GROWTH OF NORMAL AND TRANSFORMED RAT EMBRYO FIBROBLASTS

Effects of Glycolipids from *Salmonella minnesota R* Mutants

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ABSTRACT

Addition of glycolipids obtained from *Salmonella minnesota R* mutants to normal, spontaneously transformed, and SV40-transformed rat embryo fibroblasts in culture results in an inhibition of growth of transformed cells but not of normal cells. In the presence of the glycolipid with the smallest carbohydrate chain length, spontaneously transformed cells stop growing when they reach confluency. Inhibition of growth of transformed cells is inversely related to the chain length of the core sugars. Glycolipid mR595 is shown to bind with the cell membrane of transformed cells and elicits an augmentation in the intracellular level of cyclic AMP. Normal cells bind relatively less glycolipid mR595 and show a lower percent of increase in cyclic AMP due to glycolipid mR595 than do transformed cells.

INTRODUCTION

Transformed cells are characterized by loss of control mechanisms regulating ordered growth in normal cells. This loss results in multilayered growth of transformed cells in vitro and in unlimited growth, metastasis, and invasiveness in vivo. During the past years, it has been proposed (1, 2, 3, 4) that these characteristics of transformed cells depend upon alterations in cell surface/cell membrane constituents after transformation of normal cells. More recently, agglutination of transformed cells by low concentrations of plant lectins is cited as an indication of membrane alteration (5, 6, 7). Normal cells are not agglutinated by the same concentration of lectin. Although the exact mechanism of agglutination is not known, it is well established that specific sugar residues on the cell surface are involved in interaction with plant lectins (8). Interaction of trypsinized jack bean lectin (concanavalin A) with cultured cells has also been employed to control growth of polyoma transformed cells (9).

Prompted by the above studies showing that plant agglutinins can be employed as probes for cell surface studies and can also inhibit growth of transformed cells, we have employed another group of substances, namely bacterial glycolipids, because of their known ability to bind cell membranes of red blood cells (10) and their damaging action on transplanted tumors (11–15). In an initial study, one of us (V. N. Nigam) observed that injection of glycolipids obtained from *R* mutants of *Salmonella minnesota* into mice bearing Ehrlich solid tumor significantly increased their survival time. It was also observed that bacterial glycolipids of shorter length of core sugars were increasingly more effective, whereas glycolipids from wild-type *S. minnesota* were ineffective. On the basis of these preliminary findings, we have now carried out complementary studies on bacterial glycolipids utilizing normal and transformed rat embryo fibroblasts in cultures. This paper reports the effects of various glycolipids obtained from *S. minnesota R* mutants on the growth of normal and transformed cells, on the binding of

glycolipid mR595 specifically to transformed cells, and on the probable mechanism (an increase in intracellular cyclic AMP concentration) by which the glycolipid elicits its inhibitory effect on transformed cell growth.

MATERIALS AND METHODS

Cells

Primary rat embryo fibroblasts (ER) were obtained by trypsinization of 15-day old Wistar rat embryos (pregnant females were secured from Flow Laboratories, Rockville, Md.). Cells were employed after one subculture.

Spontaneously transformed cells (RST) are an established cell line which developed spontaneously from rat embryonic cultures.

SV40-transformed cell line (RSV40) is a cell line obtained after transformation of rat embryo cultures by SV40 virus. These cells carry SV40 T antigen.

Both spontaneously transformed and SV40-transformed cell lines grow in multilayers, grow in soft agar, and induce tumors in rats.

Growth of Cell Cultures

Cells were cultivated in minimum essential medium (MEM, Flow Laboratories), supplemented with 10% fetal calf serum (Flow Laboratories), 10% tryptose phosphate broth (Difco Laboratories, Inc., Detroit, Mich.), fourfold the concentration of vitamins and amino acids, L-glutamine (10 mg/ml), penicillin (200 μg/ml), and streptomycin (40 μg/ml). The cultures were prepared in roller flasks. Cells were subcultivated at a concentration of 1.2×10^6 cells/Petri dish in Falcon Petri dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) (60 mm diameter) in 4 ml medium and placed in an air-CO_2 incubator (5% CO_2). To study the effect on the growth of these cells, known amounts of bacterial glycolipids and dibutyryl cyclic AMP were added to 24-h old cell cultures after dissolution of the substances in appropriate medium. Cells were counted initially and after every 24 h in a hemocytometer (Bürker, Karl Hecht, Bayern, West Germany) after trypsinization.

