6. DISCUSSION

In the present study, investigations were aimed at the isolation, purification and characterization of breast tumor associated antigens (BTAA) from human breast tumor tissues. The serum samples from female and male breast cancer patients were screened for presence of circulating antibodies against these antigens. The investigations were further extended to analyse immunological relatedness of BTAA with murine mammary tumor associated antigens (MTAA) purified from murine mammary tumor virus (MuMTV) induced spontaneously arising mammary tumors in C₃H/Jax mice.

6.1 STUDIES ON MURINE SYSTEM

Initial studies on purification of breast tumor associated antigens were carried out using spontaneously arising murine mammary tumor (MMT) from C₃H/Jax mice. Though chemically induced murine mammary tumorigenesis does not involve a virus (245), some murine mammary cancers of spontaneous occurrence are known to be induced by the MuMTV. The virus may be exogenously infective and highly oncogenic (84) being transmitted through milk, or it may be genetically transmitted (85) via the germ cells of the host strain and is thus endogenic. These genetically transmitted MuMTV DNA sequences are MuMTV proviruses integrated into the genomic DNA of all mouse cells and are referred to as endogenous MuMTV proviruses (104). Irrespective of the route of MuMTV induction, the process of murine mammary tumorigenesis involves the integration of the MuMTV provirus into the host cell DNA at a specific chromosomal domain which is specific for the murine strain concerned (106), thus lending a selective growth advantage to the cell. This proviral integration probably activates a neighbouring oncogene, thus initiating tumorigenesis (105, 246). There are several reports describing close association of MuMTV structural antigens with human breast cancers (142, 143, 145). Thus we selected MuMTV induced spontaneously arising murine mammary tumor for purification of a tumor associated antigen (MTAA) which might serve as an ideal model for studying human BTAA. MuMTV can induce mammary tumors in susceptible mouse strains after a long latency period of 4-12 months (247).
Partial purification of MTAA was achieved by subjecting MMT, a crude mammary tumor extract to DEAE cellulose ion-exchange column chromatography. Elution of the column with a discontinuous gradient of NaCl resulted in three protein peaks $MF_1$, $MF_2$ and $MF_3$ (Figure-1). All the three DEAE fractions were screened by ELISA for presence of MTAA using rabbit anti-MMT exhaustedly absorbed with normal murine tissue 'pellet. The ELISA was standardized using positive and negative control antigens for determining the cut-off OD value for positive reaction. Following repeated experimentation it was decided that OD values above 0.1 was indicative of positive reaction and OD values below 0.1 was considered as negative. Out of the three column fractions, only $MF_1$ was found to be reactive against rabbit anti-MMT (Table-5) as evident by a high OD value produced by this fraction. A small OD value obtained following reaction of $MF_2$ with the absorbed anti-MMT is suggestive of presence of $MF_1$ components in $MF_2$ as a result of incomplete separation.

Homogeneity of $MF_1$, the antigenically reactive component of MMT, was examined by subjecting the fraction to SDS-PAGE analysis. Three distinct bands of $MF_1$ were evident from the gel electrophoresis (Figure-2). Elution of the 3 bands and their subsequent analysis in ELISA showed that the gel eluate of band 3 of $MF_1$ ($MF_1$-3) was antigenically reactive against the absorbed rabbit anti-MMT (Table-6). The OD values of less than 0.1 obtained with other two gel eluates, $MF_1$-1 and $MF_1$-2, suggested absence of immune components reactive with absorbed anti-MMT in these fractions. The reaction of the fraction $MF_1$-3 with absorbed anti-MMT suggested presence of MTAA reactivity in this fraction. By analyzing the $MF_1$-3 fraction in SDS-PAGE, the molecular weight of the MTAA was found to be of 83 Kd (Figure-3).

