THE CHARACTERIZATION OF IMMUNE CELL POPULATIONS IN RELATIONSHIP TO GROWTH OF INFILTRATING DUCTAL CARCINOMAS.

by

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ABSTRACT

The appearance of a tumor indicates that tumor cells have found a way to avoid the immune system, to survive and to proliferate and probably to metastasize into other parts of the body. A study was undertaken to examine the immune cell populations that are present in infiltrating ductal carcinomas of the breast. Thirty-two tumors were examined using immunohistochemistry with a series of monoclonal antibodies. The results revealed that one or more of the following populations of immune cells were present in all the tumors studied; T-cells, B-cells and macrophages. Subpopulations of T-cells were evident within the peripheral and the central areas of the tumors but they did not display interleukin-2 receptors, therefore those cells were not stimulated and probably unable to evoke a cytotoxic effect on the tumor cells. Macrophages were found in all the tumors examined. I detected a positive relationship between macrophages and the proliferation rate of tumor cells. In many instances, macrophages were found in direct contact with proliferating tumor cells. I hypothesize that in many instances tumor cells may require macrophages for support because of the over 100 factors that they can provide. Transforming growth factor-alpha (TGF-α), for example, can be secreted by macrophages. This growth factor can aid in tumor cells proliferation. A positive association was found between TGF-α positive staining and the presence of macrophages. In addition, cell cultures of primary breast carcinomas were used for immunohistochemical detection of cell types. It was found that three week old cultures contained mixtures of macrophages and fibroblasts in expanding colonies. Interestingly, some of macrophages were found to be undergoing cell division, indicating that macrophages and fibroblast may be closely related in breast carcinomas. In other short term cultures macrophages and presumptive tumor cell(s) formed a unit. Macrophage/tumor cell(s) unit(s) could be important in the successful establishment of metastases. This research may lead to new avenues for the treatment of breast cancer by focusing more on influencing cellular functions rather than cytotoxicity to cancer cells.

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DEDICATION

TO MY BROTHER,

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WHO HELPED ME DEVELOP MY LOVE FOR THE SCIENCES.

I AM FOREVER GRATEFUL TO YOU, FOR THE TIME AND THE PATIENCE

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LOVE BARB.

I. INTRODUCTION

PART 1. GENERAL INTRODUCTION

Cancer is a major disease worldwide, resulting in 7 million deaths per year (1). In Canada it was estimated that 109,000 people developed cancer in 1991 and approximately 56,700 deaths due to cancer occurred that year. Breast cancer is one of the leading causes of incidence and death, from cancer, among women in Canada, with 14,400 women developing breast cancer and 5,100 deaths in 1991 (2). From 1984 to 1988 the incidence of breast cancer in British Columbia rose from 1372 to 1926. Death rates increased from 439 in 1984 to 515 in 1988 (3). Breast cancer is approximately seven times more common in Northern Europe and North America than in Asia (4). The incidence of breast cancer continues to increase and no one is absolutely certain why this is happening (5).

Experimental, clinical and epidemiologic evidence indicates that hormones play a major role in the etiology of breast cancer. The known risk factors can best be understood as measures of the cumulative exposure of the breasts to various factors. The risks factors are as follows:

1. Age of menarche: There is an approximately 20% decrease of developing breast cancer for each year menarche is delayed. This includes the onset and establishment of regular menstruation. (6)
2. Age of menopause: It has been estimated that women who experience natural menopause before 45 have one half the risk of those whose menopause occurs after 55 years of age (7).
3. Age of first full-term pregnancy: Women with a first birth under the age of 20 have about one-half the risk of a woman who has never borne a child. Women who have a late first full term pregnancy are at an elevated risk compared to nulliparous women (8).
4. Weight: Women over the age of 60 who are 10kg over their weight range have an approximately 80% increase in developing breast cancer (9).
5. Dietary fat: There have been many studies done which have generally shown a

weak correlation of dietary fat to breast cancer. Hislop et al. (10) showed a positive correlation between breast cancer and the intake of fat-associated foods such as whole milk and beef in premenopausal women.

6. Family History: A women with an immediate family member such as a mother or sister that has developed breast cancer has a 20% increased chance of developing the cancer before the age of 70 (11)

7. Ionizing radiation: There is direct evidence from the atomic bomb explosion in Japan that radiation has a carcinogenic effect since women exposed had a higher incidence of breast cancer. This effect was also seen in women who were exposed to ionizing radiation for treatment of mastitis and tuberculosis (12).

There are many histological classifications of breast cancer but the two main forms seen are *in situ* (intraductal carcinoma) and infiltrating ductal carcinoma. The *in situ* component is an intermediate position between hyperplasia and invasive carcinoma. It consist of lesions without metastatic potential. It is not cancer in the full definition but it is not non-cancer (13). The cells are seen in solid or perforated masses adjacent to the wall of the duct. The center can be an area of necrotic debris. The cells have markedly atypical nuclear features and contain a large amount of cytoplasm (14). The basement membrane of the duct is intact and surrounds the malignant cells (99).

Infiltrating ductal carcinoma (IDC), also known as invasive duct carcinoma, is the largest group of malignant mammary tumors, accounting for 65% to 80% of all mammary tumors (15). IDC is often seen with other histological types of breast cancer, such as an *in situ* component, medullary carcinoma or Paget's disease (16). The tumor can be seen in three grades:

1. Well-differentiated, in which nuclear morphology is characterized by small, relatively uniform rounded nuclei with few nucleoli and few mitoses.

2. Moderately differentiated, in which there is increased nuclear size, pleomorphism and chromatin/DNA variability, and numerous mitotic figures. Nuclei contain a prominent nucleoli, often with irregular shapes.

3. Poorly differentiated, in which the nuclei are usually large with vesicular and

variable chromatin patterns, prominent nucleoli and conspicuous, often atypical mitotic figures (16). Other features are single cell infiltration intermixed with poorly cohesive islands of infiltrating cells and occasional foci of poorly formed glands. (17).

The presence of vascularity, either lymphatic or blood, indicates a lower survival rate or a shorter disease-free interval. Metastasis into the lymph nodes also indicates a poor prognosis.

Treatment for breast cancer is currently limited to the removal of the lump, or possibly the breast. This is followed by radiation. In estrogen receptor (ER) positive cancer the drug taxmoxifen is often used. ER negative cancer, chemotherapy. Not withstanding the research done on breast cancer in the last twenty-five years the absolute death rate has not changed nor has the incidence of the disease decreased (18,19,20).

PART 2. ASPECTS OF TUMOR BIOLOGY

Tumor biology encompasses a broad range of subjects, interdisciplinaries and many hypotheses. A few of these topics will be discussed here.

i. Definition of Cancer

Cancer is a disease of nonlethal genetic damage which can be a result of environmental mutagens or carcinogens, viral infection, DNA rearrangements during cell division, inherited predisposition to cancer or one of the many unknown causes. The result in each case though is basically the same; the normal genetic program of the cell is altered and this allow the cancerous cell to have a growth advantage over its normal counterpart (21).

ii. Proliferation

In order to understand how tumor cells can freely proliferate, normal cellular proliferation must be clearly understood. There are three methods in which normal tissues can renew their populations: (1) those tissues that must maintain their population by constantly renewing, for example, bone marrow and intestine, (2) those

tissues that proliferate in response to an injury, this is usually a slow proliferation, e.g.lung or liver, (3) those that are static, e.g. nerve and muscle. It has been found that most tumors are constantly renewing themselves. However, some tumors will proliferate in response to tissue injury, for example tumors caused by the irritants in cigarette smoke (22).

Renewing of tissue is performed by a small number of cells known as stem cells. These cells have the ability to proliferate quickly, however usually the division rate is slow unless tissue damage occurs or the tissue demands it. When this cell-renewal system is triggered to proliferate quickly it can lead to clonal expansion and the production of large numbers of differentiated cells. This system is under precise control which assures that the loss of mature functional cells will be replaced by newly produced cells. The molecular basis for control is still not understood, although it is believed to involve hormonal modulators. Stem cells have two major functions: the first is to be able to provide enough new cells that will differentiate and replace dying cells and secondly to be able to self-renew and therefore keep their cell numbers constant (22).

Some tissues with cell-renewal capacity may undergo expansion changes by either metaplasia or hyperplasia. Examples are the changes that occur in the normal menstrual cycle. The endometrial epithelium will respond to hormone changes by proliferating; the tissue is then shed and the epithelium returns to normal. This type of condition shows that normal cells are capable of reversing the effect of both hyperplasia and metaplasia. In the neoplastic condition those changes are permanent and self-renewal cells are an inherited property of these cells. The growth of tumors is therefore related to the heritable changes in the control of cell proliferation and differentiation (22).

There are two theories dealing with evidence that stem cells exist in tumors. The first states that the carcinogenic event may occur in a differentiated cell, thereby allowing that cell to be a proliferating cell. The cell must also maintain its ability for tissue-specific differentiation. Therefore the cell actually dedifferentiates to become a proliferating stem cell (22). Currently there is no evidence to support this theory. The

second theory states that the carcinogenic event happens in the stem cells of the involved tissue. The defect causes a change in the normal stem-cell functions of self-renewal and differentiation (23). Some tumors, once they have achieved self-renewal and differentiation, will proceed to invade the surrounding tissue.

A tumor will continue to grow because it contains a population of cells that are constantly expanding. Normal homeostatic control mechanisms have been bypassed. The growth rate of tumors varies. With the use of tritiated thymidine autoradiography and recently flow cytometry, it is possible to analyse cell kinetics.

iii. Doubling Time

It is difficult to estimate doubling times but Steel (24) examined 780 human tumors and came to five conclusions:

1. There is a wide variation in growth rates; this is even seen among tumors of the same histological nature
2. In lung metastases of common tumor the rate is about 2-3 months
3. Adult and childhood tumors that respond to chemotherapy have a tendency to grow faster than those that don't respond to chemotherapy
4. Adenocarcinomas grow more slowly than either sarcomas or squamous-cell carcinomas
5. Breast and colorectal metastasis grow more quickly than their primary tumor.

It has also been found that as growth of the tumor increases, the doubling time lengthens due to factors other than tumor cell generation time. Increased size may cause diminished blood supply resulting in a competition for certain metabolites and other necessary components. This competition will lead to an increase in cell death or differentiation (27). As the tumor size increases it will trigger tumor cells to drop out of the mitotic cycle. The cells will either enter a prolonged G_1 period or a G_0 resting state. It is important to realize that these cells are not lost to the tumor but when properly stimulated can re-enter the cell cycle. This population of cells is therefore a reserve stem cell population. (25).

Research has shown that it takes fourteen weeks for a primary breast tumor to

double. If it metastasizes into the lungs the doubling is dropped to eleven weeks. This figure drops to three weeks when it metastasizes into soft-tissues (26).

The smallest possible size for a tumor to be detected by either physical or radiologic examination is 1 cm in diameter and weighing one gram. At this size it contains 10^8 to 10^9 tumor cells and has undergone approximately 30 doublings, if it started from a single cell. To grow from one gram to one kilogram requires only 10 further doublings in cell number (26). It is during this period of doubling that the tumor is clinically evident and treatable. It also represents a short time in the tumor's life. Therefore, before the tumor is even detected there may have been ample opportunities for it to seed itself in several areas.

iv. Heterogeneity

Since some tumors arise from a single cell, it would be expected that cells in that tumor would be homogenous. Instead, it has been found that tumor cells show significant heterogeneity in the following properties: morphology, cell proliferation, karyotype, surface markers, biochemical products, metastatic behaviour and sensitivity to therapeutic agents (22). Observation has lead to the theory that tumor cells are unstable genetically when compared to normal cells and this defect leads to the cells displaying phenotypic heterogeneity (27). There are currently three mechanisms that may explain the diversity found among tumor cells as a clone expands. The first is differentiation-related heterogeneity within a clone. Studies have shown that when tumors cells are less differentiated the clones produced are more aggressive and result in rapidly growing tumors. They also have greater stem cell growth and renewal capacity. The second mechanism is nutritional heterogeneity. The supply of blood varies in different parts of the tumor. Varying amounts of oxygen, metabolites and growth factors may effects the mutation rate and therefore increase further diversity of cells. The third mechanism is the generation of new subclones during tumor progression. New subclones may arise from mutations. Some of these subclones will become extinct but those that have the growth advantage will survive and make new progeny. All subclones will share common clonal markers but will

have inherited new properties (22). In breast cancer, heterogeneity can be clearly seen. When breast tumor cells are stained for estrogen receptors via immunoperoxidase, it can be seen that some cells stain for the estrogen receptor (ER) while other cells, possibly those right beside the ER cells, will not stain for any estrogen receptor. This phenomenon will vary within the patient's own tumor as well as from patient to patient. Heterogeneity can make treatment of the cancer difficult (28).

v. Angiogenesis

Angiogenesis is the formation of new blood vessels. There is evidence to indicate that tumor growth is dependent on angiogenesis (31). Tumors are able to reach a size of a few millimeters (10^6 cells) without the need for blood vessels but to grow beyond this size requires the induction of new capillary blood vessels is required. The growth of these new blood vessels is mediated by specific angiogenic molecules which are released by the tumor cells and macrophages which are attracted to the area (29,30).

It is currently partially but not entirely understood how the angiogenic activity is initiated by a tumor. The switch is done in two stages, the first being the prevascular stage followed by the vascular phase (31). The prevascular stage has been observed in cervix, bladder, and breast carcinoma and melanoma. This condition can exist for years and is associated with limited tumor growth (32). When the vascular stage is reached or triggered, the tumor will begin growing rapidly, bleeding will occur and the potential arises for metastasis.

The development of new capillary blood vessels depends on sequential morphological and biochemical steps. These steps include degradation of the extracellular matrix by specific endothelial enzymes, endothelial migration and tube formation (33). This event is organized by multiple factors. Some factors such as fibroblast growth factor (FGF) will initiate endothelial locomotion and proliferation while other factors such as transforming growth factor-beta (TGF-B) or tumor necrosis factor-alpha (TNF-α) will cause endothelial differentiation and tube formation

(34). It is known that tumors will induce blood vessel growth from the extraluminal side of the host capillary endothelium. It is also believed that angiogenic factors can gain access to the lumen of newly generated capillaries and have a different effect. For example TNF-α extravascularly induces neovascularization but when it gains access intravascularly it provokes coagulation (29).

PART 3. CELLS INVOLVED IN THE IMMUNE RESPONSE

Lower vertebrates and invertebrates have several defense mechanisms to recognize non-self antigens found on microorganisms. The first mechanism is that a group of proteins is present that recognize and agglutinate a wide variety of microorganisms. The second mechanism is made-up of cells called phagocytes which are capable of engulfing and digesting the microbes (35).

Phagocytes were maintained and evolved with the higher vertebrates. The system developed into lymphoid organs producing lymphoid cells. These specialized cells have a high degree of specificity and can recognize non-self antigens. The cells of this system arise from the pluripotent system and two main lines of cells are differentiated. The first is the common lymphoid lineage which produces the lymphocytes, this includes the following cell types: T cells, which have subsets of cytotoxic/suppressor cells (Tc/s) and helper cells (Th); and B cells which will differentiate into plasma cells. The second lineage is the common myeloid progenitor, which consists of the following types of cells: monocytes which develop into macrophages once they leave the peripheral blood stream; mast cells; basophils; neutrophils; eosinophils; and megakaryocytes which give rise to platelets. There is a possible third type of cells which includes the following: Natural Killers (NK) and Antigen-presenting cells, however, it is still uncertain if they arise from the lymphoid or myeloid lineage (35).

i. Lymphoid cells

Lymphoid cells are produced primarily in the thymus and adult bone marrow.

They are released in the peripheral blood stream and will migrate to secondary lymphoid tissue which consists of the spleen, lymph nodes, tonsils and uncapsulated lymphoid tissue. Lymphocytes express markers on their surfaces. Some of the markers appear at specific times in cell differentiation or when the cell is activated, while other markers are characteristic of their cell lineage. Recently a systemic nomenclature was developed for the markers, it is known as cluster designation, which is abbreviated to CD (35).

ii. T-cells

T cells have several markers on their cell surfaces. One of the first markers found was CD2, which is found on all T cells. It is used to separate T cells from B cells, since CD2 will bind to sheep erythrocytes. The T-cell receptor (TCR) is a heterodimer which is composed of two chains, each having molecular weight of 40-50 kDa. There are two separate T-cell lineages. TCR1 is composed of the gamma and delta chains and it appears in ontogeny. The TCR2 contains an alpha and a beta chain and T-cells bearing these receptors will be seen throughout adult life. In all immunocompetent T-cells, the TCR is noncovalently, but still intimately linked in a complex with CD3. CD3 is a molecule composed of seven peptide chains and is found on all T-cells. The function of the CD3 is to transduce the antigen recognition signal received by the alpha and beta heterdimer on the TCR which will stimulate the T-cell (36). T-helpers (Th) carry the CD4+ marker and T-cytotoxic/suppressor (Tc/s) carry the CD8+ marker. CD4+ cells recognize antigens which are associated with major histocompatibility complex (MHC) class II molecules. CD8+ recognizes MHC class 1 antigens. The CD4+ can be further subdivided by other surface markers. CDw29+ are those Th cells which positively influence the immune response of T cells and B cells. Another surface marker is CD45R+; these Th cells will induce the suppressor inducer function of the CD8+ cells which are the cytotoxic/suppressor T cells. CD2 is also found on approximately 50% of the Natural Killers (NK), which are designated as CD3-. CD5 is expressed on all T cells and a subpopulation of B cells which are involved in autoantibody production. CD7 is seen on what is known

as the Third Population Cells (TPC) and it may be a receptor for Fc of the IgM antibody (35).

iii. B-cells

B cells form about 5 to 15% of the circulating lymphoid pool. They are well known for their production of immunoglobulins called antibodies. Antibodies will insert into the surface membrane and act as specific antigen receptors. The peripheral blood B cells mainly express both surface IgM and IgD. Those B cells that express IgG, IgA or IgE are found in large numbers in specific locations of the body; IgA for example is located in the intestinal mucosa. A majority of B cells carry the MHC II antigens, which are an important part of the cooperation mechanism with T cells. In man they are known as HLA-DP, DQ, DR antigens. Other markers are CD35, and CD21 which are complement receptors for C3b and C3d and are involved in the activation and possible homing in of B cells. CD19, CD20, and CD22 are markers used to identify B cells but their specific task is not yet known (35,36).

T, B, and TPC cells have other markers which are found on all the three types. Leucocyte function antigen (LFA)-1 is one such marker. This marker is important for cell adhesion and intercellular communication. LFA can also be found on granulocytes and macrophages (35,36).

When B or T cells are activated there is an increase in several surface markers and the appearance of several others. After a T cell is activated, the IL-2 receptor appears. It consist of a low affinity receptor, CD25, and a 70kD medium affinity molecule which combine to make a high affinity IL-2 receptor. Other markers appearing are MHC class II molecules, transferrin receptors and CD38. On B cells the high affinity IL-2 receptor appears but also receptors for growth and other factors appear, for example IL-3R, IL-4R, IL-5R and IL-6R. Transferrin receptors and some MHC IIs will also materialize. CD23 is present and is involved in driving B cells to proliferate (35,36).

iv. Myeloid cells

The other system besides the lymphoid, is the myeloid system. One part of it is the mononuclear phagocytic system. There are two main functions performed by these cells. The first is the macrophage whose job it is to remove particulate antigens. The second is to perform the function of an antigen-presenting cell (APC), whose role is to present the foreign antigen to specific lymphocytes.

