Breast cancer is one of the major public health problems and also a leading cause of death in women. Despite the recent advances made in the management of breast cancer, it is regrettable to note that the incidence of this dreadful disease is increasing and there has been no noticeable fall in the mortality rate. Statistical data have revealed that one in 9 women will develop breast cancer during her lifetime. The important risk factors associated with the development of breast cancer that cause much concern to many investigators are age, reproductive events-menarche, child birth and menopause, genetic predisposition, hormonal status, family history and lifestyle factors such as diet and alcohol consumption. It has long been acknowledged that during differentiation, the tissues associated with reproductive processes need minute amounts of steroid sex hormones for their optimal growth and function (Jensen and Jacobson, 1960). Some breast cancers require ovarian steroids to stimulate their growth. Among them, estrogens are thought to be essential for the initial development of most mammary tumors. The exact mechanism by which they modulate such growth is unknown (Osborne et al., 1990 and Gullick, 1990). The elucidation of molecular mechanisms underlying the hormonal regulation of cell proliferation in breast cancer has been the focus of much scientific interest. In other words, the importance of estrogen in the etiology of breast cancer has provided the impetus to explore the mechanism of action of this steroid hormone. Many investigators have been interested in the criteria for selecting breast cancer patients who are likely to respond to endocrine manipulations. This has been rather imprecise since 1896, when George Beatson reported that several premenopausal breast cancer patients obtained dramatic remission of metastatic disease following oophorectomy.
The endocrine ablative surgery was generally accepted for hormone dependent cancers only after the introduction of orchiectomy for the treatment of prostate cancer (Huggins and Hedges, 1941) and the use of adrenalectomy (Hufnins and Bergenstal, 1952), hypophysectomy (Luft and Olivecrona, 1953) for the treatment of metastatic breast cancer in postmenopausal women.

A major step forward in this field occurred, when animal studies revealed the nature of differences between the interaction of steroids with target and nontarget tissues and this formed the basis for clinicians to diagnose and treat breast cancer patients properly. Early studies by Folca et al., 1961 showed that the greater uptake of labelled hexestrol by breast tumors of patients who responded favourably to endocrine ablative surgery. The outcome of such in vivo study has helped to differentiate responsive and non-responsive breast cancers, but it did not provide a practical approach to routine diagnosis of the endocrine responsiveness of ablation. The extensive research carried out in many laboratories worldwide have led to a recognition that steroid hormone effects their biologic responses in target cells through the mediation of specific high affinity binding proteins called receptors (Ehrlich, 1902 and McGuire et al., 1982). Based on cell fractionation and sucrose density gradient analysis, the mechanism of steroid hormone receptors was proposed independently in 1968 by Jensen et al., and Gorski et al. According to this hypothesis, estrophilin (estrogen receptor) is thought to influence the metabolism of target cells through the following series of interactions (a) estrogens enter the cell by passive diffusion, (b) unoccupied receptor in the cytoplasm of the target cell binds the estrogen and (c) undergoes a process referred to as temperature-dependent activation, (d) that causes the hormone receptor complex to become highly nucleophilic, (e) where it interacts with chromatin and (f) induces both qualitative and quantitative changes in transcriptional patterns of mRNA. This is schematically represented in Fig 1.1. Recent studies have suggested that the steroid passes through the cell to the
Fig 1.1 Schematic representation of old model for the sequence of events in the mechanism of steroid hormone action on a target
nucleus, either unaided or perhaps bound loosely to low affinity sites in the cytoplasm, where it interacts with unoccupied receptor in the nucleus resulting in the formation of the activated steroid-receptor complex (Welshons et al., 1984). This revised interpretation is represented in Fig. 1.2. The advances made in the development of techniques have proved very useful in measuring estrogen binding activity and correlation of their levels with clinical response. The large body of knowledge has now unequivocally established that the breast cancer patients with ER+ tumors have a long disease-free interval and better survival than those with ER- cancers. In addition, they are more likely to respond successfully to endocrine therapies (Knight et al., 1977, Bishop et al., 1979 and Furmanski et al., 1980).

