Effect of Streptovitacin A on Replication of an RNA Virus (Poliovirus).* (32498)

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Cycloheximide, an antibiotic isolated from *Streptomyces griseus*, was reported to have an inhibitory effect on plaque formation by both DNA and RNA viruses(1). Streptovitacin A (STR-A), the 4-OH-derivative of cycloheximide, a rapidly acting inhibitor of protein synthesis that only partly depresses DNA synthesis and has no effect on RNA synthesis in mammalian cells was reported to have a different effect on the replication of the DNA vaccinia virus and the RNA reovirus(2). Presence of the drug in the medium for only the first two hours of exposure of cells to virus was said to result in an irreversible suppression of multiplication of the structurally complex DNA vaccinia virus but only in delayed replication of the RNA reovirus(2). With the structurally less complex DNA, SV 40 virus STR-A suppressed synthesis of both virion and non-virion antigens only as long as the drug was present in the medium, complete resumption of synthesis of these antigens and of infectious virus occurring on removal of the drug even after the virus and drug had been in contact with the cells for two days(3).

It was previously reported(3) that the cytopathic effect (CPE) of poliovirus type 1 was completely prevented when BS-C-1 cells were pretreated with STR-A (10 μg/ml) for 2 hours and then maintained in drug-containing medium. However, when cells that had been in contact with poliovirus and drug for 3 days were washed and fresh drug-free medium was added CPE appeared within 12 hours and was complete in 22 hours, *i.e.*, almost as quickly as it does in untreated cultures(3).

The purpose of the present study was to determine a) whether prevention of poliovirus CPE by STR-A was associated with complete inhibition of synthesis of viral products or only with assembly of infectious virus, and b) the periods during the infectious cycle when the drug was effective.

Materials and methods. Cells. The BS-C-1 stable line of Cercopithecus monkey kidney cells(4) was used. Monolayers were grown in 16 or 32 oz prescription bottles at the 98th to the 124th passage levels, and maintained as previously described(5). *Virus.* Poliovirus type 1 (LSc 2ab) was passaged 3× in monkey kidney cells and once in HEp 2 cells. The stock virus had 4.4×10^8 PFU per ml as measured in HEp 2 cells and 2.5×10^8 PFU per ml as measured in BS-C-1 cells. *Tests for virus replication.* 16 oz bottles were seeded with about 5×10^6 trypsinized BS-C-1 cells. Using the growth medium (199 + 0.1% yeastolate + 20% fetal bovine serum) as previously described(5) a densely confluent cell sheet of about 10^7 cells was obtained in 6-8 days. The growth medium was then poured off and the cell sheets were washed twice with 20 ml quantities of maintenance medium consisting of Eagle's basal medium (BME) + 1% heated chicken serum + 0.2% $NaHCO_3$. The wash fluid was carefully drained on sterile gauze. After the second washing 5 ml of maintenance medium and 0.1 to 0.5 ml of undiluted virus stock were added to each bottle. Depending on the experiment the bottles were then left at 37°C for 30 or 60 minutes and were rocked every 10 to 15 minutes to provide even distribution of the fluid over the cell sheet. At the end of this adsorption period the fluid was poured off and carefully drained on sterile gauze, and the cell sheets were washed 4× with 20 ml amounts of maintenance medium at 37°C, draining the residual fluid on sterile gauze between each washing. After the last washing, 20 ml of warm maintenance medium with or without drug, were added to each bottle and incubation was continued at 37°C.

When the released virus and cellular virus

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were determined separately, the culture fluid was centrifuged at 500 × *g* (1500 rpm International Horizontal Centrifuge) for 10 minutes and 5 ml of the top supernatant fluid were saved at −88°C. The sedimented cells were resuspended in a small amount of BME without serum or phenol red and added to the drained monolayer which was covered with 5 ml of the same fluid and the bottle was frozen at −88°C. The cells were frozen and thawed 4 times and the supernatant fluid after centrifugation at 500 × *g* for 10 minutes was used for titration. When the virus content in the cells and fluid was not determined separately the same procedure was used except that the cells and total original culture fluid were frozen together.

Infectivity titrations. Fully confluent HEp 2 cell sheets in rubber-stoppered 3 oz bottles were used. After adsorption of the virus for 1½ hours, the cell sheets were overlaid with 1.5% agar in BME containing 5% heated chicken serum and $NaHCO_3$ in a final concentration of 0.13%. A second overlay containing neutral red was added at 72 hours and the plaques were counted the next day.

Complement fixation (CF) tests. The procedure was the same as previously described (3) using an uncentrifuged 5% cell suspension frozen and thawed 4 times. The antiserum was derived from cynomolgus monkeys inoculated intracardially with type 1 poliovirus. *Drug.* The same lot of Streptovitacin A was used as previously described(3).