Viability of Cells

The viability of cells grown in the presence of bacterial glycolipids or dibutyryl cyclic AMP was established by two different methods. One of these was the dye exclusion test (16), and the other, the plating efficiency of cells (17). The plating efficiency of the cells was determined by plating 50, 100, 200, 500, and 1,000 cells/Petri dish (60 mm, Falcon Plastics) and 1 wk later the cells were fixed in alcohol and stained with Unna-blue. The number of colonies, their size, and the number of cells per colony were counted.

Growth in Agar

To study the effect on the growth of transformed cells in agar, cells were grown in agar medium containing glycolipid mR595. The procedure of growth in agar is described by Montagnier and MacPherson (18).

Bacterial Glycolipids

Glycolipids from *S. minnesota R* mutants were kindly supplied by Dr. O. Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg, Germany. The known features of their structures are given in Table I. Tritium labeled bacterial glycolipid mR595 was prepared as follows: heptose-less mutant of *S. minnesota* (provided by Dr. O. Lüderitz) was grown in a nutrient broth medium (Difco Antibacterial Me-

TABLE I

Structure of the Four Glycolipids Used in the Experiments

Name	Structure
mR595	$(KDO)_3$—lipid A
mR7	Hep-Hep-$(KDO)_3$—lipid A
mR5	Glc-Hep-Hep-$(KDO)_3$—lipid A
mR60	GlcNac-Glc-Gal-Glc-Hep-Hep-(KDO)—Lipid A Gal P,ETN

Abbreviations: ETN, ethanolamine; Gal, galactose; Glc, glucose; GlcNac, *N*-acetylglucosamine; Hep, L-glycero-D-mannoheptose; KDO, 2-keto-3-deoxyoctonic acid; lipid A, glucosaminyl-β-1,6-glucosamine with fatty acid substituted to hydroxyl and amino groups (Fatty acids found in lipid A are β-hydroxymyristic acid, myristic acid, palmitic acid, and lauric acid.); P, phosphate.

dium 3). It was sedimented and washed in a synthetic salt medium (19). 1–2 ml of packed bacteria were suspended in 10 ml of the above synthetic medium and 25 μCi of D-[6-^{3}H]glucosamine (sp act 1–2 Ci mmol, New England Nuclear Corp., Boston, Mass.) and 100 mg of unlabeled fructose were added. The suspension was kept at 37°C for 30 min with shaking, and later the cells were washed twice with water, and the glycolipid was isolated from the bacteria as described by Galanos et al. (20).

The yield of the glycolipid was 1–3% of the dry weight of bacteria, and the specific activity among various batches varied from 60,000 to 100,000 cpm/mg of glycolipid.

Uptake of Labeled Glycolipid by Cultured Cells

The cells were trypsinized for 15 min at 37°C, washed twice with the complete growth medium, and resuspended in the same medium at a concentration of 1.2 × 10^6 cells/4 ml. 0.10 ml of a 200 μg/ml solution of the labeled glycolipid mR595 was then added and the cell suspension was incubated for 15 min at 37°C with shaking. The cells were sedimented by centrifugation, washed twice with phosphate-buffered saline (PBS)), and dissolved in Aquasol. They were counted in a liquid scintillation spectrometer (Nuclear-Chicago Mark 1, Nuclear-Chicago, Des Plaines, Ill.). For studying the uptake of glycolipid in growing cells, the labeled glycolipid solution (0.1 ml containing 20 μg) was added to Petri dishes, 24 h after the cells had been in culture. Every subsequent 24 h the cells were scraped with a rubber policeman and treated as described above for counting their radioactivity.

Demonstration of Cell Surface Binding of Glycolipid mR595 by Immunofluorescence

Cells were grown in Leighton tubes for 24 h and were later washed in PBS. They were fixed in methanol and dried. A few drops of a solution of glycolipid mR595 (200 μg/ml) were added to the fixed cells during 20 min at room temperature. The fixed cells were washed with physiological saline and dried. To these mR595-coated cells, 1 or 2 drops of anti-mR595 rabbit antiserum (21) was added and the cells were incubated for 1 h at 35°C. The cells were washed with physiological saline, dried, and treated with 1 or 2 drops of fluorescein-conjugated sheep antirabbit globulin antiserum (Difco Laboratories, Inc.) for 45 min at 35°C. After this incubation the cells were washed in PBS, and visualized under a fluorescence microscope.