The human monocyte is approximately 10-18 μm in diameter and contains a horseshoe-shape nucleus with azurophilic granules. The ultrastructure of the monocyte is as follow: ruffled membrane, a well developed golgi complex and many intracytoplasmic lysosomes which contain several acid hydrolases and peroxidases. Monocytes/macrophages will actively phagocytose microorganisms and tumor cells. This is done by the monocytes/macrophages binding to specialized receptors for certain carbohydrates, or IgG or a complement. Macrophages have three distinct Fc receptors for IgG; Fc RI has a high affinity, Fc RII (CDw32) of medium affinity and Fc RIII (CD16) has a low affinity. It is thought these receptors have one of the following functions: triggering extracelluar killing, opsonization or phagocytosis. Some of these activities could also be performed by CD35. CD11b is involved in adhesion, and is present on activated macrophages along with leucocyte function antigen-1 (LFA-1) and CD11c. Both CD11b and CD11c are found in intracytoplasmic vesicles and rapidly go to the surface when monocytes/macrophages are activated. MHC II is present on some macrophages. A low affinity receptor for the Fc of the IgE is sometimes present. Once the monocyte leaves the peripheral blood and enters into one of the many organs it then forms the reticuloendothelial system. The monocytes then develops into many different types of macrophages (35,36).

PART 4. DEFENCE REACTIONS AGAINST TUMORS

i. Cytotoxic-T-lymphocytes

There are three discrete stages of target cell killing by cytotoxic-T-lymphocyte (CTL): 1. specific binding, with rapid formation of a strong adhesion between the two cell membranes; this takes 2 minutes. 2. the delivering of a lethal hit which is temperature and calcium dependent; this stage takes 10 minutes. 3. slower killing where the target cell is totally destroyed and the CTL is able to remove itself unharmed and go to the next target cell. (37).

The way in which an antigen is recognized by a T lymphocyte has been carefully evolved to allow an organism to define self and also to be able to distinguish non-self antigens, thereby protecting the organism from pathogens that may be life-threatening. This evolved system also allows the T-lymphocytes to proliferate when its receptors interact with antigens present on the surface of the other cell. The target cell or an antigen-presenting cell will present on its surface a complex formed by a fragment of the immunizing protein which is bound by either a major histocompatibility complex (MHC) class I or Class II (38). Some of the necessary requirements for a peptide to bind to a MHC protein have been identified, but the exact mechanism is still unknown.

The binding of the CTL to its target cell occurs via TCR. The TCR is non-covalently bound to CD3. The TCR binds to the MHC-1 on the target cell. The TCR then checks the cleft on the MHC-1 to "see" if the peptide in the cleft is a non-self processed antigen. If it is, the TCR then transmits a signal to the CD3 and the stimulation of the CTL begins (35,36). Other molecules are involved with the binding but these receptors do so in a non-specific manner. LFA-1 and LFA-2 (CD2) are thought to be involved with cell adhesion, cooperation and maybe some cytotoxic interaction. LFA-1 and LFA-2 are found only on lymphocytes whereas LFA-3 is more widely distributed on cells . LFA-2 (CD2) interacts with LFA-3 and LFA-1

forms a partnership with an intercellular adhesion molecule termed ICAM-1 (35,36).

Upon attachment to the target cell the CTL will undergo cytoplasmic rearrangement. The CTL has what is known as a polar cytoplasm, that is, the leading edge contains the nucleus which has a tapered tail carrying cytoplasmic granules. When adhesion occurs with a target cell it is at the leading edge of the CTL. This causes the nucleus to instantly move away and the area is rapidly filled by granules. These granules will fuse with the plasma membrane of the CTL as early as 4 minutes after contact has been made (37).

There are currently three potential mechanisms for cytotoxic damage of a target cell. The first, which is the most contemporary, suggests that the CTL degranulates, which causes perforin to be released and in the presence of CA^{2+} the perforin forms polyperforin channels. The second mechanism is that the CTL releases degradative enzymes, which pass through the channels on the target cell. The third mechanism which takes much longer then either 1 or 2, is one in which the CTL releases tumor necrosis factor (TNF) and interferon-gamma (IFN-g) which triggers the receptors on the target cell to change to protein synthesis which in turn causes cytotoxic damage (39,40).

ii. Macrophages

Macrophages play a central role in the cell-mediated response. They are involved in three major roles: 1. involvement in the regulatory function; 2. as an antigen-presenting cell; and 3. in the effector phase as an inflammatory, tumoricidial or microbicidal cells. Macrophages along with NK cells are regarded as part of the effector cells of natural immunity. It has also been speculated that macrophages are also a part of the immunosurveilance system which is involved in protecting the body from tumors. Macrophages have also been implicated in having some control over metastasis (35).

The exact mechanism(s) by which a macrophage is able to discriminate between tumorigenic and normal cells is currently not known. Experiments have been done to try and understand the mechanism(s). The cytotoxicity of monocytes was

assessed against three tumorigenic and nontumorigenic allogenic target cell populations. These cells were labelled with [^{3}H]TdR or [^{14}C]TdR and were plated onto a monolayer of blood monocytes. It was seen that the activated monocytes selectively lysed the three populations of tumorigenic cells while not lysing the nontumorigenic cell at all (41). Another experiment using tumoricidal monocytes, melanoma cells and time-course cytotoxicity studies led researchers to a hypothesis to explain how lysing occurs: 1. cell-to-cell contact; 2. the production of damage to target-cell membranes; 3. the development of vacuolation in the target; 4. death to target cell (41). Other studies used activated mouse peritoneal macrophages that were radiolabeled. Syngenic, allogenic and xenogenic, tumorigenic and nontumorigenic target cells were presented to the macrophages. After 72 hours the macrophages lysed the syngenic, allogenic and xenogenic tumor cells but did not harm the normal nontumorigenic cells. This suggests that target susceptibility is independent of tumor-specific antigen or transplant antigens. This statement then led researchers to hypothesize that macrophages lyse all tumor cells regardless of histocompatibility differences (41). This method is similar to that used by natural killers, whereas cytotoxic-T cells need the MHC present before they can be activated. Other projects showed that tumor cell lysing is activated by macrophages, independent of the cell cycle time. Another showed that macrophage recognition of tumor cells is independent of the tumor cell's metastatic potential (41).

Macrophages will not act as effectors with cytotoxicity activity unless they have been activated. The process is characterized by morphological changes, biochemical substances and functional change. The biochemical substances involved includes endotoxins, immune complexes, aggregated IgG, muramyl dipeptides and lymphokines. Once cell-to-cell contact is made by macrophages, they will secrete any one of the following: proteases, hydrogen peroxide and tumor necrosis factor (TNF).

PART 5. BIOLOGICAL THERAPY FOR TUMORS

When solid tumors are examined histologically, they reveal infiltration of lymphoreticular cells. Some researchers feel that heavy infiltrations are associated with a good prognosis of survival but that idea is still very debatable. The conventional methods of treating solid tumors have been surgery, radiation and/or chemotherapies. These methods are not usually completely or even tolerably successful .

In the past decade a new form of therapy has been developed called immunotherapy. This biological therapy is defined as cancer treatments which act primarily through the host's natural defense mechanisms or by the administration of natural mammalian substances (42,43). This new treatment has led to a better understanding of the immune system as researchers attempt to identify the different immune cell populations within the human and tumor-bearing animals and how those cells can identify and destroy a tumor cell. This area of immunotherapy is referred to as adoptive immune-therapy and is defined as the transfer to the tumor-bearing host of immunological reagents such as immune cells that have the ability to have an anti-tumor effect either directly or indirectly.

The major problems encountered with adoptive immunotherapy have been in the designing of a method of removing the immune cells from the tumor, selecting the correct immune cell population, stimulating that population so that a large enough population can be obtained and reintroducing them into the host (47,48,50).

Removing the immune cell population from tumors has required abundant research. The problem lies in the fact that these are solid tumors, in which the all the cell types are often tightly bound. Immune cells are bound in large amounts of collagen and are often intermixed with large clusters of tumor cells. The difficulties are to recover a viable population of cells that can be analyzed and the determination of whether those cells are representative of those immune cells that infiltrate the tumor (42-46).

i. Preparation of TILs

Two methods were investigated to determine the better way to remove the TILs from the tumor. Whiteside et al. (47) tried mechanical segregation but this yielded a population of dead tissue, tumor cells and erythrocytes and did not yield a population of TIL in any significant numbers. Therefore the method was revised and Whiteside (48) found that a combination of mechanical and enzymatic methods successfully yielded a population of viable T-cells and tumor cells but it destroyed the macrophage population.

To use TILs in adoptive immunotherapy, the population must be expanded. This expansion produces populations of 2.0 to 9.0 x 10^8 cells. The TILs were cultured with autologous serum, antibiotics and recombinant interleukin -2 (rIL-2). The cultures were kept at 37°C in humidified 5% CO2 atmosphere. Fresh rIL-2 was added weekly or as dictated by the growth rate of the culture (47,48).

Next the TIL cytotoxicity characteristics were determined. This was done using ^{51}chromium release cytoxicity assays. Topalian et al. (49,50) cultured TILs from 25 solid tumors (6 melanomas, 10 sarcomas and 9 adenocarcinomas). In a cytoxicity assay a known number of TIL effector cells are combined in with tumor target cells that have ^{51}chromium in their nucleus. This experiment included tumor cells that were fresh autologous, fresh allogenic tumor cells of same histologic type, fresh allogenic tumor cells of different histologic type, NK-sensitive K562 and NK-resistant Daudi cell line. The results showed that 19/25 TIL cultures lysed the K562 and/or the Daudi targets. Nine TIL lysed autologous tumor target, those being: 4 melanomas, 3 sarcomas and 2 adenocarcinomas TILs. The lysing of autologous targets ranged from 10.4% to 48.7% at an effector:target ratio of 40:1. The 40:1 ratios indicate that something was not right. A single cytotoxic T-cell is capable of directly killing many tumor targets. In Topalian's experiments (50), 40 TIL's to one tumor cell indicates that a substantial part of those 40 TIL's are useless.

ii. Clinical Trials

Clinical investigation of TILs involving humans began in 1987. Patients that entered any of these trial programs all had an expected survival of at least 2 months and had not recieved any treatment for their cancer for 30 days before the TIL

treatment began. Dr. Steven Rosenberg and his laboratory at the National Institute of Health in the USA has done most of the human experiment work (42-46). The first trial used twelve patients; six with melanoma, four with renal cell carcinoma (RCC) and one each with breast and colon carcinoma. Two of the patients with melanoma experienced partial regression (51). Another group of twenty patients was used in a similar trial that year. It was found that 11/20 patients had objective tumor regression. This experiment was considered to be more successful because the full dosages of interleukin-2 (IL-2) was tolerated (42,45). Rosenberg (44) completed the largest trial involving 652 patients and 7 protocols. A partial regression (a 50% reduction in tumor size) was seen in 81/652 and 27\652 had a complete regression of their tumor (an absence of their tumor for at least one month). **Of 21 patients with breast carcinomas who were involved with this trial and there was not a single case of either a partial or complete regression of the tumor.**

One clinical trial was conducted to determine where the TILs migrated within the body. Preparation of TILs were made and 10% of the cells were removed and labelled with radioactive Indium-111. The radioactive cells were mixed with the TILs and infused into the patient. Six patients received this treatment and it was found that as early as 24 hours after treatment the TILs had localized at various tumor sites (52).

CONCLUDING REMARKS

Immune cells are capable of destroying tumor cells. Many of the exact mechanisms are yet to be understood. Immunotherapy using TILs is still in the inital stages, but it holds great hope in the future as a form of cancer treatment. The incidences of breast carcinomas are currently rising, for reasons that can not be fully explained. TIL immunotherapy has currently had no success as a treatment for women with breast carcinomas. In this research project I would like to attempt the following:

1. Characterization of immune cell populations within breast carcinomas.
2. To determine if there is a relationship between tumor growth and immune cell population in order to determine which (if any) immune cell populations show a

positive or a negative correlation to tumor growth. This may give an indication of which cells should be selected and which should be avoided when attempting immunotherapy for breast cancer.

3. To understand some of the functions of the various immune cells within the tumor.

II. MATERIAL AND METHODS

PART 1. IMMUNNOHISTOCHEMISTRY

i. Preparation of tumors for sectioning

A cross section of the tumors were received fresh from the department of pathology (Royal Jubilee Hospital of The Greater Victoria Hospital Society). They had received the tumor as a biopsy, lumpectomy or a mastectomy from the operating room. The tumors used were those that the pathologist had examined for general morphology and rapid frozen section diagnosis and had been declared malignant. Once that had occurred our laboratory received a cross section of the tumor. If possible the tumor was orientated on the orientation tray (van Netten et al., 1988).

The tumor was placed on a cold tray and any excess fat was trimmed. The sample was then placed on a metal cryostat block and embedded in OCT embedding medium (Tissue-Tek, Miles). The tumor and block were then placed in the -26°C cryostat (AO Histostat Microtome). When frozen solid the tumor and block were wrapped in tinfoil, placed in a plastic bag and a plastic labelled container and placed in the -75°C freezer until they were sectioned.

ii. Cutting of sections

The tumor and block were removed from the freezer and placed in the -26°C cryostat (AO Histostat Microtome). 6 micrometers (μm) sections were taken consecutively. Two complete series of sections were cut (Table 1) from each tumor. The best series was used for analysis. Sections were placed on poly-L-lysine coated slides (Sigma P8920). From each series cut, the first and last slides were stained in hemotoxin and eosin and used to study the general morphology of the tumor.

For CD3, CD4, CD8, macrophage, IL-2R and B cell, tonsil was used as a positive control (received from the Victoria General Hospital and usually from a child under the age of ten), and for TGF-α and EGFR, foreskin tissue was used. These slides were cut at the same time and treated in the same manner as the tumor slides.

Negative controls were cut for each series.

iii. Fixation of slides

a. Ki-67, ER, and EGFR slides were cut and immediately fixed in 3.7% PBS buffered formaldehyde for 10 to 15 minutes. They were then placed in fresh phosphate buffer solution (PBS) for at least six minutes, followed by 4 minutes in -10^{0}C methanol, then 2 minutes in -10^{0}C acetone, and then at less six minutes in fresh PBS. At this point the staining procedure was either started or the slides could be placed in the storage medium for up to 3 months and then rinsed in PBS for five minutes prior to staining.

b. The CD3, CD4, CD8, macrophage, IL-2R, B cells and TGF-α slides were cut, placed on a slide tray and allowed to dry overnight at 3-5^{o}C. Immediately before staining the slide were placed in -10^{0}C acetone for 10 minutes.

iv. Staining procedure

a. This procedure was used for all the slides except TGF-α (4b) and ER (4c). Normal rabbit serum (NRS) (50μl), was added as a blocking serum, for a 20 minutes incubation. The "block" aid's in reducing nonspecific staining. The NRS was then carefully blotted off and the primary antibody added to the slides for a 60 minute incubation (see Table 1 for dilution of each primary antibody). The negative control slides received PBS instead of a primary antibody solution. After incubation, the slides were placed in a 25% solution of hydrogen-peroxide and PBS for 30 minutes. This step stops endogenous peroxidase activity. It was followed by a five minute rinse in fresh PBS. The slides then received the linking antibody; Dakopatts rabbit-anti-mouse immunoglobin, and were incubated for 30 minutes. The slides were then rinsed in two washing of PBS for five minutes each. Next, peroxidase-anti-peroxidase (PAP) was placed on the slides for 30 minute incubation, followed by two washings in PBS for five minutes each. The slides were then ready for color development. All incubation were at room temperature in a humidified slide chamber and covered with tinfoil to prevent degradation of the antibodies by the light.

b. The staining of the TGF-α slides. The slides were "blocked" for 30 minutes with normal donkey serum. The slides were then blotted off and the primary antibody was placed on the slide for a 60 minutes incubation, followed by the endogenous peroxidase bath (as above) for 30 minutes. The slides were then given a five minute rinse in PBS. The link, horseradish peroxidase conjugated donkey-anti-sheep IgG was placed on the slides for 45 minutes, followed by two rinses of fresh PBS for five minutes each. The slides were then ready for color development. The last five tumor stained for TGF-α had a change in procedure. Normal goat serum was used as the "block" for 20 minutes and the link and PAP were replaced with horseradish peroxidase conjugated goat-anti-mouse IgG for 45 minutes. (It was found this method reduced the background staining).

c. ER monoclonal antibody used the Abbott ER-ICA monoclonal kit, an immunocytochemical assay for the detection of human estrogen receptors (Abbott Laboratories #83-4502/R7). The directions for the procedure are within the kit. A short summary will be presented here. The blocking reagent was added first and incubated on the slide for 15 minutes. Next the primary antibody was added for 30 minutes followed by two washes of fresh PBS. The bridging antibody was then added to the slide for 30 minutes and followed by two washes of PBS. The PAP complex was added and incubated for 30 minutes followed by two washes of PBS. The slides were then ready for color development.

v. Color development

Slides were blotted dry and placed on the slide tray. Activated DAB mixture was then added to each slide and incubated until the desired color development was reached; maximum incubation time being 15 minutes. Once the desired color development was reached the slides were placed in a distilled water rinse to stop color development. The slides must remain in the rinse for a minimum of five minutes. The slide rack was then transferred to a solution of 10% hematoxylin (Harris Hematoxylin) bath for 10 minutes and then rinsed in running tap water until no more hematoxylin came out. The slides were then put into a blueing solution for 45

seconds.

vi. Mounting

The slides were then dehydrated through 2 baths of 95% alcohol, 2 baths of 100% alcohol and 2 baths of xylene for two minute each. The slides were then mounted with coverslips using Parmount (Fisher Scientific #SP15-500) mounting medium.

vii. Methods of analyzing the Immunohistochemical slides

a. Three independent observers were used to analyze the slides. The slide was placed on a microscope which was hooked up to a video camera so the image appeared on a video screen.

b. The slide sections were analysed for positive staining cells using a relative 4 grade scale according to the method used by Horst et al. (1987), where 1= no cells or a very small number of cells; 2=a small number of cells; 3=a moderate number of cells; and 4=a large number of cells. These categories correspond for the Ki-67 sections approximately to the following percentages: 1=0%; 2=<5%; 3=5-10%; and 4=>10%. For the estrogen receptors (ER) sections the categories correspond approximately to: 1=0%; 2=<20%, 3=20-60%; and 4=60-100%.

