

Immunochemical studies of organ and tumor lipids: VIII. comparison of human tumor and ox spleen cytosides*

MAURICE M. RAPPORT, LISELOTTE GRAF, and
NICHOLAS F. ALONZO

*Department of Biochemistry, Albert Einstein College
of Medicine, Yeshiva University, New York 61, N. Y.;
and Division of Experimental Pathology, Sloan-Kettering
Institute for Cancer Research, New York 21, N. Y.*

[Received for publication March 2, 1960]

SUMMARY

Preparations of cytolipin H, a human tissue lipid hapten containing fatty acid, sphingosine, glucose, and galactose in equimolar proportions (cytoside), have been compared with preparations of similar composition isolated from ox spleen. The iodine number and the specific rotation of preparations derived from human tumor are significantly larger than those from ox spleen. Results of other chemical analyses (based on the carbohydrate and sphingosine portions of the molecule) as well as immunochemical reactions are indistinguishable for both.

Repeated injection into rabbits of cell fractions or homogenates of foreign tissue provokes the formation of antibodies that frequently react with lipids extracted from the tissue. With human tissues this phenomenon is more readily seen with fractions derived from cancerous tissues than with similar fractions from normal organs. With many antisera, several independent antigen-antibody systems have been found to contribute to the over-all measurement (1). However, one substance, cytolipin H, and its antibody appear to be most frequently responsible for a major part of the reactions observed *in vitro* (1). Cytolipin H, a glycolipid isolated recently from human epidermoid carcinoma (H.Ep.3) grown in conditioned rats (2), is a water-insoluble substance containing four residues in equimolar proportions: fatty acid, lipid base (sphingosine), glucose, and galactose.

A molecule with this composition and similar physical properties was reported to be present in ox spleen by Klenk and Rennkamp in 1942 (3). In connection

with the isolation of spleen gangliosides, they encountered a fraction which they called "cerebroside with two hexose residues" since it contained fatty acid, sphingosine, and two different hexoses. From studies based on fermentability, rotation, and reducing power of the water-soluble hydrolytic products, and assuming the carbohydrates to be galactose and glucose, the value of 1.25 was found for the ratio of galactose to glucose.

It is the purpose of this report to present a comparison of the chemical, physical, and immunological properties of these similar glycolipids found in human tumor and in ox spleen. Small but significant differences were found in specific rotation and iodine number. On the other hand, the preparations were indistinguishable by other chemical analyses and by immunological criteria.

It may be anticipated that the finding of biological (haptenic) activity in a molecule of this type will require some simplification in nomenclature in dealing with structure-activity relationships. For this reason the term "cytoside" has been introduced (4) for a molecule composed of ceramide (fatty acid amide of sphingosine) linked to two monosaccharide residues, in strict analogy with the useful term "cerebroside" describing a molecule in which ceramide is linked to one monosaccharide. We shall accordingly refer in this

*This work was supported by grants from the National Science Foundation, the American Cancer Society, and the United States Public Health Service (Research Grant C-2316 from the National Cancer Institute). We also wish to acknowledge partial support by Interdisciplinary Grant 2M-6418, National Institutes of Mental Health, United States Public Health Service. This paper was presented at the Fiftieth Annual Meeting of the American Society of Biological Chemists.

paper to preparations of human tumor and ox spleen cytosides.

METHODS

Cytoside Preparations. Two preparations of cytolipin H were isolated as described (2) from human epidermoid carcinoma (H.Ep.3) grown in rats. Two preparations of ox spleen cytoside were isolated similarly, the essential steps involving extraction of crude lipids from ox spleen, removal of water-soluble substances, fractionation on silicic acid, fractionation on Florisil,¹ and recrystallization from pyridine-acetone (2). From 10 kg. of tissue, 50 mg. of pure cytoside was obtained. Paper chromatography of the water-soluble, acid hydrolysis products of all preparations showed the presence of only two substances, these migrating at the same rates as glucose and galactose.

Chemical Analyses. For total hexose determination, samples of 100 to 150 μ g. of cytoside were hydrolyzed for 2 hours in sealed tubes with 0.5 ml. of 3 N aqueous hydrochloric acid in a bath at 100°C. The tubes were opened, and the contents evaporated to dryness over sodium hydroxide in a vacuum desiccator. The residues were dissolved in 0.80 ml. of water, and a portion (0.60 ml.) taken for analysis. The analytical method was based on color formation with o-aminodiphenyl (5), using equimolar mixtures of glucose and galactose as standards.

