Virchows Arch [Pathol Anat] (1982) 397:327–333

Virchows Archiv A

Similarities of Antisera to Casein and Epithelial Membrane Antigen

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Summary. Antisera raised against human milk fat globule membranes and against the casein fraction of human milk have been compared. Using an immunohistochemical stain of tissue sections it has been shown that many of the antigenic determinants detected by the different antisera are identical. A radioimmunoassay for epithelial membrane antigen (EMA) showed that casein preparations are associated with small quantities of EMA. Antisera to casein frequently contained appreciable concentrations of antibodies to EMA and this accounts for the immunohistochemical staining of non-mammary tissues.

Key words: Immunohistochemistry – Casein – Epithelial membrane antigen

Introduction

Using immunohistochemical methods, it has been shown that antisera raised against human casein react with a variety of epithelial tissues (Bussolati et al. 1975; Bussolati and Pich 1975; Pich et al. 1976). This casein-like material has been located on the luminal surfaces of mammary and pancreatic ductules, sweat glands, endometrial glands in the proliferative phase, distal and collecting tubules of the kidney, in the bronchial epithelium and glands at the surface of some alveoli of the lung and in the sebaceous glands of the skin (Pich et al. 1976). More recently an antigen (named epithelial membrane antigen, EMA) with a similar tissue distribution has been defined using an antiserum raised against defatted human cream (Heyderman et al. 1979; Sloane et al. 1980; Sloane and Ormerod 1981). Immunohistochemical stain using the antiserum has been used to detect micrometastases of mammary carcinoma in aspirates of bone marrow (Sloane et al. 1980; Dearnaley et al. 1981), to help distinguish anaplastic carcinoma from non-epithelial

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0340-1227/82/0397/0327/$01.40

tumours (Sloane and Ormerod 1981) and to assist in the cytological diagnosis of serous effusions (Coleman et al. 1981).

The more recent studies had overlooked the work on casein. When the remarkable similarity of the tissue distribution of the two antigens was realised, antisera and antigens were exchanged between the two groups of workers. Experiments in both centres have now confirmed that many of the antigenic determinants detected by the different antisera are identical. Further chemical studies suggest that preparations of human casein may carry small amounts of EMA as an impurity. The EMA is probably more immunogenic than casein so that immunisation with casein may yield sera which react with EMA on tissue sections.

Materials and Methods

Antisera

The anti-casein serum was raised in rabbits against total human casein prepared as described below. Identical patterns of staining were obtained using antisera raised against β-casein including one antiserum kindly supplied by Dr. Monaco (Monaco et al. 1977).

The anti-EMA serum was prepared by injecting rabbits with defatted human cream (Ceriani et al. 1977). The antiserum was absorbed to remove unwanted antibodies according to the published schedule (Sloane and Oermerod 1981).

Antigens

One sample of human casein (Sample A) was obtained by precipitation (twice) at pH 4.5 from skimmed milk followed by affinity chromatography on thiol-Sepharose. A further sample (B) of β-casein was prepared on DEAE-cellulose. Other samples of casein, used for preparing antisera, were prepared by ultra-centrifugation.

Epithelial membrane antigen was prepared by the following schedule. Skim milk was brought to 40% saturation in ammonium sulphate, the precipitated proteins (including casein) were removed by centrifugation and the proteins in the supernatant precipitated by increasing the ammonium sulphate concentration to 80% saturation. The precipitate was dissolved in Tris-buffer, pH 8.6, 0.1% Triton X and applied to a column of Sepharose 6B. The EMA activity was eluted in the void volume. The appropriate fractions were concentrated, dialysed against 6M guanidine HCl and applied to a column of Sepharose CL-6B. Elution with 6M guanidine yielded the EMA activity in a peak close to the void volume. After treatment with 1 μg/ml trypsin at 37° for 18 h, the material was applied to a column of wheat germ-Sepharose 6MB (Pharmacia Fine Chemicals, Uppsala, Sweden). EMA was removed in 10% N-acetyl glucosamine, dialysed and applied to a column of peanut lectin from which it was eluted with 2% galactose. Finally the solution was extracted with 9 volumes of a chloroform/methanol mixture (2:1, v/v). EMA was found in the aqueous phase which was dialysed and lyophilised.

This preparation of EMA contained approximately 2% protein, the bulk of the material being carbohydrate.

Immunohistochemical Staining

Sections of formalin-fixed paraffin embedded material were stained immunohistochemically for either casein or EMA using the methods described previously (Pich et al. 1976; Heyderman et al. 1979).