PART 2. CULTURES

i. Preparation and growth of cultures

Breast carcinoma tissue was received fresh from the Pathology Department (RJH of GVHS). The tissue was placed in a sterile culture dish on gauze soaked in a wash solution. The wash solution was made-up of 100 ml of medium-199 (Gibco Laboratories #D-88) and 500 μl of gentamicin reagent solution (Flow Laboratories #16-762-45). The tissue was then taken to the Special Development Laboratory and the culture was prepared in a Laminar flow hood. The tissue was transferred into another culture dish and submerged in wash solution for 20 minutes. The fat and any

unnecessary tissue was removed. It was then transferred to another culture dish for 20 minutes. The procedure was repeated once more. In the last wash dish the best portion of the carcinoma was chosen and that portion was transferred to another culture dish, which contained a small amount of culturing medium. The culturing medium was made-up of 100 ml of medium-199 with 100 μl of gentamicin added. The tissue was then minced with two sterile scalpel blades to release the cells into the medium. A solution of culturing medium with 20% fetal calf serum (FCS) (Flow Laboratories #29-167-49) was made and 3 ml was added to each flaskette chamber (16 ml Lab-Tek flaskette, #4820). Then 200-300 μl of cell mixture was added to each flaskette. The flaskettes were incubated at 37°C for two to three weeks. When available, after three days the culture medium could be replaced with 3 ml of 20% autologous serum (culturing medium and 20% autologous serum).

ii. Staining of cultures

Cultures were rinsed with PBS and fixed with 1% paraformaldehyde solution for ten minutes. The top portion of the culture dish was then removed. The culture was then placed in two washes of PBS for five minutes each. The culture then received 2 ml of normal rabbit serum and was incubated for 20 minutes. The NRS was poured off and primary antibody was added and incubated for 60 minutes. The primaries used are indicated in Table 2. The primary was then poured off and the culture placed in an endogenous peroxidase bath for 30 minutes followed by a rinse of PBS for five minutes. The linking antibody was then added and incubated for 30 minutes, followed by two rinses in PBS for five minutes each. The PAP was added and incubated for 30 minutes, followed by two five minutes rinses in PBS. Activated DAB was added for 10-15 minutes for color development. The culture was then placed in distilled water for five minutes followed by 10% hematoxylin solution for 20 minutes and then carefully rinsed in running water until no more blue could be removed. The culture was then mounted using an aqueous mounting solution (Sigma Diagnostics #1000-4) and specially cut coverslips. The mounted culture was kept in an covered petrie dish to avoid drying out.

PART 3. FLOW CYTOMETRY

i. Preparation of the sample

The flow cytometry was done at the British Columbia Cancer Agency (BCCA) Vancouver Clinic. Tonsils were used to test the effect of teasing the tissue apart, using digestive enzymes, or mechanical segregation on the LR population. The tonsils were received fresh from the Vancouver General Hospital and placed in RPMI 1640 medium with sodium bicarbonate, antibiotics, 10nM hepes, L-glutamine (TFL Media Preparation Service) for transportation to the BCCA. Then tonsil was then cut into three equal cross sections. One piece was gently teased apart in RPMI1640, using scalpel blades. The cells that were released and the RPMI were placed in a centrifuge tube and spun down. The excess RPMI was poured off and the cell pellet was resuspended in fresh RPMI. The second cross section was cut into cubes and placed in a digestive mixture consisting of 0.1% collagenase (Sigma 5138), 0.002% DNAase (Sigma EC3.1.21.1) and 0.01% hyaluronidase (Sigma) for 60 minutes. The mixture was then poured through a wire mesh and the cell solution was spun down and resuspended. The third cross section was placed in a sterilized blender with RPMI for 10 seconds at high speed, poured through the wire mesh and spun down. A viability count was done on each of the three samples using trypan blue to determine the amount of each mixture needed for one million cells per ml.

ii. Staining

The following procedure was used for all cell mixtures except CD68 and its control. Two monoclonals were used per tube (Table 3), which were bound to fluorescence colors, one being fluorescein (FITC,green) and the other is phycoerythrin (PE, red). Ten μl of each antibody was added to the tubes, followed by the volume of cell mixture calculated in section i. The tubes were then incubated on ice in the dark for 30 minutes. After incubation, they were topped up with PBS-azide to wash out excess antibody and spun down in a refrigerated centrifuge at 1200 rpm for 5 minutes at 4^0C. The PBS-azide was then aspirated off leaving a pellet at the bottom. Then 0.5 ml of 1% paraformaldehyde was added and mixed well to fix the

cells. The tubes were then ready to be run on the flow cytometer within 24 hours.

The CD68 tube and the control tube were prepared differently because the CD68 needs to be conjugated to its fluorescence color. To each tube the correct cell mixture was added followed by 0.5 ml of 1% paraformaldehyde. The tubes were incubated for 10 minutes on ice in the dark. The tubes were then spun for five minutes as described above, decanted and 10 μl of CD68 monoclonal antibody was added to one tube and 10 μl of goat-anti-mouse (GAM) was added to the other tube. The tubes were incubated for 30 minutes, washed in PBS-azide and spun for 5 minutes and aspirated. Then, 20 μl of GAM was added to each tube, and incubated for 30 minutes, washed in PBS-azide, spun down, aspirated and 0.5 ml of 1% paraformaldehyde was added to each tube. The tubes were then ready to be run on the flow cytometer within 24 hours.

The conjugated cell-monoclonal antibodies mixtures were analyzed on the flow cytometer by either Kees Pot or Don Phillips of the British Columbia Cancer Agency in Vancouver B.C.

TABLE 1

Monoclonal Antibodies Used in Immunohistochemical Research

Monoclonal	Description	Company	CAT Code	Dilution
Ki-67	proliferating cells	Dakopatts	M722	1:50
ER	estrogen receptors	Abbott Lab	3087-18	prediluted
CD3	pan-T cells	Dakopatts	M756	1:100
CD4	helper T-cells	Dakopatts	M716	1:20
CD8	cytotoxic/suppressor T cell	Dakopatts	M707	1:20
Macrophage	macrophage	Dakopatts	M814	1:100
IL-2R	interleukin-2 receptor	Dakopatts	M731	1:50
B-cell	B-cells	Dakopatts	M740	1:50
EGFR	epidermal-growth factor receptor	Biogenex Lab	MU170-UC	1:80
NRS	normal rabbit serum	Dakopatts	X902	1:25
Link	linking rabbit-anti-mouse Ig	Dakopatts	Z259	1:25
PAP	horseradish peroxidase in mouse	Dakopatts	B650	1:350
TGF-a	transforming-growth factor-alpha	Biodesign	M301005	1:20
NDS	normal donkey serum	Sigma	D-9663	1::5
DaSPase	donkey-anti-sheep peroxidase	The Binding Site	PP360	1:25

TABLE 2

Monoclonal Antibody Combinations Used in Cell Cultures Research

Monoclonal Antibodies
1. Macrophage
2. Macrophage Ki-67
3. Macrophage Fibroblast

TABLE 3

Monoclonals Used for Flow Cytometry Research and Their Pairings

Antibody	Color	Type	Company
IgG1	FITC	Control	BD
Ig2A	PE	Control	BD
aleu	FITC	pan-lymphocytes	BD
M5	PE	mac/mono	BD
leu 12	FITC	B cells	BD
leu 4	PE	pan T cells	BD
leu 2a	FITC	CD8	BD
IL-2R	PE	IL-2R	BD
leu 3a	FITC	CD4	BD
IL-24	PE	IL-2R	BD
leu 11A	FITC	NK,grans,mac	BD
leu 19	PE	NK,act.lymphocytes	BD
GAM	FITC	control	TAGO
CD68/GAM		MAC	Dakopatts

BD = Becton-Dickson

FITC = Fluorescein

PE = Phycoerythrin

III. RESULTS

PART 1. RESULTS FROM THE IMMUNOHISTOCHEMICAL ANALYSIS.

i. EXPLANATION OF PHOTOMICROGRAPHS

Figure 1.

Patient #22 was a postmenopausal woman age 69. This tumor was a moderately differentiated infiltrating ductal carcinoma of the breast. The tumor was composed of solid sheets of large cells with moderate nuclear pleomorphism and relatively frequent mitotic figures. The tumor also had a *in situ* component seen in many of the ducts. Metastatic breast carcinoma was present in one of the seven lymph nodes. Figure 1a was a transverse sectional cut of the terminal lobular ductal unit. The tumor cells at this point are surrounding the unit. As the tumor was sectioned deeper into the duct the tumor cells can be seen filling the duct until in Figure 1i and 1j they fill the duct. It can be observed that the there are two different shapes of immune cells. One type is circular in shape; it may be a resting cell. The other type is stranding. These cells are possibly migratory.

Figure 2.

Patient #30 was a 57 year old postmenopausal woman. The tumor was a poorly differentiated infiltrating ductal carcinoma. It was composed of solid sheets and nests with no tubular formation. The cells have large nuclei displaying moderate pleomorphism and frequent mitotic figures. The metastatic tumor has almost completely replaced twelve of the fourteen axillary lymph nodes removed. In figure 2h it can be seen that some of the macrophages that surround the tumor clump are in direct contact with the tumors cells (see arrow).

Figure 6.

This figure shows two different patterns of staining for the EGFr in two

different infiltrating ductal carcinomas of the breast. A heterogenous staining pattern is seen in 6a where some tumor clusters stain positive for the EGFr (arrow) while the other clusters show no staining (double arrow). In 6b, a homogenous staining pattern is evident, nearly all tumor cells stain positive for the receptor.

ii. RESULTS FROM SEMI-QUANTITATIVE ANALYSIS OF IMMUNOHISTOCHEMICAL SLIDES

a. Table 4 lists the monoclonal antibodies used in this study and the number of tumors examined. The analytical method used is described in part vii of the material and methods section.

b. A comparison was made to determine the relationship between growth rate and macrophage concentration. The results are depicted in Table 5. and 6. In the peripheral area (Table 5) of the tumor it can be seen that 1/32 tumors had an absence of growth and a high macrophages concentration. Also 1 tumor had a low growth rate and low concentration of macrophages. 11/32 (35%) of tumors had a low growth rate with a moderate to high concentration of macrophages. The majority of tumors (19/32, 59%) had a moderate to high growth rate with moderate to high concentration of macrophages.

In the central area of the tumors, somewhat similar results were observed (Table 6). Only one tumor had an absence growth with macrophages present. One other tumor had an absence of growth with a high concentration of macrophages. Another tumor had a low growth rate and low concentration of macrophages. 35% of the tumors had a low growth rate and a moderate to high concentration of macrophages. A majority (56%) had a moderate to high growth rate and a moderate to high concentration of macrophages present.

c. The growth rate of the tumors were compared to the population of normal T-cells (CD3) present (Table 7) . In the peripheral areas of the tumors, only one tumor had an absence of growth but had a high concentration of normal T-cells present.

6/32 (19%) tumors showed a low growth rate and a low concentration of normal T-cells. Another 6/32 tumors (19%) had a low growth rate with a moderate to high concentration of normal T-cells . 3/32 (9%) tumors had a moderate to high growth rate and low T-cells concentration. One tumor had a high growth rate with an absence of normal T-cells. The largest number of tumors (15/32, 47%) had tumors had a moderate to high growth rate with a moderate to high concentration of normal T-cells.

In the central area of the tumor (Table 8), one tumor showed an absence of growth and a moderate concentration of normal T-cells. 9/32 (28%) showed a low growth rate and low concentration of normal T-cells present. 3/32 (9%) showed a low growth rate and a moderate to high concentrations of normal T-cells. 6/32 (19%) showed a moderate to high rate of growth and low concentration of normal T-cells. 2/32 (6%) showed a high growth rate with an absence of normal T-cells. 11/32 (34%) had a moderate to high growth rate with a moderate to high concentration of normal T-cells.

d. Table 9 and 10 compares the growth rate of the 32 tumors to the concentration of helper-T cells. In the peripheral area of the tumor, one tumor showed an absence of growth with a high concentration of helper T-cells. 5/32 (15%) had a low growth rate and a low concentration of helper T-cells. 7/32 (21%) showed a low growth rate with a moderate to high helper T-cells. 6/32 (19%) had a moderate to high growth rate with a low concentration of helper T-cells. 13/32 (41%) showed a moderate to high growth rate and a moderate to high concentration of helper T-cells.

In central areas of the tumors (Table 10), one tumor had an absence of growth and a high concentration of helper T-cells. 6/32 (19%) tumors displayed a low rate of growth and low concentration of helper T-cells present. 6/32 (19%) tumors showed a low growth rate with a moderate to high concentration of helper T-cells. 7/32 (22%) tumors showed a moderate to high growth rate and a low population of helper T-cells. Two tumors showed a high growth rate with an absence of helper T-cells. 10/32 (31%) tumors displayed a moderate to high growth rate and moderate to high concentration of helper T-cells.

e. The growth rate of the 32 tumors was compared to the population of cytotoxic T-cells. In the peripheral portion of the tumors (Table 11), one tumor had an absence of growth and low concentration of cytotoxic T-cells. Also one tumor had a low rate of growth and an absence of cytotoxic T-cells. 7/32 (22%) tumors displayed a low rate of growth and a low concentration of cytotoxic T-cells. 4/32 (13%) tumors showed a low rate of growth and moderate to high concentration of cytotoxic T-cells. 6/32 (19%) of tumors showed a moderate to high rate of growth and low concentration of cytotoxic T-cells. 13/32 (41%) tumors showed a moderate to high growth rate with a moderate to high concentration of cytotoxic T-cells.

In the central portion of the tumors (Table 12), one tumor displayed an absence of growth and a low concentration of cytotoxic T-cells. Another tumor had a low growth rate and 2/32 (6%) had a high rate of growth but none of those three tumors contained cytotoxic T-cells. 7/32 (22%) tumors showed a low growth rate and a low concentration of cytotoxic T-cells. 11/32 (34%) tumors showed a moderate to high growth rate and a low concentration of cytotoxic T-cells. 5/32 (16%) tumors displayed a low growth rate and moderate to high concentration of cytotoxic T-cells. 5/32 (16%) showed a moderate to high growth rate and a moderate to high concentration of cytotoxic T-cells.

f. The growth rate of the tumors in relation to the concentration of B-cells within tumors is seen in Table 13. One tumor showed an absence of growth and a moderate concentration of B-cells. 4/32 (13%) tumors showed a low growth rate, 2/32 (6%) tumors had moderate growth rate, and one tumor had a high growth rate. These 7 tumors all showed an absence of B-cells. 6/32 (19%) tumors had low growth rate, 6/32 (19%) tumors had moderate growth rate, and 5/32 (16%) had a high growth rate. These 17 tumors all had a low concentration of B-cells present. Two tumors showed a low growth rate and 4/32 (13%) tumors showed a high growth rate. Those 6 tumors had a moderate concentration of B-cells present. Only one tumor had a high growth rate and a high concentration of B-cells.

g. The relationship between growth rate of the tumors and the estrogen receptor

(ER) levels within the tumors was examined using the immunohistochemical or the biochemical results (Table 14). One tumor showed an absence of growth with a high concentration of ER. 6/32 (19%) tumors had a moderate to high growth rate and an absence of ER. 4/32 (13%) had a low growth rate with an absence to low concentration of ER present. 4/32 (13%) had a moderate to high growth rate with an absence to low concentration of ER. 8/32 (25%) tumors a low growth rate and high levels of ER present. 9/32 (28%) tumors showed a moderate to high growth rate and a high concentration of ER present.

Tables 5 to 13 depict a summary of a direct comparison between the concentration of the various immune cell population and breast cancer growth rates using absent, low, moderate or high catagories. Tables 5 to 12 show the number of tumors that exhibit both a moderate to high tumor growth rate as well as a moderate to high immune cell population within the peripheral and central areas of breast carcinomas. The highest positive association was found between tumor growth and macrophages concentration. Only one tumor had a high growth rate with an absence of immune cells.

PART 2. ANALYSIS OF CELL CULTURES.

Figures 3a and 3b.

Depicts a colony of cells from a 21 day old culture of a primary breast carcinoma. It can be seen from the staining that there are positive and negative cells for the macrophage specific antibody, and that they are intermixed through out the culture with negative staining cells. All cells appear to have a fibroblast-like morphology.

Figures 4a and 4b.

Depicts a 2-3 week old primary infiltrating ductal breast carcinoma cell culture. It is stained for macrophage specific antibody (intracytoplasmic granules will stain) and also for growth using the Ki-67 monoclonal antibody (nuclear staining). Only macrophage positive cells were also positive for nuclear specific proliferating

antibody.

Figure 5a and 5b.

Shows an interaction of cells derived from cells of a primary breast carcinoma that were cultured for two weeks. It was stained for macrophages and counterstained with hematoxylin. Figure 5a shows a number of macrophage positive cells which are in close contact with a number of other cells (presumably tumor cells). The fact that their interaction occurred after 22 days in cultures suggests that this association is not detrimental to tumor growth. In figure 5b an isolated macrophage appears to be surrounding another cell (probably a tumor cell). These interactions were seen scattered throughout the tissue culture.

PART 3. RESULTS FROM FLOW CYTOMETRY.

Two tonsils were used to detect possible differences in teasing, blending or digestion on the concentration of immune cell populations. Using the CD68 antibody specific for macrophages, it can be seen that with teasing, 28.7 to 52.3% of cells obtained were macrophages, this number decreased with blending to 8.0-15.8% and in digestion the numbers further decreased to 1.6-14.5% (Table 15). Therefore it appears that digestion and blending as methods for removing viable macrophages are not successful. The three methods did not seem to affect the separation of any of the other immune cells populations studied.

TABLE 4

Semi-quantitative Analyses of Immune Cells Populations and Other Factors Within Breast Carcinomas*.

Monoclonal antibodies	number examined	1 Absent		2 Low		3 Moderate		4 High	
Ki-67	32	1		12		8		11	
ER	33	5		3		6		19	
IL-2R	27	20		5		2		0	
B-cell	32	6		18		7		1	
TGF-Alpha	17	5		1		4		7	
		P	C	P	C	P	C	P	C
CD3	33	1	2	9	15	12	5	11	8
CD4	33	0	2	12	13	13	11	9	9
CD8	33	1	3	14	18	10	7	8	8
Macrophages	33	0	1	1	1	10	10	22	22

P=peripheral

C=centre

Explanations for absent, low, moderate, and high are in Material and Methods; section vii.

*Numbers indicated the total number of breast tumors present in each catagory.

TABLE 5

Comparison of growth rate (Ki-67) and macrophage concentration in the peripheral region of the breast tumors* (n=32).

			Ki-67			
			1	2	3	4
			Absent	Low	Moderate	High
Mac	1	Absent	0	0	0	0
	2	Low	0	1	0	0
	3	Moderate	0	6	1	4
	4	High	1	5	6	8

*Numbers indicate the total number of breast tumors present in each catagory.

TABLE 6

Comparison of growth (Ki-67) and macrophage concentration in the central portion of the tumor* (n=32).

			Ki-67			
			1	2	3	4
			Absent	Low	Moderate	High
Mac	1	Absent	1	0	0	0
	2	Low	0	1	0	0
	3	Moderate	0	6	1	3
	4	High	1	5	7	7

*Numbers indicate the total number of breast tumors present in each catagory.

TABLE 7

Comparison of the growth rate (Ki-67) and the concentration
of Normal T-cell Receptors (CD3) in the peripheral region of the breast tumors* (n=32)

			Ki-67			
			1	2	3	4
			Absent	Low	Moderate	High
CD3	1	Absent	0	0	0	1
	2	Low	0	6	2	1
	3	Moderate	0	5	3	3
	4	High	1	1	3	6

*Numbers indicate the total number of breast tumors present in each catagory.

