Immunological and radioimmunological studies of membrane antigen(s) from human breast carcinomas and non-tumoral breast tissues. II.

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Summary. The authors found that breast carcinomas contain 2 groups of antigens not detectable in normal tissue. The groups were extracted and partially purified. One responded to absorbed anti-CEA and the other only to an antibody, produced by immunizing rabbits with human breast carcinoma extract which were unabsorbed and absorbed with control breast tissue.

Introduction. Our previous paper[3] described the extraction of primary breast carcinomas (MK) and non-tumoral breast tissue (M) with 3 M KCl, the subsequent gel filtration of the crude extracts on a Sephadex G-200 column, and reported the detection of antigenic determinants for anti-CEA in the crude MK extract. This immune response was found in the descending portion (FMK) of the first peak obtained by gel filtration of the crude extract.
Rabbits were immunized with the entire first peak of MK (PMK) and with the fractions of MK showing the highest immunological activity with anti-CEA (FMK). As a control, an equal number of animals was immunized with the corresponding fractions (FM) and the first peak (PM) of M. The present paper describes attempts to further characterize the antigenic fractions with regard to their immunological activity with absorbed anti-CEA (goat 23)[4,5], and to 2 of the antibodies mentioned above (anti-PMK, anti-PM).
Material and methods. The crude extracts of MK and M were chromatographed on a DEAE-cellulose column. 200 mg of crude extract were dissolved in 40 ml of 0.005 M sodium-phosphate buffer, pH 7.5. The solution was applied to a 2.4 × 30 cm DEAE-cellulose (23 S. S. Serva) chromatography column that had been equilibrated with the 0.005 M phosphate buffer, pH 7.5. The column was eluted stepwise with a series of phosphate buffers at pH 7.5 (0.005 M, 0.025 M, 0.05 M, 0.1 M and 0.5 M). 10-ml fractions were collected at a flow rate of 20 ml/h. The absorbancy of each fraction was read at 280 nm. The fractions within the same peaks were combined and dialyzed against PBS (0.04 M sodium-phosphate + 0.1 M NaCl), pH 7.2. The protein content was checked by determining the quantity of ammonia nitrogen. The protein concentration of each peak was adjusted to 750 µg/ml and the immunological capacity of all the peaks was checked with ^{125}I-CEA and anti-CEA. The peak showing the highest immunological activity with anti-CEA and anti-PMK, previously absorbed with PM (300 γ/ml)[4], was concentrated and purified by gel filtration on a 1.5 × 70 cm Sephadex G-200 column. A 1.5-ml-portion of the

