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Epithelial Membrane Antigen (EMA) Distribution in Various Biological Fluids*

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Abstract—*Different human biological fluids, namely breast cyst fluids (five), milks (four), sera (five), were submitted to molecular sieving chromatography on Sepharose CL6B. Global protein contents of the eluted fractions were estimated by the Bradford method. Epithelial membrane antigen (EMA) was assayed by two different ELISA techniques using polyclonal and monoclonal antibodies. Various molecular species reacting with EMA (15) were found in the chromatographies with molecular weights ranging from 35 to 1500 kd. But the total amount of antigens detected using polyclonal or monoclonal antibodies was quite similar. Moreover no significant difference was found between the sera from two lactating women and the sera from three women with adenocarcinoma with respect to the molecular distribution of different molecular species of EMA.*

INTRODUCTION

RECENTLY we described an ELISA for epithelial membrane antigen (EMA) [1] in human sera [2]. It was found that this assay could be useful in various pathologies mainly in lung and breast diseases. Nevertheless, EMA belongs to a large family of antigens extracted from human milk fat globule membranes. Various antigens have been described, for example MAM6 [3], human mammary epithelial antigen (HME-AG) [4], PAS-O [5]. It was demonstrated that these antigens showed heterogeneity with respect to molecular weight [4]. Various methods have been described to detect these antigens, some using monoclonal or polyclonal antibodies. It has been previously pointed out that all these techniques do not detect the same molecular species [6].

In attempting to assess our own assay methods, various biological fluids, milks, breast cyst fluids and sera, containing EMA were submitted to molecular sieving chromatography in order to separate the different molecular species. The eluate fractions were assayed by two different techniques using polyclonal and monoclonal antibodies.

MATERIALS AND METHODS

1. *Antigen purification*

The purification method developed by Ormerod *et al.* [7] was used.

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2. *Antisera*

Polyclonal antibody anti-EMA was obtained from a rabbit as previously described [2]. Monoclonal antibody anti-EMA was purchased from DAKO (Denmark) code M613.

3. *Assay for EMA*

a. *Single polyclonal antibody assay.* This method was described previously [2]. Briefly, 0.2 ml of purified EMA solution (10 μg/ml in bicarbonate pH 9.5, 0.1 M) was used to coat a Nunc Immunoplate for at least 24 h at 4°C and then washed with PBS 0.05 M, pH 7.5, containing 0.1% Tween 20. One hundred and fifty microlitres of antiserum solution (1:10000) were added to 50 μl containing either the reference EMA solution (from 80 to 5000 ng) or the sample to be tested. Dilutions were made in the same buffer as above except that it contained 5 g BSA/l. After 48 h incubation at 4°C, the mixture was added to the EMA coated immunoplates. After 2 h at room temperature, the wells were washed and incubated with 0.2 ml of peroxidase labelled anti-rabbit IgG (Tago 1:1000). Two hours later, the wells were washed again and enzymatic activity was detected with 0.2 ml of ABTS (Boerhinger) solution (75 mg in 100 ml of 0.1 M phosphate citric acid buffer pH 4). The optical density at 405 nm was measured after 45 min in a Titertek multiscan colorimeter. Precision was estimated from the variation coefficient calculated on the different points of the standard curve made in triplicate.

b. *Sandwich polyclonal–monoclonal antibody assay.* Immunoglobulins from the polyclonal antiserum were isolated by Na_2SO_4 precipitation [8]. In the first step 0.2 ml of polyclonal immunoglobulins (10 μg/ml in bicarbonate buffer pH 9.5, 0.1 M) were used to coat Nunc immunoplates for at least 24 h at 4°C and washed with PBS 0.05 M, pH 7.5, containing 0.1% Tween 20. The 200 μl of reference EMA solution (from 80 to 5000 ng) or samples to be tested were added to the wells. Dilutions were made in the same buffer as above except that it contained 5 g BSA/l. After 2 h incubation at room temperature the wells were washed and incubated with 0.2 ml of monoclonal antibody at a dilution of 1/40. After an additional 2 h, the wells were again washed and filled with 0.2 ml of peroxidase labelled anti-mouse antibody (1/500 DAKO). Incubation time was still 2 h and a last washing was performed before adding 0.2 ml of ABTS solution (see Section 3a). OD at 405 nm was measured after 1 h in a Titertek multiscan colorimeter. Precision was estimated from the variation coefficient calculated on the different points of the standard curve made in triplicate.

