INHIBITION OF SIMIAN VIRUS 40 REPLICATION BY KANAMYCIN DERIVATIVE

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Recently, the antiviral activity of kanamycin derivatives against influenza and herpes simplex virus has been reported[1~3]. The mechanism of antiviral action of these agents is not well understood. In this report, the mechanism of antiviral effect of 1-*N*-eicosanoyl-3″-*N*-(trifluoroacetyl)kanamycin (ETK), one of these agents, was studied. We have tried to test the effect of ETK on simian virus 40 (SV40), a DNA virus. When the permissive monkey cells are infected with SV40, the following phenomena occur; expression of early genes, production of T antigen, replication of SV40 DNA, expression of late genes, production of late proteins, and finally the maturation of virus particles[4]. ETK inhibits the multiplication of SV40. The agent inhibits the production of T antigen coded by SV40 early gene, followed by inhibition of SV40 DNA replication. However, the transcription of early gene was stimulated. The complicated results may come from release of autoregulation of T antigen production by inhibition of T antigen synthesis by ETK. The monkey cell line, GC7[5] was infected with SV40 at a multiplicity of infection of 50 PFU per cell. At 40 hours after infection, the production of late proteins was examined by indirect immunofluorescence technique using anti-SV40 V antigen rabbit serum (Table 1). The intensity of fluorescence was normalized as follows: The strong fluorescence was scored +2 and weak fluorescence was scored +1. The results are shown as the sum of these two figures per plate. It is clear that production of viral late proteins were suppressed in a concentration dependent manner by ETK. The 10^{-4} M of ETK inhibited approximately 70% of production.

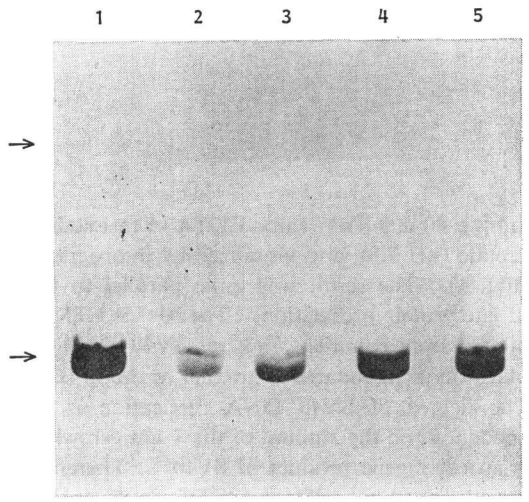
Table 1. Effect of ETK on SV40 multiplication.

ETK (M)	Fluorescence index
0	1.00
10^{-4}	0.31
10^{-5}	0.61
10^{-6}	0.81
10^{-7}	0.96

5×10^3 of GC7 cells infected with SV40 were fixed with acetone - ethanol and stained with anti-SV40 antigen rabbit serum followed by FITC-conjugated goat anti-rabbit IgG[5]. The strength of fluorescence was expressed as a fluorescence index, $(2N+n)/(2N'+n')$, where N stands for the number of cells stained strongly among ETK treated cells, n for that of the cells stained weakly among ETK-treated cells, N′ and n′ for those of the cells stained strongly and weakly among non-treated cells, respectively.

Fig. 1. Effect of ETK on SV40 DNA synthesis.

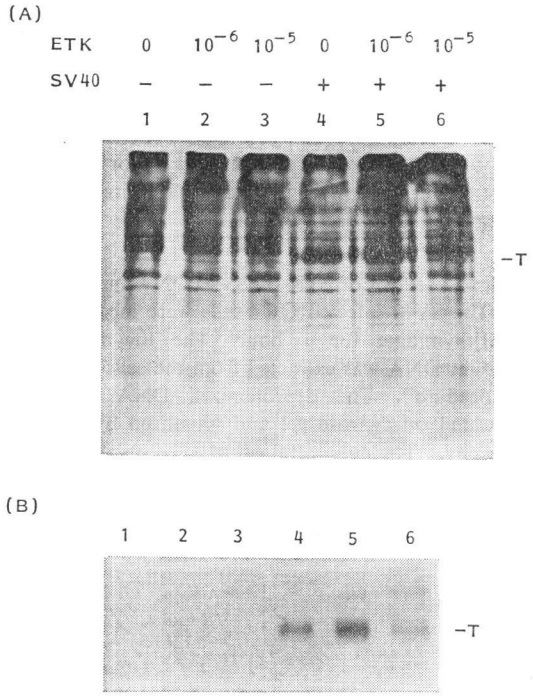
The SV40 infected GC7 cells were labeled with [^{3}H]thymidine for 1 hour. The low-molecular weight DNA was extracted from the cells by HIRT procedure[6]. The deproteinized DNA was separated on agarose gel and visualized by fluorography. The concentration of ETK used were 0, 10^{-4} M, 10^{-5} M, 10^{-6} M and 10^{-7} M from lanes 1 to 5, respectively.



We next checked the effect of ETK on SV40 DNA replication. At 16 hours after infection, the infected GC7 cells were labeled with 100 μCi of [^{3}H]thymidine per ml for 60 minutes. The low molecular weight DNA containing SV40 DNA was extracted from GC7 cells by HIRT procedure[6], separated on 1% agarose gel con-

Fig. 2. Effect of ETK on protein synthesis.

