Studies on the Mode of Action of Streptovitacin A*

CHARLES G. SMITH, WILLIAM L. LUMMIS, AND JOSEPH E. GRADY

(Research Laboratories, The Upjohn Company, Kalamazoo, Michigan)

SUMMARY

Streptovitacin A was shown to induce a lag phase in KB human epidermoid carcinoma cells which lasted approximately 24 hours. The cells then grew out at the normal rate.

At the time when KB cells overcame the streptovitacin A inhibition, cytotoxic activity was still present in the medium, and the culture was still sensitive to subsequent treatment with this agent. A possible mechanism to explain these results is presented.

The inhibition pattern of streptovitacin A on KB cells which were treated *in vitro* under conditions simulating those found *in vivo* was determined.

No reversal of streptovitacin A cytotoxicity to KB cells was observed when mixtures of purines, pyrimidines, nucleosides, vitamins, amino acids, co-factors, Krebs' cycle intermediates, glucose, metals, or versene were added to the tissue culture medium.

Streptovitacin A, 3-[2-(5-hydroxy-3, 5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-glutarimide (2, 8, 9), is an antibiotic substance which inhibits the growth of experimental tumors *in vivo* and human carcinoma cells *in vitro*. It has been reported active against Sarcoma 180 (3–6), RC-carcinoma (6), Walker 256 carcinoma (3, 5, 6), Ehrlich ascites carcinoma (3, 5, 6), Jensen sarcoma (3, 5), Murphy-Sturm lymphosarcoma (5), and leukemias L4946 and L1210 (5, 6) *in vivo* at doses of 0.1–0.5 mg/kg/day. In addition, temporary remissions of spontaneous tumors in dogs and man have also been achieved (5, 6). The ID_{50} vs. KB human epidermoid cells *in vitro* is 0.035 μg/ml (12, 13). Knowledge of the mechanism of drug action might be helpful in suggesting (*a*) other inhibitors for combination chemotherapy and (*b*) agents which might be effective in reversing toxicity in humans, thus permitting therapy at high drug doses.

Investigations of the mode of streptovitacin A activity reported in this publication include (*a*) time-course determinations, (*b*) studies on the emergence of resistant cells after drug treatment, and (*c*) reversal studies with various metabolites and mineral elements.

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MATERIALS AND METHODS

The methods used for maintenance of stock cultures, planting of tube cultures, and determination of cellular protein have been described in detail previously (11, 13). Streptovitacin A solutions were freshly prepared at approximately monthly intervals and were stored at −10° in the interim. The cells were cultured in 16 × 150 mm. test tubes containing 4 ml. of Eagle's basal medium (BME) plus 10 per cent calf serum with no intermittent medium changes unless specified otherwise. Agents under test were added to the culture tubes before the cells were planted, and assays for reversal studies were made after 3 days' incubation (13). The generation time of KB cells in 70 per cent calf serum plus 30 per cent BME fortified with glutamine (2 mM) was approximately 33 hours, compared with a generation time of 24 hours in 10 per cent serum. Nontoxic lots of calf serum were selected for all experiments.

RESULTS

Time-course of streptovitacin A inhibition.—Previous studies showed that streptovitacin A caused 50 per cent inhibition of KB cell protein synthesis (ID_{50}) at a concentration of 0.035 μg/ml in BME after 3 days' incubation (13). The time-course of streptovitacin A inhibition was investigated by determining at daily intervals the effect of various drug concentrations on the growth of

KB cells, and the results are presented in Chart 1. These data show that maximum cytotoxicity was manifest between 0 and 48 hours, after which the cells usually began to grow. Although this inhibition pattern was repeated in several experiments, the drug occasionally showed little cytotoxicity between 0 and 24 hours, followed by marked inhibition between 24 and 48 hours, after which the cells grew out at the normal rate. The per cent inhibition observed at 48 hours and thereafter was the same, however, regardless of the initial pattern of inhibition.

