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ALTERATIONS OF FORSSMAN-ANTIGENIC REACTIVITY AND OF MONOSACCHARIDE COMPOSITION IN PLASMA MEMBRANE FROM POLYOMA-TRANSFORMED HAMSTER CELLS

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SUMMARY

Plasma membrane was prepared from BHK cells and their polyoma virus transformed cells according to the procedure of WALLACH AND KAMAT.

The immunological reactivity of Forssman antigen was assayed on the isolated membranes by the hemolysis-inhibition technique. The results confirmed the previous observations that the reactivity was found in the transformed cells but not in the untransformed cells.

When the untransformed BHK membrane was treated with trypsin, the antigenic reactivity appeared. Trypsin treatment of the polyoma virus-transformed BHK membrane enhanced the reactivity to some extent. These results imply that the acquisition of the reactivity is due to the organizational changes of the plasma membrane of the BHK cells concomitant with viral transformation.

Determination of sugar contents of the membrane showed that in the plasma membrane of the transformed cells, most of the neutral and aminosugars were decreased as compared with those of the untransformed cells. This pattern of reduction in monosaccharides implies a decrease of carbohydrate moiety in glycoproteins and glycolipids in the plasma membrane of the polyoma virus-transformed BHK cells.

INTRODUCTION

Evidence has been presented that viral transformation is accompanied by the appearance of reactivities of cells with certain antigens[1,2] and phytoagglutinins[3,4] as well as by biochemical alterations[5–11] in the transformed cells. To understand the characteristic properties of tumor cells[12,13], such as invasive growth, metastasis and the loss of contact inhibition, it is important to characterize the immunological and chemical alterations occurring in the plasma membrane of the virally transformed cells.

The present investigation is concerned with the Forssman antigenic reactivity of the plasma membrane isolated from untransformed and polyoma virus-transformed

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hamster cells, and with a comparison of the sugar composition of the membrane from both cell lines.

MATERIALS AND METHODS

Cells

Cells used were generously provided by Dr. O'Neill and Miss Ullrey in this laboratory. Baby hamster kidney fibroblast of the BHK 21 line, clone C 13 (BHK), and the polyoma virus-transformed derivative of this line, clone Py 3 (BHK-Py), were grown as described by O'NEILL[14]. The cells were cultured until they formed a heavy, confluent layer. The cells were harvested by scraping with a rubber policeman. Trypsin was not used for harvesting. The cells were suspended in chilled 5 mM Tris–HCl buffer (pH 7.4) containing 0.15 M NaCl, centrifuged, washed twice by resuspension and centrifugation, and then subjected to cell fractionation.

Subcellular fractionation

The cells were ruptured by a pressure homogenizer (Artisan Metal Industries Inc.) and the resulting homogenate was separated into the following fractions by differential centrifugation; nuclear fraction precipitated at 600 $\times$ *g* for 10 min, mitochondrial fraction at 6000 $\times$ *g* for 15 min, and microsomal and supernatant fractions at 110000 $\times$ *g* for 45 min.

Plasma membrane and endoplasmic reticulum free of ribosomes

These were prepared from the microsomal fraction of the cells by the procedures of WALLACH *et al.*[15,16]. Dextran 110 (Pharmacia Fine Chemicals) was used instead of Ficoll for the density gradient method. The purity of these membrane fragments was examined by assaying (Na^+,K^+)-ATPase and NADH-dehydrogenase activities[15] as marker enzymes for plasma membrane and endoplasmic reticulum, respectively. The membranes were washed repeatedly by suspending in 1 mM Tris–HCl buffer (pH 8.1), followed by centrifugation to eliminate Dextran until the supernatants were negative to the Molisch test. The final preparations were lyophilized, except for the sialic acid determination.

Forssman antigenic reactitity

This was assayed by the sheep-cell hemolysis inhibition method as described previously[17]. The results were expressed graphically in terms of per cent hemolysis inhibition plotted against the amounts of membrane protein, rather than by expressing as the concentration of the Forssman-reactive materials at a 50 % inhibition as described previously[17], because the samples tested contained large amounts of material other than the antigen and the reactivity of some samples did not reach 50 % of hemolysis inhibition. A correction for turbidity caused by the suspension of the test substances themselves was made at each concentration.

Forssman antigen was isolated from equine kidney as described previously[17].