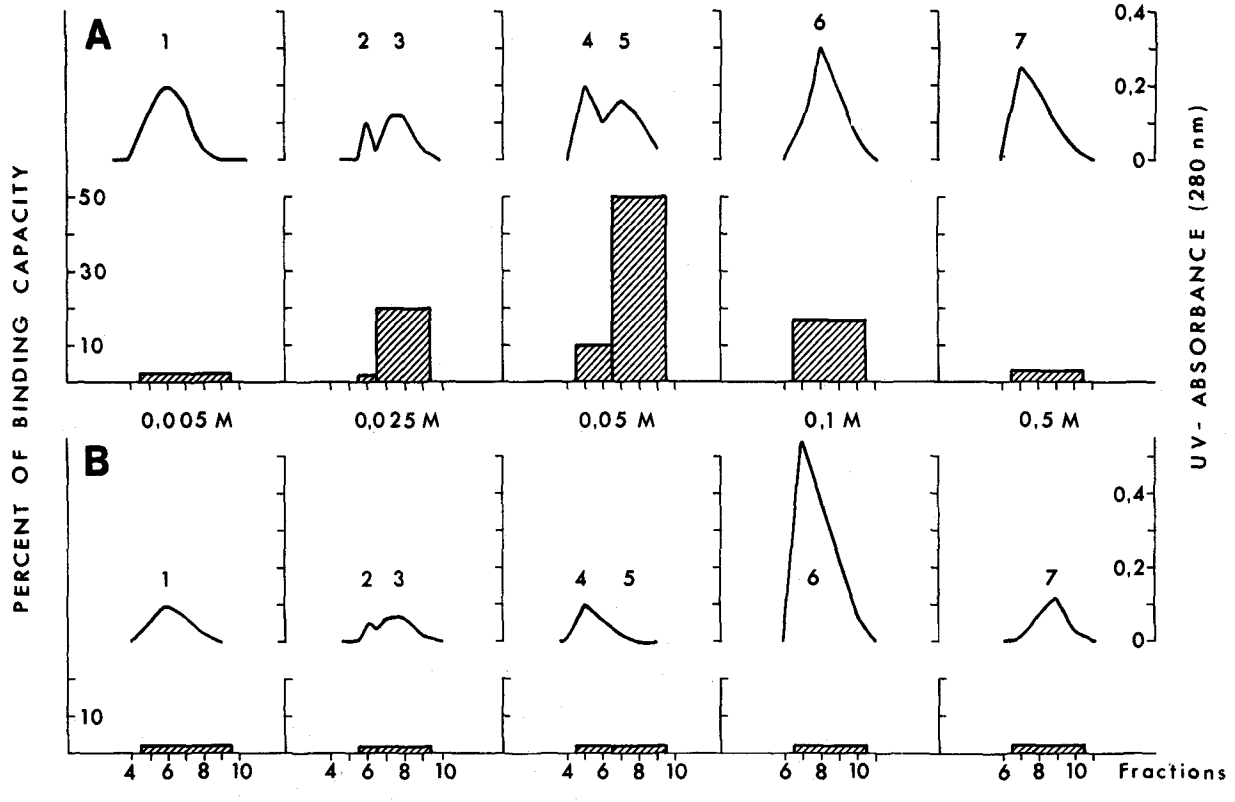

Fig. 1. Binding capacity of anti-CEA with the various peaks obtained by chromatography of the crude MK (a) and M (b) extracts on DEAE cellulose. The UV readings at 280 nm for the fractions eluted stepwise with a series of buffer solutions of increasing molarity are given at the top of each graph.

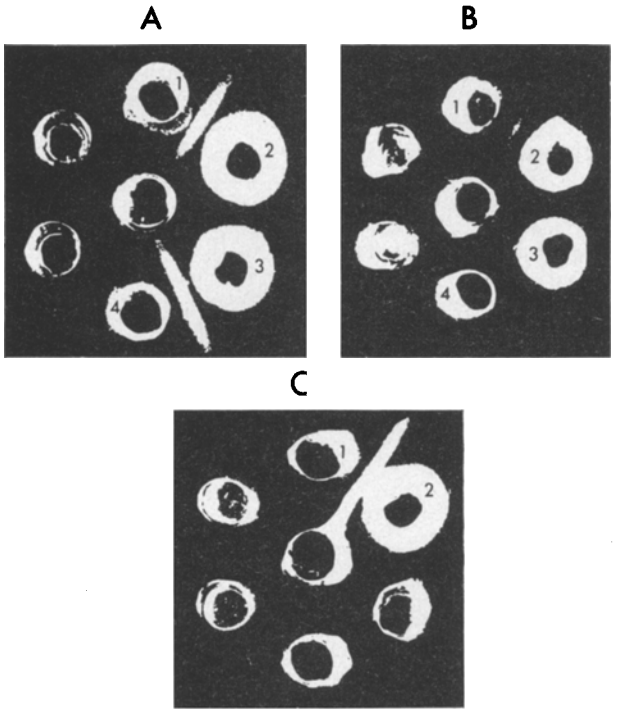

Fig. 2. Ouchterlony double diffusion test in agar. *a* (1): Peak 5 of MK obtained by chromatography on DEAE-cellulose. (2, 3): Unabsorbed anti-PMK. (4): Peak 5 of M obtained by chromatography on DEAE. *b* (1): Peak 5 of MK obtained by chromatography on DEAE. (2, 3): Anti-PMK absorbed with PM. (4): Peak 5 of M obtained by chromatography on DEAE. *c* (1): Fraction 18 obtained by gel filtration of peak 5 of MK. (2): Unabsorbed anti-PMK. – See text.

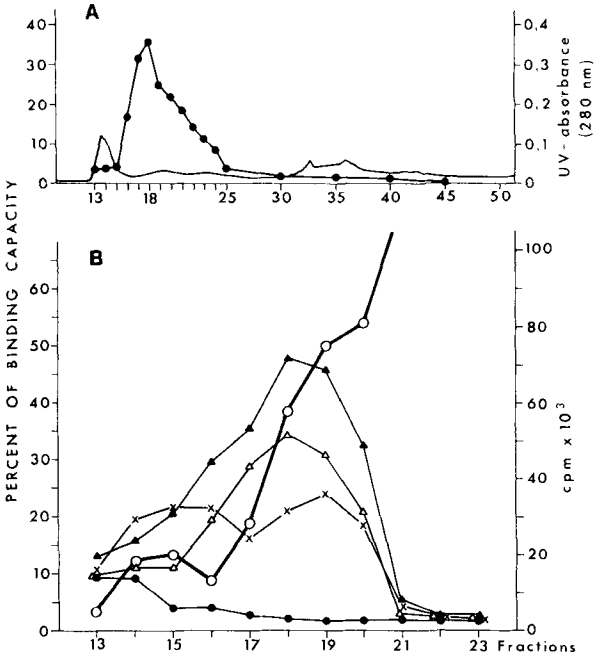

Fig. 3a. — Elution profile of fraction 5 of MK on Sephadex G-200. ●—● Binding capacity with anti-CEA.

