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Tight junctional inhibition of entry of *Toxoplasma gondii* into MDCK cells

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Abstract: Various conditions of cultures were performed to investigate the role of tight junctions formed between adjacent MDCK cells on the entry of *Toxoplasma*. When MDCK cells were cocultured with excess number of *Toxoplasma* at the seeding density of 1×10^5, 3×10^5, and 5×10^5 cells/ml for 4 days, the number of intracellular parasites decreased rapidly as the host cells reached saturation density, *i.e.*, the formation of tight junctions. When the concentration of calcium in the media (1.8 mM in general) was shifted to 5 μM that resulted in the elimination of tight junction, the penetration of *Toxoplasma* increased about 2-fold($p<0.05$) in the saturated culture, while that of non-saturated culture decreased by half. Trypsin-EDTA which was treated to conquer the tight junctions of saturated culture favored the entry of *Toxoplasma* about 2.5-fold($p<0.05$) compared to the non-treated, while that of non-saturated culture decreased to about one fifth. It was suggested that the tight junctions of epithelial cells play a role as a barrier for the entry of *Toxoplasma* and *Toxoplasma* penetrate into host cells through membrane structure-specific, *i.e.*, certain kind of receptors present on the basolateral rather than apical surface of MDCK cells.

Key words: *Toxoplama gondii*, MDCK cells, epithelium, tight junction, entry, membrane structure-specificity

INTRODUCTION

Toxoplasma gondii, an obligate intracellular protozoan parasite is an important and ubiquitous pathogen in human and veterinary medicine all over the world (Levine, 1977; Choi *et al.*, 1987). It is known to cause transplacental infections that can lead to abortion or severe neonatal malformations (Krick and Remington, 1978; Remington and Desmonts, 1983). More recently, it has acquired additional concerns as one of the major opportunistic pathogens in immunocompromised patients such as AIDS (Anderson *et al.*, 1983; Luft *et al.*, 1981; Wanke *et al.*, 1987).

Although the general features of *Toxoplasma* entry have been known for years, the mechanisms underlying these processes are not well understood. The process of penetration of parasites into vertebrate cells begins with their adhesion to the host cell surface, followed by their internalization. It has been suggested that the rhoptries and micronemes of *Toxoplasma* contain substances such as "penetration enhancing factors" or enzymes that interfere with some properties of plasma membrane of the host cell, facilitating the entry of parasite (Lycke *et al.*, 1975; Silva *et al.*, 1982; Nichols *et al.*, 1983; Schwartzman, 1986; Saffer *et al.*, 1989) into host cells. Some authors have studied parasitophorous vacuoles or intraphagosomal membrane networks which are formed during or after the internalization of the parasite (Nichols and O'Connor, 1981; Sibley *et al.*, 1986; Carvalho and Souza, 1989; Leriche and

Dubremettz, 1990) to elucidate the entry mechanism. In addition to the above studies, many reports have described host cell surface receptors in other protozoan species such as *Trypcnoscma cruzi* (Villalta *et al.*, 1983; Kierszenbaum and Stiles, 1985; Piras *et al.*, 1987; Boschetti *et al.*, 1987; Davis and Kuhn, 1990), *Plasmodium falciparum*(Jungery *et al.*, 1983), *P. knowlesi*(Bannister *et al.*, 1986), *Entamoeba histolytica*(Petri *et al.*, 1987; Arroyo and Orozco, 1987), and *Leishmania donovani* (Wilson and Pearson, 1988; Rodrigues *et al.*, 1988). Although most reports did not identify the host cell surface receptor for specific species exclusively, it might be important to introduce the concept of receptor-ligand reactions in the course of cell entry by these parasitic protozoa.

Recent availability of a stable epithelial cell line, MDCK(Madin-Darby Canine Kidney), which has been used to study tight junction between adjacent cells and/or surface membrane polarity enables us to employ preliminarily the concept of receptor-ligand interactions in the invasion of *Toxoplasma* into these cells. In this report we will demonstrate the usefulness of MDCK cells to study the entry of *Toxoplasma* and the role of epithelial tight junctions on the penetration of *Toxoplasma* which suggests the presence of membrane structure-specific, *i.e.*, certain kind of receptors for the invasion.

