CHAPTER 3

GENERATION OF MONOCLONAL ANTIBODIES TO ESTROGEN RECEPTORS

3.1 INTRODUCTION

The estimation of ER is a generally acknowledged parameter for the management of breast cancer patients. The biochemical steroid receptor assays using the radioactive ligand has been the main methodology for the relevant knowledge obtained on ER status in human breast cancer (DeSombre et al., 1979, Osborne et al., 1980 and DeSombre, 1984). Although the biochemical ER assays are of proven clinical value, they possess certain intrinsic disadvantages viz., large amounts of tissue required, which may or may not be contaminated with benign mammary epithelial cells, use of radioactive ligand and sophisticated instrumentation and in addition to the above, they are expensive and difficult to perform. These biochemical assays do not give a clear picture of tumor cell ER mosaicism since they are performed on tissue homogenates (Poulsen, 1983). As a consequence, there has been much interest in the development of histochemical techniques since such methods would circumvent many of the problems associated with the standard biochemical assays. Since then several methods have been proposed which either depend on immunocytochemical localization of receptor bound estradiol antibodies or on direct visualization of naturally bound fluorescent conjugated derivatives (Nenci et al., 1976, Lee, 1978, Pertschuk et al., 1979 and Walker et al., 1980). Though these procedures have shown to correlate well with the established methods, they are not always reproducible. Their validity in fulfilling the need for histochemical detection of ER has been questioned for their ability to detect ER and to
discriminate ER from other estrogen binding proteins (Mercer et al., 1981, Panko et al., 1982 and Pari et al., 1984).

The purification of ER from calf uterus has made tremendous progress in the development of Immunocytochemical assays. The polyclonal antibodies to the ER from calf uterus was first raised in a rabbit and subsequently in a goat (Greene et al., 1977 and 1979). These polyclonal antibodies were found to react with both occupied and unoccupied steroid receptors. It has been reported that the polyclonal antibodies raised against a high molecular weight estrogen binding fraction of ER, present in human breast cancers, could be used to identify receptor positive cells in frozen sections of such tumors (Raam et al., 1982), however, little information is available regarding their cross reactivity with non-receptor component of ER containing cells.

The advent of hybridoma technology introduced by Kohler and Milstein (1975) has revolutionised the study of steroid hormone receptors. The procedure used for the generation of monoclonal antibodies is represented in Fig. 3.1. The monoclonal antibodies to mammalian estrogen receptors were first prepared by Greene et al., (1980a) at Ben May Lab, University of California, U.S.A. by polyethylene glycol mediated fusion of splenic lymphocytes from male Lewis rats, immunized with partially purified nuclear ER from calf uterus with mouse myeloma cells. The resultant rat monoclonal antibodies have been found to recognize both cytosolic and nuclear ER except IgM monoclonal antibodies which preferentially reacted with nuclear forms. All these data accumulated so far indicate that the ten monoclonal antibodies both IgM and IgG recognize either the same epitope or mutually exclusive epitopes on the calf estrogen receptor. Considering their limitations of the specificity and recognition of only one region of the receptor molecule and also to study estrogen receptors in other species particularly in human reproductive tract and breast cancer, Greene et al. (1980b) have succeeded in the preparation of monoclonal
Fig 3.1 Schematic representation of the procedure used in the generation of monoclonal antibodies
antibodies to ER isolated from MCF-7, human breast cancer cell line, which yielded 3 hybridomas namely D58, D75, D547 each of which secretes a unique idiotype of antibody that recognizes a distinct region of ER molecule. So the development of specific monoclonal antibodies to ER of human origin has provided a new approach for the detection of ER in human breast cancer, whether or not they are occupied with hormone.

This chapter deals with the generation of monoclonal antibodies to Estrogen receptor protein by the hybridoma technology. These monoclonal antibodies have been generated for the evaluation of very specific and precise enzyme immunoassay for the estimation of ER as well as Immunocytochemical study of tissue and aspiration specimens and their application in the study of human breast cancers.

3.2 MATERIALS

3.2.1 Animals

4-6 weeks old inbred BALB/c mice were used for immunization.

