LYMPHOCYTE FUNCTION IN HUMAN BREAST CANCER

by

Alastair G. Paterson BSc MB ChB FRCS

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Department of Surgery,
University of Wales College of Medicine,
Cardiff.

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Summary

Breast cancer causes 20% of all cancer deaths in women in the U.K. The inadequacy of existing methods used to stage tumours at presentation is reflected by the high proportion of women with apparently localised disease who later die from metastases. Better ways of assessing the biological aggressiveness of the disease are therefore required.

Evidence suggests that cancers exert an inhibitory or 'blocking' effect, proportional to tumour load, on immune function. This may be mediated through the action of the combined products of tumour cell materials and host antibody, circulating immune complexes (CICs), on immune competent cells. CICs have been reported to depress % T lymphocyte counts and also under certain conditions to stimulate lymphocyte activity. A new method of assessing lymphocyte function by measuring the O_2 consumption of separated lymphocytes has been developed which utilises this possible stimulatory effect.

In the present study % T lymphocyte counts were depressed and lymphocyte O_2 consumption increased in patients with breast cancer compared with benign breast disease ($p<0.001$) and this was stage related ($p<0.05$). Neither test was affected by previously defined prognostic factors or local treatment and was not predictive of disease recurrence. Both tests returned towards control values in patients benefiting from endocrine manipulation ($p<0.02$) and this effect was seen prior to objective clinical evidence of benefit.

The factor(s) responsible for increased lymphocyte O_2 consumption was present in the sera from patients with cancer and IC related diseases and

affected the T-rich lymphocyte population only. It had many features in common with 'blocking' factor(s) and with ICs, and indeed, lymphocyte O_2 consumption correlated significantly with % T counts ($r= -0.409$; $p<0.001$) and CIC levels ($r= 0.399$; $p<0.005$) in controls and patients with benign and malignant breast disease.

This suggests that CICs may be responsible for the depression of % T counts and stimulation of T lymphocyte O_2 consumption in breast cancer patients, and can be used as a gauge of tumour activity permitting earlier evaluation of disease response to endocrine manipulation.

Chapter 1

Chapter 1

Introduction

One fifth of deaths in women dying of cancer in the United Kingdom are from carcinoma of the breast, the incidence of this disease increasing steeply with age from 35 years onwards (Forrest 1986).

Once a diagnosis of breast cancer has been made the subsequent step is to assess the extent of the disease, this being the main criterion in selection of an appropriate treatment modality. At the present time this usually entails haematological and biochemical testing of marrow and liver function, radiography of chest and breasts and where indicated, isotope bone scanning and liver scanning by isotopes, ultrasound or both. The prognosis of the patient may be better gauged by using information on, for example, oestrogen receptor content (Knight et al. 1977), progesterone receptor content (Horwitz & McGuire 1977), size, attachments and histological grade (Fisher et al. 1976), DNA content (Atkin & Kay 1979) and cell labelling index (Meyer et al. 1983; Silvestrimi et al. 1985) of the primary tumour, but the most important prognostic indicator in early breast cancer is involvement of axillary nodes (Cutler et al. 1969; Bonadonna et al. 1978). Others have attempted, by combining several such prognostic factors into a prognostic index (Blamey 1983), to obtain more sensitive mechanisms by which to predict outcome. Further information relating to prognosis in patients with recurrence of disease or metastases can be obtained from the disease free interval, the predominant site of the lesions, and the menopausal status (Kardinal & Donegan 1979).

However, at this time, there is no adequate way of demonstrating the presence of occult metastases in those with apparent locoregional

disease only, on tumour staging; many patients with involved axillary nodes and a substantial proportion of those without node invasion having widely disseminated disease at presentation (Lancet 1977). More over, it may be extremely difficult to assess the efficacy of treatment in patients given systemic therapy for some time, a trial period of at least 12 weeks normally being necessary before this can be ascertained. Occasionally it may indeed be impossible to tell by objective means whether or not the patient is responding to treatment, a substantial number having no objectively measurable disease by UICC criteria (Hayward et al. 1977). Present methods of predicting the prognosis and responsiveness of breast cancer are therefore clearly of insufficient calibre, necessitating a search for other ways of assessing the biological aggressiveness of the disease if we are to obtain information on which to base therapeutic regimes and attain speedier evaluation of any given treatment, permitting earlier selection of alternative treatment modalities.

Human chorionic gonadotrophin (HCG) produced by trophoblastic tumours (Bagshawe 1969), and alphafeto protein (AFP) often found in patients with primary liver tumours and testicular teratomas (Sell & Becker 1978) are markers which allow measurement of tumour burden and response to treatment in a systemic fashion. These reflect tumour activity more than tumour volume, for instance, patients with testicular teratomas which have responded to chemotherapy will have normal tumour marker levels, despite computerised tomographic evidence of enlarged retroperitoneal lymph nodes. These are usually found to contain differentiated tissue on surgical excision (Jones et al. 1982). Adenocarcinomas, because of their low cell turnover rates and surrounding non-malignant stroma are not capable of showing a rapid decrease in total tumour volume when measured by more routine radiological or physical methods (Parker &

Bagshawe 1985). The use of markers to measure tumour activity, rather than change in size, should therefore give a more accurate and informative assessment of what is actually happening to a tumour on treatment.

Such tumour products have been looked for in breast cancer and sporadic reports have been published of 'ectopic' hormone production, for example, parathyroid hormone (Maglivit et al. 1971), HCG (Braunstein et al. 1973) and calcitonin (Hillyard et al. 1976), unique milk products such as alpha lactalbumin (Woods et al. 1979) and casein (Hendrick & Franchiment 1974), enzymes such as placental alkaline phosphatase (Stolbach et al. 1969), and various other products such as ferritin (Marcus & Zinberg 1975), pregnancy associated alpha macroglobulin (Stimson 1975), other acute phase proteins (Cooper and Stone 1979), and carcinoma embryonic antigen (Tormey & Waalkes 1978). However, the presence of these substances is too infrequent to be of clinical value (Hilf 1985) and their specificity for breast cancer similarly disappointing.

The best tumour marker would be one specific only to the tumour and produced and released into the serum at a rate proportional to tumour turnover. A tumour specific antigen would meet this requirement but there is little evidence that such an entity exists in any of the spontaneous human tumours (Baldwin 1984). On the other hand, if it could be shown that tumour products affect the immune system in a certain manner and that the degree to which this happens is related to the volume or, better still, the activity of tumour present, we should then be able to indirectly assess tumour burden and behaviour by measuring certain parameters of immune function.

Bearing this in mind, this thesis examines firstly, the evidence linking cancer and immunity and secondly, the data indicating that immune impairment is a consequence of tumour development and progressive

growth and that such dysfunction is related to inhibitory or 'blocking' factors, these possibly being immune complexes, present in the serum of tumour bearers. Finally, the main purpose of this study is to show that these 'blocking' factors alter some particular aspects of lymphocyte function in a manner which reflects the behaviour of the breast cancers under study.

Chapter 2

Chapter 2

2. Immunology and Cancer

2.1. Introduction

The idea that tumours frequently occur but are prevented from developing by the host immunological rejection mechanism, so that they seldom become apparent, was propounded by Thomas (1959) and later elaborated by Burnet (1964; 1970a) as the 'Theory of Immune Surveillance'. Long before then, a similar theory was proposed by Ehrlich (1909) when he wrote "I am convinced that aberrant germs appear very often during the foetal and neonatal development periods, which are extremely complicated. Fortunately, these germs remain inactive in the majority of people because of the immune system. If this self protection did not exist we could expect that carcinomas would appear with overwhelming frequency." (from Beverley 1983).

The idea that the host can immunologically recognise a tumour as foreign and thereafter respond in an aggressive fashion to it, led to the hope that by stimulating the immune system, tumour rejection would occur. Furthermore, by developing hetero-antisera to tumour antigens it should be possible to use these antisera for diagnostic and therapeutic purposes.

The hypothesis of immune surveillance, however, poses several problematical questions. Firstly, is there any evidence relating immunity to cancer; secondly, do tumours carry new antigens recognisable as different from the antigens on a normal cell; thirdly, can the host recognise and respond specifically to such neoantigens and if so, what is the effector system involved; and fourthly, if such immunological responses do occur, why do tumours continue to grow in the face of these? Perhaps cancer patients have depressed immunity allowing this to happen, or indeed, is it possible that tumours themselves cause host immune depression?

2.2. Evidence Relating Immunity and Cancer in Humans

2.2.1. Clinical

2.2.1.1. Tumour histology: MacCarthy (1922) reported that one of the factors which influenced longevity in cancer was lymphocytic infiltration of the tumour. Microscopic evidence of an 'inflammatory' infiltrate in and around the tumour and sinus histiocytosis in the regional nodes have been implicated many times since then as manifestations of host resistance to tumour and have been shown to be associated with improved prognosis in cancer of the stomach (Black et al. 1954), of the breast (Black & Spear 1958; Anastassiades & Pryce 1966), and in Hodgkin's disease (Lukes et al. 1966). Berg (1956) was initially rather sceptical of the significance of sinus histiocytosis in terms of a host reaction but did find (Berg 1959) a 'peripheral plasma cellular reaction' in the primary breast cancers and the nodal metastases, correlating with degenerative change in the cancer cells, in a group of unexpected long-term survivors with anaplastic breast cancers. Tsakraklides et al. (1973) showed that regional lymph nodes could be classified into four histological patterns designated lymphocyte predominance, germinal centre predominance, unstimulated and lymphocyte depleted. Survival data from those with carcinoma of the uterine cervix (Tsakraklides et al. 1973) and carcinoma of the breast (Tsakraklides et al. 1974) correlated with these histologic patterns, lymphocyte predominance being common in those with good prognosis, lymphocyte depletion common in those with bad prognosis and the other two histological patterns common in those with intermediate prognosis. Black and Leis (1971) using the 'skin window' technique demonstrated that a hypersensitivity type of cellular response to autologous breast tissue correlated with the degree of sinus histiocytosis in the regional nodes and with stage of disease. The suggestion that a host reaction may occur

which modifies invasion can also be seen in the study by Fenoglio and Pascal (1982) on neoplastic change in colorectal adenomas. They found that tumours with a prominent lympho-plasmacytic infiltrate in which there were well formed germinal centres surrounding the invasive focus tended to be less invasive, the majority of the lesions being categorised as Dukes' A. Nind et al. (1973) found a correlation between peripheral blood lymphocyte reactivity and the type of lymphocytic infiltrate of tumours. Eighty percent of patients with anergic peripheral blood lymphocytes had diffuse stromal leucocyte infiltration of their tumours including large lymphocytes and plasma cells; whereas 50% of patients with reactive peripheral blood lymphocytes showed a tendency to discrete perivascular aggregation of small hyperchromatic lymphocytes, a finding which never occurred in the tumours of anergic patients. Surprisingly, however, there was no correlation between these findings and prognosis.

Other investigators have attempted to elucidate the relationship between cancer and such histological phenomena by looking at the cell subsets involved, their effectiveness against tumour antigens and their competence on being assayed immunologically. These in vitro studies have not shown any strong antitumour effect (Cochran 1982) although this could admittedly be a problem of appropriate cell selection.

It is possible that the importance of tumour mononuclear cell infiltration has been exaggerated and may have little to do with host resistance, but rather, defective tumour growth. Berg (1971) noted that in his experience of 20 spontaneously regressing malignant melanomas: "In no instance was there a prominent round cell reaction. The tumour cells just seemed to fade away.". He and Robbins (1964) also found that women demonstrating the morphological patterns of host resistance in one breast went on to develop a second primary in the other breast at the same rate

as those without such patterns; hardly what one would expect in a resistant host. Richardson (1965) similarly found that medullary breast cancers did just as well whether or not they contained lymphocytic infiltrates.

Such conflicting data are difficult to interpret. On balance it would seem that where there is lymphocyte infiltration it appears to be relatively inefficient, certainly by the time tumours have developed sufficiently to present clinically (Stewart 1969a). One cannot be sure whether these histological appearances are just epiphenomena of no immunological importance, or, that the changes are part of an immunological response which, in the majority of cases, is made ineffective by the local action of the tumour. The latter possibility is discussed later.

2.2.1.2. Tumour behaviour

Tumour regression: Spontaneous regression of human tumours, though rare, has been well documented (Everson & Cole 1966; Smithers 1962). Tumours most noted for this phenomenon are neuroblastoma, malignant melanoma, hypernephroma, choriocarcinoma, Burkitt's lymphoma and breast carcinoma. Minor viral and bacterial infections, fever of unknown aetiology and changes in hormonal balance sometimes appear to play a role in these inexplicable regressions (Morton et al. 1983). Examples of excision of the primary tumour followed by spontaneous regression of lung secondaries have been reported and this has been observed most commonly with hypernephromas. Choriocarcinoma (Li et al. 1958) and Burkitt's lymphoma (Burkitt 1967) occasionally regress following very low doses of chemotherapy raising the possibility of initial cell destruction being followed by a stimulated immune host response; indeed one of Burkitt's cases regressed following a diagnostic biopsy only.

Smithers (1962) felt that most regressions of breast cancer, choriocarcinoma, ovarian cancer and perhaps hypernephroma, could be explained

by changes in the host hormonal milieu. He suggested that regressing neuroblastomas and teratomas were examples of embryomal tissue undergoing maturation and that some soft tissue sarcomas in regression are embryomal in character and unstable in nature. This spontaneous differentiation, therefore, may have little or no immunological foundation. However, Everson and Cole (1966) pointed out that there was evidence for differentiation in only five of 29 spontaneously regressing neuroblastomas.

Variation in tumour growth rate: occurs between individuals with similar histological tumours, some progressing more rapidly than others. This may be the result of an individual's immunogenicity but could be due to the host's hormonal, metabolic, psychological or biological make-up, or perhaps the effect of an external factor such as infection. However, within individuals there may also be variations in growth rate in the form of waxing or waning of tumour deposits. Malignant melanomas, for example, may be seen to regress as others develop and breast cancers occasionally appear to undergo fluxes in growth rate without any obvious change in the host's hormonal status.

Subclinical incidence: The incidence of certain tumours such as neuroblastoma and carcinomas of the thyroid and prostate has been found to be higher at postmortem examination than would be expected on the basis of clinical incidence (Gordon-Taylor 1959; Currie 1980) raising the possibility that in otherwise normal healthy people tumours are developing and regressing all the time.

Dormancy: Histologically identical metastases to the original primary tumour develop in some patients 10 to 30 or more years following resection of the original lesion. It appears unlikely that these secondaries are just

extremely slow growing ones as, when they do develop, they often grow very rapidly. Hadfield (1954) found it difficult to believe that cellular proliferation in the residual tumour over such a prolonged period of time could be continuous and suggested that it was hard to escape the conclusion that the cells of the dormant growth must be in a state of temporary mitotic arrest. "Dormant cells are prone to lurk in or near the site of the original operation, in regional lymph nodes, or in deeply placed organs." (Gordon-Taylor 1959) and in at least 50% of cases the reactivated metastases are found in the haemopoietic bone marrow. Carcinoma of the breast and prostate are the most usual tumours to exhibit dormancy and it is possible that the phenomenon is hormonally based; tumour and host co-existing until an hormonal environment develops which is suitable for tumour growth. Many other cancers of organs not obviously under hormonal control have been shown capable of latent recurrence and because of this it is difficult to explain dormancy only in terms of hormonal milieu. Gordon-Taylor (1959) related instances of apparently cured women who developed late recurrences of their breast cancers following stress events such as infection or relatively trivial surgical procedures. Others have also related stress to the development of previously dormant tumours and more recently there have been reports in the literature on the effect of stress on cell-mediated immunity (CMI) (Schleifer et al. 1983; Stein et al. 1985; Denman 1986). However, what changes there are in CMI are probably brought about by changing cortisol levels and it may be that this is more pertinent to the appearance of latent cancer than any immunological upset.

Circulating tumour cells: Griffiths et al. (1973) and other authors have found tumour cells present in the peripheral blood of patients undergoing surgery, many of whom never developed metastases. This suggests the existence of a highly efficient mechanism for the destruction of

circulating tumour cells. The nature of this mechanism is speculative but a host immune defence may be one of them. Griffiths discovered that patients, in whose blood free tumour cells were seen at operation, surprisingly appeared to have a better prognosis than those whose blood contained none. This finding was verified by investigators from the same centre (White et al. 1976) in a follow-up study in which they also demonstrated that tumour cells could be maintained in the circulation for much longer periods of time by giving perioperative anticoagulants. They suggested that improved prognosis was secondary to prevention of attachment of tumour cells to the vascular endothelium, possibly as a result of increased levels of fibrinolysins. A specifically directed immunological defence mechanism acting against cancer cells in the blood should be equally effective against cancer cells within the tissues and as this is clearly not the case, it therefore seems unlikely that this is the cause of tumour cell destruction in the circulation. It is more probable that such destruction is due to tumour cell trauma and non-specific defence mechanisms in the circulation itself but to date, no one has been able to explain the mechanism satisfactorily.

Family history: There is increased risk in blood relatives of developing the same malignancies (Ponder 1984), even when inherited disorders leading to malignancy such as retinoblastoma, familial polyposis coli, xeroderma pigmentosum, immune deficiency syndromes, etc., are excluded. This may be in the order of a two to four-fold increased risk for relatives of patients when compared to the control population (Ponder 1984) for the more common adult cancers, and it is tempting to implicate weakened familial immunological host defence through perhaps inadequacy of IR gene expression. Furthermore tumour directed immunity has been shown with high frequency in the

relatives of cancer patients with some tumours when compared with controls (Cochran 1982). Examples of such tumours are osteogenic sarcoma, neuroblastoma, leukaemia, Burkitt's lymphoma and breast cancer. The vast majority of these relatives do not develop the cancer and it is again tempting to conclude that exposure to the immunogenic stimulus results in immunisation only, a satisfactory outcome for the host immune defence mechanism. Whether this tumour-directed immunity is transmitted vertically or horizontally, the latter being reminiscent of the spread of feline-leukaemia virus, is open to debate. However, it should be borne in mind that although tumour directed immunity reactions can be demonstrated in a high proportion of cancer patients, it can also be shown in 15 to 20% of ostensibly normal controls (Cochran 1982). The question of the specificity of these reactions and therefore their relevance to cancer must be in doubt.

Other perhaps more rational explanations for familial clustering can be given, the exposure of members of a family to the same environment (carcinogens) being the most obvious. Genetically, members of a family may be more susceptible to certain carcinogens in that the genetically determined differences in the metabolism of drugs between individuals may also apply to the handling of carcinogens. Additionally, factors such as dietary fat and hormonal status must be taken into account when considering carcinomas of the breast, ovary, endometrium and large bowel (Lemon 1984).

Multiple primary tumours: Although it is rare to have two synchronous tumours this does happen; but more commonly sequential tumours develop (Cochran 1982). Indeed, one primary increases the risk of a second. Barber (1984) noted that carcinoma of the breast predisposes the patient to develop colonic, rectal or ovarian cancer. Similarly, there is increased risk for patients who have carcinoma of the ovary or endometrium to present later with a breast primary. Harwood (1984) noted "The clinician

treating cancer of the head and neck is facing an epidemic of second respiratory tract malignancies of such a magnitude that it is progressively replacing the initial primary cancer as the major cause of death in these patients.". The misfortune of these patients might be attributed to defective host immune surveillance and indeed some second tumours especially of the lymphatic and reticulo-endothelial system appear to follow the rather immunosuppressive treatments of chemotherapy and radiotherapy applied to the first tumour (Whang-Peng & Sieber 1984). Such treatment, or indeed, the immunosuppressive effects of the first tumour itself (as we shall see later), might allow oncogenic viruses to exert their effect at a cellular level at that time, or perhaps after further depression of CMI following opportunistic invasion of the host by immunosuppressive viruses. These tumours may possibly be related in some way or another to defective immune surveillance.

Immunologically unrelated explanations for multiple primary tumours are just as, if not more, likely. The mutagenic effects of radiotherapy or chemotherapy may be the result of chromosomal damage. Successful treatment of the first tumour may result in patients surviving long enough to manifest a second tumour in a less carcinogen-sensitive tissue, or a second tumour to a different carcinogen altogether. These may be exogenous carcinogens such as chemical, viral or physical agents, or endogenous carcinogens such as hormones. Certainly hormones appear to be implicated in the development of certain tumours such as breast, endometrium and ovary. Dietary fat is closely correlated to the incidence of large bowel, breast and ovarian tumours perhaps as a result of its effect on bile acid metabolism and oestrogen metabolism (Hill & Thompson 1984). It seems probable that multiple cancers are the net result of exposure to carcinogens, and the effect on the host of mutagenic therapy for the first tumour. The possible role of

oncogenic viruses and their relationship to the host immune system cannot however be excluded.

2.2.1.3. Immune deficiency

According to the theory of immune surveillance, immune deficiency, whether congenital or acquired, should result in an increase in the incidence of tumours of all types of tissue, often occurring metochronously and this effect would be most marked in those with defective CMI. What actually happens can be seen when tumour development is examined in relation to age, allergic individuals, immune deficiency states, and immunologically privileged sites.

Age: and immune function are indisputably related. Indeed some, including Burnet (1970b) have suggested that the ageing process may to some degree follow impairment of the immune system. There appears to be diminished immune function in the foetus, the early infant, and the elderly; these extremes of age being times of greater risk from infectious disease. Nagel (1983), in a review of the literature on ageing and immunity, noted that humoral immunity showed little obvious change with age, but CMI appeared increasingly depressed with advancing years. The fact that most kinds of cancer, excluding those in organs directly influenced by female hormonal status around the menopause, have curves of specific age incidence which rise logarithmically with age, led to the suggestion that cancers were more common at this time in life because they were allowed to grow in the face of defective immunological surveillance (Burnet 1970b). Good (1972) further argued that because with age, CMI involved in the host defence to tumour becomes impaired whilst humoral immunity with its potential negative 'blocking' effect on this host reaction is retained, there is imbalance in the elderly favouring the development of malignancy.

The possibility exists, however, that the increasing incidence of cancers with age is merely related to the length of exposure to background and spontaneous carcinogenic stimuli. It has been shown (Peto et al. 1975) that tumour induction in mice is a feature of duration of exposure to the carcinogen rather than the age of the animal when exposed to this carcinogen. If this holds true for humans, no intrinsic effects of ageing such as failing immunological surveillance need be postulated to explain the vast increases, in old age, of the incidence rates of human cancers.

Allergy: is a cell-mediated immune phenomenon. It is conceivable that those displaying such hyperreactive cell-mediated responses to antigens might invoke the same powerful responses to tumours. Fisherman (1960) using the triad of flexural eczema in early childhood, development of hay fever at puberty and atopic asthma in middle life, was able to categorise those in his study into atopic and non-atopic types. He found that in his control population four times as many people gave a history indicating atopy as those from a group of approximately 1,300 cancer patients. Similarly, MacKay (1966) found that controls had greater than twice the number of individuals giving a history of allergy as patients with cancer but this phenomenon was seen only in women.

Congenital immune deficiency: Melief and Schwartz (1975) reported that cancer develops in approximately 10% of patients with spontaneous immune deficiency diseases. Forty-seven of the 58 patients in their series, however, had either lymphoma or acute leukaemia. Good (1972) found a similar proportion of malignancies. Those with mainly CMI deficiencies such as ataxia telangiectasia and Wiskott-Aldrich syndrome were prone to develop tumours of the lymphoreticular system, as were common variable immunodeficiency syndrome patients who have mixed humoral and cellular immune deficiency. These patients

also had a slightly higher preponderance of epithelial and rare tumours than the control population. Patients with Bruton's agammaglobulinaemia have intact CMI but deficient B lymphocyte activity and they too have an increased susceptibility to development of cancers, 10% presenting with leukaemia (Good 1972).

Acquired immune deficiency: other than secondary to nuclear accidents or to transmitable viruses is usually the result of medical manipulation in the area of autoimmune diseases or transplantation procedures. In renal allograft patients, Hoover and Fraumeni (1973) found a 350-fold risk to patients of developing reticulum cell sarcoma and a 35-fold risk of lymphomas. The overall risk of developing malignancies in other organs was hardly affected, a notable exception being tumours of the liver which possibly can be attributed to horizontal transmission of hepatitis virus, the hepatitis B virus being implicated in hepatocellular carcinoma (Lancet 1981a; Weiss 1984). Five percent of renal transplant patients reported by Melief and Schwartz (1975) developed cancers, mainly malignant lymphomas, but also epithelial cancers of the skin, lip or cervix. These investigators pointed out that the incidence of reticulum cell sarcoma in women in this study was 700 times greater than expected, yet the incidence of the most common female cancer, carcinoma of the breast, was not increased at all. They attributed the increased incidence of epithelial cancers to an increased frequency of physical examination in these patients when compared with the control population. Kinlen et al. (1979) examined, in a multicentre study, almost 4,000 renal transplant patients who had been given immunosuppressive drugs for at least three months and over 1,300 patients with other medical conditions necessitating immunosuppressive therapy. They noted in the transplant group a 60-fold increase in non-Hodgkins' lymphoma and an excess of squamous cell skin cancers and mesenchymal tumours although the total number

of these was very small. They also found the tumour induction period for lymphomas to be very short and suggested, as a consequence of this finding, that the cause of these tumours might be an oncogenic virus. Patients in Kinlen's study who had immunosuppressive therapy for medical conditions; rheumatoid arthritis, glomerulonephritis, ankylosing spondylitis etc., although having increased development of tumours, were not affected to nearly the same extent as patients who had undergone transplantation. The implication from this is that the transplant patients developed more tumours because they were more deeply immunosuppressed as a result of prolonged renal failure and prolonged period of administration of immunosuppressive drugs. Interestingly, conditions with chronically but not severe defective cell-mediated, and especially T cell, immunity as occurs in leprosy and chronic renal failure, do not appear to have an increased incidence of cancer.

Immunologically privileged sites: Areas of the body having no, or little, connection with the lymphatic system are relatively independent of the immune system. The anterior chamber of the eye in humans and the hamster cheek-pouch are characterised by absence of lymphatic drainage. Intracerebral, prostatic and testicular tissues, and to a lesser extent the liver, are areas having poor lymphatic connections. Such sites are immunologically privileged but do not have the increased incidence of cancers which one would expect if immunity were important in the prevention of tumour development.

In summary, immune deficiency does result in an increase in cancers. These, however, tend to be lymphoreticular tumours with no obvious increased evidence in the common solid cancers affecting man. It can be argued that patients with congenital immune deficiency may develop tumours as a result of associated genetic factors, or that those with acquired immune deficiency

have been subjected to immunosuppressive medication which itself is directly oncogenic. Another possible suggestion (Currie 1980) is that immunologically suppressed individuals are more prone to infections by viruses some of which are oncogenic (Weiss 1984). For instance nude mice which are athymic and cannot mount allograft or xenograft rejection reactions nor respond to viral infections also have a high incidence of lymphoreticular tumours but no increased incidence of other tumours (Rygaard & Povlsen 1974), behaving just as immunosuppressed humans do. Proponents of the theory of immune surveillance might suggest that because these mice have to be kept in a germ free environment they do not come into contact with other tumour producing carcinogens, or that these lymphoreticular cancers develop not because of defective surveillance, but secondary to a defect in cellular interactions and regulations in a defective immune system. Certainly there is no increased incidence of tumours of all kinds occurring spontaneously and synchronously in immunodepressed individuals and neither is there an increased incidence of tumours occurring in immunologically privileged sites. The tumours which do develop belong to a specialised group and may be the result of oncogenic viruses (Kinlen 1982), these being permitted to exert their effect in the face of a defective host immune response. The evidence to date suggests, however, that there is no strong link betwen impaired immunity and the development of the more common tumours in man in Western society.

2.2.2. Experimental

Tumour transplantation experiments and cutaneous testing to tumour extracts were performed on the basis that tumours had associated antigens recognisable as foreign by the host. The experiments were an attempt firstly to detect antitumour immunity *in vivo* in humans, to elucidate the mechanism of tumour rejection, to correlate immune reactivity with other

parameters of prognostic significance, and finally to define and purify, if possible, specific tumour antigens.

