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# ANTIBODIES TO THE ESTROGEN INDUCED 52 K PROTEIN RELEASED BY HUMAN BREAST CANCER CELLS

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SUMMARY - Polyclonal antibodies against an estrogen induced 52 K protein released by human breast cancer cells have been developed by injecting rabbits with a crude cellular pellet of $MCF_7$ human breast cancer cells. The rabbit antisera have been tested against ($^{35}S$) Methionine labelled proteins released by the $MCF_7$ cells followed by separation of the immune complexes with Protein A Sepharose. In spite of their low specificity and titer, these antisera allowed us to investigate the release of the 52 K protein in vitro by other mammary cancer or normal cells.

INTRODUCTION - Estrogen stimulates the synthesis of a glycoprotein (M.W. 52,000 daltons = 52 K) released into the medium (1) by the $MCF_7$ and $ZR_{75-1}$ human breast cancer cell lines. Since this protein is also present and regulated in metastatic human breast cancer grown in primary cultures, it is a potential circulating marker of hormone responsiveness. In order to determine the localisation and the tissue repartition of the 52 K protein in other tissues and to assay it in blood, it is necessary to obtain specific antibodies to this protein.

The purification of the released 52 K protein from culture medium is difficult, since it is present at an extremely low concentration in the harvested cell culture medium, which also contains serum proteins.In this study, we have used a faster approach to prove the immunogenicity of the 52 K protein by assuming that it will be also present in a crude cellular pellet of $MCF_7$ cells. We show that the resulting rabbit antisera immunoprecipitated mainly the 52 K protein from culture

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medium and are useful for detecting the release of this protein from other cells in cultures.

## MATERIAL AND METHODS

The $MCF_7$ (1), $R_{27}$ (3,4), NMU (5), RBA (6) and $T_{47}D$ (7) mammary cancer cell lines were cultured as described, in the presence of fetal calf serum which contains estrogens.

Preparation of immunogen : Confluent $MCF_7$ cells (provided by Dr Marvin RICH) were removed and suspended in phosphate-buffered saline PH 7.2 (PBS) EDTA 1 mM. They were then homogenized in 10 mM Tris HCl, PH 7.4 1 mM PMSF in a Dounce homogenizer. The homogenate was centrifuged at 205,000 x g for 40 min, and the pellet resuspended in saline (0.9 % NaCl). Another source of immunogen, kindly provided by Dr NOLAN (Abbott Laboratories, Chicago) was the 105,000 x g cellular pellet of $MCF_7$ cells, grown in 5 % calf serum, which had been saved from the purification of cytosoluble estrogen receptor (8).

Generation of antisera : Male New-Zealand rabbits were given subcutaneous injections of 3 mg of pelleted cellular proteins emulsified with Freund's complete adjuvent. Booster injections were given at 3 to 4 week intervals, and the rabbits bled by external ear vein puncture 8-10 days after each injection. Preimmune sera were obtained from the same animals before the first injection. All sera were adjusted to 0.02 % $NaN_3$, aliquoted in 1 ml fractions and stored at -80°C until use.

Preparation of labelled antigen : Cells stimulated by the estrogens present in fetal calf serum were labelled with ($^{35}S$) Methionine (S.A. = 700-1200 Ci/mmole) for 6 hours (1). The ($^{35}S$) labelled proteins released into the culture medium were used for immunoprecipitations and finally counted and analysed by SDS electrophoresis in a 12 % acrylamide gel as described (1) and by fluorography (10). Scanning of the fluorographs was carried out with a Vernon densitometer (Vernon, Paris, France).

Immunoprecipitations : The immunoprecipitation assay was performed in 1.5 ml polypropylene microfuge tubes with 50 µl of ($^{35}S$)Methionine labelled proteins of the medium (10,000-15,000 cpm TCA precipitable proteins), 10 µl of antiserum (Igi), 90 µl of TNN buffer (Tris 100 mM, pH. 8.0, NaCl 100 mM, Nonidet P 40 0.5 %, bovine serum albumin 2 % w/v). After incubation for 2 h at 37°C and overnight at 4°C, 60 µl of protein A Sepharose (Pharmacia, 6 % w/v in the same buffer) was added (9). Tubes were allowed to rotate for 90 min at 4°C and then centrifuged at 10,000 g for 2 min. The protein A Sepharose gel was washed with 1 ml of Tris LiCl buffer (Tris 100 mM pH 8.6, LiCl 500 mM, Nonidet P 40 0.5 %) and with Tris 50 mM pH 6.8. The gel was resuspended in 60 µl of sodium dodecylsulfate (SDS) analysis buffer. Control precipitations were carried out with 20 to 30 µl of preimmune serum.

