normal HRBC were collected in the presence of heparin, washed five times in PBS, and resuspended to a final concentration of 1.5% in RPMI-1640 medium, pH 7.2, containing the above-described supplements. The suspensions of live bacteria of different *Y. pestis* strains in RPMI-1640 with supplements (1×10^9 cells/ml) were added to 5.0 ml of HRBC 1.5%. After stationary incubation for 24 h at 37°C with CO₂ 5% in air the slide smears prepared from this suspension were stained by Gram's technique and studied visually with an inverted microscope (Leitz). The HRBC in RPMI-1640 with supplements incubated at 37°C for 24 h with CO₂ 5% in air were used as a control.

In all cases, at the beginning of combined incubation of bacteria with HRBC and 24 h later, specimens of culture fluids were tested for: (i) glucose activity (presence of glucose) with a standard diagnostic kit (Glucose Fs; Unimed, Russia); (ii) for the presence of Fe²⁺ or Fe³⁺ ions by the benzidine test [11] (briefly, benzidine in 50% w/v ethanol 96% v/v was mixed 1:1 v:v with the above described specimens, then 3–5 drops of acetic acid 30% v/v with 2–3 drops of H₂O₂ 3% v/v were added; the colour of the medium became blue or green in the presence of Fe³⁺ or Fe²⁺, respectively; FeSO₄·7H₂O 1% and Fe₂(SO₄)₃ 1% in RPMI-1640 with supplements were used as a control); (iii) haemoglobin according to the method of Lepehne [12]; and (iv) medium pH with universal indicator paper pH 0–12 (Dia-M, Russia).

For in-vivo experiments, five groups of 30 female, 8–10-week-old outbred mice (6 animals in each group) were given 100 × 50% lethal doses (LD50) of virulent *Y. pestis* strain 231 (LD50 = 6 cfu) only or in combination with 1.0, 2.0, 3.0, 4.0 or 5.0 mg methylene blue in 0.2 ml of distilled water by subcutaneous injection. Slide smears of blood of dead mice were prepared and stained by Gram's technique.

Plasminogen activator activity testing

Y. pestis plasminogen activator activity (PAA) was assayed as described by Beesley *et al.* [13] or in dot-

ELISA with monoclonal antibody to Pla (MAb-Fib) as described previously [5].

SDS-PAGE

Whole-cell lysates of *Y. pestis* strains EV-76, KM-218 and KM-217 or the same proteinase K-treated (PK-treated) whole-cell lysates were subjected to SDS-PAGE as described previously [10], with a stacking 4% gel and separating 12.5% gel. Electrophoresis was done at 35 mA constant current in Tris-glycine (pH 8.3) plus SDS 0.1% buffer for *c.* 2.5 h. A set of low-mol.-wt markers (Sigma) was used. The gels were either counterstained with Coomassie Brilliant Blue R 250 (Sigma) 0.2% w/v in ethanol 25% v/v, acetic acid 7% v/v or according to Tsai and Frasch [14].

Results

Bacterial survival and growth within RBC in vitro and in vivo

As shown in Fig. 1a, at the beginning of combined cultivation with HRBC *in vitro*, the *Y. pestis* cells remained outside the HRBC during a short period but by 2–3 h later they were seen intracellularly; 24 h later the bacteria appeared outside the HRBC, i.e., in the cultural medium or extracellularly on the erythrocyte surface (Fig. 1c). The same effect was observed with all bacteria independently of their plasmid composition, including plasmid-free *Y. pestis* strain PKR-133 (Fig. 1d) or bacteria of Pgm phenotype. In all cases, the erythrocytes were significantly damaged and possessed indistinct friable porous cell membranes. However, some differences in HRBC morphology were seen. When *Y. pestis* strains U-2422 and C-534 harbouring pLcr were used, HRBC were grey and more deformed than when the plasmid-free strain PKR-133 or a *Y. pestis* strain U-2377 lacking pLcr were employed. No significant changes in intact control HRBC were revealed. They appeared to be sphere-like pink cells with distinct cell membranes without any inclusions (Fig. 1b).

Consistent with in-vitro studies with HRBC, the *Y. pestis* 231 cells were found in most of the RBCs of mice infected by the relevant strain. Deformation of

mouse RBC which possessed friable porous cell membranes was also observed (Fig. 1e).

