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DETECTION IN THE BLOOD SERUM OF BREAST CANCER PATIENTS OF CIRCULATING IMMUNE COMPLEXES CONTAINING ANTIGENS SHOWING COMMON EPITOPES WITH STRUCTURAL PROTEINS OF MOUSE MAMMARY TUMOUR VIRUS (MMTV)

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Summary. — In the blood serum of a proportion of breast cancer patients (29.4%) immune complexes specifically reacting with the immunoglobulins of the serum against MMTV structural proteins were detected using indirect enzyme-linked immunosorbent assay (ELISA). The presence of such complexes could decrease the titre of antibodies reactive with MMTV in the blood serum of breast cancer patients.

Key words: human breast cancer; mouse mammary tumour virus; immune complexes

Introduction

Convincing evidence has been gained for the presence in the human breast carcinomas of antigens immunologically related to structural proteins of mouse mammary tumour virus (MMTV) (Meza-Tejada and Spiegelman, 1983; Segev *et al.*, 1985; Hehlman *et al.*, 1984a; Kryukova *et al.*, 1981; Remennik *et al.*, 1985). In the blood serum of breast cancer patients antibodies occur that react with MMTV proteins, products of genes *env* and *gag* (Zotter *et al.*, 1983; Tomana *et al.*, 1981; Day *et al.*, 1981; Litvinov *et al.*, 1984). It has been shown elsewhere that in breast cancer patients the antibodies reactive with MMTV occur less frequently at advanced stages of disease (Litvinov *et al.*, 1984) than at early (I, IIa) clinical stages. This phenomenon could depend, in particular, on the decrease of antibody titre resulting from binding with an antigen produced by the tumour, a way how the titre of antibodies to MMTV is decreased in mice of highly oncogenic strains with virus-induced tumours (Pascal *et al.*, 1975). In this study blood sera of breast cancer patients have been tested for immune complexes reactive with antibodies against MMTV proteins.

Materials and Methods

Viruses. MMTV of mouse strains RIII, M7, SSV and MPMV were from National Cancer Institute (Bethesda, U.S.A.). Sera against retroviruses SSV, RaLV and Balb/2 were supplied by National Cancer Institute (Bethesda, MD. U.S.A.) according to the cooperation agreement.

The serum against proteins of MMTV A-particles (rabbit) was kindly supplied by Dr. Zotter (Institute of Pathology, Karl-Gustav Karus Medical Academy, Dresden, G.D.R.).

Rabbit serum against MMTV of RIII mice was prepared in our laboratory. As shown elsewhere, it did not react with proteins of retroviruses type C or D (including glycoproteins) and is efficient against MMTV-related proteins expressed in some cells of stable human breast cancer lines MCF-7 and T47D (Litvinov and Golovkina, 1985).

The serum against milk proteins of C57B1 mice was prepared in our laboratory by immunization of rabbits. The serum reacted with protein gp68 in the milk and in the virus. This protein described by Sarcar and Dion (1975) was incorporated from the milk to MMTV of RIII mice.

Blood sera of women with breast cancer were collected upon admission to the breast cancer ward of the All-Union Cancer Research Centre (U.S.S.R. Academy of Medical Sciences) before surgery or treatment. The collected sera were immediately frozen and kept at −20 °C until tested. All characterizations of tumours presented hereafter are based on postsurgical epicrisis and histologic analysis of the samples. Stages and TNM are based on WHO classification from 1981. Rabbit serum against human immunoglobulins was prepared in the Gamaleya Institute of Epidemiology and Microbiology (Moscow, U.S.S.R.). Globulin fraction from these sera was precipitated with 33% ammonium sulphate; IgG rom this raction was purified on DEAE--Sephadex and after dialysis conjugated with peroxidase (Serva, 1000 units (mg) according to the method of Nakane and Kawaoi (1974).

Antibodies against MMTV were previously detected (Litvinov *et al.*, 1984) with the use of enzyme immunoassay.

