INTRODUCTION

Yersinia pestis employs a variety of mechanisms to evade or overcome the host immune system and to establish infection. These include strategies for resisting killing by phagocytes. Two main types of resistance to phagocytosis after entry into the bloodstream of plague-sensitive mammals have been found in Y. pestis. The first, the intracellular type, enables the survival of virulent bacteria within phagolysosomes inside phagocytes (Straley & Harmon, 1984). The second, the extracellular type, demonstrated previously by Burrows & Bacon (1956a, b), develops after a short period (3–5 h) in vivo or under in vitro conditions imitating those in vivo but independently of bacteria–host-phagocyte interactions and results in Y. pestis bacteria not being recognized, captured and killed by human phagocytes. The first mechanism is well studied and is expressed by the yop regulon (Brubaker, 2000; Domaradskii, 1998). Less is known of the second mechanism; it has only been reported to occur in Y. pestis, mainly in the absence of visible capsulation (Burrows & Bacon, 1956a, b; Cavanaugh & Randall, 1959), and may be a result of changes in the production of principal surface antigens, especially fraction 1 (F1).

We decided to reproduce the in vitro conditions in which the development of phagocytosis-resistance in Y. pestis was observed, using Hottinger broth (HB) and culture medium RPMI 1640 (RPMI) containing calcium and glucose in appropriate concentrations. The results showed that Y. pestis in the resistant state does not produce detectable amounts of either species-specific antigens (F1, ‘murine’ toxin or Ymt, plasminogen activator (Pla) and any surface-specific polysaccharides, resulting in unmasking of the cross-reactive epitopes of lipid A in reduced Y. pestis lipopolysaccharide. This may produce mimicry by Y. pestis of some human tissue and blood cell components, with no immune response and inflammation at the site of infection at the early stage, which enables Y. pestis to survive, extensively multiply and spread into the circulation.

METHODS

Bacterial strains and culture conditions. The vaccine strain Y. pestis EV-76 (Pgm−), its plasmidless derivative KM-218 (Pgm−) and fully virulent Y. pestis 231 (Pgm+) strains have been described previously (Feodorova & Devdariani, 2002). The strains were cultured either in RPMI, pH 7.2, containing 2 mM glutamine, 2.2 mM sodium bicarbonate and 20 mM HEPES, or in HB, pH 7.2. The media were supplemented with 2.5 mM CaCl2 or 5.5 mM glucose or both and cultured at 28°C for 24 h and then at 37°C for 24 h. For controls the same strains were cultured in the same media without any supplements. After cultivation, the bacteria were harvested and washed twice with PBS, pH 7.2. Viable cell yields were determined by turbidity measure-

Abbreviations: Pla, plasminogen activator; PLD, phospholipase D; PK, proteinase K; PPA, plague polyclonal antibody; PSD, prostaglandin; WCL, whole-cell lysate.
ments and by standard plating techniques. Viable cells were counted and, after killing with sodium merthiolate at a concentration of 1:10,000 or with 2 % formalin, used for dot-ELISA.

Plasminogen activator activity testing. Y. pestis plasminogen activator activity was assayed according to Reesley et al. (1967) or in dot-ELISA with mAbs to Pla (mAB-Pla) as described previously (Feodorova & Devdariani, 2002).

Defining of bacterial capsule. To study the ability of the Y. pestis strains to form a capsule, the bacteria were stained with India ink as described elsewhere (Burrows & Bacon, 1956b) and examined by electron microscopy. Briefly, bacteria were observed with a JEOL model JSM-U3 scanning microscope. Test samples of bacterial suspensions were placed on carbon-coated collodion grids and then treated and negatively stained as described elsewhere (McNab et al., 1999).

Determination of phospholipase D activity. Y. pestis phospholipase D (PLD) activity was determined by the method of Kouzmitchenko & Drozdovskaya (1977). Briefly, 0-1 ml suspensions of 1 X 10⁸ Y. pestis cells in 1 ml of one of the growth media described above, with or without supplements, were incubated with 0-05 ml yolk emulsion 10 % in saline, 0-13 ml Tris/HCl 0-05 M, 0-02 ml CaCl₂ 2-2 % in saline, 0-35 ml SDS 0-01 % and 0-05 ml diethyl ether at 37 °C for 4 h. Then, 0-1 ml trichloroacetic acid 20 % was added to precipitate any unbound lecithin. Slide smears of supernates with Florence reagent (KJ 50 % v/v and iodine crystalline 56 % w/v in distilled water) were prepared and examined visually for the formation of choline periodide crystals under a BioLam R-15 microscope (Lomo, Russia).