MATERIALS AND METHODS

Parasite: Virulent tachyzoites of RH strain of *Toxoplasma* were maintained and propagated by continuous passage in ICR mice, and were purified from the peritoneal exudate by centrifugation at 1,600 rpm(Beckman, TJ-6) for 5 min after washing with saline as described previously(Choi *et al.*, 1988).

Cell culture: MDCK cells(ATCC CCL 34) were maintained in Earle's MEM (EMEM) medium supplemented with 10% fetal bovine serum(FBS), 100 unit/ml penicillin, 100 μg/ml streptomycin and 10 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.4. Cells were grown at 37°C in humidified 95% air/5% CO_2 atmosphere. Cells were subcultured with trypsin-EDTA at every 5 day at a seeding density of 5×10^4 cells/ml. All cell culture materials were obtained from Gibco Laboratories (Grand Island, NY) and culture plasticwares from NUNC Co. (Denmark).

Entry of *Toxoplasma* into MDCK cells: To determine the control incubation time of coculture MDCK cells were seeded at a density of 1×10^5 cells/ml on a cover glass immersed in media for 1 day and then excess number of *Toxoplasma*(about $\times10^7$ tachyzoites) was added on the culture plate for time schedules of 10 min to 5 hr. The number of parasites penetrated was counted for 6 to 10 sights under light microscope(Vidas image analyzer, Kontron) of $\times400$ after staining with Giemsa solution. Data were expressed by the number of *Toxoplasma* per 100 host nuclei and Student's *t*-test was performed on the data.

Cell number-dependent entry of *Toxoplasma*: MDCK cells were seeded at a density of 1×10^5, 3×10^5, and 5×10^5 cells/ml respectively and were cultured to obtain serial confluent density of saturation(when they reached to the saturation density of $4\sim5\times10^5$ cell/ml, epithelial tight junctions or cell polarity were formed between adjacent cells) (Herzlinger and Ojakian, 1984; Gumbiner *et al.*, 1988) for 4 days. Every day excess number of *Toxoplasma* was cocultured for 60 or 90 min and then the number of *Toxoplasma* penetrated was counted as described above.

Effect of calcium depletion on the entry of *Toxoplasma*: To eliminate the tight junctions which were formed at a saturation density, cells were grown in media of low concentrations of calcium media (5 μM instead of 1.8 mM and supplemented with 10% FBS that had been dialyzed extensively against a solution of 10 mM Tris-HCl, pH 7.4, 120 mM NaCl; LC medium) as recited by Nelson and Veshnock(1987). Briefly, MDCK cells were maintained at a very low density(about 1×10^4 cells/ml) for 60 hr. During this period, the cells were subcultured

twice by light trypsinization and replated at a density of 1×10^4 cells/ml. To produce instant confluent monolayers of cells from these cultures, cells were cultured at a density of 5×10^5 cells/ml for 1 day. Then *Toxoplasma* was cocultured with these cells and the number of parasites entered was counted with the control of non-treated and non-saturated cultures.

Effect of trypsin-EDTA on the entry of *Toxoplasma*: To digest the tight junctions of saturated culture(culture of a seeding density of 5×10^5 cells/ml) mild trypsinization was performed on the culture for 1 min. Non-saturated culture(culture of a seeding density of 1×10^5 cells/ml) was also performed as a control. After 60 min of coculture with *Toxoplasma* intracellular parasites were counted.

RESULTS

Entry of *Toxoplasma* into MDCK cells: *Toxoplasma* penetrated into MDCK cells very massively as a time-dependent mode. As shown in Fig. 1, the number of intracellular parasites increased almost linearly as the time of co-culture was prolonged. For 24 hr coculture the number of parasites reached to about 3,500 per 100 host nuclei(data not presented). We determined the optimal duration of coculture as 60 or 90 min which might be sufficient to measure the degree of invasion and to exclude the changes of cellular state or environments of cells especially in the case of saturation-density cultures.

