Polycationic Polypeptides: a Possible Model for the Penetration-enhancing factor in the Invasion of Host Cells by *Toxoplasma gondii*

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The effect of polycationic polypeptides (polylysine, polyarginine and polyhistidine) on the invasion of mammalian cells and plant protoplasts by *Toxoplasma gondii* was studied. In JM cells, a human lymphoblastoid cell line with T cell characteristics, all polycationic polypeptides used increased the invasion rate in a concentration-dependent manner. This effect and the morphological changes revealed by electron microscopy resembled the action of the penetration-enhancing factor previously described by E. Lycke and co-workers. Plant protoplasts of *Catharanthus roseus*, which are resistant to *T. gondii* invasion, showed the same morphological changes in the presence of polycationic polypeptides as observed for JM cells, but were not invaded.

INTRODUCTION

The biochemical mechanism of host cell invasion by the parasite *Toxoplasma gondii* is still a matter of controversy. Lycke *et al.* (1975) reported on the isolation of a penetration-enhancing factor (PEF) with a molecular weight of about 70000 to 150000. This factor increased the number of infected cells and induced distinct morphological alterations in the host cell. The PEF is believed to be secreted by specific organelles, the rhoptries, located in the anterior part of the parasite (Gustafson *et al.*, 1954; Garnham *et al.*, 1962). These organelles have an unusually high concentration of polycationic peptides (De Souza & Souto-Padron, 1978). Morphological alterations of cells induced by polycationic polypeptides (PCP) have been reported (Mamelack *et al.*, 1969; Hartmann & Galla, 1978); Lycke *et al.* (1975) described similar features for the PEF. Moreover, Hughes & van Knapen (1982) reported a secretory circulating antigen of *Toxoplasma*. This antigen, Ag 5, is a basic protein which has an isoelectric point of 5.8 and a molecular weight of 324000. The authors speculated that this protein, which is in part similar to the PEF, is released by the parasite during host cell entry (Hughes & van Knapen, 1982).

In this study we examined the effect and invasion-enhancing capacity of polycationic polypeptides on the invasion of host cells by *T. gondii*. Plant protoplasts have been reported to be the only cell type resistant to infection by *Toxoplasma* parasites (Werk & Fischer, 1982). There is at present no explanation for this finding; however, a different membrane structure might be responsible for the lack of infectivity of the *Toxoplasma* parasite to plant protoplasts (Evans, 1976). It was therefore of interest to study the effect of PCP on the invasion of protoplasts by *T. gondii*.

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Abbreviations: Eagle's MEM, Eagle's minimal essential medium; PCP, polycationic peptides; PEF, penetration-enhancing factor.

METHODS

Cells. The RH strain of *Toxoplasma gondii* was obtained from Dr H. Seeliger, Institut für Hygiene und Medizinische Mikrobiologie der Universität, Würzburg. Mice were infected intraperitoneally and the parasites were harvested from the peritoneal cavity three d post-infection. The trophozoites were washed three times with phosphate-buffered saline pH 7.2 (PBS) (Dulbecco & Vogt, 1954) at room temperature, and collected by low-speed centrifugation at 1000 g. JM cells were kindly provided by Dr H. W. Kreth, Institut für Virologie, Universität Würzburg. The JM cell line is a human lymphoblastoid cell line with T cell characteristics (Schneider *et al.*, 1977). The cells were kept in a RPMI 1640 medium (Morgan, 1970) which had been supplemented with 10% (v/v) foetal calf serum, 50 µg ml⁻¹ gentamicin, and 1% (w/v) glutamine. HeLa cells, a gift of Dr G. May, Zentrum der Hygiene, Universität Frankfurt, were kept in Eagle's minimal essential medium (MEM) supplemented with 5% (v/v) foetal calf serum, 50 µg gentamicin ml⁻¹, and 1% (w/v) glutamine (all from Flow Laboratories). Plant protoplasts, of *Catharanthus roseus*, were prepared as previously described (Werk & Fischer, 1982).