Radioimmunoassay for EMA

A crude preparation of EMA was made by ammonium sulphate fractionation of skim milk, chromatography on Sepharose 6B in the presence of 0.1% Triton X (see above) followed

by fractionation on Sephadex G200. This was diluted to a protein concentration of 2 µg/ml in 0.05M bicarbonate, pH 9.7 and used to coat U-shaped Removawells (Dynatech Ltd. Billingshurst, GB).

25 µl of anti-serum diluted 1 in 200 was added to 100 µl of solution to be tested. After incubation at room temperature overnight, 50 µl was added to an EMA-coated Removawell. After 1 h, the wells were washed, and incubated with ^{125}-I-labelled sheep anti-rabbit Ig γ globulins for an hour. After further washes, the bound activity was estimated in a λ-scintillation counter.

Polyacrylamide Gel Electrophoreses

This used a modification of the method of Lammeli (1970). $7^1/_2$% polyacrylamide running gels with $3^3/_4$% polyacrylamide stacking gels were cast in 5 mm glass tubes to a total length of 65 mm. Sodium dodecyl sulphate (SDS) was omitted from the gels and lower buffer reservoir but was included in the upper reservoir at a concentration of 0.03%. The samples were dissolved in 1% SDS, 1% mercaptoethanol. After electrophoresis the gels were cut into 1 mm slices. The slices were placed individually in a well of micro-titer plate and 100 µls of antiserum at a dilution of 1 in 1,000 was added. After 16 h incubation at room temperature, 50 µls of the solution were removed from each well and added to a ›Removawell‹ coated with EMA and the procedure for the radioimmunoassay followed as described above. Gels run in parallel were stained for protein using Coomassie Brilliant Blue.

Results

Two antisera, anti-casein prepared in Turin and anti-EMA prepared in Sutton, were compared on sections of resting breast, lactating breast, skin (containing sebaceous and both eccrine and apocrine sweat glands), kidney and a mammary carcinoma. The stain from the anti-casein serum was weaker but, with the exception of lactating breast, the major features of

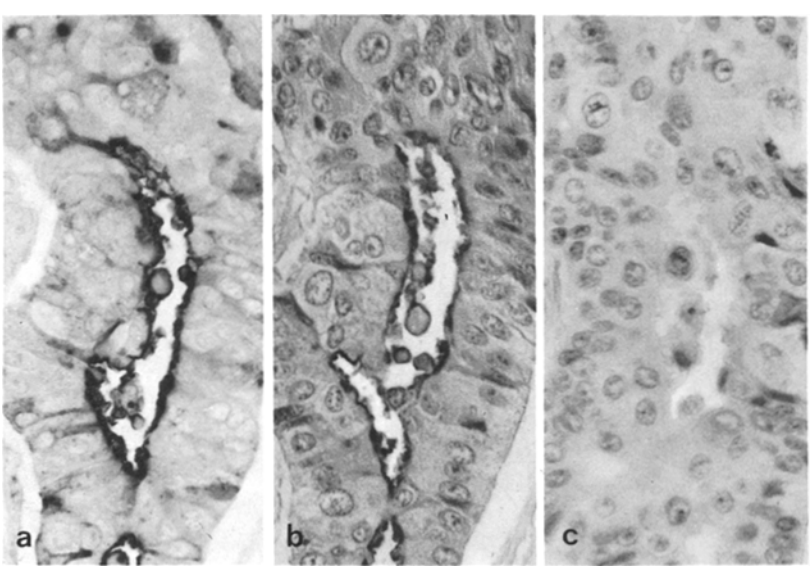

Fig. 1 a–c. Sections of infiltrating ductal carcinoma of breast stained with an immunoperoxidase stain with: (**a**) anti-EMA serum; (**b**) anti-casein serum; (**c**) anti-casein casein serum absorbed with purified EMA. Magnification: ×315. Counterstain: Mayer's Haemalum