2.2.2.1. Tumour transplantation Allografts: Southam et al. (1957) intradermally inoculated tissue cultured tumours into patients with advanced incurable cancers and short life expectancy. Many had infections, metabolic complications and cachexia but surprisingly were still able to produce antibody against viruses inoculated at the same time. Most of these patients rejected the tumours, but slowly when compared to control patients who uniformly exhibited rapid rejection of the tumour inoculum. In some cancer patients the inoculated tumour continued to grow locally and occasionally these tumours recurred locally following excision of the site of inoculation. Two of these particular patients continued to exhibit this local growth up until death from their own original tumours, six and nine weeks later, one of these patients having metastases of inoculated tumour to her axillary nodes. These investigators later showed that tumours from individuals with more aggressive disease were more likely to 'take' than more localised growths (Southam et al. 1966), and that successful growth was also related to the immune status of the recipient (Southam et al. 1965). Scanlon et al. (1965) reported the death from metastatic melanoma of an 80 year old woman who had been the recipient of an inoculum of her daughter's tumour, in an attempt to raise antimelanoma serum. Whether or not this was the result of depressed CMI related to her age must remain hypothetical.

Reports of inadvertent transplantation of tumours have come from the field of renal transplant surgery (McIntosh et al. 1965; Martin et al. 1965; Kinlen 1982) but these tumours usually regress on cessation of therapeutic immune suppression (Kinlen 1982) as indeed do most of the lympho proliferative lesions which develop secondary to immunosuppressive therapy in transplant recipients (Starzl et al. 1984).

Autografts: have a low incidence of successful growth in humans, most being rejected even in those with advanced cancer. Southam et al. (1965) showed this to be dependent on the immune status of the recipients measured by skin reactivity to tuberculin and DNFB, and macrophage mobilisation. Successful growth was also dependent on inoculum size, 10^4 cells never being successful, 10^8 cells always inducing growth, and intermediate numbers of cells successfully growing, depending on the recipient's immune status. Mixing autologous leukocytes (?lymphocytes) with the tumour cells prior to inoculation resulted in cessation or slowing of growth in half the recipients. Under these conditions to achieve successful 'take' the number of tumour cells in the inoculum had to be increased ten-fold. Similar tumour dose-related effects have been seen in animals (Old et al. 1962; Stjernsward 1968). Southam and his colleagues believed that the mechanism of tumour rejection was probably cell-mediated, most likely through the lymphocyte population, as autologous plasma had little tumour inhibitory activity in this system (Southam et al. 1966).

To summarise tumour transplantation phenomena, it would appear that although the rejection of homograft tumours is to some extent dependent on the patient's immune status, it is almost certainly related to HLA associated antigens rather than a response to tumour associated antigens. Autochthonous tumour rejection on the other hand does seem to depend on a cell-mediated reaction specific for the tumour cells. In attempting to elucidate this and to find out more about the antigens involved, investigators turned to the effects of cutaneous testing to tumour extracts, as well as to other less direct techniques consisting largely of examination of the interactions of tumour cells with various effector limbs of the immune response.

2.2.2.2 Cutaneous testing to tumour extracts: One of the earliest methods used to detect cell-mediated antitumour immunity was the induction of delayed-type skin hypersensitivity using crude extracts of autologous tumour. Previously, a humoral immediate-type skin response had been noted to autologous crude extract of tumour (Curtis et al. 1961).

Hughes and Lytton (1964) showed that autologous cytoplasmic particles from various cancers, injected intradermally, produced a positive reaction of the immediate type. This was followed in 11 out of 50 patients by a delayed-type response. Two of these patients also reacted in this fashion to extracts of benign tissue from the same organ from which the cancer was extracted. Three of 11 patients with breast cancer displayed delayed-type hypersensitivity and none of these reacted to control breast tissue extract. There was no correlation with tumour histology or nodal status.
Stewart (1969b) demonstrated that the delayed hypersensitivity reaction was to the tumour cells and not to nuclear material or bacterial antigen, and later (Stewart 1969a) that skin reactivity was proportional to lymphocytic invasion of the tumour. The same author, with Orizaga (1971), reported delayed hypersensitivity reactions in 12 of 56 patients with breast cancer on testing up to four days following surgery. Positive reactions occurred more commonly in those with anaplastic tumours and node involvement and in those tumours showing a high degree of infiltration with lymphocytes and plasma cells. Peculiarly, this reactivity appeared to be inversely related to survival in that only 42% of patients who were skin test positive were alive at two and a half years compared with 78% of those who were non-reactive. They postulated that this was because these particular tumours were so aggressive that they overcame the lymphoid reaction or even that this reaction was stimulatory. Another finding was that 50% of these patients displayed cross-reactivity on testing with allogeneic tumour extracts,

indicating perhaps a viral aetiology for these breast cancers.

Others have used membrane preparations in an effort to develop a well characterised standardised skin test for breast cancer. Alford et al. (1973) showed that approximately half of breast cancer patients were reactive to membrane preparations of their own or allogeneic tumour membrane preparations but were non-reactive to extracts of normal breast tissue. Patients with benign breast disease or other types of tumour were non-reactive. Further purification of the membrane preparation by sonication and elution on Sephadex G-200 resulted in a fraction which caused reactivity in 9 of 10 patients with breast cancer; but 2 of 4 patients with other types of cancer also responded, as did 12 of 22 with normal or benign breast disease.

Another approach to the in vivo detection of CMI to breast cancer was the use of the 'skin window' technique where a section of autologous tumour or benign disease tissue mounted on a coverslip is placed over an abraided area of skin. The nature and amount of cellular infiltration is evaluated approximately 24 hours later (Black & Leis 1971; Black & Leis 1973). Cellular infiltrates indicative of hypersensitivity reactions were induced in 40% of cancer patients as compared with 10% of patients with benign breast disease. Positive infiltrates were induced more often in those with premalignant mastopathy and in-situ cacinoma than in those with invasive carcinoma. Positive infiltrates were also more commonly seen in patients who had regional nodes displaying positive sinus histiocytosis indicating that these cryostat sections might have some degree of prognostic correlation. Studies of cross-reactivity are precluded using this technique for reasons of safety, and conclusions regarding the specificity and hence immunological nature of such observations cannot be made.

In summary, cutaneous testing using tumour or tumour extracts has

never been wholly satisfactory in that both patients and controls react in a somewhat arbitrary fashion. Interpretation is often difficult because of the subjective element attached to it, there is occasional bacterial contamination of the tumour extracts, and also a lack of appropriate normal control tissue extracts (Currie 1980).

2.3. Tumour Associated Antigens

2.3.1. Historical background

Work in the late nineteenth and early years of this century, as a result of observations that transplanted tumours frequently showed spontaneous regression, demonstrated that animals rechallenged with the same tumour displayed increased resistance. These observations resulted in many attempts at immunotherapy but with little success. The reason for this failure was acidly pointed out by Woglom (1929) who demolished the work of many who had laid the foundations of the existence of tumour-specific immunological responses by indicating the lack of genetic homogeneity in the experimental animals. These experiments were not examining immunity to tumour antigens but to allografts.

The existence of tumour antigens could only be satisfactorily decided when inbred genetically homogeneous strains of experimental animals became available. Gross (1943) induced sarcomas in the C_3H strain of mice using methylcholanthrene, and showed that these tumours could be transferred from one mouse to another by intradermal inoculation. However, a proportion of these tumour transplants regressed and regressor mice subsequently showed increased resistance to further rechallenge with the tumour. Interestingly, spontaneous tumours developing in these mice were unaffected by this immunity and he was thus able to conclude that the resistance to tumour growth was immunologically specific, and by inference, that these tumours carried antigens specific for the tumour. A decade later it was shown (Foley 1953) that increased resistance could be demonstrated following surgical excision of the tumour. These experiments continued to be criticised because of the possible influence of residual heterozygosity. Prehn and Main (1957) demonstrated, however, that antigens produced by methylcholanthrene

were peculiar to the tumour produced and were not present in cells from normal tissues of the animal. There did appear to be evidence of some degree of cross-reactivity between some of the tumours although, in the main, the tumours tended to be immunologically distinct. Using the same model Klein et al. (1960) demonstrated that methylcholanthrene-induced tumours were each antigenetically different even in the same animal. The same findings were obtained in rats and guinea pigs. Because of such clear cut specificity and the experimental methods used to show this, these antigens were termed 'tumour specific transplantation antigens' (TSTAs). Since then, many have shown that tumours produced in the laboratory by viral, chemical and physical agents have TSTAs (reviewed by Old & Boyse 1966).

Such experiments are ethically unsuitable for humans, and other in vitro methods to detect tumour specific antigens (TSAs) have been used in both human and animal experiments. Such antigens should not be called TSTAs as it is not known if they are those responsible for tumour transplant rejection. Methods used to demonstrate these antigens have been assays of cell-mediated immunity, humoral immunity and monoclonal antibody.

Hellstrom and Sjogren (1965) showed that cells taken from tumour immune animals could inhibit or lyse tumour cells in vitro. This colony inhibition test proved the basis of many further cytotoxicity assays used in studies of human tumour immunity. The first development of it was a micro-cytotoxicity assay (Tagasugi & Klein 1970) for studying lymphocyte cytotoxicity. Numerous studies of human tumour patients were performed using these assays and the principal finding was that lymphocytes from these patients could kill target cells derived from their own tumours or other tumours of the same histologic type (reviewed by Currie 1976), a surprising outcome in the face of a large body of animal data which had shown chemically and physically induced tumours to carry transplantation antigens specific

to that tumour alone, but not shared by tumours of similar histology. Other assays of CMI to tumour antigens have been those using lymphoblastogenesis, leukocyte migration inhibition, leukocyte adherence inhibition and antibody-dependent cellular cytotoxicity, all which have been well reviewed (Currie 1980; Gupta & Morton 1983; Heppner & Hager 1980) and all of which appear to suggest the existence of tumour antigens. However, there are problems with all of these tests with regard to technique, interpretation, cross-reactivity and specificity, and none have, as yet, shown unequivocally the presence of antigen specific only for a specific tumour.

Antibodies are not difficult to detect in cancer serum but proving their specificity is extremely difficult. However, using autologous, allogeneic and xenoantibody, attempts have been made to find tumour associated antigens (TAAs) serologically. Gupta and Morton (1983) stated "Using hetero-antisera a number of tumour antigens other than CEA and AFP, have been detected in various human malignancies including melanoma, sarcoma, and carcinomas. However, many of these antigens have been shown to be expressed by fetal tissues as well.". To date there is no conclusive evidence that these antigens are immunogenic in the cancer host. Autologous and allogeneic assays in humans have been beset with problems of specificity and technique, cross-reactivity between different tissues occurring commonly and antibody to cytoplasm and nuclear factor further masking the situation. Some success has occurred with serological assays in Burkitt's lymphoma and sarcomas, i.e. tumours with possible viral aetiologies, and Currie (1980) has suggested that these antibodies may be directed against viral antigen. The serological literature on human tumour antigens was reviewed by Currie (1976) and can be summarised by stating that many laboratories have shown tumour antigen systems identified by many methods and that there are many discrepancies between laboratories. Most of these have been due to the fact

that no two polyclonal sera, whether from a tumour bearing patient or immunised experimental animal, are ever identical.

Monoclonal antibodies, developed by Kohler and Milstein (1975), are extremely specific but they too have been beset with the problem of specificity, and the more carefully a TSA is investigated, the less likely it appears to be specific to the tumour cell, certainly outside the rather artificial world of highly immunogenic animal models.

2.3.2. Aetiology of tumour associated antigens

"In general the type of antigen that appears on a tumour depends on the agent that induces the tumour." (Old & Boyse 1966). In animals and man we know that chemicals, physical agents and viruses can bring about tumour induction. There is usually no obvious aetiological factor in humans and such tumours are termed spontaneous.

TSTAs induced in laboratory animals are unique in that they are specific for a specific tumour and have no strong cross-reactivity, although the tumours also appear to have weaker associated antigens which do have some degree of cross-reactivity (Gupta & Morton 1983). There is evidence that these weaker antigens may be due to viral contamination of the laboratory animals but it seems more likely that they are embryonic or foetal antigens, demonstrated as a result of derepression of an inactive gene coding for their synthesis. Chemically induced TSTAs in laboratory animals tend to be strongly immunogenic but the strength of the immunogenicity varies with other factors. Tumours induced by low doses of chemical carcinogen take longer to develop and possess little or no immunogenicity whereas large doses of carcinogen rapidly induce tumours of high immunogenicity (Prehn 1975). This phenomenon appears to be related to the associated immunosuppressive activity of the carcinogen (Prehn 1963). The host immune status is also related to the strength of TSTAs, Lappe (1968)

demonstrating that immunosuppressed animals quickly developed tumours of high immunogenicity, whereas animals with stimulated immunity took longer to develop tumours which had weak TSTAs.

Physically induced TSTAs behave as their chemical counterparts, but tend to be rather weak in comparison (Gupta & Morton 1983).

Virally induced TSTAs were first demonstrated on tumour cells by Sjogren et al. (1961) who showed that polyoma virus-induced mouse tumours contained TSTA which would cross-react with other tumours induced by the same virus; very different to the type of specificity displayed by chemical and physically induced tumours. It was subsequently demonstrated (Smith 1968) that sarcomas, carcinomas, and embryonal tumours induced by a specific virus shared the same TSTA, this cross-reactivity being therefore independent of histological tumour type and indeed of animal species, but dependent on the oncogenic virus. There was no cross-reactivity between tumours induced by different strains of virus however. Viral tumours, like physical and chemically induced tumours, are known to express weaker foetal or embryonic antigens (Gupta & Morton 1983; Coggin et al. 1970). TSTAs specific to viruses differ in immunogenicity depending on the strain of virus (Old & Boyse 1966), but are of similar antigenic strength as those induced by chemical carcinogens (Gupta & Morton 1983), the spectrum which ranges from no detectable antigenicity to very strong antigenicity (Prehn 1975).

Spontaneous neoplasms appear sporadically and infrequently in certain animal strains, leukaemia in AKR mice and mammary cancer in C_3H mice being examples. They are different to artificially induced tumours, most of which are strongly immunogenic, in that they tend to be only weakly or not at all immunogenic (Hewitt et al. 1976). Most human tumours are at best possessed of weakly immunogenic TAAs, metastasise early and extensively, and

in this fashion behave differently to animal tumours in which this behaviour is exceptional. Hewitt et al. (1976) made a plea for more research using animals with spontaneous neoplasms as these were more akin to human malignancies. They believed that because the majority of animal models were chosen for their strong TSTAs and IR genes inducive of strong immune responses, research involving such models was artificial and consequently counter productive in the understanding of the relationship between cancer and immunity in humans. Currie (1980), on the other hand, argued that animal models with spontaneous tumours were also imperfect as they displayed entirely predictable behaviour, and did not exhibit the episodes of partial regression, dormancy and clinical unpredictability which is seen in humans. Tumours developed in laboratory animals and spontaneous animal and human tumours are not entirely independent of each other however; there being evidence that some spontaneous tumours have viral aetiology (Kinlen 1982; Weiss 1984), and others occur as a result of exposure to low levels of chemical carcinogens present in the environment (Prehn 1975). Prehn's suggestion is in accord with human tumours expressing weak antigenicity and with the concept that 70 - 90% of human cancers are induced by environmental factors (Higginson 1972).

2.3.3. Role of tumour associated antigens in humans

Artifically induced animal tumours invariably have strong TSTAs with powerful rejection reactions to them, unless the animals are immuno-suppressed or given too great a tumour burden to cope with, and it would appear that the development of these neoantigens is an essential ingredient of the malignant transformation process (Old & Boyse 1966). However, in animals and humans with spontaneously occurring tumours no conclusive evidence has been presented that TSAs exist despite serological (both

monoclonal and polyclonal) and cell-mediated responses to antigens associated with tumours having been demonstrated. Are these tumour associated immunological responses important in the sense of host rejection activity, or, are spontaneously occurring tumours antigenic but not immunogenic, possessing membrane determinants capable of specific interaction with one element or another of the immune response but not inducive of cell killing? In answer to this, the degree of cross-reactivity in tumours arising spontaneously, suggests that malignant transformation may be associated with exposure of either, cell surface antigen hitherto shielded on the membrane, or intracytoplasmic antigen not previously expressed on normal tissue, that is, malignant change comprises the expression of a normally existing and non-immunogenic macromolecule to the host immunological apparatus and not the synthesis of neoantigen. On the other hand cross-reactivity occurring in viral tumours can still be explained by common viral neoantigens.

Cochran (1982) has suggested that TAAs are possibly differentiation markers, organ specific markers, re-expressed embryonic or foetal antigens such as CEA and AFP, or antigens of classes not yet identified, and are not specific for tumours. For instance, antigen belonging to normal tissue may appear initially to be a TSA because it is expressed on tumour cells in quantitatively larger numbers than on the normal cells from which the tumour is derived (Woodbury et al. 1981). Similarly, where the normal body cell population is very small, its malignant counterpart will be present in relatively greater numbers and antigens common to both will initially appear tumour specific. An example of this is the CALLA antigen initially thought to be specific for the common (nonT) ALL (Ritz et al. 1980), but later found in normal bone marrow probably on an early stage B cell (Greaves et al. 1981). Antigenic expression may also be cell cycle dependent as is the receptor for transferrin, normally present on cells

in the bone marrow and thymocytes, but which has been found also on rapidly growing tumour cells in culture (Omary et al. 1980). These are examples of the problems confronting those searching for TSAs, and certainly to date, it appears that the more critically one evaluates an apparent TSA the less likely it is found to be specific for the tumour concerned.

Although their presence has never been unequivocally proven it is possible that TSAs exist in all of us perhaps for a transient period of time during which they are recognised as foreign and the tumours carrying them rejected by our immunological apparatus, this being in accord with the theory of immune surveillance. Such supposition would permit tumours to grow successfully only where these antigens are too weak to invoke such a response although Prehn (1976) has argued strongly against this possibility. Our present knowledge of TAAs is insufficient to permit critical evaluation of their importance with regard to the host immunological response to tumour. They have, however, been useful in the understanding of the host effector systems in immunity, and are presently in use as measures of disease activity and response to treatment, examples being CEA in large bowel tumours and AFP in primary hepatomas and teratomas of the testis and ovary.

2.4. Immune Effector Systems Against Tumours

The effector systems responsible for tumour rejection are ill-defined in laboratory animal tumours and even more so in the spontaneous cancers arising in man. Host attack on tumour cells can be implemented immunologically through either the humoral or cell-mediated arms, or a combination of both. If TSAs exist there should be specific immunological responses to the cells carrying them but this does not preclude the occurrence of other non-specific responses to these cells.

Lewis et al. (1969) found specific antibodies to malignant melanoma cells, and Thompson and Linna (1973) showed that humoral immunity, as well as cell-mediated immunity, protected chickens from viral oncogenesis. Currie (1980), reviewing the role of antibody in resistance to tumour stated "As a general rule tumour-specific antibodies develop in early disease and following tumour excision; and such antibodies can be made to lyse tumour cells under a variety of artificial conditions.". He also noted that antibody may act not only in a cytolytic fashion but also by inhibiting tumour growth especially when present in lower concentrations. He warned that although antibody appeared present in some cancers, *in vitro* assays of their activity contained many technical pitfalls, thereby calling into question the importance or even relevance of that presence. Antibody also appears, *in vitro*, to act by causing inhibition of tumour cell motility (Currie & Sime 1973) and may work in concert with unsensitised lymphocytes to lyse target tumour cells *in vitro*, this phenomenon being known as antibody dependent cell cytotoxicity (ADCC), the specific cells involved being killer (K) cells. Other cells capable of antibody dependent killing are macrophages and granulocytes which, like K cells, have Fc receptors on their surface membranes. The specific antibody

involved is usually IgG and the process is independent of phagocytosis and complement. The first person to demonstrate such co-operation between antibody and cellular mechanisms was Moller (1965) who noted that the antibody was only detectable during early tumour growth and also that it was capable of 'blocking' the effect of specific cell-mediated cytotoxicity, as prior treatment of target cells with antibody abrogated the effect. Moller's suggestion of 'blocking' by antibody received support from the Hellstroms (1969) who showed that serum from tumour bearing animals could abrogate in an immunologically specific manner the antitumour effect of specifically sensitised lymphocytes, and for a time it was held that antibody was bad in that it prevented cell-mediated tumour damage, even though there was never proof that it was antibody itself which did the 'blocking'.

In addition to specific antibody, non-specific auto-antibody has been noted in patients with cancer (Whitehouse & Holborow 1971), to cellular components, presumably as a response to tumour cell necrosis, but this is also seen in normal people and increases with age and may, therefore, be a mechanism for clearing up debris from dead or dying cells. Currie (1980) has, however, suggested that tumour cells may have 'natural' antibody directed against them, perhaps by expression of surface determinants not detectable on normal cells, through defective glycolisation, in a similar manner to the opsonisation of effete red cells by immunoglobulin in normal autologous serum.

Shuster (1984) and Campbell et al. (1986) have suggested the reason that specifically directed antibodies to tumours have not to date been isolated is because the humoral response to tumour may be very limited when compared to that of cellular immunity. Further, any specific humoral response may be masked by the much greater non-specific antibody responses in the serum directed against common tissue antigens released by normal

cell destruction.

Whether or not specific or non-specific humoral responses to cancers do occur and play a part in the process of tumour rejection remains inconclusive. Indeed it is possible that their role may be a negative one because of their ability to 'block' specific cell-mediated responses, and further, there is evidence to suggest that such antibodies could even be stimulatory to tumours under certain conditions (Murasko & Prehn 1983).

It is generally held that CMI is more important in tumour rejection than its humoral counterpart, and indeed tumour specific immunity can be adoptively transferred by lymphocytes much more readily than antibody (Southam et al. 1966; Morton et al. 1983).

From allograft work it was assumed, though never proven, that the effector cells in tumour rejection were thymus-derived lymphocytes (Cerottini & Brunner 1974) and there was some evidence from animal work to suggest an in vivo role for these lymphocytes in the prevention of tumour development. Allison and Law (1968) showed that thymectomy or giving antilymphocyte serum (ALS) enhanced the incidence of virally induced tumours in neonatal mice. Grant and Miller (1965) found a decrease in the latent period for development of chemically-induced tumours in neonatally thymectomised mice, although, following tumour development, there was no difference in tumour growth rate between thymectomised animals and controls. Thymectomy in adult mice had no effect on tumour development. Allison and Law (1968) stated "Animals treated with chemical carcinogens sometimes show increased incidence of tumours after neonatal thymectomy but usually not.", and Currie (1980) in summarising the literature suggested that tumours induced by carcinogenic hydrocarbons were little influenced by immunosuppression and their incidence in

thymectomised or nude mice was no greater than in normal control mice. Such data appears to indicate that chemically-induced tumours and, by inference, spontaneous tumours are independent of T lymphocyte function but that virally induced tumours are influenced by these lymphocytes. Hellstrom et al. (1968), however, demonstrated cellular (and humoral) immunity in humans which indicated specificity, as only autochthonous tumour cells were killed by the effectors. This supported the concept of necessary effector stimulation by prior immunisation, and hence a role for the T lymphocyte, a prerequisite for its generation being previous contact with the antigen. This finding was upset later in 1973 by Tagasugi et al. who showed that prior immunisation was not necessary, by demonstrating that cells from normal individuals appeared to exert the same, or even greater, cytoxicity than lymphocytes from cancer patients. Tagasugi's work has been amply confirmed by other authors since then.

The search for effector cells against tumours was aided by the use of alloantisera in mice which allowed better definition of lymphocytes and particularly T lymphocytes (reviewed by Simpson & Beverley 1977) showing that various subsets had different functions, e.g. help and suppression of antibody responses were mediated by different T cell types. The use of monoclonal antibodies specific for these subsets further enhanced investigation of their definition and function (Reinherz & Schlossman 1980; Ballieux & Heijnen 1983). Further, a number of researchers have defined and characterised the small subset of apparent lymphocytes responsible for non-specific, antibody-independent tumour cell cytotoxicity. These natural killer (NK) cells have been well reviewed (Santoli & Koprowski 1979; Kiessling & Wigzell 1979; Herberman et al. 1979; Herberman 1983a). In summary they are non-adherent, non-phagocytic, complement-independent and carry surface markers compatible with macro-

phage or lymphocyte lineage, though Herberman and colleagues feel they are early T cells. They may be involved in ADCC through their Fc - IgG receptors, as well as having the ability to achieve tumour cytotoxicity non-specifically through tumour antigen receptors, and as such may indeed be K cells. Their activity is augmented by viruses, BCG, Corynebacterium parvum and tumour cells susceptible to their action, probably through the production of Interferon to which NK cells are very responsive. Natural killer cell activity is depressed by immunosuppression, high dose radiotherapy, chemotherapy, hydrocortisone and large tumour burden. Natural killer cells, when stimulated by Interferon, are highly cytotoxic to virus-infected cells and to tumour cells but do not damage normal cells. It is thought that they are the cells responsible for the low incidence of spontaneous and chemically-induced tumours in nude and neonatally thymectomised mice as NK activity in these animals is very high. Interestingly, this resistance to tumour can be broken in nude mice by irradiating the animals (personal communication from Rygaard to Kiesling & Wigzell 1979) which is known to depress NK activity. These cells seem to meet the criteria necessary for tumour surveillance in that they "have high spontaneous activity or can rapidly develop high activity (through Interferon) in response to tumour cells or other stimuli", whereas immune T lymphocytes and activated macrophages take longer to respond (Herberman et al. 1979) by which time the tumour may have developed sufficiently to overcome further immunological attack. It is possible that NK cells may be more important in tumour rejection than T lymphocytes but because they are present in the peripheral blood of controls as well as in cancer patients, it is almost impossible to assay for the specific cytotoxicity of T cells with any certainty.

Macrophages are also apparently capable of reacting in non-specific

fashion to tumour cells. When activated they kill tumour cells *in vitro* by direct contact and transfer of lysosomal contents and can also act to inhibit tumour growth (Alexander & Evans 1971). These processes affect tumour cells only and macrophages do not harm normal viable host cells (Currie & Basham 1975) or cells with normal surfaces (Hibbs 1973). Macrophage activation can be non-specifically effected by BCG, Corynebacterium parvum, endotoxin and other microbial products (Parr et al. 1973; Currie 1980) but they can also be specifically armed to kill tumour cells by exposure to a supernatant factor released by immune lymphoid cells (Evans & Alexander 1970), these being now recognised as lymphokines and T lymphocytes respectively. Macrophages may further play a part in tumour rejection through their ability to process antigen and present it in a highly antigenic form to other lymphoid cells (Currie 1980). It has been noted that lower vertebrates and invertebrates appear to succeed in achieving good tissue surveillance to foreign material without the sophisticated apparatus associated with elaborate immunological responses. Cells involved in the performance of such allograft reactions have many features in common with human macrophages (Currie 1980).

Because of such attributes it was postulated that antitumour resistance in nude mice and T lymphocyte-depleted mice was the result of the possession of a highly activated macrophage system compensating for the lack of T lymphocytes. However, the discovery that macrophage activating substances such as BCG and Corynebacterium parvum also stimulate NK cell activity brought this theory into question (North et al. 1980). Evidence for the the role of macrophage resistance to tumour *in vivo* has been presented by Eccles and Alexander (1974) who showed that immunosuppressed rats had lower macrophage content in their tumours than controls and that non-specific immunological stimulation resulted in a slight increase in tumour macro-

phage content and a decrease in the number of tumour metastases. They postulated that the macrophage content of tumours was inversely proportional to the tumour's ability to metastasise. Keller (1980) demonstrated, by inducing fibrosarcomas in DA rats with Dimethylbenz(a)-anthracene, that resistance to tumour induction was least in animals with lower macrophage activity, that is, young and old rats. He further showed that macrophage-stimulating agents such as BCG and Corynebacterium parvum increased the resistance to tumour induction in young and old animals, and age-diminished resistance could be restored by adoptive transfer of Corynebacterium parvum-induced peritoneal cells. However, Eccles and Alexander (1974) suggested that tumour macrophages might be "ineffectual bystanders brought in by lymphokines released in an immune process effected entirely by lymphoid cells interacting with the tumour.". North et al. (1980) who demonstrated significant systemic macrophage activation in response to tumour growth, and interestingly, local suppression of both T lymphocyte and macrophage activation by the tumour itself, argued strongly that this systemically acquired macrophage activation was a consequence of the generation of concomitant T lymphocyte-mediated antitumour immunity. These authors further called into doubt the validity of much of the previous in vitro work demonstating macrophage-induced tumour inhibition because of many and various technical pitfalls not appreciated by the researchers. The role of the macrophage in defence against tumour therefore remains unclear at the present time.

