Immunocytochemical Localisation of Peptide Hormone Binding Sites
OBJECTIVES AND PLAN OF WORK

Human breast is generally considered to be a passive organ being a 'target tissue' for both steroids (estrogen and progesterone) and peptide (PRL, GH etc.) hormones. It was reported that even in the absence of lactation there is marked rise in serum PRL when female breast are mechanically stimulated (Kolodny et al., 1972). However, in another study gentle palpation of breasts or stimulation of nipples for 10 seconds was not associated with appreciable rise in blood PRL levels (Peters et al., 1982). Further reproductive disturbances reported after mastectomy in goats raised the question as to whether human breasts were acting as endocrine organs (Diamond et al., 1982). Changes in breast epithelium and stroma are seen with the hormonal changes of the menstrual cycle. Alteration in the hypothalamic-pituitary axis function have been demonstrated in patients with benign disease (Kumar et al., 1984a&b). It is unknown, however, whether the anterior pituitary hormones have any direct action on the breast tissue. Since the breast cells were shown to possess specific binding sites for estrogen and progesteron (McGuire et al., 1977), the effect of LH and FSH on the breast tissue may be through their effect on the ovarian secretion. PRL is known to affect the milk secretion but the effect of FSH & LH is unknown in breast.

The aim of this study, therefore, is to see if there is any specific binding sites for the peptide hormone (PRL, LH, FSH) in normal and pathological breast tissue.

With this aim, the main objectives of the present study were-
i) to determine the peptide hormone binding sites in human breast tissue slices using a highly sensitive immunocytochemical localization technique.

ii) to determine if there are any functional correlation between the hormone binding and the breast.

iii) to identify 'high risk' changes in the benign breast tissue by retrospective and prospective staining of the breast tissue.

iv) to demonstrate PRL binding sites in fine needle aspirate cytology.

The binding was accomplished using DNP hapten sandwich staining (DHSS) procedure of Jasani et al. (1981). The second antibody used were labelled with hapten DNP and a monoclonal antihapten antibody was incorporated as a bridge between the primary antibody and the enzyme system. The colour reaction was developed using diaminobenzidine in the presence of nacent hydrogen peroxide generated by glucose oxidase in the presence of added glucose (Fig. 3.1).
Glucose

DNP

labelled glucose

oxidase

DNP

labelled per-oxidase

conjugate

Monoclonal DNP

Specific bridge Ab

DNP

labelled

universal 2nd Ab

Primary Ab

Antigen

Fig. 3.1: Schematic representation of DNP-Localisation System – their mode of interaction
INTRODUCTION

Out of the various approaches for studying hormonal binding sites in a tissue immunocytochemistry is potentially of great interest. A tumour may consist of a mixture of positive and negative cells for peptide binding sites. Since, the biochemical technique requires tissue homogenization, this technique is not able to distinguish between the two types of cells. Autoradiography, on other hand, requires use of radioactive material. Immunocytochemistry could identify these cells by means of a specific antigen-antibody reaction rendered visible by a suitable marker. Keeping this in view a detailed study of immunocytochemically localising the peptide hormone mainly the PRL binding sites in various breast lesions was undertaken. Such a finding will open gates for a large number of experimentation and treatment for breast disease.

Immunocytochemically demonstrable PRL Immunoperoxidase techniques for visualising PRL, human GH, human placental lactogen in human breast cancer tissue using an antibody bridge technique (de Souza et al., 1977) replaced the autoradiographical techniques of localising the binding sites (Birkinshaw and Falconer, 1972, Rajaniemi et al., 1974). The technique demonstrated the apparent presence of both PRL binding sites and endogenous immunoreactive PRL in lactating mammary gland alveolar cells of rat suggesting the presence of PRL receptors also within the cytoplasm, under physiologic condition, in addition to the cell membrane (Nolin and Witorch, 1976).

Using immunocytochemical techniques hormone binding sites in tissues other than the breast i.e. PRL, FSH, LH and GH in prostrate (Sibley et al.,
1981), PRL & 24K protein sites in secretory endometrium (Mary et al., 1986) and gonadotrophic and PRL binding sites in normal and neoplastic ovaries (Al Tumimi et al., 1986) was also demonstrated.

The presence of PRL has been demonstrated in benign and cancerous breast tissue sections using an immunocytochemical approach (Purnell et al., 1982; Dhadly and Walker, 1983). Out of the various fixative explored for immunocytochemistry to examine the effect of these on the PRL receptors, formalin fixed tissue was found to be the most suitable (Salih et al., 1979). It has also been traced in different experimental conditions such as in cryostat sections and cell suspensions of breast cancer (Marchetti et al., 1984) and also in cancer cells in tissue culture (Patterson et al., 1982), exploiting the immunocytochemical techniques.

