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Mouse Mammary Tumour Virus-related Antigens in Core-like Density Fractions from Large Samples of Women's Milk

ST. ZOTTER,* CHR. KEMMER,* A. LOSSNITZER,* H. GROSSMANN* and B. A. JOHANNSEN†

**Institute of Pathology and †Department of Nuclear Medicine, Medical Academy "Carl Gustav Carus", Dresden, German Democratic Republic*

Abstract—*Specimens of human milk* (500 ml) *were processed applying methods useful for isolating oncornavirus cores from mouse milk. Treatment with Triton X* 100 *and diethyl ether as well as banding in sucrose density gradients were included in the preparation procedure. Out of* 28 *women investigated, five exhibited mouse mammary tumour virus* (*MuMTV*)*-related antigens in fractions of density* 1.26–1.28 g/ml. *By means of a sensitive radioimmunodiffusion* (*RID*) *test, these antigens were shown to be identical to at least two main antigenic constituents of MuMTV-B particles and intracytoplasmic A particles* (*iAp*) *of mouse mammary tumours. They were precipitated by rabbit antisera against MuMTV-B particles, iAp, and isolated iAp polypeptides* (*Ap*37, *Ap*14). *The antigens were reproducibly detected in* 22 *out of* 23 *core-like density fractions prepared from the milk of the five positive women between the* 5th *and* 28th week *after delivery. By indirect immunofluorescence technique, a rabbit hyper-immune serum against antigen-positive human milk fractions was seen to react with clusters of iAp within mouse mammary tumour cells. There were no immunological cross-reactions with Mason–Pfizer monkey virus main core antigen p27 and Mason–Pfizer-like viruses replicating in a cell line of human origin* (*HEp*-2). *All women found positive for MuMTV-antigens in their milk, possessed the previously described serum antibody which reacts with murine iAp and inner constituents of B particles. Antigen-positive human milk fractions absorbed the antibody from autologous and homologous sera. Thus, in summary, the presence is indicated of an antigen–antibody system in man that is closely related to MuMTV.*

INTRODUCTION

THE EXISTENCE of a human breast cancer virus has been suggested by the demonstration of particles with many characteristics of oncornaviruses in human milk [1–6], cultivated mammary epithelial cells [7], and mammary carcinoma tissue [8–10]. By some authors, the particles have been shown to closely resemble mouse mammary tumour virus (MuMTV) morphology [11–14]. Moreover, human milk core-like structures of a density of 1.26–1.27 g/ml in sucrose or, respectively, 1.22 g/ml in cesium chloride were also reported as possessing biochemical properties of oncogenic RNA viruses including Mg^{2+}-prefering reverse transcriptase [15–17].

Here we describe the detection of at least two antigens in core-like density fractions from detergent- and ether-treated human milk that cross-react with MuMTV and intracytoplasmic A particles (iAp) of mouse mammary tumours by means of a radioimmunodiffusion (RID) method. These investigations were initiated by a very preliminary finding previously published [18]. During the course of these previous experiments one out of several pools of five 100-ml milk specimens proved to contain MuMTV-related antige-

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Abbreviations: MuMTV, mouse mammary tumour virus; iAp, intracytoplasmic A particles; PAGE, polyacrylamide gel electrophoresis; RID, radioimmunodiffusion; FITC, fluorescein isothiocyanate; TNE buffer, 0.05 M Tris, 0.5 M NaCl, 0.125 M EDTA, pH 7.4; TN buffer, 0.01 M Tris, 0.15 M NaCl, pH 7.4; SDS, sodium dodecyl sulfate.

nicity detectable by micro-immunodiffusion. Now, by demonstrating cross-reacting antigens in particular women, these preliminary data are confirmed and completed. Furthermore, these antigens seem to be related to the earlier described human antibody which reacts with iAp and disrupted murine B-type particles [19–21].

MATERIALS AND METHODS

Human milk

Altogether, 54 samples of 500 ml each were obtained from 28 individual women by repeated breast pumping over periods of not more than 36 hr. Samples were stored at 5°C until processed.