Cyclic AMP Determination

The technique employed was a modification of that described by Brøustad et al. (22). Cells were incubated in the presence or absence of glycolipid mR595. 24 h later, 1.5 μCi of [^{14}C]adenine (6.83 mCi/mmole, New England Nuclear Corp.) was added to the cultures (1 h at 37°C). The cells were washed three times with PBS, scraped from the Petri dishes with a rubber policeman, and homogenized (Vortex homogenizer) with 1 ml 0.1 N HCl followed by centrifugation (15 min, 3,000 rpm). The recovery was determined by adding a known amount of [^{3}H]cyclic AMP. 0.10 ml of the supernatant was spotted on Whatman paper no. 1. After 12 h of migration (solvent: 96% ethanol, 2 M NH_4OH, 2 M acetic acid, 100 19 21, vol/vol/vol), the cyclic AMP band was cut out, eluted with 1 ml of 0.5 N HCl, and the radioactivity was counted in a liquid scintillation spectrometer.

RESULTS

Effects of Various Bacterial Glycolipids on Growth of Cultured Cells

In Fig. 1 the effects of four glycolipids (mR595, mR7, mR5, and mR60) on the growth of normal, spontaneously transformed, and SV40-transformed rat embryo fibroblasts are shown. Growth of normal cells seeded at a concentration of 0.4 × 10^6 cells/Petri dish was not affected by these glycolipids during their five cycles of cell division. Fig. 1 *a* represents the growth of normal cells seeded at a concentration of 1.2 × 10^6 cells/Petri dish in order to compare with the growth characteristics of transformed cells. In contrast, spontaneously transformed cells (Fig. 1 *b*) show an inhibition of growth which varies according to the nature of the bacterial glycolipid. It is interesting to note that the order of decreasing inhibition of growth by glycolipids is: mR595 > mR7 > mR5 > mR60, which represents the increasing chain length. This is also true for SV40-transformed cells although SV40-transformed cells (Fig. 1 *c*) are relatively less sensitive to inhibition than spontaneously transformed rat embryo fibroblasts.

Apparently one can observe two types of effects of the bacterial glycolipids on spontaneously transformed cells. The first consists in an inhibition of cell growth after their addition, and the second seems to relate to cessation of growth which becomes explicit after the cell cultures reach a certain density. These effects are less pronounced