Detection of the PRL positive benign and malignant tumours could probably suggest the importance of PRL as a growth factor for these tumours and give further support to the use of anti PRL drugs in the therapy of the disease.

Besides applying immunocytochemistry on the tissue sections for localising PRL binding sites in breast lesions an attempt was made to localise these sites in fine needle aspirate also (FNA). By this method the cells are obtained by a small gauge needle generally with a vacuum system provided by an airtight syringe from any part of the body. This technique, now popularly used as the diagnostic tool, offers several advantages over the use of surgical biopsies for hormonal receptor studies. It is relatively easy, cost effective and non-traumatic procedure as compared to surgical biopsy that provides tissue
for hormonal studies. In cases where surgery is not possible this is the only suitable method for such studies.

Immunoperoxidase technique has already been utilized to study ER (Flowers et al., 1986; Masood S., 1989) and PgR (Lozowski et al., 1990) in FNA smears of patients with breast cancer as enough cells for demonstrating the receptors could be obtained by this technique. However, studies on the PRL binding in cytology specimens of breast tissue is hitherto undescribed.

An initial attempt has also been made to determined whether breast has any binding sites for FSH and LH in benign and malignant condition.
A. PEPTIDE HORMONE BINDING SITES IN PARAFFIN SECTIONS

MATERIALS AND METHODS

MATERIALS

Patients with benign and malignant breast conditions were studied clinically and histologically. A detailed history was taken and physical examination was done in all the patients. Age, onset of symptoms and progress of the symptoms was recorded in all the patients. Fresh breast tissues were collected in formalin from operation theatres and processed to prepare the slides for histology. Paraffin embedded sections of about 5 μm thickness were cut and picked up on gelatinized slides. Both histochemical and immunocytochemical studies were done on these slides. Also retrospective breast tissue sections comprising of both benign and malignant conditions were included in this study.

Immunocytochemical localisation of PRL, FSH and LH binding sites were carried out using a highly sensitive immunocytochemical method, the dinitrophenyl hapten sandwich staining procedure. It represents a novel versatile, indirect immunoperoxidase bridge technique designed for the detection of unlabelled primary rabbit antibodies reacted with antigen present in the sections or smears.

The histopathological type of tissue selected for localising the three peptide hormone binding sites are listed in Table 3.1. Benign breast disease were classified into two groups - i) Non proliferative lesions and ii) proliferative lesions (Dupont and Page, 1985).
The primary antisera used in this study were rabbit antihuman PRL, rabbit antihuman FSH and rabbit antihuman LH hormone. These as well as secondary detection reagent kits were obtained from Biochemical Services Ltd., PO Box 129, Cardiff CF44VU, U.K. BSA (A-8022), 3,3-Diaminobenedine tetrahydrochloride (DAB) (D-5637) and glucose (G-5250) were purchased from Sigma Chemical Corporation (USA). All the other chemicals and reagents were of the highest purity available.

Table 3.1
BREAST LESIONS STUDIED FOR LOCALISING PRL, FSH AND LH BINDING SITES.

<table>
<thead>
<tr>
<th>Breast Lesion</th>
<th>PRL</th>
<th>FSH</th>
<th>LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Malignant</td>
<td>40</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>2. Non-proliferative</td>
<td>50</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>3. Proliferative</td>
<td>7</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4. Lactating adenoma</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5. Gynecomastia</td>
<td>8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>107</td>
<td>35</td>
<td>28</td>
</tr>
</tbody>
</table>

METHODS

Five \( \mu \text{m} \) thick sections cut from the breast tissue, pituitary gland and lymph node were picked upon gelatinised slides (15\% gelatin solution was smeared on the slides and these slides were kept at 40°C overnight for proper setting of gelatin). In each case routine haemotoxylin and eosin staining was carried out along with immunocytochemical method for histopathological diagnosis. Immunocytochemical staining was carried out in several batches consisting of 8-10 slides. Each batch included a section of pituitary and a section of lymph node.
Dewaxing of paraffin section was done by treating the slides with below mentioned reagents.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylol 1</td>
<td>2-4 times at R.T. (17°C to 25°C)</td>
</tr>
<tr>
<td>Xylol 2</td>
<td>&quot;</td>
</tr>
<tr>
<td>Xylol 3</td>
<td>&quot;</td>
</tr>
<tr>
<td>Xylol 4</td>
<td>&quot;</td>
</tr>
<tr>
<td>Alcohol 1</td>
<td>&quot;</td>
</tr>
<tr>
<td>Alcohol 2</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

**Haemotoxylin-Eosin staining technique**

- Deparaffinised sections were washed with running water.
- Sections were stained in a solution of haemotoxylin for 2-5 mins. for nuclear staining.
- Washed in water till the section was blueing.
- Decolourised with a solution of acidic alcohol (1%) till the cytoplasmic staining by haemotoxylin was removed.
- Washed in running tap water for 5 to 15 mins.
- Counterstained in 1% aqueous eosin for 1 min.
- Washed rapidly in water and blotted.
- Dehydrated in several changes of absolute alcohol.
- Cleared in xylol and mounted in Canada Balsam.