Infection assay. The experimental schedule including 'target' and 'effector' cell specificity, the ratio of 'target' cells to 'effector' cells, and the concentration of the PCP is shown in Table 1. Polylysine (mol. wt 400000), polyhistidine (7000) and polyarginine (130000) (Sigma) were used. The assay for JM cells and protoplasts was done in 1 ml RPMI medium without foetal calf serum, and with or without PCP for 30 min at 37 °C. At the end of the incubation period samples were prepared as smears on glass slides and examined by phase-contrast light microscopy at 500 × magnification. The infection rate was assayed as percentage of infected cells. For each value at least 300 cells were analysed and three parallel replicate experiments were conducted.

Control experiments including tests for polypeptide-induced phagocytosis and test for viability of 'target' and 'effector' cells are shown in Table 2. Since JM cells are not very suitable for observing parasite rosette-colony formation after multiple infection, HeLa cells were used for control experiments. Overnight HeLa cell coverslip cultures were infected with *T. gondii* in the presence or absence of polypeptides for 30 min at 37 °C. The cultures were then carefully rinsed with Eagle's MEM to remove free parasites. Cells were incubated in 10 ml fresh Eagle's MEM without foetal calf serum, to prevent HeLa cell proliferation, for 18 h at 37 °C. The cultures were examined microscopically and the infection was quantified by expressing the number of parasite colonies as a percentage of the number of 'target' cells. HeLa cell coverslip cultures incubated with viable or heat-killed yeast cells (*Saccharomyces cerevisiae*) were examined directly after the infection period of 30 min.

Electron microscopy. To examine ultrastructural changes due to the PCP, 3.3×10^6 JM cells were treated with 10 or 100 ng ml⁻¹ of each of polyhistidine, polylysine and polyarginine, and infected for 30 min with 1.2×10^6 *Toxoplasma* trophozoites. Cells were collected by centrifugation and fixed with 5% (w/v) glutaraldehyde in PBS, post-fixed with 1% osmium tetroxide and embedded in Epon by the procedure of Luft (1961). Sections were cut on a Reichert OM3 ultramicrotome and stained with uranyl acetate and lead citrate, and were examined in a Zeiss EM 10 electron microscope.

RESULTS

By comparison with controls, the infection rate of JM cells in the presence of polylysine, polyhistidine and polyarginine increased with PCP concentration over a range of 0.5 to 10.0 ng ml⁻¹ (Table 1). Polylysine, at the highest concentration used, caused an increase in infection rate to 39.6% as compared to the standard infection rate of 17%. The infection rate at the corresponding concentration of polyhistidine and polyarginine was assayed to be increased to 34% and 37% respectively (Table 1). Plant protoplasts previously shown to be resistant to infection by *T. gondii* were also used as target cells, but even in the presence of 100 ng PCP ml⁻¹ no infected cells were found (Table 1). Control experiments for stimulated phagocytosis were done. PCP did not stimulate the uptake of viable or heat-killed yeast cells by JM or HeLa cells (Table 2).

Severe structural damage in most JM cells at a PCP concentration of 10 ng ml⁻¹ was revealed by electron microscopy. The effect of polylysine was especially strong, whereas polyhistidine showed less severe alterations at the same concentration. The cytoplasm of treated cells showed various stages of vacuole formation and a wide range of disorganization (Fig. 1). At high concentrations (100 ng PCP ml⁻¹) severe plasma membrane damage was apparent. The nucleus showed partial condensation of chromatin (Fig. 2).

The parasite also showed essentially similar morphological modifications including stripping of the pellicle at 100 ng PCP ml⁻¹ (Fig. 3). The results of the uptake experiments (Tables 1 and 2) revealed that at PCP concentrations up to 10 ng ml⁻¹ *T. gondii* was able to multiply intracellularly. There was no difference between infected and uninfected cells in the degree or type of

Table 1. Infection experiments with *T. gondii* in the presence of PCPs

JM cells or plant protoplasts were exposed to *T. gondii* in the presence of PCPs at the concentrations shown.