Preparation of human milk core-like density fractions

All preparation steps were performed at 5°C, unless otherwise indicated. Immediately after obtaining, the milk samples were diluted with 100 ml TNE buffer and centrifuged at 6000 ***g*** for 20 min. The clear zone between lipid and pellet was ultracentrifuged at 105,000 ***g*** for 60 min. The pellets were suspended in 12 ml TN buffer with the aid of a glass homogenisator. The supernatants were collected only from some milk specimens and lyophilized to examine them by immunodiffusion tests as described below. The pellet suspensions were then centrifuged (105,000 ***g***, 60 min) through a 20% metrizamide (Nyegaard, Oslo, Norway) cushion to prevent further influence of particle and enzyme destroying milk factors [6, 22–24]. Following this centrifugation step, the pellets were homogenized again and re-suspended in 3.0 ml TN buffer containing 0.5% Triton X 100 (Serva, Heidelberg, F.R.G.). After an incubation period of 10 min, the samples were treated with equal volumes of diethyl ether at room temperature for 30 min. Ether was allowed to evaporate during gently shaking of the mixture for another 30-min period. Each specimen was then layered on a linear sucrose gradient of 30–68% concentration and centrifuged at 105,000 ***g*** for 60 min. The density region of 1.26 to 1.28 g/ml was removed whether or not it was marked by a visible light scattering band in the gradient tube. This so-called 'core' fraction was washed in 5 ml TN buffer and pelleted under virus sedimenting conditions. The 'core' pellet resulting from this procedure was resuspended in 0.1 ml TN buffer. The protein contents of 4 samples were measured according to [25] as being about 0.1–0.4 mg/ml. For comparison, in some cases, the 1.29–1.33 g/ml density region (between the 'core' fraction and sediment) was removed from the gradient, washed, and used in immunodiffusion and immunofluorescence absorption studies. The gradient pellets ($\geqq$1.35 g/ml) were also checked for MuMTV-related antigens. Occasionally, the preparation procedure was performed without detergent and ether, and the 'core' fraction as well as sucrose density fractions of about 1.16–1.18 g/ml were tested for MuMTV-B particle antigenicity (after post-treatment of the latter fraction with diethyl ether).

Purification of MuMTV-B particles

B particles were isolated from CBA/Bln mouse breast milk as detailed earlier [26]. Two ml aliquots were defatted, treated with 0.1 M EDTA in sucrose–Tris–HCl buffer (pH 7.0), precleaned further by a 10,000 ***g*** centrifugation step, and then given on continuous sucrose density gradients (1.10–1.24 g/ml). Bands of density 1.18 g/ml, were removed and re-centrifuged on gradients. Finally, the particles were washed and suspended in 0.1 ml Tris–sucrose buffer. Virus suspensions were stored at −20°C. Protein concentration was between 0.5 and 1 mg/ml of particle suspension (estimated according to [25]). Before being used in immunodiffusion tests, particles were treated with diethyl ether.

Preparation of iAp from mouse mammary tumours

For routine preparation (cf. [26]), about 8 g of syngeneically transplanted mammary tumours grown in the thighs of 8–12 mice of the XVII/BlnfCBA/Bln strain were taken as source material. The main preparation steps were two centrifugation runs on continuous sucrose density gradients (1.13–1.35 g/ml). Visible bands of the density 1.25–1.27 g/ml were removed, washed and finally suspended in 0.1 ml TN buffer. Protein concentration was about 1mg/ml suspension. iAp preparations were stored at −20°C until use. iAp suspensions prepared according to the method described are almost free of corpuscular contaminations [26–29].

Antisera

In order to support the specificity of the immunodiffusion reactions observed, antisera against virus particles and proteins from our own laboratory, reference sera kindly provided by other tumour virologists, and commercial antisera were used. We prepared anti-

sera in rabbits known to be free of heterophilic antibody activity towards murine and human milk and MuMTV antigens. Intramuscular and foot pad injections of particles disrupted by ether (B particles) or freezing and thawing (iAp) and, respectively, of purified polypeptides were given. Viral proteins were eluted from SDS polyacrylamide slab gel sections after electrophoretical fractionation of iAp which had been previously incubated for 16 hr at 37°C and treated with 1% SDS and 1% mercaptoethanol (100°C, 3 min). Initial immunizing antigen preparations were mixed with adequate volumes of complete Freund's adjuvant. Three to six injections were necessary to get antisera strong enough for immunodiffusion. The antisera used are shortly characterized by the following data:

Anti-MuMTV-B. Rabbit antiserum against B particles isolated from CBA/Bln strain milk; absorbed with MuMTV-free XVII/Bln mouse milk, normal mouse tissue and serum as well as with human embryonic tissue; shown to give almost completely identical precipitation lines with anti-MuMTV-B (Daams, see below) in reactions performed with MuMTV virions and iAp; the main lines were identically given by anti-MuMTV-gp52 and anti-MuMTV-p27 from W. P. Parks, Bethesda, U.S.A., compare [27, 30]; lines of identity were also given by anti-Ap14 and -Ap37 (see below).

Anti-MuMTV-B (Daams). Rabbit antiserum to C3H mouse milk B particles; absorbed with milk, serum and tissue of MuMTV-negative mice; a generous gift from Drs. H. Daams and Ph. Hageman, Amsterdam, The Netherlands.

Anti-iAp. Rabbit hyperimmune serum to iAp isolated as described; absorbed with XVII/Bln mouse serum and tissue and with human embryonic tissue homogenate pellets; shown to cross-react with anti-MuMTV-B sera, anti-iAp antiserum from H. Tanaka [26] and in part (one line) with anti-p27 from W. P. Parks [27].

Anti-iAp (Tanaka). Rabbit serum to iAp isolated from DBA/2 leukemia cells; kindly provided by Dr. H. Tanaka, Kyoto, Japan, compare [31]; absorbed with normal mouse and human embryonic tissue.