TABLE 8

Comparison of the growth rate (Ki-67) and the concentration of the normal T-cell receptors (CD3) in the cenrtal area of the breast tumors* (n=32).

			Ki-67			
			1	2	3	4
			Absent	Low	Moderate	High
CD3	1	Absent	0	0	0	2
	2	Low	0	9	4	2
	3	Moderate	1	2	1	3
	4	High	0	1	3	4

*Numbers indicate the total number of breast tumors present in each catagory.

TABLE 9

Comparison of growth rate (Ki-67) and the concentration of helper T-cells (CD4) in the peripheral areas of the tumors* (n=32).

			Ki-67			
			1	2	3	4
			Absent	Low	Moderate	High
CD4	1	Absent	0	0	0	0
	2	Low	0	5	4	2
	3	Moderate	0	5	3	4
	4	High	1	2	1	5

*Numbers indicate the total number of breast tumors present in each catagory.

TABLE 10

Comparison of the growth rate (Ki-67) and the concentration of helper T-cells (CD4) in the central portion of the tumors* (n=32)

			Ki-67			
			1	2	3	4
			Absent	Low	Moderate	High
CD4	1	Absent	0	0	0	2
	2	Low	0	6	4	3
	3	Moderate	0	5	2	3
	4	High	1	1	2	3

*Numbers indicate the total number of breast tumors present in each catagory.

TABLE 11

Comparison of the growth rate and the concentration of cytotoxic T-cells (CD8) in the peripheral areas of the tumors* (n=32)

			Ki-67			
			1	2	3	4
			Absent	Low	Moderate	High
CD8	1	Absent	0	1	0	0
	2	Low	1	7	3	3
	3	Moderate	0	2	3	4
	4	High	0	2	3	3

*Numbers indicate the total number of breast tumors present in each catagory.

TABLE 12

A comparison of the growth rate (Ki-67) and the cytotoxic T-cell (CD8) in the central areas of the breast tumors* (n=32)

			Ki-67			
			1	2	3	4
			Absent	Low	Moderate	High
CD8	1	Absent	0	1	0	2
	2	Low	1	7	5	6
	3	Moderate	0	4	0	1
	4	High	0	1	2	2

*Numbers indicate the total number of breast tumors present in each catagory.

TABLE 13

Comparison of the growth rate and the concentration of B-cells* (n=32)

			Ki-67			
			1	2	3	4
			Absent	Low	Moderate	High
B-cells	1	Absent	0	4	2	1
	2	Low	0	6	6	5
	3	Moderate	1	2	0	4
	4	High	0	0	0	1

*Numbers indicate the total number of breast tumors present in each catagory.

TABLE 14

Comparison of the tumor growth and either the immunochemical or biochemical results of estrogen receptors (ER) concentration is breast carcinomas* (n=32)

			Ki-67			
			1	2	3	4
			Absent	Low	Moderate	High
ER	1*	Absent	0	0	3	3
	3*	Moderate	0	4	1	3
	4*	High	1	8	4	5

*Numbers indicate the total number of breast tumors present in each catagory.

Catagories for ER are: $\leq$5 fmoles absences of ER

6-12 fmoles moderate level of ER

$\geq$13 fmoles high levels of ER

TABLE 15

Percentages of immune cells in tonsils that have been obtained via teasing, blendin or digesting (n=2)

Monoclonal Ab	Description	Digesting		Blending		Teasing	
Aleuk	leucocyte antigen	40.6	42.2	42.9	44.1	43.1	44.4
M3	monocyte/macrophage	6.7	5.9	2.6	3.3	6.1	6.2
leu4	CD3/normal T cells	40.7	21.2	42.6	32.4	42.3	23
leu12	Blymphocytes	56.6	32.7	49.7	33.9	56.7	34.5
leu2a	CD8/T cytotoxic cells	6.9	3.5	5.7	4.2	7.0	3.1
IL-2R	interleukin-2 receptors on CD8	0.3	0.3	0.2	0	0.2	0.3
leu3a	CD4/T helper cells	30.9	6.3	31.4	5.2	30.6	7.2
IL-2R	interleukin-2 receptors on CD4	0.4	0.2	0.1	0.3	0.3	0.7
leuiic	natural killers/neeuttrophils	1.3	11.8	2.7	28.9	1.2	13
leu19	*see below	0.2	0.7	2.7	2.4	0.4	1.5
CD68	macrophages	1.6	14.5	8.0	15.8	28.7	52.3

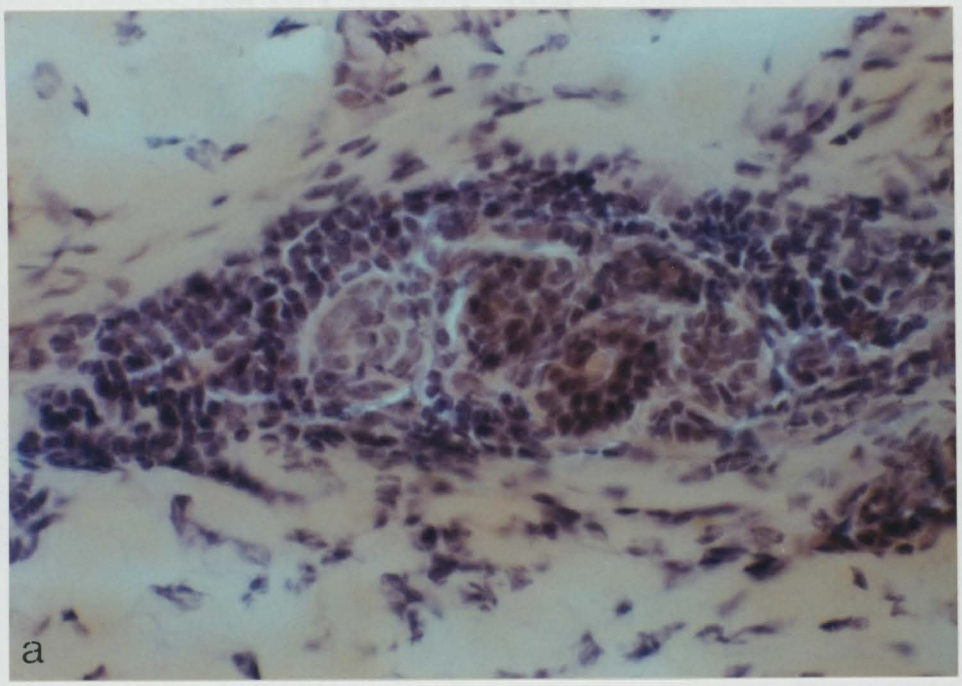
*Perpheral blood lymphocytes/resting NK/some CD4 and CD8

V. FIGURES

Figure 1. Patient #22 is a 69 year old woman with an infiltrating ductal carcinoma. There was lymph node involvement. Figures 1b to 1j show an *In situ* component of the breast carcinoma. Figures 1b to 1j were counterstained with hematoxylin.

Figure 1a. A hematoxylin and eosin stained slide, used to identify the general morphology of the tumor. This figure shows the terminal ductal lobular unit. It is a transverse cut, with tumor cells seen surrounding the unit. Magnification is 312.5x.

Figure 1b. The same duct as above but analysed for estrogen receptors (ER). The ER monoclonal antibody (brown nuclei) was analysed as high (4) for this tumor. In this duct it can be seen that a large number of the cells are positive for ER. Magnification is 312.5x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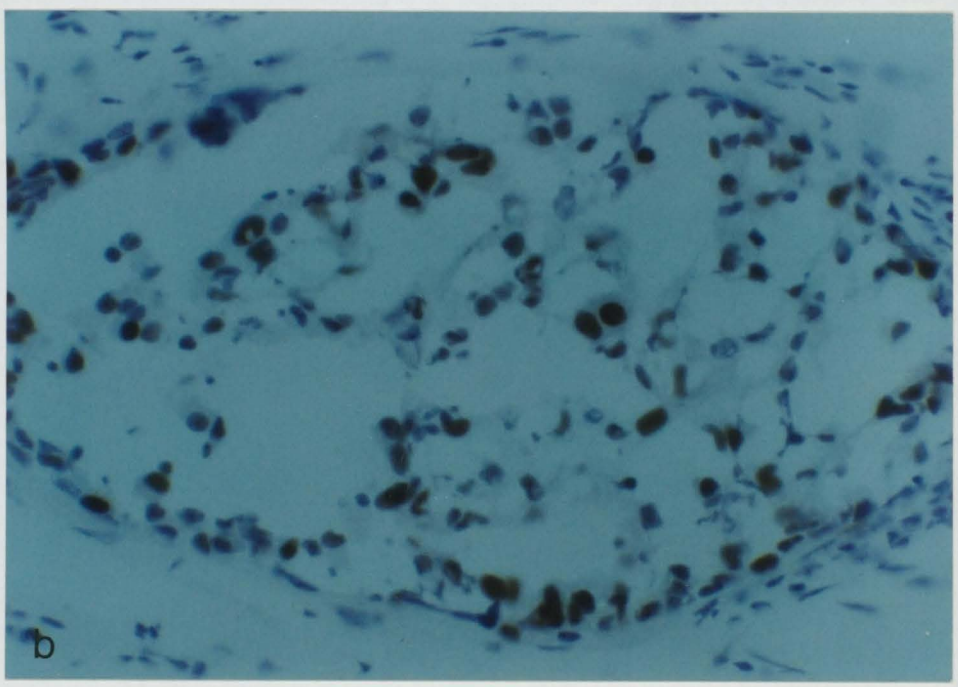
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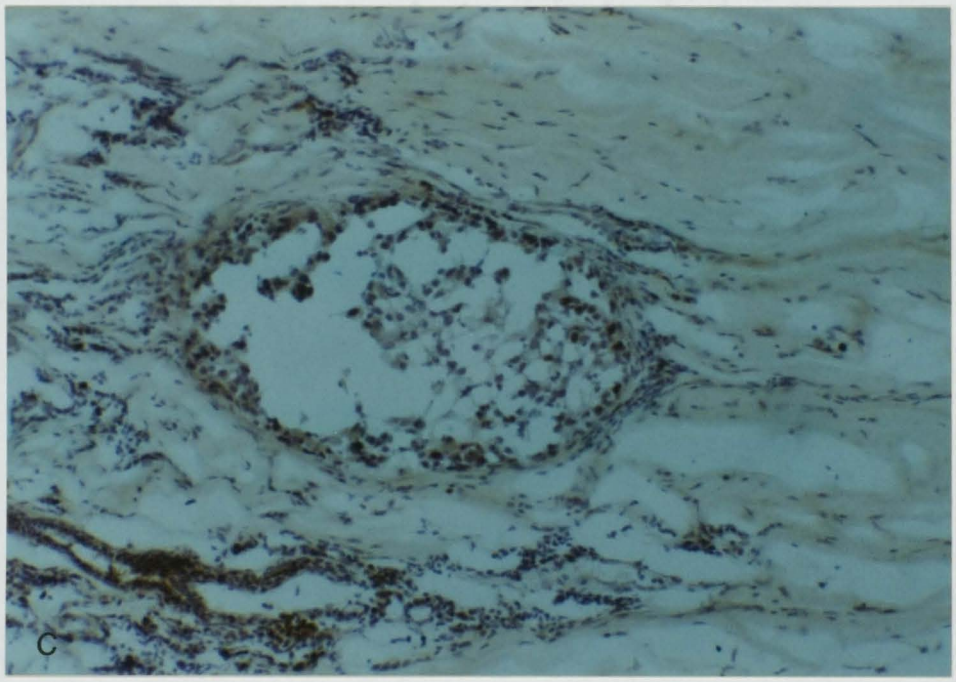
Figure 1c. Same duct as in Figure 1a, analysed for cellular proliferation using Ki-67 monoclonal antibody (brown nuclei). The proliferating cells are seen throughout the duct but concentrated in the basal region. The tumor was rated as having a high (4) tumor growth. Magnification is 125x.

Figure 1d. A higher magnification (321.5x) of Figure 1c. The proliferating cells are concentrated in the basal region of the duct.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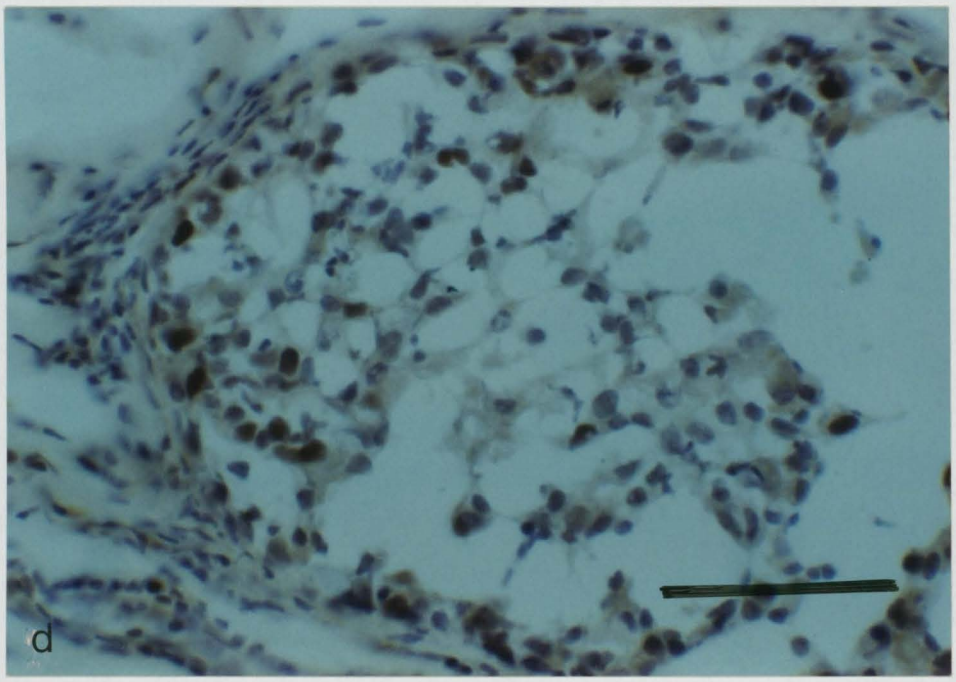
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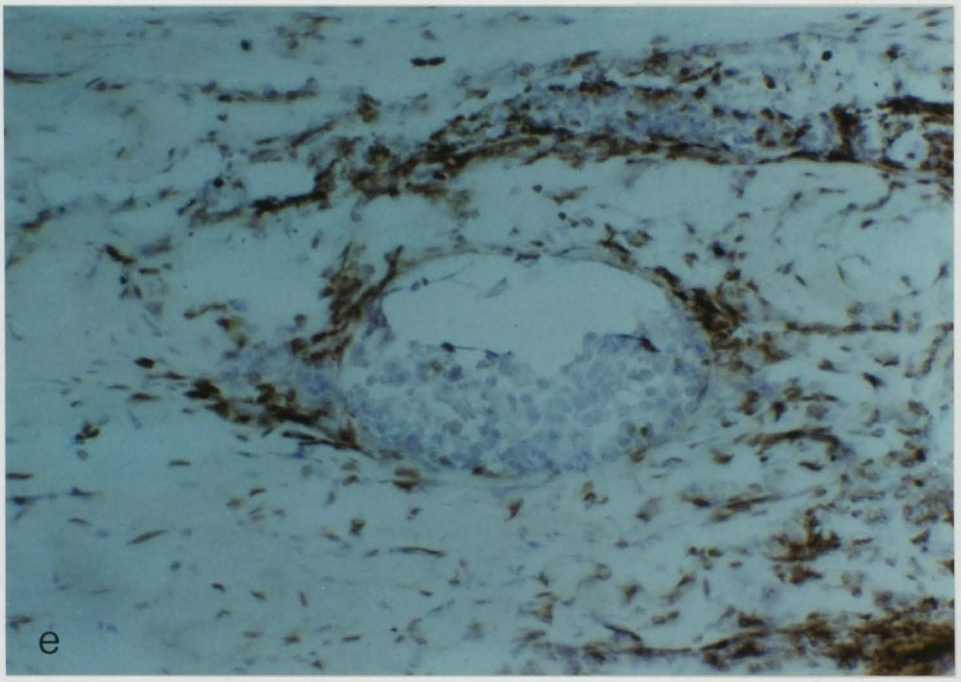
Figure 1e. The same duct as in Figure 1a, analysed for normal T-cells (CD3). This monoclonal antibody binds to the receptors on the surface of the cell and forms a brown ring around the cell. CD3 cells are concentrated at the extreme left and right of the duct. The tumor was rated to have a high (4)concentration of CD3 cells present within the peripheral and the central region of the tumor. Two different cell shapes are seen; the round cells (resting) and the strandy cells (mobile). Magnification is 125x.