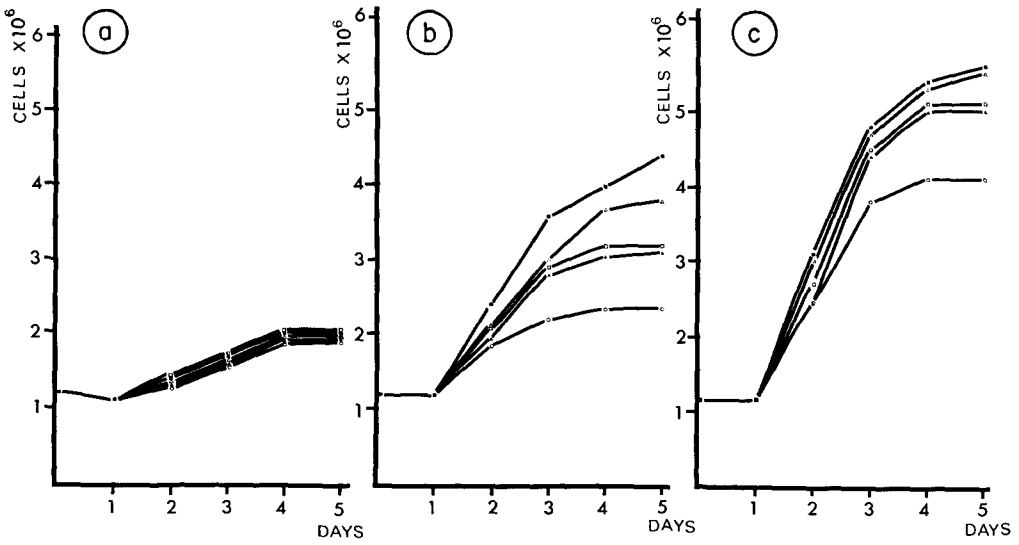

FIGURE 1 Effects of a single addition of glycolipid mR595 (○), mR7 (▲), mR5 (□), and mR60 (△) on the growth of normal (ER) (1 *a*), spontaneously transformed (RST) (1 *b*), and SV40-transformed (RSV40) (1 *c*) rat embryo fibroblasts, added 24 h after seeding. The controls are represented by closed squares. The glycolipids were dissolved in PBS by heating at 100°C for 15 min, to give a concentration of 200 μg/ml. 0.10 ml of the solution was added to 4 ml of the culture medium contained in the Petri dishes seeded with the cells. To the controls, 0.1 ml of PBS was added. Each point represents an average value obtained with 30 Petri dishes. The following t values have been obtained denoting significance of the results; t is considered significant when $t < 0.05$: (Fig. 1 *a*) mR595 $0.20 < t < 0.30$, mR7 $0.30 < t < 0.50$, mR5 $0.30 < t < 0.50$, mR60 $0.50 < t < 0.90$; (Fig. 1 *b*) mR595 $t < 0.001$, mR7 $t < 0.001$, mR5 $t < 0.001$, MR60 $t < 0.001$; (Fig. 1 *c*) mR595 $t < 0.001$, mR7 $t < 0.001$, mR5 $t < 0.001$, mR60 $0.001 < t < 0.01$.

in virally transformed cells, although they do exist in their case as well.

Since glycolipid mR595 was the most effective growth inhibitor for spontaneously transformed cells, we studied the effect of its concentrations on the growth of spontaneously transformed rat embryo fibroblasts (Fig. 2). It was observed that the inhibition was dose dependent within the range of 0.1–5 μg glycolipid/ml medium. Increase in the concentration to 20 μg/ml failed to elicit an inhibition greater than that observed with 5 μg/ml (Fig. 2).

Growth of Cells in Agar

It is known that transformed cells have the ability to grow in agar whereas the normal cells fail to do so. As a corollary to the above experiment we tried the effect of mR595 on the growth of spontaneously and virally transformed rat embryo fibroblasts in agar medium. It was observed that the presence of the bacterial glycolipid significantly inhibited the growth of these cells in agar.

Lack of Cytotoxic Effect of Bacterial Glycolipid mR595 on Cultured Cells

The morphology of cells (both normal and transformed) remained unaltered when they were treated with glycolipid mR595. The cells remained viable as demonstrated by exclusion of trypan blue by the treated cells. A more vigorous assay of viability was performed by estimating the plating efficiency of glycolipid mR595-treated and untreated cells. It was observed that the plating efficiency of both spontaneously transformed and SV40-transformed cells was the same in the presence and absence of glycolipid mR595. The only difference was that the colonies that formed in the presence of mR595 were smaller.

Uptake of Glycolipid mR595

Table II shows the uptake of the labeled glycolipid mR595 when it was added to cultured normal and transformed cells for 15 min, 24 h, and 48 h. It was observed that 15 min of exposure