Detection and purification of the 83 Kd MTAA from spontaneously arising mammary tumor of C3H/ax mice was reported earlier from our laboratory (111). Phytohemagglutination assay (PHA) was adopted to analyze the antigenic profile of the MMT and to detect MTAA (111). The present study utilized ELISA, which is more sensitive than PHA to confirm immune reactivity of the MTAA. We have earlier observed the presence of circulating antibodies against $MF_1$ in the sera of mammary tumor bearing mice but not in that of tumor free mice (111). The observation suggested presence of a mammary tumor associated antigen in $MF_1$. 
A number of studies indicate that MuMTV-induced mammary tumors possess MuMTV structural antigens and non-virion tumor associated antigens (TAA) (251, 252). Circulating antibodies to the MuMTV structural antigens have been detected in the sera of mammary tumor bearing mice (253, 254). The same antibody could also be detected, though in much lower concentration, in the sera of MuMTV positive tumor free mice. The immunological relatedness of the MTAA with MuMTV structural antigens were examined by analyzing the reaction of MF \textsubscript{1} with an antiserum produced against Triton X-100 disrupted MuMTV. An OD value of less than 0.1 suggested non-reactivity of the MF \textsubscript{1} with anti-MuMTV (Table-7). The absence of reaction of the MF \textsubscript{1} with anti-MuMTV further suggested a non-virion nature of the MTAA. However, the fraction MF \textsubscript{3} revealed a strong antigenic reactivity with the anti-MuMTV serum (Table-7) suggesting presence of MuMTV structural antigens in the fraction MF \textsubscript{3}. The data suggested that the MTAA purified from the MuMTV induced spontaneously arising mammary tumor of C\textsubscript{3}H/Jax mice might not be a MuMTV structural antigen. However, since MuMTV genome is integrated in the host cell DNA at a specific chromosomal domain (106), there is a strong possibility that the viral genome integration into the host DNA would lead to production of new proteins. The MTAA might represent such protein synthesized by the mammary tumor cells. It therefore, may be suggested that the MTAA, though might not be MuMTV structural antigens, it might be a MuMTV coded antigen. Presence of circulating antibodies against MTAA in mammary tumor bearing mice indicated that the antigen might be used as a marker for tumors induced by MuMTV or a related virus.

6.2 STUDIES ON HUMAN FEMALE BREAST TUMORS

Association of MuMTV or a similar virus with human breast cancer is being debated for long time (142, 143, 145). We attempted to purify a human breast tumor associated antigen and compare with MTAA with a view to understand viral association, if any, with human breast tumor and the process of carcinogenesis (185).

The possible viral etiology of human breast neoplasias has been considered on the basis of several evidence. Particles morphologically similar to B particles have been seen by electron microscopists in some
human breast tumors and milk samples (107). Particulate fractions from some human milks contain a DNA polymerase activity that has some characteristics of oncornavirus type RDDP (108, 109). The RDDP activity of human milk, like that of MuMTV prefers Mg$^{2+}$ as a divalent cation when synthesizing DNA using Oligo (dG), poly (rC) as a primer-template (168). Some human breast tumors have RNA sequences that are homologous to the MuMTV genome. Using CaSO$_4$ gradients to detect hybrid formation, 69% of human tumors were found to contain RNA sequences that hybridized to MuMTV cDNA (169). In human breast cancer, circulating antibodies against gp 52, the major structural protein of MuMTV have been widely recorded (161, 173, 175). The specificity of gp 52 as a breast tumor marker is however questionable since such antibodies have been found to occur in the sera of patients suffering from benign breast disease and other cancers (161, 176) and no difference was observed between the normal control and breast cancer group in direct and indirect LMI assays with MuMTV antigens (256). Regarding virus like B particles, which have occasionally been observed in human breast tumors (152), have also been observed in benign breast lesions (172). Under epidemiological conditions, it is unlikely that viral transmission via milk will even be shown to be the cause of human breast cancer since breast cancer is relatively infrequent in women raised in societies where nursing is the predominant mode of infant nurture as opposed to populations drawn from societies where artificial infant feeding is a dominant custom. Moreover, sequences related to MuMTV have been identified and cloned from normal human DNA (248). Thus, evidence of the existance of an oncornavirus similar to the MuMTV in human breast cancer is still very controvertial.

A number of human breast tumor associated antigens; protein, glycoprotein or mucine in nature and of a wide range of molecular weights have been reported in literature (133, 135, 139, 147, 162). The biological significance of these antigens are yet to be established, though a few of the markers were shown to be associated with oncogenes (132, 157), growth factor receptors (249, 250) and Laminin receptor (151). New tumor markers are continually being discovered in research laboratories (134, 140, 154), clinically evaluated and the best of them are being made commercially available. However, for the clinician, it has become very hard to objectively judge and to rapidly evaluate the performance of
the new markers as compared to the older ones in terms of clinical benefit and cost. In view of the increased incidence of breast cancer in Indian women, having different genetic make up, there is a need for local availability of specific markers. In this study, we purified an antigen which appears to be promising regarding diagnosis and prognosis of the disease as well as to understand the disease process.