Cell number-dependent entry of *Toxoplasma*: With an aim to obtain serial confluent density of saturation, MDCK cells were seeded at a density of 1×10^5, 3×10^5, and 5×10^5 cells/ml. There were significant differences in the number of intracellular parasites at day 1 according to the primary seeding densities, and when MDCK cells multiplied to fulfill the saturation density, the number of intracellular parasites was decreased rapidly as shown in Fig. 2 and Fig. 3. There were few parasites in the cytoplasm of MDCK cells from the third day in the cases of seeding density of 1×10^5 cells/ml(Fig. 3; 1T3 and 1T4), from the second day in 3×10^5 cells/ml(Fig. 3; 3T2, 3T3, and 3T4) and from the first day in 5×10^5 cells/ml (Fig. 3; 5T1, 5T2, 5T3, and 5T4). We could employ the state of cellular relationship of

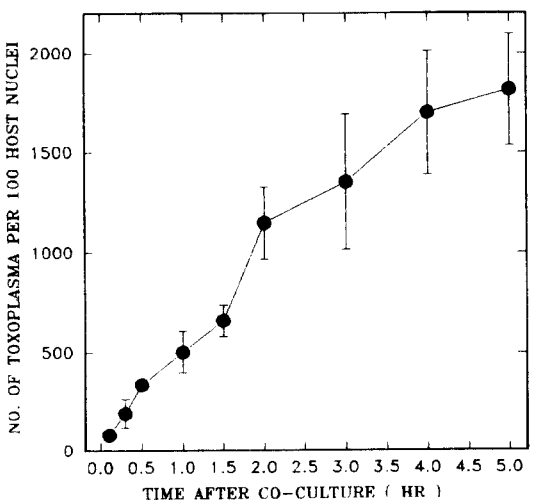


Fig. 1. Host cell entry of *Toxoplasma* into MDCK cells. MDCK cells and parasites were cocultured for 60 min and intracellular parasites were counted on 100 cells after staining with Giemsa solution.

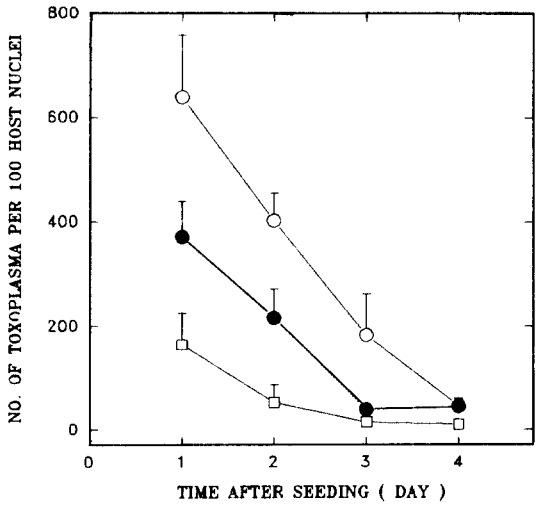
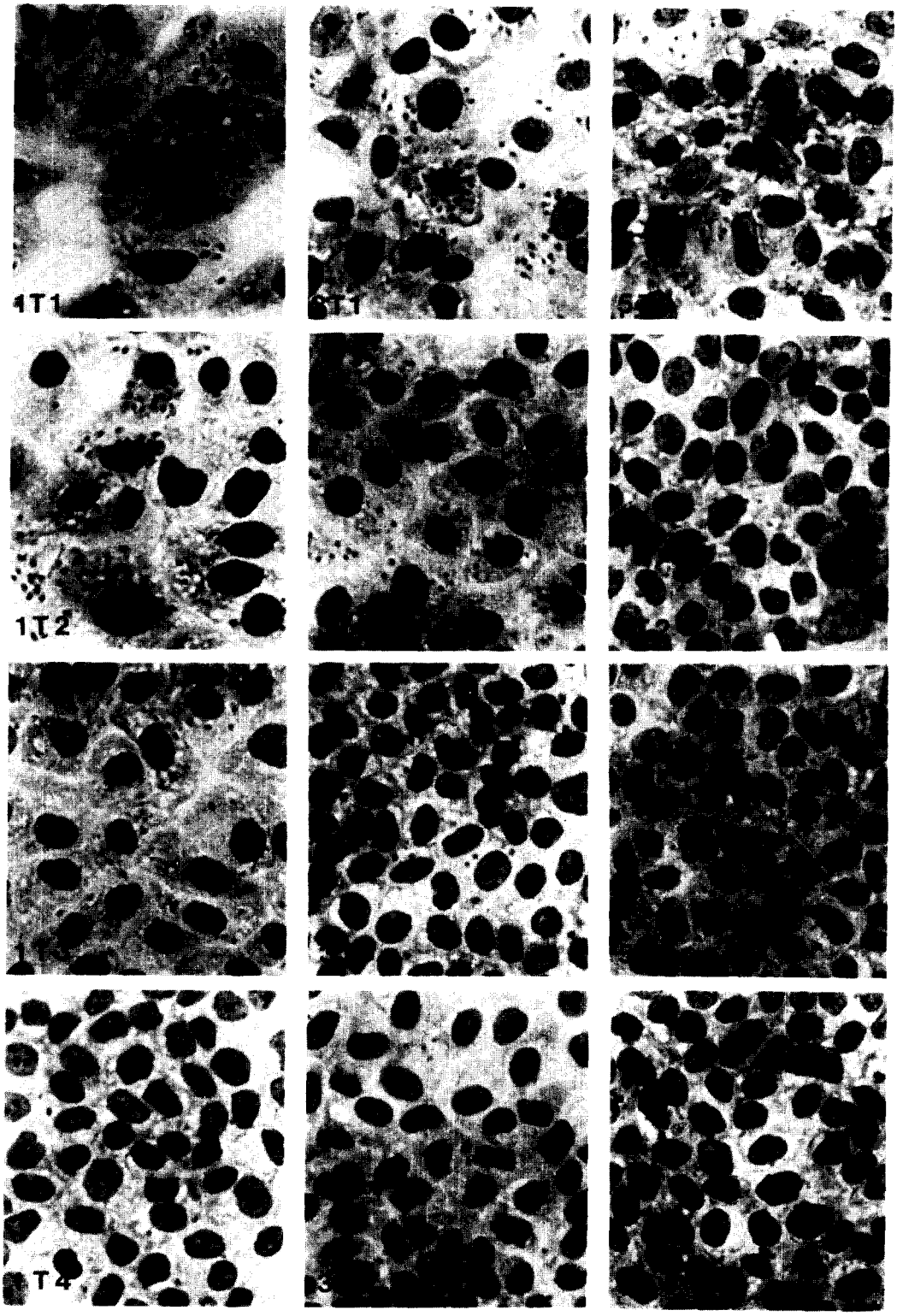


