Characteristics of Mucinlike Antigen Expressed on Epithelial Tumors

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Epithelial antigen from the membranes of fat globules of maternal milk (molecular weight of about 400 kD, carbohydrate content 40-50%) is isolated by affinity chromatography using IKO-25 monoclonal antibodies, and its characteristics are determined. It is shown that the antigen contains oligosaccharide chains bound to polypeptide with O-glycoside bonds. The antigenic determinants are situated in the polypeptide portion of the antigen molecule. The formation of high-molecular aggregates is evidently due to N-acetylneuraminic acid.

Key Words: *antigen; epithelial tumors; monoclonal antibodies; membranes of fat globules from breast milk*

Epithelial antigens of breast tumors have been identified with the use of monoclonal antibodies (MAB) (DF3, 115D8, HMFG-2, and IKO-25) obtained from fat globule membranes (FGM) from breast milk [2,5,7,10]. The use of these MAB in immunofluorescence and enzyme immunoassay helps distinguish between malignant and benign tumors. The same MAB allow epithelial tumor antigens to be detected in the serum of patients with breast tumors.

Since there is no standard for the epithelial antigen which may be used to assess the minimum concentration of test antigen in the serum of oncological patients by enzyme immunoassay (test system based on IKO-25 MAB), the aim of the present study was to isolate and characterize the antigen of FGM from breast milk.

MATERIALS AND METHODS

Fresh breast milk taken 1-3 h after lactation was used to isolate FGM. The lipid layer separated from the milk plasma by centrifugation (20,000 g, 30 min) was washed six times with a 0.01 M Tris buffer, pH 7.2 (buffer 1) containing contrical and ε-aminocaproic acid. The washed lipid layer was stored at -70°C. After thawing, the lipid layer was suspended in buffer 1 and centrifuged at 105,000 g during 1 h. Three fractions were obtained: the membrane pellet (FGM), the supernatant (a solution of proteins desorbed from the membranes), and fat. FGM were suspended in buffer 1 containing 1% sodium deoxycholate (SDC) and sonicated four times (15 sec, 0°C) using a Serva ultrasound generator, this being followed by centrifugation at 105,000 g for 1 h. Affinity chromatography of the supernatant was performed on a column (4×20 cm) with Sepharose 6B-immobilized IKO-25; the elution buffer was 0.01 M Tris, pH 7.8. The FGM antigen was eluted with 0.05 M Tris buffer (pH 11.5) containing 0.5% SDC, dialyzed against distilled water during 3 days, and lyophilized.

The protein content was determined after Bradford [4], neutral carbohydrates were assayed by the phenolsulfate method [6], and aminosaccharides hydrolyzed during 3 h with 2 N HCl on a boiling water bath were determined after Rondle

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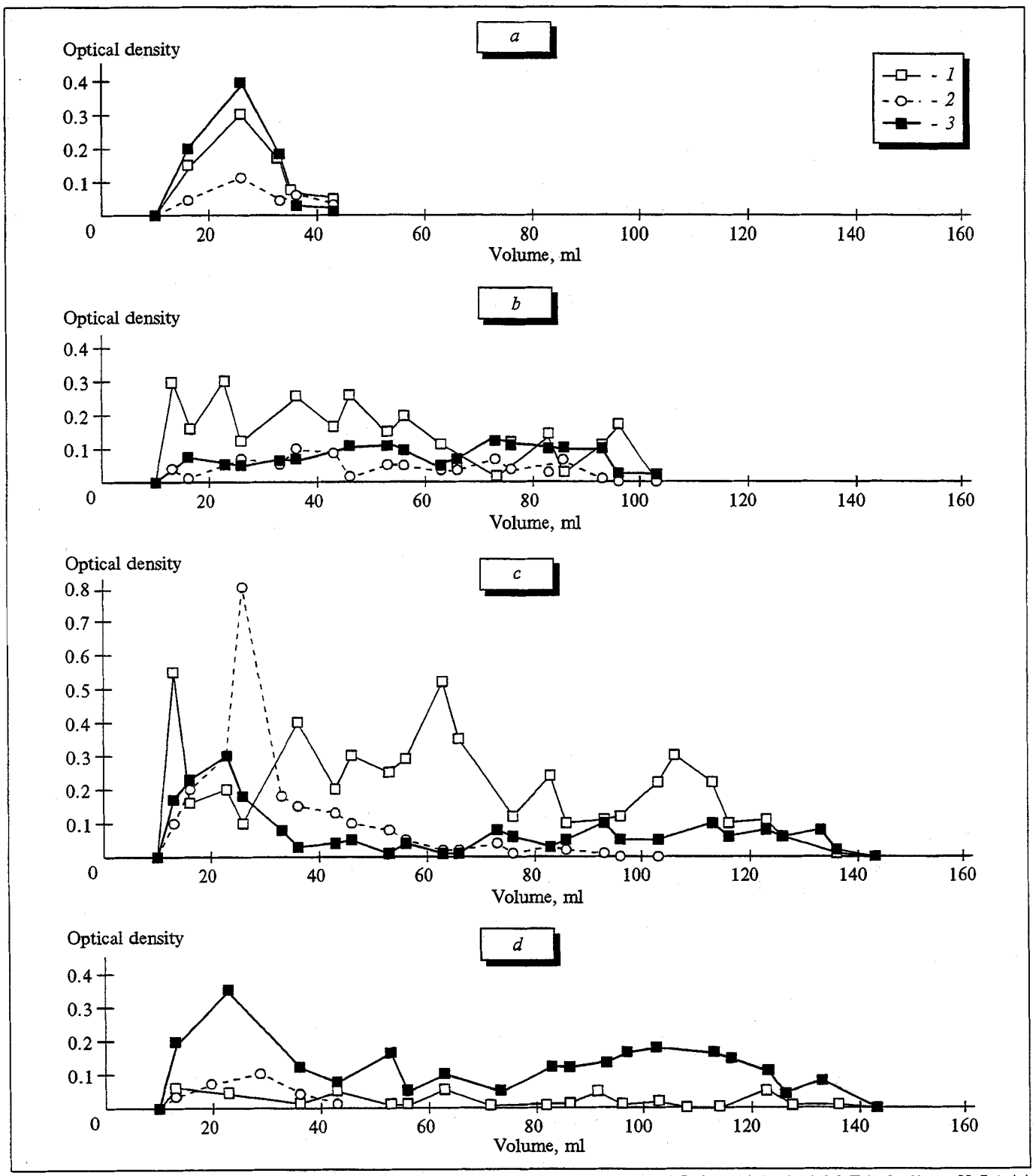

