기 생 충 학 잡 지 제29권 제 2 호
The Korean Journal of Parasitology
Vol. 29, No. 2, 121-128, June 1991

Cell cycle-dependent entry of *Toxoplasma gondii* into synchronized HL-60 cells

Ji-Hye Youn, Ho-Woo Nam, Dong-Jin Kim, Yu-Moon Park, Woon-Kyu Kim, Woon-Sik Kim and Won-Young Choi

Catholic Institute of Parasitic Diseases, Catholic University Medical College, Seoul 137-701, Korea

Abstract: The degree of attraction of *Toxoplasma gondii* to vertebrate cells varies with cell type and cell phase. Human promyelocytic leukemia cells, HL-60, were synchronized by double thymidine block method and co-cultured with *Toxoplasma* for 1 hr at each cell stage to investigate the cell cycle specific susceptibility of parasites to host cells.

For 30 hr the average number of *Toxoplasma* that invaded was a little changed except at 3 hr from G_1/S phase boundary which concurred with the peak point of DNA synthesis. At 3 hr which is a relatively short interval compared to whole S phase, modification of cells by parasitic invasion was most remarkable. The number of *Toxoplasma* that penetrated was increased to more than six times. The shape of the cells became sludgy and almost indiscernible by strong accessibility of parasites only for an hour of mid-S phase. The same fluctuation was also observed at the second peak of S phase but weakly.

This suggests that there be surface molecules concerning with the attachment of *Toxoplasma* to the host cells, which is expressed at special point of S phase. Further studies on the specific protein or similar molecules related could be carried out using synchronized HL-60 cells.

Key words: *Toxoplasma gondii*, *in vitro* culture, HL-60 cell, synchronization, double thymidine block, cell cycle dependence

INTRODUCTION

Toxoplasma gondii, an obligate intracellular protozoan parasite that causes human and animal toxoplasmosis, is able to infect many vertebrate cells *in vitro* with very low cell type specificity, such as HL-60 cells (Kim and Choi, 1988), MDCK cells (Nam *et al.*, 1990), Vero cells (Srinivasa *et al.*, 1982), HeLa cells (Valkoun, 1983), ovine fetal kidney cells (Chang and Gabrielson, 1984), a macrophage cell line J774GS (Siebley *et al.*, 1985), a human epidermoid larynx cell line HEp-2 (Hermentin and Aspock, 1987) and a murine tumor cell line YAC-1 (Noriega and Hauser, 1988), of which the mechanisms of entry are not well established.

The degree of attraction of *Toxoplasma* and other protozoan parasites to vertebrate cells varies with cell type and culture conditions (Endo and Yagita, 1990; Schenkman *et al.*, 1991). The process of penetration of protozoan parasites begins with their attachment to the host cell surface, followed by their interiorization. *Plasmodium vivax* and *P. knowlesi* enter the erythrocytes only with

Duffy antigen determinants and Duffy receptor is required in the interiorization phase of both *Plasmodium* species into their hosts (Miller and McGinniss, 1975; Adams *et al.*, 1990). In addition it has been described that host cell membrane receptors play a role in the invasion of mammalian cells by other protozoan parasites although there is little specific information available concerning host cell surface molecules (De Carvalho and De Souza, 1989; Davis and Kuhn, 1990).

In respect of membrane receptors some of surface molecules are expressed in a cell cycle related manner. As vertebrate cells progress through the cell cycle, phase-dependent surface antigen levels increase and decrease, for example, major envelope protein of murine leukemia virus in T-lymphoma cells (Reyero and Waksman, 1980), and H-2 histocompatibility complex and surface antigens of moloney leukemia virus in mouse cell lines (Cikes and Friberg, 1971). Therefore, the influence of host cell cycle on susceptibility to infection could elucidate the surface molecules of the host cells and reveal the exact entry mechanism of *Toxoplasma* relating with broad range of host cells.

In this study we have examined whether a modulation of the attachment and subsequent entry of *Toxoplasma* is dependent on the growth stage of the human promyelocytic leukemia cells, HL-60, synchronized by double thymidine block method.