4. *Biological fluids tested*

Different kinds of biological fluids were chromatographed:

four human milks at day 4 of lactation
five human breast cystic fluids
two sera from lactating women
three sera from patients with adenocarcinoma.

All these fluids were selected for their high EMA contents.

5. *Chromatography*

Gel filtration of 2 ml samples was performed on a column of Sepharose CL6B (1.5 × 96.5 cm) equilibrated in PBS (PH 7.5). The milks were defatted by centrifugation for 30 min at 3500 ***g*** before chromatography. The column was calibrated using Blue Dextran (Pharmacia), fibrinogen (mol. wt 400,000), rabbit gamma-globulin (mol. wt 160,000) and bovine albumin (mol. wt 60,000). Two millilitre fractions were collected. The OD at 280 nm of the eluate was determined. Protein content was estimated by the method of Bradford [9] and EMA was determined by the two enzyme immunoassays using single polyclonal and sandwich polyclonal–monoclonal techniques.

RESULTS

1. *Assay*

The sensitivity of the assay using the sandwich polyclonal–monoclonal method was estimated with a 95% confidence interval at the zero point. A sensitivity of 80 ng per ml sample was found: the same range as the single polyclonal antibody assay [2]. The coefficient of variation of the different points on the curve did not exceed 4%. The specificity was assessed as for the previously described single polyclonal assay. The same results were observed; a minor cross-reactivity exists with casein [10], 5% probably due to EMA contamination [11], and no cross-reaction with GCDFP [12], CEA [13] or NCA [14].

2. *Chromatography*

Five breast cyst fluids were chromatographed. The main point is that the elution profiles were complex and very different with respect to the number of peaks and protein content. In fact some peaks detected in u.v. did not seem to be protein in nature due to the fact that they did not react when the Bradford method was used. Figure 1 illustrates the elution profile of the first chromatographed breast cyst fluid (the simplest aspect found). Table 1 summarizes the results combined with the five breast cyst fluids analysed.

Four human milks were chromatographed. In this case the elution profiles were similar with respect to the number of peaks but not in terms of protein content. The main EMA peak detected by monoclonal and polyclonal antibodies was in the same position and displayed the same shape. Table 2 summarizes the major features of the chromatograms of the four milks.

Lastly, five human sera (two from lactating women, three from patients with adenocarcinoma) were chromatographed. The elution profiles observed in the sera were very similar with respect to the number of peaks and protein content. The EMA peaks detected by monoclonal and polyclonal antibodies were in the same position and were of the same extent. Table 3 summarizes the main features of the chromatograms of five human sera.

DISCUSSION

Numerous assays for EMA and related substances isolated from human milk fat globule membranes have been described [2, 3, 4, 5, 6, 11]. However, few studies have been performed to compare the results obtained with techniques using monoclonal or polyclonal antibodies. Furthermore, complex biological fluids such as breast cyst fluids have scarcely been studied with respect to EMA content. In the paper of Collette *et al.* [15], it was shown that EMA was present in this biological fluid at a concentration of 0.98–80 μg/ml with a mean value of 17.4 μg/ml; however, there were no data on the molecular weight distribution of this antigen.

The chromatograms of the five breast cyst fluids indicated that their contents were complex and very different from one another. The number of peaks detected in u.v. varied from three to six and their nature with respect to protein content was also very

