Acta virol. **24** : 311—316, 1980

SEPARATION BY AFFINITY CHROMATOGRAPHY OF HUMAN VERY LOW AND LOW DENSITY LIPOPROTEINS INHIBITING SINDBIS VIRUS HAEMAGGLUTINATION

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Received April 2, 1980

Summary. — The haemagglutination-inhibiting activity of human serum towards Sindbis virus is linked to very low and low density lipoprotein classes, in particular to their lipid component. To investigate the specific role played in this inhibition by carbohydrates contained in the molecule, inhibitor separation was carried out by Concanavalin A-Sepharose affinity chromatography. A different behaviour among the inhibiting molecules in relation to the carbohydrate content was observed. The importance of mannose for the biological activity of the inhibitor is discussed.

Key words: *Sindbis virus*; *haemagglutination inhibition*; *lipoproteins*; *mannose*; *affinity chromatography*

Introduction

Human serum lipoproteins are capable of inhibiting the haemagglutinating activity of numerous viruses (Orsi *et al.*, 1974). This effect is mainly due to the low density lipoprotein (LDL) class, and is related to the lipid component of the molecule (Gorman, 1970); (Hoo and Shortridge, 1976). The mechanism of this inhibition has not yet been elucidated, but it has been suggested that the inhibitors can bind to the surface of viral particles and impede the interaction between the superficial "recognition units" of the virus and "the receptor sites" on the red blood cells (Meager and Hughes, 1977). Concerning the chemical groups involved in this phenomenon, particular attention has been paid to the presence of glycids in the lipid component of lipoproteins. On the basis of a certain inhibiting activity shown towards rubella virus by amino and acetyl derivatives of glucose, galactose and mannose (Biddle, 1971) it has been suggested that lipid acts as carrier molecule for galactosamine-containing glycolipid which is almost certainly bound to the lipid by hydrophobic interaction (Shortridge, 1972). Moreover, it has been demonstrated that mannose and mannan inhibit the haemagglutination by Japanese encephalitis virus and that mannosidase destroys

Table 1. Relationship between chemical composition of lipoproteins and their HI activity towards Sindbis virus

Lipoprotein classes		Lipid content mg/ml	Protein content mg/ml	Neutral hexoses content mg/ml	HI titre
VLDL	whole molecule	4	0.334	0.455	36
	apolipoproteins	—	1	0.210	0
	lipid extract	4	—	0.400	26
LDL	whole molecule	4	0.647	0.980	170
	apolipoproteins	—	1	0.960	0
	lipid extract	4	—	0.389	72
HDL	whole molecule	4	1.190	0.970	12
	apolipoproteins	—	1	0.810	0
	lipid extract	4	—	0.238	0.5

the activity of purified erythrocytes and host cell receptor sites (Yasui *et al.*, 1968).

Based on these observations emphasizing the importance of mannose for the attachment of the virus to the cell receptors it has been suggested that a glycoprotein of the viral envelope can show a lectin-type activity and thus bind specifically mannose residues of cell receptors.

Previously we found that the successive action of different glycosidases on human serum lipoproteins can reduce to 1/3 their inhibiting activity towards Sindbis virus (Mastromarino *et al.*, 1980). All these data encouraged us to investigate the specific role played in this inhibition towards Sindbis virus by carbohydrate contained in the lipid component of lipoproteins. We used for this purpose Concanavalin A-Sepharose based on the binding to molecules containing α-D-mannopyranosil, α-D-glucopyranosil or fructofuranosil residues (Shore and Shore, 1973). A similar method was used with rubella virus inhibitors (Orsi *et al.*, 1978). The present paper reports the results concerning the importance of carbohydrates contained in the lipoproteins involved in the inhibiting activity towards Sindbis virus haemagglutination.

Materials and Methods

Inhibitor preparations. Very low, low and high density lipoprotein (VLDL, LDL and HDL) preparations were obtained by ultracentrifuge flotation, by addition to a pool of human sera of NaCl and KBr at final densities of 1.019 g/ml, 1.063 g/ml and 1.21 g/ml according to the technique of Havel *et al.* (1955). Apolipoprotein preparations were obtained by extraction with organic solvents according to the method proposed by Shore and Shore (1967). Lipids were extracted in a mixture of chloroform and methanol according to Sperry and Brand (1955). The purity of lipoprotein preparations was tested by analytical polyacrylamide gel electrophoresis in a Gelman apparatus by the standard procedure.