Complexes containing human immunoglobulins and antigens identified by the serum against MMTV proteins were detected by a method proposed by Hehlman *et al.* (1984*b*). Serum IgG to MMTV proteins was isolated using the above-mentioned procedure and dialysed against sodium carbonate buffer (0.01 mol/l, pH 9.5). Into each well o the microplates (Dynatech) 200 μg IgG at concentration of 20 μg/ml was added, incubated overnight at 8 °C and washed with PBS containing 0.1% Tween 20. Then 200 μl of the tested serum was added into the wells. The serum was diluted at 1 : 10 with PBS containing 0.1% Tween 20 and 10% nonimmune rabbit serum (in parallel wells the mixture contained 10% serum against MMTV). The plate was further incubated overnight at 8 °C, washed, and finally the conjugate against human immunoglobulins diluted at 1 : 1000 with PBS containing 0.1% Tween 20 and 10% normal rabbit serum was added. After 2 hr incubation at 37 °C the conjugate was washed off and substrate reagent solution — 5-aminosalicylic acid (0.8 mg/ml, pH 6.0) with H_2O_2 up to 0.01% was added thereto. The reaction intensity was determined in ELISA-reader MR590 (Dynatech) at 450 nm.

For inhibition of the reaction, various specific immune sera at decreasing amounts were added to tested human serum. Before addition into the wells these mixtures were incubated at 37 °C for 2 hr. The reaction was always performed in 2 or 3 parallel series and also in the presence of 10% normal rabbit serum.

Results

Altogether 85 blood sera of breast cancer patients have been tested (most of them had intraductal carcinomas); in addition, 3 sera were from Padgett cancer patients, 3 from women with intraductal papilloma and 15 from women with fibro-cystic mastopathy. The binding of serum components, mostly globulins, to the plate entailed a nonspecific reaction whose variability limits were established for all sera collected. That is why each serum was tested in the presence of normal rabbit serum and the serum to MMTV proteins at the same concentration (at which specific binding with IgG absorbed on the plastic was blocked). The values of nonspecific reaction for all sera were

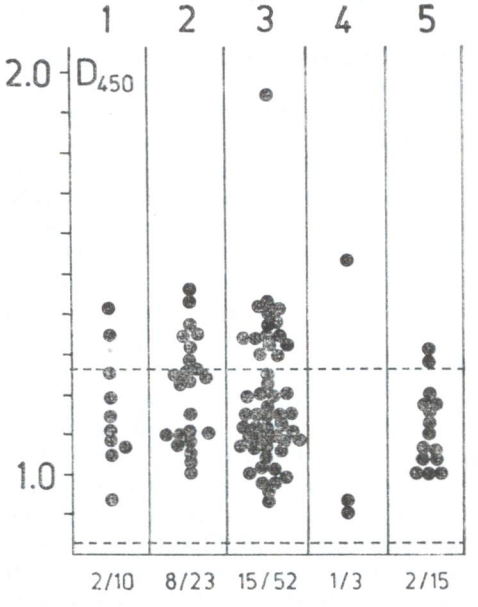

Fig. 1.
Reaction of the sera of women of different groups with IgG against MMTV proteins of RIII mice: breast cancer patients stage 1(1), stage 2(2), stage 3(3), patients with intraductal papilloma (4) and with fibro-cystic mastopathy (5).
Here and in Fig. 5: ordinate: optical density (optical units at 450 nm), dashed line indicates the limits of reaction determined by nonspecific binding.

thereby determined as well as the variance limits of this background binding ($X \pm 2\sigma^{n-1}$, where X is a mean value of reaction in the presence of serum against MMTV proteins and σ is the standard deviation). The serum was defined as positive if its value without blockade (i.e. in the presence of normal rabbit serum) was beyond the upper limit of the background ($p < 0.05$).

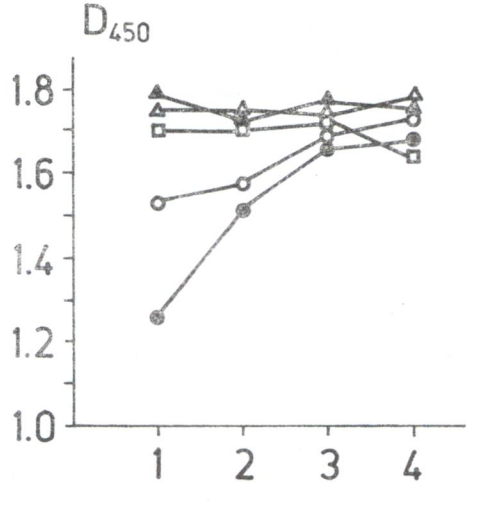

Fig. 2.
Blocking of complex binding with IgG against MMTV of RIII mice, of the sera against MMTV, intracytoplasmic A-particles and other retroviruses.
Serum against the milk of C57B1 mice and normal rabbit serum fail to block the reaction. The tested human serum was preincubated with 10 μl (1), 5 μl (2), 2 μl (3), and with 1 μl of mouse serum (4). Anti-RIII MMTV (●), anti-iAP (○), anti-p27 SSV (△), anti-p30 RaLV (□), anti-gp70 Balb2 LV (▲).