In an attempt to purify and characterize BTAA, MBT and NHB extracts were chromatographed through DEAE cellulose column in a discontinuous gradient of sodium chloride. In chromatography, MBT resolved into 3 major protein peaks - HF₁, HF₂ and HF₃ while NHB resolved into 2 major protein peaks NHF₁ and NHF₂ (Figure 4A and 4B). The additional peak HF₃, obtained in MBT suggested presence of additional proteins in the malignant tissue. All the 22 MBT samples studied presented similar chromatographic profiles. The NHB samples also showed similar protein peaks in DEAE cellulose chromatography. The strong reactions in ELISA of the HF₁, fractionated from all the samples of MBT with unabsorbed as well as NHB absorbed sera of female breast cancer patients (Tables-8 and 9) suggested presence of a tumor associated antigen - BTAA, in all the breast tumor samples studied. An OD of less than 0.1 was repeatedly observed in ELISA carried out with control sera or control antigens. Therefore, the OD value of less than 0.1 was considered as negative for determining immunological reactions in ELISA. The weak antigenic reaction of the DEAE fraction, HF₂ with breast cancer patients' sera might be due to incomplete separation of HF₂ from HF₁ in DEAE cellulose chromatography. Both HF₁ and NHF₁ produced weak reactions, as evident from the low OD values, against the sera of healthy women, other cancer patients, and patients with benign breast diseases. The observed weak reactions of both HF₁ and NHF₁ with the control sera might be primarily or exclusively due to the presence of the autoantibodies in these sera. Presence of autoantibodies against normal breast epithelial antigens is well known (123, 155). Following absorption of the breast cancer sera with NHB tissue pellets, the absorbed sera still showed presence of a high titer of circulating antibodies against HF₁ but not with NHF₁ (Table-9). These observations suggested that absorption of the sera with NHB resulted in removal of autoantibodies against normal epithelial
antigens. Persistant reaction of the absorbed sera with HF\textsubscript{1} further suggested presence of breast tumor specific antibodies in these sera. Lowering of the HF\textsubscript{1} reactive antibodies in the breast cancer sera following exhaustive absorption with NHB (Table-9) suggested that HF\textsubscript{1} shared some antigenic components with NHB against which normal autoantibodies were present. The levels of antibody titer in all other control sera against both HF\textsubscript{1} and NHF\textsubscript{1} came to negligible levels after removal of autoantibodies following absorption (Table-9).

The BTAA reactivity in HF\textsubscript{1} was further confirmed by immuno-diffusion, affinity chromatography and Western blot analysis. In immuno-diffusion experiments, a strong reaction of the IgG fractions, purified from the sera of breast cancer patients by sephadex G-200 gel filtration, with HF\textsubscript{1} was evident by formation of strong precipitin band (Figure-6). Partial purification of the BTAA was achieved by subjecting HF\textsubscript{1} through affinity column of Protein A-Sepharose, linked to IgG purified from breast cancer patients' sera. A high titer of antibodies against the affinity column eluate (ACE) observed in ELISA with NHB absorbed untreated breast cancer sera (Table-10) suggested presence of BTAA in the ACE. The ACE was non-reactive with the sera of healthy women. The Western blot analysis of HF\textsubscript{1} showed a 85 Kd band of HF\textsubscript{1} was strongly reactive with breast cancer sera, partially absorbed with NHB (Figure-7), suggesting presence of BTAA in HF\textsubscript{1}. The observed faint band of 85 Kd produced by NHF\textsubscript{1} with the same serum in Western blot analysis (Figure-7) might be due to the use of incompletely absorbed breast cancer sera. These experiments further suggested that the BTAA present in HF\textsubscript{1} was of 85 Kd molecular weight. Moreover a protein of 85 Kd reactive with the sera of breast cancer patient may also be present in NHF\textsubscript{1}. The quantity of this 85 Kd protein in NHF\textsubscript{1} was significantly less than the 85 Kd BTAA present in HF\textsubscript{1}, as was evident by the intensity of the bands produced by these two fractions in Western blot.

To see the homogeneity of HF\textsubscript{1}, it was subjected to SDS-PAGE analysis. The HF\textsubscript{1} resolved into 6 major protein components (Figure-8B). Elution of the six protein components of HF\textsubscript{1}, followed by their screening in ELISA provided evidence that the BTAA was present in the eluate of band 3 of HF\textsubscript{1} (HF\textsubscript{1}-3). Reaction of HF\textsubscript{1}-3 with the absorbed
sera of breast cancer patients, but not with the absorbed sera of normal healthy individuals confirmed retrieval of the BTAA in HF₁⁻³ (Table-11).

