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EFFECT OF ENTEROVIRUS INFECTION ON SUSCEPTIBILITY OF HELA CELLS TO *SHIGELLA FLEXNERI* INVASIVITY

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Summary. – Invasiveness of *Shigella flexneri* M90T in HeLa cells was significantly increased when cells were preinfected with poliovirus 1, coxsackievirus B3 and echovirus 6. This effect was dependent on the dose of virus used, evident at early stages of viral infection and lasted hours before the appearance of a cytopathic effect. An increase of bacterial invasion ability was also noticed when HeLa cells were incubated with UV-inactivated enteroviruses. This enhancing effect obtained with both viable and UV-inactivated enteroviruses was not observed when in coinfection experiments HN555, a mutant of *S. flexneri* M90T which lacked invasive properties, was used. The data presented here suggest that the early steps of enterovirus infection induce some alterations of HeLa cells which are responsible for the enhancing of the invasiveness of *S. flexneri* M90T, but not sufficient to promote internalization of a non-invasive strain.

Key words: enterovirus; Shigella flexneri; coinfection; cell membrane permeability

Introduction

Infection of cells by picornaviruses begins through some key events such as attachment, penetration and uncoating which induce, at early stages, significant modifications of the macromolecular metabolism and of the host cell membrane (Carrasco, 1981; Rueckert, 1990). Enteroviruses enter host cells by a mechanism of receptor-mediated endocytosis which involves the generation of clathrin-coated pits and vesicles in plasma membranes (Crowell and Landau, 1983; Zeichhardt *et al.*, 1985). Increased permeability to monovalent ions as well as to some plant toxins during early steps of picornavirus infection has also been reported (Fernandez-Puentes and Carrasco, 1980) and polioviruses have been

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shown to induce different alterations in HeLa cell membrane functions (Schaefer *et al.*, 1982).

Cellular surface modifications induced by viruses whose normal habitat is the intestinal tract may modulate *in vivo* the ability of enteropathogenic bacteria to adhere and to invade epithelial cells as well as to produce and/or to favour more severe infections in humans (Sweet and Smith, 1990). It has been reported that the invasiveness of *Salmonella typhimurium* was enhanced in HEp-2 cells preinfected both with infectious or noninfectious coxsackie B1 virus (Bukholm and Degré, 1984; Bukholm *et al.*, 1985) and that preinfection of MA-104 cells by human rotavirus promoted enterobacteria internalization (Bukholm, 1988). More recently it has also been observed that coinfections of HEp-2 cells with enteroviruses and *Campylobacter jejuni* resulted in an increase of bacterial invasion (Konkel and Joens, 1990). Coxsackie B1 virus was reported to induce an enhancement of invasion of *S. flexneri* in HEp-2 cells which correlated with an increase of induced phagocytosis (Modalsli *et al.*, 1990).

However, the mechanisms involved in this virus-induced enhancement of bacterial invasion of epithelial cells have not been fully clarified. The purpose of this study has been to examine in HeLa cells infected with poliovirus 1, coxsackievirus B3 or echovirus 6 the behaviour of *S. flexneri* with invasive phenotype which is known to enter HeLa cells through a mechanism of receptor-mediated endocytosis and requires the participation of clathrin (Clerc and Sansonetti, 1989). The results obtained in this study suggest that specific early interactions between enteroviruses and HeLa cells significantly increased internalization of *S. flexneri*.

Materials and Methods

Cell cultures. HeLa S3 and Vero cell lines were cultured in Eagle's Minimum Essential Medium (MEM) (GIBCO Laboratories) supplemented with 2 mmol/l L-glutamine, 100 UI/ml penicillin, 100 μg/ml streptomycin and 10 % of foetal bovine serum for growth or 2 % for maintenance.