As seen on Fig. 1 a specific component was involved in reactions with a number of sera.

For testing the specificity of the reaction with antibodies against MMTV a few "positive" and "negative" sera have been selected. The binding of antibodies to MMTV with the complexes containing human immunoglobulins and a certain antigen was specific because: a) it was blocked by preincubation of human serum with antibodies to MMTV and to intracytoplasmic A-particles but not with those to milk of C57B1 mice or other retroviruses (Fig. 2); b) by increasing dilution of human serum the reaction with "positive"serum approached the reaction value with "negative" serum; c) decrease of the amount of sorbed antibodies against MMTV dramatically lowered the value of reaction with "positive" serum and only insignificantly that of the "negative" one (Figs. 3-I, II).

These investigations ruled out the involvement of nonspecific components such as rheumatoid factor, antibodies against milk proteins present in the serum against MMTV and antibodies against other retroviruses. The serum prepared to proteins of RIII MMTV does not interact with glycosylated proteins of other retroviruses which suggests that its reaction with the sugar residue of the antigens circulating in blood is unlikely.

Preincubation of MMTV itself in the wells with sorbed antibodies against MMTV did not decrease the reaction, but in some cases even increased it. This seems to be due to the presence of free antibodies to MMTV in the tested sera: as we measured the binding of human immunoglobulins, the absence of

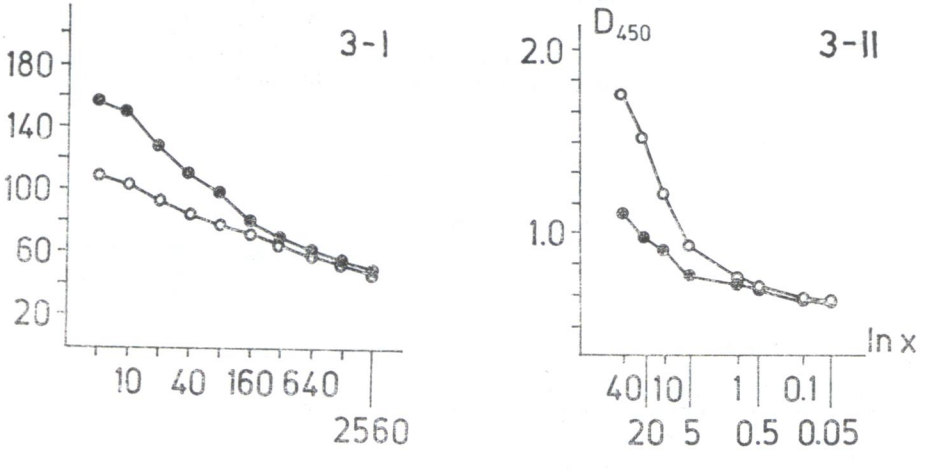

Fig. 3.

Decreased binding of immune complexes by antibodies against MMTV to „positive" and „negative" sera, caused by dilution of serum (I) and decrease of the quantity of antibodiets to MMTV adsorbed to the plastic (II)

Abcissa: dilution of human serum tested (I) and concentration (μg/ml) of antibodies to MMTV added into the well for sorption (II).

