Eur J Cancer Clin Oncol, Vol. 22, No. 10, pp. 1223–1229, 1986.
Printed in Great Britain

0277-5379/86$3.00+0.00
Pergamon Journals Ltd.

Assay of Epithelial Membrane Antigen (EMA) in Human Serum by ELISA*

J.C. HENDRICK, F. GILIS and P. FRANCHIMONT
Radioimmunassay Laboratory, Institute of Pathology, University of Liege, Belgium

Abstract—*An enzyme linked immuno sorbent assay (ELISA) for an epithelial membrane antigen (EMA) was described. Possible cross reactions with various antigens were investigated. In sera, EMA has been found in all the subjects studied. In normal population, levels were in the range of* 500 ng/ml +/− 125 *(S.D.) A significant increase was observed at the end of pregnancy and during lactation. A large number of patients suffering various benign and cancerous diseases were studied. The elevated levels found in breast and pulmonary pathology indicated that this assay could be useful in the follow-up of patients suffering from these diseases.*

INTRODUCTION

RECENTLY, many papers have been published concerning a family of antigens isolated from human milk fat globule membranes [1, 2, 4, 6–10]. These antigens are glycoproteins in which carbohydrates represent the major component whereas the protein moiety consists of less than 15% of the total molecular weight [4].

By the immunoperoxidase technique, Heyderman *et al.* [1] identified a glycoprotein which was localized on the luminal and surface membranes of most non-squamous epithelia in normal human tissues and called it epithelial membrane antigen (EMA). Increased staining was observed in a variety of non-neoplastic disease states as well as in many neoplasia.

An immunohistochemical stain for EMA was of great interest in cancer investigations. It was first used in diagnostic tumour histopathology and cytology as an indicator of epithelial differentiation [8, 11, 12]. A review of the results obtained showed that it could assist carcinoma diagnosis in 22 out of 48 cases where differential diagnosis was difficult [9].

Secondly, an immunohistochemical stain for EMA could identify minute metastatic carcinoma deposits in organs such as the liver, bone marrow, and lymph nodes [10, 13, 14]. EMA positive cells were shown in bone marrow in approx. one third of patients undergoing primary breast cancer surgery [14]. Thirdly, using a monoclonal antibody (HMGFI) the complete absence of staining was associated, in breast cancer, with an extremely poor prognosis as assessed by the duration of relapse-free survival time, whereas extracellular staining was associated with a favorable prognosis. Absence of staining was independent of histological grade, tumor size, axillary lymph node invasion, and menopausal status. In contrast, positive staining was related to low histological grade [6].

Assays of EMA or antigens isolated from milk fat globule membranes were seldom applied to serum assays of normal subjects or patients suffering from cancerous and non-cancerous diseases. Furthermore, the proposed radioimmunoassays were carried out by labelling a second system. Either anti rabbit gamma-globulin antibody [3], or avidin which was bound to biotin conjugated anti-human mammary epithelial antigen antibody [5] were used.

We propose a simple assay of EMA using ELISA (Enzyme Linked Immuno Sorbent Assay). It was applied to sera of normal subjects and patients suffering from various benign and malignant diseases.

MATERIAL AND METHODS

1. *Antigen purification*

The purification method developed by Ormerod *et al.* [4] was used. In brief: half a liter of human milk was skimmed and brought to 40% saturation with (NH4)2S04. The supernatant was brought to 80% saturation with (NH4)2S04 and the precipitate was collected and dialysed against distilled water. The material was then fractionated on a

Accepted 27 March 1986.
*Supported by grant 3.4530.81 from the F.R.S.M. and by the Cancer Foundation of C.G.E.R.

column of sepharose 6B, 0.1% triton X–100, pH 8. The active fraction in the first peak was stirred with chloroform–methanol (6 vol. of chloroform–3 vol. of methanol and 1 vol. of fraction). The aqueous phase was dialysed against water. A last purification step was performed on a column of peanut lectin. The final product was eluted with 2% galactose.

2. *Antisera*

Antisera were raised in rabbits by injecting purified EMA following the immunisation schedule of Vaitukaitis *et al.* [15]. Two milligrams of EMA were mixed with complete Freund's adjuvant and injected subcutaneously. Booster injections were given at 1-month intervals in the same way.