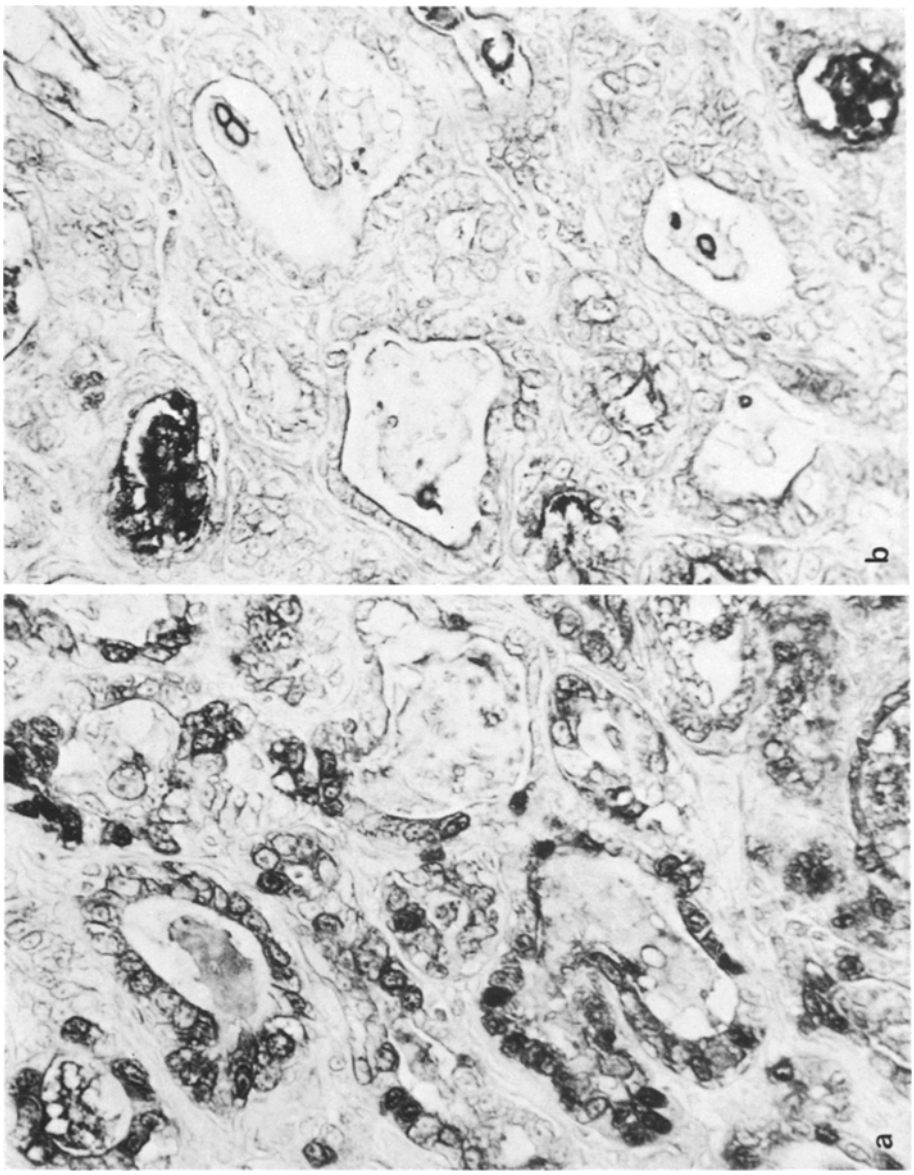

Fig. 2a, b. Sections of lactating breast stained with an immunoperoxidase stain with: **(a)** anti-casein serum; **(b)** anti-EMA serum Magnification: ×250. No counterstain. In **(b)** the EMA positive material in acini corresponds to the milk fat globules

the distribution of staining were identical. In the resting breast the luminal membranes of the ducts were positive; in the skin, the epidermis was negative, the sebaceous glands strongly positive and the sweat glands positive; in the kidney, the luminal membranes of the distal and collecting tubules stained while those of the proximal tubules were negative. The luminal membranes of acini formed in an infiltrating ductal carcinoma of the breast stained. This stain was abolished if either antiserum was absorbed with

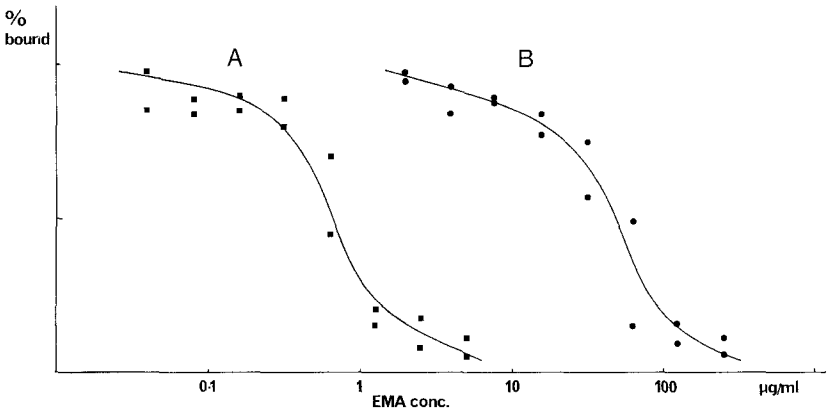

Fig. 3A, B. Radioimmunoassay for EMA. Inhibition curves for (**A**) purified EMA (50% inhibition at 0.65 µg/ml) and (**B**) casein, preparation B (50% inhibition at 50 µg/ml)