Figure 1f. Same duct as in Figure 1a, analysed for helper T-cells (CD4). The monoclonal antibody appears as a brown ring on the cells. The main concentration of CD4 cells is at the extreme right and left sides of the duct. The tumor was rated a high (4) for CD4 for both the peripheral and the central areas. Magnification is 125x.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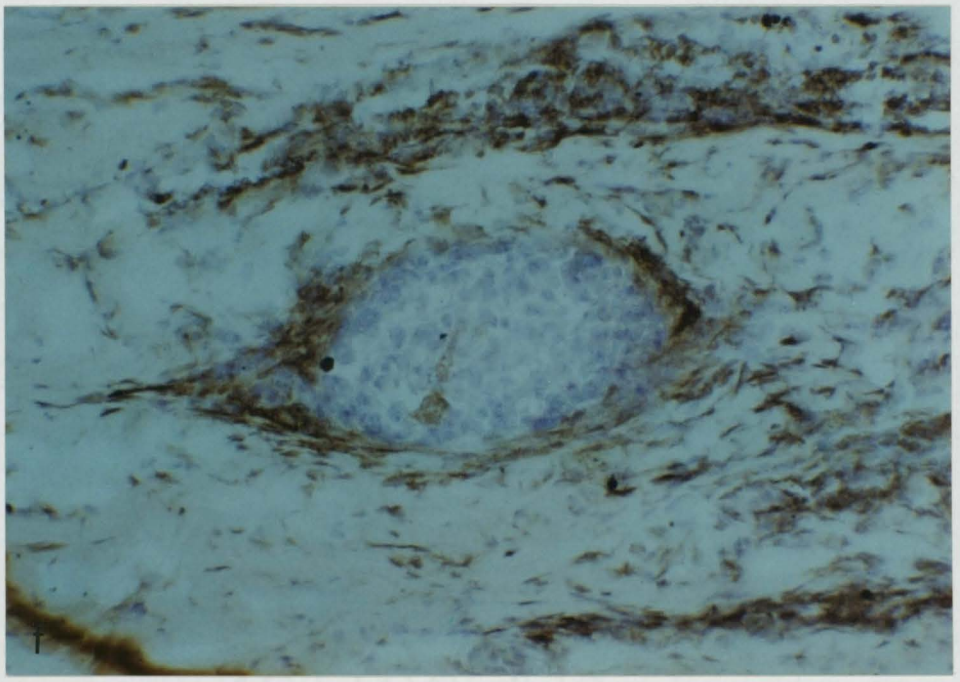
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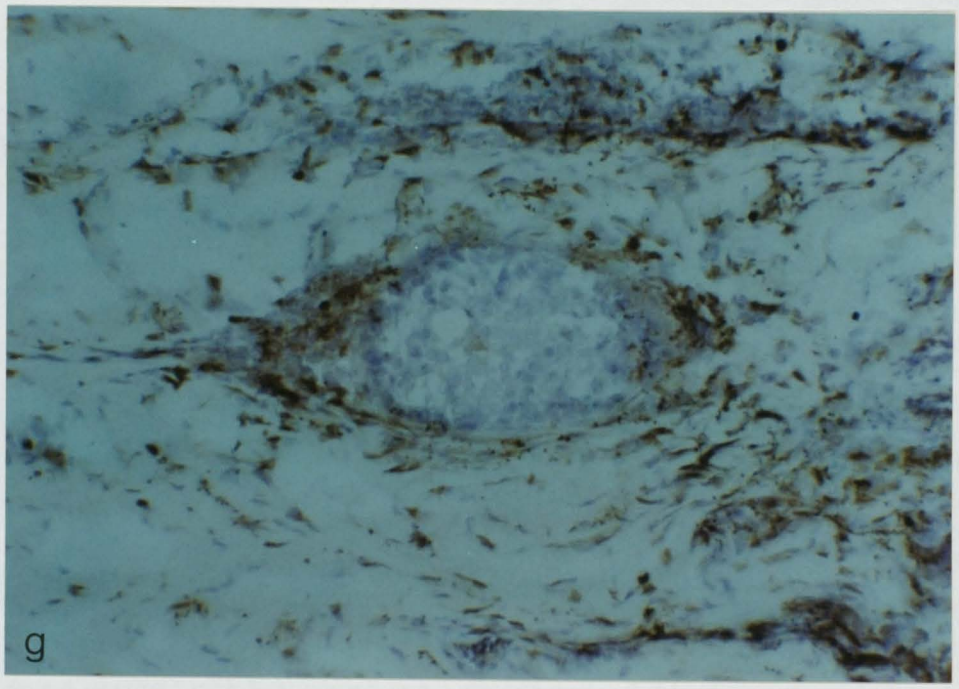
Figure 1g. Same duct as in Figure 1a, analysed for cytotoxic T-cells (CD8). The monoclonal appears as a brown stain on the cells. The main concentration of CD8 cells is at the extreme right and left side of the duct. The tumor was rated to have a moderate (3) concentration of CD8 cells in the peripheral region and a low (2) concentration in the central area. Magnification is 125x..

Figure 1h. Same duct as in Figure 1a, analysed for interleukin-2 receptors (IL2R). IL-2R appears on activated T-cells or B-cells. The monoclonal antibody appears as a brown ring on the cells. IL-2R was seen only at the right side of the duct. Magnification is 321.5x.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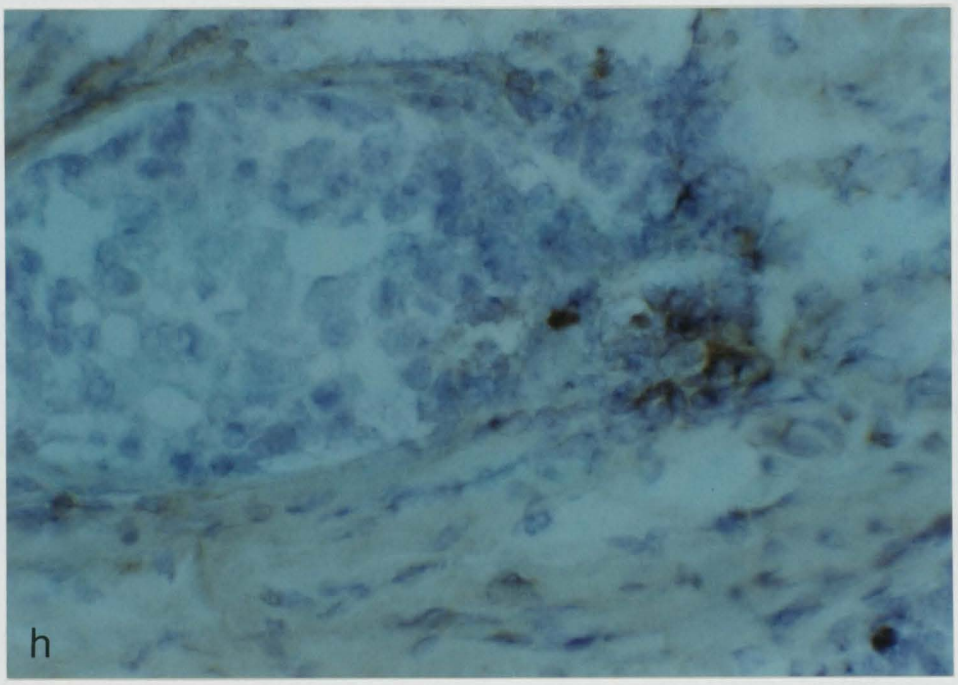
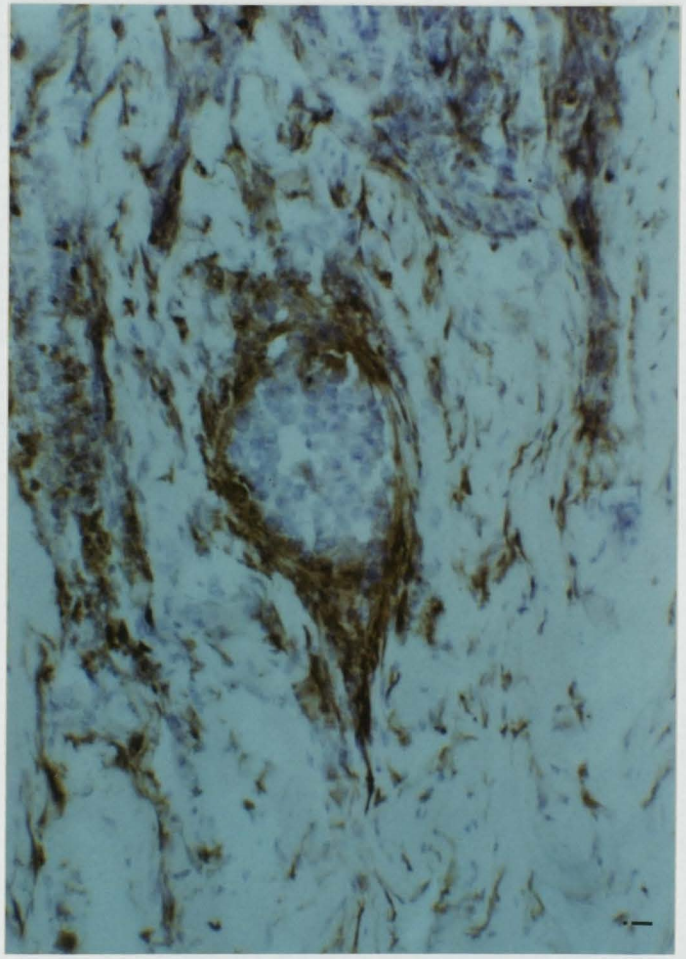

h

Figure 1i and j. Same duct as in Figure 1a, analysed for macrophages using the CD68 monoclonal antibody, which stains intracytoplasmic granules associated with lysosomes found in macrophages. The macrophages are intermixed with the basal cell layer cells within the duct (Figure 1d). Magnification is 125x (1i) and 312.5x (1j).



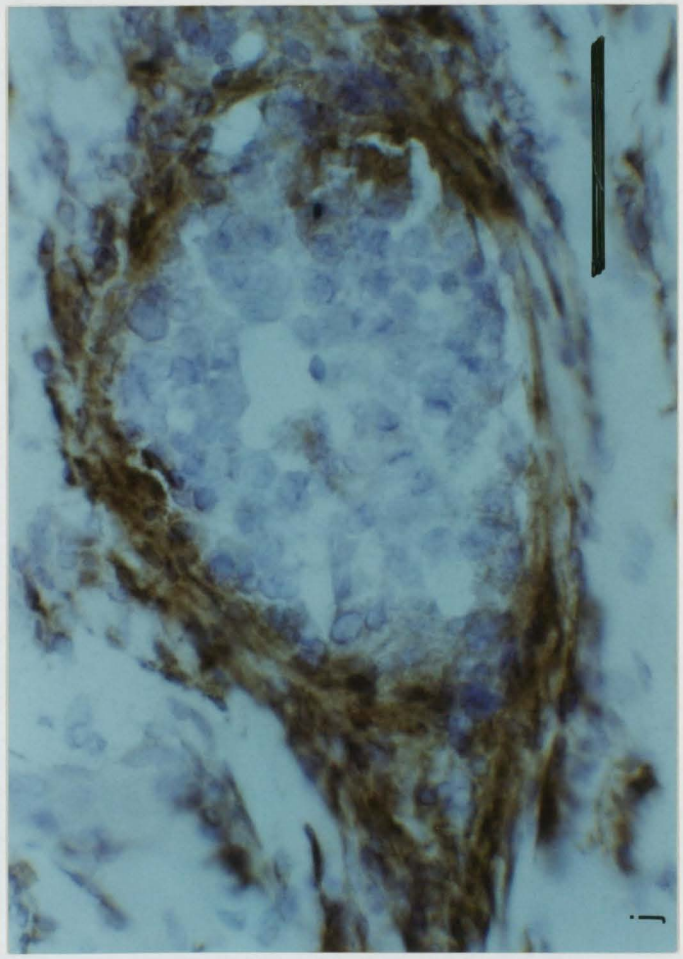
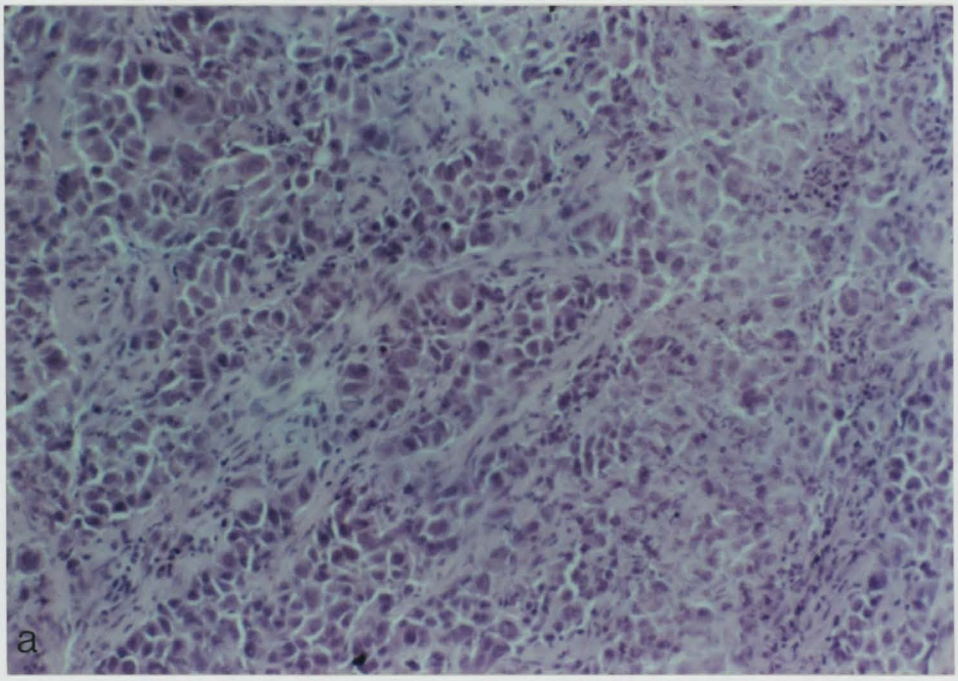


Figure 2. An example of a poorly differentiated infiltrating ductal carcinoma. Patient #30 is 57 years old with lymph node involvement. Figures 2b to 2h were counterstained with hematoxylin.

Figure 2a. A hematoxylin and eosin slide showing the general morphology of the tumor. There was no evidence of tubular formation. The whole cross section of the tumor has been replaced with infiltrating tumor cells of various sizes located within the stroma. Magnification is 125x.

Figure 2b. The same tumor as in Figure 2a, analysed for cellular proliferation using Ki-67 monoclonal antibody (brown nuclei). This tumor was classified as having a high growth rate (4). Magnification is 125x.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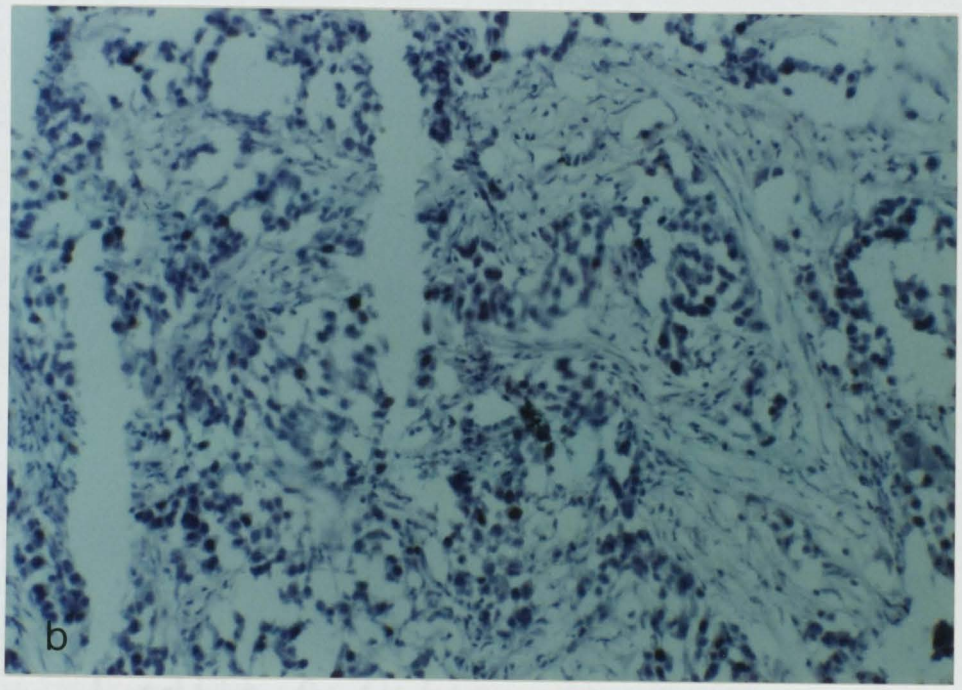
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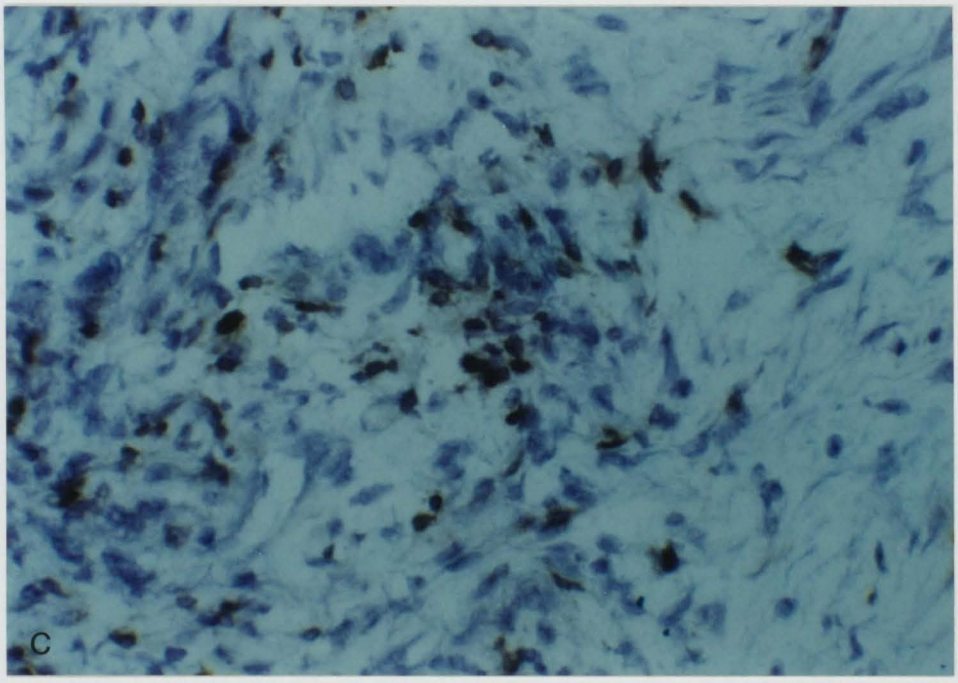
Figure 2c. Same tumor as in Figure 2a, stained for normal T-cell (CD3) populations. The CD3 cells appear with a brown stain ringing the cell. This tumor had a CD3 rating of high (4) in the peripheral part of the tumor and a rating of moderate (3) in the central part of the tumor. Magnification is 321.5x.

