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PREFACE

The first oncogenic virus to be discovered, RSV remains a major focus of interest for those concerned with the etiology of cancerous growth. At present, RSV represents the best experimentally accessible model of an oncogenic RNA virus whose effects can be observed both *in vivo* and *in vitro*.

The present two-volume collection includes papers published from 1970–1972 on host cell surface changes induced by RSV, on the enzymic machinery contained in the virion as well as on the viral nucleic acids. Current research on RSV mutants or genetic variants is also presented.

Cellular Responses to Rous Sarcoma Virus

Transformation by Rous Sarcoma Virus: Effects on Cellular Glycolipids

SEN-ITIROH HAKOMORI, TERUNOBU SAITO, AND PETER K. VOGT

INTRODUCTION

Neoplastic transformation of animal cells results in alterations of the cell surface. These changes include loss of contact inhibition of movement and of growth (for review, see Abercrombie, 1970), decrease of cellular adhesiveness (Coman, 1960), formation of tumor-specific antigens (Habel, 1967; Prehn, 1963; Sjögren, 1965), increase of negative surface charge (Forrester *et al.*, 1962), uncovering of cryptic carbohydrate haptenes (Burger, 1969, Inbar and Sachs, 1969; Häyry and Defendi, 1970), and altered patterns of cellular glycoproteins (Bosman *et al.*, 1968; Wu *et al.*, 1969; Meezan *et al.*, 1969; Buck *et al.*, 1970). The molecular mechanisms of these alterations are largely unknown. However, it is probable that not all of them represent separate and independent events. Indeed, some may be different manifestations of the same process.

In a search for transformation-induced changes which can be defined chemically, we have studied cellular glycolipids. Glycolipids are common components of animal cell membranes (Yamakawa and Suzuki, 1952; Yamakawa, 1966; Hakomori, 1964; Hakomori and Strycharz, 1968; Dod and Gray, 1968; Weinstein *et al.*, 1970; Klenk and Choppin, 1970). During virus-induced neoplastic transformation qualitative as well as quantitative changes occur in this molecular species. It has been reported that the nonreducing terminals of carbohydrate chains are deleted in transformed cells, possibly as a result of incomplete synthesis (Hakomori and Mura-

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kami, 1968; Hakomori *et al.*, 1968). Similar changes have been observed in higher gangliosides (Mora *et al.*, 1969; Brady *et al.*, 1969; Cumar *et al.*, 1970).

The glycolipid pattern of animal cells in culture is also greatly affected by the number of passages. For instance, BHK C13/21 cells (Stoker and Macpherson, 1961) of low passage number contain a large quantity of ceramide trihexoside which tends to be decreased or completely lost as these cells are passaged for longer time periods (Hakomori, 1970).

The present study is an initial survey of changes in cellular glycolipids during neoplastic transformation induced by RNA sarcoma viruses in tissue culture. Various strains of Rous sarcoma virus (RSV) infecting chick embryo fibroblasts were investigated, and cellular glycolipid patterns during and after transformation were compared with those of normal fibroblasts and of nontransformed fibroblasts infected with an avian leukosis virus. After infection with RSV neoplastic transformation of over 90% of the cells can be achieved within 1–2 days (Hanafusa, 1969; Vogt, unpublished observation). RSV-induced changes of glycolipid patterns can thus be studied before prolonged periods of cell culture superimpose additional alterations which may be unrelated to virus-induced neoplasia. Furthermore, it is possible to use the same chick embryo for infected and for control cultures; variations due to genetic heterogeneity of cells can therefore be eliminated.

MATERIALS AND METHODS

Viruses. The following avian tumor viruses, listed according to their subgroup affiliation were used. Subgroup A: RSV-(RAV-1), a pseudotype of Bryan strain Rous sarcoma virus (RSV) formed with Rous associated virus type 1 (RAV-1), Schmidt-Ruppin RSV, type 1; *f*RSV-A, a pseudotype of fusiform RSV (Temin, 1960; Yoshii and Vogt, 1970) formed with a Rous-associated virus of subgroup A; RAV-1, Rous-associated virus type 1. Subgroup C: PR RSV-C, Prague strain RSV. The preparation of these viruses has been described (Ishizaki and Vogt, 1966; Duff and Vogt, 1969).

Cell culture and virus assays. Cultures from chick embryo fibroblasts and assay of RSV followed the techniques of Rubin (1960). The chicken cells were of the C/O and C/B phenotypes (Vogt and Ishizaki, 1965). A complete account of these methods including the composition of growth media has been given recently (Vogt, 1969). Titers of RAV were determined with the fluorescent focus assay (Vogt and Rubin, 1963).

Infection with high multiplicities of virus and rapid transformation of cells. Primary cultures of chick embryo fibroblasts were suspended in growth medium at a concentration of 1×10^7 cells per ml, mixed with an equal amount of virus (1 to 5×10^7 focus-forming units per ml) and stirred on a magnetic stirrer at room temperature. Subgroup A viruses showed rapid adsorption to host cells under these conditions. Optimal adsorption of all other subgroups was achieved by adding to the virus–cell suspension 2–5 μg/ml of a polycationic enhancer, DEAE dextran or Polybrene (Vogt, 1967a; Toyoshima and Vogt, 1969). After 1 hr the cells were diluted with growth medium to 5×10^5 per ml and seeded in 10-ml amounts on 100-mm petri dishes.

