Evidence for Specific Antibodies and Antibody-independent Inhibitors in Milk which Inhibit the Virus-induced Hemagglutination

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It has been documented that mammalian and avian body fluid or milk possesses inhibitors for virus-induced hemagglutination. The inhibition of virus-induced hemagglutination is postulated to be reflection of the inhibition of virion adsorption on cell surface.

Lanni *et al.*¹⁾ described that cow's milk was capable of inhibiting virus-induced hemagglutination and presented a hypothesis that the inhibitory activity was attributed to a non-specific inhibitor which is characteristic of milk rather than a specific antibody. However, the specific antibody against poliovirus has been observed in human milk by Michaels²⁾ and Athreya *et al.*³⁾

The purpose of this article is to demonstrate the inhibitory ability of human milk against hemagglutination induced by influenza virus A₂, and is to describe experimental results which suggest that this inhibitory activity in human milk mainly depends on antibody-independent, non-specific hemagglutination inhibitors.

Human colostrum and transitional milk, which were obtained from individual mothers 1 to 10 days after parturition, were centrifuged to eliminate fats and lyophilized. Samples of cow's milk, directly supplied from a dairy farm, were used without pasteurization for the experiment and were also centrifuged to eliminate fats and lyophilized. Influenza virus A₂ (Adachi), used in this experiment, was propagated in chicken egg allantoic fluid and harvested by centrifugation. The virus

suspension was adjusted to the virion density which reveals hemagglutination activity even after 3 fold dilution; the activity is last by 4 fold dilution. (3 to 4 hemagglutination units).

Aliquots of 0.25 ml of the virion suspension of 3 to 4 hemagglutination doses were incubated for 30 min at 0°C with the same volume of the inhibitor solution which had been sequentially diluted and 0.5 ml of 0.25% chicken red cell suspension was added to the incubation mixture. The inhibition titer of the inhibitor solution was expressed in terms of the reciprocal of the maximum dilution index which is able to inhibit the hemagglutination. Dilution of the inhibitor fluid was carried out with a sterilized physiological saline throughout.

Fractionation of human milk was carried out through precipitation by means of ammonium sulfate followed by dialysis and lyophilization according to an analogous procedure as employed in the purification of the globulin fraction of cow's milk^{4,5)} as shown in Fig. 1. Inactivation of the inhibitor by receptor destroying enzyme (RDE, a lyophilized filtrate of a Cholera vibrio culture containing Penicillin G potassium salt and Streptomycin) was achieved by incubating 0.25 ml of the inhibitor fluid (8 mg/ml) with 0.25 ml of RDE (purchased from Takeda Chemical Co.) for 18 hr at 37°C and then for 30 min at 56°C. The RDE solution was prepared by diluting one vial quantity with 40 ml of the physiological saline.

Trypsin (purchased from Sigma Chemical

FIG. 1. Fractionation Procedure of Human Milk.

Every fraction was dialysed against water and then lyophilized.

^{a)} HI values per mgN was lower than 2.

^{b)} considered as casein fraction

Co.) was dissolved in 0.01 N HCl to give a concentration of 40 mg/ml and from the solution dilution was made with 0.1 M veronal buffer of pH 8.2 to prepare a solution of 8 mg/ml immediately before use.

In an experiment to examine the effect of KIO₄ on the inhibitory activity, 0.5 ml of 0.01 M KIO₄ was added to 0.25 ml of the inhibitor fluid and the mixture incubated for 16 hr at 4°C, followed by addition of 0.25 ml of 1% glycerine in order to terminate the

reaction.

Profile of transition in the inhibitory activity of human and cow's milk against hemagglutination is presented in Table I as a function of days after parturition. The inhibitory activity was significantly higher in human milk from individuals on fourth day after parturition than that of later days.

Specificity of the inhibitory activity of human milk was examined in a comparative study with cow's milk. The activity in cow's milk

TABLE I. INHIBITORY ACTIVITY OF HUMAN AND COW'S MILK AGAINST HEMAGGLUTINATION BY INFLUENZA A₂ VIRUS.

Each value is expressed as a mean of five determinants.

Milk source	Day after parturition	HI value/ mg-weight	HI value/ mgN
Human	3	32	551
	7	4	144
	8	2	69
Cow's	1	8	108
	4	8	99
	7	4	73
	8	4	75
	9	4	71

was found to be 1/2 to 1/5 that of human milk. The discrepancy in the activity between human and cow's milk was particularly remarkable in the colostrum.

Migration of poliomyelitis antibody from serum into milk was demonstrated by Michaels²⁾ and Athreya.³⁾ The authors' interest was focused on the elucidation of whether the inhibitory activity in human milk against hemagglutination by influenza virus is derived from a specific antibody or a non-specific inhibitor in human milk. It has been established that the criteria for antibodies is their insusceptibility to RDE, trypsin and KIO₄ reagents.^{6,7)} Human milk was, therefore, fractionated into 5 fractions by means of ammonium-sulfate precipitation (Fig. 1) and susceptibility of the inhibitory activity in the fractions against these reagents was tested.

The experimental results revealed that the inhibitory activity is not restricted in globulin fraction (Fraction 3), but rather distributed among the four fractions. It is probable that a portion of the activity in Fraction 3 is attributed to antibody, and each activity in Fractions 1, 2 and 4 seems to be derived from non-specific inhibitors because of their modes of the responses to the reagents (Table II).

Non-specific inhibitors in Fractions 1 and 4 can be assigned as inhibitors and those in Fraction 2 seemed to be α inhibitors according to

TABLE II. RESPONSE OF INHIBITOR ACTIVITY IN FRACTIONS OF HUMAN MILK TO RDE, TRYPSIN AND KIO₄ REAGENTS.

Fractions	HI values/ mgN	RDE	Trypsin	KIO ₄
Casein	348	+	±	+
Acid-whey	2948	+	+	—
Fraction 1	1392	—	—	+
Fraction 2	28	+	±	+
Fraction 3	1076	—	—	±
Fraction 4	1516	±	—	+

+: Inhibitory activity was diminished by the treatment.

—: Inhibitory activity was not diminished by the treatment.

the classification of non-specific inhibitors which is based on the mode of the response to the reagents^{6,7)} above described. It is generally accepted that the specific antibodies in human milk operate as inhibitors against virus multiplication in the intestine of infants fed with breast milk. It is also a plausible hypothesis that antibody-independent and non-specific hemagglutination inhibitors, found in human colostrum to a higher activity than in cow's milk, might influence the inhibitory effect against absorption of virus to their intestinal cells.

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