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Enterocyte-like Caco-2 cells as a model for *in vitro* studies of diarrhoeagenic *Providencia alcalifaciens* invasion

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ABSTRACT

The entry of *Providencia alcalifaciens* into the enterocyte-like cell line Caco-2 compared to HEp-2 was studied. Of the 22 *P. alcalifaciens* strains, 13 and 21 were invasive for Caco-2 and HEp-2 cells, respectively. In contrast to HEp-2 cells, *P. alcalifaciens* was internalised by Caco-2 cells via receptor-mediated endocytosis. Tyrosine kinases play an important role in *P. alcalifaciens* uptake, also microfilaments and microtubules are engaged in this process. Inhibition of endosome acidification by ammonium chloride did not seem to have any significant effect on *P. alcalifaciens* invasion. Similarly to *Shigella flexnerii*, the invasion of Caco-2 cells by these bacteria occurred more effectively through the basolateral pole than through the apical surface of these cells. Plasmid DNA analysis showed the presence of plasmids of 5-172 kb in 13 strains regardless of their invasive ability. The presence of extracellular bacterial protein, most likely a kind of an invasin, is required for the invasion of Caco-2 and HEp-2 cells.

1. Introduction

Providencia alcalifaciens is a Gram-negative bacterium from the family *Enterobacteriaceae* and is thought to be a cause of diarrhoea in children and travellers returning from Mediterranean countries [1, 2]. The ability of *P. alcalifaciens* to cause diarrhoea was confirmed by Albert *et al.* [3] and Mathan *et al.* [4] with removable ileal ties of adult rabbit diarrhoea (RITARD) model. These studies showed that *P. alcalifaciens* penetrated the epithelial cells in two ways: one directly by endocytosis associated with polymerisation of cytoskeletal components and the second by disruption of tight junctions with the entry into and proliferation within intracellular spaces [4]. Moreover, Albert *et al.* demonstrated that two of three *P. alcalifaciens* strains studied were able to invade the HEp-2 cells and cause diarrhoea in infected rabbits. These authors showed no evidence for the presence of enterotoxin or cytotoxin in the culture supernatant and, consequently, suggested cells invasion as the main virulence mechanism of *P. alcalifaciens* [3, 5]. Other investigators have found that *P. alcalifaciens* strains isolated from diarrhoea were able to invade with different efficiency several cultured mammalian cells *in vitro* e.g. Vero, Y1, INT-407, HEp-2, HeLa, Caco-2 [5-9].

In the invasion of HEp-2 cells by *P. alcalifaciens* cellular microfilaments excluding microtubules are engaged [5]. Moreover, the invasion was not stopped by agents inhibiting receptor-mediated endocytosis, receptor recycling or endosomes acidification. However, the study of HEp-2 model of the invasion process does not show what takes place *in vivo*, because HEp-2 cells, which are routinely used, do not establish confluent polarized monolayers similar to intestinal epithelial cells. The polarized human colon carcinoma Caco-2 cell line, which has an apical surface, which is organized like a brush border, and is separated by intracellular junctions from the basolateral area having a different protein and lipid composition [10], seems to be more helpful for the understanding of the invasive phenomenon of *P. alcalifaciens*. Earlier an *in vitro* model of Caco-2 cells was used for the investigation of the invasive mechanism of a number of enteropathogens e.g. *Salmonella*, *Shigella*, *E. coli* or *Yersinia*. *Shigella* entry into Caco-2 cells occurred in the basolateral area only when the basolateral surface was exposed after the disruption of intracellular junctions by the treatment of cells with a Ca^{2+} chelator [11], while Salmonellae invaded Caco-2 cells through their apical surface [12].

The purpose of this work was to study in a cell assay model, more closely to *in vivo* situation, the invasion of *P. alcalifaciens* into polarized enterocyte-like cell line Caco-2 in comparison to HEp-2 cells. We determined the invasiveness and the dynamics of intracellular multiplication of *P. alcalifaciens* in human epithelial cells lines Caco-2 and HEp-2 and influence of some selected metabolic

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inhibitors on the invasion process, as well as plasmid and cellular protein profiles of bacteria.