Extensive experimental studies were made in estimating the estrogen binding capacity in excised tumor specimens by incubating it with dilute solutions of tritiated estradiol in the presence and absence of an inhibitor. The slice uptake study was proved to be less sensitive, requiring large and fresh tumor samples (Jensen et al., 1971). Then the need to search for more sensitive techniques was emphasized. The concept that the receptor protein of target cells originally in the cytoplasm and moves to the nucleus after combining with the hormone has provided an improved approach to devise more sensitive and quantitative estimations of estrogen binding capacity, simply by adding an excess of radioactive estradiol to a soluble extract of broken tumor cells and determining the radioactivity specifically bound to receptor protein (Jensen et al., 1967). The separation of estrogen receptor complex from the unbound steroid may be effected in different ways viz, Sucrose density gradient centrifugation (Toft and Gorski, 1966), Agar gel electrophoresis (Jungblut et al., 1972), Dextran coated charcoal assay (Korenman and Dukes, 1970), Isoelectric focusing (Wrangel et al., 1976) and Gel filtration through Sephadex G-25 (Godefroi and Brooks, 1973). The different molecular forms of ER can be determined on 5-20% sucrose density gradients. As an alternative to sedimentation analysis, the estradiol-receptor complex of tumor cytosols can be estimated by agar gel
electrophoresis. The third approach has been employed for the separation of estradiol-receptor complex by filtration through Sephadex G-25. The widely used method for the determination of ER in breast cancer is the Dextran Coated Charcoal assay (DCC), in which unbound hormone is removed from an estradiol-cytosol mixture by adsorption on dextran charcoal. The use of a radioactive steroid as a marker for the protein to which it binds has provided essentially all of our knowledge concerning estrogen receptors and their interaction in cells. Though these methods are accurate and reliable, they require significant amounts of tissue, time consuming to perform, and sophisticated equipments.

Despite the overwhelming evidence for the usefulness of binding assays for steroid receptors, there are still some recognized intrinsic limitations to their successful application. Biochemical assays for ER suffer from a number of inherent limitations; they do not account for approximately 1/3 of ER+ breast cancer patients who do not respond to endocrine therapy. Breast cancers are frequently heterogeneous both morphologically and in terms of their behavior. This heterogeneity has been observed in estrogen receptor assays. Currently used biochemical assays for ER were carried out on homogenates which obscure the source and proportion of ER+ cells in enclosing a heterogeneous population of cells (Poulsen et al., 1981). There is a clear need for analytic procedures which are simpler, less expensive and more accurate than the conventional steroid binding assay. The desirability of developing new quantitative histochemical immunohistochemistry of the receptor protein would prove to be more reliable and clinically useful as an assay to study ERs in human breast cancer. Such an immunochemical approach will provide the added advantages of enabling the observer to correlate the ER content with tumor histopathological characteristics, to assess the heterogeneity of ER+ cancer cells within tumors and to evaluate breast lesions that are too small for conventional methods.
radioligand assays. The first immunochemical methods were reported by Nenci et al., 1976 and later on by Perschuk et al., 1976. Later, immunoperoxidase procedures were proposed by Ghosh et al., 1978 and Kurzon and Sternberger et al., 1978. These histochemical techniques have been reported as a possible means of identifying ER and PR in histologic sections. All of these methods have used steroids linked directly to fluorescein conjugates composed of fluorescein-bovine serum albumin-estradiol, rhodamine-BSA-progesterone and polyestradiol phosphate in order to localize receptor in tissue sections. These methods were reported to have a number of problems due to the low affinity and specificity of conjugates for the receptor and also low stability of the conjugates. There has been a growing need for alternative methods for detecting and measuring estrophilin which will recognize receptor whether or not it is occupied with hormone. A long effort in the purification of ER from calf uterus has resulted in the production of polyclonal antibodies to the receptor in rabbit (Greene et al., 1977) and subsequently in goat (Greene et al., 1979). These polyclonal antibodies were found to react with both cytosolic and nuclear ER complexes from calf uterus and do not react with non-specific estrogen binding proteins. Though the goat and rabbit polyclonal antibodies to the ER are very useful reagents, there are some drawbacks which limit their use in the development of certain new immunochemical assays for ER.