Fig. 1. Chromatography of affinity-purified FGM antigen on AcA 34 (column 1.5×29 cm) in 0.01 M Tris buffer, pH 7.4 (*a*) and the same after treatment of antigen with neuraminidase (*b*), with a mixture of glycosidases (*c*), and with proteinase K or collagenase (*d*). *1*) immunological activity determined by solid-phase enzyme immunoassay; *2*) protein content assayed after Bradford; *3*) neutral carbohydrates.

et al. [9]; N-acetylneuraminic acid was measured after Warren [12], and RNA/DNA by Spirin's method [1].

Horseradish peroxidase-labeled IKO-25-IgG1 conjugate was obtained after Nakane *et al.* [8]. The binding of FGM antigen with IKO-25 was

assayed in 96-well plates (Flow Lab.). Detection was performed on a Flow Multiscan photometer.

Vibrio cholerae neuraminidase and proteinase K (Serva), collagenase (MGP Bio-90), and a mixture of glycosidases (N-acetyl-β-D-hexosaminidase, β-D-galactosidase, and α-D-mannosidase) from *Canavalia gladiata* beans (Bioteknologiya) were used in the study.

RESULTS

Hybridomas producing IKO-25 MAB against the FGM components of breast milk were obtained at the Oncological Research Center of the Russian Academy of Medical Sciences and at the P. A. Gertsen Research Oncological Institute of the Ministry of Health of the Russian Federation. The fraction of FGM glycoproteins was used as immunogen. The characteristics of IKO-25 MAB were described elsewhere [2,11]; it was shown that there are three distinct types of IKO-25 reaction to sections from epithelial tumors, which differ with respect to the intensity and distribution of the label.

Each type is characteristic of a certain group of tumors. No areas entirely inactive in relation to IKO-25 were discovered in the tissues of malignant tumors of the breasts, lungs, and ovaries.

Glycoproteins were isolated from FGM by step-by-step treatment with solubilizing agents with an increasing extracting capacity by the method of Yakubovskaya *et al.* [3] with modifications. For separation of IKO-25-specific glycoproteins, the fraction of glycoproteins was then chromatographed on an affinity column with IKO-25 immobilized on Sepharose 6B by the cyanogen bromide method. FGM antigen was eluted with 0.05 M Tris buffer, pH 11.5, containing 0.5% SDC. After complete dialysis, FGM antigen was lyophilized. The immunological activity and the capacity for binding with IKO-25 were thereby preserved. After polyacrylamide gel electrophoresis in the presence of SDS after Laemmli FGM antigen did not stain with Coomassie; fuchsin staining revealed a diffuse band near the start. From a column with AcA 34 Ultragel (LKB) FGM antigen was eluted in the form of one broad peak immediately following the buffer front (Fig. 1, *a*), which corresponded to a molecular weight of some 400 kD. The composition of FGM antigen was determined (in % of dry weight): protein 2.5, hexosamines 8, N-acetylneuraminic acid 16, neutral sugars 20, and DNA/RNA 0.18.