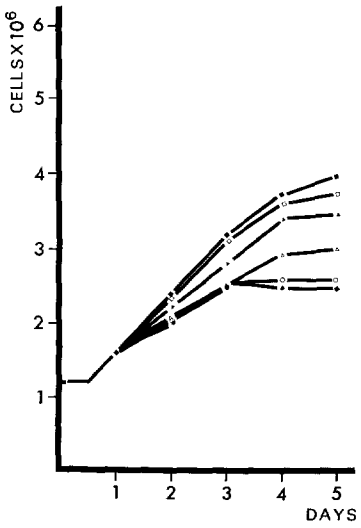

FIGURE 2 Effect of different concentrations of glycolipid mR595 on the growth of spontaneously transformed rat embryo fibroblasts (RST). 0.10 ml of different concentrations of glycolipid mR595 dissolved in PBS by heating at 100°C for 15 min was added to Petri dishes seeded 24 h earlier with spontaneously transformed cells and containing 4 ml of the culture medium. ■, PBS (control); □, 0.1 μg/ml; ▲, 1 μg/ml; △, 5 μg/ml; ○, 10 μg/ml; ◆, 20 μg/ml. Each point on the figure represents an average value obtained with 30 Petri dishes. The t values denoting the significance of the results are as follows: 0.1 μg/ml, $0.001 < t < 0.01$; 1 μg/ml, $t < 0.001$; 5 μg/ml, $t < 0.001$; 10 μg/ml, $t < 0.001$; 20 μg/ml, $t < 0.001$. The t value between 5 μg/ml and 20 μg/ml ($0.10 < t < 0.20$) indicates no significant difference between these concentrations.

gave no uptake by normal cells, but exposure for 24 h elicited some uptake which decreased if the glycolipid was left for 48 h in the culture medium. On the other hand, both transformed cells exhibited small uptake during 15 min of exposure which increased considerably after 24 h. This uptake was not decreased after an additional 24 h of exposure in the case of spontaneously transformed cells and decreased 45% in the case of virally transformed cells.

When both normal and transformed cells were trypsinized (15 min at 37°C) and the detached cells suspended in the complete growth medium, addition of labeled glycolipid mR595 gave an increased uptake during 15 min of exposure of the three types of cells (Table II, column 5). The most dramatic increase was in the case of normal rat embryo fibroblasts which took up the same amount of glycolipid as the virally transformed cells. Similarly, neuraminidase treatment gave increased uptake (experiments done in case of RST cells only). The uptake of the glycolipid by untrypsinized spontaneously and virally transformed cells was not removed by trypsin treatment (Table II, column 2).

Fixation of Glycolipid mR595 on Cell Membrane

In order to show cell surface binding, the three kinds of cells were grown in tubes for 24 h and fixed in methanol for immunofluorescence assay (see Materials and Methods). It was observed that normal rat embryo fibroblasts showed very little fluorescence (Fig. 3 *a*), whereas both transformed cell lines (Fig. 3 *b*, 3 *c*) exhibit strong fluorescence at the cell surface.

Role of Cyclic AMP and Glycolipid mR595 on Growth

During the course of our work on bacterial glycolipid action on growth, a number of investigators reported (23–26) that cyclic AMP and dibutyryl cyclic AMP alone or with theophylline inhibit growth of cells and alter the morphology and agglutination characteristics of transformed cells. It appeared to us reasonable to think that glycolipid action may be mediated in a manner similar to dibutyryl cyclic AMP plus theophylline action reported for transformed 3T3 cells by Sheppard (26). In case both glycolipid and dibutyryl cyclic AMP plus theophylline increase the intracellular concentration of cyclic AMP to the same extent, the addition of the two together should not induce an additive effect on the inhibition of growth of the transformed cells. The results from such an experiment are shown in Fig. 4. It is apparent from Fig. 4 that both glycolipid and dibutyryl cyclic AMP give a similar pattern of growth inhibition of spontaneously transformed cells. When they are added together there is slightly greater inhibition only during the two initial days of growth. Subsequently, we carried out an estimation of cyclic AMP in normal and transformed cells, with and without the treatment of cells with glycolipid mR595 for 24 h. We observed (Table III) that the cyclic AMP level was highest in normal cells, that it was about half of this value for spontaneously transformed

TABLE II

Uptake of Labeled Glycolipid mR595 by Normal and Transformed Cells
(μg of mR595 bound/10^6 cells)

Type of cells	Time of binding					
	15 min	15 min uptake followed by trypsinization	24 h	48 h	Trypsinized cells exposed to mR595 for 15 min	Neuraminidase-treated cells exposed to mR595 for 15 min
ER	0	0	0.69 ± 0.10	0.29 ± 0.05	4.7 ± 0.50	—
RST	0.74 ± 0.33	0.44 ± 0.11	2.90 ± 0.60	3.28 ± 0.40	9.12 ± 1.28	7.57 ± 0.82
RSV40	0.65 ± 0.11	0.54 ± 0.05	4.28 ± 0.75	2.25 ± 0.80	5.14 ± 0.65	—