Trypsin digestion

The digestion mixture contained 0.3 % (w/v) membrane protein, 0.01 % (w/v) crystalline trypsin (Worthington Biochemical Corp.), 10 mM Tris–HCl buffer (pH 8.1)

and 2 mM Ca^{2+}. The mixture was incubated for 30 min at 24°. At the end of the incubation, a soybean trypsin inhibitor (crystallized 5 times, Mann Research Lab.) was added to the mixture to give a final concentration of 0.01 % (w/v) in order to inactivate the trypsin. A digestion mixture in which the inhibitor had been added in advance of incubation was prepared and used as control. These mixtures were subjected to serial 2-fold dilution and assayed for Forssman reactivity. In all the experiments, the mean values from duplicate experiments are recorded.

Determination of monosaccharides

Neutral sugars were determined by gas chromatography according to the method of CLAMP *et al.*[18] with some modifications. The instrument used was the Microtek Model 220 gas chromatograph equipped with a flame ionization detector. The column used was 5 % SE-30 on Chromosorb W (80–100 mesh) packed into a glass U-shaped tube (10 ft × 1/8 inch internal diameter). Chromatography was carried out at a constant temperature of 150° or 160°.

In one case, a gas chromatographic procedure[19] different from that described above was also employed. The values obtained by both procedures agreed well within the limits of experimental error.

Hexosamine[20] and sialic acid[21] were determined colorimetrically using glucosamine HCl and *N*-acetylneuraminic acid as standards, respectively. Hydrolysis was carried out in a boiling water bath with 4 M HCl for 6 h for hexosamine, and at 80° with 0.05 M H_2SO_4 for 1 h for sialic acid. Individual hexosamine was not determined.

Protein

Protein was measured according to LOWRY *et al.*[22].

RESULTS

The results of enzyme assays shown in Table I showed that the separation of membrane fractions into plasma membrane and endoplasmic reticulum was satisfactory.

(I) Immunoreaction of isolated membranes

The plasma membranes of BHK-Py and BHK cells were assayed for the Forssman antigenic reactivity. As shown in Fig. 1, the reactivity was only detected in the transformed BHK-Py membrane. This observation is similar to that using intact cells and reported previously[2,14,23], that the Forssman antigenic reactivity appeared on the cell surface of hamster cells after polyoma virus transformation.

Some reactivity (less than 20 % of that of the plasma membrane when compared on 100 μg protein basis) was detected in the endoplasmic reticulum of the BHK-Py cells.

Distribution of Forssman antigenic reactivity in the subcellullar fractions of BHK-Py cells. The reactivity was found in the homogenate of BHK-Py cells, but not in that of BHK cells. As shown in Fig. 2, the prominent reactivity was demonstrated in the microsomal fraction of BHK-Py cells, which contain plasma membrane, whereas no reactivity was detected in the nuclear and the supernatant fractions. An appreciable reactivity, however, was also detected in the mitochondrial fraction,

though a possibility that this fraction is contaminated with some plasma membrane could not be excluded. It is noteworthy that the microsomal and mitochondrial fractions from the tissues of certain strains of mouse contained larger amounts of Forssman antigen than did the nuclear fraction[24].

TABLE I

ENZYMIC ACTIVITIES OF MEMBRANES ISOLATED FROM BHK CELLS (BHK) AND THEIR POLYOMA-TRANSFORMED CELLS (BHK-Py)

		NADH dehydrogenase (μmoles NADH used/ mg protein per min)	*(Na^+, K^+)-ATPase (μmoles P_i liberated/ mg protein per h)*
BHK	PM	0.552	6.85
	ER	1.604	0.64
BHK-Py	PM	0.411	3.05*
	ER	1.420	0.68*

Abbreviations: PM, plasma membrane; ER, endoplasmic reticulum.
* One measurement. Other values are averages of two measurements.

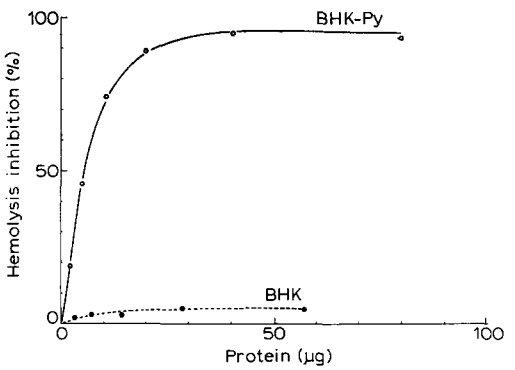

Fig. 1. Forssman-antigenic reactivity of the plasma membranes from the untransformed BHK cells (BHK) and from BHK cells transformed with polyoma virus (BHK-Py).