MATERIALS AND METHODS

Culture conditions: Virulent tachyzoites of RH strain of *Toxoplasma gondii* were maintained and propagated by continuous passage in ICR mice and were extracted from the peritoneal exudate. After extraction, the peritoneal exudate could be stored at 4°C for a week and longer.

HL-60 cells were maintained in Earle's MEM supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES, pH 7.4. Cells were grown at 37°C in humidified 95% air/5% CO_2 atmosphere. Cells were subcultured weekly at a seeding density of 3×10^5 cells/ml.

Synchronization of HL-60 cells: HL-60 cells were synchronized with respect to the cell cycle by double thymidine block method (Studzinski and Lambert, 1968; Volpe and Eremendo-Volpe, 1970; Tarella *et al.*, 1982). Cells with a density of 5×10^5 cells/ml were incubated for 24 hr in medium containing 2 mM thymidine, and then washed and resuspended in thymidine free medium for about 10 hrs until dividing cells were predominant under phase contrast microscope (AO, American Optical). They were again blocked with medium containing 2 mM thymidine for 16 hr and resuspended in fresh medium, leading to a density of 5×10^5 cells/ml. Briefly after such treatment the cells entered the synthetic (S) phase.

The rate of synchrony was monitored by determination of the degree of DNA synthesis, mitotic index (MI), and cell number at each time which was divided 13 intervals for 30 hr covering a full cell cycle. DNA synthesis was estimated by incorporation of [^{3}H]-thymidine. Cells were inoculated in 96-well plate and incubated with [^{3}H]-thymidine (1 μCi/well) for 1 hr. Then the cells were ruptured and harvested onto a filter paper using cell harvester (Titertek Co.). Radioactivity was measured in liquid scintillation counter (Kontron Co.). The MI was determined by the ratio of mitotic figures on a smear prepared with cytospin centrifuge (Shandon) and stained with Giemsa.

Cell cycle-dependent entry of *Toxoplasma*: Synchronized HL-60 cells with a density of 5×10^5 cells/ml were aliquoted to culture dish and co-cultured with 10 fold number of *Toxoplasma* for 1 hr. At each time interval the cells co-cultured with *Toxoplasma* were smeared by cytospin centrifuge and stained with Giemsa solution. The number of *Toxoplasma* which penetrated was estimated by counting about 1,000 host cells and *Toxoplasma* in their cytoplasm under light microscopy of ×400. Data were calculated as the number of intracellular

parasites per 100 host cells.

RESULTS

The high concentration of thymidine inhibits DNA synthesis and thus arrests cell progression through the S phase of the cell cycle, while cells in the G_2, M, and G_1 phases are not affected by thymidine and thus accumulate the cells at the G_1/S phase boundary (Studzinski and Lambert, 1968). As excess thymidine has severe cytotoxic effects, the optimal concentration used in cell synchronization is 2 mM, which produces approximately 50% inhibition of DNA synthesis although some heterogeneity of cells appears as the time progresses towards S and G_2 phases.

As depicted in Fig. 1, [^{3}H]-thymidine incorporation reached a peak at 3 hr and 19 hr (A), MI peak at about 9 hr (B) and cell density