3. *ELISA for EMA*

A method previously described for other antigens [16, 17], was used. Briefly 0.2 ml of purified EMA solution (10 μg/ml in bicarbonate pH 9.5, 0.1 M) was used to coat Nunc Immunoplate during at least 24 hr at 4° C and washed with PBS 0.05 M ph 7.5 containing 0.1% Tween 20. 150 μl of antiserum solution (1 : 10,000) were added to 50 μl containing either the reference EMA solution (from 80 to 5000 ng) or serum to be tested. Dilutions were made in the same buffer as above except that it contains 5 g BSA/1. After 48 hr incubation at 4° C, the mixture was added to the EMA coated previously washed immunoplates. After 2 hr 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 detected with 0.2 ml of ABTS (Boerhinger) solution (75 mg in 100 ml of 0.1 M phosphate citric acid buffer ph 4). Optical density at 405 nm was measured after 45 min in a Titertek multiscan colorimeter. Precision was estimated by the variation coefficient calculated on the different points of the standard curve made in triplicate. The specificity was assessed by investigating the possible cross reaction with different human mammary gland proteins in the assay: alpha-lactalbumin purified according to Schultz and Ebner [18], whole casein purified according to von Hippel and Waugh [19], gross cystic disease fluid protein (GCDFP: gift from Haagensen) [12], were used. Moreover CEA purified according to Gold *et al.* [21] and NCA purified according to Von Kleist *et al.* [22] were also tested (gift from P. Burtin).

4. *Types of sera assayed*

Normal serum. Forty-seven sera from blood donors without hepatic or renal disease were used as normal controls. Furthermore, serum was collected from 35 pregnant women at the beginning of the pregnancy (1–20 weeks) and 36 women at the end of the pregnancy (30–40 weeks). Nineteen lactating women were also investigated on the fourth day after delivery.

Benign diseases

Serum was collected in patients with benign diseases: 10 sera of women with benign mammary cyst and nine with fibroadenoma; 13 sera from benign digestive diseases: gastritis (1), ulcer (2), hepatitis (1), cirrhosis (3), pancreatitis (4) and ileitis (2); 19 benign affections of the lung were analysed including asthma (4), emphysema (3), bronchopneumonia (4), bronchitis (3), and pneumonia (4); 20 patients with cardiac diseases were studied: angina pectoris (8), valvulitis (5), hyposystolia (3), acute myocardial infarction (4).

Carcinomas

Serum from patients suffering from carcinomas were assayed for EMA: digestive cancer: gastric neoplasm (7), pancreatic adenocarcinoma (11), colon adenocarcinoma (55), hepatoma (1); lung cancer: oat cell carcinoma (28), squamous cell carcinoma (2), adenocarcinoma (5), mesothelioma (2); breast adenocarcinoma (13).

RESULTS

Figure 1 shows a typical response curve obtained with EMA ELISA. The sensitivity of the assay estimated within the 95% confidence interval at the zero point was 80 ng of EMA per ml of sample. The variation coefficient of the different points on the curve did not exceed 4%. The specificity of the assay is illustrated in Fig. 1. Casein showed minor cross reactivity less than 5% in EMA ELISA. Alpha-lactalbumin, CGDFP, CEA and NCA did not cross react at all.

In control sera, EMA was detectable in all samples and levels varied between 300 and 750 ng/ml. The mean value was 528 +/− 125 (S.D.). During the 20 first weeks of pregnancy, EMA levels were in the same range as the controls, but at the end of the pregnancy and at the 4th day of lactation a mean concentration of EMA was found significantly higher than the normal value mean 855 and 895 ng/ml, respectively 80% and 40% of the concentrations were out of the normal value distribution (Table 1). In mammary pathology, mean levels were normal in cases of women suffering from cysts or fibroadenomas, but were significantly increased in adenocarcinoma cases (mean level: 844 ng/ml +/− 87). 70% of the values (i.e. 9 cases out of 13) were superior to the mean plus 2 S.D. (Table 1). Four cases showed EMA levels within the range of 2 S.D. around the mean and all were ductal adenocarcinomas, weakly differentiated and complicated with metastasis in