Fig. 2. Cell number-dependent entry of *Toxoplasma*. MDCK cells were seeded at densities of 1×10^5(—○—), 3×10^5(—●—), and 5×10^5 (—□—) cells/ml. After incubation for 60 min with excess number of parasites intracellular parasites were counted.


1T1
1T2
T4

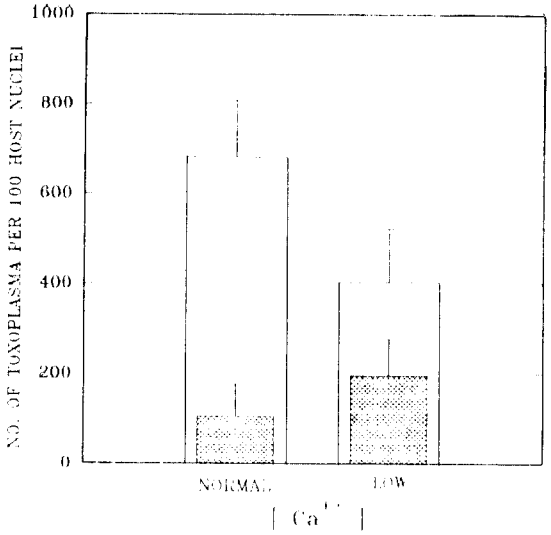


Fig. 4. Effect of calcium depletion on the entry of *Toxoplasma*. MDCK cells were seeded with densities of 1×10^5(□) and 5×10^5 (▨) cells/ml, grown in normal media or LC media, and then *Toxoplasma* was added.