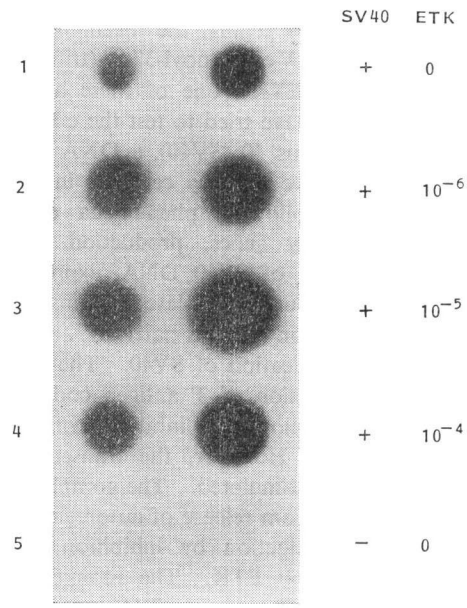
The GC7 cells were labeled with [^{35}S]methionine for 4 hours and the proteins were extracted by the buffer containig Nonidet P-40[7]. The half of each sample was separated on 10% polyacrylamide gel and vizualized by fluorography (A). The other half of each sample was immuno-precipitated with anti-T antigen hamster serum (B). The concentration of ETK used and case of SV40 infection were shown over the figures.



taining 40 mM Tris - 1 mM EDTA - 5 mM sodium acetate (pH 7.8), and visualized by fluorography (Fig. 1). The result was quite parallel to that of late protein production. The 10^{-5} M ETK inhibited approximately 70% of SV40 DNA replication as compared with that without ETK. The degree of SV40 DNA replication is dependent upon the amount of the T antigen which is an early gene product of SV40[4]. Therefore, we examined the effect of ETK on T antigen production. At 16 hours after infection, the GC7 cells were labeled with 100 μCi of [^{35}S]-methionine per ml for 4 hours. The labeled proteins were extracted from the cells by the buffer containing Nonidet P-40[7]. Total proteins were separated on 10% polyacrylamide gel and visualized by fluorography (Fig. 2A). There was little overall difference in proteins from

Fig. 3. Effect of early gene transcription of SV40.

The cytoplasmic RNA was extracted from GC7 cells, blotted on nitrocellulose filter, and hybridized with ^{32}P-labeled SV40 early gene probe. The amounts of RNA blotted on filter were 5 and 10 μg at left and right, respectively. The hybridization was carried out in a mixture containing 3×SSC (1×SSC contains 1.5 M NaCl and 0.15 M sodium citrate), 50 mM Tris (pH 7.5), 1 mM EDTA, 1× Denhardt solution (1×Denhardt contains 0.02% Ficoll, 0.02% polyvinylpyrolidone and 0.02% bovine serum albumin), 20 μg of heat-denatured salmon testis DNA per ml, 20 μg of tRNA per ml, ^{32}P-labeled probe, 0.1% sodium dodecylsulfate and 50% formamide. After 20 hours at 42°C, the filter was washed twice with 3×SSC - 0.1% sodium dodecylsulfate at 37°C for 30 minutes and then autoradiographed at 37°C at −80°C. The concentration of ETK used and case of SV40 infection were shown beside the figure.



ETK treated and non-treated cells. However, the amount of T antigen that could be seen only in SV40 infected cells was reduced in the cells treated with 10^{-5} M ETK (Figs. 2A and B). This was clearly observed after labeled proteins were immuno-precipitated using anti-T antigen hamster serum (Fig. 2B). The 10^{-5} M ETK again reduced T antigen production to 30% of that in untreated cells. Therefore, it is quite reasonable that the reason for suppression of SV40 DNA synthesis by ETK is suppression of T antigen production. It is generally known that the

degree of SV40 DNA synthesis depends upon the amount of T antigen[4].

Then, we examined whether or not transcription of early gene is also inhibited by ETK. Cytoplasmic RNA was extracted from GC7 cells by standard procedure[8], blotted on nitrocellulose filter, and hybridized with ^{32}P-labeled SV40 early gene probe which is *Pvu*II-*Pvu*II fragment nucleotide number 3,506 to 270 (Fig. 3). Hybridization was carried out under high stringent condition as described in figure legends. When the infected cells were treated with ETK, early RNA was produced at approximately 5-fold higher in SV40 infected GC7 cells treated with 10^{-5} M ETK, which was the concentration that inhibited T antigen production by 70%. This complicated results are likely to be explained as follows: When infected cells are treated with ETK, early RNA synthesis is stimulated due to release of autoregulation mechanisms induced by a low level of T antigen production.

It is not clear why ETK inhibited T antigen production while the overall protein synthesis of cells was not significantly changed. The reason for preferential inhibition of T antigen synthesis to other proteins is not clear. Identification of the precise target of ETK in T antigen synthesis remains to be determined. Suppression of T antigen, coded in early gene, by ETK is thought to be of primary importance for antiviral action against SV40. We have previously reported that ETK inhibits SV40 DNA replication *in vitro* and that the inhibition of SV40 DNA replication is attributed to the interaction between ETK and the template DNA[9]. This direct inhibition of SV40 DNA replication by ETK, which was shown *in vitro* system, may be of secondary importance.

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