Toxoplasmacidal Effect of HL-60 Cells Differentiated by Dimethylsulfoxide*

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Abstract: *In vitro* culture of *Toxoplasma gondii* in HL-60 cells and cell-mediated immunity against *Toxoplasma* in dimethylsulfoxide(DMSO)-induced HL-60 cells, *i.e.*, differentiation into granulocytes, were pursued. HL-60 cells were treated with various concentrations of DMSO, and 1.3%(v/v) for 3 day incubation was chosen as the optimal condition for differentiation into granulocytes. The degree of differentiation was assayed in physiological and functional aspects in addition to morphological point. When treated with 1.3% DMSO for 3 days, HL-60 cells did not synthesize DNA materials beyond background level, and showed active chemotactic response to chemotactic peptide, formyl-methionyl-leucyl-phenylalanine(FMLP). Morphologically promyelocytes of high nuclear/cytoplasmic(N/C) ratio changed to granulocytes of relatively low N/C ratio. The relationships between HL-60 cells or DMSO-induced HL-60 cells and *Toxoplasma* were examined after stain with Giemsa and fluorescent dye (acridine orange). HL-60 cells did not show any sign of toxoplasmacidal activity but showed intracellular proliferation of *Toxoplasma* to form rosette for 72 hr co-culture. In contrast, DMSO-induced HL-60 cells phagocytosed *Toxoplasma* within 1 hr, and performed a process of intracellular digestion of *Toxoplasma* thereafter.

With the above results, it is suggested that phagosome-lysosome fusion is one of the critical events for the parasitism by *Toxoplasma* or for susceptibility of host cells. The *in vitro* culture system of this study has offered a defined condition to study the protozoan parasite-host cell interactions.

Key words: *Toxoplasma gondii*, HL-60 cells, toxoplasmacidal effect, dimethylsulfoxide, *in vitro* culture

INTRODUCTION

Toxoplasma gondii is an obligatory intracellular protozoan parasite that infects mammals and birds with very low specificity(Levine, 1977; Choi *et al.*, 1987) and can cause abor-

tion, fetal abnormalities, or perinatal death (Krick and Remington, 1978). Recently, there have been many reports on this parasite which increased the morbidity and mortality of the immune compromised patients such as acquired immune deficiency syndrome(AIDS) (Anderson *et al.*, 1983; Luft *et al.*, 1984).

The importance of cell-mediated immunity in resistance to infection caused by intracellular pathogens has been well established. Neutro-

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phils or mononuclear phagocytes clearly play primary roles in the cellular control of such infections (McLeod and Remington, 1977; Rimoldi *et al.*, 1981). However, some intracellular pathogens, such as *T. gondii* (Jones and Hirsch, 1972), *Mycobacterium tuberculosis* (Hart and Armstrong, 1974), *Chlamydia psittaci* (Friis, 1972), and *Encephalitozoon cuniculi* (Niederhorn and Shadduck, 1980), have been reported to inhibit fusion of phagosomes with lysosomes. Others, such as *Leishmania* (Berman *et al.*, 1979) and *Mycobacterium lepraemurium* (Hart *et al.*, 1972), have been reported not to inhibit fusion and they are apparently able to survive within the normally inhospitable milieu of the phagolysosome. Still other intracellular pathogens such as *Trypanosoma cruzi* have been shown to avoid the unfavorable environment of the phagolysosome altogether by escaping into the host cell cytoplasm (Tanowitz *et al.*, 1975). Host cells in the above studies have almost invariably been mouse peritoneal macrophages or other mouse cell lines.