Y. pestis strains, independently of their plasmid composition, grew well in the medium imitating the intracellular conditions of the RBC and containing 20 mM Mg^{2+} , 5.5 mM glucose and 1 mM ϵ -aca. In all cases, bacterial yield was significantly higher in this medium than in Hottinger broth without any of these supplements. Combination of Mg^{2+} with glucose provided either the same bacterial yield in both strains KM-217 and EV-76 or its significant decrease in plasmid-free strain KM-218 (Table 2). Addition of ϵ -aca had no significant effect on bacterial growth.

Y. pestis EV-76 plasminogen activator activity

Y. pestis strain EV-76 grown at 37°C in Hottinger broth without any supplements expressed PAA, whereas the same strain grown in media containing ϵ -aca did not have this activity (Table 2). In dot-ELISA, MAb-Fib gave a strong positive reaction with *Y. pestis* EV-76 grown in media with ϵ -aca was registered (Table 2).

Changes of some main biochemical activities of HRBC

At the beginning of the combined cultivation of HRBC with *Y. pestis*, independently of plasmid composition or Pgm phenotype, the levels of glucose and haemoglobin were the same as in intact HRBC. However, 24 h later these substances were found only in intact control HRBC (Table 3).

Fe^{2+} ions were abundant before and at the beginning of the experiments with HRBC infected by *Y. pestis* bacteria but 24 h later only Fe^{3+} ions were detected. Fe^{2+} ions were detected only in intact HRBC both at the beginning and at the end of the experiments.

No changes of medium pH during combined cultivation of HRBC with *Y. pestis* bacteria, independently of their plasmid profiles or Pgm phenotype, were registered. Some medium pH increase from 7.2 to 7.8 in intact HRBC after cultivation for 24 h at 37°C in RPMI-1640 with supplements was detected (Table 3).

Inhibition of Y. pestis penetration into RBC by methylene blue

The *Y. pestis* bacteria were readily detected within the RBC of mice (MRBC) challenged with virulent strain 231 or when 1–3 mg of methylene blue was administered together with the same concentration of bacteria. However, only single bacteria were detected in the MRBC in the case of combined injection of 4.0 mg of methylene blue with *Y. pestis*. No bacteria were seen within MRBC when a higher dose (at least 5 mg) of methylene blue was used.

Release of specific proteins by Y. pestis within the RBC

Fig. 2 demonstrates the electrophoretic profiles of whole-cell lysates of *Y. pestis* strain EV-76 and its isogenic derivatives grown in the presence of 20 mM Mg^{2+} , 5.5 mM glucose and 1 mM ϵ -aca. In all cases they were not absolutely identical to those of bacteria grown in standard conditions in Hottinger broth without any supplements. Firstly, a number of proteins, 1–12 with mol. wts of 14.5, 15.8, 16.9, 19.2, 21.9, 46.8, 52.5, 69.2, 70.8, 74.1, 77.6 and 79.4 kDa, respectively, were defined in *Y. pestis* strains tested after their cultivation in standard conditions only or in the presence of ϵ -aca or in combination of ϵ -aca with glucose. However, none of these proteins was present in these bacteria when Mg^{2+} was added to the cultivation media. Thus, these proteins were chromosomally encoded polypeptides which were not produced

Table 2. Effect of the cultivation conditions on the proliferative and plasminogen activator activity of *Y. pestis* strains with different plasmid composition

Strain no.	Addition of*			Viable cell yield ($\times 10^9$)	Multiplication ratio	Plasminogen activator activity	Reaction with MAb-Fib in dot-ELISA
	ϵ -aca	ϵ -aca + glucose	ϵ -aca + glucose + Mg^{2+}				
EV-76	–	–	–	9.0	1.0	No clot	+
EV-76	+	–	–	15.0	1.7	Clot	–
EV-76	+	+	–	11.5	1.3	Clot	–
EV-76	+	+	+	13.5	1.5	Clot	–
KM-218	–	–	–	7.85	1.0	Clot	–
KM-218	+	–	–	7.9	1.0	Clot	–
KM-218	+	+	–	5.0	0.6	Clot	–
KM-218	+	+	+	15.0	1.9	Clot	–
KM-217	–	–	–	10.0	1.0	Clot	–
KM-217	+	–	–	10.0	1.0	Clot	–
KM-217	+	+	–	10.0	1.0	Clot	–
KM-217	+	+	+	16.5	1.7	Clot	–

All strains were grown in Hottinger broth.

*The concentration of each of the components is indicated in the *Materials and methods* section.