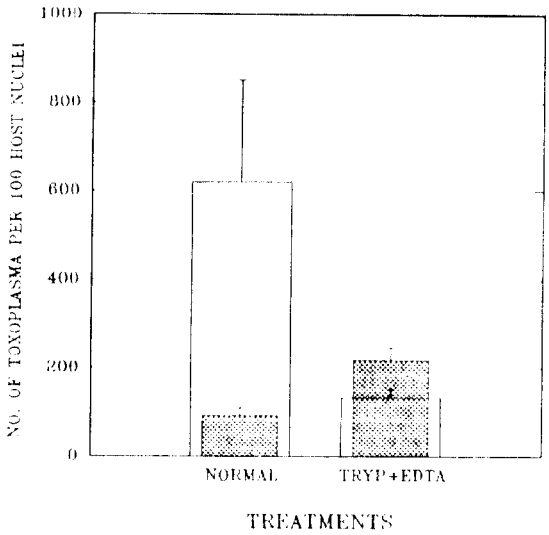


Fig. 5. Effect of trypsin-EDTA on the entry of *Toxoplasma*. MDCK cells were seeded with densities of 1×10^5(□) and 5×10^5 (▨) cells/ml, treated with trypsin-EDTA or not, and then *Toxoplasma* was added.

seeding density of 1×10^5 cells/ml as so-called 'non-saturated' culture and those of 5×10^5 cells/ml as 'saturated'.

Effect of calcium depletion on the entry of *Toxoplasma*: It was revealed that the tight junctions formed between adjacent MDCK cells inhibited host cell entry of *Toxoplasma*. To assess this result it was performed whether the prevention from formation of tight junctions in the saturated culture favored the invasion of *Toxoplasma* into MDCK cells. Cells were grown in LC media to eliminate the tight junctions. As shown in Fig. 4, the number of intracellular parasites increased significantly in the saturated culture when the cells had been grown in LC media by about 2-fold($p<0.05$) compared to the cells grown in normal media. On the contrary parasites penetrated host cells decreasingly by half in the non-saturated culture.

Effect of trypsin-EDTA on the entry of *Toxoplasma*: It was also verified that the elimination of the tight junctions promoted the invasion of parasites by digesting the tight junctions with trypsin-EDTA (Fig. 5). The number of intracellular parasites increased significantly by about 2.5 fold ($p<0.05$) in the saturated culture after mild trypsinization of the culture for 1 min, while those of non-saturated culture decreased to about one fifth.

DISCUSSION

MDCK cells might be deserved as a host cell to study the entry of *Toxoplasma* because of the accomplishment of mass penetration in relatively short times and easiness to identify parasites penetrated with conventional staining method such as Giemsa. With the results in

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Fig. 3. Photographs of MDCK cells penetrated by *Toxoplasma*. The number of *Toxoplasma*(black dots in the cytoplasms but not in nuclei of MDCK cells) decreased rapidly as the host cell reached saturation density. 1T1, at day 1; 1T2, at day 2; 1T3, at day 3; and 1T4, at day 4 after seeding with density of 1×10^5 cells/ml. 3T1, at day 1; 3T2, at day 2; 3T3, at day 3; and 3T4, at day 4 after seeding with density of 3×10^5 cells/ml. And 5T1, at day 1; 5T2, at day 2; 5T3, at day 3; and 5T4, at day 4 after seeding with density of 5×10^5 cells/ml.

this study we could conclude the tight junctions which were formed between adjacent cells in saturated culture inhibited the invasion of *Toxoplasma*. Therefore it might be suggested that *Toxoplasma* penetrated into MDCK cells through membrane structure-specific, *i.e.*, certain kind of receptors present in the basolateral surface rather than the apical.

There were notable differences in the number of intracellular parasites as the host cells became 'saturated' as shown in Fig. 2 and Fig. 3. There were many studies on the development of tight junctions(Madara, 1989; Rodriguez-Boulan and Nelson, 1989; Behrens *et al.*, 1989; Parry *et al.*, 1990). It was pointed out that as the cells grew to 'saturated' state of which the density of cells were $4\sim5\times10^5$ cells/ml so the tight junctions were formed between adjacent cells. Many researchers have studied on the polarity of MDCK cells which characterized surface membranes of the cells to be differentiated into domains such as apical and basolateral (Nelson and Veshnock, 1986; Fuller and Simons, 1986; Salas *et al.*, 1988; Bivic *et al.*, 1990; Wandinger-Ness *et al.*, 1990). It was suggested that the invasion of *Toxoplasma* be related with the formation of tight junctions or polarity of MDCK cells because the penetration of parasites was reduced with the generation of tight junctions. Deduced from the results of cell number-dependent entry, it was also suggested that the entry of parasites occur through a membrane structure-specific mode, that is, certain kind of receptors which are distributed randomly in 'non-saturated' but restrictly in basolateral membrane in 'saturated' might be concerned with the entry of parasites.