**Immunocytochemical staining technique**

A detailed stepwise description of the staining protocol is given below.
Materials & Methods

i) **Inhibition of Endogenous peroxidase:*** For this purpose pre-incubation of deparaffinised sections was done in methanol containing hydrogen-peroxide. The slides were immersed into a mixture of 47.2 ml of methanol and 0.8 ml of 33% hydrogen peroxide for 30 minutes at R.T. (Streefkerk, 1972).

ii) **Rehydration:** Slides were washed by immersing them into distilled water for 2 minutes x 3 changes. Slides were then transferred in a jar containing PBS (0.01 M, pH=7.1) for 2 mins x 3 changes. After these steps, dinitrophenyl localisation system was used for detecting primary antigenic sites.

iii) **Incubation in primary antibody:** The primary antibody was applied at the optimum dilution (dilution profile experiment was carried out for each antibody to evaluate the appropriate dilution). PRL was applied at dilution 1:15,000, FSH at 1:10,000 and LH at 1:7,000. The dilution was done with 0.6% BSA in PBS. The slides were covered with either of the required primary antibody (50 ul to 100 ul) and left overnight at 4°C in a closed moistened box.

iv) Washing in PBS was conducted by total immersion of the slides in a coplin jar (1 min. x 3 changes).

v) Incubation in excess of second antibody labelled with DNP was for 30 mins. at R.T. The second antibody used here was antirabbit IgG tagged with DNP.

vi) Washing in PBS as in step iv.

vii) **Incubation in IgM anti DNP bridge antibody** was achieved by applying 50 ul unto 100 ul of a 1:500 dilution of hybridoma asate fluid to the slides for 30 mins. at RT.

viii) Washing in PBS as in step iv.
ix) Incubation in DNP peroxidase conjugate in dilution 1:800 of a standard preparation for 30 mins. at RT.

x) Washing in PBS as in step iv.

xi) Incubation in DNP glucose oxidase (100 units/ml solution) for generation of nacent oxygen (50 ul to 100 ul) for 30 mins. at RT.

xii) Washing in PBS as in step iv.

xiii) Incubation in 25 μg DAB + 750 mg glucose dissolved in 50 ml PBS (0.1M, pH 7.1) in a coplin jar left overnight at room temperature in a dark place.

xiv) Washing in distilled water (1 min. x 3 changes).

xv) Immersion of slides in 1% acetic acid (1 min.)

xvi) Counterstaining was done as usual.

xvii) Finally sections were dehydrated and mounted in Canada balsam and studied under the microscope.

Controls
i. Sections from postmortem anterior pituitary were used as positive controls.

ii. Sections from lymph node were used as negative controls.

iii. Omission of primary antibody and substitution with appropriate dilution of BSA.
RESULTS

One hundred and seven formalin fixed paraffin embedded breast tissue sections were stained for localisation of PRL hormone receptor sites. Among these thirty five and twenty eight adjacent sections were also stained for FSH and LH binding sites respectively. These could be classified as malignant, benign non proliferative breast disease, benign proliferative breast disease, lactating adenoma and Gynaecomastia. The percentage positive immunocytochemical staining of the three above mentioned peptide in each group are given in Table 3.2. Positive immunostaining reaction was predominantly localised in the cytoplasm of the epithelial cells present in various breast lesions and was heterogeneously distributed. Sometimes there was irregular staining of the cell periphery and some nuclei. Epithelial cells of both duct and acini showed positive staining. In some, the staining was consistent in all ducts and lobules whereas in others there was a variation in intensity of staining reaction between ducts and their associated lobules.