Viruses. Poliovirus 1, coxsackievirus B3 and echovirus 6 were grown in Vero cell monolayers by infecting a 75 cm^2 tissue culture flask containing a confluent sheet of cells at a multiplicity of infection of 0.1 PFU/cell. After 48 hr incubation at 37 °C, when cytopathic effect was well developed, the cultures were frozen and thawed, centrifuged at 4000 rpm for 15 min and the supernatants stored at -70 °C.

Titrations of poliovirus 1, coxsackie B3 virus and echovirus 6 were performed in HeLa cells in 96 wells microtiter plates (Falcon). The $TCID_{50}$ was determined after 48 hr incubation at 37 °C in 5 % CO_2 by the neutral red uptake assay. After 48 hr incubation the cultures were stained for 3 hr with neutral red (50 μg/ml, 200 μl/well, 37 °C, 5 % CO_2), washed with Hank's salt solution and fixed with 4 % formaldehyde in 10 % $CaCl_2$ (200 μl/well). The dye was extracted by 1 % acetic acid in 50 % ethanol (200 μl/well) and the disruption of cells was measured at 550 nm in an ELISA-reader. In coinfection experiments virus infection was performed in HeLa cells ($3x10^5$) grown in 24 wells culture dishes (Nunc) with $1x10^4$, $1x10^5$ or $1x10^6$ $TCID_{50}$. Virus suspensions were incubated with cell monolayers for 1 hr at 37 °C; non-adsorbed particles were then removed and fresh maintenance medium was added for different time intervals.

Inactivation of viruses by ultraviolet irradiation (UV). Virus stock solutions were diluted 1:10 and

1 ml aliquots were irradiated by an UV lamp (1 mW/cm²) in a 35 mm Petri dish for 3 min at a distance of 120 mm. UV-treated virus preparations were tested in HeLa cell cultures and no cytopathic effect was obtained after 48 hr incubation.

Bacterial strains. The invasive *S. flexneri* strain M90T and the plasmid-cured non-invasive derivative HN555 were grown on trypticase soy broth and agar (TSB and TSA, respectively; BBL, Microbiology System, Cockeysville, MD.).

Invasion assays. Invasiveness was assayed in HeLa cells essentially as described by Sansonetti *et al.* (1986). Briefly, virus infected or non-infected HeLa cells grown in 24 wells culture dishes (Nunc) were incubated with 0.5 ml of exponentially growing bacteria ($6x10^7$ CFU/ml) at a multiplicity of infection of about 100 bacteria per cell for 90 min at 37° C. The monolayers were washed five times with PBS to remove extracellular bacteria, then covered with 2 ml of fresh MEM with 50 μg/ml of gentamicin (Sigma Chemical Co.) and incubated again for 90 min at 37 °C in an atmosphere of 5 % CO_2. Then the wells were washed five times with PBS and the cells were either fixed in methanol and stained with Giemsa, or trypsinized and lysed with 0.5 % sodium deoxycholate. Stained monolayers were examined using a light microscope and the number of invaded cells and that of internalized bacteria per invaded cell were evaluated by random counting of 400 cells. Dilutions of the lysed monolayers were plated on TSA. Bacterial entry was calculated as the number of the CFU recovered from $2x10^5$ HeLa cells. The results were expressed as the percentage of internalized bacteria in virus-infected cultures as compared to controls not infected by enterovirus. Under all experimental conditions the number of viable HeLa cells was determined by the neutral red uptake assay.

Results

In preliminary experiments, one day-old confluent HeLa cell monolayers were inoculated with $1x10^4$, $1x10^5$ and $1x10^6$ $TCID_{50}$ of polio 1, coxsackie B3 and echo 6 viruses. No significant decrease in the number of viable cells was observed 8 hr post infection and no evident cytopathogenic effect was recorded by microscopic examination 10 hr post adsorption.