The labeled glycolipid mR595 and the cell culture were prepared as described in Materials and Methods. For the 15 min uptake the glycolipid mR595 (5 μg/ml) was added directly in the cell culture medium. After 15 min at 37°C the cells were washed three times with PBS. Some samples were resuspended in trypsin (0.1%) and kept at 37°C for 15 min, and washed three times with PBS. In the second case of uptake, the labeled glycolipid mR595 (2 μg glycolipid/1.2 × 10^6 cells/4 ml medium) was added to Petri dishes 24 h after the cells had been in culture. Every subsequent 24 h, the cells were scraped with a rubber policeman and washed three times with PBS. For the last case of uptake, the cells were treated with trypsin or neuraminidase for 15 min at 37°C, washed twice with the complete growth medium, and resuspended in the same medium at a concentration of 1.2 × 10^6 cells/4 ml. 0.10 ml of a 200 μg/ml solution of the labeled glycolipid mR595 was then added, and the cell suspension was incubated for 15 min at 37°C with shaking. The cells were sedimented by centrifugation, and then were washed three times with PBS. For radioactivity determination, the cells were dissolved in Aquasol. They were counted in a liquid scintillation spectrometer. The values are an average ± the standard deviation obtained in three experiments. In each experiment, 10 Petri dishes were taken.

cells, and that it was lowest in SV40-transformed cells. 24 h after the addition of glycolipid mR595, the level of cyclic AMP was increased about 35% in the case of normal cells, and it was augmented to the level of normal cells in the case of spontaneously transformed cells and to half the level of normal cells in the case of SV40-transformed cells.

DISCUSSION

Factors that regulate the growth of transformed cells without affecting normal cells are apparently at the heart of the problem of control of neoplasia. During the past 3 yr reports have appeared showing that certain substances such as trypsinized concanavalin A (9), dibutyryl cyclic AMP (23), dibutyryl cyclic AMP plus theophylline (26), and dibutyryl cyclic AMP plus testosterone (27) alter the growth pattern of certain transformed cells such that these treated cells revert back to the growth pattern of normal cells. This reversion is expressed as long as the additive is present in the culture medium. Although we initially embarked on the action of bacterial glycolipids with the intention of showing their specific cytotoxic action on cancer cells, the results have demonstrated that bacterial glycolipids at the concentrations we have used are not cytotoxic. On the other hand, they show a tendency to inhibit the growth of transformed cells. Our observations with bacterial glycolipids differ from those with trypsinized concanavalin A and with dibutyryl cyclic AMP in certain respects. First, unlike trypsinized concanavalin A, bacterial glycolipids inhibit growth immediately after their addition to the culture medium rather than inhibit growth only when transformed cells reach confluency, and secondly, unlike cyclic AMP, the glycolipids have not been observed to cause any morphological alterations in cells treated with them.

One of the interesting features of our results is the demonstration that the inhibitory action of bacterial glycolipids is a function of the carbohydrate chain length. The smallest carbohydrate chain length is the most effective, whereas longer chain length of core sugars (as in mR60) is the least effective in inhibiting growth. This is in accord with the experiments of one of us (V. N. Nigam) where survival times of animals bearing Ehrlich solid tumor are also increased when the same glycolipids with gradually shorter core length are used.

We have also observed that the growth of spontaneously transformed cells is more effectively in-