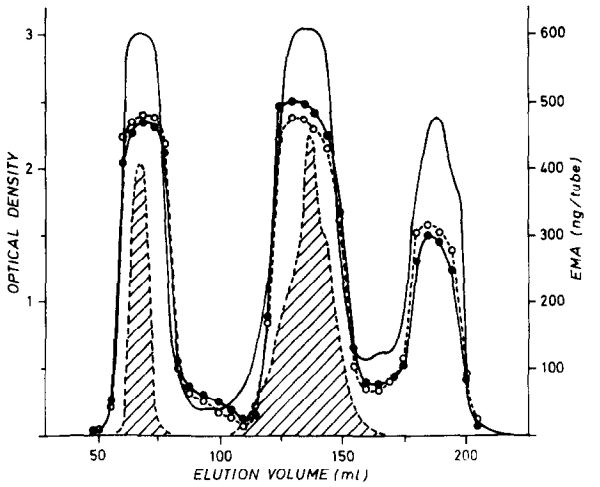

Fig. 1. Chromatography of breast cyst fluid (No. 1) on Sepharose CL6B: —— OD at 280 nm; ----- Bradford protein determination (OD at 595 nm); -○-○- monoclonal EMA concentration; -●-●- polyclonal EMA concentration.

different (Table 1).

The molecular species detected using the single polyclonal or sandwich polyclonal–monoclonal assay were in the same position and the total amount of antigen detected in both techniques was very similar. The molecular weight distribution was scattered ranging from 1,300,000 to 35,000 daltons with various intermediates (seven). Initially, the antibodies were raised against a high molecular weight EMA preparation extracted from human milk exceeding 1,000,000 daltons. It seemed, therefore, that the high enzymatic activity of breast cyst fluids induced a strong degradation of EMA yielding various subfractions that could react with antibodies. Some fractions detected were not protein in nature as shown for the five samples in Table 1. This was not surprising because the EMA antigen consists mainly of carbohydrates, 60% [7], and it has been previously pointed out that many antigenic determinants are oligosaccharide in nature [6].

On the contrary, the chromatograms of four human milks displayed a simpler aspect. Elution profiles were very similar with respect to the number of peaks but not in terms of protein content. EMA was found in a maximum of four peaks of similar molecular weights as shown in Table 2, ranging from 1,000,000 to 40,000 daltons. The total amount of antigen detected in both monoclonal and polyclonal techniques was of the same order. The main peak was of high molecular weight: 1,000,000 daltons. This was quite logical if one remembers that the purified EMA used for immunization and

Table 1. Chromatography of breast cyst fluids

Sample No.	u.v. peak	Molecular weight (kdaltons)	Presence of EMA	Nature	EMA detected (ng*100) polyc	monoc
1	1	1000	+	Protein	47.7	52.1
	2	150	+	Protein	59.3	19.8
	3	35	+	Non-protein	20.2	22.7
				Total	127.2	124.6
2	1	1300	+	Protein	24.2	28.6
	2	350	+	Non-protein	6.4	16
	3	80	+	Protein	53	62.4
				Total	83.6	107
3	1	1300	+	Protein	20	24
	2	1000	+	Protein	12.05	8.6
	3	400	+	Non-protein	14.6	10
	4	280	+	Non-protein	5	5.5
	5	100	+	Protein	14	12.6
	6	35	+	Non-protein	9	12.4
				Total	74.65	73.1
4	1	1300	+	Protein	47.5	50.1
	2	1000	+	Protein	60.1	71.3
	3	350	−	Protein	0	0
	4	35	−	Protein	0	0
				Total	107.6	121.4
5	1	1000	+	Protein	82	91.8
	2	400	+	Non-protein	20	18.7
	3	350	+	Non-protein	21.3	25.8
	4s	100	+	Protein	5	11.7
	4	35	−	Protein	0	0
				Total	128.3	148

s means shoulder of the main peak.

Table 2. Chromatography of human milks

Sample No.	u.v. peak	Molecular weight (kdaltons)	Presence of EMA	Nature	EMA detected polyc	(ng*100) monoc
1	1	1000	+	Protein	54	53
	2	325	−	Non-protein	0	0
	3	140	+	Protein	9.3	7.7
	4	40	+	Protein	3.4	2
				Total	66.7	62.7
2	1	1000	+	Protein	67	70
	2	325	−	Non-protein	0	0
	3	140	+	Protein	16.5	9
	4	40	+	Protein	14	4.6
				Total	97.5	83.6
3	1	1000	+	Protein	135	147
	2	325	+	Protein	22.7	37
	3	140	+	Protein	7	10.3
	4	40	−	Protein	0	0
				Total	164.7	194.3
4	1	1000	+	Protein	128	102
	2	325	+	Protein	15.3	11.3
	3	140	+	Protein	2	5.2
	4	40	+	Protein	3.5	10
				Total	148.8	128.5