Other techniques : Proteins were assayed by the method of LOWRY et al (11), using bovine serum albumin as the standard. Standard unlabelled and labelled proteins were from Biorad Laboratories and New England Nuclear respectively.

## RESULTS

Evidence for antibodies against the 52 K protein in $MCF_7$ lines : The antibodies raised against a high speed pellet of $MCF_7$ cells were tested on ($^{35}S$) Methionine labelled proteins

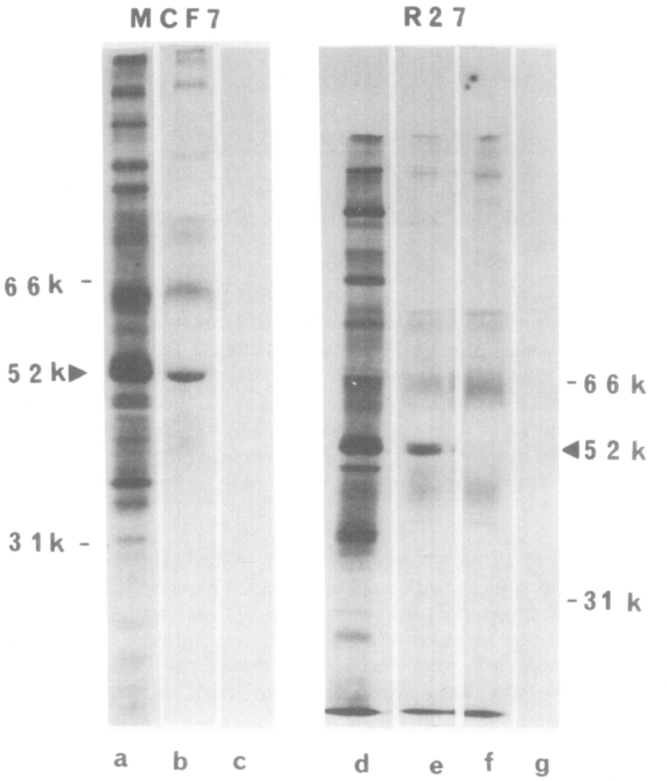
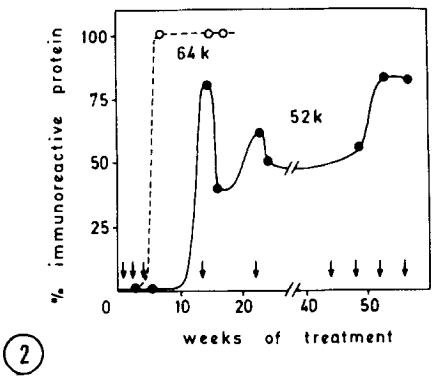



FIGURE 1 - Immuno-precipitation of ($^{35}$S) Methionine-labelled proteins released by $MCF_7$ and $R_{27}$ cells : Human $MCF_7$ and $R_{27}$ cells were cultured and radiolabelled as described (1,4). Immuno-precipitates of the released proteins were analyzed by electrophoresis in SDS polyacrylamide gels as described in the Methods. Proteins released by the wild type $MCF_7$ (a-c) : Total released proteins (a), immunoprecipitate with the immunized (b) or the preimmune control serum (c) of rabbit n°5. Proteins released by the $R_{27}$ clone of $MCF_7$ (d-g) : Total released proteins (d), immuno-precipitate with antisera of rabbit n°5 (e), rabbit n°6 (f) and preimmune control serum of rabbit n°5 (g). All antisera were used at 1:15 dilution.

released by wild type $MCF_7$ cells (from Marvin RICH) and a cloned variant ($R_{27}$) which is resistant to antiestrogen (3). The immunoprecipitates were analysed by SDS electrophoresis. Five of the 6 immunized rabbits gave antisera which reacted with the 52 K protein, a 64 K protein and other minor proteins released into the medium from $MCF_7$ (Fig. 1, b) and $R_{27}$ cells (Fig 1, e). No precipitate could be detected when preimmune serum was used (Fig. 1, c and g). The 6th rabbit produced no antibodies against the 52 K protein even after 6 injections, even though the same pattern was obtained for the other precipitated proteins (Fig 1, f).



