Invasive Characteristics of Apathogenic *Shigella flexneri* 5a2c Mutant Obtained under the Effect of Furazolidone

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Apathogenic *Shigella flexneri* 5a2c mutant treated with furazolidone can infect eucaryotic cells. These bacteria contain no virulence genes responsible for *Sh. flexneri* invasion, which seems to be the cause of their apathogenicity. The capacity of bacteria to penetrate into eucaryotic cells correlates with the appearance of ECP 32 protease specifically cleaving actin.

Key Words: *bacterial invasion; Shigella flexneri L forms; ECP 32 actin-specific protease*

Pathogenicity of many bacteria, including *Shigella flexneri*, is based on their capacity to penetrate into eucaryotic cells and utilize host cell cytoskeleton proteins for construction of microfilament structures ("comet-like tails") providing bacterial movement inside the cell and from cell to cell and thus promoting propagation of infection [9,11,13,15]. This capacity is determined by the cluster of virulence genes situated in the plasmid [5,11]. Incorporation of virulence genes transforms apathogenic bacteria into pathogenic [12]. On the other hand, loss of pathogenicity can result from modification or loss of virulence genes [10,14]. This phenomenon underlies the therapeutic effects of many drugs used in the treatment of dysentery. It was previously shown that single treatment with furazolidone (nitrofuran drug) leads to the formation of *Sh. flexneri* L forms, whose revertants are not pathogenic for mice upon intraperitoneal infection and for guinea pigs in the Cherene test [3]. The aim of this study was to clear out whether apathogenic *Sh. flexneri* mutants obtained after furazolidone treatment contain known virulence genes of *Sh. flexneri* and whether these bacteria retain their invasive potential.

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MATERIALS AND METHODS

The study was carried out on two apathogenic *Sh. flexneri* strains. *Sh. flexneri* strain 2a 4115 was isolated from a patient with dysentery as pathogenic [3] and after many-year culturing under laboratory conditions it lost its pathogenicity [4]. *Sh. flexneri* strain 5a2c was obtained after single treatment of pathogenic *Sh. flexneri* 2a 4115 with furazolidone (0.05 μg/ml in 2% serum-saline nutrient agar) at 28°C [3]. Pathogenic *Sh. flexneri* freshly isolated from a patient with dysentery was also used in the study. The bacteria were cultured in LB medium for 24-28 h at 37°C without aeration. Hep-2 laryngeal epidermoid carcinoma cells (from Russian Collection of Cell Cultures, Institute of Cytology) were cultured in DME medium with 10% fetal calf serum for 2 days at 37°C and 5% CO_2 until monolayer formation.

In order to detect invasive activity, bacterial suspension in the growth medium was centrifuged at 10,000 rpm for 10 min. The precipitate was resuspended in DME medium and added to the cells after replacement of the medium. The cells and bacteria were co-cultured for 1 h at 37°C and 5% CO_2. Then the nutrient medium was replaced with medium containing gentamicin and the cells were further incubated for 4, 8, and 24 h after the start of the experiment.

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For visualization of microfilaments, the cells were washed in phosphate buffered saline, fixed in 3.7% formalin for 15 min, incubated for 20 min in 0.1% Triton X-100 solution, and stained with rhodamine-phalloidine. For electron microscopy the cells were processed routinely. After centrifugation of the growth medium cell precipitate was fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 40-60 min, washed in phosphate buffer, and postfixed in 1% osmic acid in the same buffer for 30 min. The cells were embedded in epon-araldite. Ultrathin sections were contrasted with 2% uranyl acetate in 50% ethanol and with lead citrate and examined under a JEM-100C electron microscope at 80 kV.

Proteolytic activity of bacterial extracts was evaluated by their capacity to cleave actin [8]. The cells were destroyed in 5-7 frosting—defrosting cycles. The extract was clarified by 20-min centrifugation at 15,000 rpm and added to actin solution (1 mg/ml). The mixture was incubated for 1 h at 20°C or 20 h at 4°C and analyzed by SDS-PAGE. Proteolytic activity was evaluated by the appearance of a 36 kDa actin fragment [8].