3.2.2 Reagents Required

1. Dulbecco's Modified Eagle's Medium (DMEM) 1 x 10 litres packet (Flow laboratories, U.K.)

2. HAT solution and Fetal Calf Serum (FCS) were obtained from Flow laboratories, U.K.

3. Freund's complete and incomplete adjuvants (Sigma Chemical Co., U.S.A.)

4. Disposable plastic items were obtained from Dr. David Golde, U.S.A., and M/s. Greiner Co., West Germany.
3.2.3 Preparation of reagents

3.2.3.1 Fusing Agent-Polyethylene Glycol 50% (PEG 1500)

10 gm Polyethylene glycol was taken in a small stoppered bottle and autoclaved. The melted solution was kept at 37°C in a water bath. 10 ml of warm plain DMEM was added and mixed well. The solution was stored in aliquots at -20°C.

3.2.4 Preparation of Tissue Culture Medium

3.2.4.1 Dulbecco's Modified Eagle's Medium (DMEM)

The preparation of DMEM was detailed in Chapter 2.

3.2.4.2 HAT Medium (100 X)

1 ml of sterile HAT solution was added to 80 ml of plain DMEM followed by 2 ml 2mM L-glutamine and 20 ml of Fetal Calf Serum.

3.2.3 Immunogen

The purified estrogen receptor protein from MCF-7 cells and breast tumor tissues were used as immunogen.

3.2.3.1 In vivo Immunization of BALB/c Mice

50-100 μg ER protein/50 μl PBS emulsified with 50 μl of Freund's complete adjuvant was injected i.p., followed by two subsequent weekly injections of the same dose, emulsified with incomplete Freund's adjuvant. After a pause of 4 weeks, one immunized mouse was injected with a booster dose of the same concentration, four days prior to fusion.
3.2.4 Maintenance of Mouse Myeloma Cells

*SP*₂/O Ag. 14 myeloma cells, a non producer variant of BALB/c origin with an initial density of 2 x 10⁵ cells / ml were grown in DMEM containing 10% FCS for a period of one week in a humidified CO₂ incubator with 5% CO₂ and 95% air at 37°C. Confluent flasks with viable cells were used for fusion.

3.2.5.1 Preparation of Immune Spleen Cells

The immunized BALB/c mouse was sacrificed by cervical dislocation and cleaned with 70% ethanol. The superficial skin over the left side of the abdomen was pinched up with the help of a fine forceps. A small incision was made over the spleen. The spleen was aseptically removed through the incision and placed inside the metal sieve in a sterile petridish. Then 5 ml of plain DMEM was added into the petridish and the spleen was gently pressed through the sieve using the plunger of a glass syringe. The immune spleen cells were collected in a sterile 50 ml conical tube, centrifuged at 1200 rpm for 5 min and the cell button was washed twice by centrifugation. The immune spleen cells were finally suspended in the plain DMEM. The cell count was determined using a small aliquot of the cell suspension by trypan blue exclusion method (0.03% trypan blue in PBS).

3.2.5.2 Preparation of Mouse Myeloma Cells

The mouse myeloma cells in the log phase of growth were collected on the day of fusion by gently tapping the sides of the tissue culture flask. The collected myeloma cells were washed twice in plain DMEM by centrifugation at 1200 rpm for 5 min. The cell count was determined by trypan blue exclusion method.
3.2.5.3 Preparation of Feeder Cells

The spleen cells from a normal BALB/c mouse were separated as per the procedure detailed earlier under the preparation of immune spleen cells. $10^5$ normal spleen cells were added to each well.

3.2.6 Generation of Hybridomas

3.2.6.1 Fusion protocol

The fusion of immune splenocytes and Sp2/O myeloma cells was performed using the method of Galfre and Milstein (1981). $10^5$ spleen cells per well were used for each plate. The spleen cells and myeloma cells were used for fusion in the ratio of 5:1. The spleen cell suspension and the myeloma cell suspension were mixed in appropriate volumes, mixed well, centrifuged at 1200 rpm for 5 min and the cell button was used for fusion. All reagents used for fusion were maintained at 40°C. 1 ml PEG was slowly added to the cell button by mixing and starting a stopwatch simultaneously. After 1 min, the concentration of PEG was gradually reduced by adding plain DMEM at various time intervals as indicated below in Table 3.1.