Harvest of cells and extraction of glycolipids. All harvests were made from confluent cultures with the exception of normal chick embryo fibroblasts which were studied in sparsely seeded cultures as well. At the time of harvest the culture medium was withdrawn from the dishes, the cells were twice gently rinsed with phosphate-buffered saline, pH 7.2 (PBS), and scraped with a rubber policeman into a small amount of PBS. The cell suspension was collected in a graduated conical centrifuge tube and sedimented at 2000 rpm for 5 min. The PBS-supernatant was saved (Sup-A). The volume of the cell pellet ($\times$ ml) was recorded, and the cells were throughly suspended in 4 $\times$ ml of PBS. A small portion (0.05–0.1 ml) was used for protein determination (Lowry *et al.*, 1951). The rest of the cell suspension was extracted with 80 $\times$ ml of chloroform–methanol (2:1) by homogenization for 3 min in a Sorvall "Omnimixer" and filtered (Ext-1). The cell residue on the filter was again extracted with 80 $\times$ ml of chloroform–methanol (1:2) (Ext-2).

Sup-A was dialyzed, lyophilized, and extracted with chloroform–methanol. No glycolipids were recovered from this fraction.

Fractionation of lipids and quantitative separation of glycolipids. Ext-1 and Ext-2 were combined and evaporated to dryness in a rotary evaporator. The dried residue was dissolved in 20 × ml of chloroform–methanol (2:1), and the insoluble material was filtered off. The filtrate was fractionated according to a modification of Folch's partition into upper and lower phase glycolipids (Svennerholm, 1963). The partition was repeated 10 times by this modified method. All gangliosides and 90% of hematosides were extracted in the upper phases. They were combined, evaporated to a small volume and dialyzed against ice-water. The dialyzed solution was evaporated to dryness, the residue was extracted with small volumes of chloroform–methanol (2:1). The extracts were transferred to a conical tube and again evaporated to dryness under a stream of nitrogen (fraction I). The lower phase glycolipids (neutral glycolipids having less than 4 carbohydrate units per molecule, a small portion of hematosides and ceramide) were quantitatively separated from the bulk of phospholipids and cholesterols by an acetylation procedure (Saito and Hakomori, 1971). Briefly, the lower phase lipids were dried, acetylated, and placed on a magnesium silicate column in 1,2-dichloroethane (DCE). The acetylated glycolipids and ceramides were quantitatively eluted from the column by DCE–methanol (9:1) or by DCE-acetone (1:1). Deacetylation in chloroform–methanol–0.5% sodium methoxide (2:1:0.6) at room temperature for 30 min recovered all glycolipids and ceramides without any loss or structural changes. The fraction was neutralized with methylacetate and evaporated under nitrogen (fraction II). Fractions I and II were characterized by chemical analysis, sugar determinations, and thin-layer as well as gas–liquid chromatography.

Characterization of lipids. Four major glycolipids found in fraction I were separated by preparative thin-layer chromatography on silica gel H. The molar ratios of glucose, galactose, and sialic acids and those of glucose, galactose, glucosamine, and galactosamine were analyzed by gas chromatography according to the methods of Sweeley and Walker (1964) and of Yang and Hakomori (1971), respectively. *N*-Glycolyl- and *N*-acetylneuraminic acids were determined by mild methanolysis (0.01 *N* methanolic hydrochloric acid) followed by gas chromatography (Yu and Ledeen, 1970). The migration rates of these glycolipid components on thin-layer chromatography were compared before and after partial hydrolysis with the reference glycolipids shown in Table 1. Also included as standards were ceramides with or without hydroxylated fatty acids which had been purchased from Applied Science Laboratories, Inc., State College, Pennsylvania.

Some glycolipid components were degraded according to a published technique (Smith and Unrau, 1959) in order to determine the terminal carbohydrate unit. Partial hydrolysis was carried out in 0.1 *N* sulfuric acid in 50% methanol at 80° for 1 hr.

Quantitative determination of glycolipids. Glycolipid fractions were diluted serially in 2-fold steps and chromatographed together with known amounts of reference glycolipids diluted in the same fraction. The parallel chromatographs were sprayed with the Svennerholm's resorcinol reagent (1957), covered with a glass plate, and heated at 130° for 20 min. The dilution end points of the unknown sample were determined visually by comparison with the standards which contained known quantities of sialic acid. This method was superior to direct densitomitry of resorcinol positive spots and to spectrophorometric determinations of resorcinol reaction in test tubes applied to individual spots scraped off the chromatography plates. For determination of glucosylceramide and free ceramides, the dilution method was used after the plates were developed with hypochloride-benzidine method (Bischel and Austin, 1963).

Throughout this communication glycolipid concentrations are standardized by relating them to equal amounts of cellular proteins. As has been shown by Goldé (1962), the protein content per cell increases about 2-fold after transformation with RSV. Therefore, glycolipid values listed in the paper do