Coinfection with invasive *S. flexneri* was carried out during the virus adsorption period (Fig. 1A) and at a later stage (Fig. 1B). The bacterial invasion efficiency was evaluated by determining the CFU (Fig. 1) and by microscopic

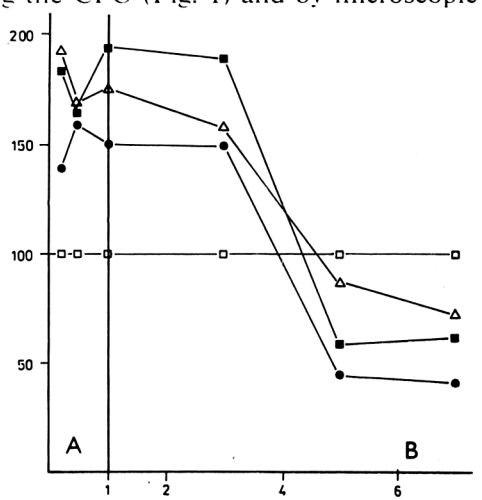

Fig. 1
The effect of enterovirus infection on the invasiveness of *S. flexneri* M90T
Non-infected HeLa cells (—□—) and infected with poliovirus 1 (—·—), coxsackievirus B3 (—■—) and echovirus 6 (—●—).
Invasivity was measured by determining the percentage of viable internalized bacteria with respect to controls.
A - viral adsorption period; B - viral post adsorption period. Each point represents the mean value from five independent experiments; standard deviation < 14 %.
Ordinate: % of viable internalized bacteria.
Abscissa: time post infection (hr).

counts of internalized bacteria (Table 1). The data reported were obtained using $1x10^6$ $TCID_{50}$ of viruses, i. e. a dose which was found to be the most effective in promoting bacterial penetration. Fig. 1A depicts the penetration efficiency of *S. flexneri* added to virus-infected HeLa cells at different times during the adsorption period. The efficiency of bacterial invasion of HeLa cells was already significantly influenced 15 min after virus addition. Fig. 1B shows that when the invasion assay with *S. flexneri* was performed at different times after viral adsorption, the augmented efficiency of bacterial internalization remained almost constant for at least 3 hr post virus infection. As compared to the virus non-infected controls the percentage of internalized bacteria in virus infected cultures 3 hr after virus infection was 158 %, 190 % and 150 % for poliovirus 1, coxsackievirus B3 and echovirus 6, respectively. Five hr post virus infection an apparent decrease of viable bacteria recovered from coinfected monolayers was observed.

Duplicate wells were also examined microscopically at intervals from 15 min to 7 hr post virus addition and the results are reported in Table 1. Bacterial entry in virus-infected cells was increased with respect to controls up to 7 hr post virus infection as shown by both the percentage of invaded cells and the number of internalized bacteria per invaded cell.

Experiments were also performed with UV-inactivated viruses. Invasion assays were carried out 1 hr and 3 hr post virus addition (Table 2). The increase

Table 1. The invasiveness of S. flexneri M90T in HeLa cells infected with different enteroviruses

Viruses	Incubation time	Percentage* of invaded cells	Mean value of internalized bacteria per invaded cell
none	0	23	1.3
polio	15 min	28	1.4
	1 hr	27	1.6
	5 hr	32	2.4
	7 hr	33	2.0
coxsackie B3	15 min	35	2.0
	1 hr	42	1.9
	5 hr	44	1.5
	7 hr	48	2.0
echovirus 6	15 min	24	2.0
	1 hr	45	2.7
	5 hr	49	2.6
	7 hr	50	2.5

* Each point represents the mean value from five independent experiments; standard deviation < 4 %.