Figure 2d. Same tumor as in Figure 2a, which was stained for helper T-cells (CD4), seen as brown ring. The CD4 population of this tumor was rated as high (4) in the peripheral and moderate in the central part of the tumor. Magnification is 312.5x.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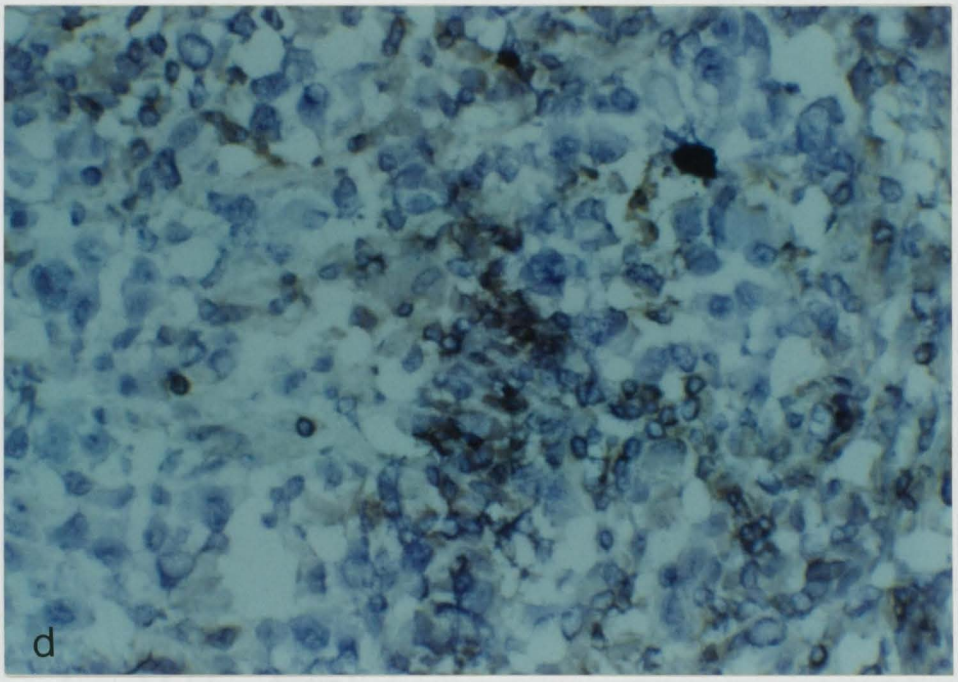
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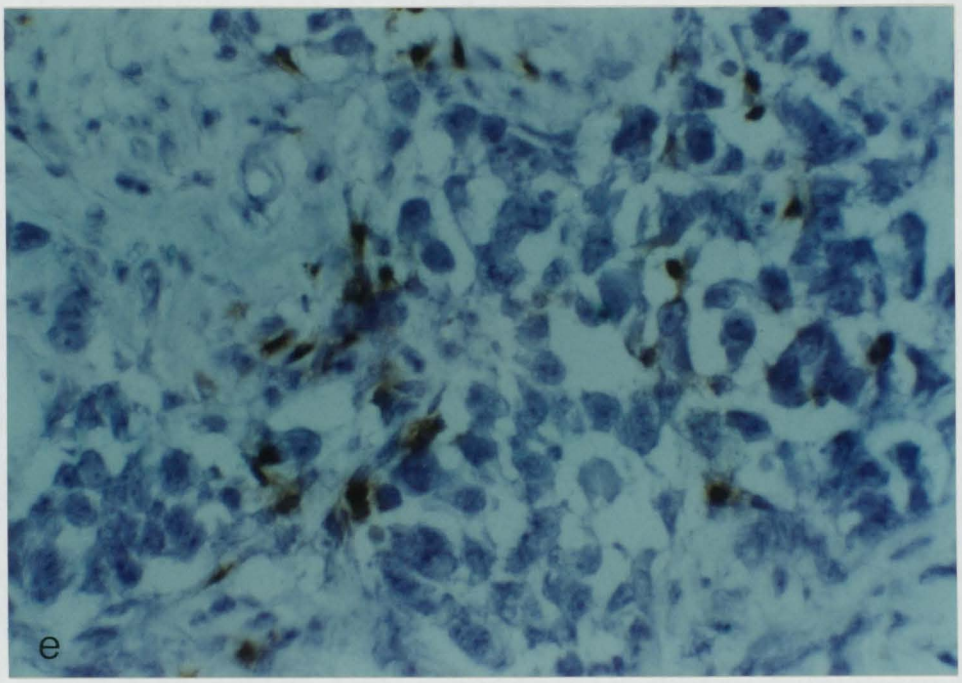
Figure 2e. Same tumor as in Figure 2a, which was stained for cytotoxic T-cells. This tumor was rated as moderate (3) in the peripheral and low (2) in the center part of the tumor. Magnification is 312.5x.

Figure 2f. Same tumor as in Figure 1a, stained for transforming growth factor-alpha. It is difficult to identify the cell type that is expressing this growth factor although many positive cells are present. Magnification is 312.5x.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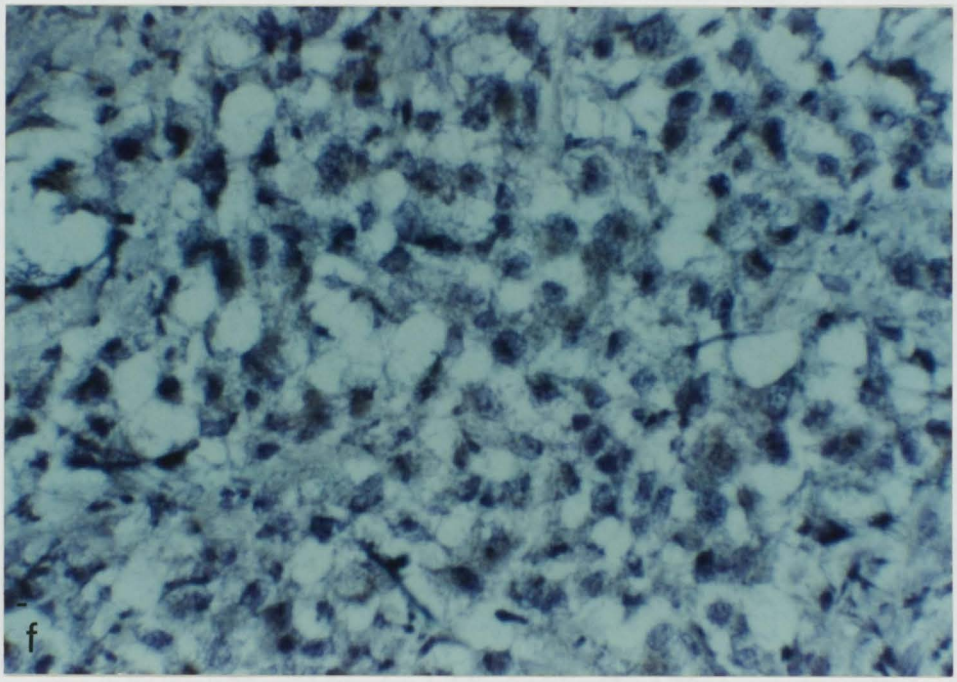
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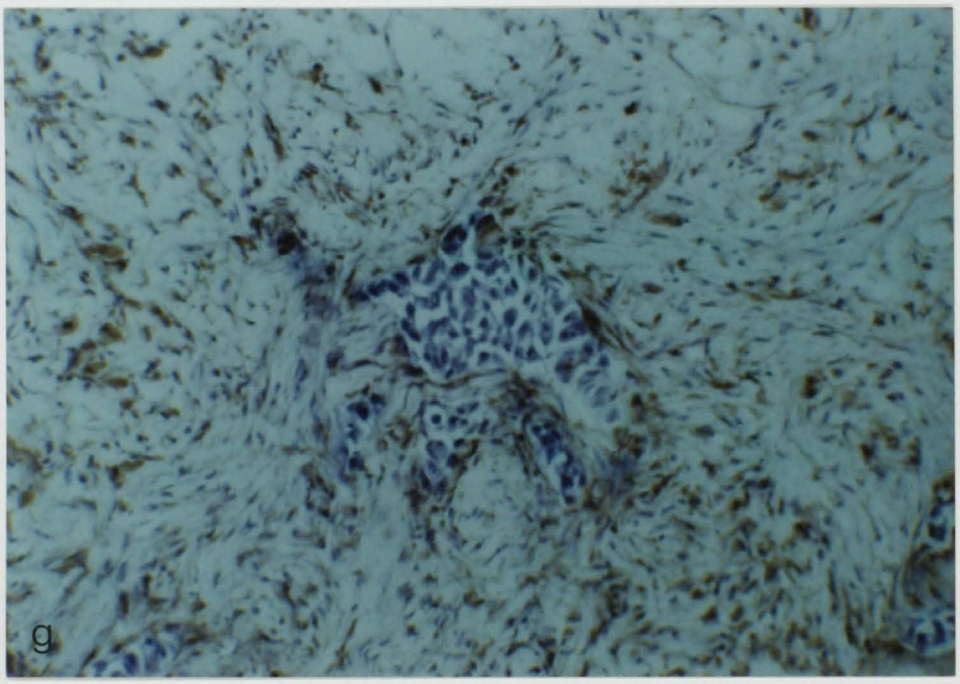
Figure 2g and h. Same tumor as in Figure 2a. Figure 2g is at 125x magnification and Figure 2h is at 312x magnification. This tumor was analysed for macrophages using the CD68 monoclonal antibody. The macrophages are surrounding a cluster of tumor cells and no necrosis is present. Note that several of the macrophages seem to be directly in contact with the basal cells of the tumor cluster (Figure 2h, arrow). The concentration of macrophages for this tumor was rated at high (4) in both the peripheral and center portion of the tumor.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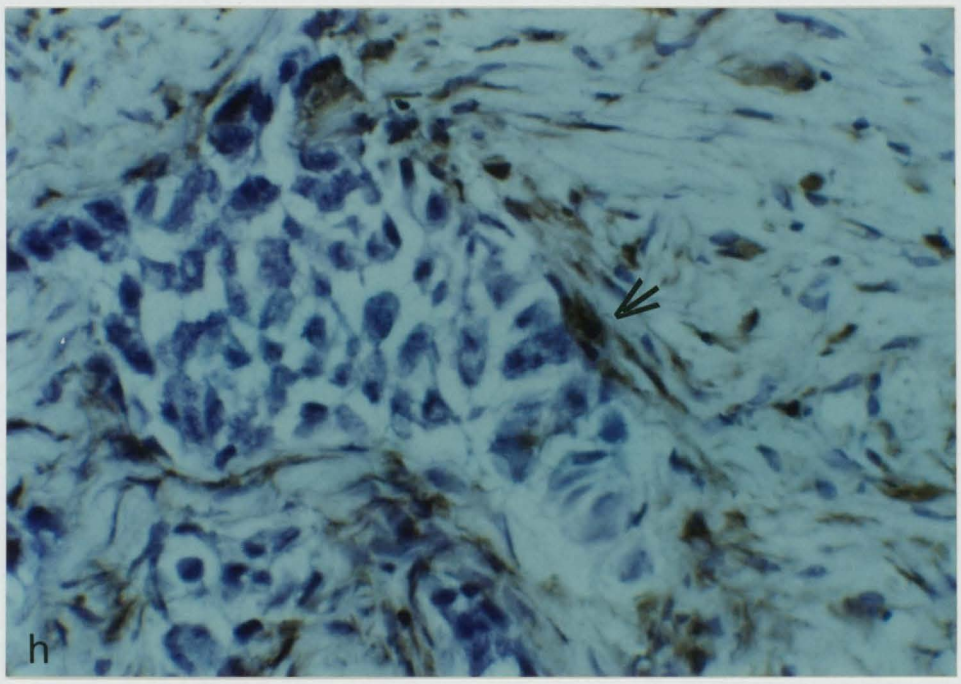
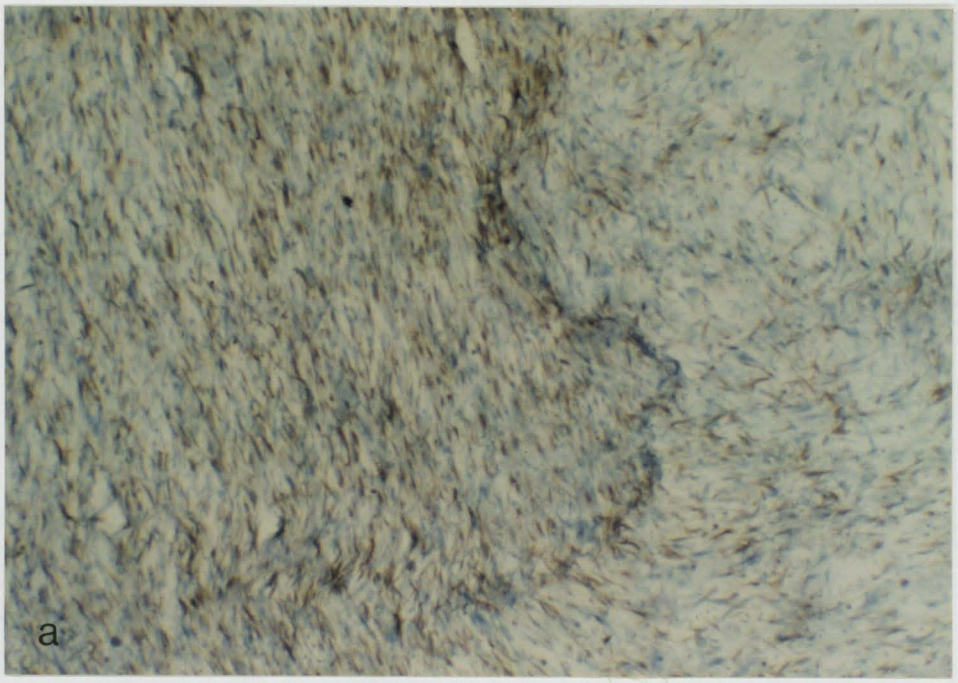

h

Figure 3a. A low power photograph of a cell culture derived from a primary breast carcinoma. The cell culture was stained with the macrophage antibody after it had grown for 3 weeks. The monoclonal CD68 identifies the macrophages present within the culture (brown stain). Counterstained with hematoxylin. Magnification is 125x.

Figure 3b.The same cell culture as in Figure 3a. It can be seen that the macrophages are interdispensed with the fibroblasts-like cells. Counterstained with hematoxylin. Magnification 312x.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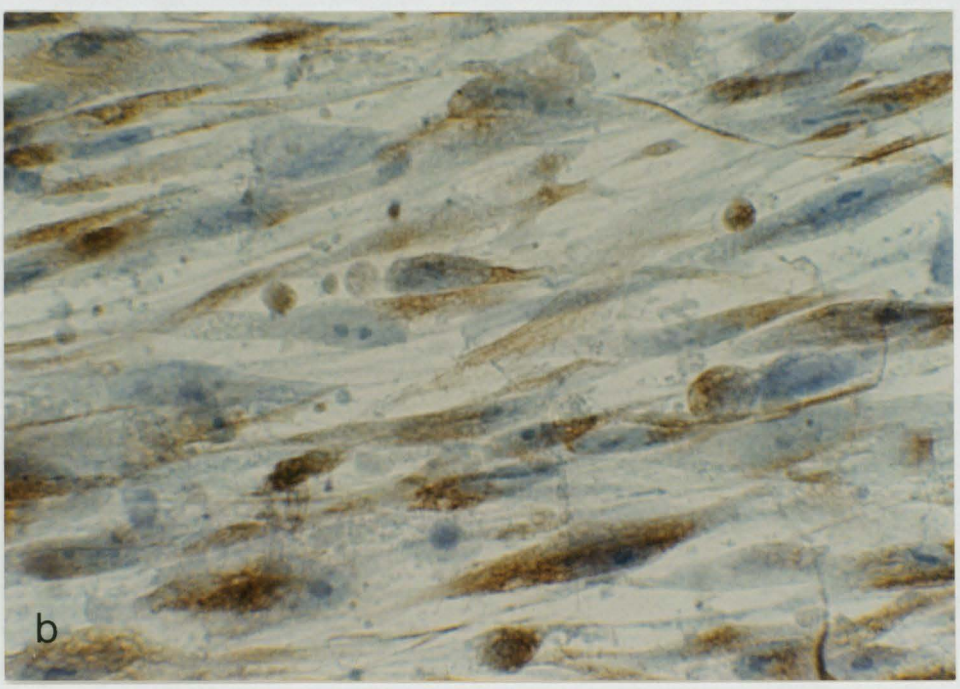
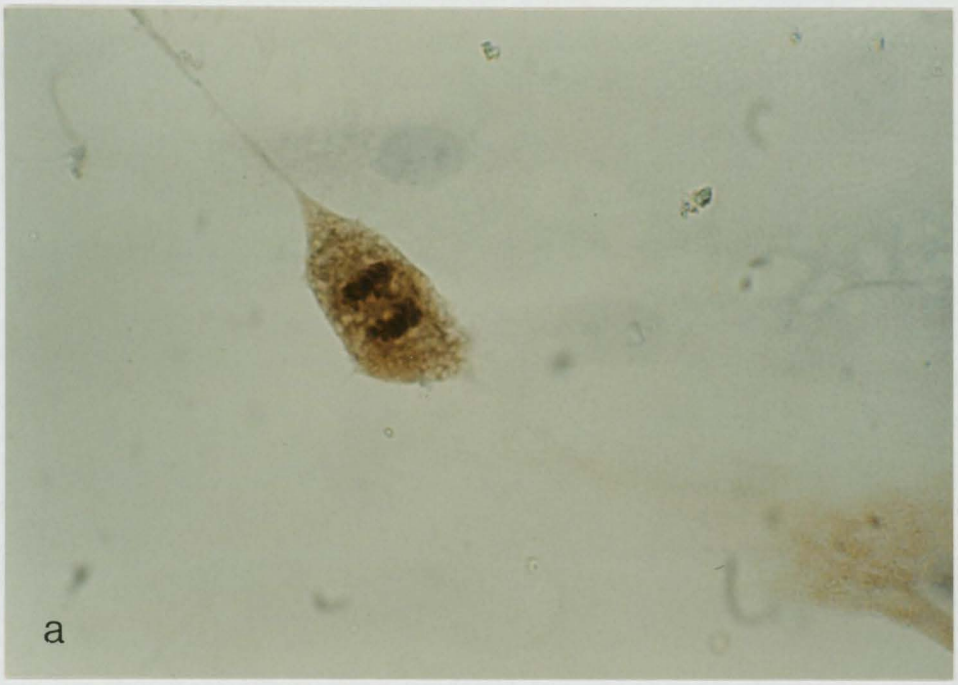

b

Figure 4a. This cell was a part of a 2-3 week old cell culture from a primary breast carcinoma. This culture was "double" stained with CD68 monoclonal antibody that stains the intracytoplasmic granules of lysosomal origin and the Ki-67 monoclonal that stains the nuclei of proliferating cells. Magnification is 500x.