The data in Chart 1 show that streptovitacin A can completely inhibit cell multiplication when in contact with KB cells over a 3-day period at a concentration of 0.64 μg/ml. If a quantitative correlation exists between the inhibition of growing human tumor cells *in vitro* and *in vivo*, useful

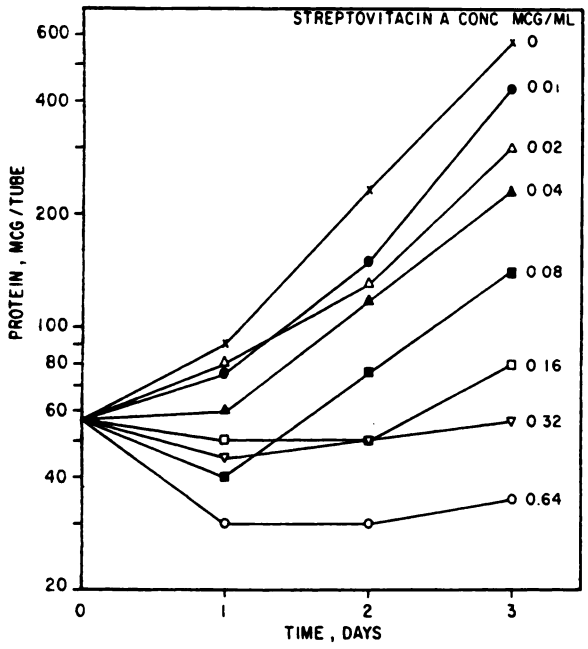

CHART 1.—Time-course of streptovitacin inhibition of KB cells.

information on effective blood levels in humans under therapy with streptovitacin A can be obtained from tissue culture studies. To this end, the conditions under which the tumor cells were cultured were modified to more closely approximate conditions found *in vivo*. Thus, the serum concentration was increased from 10 to 70 per cent, and streptovitacin A was allowed to remain in contact with the cells for 1 day rather than 3. Following removal of drug by washing in BME plus 70 per cent calf serum, the cells were incubated in the presence of fresh medium for an additional 3 days' growth. The results of this experiment are presented in Table 1.

Almost identical results were obtained when the cell count was determined in addition to protein content. It should be pointed out that KB began to grow out from drug control at 0.16 μg/ml, even when the agent remained in contact with the cells over the entire incubation period. When treated with 0.64 μg/ml for only 24 hours, KB cells partially recovered from drug inhibition and grew out to a lower population than that found in control tubes. These data show that somewhat higher concentrations of streptovitacin A are required to suppress completely the growth of mammalian cells when treated under simulated *in vivo* conditions than are required under the usual tissue culture assay conditions (13). Peak streptovitacin A levels of 0.4–1.0 μg/ml, observed in the blood of humans under streptovitacin A treatment (8), might therefore be expected to exert marked carcinostatic effects but probably not true carcinolytic activity. This actually seems to be the case in clinical practice (5, 14).

TABLE 1

EFFECT OF STREPTOVITACIN A ON KB CELLS DURING LONG- AND SHORT-TERM EXPOSURE

EXPOSURE TO DRUG*	PER CENT INHIBITION OF GROWTH†			
	Day 1	2	3	4
Long-term:				
0.04 μg/ml	15	66	63	51
0.16 "	25	83	106	87
0.64 "	45	107	106	95
Short-term:				
0.04 "	15	60	41	41
0.16 "	25	67	57	70
0.64 "	45	70	65	83

* Long-term exposure means that the drug was allowed to remain in contact with the cells over the entire incubation period. In the short-term treatment, the agent remained in contact with the cells for only 1 day as described in text. Duplicate tubes were assayed daily. Cells were grown in BME plus 70 per cent calf serum.

† Calculated as follows:

$$\text{Per cent inhibition} = \frac{\text{Final protein in control} - \text{final protein in experimental}}{\text{Final protein in control} - \text{initial protein in control}} \times 100;$$

Values greater than 100 per cent indicate complete inhibition of growth plus lysis of the inoculum.