Total and plasmid bacterial DNA was isolated by the standard method [2]. The following primers were used for amplification of virulence genes: actcaagcgccaacattctt/tttggcatacgcattcattg (*ipaA*), tgacttccactgagcttgga/ttttgctgtcttgcctgttg (*ipaB*), tcccgatatttctttgcagg/cgcttgggatgcttctttag (*ipaC*), tgatggactttctcccttgg/ccgctaccaccaagaatcat (*icsA*). PCR was carried out in 20 μl reaction mixture containing PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5% Triton X-100, 1.5 mM $MgCl_2$), 25 mM deoxynucleotide triphosphatase, 30 pmol of each primer, matrix DNA, 5 U Taq-DNA polymerase (SibEnzyme), and mineral oil. The reaction consisted of 30 cycles: 3 min at 94°C, 1 min at 55-60°C (depending on the primer), and 1 min at 72°C. After the reaction the samples were separated by electrophoresis in 1% agarose gel in the presence of ethidium bromide. The gels were examined in UV and photographed.

RESULTS

Invasion of bacteria in eucaryotic cells includes 2 processes: penetration of bacteria into the cell and their propagation. The first process is associated with expression of *ipaA-C* genes [11-13], while the second process requires *icsA* gene-encoded protein initiating assembly of actin "tails" on the surface of bacteria [7]. Pairs of primers were constructed and PCR was carried out in order to detect these genes in *Sh. flexneri* strains 2a 4115 and 5a2c (Fig. 1). DNA of virulent *Sh. flexneri* strain isolated from a patient with dysentery served as the control. Freshly isolated *Sh. flexneri* strain, but not *Sh. flexneri* 2a 4115 and 5a2c strains contained *ipaA-C* and *icsA* genes (Fig. 1, *a*). Moreover, only *Sh. flexneri* strain isolated from a patient with dysentery contained the plasmid, which seemed to correspond to the virulence plasmid (Fig. 1, *b*).

Hence, *Sh. flexneri* 2a 4115 and 5a2c strains do not contain the genes, which are considered obligatory for *Sh. flexneri* invasion [10,13,15]. Therefore it could be expected that these bacteria cannot penetrate into eucaryotic cells. Indeed, incubation of Hep-2 cells with *Sh. flexneri* 2a 4115 strain did not change cell morphology or organization of their cytoskeleton (Fig. 2, *a*). Electron microscopy showed bacteria only in the intercellular space (Fig. 2, *c*). However, incubation of Hep-2 cells with *Sh. flexneri* 5a2c strain led to loosening of the cell monolayer and appearance of numerous protrusions on the cell surface and reorganization of the cytoskeleton (Fig. 2, *b*), which indicates interactions between the bacteria and the cell. Electron microscopy confirmed penetration of *Sh. flexneri* 5a2c strain into Hep-2 cells. Hence, these bacteria are capable of invasion despite the absence of virulence genes.

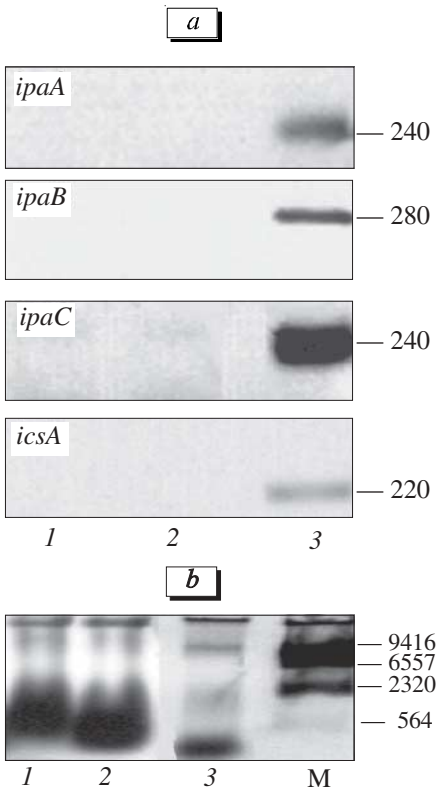

Fig. 1. Analysis of *Sh. flexneri* DNA. *a*) electrophoresis of PCR products with primers to *ipaA-C* and *icsA* genes. Total DNA isolated from *Sh. flexneri* 2a 4115 (*1*), 5a2c (*2*), and pathogenic strains (*3*) served as the matrix; *b*) electrophoresis of preparations obtained in isolation of plasmid DNA from *Sh. flexneri* 2a 4115 (*1*), 5a2c (*2*), and pathogenic strains (*3*). M: molecular weight marker λ/HindIII (Fermentas); the figures show the size of fragments in nucleotide pairs.