Table 3.1

Fusion Protocol

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1 ml 50% PEG was added</td>
</tr>
<tr>
<td>2.0</td>
<td>1 ml plain DMEM was added</td>
</tr>
<tr>
<td>2-4</td>
<td>2 ml plain DMEM was added</td>
</tr>
<tr>
<td>4-6</td>
<td>3 ml plain DMEM was added</td>
</tr>
<tr>
<td>6-8</td>
<td>5 ml plain DMEM was added</td>
</tr>
<tr>
<td>8-10</td>
<td>10 ml plain DMEM was added</td>
</tr>
<tr>
<td>10-12</td>
<td>10 ml plain DMEM was added</td>
</tr>
</tbody>
</table>
The tube was centrifuged at 500 rpm for 5 min. The supernatant was aspirated off and the cell button was suspended in HAT medium with 20% FCS (about 6 ml/plate). One drop was delivered to each well of 96 well falcon plates. To each well, $10^5$ feeder cells in HAT medium were added and the plates were incubated at 5-7% CO$_2$ at 37°C. After 4 days, one drop of medium was aspirated off from each well and one drop of fresh HAT medium added to each well. The plate was examined in an inverted microscope for any contamination before refreshing the medium. After a week, when small clones started appearing in the wells, wells with single clones were noted in each plate. When the clones were sufficiently large, they were screened for the presence of specific antibody by testing the culture supernatant by ELISA.

3.2.7 Screening of Clones

3.2.7.1 Enzyme Linked Immunosorbent Assay (ELISA)

Before fusion, an ELISA was perfected using 96 well falcon plates coated with 50 μl of the ER protein (3-5 μg) per well using a multichannel pipette and allowed to dry at 37°C. The plates were washed thrice in PBS/Tween 20 (0.5 ml Tween 20/litre PBS) and then thrice in PBS. 200 μl of 2% NRS in PBS was added to each well and incubated for 45 min at 37°C to block the nonspecific binding. After washing, the plates were stored at -70°C until use.

3.2.7.2 Materials

Tween 20 and Dimethylsulfoxide (DMSO) were purchased from E. Merck, 3,3',5,5' Tetramethyl benzidine (TMB) was purchased from Sigma Chemical Co., U.S.A.

Normal rabbit serum: Blood was collected from normal inbred healthy rabbits by ear vein puncture. The blood was allowed to clot,
centrifuged at 3000 rpm for 10 min and the serum was stored in aliquots at 
-70°C.

Peroxidase labelled rabbit antimouse Ig. (DAKO P260) was supplied as a gift item from Dakopatts, Denmark.

### 3.2.7.3 Preparation of reagents

#### 3.2.7.4 Phosphate Buffered Saline (PBS)

<table>
<thead>
<tr>
<th>NaCl</th>
<th>- 200 gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>- 5 gm</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>- 28.5 gm</td>
</tr>
<tr>
<td>Na₂HPO₄·2H₂O</td>
<td>36 gm</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>- 5 gm</td>
</tr>
</tbody>
</table>

Dissolved in 25 litres of deionized water. pH adjusted to 7.2-7.4.

#### 3.2.7.5 Substrate buffer

**Solution A**

27.8 gm monobasic sodium phosphate was dissolved in one litre deionised water.

**Solution B**

53.65 gm dibasic sodium phosphate was dissolved in one litre deionised water.

87.7 ml of solution A was mixed with 12.3 ml of solution B to get 0.1 M sodium phosphate buffer pH 6.0.
3.2.7.6 TMB reagent

3,3', 5,5' Tetramethyl benzidine (TMB)

6 mg TMB was dissolved in 1 ml of hot DMSO and stored in aliquots at -70°C.

3.2.7.7 Substrate for ELISA

The substrate solution was prepared fresh by adding 100 μl of TMB reagent to 10 ml of substrate buffer, followed by 10 μl of 30% H₂O₂.

3.2.7.8 Procedure

50-100 μl of the culture supernatant from the wells of the culture plate were transferred to the corresponding wells in the ELISA plate, incubated for 45 min at 37°C in a humidified incubator. In each assay, a positive and negative controls were included. The solution was discarded and the plates were washed thrice in Tween 20/PBS and then in PBS and incubated with 50 μl of peroxidase labelled rabbit antimouse Ig (1:1000 in 10% NRS) for 30 min at 37°C. The plates were again washed and 100 μl of the substrate solution was added and kept in the dark until the positive control developed maximum colour and the negative control did not develop any colour. The reaction was arrested by adding 50 μl 2M H₂SO₄. The plates were scanned at 450 nm in a BIO-TECH ELISA reader. The positive clones so identified were screened for further specificity by peroxidase-anti-peroxidase method (PAP) using MCF-7 cells from culture and frozen tissue sections of ER+ breast tumors.
3.2.8 Immunocytochemical assay
3.2.8.1 Materials