Anthrone analysis was performed according to the method of Radin *et al.* (6). In this method glucose produces about 40 per cent more color than galactose, and a slightly larger difference (47 per cent) is observed with the corresponding cerebroside. The color yield of cytoside is close to the predicted value for a molecule with one residue of each hexose.

For the determination with glucose oxidase, samples were hydrolyzed and evaporated to dryness as in the analysis for total hexose, and the residue was dissolved in 0.40 ml. of water. To 0.20 ml. of this solution was added 0.05 ml. of an aqueous solution of glucose oxidase in 0.05 M phosphate buffer (vial of Worthington "Glucostat" dissolved in water and made up to 10 ml.). After 2 hours at room temperature (20° to 25°C), 0.35 ml. of water was added, and analysis for residual hexose was made with o-aminodiphenyl (5), using galactose as the standard. Results are expressed as per cent of total hexose present as glucose.

Kjeldahl N was determined according to the modification of Tompkins and Kirk (7). Samples of ap-

proximately 500 μ g. of cytoside were analyzed, yielding about 7 to 8 μ g. of N.

Ninhydrin color was determined as follows: Samples of 100 μ g. of cytoside were hydrolyzed in 13 \times 150 mm. tubes (covered by "tear drop"-shaped glass caps) for 2 hours at 90°C with 0.5 ml. of 1.2 N hydrochloric acid in 90 per cent ethanol (8). Evaporation was reduced to a minimum by inserting the tubes into the bath only to the level of liquid in the tubes. Samples were carefully evaporated to dryness in a vacuum desiccator over sodium hydroxide and then analyzed without transfer by the method of Lea and Rhodes (9). The molar color yield of sphingolipids is about 75 per cent of that of leucine. It was essential that every trace of free hydrochloric acid be removed to obtain correct analytical values. With ethanol, this step frequently required prolonged periods of drying. This analytical method worked very well with cerebroside and sphingomyelin preparations isolated from animal tissues. It has not given satisfactory results with synthetic derivatives of dihydrosphingosine.

The iodine number on samples of 1.5 to 2 mg. of cytoside was determined by a micromodification of the Yasuda method (10). Specific rotation was measured in a Keston polarimeter at 589 $m\mu$. at a concentration of about 2 per cent in pyridine.

Immunological Analysis. Isofixation curves (11) were obtained for the four cytoside preparations by determining 2 points in the zone of antibody excess and 3 points in the zone of antigen excess, using a rabbit antiserum (No. 161) prepared by injecting a particulate fraction of human cervix carcinoma (1). The complement level was six 50 per cent units (0.0085 ml. of guinea pig serum). Auxiliary lipid (100 parts by weight of a mixture of equal quantities of lecithin and cholesterol) was combined with cytoside in organic solvent (1). The solvent was evaporated in a stream of nitrogen. The dry residue was taken up in 0.1 ml. of methanol and then 0.9 ml. of saline was added. Dilutions were made with saline. Further details of the complement fixation test appear in preceding publications (1, 11, 12).

RESULTS

Chemical Analysis. The chemical analysis of two preparations of cytolipin H and two preparations of ox spleen cytoside are shown in Table 1. Values are referred to weight units rather than mole units because the fatty acid residues present in these preparations are mixed.² Although small differences are observed

¹ Floridin Company, Tallahassee, Fla.

² V. P. Skipski and M. M. Rapport, unpublished studies.

TABLE 1. CHEMICAL ANALYSIS OF HUMAN TUMOR AND OX SPLEEN CYTOSIDES

Cytoside Preparation	Hexose	N	Anthrone As*	Ninhydrin As†	Glucose	Iodine Number	$[\alpha]_D^{\ddagger}$
	<i>per cent</i>	<i>per cent</i>			<i>per cent hexose</i>		<i>degrees</i>
Human tumor I	36.8	1.38	125	135	49.7	32.0	-10.8
Human tumor II	36.2	1.50	128	137	51.0	35.4	-9.1
Ox spleen I	34.0	1.36	130	137	48.9	22.6	-7.3
Ox spleen II	34.5	1.43	125	139	52.9	27.0	-6.9

* Absorbancy of solution (1%, 1 cm., 625 m μ).