Results. The data shown in Table I indicate that with an IMV of 4-5 and about 10^7 cells there was no assembly of new infectious virus for 5 hours and that in the subsequent hour about 20% of that present in the cells at 14-16 hours appeared. Although at 8 hours after infection the cells already contained about 75% of that present at 16 hours, virus release into the culture fluid had only just begun and even at 16 hours the fluid on two occasions contained only about 12% of that in the cells. When STR-A was added at ½, 2 or 4 hours after virus and left in the culture fluid, no new infectious virus appeared. When STR-A was first added at 5 hours after virus (Exp. D) when no new infectious virus had yet appeared, a very small amount of new infectious virus was assembled by 16 hours (21×10^5 PFU *vs* $8{,}250 \times 10^5$ PFU or only 0.24% of that in the untreated cells, and 8×10^5 PFU *vs* $1{,}000 \times 10^5$ PFU, or 0.8% of that released in the fluid). When STR-A was added at 6 hours after virus (Exp. E) at a time when about $2{,}600 \times 10^5$ PFU of new virus was already present in the cells and only 6×10^5 PFU in the fluid, an additional $2{,}400 \times 10^5$ PFU appeared in the drug-treated cells in the next 10 hours *vs* $11{,}100 \times 10^5$ PFU in the untreated cells and 580×10^5 PFU *vs* $5{,}360 \times 10^5$ PFU in the fluid. When STR-A was added at 8 hours after virus (Exp. C), at a time when the cells contained $3{,}750 \times 10^5$ PFU and the fluid only 20×10^5 PFU, there was no additional assembly of new virus in the subsequent 8 hours but only release of some of the intracellular virus into the fluid—$3{,}000 \times 10^5$ PFU in the cells and 580×10^5 PFU in the fluid or a total of $3{,}580 \times 10^5$ PFU at 16 hours compared with a total of $3{,}770 \times 10^5$ PFU that was present at 8 hours before addition of the drug; the total virus/16 oz bottle in the untreated cultures was $5{,}660 \times 10^5$ PFU at 16 hours. The addition of STR-A ½ hour before the virus and its removal ½ hour later, when the unadsorbed virus was washed out (Exp. B), did not prevent subsequent synthesis of infectious virus but resulted in a significant reduction in the total amount synthesized in the cells and released into the fluid—830×10^5 PFU (intracellular) + 133×10^5 PFU (extracellular) in the drug-treated cultures *vs* $2{,}000 \times 10^5$ PFU (intracellular) + 500×10^5 PFU (extracellular) in the untreated cultures at 14 hours.

The experiment recorded in Table II was carried out to correlate the appearance of new infectious virus with visible destruction of cells (CPE) and the accumulation of virion components measurable by the CF test. In this experiment, with a similar IMV but a somewhat larger number of cells (per 32 oz bottle), a very small amount of newly assembled virus (46×10^5 PFU) was already present at 5 hours, at a time when no CPE was visible and no CF antigen was detected. Moreover since there was a marked increase

TABLE I. Effect of Addition of Streptovitacin A (10 μg/ml) at Different Times After Infection of BS-C-1 Cells* on Synthesis of Infectious Type 1 Poliovirus (IMV† = 4–5).

Drug in medium	Hr pre- or post virus drug added	Exp.	Virus in cells or fluid/16 oz bottle at indicated hr after infection – PFU × 10^5															
			Cells								Fluid							
			0.5	2	4	5	6	8	14	16	24	0.5	2	4	5	6	8	14	16	24
None	—	A	7.7	—	1.9	—	—	—	4,625	—	—	<0.1	—	0.1	—	—	—	600	—	—
"	—	B	0.1	—	—	—	—	—	2,000	—	—	0.2	—	—	—	—	—	500	—	—
"	—	C	—	7.5	5	—	—	3,750	—	5,000	—	—	0.6	2	—	—	20	—	660	—
"	—	D	—	—	2.5	3.0	—	—	—	8,250	—	—	—	0.1	0.3	—	—	—	1,000	—
"	—	E	14.5	—	—	—	2,600	—	—	13,700	—	<0.1	—	—	—	6	—	—	5,360	—
STR-A‡	0.5 post and left	A	—	—	1.7	—	—	—	1.4	—	0.9	—	—	0.1	—	—	—	0.1	—	0.4
"	2 " "	C	—	—	—	—	—	—	—	1.6	—	—	—	—	—	—	—	—	<0.1	—
"	4 " "	C	—	—	—	—	—	—	—	2.2	—	—	—	—	—	—	—	—	1.2	—
"	5 " "	D	—	—	—	—	—	—	—	21.0	—	—	—	—	—	—	—	—	8.0	—
"	6 " "	E	—	—	—	—	—	—	—	5,000	—	—	—	—	—	—	—	—	640	—
"	8 " "	C	—	—	—	—	—	—	—	3,000	—	—	—	—	—	—	—	—	580	—
"	0.5 pre, removed 0.5 post	B	0.3	—	—	—	—	—	830	—	—	0.6	—	—	—	—	—	133	—	—