The revolutionary hybridoma technology introduced by Kohler and Milstein in 1975 has represented a quantum leap in molecular biology and tumor immunology. It has thrown a new light on the etiology, detection, treatment and prognosis of the breast cancer. For the first time, B lymphocytes from immunized host could be immortalized by fusing with drug selected non-immunoglobulin secreting murine myeloma cells. The supernatant fluids of cultures from the cloned cell populations termed hybridomas could be assayed for the presence of homogeneous populations of immunoglobulins with the desired reactivity. The first monoclonal
antibodies to mammalian ER were prepared using polyethylene glycol mediated fusion of splenic lymphocytes from male Lewis rats, immunized with the partially purified nuclear estrophilin from calf uterus, with the mouse myeloma cells (P3-X63-Ag8, P3-NS1/1-Ag4-1 and Sp2/O-Ag14) (Greene et al., 1980a). Monoclonal hybridoma preparations of IgM and IgG2a were obtained. Unlike the polyclonal antibodies, these MAbs were species specific, and they recognized 4S cytosol E*R and 5S nuclear E*R only from calf uterus. Subsequent efforts have been directed to develop immunochemical reagents for the detection and measurement of ERs in tissues of other species. With the advancement in the affinity chromatography system, the rapid purification of cytosol receptor from MCF-7, human breast cancer cells has become feasible. The MAbs to human ER were prepared by fusing immune splenic lymphocytes of male Lewis rats immunized with affinity purified estrophilin from MCF-7 breast cancer cells with two different mouse myeloma lines Sp2/0.Ag.14 and P3-X63-Ag8. Three cloned hybridomas D58, D75, D547 were obtained, each of which secretes a unique idiotype of antibody that recognizes a distinct region of the ER molecule (Greene et al., 1980b). Subsequent fusions carried out by Greene et al., (1984) and Dr.L.S.Miller (1982) at Abbott Laboratories have resulted in 13 MAbs to ER, all of which (with one possible exception) recognize distinct regions of the receptor molecule. These MAbs have high affinity for both steroid occupied and unoccupied ER and recognize nuclear as well as cytosolic forms of the receptor molecule. Moncharmont et al., (1982) have reported that their ER MAb recognized only the steroid occupied form of the receptor.

Based on sucrose density gradient and immunoblot analyses, all antibodies appear to be completely specific for the 65,000-70,000 dalton steroid binding protein from either nuclear or cytosolic fraction. The cross-reactivity patterns indicate both sequence homology and heterogeneity among mammalian and nonmammalian estrogen receptors. Several antibodies appear to recognize determinants common to all tested ERs
(H222 & H226). D547 and D58 were found to be specific for determinant present only in mammalian receptors whereas D75 appears to be restricted to primate ER. With the help of limited proteolytic digestion and sucrose density gradient analysis of steroid binding as well as the more recent use of restriction fragments of ER cDNA, the distribution of various epitopes in relation to each other and to steroid and DNA binding domain has been determined. Epitopes for D75, D547 and H226 ER MAbs are susceptible to selective cleavage by papain, chymotrypsin and trypsin. It is interesting to note that those epitopes which are best conserved across all tested species are located either near steroid binding domain (H23, H142, H165, H221, H222) or the DNA binding domain (H226). D75 recognized an epitope near the carboxy terminus and D547 recognized an epitope between the steroid binding and DNA binding domains (Greene, 1987). A combination of two monoclonal antibodies which react with independent antigenic sites on the receptor molecule and associating with the receptor simultaneously has been used to devise immunoassays for ER viz., immunoradiometric assay (IRMA) and enzyme immunoassay (EIA) (Nolan et al., 1984). The principle of immunoassays is depicted in Fig. 1.3. A solid phase, sandwich type enzyme immunoassay has been developed by Abbott laboratories using two monoclonal antibodies (D547 and H222) which recognize different antigenic sites on the ER molecule (Nolan et al., 1984). The introduction of this immunoassay was meant to represent an alternative method and a competitive to the established steroid binding assays. Parallel assays of tumor cytosolic fractions using the ER-EIA and ER-DCC revealed an excellent linear correlation between the assays at low and medium receptor levels (Spona et al., 1986).

In order to overcome major disadvantages in the quantitative receptor assays performed on tissue homogenates, numerous attempts have been made in developing immunocytochemical assays for visualizing these steroid receptor proteins directly in tissues and cells. With the availability of specific ER MAbs, specific and very sensitive methods for
SANDWICH TECHNIQUE FOR IDENTIFYING ESTROGEN RECEPTORS

Ab, attached to ER molecule at second binding site. The antibody is tagged with either radioactive label or peroxidase enzyme.