Anti-Ap14 and Anti-Ap-37. Rabbit hyperimmune sera against iAp polypeptides eluated from polyarcylamide gel electrophoresis (PAGE) as described above; absorbed with human embryonic tissue; shown to give incomplete cross-reactions with anti-MuMTV-B antisera, anti-iAp (see Fig. 3e), and anti-iAp (Tanaka). Anti-Ap14 was also demonstrated to give a line of identity with anti-MuMTV-p14 antiserum prepared by W. P. Parks (cf. [32]; RID tests performed in collaborative studies with Dr. I. N. Kryukowa, Moscow, U.S.S.R., unpublished).

Anti-DV (Ilyin). Rabbit antiserum to the Mason–Pfizer-like D-type virus of HEp-2 cells; prepared and provided by Dr. K. V. Ilyin [33, 34], Moscow, U.S.S.R.; absorbed with calf serum and human embryonic tissue; shown to cross-react in part (one line) with anti-MPMV-p27 (see below) by double immunodiffusion tests with HEp-2 D-type oncornaviruses.

Anti-MPMV-p27. Goat antiserum to the main core polypeptide of MPMV (serum No. 5S 148 from Flow Laboratories, Rockeville; courtesy of K. V. Ilyin); unabsorbed.

Anti-human core-like fraction. Antiserum from rabbit immunized by three injections (0.1 mg protein each, of MuMTV antigen-positive 1.26–1.28 g/ml milk fractions of different women, absorbed with human embryonic tissue homogenate pellet and pooled MuMTV-negative normal tissue from XVII/Bln mice.

All these antisera lack nonspecific reactions in RID tests performed with equal volumes of homogenate pellets of normal tissues (liver, kidney, lung, muscle, heart, spleen, brain), healthy donors' sera, freshly collected human milk (without any previous preparation procedures), and embryonic tissue homogenate pellet of human origin, normal tissue homogenate pellets, serum, and milk from MuMTV-free mice, calf serum, defatted bovine milk, bovine lactalbumin (0.25 mg/ml protein; kindly provided by Dr. Trübsbach, Medical Academy, Dresden), and alpha-lactalbumin and casein from human milk (isolated according to [35]; protein contents 0.8 mg/ml and 1.0 mg/ml, respectively). Furthermore, all the sera proved negative if tested by RID against gradient fractions of density 1.26–1.28 g/ml prepared from 300 g of pooled human liver, kidney, heart, muscle (M. psoas) and spleen tissue in the very same way as done with milk.

Anti-human serum ("polyspecific"), anti-human IgG and anti-human albumin antisera. These were obtained from rabbits (Sifin, Berlin, G. D. R.) and were used for control purposes.

Human sera

Sera from lactating women were stored at −20°C until use. Before being tested by im-

munofluorescence, they were absorbed with normal mouse tissue and, additionally, *in vivo* in XVII/Bln mice as described [19–21]. Sera from a blood donor (No. 12) and a breast cancer patient (C303) known to possess antibodies reacting with iAp in immunofluorescence tests [21, 36] were taken as reference material and for absorption studies.

Radioimmunodiffusion (RID) test

The method was performed as described in detail earlier [26]. Micro-Ouchterlony technique was employed according to [37] and [38]. A thin 0.75% agar layer was mounted with plastic templates bearing funnelled holes for 30 µl-test samples each. After double-immunodiffusion was finished, the templates were removed. Washed test slides were then incubated with ^{125}I-labelled anti-rabbit IgG antibody. After washing and drying, slides were exposed on ORWO NP27 films (16 hr) for autoradiography. Iodination of antibody was performed according to [39] using Chloramin T as oxidative reagent. Four mCi were coupled to 4 mg of purified anti-rabbit IgG antibody (protein estimation according to [25]). As measured by the determination of endpoint dilutions, the radioimmunological reaction step was shown to enhance the sensitivity of the microimmunodiffusion method about 10–20-fold in dependence on the antigen-antibody system used.

Immunofluorescence tests

Tests were performed on frozen cut slices of the mouse mammary tumour XVIIf10 known to contain paranuclear clusters of iAp (cf. [25, 28, 40]). These virus accumulations have been shown to be specifically labelled by rabbit hyperimmune antisera as well as by mouse and human natural antibodies resulting in characteristic intracellular granular fluorescence reactions [21, 28]. In this study, we monitored the sera of all milk donor women for anti-iAp antibody activity after being absorbed *in vitro* and *in vivo* as methodically described. For reference, anti-iAp and anti-Ap14 antisera were also used on slices of the same mammary tumour after absorption by both methods. The rabbit hyperimmune serum against human milk fractions positive for MuMTV antigenicity (antihuman core-like fraction; see above) was also tested by immunofluorescence after *in vitro* and *in vivo* absorption. Before use, FITC-labelled anti-rabbit IgG (Sifin, Berlin, G. D. R.) and antihuman gamma chain (Behringwerke, Marburg, F.R.G.) antisera were exhaustively absorbed with A and B particle-containing murine mammary tumour tissue homogenate.