Despite all, we remain unsure of the effector mechanisms against spontaneous cancers. The consensus of opinion appears to have swung towards the role of 'natural' cellular cytotoxicity as being of importance, and perhaps even rescuing the theory of immune surveillance, but whether these cells are NK cells, macrophages, cells of the granulocyte series or some,

as yet, undiscovered cell, is unknown. It would seem more likely, however, that immune resistance to tumour is a combination of humoral and cellular immunity; these two arms and the various cell types interreacting with each other in the complex fashion with which they resist foreign materials and infection. There is, however, evidence that some particular effector mechanisms are more important than others with regard to certain cancers, that is, the relationship between cancers of possible viral aetiology and T lymphocyte function.

The study on nude mice by Rygaard and Povlson (1976) and studies of immunosuppressed humans (reviewed by Kinlen 1982) showed no increase in the incidence of the common cancers but noted a large rise in the numbers of lymphoid and other possibly virus-related tumours. Together with the fact that immunosuppressed animals and humans are prone to viral infections, these findings have led to the view that T lymphocytes defend against viruses and not tumours; therefore, as some viruses are oncogenic, one would expect an increase in virally induced tumours in the immunosuppressed. Added support comes from the study of Simpson and Nehlsen (1971) who found no general increase in tumours in mice immunosuppressed with antilymphocyte serum (ALS); but did find a group who developed polyoma-virus-induced cancers. This virus was found to have been administered in a batch of ALS. There is compelling evidence that some cancers, and especially those which are commonly found with increased incidence in immunosuppressed individuals, are of viral aetiology. Epstein Barr (E.B.) virus has been implicated as the cause of lymphomas in the immunosuppressed (Crawford et al. 1980; Kinlen 1982), in Burkitt's lymphoma and nasopharyngeal carcinoma (Klein 1975). Human T cell leukaemia retrovirus, was first isolated from a patient with Sezary T cell leukaemia by Poiesz et al. (1981); hepatitis B virus is strongly suspect in the aetiology of hepatocellular

carcinoma (Lancet editorial 1981a) and herpes simplex type II and papilloma virus types 6 and 11 may be implicated in squamous cell carcinoma of the cervix (Weiss 1984). More topically, there is a link between cytomegalovirus (CMV) and Kaposi's sarcoma (Lancet editorial 1981b), a common tumour in Africa but now seen in patients with acquired immune deficiency syndrome (AIDS). Gottlieb et al. (1981) and Masur et al. (1981) demonstrated evidence of CMV infection in homosexuals and drug abusers who were anergic to a battery of delayed hypersensitivity recall antigens, had poor *in vitro* lymphocyte function and profound depletion of T helper lymphocytes with reversal of helper/suppressor ratio but with preservation of B cell function. The CMV is probably permitted to exert its oncogenic effect as a result of this T lymphocyte immunosuppression (Weiss 1984), the virus responsible for the latter being retrovirus human T lymphotrophic virus type III (HTLV-III) also know as lymphadenopathy associated virus (LAV), AIDS associated retrovirus (ARV) and more recently human immunodeficiency virus (HIV) (reviewed by Curran et al. 1985; Melbye 1986).

Such evidence together with previously discussed data (showing that virally-induced tumours can be affected by changes in T cell function whereas chemically-induced tumours tend not to be, indeed Greenberg and Greene (1976) showed the latter to be independent of adaptive immunity) suggests that T lymphocytes, by their immunosurveillance against viruses, may prevent the formation of virally-induced tumours. It may be that a period of immunosuppression is necessary for induction of such tumours, at least until the cancer grows to sufficient size to defy rejection.

It is indeed possible that virally-induced tumour surveillance is the only form of immune surveillance in man and that there is no adequate effector system working against the more common spontaneous tumours. The Mollers (1976) argue that because most tumours are monoclonal, genetic change,

with failure of repair of such mutation occurring in one cell, is the most likely explanation for in vivo carcinogenesis. They further argue that carcinogens only accelerate the appearance of these rare genetic changes leading to neoplasms and therefore host resistance to carcinogenesis is more likely to involve genetically determined repair mechanisms rather than hypothetical immunological surveillance mechanisms. Such criticism of the immune surveillance hypothesis in relation to spontaneous cancers has received wide support, some of the best evidence coming from Prehn (1970; 1976) and Rygaard and Povlsen (1976). Despite the fact that spontaneous tumours may therefore not require to modify themselves to escape hypothetical effector mechanisms, there is evidence - some theoretical and some real - that tumours can modify both themselves and the immunological reactions of the host. This is discussed in the following sections.

2.5. Immunological Escape of Tumours

If we accept that the host immunological apparatus is able to recognise and respond to TSAs, it must be asked why tumours, even strongly immunogenic ones such as those induced in animals, can grow and metastasise in the face of such an attack.

Insufficient tumour immunogenicity: If TSAs are absent or very weak, as is probable in the case of spontaneous tumours, cancers would not be recognised as foreign and there would be no immunologically directed response raised against them. The tumours would not have to escape as there is nothing to escape from. However, cancer patients are able to display reactivity to autologous tumour cells and show evidence of cellular and humoral cytotoxicity to tumour cells thereby demonstrating that there is some degree at least of tumour immunogenicity in spontaneous cancers.

Immunological tolerance: Some animals associated with vertically transmitted viruses such as the Gross leukaemia virus in AKR mice, appear to raise no immunological response to the virus or to the induced tumour cells whereas they can respond immunologically to other viruses and tumours. It may be that this is due to exposure to the virus during embryonic or foetal life when surveillance systems do not afford protection. Similarly, mice infected with mammary tumour virus (MTV) as neonates become tolerant and cannot be immunised against MTV-induced tumours (Morton 1969). However, this phenomenon, which is akin to insufficient tumour immunogenicity, has not yet been demonstrated in man.

'Sneaking through' was the term devised by Klein (1966) to explain the phenomenon first noticed by Old et al. (1962) who showed that small inocula of tumour cells would grow whereas intermediate doses were rejected by mice

suggesting that low doses were too small to provoke an immune response. This hypothesis only provides a mechanism of escape during the earliest phase of tumour growth and offers no explanation for the successful growth of the tumour at a later time.

Immunoselection: Resistant tumours might occur in the presence of a host response because immunogenic tumours are gradually deleted. Thus, surveillance mechanisms would eliminate all but a small minority that would constitute the spontaneous tumour population. Surveillance could thus be postulated to be the reason for the low immunogenicity of spontaneous tumours. This hypothesis was reviewed by Prehn (1976) who noted that spontaneous tumours arising in tissue cultures or in diffusion chambers, and hence not in contact with surveillance mechanisms, were also of low immunogenicity. He concluded "that the immunogenicity of a tumour is the result of the direct action of the oncogen on the tumour cells. When little or no action is present, as in spontaneous tumours, there is little or no immunogenicity upon which surveillance can act.". It appears unlikely, therefore, that immunoselection of tumours is a method of tumour escape in man.

Antigenic modulation: Tumours appear to have an ability to change their presenting 'face' to the immunological environment. This, antigenic modulation was first described by Boyse and Old (1969) and originally detected in the case of thymus leukaemia (TL) antigen in mice. It was noted that when murine lymphoma cells were transplanted into tissue culture containing specific antibody or into an immunised host they lost their TL antigen. The antigen reappeared on transplantation back into tissue cultures containing no antibody or into the unimmunised host. The mechanism whereby this occurs is unclear and the phenomenon has not yet been demonstrated

in human tumours.

Immunostimulation: This hypothesis was first presented by Prehn and Lappe (1971) who suggested that low levels of immune reactivity had the potential to stimulate growth of both normal and tumour tissues. They reasoned that tumours in nude mice grew poorly, tending to regress or to remain localised because there is a lack of immune stimulus to growth. Prehn (1976), by manipulating the immunity of normal adult mice was able to show that tumours developed more quickly in partially immunosuppressed mice than in either fully immunosuppressed mice or mice with fully restored immunity. He went on to suggest that spontaneous tumours, being weakly immunogenic, were prone to stimulation rather than rejection by the host immune system. The actual mechanism of the stimulatory activity is still unknown (Murasko & Prehn 1983) but suppressor cell activity, 'blocking' factors or direct tumour cell stimulation by T cells, macrophages and antibody, have all been implicated.

Antigen shedding: The glycocalyx of all nucleated mammalian cells is constantly being shed and resynthesised and the normal transplantation antigens - HLA in man and H2 in mice, are found in the serum in soluble form. Davey et al. (1976) demonstrated that mouse lymphoma cells shed their H2 antigens with increased frequency, suggesting that tumour cells shed their surface components more extensively and rapidly than normal cells. Gold and Freedman (1965) described CEA in patients with colonic carcinoma, showing that this antigen which is a component of the tumour cell glycocalyx, is shed in large quantities and can be demonstrated in the serum of the patients. The cancers of experimental animals with readily metastasising *in vivo* tumours, rapidly shed TSTA also *in vitro*, whereas non-metastasising or poorly metastasising tumours do not (Currie &

Alexander 1974). "A lethal interaction between a cytotoxic effector cell or an immunoglobulin molecule plus complement and a tumour cell necessitates some local stability of the target antigen on the cell surface." (Currie 1980), and it is tempting to correlate the evasive and metastasising behaviour of tumours of low immunogenicity with their capacity to shed surface antigen so readily. Furthermore, such shedding will create an antigen 'smoke screen' in the vicinity of the tumour, attractive to host immune effectors and distracting them from the appropriate target. The resulting complexes between antigen and effectors are thought to be responsible for the phenomenon of 'blocking' which will be discussed in greater detail in the next chapter.

Host immunodeficiency: Immunosuppressive manipulations using steroids, ALS, radiotherapy and chemotherapy, can modify graft rejection. Tumours are more easily induced in animal models when these manipulations or thymectomy are performed in neonates. Patients with cancer and concomitant general immune hyporeactivity have more rapidly growing tumours and following surgical procedures have a poorer prognosis. However, with the exception of the immunologically compromised, those with disease of the lymphoid or reticuloendothelial systems, and patients with bronchogenic or head and neck cancer (Hersh et al. 1976), depressed immunity tends to be a feature of advanced cancer only. When other features such as age are taken into consideration, patients with early cancer have almost normal immunity. It, therefore, appears that tumour dissemination appears to be the cause rather than the result of immune depression (Stein et al. 1976) and this will be discussed more fully in the next chapter.

2.6. Summary

There is evidence of a relationship between host immunity and cancer in humans but much of this can be explained on non-immunological grounds, for instance, on the basis of physiological or hormonal events.

There is growing evidence that some human tumours are virally induced but it is probable that the majority of spontaneous tumours are a result of chronic exposure to physical and chemical agents.

Tumours carrying TSAs can be induced in laboratory animals and the strength of these antigens is dependent on the dose and method of application of the inducing agent. Definite host immunological reactions resulting in tumour rejection can be demonstrated in these animals but these reactions can also be overcome permitting tumours to thrive.

The occurrence of TSAs in spontaneous tumours have never been unequivocally shown in animals or man. These tumours have no, or at best, weak, immunogenicity and it seems improbable that specifically directed immune surveillance acts against them. Further, spontaneous tumours nearly always predate the appearance of measurable depression of immunity, making it unlikely that they arise because of defective immunity. On the other hand, tumours of probable viral aetiology occur reasonably often in hosts with depressed T lymphocyte function and it seems likely that such tumours are rare in normal individuals because viruses and their induced TSAs are under the immune surveillance of the T lymphocyte system.

Chapter 3

Chapter 3

3. The Effect of Cancer on Immune Function

3.1. Introduction

There is general acceptance that host immunity is impaired in tumour bearers and that CMI is more affected than humoral immunity. In this chapter the evidence for this will be reviewed with regard to the more common spontaneous neoplasms of man, evidence which also indicates that the impaired immunity in such cancers is a secondary phenomenon rather than the primary event by which immunodeficiency permits the development of virally-linked cancers for instance. Evidence will also be presented which suggests that this effect on the host immune system is a result of the action of serum blocking or inhibitory factors which are products of the tumour and host immune responses.

3.2. Tests of Immune Function in Cancer

3.2.1. Humoral immunity

Humoral immunity may be assessed by measuring circulating antibodies, complement and its components and possibly 'blocking' or 'inhibitory' serum factors. These 'blocking' factors will be discussed later.

As previously noted, apparent tumour-specific antibodies have been demonstrated in patients with Burkitt's lymphoma, some sarcomas, malignant melanoma and other tumours (Ioachim 1980). It is not surprising that this may be the case where tumours linked with a viral aetiology are concerned, Thompson and Linna (1973) having demonstrated the protective role of antibody against oncogenic viruses in chickens. However, with regard to other tumours, the specificity of such antibodies is not clear (Heppner & Hager 1980). Tests of humoral immunity in the context of measuring serum antibody levels in cancer patients have, therefore, for the most part, comprised the measurement of immunoglobulin levels in the serum, most commonly IgG, IgA and IgM.

Teasdale et al. (1979a) on examining these immunoglobulins in various cancers found no significant differences between cancer patients and controls, and similar conclusions have been published by others concerning patients with breast cancer (Bolton et al. 1976; Wanebo et al. 1978; Toivanen et al. 1984; Shukla et al. 1986a). Hughes (1971) however, found changes in some immunoglobulin levels in patients with epithelial cancers but concluded that these were explained by bacterial invasion through the malignant epithelium. Roberts et al. (1975), on the other hand, reported significantly raised levels of IgA and depressed levels of IgG in all stages of breast cancer but their numbers were small and their control group much younger than the cancer

patients; Bolton et al. (1976) having demonstrated that IgA levels increase with age, and when this factor is taken into consideration, that previously significantly elevated levels in patients with metastatic disease, become insignificant. Toivanen et al. (1984) found no changes in immunoglobulin levels relating to treatment or outcome of breast cancer but Webster et al. (1979) and more recently others (Shukla et al. 1986b), reported a drop in IgM levels following treatment in all breast cancers irrespective of treatment modality and outcome. Shukla et al. (1986b) further noted a rise in IgG levels in these patients just prior to, or at development of, breast cancer recurrence.

In short, immunoglobulin levels do not appear to be altered dramatically by cancer itself but when levels do change this may be a reflection of age, infection or malnutrition, making interpretation of results difficult, if not impossible (Cochran 1982).

A humoral response to tumour should result in the consumption of complement components and although abnormalities have been detected in cancer patients they are by no means uniform, perhaps because of the activation of the alternate pathway by endotoxin from intercurrent infection or the effects of tumour necrosis (Cochran 1982).

In conclusion, there is no substantial evidence at present to indicate that patients elicit specific antibody responses to malignant cells of the more common solid tumours occurring spontaneously in man. The measurement of non-specific immunoglobulins or complement does not appear to be of any significant benefit in assessing these tumours.

3.2.2. Cell-mediated immunity

Much of the investigative work on the relationship between the immune system and cancer has been based on *in vitro* studies using

tumour cells or cells from tumour cell lines and peripheral blood lymphocytes, the supposition being that tumour cell destruction was mediated by cytotoxic T cells. Serious doubt presently exists about this interpretation as it is now recognised that cytotoxicity tests detect a range of cell-mediated responses including those affected by NK cells. Nevertheless, both lymphocyte numbers and lymphocyte function appear to be affected in patients with neoplasms; the most commonly used tests being those relating to T lymphocytes; skin testing to recall or primary antigens, in vitro blastogenesis of lymphocytes and enumeration of their numbers.

3.2.2.1 Delayed hypersensitivity testing

The logic behind delayed hypersensitivity testing to dermal antigens was that if a patient were able to respond to an antigen to which he had previously been sensitised, he should be able to mount an effective response to TSA and hence against tumour. This indirect assay of host response to tumour has followed two major approaches. The earlier approach utilised common antigens to which there was a high probability of the patient having had previous exposure, examples being purified protein derivitive (PPD), candida, varidase (streptokinase-streptodornase), mumps, diptheria toxoid, etc. There are, however, difficulties in interpreting responses to recall antigens because of variables such as age and ill health, and that there may be doubt as to whether or not the patient has had previous exposure to the antigens being tested, how long previously and how intense the exposure. A second approach was, therefore, developed which utilised the generation of a primary immune response against an antigen to which the patient had had no previous exposure, such as 3,4-dinitro chlorobenzene (DNCB), an antigen which can sensitise a patient upon contact and will cause

approximately 90% of controls to display a positive skin hypersensitivity reaction on rechallenge two weeks later (Bolton 1975b), i.e. a delayed hypersensitivity reaction (DHR).

Recall antigens: Testing with recall antigens has been demonstrated to be of little use in assessing cancer status and prognosis by some (Eilber & Morton 1970; Wanebo et al. 1978; Webster et al. 1979; Shukla et al. 1986a). Others have found depressed DHR to recall antigens only in women with terminal breast cancer (Nemoto et al. 1974) commenting that this was most significant when a battery of antigens was used rather than just one. Kopersztych et al. (1976) in a comparison of various tumours with controls also found depressed DHR in patients only with metastases using tuberculin; however, reactivity to candida albicans was depressed in all stages of cancer, and varidase although depressed in both, was able to discriminate between local and disseminated tumours. Depressed DHR has been reported throughout all stages of breast, gastric and colonic cancer (Bolton et al. 1975) although it was noted that reactivity was most depressed in colonic tumours and least affected in breast cancer apart from Stage IV disease where it was also markedly depressed. Reports of depressed DHR to recall antigens in early cancer have been published by Hughes and MacKay (1965), Roberts and Jones-Williams (1974), Stein et al. (1976), Mandeville et al. (1982), and further, that impairment increased with advancing disease in a stage related manner. Hortobagyi et al. (1981) showed that recall antigen reactivity was weakly predictive of response to chemotherapy and survival time in breast cancer patients but interestingly this did not correlate with other prognostic factors such as age, menopausal status, tumour load, previous irradiation therapy, absolute lymphocyte count or performance status. The relationship to prognosis has also been noted by Mandeville et al.

(1982) who reported that recall antigens were more favourable in this instance than DNCB reactivity. Sequential testing with recall antigens has been said to be unhelpful in assessing treatment efficacy (Webster et al. 1979; Hortobagyi et al. 1981; Shukla et al 1986b) although Shukla et al. (1986b) using PPD, and Roberts and Jones-Williams (1974) using varidase, noted that some patients became less reactive as disease spread, but not before this was clinically obvious. Roberts and Jones-Williams (1974) further described a sharp drop in DHR in patients just prior to death. On the opposite side of the coin, depressed PPD responses have occasionally been reversed following curative surgery (Hughes & MacKay 1965).

Primary antigens: Wanebo et al (1978) reported that patients with node negative operable breast cancer had no impairment of DNCB response and that those with four or more involved nodes had only slight impairment. Bolton (1975) also noted only slight impairment in early breast cancer, and others have shown on testing patients with many different solid tumours that those with localised disease have normal DHR (Simo-Camps et al. 1976; Kopersztych et al. 1976). However, Stein et al. (1976) found impaired DHR in a significant number of women with early breast cancer and Teasdale et al. (1976b) observed that depressed reactivity to DNCB was the only consistent marker of immunodepression in early breast, gastric and colonic cancer, especially when corrections were made for age and ill health. Impairment of DHR with increasing stage of disease has been demonstrated in patients with breast cancer (Bolton et al. 1975; Bolton 1976) with the exception of Stage III disease in which DHR is virtually normal. These authors, however, found that all stages of stomach cancer and even more profoundly, colonic cancer, had markedly depressed

reactivity to DNCB, this being most severe in cases with disseminated disease. Similar findings with regard to Stage III breast cancer were published by Teasdale et al. (1979b) but Stein et al. (1976) found straight forward increasing depression of DHR with increasing stage of the disease, and most authors have agreed that this depression is greatest in patients with metastases (Stein et al. 1976; Teasdale et al. 1979b; Shukla et al. 1986a) and in those with shortest survival times (Shukla et al. 1986b), reflecting perhaps a relationship between DHR and tumour load. Other authors, although finding normal DHR in a variety of localised cancers, also concurred that impairment appears with disseminated disease (Simo-Camps et al. 1976; Kopersztych et al. 1976). Mandeville et al. (1982) however, found no such stage relationship even in patients with metastatic cancer. It has been reported that pre-treatment DNCB reactivity has no prognostic significance (Wanebo et al. 1978; Teasdale et al. 1979a; Mandeville et al. 1982; Shukla et al. 1986a) in early breast cancer. Similarly, Stein et al. (1976) noted no difference in DHR response between patients with early breast cancer who remained disease free and those developing metastases within two years, and Davies et al. (1978) found no correlation of DHR with either early treatment failure or axillary node metastases. Other authors, however, have found DNCB responses to be predictive of outcome in advanced breast cancer treated with chemotherapy or hormonal manipulation (Webster et al. 1979); anergy usually being associated with poor prognosis. Eilber and Morton (1970) also noted on pre-operative testing of 83 patients with a wide variety of tumours, a highly significant correlation between DNCB reactivity and disease outcome, although later follow-up of these patients demonstrated that much of this significance was lost (Bolton 1975). Hortobagyi et al. (1981), using the primary antigen KLH, reported that

reactivity to this was predictive not only of survival but also of response to subsequent chemotherapy. These authors noted that serial measurements of DHR, using both primary and recall antigens, were no more advantageous than pre-operative testing alone. Webster et al. (1979) however, have shown that DNCB responses can parallel clinical course, anergic patients becoming reactive with successful therapy and becoming anergic again on recurrence of their advanced breast cancer.

Not all cancers affect DHR to the same degree, impairment being greatest in lymphomas such as Hodgkin's disease and in squamous cell carcinomas, and varying in different adenocarcinomas (Bolton 1975). For instance, DHR is impaired, stage for stage, less in breast carcinoma than in gastrointestinal cancer, and colonic carcinoma patients have more depressed DHR than those with gastric cancer. This seems somewhat anomalous when it is considered that those with colonic tumours have a better prognosis than those with gastric cancer.

Other influencing factors on DHR which complicate the issue may be external ones such as the depressant effect of radiotherapy (Shukla et al. 1986b) or those which are an integral part of the patient's physiology. Although there is no sex difference in DHR responses to primary or recall antigens (Whitehead et al. 1979b), increasing age is associated with diminishing DHR to primary antigens (Gross 1965; Bolton et al. 1976; Davies et al. 1978; Teasdale et al. 1979b) and recall antigens (Roberts-Thomson et al. 1974; Roberts & Jones-Williams 1974; Teasdale et al. 1979b). There is, however, considerable evidence to suggest that this age related depression is a reflection of the associated general debility of age, rather than age per se. For instance, Roberts-Thomson et al. (1974) showed that elderly people who were hyporeactive compared with others in the same age group had significantly

higher mortality over the ensuing two years, indicating perhaps poorer health in these particular people. Teasdale et al. (1979b) also noted that healthy controls had no age related impairment of DHR but that those with benign disease did; patients with gastrointestinal disease who tended to be more ill, being worse affected that those with benign breast disease. Further, in the communication published by Gross (1965) noting age related hyporeactivity, young healthy adults were compared with older patients who had 'chronic active disease ' and it is likely that illness and not age influenced the DNCB responses in that study (Bolton 1975). That debility itself can cause impaired DHR is beyond doubt, an example being the depressed tuberculin responses seen in severely protein deficient children (Sinha & Bang 1976).

In summary, tests of DHR have been found to be of varying usefullness in the assessment of disease status and prognosis in patients with solid tumours. The consensus opinion appears to be that impairment varies with the type of tumour; that DHR is often depressed in early cancer and this depression, to some extent, parallels advancing stages of disease. Nutritional status and general debility affect DHR in a substantial manner and accordingly the degree of impairment seen in many cancer patients may be due to a combination of the effects of tumour and these factors. Consequently it may be difficult to discriminate between early and advanced neoplasia and even between benign and malignant conditions and because of this lack of specificity the place of delayed hypersensitivity skin testing in assessing cancer patients has perhaps become less apparent.

3.2.2.2. In vitro lymphocyte blastogenesis

Contact between a lymphocyte coded for a particular antigen and that antigen results in transformation of the lymphocyte into a blast cell

which undergoes mitosis, forming a clone of lymphokine producing lymphocytes specifically directed against the antigen. By stimulating lymphocytes in this way, their functional ability can be assessed either by measuring lymphokine production or the number of blast cells formed. Stimulation can be affected specifically by using common recall antigens, tumour cells or foreign lymphocytes, or non-specifically by extracts from various bean species known as mitogens, the three most commonly used being phytohaemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM), the first two which stimulate T lymphocytes and the last, mainly B lymphocytes.

Breast cancer patients have been reported to have normal PHA responses (Roberts & Jones-Williams 1974; Bolton et al. 1976) and normal PHA and Con A responses in all but elderly patients with disseminated disease (Ludwig et al. 1985). It has been demonstrated that gastric, colorectal and breast cancer patients have normal PHA and Con A responses but that patients with Stage III breast cancer have impaired blastogenic responses to PHA (Teasdale et al. 1979b). Other authors have demonstrated depressed PHA responses in many solid tumours but only in those which have metastasized (Simo-Camps et al. 1976; Kopersztych et al 1976) whereas Nemoto et al. (1974) reported hyporeactivity solely in patients who were terminally ill. Yet others have demonstrated impaired responses to PHA in various solid cancers (Gatti et al. 1971; Whittaker et al. 1971a; Suciu-Foca et al. 1973; Semenzato et al. 1980) including early disease (Whitehead et al. 1975; Stein et al 1976; Mandeville et al. 1982), and reports of abnormal PHA responses in early disease becoming more obviously depressed in disseminated disease have also been published (Whittaker et al. 1971b; Stein et al. 1976). Such stage related depression has also been noted by Bolton et al. (1976) using suboptimal doses of PHA but this discriminatory ability was lost

when correction was made for the age of the patients. Mandeville et al. (1982) have reported stage related lymphocyte response depression to Con A, and recall antigens as well as to PHA, and Suciu-Foca et al. (1973) depressed stage related, mixed lymphocyte culture (MLC) responses. Wanebo et al. (1978) examining risk groups in early breast cancer noted significant depression of PHA responses only in the high risk group, but surprisingly, increased responses to PWM, Con A and recall antigens also in this group, suggesting that lymph node metastases might be associated with stimulation of certain lymphocyte responses. Using suboptimal doses of PHA, Whitehead et al. (1975) reported that pre-treatment responses correlated well with prognostic groups in breast cancer, and similar findings using both PHA and recall antigens have been published by Mandeville et al. (1982), though neither group used corrections for patient age differences. Teasdale et al (1979a) on the other hand found no correlation between prognosis and blastogenic responses. PHA responses have not been found predictive of response to chemotherapy or hormonotherapy in advanced breast cancer (Webster et al. 1979) and although these authors found no associated changes in PHA responsiveness with clinical outcome, Braun et al. (1983) did so having demonstrated increased PHA responsiveness following successful surgery for lung tumours, this diminishing prior to disease recurrence, remaining low or dropping even further as disease progressed, and appearing therefore to correlate with tumour burden.

Lymphocyte stimulation is also affected by factors other than cancer and although there are no sex related differences (Teasdale et al. 1979b) responses are profoundly affected by ageing (Roberts-Thomson et al. 1974; Weksler & Hutteroth 1974; Bolton et al. 1976; Teasdale et al. 1979b; Mascart-Lemone et al. 1982). General ill health also depresses blastogenic responses (Whitehead et al. 1975; Teasdale et al. 1979b) as do many

specific disease states such as tuberculosis, multiple sclerosis, hepatitis, chronic mucocutaneous candidiasis, ataxia telangiectasia and syphilis (Gatti et al. 1971). Various forms of therapy such as surgery, irradiation and cytotoxic drugs have been reported to have no effect on lymphocyte stimulation (Whitehead et al. 1975) but Espanol et al. (1974) have shown transiently reduced PHA responses following anaesthesia, and longer lasting depression following radiotherapy has been demonstrated by others (Stjernsward et al. 1972; Toivanen et al. 1984).

In essence, lymphocyte blastogenesis appears to be depressed in patients with disseminated cancer and occasionally, to a lesser degree, in those with early disease, where it may have some prognostic implications. Further there is some evidence that responses may change in line with clinical responses to treatment and occasionally precede objective evidence of such changes in disease status, implying a relationship between lymphocyte reactivity and tumour burden. However, the influencing effects of age, ill health, and therapeutic manipulations on in vitro lymphocyte stimulation, the complexity of the techniques, and the different methodologies used between laboratories, make it necessary to interpret results with extreme caution.