ELISA. Indirect dot-ELISA was used for studying the ability of all Y. pestis strains to produce F1 antigen. Briefly, several drops of killed bacterial suspension were placed onto nitrocellulose membranes, pore size 22 μm (Schlescher & Schuell), and fixed at 110 °C for 10 min. After washing three times in PBS and blocking with 3 % skimmed milk for 15 min, the nitrocellulose membranes were incubated for 15 min with mAbs to F1 (mAB-F1) or plague polyclonal antibodies (PPAs) (Devdariani et al., 1993). Peroxidase-labelled anti-mouse or anti-horse IgG (Gamaley Institute, Russia) were employed as conjugates. O-Dianisidine (Sigma) was used as a substrate. Finally, the nitrocellulose membranes were dried and observed for colour differentiation.

SDS-PAGE. Proteinase K-treated (PK-treated) whole-cell lysates (WCLs) of Y. pestis strains EV-76, KM-218 and 231 were subjected to SDS-PAGE as described previously (Feodorova & Devdariani, 2002), with a stacking 4 % gel and separating 12.5 % gel. Electrophoresis was done at 35 mA constant current in 25-192 mM Tris/glycine (pH 8-3) plus 0-1 % SDS buffer for approximately 2-5 h. A set of low-molecular-mass markers (Sigma) was used. The gels were stained according to Tsai & Frasch (1982).

RESULTS AND DISCUSSION

According to Burrows & Bacon (1956a, b), virulent Y. pestis organisms acquire resistance to phagocytosis and are not recognized by mammalian phagocytes in vivo and in vitro at 37 °C under defined conditions. Glucose was found to be critical for the phenomenon to develop in vitro (Burrows & Bacon, 1956a). On the other hand, the presence of calcium ions in culture media might favour the maintenance of virulence and active growth of virulent Y. pestis strains (Higuchi et al., 1959). Taken together, these conditions correspond to those in the bloodstream of mammals (Higuchi et al., 1959), where Y. pestis extensively multiplies and spreads to the liver, spleen and other organs, resulting in septicemia (Brubaker, 2000; Domaradskii, 1998).

To simulate the bloodstream environment in this study, two standard basal media were used. One of them was HB, which is routinely used for growth of Y. pestis in vitro (Devdariani et al., 1993; Feodorova & Devdariani, 2002), and the other was RPMI 1640, which simulates the human extracellular environment (Fasthman & Fitch, 1984). Calcium and glucose were added to the growth media in concentrations similar to those in the human bloodstream. To study the influence of each component on the proliferative activity of the Y. pestis strains, calcium and glucose were also tested independently, at the same concentrations.

Behaviour of Y. pestis in non-supplemented growth media

Firstly, the behaviour of the vaccine strain and fully virulent Y. pestis bacteria in HB and RPMI without supplements was compared. As shown in Table 1, both of the Y. pestis strains grew significantly better in RPMI than in HB. However, the bacterial yield of the fully virulent Y. pestis strain was apparently higher in comparison with the vaccine strain. Definite growth restriction was found when strain 231 was cultured in HB.

Phenotypic behaviour in supplemented growth media

The combined addition of calcium and glucose to the growth media resulted in a greater increase in bacterial yield of strain EV-76 than of strain 231 in both media. No significant effect of the supplements on bacterial proliferation of strain 231 in RPMI was observed (P > 0.05). Both strains grew better on HB with glucose alone than HB without glucose; however, the addition of glucose to RPMI had no significant effect upon the growth of either strain (P > 0.05). The same results were obtained when calcium alone was added to the growth media. No significant change of media pH was found after cultivation of Y. pestis in any experiment.

As expected, RPMI was found to be more suitable than HB for imitating bloodstream conditions. This was due to its balanced composition, including adequate concentrations of calcium and glucose as well as most nutrients probably necessary for Y. pestis growth at 37 °C, for instance cystine (Burrows & Bacon, 1956a). HB permitted less bacterial growth even when it was enriched with the same concentrations of calcium and glucose. However, both components were essential for bacterial proliferation at 37 °C because each of them exerted a significant stimulatory effect (calcium to a higher degree and glucose to a lesser degree) on Y. pestis growth in HB (Table 1).