* 16 oz bottles with 1.0 to 1.4 × 10^7 cells per bottle and 20 ml of culture fluid.
† IMV = input multiplicity of virus. Unadsorbed virus washed out at 30 min.
‡ STR-A = Streptovitacin A.

in intracellular virus and CF antigen between 16 hours (4,600 × 10^5 PFU and 2 CF units) and 40 hours after infection (78,000 × 10^5 PFU and 16 CF units), it is obvious that an IMV of 4 did not provide simultaneous infection of almost all cells. It is nevertheless evident that STR-A added 1 hour after infection and left in for 40 hours, completely prevented synthesis of detectable CF antigen as well as of infectious virus. The synthesis of a very small amount of new infectious virus when STR-A was added at 4 and 5 hours after virus confirms observations recorded in Table I, and the absence of demonstrable CF antigen under these conditions again suggests that the virion products measurable by this CF test accumulate concurrently with the assembly of infectious virus.

The experiments recorded in Table III were carried out with an IMV of 20 to obtain simultaneous infection of almost all cells and perhaps also a larger input of preformed viral RNA per cell. The effect of adding and removing STR-A at different times during the eclipse phase and during the period of exponential assembly of infectious virus was tested. Under the conditions of these experiments the eclipse phase lasted 4 hours and the exponential assembly of infectious virus began before the fifth hour and was complete in about 8 hours. It is noteworthy that CPE occurred many hours after the appearance of infectious virus. CPE was absent during the first 6 hours; at 8 hours about 10% of the cells in Exp. F and 30% of the cells in Exp. G exhibited CPE; in Exp. F CPE affected only 50% of the cells at 16 hours and was complete only at 40 hours, while in Exp. G it was complete at 16 hours.

When STR-A was added at 1 hour or 2 hours after infection and left in the medium until harvest at 16 hours, no new infectious virus appeared. When STR-A was added at 4 hours before new infectious virus had appeared (Exp. F) there was again a definite but small increase in infectious virus by 6 hours (29 × 10^5 PFU at 4 hours *vs* 470 × 10^5 PFU in the drug-treated cultures and 3,500 × 10^5 PFU in the untreated cultures) but no further increase up to 40 hours; the continuing drop in the amount of virus re-

TABLE II. Effect of Streptovitacin A (10 μg/ml) on CPE, Synthesis of CF Antigen and Infectious Virus in BS-C-1 Cells Infected with Type 1 Poliovirus.

Drug in medium	Hr post virus* drug added	Hr post virus	Viral CPE % of cells	Virus/ 32 oz bottle† PFU × 10^5	CF antigen‡
None	—	0.5	0	4	0
"	—	5	0	46	0
"	—	16	25	4,600	2
"	—	40	100	78,000	16
Streptovitacin A	1	40	0	<1	0
"	4	16	0	17	0
"	5	16	0 ?	200	0

* Input multiplicity of virus = 4. Virus washed out at 30 min.
† Only intracellular virus measured; each 32 oz bottle contained about 1.75 × 10^7 cells.
‡ Units in 5% cell suspension as measured with 2 units of antipolio 1 monkey serum.

TABLE III. Effect of Addition and Removal of Streptovitacin A (10 μg/ml) at Different Times on Replication of Type 1 Poliovirus in BS-C-1 Cells (IMV* = 20).

	Drug in medium		Virus in cells + fluid/16 oz bottle — PFU × 10^6 hr after addition of virus							
Exp	Hr after virus drug added	Hr after virus drug removed	1	2	4	5	6	8	16	40
F	none	—	49	35	29	1,130	3,500	9,000	6,000	7,100
G	"	—	92	91	—	—	—	25,000	37,500	—
G	1	at harvest	—	—	—	—	—	—	14	—
"	1	2	—	—	—	—	—	1,400	11,000	—
"	1	3	—	—	—	—	—	37	11,400	—
"	2	at harvest	—	—	—	—	—	—	15	—
"	2	3	—	—	—	—	—	440	12,260	—
"	2	4	—	—	—	—	—	29	16,600	—
F	4	at harvest	—	—	—	—	470	360	250	150
"	5	" "	—	—	—	—	1,950	1,650	3,000	2,900
"	6	" "	—	—	—	—	—	4,000	5,600	4,600
"	8	" "	—	—	—	—	—	—	9,200	6,900

* IMV = input multiplicity of virus. Virus washed out at one hour.

covered from the cultures harvested at 8, 16 and 40 hours may represent the effect of the 37°C temperature on infectivity. When STR-A was added at 5 and 6 hours after virus (Exp. F) during the exponential phase of formation of infectious virus, there was again an increase in infectious virus during the subsequent hours, although less than that occurring in the untreated cultures. When STR-A was added at 8 hours (Exp. F) when viral replication had reached its peak, there was no significant effect on the amount of virus recovered at 16 hours and 40 hours.

