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Environmental Toxic Agents on Genetic Material and Cellular Activity
III. DNA Polymerase Inhibitors on Repair of Mutagen-Induced DNA Damage in Mammalian Cells

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The effects of aphidicolin (APC), an inhibitor of DNA polymerase α, or 2',3'-dideoxythymidine 5'-triphosphate (ddTTP), an inhibitor of DNA polymerase β, on the repair of DNA damage induced by ethyl methanesulfonate (EMS) or bleomycin (BLM) were investigated in Chinese hamster ovary (CHO)—K1 cells. Three assays were employed in this study: unscheduled DNA synthesis, alkaline elution and alkaline sucrose gradient sedimentation. It was shown that APC or ddTTP inhibited DNA repair induced by EMS, and thus, the post-treatment with APC or ddTTP following EMS treatment was resulted in the more amount of unscheduled DNA synthesis, and the more accumulation of DNA single-strand breaks than the cells post-incubated without APC or ddTTP. While, in the BLM-induced DNA repair, only ddTTP inhibited DNA repair induced by BLM. And thus, the groups post-incubated with or without APC after BLM treatment had the same value in the amount of unscheduled DNA synthesis and of DNA single-strand breaks, while post-treatment with ddTTP was resulted in the increased amount of unscheduled DNA synthesis and the increased DNA single-strand breaks than the group without ddTTP. These results suggested that both of DNA polymerase α and β participated in the repair of DNA damage induced by EMS, but in BLM-induced DNA repair, polymerase β participated.

INTRODUCTION

Many enzymes are known to be associated with the recognition of specific lesions and incision of the DNA strand in their vicinity (Clarkson and Mitchell, 1983), and several enzymes concerned with excision and polymerization have also been identified (Collins & Johnson, 1984; Collins *et al.*, 1984; Downes *et al.*, 1985; Mattern *et al.*, 1982). In a number of studies, controversial

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results have been obtained on whether DNA polymerase α is (Cleaver, 1984; Collins *et al.*, 1982; Dresler and Frattini, 1986; Dresler and Kimbro, 1987; Johnson *et al.*, 1987; Snyder and Reagan, 1981, 1982) or is not (Giulotto and Mondello, 1981; Hardt *et al.*, 1981; Seki *et al.*, 1980) important in DNA repair, while DNA polymerase β has been postulated to have a role in the repair of DNA (Cleaver, 1983; Dresler and Lieberman, 1983; Miller and Chinault, 1982; Spadari *et al.*, 1982).

Many conclusions concerning the functions at these enzymes can be obtained using enzyme inhibitors. Among them, the commonly used inhibitors have been aphidicolin (APC), an inhibitor of DNA polymerase α (Smith and Paterson, 1983; Th'ng and Walker, 1985), and 2′,3′-dideoxythymidine (ddThd) or 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP), an inhibitor of DNA polymerase β (Dresler and Kimbro, 1987; Th'ng and Walker, 1985; Yamada *et al.*, 1985). On the other hands, the studies using APC and ddTTP yielded results that suggest the participation of both polymerases in DNA repair depending on the agent used and the dosage applied (Cleaver, 1983; Dresler and Liberman, 1983; Yamada *et al.*, 1985). Therefore, the same approach, using three different assays for DNA repair and two inhibitors, has been undertaken in this study with the view to give insight into the role of specific enzymes in the repair process.

The purpose of this study is to elucidate the involvement of DNA polymerase α or β in DNA repair synthesis induced by EMS, an alkylating agent, or BLM, an antibiotics and X-ray mimetic agent, in CHO cells.

MATERIALS AND METHODS

Cell Culture

Chinese hamster ovary (CHO)–K1 cells were used throughout this investigation. Monolayer cultures of this cell line were grown at 37°C in humidified 5% CO_2 incubator using Eagle's minimum essential medium (MEM; Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% newborn calf serum and gentamycin (50 μg/ml).

Chemical Treatment

Ethyl methanesulfonate (EMS, Tokyo Kasei Co., Tokyo, Japan) or Bleomycin (BLM, Nihon Kayaku, Japan) was dissolved in the serum-free medium prior to use and exposed to cells at 37°C for desired times.