<table>
<thead>
<tr>
<th>Lesions</th>
<th>PRL No.</th>
<th>PRL %</th>
<th>FSH No.</th>
<th>FSH %</th>
<th>LH No.</th>
<th>LH %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant</td>
<td>33/40</td>
<td>82.5</td>
<td>9/15</td>
<td>60</td>
<td>4/10</td>
<td>40</td>
</tr>
<tr>
<td>Non-proliferative benign breast lesion</td>
<td>32/50</td>
<td>64</td>
<td>7/14</td>
<td>50</td>
<td>6/12</td>
<td>50</td>
</tr>
<tr>
<td>Proliferative benign breast disease</td>
<td>3/7</td>
<td>48</td>
<td>1/3</td>
<td>33</td>
<td>1/3</td>
<td>33</td>
</tr>
<tr>
<td>Lactating adenoma</td>
<td>2/2</td>
<td>100</td>
<td>0/1</td>
<td>0</td>
<td>0/1</td>
<td>0</td>
</tr>
<tr>
<td>Gynaecomastia</td>
<td>1/8</td>
<td>12.5</td>
<td>1/2</td>
<td>50</td>
<td>1/2</td>
<td>50</td>
</tr>
</tbody>
</table>
PRL binding

*Malignant (Carcinoma):* Majority (82.5%) of the breast carcinoma sections (33/40) revealed positive staining for PRL binding sites (Fig. 3.2). In some carcinoma cases, majority of cells were positive whereas in others the positive staining cells were intermixed with negative cells thus showing heterogeneous reaction (Fig. 3.3). Omission of primary antibody resulted in negative staining (Fig. 3.4a and 3.4b).

### Table 3.3

<table>
<thead>
<tr>
<th>Different Histological Types of Carcinoma</th>
<th>No. of Cases</th>
<th>Immunocytochemical reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Weak</td>
</tr>
<tr>
<td>Well differentiated (ductal carcinoma)</td>
<td>19</td>
<td>7 (36.8%)</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>2</td>
<td>1 (50%)</td>
</tr>
<tr>
<td>Papillary adenocarcinoma</td>
<td>3</td>
<td>1 (33.3%)</td>
</tr>
<tr>
<td>Lobular carcinoma</td>
<td>6</td>
<td>1 (16.6%)</td>
</tr>
<tr>
<td>Carcinoma associated with Paget's disease</td>
<td>2</td>
<td>2 (100%)</td>
</tr>
<tr>
<td>Metastasising into lymph node</td>
<td>8</td>
<td>-</td>
</tr>
</tbody>
</table>

On further analysis of grade of malignancy in relation to immunocytochemical staining it was observed that well differentiated ductal tumours showing stronger positive staining and had more cells reacting as compared to poorly differentiated tumours having small number of cells that were stained (Table 3.3). Out of three cases of papillary carcinoma one showed weak reaction with only few cells stained while other two cases were
Fig. 3.2: Breast carcinoma - Section showing positive staining for PRL binding sites.

Fig. 3.3: Breast carcinoma - showing heterogeneity of immunocytochemical staining.
Fig. 3.4a: Carcinoma breast - Positive for PRL binding sites.

Fig. 3.4b: Above case - Negative staining when primary antibody is omitted.
Materials & Methods

completely negative. Similarly, lobular carcinoma and carcinoma associated with Paget’s disease were weakly positive. However, the metastasing tumours showed stronger reaction with more number of cells showing positive reaction whereas the lymphocytes were negative (Fig. 3.5). The normal epithelial cells within the tumour areas were generally negative.

A relationship between age of the patient and intensity as well as number of cells showing positive staining for PRL binding sites was observed. Majority of the breast carcinoma slides showed positive reaction, with many cells reacting, in tumours removed from patients above 41 years of age i.e. in the peri menopausal and post menopausal patients, whereas tumours removed from patients in younger age, the intensity of staining reaction was either poor or absent (Table 3.4).

Table 3.4
Relationship of immunocytochemical reaction in breast carcinoma with age of patients

<table>
<thead>
<tr>
<th>Lesions</th>
<th>Age Group</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>&lt;30</td>
<td>2/3</td>
<td>66.66</td>
</tr>
<tr>
<td></td>
<td>31-40</td>
<td>6/9</td>
<td>66.66</td>
</tr>
<tr>
<td></td>
<td>41-50</td>
<td>14/16</td>
<td>87.5</td>
</tr>
<tr>
<td></td>
<td>51-60</td>
<td>5/6</td>
<td>83.3</td>
</tr>
<tr>
<td></td>
<td>&gt;60</td>
<td>6/6</td>
<td>100</td>
</tr>
</tbody>
</table>