In order to get information about the cross-reactivity between the MuMTV-related antigens found in human milk and the human anti-iAp antibody, absorption experiments were performed with the use of different milk fractions and, as reference materials, with murine iAp and B particles. Samples (0.05 ml) of *in vivo* absorbed blood donor (No. 12) or breast cancer (C303) sera known to possess anti-iAp activity with an endpoint dilution of 1 : 32 were mixed with adequate volumes of the absorption materials and incubated at room temperature for 1 hr and, in addition, at 5°C for 3 hr. Serum titers then were determined. In order to exclude the influence of proteolytic effects of the human milk fractions, control tumour slices were incubated with these milk materials prior to incubation with human sera. However, according to these control procedures, there was no detectable tissue-destroying activity associated with the milk fractions tested.

RESULTS

Detection of MuMTV-related antigens in women's milk

Simulating oncornavirus core preparations from human milk samples of 500 ml each, we found distinct light scattering bands of density 1.26–1.28 g/ml in sucrose gradients. These bands were seen in 43 of 54 milk preparations performed from 22 of 28 women investigated. Whether there have been visible bands or not, these so-called 'core' fractions as well as fractions of density 1.29–1.33 g/ml, and gradient sediments ($\geqq$ 1.35 g/ml) were tested by RID technique for the presence of MuMTV and iAp antigenicity. Positive results were seen in freshly prepared milk specimens from 5 of 28 women tested. The antigenicity was mainly found in association with the core-like density fraction. Nevertheless, occasionally, sediments of two preparations and the fraction of density 1.29–1.33 g/ml (one of these cases) also contained antigenic activity. Out of 23 core-like fractions prepared from the 5 positive women between the 5th and the 28th week after delivery, 22 gave positive RID results. The intensity of the cross-reacting antigenicity, however, varied from day to day. The milk of one woman (C.T.) was found to be positive at the 12th week after delivery, whereas it did not contain detectable

MuMTV antigenicity 8 days later. On the other hand, as a point of importance, the comparatively weak antigenicity of some samples was abolished after they had been frozen at −20°C overnight or for longer periods. Thirty-one core-like preparations from the milk of 23 women gave negative RID tests.

Table 1 summarises the results obtained in positive human milk core-like fractions with a spectrum of antisera to viral particles and purified polypeptides. The reactions are in part demonstrated in Fig. 1. At least one or two distinct precipitin lines were seen in the autoradiographs of reactions between human core-like density fractions and antisera against cipitated by antisera directed to the A particle polypeptides Ap14 (Fig. 1f) and Ap37 (Table 1). Exceptionally, some of the precipitin reactions given by human milk core-like preparations were already visible on micro-Ouchterlony immunodiffusion slides without autoradiographic development (Fig. 2). RID tests performed with antisera to oncornavirus type D particle antigens were negative (Table 1). No precipitin reactions occurred by testing human milk fractions with anti-human albumin antiserum. Out of seven MuMTV-positive milk specimens tested, four gave one or two weak immunoprecipitin lines (reactions not shown) with anti-human IgG antiserum and anti-human serum antiserum ("polyspe-

Table 1. *Results of RID tests for oncornaviral antigens in human milk core-like preparations*

	Milk core-like preparations from women (initials)				
Antisera	E.G.	E.H.	C.T.	S.L.	M.K.
Anti-MuMTV-B	+*	+†	+, −‡	+§	+‖
Anti-MuMTV-B (Daams)	+	+	n.t.	n.t.	n.t.
Anti-iAp (MT)	+*	+†	+, −‡	+§	+‖
Anti-iAp (Tanaka)	+	+	n.t.	n.t	n.t.
Anti-Ap14	+	+	−‡	+	n.t.
Anti-Ap37	n.t.	+	n.t.	+	n.t.
Anti-DV (HEp2, Ilyin)	−	−	−	−	n.t.
Anti-MPMV-p27	n.t.	−	−	−	n.t.

n.t. = not tested.

*RID tests of core-like density fractions prepared between the 14th and the 18th week after delivery, with anti-MuMTV-B (6 ×) and anti-iAp (7 ×).

†Tests with anti-MuMTV-B (4 ×) and anti-iAp (5 ×) with core-like density fractions prepared between the 18th and the 24th week after delivery.

‡Positive tests at the 12th week after delivery, negative tests 8 days later (also with anti-Ap14).

§Preparations performed twice at the 5th and the 9th week after delivery.

‖Core-like preparations proved RID-positive at the 14th, 15th, 16th and 18th week after delivery.

MuMTV-B type virions or iAp (Figs. 1a, b, c, and f). With the above mentioned exception, there were no such lines in tests performed with 1.29–1.33 g/ml fractions (demonstrated for comparison in Fig. 1d). The antigens detected in human milk were identical to the main antigenic components of purified MuMTV-B particles (treated with ether) and iAp as indicated by confluence of precipitin lines (Figs. 1a, b and c). The cross-reaction between human material and mouse viruses was almost complete. In some of the RID tests performed, however, there have been singular precipitation lines only given with MuMTV type B viruses or iAp (Fig. 1b). Human cross-reacting antigens were also pre-cific"). These lines were not identical to those resulting from reactions with anti-viral antisera and were also given, in two out of four cases tested, by milk specimens which were negative for MuMTV-related antigens. The lyophilized supernatants which result from the first ultracentrifugation step (see Materials and Methods) of two MuMTV-positive milk preparations (E.G., E.H.) were resolved in 1 ml distilled water each and then subjected to RID tests. These preparations were negative with respect to viral antigens. Moreover, sucrose gradient fractions of density 1.16–1.18 g/ml were prepared from three human milk specimens (E.H., S.L., M.K.) that had not been pre-treated with detergent and ether. These

fractions did not give positive RID reactions with anti-MuMTV-B antiserum (after shaking the preparations with diethyl ether immediately before being tested).