Fig. 1. Infection of mammalian RBC by *Y. pestis* with consequent cell damage: **a**, **b**, the intact (control) undamaged HRBC cultivated *in vitro*; **c**, different stages of damage to HRBC caused by *Y. pestis* EV-76: bacteria surround the HRBC, form pores in their membranes, invade and then leave the HRBC and appear extracellularly; **d**, damage to HRBC produced by *Y. pestis* PKR-133: showing pore formation, intracellular location of bacteria, with chains of organisms outside the HRBC; **e**, total destruction of RBC of mice by virulent *Y. pestis* strain 231, showing intracellular location and apparent multiplication of *Y. pestis* within the MRBC. **a**, 0 h; **b**, **c**, **d**, 24-h incubation; **e**, 48-h infection.

by *Y. pestis* within the RBC, but their synthesis was regulated by Mg^{2+} .

Proteins 13–15 with mol. wts of 42.7, 47.9 and 50.1 kDa, respectively, appeared in strain EV-76 grown in the presence of ϵ -aca independently of any other supplements and were absent when this strain was grown in routine conditions (Hottinger broth only), being completely degraded by the pla protease [1,

3–5]. All these proteins were found in both strain KM-218 and KM-217 grown in any conditions. Thus, they were also chromosomally encoded proteins.

Protein 16 with mol. wt 12.6 kDa was seen in all *Y. pestis* strains independently of their plasmid profile only in Hottinger broth with all the above-described supplements. Thus, this protein was encoded chromosomally and synthesised by *Y. pestis* within the RBC only.

Fig. 1. (continued)

Protein 17 with mol. wt 19.9 kDa was visualised only in strain KM-218 grown in Hottinger broth with all supplements (Fig. 2, lane 6) and was absent from this strain grown in medium without Mg^{2+} (Fig. 2, lanes 5 and 11). Thus, it was a product of the *Y. pestis* chromosome whose synthesis is regulated by Mg^{2+} in combination with ϵ -aca and glucose.

Interestingly, proteins 18–20 with mol. wts of 11.08, 13.12 and 13.61 kDa, respectively, were found in strain KM-217 grown in medium with all supplements (Fig. 2, lane 9) and were absent when this strain was grown in medium without Mg^{2+} (Fig. 2, lanes 7, 8 and 10). Thus, these proteins were plasmid-encoded (pLcr) polypeptides which could be produced by *Y. pestis* within the RBC.

Modification of *Y. pestis* R-LPS to S-LPS within the RBC

LPS profiles of the PK-treated *Y. pestis* EV-76 differed significantly one from another depending on the

cultivation conditions of bacteria. When they were grown in Hottinger broth only or in combination with ϵ -aca, the predominant lower-mol.-wt component corresponding to the core lipid A region and several bands of intermediate mol. wt were seen (Fig. 3, lane 1). If glucose with ϵ -aca was added to cultivation medium, a single predominant high-mol.-wt component appeared in the profile of this strain (Fig. 3, lane 2). Interestingly, when the same bacteria were cultured in medium containing the combination of Mg^{2+} with ϵ -aca and glucose, a ladder pattern of high mol. wt which is typical of micro-organisms with S-type LPS was seen [15]. The same tendency was found in both strains KM-218 and KM-217. Moreover, in strain KM-217 the high-mol.-wt component appeared as a diffuse band but not a ladder pattern occupying more space and stained more intensively than in both strains EV-76 and KM-218. Thus, O-specific polysaccharide chains of *Y. pestis* LPS could shorten or lengthen depending on the cultivation conditions of the bacteria. Thus, LPS (namely, core lipid A and mostly O antigen) is mainly

Table 3. Effect of combined incubation of HRBC and *Y. pestis* strains with different plasmid composition and Pgm phenotype on some biochemical activities of HRBC

Strain no.	Presence of RBC components at (h)									
	glucose		Hb		medium pH		Fe^{2+}		Fe^{3+}	
	0	24	0	24	0	24	0	24	0	24
EV-76	+	–	+	–	7.2	7.2	+	–	–	+
231	+	–	+	–	7.2	7.2	+	–	–	+
U-2377	+	–	+	–	7.2	7.2	+	–	–	+
U-2422	+	–	+	–	7.2	7.2	+	–	–	+
PKR-133	+	–	+	–	7.2	7.2	+	–	–	+
Intact HRBC (uninfected control)	+	+	+	+	7.2	7.8	+	+	–	–

Hb, haemoglobin.