Affinity chromatography on Concanavalin A-Sepharose. Concanavalin- A-Sepharose 4B was purchased from Pharmacia, Uppsala. Pharmacia columns K/9/15 were used for affinity chromatography of LDL and VLDL. The column was equilibrated at room temperature in a 0.2 M NaCl + 0.1 M sodium acetate solution adjusted to pH 6.8 with acetic acid. The lipoprotein preparations were dialysed against the equilibrating solution and then added (10 mg/ml lipid) on the top of the column. The column was washed with several bed volumes of the equilibrating

buffer and afterwards by salt solutions containing 0.1 M glucose, 0.1 M mannose, 0.1 M methyl α-D-glucopyranoside, 0.1- M methyl α-D-mannopyranoside, 0.3 M methyl α-D-mannopyranoside, 0.1 M Tris, pH 9.5. and finally by 6 M urea. The column effluent was monitored continuously at 280 nm and 4 ml fractions were collected and titrated for inhibitory activity.

Chemical determinations. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Samples of high lipid concentration were clarified by adding an equal volume of a 2% sodium deoxycholate solution. In protein deter-

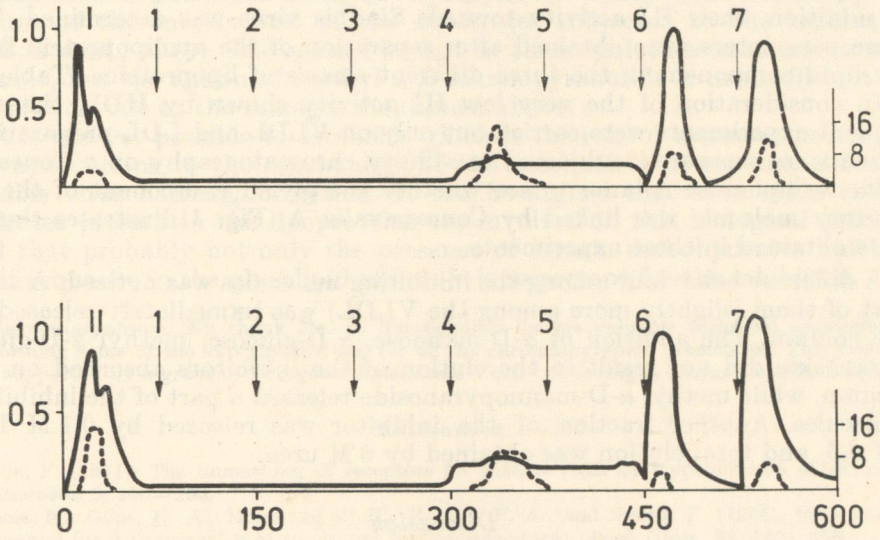

Fig. 1.

Affinity chromatography of LDL (I) and VLDL (II) on a Concanavalin A-Sepharose column
——— Optical density at 280 nm (left ordinate)
------ HI activity towards Sindbis virus (right ordinate)
Abscissa: effluent (ml)
Chromatography conditions: flow rate 20 ml/h; elution with starting buffer 0.1 M acetate, pH 6.8; 1 — 0.1 M glucose; 2 — 0.1 M mannose; 3 — 0.1 methyl-α-D-glucopyranoside; 4 — 0.1 M methyl-α-D-mannopyranoside; 5 — 0.3 M methyl-α-D-mannopyranoside; 6 — 0.1 M Tris, pH 9.5; 7 — 6 M urea.

mination of lipoprotein, a 0.77 correction value was used as proposed by Margolis (1966). Lipid concentration was determined by the phosphovanillin procedure (Frings *et al.*, 1972) using olive oil as standard. Total neutral hexose was estimated by the phenol sulphuric acid method (Dubois *et al.*, 1956) using D-galactose as standard.