Figure 4b. The same cell culture as in Figure 4a. The dark brown cell is in the finishing stage of dividing. The cell is stained with both the macrophage monclonal antibody (intracytoplasmic staining) and the nuclei of those cells that are dividing are also positive for the nuclear proliferation protein, using the Ki-67 monoclonal antibody. Note that the two cells to the right are staining positive for the macrophage monoclonal antibody but not the Ki-67 monoclonal antibody (arrows). Magnification is 500x.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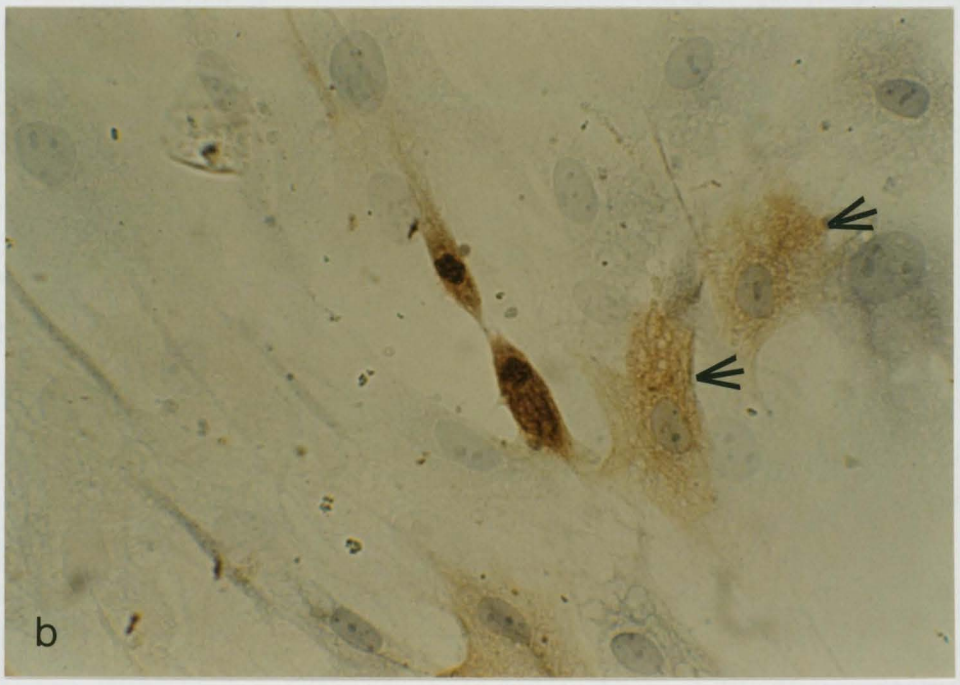
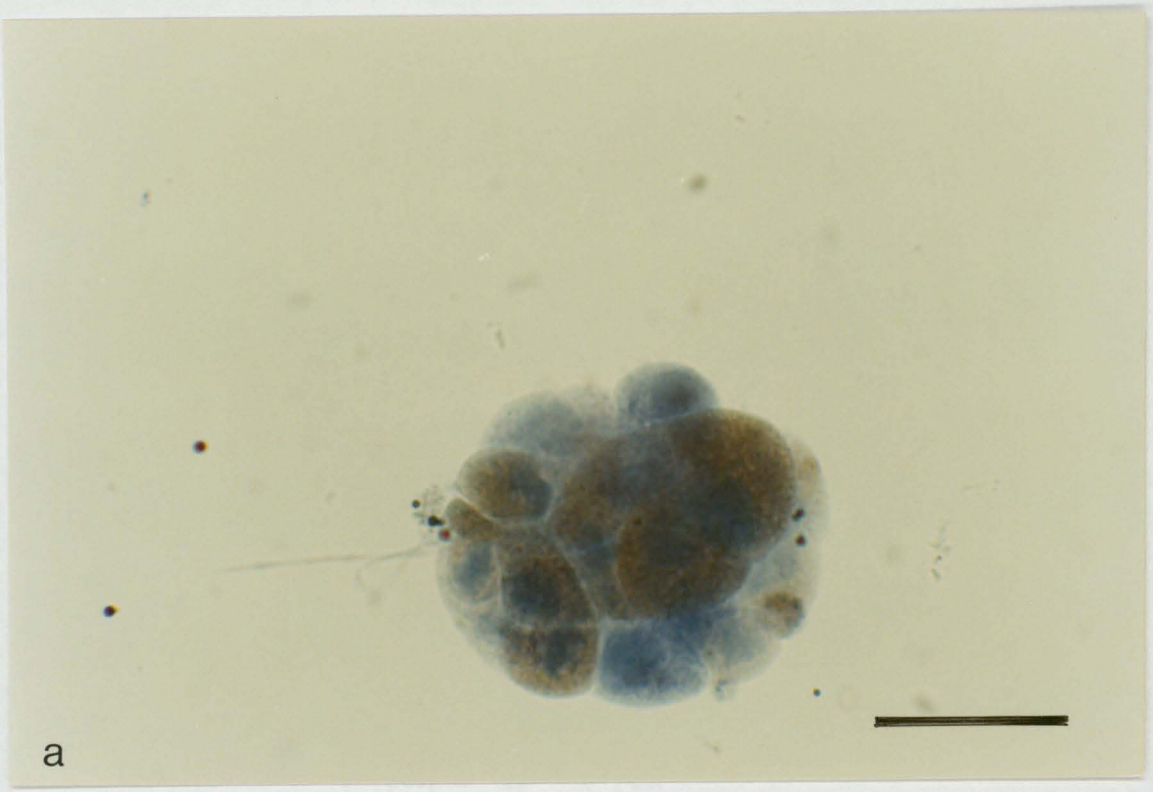

b

Figure 5a and b. Cellular association between macrophages (brown), using CD68 monoclonal, and other cell types, probably cancer cells (purple). Breast cancer cells were mechanically separated from an infiltrating ductal carcinoma. Bar is 20 μm.



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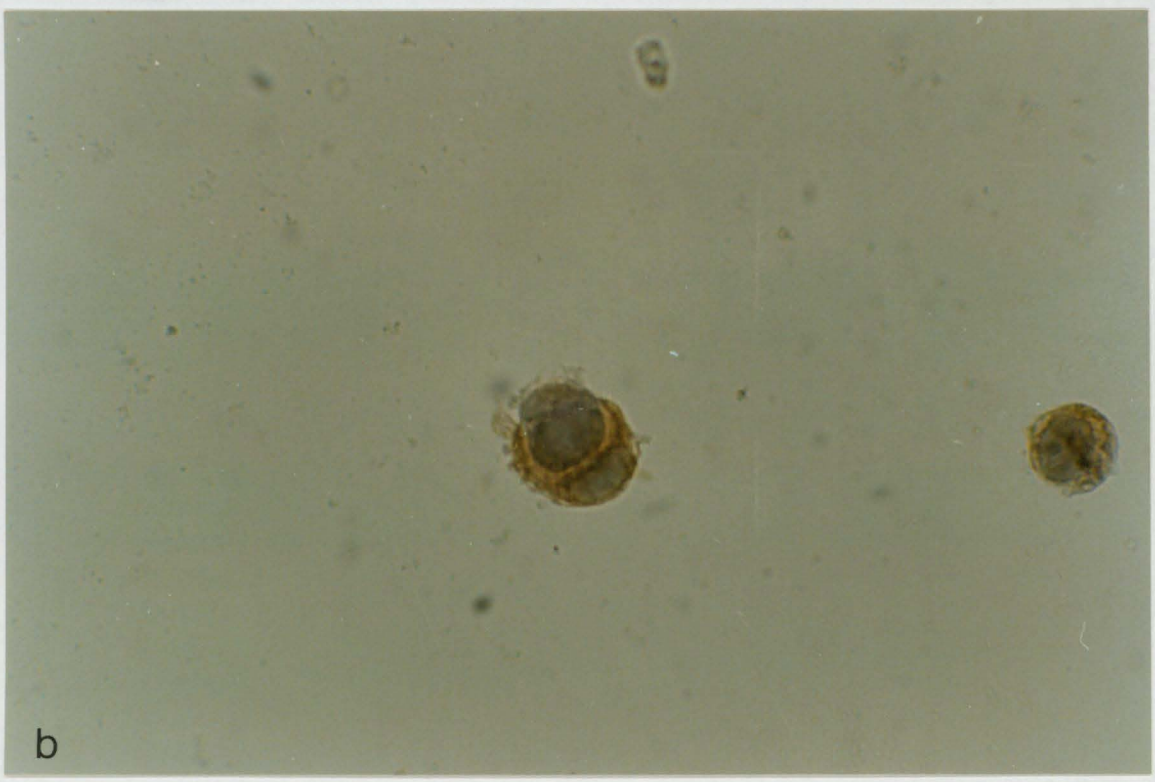
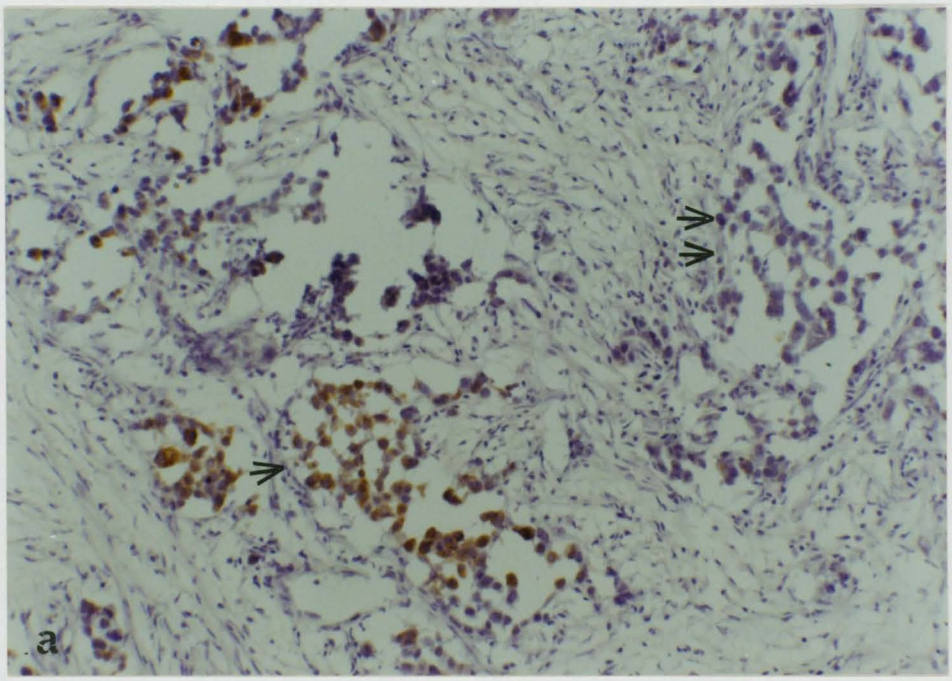


b

Figure 6a. Infiltrating ductal carcinoma showing a heterogenous staining pattern for EGFr. Some tumor clusters stained positively for the receptors (arrow) while others stained negatively (double arrow). Magnification is 312x.

Figure 6b. Shows a homogeneous staining pattern for EGFr in an infiltrating ductal carcinoma. Nearly all tumor cells stained positively for the receptors. Magnification is 125x. (Photograph 6a and b taken by Robin Parker)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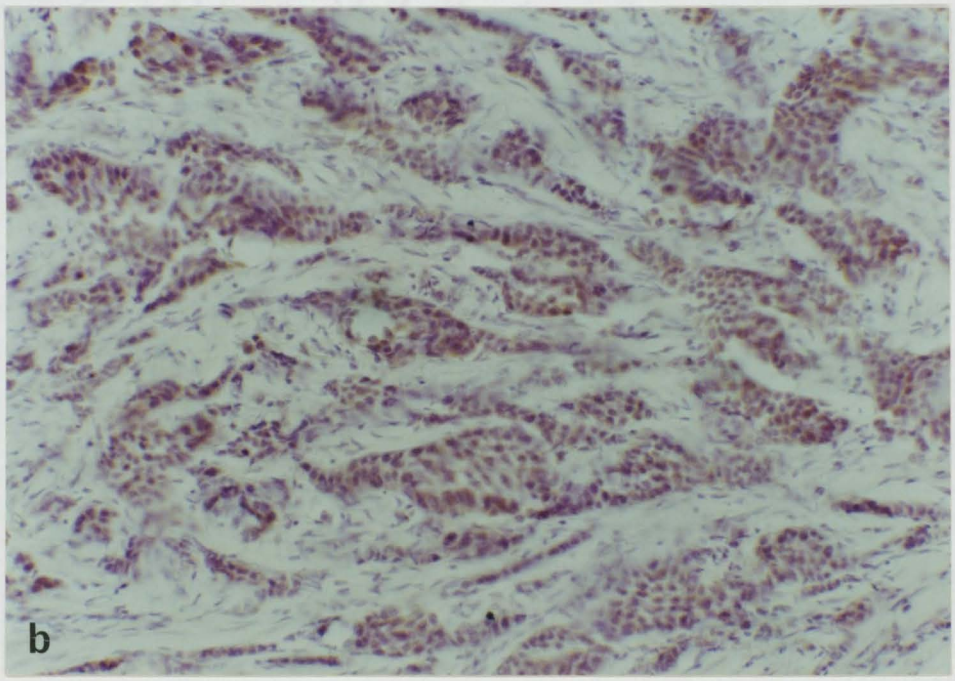
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VI. DISCUSSION

i. Presence of immune cells within breast tumors

It has been known for many years that malignant tumors are usually infiltrated by immune cells. At one time it was considered that the presence of immune cells indicated a favourable prognosis (56,57). However, in recent years researchers have speculated that the presence of immune cells may indicate a less favourable prognosis (58,59). Thoughts as to the major immune cell population present in tumors varied. Some felt that T-cells are the major immune cell population present (60), while others believed it was helper T-cells (CD4) (61), and still other thought it was cytotoxic T-cells (CD8). There were also those that believed macrophages are the main immune cell type present (62,63). Our research indicates that macrophages are by far the major immune cell population found in breast carcinomas examined at the Royal Jubilee Hospital in Victoria, B.C. Macrophages appeared in the majority (94%) of tumors in moderate to high concentrations in both the peripheral and the central areas. T-cells were also present in a majority of tumors but not to the same degree as macrophages. Normal T-cells for example appeared in a moderate to high concentrations in the peripheral areas in 69% of the tumors and in the central area in 39% of the tumors. Using monoclonal antibodies to identify subpopulations of T-cells; helper T-cells appeared in moderate to high concentrations in the peripheral area of 69% of the tumors and in the central portions in 55% of the tumors. Cytotoxic T-cells, appeared in moderate to high concentrations in the peripheral portions of 56% of the tumors and in the central portions in 36% of the tumors. These observations indicate that T-cells are able to infiltrate into tumors but appear to be less able to enter the central areas of the tumors. B-cell infiltration was present in moderate to high concentrations in 25% of tumors with insignificant differences between central and peripheral areas.

ii. T-cells in relation to tumor growth

Balch et al. (64) hypothesized that because of the heterogenous nature of different cancers there are a variety of immune responses among individuals. His research showed a low immune response in breast cancer, colon cancer and sarcomas whereas melanoma and renal cell carcinoma had a relatively high immune response. My work indicated that 100% of the breast carcinomas examined exhibited a moderate to high macrophage and T-cell concentration.

I theorized at the beginning of this project that I would find an inverse relationship between immune cell infiltration and tumor growth and a positive relationship between the presence of immune cells and tumor necrosis. This was not what I found! In the 32 breast carcinomas that we examined for growth, 97% of the tumors showed some tumor cell division. Only one tumor showed no cell division occurring, while approximately one-third of the 32 tumors had low tumor cellular proliferation rate; approximately one-quarter of the tumors had moderate tumor cell division rate and the rest, about one third of the tumors, had high tumor cell division rate.

From the assessment of the relationship between tumor growth and the normal T-cells infiltration into various areas, it can be seen that in the peripheral areas (Table 7) about half (47%) of the tumors had moderate to high concentrations of T-cells with a low tumor cell division rate. In the central areas (Table 8) of those same tumors, one-third of the tumors had moderate to high infiltration of normal T-cells and the tumor cells were proliferating at a moderate to high rate. This pattern was also evident with the T-cell subpopulations of helper (CD4) and cytotoxic (CD8) T-cells. Forty-one percent (Tables 9&11) of the tumors had both CD4 and CD8 populations in the peripheral areas in moderate to high concentrations while the tumor cells were proliferating at a low rate. In the central portion of the tumors, (Tables 10 and 12) 31% and 16% of the tumors had CD4 and CD8 populations that had infiltrated into those tumors respectively and had tumor cells dividing at a moderate to high rate. My observations indicated that instead of a negative relationship between T-cell infiltration and dividing tumor cells, as I originally expected, I found the opposite, a positive relationship between T-cells and the rate of tumor cell division.

iii. Are T-cells activated within the tumors?

Balch et al. (64) observed that even when immune cells have infiltrated into a tumor site they maybe unable to invoke an immune response. T-cells play a major role in the specific acquired immune system. Helper T-cells are activated when they have recognized an antigenic fragment presented by an antigen presenting cell (APC). This recognition involves the CD3 and CD4 receptors on the helper T-cell and the major histocompatibility complex (MHC) class II proteins on the APC. Once this has occurred, the stimulated helper T-cells will release interleukin-2, a lymphokine that will cause an up-regulation of interleukin-2 receptors (IL-2r) on the cells and trigger cell division in both the helper and cytotoxic T-cells. This will result in further release of IL-2 (65,66,67). IL-2 is both an autocrine and paracrine lymphokine. In my research, I used a monoclonal antibody specific for a portion of the interleukin-2 receptor. In 27 tumors, 20 had no IL-2r staining, 5 had IL-2r in low concentrations and 2 had IL-2r in moderate concentration. Following activation by IL-2, cytotoxic T-cells will start the process of destroying non-self cells. My observations indicated that both helper and cytotoxic T-cells are present within tumors but they lack interleukin-2 receptors on their cell surface. This indicates that the majority of these T-cells were not stimulated and therefore were unable to destroy the surrounding tumor cells. The ability of the tumor to escape immunological attack is not currently fully understood. There are several hypotheses to possibly explain why T-cells are not activated. The tumor may be producing a toxic environment which inactivates T-cells once they have entered the tumor or the tumor cells themselves maybe resistant to lymphokines released by the T-cells (35,64,69). Tumor antigens are usually recognized by T cells via the major histocompatibility complex (MHC) associated peptides. Currently there are three hypotheses proposed by Wunder and Hodes (68) to explain why tumor cells that express their MHC can avoid destruction even if the tumor cells are endogenously synthesizing the non-self peptide for the MHC groove. The first hypothesis is that intracellular proteolysis occurs and results in the destruction of the non-self peptide segment before it can sit in the groove. The second possibility is that the non-self peptide survives but is unable to sit in the groove. The third hypothesis is that

the non-self peptide it made, survives, reaches the MHC and is able to sit in the groove, but the cell is able to escape T cell recognition by failing to express levels of MHC molecules on its surface that will trigger a T cell response. My work indicates that very few of the T-cell are stimulated. It is possible that one of the above hypotheses may be involved.

Two different shapes of immune cells were seen within the tumors I examined: circular and strandy. The circular cells appeared to be in a stationary phase which may have been due to specific stimuli from tumor cells. The strandy cells appeared to be in a migratory state, possibly attracted by specific stimuli from tumor cells. Whether or not the circular state is the "end point" of the migrating cells is not yet known.

iv. Macrophages in Cancer

Macrophages belong to the mononuclear phagocyte system. The main function of macrophages is to recognize any cell that has become an altered-self and destroy it, thus normally maintaining homeostatis within the system. Macrophages are involved in tissue turnover, which includes tissue remodelling such as that which occurs in embryogenesis or metamorphosis such as tissue destruction and repair due to injury or infection or tissue renewal such as the removal of damaged or senescent cells (70)

Macrophages can be activated by two pathways. The first is poorly understood but involves reaction to, substances such as cell walls or their products (endotoxins) of certain micro-organisms. The other type of activation is via lymphokines such as the macrophage activation factor (MAF)

Macrophages originate in the bone marrow as monocytes, travel through the bloodstream and migrate into tissues to become fixed or free macrophages. It was once thought that when a monocyte differentiates into a macrophage it lost its ability to proliferate but research by Marino and Adams (71) indicates that macrophage progenitor cells exist in a variety of peripheral tissue and that they contribute to maintaining macrophage levels. This may explain the dividing macrophages seen in Figure 4. Heterogeneity exists among macrophages. This may be caused by functional or morphological differences at various stages of differentiation due to micro-environmental

changes. This modulation can cause reversible changes by variations in the micro-environmental caused by secretion of chemotactic or other factors. Therefore different types of macrophages may arise depending on the modulation of the microenvironment (72).

Macrophages found within tumors can be referred to as tumor-associated macrophages (TAMs). In a tumor cell suspension it has been found that TAMs can vary from 0 to 80% of the cells, with an average of 20-30%. For example, if a tumor contains 10^9 cells there will be several hundred million macrophages. Within a tumor, TAMs may be operating under such restrictive conditions as hypoxia or nutrient deficiency. These conditions are very different from those found in normal healthy tissue (72).

In my research the majority (97%) of tumors examined contained macrophages in moderate to high concentrations. Because of this and the fact that macrophages can kill in a nonspecific manner it was expected that tumors would be slow growing, but again, as seen with the T-cells, this did not occur. Over half of the tumors had a moderate to high concentration of macrophages in the peripheral and central region. Only one tumor out of thirty-two had the expected relationship of high macrophages and an absence of growth. Thus, it is possible that the macrophages present in the tumor are there for reasons other than the destruction of the tumor.