Aphidicolin (APC, Sigma Chemical Co., St. Louis, Mo), inhibitor of DNA polymerase α, was dissolved in dimethyl sulfoxide and 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP, Sigma Chemical Co., St. Louis, Mo), inhibitor of DNA polymerase β, was dissolved in distilled water and further diluted to working concentrations. The cells were treated to these inhibitors for 1 hour.

Unscheduled DNA Synthesis

CHO cells grown on coverslips in plastic petridishes were exposed to chemicals. The cells were then labeled with 10 μCi/ml ^{3}H-thymidine (specific activity; 77.9 Ci/mmol, Amersham Co., England) for 1 hour after treatment with inhibitor.

Labeling with ^{3}H-thymidine was terminated by washing the cells three times in cold Hank's balanced salt solution (HBSS) containing 100 μg/ml of unlabeled thymidine. Autoradiograms were prepared by using Kodak NTB liquid nuclear track emulsion. Silver grains over nuclei of evenly and lightly labeled cells were counted.

Alkaline Elution Experiments

Cells were labeled with 0.2 μCi/ml of ^{3}H-thymidine for 24 hours and then exposed to chemicals. The cells were harvested with cold PBS-Merchant solution (150 mM Nacl, 4.28 mM K_2HPO_4, 0.71 mM KH_2PO_4), filtered onto 2 μm pore size polycarbonate filter (nuclepore Co., Pleasanton, CA.), and lysed with lysing solution (2% SDS, 0.1 M glycine, 0.025 M Na_2-EDTA, pH 10.0). And cells were eluted in the dark with eluting solution (30 mM tetrapropylammonium hydroxide, 0.02 M EDTA, 1% SDS, pH 12.1) at a flow rate of 0.035 ml/min. Fractions were collected at 90 minutes intervals. The radioactivities remaining on filters were counted using liquid scintillation counter after treating them with 1 N HCl at 60°C for 45 minutes. Fractions of radio-activity remaining on filter were plotted against elution time.

Alkaline Sucrose Gradient Sedimentation

CHO cells were labeled with 0.2 μCi/ml of ^{3}H-thymidine for 24 hours and then exposed to chemicals. Cells were harvested with SSC (0.15 NaCl, 0.015 M sodium citrate) and resuspended in 1 ml of PBS-Merchant solution (150 mM NaCl, 4.28 mM K_2HPO_4, 0.71 mM KH_2PO_4). The sample was added onto 5-20% sucrose gradient on which alkaline lysis solution (0.5 M NaOH, 0.02 M Na_2EDTA) was layered and lysed for 4 hours at 20°C. DNA was sedimented in RPS 27-2 rotor (Hitachi, SCP 7OH, Japan) at 23,000 rpm for 5 hours at 20°C. The gradient were fractionated with Density Gradient Fractionator (Hitachi DGF-U, Japan) from top of the tube. Each fractions were filtered on Whatman GF/C glass fiber filters and dried. The radioactivity of samples was counted with liquid scintillation spectrometer.

RESULTS

The effects of APC, ddTTP, EMS or BLM on unscheduled DNA synthesis are shown in Table 1. When cells were treated with 5 μg/ml APC or 10, 20 and 200 μM ddTTP, the amount of unscheduled DNA synthesis was not much

Table 1. Effects of APC, ddTTP, EMS, or BLM on unscheduled DNA synthesis in CHO cells.

Treatment*		Grains per Cell** (Mean ± S.E.)
Chemical	Inhibitor	
0	0	8.8 ± 1.2
0	APC 5 μg/ml	8.2 ± 1.1
0	ddTTP 10 μM	8.6 ± 1.0
0	ddTTP 20 μM	8.4 ± 1.2
0	ddTTP 200 μM	10.8 ± 1.2
EMS 2 mM	0	25.7 ± 2.0
BLM 40 μg/ml	0	26.9 ± 1.7

* APC, ddTTP or EMS for 1 hour, and BLM for 2 hours.