Fig. 2. SDS-PAGE of whole-cell lysates of *Y. pestis* strains EV-76 (lanes 1–4), KM-218 (5, 6, 11) or KM-217 (7–10) grown in the presence of ϵ -aca (lanes 2, 5, 7) or in combination with glucose (lanes 3, 8) and Mg^{2+} (lanes 4, 6, 9) or without any supplements in Hottinger broth only (lanes 1, 10, 11).

encoded by chromosomal genes but its O-side chains may be partly expressed by a plasmid.

Discussion

It is known that iron is an essential nutrient for bacterial growth [1, 3, 4, 16–19]. Plague bacilli utilise several ways of scavenging iron from mammalian haemoproteins and haemin [1, 3, 17, 18] which is an abundant iron-containing compound found *in vivo* in the form of haemoglobin [16, 20]. Thus, haemoglobin-

containing mammalian RBC could be attractive to *Y. pestis*, as sources of iron or other nutrients. This hypothesis was confirmed in the present study. The *Y. pestis* bacteria quickly invaded the human or murine RBC *in vitro* and *in vivo*, respectively, and could be seen easily intracellularly inside them (Fig. 1) when their resistance to mammalian phagocytes had already developed [6]. Furthermore, *Y. pestis* strains could grow and proliferate *in vitro* in the conditions simulating the intracellular environment of mammalian RBC (Table 2). The data obtained indicated that *Y. pestis*, a facultative intracellular parasite, attacked and invaded the mammalian RBC but not the macrophages.

Fig. 3. SDS-PAGE of proteinase K-digested whole-cell lysates of *Y. pestis* strains EV-76 (lanes 1–4), KM-218 (5, 6, 11) or KM-217 (7–10) grown in the presence of ϵ -aca (lanes 2, 5, 7) or in combination with glucose (3, 8) and Mg^{2+} (4, 6, 9) or without any supplements in Hottinger broth only (1, 10, 11).

In vivo, *Y. pestis* is able to scavenge mainly Fe^{3+} but not Fe^{2+} [1, 3, 4, 17, 18]. However, oxygenated haemoglobin in all cases possesses only Fe^{2+} without any oxidation to Fe^{3+} [16, 20]. In contrast, haemin has Fe^{3+} [16, 20] and can be used easily by *Y. pestis* [1, 3, 4, 17, 18] although there is no free haemin in normal mammalian RBC *in vivo* [16, 20, 21]. Thus, *Y. pestis* must possess a special mechanism to transform haemoglobin iron to a scavengeable form. Mammalian haemoglobin exists as a very stable molecule consisting of haem and globin, the detachment of which is possible under strong acidic or alkaline conditions [16, 20]. However, the combined cultivation of HRBC with *Y. pestis* did not result in any alteration of the pH of the medium (Table 3) which remained neutral or weakly alkaline. This finding led to the conclusion that *Y. pestis* used some other means of transforming haemoglobin iron.

As found previously, glucose plays the main role in metabolism of mammalian RBC [16, 20]. *Y. pestis*

lacks glucose 6-phosphate dehydrogenase [1, 3] which makes it difficult for these bacteria to utilise glucose because of its prompt conversion to organic acid [3]. However, due to *Y. pestis* oxidase activity, glucose may be easily oxidised with the generation of H_2O_2 in large concentration [16] leading to oxidative denaturation of haemoglobin [20]. Furthermore, the H_2O_2 concentration may be significantly increased by *Y. pestis* by catalase-peroxidase activity which in *Y. pestis* is the highest reported among prokaryotes [3, 9]. Being a bifunctional enzyme [22], catalase may not only degrade H_2O_2 , protecting the *Y. pestis* from its bactericidal activity, but it can also promote the increase of H_2O_2 concentration [23]. Inhibition of *Y. pestis* proliferative activity in media containing glucose (Table 2), in line with other reports [3, 4], make this hypothesis feasible. It is clear that H_2O_2 as an oxidation product of glucose caused a decrease of *Y. pestis* bacterial concentration but not acidification of cultural media that, in contrast, stimulated *Y. pestis* bacterial growth [5]. Thus, high concentration of H_2O_2 inside the RBC could be the main reason for detachment of globin from haem with the resultant immediate oxidation of haem iron from Fe^{2+} to Fe^{3+} , i.e., haemin formation [16]. The results of testing HRBC for biochemical activities during their combined incubation with *Y. pestis* strains provided strong evidence that glucose is directly involved in haemoglobin destabilisation without its conversion to organic acid (Table 3). Finally, generated Fe^{3+} is suitable for utilisation by *Y. pestis* which possesses a haemin utilisation system [1, 3, 4, 17, 18].