Resistance of KB cells to streptovitacin A.—The sensitivity of KB cells which had grown out after 24 hours' inhibition by 0.06 μg/ml of streptovitacin A was investigated by renewing the culture medium on day 2 (T_2) with fresh BME (10 per cent serum) containing the same level of strepto-

vitacin A. Cells which had previously grown out of streptovitacin A control were still sensitive to a second treatment with the drug which caused 55 per cent inhibition of growth of previously treated cells and 52 per cent inhibition of untreated cultures. When an additional 0.06 μg/ml of the agent was added on T_2 without concomitant medium change, the inhibition pattern which followed was essentially the same as that observed when the entire medium was renewed. Greater inhibition was sometimes observed when streptovitacin A was added to an untreated culture in fresh BME than when the drug was added directly to the medium in which the cells were growing. When higher concentrations of streptovitacin A were added on T_2 or T_3 (0.16–0.64 μg/ml) to cultures which had grown out of drug control, complete inhibition and lysis were observed, again showing that the cells were still sensitive.

TABLE 2

COMPOUNDS WHICH WERE UNABLE TO REVERSE THE CYTOTOXICITY OF STREPTOVITACIN A TO KB CELLS

Compounds*	Concentration range (μg/ml)
1. Nucleic acid derivatives:	
a) Adenine, guanine, cytosine, uracil and thymine	5–50
b) Adenosine, guanosine, cytidine and uridine	5–250
c) Deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine	5–250
2. Amino acid mixtures:	
a) Eagle's amino acid mixture	6× and 11×†
b) L-glutamine/glutathione	1500/50
c) L-glutamine	500–2500
d) L-methionine, choline, L-serine and glycine	100
e) L-threonine, L-aspartic acid, L-β-alanine, L-α-alanine and L-proline	100
f) D-glucose/L-serine + glycine	7000/200
3. Vitamin mixtures:	
a) Eagle's vitamine mixture	6× and 11×†
b) DPN, ATP, CoA and FMN	5–50
4. Salts:	
a) $CaCl_2 \cdot 2H_2O$	100
b) $CoCl_2 \cdot 6H_2O$	0.1
c) $CuSO_4 \cdot 5H_2O$	0.1
d) $FeSO_4 \cdot 7H_2O$	100
e) $MgCl_2 \cdot 6H_2O$	100
f) $MnCl_2 \cdot 4H_2O$	2
g) $ZnCl_2$	10
5. Miscellaneous:	
a) Versene	100
b) Glucose/citric, succinic, DL-malic and fumaric acids	4000/300

* Added to BME plus 10 per cent calf serum. Streptovitacin A present at 0.04–0.12 μg/ml. Inhibition determined after 72 hours, with duplicate tubes/test. Reversing agents tested in various combinations over the dosage ranges indicated.

† Concentrations used were 6- and 11-fold greater than normally present in BME.

Effect of KB extracts on streptovitacin A cytotoxicity.—When BME plus 10 per cent calf serum, which was harvested from the streptovitacin-treated cultures on T_1, was transferred to a control culture which was not previously treated, the latter was inhibited to a degree approximating that seen with fresh drug. Thus, although KB cells were able to grow out from the control of streptovitacin A after 24 hours of complete inhibition, active drug appeared to be present in the culture medium. Since active drug was present and the culture had not developed any marked resistance, it appeared possible that cellular outgrowth was due to the presence of a reversing agent which accumulated in the cells during inhibition. To test this hypothesis, the culture was grown for 2 days in BME with 10 per cent calf serum in the presence and absence of streptovitacin A (0.05 μg/ml), and the resulting cells were sonicated (10 KC for 5 minutes) after washing with saline. When 400 μg. of extracted KB protein were added per ml. of growth medium and the cytotoxicity of streptovitacin A determined, no marked difference in activity was observed which could be correlated with previous drug exposure. Although no evidence for reversal was obtained, the postulated reversing agent may not be freely diffusible.