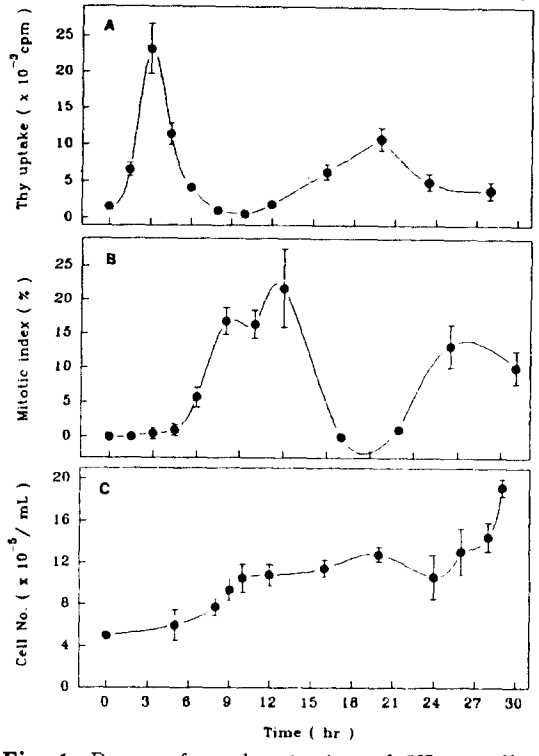

Fig. 1. Degree of synchronization of HL-60 cells. A : [^{3}H]-thymidine uptake of HL-60 cells which reached peaks at 3 hr and 19 hr, B : Mitotic index which increased from 8 hr, and C : Increase of cell number.

was doubled at about 10 hr (C) and the total cell cycle time was 17 hr. The length of each phase was estimated in this experimental conditions, of which the S phase was 4 hr; G_2, 5.5 hr; M, 1.5 hr and G_1, 6 hr (Volpe and Eremento-Volpe, 1970). Briefly after thymidine was removed from the media, cells started to progress through the synthetic phase but [^{3}H]-thymidine was less incorporated at initial time probably because the cellular level of thymidine was high enough not to absorb exogenous thymidine. Although the second peak of DNA synthesis was widely spread to permit its precise determination, the sharp first peak of S phase and subsequent M phase clearly demonstrated the high rate of synchrony achieved by double thymidine block method, which is easy to perform compared to other methods (Clowick and Kaplan, 1979; Yen and Albright, 1984).

To investigate the cell stage dependency the kinetics of the cell cycle modulation of entry by *Toxoplasma* was described when the synchronized population of HL-60 cells were exposed to *Toxoplasma* (Fig. 2). For about 30 hr the average number of parasites that invaded into the synchronized HL-60 cells was a little varied except at 3 hr of mid-synthetic phase. Approximately one parasite was invaded into one host cell in a uniform pattern and the shape of the cells was not changed by the penetration (Plate I), which distinguish HL-60 cells from

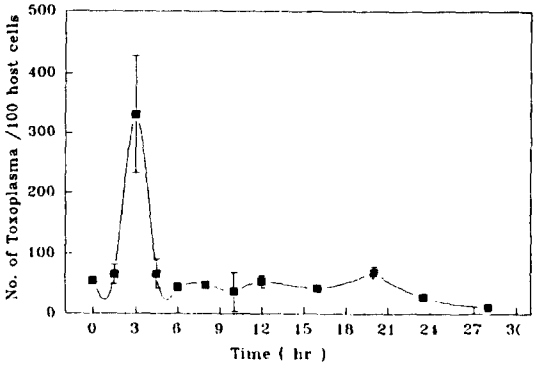

Fig. 2. Cell cycle-dependent entry of *Toxoplasma* into synchronized HL-60 cells. After coculture for 1 hr the number of intracellular parasites were counted at each time interval.