It is already well-established that calcium is metabolized by yersiniae (Perry & Brubaker, 1987). Apparently, calcium-specific ion channels of the Y. pestis bacterial membrane open in media containing calcium in concentrations similar to that...
Table 1. Effect of the cultivation conditions on the proliferative and main phenotypic characteristics of *Y. pestis* EV-76 and 231 strains

Symbols: +, positive reaction; −, negative reaction; ±, trace reaction.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Basal medium</th>
<th>Supplement*</th>
<th>Viable cell yield (no.)</th>
<th>Multiplication ratio</th>
<th>Capsule production activity</th>
<th>PLD activity</th>
<th>Plasminogen activator activity</th>
<th>mAb-F1</th>
<th>mAb-Pla</th>
<th>PPA</th>
<th>mAb-LPS</th>
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<tr>
<td></td>
<td>Calcium</td>
<td>Glucose</td>
<td>10⁻⁹</td>
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<td>−</td>
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<td>Clot</td>
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*The concentration of each of the components is indicated in Methods.
in the mammalian bloodstream environment (Metzler, 1980), resulting in the influx of calcium from extracellular sources into the bacterial cells and then, via some consequent cascade reactions, stimulation of DNA biosynthesis and bacterial proliferation (Janeway & Travers, 1997). Glucose is reported to be the main carbohydrate source for actively growing Y. pestis because only in its presence do both the Embden-Meyerhof scheme and the hexose monophosphate shunt pathway operate (Santer & Ajl, 1955). Indeed, uptake of glucose by Y. pestis occurred more effectively when it was added to the media in combination with calcium, which obviously stimulated the bacterial growth, i.e. giving actively growing cells, and in this case exhaustion of glucose during the incubation period was observed as described previously (Burrows & Bacon, 1956a). In contrast, addition of glucose alone in HB with no added calcium, i.e. to ‘resting’ cells (not stimulated by calcium), when it can be fermented almost exclusively via the Embden-Meyerhof scheme, resulted in a weak increase in bacterial yield.

The data obtained support the hypothesis that an adequate concentration of calcium in the mammalian bloodstream initiates the operation of an alternative pathway of dissimilation of glucose, enabling effective multiplication of Y. pestis in vivo at the extracellular stage. It is likely that these observations are correlated with the mutation in the gene encoding a glucose-6-phosphate dehydrogenase (Brubaker, 2000), the main enzyme of the pathway, during the divergence of Y. pestis from Yersinia pseudotuberculosis, resulting in the production of only two isoforms of glucose-6-phosphate dehydrogenase by Y. pestis, in contrast to three in Y. pseudotuberculosis (Kouzmitchenko & Naumov, 1989), and in small or undetectable amounts of this enzyme in routine media (Brubaker, 2000; Kouzmitchenko & Naumov, 1989; Santer & Ajl, 1955). Strong evidence for Y. pestis retaining production of the enzyme in this case was the presence of the enzyme intracellularly in dried bacterial cells (Kouzmitchenko & Naumov, 1989; Santer & Ajl, 1955). We believe that this reflects adaptation of Y. pestis to the bloodstream environment abundant in glucose because a direct correlation between active metabolism of the enzyme and the development of resistance to phagocytosis has been found (Burrows & Bacon, 1956a).

Surface antigen modifications in supplemented growth media

If Y. pestis bacteria are not recognized by mammalian phagocytes as ‘foreign’ substances, these organisms probably do not produce species-specific antigens in the bloodstream, providing host immunological tolerance, as was observed by FACS-analysis in both strain EV-76 and strain 231 isolated after a contact with human phagocytes (Kravtsov, 1997) as well as microscopically after their combined cultivation in RPMI (data not shown). This seems logical because Y. pestis bacteria in a resistant state, both in vitro and in vivo, have no visible capsule and do not produce detectable amounts of F1 (Burrows & Bacon, 1956a; Cavanaugh & Randall, 1959). This phenomenon can be produced by the presence of a glucose concentration similar to that in the bloodstream, which was earlier shown to inhibit production of the Y. pestis F1 in vitro (Veinblat & Borisova, 1968). This antigen together with PLD, previously characterized as ‘murine’ toxin (Ymt), and Pla are known as the main surface species-specific antigens synthesized by Y. pestis at 37°C and are encoded by two plasmids, pFra and pPst (Brubaker, 2000; Domaradskii, 1998; Rudolph et al., 1999). Therefore the production of each of them was carefully checked in this study in several independent tests.