** Fifty cells were analyzed for grain counting.

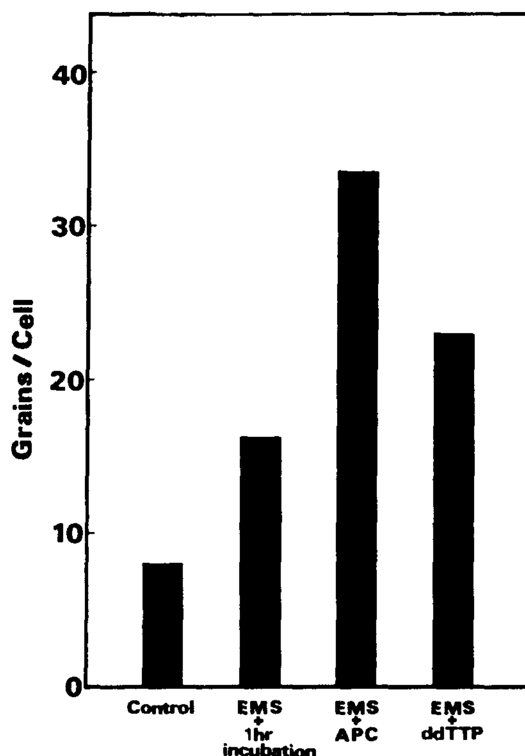

Fig. 1. Effects of 5 μg/ml APC or 20 μM ddTTP on unscheduled DNA synthesis induced by 2 mM EMS for 1 hour in CHO cells.

different from the control. But 2 mM EMS or 40 μg/ml BLM induced the unscheduled DNA synthesis.

The effects of 5 μg/ml APC or 20 μM ddTTP on unscheduled DNA synthesis induced by 2 mM EMS are shown in Fig. 1. The post-treatment with APC or ddTTP inhibited EMS-induced excision repair and resulted in the higher degree of unscheduled DNA synthesis than the group post-incubated without APC or ddTTP.

Fig. 2 represents the effects of 5 μg/ml APC or 20 μM ddTTP on unscheduled DNA synthesis induced by 40 μg/ml BLM. As shown in the figure, the post-treatment with ddTTP inhibited BLM-induced excision repair, and resulted in the increment of the amount of unscheduled DNA synthesis compared with the group post-incubated without ddTTP. But APC did not affect the unscheduled DNA synthesis induced by BLM, the amount of unscheduled DNA synthesis of cells post-treated with APC was the same as the group post-incubated without APC.

Table 2 represents the effects of APC, ddTTP, EMS or BLM on DNA single-strand breaks using alkaline elution technique. When cells were treated with 5 μg/ml APC or 10, 100 and 200 μM ddTTP, there was no increment of DNA

Table 2. Effects of APC, ddTTP, EMS or BLM on DNA single-strand breaks in CHO cells

Treatment*		Percent of DNA remaining on filter after elution for 9 hours
Chemical	Inhibitor	
0	0	89.4 ±0.1
0	APC 5 μg/ml	90.5 ±1.0
0	ddTTP 10 μM	89.1 ±0.2
0	ddTTP 100 μM	90.0 ±1.4
0	ddTTP 200 μM	89.1 ±0.6
EMS 40 mM	0	56.0 ±1.0
BLM 400 μg/ml	0	57.1 ±1.4

* APC or ddTTP for 1 hour, BLM for 2 hours, and EMS for 3 hours.

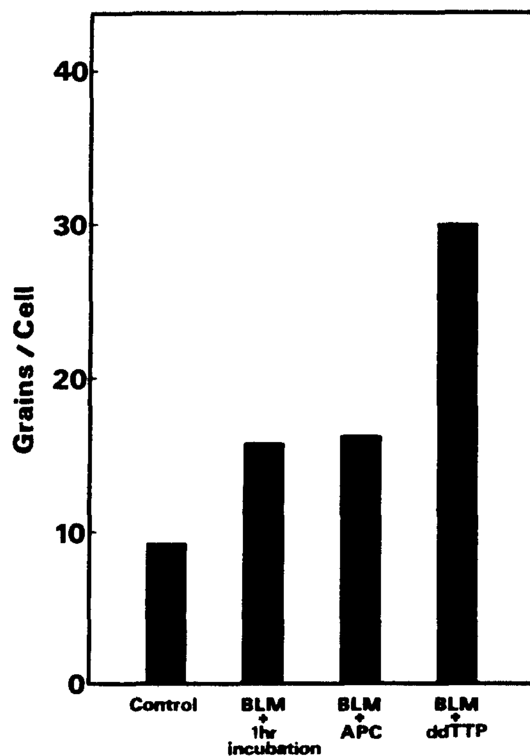

Fig. 2. Effects of 5 μg/ml APC or 20 μM ddTTP on unscheduled DNA synthesis induced by 40 μg/ml BLM for 2 hours in CHO cells.