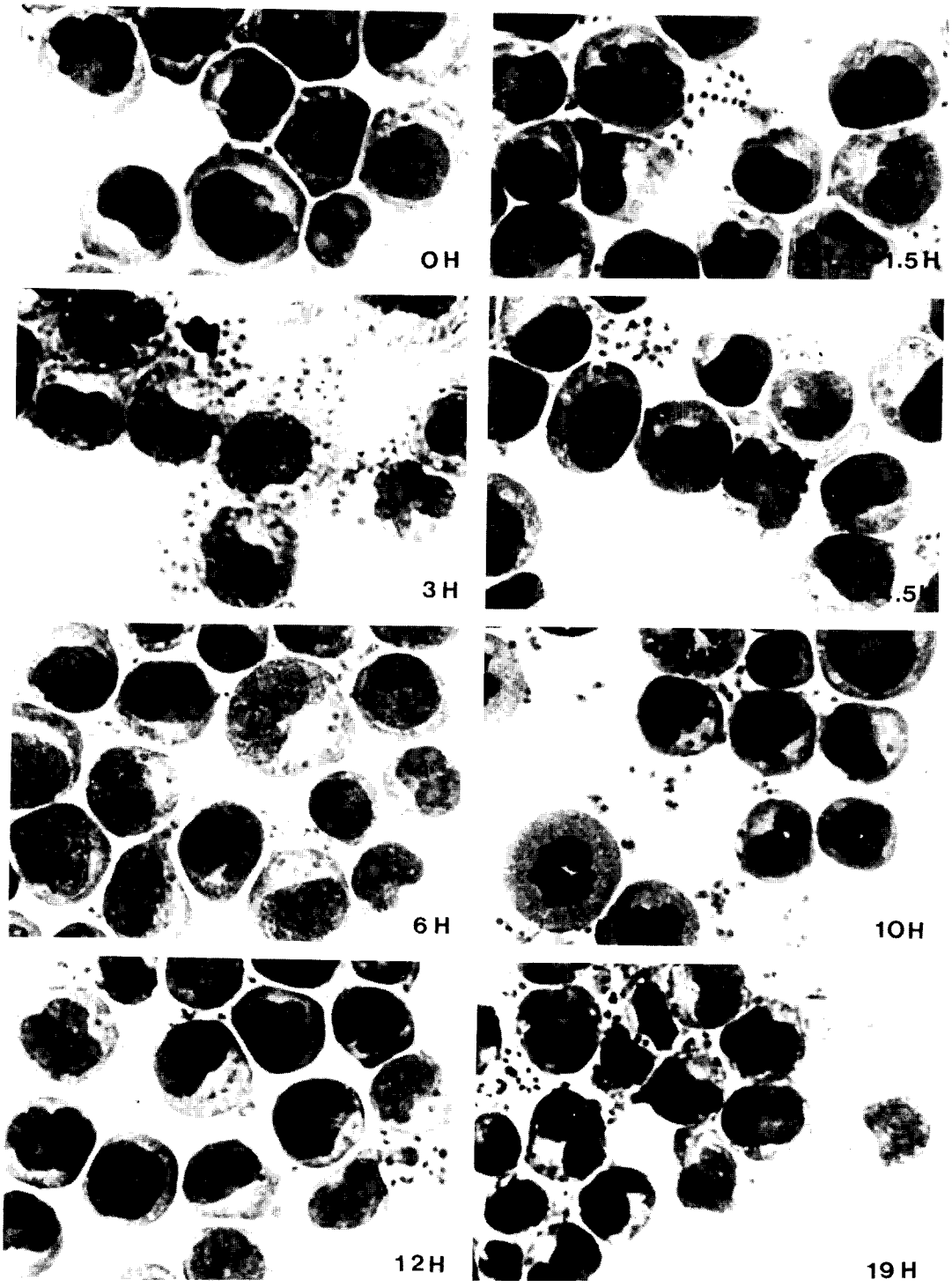

Plate I. Giemsa stained photographs of synchronized HL-60 cells co-cultured with *Toxoplasma* at each cell stage, which showed cell features at 0, 1.5, 3, 4.5, 6, 10, 12 and 19 hr from G_1/S boundary.