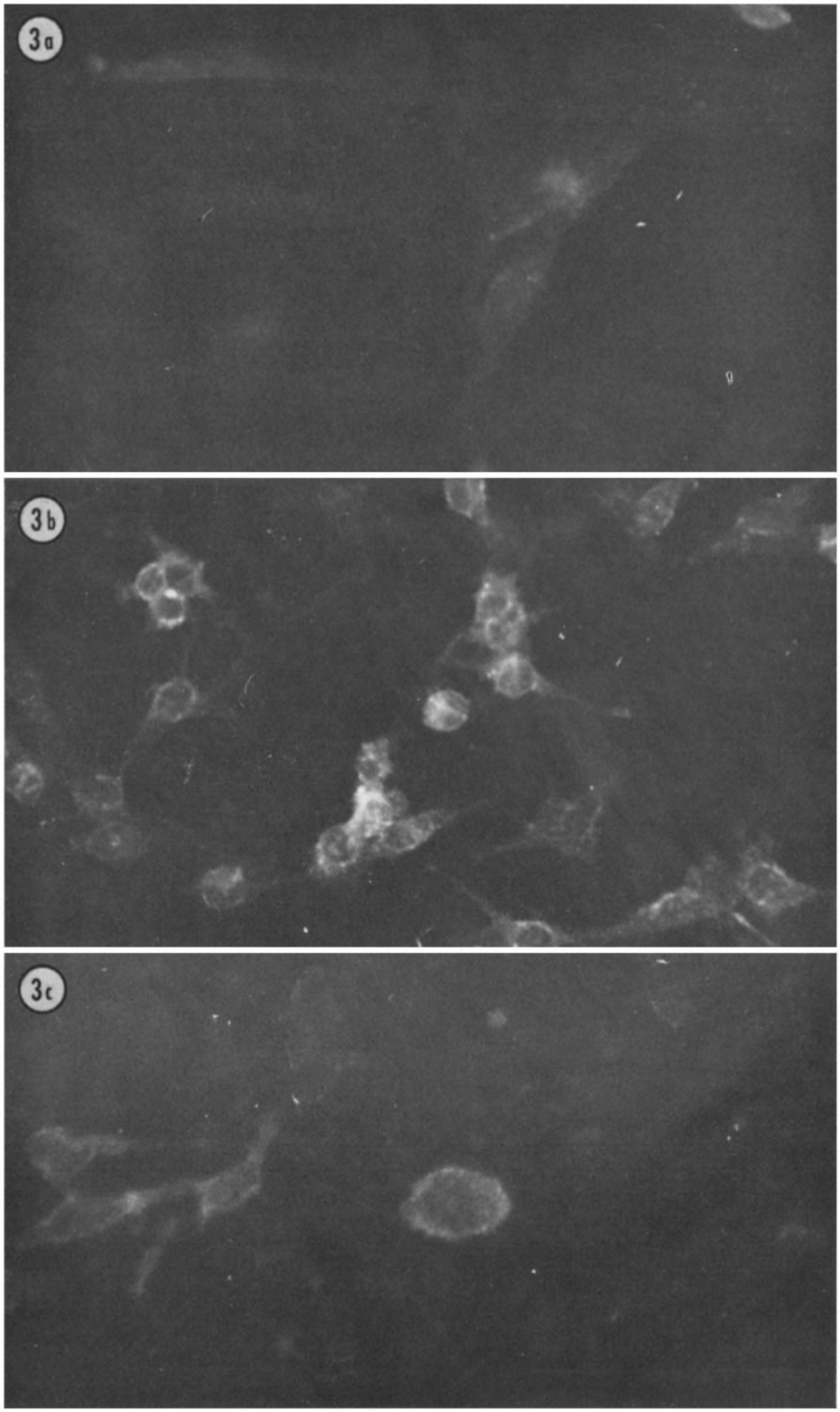

FIGURE 3 Fluorescence pattern of normal (*a*), spontaneously transformed (RST) (*b*), and SV40-transformed rat embryo fibroblasts (RSV40) (*c*). The figure shows an indirect immunofluorescence assay for the binding of glycolipid mR595 on transformed cells. Normal cells show poor fluorescence. Details of the experimental procedure are provided in Materials and Methods. Fig. 3 *a*, × 500; Fig. 3 *b*, × 320, Fig. 3 *c*, × 500.

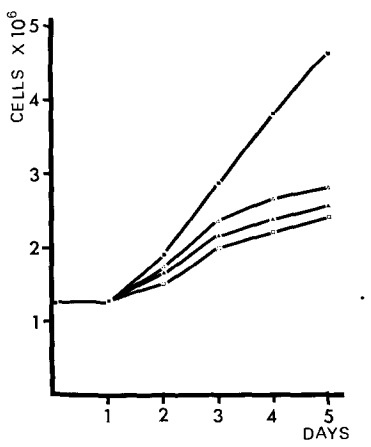

FIGURE 4 Effects of dibutyryl cyclic AMP + theophylline(▲), glycolipid mR595(△), and a mixture of dibutyryl cyclic AMP, theophylline, and glycolipid mR595(□) on the growth of spontaneously transformed rat embryo fibroblasts (RST). Dibutyryl cyclic AMP and theophylline were added after dissolution in PBS (0.1 ml) to give a final concentration of 0.1 mM and 1 mM/Petri dish, respectively. To the control (■), 0.2 ml of PBS was added. The conditions of the experiment were the same as described in Figs. 1 and 2. Each point in the figure represents an average value obtained with 30 Petri dishes; t is considered significant when the value is less than 5% ($0.05 > t$): mR595, $0.001 > t$; cAMP, $0.001 > t$; mR595 − cAMP, $0.001 > t$; cyclic AMP with mR595 − cAMP, $0.20 > t > 0.10$.