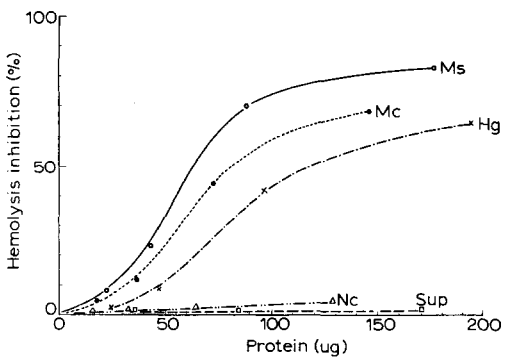

Fig. 2. Forssman-antigenic reactivity of the subcellular fractions of the BHK cells transformed with polyoma virus. Ms, microsome; Mc, mitochondria; Hg, homogenate; Nc, nuclei; Sup, supernatant fraction at 110000 × *g*.

When the BHK microsome was incubated with the BHK-Py supernatant at 37° for 2 h, or the BHK-Py microsome with the BHK supernatant, there was no difference in the Forssman reactivity from that in the non-incubated microsomes, respectively. These observations may exclude the possibility that an action of proteolytic enzymes of the cells could influence the reactivity.

Effect of lipid extraction on the reactivity. In mammalian tissues, the site of antigenic reactivity is either glycolipids[17,25] or glycoproteins[26], containing an α-linked *N*-acetylgalactosaminoyl residue in their terminal carbohydrate chains. In order to determine the nature of the reactivity site, lipid extraction was made on the membrane according to the method of FOLCH *et al.*[27]. The lipid fraction and lipid-free membrane were assayed for the Forssman reactivity. As shown in Fig. 3, the reactivity was found only in the lipid fraction. This indicates that the Forssman-reactive substance present in the cultured cells may be identical with the Forssman antigen of equine tissues[17].

Effect of treatment of the membrane with trypsin on the reactivity. In order to

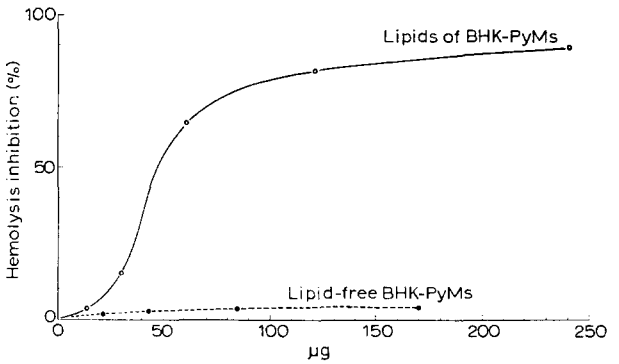

Fig. 3. Forssman-antigenic reactivity of lipids and lipid-free membrane from the microsome of the transformed BHK cells (BHK-Py Ms). In this figure, the abscissa indicates the amounts of the microsome protein from which lipids and lipid-free material were derived, but does not give the actual amounts of lipids or lipid-free material.

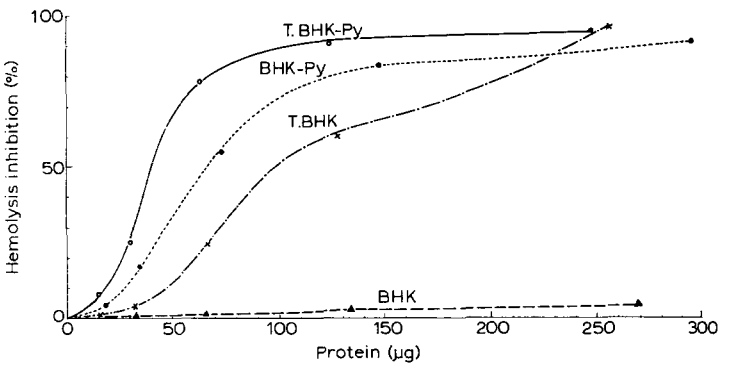

Fig. 4. Forssman-antigenic reactivity of the plasma membranes with and without treatment by trypsin. BHK, intact membrane of the untransformed BHK cells; T.BHK, trypsin-treated membrane of the untransformed cells; BHK-Py, intact membrane of the cells transformed with polyoma virus; T.BHK-Py, trypsin-treated membrane of the cells transformed with polyoma virus.

examine a possibility of the mechanism of the antigenic alteration observed between the BHK and BHK-Py membranes, the membranes were treated with trypsin. As shown in Fig. 4, a trypsin treatment was found to convert the isolated plasma membrane of the untransformed BHK cells from a state of nonreactivity to a state of reactivity. The reactivity thus attained, however, was still less than that of untreated BHK-Py membrane. When Forssman-positive BHK-Py membrane was treated with trypsin, the reactivity was further increased to some extent.