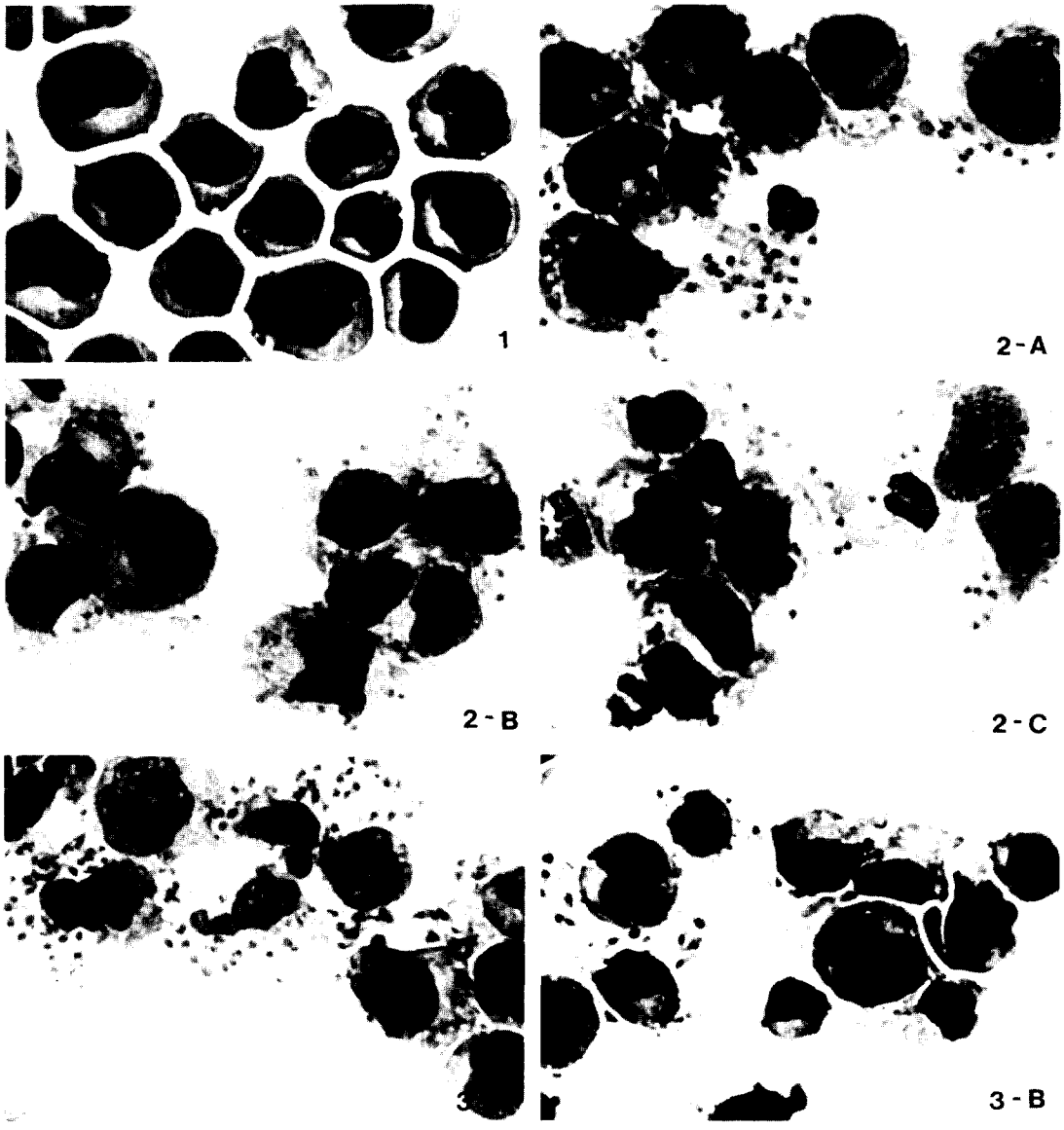

Plate II. Giemsa stained photographs of characteristic patterns of *Toxoplasma* invasion at mid-S phase. 1 : synchronized HL-60 cells only, 2 : HL-60 cells invaded by *Toxoplasma* at mid-S phase, 3 : HL-60 cells invaded at second peak of DNA synthesis.

other cell lines massively infected, such as HeLa and MDCK cell. But at 3 hr from G_1/S phase boundary the number of parasites that penetrated was increased to more than six times and this has a significant implication that this stage concurred with the peak point of DNA synthesis. Whereas at 1.5 and 4.5 hr near the 3 hr the number and the shape was almost the same as that in M, G_1 and G_2 phases.

The modification of cells at 3 hr was most remarkable not only in their average number of parasites but also in their microscopic morphology only when the cells were co-cultured with *Toxoplasma* (Plate II). By microscopic observations synchronized HL-60 cells which was not attacked at this stage were shown and

their boundary was clear. Comparatively in co-cultured HL-60 of mid-S phase with *Toxoplasma* most parasites were very accessible to cytoplasmic membrane of HL-60 (Plate II, 2-A). The cells protruded cytoplasmic pseudopods and their membrane lost exact boundary showing almost sludged and indiscernible features from the cell membrane of *Toxoplasma*, which made it difficult to count the parasites inside the cell cytoplasm discriminating *Toxoplasma* not infected (Plate II, 2-B). Some cells were ruptured by massive invasion and free nuclei were scattered reducing the density of the cells (Plate II, 2-C). The characteristic at 3 hr was the case of the second peak of S phase (Plate II, 3-A and B) but accurate time of the fluctuation was varied and the morphology was less remarkable than the first one because the cell state might become heterogeneous gradually. This suggested that certain changes during the mid-S phase only for about 1 hr induce strong interactions between host cells and *Toxoplasma*. At least it seems that the state and the density of *Toxoplasma* did not affect the process, because we also attempted the experiments repeatedly with various density and stages of *Toxoplasma* (data not shown).

