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Isolation and Characterization of an immunomodulatory Protein from Bovine Colostrum

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A colostral protein that augments natural killer (NK) cell activity was isolated from bovine colostrum. This protein, designated maternal immunity enhancing factor (MIEF), increased NK cell-mediated cytotoxicity against human tumor targets when added to cultures of resting peripheral blood lymphocytes. The NK cell stimulatory activity of the MIEF was demonstrated at the concentrations as low as 0.1-0.01 μg/ml. Purified MIEF showed an apparent molecular weight of 22,000 in SDS-polyacrylamide gel electrophoresis. The unusual biochemical characteristics of the MIEF distinguish it from other cytokines. The MIEF was soluble at a cold temperature, and precipitated by raising the temperature. This thermal precipitability was reversible, and dependent on the concentration, pH, and ionic strength. Maximal precipitation was observed at neutral pH, and higher ionic strength.

Key words: Bovine colostrum, Immunomodulation, Natural killer cell, Reversibly heat precipitable protein

INTRODUCTION

The newborn mammal enters a world of pathogenic microorganism from maternal protection. The neonate must defense against infections by itself. Since the immune system of the neonate of most species is immunologically immature at birth, maternally transferred passive immunity confers a transient protection against most viral and bacterial assaults until the neonate's immune system matures and immunocompetent (Ogra and Ogra, 1978). Whereas colostrum has been well characterized for its ability to provide maternal passive immunity (Arnold *et al.*, 1977; Goldman *et al.*, 1982), very little is known for the development of the immune response in the neonate. The ontogeny and maturation of the immune system may be dependent on the several factors such as the information encoded in the germline DNA of an individual, the maternally transmitted immunoglobulins, and types of antigens encountered (Jerne, 1974; Kim, 1975; Tonegawa *et al.*, 1978).

Colostrum have been shown to affect the development of the immune response not only providing a transient protection. In studies on the effects of colostrum on the development of the immune response in pigs, it has been shown that immunologically virgin piglets obtained by historectomy 3 to 5 days before term and fed Mulsoy instead of colostrum in a germ-free condition lack serum immunoglobulin, plaque forming cells, and NK cells (Kim, 1975; Hur *et al.*, 1981). When those animals fed with colostrum, the level of serum immunoglobulin increased dramatically and in a few days become immunocompetent (Kim, 1975; Hur *et al.*, 1981). This may simply due to the influx of maternal antibodies that as antigens interact or activate lymphocytes and generate a cascade of cross-reacting idiotype networks as proposed by Jerne (Jerne, 1974). Alternatively, the rapid establishment of the immune responsiveness may in part due to colostral factors other than immunoglobulins which can stimulate the growth and differentiation of the neonate's lymphocytes.

The present study describes a colostral protein that appears to play a role in the generation of the active immunity of the neonate. This factor, designated maternal immunity enhancing factor (MIEF), was purified from bovine colostrum. Purified MIEF augmented natural cytotoxicity of peripheral blood lymphocytes against human tumor targets.

MATERIALS AND METHODS

Cell line and cell culture

Human erythroleukemic cell line K-562 and periphe-

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ral blood lymphoid cells were cultured in RPMI-1640 (GIBCO) media supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma), here after referred to as complete medium, at 37℃ under humidified 5% CO_2 in air.

Purification of MIEF

Fresh cow colostrum (provided from Chungbuk Livestock Bleeding Station) was diluted with an equal volume of saline and centrifuged at 45,000 g for 2 hr at 4℃ to remove lipids and casein. The milk whey protein was precipitated at 4℃ by addition of an equal volume of saturated ammonium sulfate solution. The resulting precipitates was dissolved, and dialyzed against 0.01 M sodium phosphate buffer, pH 7.4, at 4 ℃ using cellulose membrane (Spectra, MWCO 12,000-14,000). The dialysate was applied to a DEAE-cellulose (Wattman) column (2.5×40 cm) equilibrated with the same buffer. The protein fractions passed through the DEAE-cellulose column was collected, warmed to 37℃ for 15 min, and the resulting precipitate was collected by centrifugation at 1500 g for 10 min at 37℃. The precipitate was redissolved in 10 ml of 0.01 M sodium phosphate buffer, pH 7.4, cooled to 0℃ and insoluble precipitates was removed by centrifugation at 10,000 g for 10 min at 4℃. The warm and cold treatment was repeated, and finally resuspended in 2 ml of 0.01 M sodium phosphate buffer, pH 7.4. Further purification of the protein was carried out on Sephadex G-75 column chromatography. To column of 1.5×45 cm, equilibrated with 0.01 M sodium phosphate buffer, pH 7.4, 5 mg of the protein was applied. The flow rate was 8 ml/hr, and fractions of 0.85 ml were collected.