In SDS-PAGE analysis, NHF₁ also resolved into 6 major protein bands (Figure-8B). The band patterns of the first 4 bands were very similar to that of HF₁. But the band 3 of NHF₁ (NHF₁⁻³) was of much lower intensity in comparison to the band 3 of HF₁ (HF₁⁻³). This observation was similar to the findings in Western blot analysis and it could be suggested that BTAA or a related protein is present in very low amount in normal breast tissues. It may be conceived that the BTAA was an overexpressed normal protein associated with malignancy of breast. There are several reports available, describing overexpression of normal protein in malignancies (123, 155). Pancino et al (155) found a 76 Kd glycoprotein antigen of unknown function in breast cancer, which was also a component of normal breast. This 76 Kd glycoprotein was also expressed in colon-adenocarcinomas and serous ovarian carcinomas. Overexpression of the BTAA protein might be restricted to the malignancy of breast as is suggested by the lack of reaction of HF₁ with the sera of healthy individuals and with the sera of patients with other malignancies and benign breast diseases. However, the reaction of HF₁ with the serum of one patient with advanced carcinoma of uterine cervix suggested presence of circulating antibodies against BTAA. In this patient presence of the anti-BTAA antibodies might be associated with secondary involvement of other organs for which no confirmed history was available. Moreover, this patient might have expressed antibodies, reactive to the proteins other than BTAA, present in HF₁. Qualitative difference in antigen expression or only a modification of a normal protein in malignancy of breast is suggested earlier by Ronai and Sulitzeanu (123), who by immunoblotting detected presence of high titer of autoantibodies in human breast cancer sera and a low titer of similar antibodies in normal women.

Purification of BTAA was also attempted by subjecting HF₁ to Sephadex G-100 gel filtration and eluting out the proteins with PBS. Out of 2 major protein peaks of HF₁, HF₁⁻S₁ and HF₁⁻S₂ (Figure-9), resolved by sephadex gel filtration, HF₁⁻S₂ was found to produce high OD values in ELISA against the NHB absorbed sera of breast cancer patients, suggesting the presence of BTAA in this fraction. The lack of reac-
portion of \( HF_1-S_2 \) with the NHB absorbed sera of normal healthy individuals (Table - 12) also supported presence of the BTAA in \( HF_1-S_2 \). On SDS-PAGE analysis, \( HF_1-S_2 \) resolved into 4 bands, one of which was found to be of 85 Kd and was similar to the band 3 of \( HF_1 \) or BTAA (data not shown). For purification of the BTAA from \( HF_1-S_2 \), the fraction was subjected to SE-HPLC. As shown in the figure-10, in HPLC, \( HF_1-S_2 \) resolved into 9 major protein peaks. Screening of the 9 protein peaks in ELISA against NHB absorbed sera of breast cancer patients resulted in retrieval of the BTAA in the peak 5 (SE\(_5\)) of the column eluates. The SE\(_5\) was strongly reactive with the NHB absorbed breast cancer sera but not with that of healthy women (Table-13).

In an attempt to physicochemically characterize the BTAA, its molecular weight, enzyme susceptibility and thermostability were determined. SDS-PAGE analysis of SE\(_5\), \( HF_1-3 \) and \( HF_1-S_2 \), indicated that the BTAA is of MW 85 Kd (Figure-11A). Enzymatic degradation of \( HF_1 \) with trypsin, papain and neuraminidase, associated with loss of reactivity of the fraction with NHB absorbed sera of breast cancer patients (Table-15) suggested that the reactive antigen is a glycoprotein and both carbohydrate and protein moieties are involved in expression of its antigenicity. Loss of BTAA activity of \( HF_1 \) using neuraminidase treatment was suggestive of possible involvement of sialic acid in the antigenic epitope of the BTAA. High thermostability of the BTAA was evident in retention of reactivity of \( HF_1 \) with the NHB absorbed breast cancer patients' sera even at 80°C (Table-15). This is in confirmation with the fact that glycoproteins are highly thermostable.

To determine the identity of the BTAA, the physicochemical characteristics of the molecule was compared to that of breast tumor markers, reported in the literature, having similar MWs. A 76 Kd breast cancer associated glycoprotein antigen, described by Pancino et al (155) resembles closely with the BTAA in terms of MW and chemical nature. However, involvement of sialic acid in determining antigenicity differed in these two molecules, though both are glycoproteins. In the 76 Kd breast TAA, sialic acid was not present in its epitope. Sanchez et al (154) described a lactoferrin antigen having a MW of 80 Kd in the breast
secretions of women with malignant breast disease. The BTAA though closely resembled in MW to this recently identified lactoferrin antigen, it appeared to be a different molecule. We have reported earlier purification of a 83 Kd tumor associated glycoprotein antigen, MTAA, from MuMTV induced murine mammary tumors of C3H/Jax mice (111). The BTAA closely resembled in MW, enzyme susceptibility and thermostability to the MTAA.