Table 3. Chromatography of human sera

Sample No.	u.v. peak	Molecular weight (kdaltons)	Presence of EMA	Nature	EMA detected polyc	(ng*100) monoc
1	1	1500	+	Non-protein	3.4	7.7
	2s	750	+	Protein	23	23
	2	200	+	Protein	4.5	7
	3	40	+	Non-protein	1	2.6
				Total	31.9	40.3
2	1	1500	+	Non-protein	7.3	4
	2s	750	+	Protein	25	27
	2	200	+	Protein	6	5
	3	40	+	Non-protein	10	7.2
				Total	48.3	43.2
3	1	1500	+	Non-protein	1.4	2
	2s	750	+	Protein	12	10
	2	200	+	Protein	5.3	3.5
	3	40	+	Non-protein	4.5	4.7
				Total	23.2	20.2
4	1	1500	+	Non-protein	3	4.7
	2s	750	+	Protein	28	32
	2	200	+	Protein	10	18
	3	40	+	Non-protein	13.5	7.5
				Total	54.5	62.2
5	1	1500	+	Non-protein	4	2.9
	2s	750	+	Protein	35.7	30
	2	200	+	Protein	11	9
	3	40	+	Non-protein	2.6	7
				Total	53.3	48.9

s means shoulder of the main peak.
Samples 1,2: lactating women.
Samples 3, 4, 5: patients with adenocarcinoma.

standardization was extracted from milk and selected for high molecular weight. Nevertheless, three fractions of lower molecular weight were detected and they contained significantly lower amounts of antigen than the first (approx. 15–30% of the total). The total amount of antigen detected by the polyclonal–monoclonal assay was slightly lower than by the single polyclonal assay (except in sample 3).

Lastly the chromatograms of the five human sera displayed a different picture. In this case only three reactive fractions were detected with both assays, their molecular weights were respectively 1,500,000, 750,000 and 40,000 daltons (the presence of EMA in the peak 2 could be due to the tail of the main fraction). The main peak was the second and had a molecular weight lower than the EMA reference extracted from milk, indicating that the circulating antigens were different to the antigen extracted from milk. The total amount of EMA detected by the two assay techniques was the same. The antigenic distribution of EMA was the same in the sera of lactating women as in the sera of patients with cancer.

These results from sera were not concordant with the paper of Ceriani *et al.* [4]. In their assay of human mammary epithelial antigens (HME-Ags), they found polyclonal antibody antigens of molecular weights respectively 150,000, 70,000 and 46,000 daltons only in breast cancer patients. Under the same conditions using a monoclonal antibody, they found only one antigen with a molecular weight of 46,000 daltons. This discrepancy can be due to various factors: (1) The antigen used for immunization may be very different from ours [6]; (2) the assay method used by Ceriani *et al.* involved an extraction procedure whereas our assays were directly performed on the chromatographed fractions.

CONCLUSION

Fourteen chromatographies on three different biological fluids were performed. In the breast cyst fluids the EMA antigenic distribution was complex and seemed to be very different from one fluid to another. Numerous subfractions (nine) were found ranging from 1,300,000 to 35,000 daltons. This could be explained by the high enzymatic activity encountered in this kind of fluid.

In the milks, the EMA antigenic distribution was more constant, the main EMA peak showed a high molecular weight, 1,000,000 daltons, corresponding to the reference EMA preparation.

Lastly, in the sera, the EMA antigenic distribution was also constant. But the main peak had a molecular weight of 750,000 daltons, lower than the reference preparation.

A single monoclonal or sandwich polyclonal–monoclonal assay detected a total amount of EMA that was very similar in all the chromatographies indicating that both techniques were comparable in terms of specificity. There was apparently no difference in EMA molecular weight distribution in the sera of cancer and disease-free patients.

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