Preparation of lymphoid cells

Peripheral blood mononuclear cells (PBMC) of adult miniature swine were prepared by the Ficoll-Hypaque density centrifugation method of Boyum (Boyum, 19 68). PBMC were washed three times with Balanced Salt Solution (BSS), and then resuspended in complete media. The viability of the PBMC were measured by trypan blue-dye exclusion.

Stimulation of PBMC

PBMC were cultured for 18 hr at 37℃ at the density of 5×10^6 cells/ml in complete media in the presence or absence of different concentration of MIEF or a source of interleukin IL-12. The cell-free supernatant of the human cell line RPMI 8866 was used as a source of IL-12 (Kobayashi *et al.*, 1989).

Cytotoxicity assay

Cytotoxicity assay was carried out in triplicate in 96-well U-bottom microtiter plates (Falcon). Briefly, human erythroleukemic cell line K-562 was used as a target for spontaneous cytotoxicity assay. Between 10 to 20×10^6 target cells were resuspended in 100 μl of complete media, added 100 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear), and then incubated in a 37℃ water bath for 1 hr. Labelled target cells were washed three times with BSS and finally resuspended in complete media. To 10^4 target cells, stimulated PBMC was added at different effector to target ratios and incubated for 3 hr. A total of 100 μl of the supernatant was collected after centrifugation at 500 g for 5 minutes, and counted in a gamma counter (Packard, 5550). Percent specific lysis was calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{cpm experimental} - \text{cpm SR}}{\text{cpm MR} - \text{cpm SR}} \times 100$$

Spontaneous release (SR) was defined as the counts per minute (cpm) released from targets incubated with medium alone. Maximum release (MR) was defined as the cpm in the supernatant after lysis of the targets with 1% Triton X-100.

Determination of protein concentration

Protein content was determined by Bicinchoninic acid protein assay method (Smith *et al.*, 1985). Bovine serum albumin was used as a standard protein.

Soluble properties of the MIEF

The precipitation of the MIEF was monitored by measurement of turbidity at 530 nm in a Beckman model DU60 spectrophotometer with 10 mm cuvettes. The required temperature was maintained by circulating water through a jacketed cell holder. To test the effect of pH on the precipitation, MIEF solutions in 10 mM sodium phosphate buffer, pH 7.4, were adjusted to various pH values by 1.0 M H_3PO_4 or 1.0 M NaOH. The final concentration of the MIEF was 0.1 mg/ml. To assay the effects of ionic strength on the precipitation, MIEF solutions in 10 mM-Sodium phosphate buffer, pH 7.4, were adjusted to various ionic strength by addition of NaCl. The final concentration of the MIEF was 0.3 mg/ml.

Polyacrylamide gel electrophoresis

Nonreducing 15% polyacrylamide gels containing 0.1 % sodium dodecyl sulfate (SDS) were prepared according to the methods described by Laemmli (Laemmli,

1970). The gels fixed in 50% methanol, and subjected to silver staining (Sambrook *et al.*, 1989).

RESULTS

Isolation of MIEF

To search for immunoregulatory proteins contained in colostral whey proteins were precipitated by 50% ammonium sulfate and divided into several fractions by DEAE-cellulose column chromatography. When those fractions were added to cultures of resting PBL and tested for their ability to stimulate lymphoblast-formation, a low but significant level of stimulatory activity was identified from the protein fraction that was not retained on the DEAE-cellulose column (data not shown). Thus, the proteins that were not retained on the DEAE-cellulose column were subjected to further purification. During our further purification procedure, we found a protein with a unique thermoprecipitability from the protein fraction that was not retained on the DEAE-cellulose. When the protein fraction which was not retained on the DEAE-cellulose column was warmed to room temperature or higher, visible precipitates were formed, and the precipitates were disappeared again when they were cooled to 4℃. Thus, by the warming and then centrifugating at 37℃, the proteins that were not retained in the column were divided into two subfraction-heat perceptible or not. Further purification of the protein showing unusual thermal precipitability was performed on Sephadex G-75 column chromatography. Two major peaks, designated protein-A or protein-B, respectively, were obtained as shown in Fig. 1.