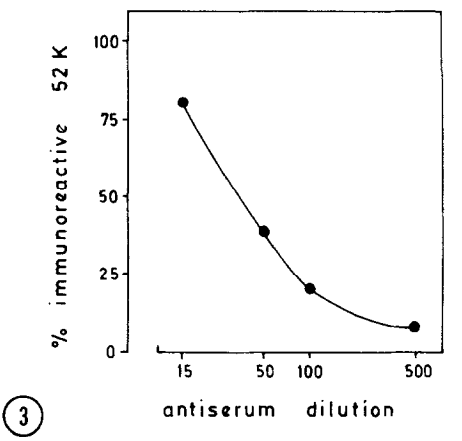




FIGURE 2 - Time course of an immunization : Rabbit n°5 was treated with 3 mg of immunogen as described in the Methods at the times indicated by arrows and bled 10 days and 20 days after each injection. Antisera at 1:15 dilution were assayed by immunoprecipitation for the presence of antibodies to ($^{35}$S) Methionine labelled proteins released by $MCF_7$ cells. Immunoreactive labelled proteins were analyzed by SDS polyacrylamide gel electrophoresis and estimated by scanning the fluorographs as described. 100 % is the amount of 52 K or 64 K protein present in samples before immunoprecipitation.

FIGURE 3 - Percentage of the ($^{35}$S)-Methionine 52K protein by antiserum at different dilutions : The ($^{35}$S) Methionine proteins released by $MCF_7$ wild type, were assayed by immunoprecipitation at the indicated antiserum dilution of the rabbit n°5 (logarithmic scale) and analysed as indicated in Methods and legend of fig. 2.

As seen in Fig. 2 antibodies against the 52 K protein appeared after the 4th injection but their titer dropped by 30 % within 9 days of the first bleeding. Repeated injections of immunogen were necessary to maintain the titer. Antibodies against a 64 K protein of the medium appeared earlier than those against the 52 K protein.

The titer of the antibody to the 52 K protein was low as shown by an antiserum dilution curve (Fig 3). 80 % and 40 % of the ($^{35}$S) labelled 52 K protein was precipitated at an antiserum dilution of 1:15 and 1:50 respectively. For the same preparation of antigen, the reactivity of the antisera varied markedly according to the rabbit (from 0 for rabbit n°6 to 67 % for rabbit n°10). Antibodies of similar titer and specificity were obtained from $MCF_7$ cells provided by our or Abbott Laboratories