We found that Y. pestis EV-76 and 231 strains grown in similar conditions both demonstrated the same changes in phenotypic properties and immunoreactivity, as shown in Table 1. These data were confirmed in dot-ELISA with commercial PPAs containing antibodies to F1, Pla and Ymt (Devdariani et al., 1993; Domaradskii, 1998). Thus, the reaction of PPAs was positive with the bacteria grown in HB with or without calcium or glucose, and negative with those cultured in RPMI with and without supplements. Consequently, the Y. pestis bacteria in the resistant state were not identified by commercial PPAs and no F1, PLD and Pla in detectable amounts were found in either the vaccine or the virulent Y. pestis strain cultured under in vitro conditions imitating the bloodstream environment.

Polysaccharide modifications in supplemented growth media

Using mAbs and polyclonal antisera on Y. pestis, species-specific epitopes have been found on core and some surface-specific polysaccharides (Bakhrakh & Veinblat, 1972; Feodorova et al., 2004; Feodorova & Devdariani, 1998; Prior & Titball, 2002), i.e. truncated O-antigen (Bakhrakh & Veinblat, 1972), enterobacterial common antigen (ECA) (Vinogradov et al., 1994) and capsular polysaccharide (Glosnicka & Gruszkiwicz, 1980), which can be produced in defined conditions in culture media (Bakhrakh & Veinblat, 1972; Feodorova & Devdariani, 2002; Veinblat & Borisova, 1968). Although genetic control of the polysaccharides has not been well-studied at present, the relevant preparations have been characterized biochemically in previous reports (Bakhrakh & Veinblat, 1972; Vinogradov et al., 1994), and PK-treated Y. pestis WCL seems to be indistinguishable from S-LPS in SDS-PAGE (Dodgson et al., 1996). That is why we analysed both specificity in dot-ELISA and modification in SDS-PAGE of PK-treated WCL in the Y. pestis strains grown in conditions simulating the mammalian bloodstream. For this, we used the mAb-LPS that recognizes the same epitope located on lipid A/core/O-antigen/ECA (Feodorova & Devdariani, 1998) and is synthesized independently of cultivation temperature by all virulent and vaccine Y. pestis strains (Devdariani et al., 1993). We tested in dot-ELISA both the Y. pestis microbial cells and the culture fluids.

The bacteria grown in HB with or without supplements produced in abundance outer-membrane-bound components of LPS, while only trace amounts of these were present on the bacterial surface of the strains grown in RPMI.
irrespective of supplements (Table 1). Addition of glucose did not affect the production of any polysaccharides in the strain EV-76 grown in HB; however, calcium alone or in combination with glucose significantly reduced their production in EV-76 but not in strain 231. In RPMI, with or without supplements, neither strain produced detectable polysaccharides in the culture medium. These results correlated with the subsequent examination of the Y. pestis LPS profiles, which apparently differed from one another depending on the cultivation conditions of the bacteria. So, the predominant lower-molecular-mass component, several major bands of intermediate-molecular-mass and a single higher-molecular-mass one corresponding to lipid A/core region and any specific polysaccharides (Dodgson et al., 1996; Skurnik & Bengoechea, 2003; Whitfield et al., 1997), were seen in LPS profiles of the bacteria cultured in HB (Fig. 1, lane 1). Four large bands of lower- and intermediate-molecular-mass were visualized when the bacteria were cultured in HB with added calcium (Fig. 1, lane 3), suggesting that the concentration of calcium might modulate the production of Y. pestis-specific polysaccharides as reported for other pathogenic yersiniae (Bengoechea, 2002). Remarkably, when cultured in RPMI, the bacteria of the strain EV-76 had a significantly reduced LPS profile represented by a single lower-molecular-mass component of smaller size and less intensively stained (Fig. 1, lane 2), indicating that Y. pestis produced less core/lipid A and, probably, phospholipids which typically migrate as the fast-moving broad band at the bottom of the gel similar to Gram-negative bacteria (Barr et al., 1999) and, probably, no other surface specific polysaccharides as shown here (Fig. 1, lanes 2, 5). The same tendency was revealed in the strains 231 and plasmidless KM-218 (Fig. 1, lanes 4–6) supposing possible repression mainly of chromosomal genes/clusters controlled by PhoPQ two component signal transduction system (Oyston et al., 2000). On the other hand, based on heterogeneity of the population of LPS molecules varying in the O-antigen chain lengths, including those lacking O-antigen (Skurnik & Bengoechea, 2003) and, probably, other surface specific polysaccharides (Metzler, 1980; Whitfield et al., 1997), the bacteria producing reduced LPS have a survival advantage in conditions simulating the bloodstream environment.