Fuctional assay

The proteins contained in the first two major peaks of Figure 1 were tested for their NK cell-stimulatory activity. The protein contained in the first peak showed

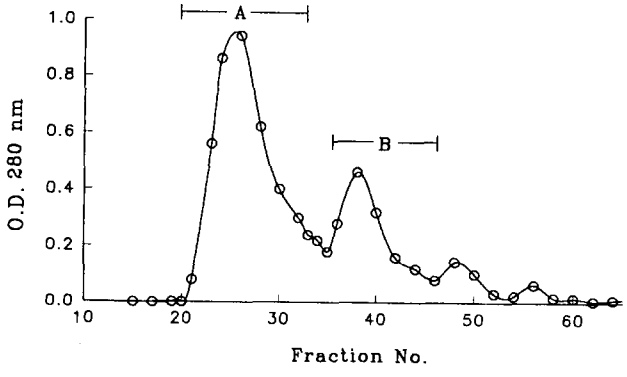


Figure 1. Sephadex G-75 fractionation of the heat-precipitated proteins. The proteins contained in the first two peaks were designated as protein-A and protein-B, respectively.

Table I. Identification of NK cell-stimulatory activity

Stimulus[a]	% specific lysis of target cell[b] Effector to target ratio	
	100 : 1	50 : 1
Medium	44.5± 3.0	23.4± 1.4
IL-12	64.7± 1.8*[c]	40.3± 3.0*
Protein-A (MIEF)		
10 μg/ml	56.7± 1.0	35.1± 0.8*
1 μg/ml	62.7± 1.0*	38.2± 1.4*
Protein-B		
10 μg/ml	48.3± 3.1	28.2± 1.1
1 μg/ml	40.1± 1.0	26.4± 1.0

[a] Peripheral blood lymphocytes were cultured with the indicated amounts of stimulus for 18 hr, washed with BSS, and then added to 1×10^4 target cells in a 3 hr ^{51}Cr-release assay. For IL-12, a maximally enhancing amount of the culture supernatant of IL-12-producing cell line RPMI-8866 was used.
[b] A 3 hr ^{51}Cr-release assay was performed in triplicate and expressed as mean percent± SE.
[c] *Significantly different from contol group ($p<0.01$).

Table II. Enhancement of PBL-mediated spontaneous cytotoxicity by MIEF

Exp. No.	Stimulus[a]	% specific lysis of target cells[b] Effector to target ratio		
		100 : 1	50 : 1	25 : 1
1	Medium	19.4± 0.8	10.4± 0.5	6.0± 1.0
	IL-12	59.1± 2.0*c	36.8± 0.2*	22.7± 1.1*
	MIEF			
	1 μg/ml	45.9± 0.8*	31.1± 1.2*	18.6± 0.9*
	0.1 μg/ml	33.8± 1.1*	28.3± 0.3*	14.2± 0.5*
	0.01 μg/ml	23.3± 0.8*	13.7± 0.2*	10.7± 0.8
	0.001 μg/ml	20.4± 0.9	10.7± 0.8	7.8± 0.6
2	Medium	56.4± 1.3	39.7± 2.1	20.2± 1.2
	IL-12	71.5± 0.8*	57.8± 1.1*	39.6± 1.2*
	MIEF			
	1 μg/ml	69.8± 1.7*	55.9± 2.0*	37.8± 0.8*
	0.1 μg/ml	72.2± 2.7*	58.0± 1.6*	43.3± 1.3*
	0.01 μg/ml	69.2± 1.1*	50.8± 0.8*	36.0± 1.2*
	0.001 μg/ml	53.8± 0.6	37.5± 1.4	22.1± 0.8
3	Medium	31.9± 1.1	18.3± 0.3	10.2± 0.1
	IL-12	53.9± 0.8*	38.1± 1.7*	18.7± 0.8*
	MIEF			
	1 μg/ml	46.3± 1.2*	34.1± 1.1*	17.5± 1.6*
	0.1 μg/ml	39.7± 1.2*	29.4± 0.6*	14.8± 0.5*
	0.01 μg/ml	33.7± 1.4	19.9± 1.1	11.2± 0.6
	0.001 μg/ml	32.5± 1.2	19.5± 0.6	9.8± 0.9