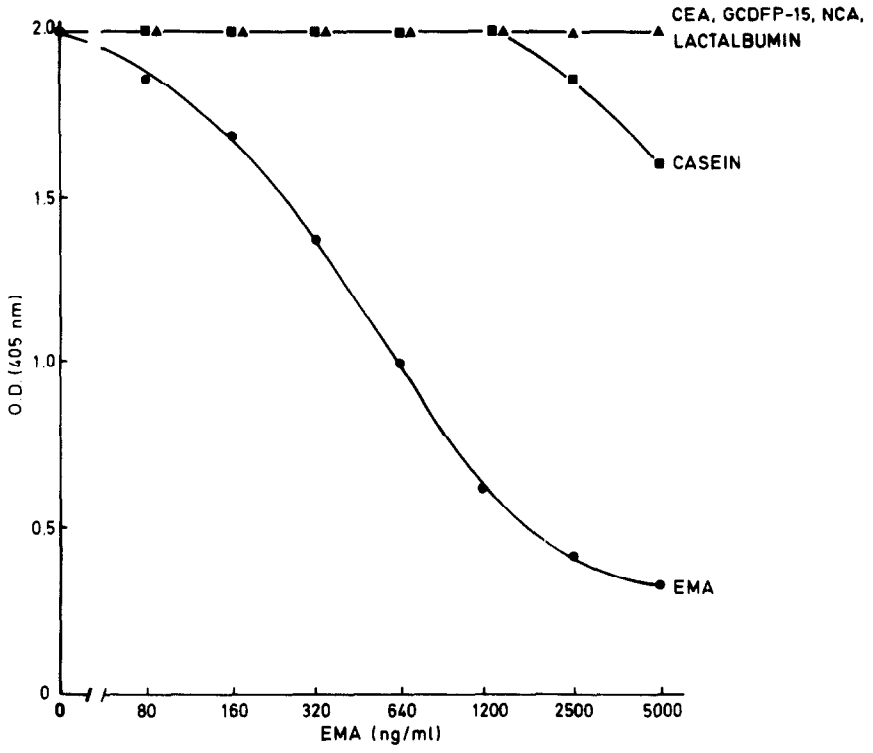

Fig. 1. EMA reference curve with cross reaction of casein, lactalbumin, CEA, NCA, and GCDFP-15.

Table 1. EMA levels in serum of normal subjects in various physiological conditions and with benign and malignant mammary diseases

	n	Mean (ng/ml)	S.D.	% values > normal mean + 2 S.D.
Normal subjects	47	528[a]	125	0
Pregnant women:				
0–20 weeks pregnancy	35	511[a]	104	0
20–40 weeks pregnancy	36	894[b]	155	80
Lactating women	10	847[b]	390	40
Benign masthopathy	19	597[a]	270	15
Adenocarcinoma	13	844[b]	315	70

a,b,c: Groups with identical letters were not significantly different whereas those with different subscripts were significantly different. (Kruskal–Wallis test.)

Table 2. Serum EMA levels in normal subjects and in patients suffering from benign diseases

	n	Mean (ng/ml)	S.D.	% values > normal mean + 2 S.D.
Normal subjects	47	528[a]	125	0
Benign pathology:				
Cardiac diseases	30	521[a]	188	0
Digestive diseases	14	662[a]	164	28
Breast diseases	19	597[a]	270	15
Lung diseases	19	843[b]	188	52

a,b,c: Groups with identical letters were not significantly different whereas those with different subscripts were significantly different. (Kruskal–Wallis test.)

Table 3. Serum EMA levels in normal subjects and in patients with digestive cancers

	n	Mean (ng/ml)	S.D.	% values > normal mean + 2 S.D.
Normal subjects	47	528[a]	125	0
Digestive carcinoma				
hepatoma	1	561[a]		0
gastric carcinoma	7	814[c]	184	57
pancreatic carcinoma	11	700[b]	181	36
colon carcinoma	55	721[b]	313	43

a,b,c: Groups with identical letters where not significantly different whereas those with different subscripts were significantly different. (Kruskal–Wallis test.)