To assess the inhibitory role of tight junctions on the invasion of *Toxoplasma* we evaluated the degree of penetration after elimination of the tight junctions by calcium depletion in 'saturated' culture. Tight junctions of MDCK cells were very sensitive to the concentration of calcium reversely(Nelson and Veshnock, 1987). While the number of *Toxoplasma* was reduced in 'non-saturated' culture by shifting the concentration of calcium to 5 μM instead of 1.8 mM, those of 'saturated' culture was increased significantly as shown in Fig. 4. It was suggested that calcium deprivation in 'saturated' culture abolished tight junctions, which favored the entry of parasites. We thought that calcium played a role on the entry of *Toxoplasma*(as depicted by 'non-saturated' culture) as well as on the destruction of tight junctions, which resulted in less increase of invasion of parasites in 'saturated' culture than expected. Cesbron-Delauw *et al.*(1989) and Leriche and Dubremetz (1990) reported the role of calcium binding proteins on and after host cell invasion.

It was further verified that the elimination of tight junctions favored the penetration of *Toxoplasma* by treatment with trypsin-EDTA. When cells of 'saturated' were digested mildly with trypsin-EDTA, the number of intracellular parasites was increased significantly as shown in Fig. 5. But when cells of 'non-saturated' were treated, the number of intracellular parasites was decreased enormously. These suggested that mild trypsinization digest tight junctions of MDCK cells, therefore *Toxoplasma* could contact with receptors present in basolateral membrane which might be digested more in 'non-saturated' culture.

There had been many reports to support the existence of host cell surface receptors in other protozoan species in view of the parasites, manifestation to prefer some host cells, the alterations of capacity to associate with certain host cells by mild enzymatic treatments, and the inhibitory association by prior binding of lectin to the host cell surface of modification of sialoglycoproteins of host cells. Although most reports did not characterize the host cell surface receptors for specific species exclusively, it might be meaningful to propose the concept of receptor-ligand interactions in the course of cell entry by parasitic protozoa. In this study we could also suggest the existence of receptors for *Toxoplasma*. We wish to investigate furthermore the receptor for this parasite in respect to purification, biochemical characterization, and

cellular physiology of the molecule.

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=국문초록=

MDCK세포의 tight junction 형성이 *Toxoplasma gondii*의 숙주세포 침투에 미치는 효과

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MDCK세포간에 형성된 tight junction이 *Toxoplasma gondii*의 숙주세포 침투에 미치는 영향에 대하여 고찰하기 위해 여러 조건의 세포배양을 시행하였다. MDCK세포를 25 well 배양기 내의 18-mm cover glass에 1×10^5, 3×10^5 및 5×10^5 개로 분주한 후 배양 1, 2, 3 및 4일에 다수의 *Toxoplasma*를 접가하여 1시간 동안 배양한 다음 Giemsa 용액으로 염색한 후 관찰하였을 때, 각 실험군에서 침투한 원충의 숫자는 숙주세포가 포화밀도를 이루면서 급격히 감소하였다. 배양액 내의 calcium 농도를 일반적인 1.8 mM에서 5 μM로 낮추어 포화밀도의 MDCK 세포간에 tight junction 형성을 억제하였을 때, 원충의 침투가 약 2배 증가하였으며($p<0.05$) 포화밀도 이전의 배양에서는 원충의 침투가 감소하였다. Trypsin-EDTA를 처리하여 포화밀도의 배양에서 tight junction을 소화시킨 경우, 원충의 침투가 약 2.5배 증가하였으며 ($p<0.05$) 포화밀도 이전의 배양에서는 급격히 감소하였다.

이상의 결과들로 볼 때, 포화밀도의 MDCK세포간에 형성된 tight junction이 *Toxoplasma*의 침투를 억제하는 것을 알 수 있었다. 이는 *Toxoplasma*가 숙주세포로 침투할 때 숙주세포의 막구조에 대한 특이성이 있음을 시사하며, 상피세포에서는 tight junction 위의 막(apical membrane) 보다 옆 및 아래의 막(basolateral membrane) 상의 구조를 이용하는 것으로 추정되었다.

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