To analyse immunological relatedness between BTAA and MTAA the antigens were analysed in ELISA for presence of antibodies against them in NHB absorbed sera from breast cancer patients and absorbed anti-MMT. Presence of circulating antibodies against MTAA in NHB absorbed sera of breast cancer patients and against BTAA in the absorbed anti-MMT sera (Table - 21) suggested sharing of antigenicity between BTAA and MTAA. The MW of BTAA (85 Kd) was close to that of MTAA (83 Kd). Both the antigens were glycoproteins, susceptible to proteolytic enzyme and neuraminidase degradable. These findings strongly suggest BTAA being identical to the MTAA.

The viral etiology of human breast cancer is still a debatable issue. Implication of MuMTV or a similar virus in human breast cancer has been suggested by many (142, 145, 152). Virus like particles in human milk fractions and breast tumors (107), RDDP like activity of MuMTV in human milk (108, 109), the MuMTV genome RNA sequence homology in some human tumors (169) and cellular immune responses in many breast cancer patients, elicited against the MuMTV gp 52 (161, 174) strengthened the idea. The observed similarity of the BTAA with MTAA led us to examine relatedness of the human antigen with MuMTV.

BTAA was tested in ELISA against highly purified anti-MuMTV serum. Lack of reaction of the BTAA with anti-MuMTV (Table-14), as was also observed with MTAA (111), excluded the possibility of both BTAA and MTAA being MuMTV structural proteins (143, 149). However, the finding that BTAA was not a MuMTV structural antigen, did not rule out the possibility of the molecule being coded by a virus, same as or close to MuMTV. The MTAA, which is also not a MuMTV structural protein, was purified from a MuMTV induced tumor. The molecule is not
present in normal mouse tissues and is possibly produced by the mammary tumor as a result of incorporation of MuMTV genome in its DNA material (106). The suggested similarity of the BTAA with MTAA indicated possibility of the BTAA also being produced following association of MuMTV or a related virus with induction of malignancy in breast. In a recent study, Holland et al (255) indicated a viral etiology of human breast cancer. A candidate virus might well resemble MuMTV and that, like it, would be integrated in DNA. They constructed polymerase chain reaction (PCR) primers and probes for a 660 base pair segment of MuMTV env gene, which was non-homologous with any known human DNA sequence. Out of 410 female patients which they have screened, 35% contained the MuMTV env sequences, with 90 to 98% homology to authentic MuMTV. After random priming with $^{32}$P labeled nucleotides, and using the sequence as a probe, an 8 Kb fragment was found by Southern blot in DNA of positive breast cancer specimens. In addition, an RNA of 8 Kb transcript was found in Northern blots. The env sequences were not found in lymphocytes of patients, other cancers, or normal tissues. However, to confirm viral association in human breast tumor, lot more studies at cellular and molecular levels are required.

There are several reports, describing presence of circulating tumor specific antibodies in breast cancer patients (203, 204). These antibodies were specific largely or exclusively for oncofetal antigens. The most widely studied antigen, against which autoantibodies were found in the breast cancer patients is carcinoembryonic antigen or CEA (141, 205). The other important oncofetal antigen, reported to be associated with human breast tumor is placental isoferritin (167). To determine whether the circulating antibodies observed in breast cancer patients against BTAA has cross reactivity with fetal antigens, reaction of the NHB absorbed breast cancer patients' sera with a crude tissue extract of a 10 weeks old human fetus was examined in ELISA. Lack of reaction between the fetal tissue antigens and the NHB absorbed sera from female breast cancer patients (Table-17) suggested no immunological relatedness between fetal antigens and BTAA. This was further confirmed by the observed strong reaction of $HF_1$ with the sera of breast cancer patients.
absorbed repeatedly and exhaustively with NHB and fetal tissue pellets (Table - 16). From these observations the possibility of BTAA of being an oncofetal antigen may be excluded.

Regarding localization of the BTAA, no different experiments were carried out. But BTAA was purified from the 15,000 g supernatant of MBT. It also closely resembles to MTAA (111). Thus it can be suggested that like MTAA, BTAA might be localized in plasma membrane of the cell.