Immunofluorescence studies on serum anti-iAp antibody activity

Table 2 summarises the results obtained after checking the milk donor sera by immunofluorescence technique on mouse mammary tumour slices. Out of the 28 women tested, 5 were seen to have anti-iAp antibody activity. These sera labelled intracellular iAp accumulations within mouse tumour cells resulting in the characteristic fluorescence pattern described earlier ([21]; see Fig. 3c). For comparison, similar reactions were found to be given by anti-iAp and anti-Ap14 antisera (Figs. 3a and b). There was a close correlation between the presence of detectable human serum antibody and women's milk-bound MuMTV antigenicity. Positive serum reactions were only observed in women who also exhibited MuMTV antigens in their milk, whereas all women without detectable milk antigens failed to have serum antibody. In order to compare the antibody activity in milk donor sera with that of positive human standard sera and specific antiviral antisera, serum titers were determined as shown in Table 2. The milk donor sera had positively reacting endpoint dilutions ranging from 1 : 4 to 1 : 16, which is very low in comparison with the titers of rabbit antisera on the one hand, and also beyond the titers of the human standard sera on the other. In order to give support to the specificity of the antibody reaction, limited absorption studies were performed as described above. In these experiments, iAp of mouse origin completely absorbed the antibody activity of all positive milk donor sera, whereas the pre-absorption with normal mouse tissue did not. Moreover, in Fig. 3d, the anti-iAp reaction of rabbit hyperimmune serum against human milk core-like preparations is demonstrated. This antiserum had an endpoint dilution of 1 : 16. However, there was no clear evidence of precipitating anti-MuMTV antibody activity in the RID tests performed with this serum so far. Nevertheless, indicating the reaction specificity, the fluorescence antibody activity of this serum was completely absorbed by incubation with the purified iAp preparations from mouse mammary tumours or with ether-disrupted MuMTV-B particles, whereas absorption procedures with human embryonic and mouse normal tissue homogenate pellets failed to diminish the fluorescence reaction.

Table 2. Anti-iAp antibody titers in rabbit hyperimmune antisera and human sera as determined by immunofluorescence tests on mouse mammary tumour slices

Sera*	Titer†
Rabbit antisera	
Anti-iAp	512
Anti-MuMTV-B	256
Anti-Ap14	128
Anti-Ap37	128
Anti-human core-like fraction	16
Human sera	
No. 12‡‡	32
C 303‡‡	32
S.L.§	16
E.H.§	8
M.K.§	8
E.G.§	4
C.T.§	4
Other milk donor women‖	0

*Sera incubated on slices of the XVII/BlnfCBA/Bln tumour f10 after absorption *in vitro* and *in vivo* for 16 hr (FITC labelled anti-globulin antisera for 2 hr).

†Titers are given as reciprocals of the positively reacting endpoint dilution (titer 0 corresponds to negative reactions).

‡‡Standard blood donor (No. 12) and breast cancer (C 303) sera known to specifically react with iAp and taken for absorption studies with human milk preparations (compare to Table 3).

§Sera from women with MuMTV-related antigens in their milk samples (compare to Table 1).

‖Sera from 23 women without detectable MuMTV antigenicity in their milk specimens.

Absorption efficiency of human milk fractions to the human anti-iAp antibody

As shown in Table 3, human milk core-like fractions and gradient sediments exhibiting MuMTV antigenicity detectable by RID tests were seen to absorb the anti-iAp activity of human standard blood donor and breast cancer sera. In comparison with MuMTV and iAp preparations which completely abolished the serum reaction, the absorption efficiency of human milk fractions was less strong. The diminishing effect on human anti-iAp reaction, however, was mainly seen in correspondence to the RID results obtained with respect to the MuMTV antigenicity of the respective milk fractions. As an exception, very weak absorbing activity seemed to be associated with one gradient sediment that did