Recent availability of a stable human promyelocytic leukemic cell line (HL-60) which can be maintained in continuous culture (Collins *et al.*, 1977) has enabled the study on the function of neutrophils in the defence mechanism against intracellular pathogens. When cultured in the presence of dimethylsulfoxide (DMSO), HL-60 cells differentiate into neutrophils or granulocytes (Collins *et al.*, 1978) as shown by the increase of complement receptor or Fc receptor (Miyaura *et al.*, 1981) and by the acquisition of the capacity to respond to chemotactic stimuli (Niedel *et al.*, 1980). In this report we demonstrate the possibility of *in vitro* culture of *T. gondii* by using HL-60 cells as host cells, and the toxoplasmodicidal effect of HL-60 cells differentiated by DMSO.

MATERIALS AND METHODS

Parasite:

Virulent tachyzoites of RH strain of *T.*

gondii 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.

Cell Culture:

HL-60 cells were maintained in Earle's MEM (EMEM) medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 10 mM HEPES, pH 7.4. Cells were grown at 37°C in a humidified 95% air/5% CO₂ atmosphere. Cells were subcultured weekly at a seeding density of 3 × 10⁵ cells/ml.

Assessment of Cell Growth:

To examine the effect of DMSO on cell growth, 10 ml of cells at 3 × 10⁵ cells/ml were seeded in the presence of various concentrations of DMSO in culture medium. After shaking to loosen any adherent cells, aliquots of each well culture were removed every 24 hrs for 7 days and viable cells were determined by trypan blue exclusion method and counted in a hemocytometer.

DNA Synthesis and Chemotaxis:

DNA synthesis was determined by incorporation of ³H-thymidine. Two hundred µl of cell suspension was incubated at a concentration of 1 × 10⁶ cells/ml with ³H-thymidine in the 96 well culture plate (10 µCi/ml; specific activity, 5 Ci/ml) for 1 hr at 37°C. They were then harvested on the filter paper using cell harvester (Titertek. Co.), and counted the radioactivity in a liquid scintillation counter (Packard Co.).

Chemotaxis was assayed as described by Imaizumi and Breitman (1986) with 48-well microchemotaxis chamber (Neuro Probe Inc.) and with polyvinyl-pyrrolidone-free polycarbonate filters (5 µm pore). The lower compartment of the wells was filled with 25 µl of the chemotaxis buffer (PBS) containing various concentrations of formyl-methionyl-leucyl-phenylalanine (FMLP), and the upper compartment of the wells was filled with 40 µl of a cell suspension (2 × 10⁶ viable cells/ml in EMEM medium). The upper and lower compartments were

separated by the filter. The chamber was incubated for 1 hr at 37°C in a humidified atmosphere of 5% CO₂ in air. After incubation the cells on the upper surface of the filter were removed gently by scraping the filter with a wiper blade. The filters were fixed in methanol and stained with Giemsa. The number of cells that migrated to the underside of the filter was counted under $\times 400$ microscope.

Fluorescence Assay of Phagosome-Lysosome Fusion:

Phagosome-lysosome fusion (P-L fusion) in the HL-60 and DMSO-differentiated HL-60 cells was assessed as described by Hart and Young (1975) with some modifications. Cells were exposed at 37°C in EMEM medium to acridine orange (AO; 5 $\mu\text{g}/\text{ml}$) for 30 min. Cells were washed and resuspended in a fresh medium, then the same number of *T. gondii* was added. Cells were examined by fluorescent microscopy (Nikon) after the preparation of slide by centrifugation at 7,000 rpm for 5 min with cytopsin 2 (Shandon) and by light microscope after stained with Giemsa.

RESULTS

Assessment of Cell Growth:

Untreated HL-60 cells proliferated exponentially when initiated in culture and showed no significant functional differentiation. HL-60 cells showed a time-dependent, biphasic dose response when grown with added DMSO (Fig. 1). Significant inhibition of growth was seen as low as 1.3% DMSO for 3 day treatment and maintained thereafter, until the growth was slowed to $<30\%$ of the control. However, at concentration of 0.5~1.0% the growth of HL-60 cells remained almost unaffected. At concentration of $>1.5\%$ the growth was inhibited at the early times of treatments.