Fig 1.3 Schematic presentation of immunometric and enzyme-linked immunoassay for detection of ER.
immunocytochemical assay of receptor became possible. These ER MAbs have been successfully used in experimental studies to demonstrate ER cytochemically in several target tissues using both frozen and paraffin embedded sections (King et al., 1982 and Ozzello et al., 1985). It has also been shown that the immunocytochemical visualization of ER in tissue sections correlates with the biochemical assays for ER in tumor homogenates and appears to be a predictor of response to antiestrogen therapy and of the disease-free interval or survival (King et al., 1985).

It is already demonstrated by Masood and Johnson (1987) that the assessment of ER using imprint preparation is comparable to that obtained from conventional cytochemical techniques on frozen sections as well as with DCC. The application of fine needle aspiration (FNA) biopsy has gained wide acceptance as a useful technique in the evaluation of primary and metastatic lesions. FNAB of the breast is sensitive and accurate in detecting breast carcinoma (Silversward and Humla, 1980). It has been reported to show significant correlation between the biochemical and cytochemical analysis by Katz et al., 1990. The prognostic, diagnostic and therapeutic implications of estrogen receptors in the management and follow up of patients with gynaecological and other carcinomas have been well documented by Ehrlich et al., 1981, Kauppila et al., 1983 and Gao et al., 1983.

Besides estrophilin, ER related proteins have also proved to be of clinical value in the assessment of hormone dependency of breast cancer. Two estrogen regulated peptides of molecular weight 24,000 and 52,000 have been characterized in breast cancer cells (Westly and Rochefort, 1980 and Ciocca et al., 1983). These estrogen regulated proteins are of potential interest since their detection might increase the degree of predictability of receptor assays and improve our understanding of the mechanism(s) by which estrogens favour cell proliferation and mammary carcinogenesis.
The role of the estrogen receptors as mediators of estrogenic action in target organs is today unquestioned. The determination of ER content in human mammary tumors has a clinical value in that it provides guidance for the selection of suitable therapy (Horwitz et al., 1975). Thus, the management of patients with breast cancer needs reliable tests of therapeutic responsiveness. A wide variety of approaches have been used, which have led to the search for estrogen activated cellular enzymes, genes, autocrine growth factors as well as extrinsic factors which may modulate the effect of estrogen or the target response to estrogen (Butler et al., 1981, Aitken and Lippman, 1985, Bates et al., 1985 and Levine et al., 1985).

The measurement of an estrogen induced product is an undoubted improvement in comparison to the simple estrogen receptor assay. The polyamines, putrescine (PU), spermidine (Spd) and spermine (Spm) are intracellular molecules that play an important role in the mitotic cellular growth of normal and neoplastic tissues (Janne et al., 1978). It was reported that the inhibition of these polyamines accumulation parallels the attenuation of the growth rate of tumor. It has been consistently observed that the polyamines play an essential role in mediating the estrogen effect on hormone responsive N-methyl-N-nitrosourea induced rat mammary tumor (Manni and Wright, 1984). It has been suggested that the antitumor effect of tamoxifen is mediated through the induction of polyamine depletion (Metcalf et al., 1978). The depletion of polyamines has been studied extensively with the development of specific inhibitors of polyamine biosynthetic enzymes. It is not altogether surprising that early efforts were focused on inhibitors of ornithine decarboxylase (ODC), a key enzyme in the biosynthesis of polyamines. It has been established that ODC activity is stimulated by estrogens in their target cells (Cohen et al., 1970). The suppression of polyamine biosynthesis through the specific inhibitors play a critical role in understanding the molecular mechanism of action of antiestrogens. Thus
the use of a combination of antiestrogens and polyamine inhibitors in *in vivo* and *in vitro* studies may prove to be beneficial in the clinical management of estrogen responsive breast cancer.

In the course of this introduction, salient features relating to the application of estrogen receptor in the management of carcinoma of the breast using monoclonal antibodies has been detailed with all recent developments in the area of steroid receptor studies. A brief account of the experimental study relating to the significance of antiestrogen and antipolyamine therapy has also been included.