Apparently, the reduction of Y. pestis LPS molecule up to the inner core and/or the lipid A structurally and genetically highly conserved between bacterial species (Skurnik & Bengoechea, 2003; Whitfield et al., 1997) can unmask mainly cross-reactive epitopes common with surface receptors of the outer cell membranes of phylogenetically diverse eukaryotic cells, human B lymphocytes, macrophages (phagocytes), red blood cells, endothelial cells of mammals, etc. (Raetz & Whitfield, 2002). Taken together with the production by Y. pestis of some heterogeneous substances serologically related to above-mentioned antigens, and inability to synthesize other species-specific antigens (i.e. F1, Pla, Ymt), this can, probably, provide mimicry of Y. pestis to some human tissues and blood cells (Zhukov-Verezhnikov et al., 1972) resulting in the absence of immune response and inflammation in the site of infection at the early stage of the disease (Domaradskii, 1998). The resistant Y. pestis bacteria were more round than those grown in routine conditions and demonstrated a significantly thinner envelope with alteration of the production of capsular substance and LPS architecture. These findings are in agreement with earlier reports that Y. pestis bacteria in the resistant state were very poor antigenically (Burrows & Bacon, 1956b) and that virulent yersinia survived and disseminated via the bloodstream regardless of LPS-phenotype, including deep R-mutant (Najdenski et al., 2003), strongly indicating the critical role of lack of O-antigen as well as of other surface polysaccharides for this phenomenon. These changes affected the expression of other yersinia virulence factors (Bengoechea et al., 2004) responsible for invasiveness of Y. pestis, i.e. (i) activated Pla-mediated adhesion and invasion into human endothelial cells with no proteolysis (Kuukkonen et al., 2004); (ii) increased expression and secretion of phospholipase A (Bengoechea et al., 2004) which hydrolyses phosphatidylcholine and sphingomyelin, the essential phospholipid components of eukaryotic cell membranes (Kramer & Sharp, 1995; Rudolph et al., 1999); (iii) unmasked the lipid A epitopes of reduced Y. pestis which were found to be identical to those of surface I or i antigens of an adult and cord mammalian red blood cells (Bhat et al., 1993), allowing Y. pestis bacteria to bind and invade them for use as a main source of nutrients in vivo (Feodorova & Devdariani, 2002).

**Conclusion**

Possibly, the absence of bacterium–host phagocyte cell interaction can last a long period, at least, not less than 17-hours cultivation in vitro or 24 h after Y. pestis enters the bloodstream (Burrows & Bacon, 1956a, b), resulting in two main scenarios: (i) the host immune system is unable to cope with large numbers of multiplying Y. pestis cells, even if the biosynthesis of some species-specific antigens including F1 is

![Fig. 1. SDS-PAGE of PK-digested whole-cell lysates of Y. pestis EV-76 (lanes 1–3) and KM-218 (lanes 4–6) grown in HB only (lanes 1, 4) or in HB supplemented with calcium (lanes 3, 6) or RPMI (lanes 2, 5).](http://jmm.sgmjournals.org)
restored later; or (ii) in plague patients, fatalities from septicaemia or septic shock mediated by Y. pestis lipid A accompanied by progressive tissue hypoxia (Domaradskii, 1998; Feodorova & Devdariani, 2002; Raetz & Whitfield, 2002) occur, probably, before host innate immune response can be induced (Janeway & Travers, 1997). From our point of view both could be possible.

In summary, our data led us to the conclusion that Y. pestis bacteria develop capsule-independent type of resistance to phagocytosis under the defined conditions simulating the bloodstream of mammals, probably, in the early stage of plague infection due to the capability of Y. pestis bacteria to down-regulate species-specific antigens that enable the survival, extensive multiplication and spread of Y. pestis into the circulation while staying predominantly extracellularly during infection (Burrows & Bacon, 1956a, b; Domaradskii, 1998). These findings may evidence the high environmental adaptability of Y. pestis bacteria during their life cycle, due to fluidity of their outer membrane modification in production of surface antigens.

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