[a] Peripheral blood lymphocytes were cultured with the indicated amounts of stimulus for 18 hr, washed with BSS, and then added to 1×10^4 target cells in a 3 hr ^{51}Cr-release assay. For IL-12, a maximally enhancing amount of the culture supernatant of IL-12-producing cell line RPMI-8866 was used.
[b] A 3 hr ^{51}Cr-release assay was performed in triplicate and expressed as mean percent± SE.
[c] Significantly different from contol group ($p<0.01$).

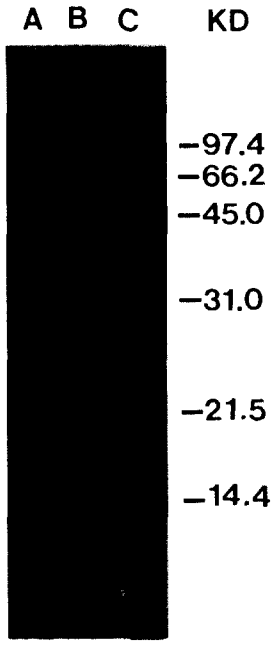


Figure 2. Polyacrylamide gel electrophoresis of the MIEF in the presence of 0.1% sodium dodecyl sulfate. The gel was subjected to silver stain. A, total protein obtained by 50% ammonium sulfate precipitation of the colostral whey; B, proteins that were not retained on DEAE-cellulose; C, purified MIEF.

Table III. Effect of pre-incubation of MIEF with bovine serum albumin

Stimulus[a]	% specific lysis of target cell[b]	
	Effector to target ratio	
	50 : 1	25 : 1
Medium	36.8± 0.8	16.4± 2.0
BSA (100 μg/ml)	39.7± 3.7	20.2± 2.5
IL-12	57.8± 1.9	39.6± 2.1
MIEF-BSAc		
10 μg/ml	57.9± 1.7	40.6± 2.1
1 μg/ml	55.9± 3.5	37.8± 1.4
0.1 μg/ml	58.0± 2.7	43.2± 2.2
0.01 μg/ml	50.8± 1.3	36.0± 3.0
0.001 μg/ml	37.5± 2.5	22.1± 1.4

[a] Peripheral blood mononuclear cells of miniature swine were cultured with the indicated amounts of stimulus for 18 hr, washed with BSS, and then added to 1×10^4 target cells in a 3 hr ^{51}Cr-release assay. For IL-12, a maximally enhancing amount of the culture supernatant of IL-12-producing cell line RPMI-8866 was used.

[b] A 3 hr ^{51}Cr-release assay was performed in triplicate and expressed as mean percent± SD.

[c] MIEF was pre-incubated with 2% bovine serum albumin for 18 hr at 4℃ and then added to the culture.

NK cell stimulatory activity as shown in Table 1, and termed maternal immunity enhancing factor (MIEF). The NK cell stimulatory activity of the MIEF was confirmed by several repeated experiments. Because the NK cell stimulated activity of the MIEF was higher in 1 omg/ml concentration than in 10 μg/ml (Table I), the NK cell stimulatory activity was examined in lower concentrations at the different effector to target ratios. As shown in Table II, the Nk cell stimulatory activity of the MIEF was identified at concentrations of the range of 0. 1-0.01 μg/ml.

Preincubation of the MIEF in the concentration of 0.1 mg/ml with 2% bovine serum albumin overnight at 4℃ inhibited completely the formation of precipitates by warming at 37℃ (data not shown). Thus, to exclude the possibility that the NK cell stimulatory activity of the MIEF might simply due to the activation of the phagocytic cells nonspecifically by the protein precipitates, the MIEF was preincubated with 2% bovine serum albumin for 18 hr at 4℃, and then tested for its NK cell stimulatory activity. As shown in Table III, preincubation of the MIEF with bovine serum albumin did not affect the NK cell stimilatory activity of the MIEF.