Fig. 3b. ○—○ Elution profile of labeled fraction 18 on Sephadex G-200 (radioactivity in 50 µl of each fraction). Percentage binding capacity with various antibodies: ●—● anti-CEA; ▲—▲ unabsorbed anti-PMK; △—△ absorbed anti-PMK; ×—× anti-PM.

concentrate was applied to the column and eluted with PBS, pH 7.2; 4-ml-fractions were collected at a flow rate of 4 ml/cm²/h. The absorbancy was determined as described previously. The immunological capacity of each fraction was checked by R.I.A. with ^{125}I-CEA and anti-CEA and by double diffusion against unabsorbed and absorbed anti-PMK.

The fraction showing the highest activity with anti-CEA was concentrated to 1 mg/ml and 10 µl were labeled with Na^{125}I according to the modified method of Hunter and Greenwood[6]. The labeled material was chromatographed by gel filtration on a 1 × 100 cm Sephadex G-200 column with PBS, pH 7.2.

Fractions of 1.5 ml were collected at a flow rate of 8 ml/cm²/h. The radioactivity of 50-µl-portions of each fraction was counted with a gamma counter (Auto gamma Packard). 0.1-ml-portions of each fraction, diluted to 12,000 cpm/0.1 ml, were incubated at 37 °C for 24 h with anti-CEA, unabsorbed anti-PMK, absorbed anti-PMK and anti-PM. At the end of the first incubation, the immune complexes were precipitated using a double antibody method[6]. Unabsorbed and absorbed anti-PMK and anti-PM were checked with ^{125}I-CEA using a R.I.A.

Results. At 280 nm, the crude extracts of MK and M chromatographed on a DEAE-cellulose column showed a variety of peaks which have been numbered progressively. After elution, fractions 7, 8 and 9 of the crude MK extract are contained in peak 5 (0.05 M phosphate buffer) (figures 1a and 1b). The corresponding fractions of M did not produce this peak.

The R.I.A. showed that the peaks of MK, eluted with the 0.025, 0.05 and 0.1 M sodium-phosphate buffers contained components that crossreacted with anti-CEA (goat 23) (figure 1a). On the other hand, no reaction was observed between this antibody and any of the eluted fractions of M (figure 1b). In the double diffusion test, all of the peaks obtained by elution of MK and M from the DEAE-cellulose column produced a precipitation line with unabsorbed anti-PMK antibody (figure 2a). After absorption, however, only peak 5 of MK produced a visible, although weak, precipitation line, whereas the corresponding fractions of M produced no line with the absorbed antibody (figure 2b). No precipitation line was observed between anti-CEA and peak 5 and all the above-mentioned peaks of M and of MK, too.

Peak 5 was then concentrated and purified by gel filtration on a Sephadex G-200 column; the elution profile is illustrated in figure 3a. Fraction 18 showed the highest anti-CEA activity in the R.I.A. (figure 3a) and produced an intense line of precipitation with unabsorbed anti-PMK in the double diffusion test (figure 2c). No line was visible with absorbed anti-PMK.

Fraction 18 was labeled with Na ^{125}I and the labeled proteins were separated from free iodine by gel filtration on Sephadex G-200 (figure 3b). The fractions obtained were assayed against the various antisera. The binding capacity for unabsorbed and absorbed anti-PMK was highest with

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2 II Cattedra di Radiologia dell'Università di Milano.

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fraction ^{125}I-18 (48% for the unabsorbed and 35% for the absorbed antiserum). That for anti-PM was high with fractions ^{125}I-15 and ^{125}I-16 (21%) and fraction ^{125}I-19 (23%), and lower (15%) with fraction ^{125}I-18.

The binding capacity for anti-CEA was 9% with fractions ^{125}I-13 and ^{125}I-14 and nil with fractions ^{125}I-18 (figure 3b). No binding capacity was detected when unabsorbed and absorbed anti-PMK and anti-PM were assayed with ^{125}I-CEA.

Conclusion. In the double diffusion test, peak 5 obtained by chromatography of MK on DEAE-cellulose reacts with absorbed anti-PMK midway between the 2 wells. The failure of peak 5 to produce precipitation lines with anti-CEA and, after further purification, with absorbed anti-PMK can be attributed to the poorer sensitivity of this method compared to radioimmunology.

The results of the double diffusion test thus indicate that the precipitation line between peak 5 and absorbed anti-PMK is due to antigen or antigens associated with breast tumors that do not crossreact with anti-CEA in this method, in spite of the considerable crossreactivity observed in the R.I.A.