† Absorbancy of solution (1%, 1 cm., 575 m μ ., aqueous methylcellosolve).

‡ In pyridine. See text for temperature and concentration.

among the four samples, five of these methods do not permit the differentiation of cytoside preparations of human tumor from those of ox spleen. For purposes of reference, it may perhaps be of value to record that a sample of phrenosine with a nitrogen content of 1.68 per cent gave a specific anthrone absorbance of 64 and a specific ninhydrin absorbance of 165. The small differences in total hexose are not reflected in the anthrone values, and are very likely the result of small variations in both extent of hydrolysis of the cytoside sample and stability of the hydrolytic products.

Within experimental error, all four preparations contain 50 per cent of the total hexose as glucose. It would appear, therefore, that the value of 1.25 for the ratio of galactose to glucose reported by Klenk and Rennkamp (3) is incorrect. The most probable explanation for their result is that on hydrolysis, galactose is released more readily than glucose (2); incomplete hydrolysis would thus produce more free galactose than free glucose.

With regard to iodine number, both preparations of cytolipin H (human tumor cytoside) show the presence of more than one double bond per mole. One double bond would give an iodine number of about 26. The ox spleen cytosides are more saturated, and since the value for one of the preparations is less than 26, the lipid base may in part be dihydrosphingosine. These differences in unsaturation are largely reflections of differences in the fatty acid residues of these particular preparations, and possible variations in the saturation of the sphingosine residue cannot contribute more than a small part.

The concentrations, temperatures, and observed absorbancies for the specific rotations recorded in column 8 are as follows: human tumor I, 2.07 per cent, 24°,

0.036; human tumor II, 1.77 per cent, 27°, 0.025; ox spleen I, 2.15 per cent, 27°, 0.024; ox spleen II, 1.83 per cent, 27°, 0.024. The value for the specific rotation

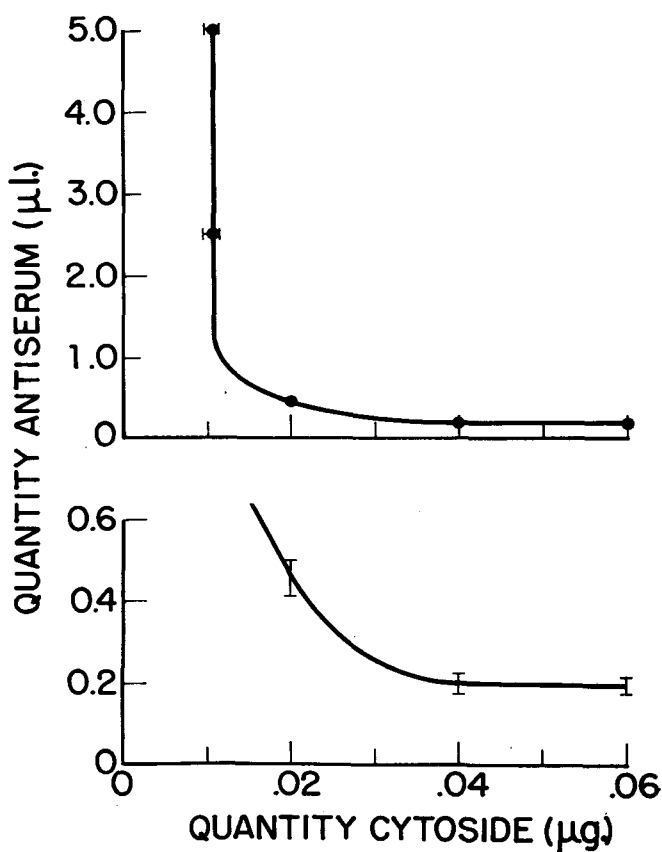


FIG. 1. Isofixation curve drawn through points representing averages of values obtained with two preparations of cytolipin H and two of ox spleen cytosides. The range of the four values at each point is shown by the short horizontal line in the upper figure (region of antibody excess) and by the short vertical line in the lower figure (region of antigen excess shown with the ordinate magnified fivefold). See text for details of complement-fixation test.

at 20° recorded by Klenk and Rennkamp for their preparation of ox spleen cytoside is -6.81° (3). Although the rotations of all preparations are in the same direction and of the same order of magnitude, the human tumor cytosides have appreciably larger rotations than the ox spleen cytosides. Much of this difference may, as before, reflect differences in the fatty acid residues. None of these four cytoside preparations contained phosphorus (less than 0.02 per cent). None was chromogenic with ninhydrin before hydrolysis.