When STR-A was added at 1 or 2 hours after infection and washed away after it had been in the medium for only 1 hour (Exp. G), the amount of virus recovered at 8 and 16 hours indicated that while there was no suppression of viral synthesis there was a significant diminution in the amount that was formed. When STR-A was added at 1 or 2 hours after infection and left in the medium for only 2 hours, no virus synthesis was evident at 8 hours but at 16 hours the drug-treated cultures already contained 30-36% of the amount present in the untreated cultures. If the synthetic process had merely been halted for 2 hours and resumed within minutes after washing out the drug, one would have expected to find new virus at 8 hours.

Discussion. Recent studies on protein synthesis in rabbit reticulocytes, which lack a nucleus and the capacity to synthesize DNA and RNA, indicated that STR-A specifically inhibited the formation of the peptide bond (6,7) and thus could be expected to interfere with any step in viral replication requiring the synthesis of new protein. However, in order to be able to interpret the significance of the observed data when this drug

was introduced during certain periods in the cycle of viral multiplication and left in the culture until the time of harvest, or when it was in the medium for only 1 or 2 hours during certain periods of the eclipse phase, it is necessary to know how rapidly STR-A completely stops protein synthesis and for how long the inhibitory effect persists after the drug is washed out in the cells used for viral replication. Unfortunately we have no such data for BS-C-1 cells and the data for other cells vary. In the rabbit reticulocyte system, cycloheximide as well as STR-A were reported to exhibit maximal inhibition of protein synthesis in 30 seconds or less; however, the effect of cycloheximide was completely reversible on washing away the drug while the inhibitory effect of STR-A persisted apparently because it did not readily diffuse out of the cells(6). In L cells STR-A acted more slowly requiring 15 minutes to effect 85-90% inhibition of protein synthesis and 30 minutes for 95-98% inhibition; after washing the drug-treated cells almost complete inhibition persisted for at least 2 hours and about 50% inhibition was still evident at 10 hours(2).

The effects of STR-A on the replication of poliovirus in BS-C-1 cells observed in the present studies, as well as those recently reported on the replication of another RNA virus (Mengovirus) in L cells(8), are best explained on the basis that protein synthesis, though diminished, nevertheless continues for 15-30 minutes after addition of the drug and that the inhibitory effect continues for a number of hours after the drug is washed away. There is no need to assume that a pool of unassembled virion protein exists when the drug is added at certain times at the end of the eclipse phase or the early stages of exponential increase in the amount of infectious virus. The statement contained in the discussion of the mengovirus experiments(8) that "presence of the drug [STR-A] for a short 60 minute period from 2 to 3 hours after infection was sufficient to suppress virus replication and to stop almost completely (for at least 14 hours) cell lysis" is misleading because virus replication did occur during this period although at a reduced level. The results of the experiments with STR-A and the RNA viruses studied indicate that interference with protein synthesis during any phase of the viral replication cycle does not result in an irreversible progression of viral synthesis.

Summary. Streptovitacin A, a highly efficient inhibitor of protein synthesis, at 10 μg/ml of medium completely prevented synthesis of infectious poliovirus (type 1) and of CF antigen, as well as of CPE, in BS-C-1 cells when it was added up to about an hour before the end of the eclipse phase and was then left in the medium for the duration of the experiment. When the drug was added at the very end of the eclipse period, before new infectious virus had appeared, and was then left in the medium, a small amount of new virus appeared in the cells. When the drug was added during the exponential phase of viral multiplication. there was always some further increase in infectious virus but never at the level achieved in the untreated cultures. When the drug was added one or two hours after infection and washed away one or two hours later, the subsequent delay in viral synthesis was always considerably longer than the time the drug was present in the culture medium. These results fit the assumption that protein synthesis in BS-C-1 cells is not completely inhibited for 15-30 minutes after the addition of the drug and is not restored for a number of hours after its removal, as was found by Dales for L cells. The data also indicate that the drug-induced interference with protein synthesis during any phase of the poliovirus replication cycle did not result in an irreversible progression in the synthesis of infectious virus.

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