DISCUSSION

It has long been studied that the cell cycle modulates infection by *Toxoplasma* or by other protozoan parasites (Dvorak and Crane, 1981), and in this process the cell membrane receptor should be mediated although specific information is not yet available. HL-60 cells are invaded by less number of *Toxoplasma* than others such as HeLa and MDCK cells, and therefore the variation of parasite numbers penetrating into host cells is easily detected as the cell cycle progressed. In this experiment not only changes of the number but also of microscopic morphology at certain point of S phase were simply distinguished from that at other points of S phase and were very crucial as the time prolonged was so short. *Toxoplasma* showed strong affinity to the host cell membrane of mid-S phase specifically. According to the results the cell cycle of HL-60 has an uncommon implication closely related with the invasion process whether it is caused by cell surface molecules expressed only for a short interval or by other unknown factors.

In respect of surface molecules concerning with *Toxoplasma* invasion laminin binding proteins appear to be involved in the attachment of tachyzoites to the host cells (Joiner *et al.*, 1989 & 1990; McLeod *et al.*, 1991) and cholesterol might also be used for its attachment, which is ubiquitous and conform its low specificity to host cells, but this has not been identified. In addition, it has been reported that the attachment process is polarized and mediated by receptors located in the basolateral domain of epithelial cells, MDCK (Nam *et al.*, 1990; Schenkman *et al.*, 1991). Besides the above explanations, considering cell surface molecules Endo and Yagita (1990) asserted that alteration of extracellular ion concentration affect the motility of *Toxoplasma*, inhibiting and enhancing the invasion. Thus as cell cycle progresses pH fluctuation might have some significance in addition to membrane receptors.

De Carvalho and De Souza (1990) analyzed that phagocytosed *Toxoplasma* was internalized with some membrane components of host cells. *Toxoplasma* stimulates the enzyme activity, for example, Na^+, K^+-ATPase during the initial attachment phase. This activity remains inside the parasitophorous vacuole associated with fusion incompetence while some enzymes of the surface membrane excluded for the parasite survival. In this respect we could suppose that many surface molecules be participated in parasitic way of life such as accessment, attachment, interiorization and survival.

Live *Toxoplasma* acquires their intracellular habitat primarily by active invasion of host cells not by phagocytosis (Nichols and O'Connor, 1981; Endo and Yagita, 1990) excluding some exceptional cases. In such invasion process breakage of plasma membrane of host cells is

induced by penetration enhancing factor (PEF) secreted from rhoptry organelles of *Toxoplasma* and then vacuolization of parasite begins (Lycke *et al.*, 1975; Hennere and Nichols, 1983; Nichols *et al.*, 1983). At the specific point of S phase the plasma membrane of HL-60 cells was crumbled, and almost fused with that of *Toxoplasma* especially, which might be induced by PEF. The stage of *Toxoplasma* did not affect the procedure when we used *Toxoplasma* stored at 4°C for 1 or 3 days. The change of HL-60 cells at S phase might induce the secretions from rhoptries.

To analyze accurate molecular interactions between *Toxoplasma* and host cells further studies are expected such as protein analysis, componential western blotting with *Toxoplasma* antibody and transmission electron microscopy using synchronized HL-60 cells.

REFERENCES

Adams, J.H., Hudson, D.H., Motomi, T., Ward, G.E., Aikawa, M. and Miller, L.H. (1990) The Duffy receptor family of *Plasmodium knowlesi* is located within the micronemes of invasion malaria merozoites. *Cell*, **63**:141-153.