It is clear, that transformation/oxidation of Fe^{2+} to Fe^{3+} causes loss of ability of RBC to transport O_2 in mammalian organisms [16, 20] resulting in significant oxygen deficiency in host tissues. These metabolic changes correspond with typical clinical symptoms, i.e., headache, chills, nausea, vomiting, dizziness, drowsiness or coma, cyanosis, vasomotor excitement or fear of death, etc. [21]. All of them were found in cases of poisoning with aniline or CO or nitrobenzene, which are characterised by the transformation of haemoglobin Fe^{2+} to Fe^{3+} [21]. Interestingly, these clinical symptoms are identical to most of the clinical disease features of plague [1] including those previously called 'facies pestica' [1]. Moreover, the strong cyanotic discoloration of the skin in plague patients [1], becoming dark brown or black in the dead body, produces the appearance for which the disease was named Black Death [1, 4]. One more strong argument that transformation of haemoglobin Fe^{2+} to Fe^{3+} plays a significant role in plague pathogenesis was found when methylene blue, which blocks this process, was used (see *Results*). Moreover, the multiple petechiae (on the skin and mucosa), haemorrhages or sometimes 'scarlet blood' [4] as well as a number of HRBC haemolysed both inside and outside the human bloodstream, or significant vascular lesions with generation of haemosiderin granules in vessels or in the

liver and spleen [4, 24], could be a result of damage to RBC only.

Thus, death in plague is, apparently, a result of progressive tissue hypoxia but not of defeat of phagocytes, because in the latter case symptoms of immunodeficiency should be found. However, immunodeficiency cannot take a fulminant fatal course which is characteristic of septicaemic or pneumonic forms of the disease [1, 4]. In the case of pneumonic plague or aerogenic challenge, the *Y. pestis* attacks the RBC in the alveolar vessels just when oxygenation of haemoglobin takes place. The damaged RBC do not transport O_2 to host tissue, resulting in severe hypoxia leading to death in 2–6 h [4]. With aerogenic challenge, the same process is probably developed.

Recently, Garcia *et al.* [22] showed that catalase is at least partially hydrolysed by Pla. However, inside the RBC anti-fibrinolysin was revealed [25] which, probably, may completely inhibit the *Y. pestis* fibrinolytic activity. In the current study ϵ -aca was employed successfully for this aim (Table 2), likewise in the experiments of Beesley *et al.* [13]. This observation is one more piece of evidence that *Y. pestis* possesses a special regulatory mechanism of blocking its PAA similar to that when bacteria reside within the phagolysosome [5]. This phenomenon allows *Y. pestis* to synthesise a set of specific proteins encoded mainly by chromosomal genes although a few pLcr-encoded polypeptides were also revealed (Fig. 2). Based on mass comparison they were, probably, proteins orfs 42, 44 and LcrG, respectively, described by Perry *et al.* [2], and which were not released by *Y. pestis* within the macrophage [5]. These data make us suggest that some Yops, especially, those involved in destroying actin microfilaments, or in translocation of bacteria through the RBC outer membrane, may be secreted within the RBC because of its Ca^{2+} deficiency [16, 20]. It is quite possible, because RBC were more deformed when *Y. pestis* strains bearing pLcr were used (Fig. 1). On the other hand, due to high homology of some components of FI, capsular antigen and catalase (KatP) to *Vibrio cholerae* [26] or enterohaemorrhagic *Escherichia coli* [22] haemolysin, respectively, they could promote *Y. pestis* translocation through the RBC membrane. The identification in *catY* of a putative Ca^{2+} binding site [22] strongly suggests the possibility of *Y. pestis* interaction with the RBC membrane because Ca^{2+} is present there but not intracellularly in mammals [16]. *Y. pestis* is able to produce some effector proteins involved in realisation of this probable mechanism.