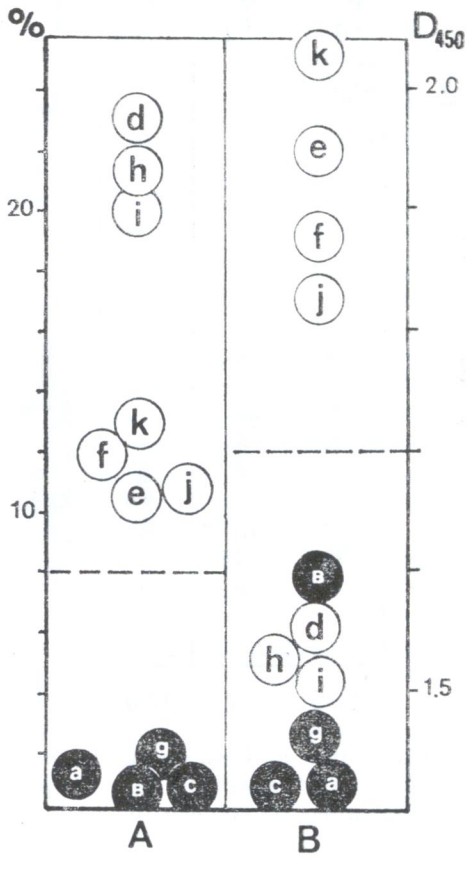

Fig. 4.
The presence in the blood serum of breast cancer patients of antibodies to MMTV (A) and complement bound by antibodies to MMTV (B).
Ordinate — A: difference between the intensity of the reaction of human blood serum with MMTV and the reaction of the same serum readsorbed to MMTV preparation (percentage), B: intensity of the reaction of serum with antibodies against MMTV. Dashed line — upper limit if the variability of nonspecific reaction. White colour marks "positive" sera containing antibodies to MMTV, black colour — "negative" sera.

the binding of immune complexes could be compensated for by the binding of human antibodies against MMTV with viral proteins that had reacted with adsorbed antibodies. This assumption is confirmed by the fact that the reaction level was increased on addition of MMTV to sera, whereas the reaction without MMTV was decreased at the same serum dilution. The reaction was not blocked by preincubation of other retroviruses or milk of Balb/c mice with sorbed antibodies against MMTV.

For some sera tested the intensity of in the reaction with MMTV preparation was previously determined. Fig. 4 illustrates the presence of antibodies against MMTV and complexes bound by antibodies against MMTV in some "positive" and "negative" (according to the presence of antibodies to MMTV) sera. It can be seen that "negative" sera do not contain complexes reactive with antibodies against MMTV either; "positive" sera with high titre of antibodies against MMTV were characterized by low content of the complexes, whereas their content was higher in "positive" sera with low antibody titre (for "positive" sera the correlation coefficient between the presence of

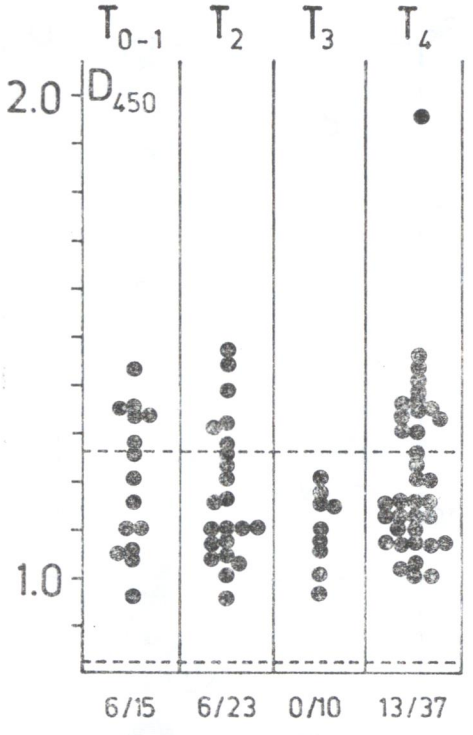

Fig. 5.
The incidence of "positive" and "negative" sera in the groups of breast cancer patients classified according to the size of the tumour ($T_0 - T_4$).

For explanations see legend to Fig. 1.

free antibodies and the complexes is $N = -0.9215$; the probability of correlation is $P > 99\%$). The comparative data obtained suggest that: a) the appearance in the serum of immune complexes bound by antibodies against MMTV is associated with the presence of humoral antibodies reactive with proteins of this virus; b) the antibodies in question are present in the complexes; c) the titre of antibodies to MMTV in the blood serum of breast cancer patients occurred to be decreased because of immune complex formation.

It should be noted (data not shown) that we did not observe cases of complete absence of antibodies to MMTV in human sera with the highest content of complexes. We do not know, therefore, whether or not a complete neutralization of the antigens tested can be achieved. On the other hand (Fig. 4), the sera without antigens against MMTV did not contain complexes either. Immune complexes adsorbed by rabbit antibodies to MMTV structural proteins were also found in blood serum of patients both at early and late clinical stages (Fig. 1).

In one blood serum out of three taken from Padgett cancer patients we, in addition, detected complexes reactive with antibodies to MMTV. In women with fibro-cystic mastopathy we also detected 2 positive sera, but the intensity of reaction was close to the threshold level. In one serum out of three collected from women with intraductal papilloma the reaction with

antibodies against MMTV appeared to be rather intensive. This is noteworthy, for intraductal papilloma is considered to be a precancerous condition (Wellings *et al.*, 1975).

