

Preliminary Communication

Occurrence of the Major Food Allergen, Ovomucoid, in Human Breast Milk as an Immune Complex

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The major food allergen, ovomucoid (molecular weight of 28 kDa) could be detected in 12 of 37 human breast milk samples by using three types of enzyme-linked immunosorbent assay. By gel-filtration, ovomucoid in breast milk was only eluted in the fractions corresponding to a molecular weight of about 450 kDa, suggesting its occurrence as an immune complex with IgA. In fact, almost the same elution profile as that for ovomucoid was obtained for its immune complex with IgA by gel-filtration.

Key words: human breast milk; ovomucoid; food allergy; monoclonal antibody; secretory IgA

The increase of food allergy in infants is one of the most severe problems for immediate solution in the developed countries. Although breast milk is considered to be the most appropriate food for infants nutritionally, immunologically, physiologically, and mentally, there are some reports that unfortunately suggest allergic sensitization *via* breast milk. Infants who had not taken any baby food have shown allergic symptoms.¹⁾ Eczema in 10 breast-fed babies disappeared within 11 days when their mothers abstained from egg and reappeared quickly when either the mothers or infants were given egg to eat.²⁾ Food allergens such as β -lactoglobulin,³⁻⁵⁾ ovalbumin,^{4,5)} ovomucoid,⁴⁾ and gliadin⁶⁾ have been detected in breast milk from lactating women who consumed them before sampling and who followed a normal diet without any enforced dose of these antigens.^{7,8)} However, the relationship between allergic symptoms and the existence of allergens in breast milk, the mechanism for their transport into breast milk, and their molecular features in breast milk are not well understood.

Hen's egg is the most frequent cause of food allergy.^{9,10)} Ovomucoid, a glycoprotein with a molecular weight of 28 kDa in egg white, is thought to be the major allergen,^{11,12)} despite the evidence presented in a controversial report.¹³⁾ Its immunological properties have been revealed¹⁴⁻¹⁶⁾ and the methods for producing low-ovomucoid egg white preparations have recently been proposed.^{12,17)} We describe here a unique molecular feature of ovomucoid in human breast milk which is meaningful to understand the digestion, absorption, transfer and secretion processes of food proteins taken orally, and their immunological effects on infants.

Ovomucoid was chromatographically purified from fresh hen's eggs. Antisera were obtained from rats that had been immunized with ovomucoid. Hybridomas producing monoclonal antibodies (mAbs) were established by the fusion of splenocytes of BALB/c mice immunized with ovomucoid and myeloma cells (NSI/1-Ag4-1) essentially as described previously.¹⁸⁾ IgG fractions were purified in Protein A-Sepharose (Amersham Pharmacia Biotech, Buckinghamshire, England) columns from the rat antisera and the ascitic fluids from BALB/c mice injected with hybridoma clone 1H or 7D. They are designated as polyclonal IgG (pIgG) and mAbs 1H and 7D. As far as tested by an enzyme-linked immunosorbent assay (ELISA) with several proteins involving ovalbumin, ovotransferrin and lysozyme, these three antibodies did not react with proteins other than ovomucoid. Portions of pIgG and mAb 1H were biotinylated with EZ-Link sulfo-NHS-LC-Biotin (Pierce, IL, U.S.A.). Ovomucoid in breast milk was determined indirectly with alkaline phosphatase conjugated with streptavidin (Oncogene, NY, U.S.A.) and *p*-nitrophenylphosphate by three sandwich-types of ELISA.¹⁹⁾ pIgG as a solid-phase antibody and its

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; pIgG, polyclonal IgG

biotinylated derivative as a second antibody were used for Assay I, pIgG and biotinylated mAb 1H for Assay II, and mAb 7D and biotinylated pIgG for Assay III. The detection limits for ovomucoid were approximately 0.3, 1.0 and 1.0 ng/ml for Assays I, II and III, respectively.

Table 1 summarizes ovomucoid contents in breast milk obtained from 37 healthy mothers with informed consent. Less than several ng/ml of ovomucoid was detected in 11 samples, and an exceptionally high value (37 ng/ml) was detected in No. 29 by Assay I with the highest sensitivity. These mothers had been attending hospital to find a cure for the food allergy of their babies. Therefore, 19 of the 37 mothers, involving Nos. 13, 20, 21, 23, 29, 33 and 35 in Table 1, abstained from eating egg according to the doctor's direction. However, no definite correlation was apparent between the level of ovomucoid in breast milk and the egg consumption.

The presence of ovomucoid in human breast milk was confirmed by the two mAbs with different epitopes. MAb 7D is capable of equally recognizing native ovomucoid and denatured ovomucoid heated at 100°C for 30 min, while, mAb 1H reacts only with the native form (H. Narita, unpublished data). The results obtained by Assay II with mAb 1H and by Assay III with mAb 7D gave a similar pattern, but rather higher values irrespective of their lower sensitivity in comparison with those by Assay I (Table 1). Ovomucoid in breast milk would have heterogeneity in its carbohydrate content, heat-denaturation, digestion, and complex formation with other proteins which would affect its determination by these types of sandwich ELISA.