3.2.2.3. Lymphocyte counts

The white blood cell count (WBC), as a whole, does not seem much influenced by cancer (Teasdale et al. 1979a; Bolton et al. 1976) and has no stage related or prognostic significance (Shukla et al. 1986a) although it is depressed following radiotherapy (Toivanen et al. 1984; Shukla et al. 1986b) and chemotherapy (Webster et al. 1979). With regard to the cell populations of the WBC, a negative correlation between the peripheral neutrophil count and the curability of various cancers has

been noted by Riesco (1970) but no correlation with monocyte, eosinophil or basophil counts was seen. The monocyte count has been reported to increase in patients with metastatic breast cancer (Ludwig et al. 1985) and non-significantly in recurrent lung cancer (Braun et al. 1983). Most authors however, have demonstrated changes only in the lymphocyte population in relation to cancer.

1. Total lymphocyte count: The total lymphocyte count is an inexpensive, readily available test which is also a rough reflection of T lymphocyte numbers. There have been reports of normal lymphocyte counts in cancer patients (Nemoto et al. 1974; Teasdale et al. 1979b; McCluskey et al. 1983; Ludwig et al. 1985), of depressed counts only in patients with disseminated tumours (Stein et al. 1976; Bolton et al. 1976; Kopersztych et al. 1976; Ownby et al. 1983; Shukla et al. 1986a), and of stage related depression in colorectal cancer (Slater et al. 1979) although Dukes' C tumours in these patients had higher lymphocyte counts than Dukes' B cancers. This increase in lymphocyte counts in bulky local colorectal disease is similar to the elevated counts noted in breast cancer patients with three or more axillary nodes involved (Rotstein et al. 1985) and reminiscent of the normal DHR (Bolton et al. 1975; Teasdale et al. 1979b) and T lymphocyte counts (Whitehead et al. 1976) seen in Stage III breast cancer. Total lymphocyte counts do not appear affected in early breast cancer although Wanebo et al. (1978) have reported a small but insignificant depression with increasing risk factors and Bainbridge et al. (1978) have shown significantly low counts in Stage II disease but similar counts to controls in all other stages. In gastrointestinal cancers however, counts are depressed both in operable and inoperable disease (Shukla et al. 1979), counts remaining constant following surgery

in operable patients but falling steadily as disease progresses in inoperable cancer. Some authors have reported a positive correlation between total lymphocyte counts and improved prognosis in various cancers (Riesco 1970) and breast cancer (Papatestas et al. 1976; Ownby et al. 1983) but Shukla et al. (1986a) surprisingly have found that elevated total lymphocyte counts in early breast cancer were more likely to indicate future recurrence. The total lymphocyte count has been reported to be both predictive of response of disseminated breast cancer to endocrine manipulation (Franks & Williams 1978), and to be unhelpful in this regard (Webster et al. 1979). Serial total lymphocyte counts have been shown (Shukla et al. 1986b) to rise slowly post-operatively in patients whose breast cancers do not recur but to drop as patients develop systemic recurrence. Total lymphocyte counts also increase on serial testing in patients with advanced breast cancer who respond to endocrine treatment (Franks & Williams 1978) but decrease following successful chemotherapy (Webster et al. 1979). It has also been noted that recurrence of lung cancer is often heralded by a decrease in total lymphocyte count, and death, preceded by a sharp fall (Anthony et al. 1975b).

Factors other than cancer also affect the total lymphocyte count, for instance, with increasing age the count falls (Bolton et al. 1976; Alexopoulos & Babitis 1976; Teasdale et al. 1979b) although there are reports that no age related changes in total lymphocyte counts exist (Weksler & Hutteroth 1974; Smith et al. 1974; Nagel et al. 1981; Mascart-Lemone et al. 1982). The total lymphocyte count is unaffected by the sex of the patient (Smith et al. 1974; Alexopoulos & Babitis 1976) but general ill health causes elevated counts (Teasdale et al. 1979b) and physical exercise transiently increase the number (Steel et al. 1974a) probably through an increase in the B cell population. Chemotherapy

(Petrini et al. 1984; Sheard et al. 1986) and, more chronically, radiotherapy (Stjernsward et al. 1972; Shukla et al. 1980; Shukla et al. 1986b; Petrini et al. 1983; Toivanen et al. 1984) both depress the total lymphocyte count, but the depressive effect of surgery is transient (Shukla et al. 1980; Rotstein et al. 1985) recovering fully within a week (Lynch & Kirov 1986). The anti-oestrogen, tamoxifen, on the other hand, elevates the total lymphocyte count and indeed counteracts the depressive effect of chemotherapy (Sheard et al. 1986).

In summary, the total lymphocyte count is not absolutely stage related but is usually depressed in disseminated cancer. It may be decreased or increased in locally advanced cancer and although the prognosis of a tumour is sometimes improved if the count remains elevated this is certainly not always the case. Such variability may be due to other factors e.g. age and ill health, and difficulties with serial testing are exacerbated by the effects of chemotherapy, hormonotherapy and radiotherapy.

2. T Lymphocytes: The thymus-dependent proportion of the lymphocyte population (T lymphocytes) accounts for between 52 and 81% of peripheral blood lymphocytes in the control population (Jondal et al. 1972). More recently this group of cells has been recognised using monoclonal surface markers which are particularly useful in enumerating the subsets of T lymphocytes (Reinherz & Schlossman 1980) but initially they were defined by their ability to bind sheep red blood cells (SRBC) in a specific but non-immunological manner (Bianco et al. 1971; Jondal et al. 1972) and most research pertaining to the relationship between these cells and cancer has been performed using this particular method (E-rosetting).

Some laboratories have attempted to achieve finer degrees of discrimination using the E-rosetting technique by modifying the methods

such that only lymphocytes with higher affinity for SRBC are counted ('active' T lymphocytes). Using this technique Braun et al. (1983) found no difference in 'active' T counts between controls and lung cancer patients and between stages of the disease. However, Wybran and Fudenberg (1973) reported depressed 'active' T counts even in early tumours and this depression became more profound the more advanced the stage of disease. This correlation with the tumour load in various cancers was made more apparent when on sequential testing 'active' T counts increased in those patients responding to treatment. Furthermore, Semenzato et al. (1980) demonstrating 'active' T count depression in glioblastomas and oesophageal and lung cancers, showed that this depression correlated well with depressed PHA responses in these patients suggesting that these particular T cells were important functionally against cancer. Djeu et al. (1977), using a different method of 'active' E-rosetting, were also able to show in several types of cancer a stage related fall in 'active' T counts, workers from the same laboratory having previously reported that clinical relapse of disease was often preceded by a fall in 'active' T counts by as much as three months (Oldham et al. 1976).

There is no uniform agreement on the relationship between total T lymphocyte counts and cancer, some authors having reported no change in percent or absolute counts in untreated tumours (Stjernsward et al. 1972; Nemoto et al. 1974; Wanebo et al. 1978; Mandeville et al. 1982; Brown et al. 1983; McClusky et al. 1983; Ludwig et al. 1985). However, depressed T lymphocyte counts have been reported in early cancers of lung (Gross et al. 1975a; Dellon et al. 1975), stomach, colon and rectum (Shukla et al. 1979), breast (Stein et al. 1976; Keller et al 1976), oesophagus and gliomas (Semenzato et al. 1980) as well as in early head and neck tumours (reviewed by Katz 1984) but other authors have only noted this depression

in advanced disease (Anthony et al. 1975a; Bolton et al. 1976; Kopersztych et al. 1976). There have also been claims that T lymphocyte counts correlate with extent of disease in lung cancer (Gross et al. 1975a; Dellon et al. 1975) but not in lung adenocarcinomas (Dellon et al 1975), and in breast cancer (Whitehead et al. 1976) apart from advanced locoregional disease where T counts like DHR are virtually normal. Conflicting reports exist on the prognostic value of T lymphocyte counts. Anthony et al. (1975a) showed that squamous cell lung cancer patients with depressed counts had a poorer prognosis, whereas Rotstein et al. (1985) surprisingly found that patients with breast cancer had a worse prognosis if T counts were elevated. Others have suggested that the pre-treatment T lymphocyte count has no prognostic significance (Stein et al. 1976; Teasdale et al. 1979a). Serial T lymphocyte counts, however, do appear to change in relation to clinical outcome, increasing or remaining stable with successful treatment (Gross et al. 1975a; Dellon et al. 1975) and falling as disease progresses (Anthony et al. 1975b; Dellon et al. 1975). Indeed changes in T counts may precede clinical evidence of outcome, increasing prior to objective response (Yonemoto et al. 1977) and falling by as much as three months prior to obvious recurrence (Anthony et al. 1975b; Dellon et al. 1975).

It is well appreciated that the results of T lymphocyte assays, especially those using the E-rosetting properties of lymphocytes, are to some extent dependent more on the techniques used to perform the assay rather than the status of the cancer patient and it is probable that much of the conflicting data presented here is the consequence of variation in technique between laboratories. Other factors, however, also compound this problem.

The sex of the patient does not affect T counts (Smith et al. 1974; Alexopoulos & Babitis 1976) but with increasing age, percentage counts

become depressed (Smith et al. 1974; Holland et al. 1975; Whitehead et al. 1978, Nagel et al. 1981; Mascart-Lemone 1982) although not all agree that there is an age associated relationship (Weksler & Hutteroth 1974; Alexopoulos & Babitis 1976; Djeu et al. 1977). Wybran and Fudenburg (1973) reported that viral but not bacterial infection depressed 'active' T counts and illnesses such as myocardial infarction, cirrhosis, sarcoidosis and vasculitis had no effect. Viral illness has also been demonstrated to depress total percentage T lymphocyte counts (Anthony et al. 1975a; Chisari & Edgington 1975; Niklasson & Williams 1974) as do benign diseases such as 'Crohn's' when in an 'active' state (Whitehead et al. 1978). Cancer therapies have widely differing effects, surgery having a short lived depressive effect of between four days to two weeks (Hamid et al. 1984; Lynch & Kirov 1986; Shukla et al. 1980), radiotherapy depressing T counts for at least one to ten years (Stjernsward et al. 1972; Toivanen et al. 1984; Petrini et al. 1983) when applied to the chest, and chemotherapy for two to three years (Petrini et al. 1984). On the other hand, hormonal manipulation with tamoxifen increases T lymphocyte counts and will, to some extent, neutralise the depressive effect of chemotherapy (Sheard et al. 1986). Indeed, hormonally induced increases in T lymphocyte counts have been claimed to be the cause of treatment success in breast cancer through immune mechanisms (Yonemoto et al. 1977).

It has been shown that markers for Fc - Ig receptors, used to differentiate T helper (Th) from T suppressor/cytotoxic (Ts) lymphocytes (Moretta et al. 1977), give an inaccurate picture and that monoclonal markers reflect the situation more truly (Reinherz et al. 1980) although even these do not accurately reflect subset function completely (Robbins & Fudenberg 1983). Using monoclonal antibodies, researchers have reported

T lymphocyte subsets to be the same in all stages of breast cancer as in controls (Petrini et al. 1984; Ludwig et al. 1985) although McCluskey et al. (1983) were able to demonstrate a difference between disseminated breast cancer and the other stages of disease and control patients, those with metastases having decreased numbers of Th and increased numbers of Ts lymphocytes with consequent reduction in the Th to Ts ratio.

Measurement of T lymphocyte subsets is unfortunately also prone to the complicating effects of other variables. For instance, Nagel et al. (1981) have reported that increasing age affects the Th population only slightly but that Ts lymphocytes decrease significantly, resulting in elevated Th/Ts ratios and this is especially so in men. Very different findings have been published by others (Mascart-Lemone et al. 1982) who found that with increasing age in men the ratio falls, due mainly to a striking reduction in Th cells and a less pronounced increase in Ts lymphocytes, but no such changes occur in women. The Th/Ts ratio has been reported to be decreased for up to ten years following radiotherapy due to a fall in Th lymphocytes (Petrini et al. 1983) but Toivanen et al. (1984) have demonstrated that the Ts population also falls and at three years post-treatment, although both subsets are reduced, the ratio remains constant. The effect of chemotherapy appears to be to reduce both subpopulations but more so, Th lymphocytes, with consequent reduction in ratio even two to three years after treatment (Petrini et al. 1984). Tamoxifen, however, increases both subpopulations, especially Th cells, and also counteracts the depressive effects of cyclophosphamide (Sheard et al. 1986). Surgery is reported to depress both subpopulations equally without disturbance of the ratio (Sheard et al. 1986) but this effect returns to normal within a week (Hamid et al. 1984; Lynch & Kirov 1986).

It is apparent that T lymphocyte counts bear a relationship to cancer, most authors agreeing on depressed absolute and percentage counts in patients with disseminated disease but there is less consensus regarding early tumours. Some evidence points to stage related changes in T lymphoycte counts and even that variation in counts may precede clinical evidence of change in disease status. Data supporting T subset fluctuations in cancer is however, poor. The changes in T lymphocytes may be masked by other variables such as age, illness and the effects of treatment directly on the immune system but more profoundly by the variation in techniques used by different laboratories, some of which may nullify the discriminatory value of this test and which will be discussed fully in the following chapter.

3. B Lymphocytes: comprise 20% (Anthony et al. 1975a) to 28% (Jondal et al. 1972) of the lymphocyte population. There have been reports of decreased B cell counts in patients with locally advanced breast cancer (Rotstein et al. 1985) and of increased counts in disseminated tumours (Kopersztych et al. 1976) but the consensus of opinion is that B lymphocyte proportions remain unaffected by cancer (Stjernsward et al. 1972; Gross et al. 1975a; Teasdale et al. 1976; Whitehead et al. 1976; Shukla et al. 1979, Shukla et al. 1980; Mandeville et al. 1982). Neither age nor sex differences affect B cell numbers (Teasdale et al. 1979b) but exercise transiently increases the B cell population (Steel et al. 1974a) as does surgery (Lynch & Kirov 1986), the effect of the latter lasting for less than a week. Hamid et al. (1984) have suggested that these stress induced changes are due to redistribution of lymphocytes from blood to tissues by cortisone. Radiotherapy reduces absolute numbers but not the proportion of B lymphocytes

(Shukla et al. 1980; Toivanen et al. 1984) although Stjernsward et al. (1972) have found a percentage increase for at least one year following this treatment.

In short, B lymphocytes appear to be a fairly constant proportion of the lymphocyte population and changes in absolute numbers in cancer or following therapy are really a reflection of the variation in total lymphocyte counts. They are, therefore, not particularly useful in assessment of the cancer patient.

4. Null cells: the null cell compartment comprises the third component of the total lymphocyte population. Null cells probably contain myeloid precursors (Richman et al. 1978), erythroid precursors (Nathan et al. 1977) and the major effector cells of ADCC and NK activity (Reinherz et al. 1980). As such, cells of the null compartment may not be lymphocytes at all but whether K cells and NK cells, which are probably the same cell anyway (reviewed by Herberman 1983a), are lymphocytes, monocytes, immature forms of either, or totally different cells is controversial. More recently it has been suggested (Lanier et al. 1986) that NK activity is mediated by non-lymphocytes, i.e. NK cells proper, accounting for approximately 10% of the total peripheral blood lymphocyte population, and by non-MHC-restricted lymphocytes which make up about 5% of the peripheral blood lymphocytes. It certainly seems that NK cells straddle both null cell and T lymphocyte populations, with 50% of NK cells having low affinity receptors for SRBC (Herberman & Ortaldo 1981; Timonen et al. 1981) as do K cells (West et al. 1978). The method of E-rosetting is, therefore, of crucial importance in assessing the true number of null cells, the presence of neuraminidase (Sandilands et al. 1975), bovine serum albumin (BSA) (Anthony et al. 1975a), foetal calf serum (FCS) (Whitehead et al. 1976; Byrom et al 1978) and prolonged

incubation of lymphocytes (Whitehead et al. 1978) increasing the number of E-rosetting lymphocytes, perhaps by providing optimal conditions for low avidity receptors to combine with SRBC (see Chapter 4).

The null cell population changes which occur in cancer patients (Byrom et al. 1978) tend to reflect in an inverse manner the changes in the proportion of T lymphocytes (Whitehead et al. 1976; Shukla et al. 1979; Shukla et al. 1980) and increases proportionally with the decrease in T cells related to age, debility, treatment regimes and disease (Whitehead et al. 1978). This relationship may be due to part of the null cell population being converted into T lymphocytes and vice versa (Whitehead et al. 1976) through blocking mechanisms present in the sera of the aged, the ill and the cancerous (Whitehead et al. 1978; Shukla et al. 1980).

These common tests of immune competence show in most solid neoplasms of man that host immunity in early disease is usually normal or only slightly impaired. However, as disease advances this impairment becomes more profound affecting most severely those with disseminated tumours and those in the terminal stages of cancer. It therefore appears that cancer precedes depressed immune function, unlike the situation with virally linked tumours, and that the degree of immunological impairment parallels the extent of disease. That impaired host immunity appears to be a secondary phenomenon rather than a primary event allowing cancer to develop, is in accord with the observation that the common solid neoplasms such as carcinoma of the breast do not occur with any greater frequency in the immuno-compromised population. Why then do tests of immune function become depressed and why does such impairment become more obvious in patients carrying greater

tumour loads?

Possible mechanisms for this (reviewed by Herberman 1983b) are: suppressor T cells, suppressor macrophages, immunosuppressor products of tumour cells, prostaglandins, corticosteroids and serum inhibitory factors, and these may be interlinked, for instance, immune complexes (ICs), a putative serum inhibitory factor, may also stimulate suppressor cell activity (Gershon et al. 1974; Moretta et al. 1977). There is good evidence that serum inhibitory factors directly affect the tests of host immunity previously discussed by 'blocking' the T lymphocytes upon which these assays are dependent.

3.3. Blocking

3.3.1. The effects of cancer sera on immune function

Control lymphocytes incubated in cancer serum have diminished blastogenic responses compared with those incubated in control serum, this depression being most marked in serum from patients with advanced disease (Whittaker et al. 1971a) and recurrent disease, whilst PHA responses are normal in patients remaining clinically free from tumour (Whittaker et al. 1971b). These authors and others (Suciu-Foca et al. 1973) also showed that PHA responses of cancer patients' lymphocytes improved following incubation in control serum. Allogeneic inhibition of control lymphocytes by cancer sera has also been demonstrated by Gatti (1971), Tanaka et al. (1978) and Suciu-Foca et al. (1973) who reported that the depth of depression was related to the extent of disease, and further, repeating these experiments using mixed lymphocyte culture (MLC) instead of PHA to induce blastogenesis, that cancer sera mediated depression of MLC responses was even more pronounced and bore a remarkable relationship to stage of disease. Both specific and non-specific 'blocking' has also been demonstrated using autochthonous and allogeneic cancer sera in an assay in which tumour cells were used to stimulate autochthonous lymphocytes (Vanky et al. 1971) and, as a rule, this 'blocking' effect tended to decrease or to disappear after tumour removal or regression. With this form of assay it was also shown that the 'blocking' factor could be removed from the lymphocyte surface by washing (Hattler & Soehnlen 1974) with resultant improvement of lymphocyte function. A note of caution was introduced about the use of allogeneic sera in such experiments when it was demonstrated that non-autologous sera have a non-specific depressive effect on PHA responses in general (Whitehead et al. 1974). However,

Suciu-Foca et al. (1973) using PHA and MLC, and Vanky et al. (1971) using autochthonous tumour, to induce lymphocyte blastogenesis, reported that although such an effect was seen it was much less obvious than the changes induced by cancer sera.

The fact that cancer sera depress control lymphocyte function and that incubation of cancer lymphocytes in control sera improves their blastogenic capability, taken together with the observation that the inhibitory factor can be removed from the lymphocyte surface by washing, implies that the cancer associated defect lies in the serum and not with the lymphocyte itself. Roberts and Jones-Williams (1974) suggested that this was the case when they noted that patients with impaired DHR to varidase surprisingly had normal PHA blastogenesis when the lymphocytes were incubated in foetal calf serum (FCS) instead of autologus serum, that is, although lymphocyte function was obviously impaired as measured by DHR, removing these cells from their serum permitted normal function as measured by blastogenic response to PHA. This loss of PHA response impairment following removal of lymphocytes from autologous plasma and placement in normal AB plasma has also been shown in patients with colonic cancers (Shafir et al. 1980). Correlation between skin reactivity and lymphocyte blastogenesis has been observed (Nemoto et al. 1974; Simo-Camps et al. 1976; Kopersztych et al. 1976) and it has been demonstrated that the link may be due to a serum factor, Nimberg et al. (1976) having shown that 66% of the sera of cancer patients who lacked DHR were capable of inhibiting PHA stimulation of normal lymphocytes.

Finally, however, it must be remembered that serum depression of lymphocyte blastogenesis is not cancer specific, and PHA responses for instance, have been shown to be inhibited by sera from patients with tuberculosis, multiple sclerosis, hepatitis, candidiasis and syphilis,

as well as from healthy multigravida women (Gatti 1971).

Serum from patients with benign and malignant disease also has the ability to prevent T lymphocytes forming E-rosettes with SRBC, for instance, sera from patients with active systemic lupus erythematosis (SLE) were able to 'block' in a non-specific manner the formation of rosettes by a proportion of T lymphocytes (Popovic et al. 1974) and further, the extent of this inhibition was related to the degree of disease activity as measured by DNA antibody activity. Such disease related inhibition of rosette formation was also reported in lung cancer (Gross et al. 1975a) and in patients with untreated Burkitt's lymphoma (Gross et al. 1975b), the inhibition disappearing following treatment induced remissions in the latter disease. The 'blocking' factor is removable from the lymphocyte surface by brinase (Holland et al. 1975), papain (Whitehead et al. 1976; Whitehead et al. 1977; Shukla et al. 1979; Shukla et al. 1980), washing (Holland et al. 1975; Whitehead et al. 1976; Whitehead et al. 1977), or plasmapheresis (Browne et al. 1976) and can be replaced by reincubation in autologous cancer serum (Whitehead et al. 1976; Whitehead et al. 1977). Further, incubation of control lymphocytes in allogeneic cancer sera results in inhibition of E-rosetting (Whitehead et al. 1977; Shukla et al. 1979; Shukla et al. 1980), the degree of depression being related to the tumour load of the donor patient (Shukla et al. 1979). It has also been observed that there is a high degree of correlation between depressed control percentage T lymphocyte counts when control lymphocytes are incubated in cancer sera and the percentage T lymphocyte counts of the cancer patients from whom the sera is taken (Shukla et al. 1979; Shukla et al. 1980). Whitehead et al. (1977) also noted this correlation stage for stage in patients with breast cancer. It has further been reported that 'blocking' factor, measured by T lymphocyte rosetting inhibition decreases significantly

in those with Stage III breast cancer following mastectomy (Shukla et al. 1980) and in those with early breast cancer, who do not recur, following mastectomy (Haffejee et al. 1983). It has been further shown that patients with little post-operative serum inhibitory factor are unlikely to develop recurrent disease whereas 50% of those with significant post-operative inhibitory levels recur within four years (Haffejee et al. 1983). The inhibitory effect of allogeneic control sera on lymphocyte blastogenic function has also been demonstrated in T lymphocyte rosetting but this appears to be limited at most to 15% of T lymphocytes (Shukla et al. 1980), cancer sera invariably causing greater depression.

Evidence that the link between the depression in DHR and percentage T lymphocyte counts in cancer patients may be a lymphocyte inhibiting serum factor has been produced by Holland et al. (1975) who showed that slow infusion of brinase, which removes inhibitory factor from the lymphocytes in vitro, into patients with malignant disease, results in increased T lymphocyte counts paripassu with a restoration of skin allergy.

The serum factor which inhibits E-rosetting is, like that affecting blastogenesis, not specific to cancer, and has been demonstrated in patients with SLE (Popovic et al. 1974), acute viral hepatitis (Chisari & Edgington 1975), and in the elderly, and in those with chronic benign disease (Whitehead et al. 1978).

Added evidence that cancer sera contains inhibitory factors affecting lymphocyte function comes from the work using assays of lymphocyte cytotoxicity against tumour target cells, much of it undertaken by the Hellstroms who, following Moller's demonstration in 1965 that autologous antibody was capable of 'blocking' cell mediated cytotoxicity, were able to show that serum from tumour bearing animals could abrogate, in an

immunologically specific manner, the antitumour effects of specifically sensitised lymphocytes (Hellstrom & Hellstrom 1969). They and others later demonstratead that the 'blocking' capacity of the serum in animals and humans was lost following complete excision of the tumour (Hellstrom et al. 1970; Hellstrom 1971; Hellstrom & Hellstrom 1974a, Hellstrom & Hellstrom 1974b; Baldwin et al. 1972; Baldwin et al. 1973a; Currie 1973) but not on partial excision. Serum from such tumour-free individuals was shown to have the capacity to neutralise the inhibitory activity of tumour bearer serum, this 'unblocking' phenomenon having previously been reported by Hellstrom and Hellstrom (1970) and Bansal and Sjogren (1971). It was also demonstrated that lymphocyte mediated cell cytotoxicity was more pronounced in patients with small tumours than in those with large tumours and that the sera of patients with growing neoplasms could 'block' cytotoxicity against tumours of the same histologic type (Hellstrom & Hellstrom 1974a) and even that such 'blocking' activity was often detectable before clinically overt tumour recurrence (Hellstrom & Hellstrom 1973; Hellstrom & Hellstrom 1974a). For instance, of patients clinically free of tumour following surgery, 92.9% who showed post-operative 'blocking' went on to develop recurrence within the first year whereas only 13% of those with no significant post-operative 'blocking' did so. Currie and Basham (1972) also showed that this inhibitory factor could be removed from the lymphocyte surface by washing, greatly enhancing lymphocyte cytotoxicity to autologous and allogeneic tumour cells of similar histologic, but not other histologic, origin. They further showed that this cytotoxicity was abolished following reincubation in cancer serum, that the inhibitory factor acted only on the lymphocyte and did not 'block' the target cell, and that this factor was present in patients with more advanced disease but absent in those with early disease,

suggesting a correlation between tumour load and the presence of inhibitor.

Interestingly, this 'blocking' phenomenon appears to be even stronger within the tumour and locoregional lymph nodes, Nind et al. (1973) having demonstrated that lymphocytes derived from tumours displayed no cytotoxicity against target cells whereas more than 50% of patients had peripheral lymphocytes cytotoxic to the tumours. They further reported, using a greater number of patients, that only 7% of these had lymphocytes from regional nodes capable of tumour cell killing whereas 33% of peripheral blood lymphocytes retained their cytotoxic ability. Currie and Gage (1973) working with animals also found similar local and regional node paralysis, later followed by peripheral blood anergy, this invariably preceding the development of micrometastases. More recently Hoon et al. (1987a) demonstrated depressed in vitro responses to PHA, MLC and interleukin-2, by the lymphocytes from lymph nodes nearest to primary malignant melanoma and breast tumours in comparison with responses by lymphocytes from more distant nodes, indicating decreasing immunosuppression of node lymphocytes with increasing distance from the tumour.

In summary, the properties of this inhibitory or 'blocking' factor appear to be that it acts on the lymphocyte to depress, the formation of rosettes with SRBC, blastogenic transformation, cytotoxicity against target cells and responses to skin test antigens. It can be removed from the lymphocyte by washing, enzyme digestion and by incubation in control sera and can be reapplied by incubation in cancer sera. There is some evidence that it is specific for tumour histologic types implying the presence of TSAs, but others have noted no immunological specificity. Further, the 'blocking' activity of cancer sera appears to reflect the tumour burden of the host in a directly proportional

fashion. It also appears that normal sera have an inhibitory capacity, to a lesser degree than cancer sera, when incubated with non-autologous lymphocytes and that sera from patients with benign active disease, the elderly, multiparous and pregnant women (Carpentier & Miescher 1983), also exert 'blocking' activity.