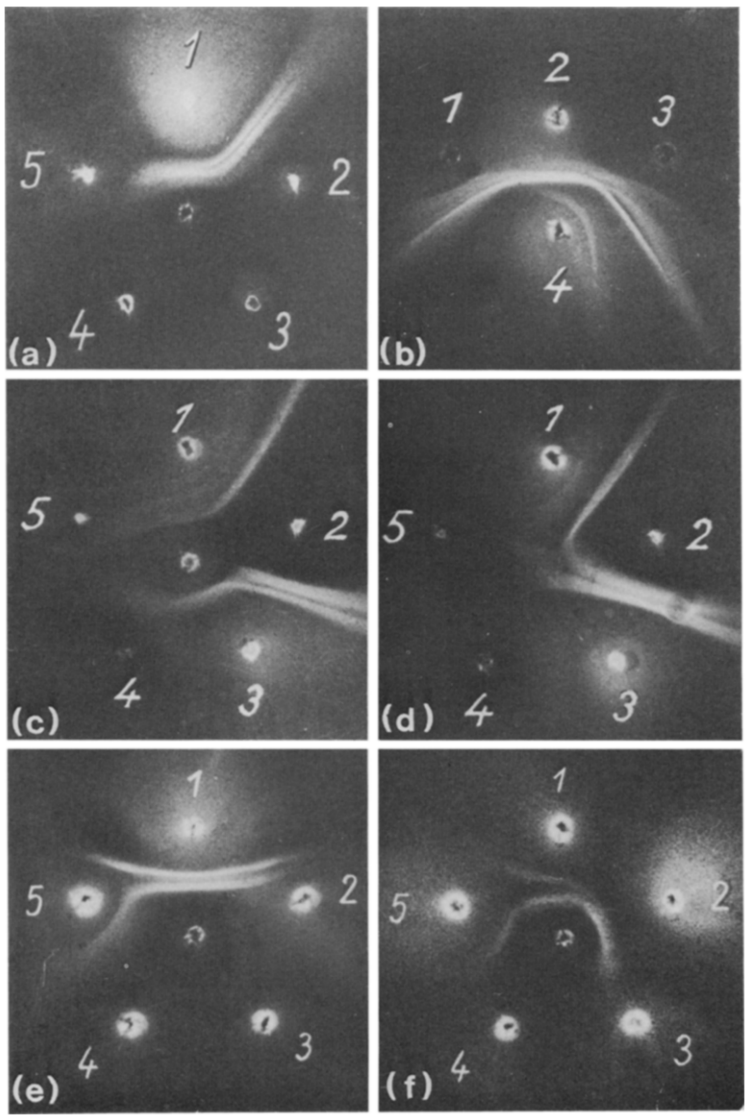

Fig. 1. *Autoradiographs from micro-Ouchterlony radioimmunodiffusion tests.*

(*a*) *Central well—core density fraction from E.G.* 1—*anti-iAp;* 2—*iAp;* 3,4,5—*rabbit control sera* (*pre-bleeds*).

(*b*) 1—*core density fraction from E.G.* 2—*iAp;* 3—*MuMTV-B particles* (*ether-tested*); 4—*anti-iAp.*

(*c*) *Central well—core density fraction from E.H.* 1—*anti-MuMTV-B;* 2—*iAp;* 3—*anti-iAp;* 4,5—*human sera.*

(*d*) *Central well—fraction of density* 1.29–1.33 g/ml *from E.H.* 1–5—*as in Fig.* 3*c* (1–5).

(*e*) *Central well—iAp;* 1—*anti-iAp;* 2,3,4—*rabbit control sera* (*pre-bleeds*); 5—*anti-iAp*14.

(*f*) *Central well—core density fraction from E.G.* 1—*anti-MuMTV-B* (*Daams*); 2—*anti-iAp;* 3,4—*rabbit control sera;* 5—*anti-Ap*14.

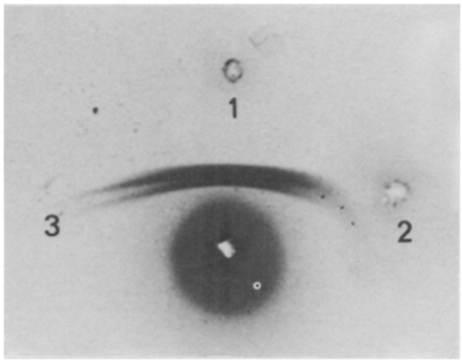

Fig. 2. Micro-Ouchterlony test. Stained with Amidoblack. Central well—anti-iAp. 1—iAp; 2—core density fraction from M. K.; 3—MuMTV-negative XVII *strain mouse milk.*