Benign Non-Proliferative breast disease: This group included fibroadenoma, apocrine metaplasia, cysts and mild hyperplasia of usual type. Fibroadenoma (no. of cases 43) comprises the major group of non-proliferative lesions in this study. Among the forty three cases of fibroadenoma two
Fig. 3.5: Metastatic carcinoma lymph node - Showing positive staining for PRL binding sites. Note that the carcinoma cells are positive whereas the lymph node parenchyma is negative.
were associated with apocrine metaplasia while two were associated with lactating breast. Leaving aside these four cases, twenty one cases (54%) of fibroadenoma out of 39 showed positive staining for PRL binding sites (Fig. 3.6). The rest were negative (Fig. 3.7). The apocrine metaplasia associated with fibrodaenoma were more intensely stained whereas ductular lining epithelial cells of fibroadenoma were either negatively or weakly stained (Fig. 3.8). The lactating breast associated with fibroadenoma were also positively stained. Three other cases of apocrine metaplasia associated with either epitheliosis, or papillomatoid hyperplasia were also positive for PRL binding sites (Fig. 3.9a,b,c). Epitheliosis and papillomatoid hyperplasia were heterogeneously stained. Other non-proliferative lesions showing duct ectasia, galactocele and benign cystic mastitis were also positive for PRL binding sites.

Proliferated benign breast disease: This group included atypical lobular hyperplasia, atypical "ductal" hyperplasia, moderate and florid hyperplasia of the usual type (epitheliosis), papillomas, ductal involvement with cells of atypical lobular hyperplasia and sclerosing adenosis. Out of 7 cases only three showed positive staining for PRL, the staining was weak and heterogeneously distributed. Epitheliosis present in three cases, two showed heterogenous staining. Lobular hyperplasia were positively stained (Fig. 3.10).

Lactating adenoma: Two cases of lactating adenoma gave intense intracellular positive staining for PRL binding sites (Fig. 3.11a,b).

Gynaecomastia: All the cases of gynaecomastia included in the study except one were negatively stained for PRL binding sites.
**Fig. 3.6:** Fibroadenoma breast - Showing positive staining for PRL binding sites.

**Fig. 3.7:** Fibroadenoma breast (another case) - Showing negative staining for PRL binding sites.
Fig. 3.8: Fibroadenoma associated with apocrine cyst. Note that the apocrine cells are positive for PRL binding sites whereas ductular lining epithelial cells of fibroadenoma are either negative or weakly positive.
Fig. 3.8
Fig. 3.9a: Apocrine cyst - Showing intense positive reaction of the metaplastic epithelial cells.

Fig. 3.9b: Higher magnification of the same case.

Fig. 3.9c: Same case, negative staining when primary antibody is omitted.
Fig. 3.10: Lobular hyperplasia breast - Showing positive reaction for PRL binding sites.
Fig. 3.11(a): Lactating adenoma breast - H.E. staining

Fig. 3.11(b): Positive reaction for PRL binding sites.
**FSH and LH binding**

Staining intensity for FSH and LH binding sites was not as much as that for PRL binding sites. In most of the carcinoma cases LH staining was absent whereas FSH staining showed weak reaction (Table 3.2). In one case out of three of benign proliferative breast disease there was positive reaction for FSH and LH binding sites. Fibroadenoma cases showed mixed reaction for FSH and LH binding sites. There was no positive staining of FSH and LH binding sites in the benign cystic lesion of the breast.

The control anterior pituitary sections were stained positive whereas sections of lymph node were not stained at all. These two tissues thus acted as positive and negative controls respectively in the present study.
DISCUSSION

Immunocytochemistry has been extensively used as a tool to localise peptide hormone binding sites in the breast tissue. A fairly consistent presence of binding of peptide hormone in both benign and malignant breast tissue had been indicated. Sections of more than 5 years old paraffin blocks of formalin fixed breast tissue could be stained as we had employed a novel and a highly sensitive method of immunocytochemistry. Earlier the use of fresh frozen material was found to be essential for localising PRL binding sites in human breast tissue (de Souza et al., 1977; Dhadly and Walker, 1983). However, in the present study PRL, FSH and LH binding sites have been detected in paraffin embedded tissue and thus is contrary to the previous reports. Nevertheless, Purnell et al. (1982) and Marchetti et al. (1984) were able to demonstrate cytoplasmic PRL binding in paraffin wax embedded tissue but using an antiserum against bovine PRL.

The binding sites for PRL were visualised by the antibodies against the hormone as the endogenous hormone is not destroyed after binding to its receptor. It remains bound to its receptors due to the very slow dissociation rate of the hormone from the receptor (Birkinshaw and Falconer, 1972).

This study had demonstrated positive staining for PRL binding sites in 82% of the malignant cases. However, only 58% positivity was obtained in the earlier study using paraffin section (Purnell et al., 1982). This variation could be attributed to the difference in the antisera, fixative and the technique used by them. The DHSS technique followed through out our study is more sensitive than PAP method. The dilution of primary antibody required for
staining was also almost 100 times greater as compared to PAP techniques. The use of monoclonal IgM type of immunoglobulins as bridge antibody results in higher staining sensitivity (Jasani et al., 1983). Using the same technique as ours Kumar et al. (1987) had reported 73% positivity for PRL binding in carcinoma but the number of cases were too small (eight) for comments.