Using monoclonal antibodies against different subclasses of immunoglobulins in ELISA, the BTAA reactive circulating antibodies present in the breast cancer patients sera was found to be of IgG₂ subtype (Table - 18). Recently, a monoclonal antibody BCA 227, produced against a breast carcinoma cell line MCF7 and capable of detecting a 71 Kd glycoprotein antigen has been reported which is of IgG₂ₐ subtype (140). The BCA 227 is not able to mediate antibody dependent cell mediated cytotoxicity (ADCC). It remains to be investigated whether the IgG₂ subtype antibodies of the BTAA are capable to mediate ADCC.

The BTAA reactive antibody titer was found to be significantly lowered in post-operated breast cancer patients as compared to that of untreated patients (Table-19). It was also observed that the BTAA reactive antibody titer in post-operated breast cancer patients gradually decreased with the increase of time interval of the post-surgical period (Table-20). It may be conceived that the BTAA is shaded by the tumor into the circulation and the hosts respond with production of antibodies. With removal of the tumor load, the BTAA induced stimulation is less, resulting in significant lowering of the antibodies. As the post-surgical period increased, the antigenic stimulation being absent, a gradual decrease of the antibody level is observed. It would be interesting to determine the presence of BTAA antibodies in the patients with metastasis of breast tumor. The observation that the BTAA antibody titer decreases with surgical removal of the breast tumor and the lowering of the antibody is co-related with post-surgical interval suggested a prognostic value of the BTAA antibodies.
A large number of breast tumor markers of a wide range of MWs have been reported. CA 15.3, CA 125, serum alpha lactalbumin, DF₃, etc., are of high MW (146, 156, 158, 159). CA 15.3 is a carbohydrate molecule having a MW of 290 Kd (158). To assess the prognostic value of this marker, tests were conducted on a group of patients with metastatic breast cancer whose CEA level was negative when metastasis was diagnosed. It has been found that CA 15.3 is more sensitive than CEA when the primary tumor is diagnosed and when metastasis is discovered. CA 15.3 levels were elevated more frequently than CEA levels, and CA 15.3 thus appears to be a superior marker than CEA in breast cancer prognosis (158). CA 125 is more commonly elevated in metastatic breast carcinoma patients (146) and can serve as a marker to monitor disease course if CA 15.3 is not elevated. However, the performance characteristics of CA 125 in breast cancer are not as satisfactory as for CA 15.3, which should be considered the marker of choice in patients with disseminated breast carcinoma. Abe and Kufe (156) showed that the MAb DF₃ reacted with a high MW glycoprotein detectable in human breast carcinomas and human milk. They analyzed the structure of DF₃ protein produced by human BT-20 and MCF-7 breast carcinoma cells. A core protein size of approximately 160 Kd was obtained for DF₃ protein in BT-20 cells. In contrast, two DF₃ proteins of approximately 160 Kd and 230 Kd were detectable in MCF-7 cells. Larocca et al (153) identified a 46 Kd human milk fat globule protein, highly expressed in human breast tumors, using MAbs. The molecule has been found to be useful for both breast cancer diagnosis and therapy. The 67 Kd laminin receptor is a cell surface protein that binds laminin with high affinity. Martignone et al (151) in vitro studies suggested that this protein is involved in the progression of human tumors to invasive breast cancers and associated with the metastatic process. Overexpression of C-erbB-2 oncoprotein occurs in 60% of in situ and 25% of infiltrating ductal carcinomas (132, 157). The biological significance of these markers as well as of 43 Kd epidermal growth factor related protein (250), insulin like growth factor binding protein (249) and the 66 Kd antichymotrypsin like protein (165), present in breast tumor is not fully known. Though the BTAA appears to be different from these markers, further characterization of the molecule is needed to properly examine the relationship of the BTAA with these breast tumor markers. The significance of the BTAA cannot be ascertained in the present
study and needs exploration. It appears that BTAA might be potentially used as a diagnostic and prognostic agent for breast cancer.

6.3 STUDIES ON HUMAN MALE BREAST TUMORS

Cancer of the male breast is a rarity. Though a number of reports are available on the familial and hereditary aspects of the male breast cancer (257, 273), not much is known about the etiology of the disease. Gynecomastia (278), estrogen therapy (272), high endogenous estrogen level (279), Kleinfelter's syndrome (268), trauma (280) and irradiation (273) have been reported to have possible association with the disease. Alteration of oncogene products are also associated with male breast cancer (274-277).