Table 4. Serum EMA levels in normal subjects and in patients with lung cancer

	n	Mean (ng/ml)	S.D.	% values > normal mean + 2 S.D.
Normal subjects	47	528[a]	125	0
Lung cancers				
oat cell carcinoma	28	973[b]	350	50
squamous cell carcinoma	2	988[b]	347	50
adenocarcinoma	5	979[b]	379	60
mesothelioma	2	521[a]	74	0

a,b,c: Groups with identical letters where not significantly different whereas those with different subscripts were significantly different. (Kruskal–Wallis test.)

lymph nodes and in other organs.

In non-neoplastic diseases, (Table 2) the mean values did not increase, neither in digestive nor cardiac pathology. Four cases out of 14 showed elevated levels in digestive pathology. On the other hand, in pulmonary non-malignant diseases 10 patients out of 19 with bronchial disease (bronchitis or bronchopneumonia) and with acute pneumonia showed levels greater than the mean + 2 S.D. and a mean level higher than the mean level of normal subjects. In contrast, patients suffering from asthma, emphysema and pulmonary fibrosis had normal values of serum EMA.

In digestive cancers, the mean values of EMA were significantly increased for gastric, pancreatic and colon cancers. For hepatoma the level was normal (Table 3).

It is in cancerous and non-cancerous respiratory diseases that the mean and individual levels of EMA were the most elevated (Table 4 and Fig. 2). In all the kinds of lung cancer the mean values were elevated except in 2 mesotheliomas. In each group some values were within the 95% confidence interval of normal values and others above the confidence interval.

DISCUSSION

Radioimmunoassay for EMA and apparented substances isolated from human milk fat globule membranes have been rarely described and mainly used for assessing purification and characterisation of these materials. A radioimmunoassay for EMA was carried out by Ormerod *et al.* [3], it was used to demonstrate contamination of casein preparation with EMA.

Furthermore, Hilkens *et al.* [7] proposed a sandwich radioimmunoassay for an antigen (MAM–6) present in the serum of patients with carcinomas. Moreover a solid phase radioimmunoassay was proposed for a similar, though not identical family of antigens called human mammary epithelial antigens (HME–Ag) also prepared from human milk fat globule membranes (Ceriani *et al.* [5]). Using this system it was shown that breast cancer patients carried high levels of HME–Ag in their circulation whereas patients with disseminated non-breast cancers as well as normal female controls did not.

The assay we propose is a classical ELISA, giving satisfactory sensitivity and precision. The assay showed no cross reaction with alpha-lac-

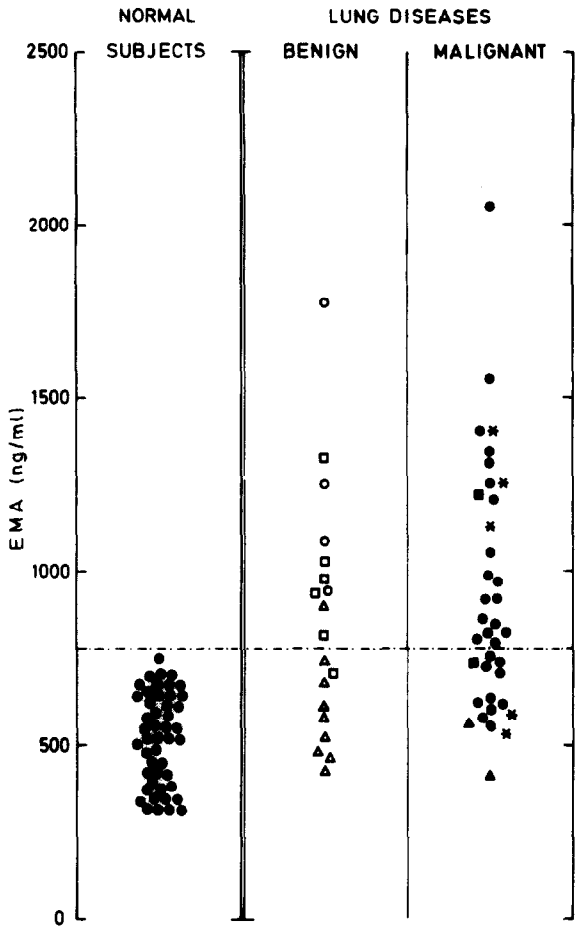

Fig. 2. EMA serum levels in normal subjects (n = 47), *benign* (n = 19) *and malignant* (n = 37) *lung diseases.* ○, *pneumonia*; △, *major fibrosis and sclerosis of the lung*; □, *bronchial inflamation*; ●, *oat cells carcinoma*; ■, *squamous cell carcinoma*; *, *adenocarcinoma*; ▲, *mesothelioma.*