Rat monoclonal antibody to human estrophilin D75P$_3$Y and rabbit antirat IgG (bridging antibody) were kindly supplied by Dr. G.L. Greene, Ben May Laboratory, U.S.A. Rat PAP was a generous gift from Dr. L. Ozzello and Dr. Clifford Welsch, U.S.A. Goat antimouse Ig and Mouse PAP were kindly supplied as gift items by Dakopatts, Denmark. 3-Amino 9-ethylcarbazole (AEC) and hematoxylin were purchased from Sigma Chemical Co., U.S.A.

3.2.8.2 Preparation of reagents
3.2.8.3 Phosphate Buffered Saline (PBS)

0.01 M Phosphate buffered saline pH 7.4.

The following chemicals were dissolved in 800 ml distilled water.

\[
\begin{align*}
\text{NaCl} & \quad - & \quad 8.5 \text{ gm} \\
K_2\text{HPO}_4 & \quad - & \quad 1.43 \text{ gm} \\
\text{KH}_2\text{PO}_4 & \quad - & \quad 0.25 \text{ gm}
\end{align*}
\]

The pH was adjusted to 7.4 and made upto 1000 ml.

3.2.8.4 AEC Substrate

5 mg of AEC was dissolved in 1 ml dimethyl formamide.

Substrate Buffer

0.05 M pH 5.0 sodium acetate buffer.
1 ml of stock buffer was diluted to 5 ml with deionised water. 200 μl of AEC solution was added to the above buffer to get a clear yellow solution followed by 5 μl of 30% H₂O₂. The solution was prepared fresh before use.

3.2.8.5 Breast Cancer Cells for Immunocytochemistry

The flasks with monolayer of cells were trypsinised, the cells were harvested by centrifugation and washed in PBS. The cell suspension in PBS of appropriate dilution was used for preparing the slides. One drop of the cell suspension was added to microscopic slides, allowed to dry at RT, fixed in cold methanol for 4 minutes and then in cold acetone for 1 min at -20°C. The slides were rinsed in ice cold PBS and stored at -70°C.

3.2.8.6 Preparation of Frozen Breast Tumor Sections

4-6 μm thick frozen sections were cut using a Leitz cryostat. The sections were fixed and stored as described earlier.

3.2.8.7 Procedure

The immunostaining of ER in frozen sections of breast carcinomas has been visualized using the peroxidase-Anti-peroxidase (PAP) technique. The principle of PAP technique is illustrated in Fig. 3.2. The frozen sections and MCF-7 cells were first incubated with 100 μl of 2% NRS in PBS for 15 min in order to reduce the non specific staining. After rinsing the slides in PBS, they were then incubated with 100 μl of culture supernatant for 1 hr, followed by 1:50 dilution of goat antimouse Ig for 1 hr. The slides were washed in PBS and then incubated with mouse PAP (1:100) for 1 hr. The sections were then incubated with substrate solution containing AEC for 10 min. The sections were washed in PBS and lightly counterstained in hematoxylin. The slides were washed in tap water and mounted. The
Fig 3.2 Schematic illustration of Peroxidase - Anti-Peroxidase (PAP) technique
ERMAb D75 supplied by Dr. G.L. Greene was used as positive control and normal mouse serum was used as negative control.

3.3 CLONING BY LIMITING DILUTION

The positive hybrids were cloned twice in the presence of feeder cells. The normal spleen cell suspension was diluted in HAT medium and one drop containing $10^5$ cells was added to each well of 96 well falcon plate. The cells from a single clone of positive cells were collected in plain medium and the cell count was determined by trypan blue exclusion method. The cell suspension was added to the plates containing feeder cells. The plates were incubated at 5-7% CO$_2$.

The medium was refreshed twice a week. After a week, the culture supernatant was tested for the presence of antibody activity by immunocytochemical analysis using frozen tumor sections. The culture supernatant was collected, centrifuged and stored in aliquots at -70°C with sodium azide as preservative.