2. Results

2.1. Entry of P. alcalifaciens into Caco-2 and HEp-2 cells

In the first stage of the investigation, the invasion of enterocyte-like cell line Caco-2 and HEp-2 cells was estimated. The results are shown in Table 1. The *P. alcalifaciens* strains demonstrated different invasive abilities and they were classified into three groups: highly invasive strains – above 0.1% of invasiveness, moderately invasive strains between 0.01 and 0.099% of invasiveness and non-invasive strains below 0,0099%. Based on preestablished criteria, three strains (13.6%) and 10 strains (45.5%) were found to be highly invasive into Caco-2 cells and HEp-2 cells, respectively. A similar percentage of strains 45.5% (10) and 50% (11) were moderately invasive to Caco-2 and HEp-2 cell lines, respectively. Consequently, 40.9% (9) and 4.5% (1) of *P. alcalifaciens* did not show an invasive ability to Caco-2 and HEp-2 lines, respectively. These findings indicated that invasiveness of *P. alcalifaciens* is strain and cell line type dependent. The positive control, *Salmonella* Toucra PMC 2359, was more invasive than *P. alcalifaciens* strains, and the negative control *E. coli* DH5α was not invasive to Caco-2 cells but, surprisingly, invasive to HEp-2 cells.

Microscopic examinations of crystal violet coloured Caco-2 and HEp-2 cells infected with selected *P. alcalifaciens* strains showed internalised bacteria in cellular cytosol or sometimes enclosed in vacuoles, similar to *Salmonella* Toucra PMC 2359 (Fig. 1.).

2.2. Dynamics of the adherence and invasion of P. alcalifaciens into Caco-2 and HEp-2

In the second stage of the study several strains with different phenotypes: highly invasive strains (35i/59, 101i/59, 352i/59 and 125), as well as non-invasive strains (208/60 and 3991) were chosen for the determination of the infection dynamics. The results are shown in Fig. 2. All six *P. alcalifaciens* strains have shown an adhesion ability to Caco-2 and HEp-2 cell lines (Fig. 2. A, B). Interestingly, the time-courses of the adhesion of highly invasive and non-invasive strains were similar, the numbers of bacteria attached to the cells were also comparable. Furthermore, bacteria were internalised more efficiently into Caco-2 than HEp-2 cells within first hour of incubation (Fig. 2. C, D), although similar numbers of bacteria attached to both lines were noticed. This indicates that specific bacterial factors possessed by invasive *P. alcalifaciens* strains are involved in the penetration of Caco-2 and HEp-2 cells. These results correspond to the microscopic observation of crystal violet stained Caco-2 cells incubated with *P. alcalifaciens* 101i/59 bacteria for different time periods. It was visible that the entry of bacteria occurred by the outer edge of the cells after the first hour of incubation (Fig. 3.). The number of bacilli present on/in/among cells increased with every passing hour. After 2 hours of incubation (Fig. 3. B) characteristically side by side arranged bacteria were visible in cellular cytoplasm which suggested that they divided. Caco-2 cells hyperinvaded by *P. alcalifaciens* 101i/59 were observed after 4 and 6 hours of incubation (Fig. 3. D, E); the bacteria were enclosed in the vacuoles and they were present in the cytoplasm.

2.3. The determination of bacterial and host factors important in the invasion

Based on the knowledge about the mechanisms of invasion into epithelial cells by enteric pathogens e.g. *Shigella* or *Salmonella* [13], we determined the process of Caco-2 and HEp-2 cells penetration by *P. alcalifaciens* using selected inhibitors of bacterial and cellular factors involved in this phenomenon (Table 2). For this study the four highly invasive *P. alcalifaciens* strains: 35i/59, 101i/59, 352i/59, 125 were chosen. The effects of the used metabolic inhibitors on the invasion are presented in Fig. 4.