Whitehead et al (1977) have further shown that the inhibitory factor in cancer sera does not affect B lymphocytes, appears like the hepatitis associated lipoprotein demonstrated by Chisari and Edgington (1975) to affect only a subpopulation of T lymphocytes, and that its effectiveness is lost by dilution to one fourth of original concentration. They further demonstrated that the cancer serum inhibition was not due to C-reactive protein which can block E-rosetting formation in its own right (Mortensen et al. 1975), anti-HLA activity also known to inhibit E-rosetting (Pyke et al. 1975), anti-blood group activity, nor to hepatitis associated lipoprotein (Chisari & Edgington 1975). Others who have analysed the inhibitory factor have claimed it to be an alpha macroglobulin, also seen in the serum of pregnant patients (Stimson 1975), or an immunosuppressive peptide (Nimberg et al. 1975). Perhaps the strongest claim, however, is that serum inhibition is due to antigen or immune complexes.

3.3.2. Immune complexes

Initially, because Kaliss (1958) had shown that injection of specific allo-antibody facilitated growth of allografted tumours in situations where rejection would normally occur and Moller (1965) had demonstrated that autologous antibody was capable of blocking ADCC, it was thought that antibody might be the inhibitory substance. Added support was supplied by the Hellstroms (1969) who observed that serum from tumour bearing animals could abrogate in an immunologically specific manner the

antitumour effects of specifically sensitised lymphocytes, this 'blocking' activity being held in the 7S fraction of serum and being neutralised by the addition of heterologous antimouse immunoglobulin. The logic of this is lost however, when it is appreciated that antibody itself appears to have an antitumour effect. For instance, it combines with lymphocytes to effect cytotoxicity (Moller 1965; MacLennan & Loewi 1968), has a protective anti-oncogenic virus role in chickens (Thompson & Linna 1973), and antibody is detectable in the sera of patients with early cancers only to disappear with advancing disease (Lewis et al. 1969) being replaced by 'blocking' activity (Basham & Currie 1974). Further, Baldwin et al. (1973a) showed that antibody not detectable in tumour bearing animals could be demonstrated in the sera of these animals following excision of the tumour. It was postulated that the explanation for this phenomenon (Thomson et al. 1973) is that antibody to TSAs is produced early in disease but as tumour bulk increases and the nodal architecture becomes disrupted, free soluble TSA is released into the circulation with resultant formation of immune complex (IC) and disappearance of antibody. Removal of the tumour, the antigen source, would permit re-expression of antibody in the serum. These authors further demonstrated that the disappearance of antibody from the serum was not due to either induction of tolerance in the animal or absorption of antibody by the tumour mass.

Evidence that complexes are involved in the 'blocking' phenomenon, was produced by elution and ultrafiltration experiments, dividing 'blocking' sera (Sjogren et al. 1971) into heavy and light molecular weight (MW) fractions. It was seen that the heavy MW fraction had no 'blocking' capability and the light MW fraction could inhibit the effector cell only but that a recombination of both fractions could inhibit at both effector and target cell levels. Hattler and Soehnlen (1974) removed the 'blocking' component from the lymphocyte membrane by washing and, by crude fractionation of this, into heavy and light MW fractions which they suggested might be antibody and antigen respectively, showed that these independently could not produce the 'blocking' effect but that recombination of the fractions resulted in inhibition of lymphocyte reactivity, the implication being that ICs were the 'blocking' factors. Unequivocal proof that antibody-antigen complexes participate in 'blocking' reactions was obtained in the rat hepatoma system (Baldwin et al. 1972, reviewed Baldwin et al. 1974) when it was demonstrated that papain-solubilised tumour, antigen and its antibody, although individually incapable of 'blocking' at the target cell level, could 'block' either at the lymphocyte or target cell surface when recombined 'in the right mix'. It was noted that post-excision serum, i.e. in antibody excess, did not cause 'blocking' until sufficient antigen was added to form complexes in the right proportions but continued addition of antigen resulted in loss of 'blocking', presumably because the ICs would then bind to free antigen instead of tumour cell or lymphocyte membranes. In the rat hepatoma model the 'right mix' for optimal 'blocking' appears to be free circulating TSA in excess together with specific ICs (Baldwin et al. 1973c). These authors, however, also noted that antigen itself

can be inhibitory at the lymphocyte (but not the tumour cell) surface, although others (Tanaka et al. 1979) have found that antigen alone does not inhibit lymphocyte function. Gorczynski et al. (1975) have suggested that immunologically specific 'blocking' may be carried out by antigens and ICs but that non-specific inhibition is a feature of ICs only. Baldwin et al. (1974) have also suggested that of the two mechanisms of 'blocking', IC inhibition is the more important. They have postulated that antigen produces lymphocyte 'blocking' in the earlier stages of tumour growth until the production of antibody occurs which, by forming complexes with antigen, results in decrease of free antigen and 'blocking' by ICs in the later stages of disease.

Further evidence that circulating immune complexes (CICs) are responsible for 'blocking' has been produced by Tanaka et al. (1979) who were able to show using assays of PHA blastogenesis and leukocyte adherence inhibition (LAI) that the inhibitory effect of cancer sera, already demonstrated by their laboratory to be tumour stage-related (Yonemoto et al. 1978) correlated with ICs precipitated out of the sera by polyethylene glycol.

There is evidence, both indirect and direct, that ICs are produced by patients with malignancy. Indirect evidence comes from well recognised but uncommon secondary immunopathological manifestations of immune complex disease thought to be a consequence of malignancy. These tend to occur in conjunction with lymphomas and leukaemias but occasionally present in solid tumours, especially those of lung and ovary. Tumour associated cases of autoimmune disease such as cerebellar degeneration, carcinomatous neuromyopathy, dermatomyositis and nephritis have been reported (reviewed by Burnet 1970a) and more recently autoimmune tumour associated phenomena have been noted; haemolytic anaemia (Laszlo 1982), vasculitis

(Doyle & Perry 1982), nephrotic syndrome (Higgins et al. 1974), and neuropathy and myelopathy (Richardson 1982). Further, Twomey et al (1976) have reported an increased incidence of rheumatoid factor (RhF) in the sera of lung and breast cancer patients following chemotherapy or irradiation therapy and Pascal et al. (1976) have shown an increased incidence of subclinical glomerular IC deposits in patients with malignancies. It has been suggested that these may be complexes between antibody and either TSA (Pascal et al. 1976) or cellular breakdown products (Higgins et al. 1974). Theofilopoulos and Dixon (1979), reviewing this subject, have proposed that the infrequency of overt immune complex disease in cancer patients is explained by the intermediate size of the ICs in the serum of such patients, these ICs not being of appropriate size or composition for deposition in the tissues.

Direct evidence that CICs are produced by many patients with spontaneous neoplasms has also been obtained by measuring these complexes in patients' sera. Soluble CICs have frequently been reported to be elevated in patients with leukaemias, lymphomas and a wide variety of solid cancers (Heimer et al. 1976; Theofilopoulos et al. 1976; Rossen et al. 1977; Teshima et al 1977; Poulton et al. 1978; Brandeis et al. 1978; Mihas et al. 1981; Vellacott et al. 1981; Poskitt & Poskitt 1985), and elevated levels shown to be related to pathological stage of disease (Brandeis et al. 1978), tumour load (Heier et al. 1972; Rossen et al. 1977; Amlot et al. 1978; Poulton et al. 1978; Brandeis et al. 1978; Poskitt & Poskitt 1985), symptomatic lymphomatous disease (Heier et al. 1977; Amlot et al. 1978), and lymphomas of poor histological grade (Amlot et al. 1978). Levels of CIC have further been reported to predict outcome (Hoffken et al. 1977; Rossen et al. 1977; Rossen et al. 1983; Poskitt & Poskitt 1979; Amlot et al. 1978; Gupta & Morton 1983) and

to vary with change in disease status (Hoffken et al. 1977; Poulton et al. 1978; Brandeis et al. 1978: Amlot et al. 1978) and sometimes before clinical evidence of this is evident (Gupta & Morton 1983). Others, however, have reported little or no difference in CIC levels between patients and controls (Herberman et al. 1981; Clarke et al. 1982; Krieger et al. 1983) and that levels do not reflect tumour burden (Heimer & Klein 1976; Vellacott et al. 1981). Indeed, although there is a tendency for patients with more advanced or aggressive tumours to have measurable or elevated levels of CIC, such patients may have CICs within the normal control range.

There are many possible reasons for such discrepencies between laboratories but one important possibility suggested (Lambert et al. 1978), [because of poor correlation between measured CIC levels by different techniques (Lambert et al. 1978; Herberman et al. 1981)], is that some techniques are more suitable for detecting the IC of certain diseases, and as there tends to be heterogeneity of IC in the circulation, not all of which may be related to the disease under study (Jones & Orlans 1981), it is quite possible to measure ICs unrelated to the disease process under investigation.

3.3.3. Possible mechanism of blocking

The relationship between tumour burden and 'blocking' capacity of the serum in animals could be explained on the following lines: antibody against TSA would be formed in the regional nodes, such antibody being measureable in the peripheral blood. However, with increase in tumour bulk the nodal architecture would be disrupted allowing free TSA access to the circulation where, by combining with antibody it would form ICs, the antibody disappearing from the blood.

Early in disease the resulting anergy would be localised but as more TSA and IC was released into the circulation it would become generalised.

Baldwin et al. (1973) have suggested that the relative concentration of substances with the potential to 'block' or inhibit the immune response, i.e. antigen and ICs, are dependent on the strength of the host immune response and the extent of tumour antigen release. Tumour antigen release is essentially a characteristic of the particular tumour. It is known that tumours have unstable membranes and shed surface antigen more readily than normal cells (Davey et al. 1976) and that those which shed their surface components more readily and extensively are those which metastasise more easily (Currie & Alexander 1974), so it would seem reasonable to conclude that very active cancers such as anaplastic tumours, which we know to be highly metastatic, are those which produce greatest amounts of antigen and IC.

The mechanism may be slightly different with regard to the spontaneous tumours of man, which do not have apparent TSA. However, man does produce autoantibodies to cellular components (Whitehouse & Holborrow 1971) probably as a mechanism for clearing debris of dead or dying cells, and this may explain the relationship between tissue necrosis and the serum suppressive activity demonstrated in patients with tuberculosis, syphilis, candidiasis, Crohn's disease, ulcerative colitis and acute viral hepatitis (Gatti et al. 1971; Whitehead et al. 1978; Chisari & Edgington 1975). Further evidence on this point relating to tumour has been presented by Shukla et al. (1980) who attributed the increase in serum 'blocking' activity in patients undergoing radiotherapy for advanced locoregional inoperable breast cancer to tissue breakdown products from the radiation induced necrosis. Currie (1980) however, has also suggested that tumour cells may have 'natural' antibody directed against

surface determinants not detectable on normal cells as a result of defective glycolysation. Thus, in man rapidly growing anaplastic cancers with their high degree of membrane shedding and production of tissue necrosis breakdown products, would be those most prone to contain high concentrations of non-specific antigen and IC in their host's serum. The more aggressive the tumour, the more 'blocking' activity in the patient's serum and the greater the depression of tests of immune competence.

As a general rule, the heavier the tumour load, the more profound the anergy but very bulky localised lesions without evidence of dissemination might be expected to have relatively stable surface membranes, shedding little antigen into the blood and, as a consequence, exhibiting only moderate 'blocking' activity. This would explain the apparent anomally in Stage III breast cancer where DHR, T lymphocyte percentage counts and serum 'blocking' activity are virtually identical to that of the control population. Small highly active cancers, while still in the early phases of their development, on the other hand, might, by their ability to rapidly shed large amounts of debris into the circulation, exert a high degree of inhibitory activity. These features may well explain why some authors have been unable to detect differences in serum inhibitory capacity between the various stages of disease (Matthews & Whitehead 1976; Whitehead et al. 1977). Therefore, although serum 'blocking' activity appears to be related to tumour load it is probably much more importantly a reflection of tumour cell turnover.

3.3.3.4. Possible use in assessment of cancer activity

Any mode of therapy causing a decrease in tumour load or activity should effectively reduce serum 'blocking' capacity. For instance, it

might be expected that serum 'blocking' activity would disappear following complete surgical ablation of all tumour, whereas surgery leaving residual tumour would result in continuing serum 'blocking' activity. Therefore, by measuring the post-operative 'blocking' capacity of sera it should be possible to assess the efficacy of surgical treatment or the presence of occult metastatic disease (see Haffejee et al. 1983). This of course has important implications not only with regard to prognosis but also to the application of adjuvant therapy. However, it must always be borne in mind that a small focus of active tumour may be as effective as a larger volume of equally active disease if sufficient 'blocking' material is produced to saturate the proportion of T lymphocytes affected by these factors, Whitehead et al. (1977) having shown that further exposure of lymphocytes, already 'blocked' by one cancer serum to a second cancer serum, resulted in no increase in lymphocyte inhibition. Once maximal 'blocking' is attained it becomes impossible to assess the true extent of disease.

On the other hand, systemic forms of therapy generally apply to all foci of cancer throughout the host, so serum 'blocking', as measured by its effect on T lymphocytes, may reflect whole tumour volume. Hormonal manipulation of breast cancer, when effective, reduces disease load but more significantly, by suppressing the production of constituents necessary to maintain cancer cell survival and replication, it reduces tumour cell turnover and consequently should diminish the shedding of antigenic material. The efficacy of this mode of therapy should, therefore, be particularly amenable to assessment by measuring the effect of serum 'blocking' activity on lymphocytes.

3.4. Summary

It is generally accepted that the immune response is impaired in many patients with solid cancers as well as in those with virally-linked tumours. When immunodeficient, they manifest depressed CMI more so than impaired humoral immunity. A vast quantity of literature has been published on the relationship between various solid tumours and tests of CMI in an attempt to predict prognosis, tumour load and efficacy of treatment but these tests have been found wanting, perhaps because of the many variables which can affect CMI and the differences in results which even slight variation in technique in the performance of these tests will give.

As a general rule the development of spontaneous cancers appear to precede impairment of CMI but as the extent of disease increases such impairment becomes more profound indicating that it is a secondary phenomenon rather than a primary event. It would seem from the evidence previously presented that assays of CMI are depressed as a consequence of inhibitory or 'blocking' factor(s) present in the sera of tumour bearers, and that these factors may be immune complexes of antibody and their antigens in man, the latter probably being non-specific products of cell turnover and necrosis.

Finally, it appears that the more actively a tumour produces antigen, either because of rapid cell turnover or as a consequence of sheer tumour bulk, the greater the amount of CIC formed and the more profound the inhibitory effects of the sera on host CMI. Hopefully, we may be able to utilise this relationship between IC production and its effect on lymphocyte function to measure tumour load or disease activity more efficiently than at the present time, perhaps even prior to clinical evidence of change in disease status, permitting earlier modification

of treatment schedules with subsequent improvement in therapeutic outcome.

Chapter 4

Chapter 4

4. Lymphocyte Counts and Carcinoma of The Breast

4.1. Introduction

As indicated in the preceding chapter absolute lymphocyte and absolute percentage T lymphocyte counts have been reported by many authors to be depressed in patients with breast cancer and various other solid tumours and the degree of depression is roughly related to the extent of disease. It has also been demonstrated (Shukla et al. 1980) that cancer and treatments which depress T lymphocyte counts such as radiotherapy, may do so in two ways: a) by reduction of the absolute number of circulating lymphocytes in the peripheral blood; and b) by depressing the proportion of T lymphocyte counts in the blood, probably by the action of serum inhibitory or 'blocking' factor(s) which convert them into null cells.

It will be shown here that T lymphocyte counts are depressed in patients with breast cancer and that this depression is related to tumour load, while recognising that tumour activity is probably even more important. Furthermore, it will be demonstrated that while local treatment makes little, if any, difference to lymphocyte and T lymphocyte counts that systemic hormonal treatments, when successful, increase the proportion of lymphocytes often before there is objective evidence of disease remission.

An attempt will be made to show that many authorities have failed to demonstrate such a relationship because of variations in techniques used

in counting T lymphocytes. In particular it is possible that much of the discriminatory power of percentage T lymphocyte counts is lost because certain methodologies in general use permit sheep red blood cells (SRBC) to rosette with low avidity receptor cells from the null cell population thereby increasing apparent T cell numbers significantly.

Finally, the relevance of circulating immune complexes, assayed by the CIq deviation tests, to breast cancer and their relationship to depressed % T lymphocyte counts, will be explored.

4.2. Part I

The Effect of Breast Disease and Treatment Response on Lymphocyte Counts

4.2.1. Materials and methods

Patients [Table 4.1]

Women studied attended the Breast Clinic at the University Hospital of Wales, Cardiff, and the Combined Breast Clinic, Velindre Hospital, Cardiff. Fifty of these patients had benign breast disease; 10 underwent mastectomy for Stage I breast cancer and 32 for Stage II breast cancer, 12 of the latter being given a two year post-operative course of adjuvant tamoxifen 10mg b.d. Fifteen patients also underwent mastectomy for Stage III breast cancer. Five patients with Stage I and II and 10 patients with Stage III disease were treated solely with tamoxifen 20mg b.d. Sixty patients with Stage IV breast cancer were treated by hormonal manipulation; 47 with tamoxifen 20mg b.d. alone, 7 by oophorectomy, 1 by adrenalectomy, and 5 with a combination of tamoxifen 20mg b.d. and prednisolone 10mg t.d.s.

Evidence of extent of disease was obtained by clinical examination, routine blood tests, mammography, chest x-ray and when indicated, bone scans and ultrasonography of liver. Histological proof of diagnosis was always obtained either by open biopsy or trucut needle biopsy.

Pre- and post-treatment lymphocyte counts were performed as often as feasible and the number of tests in each subgroup are indicated in the results tables.

Treatment response

Response of breast cancer patients to hormone therapy was

Table 4.1

Breast Disease - Treatment Regimes Used in Each Category

Treatment	Benign	Stage I	Stage II	Stage III	Stage IV	
					Locoregional recurrence	Disseminated
Biopsy	50	0	0	0	0	0
Mastectomy	0	10	20	15	0	0
Mastectomy plus adjuvant Tamoxifen	0	0	12	0	0	0
Tamoxifen	0	1	4	10	14	33
Oophorectomy	0	0	0	0	0	7
Adrenalectomy	0	0	0	0	0	1
Tamoxifen plus Prednisolone	0	0	0	0	0	5
Total	50	11	36	25	14	46

assessed applying U.I.C.C. criteria (Hayward et al. 1977), using data from physical examination, photographs, radiographs, mammographs, and measurement of lesions over a period of at least three months.

Lymphocyte separation

Approximately 15 to 20mls of venous blood was withdrawn from each patient at the time of testing and placed in a sterile container containing 50 U./ml preservative-free heparin. Eight ml aliquots were layered over 3ml ficoll-hypaque (4.8ml, 10.8% ficoll and 2ml, 33.3% hypaque specific gravity 1.078, after Boyum 1968) and the mononuclear cell layer, comprising mainly lymphocytes, isolated by buoyant density centrifugation at 400g for 30 mins (Fig. 4.1). The mononuclear layer was immediately removed and washed twice in sterile isotonic saline by centrifugation at 400g for 5 mins, following which the cells were suspended in 1ml of sterile isotonic saline. A small proportion (0.05ml) of this suspension was added to an equal volume of trypan blue stain and counted on the improved Nebauer haemocytometer to assess lymphocyte numbers, viability and contamination by granulocytes. The lymphocytes were then adjusted to a concentration of $2x10^6$ cells/ml.

Preparation of sheep red blood cells (SRBC)

Normal sheep erythrocytes for the rosetting experiments were obtained from Wellcome sheep. Blood between 10 and 14 days old and containing no preservative was washed three times at 500g for 5 mins and made up to a 2% suspension in sterile isotonic saline. A fresh suspension was made up daily.

Preparation of ficoll-hypaque

Twenty-one gms ficoll (Pharmacia, Great Britain Ltd.) was added to 216 mls of water, boiled at 15lbs/sq inch for 15 mins in a pressure

Fig. 4.1

Ficoll-Hypaque Separation of Mononuclear Cells from Whole Blood

cooker and then allowed to cool to room temperature. Sixty mls of 45% hypaque (University Hospital of Wales Pharmacy) was added and this solution stored at 4°C until use.

E-rosetting test [after Anthony et al. 1975a]

A volume of 0.25ml of 2% SRBC suspension was added to 0.25ml of lymphocyte suspension in small bottomed glass tubes (Fig. 4.2). The tubes were covered with Nescofilm (Bando Chemical Industries Ltd., Kobe, Japan), gently shaken and incubated at 37°C for 10 mins following which they were centrifuged at 100g for 5 mins and then placed in a refrigerator at 4°C for an hour and a half. After chilling, the top layer of the pellet was resuspended with great care and a drop of this suspension gently placed on a cooled haemocytometer. A cooled coverslip was then gingerly lowered, using a fine needle, over the cells. Lymphocytes were then counted to a total of 200 to ascertain the percentage of rosetting cells, a rosette being defined as a lymphocyte with three or more SRBC firmly attached to it (Fig. 4.3). Polymorphonuclear cells, which were usually few in number, could be easily distinguished by their size and multinucleated appearance. Because of the difficulty in differentiating large lymphocytes from monocytes in unstained preparations, only small and medium sized lymphocytes were counted. Duplicate tubes were invariably used and the mean of the results taken but occasionally drops of suspension from one tube were counted in duplicate on separate haemocytometers, following which the mean of these results was calculated.

Absolute lymphocyte and absolute T lymphocyte counts

Full blood counts were performed in the Department of Haematology, University Hospital of Wales, Cardiff, on the Coultor counter. Differential films were performed using haemotoxylin and eosin staining. The absolute

Fig. 4.2

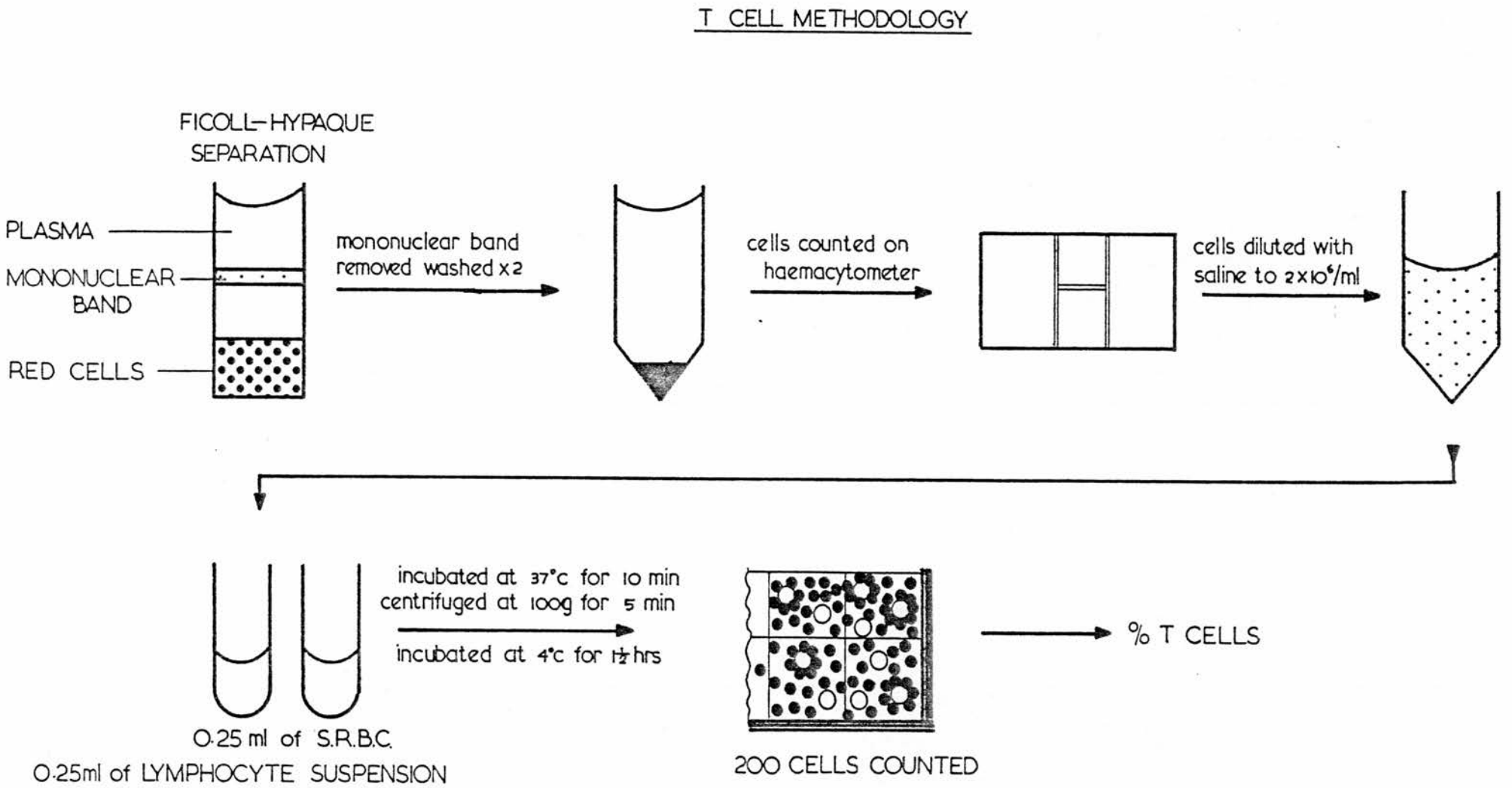
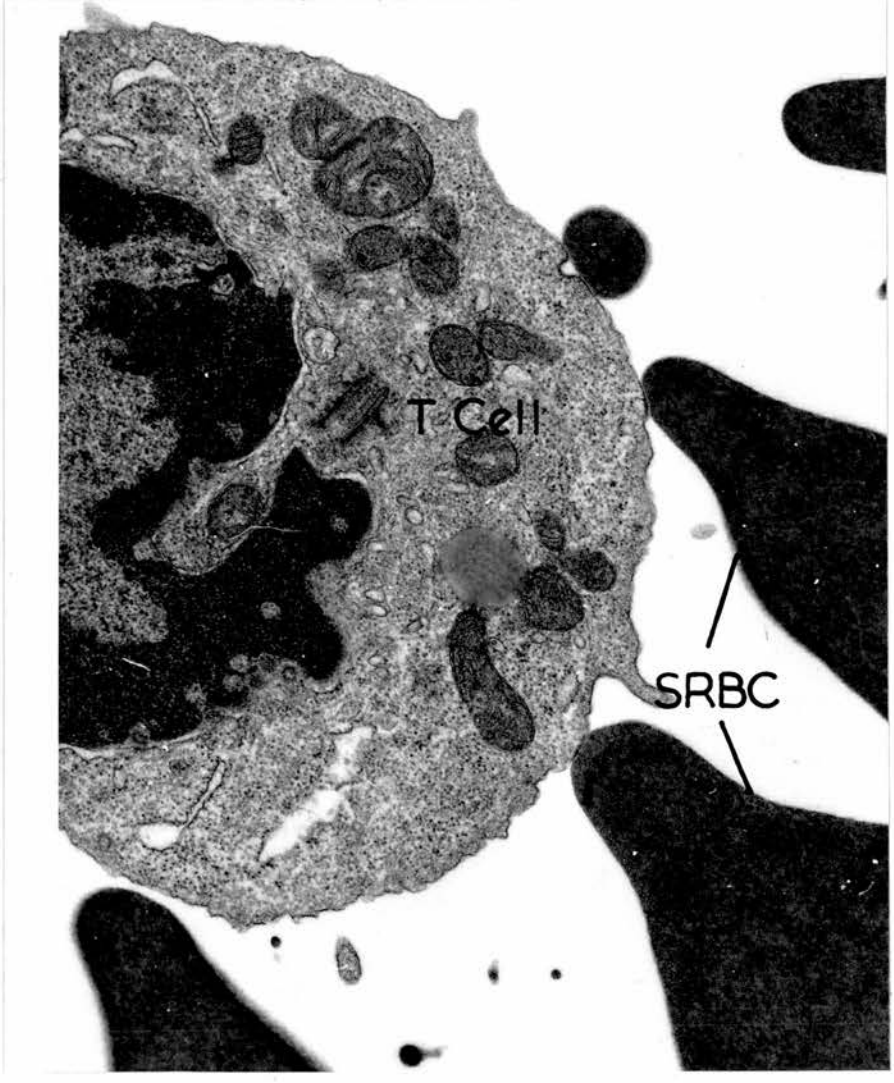

T CELL METHODOLOGY
FICOLL-HYPAQUE SEPARATION
PLASMA
MONONUCLEAR BAND
RED CELLS
mononuclear band removed washed x2
cells counted on haemacytometer
cells diluted with saline to 2x10⁶/ml
incubated at 37°C for 10 min
centrifuged at 100g for 5 min
incubated at 4°C for 1½ hrs
0·25 ml of S.R.B.C.
0·25ml of LYMPHOCYTE SUSPENSION
200 CELLS COUNTED
% T CELLS

Fig. 4.3

Electron Microscopy Photomicrograph of T Cell with Three SRBC Attached

[mag. x 18,000]



lymphocyte count was taken as the multiple of the white cell count and the percentage of white cells which were lymphocytes. The absolute T lymphocyte count was taken as the multiple of the absolute lymphocyte count and the percentage of lymphocytes which were T lymphocytes.