The specific clones identified by immunocytochemical assay was taken into large cultures by growing them in large culture flasks. The cells were used for cryopreservation in liquid N$_2$ and for the generation of hybridomas in BALB/c mice.

3.4 CRYOPRESERVATION

The cells from culture were collected by centrifugation. After aspirating the supernatant, the cells were suspended gradually in an equal volume of DMEM with 50% FCS and 20% DMSO. The cell suspension of $2\times10^5$/ml were then aliquoted in sterile vials and stored in liquid N$_2$ with gradual cooling. The viability and sterility were checked by rapidly thawing one vial in a water bath at 37°C, centrifuging for one min and washed to
remove the DMSO. The cells were taken in culture flasks and checked for sterility and viability.

### 3.5 GENERATION OF HYBRIDOMAS IN BALB/c MICE

Cells from positive clones were injected i.p into pristane (2,6,10,14 tetra methyl penta decane) primed BALB/c mouse. After 8-12 days, ascites fluid was collected from the peritoneal cavity, allowed to clot by keeping in a cold room, centrifuged at 3000 rpm for 5 min. The supernatant was stored in aliquots at -70°C with sodium azide as preservative.

### 3.6 PURIFICATION OF MONOCLONAL ANTIBODIES

#### Concentration by Ammonium Sulphate Precipitation

Equal volume of culture supernatant was added to equal volume of saturated ammonium sulphate solution, pH 6.5 under continuous stirring. The mixture was equilibrated at 4°C for 1 hr and centrifuged at 10,000 x g for 10 min. The supernatant was aspirated off and the pellet was dialysed against running water for at least 3 hr followed by 2 changes of PBS, pH 7.2.

#### Purification of antibodies by Protein A-Sepharose column

1.5 g of Protein A - Sepharose was allowed to swell in 10 ml PBS pH 8.0 containing 0.1% NaN₃ for 1 hr at room temperature and packed into a small column using a 10 ml pipette. The column was equilibrated with 0.1 M phosphate buffer pH 8.0. Concentrated samples were equilibrated at pH 8.0 by dialyzing against PBS. Then the samples were added to the column and eluted using 0.1 M pH 6.0. The elution of protein fractions was monitored at 280 nm by connecting a UV cord and fractions were collected using a fraction collector.
3.7 ISOTYPING OF MONOCLONAL ANTIBODIES BY OUCHTERLONY’S TEST

The heavy chain specificity of the antibodies produced by the hybrid cells was determined by double immunodiffusion test (Ouchterlony, 1949) in agar using specific antisera to heavy chains of immunoglobulins.

Materials

Agarose and goat antimouse α, μ, G₁, G₂α, G₂β and G₃ specific antibodies were obtained from Sigma Chemical Co., U.S.A.

Reagents

1 gm of Agarose was dissolved in 100 ml PBS and melted in a water bath with 0.1% sodium azide as preservative.

Coomassie Brilliant Blue Staining Solution

Destaining solution

Methanol 30%
Acetic acid 7%
in distilled water.

Procedure

3 ml of 1% hot agarose in PBS was poured on a 7.5 x 2.5 cm glass slide and allowed to set. The small peripheral wells of 1-2 mm diameter and large central well of 2-3 mm were punched using gel punch and suction device (LKB) 5 μl of purified monoclonal antibody was added to the central well and specific antisera to heavy chains were added to the peripheral wells. A control serum was included in each test and incubated at 37°C in
a humid atmosphere for 24 hrs. The slides were washed in deionised water for 48 hrs and kept in between filter paper at 37°C for 2 hr for drying. The dry film of gel was removed and washed in PBS, stained for 10 min with Coomassie brilliant blue staining solution and then destained using destaining solution.