'Target' cell (no.)	'Effector' cell (no.)	PCP	PCP concn (ng ml ⁻¹)	Percentage infection of 'target' cells (±SE)
JM cells (3.5 × 10 ⁵)	<i>Toxoplasma gondii</i> (1.2 × 10 ⁶)	None	—	17.0 ± 3.5
		Polylysine	0.5	20.3 ± 5.0
			1.0	27.6 ± 2.6
			5.0	36.6 ± 4.6
			10.0	39.6 ± 3.0
		Polyarginine	0.5	19.6 ± 3.6
			1.0	21.6 ± 5.6
			5.0	24.0 ± 7.0
			10.0	37.0 ± 4.6
		Polyhistidine	0.5	19.6 ± 4.6
			1.0	NT
			5.0	24.5 ± 6.5
			10.0	34.0 ± 5.0
<i>Catharanthus roseus</i> (10 ⁵)	<i>Toxoplasma gondii</i> (2 × 10 ⁶)	Polylysine	0.0	0.0
		Polyarginine	1.0	
		Polyhistidine	10.0	
			100.0	

NT, Not tested.

Table 2. Experiments to test for PCP-induced phagocytosis in HeLa or JM cells

HeLa cells or JM cells were exposed to *Toxoplasma gondii* or *Saccharomyces cerevisiae*. Development of *Toxoplasma* trophozoites in HeLa cells and uptake of viable and heat-killed cells are shown as number of 'effector' cells per 100 'target' cells.

'Target' cell (no.)	'Effector' cell (no.)	PCP	PCP concn (ng ml ⁻¹)	No. of 'effector' cells per 100 'target' cells (±SE)
HeLa cells (5 × 10 ⁵)	<i>Toxoplasma gondii</i> (2 × 10 ⁶)	None	—	53.0 ± 3.0
		Polylysine	2	65.8 ± 4.8
			5	84.6 ± 5.4
			10	> 90
		Polyarginine	2	65.2 ± 3.2
			5	82.1 ± 6.1
			10	> 90
		Polyhistidine	2	61.0 ± 5.0
			5	78.8 ± 4.8
			10	> 90
HeLa cells (5 × 10 ⁵)	<i>Saccharomyces cerevisiae</i> *	Polylysine	5	0.0
		Polyarginine	10	0.0
		Polyhistidine		
		Polylysine	5	0.0
		Polyarginine	10	0.0
		Polyhistidine		

* Control experiments without PCP gave values of 0.0 for uptake.

morphological alterations following PCP treatment. All three PCPs induced marked membrane damage of *Catharanthus roseus* protoplasts (Fig. 4). Indeed, at high concentration (100 ng PCP ml⁻¹) the plasmalemma disappeared. However, as indicated in Table 1 damage to the plant protoplast membrane did not result in invasion by *Toxoplasma*.

Fig. 1. Effect of polyhistidine on the invasion of JM cells by *T. gondii*. JM cells were exposed to *T. gondii* without polycationic polypeptides (a) or in the presence of 10 ng polyhistidine ml⁻¹ (b). T, *Toxoplasma gondii*; V, vacuole; N, nucleus. Bar markers 1 µm.

Fig. 2. Effect of polyarginine (100 ng ml⁻¹) on JM cells exposed to *T. gondii*. Note the extensive vacuolization of the cytoplasm and the condensation of the chromatin of both host and parasite cells. Abbreviations as in Fig. 1. Bar marker 1 µm.

Fig. 3. Effect of polylysine (100 ng ml^{-1}) on *T. gondii* trophozoites. Note the stripping of the pellicle. A, apical pole; P, pellicle. Bar marker $1 \mu\text{m}$.