talbumin, GCDFP, NCA and CEA. A weak cross reaction with whole casein was observed and seemed to be related to the contamination of the casein preparation with EMA as already demonstrated by Ormerod *et al.* [3]. With this assay EMA was found in all serum tested and several sera contained concentrations of EMA largely superior to the 95% confidence limit of the normal value (i.e. mean + 2 S.D.).

The identical mean level of EMA in normal subjects (majority of males: 66%) and pregnant women at the early stage of pregnancy indicated that sex did not influence very much levels of this protein. The elevated values found in pregnant women at the end of pregnancy and during lactation were to be connected to the activity of mammary gland.

The numbers of studied cases in mammary pathology was obviously low but suggested an elevation of EMA levels in cancerous patients. Normal values in breast cancer were found in four advanced cancers with axillary lymph node invasion and metastasis at distance. This fact could be related to the observations by Wilkinson *et al.* [6] who demonstrated the absence of staining for HMGF1 in breast cancers with an extremely poor prognosis.

The high levels of EMA in malignant and benign lung diseases were of interest. These data could be related to the immunohistological staining of alveolar and bronchial epithelial structures found by Sloane and Ormerod [8].

The anaplastic lung cancers were positive in more than 50% of all cases; this is in accordance with the immunohistological data from Sloane *et al.* [8, 9]. Thus it seems to us that EMA assay could prove to be of interest in pulmonary pathology where reliable markers are lacking. Work is now in progress to confirm this point of view.

But up to now we do not know if the EMA assay could be useful in clinical follow-up procedures, and no correlation has been made between EMA levels and local or metastatic cancer extention. ELISA for EMA should be used to screen larger numbers of samples and should be correlated with other markers useful in breast cancer for diagnosis and follow-up: CEA, NCA, etc.

This EMA assay could be complementary to the interesting findings by Sloane *et al.* [8, 9], where immunohistological staining allowed an evaluation

of tumoral epithelial differentiation, detection of microscopic metastatic extention [14], and prognosis assessment [6].

CONCLUSION

An ELISA for EMA was carried out. Its sensitivity was 80 ng/ml of serum. The coefficient of variation for each point of the curve was less than 4% and, in terms of specificity no cross reaction with CEA, NCA, alpha-lactalbumin, and GCDFP were shown. Patients suffering from various non-neoplastic diseases showed mean levels which were not significantly different from normal subjects (528 +/− 125 ng/ml), except for benign respiratory diseases. Patients with secretory pulmonary diseases with malignant and non-neoplastic origins displayed elevated levels of EMA. Although clinical specificity was weak, the high levels found in lung cancers, could reveal EMA as an interesting marker for assessing the follow-up of these cancers, but further investigations are necessary.

The high levels of EMA in neoplastic breast diseases and normal levels found in non-neoplastic disease could better mammary pathology diagnosis.

In digestive cancers, clinical sensitivity and specificity did not allow us to consider EMA as a valid marker. Nevertheless, high levels could be useful in clinical follow-up.