Size classification of tumours allowed us to detect a certain regularity: in tumour group T_1 "positive" sera were more numerous than in groups T_2 or T_3 (Fig. 5). This might probably be also true for group T_4, if real sizes of tumours could be established in all cases. Group T_4 tumours being associated with oedema, their classification size is not the same as their true size.

Discussion

We presented evidence for the presence of complexes containing immunoglobulins and antigens reactive with antibodies to structural MMTV proteins in the blood sera of 30% of breast cancer patients, in 1 out of 3 patients with intraductal papilloma and in 2 out of 15 women with fibro-cystic mastopathy. Studies on the specificity of reaction have shown that antibodies against structural MMTV proteins bind to the complexes. Besides, it has been found that this reaction can be blocked by addition of antibodies to MMTV proteins or intracytoplasmic A-particles, but not by antibodies to mouse milk proteins or other retroviruses.

About equal percentage of women with breast cancer at early and late stages appeared to have these complexes in the blood serum. In cases when a true nature of the tumour could be determined, it was found that they more often occurred in patients with small tumours.

In some sera a relatively high level of these complexes correlated ($p < 0.01$) with a low titre of antibodies to MMTV. This can probably account for the previously reported low incidence of antibodies to MMTV in breast cancer patients at late clinical stages (Litvinov *et al.*, 1984). It should be noted that so far we have not observed any cases when the serum would contain complexes reactive with rabbit antibodies to MMTV, but would not have antibodies interacting with MMTV proteins. Moreover, the percentages of patients with complexes and of those with antibodies against MMTV appeared to be about the same in breast cancer of stage III. Apparently, the tumours with MMTV-antigenicity are clinically recegnized at earlier stages owing to a specific biologic behaviour. And therefore, the proportion of breast cancer patients admitted to the hospital at early stage of the disease is much higher than their percentage at late stages.

If this is true, at early stages of breast cancer only a part of mammary tumour patients would have immune complexes reactive with antibodies against MMT in their serum, whereas at later stages these would appear in most patients. It is hard to say what is the nature and the source of that component of serum which reacts with specific antibodies against MMTV structural proteins and is involved in the formation of these complexes.

Müller and Grossmann (1972) have described an antigen interacting in precipitation reaction with the serum against MMTV in 10 out of 30 sera from breast cancer patients, a finding similar to ours. However, the data of

Zangerly *et al.* (1977) and Hendrick *et al.* (1978) who studies human sera by the method of competitive radioimmunoassay did not confirm these findings: they detected no MMTV-related antigens in human serum. It seems that this discrepancy can be related, among other things, to the high sensitivity of competitive radioimmunoassay allowing already small amounts of specific antibody to participate in the reaction. Because the number of cross-determinants between MMTV proteins and related human antigens is small (Segev *et al.*, 1985) and, furthermore, cross-reactive antibodies are also present in a low level in the specific serum (which is its individual characteristics), their amount participating in competitive radioimmunoassay can be undetectable. Anyhow, it seems probable that an antigen detectable by antibodies against MMTV can be present in the serum. These are not necessarily the same antigens that are detected in the breast cancer tumour. However, Keydar's group (Segev *et al.*, 1985) has demonstrated that T47D cells release into the culture medium a gp60 protein detectable with antibodies to MMTV gp52. We found large amounts of a similar protein with a molecular weight of 60 kD in some breast cancer extracts (Remennik *et al.*, 1985). Therefore, it can be assumed that such protein enters the blood flow and reacts with antibodies to MMTV similarly as described in virus-induced mouse mammary carcinomas.

It is also possible that complexes are formed by antibodies to MMTV binding with anti-idiotypes simulating epitopes of MMTV-related antigens. Free valence of these anti-idiotypic immunoglobulins can react with rabbit antibodies against MMTV sorbed to plastic. The likelyhood of this suggestion is supported by the presence in many systems of complexes formed by antibodies against tumour antigens with anti-idiotypes (Morgan *et al.*, 1979).