Interestingly, *Y. pestis* produced S- but not R- or SR-type LPS when bacteria were grown in the medium simulating intracellular conditions of the mammalian RBC (Fig. 3). SDS-PAGE showed regular lengthening of carbohydrate components of *Y. pestis* EV-76 and its isogenic derivatives. This phenomenon did not occur in bacteria grown in conditions simulating the

phagolysosomal conditions [5]. In contrast, in the latter case the *Y. pestis* O-side chains became shorter [5]. In light of the observations that almost all gram-negative bacteria are virulent when they produce S- but not R-type LPS [15], the ability of *Y. pestis* to synthesise S-type LPS inside the RBC probably indicates that the mammalian RBC is the target cell for plague bacilli.

The question remains as to whether *Y. pestis* uses haemin only as an iron source or as a source of porphyrin as well, which is necessary for *de-novo* synthesis of purines by these bacteria. The last supposition seems quite possible because host tissues do not contain enough free purines to support growth of *Y. pestis* *in vivo* and the haemin utilisation system but not an iron only utilisation system was found in *Y. pestis* [1, 3, 4, 17, 18]. These findings, apparently, are consistent with the ability of *Y. pestis* to adsorb large amounts of exogenous haemin that cause formation of dark brown or 'pigmented' colonies [1, 3, 4, 19] due to the oxidation of any haem-containing proteins in the presence of O₂ [16]. Interestingly, the 'active-site motif' involved in haem binding was recently revealed in *Y. pestis* catalase (*catY*) produced mainly at 37°C [22]. Taken together these data indicate that this antigen is somehow involved in haemin utilisation by *Y. pestis* at 37°C, i.e., *in vivo*, resulting in the formation of non-pigmented colonies *in vitro*. It is quite possible, because Pgm⁻ *Y. pestis* strain EV-76 possessing catalase activity [9] showed similar biological activities to Pgm⁺ strain 231 (Tables 2 and 3, Fig. 1c and e). These observations strongly agree with some reports that under certain conditions the *Y. pestis* vaccine strain EV-76 is able to regain virulence [8, 27], becoming a low-virulent or high-virulent *Y. pestis* strain but not absolutely avirulent. Thus, *Y. pestis* probably possesses a complicated mechanism permitting these bacteria to use haemin as a significant source of essential nutrients that permits their survival and effective gross multiplication in host mammals. This phenomenon is, apparently, common for all plague bacilli because it was observed in *Y. pestis* strains independently of their plasmid composition including the plasmidless Pgm⁺ strain PKR-133 (Tables 2 and 3). That is why probably high concentration of *Y. pestis* PKR-133 bacteria caused experimental plague in mice or guinea-pigs, although being pLcr⁻ they were easily ingested by phagocytes (data not shown).

Finally, the data obtained led us to the conclusion that the mammalian RBC but not the phagocyte is a target cell for *Y. pestis*. In contrast, plague bacilli defend themselves from the bactericidal systems of phagocytes, destroy them and become resistant to phagocytosis during a short period *in vivo*, to effectively overcome the host defence system but invade RBCs as a main source of nutrients and a means of spread in the bloodstream of mammals.

We thank Dr N. Ye. Teryoshkina for help in preparing the manuscript. We are grateful to Mrs M. N. Islyayeva and Mrs Ye. V. Mitina for excellent technical assistance.