REFERENCES

1. Heyderman E, Steele K, Ormerod MG. New antigen on the epithelial membrane: its immunoperoxidase localisation in normal and neoplastic tissues. *J Clin Pathol* 1979, **32,** 35–39.
2. Ormerod MG, Monaghan P, Easty GC. Asymetrical distribution of epithelial membrane antigen on the plasma membranes of human breast cell lines in culture. *Diagn Histopathol* 1981, **4,** 89–93.
3. Ormerod MG, Bussolati G, Sloane JP, Steele K, Gulioti P. Similarities of antisera to casein and epithelial membrane antigen. *Virchows Arch [Pathol Anat]* 1982, **397,** 327–33.
4. Ormerod MG, Steele K, Westwood JH, Mazzini MN. Epithelial membrane antigen: Partial purification, assay and properties. *Br J Cancer* 1983, **48,** 533–541.
5. Ceriani RL, Sasaki M, Susmann H, Wara WM, Blank EW. Circulating human mammary epithelial antigens in breast cancer. *Proc Natl Acad Sci USA* 1982, **79,** 5420–5424.
6. Wilkinson MJS, Howell A, Harris M, Taylor-Papadimitriou J, Swindell R. The prognostic significance of two epithelial membrane antigens expressed by human mammary carcinomas. *Int J Cancer* **33,** 299–304.
7. Hilkens J, Kroezen V, Bonfrer H, Bruning P, Hilgers J, van Eukeren M. A sandwich radioimmunoassay for an antigen (MAM–6) present in the serum of patients with carcinomas. In: Protides of Biological Fluids, Proc. 32nd Colloq (1984), 651–653.
8. Sloane JP, Ormerod MG. Distribution of epithelial membrane antigen in normal and neoplastic tissues and its value in diagnostic tumor pathology. *Cancer* 1981, **47,** 1786–1795.
9. Sloane JP, Hughes F, Ormerod MG. An assessment of the value of epithelial membrane antigen and other epithelial markers in solving diagnostic problems in tumour histopathology. *Histochem J* 1983, **15,** 645–654.
10. Sloane JP, Ormerod MG, Imrie SF, Coombes RC. The use of antisera to epithelial membrane antigen in detecting micrometastases in histological sections. *Br J Cancer* 1980, **42,** 392–398.
11. To A, Coleman DV, Dearnaley JP, Ormerod MG, Steel K, Neville AM. Use of antisera to epithelial membrane antigen for the cytodiagnosis of malignancy in serous effusions. *J Clin Pathol* 1981, **34,** 1326–1332.
12. To A, Dearnaley DP, Ormerod MG, Centi G, Coleman DV. Epithelial membrane antigen: its use in the cytodiagnosis of malignancy in serous effusions. *Am J Clin Pathol* 1982, **78,** 214–219.
13. Dearnaley DP, Sloane JP, Ormerod MJ, Steele K, Coombes RC, Clink Mc DH, Powles TJ, Ford HT, Gazet JC, Neville AM. Increased detection of mammary carcinoma cells in marrow smears using antisera to epithelial membrane antigen. *Br J Cancer* 1981, **44,** 85–90.
14. Dearnaley DP, Ormerod MG, Sloane JP, Lumley H, Imries S, Jones M, Coombes RC, Neville AM. Detection of isolated mammary carcinoma cells in marrow of patients with primary breast cancer. *J R Soc Med* 1983, **76,** 359–364.
15. Vaitukaitis J, Robbins JB, Nieschag E, Ross GT. A method for producing specific antisera with small doses of immunogen. *J Clin Endocrinol* 1971, **33,** 988–991.
16. Renard SI, Berg R, Martin GR, Foidart JM, Gehron Robey P. Enzyme linked immunoassay (ELISA) for connective tissue components. *Anal Biochem* 1980, **104,** 205–209.
17. Gysen Ph, Hendrick JC, Franchimont P. Dosage enzymoimmunologique des protéoglycanes carthilagineux humains et de leurs anticorps. *C Rend Soc Biol* 1983, **177,** 243–251.

18. Schultz GS, Ebner KE. Alpha-lactalbumin levels in human mammary tumors, sera and mammary cell culture lines. *Cancer Res* 1977, **37,** 4489–4494.
19. von Hippel PH, Waugh DF. Casein: monomers and polymers. *J Am Soc* 1955, **77,** 4311–4316.
20. Hagensen DE, Mazoujian G, Dilley WG, Pedersen CE, Kester SJ, Wells SA Jr. Breast gros cystic disease fluid analysis. 1. Isolation and radioimmunoassay for a major component protein. *J Natl Cancer Inst* 1979, **62,** 239–244.
21. Gold P, Friedman SO. Specific carcinoembryonic antigen of the human digestive system. *J Exp Med* 1965, **122,** 467–481.
22. Von Kleist S, Chavanel G, Burtin P. Identification of an antigen from normal human tissue that cross-reacts with the carcinoembrionic antigen. *Proc Nat Acad Sci USA* 1972, **69,** 2492–2494.