DNA Synthesis and Chemotaxis:

To determine the degree of physiological differentiation of HL-60 cells, DNA synthesis was assayed by the uptake of ³H-thymidine in the presence of 1.3% DMSO. There was a

Fig. 1. Dose-response growth of HL-60 cells in DMSO. Cells were inoculated at 3×10^5 cells/ml in the presence of various concentrations of DMSO.

significant decrease in the incorporation of ³H-thymidine at the time of 3 day treatment of about 15%, and decrease of 10% thereafter, as shown in Fig. 2.

In order to identify the functional differentiation of HL-60 cells into terminal granulocytes, the chemotactic response to FMLP was assayed. Unlike the uninduced cells, 1.3% DMSO-induced HL-60 cells demonstrated marked ability to migrate through a 5 μm pore polycarbonate filter in response to a chemotactic stimulus as shown in Fig. 3. The migration rate through the filter was highest, 35~40%, when the concentration of chemotactic peptide was $1 \times 10^{-8} \sim 1 \times 10^{-9}$ M. Some chemotactic responsiveness was also observed in uninduced HL-60 cells, although this was in all cases minimal when compared with the chemotaxis of induced cells.

Fluorescence Assay of P-L Fusion:

HL-60 cells incubated with 1.3% DMSO

Fig. 2. ^3H -thymidine incorporation by HL-60 cells in the presence of 1.3% DMSO. Untreated or DMSO-treated cells were labeled pulsely with ^3H -thymidine for 1 hr, then the percentages were calculated.

underwent progressive morphologic change from predominantly promyelocytes of high nuclear/cytoplasmic(N/C) ratio to granulocytes of relatively low N/C ratio as shown in Fig. 4, HC and DC. When uninduced cells or DMSO-induced cells were co-cultured with *Toxoplasma*, uninduced cells did not show any signs of toxoplasma activity until 24 hrs(Fig. 4, H1, H2, and H3), but showed intracellular proliferation of *Toxoplasma* to form rosettes at 72 hrs(Fig. 4, H4). On the while, as shown in Fig. 4, D1, D2, and D3, DMSO-induced HL-60 cells showed to contain one or two *Toxoplasma* which suggested phagocytosis and intracellular digestion by the cells for 24 hrs and Fig. 4, D4 indicated that the process of intracellular digestion was completely accomplished.

In addition to the above data, to judge for the effect of potentially lethal conditions on *Toxoplasma*, a reliable method of assessing parasite viability was required. Acridine orange

Fig. 3. Chemotactic response of HL-60 cells or DMSO-induced HL-60 cells to FMLP. Percentages of the cells migrated through the filter to initially inoculated were calculated.

Fig. 4. Giemsa-stained photographs of co-culture of HL-60 cells (HC, H1, H2, H3, and H4) or DMSO-induced HL-60 cells (DC, D1, D2, D3, and D4) with *Toxoplasma*. HC and DC stand for the controls of HL-60 cells and DMSO-induced HL-60 cells. H1 and D1 represent the co-culture of HL-60 cells or DMSO-induced HL-60 cells with *Toxoplasma* after 1 hr; H2 and D2, 5 hr; H3 and D3, 24 hr; and H4 and D4, 72 hr; respectively. (→)

(AO) was selectively concentrated by lysosomes of intact cells as shown in Fig. 5, HFC and DFC. Because *Toxoplasma* lysosomes had been reported to fluoresce after exposure to AO, this study performed the examination of the usefulness of this simple technique as an indicator of parasite viability. As shown in Fig. 5, HF1, HF2, HF3, and HF4, all cells exhibited light orange-red cytoplasmic fluorescence and no lysosomal staining of *Toxoplasma*. In contrast, DMSO-induced HL-60 cells exhibited one or two *Toxoplasma*-shaped bright orange colors which indicated the intracellular digestion after the phagocytosis of *Toxoplasma* by these cells as shown in Fig. 5, DF1, DF2, and DF3. Furthermore, Fig. 5, DF4 showed no *Toxoplasma*-shaped orange color but background fluorescent lysosomes similar to the control (Fig. 5, DFC), which indicated the lysosomal recycling after the perfect accomplishment of intracellular digestion.