The positive staining in the epithelial cells on the breast tissue sections was present intracellularly rather than at the periphery only. This was in consonance with the observation made by earlier workers (Nolin and Witorsch, 1976) and is in agreement with the concept of internalisation of PRL receptor complex (reviewed in chapter IV). Purnell et al. (1982) had demonstrated cytoplasmic immunocytochemical staining for PRL binding sites but with different technique. Dhadly et al. (1983) by treating the sections with purified human PRL prior to immunostaining steps obtained positive reaction at the periphery of both benign and malignant epithelial cells. Marchetti et al. (1984) in their study had observed that plasma membranes of these cells were never stained with PRL-antibody if the tissue were not pre-exposed to human PRL. However, we have in few cases obtained irregular staining at the periphery in addition to diffused cytoplasmic staining. The light microscopic studies did not permit us to identify the subcellular sites that contain the internalised PRL and the cytoplasm was seen to be stained giving positive reaction.

In the present study, while studying the immunocytochemical reaction in the breast tissue, it was observed that the breast responds to the staining reaction heterogenously i.e. the immunostained cells were found generally
intermixed with variable number of unstained cancer cells. This observation is compatible with the previous immunocytochemical results, suggesting a heterogenous population of cells with respect to the PRL binding (Patterson et al., 1982; Purnell et al., 1982; Dhadly et al., 1983). A high degree of tumour cell heterogenicity in breast carcinoma was suggested by the observation that significantly different proliferative rates were found for different epithelial cells cloned for different tumours (Schmidt-Ullrich, 1986).

No previous immunohistochemical studies have however commented on staining reaction variation with menopausal status for cancer patients. In the present study, it was observed that majority of the breast carcinoma slides of patients above the age of 41 years showed positive reaction. The finding of the PRL binding sites in greater proportion in peri and post-menopausal patients is of importance and is in consonance with the presence of estrogen receptor in breast cancer. Older women were more likely to be estrogen receptor positive than younger women (Clark et al., 1984; Bonneterre et al., 1986). It was also observed by Kwa et al. (1981) that post-menopausal patients who subsequently developed breast cancer have elevated levels of PRL upto 5 years before the clinical diagnosis of the disease and this elevated PRL levels in post menopausal women may result in increase number of PRL receptor in the mammmary tumour tissue that may cause the regrowth of epithelial tissue (Jensen HM, 1981). In addition estrogen, in the peri and post menopausal period, hypothetically, is considered to be the key determinant of subsequent risk for breast cancer, may be indirectly by stimulating PRL receptors in breast, thus promoting cellular susceptibility to neoplastic transformation (Kelly et al., 1980). Willis et al. (1977) suggested that in addition
to the tamoxifen treatment given to postmenopausal breast cancer patients for lowering estrogenic effect, their PRL production should also be suppressed by the drug such as bromocriptine. This would decrease the endogenous PRL levels which in turn will decrease proliferation of breast tissue.

It was also interesting to note in the present study that histopathological differentiation of breast tumours had got direct relationship with the presence of PRL binding sites. The well differentiated tumours showed staining reaction, the stronger reaction shown by areas of ductular differentiation, whereas poorly differentiated carcinoma either had only few cells that were stained or were completely negative. Similar observation was also made by Dhadly and Walker (1983). The results are comparable with the presence of ER that are more often found in well differentiated tumours (McCarty et al., 1980).

Metastasising tumours showed stronger reaction to PRL immunocytochemical staining in our study. This finding could be explained with respect to the assumption of Barni et al. (1987). PRL level rises more frequently after mastectomy in patient without node involvement, and if there was a stimulating role of PRL on breast cancer cells in humans, the high levels of PRL after mastectomy could stimulate the growth of micrometastases and this might represent a risk factor for relapse. Correlating the two, it could be stated that increased PRL level at any stage of women's life (including mastectomy) may have a stimulatory role on breast cancer cells. This could be manifested by increased binding sites in metastasing tumours. Waseda et al. (1985) reported that patients with tumours having high PRL-R have significantly reduced survival rate and thus have poor prognosis. However,
Bonnerette et al. (1986) have presented opposite results. The serum PRL level estimated by various workers also supports the above view, the pre and post operative PRL level when related with prognosis, the highest one was found to have poor prognosis (Wang et al., 1986). The response to chemotherapy or endotherapy fails or becomes ineffective when serum PRL levels were increased in post menopausal women with advanced breast cancer (Dowsett et al., 1983). The hyperprolactenemia being associated with the progressive disease in majority of advanced breast cancer patients (Holtkamp et al., 1984). Though it has been shown in experimental specimen that concentration of PRL-R in mammary tumours is independent of PRL concentration (DiCarlo et al., 1988) yet precautions must be taken prior to applying these data to human.