References

- Perry RD, Fetherston JD. *Yersinia pestis* – etiologic agent of plague. *Clin Microbiol Rev* 1997; **10**: 35–66.
- Perry RD, Straley SC, Fetherston JD, Rose DJ, Gregor J, Blattner FR. DNA sequencing and analysis of the low-Ca²⁺-response plasmid pCD1 of *Yersinia pestis* KIM5. *Infect Immun* 1998; **66**: 4611–4623.
- Brubaker RR. *Yersinia pestis* and bubonic plague. In: Dworkin M (ed) *The prokaryotes*. Michigan State University. 2000.
- Domaradskii IV. Plague. *Medicina* (Moscow). 1998: 50–106.
- Feodorova VA, Devdariani ZL. Expression of acid-stable proteins and modified lipopolysaccharide of *Yersinia pestis* in acidic growth medium. *J Med Microbiol* 2001; **50**: 979–985.
- Burrows TW, Bacon GA. The basis of virulence in *Pasteurella pestis*: the development of resistance to phagocytosis *in vitro*. *Br J Exp Pathol* 1956; **37**: 286–299.
- Kravtsov AL. Microfluometric examination in the flow of heterogenic bacterial populations of *Yersinia pestis* and immune system cells of plague-infected mammals. DSci Thesis, Russia State Anti plague Research Institute 'Microbe', Saratov, Russia, 1997.
- Meyer KF. Immunity in plague: a critical consideration of some recent studies. *J Immunol* 1950; **64**: 139–163.
- Burrows TW, Farrell JMF, Gillett WA. The catalase activities of *Pasteurella pestis* and other bacteria. *Br J Exp Pathol* 1964; **45**: 579–588.
- Feodorova VA, Devdariani ZL. Immunogeneity and structural organisation of some pLcr-encoded proteins of *Yersinia pestis*. *J Med Microbiol* 2001; **50**: 13–22.
- Ronin BS, Starobinets GM. Manual for experimental studies of methods of clinical laboratory tests. *Medicina* (Moscow) 1989: 25–30.
- Hayhoe FGJ, Quaglini D (eds). *Haematological cytochemistry*. *Medicina* (Moscow) 1983: 56. [Translated from Hayhoe FGJ, Quaglini D. *Haematological cytochemistry*. Edinburgh, Churchill Livingstone. 1980].
- Beesley ED, Brubaker RR, Janssen WA, Surgalla MJ. Pesticins. III. Expression of coagulase and mechanism of fibrinolysis. *J Bacteriol* 1967; **94**: 19–26.
- Tsai C-M, Frasch CF. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* 1982; **119**: 115–119.
- Knirel YuA, Kochetkov NK. [Structure of lipopolysaccharides from gram-negative bacteria. 1. Common characteristics of lipopolysaccharides and lipid A structure (Review).] *Biokhimiia* 1993; **58**: 166–181.
- Metzler DE (ed). *Biochemistry: the chemical reactions of living cells*. *Mir* (Moscow) 1980: 361–370. [Translated from Metzler DE. *Biochemistry: the chemical reactions of living cells*. New York, Academic Press. 1977.]
- Hornung JM, Jones HA, Perry RD. The *hmu* locus of *Yersinia pestis* is essential for utilization of free haemin and haem-protein complexes as iron sources. *Mol Microbiol* 1996; **20**: 725–739.
- Thompson JM, Jones HA, Perry RD. Molecular characterization of the hemin uptake locus (*hmu*) from *Yersinia pestis* and analysis of *hmu* mutants for hemin and hemoprotein utilization. *Infect Immun* 1999; **67**: 3879–3892.
- Jackson S, Burrows TW. The pigmentation of *Pasteurella pestis* on a defined medium containing haemin. *Br J Exp Pathol* 1956; **37**: 570–576.
- Beutler E. Hemolytic anemia in disorders of red cell metabolism. Moscow, *Medicina* (Moscow) 1981: 13–128. [Translated from Beutler E. *Hemolytic anemia in disorders of red cell metabolism*. (Topics in Hematology ser.) New York, Plenum Medical Book Co. 1978].
- Komarov FI (ed). *Manual of a physician*. Minsk, USSR. 1983: 466–482.
- Garcia E, Nedialkov YA, Elliott J, Motin VL, Brubaker RR. Molecular characterization of KatY (Antigen 5), a thermo-regulated chromosomally encoded catalase-peroxidase of *Yersinia pestis*. *J Bacteriol* 1999; **181**: 3114–3122.
- Lal AK, Ansari NH, Awasthi YC, Snyder LM, Fortier NL,

- Srivastava SK. Defense of mouse red blood cells against oxidative damage by phenylhydrazine. Glutathione peroxidase and catalase deficiency. *J Lab Clin Med* 1980; **95**: 536–552.
24. Lobanov VN. The pathological anatomy and plague pathogenesis. Moscow, State Press of Medical Literature. 1956: 31–56.
25. von Bayerle H, Kammenhuber K. Über Fermenteffektoren der Fibrinolyse. II. Mitteilung die Bedeutung der Erythrozyten für die Fibrinolyse. *Blut* 1958; **4**: 78–85.
26. Denesyuk AI, Zav'yalov VP, Abramov VM, Zav'yalov VP. The computer analysis of structure and immunological activities of *Yersinia pestis* FI. Abstracts of the Scientific Conference on Prophylaxis of Dangerous Infections, Kirov, Russia, March 26–28, 1991: 175–176.
27. Anisimov PI, Anisimova TI, Aparin GP *et al.* Changes of *Y. pestis* EV vaccine strain properties after passages in the organisms of pikas. Problems of particularly dangerous diseases, vol 5. Saratov, Russia. 1969: 68–74.