Haemagglutination inhibition (HI) test. Sindbis virus grown in BHK 21 cells was used as haemagglutinating antigen. The tests were performed in "V" microplates (Cooke Microtiter System). Twenty-five μl of inhibitor were placed in the first well of the plate and then diluted in twofold steps in 25 μl of BABS (0.4% bovine albumin, 0.12 M NaCl, 0.05 M H_3BO_3, 0.024 N NaOH, pH 9.0) using a Gilson micropipette. To each well, 25 μl of Sindbis virus (4 haemagglutinating units) were added. After 12 hr of contact at 4° C, to each well were added 50 μl of a 0.2% goose erythrocyte suspension 0.2 M phosphate saline buffer, pH 5.75. The plates were held at room temperature and the results read after 2 hr. The inhibitory titre of the sample was calculated as the reciprocal of the highest dilution of inhibitor which gave a 50% inhibition of haemagglutination. The essay for each inhibitor was performed in duplicate.

Results

First, we investigated lipoprotein preparations from a pool of human sera by successive ultracentrifuge flotations at the final densities of 1.019, 1.063 and 1.21 g/ml to separate VLDL, LDL and HDL respectively. These preparations were subjected to polyacrylamide gel electrophoresis to check their purity and analyzed for their protein, lipid and carbohydrate contents. In addition, their HI activity towards Sindbis virus was determined. The same parameters were obtained after separation of the apolipoprotein from the lipid component in the three different classes of lipoproteins (Table 1).

In consideration of the very low HI activity shown by HDL, the subsequent experiments were carried out only on VLDL and LDL preparations which were separately subjected to affinity chromatography on a Concanavalin A-Sepharose column to see whether the glycid component of the inhibitory molecule was linked by Concanavalin A. Fig. 1 illustrates the results obtained in these experiments.

A different behaviour among the inhibiting molecules was noticed. A small part of them (slightly more among the VLDL) was immediately released by the column. The addition of α-D-mannose, α-D-glucose, methyl α-D-glucopyranoside did not result in the elution of the inhibitors absorbed on the column, while methyl α-D-mannopyranoside released a part of the inhibiting molecules. Another fraction of the inhibitor was released by 0.1 M Tris, pH 9.5, and total elution was obtained by 6 M urea.

Discussion

The present results showed that the HI activity towards Sindbis virus is mainly linked to the VLDL and LDL classes. The very small activity shown by HDL could also be due to the presence of minimal quantities of contaminant VLDL and LDL. The separation of the apolipoprotein and of the lipid component demonstrated that the HI activity is linked to the latter as already observed in previous research on the inhibition of togavirus haemagglutination due to lipoprotein (Gorman, 1970; Hoo and Shortridge, 1976; Mastromarino *et al.*, 1980).

As to the behaviour of these inhibitors towards Concanavalin A-Sepharose, it obviously depends on the type of carbohydrate (N-acetyl-glucosamine, fucose, glucose, galactose, mannose, N-acetyl-neuraminic acid) contained in the lipoprotein molecule in a total percentage ranging between 5 and 12%.

With our chromatographic separations it was possible to distinguish three different types of inhibiting molecules, namely those

a) which are not absorbed by the column and therefore do not contain any mannose or methyl-mannose;

b) with a noticeable mannose content which are selectively linked to Concanavalin A-Sepharose and consequently eluted by methyl α-D-mannopyranoside; this type of inhibitor was found in fractions with a very low protein content and could include glycolipids present in the serum, flotating

with lipoproteins and which can be bound by Concanavalin A-Sepharose (Guerbette *et al.*, 1979); and

c) which are absorbed on the column but are eluted only with a higher ionic strength buffer or with 6 M urea. For this type of molecules two different hypotheses may be proposed. The first is that these inhibitors do not contain any mannose but are equally absorbed on the column by strong non-specific interactions such as hydrophobic bonds between non-polar groups of the matrix and of the substrates subjected to the separation (Ochoa *et al.*, 1979). The second is that in these molecules mannose is also present, but the action of methyl α-D-mannopyranoside is not sufficient for the elution due to the linkages mentioned above.

It is not yet possible to establish which is the role of mannose present in the lipoproteins in the mechanism of the mechanism of the inhibition of Sindbis virus haemagglutination. It can be argued that even other carbohydrates present in the lipoproteins are involved in this biological activity and that probably not only the presence of certain carbohydrates but also their sequence in the glycolipid molecule is important for the inhibition.

Acknowledgements. We thank Mr. R. Sampalmieri for his valuable technical assistance in performing some of the experiments and for all the chromatographic procedures. This research was supported by a grant of "Progetto Finalizzato Virus" of Consiglio Nazionale delle Ricerche.

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