3.8 RESULTS

Monoclonal antibodies to ER protein have been generated using ER isolated from MCF-7 cells (Human breast cancer cells) and human breast tumor tissues. Of the several fusions performed using immune spleen cells of BALB/c mice immunized with MCF-7 cell cytosolic and nuclear ER, 20 positive clones have been identified by ELISA using falcon plates coated with 3-5 µg of ER protein per well. Of these by immunocytochemical analysis by PAP method, 7 clones designated A4, B3, B6, D2, D7, E8 and G2 have been found to exhibit specific staining of cytosolic protein with MCF-7 cells and frozen breast tumor tissue sections. The clone ERD7 which has been found to exhibit maximum optical density in ELISA and intense cytoplasmic staining of cells or tissue sections has been extensively studied and characterized. However their was no staining of any nuclear ER protein. In the case of fusions performed using breast tumor cytosolic ER as immunogen, 34 clones have been found to be positive by initial screening by ELISA. Of these clones, 2 hydrids designated ERE5 and ERE10 have been found to react specifically with MCF-7 cells and frozen breast tumor tissue sections by PAP method. These 2 clones have been extensively characterized. The ER MAbs (ERD7, ERE5 and ERE10) have been found to exhibit cytoplasmic staining of frozen breast cancer cells (MCF-7) and found to be negative in ER breast cancer cells (MDA-MB-231) as also in other control cell lines. The staining patterns of ERMAbs are represented in Table 3.2. The staining patterns of these 3 clones in frozen sections of breast carcinomas are represented in Figure 3.3, 3.4, 3.5. Table 3.3.
Table 3.2: Reactivity of ER MAbs with breast carcinoma and other cell lines by an indirect immunoperoxidase method

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Reactivity with ER MAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ERD7</td>
</tr>
<tr>
<td>Breast Carcinoma</td>
<td></td>
</tr>
<tr>
<td>MCF-7 (ER⁺)</td>
<td>+++</td>
</tr>
<tr>
<td>MDA-MB 231 (ER⁻)</td>
<td>-</td>
</tr>
<tr>
<td>Other cell lines</td>
<td></td>
</tr>
<tr>
<td>Hep G₂</td>
<td>-</td>
</tr>
<tr>
<td>Jurkat</td>
<td>-</td>
</tr>
<tr>
<td>Dalton lymphoma</td>
<td>-</td>
</tr>
</tbody>
</table>

Staining pattern: ++ - Strong, +++ - Very strong, - - Negative
Fig. 3.3 Immunoperoxidase staining of frozen breast tumor section using culture supernatant of clone ERD7 x 250.
Fig. 3.4 Immunoperoxidase staining (PAP) of frozen tumor tissue section using culture supernatant of clone ERE5. A case of infiltrating ductal carcinoma X 300.
Fig. 3.5 Immunoperoxidase staining (peroxidase – antiperoxidase) of frozen tumor tissue section using culture supernatant of clone ERE10. A case of infiltrating ductal carcinoma X 250.
Table 3.3: Reactivity of ER MAbs with Breast Tissue Sections by Immunocytochemical Assay (PAP Method)

<table>
<thead>
<tr>
<th>Histological type</th>
<th>No. of tumors studied</th>
<th>Immunostaining of ER MAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ERD7</td>
</tr>
<tr>
<td><strong>Benign</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(++)</td>
<td>(++)</td>
</tr>
<tr>
<td>Cystic hyperplasia</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td><strong>Malignant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infiltrating ductal carcinoma</td>
<td>20</td>
<td>13</td>
</tr>
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<td></td>
<td>(++)</td>
<td>(+++)</td>
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<td>Intraductal carcinoma</td>
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<td>(++)</td>
<td>(+++)</td>
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<td>(+)</td>
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<td>Mucinous carcinoma</td>
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<td>1</td>
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<tr>
<td></td>
<td>(++)</td>
<td>(+++)</td>
</tr>
</tbody>
</table>

Staining intensity:  + - Moderate, ++ - Strong, +++ - Very Strong, - - Negative
The ER status of female breast tumors has been evaluated by the conventional biochemical ligand assay. There are certain limitations to the ligand assay. Since part of estrophilin molecules have been found to be saturated with circulating 17β-estradiol (Jordan et al., 1986 and Goussard et al., 1986). Also the tissue specimen used for ER assay may not be representative of the breast tumor ER status as a whole resulting in less response to hormonal therapy. In order to overcome these drawbacks, monoclonal antibodies have been generated to ER protein which have found potential application in the evaluation of immunoassays like EIA for tumor cytosol and ICA for cell or tissue cytochemical study (Greene and Jensen, 1982). In earlier studies, polyclonal antibodies have been generated to ER isolated from calf uterus, which have been found to exhibit of specific staining of both cytosolic and nuclear ER.

The polyclonal antibodies have been found to possess reactivity to some extent with other ER related proteins present in various other organs (Greene et al., 1977). In order to achieve generation of very specific antibodies of predefined specificity, the hybridoma technology has been adopted for the generation of monoclonal antibodies to ER protein (Kohler and Milstein, 1975).