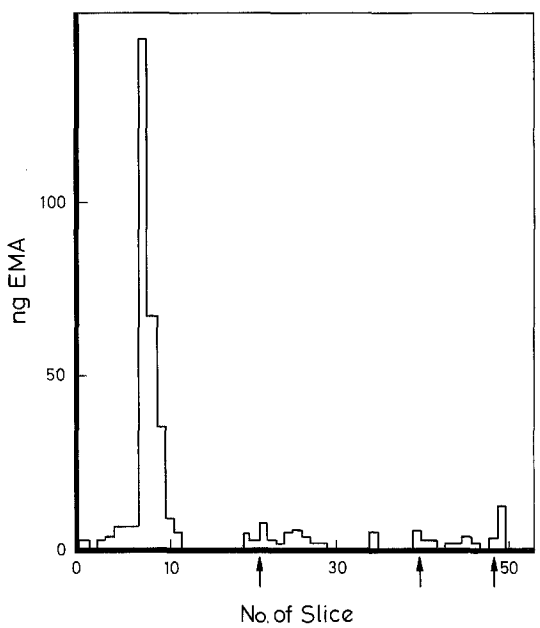

Fig. 4. SDS-polyacrylamide gel electrophoresis of casein, preparation A. The gel slices were assayed for EMA activity. The arrows mark the position of the major protein bands as revealed by Coomassie Billiant Blue staining of a similar gel run in parallel

purified EMA or a sufficient quantity of casein (Fig. 1). On lactating breast, the anti-EMA serum stained only the luminal membranes and the surface of the fat globules, not the cell cytoplasm. In contrast, the anti-casein sera additionally stained the cell cytoplasm. The cytoplasmic stain was not removed by absorption with milk fat globule membranes (Fig. 2).

Since the removal of immunohistochemical staining activity is essentially unquantitative, the reaction of casein with an anti-EMA serum was measured in a radioimmunoassay for EMA. The two samples of casein tested both reacted in the assay and the inhibition curves were approximately parallel to those for a preparation of EMA; 1 mg of preparation A of

human casein contained the equivalent of 40 μg of EMA while the purer preparation, B, contained 13 μg EMA per mg (the curves for preparation B are shown in Fig. 3).

Casein sample A was applied to a SDS-polyacrylamide gel. After electrophoresis, the gel was sliced and the EMA activity assayed by radioimmunoassay. Another gel was run in parallel and stained for protein. The EMA activity was concentrated at the top of the gel while the major casein proteins migrated further, well clear of the EMA (see Fig. 4).

Discussion

The immunohistochemical results clearly demonstrate that EMA studied in Sutton and the 'casein-like' antigen in non-mammary tissues described previously by workers in Turin are identical.

The preparation of EMA used in this study contained about 2% protein and it has been shown that the antigenic determinant resides in the carbohydrate residues (N. Mazzini and J.H. Westwood, work in progress). It seems improbable, though it is not impossible, that the results can be attributed to contamination with casein. Alternative explanations are either that casein and EMA carry similar antigenic determinants or that the casein preparations are associated with small amounts of EMA. The latter explanation is the most likely since the EMA activity was separated from the casein proteins on an SDS-polyacrylamide gel.

In our experience casein is a poor antigen in rabbits and a long course of immunisation is needed to obtain antisera to casein. It is hardly surprising if antibodies are also produced to a minor contaminant. This would not affect a radioimmunoassay for casein since reaction with the major labelled protein (casein) is all that will be detected. Application of an antiserum to a tissue section does not have the advantage of this selectivity; staining by all antibodies present will be observed. This is why it can be more difficult to establish the specificity of an immunohistochemical stain.

In addition to anti-EMA activity, the anti-casein sera also contained antibodies to casein. This was demonstrated by the cytoplasmic stain of epithelial cells in the lactating breast.

Acknowledgements. We thank Dr. G.F. Voglino who prepared some of the samples of casein and Mrs. S.F. Imrie for her technical assistance. G. Bussolati was supported by C.N.R. (Rome) (Grant No. 81.01310,96) and M.G. Ormerod and K. Steele by the Medical Research Council Great Britain.

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Accepted August 17, 1982