Chang, G.N. and Gabrielson, D.A. (1984) *Toxoplasma gondii*: Growth in ovine fetal kidney cell culture. *Exp. Parasit.*, **57**:81-85.

Cikes, M. and Friberg, S. (1971) Expression of H-2 and Moloney leukemia virus-determined cell-surface antigens in synchronized cultures of a mouse cell line. *Proc. Natl. Acad. Sci. USA*, **68**:566-569.

Colowick, S.P. and Kaplan, N.O. (1979) Methods in enzymology. Vol. 58. cell culture. pp. 248-262. Academic press.

Davis, C.D. and Kuhn, R. (1990) Selective binding of *Trypanosoma cruzi* into host cell membrane polypeptides. *Infect. Immun.*, **58**:1-6.

De Carvalho, L. and De Souza, W. (1989) Cytochemical localization of plasma membrane enzyme markers during interiorization of tachyzoites of *Toxoplasma gondii* by macrophages. *J. Protozool.*, **36**(2):164-170.

Dvorak, J.A. and Crane, M. (1981) Vertebrate cell cycle modulates infection by protozoan parasites. *Science*, **214**:1034-1036.

Endo, T. and Yagita, K. (1990) Effect of extracellular ions on motility and cell entry in *Toxoplasma gondii*. *J. Protozool.*, **37**(2):133-138.

Hennere, E.P. and Nicholas, G. (1983) Are rhoptries of coccidia really extrusomes? *J. Ultrastruct. Res.*, **84**:194-203.

Hermentin, K. and Aspock, H. (1987) Higher yields and increased purity of *in vitro* grown *Toxoplasma gondii*. *Zbl. Bakt. Hyg.*, **A267**:272-276.

Joiner, K.A., Furtado, G., Mellman, I., Kleiman, H., Miettinen, H., Kasper, L.H., Hall, L. and Fuhrman, S.A. (1989) Cell attachment and invasion by tachyzoites of *Toxoplasma gondii*. *J. Cell. Biochem.*, **13E**:64.

Joiner, K.A., Fuhrman, S.A., Miettien, H.M., Kasper, L.H. and Mellman, I. (1990) *Toxoplasma gondii*: Fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science*, **249**:641-646.

Kim, K.A. and Choi, W.Y. (1989) *In vitro* culture of *Toxoplasma gondii* and changes of the membrane proteins in HL-60 cells. *J. Catholic Med. Coll.*, **42**:407-418.

Lycke, E., Carlberg, K. and Norrby, R. (1975) Interactions between *Toxoplasma gondii* and its host cells: Function of the penetration-enhancing factor of *Toxoplasma*. *Infect. Immun.*, **11**(4):853-861.

McLeod, R., Mack, D. and Brown, C. (1991) *Toxoplasma gondii*—New advances in cellular biology. *Exp. Parasit.*, **72**:109-121.

Miller, L.H. and McGinniss, M.H. (1975) Erythrocyte receptors for (*Plasmodium knowlesi*) malaria: Duffy blood group determinants. *Nature* (*London*), **15**:561-563.

Nam, H.W., Youn, J.H., Kim, D.J. and Choi, W.Y. (1990) Tight junctional inhibition of entry of *Toxoplasma gondii* into MDCK cells. *Korean J. Parasit.*, **28**(4):197-205.

Nichols, B.A. and O'Connor, R. (1981) Penetration of mouse peritoneal macrophages by the protozoon *Toxoplasma gondii*. *Lab. Invest.*, **44**(4):324-335.

Nichols, B.A., Chiappino, M.L. and O'Connor, G.R. (1983) Secretion from the rhoptries of *Toxoplasma gondii* during host-cell invasion. *J. Ultrastruct. Res.*, **83**:85-98.

Noriega, F.R. and Hauser, W.E. (1988) *Toxoplasma gondii* maintenance in tissue culture: A new efficient method for culturing RH tachyzoites. *J. Parasitol.*, **74**:495-499.

Reyero, C. and Waksman, B.H. (1980) Cell cycle-dependent expression of surface antigen in murine T-lymphoma cells. *Exp. Cell Res.*, **130**:275-280.