Physical characteristics

The yield of the MIEF was 3.9 mg from 100 ml of the colostral whey. The purified MIEF was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for the estimation of purity and molecular weight. As shown in Fig. 2, only one protein band was revealed in SDS-PAGE by silver staining. The molecular weight was estimated to 22,000 dalton in SDS-PAGE.

The MIEF is soluble at 4℃, but is reversibly precipitated on warming the temperature. The optimum pH for the precipitation appeared to be around pH 7.0 as shown in Fig. 3. The precipitation was also dependent on the temperature and ionic strength. As shown in Table IV, the higher the temperature or ionic strength, the greater amount of precipitate was formed as measured by turbidity changes.

DISCUSSION

A colostral protein that augments NK cell-mediated cytotoxicity was purified from bovine colostrum. This protein, designated maternal immunity enhancing factor (MIEF), demonstrated a significant enhancement of NK-cell mediated cytotoxicity at concentrations as low as 0.1-0.01 μg/ml. The intensity of the NK cell stimulatory effect of the MIEF was high when the NK cell activity of resting PBL was low, and there was variability in NK cell activity between individual animals as previously noted (Hur *et al.*, 1981).

The molecular weight and the physicochemical characteristics of the MIEF distinguish it from other known cytokines which have been characterized to stimulate

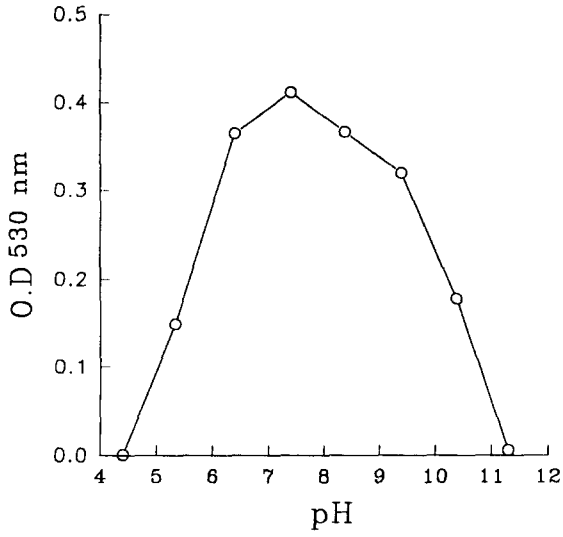
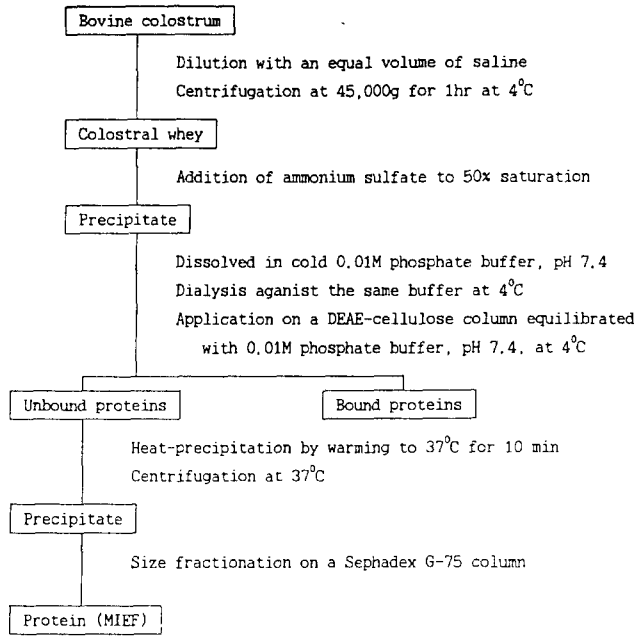


Figure 3. Effects of pH on the precipitation of the MIEF. MIEF solution in 10 mM Sodium phosphate buffer, pH 7.4, were adjusted to various pH by addition of 1.0 M H_3PO_4 or 1.0 M NaOH. The final concentration of the MIEF was 0.1 mg/ml.



Scheme 1. Isolation of maternal immunity enhancing factor.

Table IV. Effects of temperature and ionic strength on the precipitation of MIEF

Ionic strength[a]	Absorbance at 530 nm[b]			
	4℃	20℃	37℃	Recooling to 4℃
0.05	0.00	1.55	1.73	0.00
0.15	0.00	1.63	1.70	0.00
0.30	0.00	1.65	1.69	0.00

[a] Ionic strength was adjusted by the addition of NaCl.
[b] The required temperature was maintained for 15 minutes by circulating water through a jacketed cell holder. The final concentration of MIEF was 0.3 mg/ml.