hibited by certain glycolipids (mR595, mR5, and mR7) than that of virally transformed cells. However, the significance of this result must await further testing with a variety of spontaneously and virally transformed cell lines.

The action of bacterial glycolipid mR595 seems to be mediated by its initial interaction with the cell membrane. From the binding data with ^{3}H-labeled glycolipid mR595, it appeared that untreated normal cells lack some receptor sites for the glycolipid whereas transformed cells have these sites. This has not been found true. The fixation of the glycolipid on normal cell membrane seems to be hindered by the presence of sialic acid. The sialic acid in question is the glycoprotein sialic acid, since trypsin which removes glycopeptides also allows free access of glycolipid to the cell membrane, and its uptake by the cell. In fact it has been observed that protein-bound sialic acid is lower in transformed cells as compared to normal cells. On the other hand, ganglioside sialic

TABLE III

Alteration in Intracellular Cyclic AMP Concentration by Glycolipid mR595

Expt no.	Type of cell	Cyclic AMP (pmol/mg proteins) Control (−595)	Experimental (+595)	Ratio
1	ER	18	24	1.3
	RST	11	27	2.5
	RSV40	4	11	2.6
2	ER	20	33	1.6
	RST	9.7	22	2.2
	RSV40	4.5	12	2.6

Details of the determination of cyclic AMP are described in Materials and Methods.

acid is the same in normal and spontaneously transformed cells and is lower in virally transformed cells. Quantitatively the uptake of glycolipid mR595 after trypsinization of the cells differs between the three kinds of cells, insofar as normal cells show least uptake, whereas spontaneously transformed cells show nearly double that amount. How far this difference could be responsible for growth inhibition is difficult to assess at the present time.

Although experiments with ^{3}H-labeled glycolipid mR595 can only be described as experiments demonstrating uptake, immunological experiments suggest that the glycolipid is in fact bound to the cell surface and/or cell membrane. We tend to believe that the glycolipid binds to the lipid bilayer of the membrane since the lipid group of the bacterial glycolipid would have greater affinity for lipids of the cell membrane, and secondly, we have seen that bound glycolipid is not removed from the cells even if they are treated with trypsin. In this respect, the binding of bacterial glycolipid with cells differs from that of concanavalin A, which is known to bind with cell surface glycoprotein-hexose. Thus, in our case the cryptic nature of bacterial glycolipid binding sites on normal cells is due to cell surface glycoprotein, whereas in the case of concanavalin A-mediated agglutination of normal cells it is due to possible exposure of masked hexose residues of glycoprotein on mild trypsin treatment. Further, in our case transformed cells appear to have certain regions of exposed cell membrane (not recovered by glycoprotein), whereas normal cells have very few or none. In both cases, trypsin treatment uncovers areas so

that bacterial glycolipid can bind to the cell membrane.

Although at present we do not know whether there are specific receptors for bacterial glycolipids on the cell membrane, it is nevertheless apparent that the binding of the bacterial glycolipid to either the lipid bilayer or to a membrane-associated protein alters the activity of the membrane-bound adenyl cyclase-cyclic AMP phosphodiesterase system such that the intracellular concentration of cyclic AMP is enhanced. Because of the known growth inhibitory effect of dibutyryl cyclic AMP on transformed cultured cells, we tend to believe that the mechanism of action of bacterial glycolipid-mediated inhibition of transformed cells is due to the increase of the intracellular level of cyclic AMP. Our future work on the isolation of bacterial glycolipid receptors in isolated cell membranes from normal and transformed cells as well as on the alterations in the activities of membrane-bound adenyl cyclase-cyclic AMP phosphodiesterase is likely to throw light on the mode of action of bacterial glycolipids on normal and transformed cultured cells.

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