These findings indicate that the membrane of the untransformed cells already contains Forssman antigen in such a fashion that it becomes available to the antibody only after treatment of the membrane with trypsin. Similar observations on trypsin action have been reported previously[4,28,29].

The assay of a transferase[30] which catalyzes the transfer of *N*-acetylgalactosamine from labeled UDP-*N*-acetylgalactosamine to hexosyl ceramide was undertaken to compare the formation of the Forssman antigen between BHK and BHK-Py cell lines. However, the enzymic activity found in both the cell lines was too low to evaluate.

Forssman reactivity in hamster tissue. This was assayed for comparison with cells in culture. A microsomal fraction was prepared from the kidney of normal adult and 3-day-old Golden Syrian hamsters, and from whole viscera of the embryo at about the 15th day of gestation.

The antigenic reactivity of these hamster tissues *in vivo* was much higher, compared with that of BHK-Py cells in culture (Fig. 5). When compared at various stages of tissue development, the reactivity was highest in the embryo and decreased with age.

(II) Monosaccharide contents of BHK and BHK-Py membrane

The sugar content of the isolated membrane was determined by gas chromatographic and colorimetric methods.

The results are summarized in Table II. The amounts of neutral and aminosugars were decreased in the plasma membrane of the transformed BHK-Py cells, as compared with those of the untransformed BHK membrane. Exceptions were ribose and xylose.

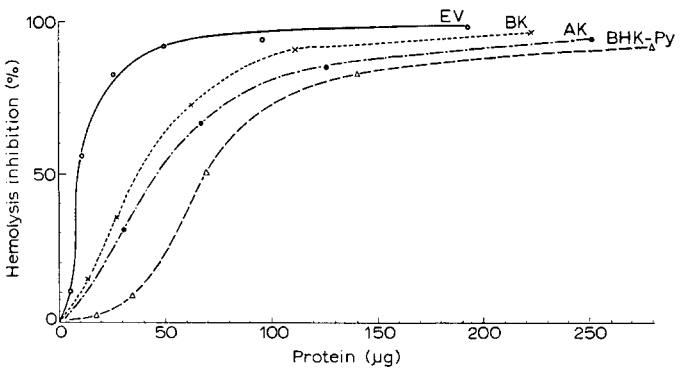

Fig. 5. Forssman-antigenic reactivity of hamster cells in tissue and in culture. Microsomes of whole viscera from embryo (EV), baby kidney (BK), adult kidney (AK) and BHK cells transformed with polyoma virus (BHK-Py) were assayed by hemolysis-inhibition technique.

TABLE II

MONOSACCHARIDE COMPOSITION OF THE PLASMA MEMBRANES OF BHK AND THE POLYOMA VIRUS-TRANSFORMED BHK CELLS (BHK-Py)*

Values are average of two measurements.

Cell line	*Monosaccharides (nmoles/mg of dry membrane)*							
	Fucose	*Ribose*	*Xylose*	*Mannose*	*Galactose*	*Glucose*	*Hexos-amine*	*Sialic acid**
BHK	4.3	30.0	2.0	39.5	50.5	42.7	32.4	34.5
BHK-Py	1.2	40.6	3.1	28.4	25.6	38.2	17.2	13.1

* Sialic acid was determined on the basis of nmoles/mg of protein.

DISCUSSION

Several reports using whole cells have indicated that oncogenic virus transformation converts the Forssman-negative cells to the positive form[2,14,23]. This antigenic reactivity could be demonstrated not only in the cells transformed with DNA viruses such as polyoma[2,14,23] and SV40[14,23], but also in the transformant with the RNA vrius, Rous sarcoma virus[14]. However, the acquisition of this antigenic reactivity is not a common phenomenon characteristic of viral transformation. ROBERTSON AND BLACK[23] presented evidence that hamster cells transformed with adenovirus 12 and mouse cells transformed with SV40 or polyoma virus did not exhibit this reactivity. In the present studies, the Forssman antigenic reactivity was demonstrated, by the hemolysis inhibition technique, in the plasma membrane isolated from polyomavirus-transformed BHK cells (BHK-Py), but not in the membrane from the nontransformed cells (BHK). These observations confirm O'NEILL'S[14] findings that the reactivity was demonstrated by the use of antisera against the isolated plasma membrane and of an immune adherence technique.