Macrophages may be attracted to tumors by the tumor cells releasing a factor called tumor-derived chemotactic factor (TDCF) (62). It is currently unknown if this factor is released by breast carcinoma cells. It is known, however,that tumor cells have the ability to release transforming growth factor-beta (TGF-B) which will attract macrophages (62,96).

v. Macrophages and their involvement with growth factors

Macrophages have the ability to secrete over 100 different factors. Many of these factors may be involved in creating a microenvironment that allows tumors to successfully grow (73). Some of these factors are growth factors. Growth factors secreted from target cells can either stimulate or inhibit cell proliferation of target cells depending on the nature of the growth factor, the cell type receiving it or the physiological

conditions and the surrounding environment of the responding cell. Therefore growth factors can have a profound effect on wound healing, tissue formation and in the development, formation and maintenance of tumors (74).

One growth factor in particular, transforming growth factor-alpha (TGF-α) plays a significant role in proliferation of normal cells and is currently thought to be important in tumor development by stimulating cell proliferation. TGF-α is a member of a small family of structurally related growth factors. The family includes epidermal growth factor (EGF), amphiregulin and heparin-binding-EGF-like growth factor and three virally encoded polypeptide factors, vaccinal virus factor (VGF), myxoma virus-growth factor (MGF), and shope fibroma growth factor (SFGF) (75,76,77). Activated macrophages have the ability to secrete TGF-α and since macrophages are present in large numbers in areas of inflammation and wound healing it has been strongly suggested that secreted TGF-α is involved in the wound healing process and also in the stimulation of the proliferation of epithelial cells. Also, TGF-α can induce proliferation of other cell types that carry EGF receptors, such as fibroblasts and endothelial cells (78).

The "EGF" is a sequence of about 45-50 amino acids containing six characteristically spaced cysteines. TGF-α is similar to this "EGF" unit and shares a 35% sequence identity of with the cysteines. This allows TGF-α to interact with the same receptor as EGF, the EGF receptor (EGFr). It is possible that there may be a specific TGF-α receptor, but currently all that is known is that TGF-α effects are mediated via the EGF receptor (78). With the exception of hemopoietic cells, nearly all cell types express EGFrs. The levels vary from 20,000 to 200,000 receptors per cell on a normal, nonmalignant cell. EGFrs have been seen in elevated numbers in primary breast tumors (80), squamous cell carcinomas (81), some glioblastomas (82) and melanomas (83).

TGF-α was evident in moderate to high concentrations in two-thirds of the tumors examined (n=17), with one tumor having a low concentration of TGF-α. One-third of the tumors had an absence of this factor. In other work done in our laboratory it appeared that close to half of the tumors (n=11) that had intense staining for TGF-α also had the same cells staining for macrophages (79). We also examined 11 tumors for EGF receptors. In five of the tumors staining was limited to tumors cells (Figure 6a&b) and

no staining was present in the other six tumors. Therefore I suggest the possibility that macrophages in some of the breast tumors are producing TGF-α, which is released into the surrounding tumor environment. This growth factor can then bind to the EGFr which we detected on some of the tumor cells. These events can trigger tumor cell division, eventually increasing the size of the tumor. This is typical of paracrine growth regulation.

TGF-α can also remain as a transmembrane factor, which is a precursor of soluble TGF-α. The transmembrane form can interact with EGF-receptors on neighbouring cells and induce mitogenic responses without the release of TGF-α. TGF-α is only one of many factors that has a transmembrane cell surface polypeptide. Others include colony stimulating factor-1 (CSF-1), tumor necrosis factor (TNF) and other members of the EGF family. This type of interaction results in a highly localized mitogenic stimulation of the target cell (78). In figure 2h it can be seen that the macrophages in several areas are in very close contact with the neighbouring tumor cells that may be undergoing cell division. This may illustrate a transmembrane TGF-α bound to an EGFr which in turn activates proliferation.

My results suggest that within the breast carcinomas that I examined there was a moderate to high concentration of macrophages in the majority of the tumors. There was also a positive correlation between tumor growth and high a infiltration of macrophages. I also detected a relationship between the production of TGF-α and macrophages. Lastly we found that some tumors are bearing EGF receptor, which can bind TGF-α, which in turn stimulates tumor cells to divide.

vi. Macrophages and their possible transformation into fibroblasts

Macrophages also have other roles within tumors. They may be able to produce the collagen needed for the tumor. Bearing in mind that the macrophage populations were moderate to high in concentration, in a majority of the tumors, that they were preferentially located in the tumor stroma, and they correlated positively with tumor growth rate. Their presence produced little if any detrimental effect on the tumor cells. In fact, cell growth was often more pronounced under these conditions. These results

were originally surprising. Further work with primary cell cultures suggested that macrophages may be transforming into fibroblasts. Cultures were grown for three to four weeks and then stained with the macrophage monoclonal antibody, which stains the intracytoplasmic granules of lysosomal origin (Figures 3 and 4). Two different staining patterns were seen: a cluster pattern, in which cells positive and negative for macrophage monoclonal antibody are grouped together within the same colony; and a checkerboard pattern, in which the two types of cells are completely intermixed. Evidence indicating macrophage to fibroblast transformation was taking place under these conditions included:

1. The presence and similarity of the two cell types within the same expanding colony.
2. The intracellular staining pattern ranging from total cytoplasmic staining through partial cytoplasmic staining to complete absence of staining, within cells of the same colony.
3. The monoclonal antibody for proliferating cells, Ki-67, was staining mitotic figures in the cultures and these cell types were also staining positive for macrophages (Figure 4).

These observations suggest that under culture conditions some of the fibroblasts in these human breast carcinomas may be derived from macrophages. Vaage and Lindblat (87) reported that Metchnikoff, as early as 1891, proposed that blood monocytes could become "fixed connective tissue cells" at sites of inflammation, but that this idea never gained acceptance. Fibroblasts derived from macrophages could be regarded as potentially dangerous fibroblasts when they take up permanent residence within complex cell systems. This is especially true when these fibroblasts are present in large numbers (88). In solid tumors, for example, abnormal stimuli by cancer cells may trigger production of specific growth factors by stromal cells, mimicking a continuing wound healing process (101). Alternatively, the positive relationship between macrophages and tumor growth that we have observed raises the possibility that macrophage to fibroblast transformation may reduce a stimulatory effect on tumor cells, thus inhibiting tumor growth. Evidence for a possible transformation of this type can be derived from the work of Adam et al.(84) who showed that fibroblasts from human breast cancers are different

from fibroblasts derived from normal breast tissue. Fibroblasts from human breast cancers secreted a growth factor that stimulated the growth of MCF-7 cells *in vitro*, but fibroblasts from normal human breast tissue did not. Presently there is no evidence that such a transformation occurs *in vivo*.

vii. Macrophages and their possible involvement in metastasis

At present, we lack a satisfactory explanation for the onset and progression of the metastatic process. For example, many highly specific, sequential functions have been assigned to a single tumor cell or a group of tumor cells to successfully establish a metastasis. These include: detachment from the main tumor mass, increased motility, traversal of the extracellular matrix, crossing of the basement membrane of a capillary or lymphatic vessel, passage through or between the endothelial cells and evasion of the immune system, particularly during transit trough the blood or lymphatic system. Upon reaching a potential metastatic site, many if not all of those functions have to be performed again in reverse order (89,90).

It is difficult to imagine that a cancer cell or a group of cancer cells that are engaged in a self-replicating mode can, in addition, be involved in such complex cellular differentiation processes. Evidence suggesting that tumor cells are capable of performing some of these functions has been obtained primarily from work on cancer cell lines metastasizing in animal systems (90,99-101). However, while studies using such systems may provide answers to some of the questions on the metastatic process, they do not fully address the potential impact of cellular relationships and their interactions that may occur in spontaneous human tumors.

Considering the high concentration of macrophages in the connective tissues of breast cancers and the affinity of macrophages for tumors cells that I observed in the thirty-two tumors I examined, it is possible that direct tumor cell-macrophage contact would occur in the initial stages of the metastatic process. Evidence for such an interaction has been obtained from short term cell cultures of breast cancers. We observed macrophage association with other cell types, probably cancer cells. These interactions range from abutting to complete encirclement of cancer cells by macrophages

(Figure 5). We do not know at present if such interactions lead eventually to phagocytosis and death of the enclosed cell but evidence from engulfment of thymocytes by macrophages (91,92) and from our work (93) on the growth potential of cancer cells in close contact with macrophages leads us to believe that such interactions with macrophages are not detrimental to cancer cells and may even aid in cancer cell metastasis. This concept of cellular parasitology is still very hypothetical, but there is circumstantial evidence from a variety of sources (32,91,92,95-99) to make this a concept worthwhile investigating in the future. Both the growth potential (85,86) and the metastatic process may be intimately dependent on the macrophage population. My work suggests that the high concentration of macrophages in the stroma, their close contact with tumor cells and their ability to produce a multitude of factors indicates these cells may well aid tumor cells in growth and the metastatic process of breast carcinomas.

VII. CONCLUSION

An appearance of a tumor within the body could be seen as a failure on the part of the immune system. Tumor cells are able to escape destruction, and are therefore able to freely proliferate and to possibly metastasize.

My research indicated that one hundred percent of the thirty-three tumors we examined were infiltrated with at least one type of immune cell population. Helper T-cells were present in moderate to high concentrations in 65% of the periphery and 55% in the center portion of the tumors. Cytotoxic T-cells were observed in the periphery of 56% of the tumors and in 36% of the center areas of the tumors. Both helper and cytotoxic T-cells were observed to be surrounding or partially surrounding cancerous ducts, and in some tumors they were intermixed with tumor cells. Our work demonstrates that even though T-cell were evident in both the periphery and central portion of the tumors, they did not seem to be activated. Activated T-cells carry interleukin-2 receptors on their surfaces. Only one-quarter of the tumors examined (n=27) had the presence of IL-2R, and only in low to moderate concentrations. It can be concluded that even with the presence of T-cells within the tumors they are only capable of causing very minimial damage to the tumor cells.

Macrophages seem to play a very important role in the growth and possibly the metastatic process of tumors. My work showed the following relationships macrophages and tumor cells:

1. Macrophages are found in moderate to high concentration in a majority of the breast tumors examined.
2. The majority of tumors that have moderate to high infiltrations of macrophages also have high tumor cell proliferation.
3. Macrophages appear to be able to produce the growth factor TGF-α. We observed that cells that stained for TGF-α were in close proximity to macrophages and may in fact be the same cells.
4. Some of the tumor cells were staining positive for EGF-receptors.
5. Macrophages were seen in direct contact with dividing tumor cells.

6. Macrophages in cell cultures appear to be able to transform into fibroblasts.

7. Macrophages appear to be the only cell type in division in our culture system.

8. Macrophages were observed in cell culture to be abutting to or encircling other cell types, possibly tumor cells.

Considering these observations we created a hypothetical model to illustrate what could be occurring in the breast tumors. Figure 7, shows possible relationships between macrophages and tumors cells that may occur. Macrophages are attracted to tumor areas possibly by transforming growth factor-beta, which is released by the tumor cells. This stimulation has several effects on the macrophages. Macrophages may transform into fibroblasts and multiplication of these cells may provide the stromal elements neccessary for tumor growth. Another effect that may occur is that stimulated macrophages may release TGF-α which can stimulate tumor cells to divide. The third effect is that tumor cells and macrophages may form units consisting of macrophage(s) enclosing tumor cell(s). This would afford the tumor cell protection from immune attack during transit through the bloodstream and could stimulate cell proliferation at the metastatic site. This may occur via the transmembrane TGF-α/EGFr axis.

The final conclusion of my work is that there is evidence that several immune cell populations were present in substantial numbers in the 32 breast carcinomas that I examined. There was very little evidence of B-cells infiltration, therefore they may not play a large role in either tumor development or destruction. Both subpopulation of T-cells, helper and cytotoxic T-cells were present. These cell populations were in high concentrations in some tumors but there was no evidence that they are stimulated and are therefore probably inactivated. Macrophages were the biggest surprise. They were found predominantely in the majority of the tumors. A positive relationship between macrophage infiltration and tumor growth existed. Macrophages can also express TGF-α which binds to EGF-receptors on tumor cells, possibly triggering cell division. Macrophages could also be undergoing transformation into fibroblasts, which in turn may provide stromal elements neccessary for tumor survival. The most interesting role of macrophages may be that of cellular parasitology, evidenced by tumor cell(s) encircled or engulfed by macrophage(s). Such a unit may then be able to move to other areas of

the body to seed itself, causing a metastasis. I believe that macrophages may be very damaging for the patient who has developed breast carcinoma.

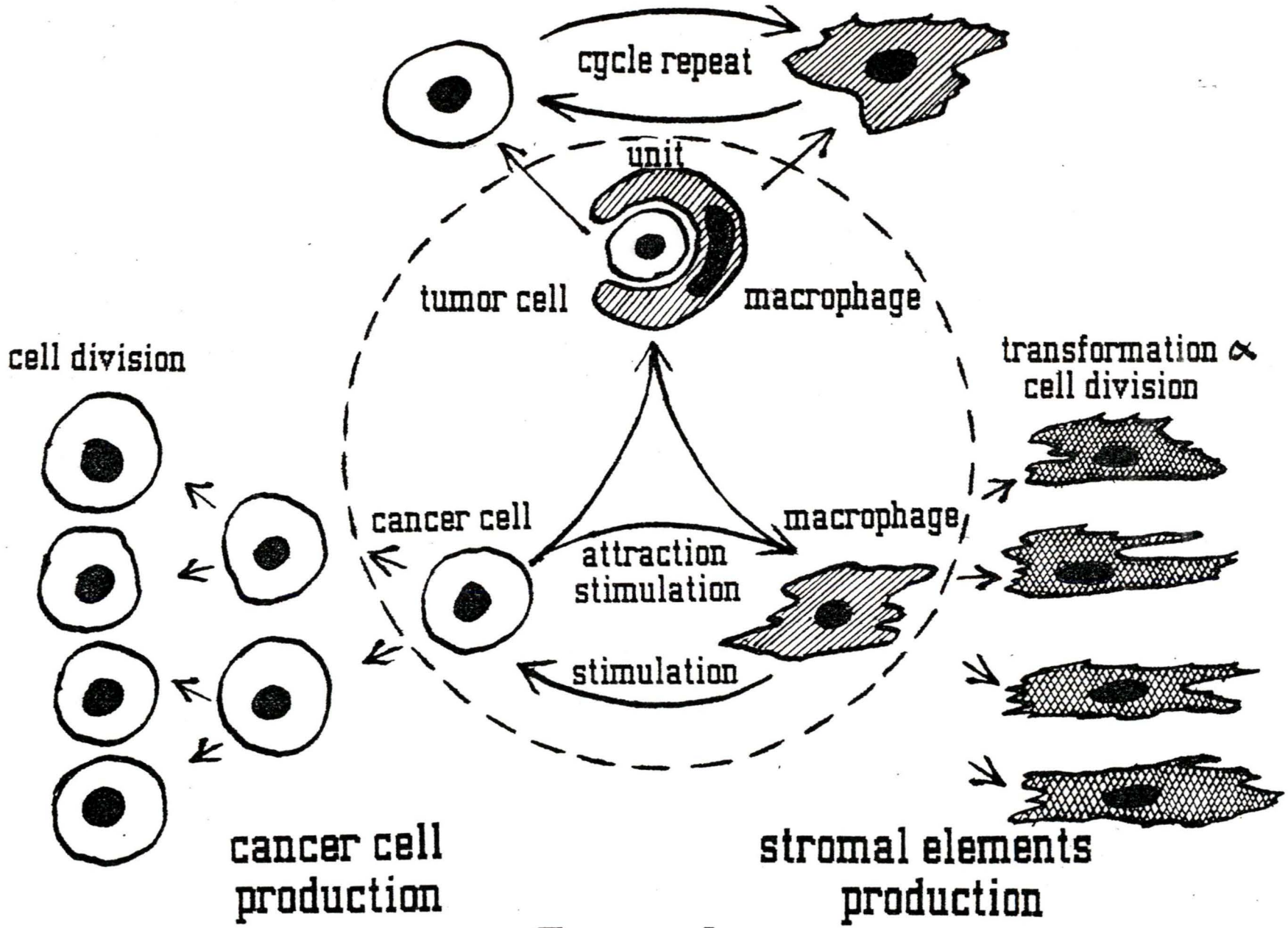
Presently the main treatment regimen for breast carcinoma is targeted at the destruction of tumor cells with chemotherapy and/or radiation and/or hormonal therapy. Not withstanding the fact that over the last 25 years the 5 year survival rate for breast carcinoma has increased to 70%, the absolute death for this disease has not changed significantly. Thus there is a dire need for a search for alternate treatment. Rather than focussing on cytotoxicity to cancer cells, our work directs more emphasis on influencing cellular function aa a form of treatment for cancer of the breast.

VIII. FUTURE WORK

1. More detailed work must be done on the macrophage's role in breast carcinomas. It may be that there are subpopulations of macrophages, each with different functions.
2. Investigations on the possiblity of changing the function of macrophages within the tumor from working on behalf of the tumor to working against the tumors possibly with the use of different cytokines or factors such as vitamins or hormones.
3. Chemicals that might block further recruitment of the macrophages into tumors should be investigated.
4. The possibility of activation of the T-cell subpopulation already present within the tumor should be investigated.

Figure 7. Diagram showing hypothetical cancer-tissue-generating-triad (cancer cell, macrophage and cancer cell/macrophage unit) inside circle. Production of this triad generates cancer cells and stromal elements as well as met metastatic units in breast carcinomas.

Metastasis



Growth

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X. APPENDIX

0.01M Phosphate Buffered Saline (PBS).

pH 7.2-7.4

8.5g Sodium Chloride

1.43g Potassium Phosphate (anhydrous) Dibasic ($K_2HPO_{4)}$

0.25g Potassium Phosphate (anhydrous) Monobasic (K_2HPO_4)

make-up in a 1 litre volumetric flask using distilled water.

Specimen Storage Medium.

42.8g sucrose

0.70g magnesium chloride (hexahydrate)

adjust volume to 250ml with PBS

add 250ml of glycerol

mix well by stirring

store at -10 to -20°C

Endogenous Peroxidase Rinse.

50 ml of 3%w/v hydrogen peroxide

200ml of PBS

mix together and make fresh daily.

3.7% Formaldehyde-PBS Solution.

1 volume of 37% formaldehyde

9 volume of PBS

mix together and make fresh daily.

10% (v/v) Harris Hematoxylin.

10 ml Harris Hematoxylin (Fisher Scientific #SH26-4D)

90 ml of distilled water

mix together, solution lasts for 1 week.

3'3' Diaminobenzidine Tetrahydrochloride (DAB).

10mg DAB tablet (Sigma #D5905)

12 ml of PBS

warm tablet up to room temperature, dissolve into PBS (needs several hours to dissolve). Filter before using. To activate add 10ul of 3% hydrogen peroxide for each 1ml of DAB, use within 15 minutes. To deactivate DAB, add bleach.

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Title of Thesis: The Characterization of Immune Cell Populations in Relationship to Growth of Infiltrating Ductal Carcinomas.

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