In female breast cancer, a virus as an etiologic factor has been widely studied with varied results (143, 145, 152, 161). Association of MuMTV related molecules with female breast cancer have already been discussed earlier. A highly significant difference was found in breast tumor incidence in reciprocal matings between high and low breast cancer strains of inbred mice. Only the female offspring of mothers of high incidence strain had a high incidence of breast tumors. This strong maternal influence indicated that a non-genetic factor was transmitted to offspring from mother. This extrachromosomal factor, transmitted via the mother's milk, was shown to be a virus (80). In addition to milk spread, an occasional extra-chromosomal male transmission of MuMTV was also observed (184).

There are reports on mammary tumor induction by pituitary grafting in male mice (282). This serves as an animal model for male breast cancer. According to Kagaswara et al. (282), isologous anterior pituitary grafting, 4 each, to 3-4 months old SHN and SLN mice resulted in an appearance of mammary tumors from 8 months of age and the incidence at 12 months reached 53.8% in each strain. All tumors were diagnosed as type B adenocarcinomas. Normal mammary gland growth and mouse mammary tumor virus (MuMTV)-gp52 antigen levels in the submaxillary glands were stimulated by the pituitary grafting in these strains. The effect of pituitary grafting was much less in GR/A male mice in which no mammary tumors appeared.
The mechanism of carcinogenesis in male breast is not known. It is also not clear whether similar mechanisms of carcinogenesis are involved in male and female breast. In this work an attempt has been made to make a comparative study between female and male breast cancers in respect to tumor associated antigens and circulating antibodies against them, in view of elucidating mechanisms of carcinogenesis.

MuMTV as mentioned earlier is a potent inducer of mammary tumors in susceptible mice. As already pointed out, the female breast tumors express MuMTV-related macromolecules which are capable of inducing virus specific humoral and cell-mediated immune responses in the patients (143, 145). Studies to explore association of MuMTV or related agents in male breast cancer is lacking. The present work addressed the question of possible viral association in male breast cancer.

The purification of a mammary tumor associated non-viral glycoprotein (MTAA) from MuMTV induced murine mammary tumors has been reported earlier from this laboratory (111). In this study we have shown that BTAA, which was expressed by female breast tumors closely resembled MTAA (Table-21). Antibodies to this protein were also observed in the female breast cancer patients. Presence of circulating antibodies specifically reactive to the BTAA or MuMTV related antigens in male breast cancer patients was studied in view of establishing expression of MuMTV or BTAA related antigens by male breast cancer tissues.

While purifying MTAA and BTAA by subjecting MMT and MBT respectively to DEAE cellulose chromatography, 3 protein fractions were obtained from both the mouse mammary tumor tissue and human breast tumor tissues (Sections - 5.1.2.1 and 5.2.1.2). The fraction 1 of MMT and MBT, MF₁ and HF₁, yielded MTAA and BTAA, respectively (Tables - 5 and 8). In the studies with mouse mammary tumor and female breast tumor it was observed that none of the antigens MTAA and BTAA was reactive with anti-MuMTV serum (Tables-7 and 14). However, as evident from the OD values (Tables - 7 and 14) DEAE cellulose fractions 3 of MMT and MBT, the MF₃ and HF₃ respectively were strongly reactive with anti-MuMTV serum. This observation suggested that MF₃ and HF₃ might have some components which were antigenically related to the structural components of MuMTV.
In an attempt to analyse the type of circulating antibodies present in 4 male breast cancer patients, their sera were screened in ELISA for reaction with all the three fractions of MMT and MBT. The results presented in the tables 27 and 22, showed that all the 4 patients presented circulating antibodies strongly reactive with MF\textsubscript{3} and HF\textsubscript{3}. However, no reaction of these 4 patients' sera were evident with MF\textsubscript{1} or MF\textsubscript{2}. No reaction of HF\textsubscript{1} and HF\textsubscript{2} with the patients' sera could also be seen. These results strongly indicated that the male breast tumor express tumor associated antigens closely related to the antigenic components of MF\textsubscript{3} or HF\textsubscript{3}. The present work as well as our earlier report have indicated presence of MuMTV structural components or related proteins in MF\textsubscript{3} or HF\textsubscript{3}. Therefore, the reaction of male breast cancer patients' sera with MF\textsubscript{3} and HF\textsubscript{3} suggested expression of MuMTV structural antigens or related antigens by the male breast tumor tissues against which the patients were generating antibodies. On the other hand, absence of reaction of these patients' sera with MF\textsubscript{1} or HF\textsubscript{1} suggested that their breast tumors did not express BTAA or related antigens.