The antigenic reactivity was almost completely removed by lipid extraction from the membrane, suggesting a glycolipid nature for this antigen. This antigen was present not only in the plasma membrane, but also in the mitochondrial fraction of the BHK-Py cells. The antigen is also localized in cytoplasm[2] and in the mitochondrial fraction[24], as well as on the cell surface.

As to the difference in the antigenic reactivity between BHK and BHK-Py cell lines, the present studies on the trypsin treatment of the plasma membrane indicate that the appearance of the reactivity is most probably due to the changes of membrane organization occurring with viral transformation, but not to the induction of synthesis of Forssman antigen.

On the other hand, a conciderable decrease in the carbohydrate content including the constituents of Forssman antigen, in the plasma membrane of BHK-Py cells (Table II) leads us to postulate that the synthesis of Forssman antigen, though it was not demonstrable, might be depressed in the BKH-Py cells. These seemingly contradictory findings are similar to those of HAKOMORI *et al.*[28]. They showed that while the hematoside (sialyl lactosyl ceramide) content was much lower in the

virally transformed cells than in their untransformed parent cells (BHK and 3T3 cells), the antigenic reactivity to the anti-hematoside serum was almost the same between the transformants and the nontransformed cells whose reactivity had been enhanced by trypsin treatment.

Studies that give support to architectural alterations of the surface of virally transformed cells have been reported using whole cells of several lines, plant agglutinins[4,29] and anti-serum against a membrane component[28].

However, some differences can be seen between the reactivities shown in previous papers and the Forssman antigenic reactivity herein. For instance, the reactivity of transformed cells with wheat germ agglutinin did not change after protease treatment[29], and trypsin treatment of the transformed cells resulted in a decrease of the agglutinability with concanavalin A[4], while the Forssman reactivity of the BHK-Py membrane was increased after the treatment (Fig. 4). These differences might reflect the discrepancies of the localization in the plasma membrane of different substances with various reactive sites.

FOGEL AND SACHS[31], using a fluorescent antibody technique, reported that the Forssman antigen appeared *iv vitro* when normal cell contacts were disturbed, and disappeared when these contacts were restored.

Since the experiments reported here were carried out with isolated membranes, the changes in the antigenic reactivity could be accounted for by organization of membranes, but not by cellular interaction. A comparative study showed a marked difference in the reactivity between the membrane of untransformed BHK cells in culture (Fig. 1) and that of tissue (Fig. 5). This suggests that cell plating *in vitro* converted the membrane from the Forssman-positive state to the negative state.

It has previously been demonstrated that viral transformation is accompanied by alterations of carbohydrate composition as well as changes in immunological reactivity. According to WU *et al.*[8], the plasma membrane of the mouse fibroblast transformed with SV40 virus (3T3–SV40) showed much lower sialic acid and galactosamine, and a reciprocal increase in the relative content of glucosamine, as compared with their untransformed parent cells (3T3). Also, the contents of neutral sugars (fucose, mannose and glucose) of the 3T3-SV40 microsome were reduced as compared-with the 3T3. Since the contents of neutral sugars in the submicrosomal fractions were not presented for the mouse cell lines and a differential analysis of hexosamines was not made in this study, precise comparison is difficult. Nevertheless, the overall feature of the changes appeared to be similar between the hamster and the mouse cell systems. Some differences in the changes, however, can be seen between the two systems. In the 3T3 microsome, an increased content of galactose was noted as a result of SV40 transformation. In contrast, a significant decrease of galactose occurred in the plasma membrane after polyoma virus transformation.

It has been reported that the isolated plasma membrane contains RNA, regardless of the methods of isolation[15,32]. The content of ribose, therefore, could not account for the observed changes in carbohydrates of the membrane structure. On the basis of the content of xylose, which is known to be a constituent of mucopolysaccharides[33], it is likely that mucopolysaccharides are present on the cell surface. The increased content of xylose in BHK-Py plasma membrane compared with the BHK membrane suggests that mucopolysaccharides increase on the cell surface concomitant with viral transformation.

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