Numerous antibodies to ER protein have been reported by various research workers of which Dr. G.L. Greene and Dr. L.S. Miller have succeeded in generating highly specific ER monoclonal antibodies (Greene et al., 1980a and Miller et al., 1982) MCF-7, human breast cancer cell line has been used as a very rich source of ER protein (Soule et al., 1973).

In the present study, ER isolated from both cytosolic and nuclear ER of MCF-7 cells and ER+ breast tumor tissues has been used as immunogen. Of the positive clones identified resulting from several fusions, 3 clones designated ERD7 against MCF-7 cells and ERE5 and ERE10 against human breast tumor tissues have been generated. Studies on
These 3 clones have been stabilized by subcloning thrice by limiting
dilution and have been found to exhibit monoclonality as revealed by very
specific staining of frozen breast tumor tissue sections. These three clones
have been taken into large cultures, cryopreserved in liquid N\textsubscript{2} and also
used for the generation of hybridomas in BACB/c mice. 2-5 x 10\textsuperscript{7} cells
suspended in PBS were injected i.p into pristane primed BACB/c mice.

The ascitic fluid was collected by sacrificing the animal when there
was maximum bulging of the abdomen, allowed to clot and centrifuged at
3000 rpm for 10 min in a cold centrifuge and stored in aliquots at -70\textdegree C
using sodium azide as preservative. The culture supernatant of each clone
has been collected, centrifuged and stored in aliquots with preservative at
-70\textdegree C.

The culture supernatant as well as the ascitic fluid have been
purified by ammonium sulphate precipitation (50% saturation) followed by
Protein A-Sepharose Affinity Chromatography. The purification profile for
monoclonal antibody ERE5 is represented in Fig. 3.6. The fractions were
collected in LKB fraction collector and the immune reactive fractions,
identified by ELISA pooled, lyophilized and stored.

Isotyping has been carried out by outerchonly agar gel diffusion
using specific goat antimouse Ig classes. These antibodies have been found
to be of IgG\textsubscript{1} isotype.

3.8.1 Discussion

The development of monoclonal antibodies against ER has opened
a new perspective in the endocrinological evaluation of breast cancer
patients.
various types of ER+ and ER- cell lines and normal, benign and breast tissue sections by PAP method have been carried out as a study has revealed that these 3 MAbs exhibited specific cytoplasm of only ER+ cell lines and benign and malignant breast tumors, the of staining being different in individual cases. These monoclonal have identified only cytosolic ER.

No monoclonal antibody with staining of nuclear ER identified among the positive clones resulting from various fusio

Subcloning by limiting dilution has been found to rendering these antibodies more specific thereby exhibiting reactivity in ELISA. They have been found to produce abcites in mice when injected i.p. more than 60% of animals developed ascit 3 MAbs have been purified by Protein A-Sepharose Affinity Chrom and found to be IgG1 isotype. Purification of monoclonal anti affinity chromatographic procedures have been reported by othe

By Protein A-Sepharose Affinity Chromatography, the purification with good yield of MAbs has been achieved. Stud carried out have indicated that these three MAbs generated to E might prove useful in the study of ER+ breast tumors. Further has been ascertained by performing very sensitive and immunological and immunochemical studies.

3.8.2 Conclusion

ER protein isolated from both cytosolic and nuclear fr MCF-7 cells and ER+ breast tumor tissues was used as immun immune spleen cells and mouse myeloma cells were fused in the of 50% PEG. Out of several fusions performed positive clones were by the screening assay ELISA using 96 well falcon plates coated w
of ER protein per well. The positive clones identified by ELISA were studied by PAP method for the immunocytochemical analysis using both frozen and breast tumour tissues. Three hybridomas designated ERD7, ERE5 and ERE10 were found to exhibit strong cytoplasmic staining. These clones were subcloned by limiting dilution and taken into large cultures for the production of specific antibodies. 2-5 x 10⁷ cells were injected i.p into pristane primed BALB/c mouse. The ascites fluid was drained after 10 days. One portion of the hybrid cells were preserved in liquid nitrogen for future use. The culture supernatant and ascitic fluid were purified by using 50% ammonium sulphate precipitation and followed by Protein A-Sepharose Affinity Chromatography. All the three monoclonal antibodies were found to be IgG₁ isotype.