Oestrogen receptor assay

The oestrogen receptor content of tumours was determined using the dextran coated charcoal assay described by Korenman and Dukes (1970) at the Tenovus Institute for Cancer Research, Cardiff. Samples of tumour were taken at operation, warm ischaemic time, if any, being kept to an absolute minimum, placed in ice and delivered to the laboratory immediately. The results for patients included in this study were collected from a special register kept in the University Department of Surgery, Cardiff. Tumours were considered positive for oestrogen receptors only when they contained more than 5 f mol specific oestradiol binding per mg cytosol protein.

Statistical methods

The significance of the difference in mean lymphocyte counts and in mean age of patients between various disease stages and between various response groups was determined using the two-tailed Mann-Whitney U test.

The results quoted in the tables are the mean $\pm$ standard deviation of the lymphocyte counts.

4.2.2. Results

Little difficulty was usually encountered with the mononuclear band separation over ficoll-hypaque. However, when patients were very ill there was often severe contamination of the band by granulocytes

and red blood cells and sometimes, though rarely, in such patients no separation was possible at all. Granulocyte contamination in the various groups prior to treatment was 3.1±1.5% for controls; 5.4±4% for benign breast disease; 5.3±3.2% for Stage I and II breast cancer; 8.0±3.8% for Stage III breast cancer; 6.6±2.9% for metastatic breast cancer. Viability was always greater than 95% of the total lymphocytes.

Effect of age on lymphocyte counts [Appendix 4.1 - 4.4]

Percentage T lymphocyte counts (% T) did not vary with age in patients with benign breast disease (Table 4.2) but the oldest woman assessed was only 62 years. Absolute T lymphocyte (Abs T) and absolute lymphocyte (Abs L) counts tended to decrease slightly with increasing age but there was a significant difference only between women younger than 30 years and those over 50 years ($p<0.01$).

There was no significant change in % T, Abs T or Abs L counts with age in women with operable breast cancer until the age of 75 years when there was a sharp and significant ($p<0.05$) drop in % T counts and a non-significant decrease in Abs T and Abs L counts (Table 4.3). Patients with Stage IV breast cancer similarly had no significant age related change in % T, Abst T or Abs L count although, as in those with operable disease, both Abs T and Abs L tended to be lower in women older than 75 years (Table 4.4).

Effect of disease status on lymphocyte counts [Appendix 4.1 - 4.4]

Patients with benign breast disease were significantly younger (39.1±9 years) than those with breast cancer but between the stages of cancer no significant age difference was noted (Table 4.5). Patients with breast cancer had significantly depressed ($p<0.001$) % T counts

Table 4.2

Variation in % T, Abs T, and Abs L, with Age in Patients with Benign Breast Disease

	15-30 yrs	31-40 yrs	41-50 yrs	50-65 yrs
% T	61.9±4.8 (n=10)	59.7±6.3 (n=16)	59.6±4.5 (n=20)	59.5±5.7 (n=4)
Abs T	1.454±0.196 (n=6)	1.264±0.430 (n=11)	1.278±0.581 (n=10)	0.855±0.206* (n=3)
Abs L	2.414±0.207 (n=6)	2.150±0.743 (n=11)	2.125±0.948 (n=10)	1.512±0.387* (n=3)

*Compared with patients 15 to 30 years old ($Z=0$, $p<0.01$)

Mann-Whitney U test

Table 4.3

Variation in % T, Abs T, and Abs L with Age in Patients with Operable Stage I, II, & III, Breast Cancer

	30-45 yrs	46-55 yrs	56-65 yrs	66-75 yrs	75+ yrs
% T	52.7±8.0 (n=9)	54.5±6.8 (n=13)	50.1±8.4 (n=8)	56.7±10.3 (n=9)	41.3±11.5* (n=6)
Abs T	0.985±0.265 (n=9)	0.979±0.281 (n=12)	1.241±0.949 (n=6)	0.971±0.457 (n=9)	0.723±0.431 (n=5)
Abs L	1.863±0.456 (n=11)	1.845±0.451 (n=14)	2.161±1.243 (n=8)	1.695±0.675 (n=9)	1.598±0.494 (n=5)

* Compared with

30-45 yrs	U = 11.5,	p<0.05
46-55 yrs	U = 11.5,	p<0.05
56-65 yrs	U = 13.0,	NS
66-75 yrs	U = 7.5,	p<0.05

Mann-Whitney U test

Table 4.4

Variation in % T, Abs T, and Abs L with Age in Patients with Stage IV Breast Cancer Pre-Treatment

	30-45 yrs	46-55 yrs	56-65 yrs	66-75 yrs	75+ yrs
% T	45.2±10.5 (n=13)	46.6±12.1 (n=8)	50.8±9.1 (n=8)	49.5±11.6 (n=11)	47.6±16.8 (n=5)
Abs T	0.748±0.480 (n=13)	0.710±0.459 (n=6)	0.814±0.694 (n=7)	0.854±0.494 (n=7)	0.534±0.291 (n=4)
Abs L	1.660±0.994 (n=13)	1.443±0.829 (n=6)	1.536±1.230 (n=7)	1.730±0.682 (n=7)	1.095±0.483 (n=4)

Table 4.5

Variation in % T Lymphocyte Counts Between Different Stages

Stage	Age	% T	Mann-Whitney U test
Benign Breast Disease	39.1±9.0	60.1±5.2 (n=50)	
Stage I & II Breast Cancer	58.4±15.0	52.7±10.0* (n=34)	Z=3.77 p<0.001
Stage III Breast Cancer	64.2±12.8	50.9±9.7* (n=21)	Z=3.69 p<0.001
Stage IV Breast Cancer	57.9±14.8	47.8±11.3*+ (n=45)	Z=5.89* p<0.001 Z=1.78+ p<0.05

* Compared with benign breast disease

+ Compared with Stage I and II breast cancer

compared to those with benign breast disease (Table 4.5, Fig. 4.4) and patients with Stage IV disease had significantly lower ($p<0.05$) % T counts than those with Stage I and II breast cancer. Abs T counts were also significantly depressed (Table 4.6) in those with breast cancer compared with benign breast disease ($p<0.02$), this depression being most marked in patients with advanced disease ($p<0.001$). Abs T counts were significantly lower in patients with metastatic disease than in those with Stage I and II cancer ($p<0.02$). There was also a trend for the Abs L counts to be depressed in cancer patients in comparison to those with benign breast disease but this only attained significance ($p<0.01$) in those with Stage IV breast cancer (Table 4.7). Comparison between operable and metastatic breast cancer within each group (Tables 4.8, 4.9 & 4.10) showed a trend to depressed % T, Abs T and Abs L counts in those with Stage IV disease; apart from patients over 75 years of age with operable disease, whose % T counts were lower than those with metastatic cancer.

Effect of surgery on lymphocyte counts [Appendix 4.2 - 4.6]

All patients studied here had Patey mastectomy without any additional adjuvant treatment and counts were performed at least 6 weeks after surgery. There was no difference between pre- and post-operative % T, Abs T or Abs L counts in patients undergoing mastectomy for either Stage I and II or Stage III breast cancer (Table 4.11 & 4.12) (Fig. 4.5).

Relationship between prognostic factors and pre-operative lymphocyte counts [Appendix 4.7 & 4.8)

Tumour histology was not sufficiently specific in the majority of patients to allow useful comparison with pre-operative lymphocyte counts. Histological status of axillary lymph nodes and oestrogen

Table 4.6

Variation in Absolute T Lymphocyte Counts Between Different Stages

Stage	Age	Abs T	Mann-Whitney U test
Benign Breast Disease	39.3±9.9	1.266±0.451 (n=30)	
Stage I & II Breast Cancer	57.6±15.0	0.993±0.513* (n=32)	Z=2.3 p<0.02
Stage III Breast Cancer	64.3±13.1	0.949±0.291* (n=20)	Z=2.59 p<0.01
Stage IV Breast Cancer	56.3±15.1	0.751±0.492*+ (n=37)	Z=4.24* p<0.001 Z=2.73+ p<0.01

* Compared with benign breast disease

+ Compared with Stage I and II breast cancer

Table 4.7

Variation in Absolute Lymphocyte Counts Between Different Stages

Stage	Age	Abs L	Mann-Whitney U test
Benign Breast Disease	39.3±9.9	2.149±0.753 (n=30)	
Stage I & II Breast Cancer	56.3±14.7	1.842±0.737 (n=36)	
Stage III Breast Cancer	64.1±12.5	1.849±0.501 (n=22)	
Stage IV Breast Cancer	56.3±15.1	1.554±0.900* (n=37)	Z=3.03* p<0.005

* Compared with benign breast disease

Table 4.8

% T: Variation with Age, Stage I, II, III, and IV Breast Cancer Pre-Treatment

Age	Stage I, II, III Operable Breast Cancer % T	Stage IV Breast Cancer % T	Mann-Whitney U test
30-45 yrs	52.7±8.0 (n=9)	45.2±10.5 (n=13)	NS
46-55 yrs	54.5±6.8 (n=13)	46.6±12.1 (n=8)	NS
56-65 yrs	50.1±8.4 (n=8)	50.8±9.1 (n=8)	NS
66-75 yrs	56.7±10.3 (n=9)	49.5±11.6 (n=11)	NS
75+ yrs	41.3±11.5 (n=6)	47.6±16.8 (n=5)	NS

NS = non-significant

Table 4.9

Abs T: Variation with Age, Stage I, II, III, and IV Breast Cancer Pre-Treatment

Age	Stage I, II, III Operable Breast Cancer Abs T	Stage IV Breast Cancer Abs T	Mann-Whitney U test
30-45 yrs	0.985±0.265 (n=9)	0.748±0.480 (n=13)	NS
46-55 yrs	0.979±0.281 (n=12)	0.710±0.459 (n=6)	NS
56-65 yrs	1.241±0.949 (n=6)	0.814±0.694 (n=7)	NS
66-75 yrs	0.971±0.457 (n=9)	0.854±0.494 (n=7)	NS
75+ yrs	0.712±0.374 (n=5)	0.534±0.291 (n=4)	NS

NS= non-significant

Table 4.10

Abs L: Variation with Age, Stage I, II, III, and IV Breast Cancer Pre-Treatment

Age	Stage I, II, III Operable Breast Cancer Abs L	Stage IV Breast Cancer Abs L	Mann-Whitney U test
30-45 yrs	1.863±0.456 (n=11)	1.660±0.994 (n=13)	NS
46-55 yrs	1.845±0.451 (n=14)	1.443±0.829 (n=6)	NS
56-65 yrs	2.161±1.243 (n=8)	1.536±1.230 (n=7)	NS
66-75 yrs	1.695±0.675 (n=9)	1.730±0.682 (n=7)	NS
75+ yrs	1.598±0.494 (n=5)	1.095±0.483 (n=4)	NS

NS = non-significant

Table 4.11

Stage I & II Breast Cancer

Comparison of Pre- and Post-Operative Lymphocyte Counts

	Pre-Operative	Post-Operative	Mann-Whitney U test
% T	52.9±8.2 (n=27)	52.0±7.5 (n=21)	NS
Age	54.3±12.3	57.7±12.2	
Abs T	1.025±0.488 (n=25)	1.199±0.408 (n=19)	NS
Age	53.1±11.7	57.4±11.3	
Abs L	1.900±0.702 (n=29)	2.202±0.726 (n=20)	NS
Age	52.0±11.3	56.8±11.3	

NS = non-significant

Table 4.12

Stage III Breast Cancer

Comparison of Pre- and Post-Operative Lymphocyte Counts

	Pre-Operative	Post-Operative	Mann-Whitney U test
% T	49.9±8.7 (n=10)	50.5±8.9 (n=8)	NS
Age	58.3±11.8	60.0±6.8	
Abs T	0.953±0.308 (n=9)	0.904±0.352 (n=7)	NS
Age	57.8±12.4	59.6±7.3	
Abs L	1.845±0.511 (n=11)	1.735±0.561 (n=7)	NS
Age	58.6±11.2	59.6±7.3	

NS = non-significant

Fig. 4.4

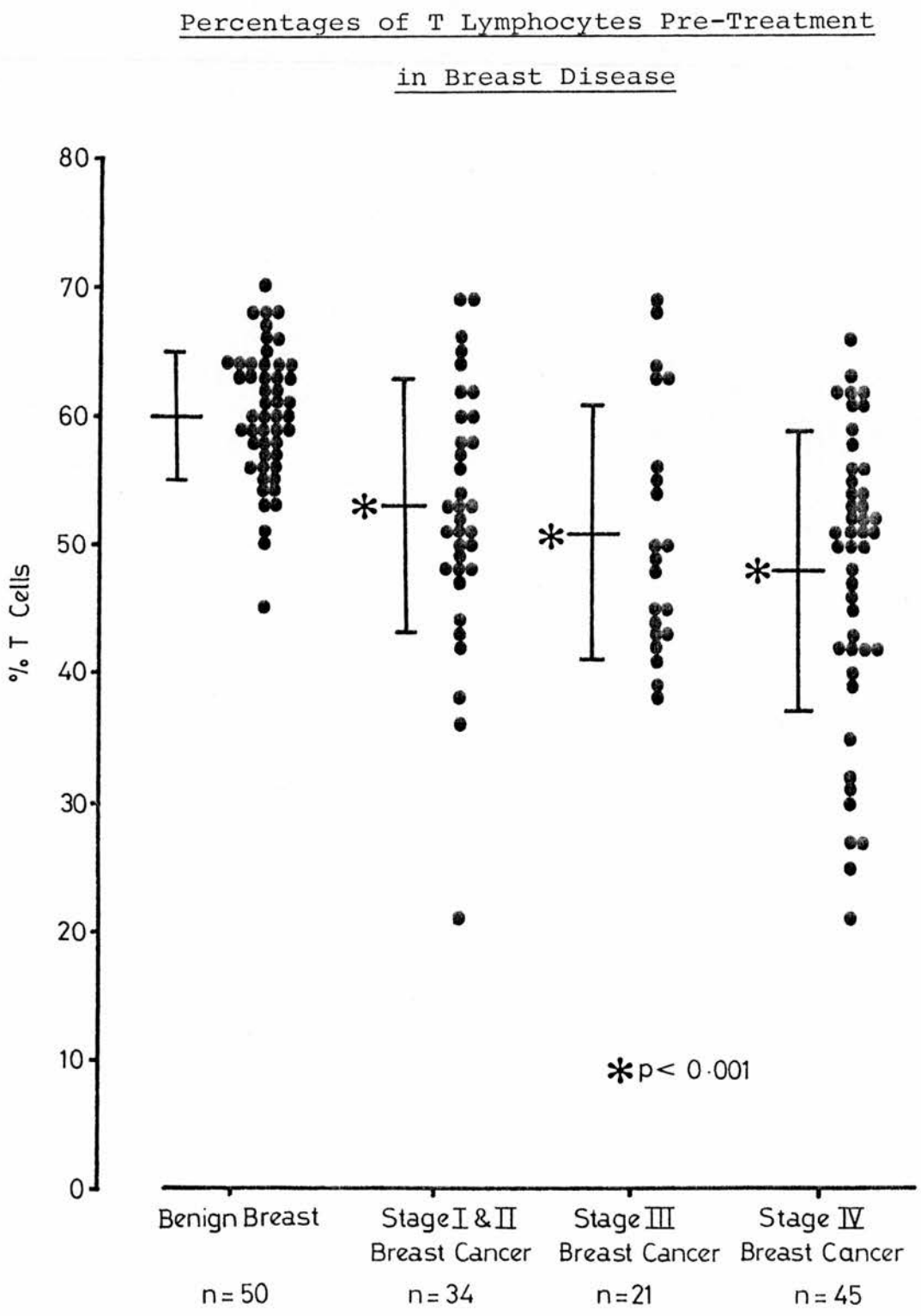
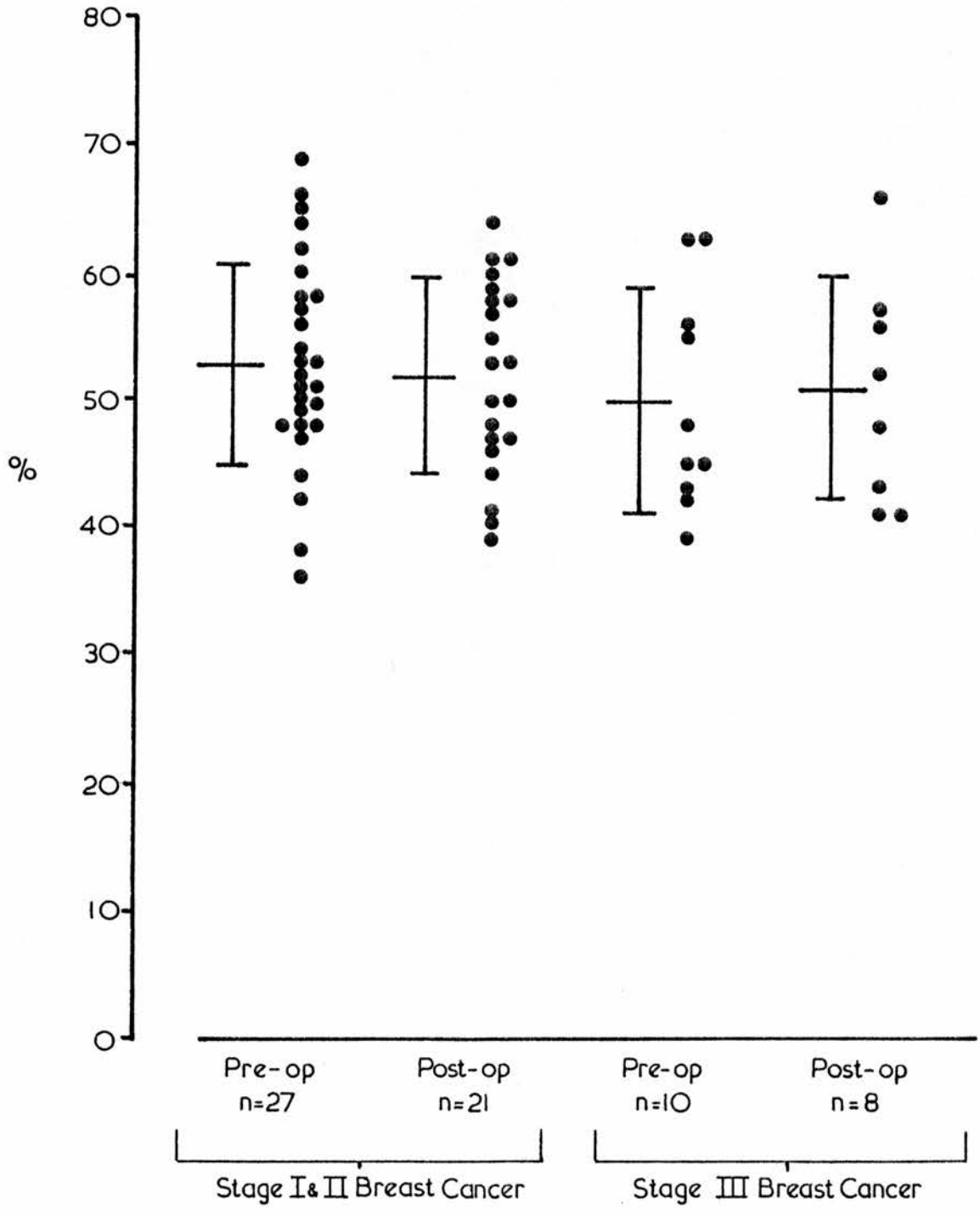

Percentages of T Lymphocytes Pre-Treatment
in Breast Disease
% T Cells
80
70
60
50
40
30
20
10
0
*p< 0·001
Benign Breast
n = 50
Stage I & II
Breast Cancer
n = 34
Stage III
Breast Cancer
n = 21
Stage IV
Breast Cancer
n = 45

Fig. 4.5

% T Cell Counts Pre- and Post-Operatively



receptor (ER) status of primary tumours were reported in sufficient numbers to permit such comparison.

Patients with node metastases tended to be younger than those with tumour free nodes, this age difference being significant in the group of women undergoing % T count assessment ($p<0.05$). No difference was noted in pre-operative % T, Abs T or Abs L counts between patients found to have tumour invasion of axillary nodes and those without node metastases (Table 4.13)

Women with ER negative tumours were significantly younger ($p<0.05$) than those with ER positive tumours. No difference in pre-operative % T, Abs T or Abs L counts was seen between the two groups (Table 4.14).

Relationship of pre- and post-operative lymphocyte counts to later tumour recurrence [Appendix 4.9 & 4.10]

Lymphocyte counts were examined pre-operatively and between six and 12 weeks post-operatively in patients undergoing Patey mastectomy without additional adjuvant therapy. These were related to recurrence within a four year period from surgery, the minimum follow-up time, to see if depressed pre-operative lymphocyte counts predicted earlier recurrence, or if post-operative lymphocyte count depression indicated residual disease in the form of micrometastases.

There was no significant age difference between patients developing recurrence or those remaining disease free at 4 years. Those who developed later recurrence tended to have slightly higher, but not statistically significantly higher, lymphocyte counts pre-operatively (Table 4.15). No difference was noted between lymphocyte counts post-operatively in patients developing recurrence or remaining free of disease at four years (Table 4.16).

Effect of adjuvant tamoxifen on lymphocyte counts [Appendix 4.11]

Table 4.13

Stage I, II, & III Breast Cancer

Pre-Operative Lymphocyte Counts Related to Axillary Node Histology

	Node Negative	Node Positive	Mann-Whitney U test
% T	53.4±9.7 (n=24)	53.8±7.6 (n=12)	NS
Age	60.1±12.4	50.9±10.9	
Abs T	1.058±0.566 (n=22)	1.020±0.339 (n=11)	NS
Age	59.2±12.3	51.7±11.0	
Abs L	1.905±0.818 (n=22)	1.872±0.624 (n=13)	NS
Age	59.2±12.3	53.4±10.8	

NS = non-significant

Table 4.14

Stage I, II & III Breast Cancer

Pre-Operative Lymphocyte Counts Related to Oestrogen Receptor Status of Primary Tumour

	ER Positive	ER Negative	Mann-Whitney U test
Patients	18	10	
Age	56.8±10.0	47.7±9.9	
% T	57.3±8.0	53.3±7.4	NS
Abs T	1.084±0.579	1.015±0.413	NS
Abs L	1.886±0.877	1.887±0.645	NS

Table 4.15

Stage I, II & III Breast Cancer

Pre-Operative Lymphocyte Counts Related to Tumour Recurrence Within 4 Years from Mastectomy

	No Recurrence	Recurrence	Mann-Whitney U test
Patients	15	11	
Age	54.3±12.7	54.5±12.5	
% T	52.9±7.4	54.9±8.3	NS
Abs T	0.882±0.266	1.027±0.270	NS
Abs L	1.666±0.445	1.893±0.435	NS

NS = non-significant

Table 4.16

Stage I, II & III Breast Cancer

Post-Operative Lymphocyte Counts Related to Tumour Recurrence

Within 4 Years from Mastectomy

	No Recurrence	Recurrence	Mann-Whitney U test
% T	52.0±7.2 (n=17)	52.2±8.7 (n=10)	NS
Age	55.8±12.1	60.2±11.3	
Abs T	1.107±0.455 (n=13)	1.093±0.411 (n=9)	NS
Age	59.0±11.8	58.1±9.8	
Abs L	2.101±0.856 (n=13)	2.027±0.650 (n=9)	NS
Age	59.0±11.8	58.1±9.8	

NS = non-significant

A group of post-menopausal patients with Stage II breast cancer were given tamoxifen 10mg for 2 years following mastectomy as part of a controlled randomised study. Data on lymphocyte counts was available in 12 of these women but there was insufficient information on pre-operative lymphocyte counts to compare these with post-operative results. A reasonably comparable group of post-menopausal patients undergoing mastectomy for Stage II breast cancer, who were given no form of adjuvant therapy, was selected and their post-operative lymphocyte counts compared with those of the adjuvant tamoxifen group. The two groups were comparable for age and axillary node histological status (Table 4.17) but there was some discrepancy in that a larger number of patients given adjuvant tamoxifen post-operatively had ER positive tumours. Interestingly, only one of 12 patients on tamoxifen developed tumour recurrence whereas 4 of 10 patients in the control group did so within 4 years.

There was a trend for patients on tamoxifen to have higher Abs T and Abs L counts but % T counts were significantly elevated (Fig. 4.6) in these patients compared with the non-adjuvant group ($p<0.05$), and indeed were comparable to % T counts of patients with benign breast disease. Bearing in mind that earlier results here have shown that surgery does not result in an increase post-operatively from pre-operative % T counts, tamoxifen by itself appears to have the effect of increasing % T counts.

Effect of tamoxifen on primary locoregional breast cancer and on lymphocyte counts in such patients

Fifteen patients were treated with tamoxifen 20mg b.d. as they were considered unfit for any other form of therapy. Seven achieved partial remission (PR) of median length 28 (5-50) months; 3 died of intercurrent illness soon after the start of treatment and disease continued to progress in the remaining 5 patients. Pre-treatment lympho-

Table 4.17

Stage II Breast Cancer - Post-Menopausal Women

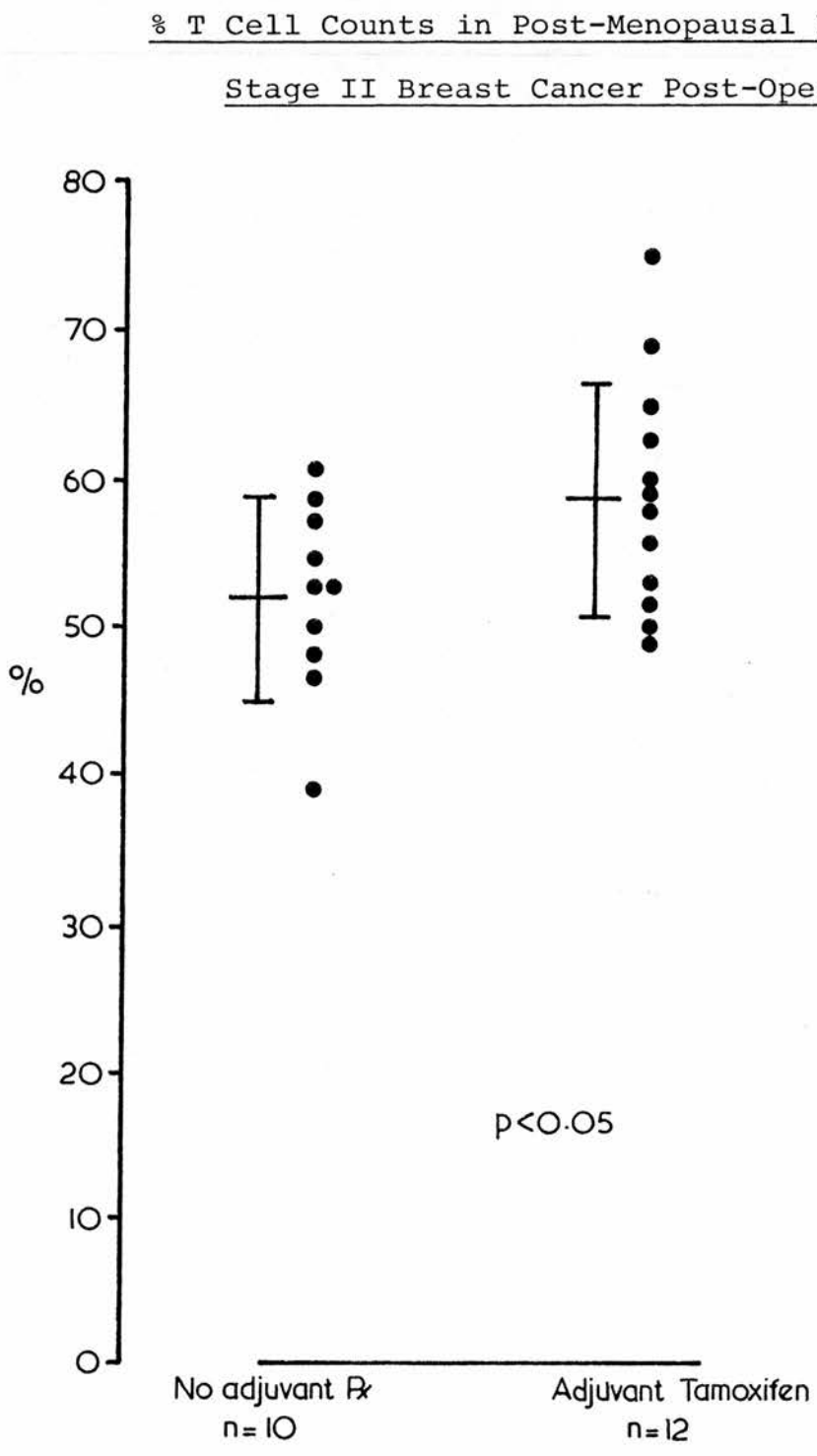
Post-Operative Lymphocyte Counts Related to

Adjuvant Tamoxifen Therapy

	No Adjuvant Therapy	Adjuvant Tamoxifen	Mann-Whitney U test
Patients	10	12	
Age	64.3±10.1	63.3±7.3	
Recurrence <4yrs	4	1	
ER Status	2+ve;4-ve;4N/A	7+ve;1-ve;4N/A	
Nodal Status	7-ve;3+ve	9-ve;3+ve	
% T	52.2±6.5	59.1±7.9	$U=29.0, p=^{0}.05$
Abs T	1.206±0.457	1.480±0.659	NS
Abs L	2.294±0.842	2.553±1.141	NS

NS = non-significant

Fig. 4.6


% T Cell Counts in Post-Menopausal Patients with Stage II Breast Cancer Post-Operatively
80
70
60
50
%
40
30
20
10
0
p<0.05
No adjuvant Rx
n=10
Adjuvant Tamoxifen
n=12

cyte counts were available in 12 patients and post-treatment data in 5 patients undergoing PR and in 5 with continuing disease progression (Table 4.18).