References

Day, N. N., Witkin, S. S., and Sarkar, N. H. (1981): Antibodies reactive with murine mammary tumour virus in sera of patients with breast cancer: geographic and family studies. *Proc. natn. Acad. Sci.* **78**, 2483

Hehlmann, R., Schreiber, M. A., Kreeb, G., Luz, A., Mesa-Tejada, H., Lohe, K. J., and Prechtel, K. (1984a): Monoclonal antibodies against virus-like particles from a human breast cancer cell line detect a cross-reacting antigens in human breast carcinomas. *Zbl. Bact., I Abt. Orig.* **210**.

Hehlmann, R., Erfle, V., and Schetters, H. (1984b): Antigens and circulating immune complexes related to the primate retroviral protein SiSV gp70. *Cancer* **54**, 2927.

Hendrick, J. C., Francois, C., and Calberg-Bacq, C. M. (1978): Radioimmunoassay for protein p28 of murine mammary tumour virus in organs and serum o mice and search for related antigens in human sera and breast cancer extracts. *Cancer Res.* **38**, 1826.

Kryukova, I. N., Komarova, E., Zotter, S., and Grossmann, H. (1981): Use of electroimmunodiffusion method on cellulose acetate films for the detection of antigenicity in human breast cancer that is related to the major core antigen of mouse mammary tumour virus. *Arch. Geschwuelstforsch.* **51**, 517.

Litvinov, S. V., Remennick, L. V., and Kryukova, I. N. (1984): Expression of humoral antibodies to MMTV-related antigens in breast carcinoma patients and control subjects. *Bull. exp. Biol. Med.- Eng. Trans.* **97**, 649.

Litvinov, S. V., and Golovkina, T. V. (1985): The expression of MMTV-related antigens in breast cancer cells (in Russian). *Vopr. Oncologii* **31**, 47.

Meza-Tejada, R., and Spiegelman, S. (1983): Retroviruses and human breast cancer. p. 473- In L. Phillips (Ed.): *Viruses Associated with Human Cancer*, M. Dekker, New York.

Morgan, A. S., Rosen, R. D., and Twomey, J. J. (1979): Naturally occuring circulating immune complexes: normal human serum contains idiotype-anti-idiotype complexes dissociable by certain IgG antiglobulins. *J. Immunol.* **122**, 1672.

Müller, M., and Grossmann, H. (1972): An antigen in human breast cancer sera related to the murine mammary tumour virus. *Nature (London)* **231**, 116.

Nakane, P., and Kawaoi, A. (1974): Peroxidase-labelled antibody. A new method of conjugation. *J. Histochem. Cytochem.* **22**, 1084.

Pascal, R., Rollwagen, F., Haroling, T., and Schiavone, W. A. (1975): Glomerular immune complexes deposits associated with mouse mammary tumour. *Cancer Res.* **35**, 302.

Remennik, L. V., Litvinov, S. V., Kryukova, I. N., and Chuev, Yu. V. (1985): Expression of antigens immunologically related to antigens of mouse mammary gland carcinoma virus in human mammary tumours. *Bull. exp. Biol. Med. — Eng. Trans.* **99**, 641.

Sarkar, N. H., and Dion, A. S. (1975): Polypeptides of the mouse mammary tumour virus. I. Characterization of two group-specific antigens. *Virology* **64**, 471.

Segev, N., Hizi, A., Kirenberg, F., and Keydar, I. (1985): Characterization of a protein, release by T47D cell line, immunologically related to the major envelope protein of mouse mammary tumour virus. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1531.

Tomana, M., Kajdos, A., Niedermeier, W., Durkin, W., and Mesteeky, J. (1981): Antibodies to mouse mammary tumour virus-related antigen in sera of patients with breast carcinoma. *Cancer* **47**, 2696.

Wellings, S. R., Jensen, H. M., and Marcum, R. G. (1975): An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions. *J. natn. Canc. Inst.* **55**, 231.

Zangerle, R. F., Calberg-Bacq, C.-M., Colin, C., Franchinont, P., and Francois, C. (1977): Radioimmunoassay or glycoprotein gp47 of murine mammary tumour virus in organs and serum of mice and search for related antigens in human sera. *Cancer Res.* **37**, 4226.

Zotter, S., Grossmann, H., Francois, C., Kozma, S., Hainaut, P., Calberg-Bacq, C., and Osterrieth, P. (1983): Among the human antibodies reacting with intracytoplasmic A-particles of mouse mammary tumour virus, some react with p14, the nucleic-acid binding protein and others with MMTV p28, the main core protein. *Int. J. Cancer* **32**, 27.