Schenkman, S., Diaz, C. and Nussenzweig, V. (1991) Attachment of *Trypanosoma cruzi* trypomastigotes to receptors at restricted cell surface domains. *Exp. Parasit.*, **72**:76-86.

Siebley, L.D., Krahenbuhl, J.L. and Weidner, E. (1985) Lymphokine activation of J774G8 cells and mouse peritoneal macrophages challenged with *Toxoplasma gondii*. *Infect. Immun.*, **49**:760-764.

Srinivasa, H., Mithal, S. and Balaya, S. (1982) Maintenance of *Toxoplasma gondii* in Vero cell line. *Indian J. Med. Res.* **75**:521-528.

Studzinski, G.P. and Lambert, W.C. (1968) Thymidine as a synchronizing agent 1. Nucleic acid and protein formation in synchronous HeLa cultures treated with excess thymidine. *J. Cell. Physiol.*, **73**:109-118.

Tarella, C., Ferrero, D., Gallo, E., Pagliardi, G.L. and Ruscetti, F.W. (1982) Induction of differentiation of HL-60 cells by dimethyl sulfoxide: Evidence for a stochastic model not linked to the cell division cycle. *Cancer Res.*, **42**:445-449.

Valkoun, A. (1983) Continual cultivation of *Toxoplasma gondii* on HeLa cells. *Folia Parasit.*, **30**: 289-294.

Volpe, P. and Eremendo-Volpe, T. (1970) A method for measuring the length of each phase of the cell cycle in spinner's cultures. *Exp. Cell Res.*, **60**:456-458.

Yen, A. and Albright, K.L. (1984) Evidence for cell cycle phase-specific initiation of a program of HL-60 cell myeloid differentiation mediated by inducer uptake. *Cancer Res.*, **44**:2511-2515.

=국문초록=

세포 주기 변화에 따른 *Toxoplasma gondii*의 침투 양상

가톨릭의대 기생충병연구소

윤지혜 • 남호우 • 김동진 • 박유문 • 김운규 • 김운식 • 최원영

*Toxoplasma gondii*를 HL-60 세포와 함께 *in vitro* 배양시 *Toxoplasma* 침투 정도가 각 세포에서 균일하지 않으므로 세포의 주기에 따른 일정 phase가 그 침투에 좋은 환경을 제공할 것이라는 추론으로 HL-60 세포 주기를 동시화(synchronization)하여 각 stage에서 변화를 관찰하였다.

동시화는 과량의 thymidine이 DNA 합성을 억제함을 이용하여 2 mM thymidine을 10시간 간격으로 각 24, 18시간 동안 처리하여 (double thymidine block method) S (synthetic) phase를 진행하는 세포를 얻었고 이후 30시간 동안 13회의 간격을 두고 5×10^6/ml의 *Toxoplasma*를 첨가하여 1시간 동안 배양하였다. 숙주세포의 동시화 정도는 (1) ^{3}H-thymidine의 표지량 (2) mitotic index 측정 및 (3) 세포수의 증가를 통해 확인하였다.

*Toxoplasma*의 침투 정도는 S phase 중에서도 배지에서 thymidine을 제거한 후 3시간 경과시가 그 전후에 비해 6배 이상 높았으며 특히 이 시기는 DNA 합성이 최고가 되는 점과 일치하였다. 침투된 *Toxoplasma* 수의 변화 외에 세포의 모양도 상당한 변화가 있었고 이는 19시간 후 2번째 S phase에서도 약하나마 관찰되었다. 실험 결과를 통해 특정 약 1시간 동안 일어나는 어떤 세포의 변화가 *Toxoplasma* 침투에 중요한 역할을 한다는 것을 알 수 있었다. 이는 원충 기생충의 숙주세포 흡착과 interiorization 과정에 receptor가 관련되고 몇 receptor는 세포주기에 따라 발현이 조절되는 사실로부터 G_1/S 경계부터 3시간째에 발현되면서 *Toxoplasma*를 유인하는 receptor molecule의 존재 가능성을 시사하였다.

〔기생충학잡지, 29(2):121-128, 1991년 6월〕