Table 2. The effect of UV-inactivated enteroviruses on the susceptibility of HeLa cells to S. flexneri M90T invasiveness

	Percentage of viable internalized bacteria	
	Time post virus addition	
	1 hr	3 hr
Infectious poliovirus 1	176	158
UV-inactivated poliovirus 1	132	94
Infectious coxsackievirus B3	195	190
UV-inactivated coxsackievirus B3	143	96
Infectious echovirus 6	151	150
UV-inactivated echovirus 6	124	93
Bacterial control	100	100

Each point represents the mean value from five independent experiments; standard deviation < 14 %.

in the number of viable internalized bacteria observed in HeLa cells infected with non-infectious virus particles was similar to that obtained with the same number of infectious virus particles but lasted only 1 hr post virus infection.

Finally, the effect of enterovirus infection on the internalization of the non-invasive mutant of *S. flexneri* M90T, HN555, was evaluated under the same experimental conditions as those followed for *S. flexneri* M90T. The non-invasive strain HN555 did not show any invasive ability in HeLa cells preinfected with $1x10^6$ $TCID_{50}$ of poliovirus 1, coxsackie B3 or echovirus 6 (results not shown).

Discussion

In this study we observed that poliovirus 1, coxsackievirus B3 and echovirus 6 replication influenced the susceptibility of HeLa S3 cells to the invasion by *S. flexneri* M90T.

A significant increase of the efficiency of invasion was obtained at an early stage (15 min) after virus addition, which lasted at least 7 hr post virus infection and was similar for polio, coxsackie or echo viruses. Enhancement of bacterial entry was less evident if viral infection was carried out with a reduced viral inoculum. Our results are in agreement with those of other authors who noticed a general increase of the invasive ability of different enterobacteria towards various human cell lines infected with coxsackie B1, measles, rotavirus and vesicular stomatitis viruses (Bukholm *et al.*, 1984; Bukholm *et al.*, 1986;

Bukholm, 1988; Bukholm *et al.*, 1988). However, in these studies the increase in the efficiency of invasion was found to peak at later stages of viral multiplication.

Nevertheless, in our experiments controversial results were obtained 5 and 7 hr post virus infection (before the onset of an evident cytopathogenic effect) when the invasion was recorded as internalized bacteria microscopic counts or as CFU recovered from lysed monolayers. In fact the percentage of invaded HeLa cells, if determined microscopically, was further enhanced 7 hr post virus infection, while a decrease of viable internalized bacteria was observed by CFU counts. It might be assumed that a virus-induced modification of cell membrane allows gentamicin penetration into HeLa cells. According to Bukholm *et al.* (1985) and Konkel and Jones (1990) we noticed that some enhancement of invasion was also induced by non-infectious virus particles. Moreover, the increased invasion of virus-infected cells was restricted to invasive *S. flexneri* M90T since coinfection experiments performed with the non-invasive *S. flexneri* HN555 strain did not induce internalization. It might be argued that some unknown virus-induced factors could be used by invasive microorganisms in promoting the penetration in susceptible host cells. Similar observations have been reported by Bukholm and Degré (1984) and by Konkel and Jones (1990) who found that non-invasive *E. coli* and *Campylobacter* isolates were not capable of invading virus-infected cells.

It is well known that different mechanisms are involved in the interactions between virus, bacteria and host cells. The early steps of enterovirus multiplication in susceptible cells correlate with altered ionic conditions and consequent changes in membrane electric potential and permeability (Schaefer *et al.*, 1982; Carrasco, 1981). Moreover, virus-induced alterations in cell membrane may cause a decrease of aminoacid uptake and then an inhibition of host cell protein synthesis (Schaefer *et al.*, 1982). These modifications of virus-infected cell membranes at an early stage of the virus infective cycle could lead to more suitable membrane ionic conditions for *S. flexneri*-HeLa cell early interactions which promote internalization. This hypothesis appears to be supported by the fact that among these modifications there are those concerning the permeability to cations, the level of which increases within the cells (Schaefer *et al.*, 1982). Furthermore, it cannot be excluded that the increase of invasion efficiency could also be correlated to virus-induced mobilization of clathrin which is also known to participate in the internalization process of *S. flexneri* (Clerc and Sansonetti, 1989).

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