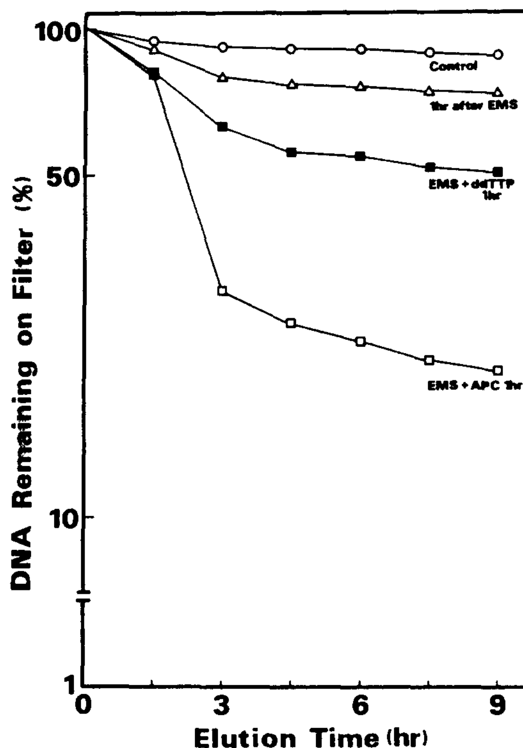

Fig. 3. **Effects of 5 μg/ml APC or 200 μM ddTTP on DNA single-strand breaks induced by 40 mM EMS for 3 hours in CHO cells.**

single-strand breaks compared with the control. But 40 mM EMS or 400 μg/ml BLM markedly increased the DNA single-strand breaks compared with the control.

Figs. 3 and 4 show the effects of 5 μg/ml APC or 200 μM ddTTP on DNA single-strand breaks induced by 40 mM EMS or 400 μg/ml BLM using alkaline elution technique. The post-treatment with APC or ddTTP inhibited the rejoining of DNA single-strand breaks by EMS and resulted in the much more accumulation of DNA single-strand breaks than that of the group post-incubated without APC or ddTTP. While the DNA single-strand breaks induced by BLM were not inhibited by APC, thus, the percentage of DNA remaining on filter after 9 hr elution of post-incubated group with APC was the same as the group post-incubated without APC. But the post-treatment with ddTTP inhibited the rejoining of DNA single-strand breaks by BLM.

Fig. 5 represents the effects of 5 μg/ml APC or 200 μM ddTTP on DNA single-strand breaks induced by 40 mM EMS using alkaline sucrose gradient sedimentation. The major peak was shown at fraction 20 in control, and at

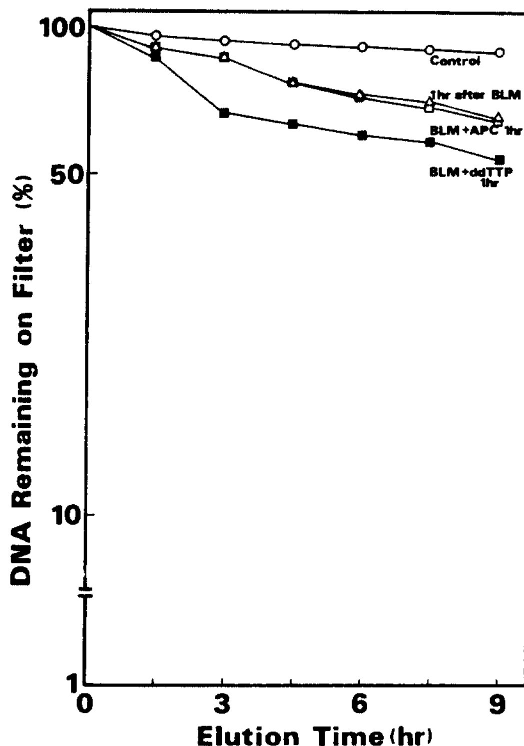

Fig. 4. Effects of 5 μg/ml APC or 200 μM ddTTP on DNA single-strand breaks induced by 400 μg/ml BLM for 2 hours in CHO cells.

fraction 13 in EMS-treated group, respectively. But in the group of post-treatment with APC or ddTTP, the major peak was shifted to the fraction 10. These facts mean that APC or ddTTP inhibits the DNA repair induced by EMS.