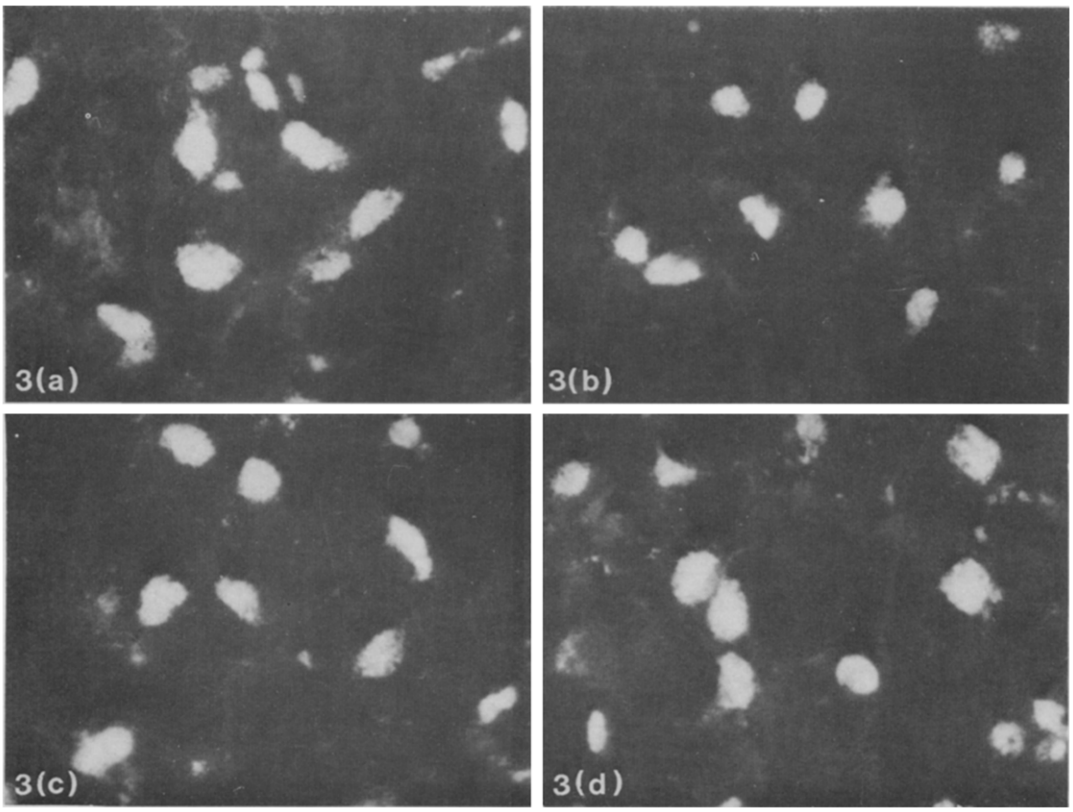

Fig. 3. Indirect immunofluorescence reactions on slices of a mouse mammary tumour with abundant paranuclear clusters of iAp (tumour XVII*f*10*). Characteristic fluorescent labelling of iAp accumulations.* ×1500. *(a) Anti-iAp. Dilution* 1:8 *(b) Anti-Ap*14 *(undiluted). (c) Anti-human core fraction (undiluted). (d) Serum of milk donor woman S. L. (undiluted).*

Table 3. Absorption efficiency of human milk fractions to the human anti-iAp antibody (immunofluorescence tests on slices of the mouse mammary tumour XVII *f*10)

Absorption material* Women (initials)	Density fraction of milk (g/ml)	MuMTV antigenicity†	Titer‡ after absorption
E.G.§	1.26–1.28	+	2
	1.29–1.33	+	4
	≧1.35	+	4
E.H.	1.26–1.28	+	4
	1.29–1.33	−	16
	≧1.35	−	8
H.T.	1.26–1.28	−	16
	1.29–1.33	−	16
	≧1.35	−	16
K.G.	1.26–1.28	−	16
	1.29–1.33	−	16
	≧1.35	−	16
B.H.	1.26–1.28	−	16
	1.29–1.33	−	16
	≧1.35	−	16
Purified iAp‖		+	0
Purified MuMTV-B particles‖		+	0
PBS (control)		−	16

*Sucrose gradient fractions after pre-treatment of 500 ml milk with EDTA, Triton × 100; and ether (see Materials and Methods); each fraction pelleted and washed with PBS before taken as absorption material.

†As determined by radioimmunodiffusion tests (compare to Table 1).

‡Titers are given as reciprocals of the positively reacting endpoint dilution of the human serum No. 12 (titer without dilution: 32; dilution due to incubation with absorption material is twofold).

§Absorption tests with milk preparations of this woman have been repeated three times with similar results (with the serum No. 12 and, additionally, with the breast cancer serum C303).

‖Purification from mouse tumour tissue and milk as described in Materials and Methods; B particles after disruption with ether.

not exhibit viral antigens detectable by RID technique (sediment from milk of E.H., being antigen-positive in the core-like fraction). On the other hand, fractions of density 1.29–1.33 g/ml, which were negative in RID tests, did not exhibit absorbing activity. The corresponding milk fractions of three RID-negative women did not show any absorption effect.

Large-scale absorption studies could not be performed because of the minimal amounts of antigenic human material resulting from each milk preparation. However, in order to find out whether or not the milk core-like fractions were able to absorb the antibody activity from autologous serum, a limited study was performed with two sera. After *in vitro* and *in vivo* absorption, sera from E.H. (endpoint dilution 1:8) and C.T. (1:4) were additionally incubated with autologous core-like fractions as usually done in the absorption experiments. In both cases, an absorbing effect was seen resulting in complete abolishing the fluorescence reaction.

DISCUSSION

We report here the presence in human milk core-like preparations of at least two antigenic determinants which are related to MuMTV-B particles and also to iAp. During the last few years, evidence has been accumulated demonstrating these iAp to doubtlessly represent the intracellular particulate structures of MuMTV [25, 29, 31, 41–43] and, especially, to share antigenicity with B particle nucleoids [27, 44–46]. Therefore, the human milk antigens described herein are regarded as related to inner constituents of the mature murine type B virus.

In our opinion, the following details of the preparation procedure have been prerequisite or substantially helpful for the detection of the antigens in question:

Because of the comparatively low content of morphologically detectable type B particle structures within human milk [6], we used large amounts of milk (500 ml) as source material for each preparation. It is to be mentioned here, that we were not able to detect MuMTV antigens in some preparations done with comparatively small human milk samples of up to 50 ml (detailed data not shown).