Attempt was made to purify the antigenic component present in HF\textsubscript{3} which was reactive with male breast cancer patients' sera by subjecting HF\textsubscript{3} to sephadex G-100 gel filtration. Out of the 2 resultant protein peaks - HF\textsubscript{3}\textsubscript{S\textsubscript{1}} and HF\textsubscript{3}\textsubscript{S\textsubscript{2}} (Figure-13), the former was found to be antigenically reactive against the sera of male breast cancer patients. When HF\textsubscript{3}\textsubscript{S\textsubscript{1}} was electrophoresed in SDS-PAGE with a view of identifying and further purifying the reactive antigenic component, 5 major protein bands were observed (Figure-12). Elution of the gel bands and subsequent screening in ELISA, showed that the reactive antigen was present in the gel eluate of band 2 (B\textsubscript{2}) of HF\textsubscript{3}\textsubscript{S\textsubscript{1}} (Table-24). By SDS-PAGE analysis of HF\textsubscript{3}\textsubscript{S\textsubscript{1}}, the MW of B\textsubscript{2} was determined to be of 72 Kd. The absence of reaction of B\textsubscript{2} with the sera of healthy male volunteers suggested that B\textsubscript{2} is associated with the malignant disease of male breast. The reaction of HF\textsubscript{3} and B\textsubscript{2} with male breast cancer patients' sera even after their exhaustive absorption with packed blood cells obtained from normal healthy donors, suggested tumor specificity of the circulating antibodies of the male breast cancer patients' sera (Table-26).
Partial purification of a 70 Kd protein reactive with male breast cancer patients' sera was achieved by subjecting MF\textsubscript{3} to SDS-PAGE and eluting the gel bands. Out of the 3 major protein bands (Figure-14), the band 2 (MF\textsubscript{3}-2) was reactive and the 70 Kd antigen was retrieved in the eluate of band 2 (MF\textsubscript{3}-2). The lack of reaction of the MF\textsubscript{3}-2 with the sera of healthy male volunteers and its positive reaction with male breast cancer patients' sera (Table-28) suggested that the 70 Kd antigen present in MF\textsubscript{3}-2 was associated with male breast tumor.

As discussed earlier, a number of structural antigens of MuMTV such as, gp68, gp52, gp34, p28, p24, p18 and p14 are present (97-100). However, there are differences in the polypeptide patterns between tissue culture produced and milk produced MuMTV viruses. The antigenic components B\textsubscript{2} and MF\textsubscript{3}-2 having MWs of 72 Kd and 70 Kd respectively might be considered as analogous to the envelope glycoprotein gp68 of MuMTV. The present observations suggesting presence of MuMTV related antigens and circulating antibodies to these antigens in male breast cancer patients could be due to either exposure of these patients to exogenous MuMTV or related agents and/or activation of endogenous MuMTV related DNA sequences. Dion et al (142) has reported serologic responses to the MuMTV structural antigens in the laboratory personnel having exposure to MuMTV. However, absence of these antibodies in the groups of age matched male and female healthy volunteers and in female breast cancer patients presently studied, rules out the exposure to exogenous MuMTV as a possible explanation for the presence of anti-MuMTV antibodies in male breast cancer patients. The existing evidence for presence of MuMTV related sequences in human DNA (248, 281) suggest activation of endogenous MuMTV related sequences as a plausible explanation.

A few antigenic components have been demonstrated in male breast cancer patients using monoclonal antibodies (270). Immunohistochemical assays using the monoclonal antibodies B72.3 and B6.2, recognizing two distinct and independently expressed breast tumor associated antigens, recently have been shown to significantly improve the accuracy of cytodiagnosis of breast nodules by fine needle aspiration (270). It has also
been found that these two monoclonal antibodies can be used diagnostically to distinguish gynecomastia from breast cancer in men. Blin et al. (274) investigated 38 samples of carcinomas of the male breast for P185 erbB-2 expression by using monoclonal antibody. P185 erbB-2 is a transmembrane kinase receptor, coded by erbB-2. In the above study, although most of the cases were immunopositive (36/38), no correlation to tumor grading and survival spans was notable. Therefore, erbB-2 activity fails to add a new prognostic parameter in male breast carcinomas.

The possible expression of MuMTV 70-72Kd antigen by the male breast cancer raises the questions regarding the role of MuMTV in male breast carcinogenesis and the biological significance of the antigenic molecule. These questions need to be addressed in detail. The present work strongly indicated possible association of MuMTV with breast cancer of both men and women. However expression of different molecules as tumor associated antigens by the male and female breast tumors suggested that though MuMTV may be involved in carcinogenesis, the process of malignant transformation of the breast epithelium is different in these two sexes. Influence of sex hormones might be a guiding factor for such difference in carcinogenesis in breast of men and women.