Fig. 4. Effect of polylysine on plant protoplasts. Protoplasts of *Catharanthus roseus* were exposed to *T. gondii* without basic polypeptides (a) or in the presence of $100 \text{ ng polylysine ml}^{-1}$ (b); no invasion was detected. At this concentration of polylysine nearly all protoplasts exhibited destruction of the plasma-lemma and the thylakoid membranes. TG, thylakoid granule; TM, thylakoid membranes. Bar markers $1 \mu\text{m}$.

DISCUSSION

The PEF described by Lycke *et al.* (1975) for *Toxoplasma gondii* is a protein with a molecular weight of 70000 to 150000 and is probably secreted by the rhoptries (Norrby, 1970). The physiological effect of the PEF is reported to be enhanced parasite invasion into target cells, which then undergo severe structural alterations. These changes resemble the action of polycationic polypeptides on target cells, as reported, for example, by Mamelack *et al.* (1969) using polylysine in toad bladder cells. The exact chemical nature of the PEF is, however, unknown. Experiments by De Souza & Souto-Padron (1978) suggested that the PEF might be a polycationic protein: phosphotungstic acid staining, known to bind preferentially to PCPs (e.g. polyhistidine), revealed rhoptries containing an unusually high concentration of basic proteins. Recently, Hughes & van Knapen (1982) demonstrated a secretory circulating antigen in acute toxoplasmosis, which they isolated from *Toxoplasma* trophozoites. The protein is basic. The authors suggested that this protein may participate in the invasion events.

A histidine-rich protein isolated by Kilejian (Kilejian & Jensen, 1977) was shown to induce invagination of the erythrocyte plasmalemma and it was suggested, although not proved, that it participates in malaria parasite invasion (Bannister *et al.*, 1977). An explanation of the action of polycationic substances is derived from the experiments of Sheetz & Singer (1974). They observed changes in the microviscosity and morphology of red blood cell membranes after exposure to cationic substances. Based on these results Bannister *et al.* (1977) suggested that the histidine-rich protein either introduced energy dependent alterations of membrane proteins or changed the membrane microviscosity by intercalation with the lipid bilayer, and that this would result in an inward extension of the membrane.

Each of the three PCPs tested in this study increased the infection rate over the range 0.5 to 10.0 ng PCP ml⁻¹. This indicated that the invasion-enhancing effect of the PCPs is concentration dependent. However, the effect was not due to PCP-stimulated phagocytosis, since viable and heat-killed yeast cells were not internalized either by JM cells or by HeLa cells in the presence of PCPs. The physiological effect was accompanied by morphological changes of the target cells. Electron micrographs revealed severe structural damage such as vacuolization and stripping of the membrane as well as morphological changes of the parasite. PEF produced similar alterations of HeLa cells (Lycke *et al.*, 1975). Nevertheless, the trophozoites were still able to invade host cells. The data presented show the strongest invasion-enhancing effect to be at 10 ng PCP ml⁻¹. Morphological examination revealed that the parasites were damaged to some extent at this concentration, but to a far less extent than the target cells. Moreover, uptake experiments demonstrated that PCPs did not interfere with multiplication of parasites in HeLa cells.

The similarities of physiological and morphological actions shown by the basic polypeptides and the PEF prompt us to suggest them as a possible model for the PEF. This does not imply a chemical similarity.

Werk & Fischer (1982) reported that plant protoplasts were the only cell line definitely resistant to infection by *T. gondii*. A different membrane structure might be responsible for this resistance (Evans, 1976). Polylysine, polyhistidine and polyarginine caused the typical morphological changes reported above for JM cells. Nevertheless, *Toxoplasma* parasites failed to invade protoplasts in the presence of low or high concentrations of these polypeptides. Since plant protoplasts remained refractory to invasion by *Toxoplasma* it seems that PCP-induced membrane changes are not sufficient to ensure successful invasion. It has to be assumed that additional factors, missing from plant protoplasts, are necessary for invasion.

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