There was no difference in lymphocyte counts between patients pre-treatment and patients with progressive disease on treatment. There was a non-significant rise from pre-treatment levels in % T, Abs T and Abs L counts in patients developing PR of disease. When patients with progressive disease were compared with those achieving PR at 3 months after treatment, there was a significant difference in % T counts ($p<0.05$) and rises approaching significance in Abs T and Abs L counts in patients benefiting from treatment.

Effect of hormonotherapy on metastatic breast cancer

Two of 5 patients treated with a combination of tamoxifen 20mg b.d. and prednisolone 10mg t.d.s. responded, 1 PR of 13 months duration and 1 achieving no change (NC) for 9 months. The remaining 3 patients were all dead within 6 months. None of the 5 patients were assessed for lymphocyte counts following treatment.

The remaining 55 patients were assessed in terms of age, menopausal status, oestrogen receptor status and predominant site of disease, with regard to outcome to treatments shown in Table 4.1. Twelve patients achieved PR of median duration 15 to 16 (6-43) months; 4 developed NC and 9 were not assessable (NA) in terms of UICC criteria, 3 having local ulcers, 2 having lung effusions, 1 having multiple disease with bilateral lung effusions, 1 having radiotherapy for femoral secondaries, 1 having excision of a spinal dural lesion followed by radiotherapy and 1 having excision of supraclavicular nodes. All of these 9 patients had resolution of ulcers, effusions and symptoms and none developed any symptom or sign

of progression for at least 6 months whilst on treatment. The median duration of cessation of disease progression in those with NC or NA was 14 (6-68) months, apparently doing as well as those achieving PR on treatment. Overall the median delay to progression in disease for PR, NC, NA, was 15 (6-68) months. Thirty patients did not benefit from hormonal manipulation and their disease continued to steadily progress.

The mean age of those with progressive disease was 52.7±13.4 years, significantly younger ($p<0.01$) than those in whom the disease process was halted by treatment; 62.4±11.8 years. This is in accord with the finding that 54% of patients with Stage IV disease who benefited from hormone therapy were post-menopausal compared with only 21% of pre-menopausal women (Table 4.19).

Oestrogen receptor data was available in only 28 of 55 patients (Table 4.20) but it was notable that no patient whose tumour was ER negative achieved benefit from treatment.

Patients with locoregional or bone disease tended to benefit more than those with visceral or multiple disease (Table 4.21).

Effect of extent of Stage IV disease dissemination on pre-treatment lymphocyte counts [Appendix 4.12]

Forty-one patients had clinically proven disseminated disease and 14 had locoregional recurrence only, but of these only 26 with dissemination and 12 with locoregional recurrence had pre-operative lymphocyte count data. Those with locoregional disease were older (64.3±15.3) and tended to have higher % T, Abs T and Abs L counts although not significantly so (Table 4.22).

Effectiveness of hormone therapy on Stage IV breast cancer related to lymphocyte counts [Appendix 4.13]

Patients had lymphocyte counts performed prior to start of treatment,

Table 4.18

Stage I, II & III Breast Cancer Treated with Tamoxifen

	Pre Rx	Progression	PR	Mann-Whitney U test: Pre Rx vs Prog.	Pre Rx vs PR	Progression vs PR
	(n=12)	(n=5)	(n=5)			
Age	79±8	74±6	81±5			
% T	49.4±13.6	49.2±4.1	59.2±6.4	NS	NS	U=3.0; p<0.05
Abs T	0.851±0.337	0.835±0.326	1.374±0.566	NS	NS	NS
Abs L	1.673±0.424	1.700±0.537	2.299±0.840	NS	NS	NS

NS = non-significant

Table 4.19

Stage IV Breast Cancer. Menopausal Status Related to Cessation of Progression of Disease

	PR	NC/NA	Progression	Cessation of Disease Progression (% of Patients)
Pre-Menopausal	2	1	11	21
Post-Menopausal	10	12	19	54

Table 4.20

Stage IV Breast Cancer. Oestrogen Receptor Status

	PR	NC/NA	Prog.
Positive	3	5	14
Negative	0	0	6
Not Performed	9	8	10

Table 4.21

Stage IV Breast Cancer. Predominant Site of Disease Related to Cessation of Progression of Disease

	PR	NC/NA	Prog.	Cessation of Disease Progression (% of Patients)
Locoregional	4	5	7	56
Bone	6	4	11	48
Visceral	2	3	9	36
Multiple	0	1	3	25

Table 4.22

Stage IV Breast Cancer

Pre-Treatment Variation in Lymphocyte Counts with Locoregional and Disseminated Disease

	Locoregional Disease	Disseminated Disease	Mann-Whitney U test
Age	64.3±10.9	55.6±15.3	NS
% T	50.3±10.6 (n=12)	46.3±10.9 (n=26)	NS
Abs T	1.011±0.589 (n=9)	0.743±0.485 (n=24)	NS
Abs L	1.967±0.998 (n=9)	1.536±0.891 (n=24)	NS

NS = non-significant

6 weeks later, and a mean of counts performed later than 3 months from the beginning of treatment was also calculated for each individual. With regard to patients who appeared to benefit from treatment any lymphocyte count within 2 months of objective recurrence was termed the pre-recurrence count. Lymphocyte counts were also performed in these patients following objective recurrence.

Where patients benefited the Abs L count tended to rise slightly (Table 4.23) but this effect was not seen for at least 3 months. This increase, although never significant, was most marked in patients who achieved PR on treatment.

Abs T counts increased but not significantly in those showing PR (Table 4.24). However, there was a significant difference ($p<0.05$) by 6 weeks between this group of responders and patients in whom disease progression continued, this difference becoming more marked still ($p<0.02$) after 3 months' treatment.

Percentage T counts also increased significantly ($p<0.002$) by 6 weeks in patients in whom disease progression appeared to be halted by treatment (Table 4.25) and this increase was maintained ($p<0.05$) during the period of benefit. The difference between this group of patients and those with progressing disease was highly significant ($p<0.001$) by 6 weeks of therapy and continued to be marked during the period of benefit (Fig. 4.7).

In those patients benefiting from hormone therapy Abs L counts maintained their slightly increased value right up to and indeed following objective evidence of recurrence (Table 4.26). Percentage T counts on the other hand, in 8 patients in whom lymphocyte counts were performed within the two month period prior to objective disease progression, dropped significantly ($p<0.001$) in comparison to their counts during response and

Table 4.23

Stage IV Breast Disease -

Hormone Therapy Abs L Count Change with Treatment

	Pre-Treatment	Treatment 6/52	Treatment Mean >3/12
PR	1.630±0.733 (n=6)	1.865±0.723 (n=6)	2.219±0.533 (n=8)
PR/NC/NA	1.595±0.718 (n=13)	1.513±0.732 (n=13)	1.888±0.745 (n=20)
Progression	1.692±1.056 (n=20)	1.550±0.677 (n=17)	1.968±0.692 (n=8)

Mann-Whitney U test

Table 4.24

Stage IV Breast Cancer -

Hormone Therapy Abs T Count Change with Treatment

	Pre-Treatment	Treatment 6/52	Treatment Mean >3/12
PR	0.819±0.431 (n=6)	1.125±0.490 (n=6)	1.259±0.306 (n=8)
PR/NC/NA	0.798±0.466 (n=13)	0.885±0.454 (n=13)	1.040±0.415 (n=20)
Progression	0.828±0.565 (n=20)	0.717±0.336* (n=16)	0.769±0.280+ (n=9)

* Compared with PR at 6/52 (U=21.0, $p<0.05$)

\+ Compared with PR mean >3/12 (U=8.0, $p<0.02$)

Mann-Whitney U test

Table 4.25

Stage IV Breast Cancer -

Hormone Therapy. % T count Change with Treatment

	Pre-Treatment	Treatment 6/52	Treatment >3/12
PR	49.4±5.7 (n=10)	60.6±6.1** (n=7) (U=3.5)	56.9±5.8* (n=8) (U=13.5)
PR/NC/NA	48.9±10.9 (n=19)	59.5±5.0** (n=15) (U=48.0)	55.4±5.4* (n=21) (Z=2.18)
Progression	46.2±10.9 (n=21)	46.6±9.4+ (n=19) (U=13.0)	40.6±9.4++ (n=8) (U=5.5)

** Compared with pre-treatment count (p<0.002)

* Compared with pre-treatment count (p<0.05)

+ Compared with PR/NC/NA at 6/52 (p<0.001)

++ Compared with PR/NC/NA at >3/12 (p<0.001)

Mann-Whitney U test

Table 4.26

PR/NC/NA Stage IV Breast Cancer - Hormone Therapy. Change in Lymphocyte Counts with Cessation of Progression and Following Recurrence of Progression

	Pre-Treatment	Treatment 6/52	Treatment Mean>3/12	Pre-Recurrence	Recurrence
% T	48.9±10.9 (n=19)	59.5±5.0 (n=15)	55.4±5.4 (n=21)	46.4±3.5* (n=8)	46.0±4.8+ (n=9)
Abs T	0.798±0.466 (n=13)	0.885±0.454 (n=13)	1.040±0.415 (n=20)	0.786±0.401 (n=5)	0.951±0.462 (n=8)
Abs L	1.595±0.718 (n=13)	1.513±0.732 (n=13)	1.888±0.745 (n=20)	1.778±0.952 (n=5)	2.028±0.978 (n=8)

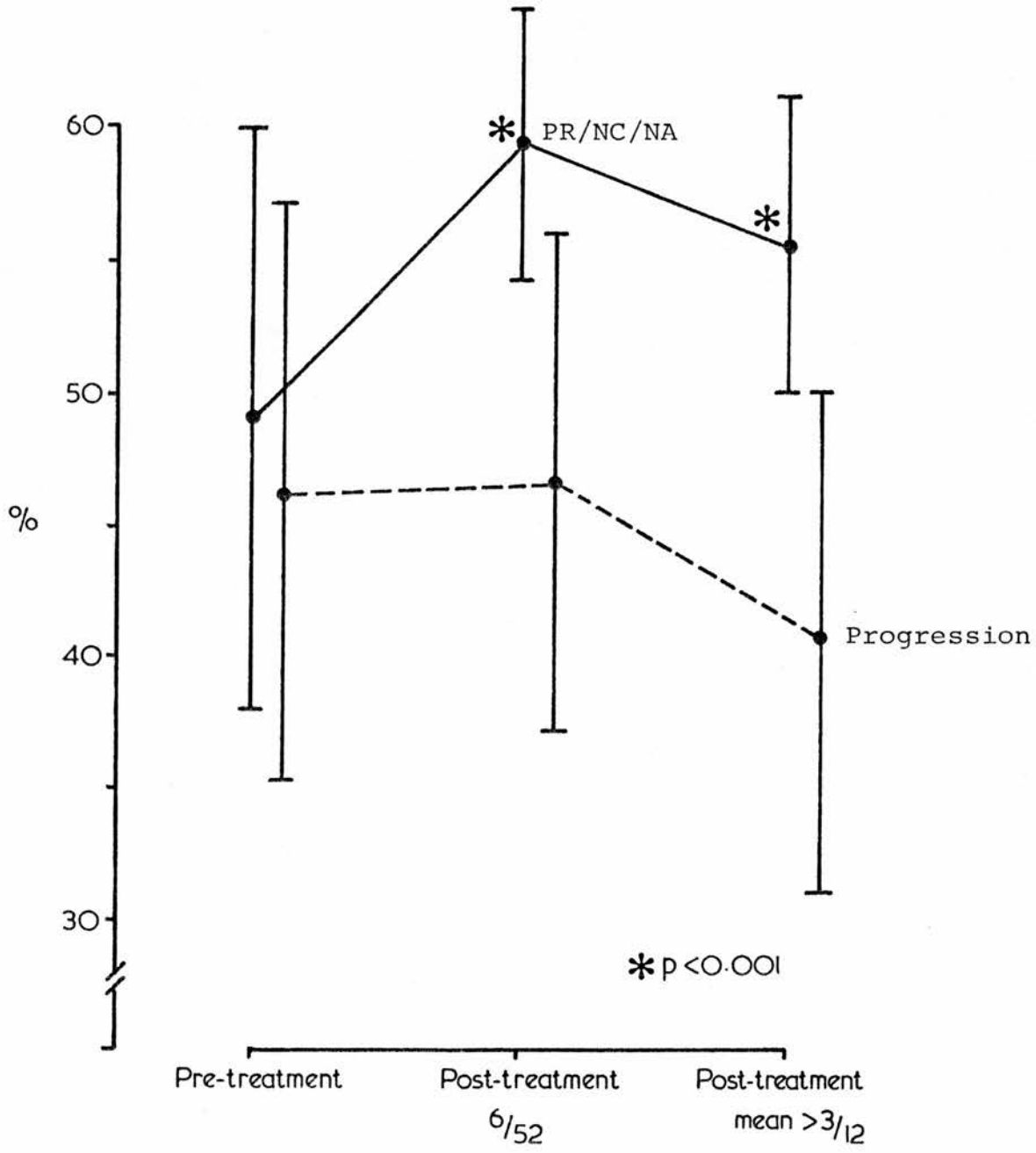
* Compared with treatment result at 6/52 (U=1.0) and at >3/12 (U=2.0) ($p<0.001$)

\+ Compared with treatment result at 6/52 (U=1.0) and at >3/12 (Z=3.7) ($p<0.001$)

Fig. 4.7

% T Cell Counts in Stage IV Breast Cancer

PR/NC/NA vs Progressive Disease



this depression was maintained at time of clinical recurrence of disease (Fig. 4.8). Objective evidence of clinical recurrence was therefore pre-dated by a significant drop in % T counts.

The changes in % T lymphocytes in individual patients with Stage IV breast cancer showing PR are demonstrated in Fig. 4.9. Changes in % T counts of all patients with Stage IV breast cancer, related to response to hormonal therapy are demonstrated in Fig. 4.10.

Fig. 4.8

% T Cell Counts in Patients with Stage IV Breast Cancer. Cessation of Disease Progression followed by New Progression

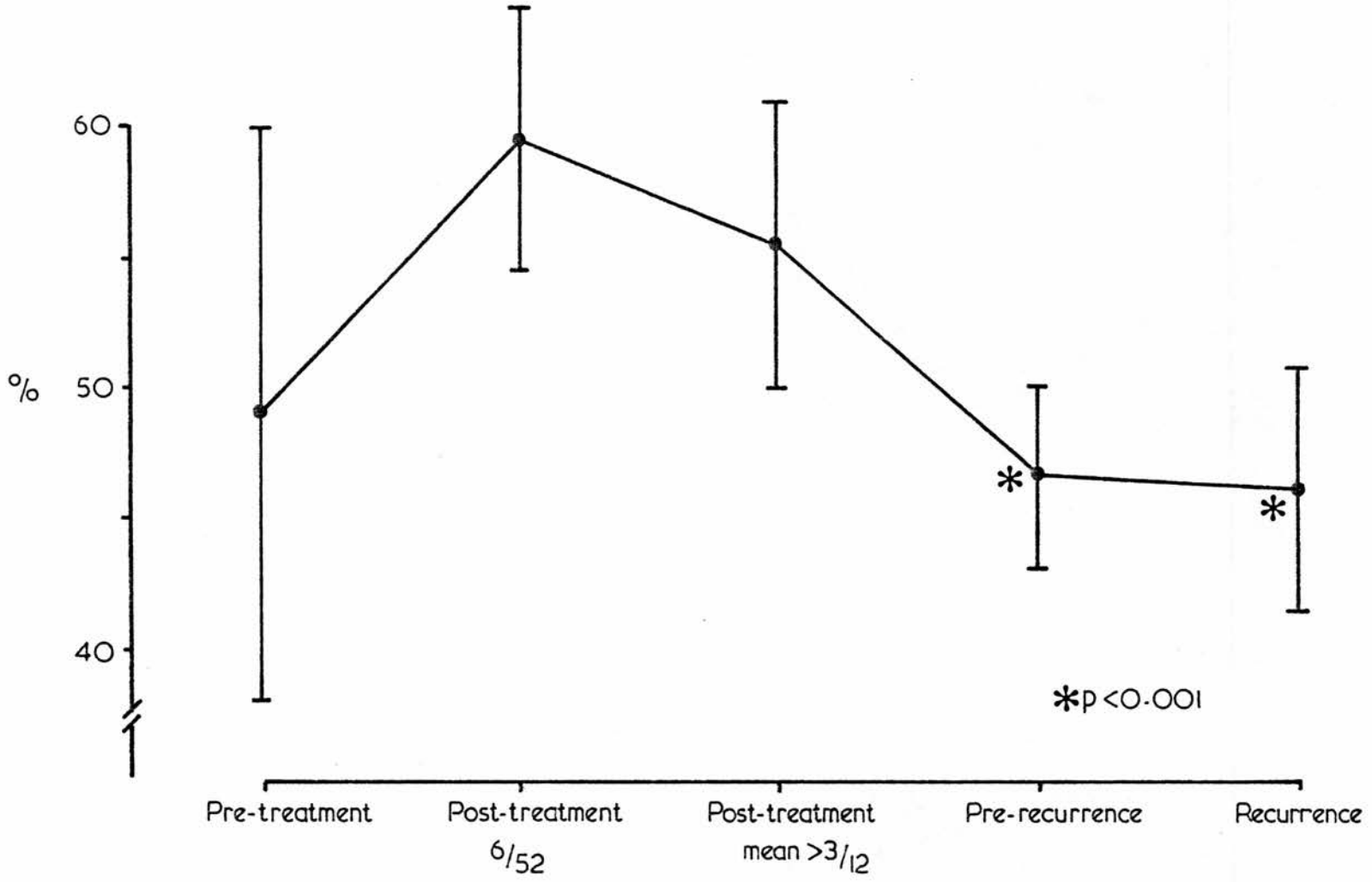


Fig. 4.9

Changes in % T Cell Counts in Individual Patients with Stage IV Breast Cancer showing P.R. on Hormone Therapy

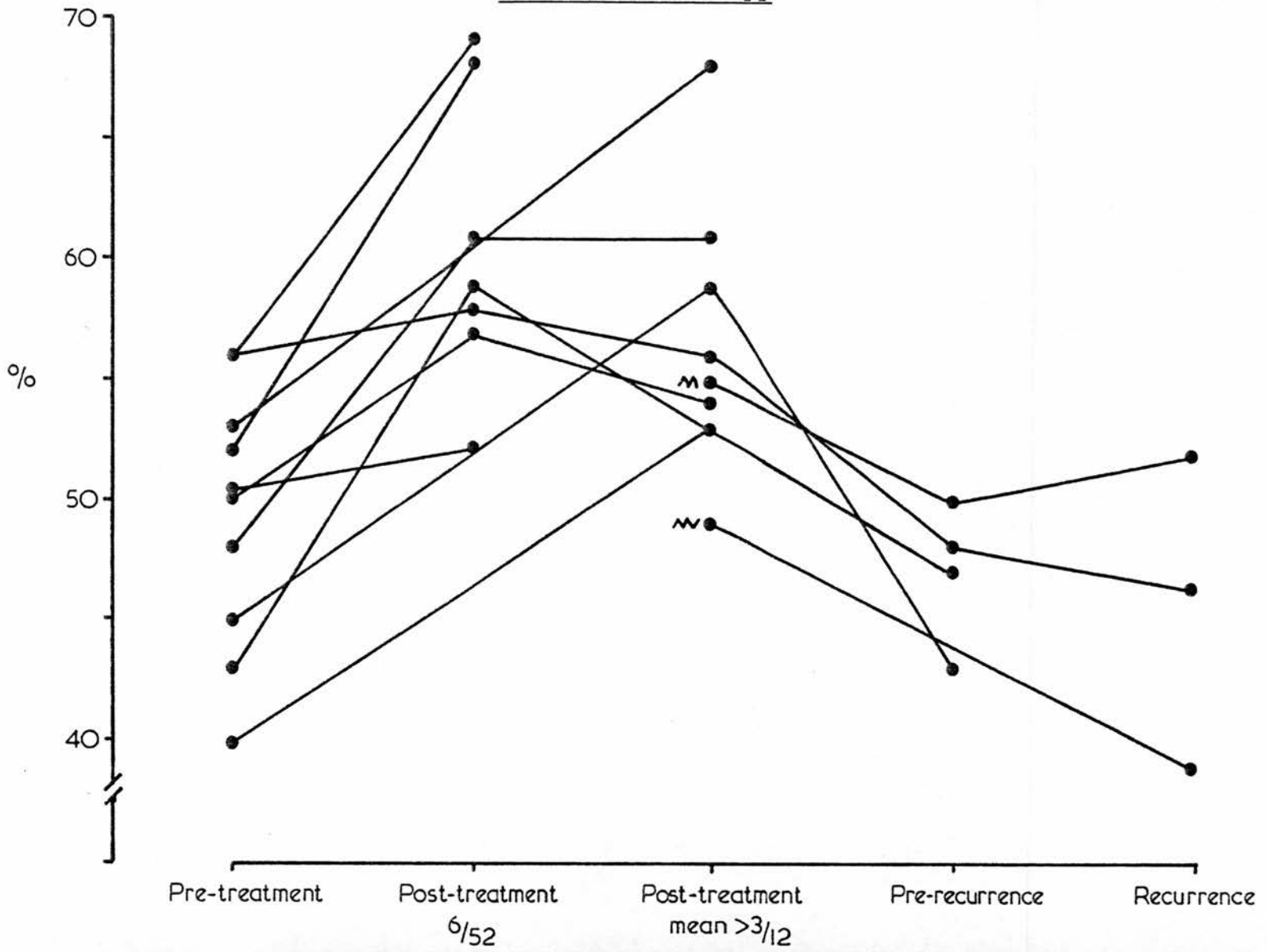
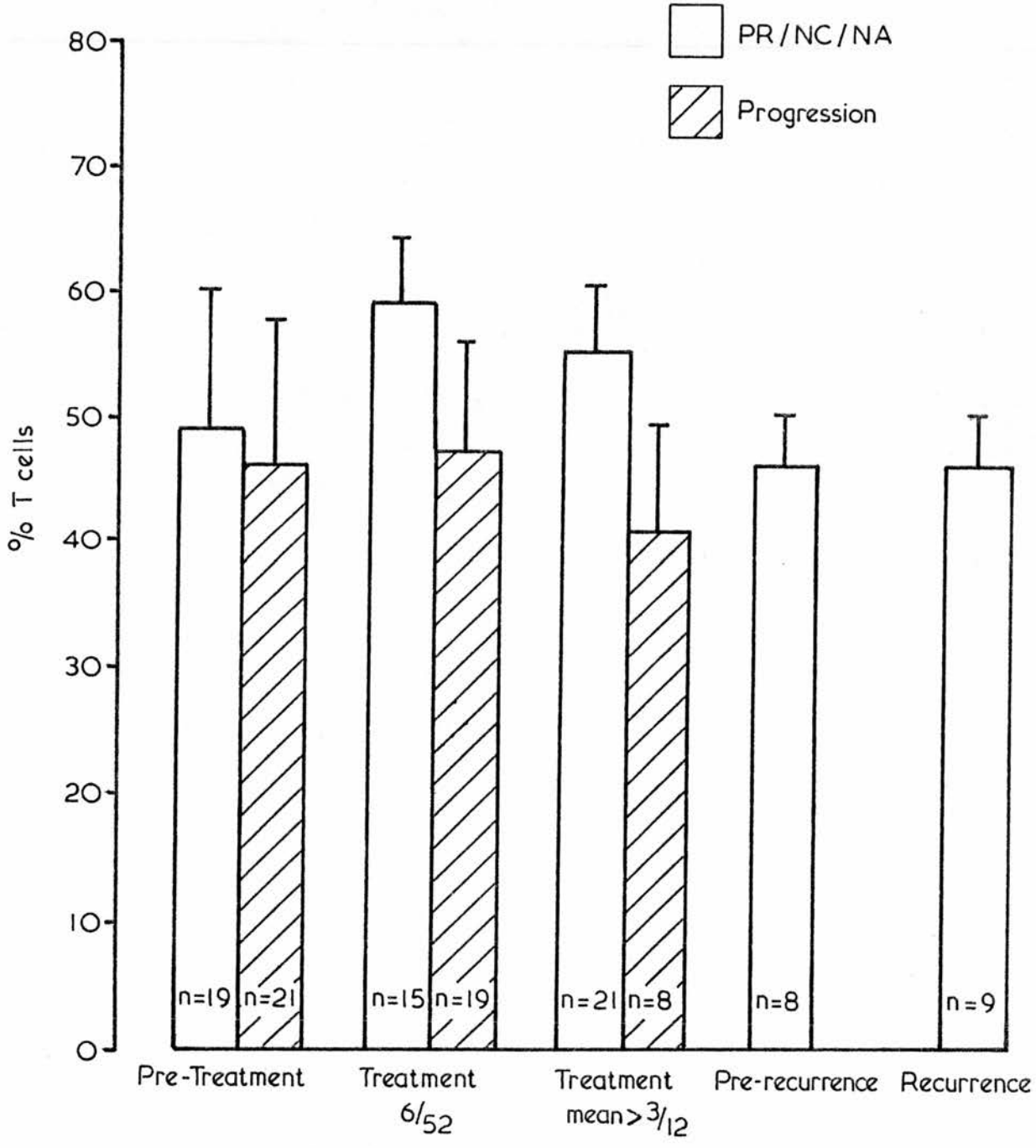


Fig. 4.10

Stage IV Breast Cancer - Hormone Therapy

% T Counts with Disease Response



4.3. Part II

To Show that Variation in E-Rosetting Methodology Alters T Lymphocyte Counts

4.3.1. Materials and methods

Peripheral blood was obtained from 5 groups:

(a) 9 healthy laboratory staff; b) 16 patients with benign breast disease; c) 41 patients with Stage I or II breast cancer; d) 9 patients with Stage III breast cancer; e) 31 patients with Stage IV breast cancer.

Assessment of patient responses, collection of blood, preparation of SRBC, and lymphocyte separation were carried out as previously described.

E-rosetting techniques:

1. Day 1 counts: This technique was the normal one used in the previous section to assess % T counts in patients with varying stages of breast disease, an aliquot of the lymphocyte suspension being rosetted in the usual fashion and counted after being chilled at $4^{o}C$ for one and a half hours.