Fig. 6 shows the effects of 5 μg/ml APC or 200 μM ddTTP on DNA single-strand breaks induced by 800 μg/ml BLM using alkaline sucrose gradient sedimentation. The major peak was appeared at fraction 20 in the control, and at fraction 3 in BLM-treated group, respectively. But in the group of post-treated with APC, the major peak was shifted to the right. The results suggested that APC did not affect the repair of BLM-induced DNA damage. While the post-treatment with ddTTP inhibit the BLM-induced DNA repair, the major peak was appeared at fraction 2.

The above results suggested that both of DNA polymerase *α* and *β* were participated in EMS-induced DNA repair, while only the polymerase *β* was involved in the repair of DNA damage by BLM.

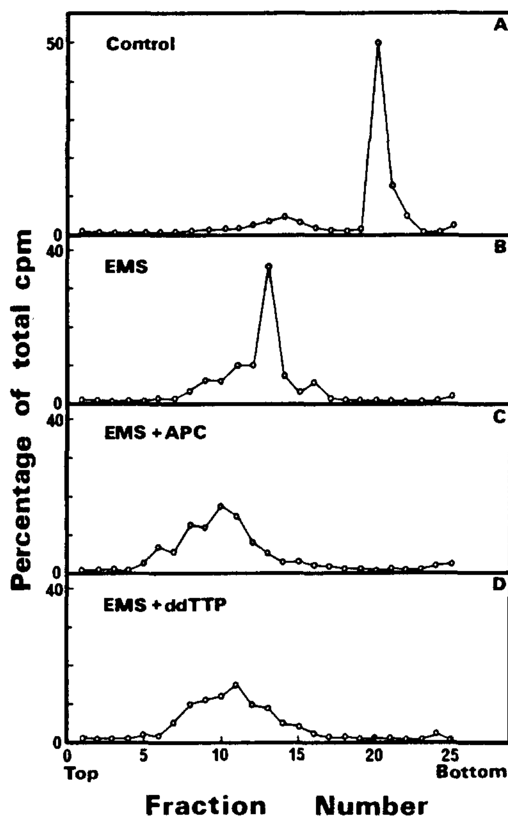

Fig. 5. Alkaline sucrose gradient sedimentation profiles of DNA single-strand breaks in CHO cells. Each profile represents control(A), 40 mM EMS for 3 hours(B), post-treatment with 5 μg/ml APC for 1 hour after EMS treatment(C), and post-treatment with 200 μM ddTTP for 1 hour after EMS(D).

DISCUSSION

The results of this paper demonstrated that APC or ddTTP inhibited the DNA repair induced by EMS, while only ddTTP affect on the DNA repair induced by BLM in CHO cells. These results suggested that DNA polymerase α and β participated in the repair of DNA damage by EMS, but polymerase β was involved in BLM-induced DNA repair.

There were reports that DNA polymerase α required a larger gap for initiating synthesis, whereas polymerase β required a smaller gap (Grossman, 1981;