Immunological Characterization. Figure 1 shows the quantities of each cytoside preparation that react with various quantities of antiserum to produce the same end point (50 per cent hemolysis) in the presence of a constant quantity of guinea pig serum (complement) and a constant number of sensitized sheep red cells. Each point in the upper isofixation curve represents the average of the four values obtained with the four different cytoside preparations. The range of values for the two points in the region of antibody excess is shown by the short horizontal lines drawn through each point in the upper curve. In order to show this variation for the three points in the region of antigen excess, the lower curve is presented. It is the same as the upper curve except that the ordinate axis is magnified fivefold. The range of values is indicated by the short vertical lines through each point. It is evident that the four cytoside preparations are indistinguishable in this complement fixation test.

DISCUSSION

This study shows that cytoside preparations from human tumor and ox spleen have very similar chemical properties and are serologically identical in the complement fixation test with specific antibody. Since these preparations each contain a range of fatty acid residues (one that differs between individual preparations because of solubility selection during purification), it seems probable that an explanation for the observed differences will be found in the fatty acid portion of the molecule. In any case, the immunological method is not sensitive to this difference, suggesting that the specificity for the interaction with antibody resides principally in the carbohydrate portion of the structure. An excellent opportunity should thus be at hand for determining the configuration of this structure using the method of hapten inhibition, and such studies are in progress.

The finding that ox spleen and human tumor cytosides exhibit identical immunological behavior is of great importance for the further study of lipid haptens

of human tissues. One reason is that it offers a solution to the problem imposed by limitations in the availability of human tissues. Despite the low concentrations of cytoside and complications in isolation arising from the presence in spleen of such closely related compounds as ceramide, cerebroside, and ganglioside, this tissue is an easily accessible, unlimited source of supply. A second reason is that it confirms the fact that hapten activity is an integral property of a molecule composed of fatty acid, sphingosine, glucose, and galactose. The observation that a high degree of biological activity is associated with a natural product and is retained through a limited number of purification steps does not exclude the possibility that an impurity in the final preparation may be the active principle. The most rigorous proof requires unequivocal synthesis of the compound in question. However, the fact that, as an immediate consequence of the isolation of cytolipin H, our attention was directed to a previously unsuspected source of material, and that this improbable result has been fully realized, goes far in establishing the validity of the original observation. It thereby offers a promise that an additional discipline, namely, immunochemistry, can be brought to bear on the study of one of the most interesting and complex classes of cellular substances, the sphingolipids.

We wish to thank Mr. Richard Brandt for valuable technical assistance.

REFERENCES

1. Graf, L., and M. M. Rapport. *Cancer Research* **20**: 546, 1960.
2. Rapport, M. M., L. Graf, V. P. Skipski, and N. F. Alonzo. *Cancer* **12**: 438, 1959.
3. Klenk, E., and F. Rennkamp. *Z. physiol. Chem., Hoppe-Seyler's* **273**: 253, 1942.
4. Rapport, M. M., L. Graf and N. F. Alonzo. *Federation Proc.* **18**: 307, 1959.
5. Timell, T. E., C. P. J. Glaudemans and A. L. Currie. *Anal. Chem.* **28**: 1916, 1956.
6. Radin, N. S., F. B. Lavin and J. R. Brown. *J. Biol. Chem.* **217**: 789, 1955.
7. Tompkins, E. R., and P. L. Kirk. *J. Biol. Chem.* **142**: 477, 1942.
8. Carter, H. E., and Y. Fujino. *J. Biol. Chem.* **221**: 879, 1956.
9. Lea, C. H., and D. N. Rhodes. *Biochem. J.* **56**: 613, 1954.
10. Yasuda, M. *J. Biol. Chem.* **94**: 401, 1931-32.
11. Rapport, M. M., and L. Graf. *Ann. N. Y. Acad. Sci.* **69**: 608, 1957.
12. Rapport, M. M., and L. Graf. *Cancer* **8**: 538, 1955.