To clarify the molecular features of ovomucoid in breast milk, a gel-filtration analysis of No. 29 milk was carried out with Assay III. Surprisingly, the peak of ovomucoid emerged only at a position corresponding to a molecular weight of about 450 kDa apart from its normal value of 28 kDa (Fig. 1). Similar results were obtained with milk No. 21 and No. 33. Secretory IgA is a major immunoglobulin in human breast milk, and the existence of IgAs specific to food proteins have been reported in human breast milk.^{5,8)} Provided that ovomucoid(s) and its specific secretory IgA forms an immune complex, the speculated molecular weight is around 450 kDa, this being dependent on the numbers of ovomucoid, which agrees with the value for ovomucoid in breast milk determined by gel-filtration. The immune complex of ovomucoid and its specific IgA was then analyzed in the same fractions by using alkaline phosphatase-conjugated goat anti-human IgA (American Qualex, San Clemente, U.S.A.) instead of biotinylated pIgG and alkaline phosphatase-conjugated streptavidin in Assay III. Almost the same elution profile was obtained for the immune complex as that for ovomucoid (Fig. 1), indicating the occurrence of ovomucoid

Table 1. Determination of Ovomucoid in Human Breast Milk

Sample No.	Ovomucoid (ng/ml)		
	Assay I	Assay II	Assay III
1	0.33	—	—
10	0.50	0.84	1.06
12	0.68	—	—
13	0.33	—	—
14	0.39	—	—
20	0.50	4.00	0.96
21	1.53	4.00	3.57
23	0.44	2.00	0.94
29	37.0	28.0	135
33	2.00	3.52	5.85
35	0.32	—	0.99
37	1.74	—	2.04

Breast milk samples were collected in sterile containers and stored at -20°C . Before the assay, they were thawed, adjusted to 0.02% NaN_3 , and centrifuged at $10,000 \times g$ for 15 min at 4°C to remove particulate materials and fat. Ovomucoid was determined with alkaline phosphatase conjugated with streptavidin by three specific types of sandwich ELISA. Polyclonal IgG as a solid-phase antibody and its biotinylated derivative as a second antibody were used for Assay I, polyclonal IgG and biotinylated monoclonal antibody 1H for Assay II, and monoclonal antibody 7D and biotinylated polyclonal IgG for Assay III.

—: Not detected.

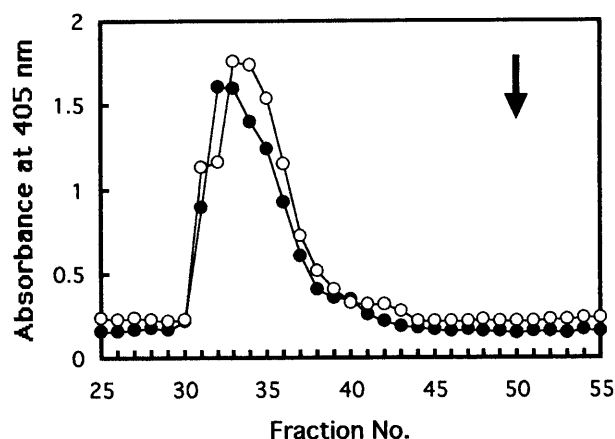


Fig. 1. Gel-filtration of Breast Milk.

After pretreating with $0.45 \mu\text{m}$ Millex-HA (Millipore, MA, U.S.A.), 0.2 ml of a sample was applied to a column of Cosmosil 5Diol-300 ($7.5 \text{ mm} \times 300 \text{ mm}$, Nacalai Tesque, Kyoto, Japan) and eluted with a 10 mM Na-Pi buffer at pH 7.4 containing 150 mM NaCl and 0.02% NaN_3 at room temperature. The flow rate was 0.4 ml/min, and fractions were 0.2-ml each. The column was calibrated with gel filtration calibration kits (Amersham Pharmacia Biotech). Blue dextran 2000, a marker of the void volume, was eluted at fraction No. 29. Ovomucoid (open circle) was determined by Assay III, and its IgA-immune complex (closed circle) was determined by monoclonal antibody 7D and alkaline phosphatase-conjugated goat anti-human IgA. The arrow indicates the position where free ovomucoid was eluted.

as an immune complex with its specific IgA in breast milk. The difference among the assays shown in Table 1 is mainly attributable to the competition of binding to ovomucoid between the mother's sIgAs and the antibodies used for the assays. Fukushima

et al. have determined ovalbumin and its specific IgA separately in human breast milk.⁵⁾ On the other hand, Kilshaw *et al.* have demonstrated that while ovalbumin was present partially as an immune complex in the blood stream, it was in a free form in breast milk after the ingestion of one raw egg.⁴⁾ This is the first report to show the occurrence of a food allergen as an immune complex with its specific IgA in human breast milk. Further analyses are necessary to reveal whether the phenomenon we have clarified here is specific to ovomucoid and how it is related to the condition or constitution of the mother.

Orally administrated ovomucoid can appear in breast milk only after passing through many barriers such as digestion, absorption, the immune system, and transcytosis. What is the mechanism to accomplish this and how does the immune complex in breast milk affect babies? It is possible that the absorption of allergens in infants with immature digestion and absorption systems is suppressed by making allergens to the immune complexes in breast milk. The relationship between the presence of allergens in a mother's milk and the allergic symptoms of their baby should be addressed further in consideration of not only the level but also the molecular features of the allergen.

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