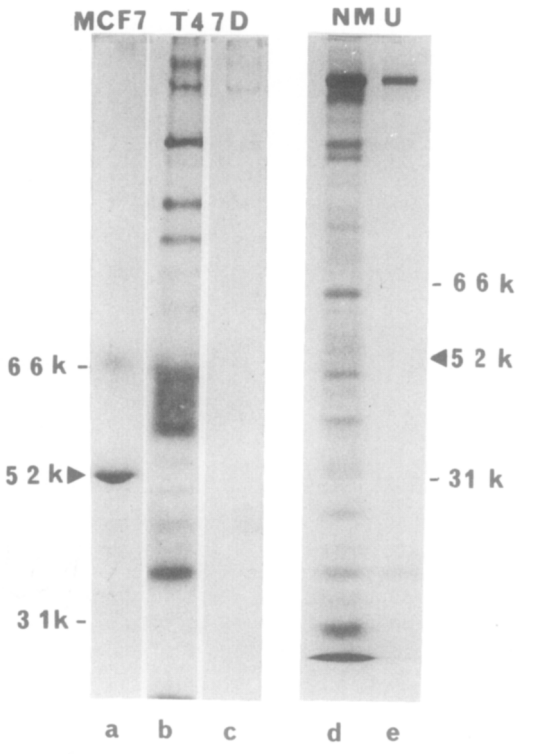



FIGURE 4 - Immunoprecipitation of ($^{35}$S) labelled proteins released by $T_{47}D$ and NMU cells as compared to $MCF_7$ cells : $T_{47}D$ and NMU cells were cultured, $E_2$ stimulated and labelled as described in Methods. After immunoprecipitations of the released proteins with antiserum of rabbit n°5 at 1:15 dilution, the immunoprecipitates were analysed by SDS polyacrylamide gel electrophoresis. Total proteins released by $T_{47}D$ (b) and NMU (d) cells. Immunoprecipitates of proteins released by $MCF_7$ (a), $T_{47}D$ (c) and NMU (e) mammary cancer cells.

We conclude that an antigen present in crude cellular pellet of $MCF_7$ cells allows antibodies to be developed against the released 52 K protein.

Use of the antisera to detect the 52 K protein in other cells : Antisera were used to detect the 52 K protein (or an antigen immunologically related to the 52 K protein) among ($^{35}$S) labelled proteins released by other cells in culture. Estradiol stimulates the synthesis of secretory proteins ranging from 55 to 66 K daltons MW (6) in the estrogen responsive clone of the $T_{47}D$ cell line which is also derived from a pleural effusion of human breast cancer (12). These proteins were not precipitated by the anti-52 K antibodies (Fig. 4) showing that the estrogen

regulated 52 K and 60 K proteins released by the $MCF_7$ and $T_{47}D$ cell lines are not related, and that $T_{47}D$ cells do not release the 52 K protein into the medium. Also, no immuno reactive 52 K protein was released by normal human fibroblasts grown in primary culture (not shown), or by the NMU rat mammary cancer cell line established from a tumor induced by nitroso-methyl-urea (NMU) (5) (Fig. 4, d,e).

## DISCUSSION

In this study, we show that a cellular pellet of $MCF_7$ cells contains an antigen able to induce antibodies against an estrogen regulated 52 K protein released by $MCF_7$ cells. In an attempt to demonstrate the precursor of the 52 K protein in the cellular pellet of $MCF_7$, we have tested the reactivity of the antisera against ($^{35}S$)labelled proteins of this pellet which had been sonicated in the presence of 0.3 % Triton X 100. These antisera immuno precipitated many cellular proteins, but the cellular precursor of the 52 K protein could not be identified using this approach. In contrast, a putative cellular precursor of the 52 K protein has recently been identified by two dimensional gel analysis as a 52 K protein corresponding to 0.5 % of the total ($^{35}S$)labelled cellular proteins in a crude cellular pellet of $MCF_7$ cells which had been treated or not by estradiol (M. Garcia, unpublished data). Preliminary results from competition experiments with soluble antigen or absorption of antibodies by different subcellular fractions suggest that the antigen reacting with the antiserum is more abundant in membrane and particulate fractions than in the cytosol.

Even though these antisera were not specific when tested on cellular proteins, they precipitated a limited number of proteins of the medium and could therefore be used to study proteins released by several cell lines.

The antisera precipitated a 52 K protein released from the $MCF_7$ human breast cancer cell line but not released from $T_{47}D$ cells that are also estrogen receptor positive. An immuno reactive 52 K protein has also been found to be released by pleural metastatic breast cancer cells which had been grown in primary culture (2). These antisera were unable to immuno precipitate any 52 K protein released from human fibroblasts (2) or from the NMU (Fig. 4) and RBA (2) rat mammary cancer cell lines. Therefore, in addition to other criteria used to identify the 52 K protein such as its estrogen inducibility, 2D gel analysis, and Concanavalin A reactivity, the antibodies described here can be used to detect the presence or absence of the 52 K protein released by other cells in culture. In this respect, we are now studying the proteins released by other tissues in order to determine whether the 52 K protein is specific for hormone responsive breast cancer cells. Recently, an antiserum against the 52 K protein has been raised in mice, and work is in progress to prepare monoclonal antibodies against the 52 K protein. These will have considerable interest to specify the cell localisation, tissue repartition, biological function and gene regulation of this protein.

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