In the present study, the positive reaction for PRL binding sites was seen in good percentage of fibroadenoma cases (54%). This is contrary to earlier reports where less percentage or zero percentage of positivity was shown (Purnell et al., 1982; Kumar et al., 1987). The number of cases included in these studies were less. However, our finding is in agreement with the study of l'Hermite Baleriaux and l'Hermite (1984) which have shown 50% positivity in fibroadenoma. In the present study cases of lactating breast and apocrine metaplasia exhibited positive staining. Apocrine metaplasia was reported to be associated with increased cancer risk (Page et al., 1978; Robert et al., 1984) though later it was considered to be non-proliferative (Dupont and Page, 1985). The positive reaction in apocrine metaplasia was also shown by Kumar et al. (1987) who considered the presence of PRL binding in these to be of significance in view of the hormone's known growth stimulating effect.
on the breast epithelium. Considering the observations of Robert et al. (1984) and Kumar et al. (1987) our finding could be useful for suggesting endocrine therapy to the patients with benign disease.

A single case of duct ectasia showing positive reaction for PRL binding sites is in agreement with the finding of Shousha S et al. (1988) where higher levels of PRL were reported in these patients, indicating increased PRL to be the causative agent of duct ectasia.

The weak and rather heterogenously distributed PRL staining in other types of both proliferative and non-proliferative benign breast lesions is difficult to rationalise. The classification of benign breast disease into non-proliferative and proliferative breast disease (Dupont and Page, 1985) might be of clinical importance but when PRL binding sites was considered no definite conclusion could be made to distinguish between the two, may be because of small number of proliferative cases included in the study.

Regarding other peptide hormones FSH and LH, this is only a preliminary study which have indicated that there are binding sites for these hormone in the tumour tissues of breast. The presence of high levels of PRL and rather low levels of FSH and LH in cystic fluid was reported (Bradlow et al., 1983). Our observation of positive staining for PRL and negative reaction for FSH and LH binding sites in cases of cystic disease is in agreement with this study. Not much work had been done relating to the role of these hormones in the etiology of breast cancer through analysis of LH-RH are used for the treatment of hormone dependent breast cancer.
B. PRL BINDING SITES IN FNAC SMEARS

MATERIAL AND METHODS

The specimens were collected from sixty eight breast lumps in women by FNAC using 20 ml disposable syringe and 23/24 gauge needle. The aspirated material was smeared on multiple glass slides. These were fixed immediately in 95% alcohol. In all the cases, one or more smears were stained with routine haemotoxylin eosin for cytological diagnosis. The immunocytochemical staining was done on smears showing tumours, benign or malignant and benign diseases of the breast. Inflammatory lesions of the breast and the smears containing scanty material were excluded from the study. In sixteen cases only it was possible to study paraffin embedded sections of corresponding FNAC smears for PRL binding sites. Squash smears prepared from fresh pituitary gland obtained from postmortem room was included in each batch of staining as a positive control. For negative control, a lymph node aspirate was used. Also in randomly selected smears primary antibody was substituted by BSA for second negative control. Positive reaction appeared as brown granules in the cytoplasm of the cells.

The immunocytochemical technique utilized here was the same as previously described for paraffin sections except the dewaxing step which was excluded here.
RESULTS

The immunocytochemical studies in FNAC for PRL binding sites were carried out in 68 cases (Table 3.5). There were forty seven cases of carcinoma and twenty one cases of benign breast disease. Benign breast disease were classified according to Dupont and Page (1985) into non-proliferative and proliferative benign breast lesions.

<table>
<thead>
<tr>
<th>Lesions</th>
<th>Total</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Malignant</td>
<td>47</td>
<td>34</td>
<td>13</td>
</tr>
<tr>
<td>2. Non-proliferative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign breast disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Blunt duct Adenosis</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Apocrine cyst</td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>3. Proliferative benign breast lesions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sclerosing adenosis</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Lobular hyperplasia</td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Atypical epithelial hyperplasia</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

Malignant (Carcinoma): This group included 47 cases of invasive ductal carcinoma. Of these thirty four (72.4%) cases of carcinoma gave intense positive reaction (Fig. 3.12) while thirteen cases (27.6%) were negative for PRL binding sites. However, six of the former cases showed variable reaction giving positive reaction in some neoplastic cells while others were negative.