NK cell activity such as interleukin IL-2, IL-12 and interferon-gamma (Paul, 1989; Kobayashi *et al.*, 1989). Purified MIEF yielded one polypeptide with approximate molecular mass of 22 KD in reducing or nonreducing SDS-polyacrylamide gel electrophoresis. However, during purification, MIEF was precipitated with 50%ammonium sulfate saturation, and eluted from Sephadex G-75 column right after void volume. These results suggest that the native molecule is a homopolymer of 22 KD polypeptide. The MIEF was not bound to DEAE-cellulose anion exchanger equilibrated with 0.01 M-sodium phosphate buffer, pH 7.4, showing that the MIEF is a very basic protein. Moreover, the unusual thermal precipitability has not been noted for other known cytokines. The MIEF was soluble at a cold temperature, and precipitated by raising the temperature. This thermal precipitation is reversible, and dependent on the concentration of the MIEF, pH, and ionic strength. Maximum precipitation was observed at neutral pH, and at higher ionic strength.

Search of the literature on colostrum or milk has revealed several previous reports identifying the existence of a protein having the unusual thermal precipitability. Reversibly heat precipitable protein have been described from the colostrum or milk of several species of animals (Schade and Reinhard, 1970; Janusz *et al.*, 1974; Seto *et al.*, 1975). Although they have some similar properties such as reversible heat precipitability and a high content of proline, other physicochemical properties were different. The molecular weight of the heat precipitable proteins isolated from sheep colostrum was 17 KD in its native form, and further dissociated into 6 KD peptides in the presence of sodium dodecyl sulfate or guanidium chloride (Janusz *et al.*, 1981). The optimum pH for heat precipitation appeared to be 4.5 (Janusz *et al.*, 1981). The heat precipitable protein isolated from human colostrum was reported to be a single polypeptide molecule with Mr of 11 KD in ultracentrifugal analysis (Schade and Reinhard, 1970). The molecular weight of the heat precipitable protein isolated from bovine milk was 60 KD in its native form, and further dissociated into two components having a molecular weight of 19 and 10 KD by sodium dodecyl sulfate (Seto *et al.*, 1975). The MIEF, which was isolated by us from bovine colostrum provided from Livestock bleeding Station, Chungbuk, Korea, showed a single band in SDS-polyacrylamide gel electrophoresis with an approximate molecular weight of 22 KD. This obvious discrepancy of the molecular weight remains to be clarified.

Even though the presence of reversibly heat precipi-

table proteins in the colostrum has been known for approximately 20 years, little is known for its biological activities. The heat precipitable protein isolated from sheep colostrum has been reported to increase the permeability of skin vessels (Zimecki *et al.*, 1979). Its effect on the humoral immune response against sheep red blood cells in mice has been tested, but appeared to stimulate or suppress the response depending on the intensity of the immune response (Zimecki *et al.*, 1979). When the intensity of the immune response of the control experiment was high, it lowered the humoral immune response, and vice versa. Recently, Julius *et al.* reported that heat precipitable protein isolated from sheep colostrum has a mitogenic activity against B lymphocyte (Julius *et al.*, 1988). We report for the first time that the heat precipitable protein isolated from bovine colostrum has NK cell stimulatory activity. There are numerous studies on NK cells for its significance in the immune surveillance, especially in natural resistance against microbial infections (Bukowski *et al.*, 1985) and tumors (Hanna and Burton, 1981; Reynolds and Ortaldo, 1987). The NK cell stimulatory activity of the MIEF may help self-defense in the immunologically immature neonates. To exert its biological activity in vivo, however, the MIEF should not be hydrolyzed by gastric peptidases before absorption. This part of experiment remains to be clarified, but already knowing that the maternal immunoglobulins in the colostrum are absorbed intact in neonates (Kim, 1975), there is possibility of absorption of the MIEF in its intact form. Our findings also support the notion that the mother provides not only a transient protection, but also an active immunity enhancing factor(s) that helps the initiation and maturation of the immune response to its immunologically immature offsprings.

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