The application of methodical details commonly used for the isolation of subviral cores including treatment with detergents and ether seemed also to be useful. In the experience of other authors [10, 15] the comparatively high-density fractions resulting from such preparations do not contain so many cellular contaminants as, e.g., the B particle fraction does. According to our own preliminary data, there was no distinct MuMTV-related antigenicity in density fractions of about 1.16–1.18 g/ml prepared from milk specimens of three women known to possess cross-reacting antigens in the core-like fractions. Moreover, there was no detectable antigenicity in density fractions of 1.26–1.28 g/ml prepared from two positive women without ether and detergent treatment (detailed data not shown).

An ultracentrifugation run with the use of a metrizamide cushion was included into the preparation method to eliminate milk particle-destroying factors [24].

Human milk particulate fractions were subjected to immunodiffusion tests immediately after they had been isolated. After the fractions were frozen and thawed once or repeatedly, the immunological activity of the cross-reacting antigens was substantially decreased or completely abolished. This phenomenon might be explained, e.g., by denaturation of antigens or liberation of proteases due to the freeze–thaw process.

Finally, a radioimmunodiffusion method was applied known to have a sensitivity which is 10–20-fold higher when compared with the non-radioimmunologically completed micro-Ouchterlony test and about 100-fold higher in comparison with the ordinary double immunodiffusion method. However, some of the strongest precipitation lines given by human milk preparations have also been seen without autoradiography of the test slides.

The most important point of discussion, however, is the specificity of the immunological findings reported in this paper. In our opinion, the human milk antigens described herein are related to the MuMTV for the following reasons: The MuMTV-B particle and iAp immunodiffusion test systems in our hands are specific for the main antigens of these viral particles on the basis of repeated comparison with reference test systems of other laboratories (see Materials and Methods; cf. [26, 27]). Moreover, antisera to isolated A particle polypeptides (Ap14, Ap37) also gave precipitin reactions with positive human milk fractions. Ap14 and Ap37 represent major polypeptides of iAp. In correspondence with recently published data [45], Ap37 is regarded to result from partial degradation of the main precursor protein of iAp (Ap73 in our experience, Ap70 in the papers of Tanaka). Ap14, on the other hand, seems to represent one of the most important polypeptides occurring after proteolytic cleavage of iAp. This protein is immunologically related to the 14,000 mol. wt polypeptide of B particles as indicated in Materials and Methods, and as has been recently suggested by [47]. Moreover, as a further indication of the specificity of the RID reactions observed, the anti-viral antisera employed did not react with subcellular fractions of density 1.26–1.28 g/ml prepared from normal human tissue nor with pellets of normal organ tissues, embryonic tissue, serum and milk proteins of human and murine origin after they had been absorbed as described in Materials and Methods. Furthermore, some of the sera failed to react with several common mycoplasms [18]. A rabbit antiserum prepared against antigen-positive human core-like fractions and subjected to immunofluorescence studies was found to react with murine iAp, which also gives support to the specificity of the MuMTV antigenicity associated with the human milk components investigated. As another point of importance, we did not obtain evidence of the presence of type D oncornavirus-related antigens in the human milk fractions tested (cf. [48]). The specificity was also emphasized by the reproducibility of the findings in 22 of 23 milk samples prepared from the 5 positive women.

In two particular milk preparations, fractions of higher density (1.29–1.33 g/ml, $\geqq 1.35$ g/ml) were also antigen-positive. This might well be due to an overloading of the gradients with milk material. In both cases the core-like fraction revealed maximum antigenic activity and the light scattering material in the gradient tubes was not only associated with the core density region, but also visible in the gradient portion below. On the other hand, these observations might explain the weak

absorbing activity of one human milk fraction (of density 1.29–1.33 g/ml) which proved negative by RID.

RID studies performed to analyse the core-like fractions with respect to the presence of serum proteins revealed evidence of IgG and, possibly, some other serum protein antigenicities in several milk specimens. However, these antigens were not related to antigenic determinants of the MuMTV. Albumin was not detectable in the fractions analysed.

Another finding of interest reported in this paper is the correlation of the human milk antigen with the serum antibody shown to specifically react with murine iAp in mammary tumour slices (cf. [19–21]. More detailed studies are necessary to see how close this correlation is in a greater number of persons. Nevertheless, so far investigated, all women with MuMTV-positive milk core-like fractions have detectable antibody activity in their sera, whereas antigen-negative women have not. Moreover, antigen-positive human milk preparations absorbed the human antibody from autologous and homologous sera. The antibody itself is correlated with physiological and pathological proliferations of the mammary gland as seen in epidemiological studies in more than 1500 persons tested by us so far (cf. [21, 36]) and as in part confirmed by Ogawa and Tanaka [49]. Thus, in summary, the existence in man is suggested of an antigen–antibody system that is related to internal constituents of the murine oncornavirus type B particles and iAp on the one hand, and to the human mammary gland and milk particulates of core-like density on the other. However, the meaning and significance of these findings are at present difficult to evaluate.

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