DISCUSSION

The morphological changes that occurred in HL-60 cells during DMSO induction mimicked normal myeloid differentiation (Collins, 1987). As shown in this study, HL-60 cells were induced to acquire those attributes that were necessary for the integrated functions of terminally differentiated leukocytes. The pattern of differentiation induced in cultured HL-60 cells by DMSO was determined using both physiological and functional criteria. A detectable increase in the percentage of differentiated cells was observed only after 3 day of continuous incubation in the presence of 1.3% DMSO (Fig. 1). When treated with DMSO, HL-60 cells showed reduced uptake of ^3H -thymidine (Fig. 2) which indicated the halt of DNA synthesis (Rovera *et al.*, 1980; Yen and Albri-

ght, 1984), that suggests the physiological differentiation into terminal cells. To identify the degree of differentiation of HL-60 cells over the various treatments, chemotaxis, phagocytosis, and superoxide anion production were performed (Fontana *et al.*, 1980; Tarella *et al.*, 1982; Mangelsdorf *et al.*, 1984; Imaizumi and Breitman, 1986). In this study, chemotaxis was chosen to identify the functional differentiation of HL-60 cells treated with DMSO, chemotaxis was an integration of phagocytic cells consisting of various properties such as adhesiveness, orientation, presence of chemoattractant receptors, and directed mobility. Alteri and Leonard (1983) reported that normal human peripheral monocytes exhibited a maximal chemotactic response at concentrations of 10 nM to 100 nM FMLP, and the highest chemotactic activity of normal granulocytes was at 1 μM FMLP (Harbath and Leonard, 1982), a concentration that markedly inhibited chemotaxis of monocytes (Alteri and Leonard, 1983). HL-60 cells induced by retinoic acid plus human T cell-derived lymphokine (DIA), with or without glucocorticoid dexamethasone, exhibited maximal chemotaxis at concentrations of 10 nM to 100 nM FMLP, and 1 μM FMLP was inhibitory (Imaizumi and Breitman, 1986). As shown in Fig. 3, 1.3% DMSO-induced HL-60 cells represented the highest chemotactic activity at concentrations of 10 nM to 100 nM FMLP, and 1 μM FMLP was inhibitory, which agreed to the results of Imaizumi and Breitman (1986). Therefore, it was concluded that 1.3% DMSO treatment for 3 days was necessary for the differentiation of HL-60 cells into terminal granulocytes with morphological, physiological, and functional activities.

The *in vitro* interaction of *Toxoplasma* with animal and human mononuclear phagocytes had been used extensively to investigate the role of

Fig. 5. Fluorescent photographs of co-culture of HL-60 cells (HFC, HF1, HF2, HF3, and HF4) or DMSO-induced HL-60 cells (DFC, DF1, DF2, DF3, and DF4) with *Toxoplasma*. HFC and DFC stand for the controls of HL-60 cells and DMSO-induced HL-60 cells. HF1 and DF1 represent the co-culture of HL-60 cells or DMSO-induced HL-60 cells after 1 hr; HF2 and DF2, 5 hrs; HF3 and DF3, 24 hrs; and HF4 and DF4, 72 hrs; respectively. (←)