In order to reveal the nature of FGM antigen binding specifically to IKO-25, the antigen was treated with enzymes, and enzyme hydrolysates

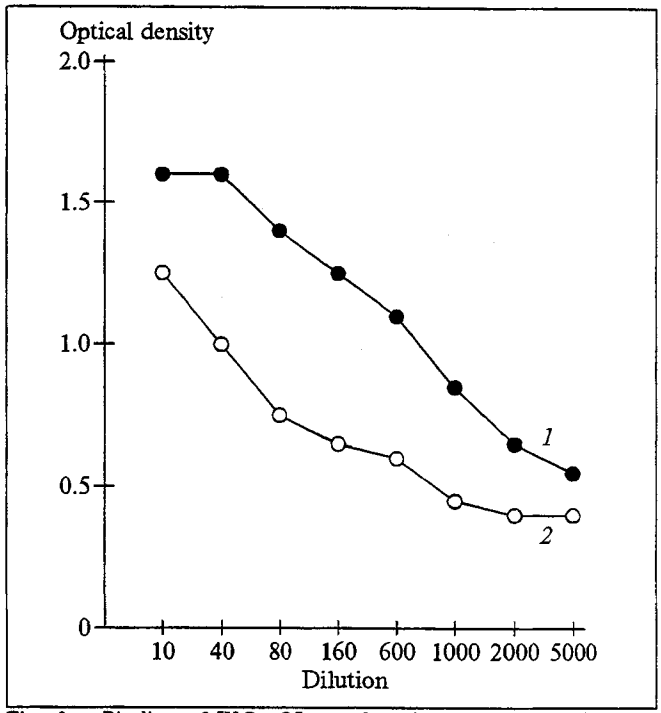

Fig. 2. Binding of IKO–25 as a function of dilution of native FGM antigen (*1*) and FGM antigen treated with 0.1 M NaOH and 1 M $NaBH_4$ (*2*).

were chromatographed on AcA 34 Ultragel (Fig. 1, *a-d*). Treatment of the FGM antigen with *Vibrio cholerae* neuraminidase did not affect its immunological activity, which indicated that the antigenic determinants of the antigen were preserved. Hence, N-acetylneuraminic acid is not a component of the antigenic determinants of FGM antigen. The heterogeneity observed after treatment with neuraminidase seemed to be due to the fact that N-acetylneuraminic acid contributes to the formation of stable high-molecular aggregates owing to additional ionic-type bonds. Splitting-off of N-acetylneuraminic acid destroyed these bonds, and the stable mucin aggregate was partially destroyed (Fig. 1, *b*).

Treatment with a mixture of glycosidases also changed the chromatographic pattern of FGM antigen, but the antigenic determinants were preserved and, moreover, unmasked after the splitting-off of some carbohydrates. Low-molecular fragments of FGM antigen were found which exhibited the capacity for binding with IKO-25 (Fig. 1, *c*).

Limited proteolysis with proteinase K, which predominantly splits bonds formed by glycine, leucine, and alanine, destroyed the antigenic determinants of FGM antigen, and its ability to bind with IKO-25 was markedly reduced (Fig. 1, *d*).

Similar changes in the chromatographic pattern of FGM antigen were observed after its treatment with *Cl. histolyticum* collagenase, which destroys

bonds formed by glycine and proline. We discovered a number of low-molecular fragments containing hexoses. As in the case of treatment with proteinase, immunological activity assessed from binding with IKO-25 disappeared. It should be mentioned that the fragments formed in this case exhibited an affinity for the ultragel, which is a polyacrylamide gel network inside the rigid agarose matrix.

Fragments of FGM antigen may bind to polyacrylamide gel since N-acetylneuraminic acid has a negative charge.

In order to establish the type of oligosaccharide bonds in FGM antigen we treated the preparation with 0.1 N NaOH and 1 M $NaBH_4$ at 37°C for 25 h, after which the reaction products were separated on AcA 34 Ultragel.

Splitting-off of carbohydrates, which was detected by the phenolsulfate method, demonstrated that the oligosaccharide chains of the FGM antigen are bound to the polypeptide skeleton by means of O-glycoside bonds. This caused the immunological activity to drop slightly (Fig. 2).

Periodate treatment, leading to oxidative destruction of C-C bonds in hexoses, resulted in a marked decrease of the IKO-25 binding capacity. Evidently, oligosaccharide chains are implicated in the formation of the antigenic structure of FGM antigen, but the antigenic determinants are composed of an amino acid sequence.

Thus, the FGM antigen recognized by IKO-25 is a glycoprotein with a molecular weight of about 400 kD; its antigenic determinants are situated on the polypeptide skeleton, but its hydrocarbon groups are involved in the formation of the antigenic structure. Evidently, rather stable mucin aggregates are formed due to N-acetylneuraminic acid carrying a negative charge.

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