Only the bacteria collected during the stationary phase were capable of penetrating into the cells. We previously found that *Sh. flexneri* strain 5a2c produced ECP 32 protease specifically cleaving actin [4]. Proteolytic activity manifested in *Sh. flexneri* 5a2c bacteria at the stationary phase of culture growth (Fig. 3, *a*). Actin-cleaving protease was not detected in *Sh. flexneri* 2a 4115 strain (Fig. 3, *b*). These data suggest that the presence of actin-specific protease in the bacteria coincides with manifestation of invasive activity. Similar results were previously obtained in studies of the invasive characteristics of apathogenic *E. coli* A2 strain producing ECP 32 protease [1,6].

Our findings indicate that furazolidone treatment leads to the loss of virulence plasmid. Genes *ipaA-C* responsible for penetration of *Sh. flexneri* into eucaryotic cells are situated in the virulence plasmid [11-14]. Loss of the plasmid after furazolidone treatment led to the loss of these genes, which was confirmed by PCR analysis of total DNA isolated from the studied bacteria. *Sh. flexneri* 2a 4115 now does not contain the virulence plasmid, which seems to be due to long-term culturing of the strain under laboratory conditions [14]. However, in contrast to strain 2a 4115, *Sh. flexneri* strain 5a2c retained the capacity to penetrate into eucaryotic cells. This capacity correlates