Fraction 18 obtained by gel filtration of peak 5 of MK was chosen because of its high response to anti-CEA in the radioimmunological study. Fraction 18 was labeled and by gel filtration yielded fractions (^{125}I-13 and ^{125}I-14) that crossreacted with anti-CEA and also reacted with anti-PMK.

Antigen(s) in these fractions do not, however, appear to be CEA, since anti-PMK, which binds ^{125}I-13 and ^{125}I-14 to a fair extent (13% binding capacity), does not bind ^{125}I-CEA, a very pure material. On the other hand, fraction ^{125}I-18 reacts only with anti-PMK. It is therefore probable that fractions ^{125}I-13 and ^{125}I-14 contain antigens associated with breast carcinoma that crossreact with anti-CEA. It can instead be postulated that fraction ^{125}I-18 contains antigens associated with breast carcinoma that do not crossreact with anti-CEA and react only with an antibody produced using antigens extracted from this type of tumor.

Immuno-electronmicroscopic study of human EA rosettes

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Summary. The immuno-electronmicroscopic study of human EA rosettes, using ferritin-labelled anti-human IgG antiserum, showed clusters of ferritin granules at the points of contact between erythrocytes and lymphoid cells, indicating that the links between Fc fragment of IgG on the surface of erythrocytes and specific receptor on the surface of lymphoid cell correspond to the sites of morphological interaction between the 2 cell types.

In previous studies, we described the ultrastructural appearance of the interaction between rosetting lymphoid cells and erythrocytes in different types of rosettes. The highest degree of interaction, consisting of interdigitations between the 2 cell types, was observed in EA rosettes[2].

In this article, we report the results of an immuno-electronmicroscopic study of human EA rosettes using ferritin-labelled anti-human IgG antibody.

Materials and methods. Group 0 Rh positive human normal erythrocytes sensitised with a human anti-D antiserum and peripheral blood normal human lymphocytes were used for the preparation of human EA rosettes according to techniques already described for EA ox rosettes[2]. After preparation of rosettes, the cell mixture was incubated for 15 min at room temperature with a ferritin-labelled anti-human IgG antibody, washed 3 times with buffered saline and then processed for electronmicroscopy. For control, the same erythrocytes, either untreated or sensitised with the anti-D antiserum, were incubated with ferritin alone or with ferritin-labelled anti-mouse IgG antibody.

Results. Rosetting lymphocytes, in several experiments, averaged 12%. EA rosettes, observed in the elec-

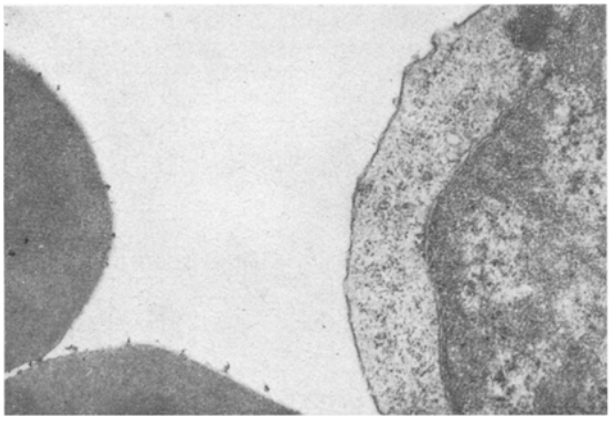

Fig. 1. Parts of 2 erythrocytes and of a lymphoid cell which are not part of a EA rosette. Clusters of ferritin granules, arranged at fairly regular intervals, are present at the erythrocytes surface. No ferritin granules are visible on the surface of the lymphoid cell. × 29000.

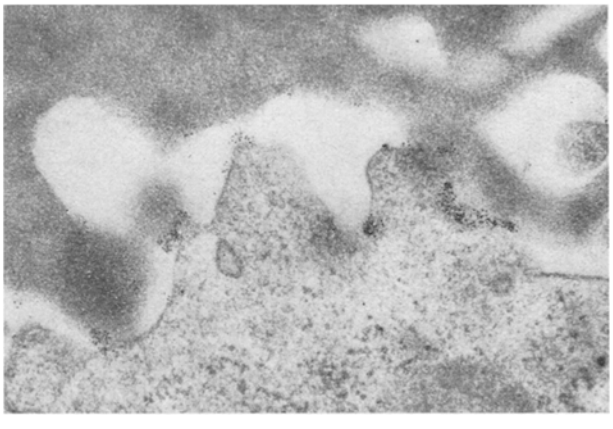

Fig. 2. Part of EA rosette. Interdigitations between an erythrocyte and a lymphoid cell are visible. The points of contact between the 2 cell types are heavily labelled with ferritin. × 46000.