Non proliferative benign breast disease: There were five specimen of fibroadenoma. The cytodiagnosis of fibroadenoma was given when smears contained sheets or clumps of monomorphic small cells with scant amount of
Fig. 3.12i: FNAC smear of breast carcinoma - Showing positive staining for PRL binding sites.

Fig. 3.12ii: FNAC smear of breast carcinoma (another case) - Showing positive staining for PRL binding sites.
cytoplasm. On immunocytochemical staining, three of these showed positive reaction though it was lighter as compared to malignant ones, whereas the other two cases were completely negative. Three cases of blunt duct and five cases of apocrine change were studied. In three instances of blunt duct adenosis the smears were hypocellular. They contained epithelial cells of variable types and occasional spindle shaped cells. Two of these cases were positive for PRL binding sites whereas one was negative. In five cases of apocrine cyst the smears were hypercellular. The cells were oval or polyhedral in shape having small nuclear cytoplasmic ratio, well demarcated cell borders and granular cytoplasm. They were arranged either in sheets or were isolated. Four of these gave positive reaction while one was negative (Fig. 3.13).

Proliferative benign breast disease: The proliferative benign breast lesion includes sclerosing adenosis (1 case), lobular hyperplasia (6 cases) and atypical epithelial hyperplasia (1 case). The smears in a solitary case, cytologically diagnosed as sclerosing adenosis, contained cells clumps showing anisonucleosis and hyperchromasia. This was negative for PRL binding sites. The six smears of lobular hyperplasia contained epithelial cells of variable sizes with moderate to abundant cytoplasm. The cells were arranged in clumps presenting branching pattern. Four of these cases showed positive staining (Fig. 3.14) while two were negative. In a solitary case of atypical epithelial hyperplasia, a few atypical epithelial cells were present in the smears. This case was also negative for PRL binding sites.

In sixteen instances immunocytochemical staining reaction in FNAC smears could be compared with corresponding paraffin sections. There were nine cases of carcinoma, three of fibroadenoma and four were benign cystic...
Fig. 3.13: FNAC smear of apocrine cyst - isolated apocrine cells showing positive immunocytochemical reaction.

Fig. 3.14: FNAC smear of lobular hyperplasia - Showing positive reaction.
disease with Apocrine metaplasia. In all the sixteen specimens in which corresponding paraffin section was stained a positive aPRL staining was obtained. However, on reviewing the FNAC smear slides of the sixteen cases, in two specimens the PRL staining was reported negative. These two cases were of carcinoma.

The optimal staining reaction was obtained at the dilution of 1:15,000 of a PRL diluted in BSA. A positive reaction for PRL was seen in all instances with section of anterior pituitary but there was no evidence of PRL binding in lymph node smears. Also omission of a PRL and its substitution with BSA abolished specific staining.
DISCUSSION

The PRL binding sites in breast cancer cells have already been demonstrated using immunocytochemical techniques in various preparations such as in tissue culture (Patterson et al., 1982), frozen sections (Dhadly & Walker, 1983) and paraffin sections (Purnell et al., 1982; Kumar et al., 1987). However, till date we had not come across any study demonstrating PRL binding sites immunocytochemically in Fine Needle Aspiration Cytology (FNAC) though estrogen (Coombs et al., 1987) and progesterone receptors (Lozwski et al., 1990) in smears of breast have been studied and its usefulness had been stressed. The prognosis significance of PRL receptor in human breast cancer has already been described (Waseda et al., 1985; Bonneterre et al., 1986). The breast carcinoma cells in FNAC smears in this study presented heterogeneity as was demonstrated earlier in paraffin sections (Purnell et al., 1982; Kumar et al., 1987). The staining reaction was observed intracellular which is in agreement with the concept of internalization of the hormone receptor complex (Djiane et al., 1981).

The PRL binding sites observed in four cases of apocrine cyst in the present study is in agreement with the results of paraffin sections.

When the results of immunocytochemical staining reaction were compared with that of FNAC smears correlation was observed in about 80% of cases. In two instances where the FNAC smears were negative and the paraffin section showed heterogeneity of staining, probably the specimens with the needle were obtained from the negative part of the tumour. To overcome this fallacy multiple smears from different parts of the tumours and metastatic sites may be prepared and stained with same immunocytochemical technique.
In view of these findings demonstration of PRL binding sites may be proved a useful marker for determining high risk benign breast disease by immunocytochemical staining even in FNAC smears, which is a very simple cost effective, and can be utilized repetitively without causing any stress of operation to the patient. The procedure has been adopted as routine diagnostic tool for breast lumps in many laboratories.