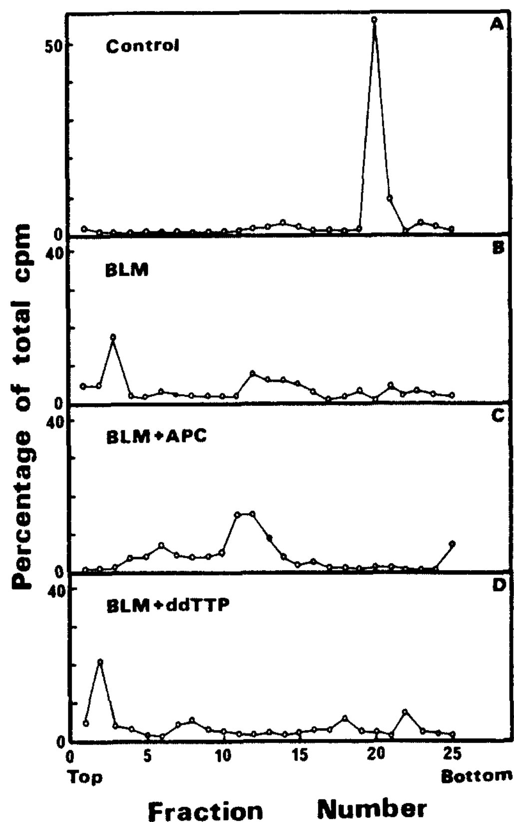

Fig. 6. Alkaline sucrose gradient sedimentation profiles of DNA single-strand breaks in CHO cells. Each profile represents control(A), 800 μg/ml BLM for 2 hours(B), post-treatment with 5 μg/ml APC for 1 hour after BLM treatment(C), and post-treatment with 200 μM ddTTP for 1 hour after BLM(D).

Mosbaugh and Linn, 1984). Miller and Chinault (1982) also reported that DNA polymerase α functioned more prominently on damage resulting from N-methyl-N′-nitro-N-nitrosoguanidine or N-nitroso methyl urea, while polymerase β was the primary enzyme utilized for the repair of damage induced by BLM or neocarcinostatin in CHO cells. The report of Mosbaugh and Linn (1984) revealed that DNA polymerase α might initiate incorporation into the gapped DNA, however, once the gaps were reduced to 10-20 nucleotides, polymerase β would appear to become required. Yamada *et al.* (1985) reported that UV irradiation generated larger gaps and only DNA polymerase α was involved,

while, dimethyl sulfate (DMS) generated middle sized gaps and both polymerase α and β participated in their repair, and BLM generated smaller gaps and polymerase β was more important in the repair.

Considering above others' reports, a possible explanation on our results was that EMS might generate middle sized gaps, because EMS was similar alkylating agent, DMS, thus, the both DNA polymerase α and β participated in this repair, whereas BLM generated small sized gap, polymerase β was involved in this repair. From overall results, it can be suggested that the participation of DNA polymerase α or β in DNA repair was dependent on different modes of DNA damage.

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환경성 유해요인이 유전물질과 세포활성에 미치는 영향
Ⅲ. 포유동물세포에서 돌연변이원에 의한 DNA 상해의 회복에 미치는 DNA 중합효소저해제의 영향

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본 연구는 Ethyl methanesulfonate (EMS) 혹은 Bleomycin (BLM) 에 의해 유발된 DNA상해의 회복에 미치는 DNA 중합효소 α 저해제인 Aphidicolin (APC) 과 DNA 중합효소 β의 저해제인 2′, 3′-dideoxythymidine 5′-triphosphate (ddTTP) 의 영향을 조사하기 위하여 Chinese hamster ovary (CHO)-K1 세포를 재료로 비주기성 DNA 합성법과 알칼리유출법 및 스칼리 자당구배침강법으로 수행하여 얻은 결과는 다음과 같다.

APC와 ddTTP는 EMS에 의해 유발된 DNA 상해의 회복을 저해하여 APC 혹은 ddTTP를 처리하지 않고 배양한 실험군 보다 비주기성 DNA 합성율과 DNA단사 절단율이 증가되었다. 한편 BLM에 의해 유발된 DNA 상해의 회복에서는 ddTTP를 처리했을 경우에만 저해되었다. 즉 BLM 처리후 ddTTP를 후처리한 실험군의 비주기성 DNA 합성율과 DNA단사 절단율은 ddTTP를 처리하지 않은 군보다 증가되었고, BLM 처리후 APC를 후처리할 경우에 비주기성 DNA 합성율과 DNA단사 절단율은 APC를 처리하지 않은 군과 유사하였다.

이상의 결과들에서 EMS에 의해 유발된 DNA 상해의 회복에는 DNA 중합효소 α와 β양자가 관여하나 BLM에 의해 유발된 DNA 상해의 회복에는 중합효소 β가 관여하는 것으로 추측된다.