Reversal studies with various metabolites.—The effects of several nucleic acid metabolites, amino acid and vitamin mixtures, inorganic salts, and Krebs' cycle acids on the inhibition of KB cells by streptovitacin A were investigated. The metabolites were studied in combination to decrease the number of runs necessary to investigate several materials at multiple doses, and the results are presented in Table 2. It is interesting that none of the agents tested was able to reverse significantly the cytotoxicity of streptovitacin A under the conditions of these experiments. Although the concentrations used were limited by the most toxic member of the group, most of the metabolites were tested over a 5–50-fold concentration range.

Recently, several metal ions were reported to reverse the toxicity of cycloheximide to yeast (1). Since cycloheximide is cytotoxic to KB cells in culture (12, 13) and streptovitacin A is 4-hydroxycycloheximide (10), it seemed entirely possible that metals might also reverse the activity of this agent vs. mammalian cells. The data in Table 2 show that neither the metals which were

investigated nor versene affected the cytotoxicity of this agent to KB cells *in vitro*. In the same experiment it was found that the metals shown in Table 2 did not reverse the toxicity of cycloheximide to KB cells either. These data suggest that streptovitacin A and cycloheximide may inhibit mammalian cells by a mechanism which is different from the inhibition of fungi by cycloheximide, although permeability factors and medium participation may be involved.

DISCUSSION

In KB carcinoma cells, streptovitacin A induced a lag phase which lasted approximately 24 hours, after which the cells grew out at a rate comparable to untreated cultures. Cells which had outgrown the control of streptovitacin A were fully sensitive to a second application of drug, indicating that selection of resistant stains was not the mechanism of outgrowth. Cytotoxic activity was present in the medium at the time when the cells overcame the inhibition. These data suggest that resting KB carcinoma cells, under the influence of streptovitacin A, accumulate a metabolite which reverses drug toxicity. In such a system, the cells would be expected to grow out when the ratio of reversing agent to streptovitacin A was just sufficient to allow multiplication. A second addition of drug would be expected to induce another period of inhibition, since excess reversing agent would probably not be present. These postulates fit the experimental facts. When the fluids from treated cells which have just begun their outgrowth were transferred to fresh KB cells, the latter were inhibited to approximately the same degree as was found with fresh drug. This observation can also be explained under the above hypothesis, if one postulates that the concentration of reversing agent is low in untreated cells.

Combinations of normal metabolites representing a variety of metabolic pathways were unable to reverse the cytotoxicity of streptovitacin A to KB cells under the conditions used in these laboratories. In most of the reversal studies, the metabolites under investigation were added at both nontoxic and toxic levels to insure that the compound was tested at maximum dose. When testing a mixture, the dose is limited by the most toxic drug present, and possibly some of the agents may have influenced the cytotoxicity of streptovitacin A if tested singly at higher dosage. It should be pointed out, however, that all metabolites (except metals) were tested at concentrations at least 100 times greater (by weight) than that of streptovitacin A, without showing significant reduction in cytotoxicity. It appears from this investigation that streptovitacin A must join the ranks of other antibiotics as a compound whose mechanism of action can be unmasked only through much laborious investigation. J. S. Evans *et al.* (3) have investigated the effects of various vitamins and amino acids on the cytotoxicity of streptovitacin A to experimental tumors *in vivo*. They too observed no reversal of tumor inhibition by any of the metabolites tested.

These studies have shown that streptovitacin A is less inhibitory to mammalian cells when it is removed from their environment after 24 hours than when it remains in contact for several days. If one determines the effect of an antitumor agent or combination of agents on the growth of tumor cells in culture under conditions which simulate those found *in vivo*—e.g., drug concentration = human blood level, drug removed after short contact time and cells incubated in fresh medium for several days before retreatment, serum concentration in medium = 50–75 per cent, data which can be translated to the inhibition of tumors *in vivo* may be obtained. Thus, with streptovitacin A, our data predict cytostatic rather than cytocidal effects in humans, since the drug did not kill KB cells at levels comparable to those found in the blood of patients under therapy (8). Previous studies have shown a good correlation with the streptovitacin family between antitumor activity against KB cells *in vitro* and Walker 256 carcinoma in rats (13). Further studies with drug combinations will be carried out in an effort to predict carcinolytic activity *in vivo* with tissue culture methods.

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