cell-mediated immunity in resistance to intracellular pathogens (Murray and Cohn, 1979; McLeod *et al.*, 1980). However, the mechanism by which these phagocytes exert antitoxoplasmal activity had not been defined. Moreover, there had been many difficulties in analyzing the data obtained from the above host cells because of their heterogeneities in cell kinds and of unknown processes or state which might modulate the functions of the cells. But, in this study, it could be overcome by using established cell line, HL-60. In order to approximate more closely the events that occurred in the protozoan parasite-host cell interactions, *in vitro* system might be more useful. The results of this study which were obtained using HL-60 cells and DMSO-induced HL-60 cells as host cells agreed well to the previous reports concerned with the parasitism of *Toxoplasma* (Jones *et al.*, 1972; Jones and Hirsch, 1972; Anderson and Remington, 1974). As shown in Fig. 4 and 5, HL-60 cells provided a good place for the proliferation of *Toxoplasma*. In contrast, DMSO-induced, terminally differentiated HL-60 cells showed toxoplasmacidal activity, which suggested phagosome-lysosome fusion might be the critical events of parasitism by *Toxoplasma*. The mechanism by which intracellular parasites inhibit phagosome-lysosome fusion is not known (Goren, 1977; Kielian and Cohn, 1980, 1981; Horwitz, 1983). It might offer some well restricted and defined conditions to analyze the mechanism of intracellular parasitism such as the inhibition of phagosome-lysosome fusion by using HL-60 cells as the host cell. On the while, toxoplasmacidal effect of DMSO-induced HL-60 cells might exert on the revelation of mechanisms such as receptor-mediated phagocytosis (Johnson *et al.*, 1983; Ofek and Sharon, 1988), intracellular digestion (Johnson and Eskeland, 1983), and further the action of anti-protozoan parasitic drugs.

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＝국문초록＝

Dimethylsulfoxide로 분화시킨 HL-60 세포의 *Toxoplasma* 파괴 효과

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HL-60 세포에서 *Toxoplasma gondii*의 *in vitro* 배양과 HL-60 세포를 DMSO로 처리하여 과립세포로 분화시킨 세포에서 *Toxoplasma*에 대한 세포매개성 면역 기능을 검토하였다.

먼저, HL-60 세포를 여러 농도의 DMSO로 처리하였는데, 1.3%(V/V)로 3일간 처리하였을 때, HL-60 세포의 적정 분화가 이루어졌다. 분화의 정도는 형태적, 생리적, 및 기능적 관점에서 검사되었는데, DMSO를 처리한 경우, ³H-thymidine의 흡입이 감소하는 것으로 보아 DNA 합성이 억제됨을 알 수 있었으며, 기능적으로는 주화성 물질인 FMLP에 대해 이동하는 성질을 보였으며, 형태적으로는 핵/세포질의 비가 큰 promyelocyte에서 작은 비를 갖는 과립 세포로 변화하여 분화를 입증하였다.

이후, HL-60 세포나 DMSO로 분화를 유도한 HL-60 세포와 *Toxoplasma*를 같이 배양하면서 이들의 관계를 관찰하였다. Lysosome에 선택적으로 흡입되는 형광 물질(acridine orange)로 전처리한 표본은 형광현미경하에서 관찰하였으며, 다른 표본은 Giemsa로 염색하여 광학 현미경하에서 관찰하여 비교하였다. HL-60 세포에서는 72시간의 배양으로 *Toxoplasma*가 세포질내에서 증식하여 rosette를 형성하였으며, DMSO로 분화시킨 HL-60 세포에서는 배양 초기 1시간째에 phagocytosis가 일어났으며 이후 세포내 소화가 이루어져 72시간째에는 lysosome이 원상태로 되돌아 오는 것이 관찰되었다.

이상의 결과들로 볼 때, *Toxoplasma*의 숙주 세포내에서의 기생 혹은 면역 세포에 의한 감수성에 phagosome과 lysosome의 융합이 결정적인 인자임을 알 수 있었으며, 아울러 HL-60 세포에서의 *Toxoplasma*의 증식 가능성과 DMSO로 분화시킨 HL-60 세포의 *Toxoplasma* 파괴 효과가 원충 기생충과 숙주의 상호 관계를 규명하는 좋은 모델임을 제시하였다.