Chloramphenicol, the inhibitor of bacterial protein synthesis inhibited the invasion of *P. alcalifaciens* to Caco-2 and HEp-2 cells, which suggests that specific bacterial proteins take part in the process. Monodansylcadaverine (MD) is an inhibitor of

Table 1
Invasion of Caco-2 and HEp-2 cells by *P. alcalifaciens* strains and control bacteria.

P. alcalifaciens	Caco-2			HEp-2		
	Inoculum $\times 10^6$	Intracellular bacteria $\times 10^2$	Invasion (%)[a]	Inoculum $\times 10^6$	Intracellular bacteria $\times 10^2$	Invasion (%)
126/66	3.8 ± 1.4	0.71 ± 0.5	0.002	2.4 ± 0.9	34.67 ± 12.99	0.142
167/60	5.1 ± 0.4	2.98 ± 1.0	0.006	2.5 ± 0.8	7.15 ± 0.91	0.029
180/60	4.1 ± 0.6	10.5 ± 4.09	0.026	3.1 ± 1.0	27.0 ± 17.06	0.088
207/60	5.6 ± 2.5	2.9 ± 1.3	0.005	3.5 ± 1.3	35.0 ± 23.03	0.101
208/60	4.0 ± 1.4	0.68 ± 0.67	0.002	2.6 ± 0.8	2.4 ± 0.8	0.009
29i/59	5.0 ± 1.6	12.4 ± 3.56	0.025	2.9 ± 1.3	28.88 ± 9.22	0.100
35i/59	4.5 ± 1.5	146.67 ± 35.02	0.326	3.1 ± 0.6	57.5 ± 17.25	0.188
101i/59	5.0 ± 0.6	300.0 ± 73.21	0.600	3.1 ± 1.1	37.67 ± 16.62	0.122
166i/59	3.9 ± 1.7	25.75 ± 13.48	0.067	2.3 ± 0.9	47.33 ± 12.93	0.203
300i/59	8.9 ± 1.1	1.7 ± 1.05	0.002	3.0 ± 1.0	63.88 ± 24.01	0.211
352i/59	3.8 ± 1.1	53.0 ± 27.84	0.141	1.6 ± 0.7	74.0 ± 15.15	0.463
5/58	4.5 ± 1.4	10.66 ± 7.73	0.024	3.4 ± 1.6	9.17 ± 4.62	0.027
537/58	4.1 ± 0.4	3.89 ± 3.8	0.009	2.5 ± 0.5	11.53 ± 5.21	0.046
11B	2.5 ± 1.5	2.87 ± 1.87	0.012	1.2 ± 0.7	14.7 ± 3.75	0.118
29B	5.1 ± 1.5	15.26 ± 12.12	0.030	2.2 ± 0.2	17.0 ± 4.8	0.076
76	6.9 ± 1.6	4.73 ± 2.38	0.007	2.4 ± 0.9	10.35 ± 3.83	0.044
125	3.4 ± 1.9	17.73 ± 13.63	0.052	2.1 ± 0.9	68.5 ± 8.71	0.321
150	5.6 ± 1.6	17.89 ± 18.01	0.032	2.0 ± 0.1	11.38 ± 4.46	0.056
368	4.4 ± 1.2	4.3 ± 2.07	0.010	1.9 ± 0.2	10.16 ± 3.42	0.053
372	4.3 ± 0.7	1.73 ± 1.99	0.004	1.7 ± 0.2	11.85 ± 4.96	0.070
425	5.6 ± 2.2	20.5 ± 7.31	0.037	2.3 ± 0.2	14.33 ± 12.35	0.064
3991	5.4 ± 0.6	86.67 ± 46.76	0.006	2.4 ± 0.1	4.27 ± 1.2	0.018
Salmonella Toucra PMC 2359	2.0 ± 0.9	665.0 ± 341.18	3.393	1.8 ± 1.0	370.0 ± 224.98	2.011
E. coli DH5α	2.7 ± 3.6	0.89 ± 0.8	0.003	1.1 ± 0.4	35.0 ± 10.38	0.321

[a] The invasiveness (%) of strains was defined as a ratio of Gm^R (cfu)/total inoculum (cfu) x 100.