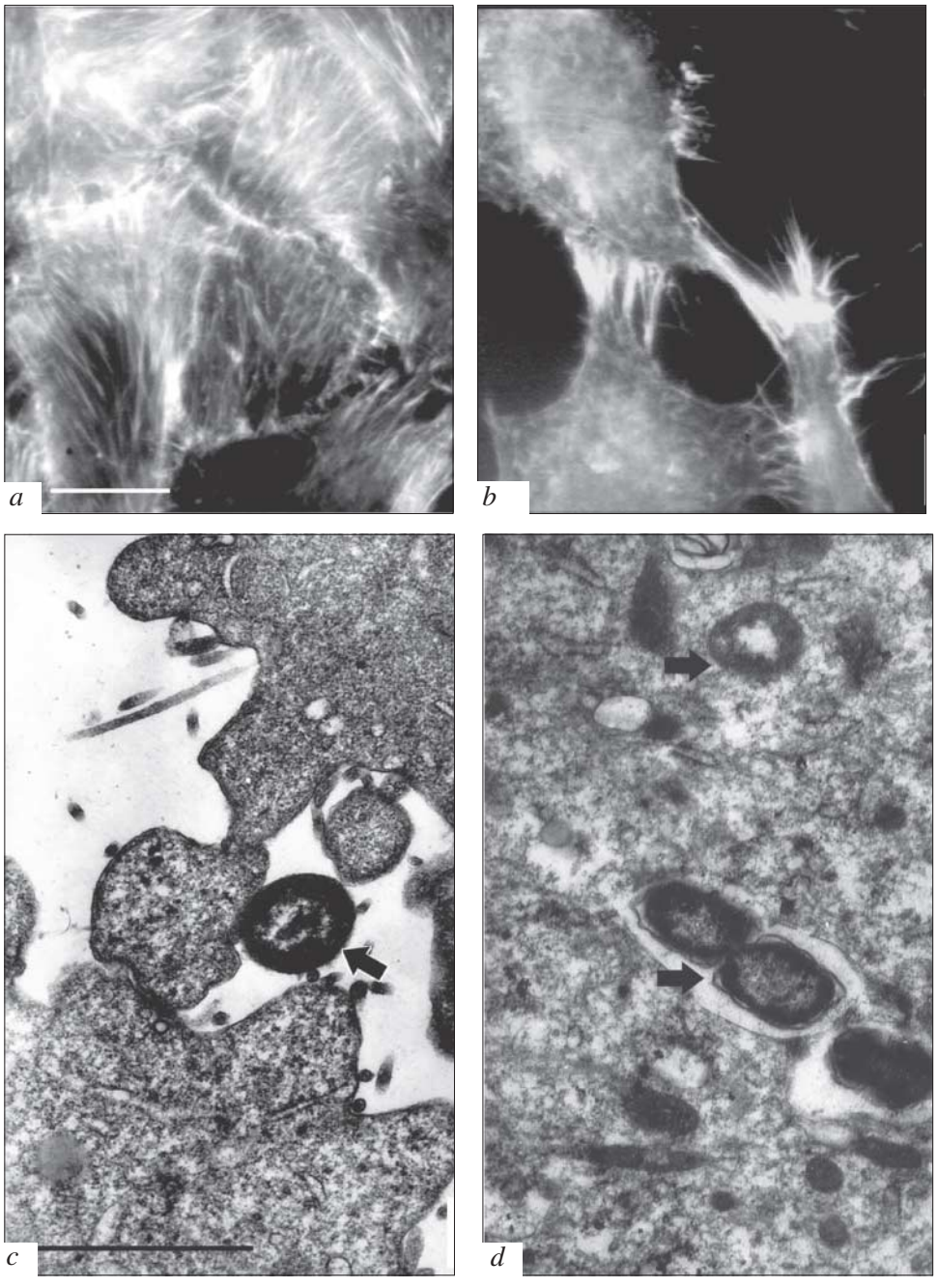

Fig. 2. Interactions of *Sh. flexneri* with Hep-2 laryngeal carcinoma cells. After incubation with *Sh. flexneri* 2a 4115 (*a*, *c*) or 5a2c (*b*, *d*) strains Hep-2 cells were stained with rhodamine-phalloidine (*a*, *b*: 9 μ line) or fixed for electron microscopy (*c*, *d*: 0.5 μ line).

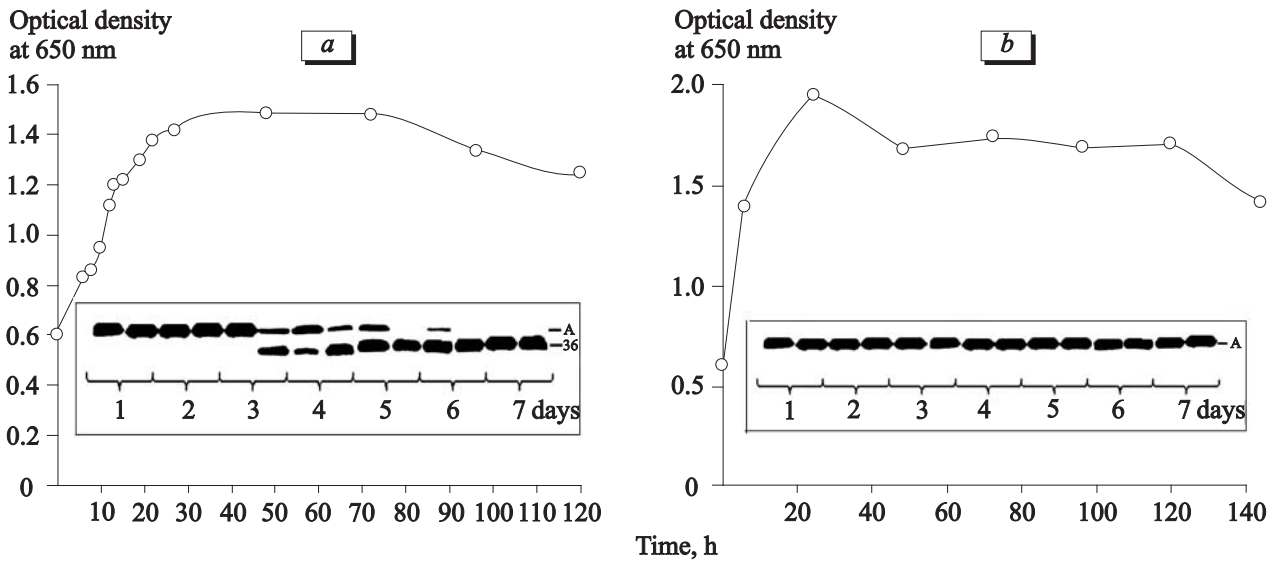

Fig. 3. Bacterial culture growth and proteolytic activity of *Sh. flexneri* strains 5a2c (*a*) and 2a 4115 (*b*). Proteolytic activity was evaluated by the capacity of bacterial extracts to cleave actin; 36: actin fragment with a molecular weight of 36 kDa.

with production of actin-specific ECP 32 protease, which is not synthesized by *Sh. flexneri* 2a 4115 strain. Moreover, we found no ECP 32 protease in intracellular extracts of freshly isolated *Sh. flexneri* from a patient with dysentery. We therefore hypothesize that penetration of *Sh. flexneri* 5a2c into the cells is associated with ECP 32 protease, which appears in the bacteria as a result of furazolidone-induced gene mutation or as a result of reversion of bacterial L forms.

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