2. Day 2 delayed counts: This technique was as above but the rosettes were not counted for at least 16 hours, being incubated at $4^{o}C$ overnight.

3. Day 2 counts (fresh rosettes): The lymphocyte suspension was left overnight at $4^{o}C$ for at least 16 hours, centrifuged at 400g for 5 mins and the supernatant removed. Fresh sterile isotonic saline was then added and the pellet resuspended vigorously and adjusted to a concentration of 2×10^{6} cells/ml and assessed for viability. A sample of this fresh suspension was then rosetted in the usual manner with freshly made-up SRBC, and lymphocytes and rosettes counted after

chilling at 4°C for one and a half hours.

4. Day 2 small pellet of lymphocytes: In some cases a third aliquot of the lymphocyte suspension was removed on the first day, pelleted without SRBC in a small round bottomed tube and incubated overnight at 4°C. On the following day only the uppermost layers of the pellet were gently resuspended, removed and rosetted in the usual manner with freshly made-up SRBC, and lymphocytes and rosettes counted after chilling at 4°C for one and a half hours.

Statistical methods:

The significance of the difference in mean % T lymphocyte counts within each group using different E-rosetting techniques was determined using paired 't' tests.

The significance of the difference in mean % T lymphocyte counts between groups for any particular E-rosetting technique was determined using the two-tailed Mann-Whitney U test. This non-parametric test was used to avoid the assumption that the data being analysed were parametric with a normal distribution.

All analyses were carried out on a BBC Master microcomputer using Microtab Statistical Software and probability values obtained from standard tables (Documenta Geigy, 7th Edition, 1970).

4.3.2. Results of variation in E-rosetting techniques

[Appendix 4.14]

The mean ± standard deviation of E-rosetting cells for each of the control and patient groups using the four different techniques of rosetting is set out in Tables 4.27, 4.28 & 4.29.

Viability of lymphocytes stored overnight at 4°C in isotonic saline was not impaired in comparison to that of freshly separated

lymphocytes.

The effect of overnight incubation of lymphocytes with SRBC

Some patients had % T counts performed on more than one occasion. The number of experiments in each disease group and the results are shown in Table 4.27.

There was a significant increase in % T counts in all groups following overnight incubation of rosetted lymphocytes ($p<0.001$) with resultant loss of any discriminatory differences in % T counts between the groups. This was particularly notable in patients with Stage IV breast cancer where the significant difference ($p<0.002$) in Day 1 counts between depressed % T counts in those with progressive disease and those with either early breast cancer or with disease responding to hormonal manipulation was lost after prolonged incubation. Indeed all breast disease patients had similar counts on the second day.

The effect of incubating lymphocytes alone overnight

The number of experiments in each disease group and the results are shown in Table 4.28.

Percentage T counts in lymphocytes incubated at $4^{o}C$ overnight and then rosetted with SRBC on Day 2 did not rise to the levels seen when rosettes were incubated overnight, the difference being significant ($p<0.001$). Although these freshly rosetted Day 2 counts were very similar to results seen on Day 1, they tended to be higher and there was an increasing trend for this to happen with increasing stage of disease, the difference in counts between the two methods being significant in the group of patients with progressing Stage IV cancer ($p<0.05$).

Table 4.27

Comparison of % T Counts Performed on First Day and Following Prolonged Incubation of Lymphocytes with SRBC

Group	Experiments	Mean age	Day 1 Counts	Day 2 (delayed counts)	Paired test
Control	9	38±5	63.9±4.4	74.7±6.9	t=5.789 p<0.001
Benign Breast Disease	16	38±10	57.5±5.2	68.0±7.8	t=6.392 p<0.001
Pre Rx Stage I & II Disease	23	55±13	56.3±6.1	69.4±7.1	t=9.273 p<0.001
Post Rx Stage I & II Disease	47	60±13	54.1±8.3	69.6±7.7	t=11.371 p<0.001
Stage III	13	62±10	53.8±8.0	70.1±11.5	t=6.641 p<0.001
Progressive/ New Stage IV Disease	21	59±16	44.2±10.0*	66.5±7.0	t=11.945 p<0.001
Post Rx Stage IV Disease in remission	20	68±10	53.4±6.6	69.5±8.4	t=7.184 p<0.001

* Comparison of Day 1 counts: Progressive/New Stage IV disease against Stage IV disease responding to hormone therapy (Z=3.07 p<0.002).

Mann-Whitney U test

Table 4.28

Comparison of % T Counts Performed on First Day, Following Fresh Rosetting on Day 2, and Following Prolonged Incubation of Lymphocytes with SRBC

Groups	No. of Experiments	Mean Age	Day 1 Counts	Day 2 Counts (Fresh Rosettes)	Day 2 (Delayed) Counts	Paired 't' test: Day 1 vs. Day 2 Fresh Rosettes	Paired 't' test: Day 1 vs. Day 2 Delayed	Paired 't' test: Day 2 Fresh Rosettes vs. Day 2 Delayed
Control	9	38±5	63.9±4.4	63.7±6.3	74.7±6.9	NS	t=5.789 p<0.001	t=5.702 p<0.001
Benign Breast Disease	16	38±10	57.5±5.2	58.6±8.1	68.0±7.8	NS	t=6.392 p<0.001	t=4.593 p<0.001
Stage I & II Breast Cancer	36	57±12	53.6±7.7	56.6±9.7	69.2±8.0	NS	t=10.872 p<0.001	t=7.189 p<0.001
Stage III Breast Cancer	8	62±13	53.4±9.0	57.0±11.3	68.1±13.6	NS	t=4.076 p<0.005	t=5.995 p<0.001
Progressive/New Stage IV Breast Cancer	11	59±15	41.0±12.2	50.1±9.5	68.6±7.5	t=2.639 p<0.05	t=12.632 p<0.001	t=5.961 p<0.001
Post Rx Stage IV Breast Cancer in Remission	11	63±11	50.7±6.2	55.0±5.5	67.1±8.6	NS	t=4.589 p<0.001	t=4.665 p<0.001

NS= non-significant

The effect of incubating small pellets of lymphocytes alone overnight

A possible explanation for the increase in % T counts seen following prolonged incubation of lymphocytes with SRBC is that cells with adherent properties, such as small monocytes, may be counted as non-rosetting lymphocytes on Day 1. Following overnight incubation such cells might remain adherent to the glassware during the gentle resuspension required to prevent rosette disruption, with consequent imbalance occurring in the numbers of individual cells and rosettes being counted compared with Day 1. To investigate this, small pellets of lymphocytes were incubated in round bottomed tubes overnight as described and then gently manipulated so that only the top layers of cells were resuspended and removed such that any glass adherent cells should remain behind. These resuspended cells were then rosetted with SRBC and counted in the usual manner.

Nine experiments were performed comparing this method to all three other methods in patients with benign breast disease and 19 experiments were similarly performed in patients with breast cancer. The results shown in Table 4.29 demonstrated that E-rosette counts were similar to those of Day 1 and freshly rosetted Day 2 counts, and lower than Day 2 delayed counts, significantly so in cancer patients ($p<0.001$), indicating that adherent cells do not interfere with the results of rosetting.

Table 4.29

Comparison of % T Lymphocyte Counts from Lymphocyte Pellets Incubated Alone Overnight with % T Counts from Other Methods

Group	Number of Experiments	Lymphocyte Pellets (small)	Day 1	Day 2 (Fresh Rosettes)	Day 2 (Delayed Counts)
Benign Breast Disease	9	59.8±13.0	56.2±5.4 NS	58.2±9.9 NS	66.1±7.3 NS
Breast Cancer	19	49.4±16.3	48.6±11.7 NS	53.6±9.7 NS	63.6±11.0 t=4.354 p<0.001

Paired 't' test

4.4. Part III

To Assess the Relationship Between % T Lymphocyte Counts and Circulating Immune Complexes

4.4.1. Materials and methods

Collection of sera

Sera was obtained from blood clotted at room temperature usually within 2 hours of venepuncture, placed in sterile containers in aliquots and stored at -70°C until use. Sera were not heat inactivated.

Assay for circulating antigen-antibody complexes

The assay used was the CIq deviation test and this was performed by Dr. Victor Danis PhD, University Department of Surgery, University Hospital of Wales, Cardiff. Circulating antigen-antibody complexes were assayed as previously described by him (Danis et al. 1980) with the following modifications. Sera were diluted ten-fold in veronal buffered saline (VBS) containing 1% BSA and normal human serum to a final concentration of 1%. Aggregated human IgG diluted in VBS - 1% BSA, 1% normal human serum was used as a reference immune complex in the assay. To 0.1ml of sample was added 0.1ml of radioactive iodine labelled purified CIq (containing 20,000 cpm radioactivity) and incubated for 30 mins at room temperature. Then 0.1ml of IgG coated latex (Behring - RF latex) diluted in VBS - 1% BSA was added and incubated for 30 mins at 4°C. Tubes were supported in microtitre trays while centrifuged at 400g for 40 mins at 4°C. The supernatants were discarded and the radioactivity in the latex pellets counted. Inhibition of the binding of the CIq to the latex particles by putative immune complexes in the serum was related to an equivalent inhibition produced by a known amount of aggregated IgG from a reference curve shown in Appendix 4.15.

All samples were assayed in duplicate and the results averaged. The degree of sensitivity depended on the proportion of CIq that bound to the latex and was dependent on the amount of latex used and also on the presence of normal serum factors, since the amount of CIq was kept constant. The figure of 40% binding in the presence of normal human serum was chosen because it gave maximum sensitivity with sufficient radioactivity to require only a two minute counting time on the gamma counter.

4.4.2. Results [see Appendix 5.1 - 5.5.]

Assays were performed on sera from 3 healthy females and 10 healthy males for comparison with % T counts and lymphocyte oxygen consumption (see Chapter 5). Controls used here, however, were 31 women with benign breast disease and their CIC levels compared with those from 20 patients with Stage I and II breast cancer, 15 with Stage III breast cancer, and 18 with Stage IV breast cancer. All were assayed prior to treatment for this comparison. Levels of CIC were also compared in pre- and post-operative patients with Stage I and II breast cancer and patients with Stage IV disease had CICs assessed in relation to their response to hormone therapy. The mean results and standard deviations are shown in Table 4.30 which demonstrates significantly higher levels of CIC in pre-treatment Stage I and II breast cancer ($p<0.05$) and Stage IV breast cancer ($p<0.001$) than in benign breast disease. There was no change in CIC levels following mastectomy for Stage I and II disease, nor following successful hormonal manipulation for metastatic cancer. Because some patients have such high levels of CIC compared to others even within the same disease group; a better way of assessing the relevance of CIC is to look at the number of patients within each group with CIC levels over a certain value. The number within each group with

Table 4.30

Breast Disease - Mean Levels of Circulating Immune Complexes Within Each Disease Group

	Age	Patients	Sera	CIC (µg/200µl AHG equivalents)	Mann-Whitney U Test
Benign	38±8	31	31	36.9±16.3	
Pre-operative Stages I & II	54±13	20	20	51.8±20.1	vs. BBD Z=2.77; p<0.05
Post-operative Stages I & II	58±11	29	41	53.4±29.3	vs. Pre-op. I & II: NS
Pre Rx Stage III	58±14	15	15	48.8±24.5	vs. BBD: NS
Pre Rx Stage IV	58±14	18	18	75.8±38.3	vs. BBD: Z=4.48; p<0.001 vs. Pre-op. I & II Z=2.11; p<0.05
Progressive Stage IV	57±15	13	14	88.0±42.0	vs. Pre Rx IV: NS
PR/NC/NA Stage IV	70±10	14	16	75.1±23.3	vs. Pre Rx IV: NS

NS= non-significant

levels of CIC above one and two standard deviations of CIC levels in benign breast disease are shown in Table 4.31. At two standard deviations above the mean for CIC levels in benign breast disease, between a quarter and a third of patients with locoregional breast cancer and half of women with metastic breast disease, had positive levels of CIC. Again, as with mean CIC levels, there was no apparent change with treatment.

To assess the relationship between % T counts and CICs, these were correlated in 139 blood samples from healthy adults and patients with benign and malignant breast disease who had both % T counts performed and serum taken for CIC assay on the same day. The correlation coefficient was -0.049 showing the negative coefficient value expected but the degree of correlation was not significant.

Table 4.31

Breast Disease - Number of Patients Positive for CIC Within Each Disease Group

	Age	Positive at 1 SD (=53µg/200µl AHG equivalents)	Positive at 2 SD (=69µg/200µl AHG equivalents)
Benign	38±8	$^{3}/_{31}$ (10%)	$^{1}/_{31}$ (3%)
Pre-operative Stages I & II	54±13	$^{11}/_{20}$ (55%)	$^{5}/_{20}$ (25%)
Post-operative Stages I & II	58±11	$^{11}/_{29}$ (38%)	$^{10}/_{29}$ (34%)
Pre Rx Stage III	58±14	$^{6}/_{15}$ (40%)	$^{5}/_{15}$ (33%)
Pre Rx Stage IV	58±14	$^{13}/_{18}$ (72%)	$^{8}/_{18}$ (44%)
Progressive Stage IV	57±15	$^{10}/_{14}$ (71%)	$^{7}/_{14}$ (50%)
PR/NC/NA Stage IV	70±10	$^{14}/_{16}$ (88%)	$^{8}/_{16}$ (50%)

4.5. Discussion

There was a tendency for women in the older age groups to have decreased absolute lymphocyte counts but this was significant only in the eldest patients with benign breast disease. However, this group was very small (3 patients) and cautious interpretation is therefore required. Percentage T counts did not vary with age in those with benign breast disease and appeared to reflect age changes noted by others (Smith et al. 1974; Holland et al. 1975; Whitehead et al. 1978; Nagel et al. 1981; Mascart-Lemone et al. 1982) only in patients with operable breast cancer over the age of 75 years. There was no variation of % T counts with age in patients with metastatic disease but such variation if present may have been masked by the general suppressive effect of such cancer on % T lymphocytes. Other investigators have similarly found little variation in lymphocyte counts with age (Weksler & Hutteroth 1974; Alexopoulos & Babitis 1976; Djeu et al. 1977) and it would appear that up to the age of 75 years no correction of lymphocyte counts need be made for age.

No variation was found in % T, Abs T or Abs L counts between Stage I, II and III breast cancer but these patients tended to have lower Abs L counts than patients with benign breast disease and higher Abs L counts than those with metastatic breast cancer. There was significant depression in Abs L counts in Stage IV patients compared to those with benign breast disease and this finding is compatible with that of other authors (Stein et al. 1976; Bolton et al. 1976; Kopersztych et al. 1976; Ownby et al. 1983; Shukla et al. 1986a) who found depressed Abs L counts only in patients with disseminated breast cancer.

Patients with Stage I, II and III breast cancer had significantly

depressed Abs T and % T counts compared to those with benign breast disease, and patients with Stage IV disease, had significantly depressed Abs T and % T counts compared to those with Stage I and II cancer. Indeed even within the group of patients with Stage IV disease, there was a trend for those with disseminated metastatic disease to have lower lymphocyte counts than those who apparently had only locoregional recurrence.

There is thus a relationship between cancer and depressed lymphocyte counts and this depression appears to be proportional to disease load, significantly so in terms of % T and Abs T counts. Whether the decrease in Abs L counts in cancer is due to general debility or to some other mechanism is unknown but depressed T counts are probably a consequence of increased blocking by serum in patients with larger tumour masses as suggested in the previous chapter. Other authors have found depression of T lymphocyte counts in early breast cancer (Stein et al. 1976; Keller et al. 1976) and a correlation between depressed T counts and breast cancer load (Whitehead et al. 1976), similar to findings reported here. However, others have been unable to demonstrate any change in % T or Abs T counts in untreated tumours (Stjernsward et al. 1972; Nemoto et al. 1974; Wanebo et al. 1978; Mandeville et al. 1982; Braun et al. 1983; McCluskey et al. 1983; Ludwig et al. 1985). The factors believed to be the cause of such differences in results are discussed later in this section.

Surgery in operable cancer did not result in any post-operative change from pre-operative lymphocyte counts and perhaps this is not surprising as many patients with apparently operable disease have occult metastases which may not present for many years. Such metastatic disease would still be able to exert a 'blocking' or other suppressive effect

on lymphocytes following removal of the primary tumour. Taking this a step further, examination of lymphocyte counts both pre- and post-operatively was undertaken to see if depression of pre-operative counts predicted early recurrence or if depressed post-operative counts indicated residual disease with serum blocking capability as demonstrated by Haffejee et al. (1983). Although insignificant, there was a trend for patients whose disease recurred within 4 years to have higher pre-operative absolute counts, a surprising finding and similar to the observation of Shukla et al (1986a) who noted that elevated Abs L counts in early breast cancer were more likely to indicate future recurrence. The reason for this is unknown and at odds with the findings of others (Papatestas et al. 1976; Ownby et al. 1983) who reported a positive correlation between pre-operative Abs L counts and prognosis in operable breast cancer. No difference occurred between pre-operative % T counts in tumour recurrers and non-recurrers and is in agreement with the findings of Stein et al. (1976) and Teasdale et al. (1979a) who suggested that this test has no prognostic significance. There was no difference in post-operative Abs L, Abs T or % T counts between patients whose tumour recurred within 4 years and those who remained disease free and hence these tests do not appear to be of use as an indicator of residual occult disease. However, the numbers studied here are admittedly small and follow-up is short at 4 years.

The prognostic indicators measured, axillary node histology and ER status of the primary tumour, also bore no relationship to pre-operative lymphocyte counts. This is in agreement with findings published by Wanebo et al. (1978) who found no significant change in lymphocyte counts with increasing risk factors in early breast cancer.

An interesting finding in this study was the effect of adjuvant

tamoxifen on post-operative lymphocyte counts. There was a trend to increased Abs L and Abs T counts in patients given this adjuvant therapy, although admittedly, despite being well balanced by the non-adjuvant group in terms of axillary node status and age, these patients contained a disproportionate number of ER positive tumours and a lesser number of tumour recurrers. The latter fact, of course, may be explained by the effects of adjuvant tamoxifen. The most notable finding, however, was that % T lymphocyte counts were significantly elevated in patients on tamoxifen and indeed were of comparable levels to patients with benign breast disease. Sheard et al. (1986) have also reported increases in Abs L, Abs T and % T, and additionally NK cell counts in patients given tamoxifen, and that this drug even neutralises the depressive effect exerted on lymphoycte counts by chemotherapy. The reason for this is unknown but these authors implied that the favourable influence of this adjuvant therapy on the course of early breast cancer; reported by Baum (1985) to be unconnected with nodal, menopausal, or ER status, might be related to these changes in lymphocyte numbers. Indeed, hormonally induced increases in T lymphocyte counts have been claimed to be the cause of treatment success in breast cancer through immune mechanisms (Yonemoto et al. 1977). A more likely explanation is that any occult metastatic disease present in those given adjuvant tamoxifen is suppressed by this systemic treatment, thereby preventing tumour from exerting its 'blocking' effect on T lymphocyte receptors, while concurrently postponing early recurrence of disease. Such neutralisation of tumour may also permit Abs L counts to increase towards normal levels, although as yet, it is not known by what mechanism tumour causes Abs L depression. It is of course possible that tamoxifen may directly effect an increase in Abs L through perhaps a 'steroid effect' but this appears unlikely

as patients with various stages of breast cancer who underwent hormone treatment, the majority of whom were given tamoxifen, did not increase their Abs L counts when disease progression continued. The trend to increase in Abs L appeared therefore to be a secondary event following cessation of cancer growth.

Patients who underwent successful hormonal manipulation, whether for primary locoregional or metastatic disease tended to be older, to be ER positive and to have soft tissue or bony disease rather than visceral or multiple metastases, and these findings are in keeping with other published data (Kardinal & Donegan 1979).

Pre-treatment lymphocyte counts were not predictive of outcome in patients undergoing hormone therapy in this series, similar conclusions having been drawn by Webster et al. (1979). On the other hand, others (Franks & Williams 1978) demonstrated that the total lymphocyte count was predictive of response of disseminated breast cancer to endocrine manipulation.

Sequential studies demonstrated that there was a rise in Abs L counts in patients in whom disease actually regressed, although this did not quite reach statistical significance. The rise was slow, taking at least three months. Over the same period of time Abs T counts rose in these patients also and the difference from Abs T counts in patients whose disease continued to progress was significant by 6 weeks ($p<0.05$) and more so by 3 months or more of treatment ($p<0.02$). The most marked effect seen in patients achieving PR, or indeed in those in whom the progression of disease was halted, was a rapid, significant, rise in % T counts, predating objective evidence of tumour remission or change in disease status and being maintained during the period of benefit to the patient. Patients in whom disease continued to progress despite endocrine

manipulation had no increase in lymphocyte counts indicating that such increases are secondary to the efficacy of treatment. All patients who benefited from treatment and who had lymphocyte counts performed within a two month period prior to objective evidence of recurrence showed a return of % T counts to pre-treatment levels and this depression was maintained following overt recurrence. Abs L counts did not drop with such rapidity, a reflection of the apparently slower response by absolute numbers to changes in disease status. It is possible that absolute numbers reflect the slower changing, bulk of disease which may perhaps exert a direct suppressive effect on the lymphoreticular system or act by trapping lymphocytes in its own mass, whereas % T count fluctuations indicate the more rapid changes in disease activity which should occur with successful therapies, these changes being reflected by variations in release of 'blocking' factor(s). Some support for this comes from the fact that rising absolute counts were seen in patients who achieved PR, i.e. in whom disease bulk decreased, whereas those in whom disease only ceased to progress had no such rise in absolute counts. In all patients in whom disease progression was halted, i.e. disease activity was slowed, there was a rise in % T lymphocyte counts.

Others have also noted sequential changes in lymphocyte counts in cancers responding to treatment. Increased Abs L counts have been noted in advanced breast cancer responding to endocrine manipulation (Franks & Williams 1978) and Abs L counts reported to drop prior to disease recurrence in lung cancer patients (Anthony et al. 1975b). Serial T counts have also demonstrated change in relation to clinical status, increasing or remaining stable with successful treatment (Gross et al. 1975a; Dellon et al. 1975), falling as disease progresses (Anthony et al. 1975b; Dellon et al. 1975), rising in a small series of breast

cancers responding to adrenalectomy (Yonemoto et al. 1977,) and falling by as much as three months before objective evidence of recurrent lung cancer (Anthony et al. 1975b; Dellon 1975). This however, is the first large series of patients with breast cancer, mostly treated with tamoxifen, which demonstrates the inverse relationship between disease load and T lymphocyte counts and the predictive value of % T counts in the assessment of treatment response or disease recurrence prior to objective evidence of either.

Why then have some authorities (Stjernsward et al. 1972; Nemoto et al. 1974; Wanebo et al. 1978; Mandeville et al. 1982; Braun et al. 1983; Ludwig et al. 1985) been unable to show any relationship between T lymphocytes and cancer? One reason for this may be the variation in methodology used to separate and count T lymphocytes, these being very sensitive and susceptible to many variables. For instance, the use of vacutainers to remove blood, preservatives in heparin, active resuspension of rosettes, increased centrifugal forces on rosettes and the holding of blood for prolonged periods of time prior to separation, result in decreased % T counts (Djeu et al. 1977). Variable results within a laboratory using a single standardised technique will also occur if SRBC from different sheep are used (Steel et al. 1974b; Djeu et al. 1977) or if FCS sources vary (Djeu et al. 1977). Nylon wool columns which favour T lymphocytes at the expense of B cells during separation (WHO/IARC Workshop 1974) were used by Nemoto et al. (1974) and Stjernsward et al (1972). Braun et al. (1983) and Mandeville et al. (1982) used FCS in the E-rosette incubation phase but FCS has been demonstrated to cause a loss in the marked difference in % T counts between cancer patients and controls (Whitehead et al. 1976; Whitehead et al. 1978; Byrom et al. 1978) by increasing cancer counts to control levels. Increased

E-rosetting has similarly been associated with the use of bovine serum albumin (Anthony et al. 1975a) and neuraminidase-treated SRBC (Sandilands et al. 1975). Some investigators (Stjernsward et al. 1972; Wanebo et al. 1978; Ludwig et al. 1985) incubated their lymphocytes with SRBC for prolonged periods of time, this technique having been demonstrated (Whitehead et al. 1976; Whitehead et al. 1978) to bring about an increase in % T counts of cancer patients to control levels, neutralising the previously noted differences in % T counts between the two groups.

The fact that FCS stabilises and enhances E-rosette formation (Steel et al. 1974b; Anthony et al. 1975a) and increases E-rosetting of cancer patients' lymphocytes to control levels implies that lymphocytes with lower avidity receptors for SRBC could be forming E-rosettes under more optimal conditions brought about by FCS. The similar effect on cancer lymphocytes following overnight incubation with SRBC has been verified here. Whitehead et al. (1976) suggested that this was the outcome of dissociation of 'blocking' factor(s) from the lymphocyte surface into the surrounding medium, thereby exposing surface membrane receptors to SRBC. However, it has also been shown in this work that % T counts rise significantly in healthy controls and in patients with benign breast disease as well as in those with breast cancer. Such patients would not be expected to carry 'blocking' factor(s) on their lymphocyte surface membranes. Further, if Whitehead's hypothesis is correct, lymphocytes incubated alone in isotonic saline for a prolonged period of time should also lose 'blocking' factor by dissociation. Yet on removal of isotonic saline on the following day and mixing and incubating the lymphocytes with SRBC in the usual manner, the number of lymphocytes forming E-rosettes is only slightly higher than the first day counts and significantly lower than the number forming E-rosettes following prolonged

incubation in the presence of SRBC. It seems probable therefore, that the increase in % T counts following overnight incubation is mainly an effect of the prolonged period of time lymphocytes spend in the presence of SRBC. One possible explanation for this is that cells with low avidity receptors for SRBC are permitted to form E-rosettes during the time of prolonged incubation. What cells have this property?

It is known that B lymphocytes (Jondal et al. 1972), granulocytes and monocytes (Bianco et al. 1970), do not form spontaneous rosettes with SRBC, unless SRBC are first treated with neuraminidase (Sandilands et al. 1975). The majority of null cells have been demonstrated to be smaller than monocytes, non-phagocytic, non-adherent, OKM1+, Ia- cells. However, they have counterparts with similar characteristics accounting for approximately 14% of the E-rosetting population (Breard et al. 1980) the vast majority of these belonging to, and accounting for most of, a subgroup of T lymphocytes with Fc receptors for IgG (Kay & Horwitz 1980; Reinherz et al. 1980), the T gamma (Tg) cells. When looked at more closely with the T lymphocyte specific monoclonal antibody OKT3, this T population has been found to contain few lymphocytes, and is not enriched for T helper (OKT4+) or T cytotoxic/suppressor (OKT5+) sub-populations, these virtually all being contained in the Tu population which have Fc receptors for lgM (Reinherz et al. 1980). More wide ranging tests of the Tg population with markers OKT3, OKT4, OKT5, OKT8, OKT9 and OKT10 by Kay and Horwitz (1980) displayed similar findings; although Fox et al. (1981), on the other hand, demonstrated that three other monoclonal anti-bodies specific for T cell associated antigens did react with Tg cells, and suggested that because they were E+, non-adherent, non-phagocytic and esterase negative, that they, despite being OKM1+, must be T lymphocytes. Rosenberg et al. (1981) further demonstrated that the T cells did not

react with the specific monoclonal marker for monocytes, 63D3 and as such could not be monocytes. The Tg cells are therefore probably intermediate cells ontogenically and phylogenically, as suggested by Kay and Horwitz (1980) and together with their E-Ig- counterparts in the null cell population probably comprise the group of cells known as K or NK cells, these similarly having been shown to be OKMI+, Ia-, carry Fc-IgG receptors, 90% of NK activity being exhibited by cells with Fc-IgG receptors (Masucci et al. 1983), do not stain for T cell specific monoclonal antibody and do not become phagocytic or esterase positive on culture (Kay & Horwitz 1980). Tg cells are also radiosensitive (Moretta et al. 1977) like NK cells (Kiessling & Wigzell 1979; Herberman et al. 1979) and at least 50% have similar morphology to NK cells (Timonen et al. 1981). Finally approximately 50% of NK or K cells carry low avidity E-receptors (Herberman & Ortaldo 1981; Timonen et al. 1981) as do Tg cells (Herberman & Ortaldo 1981). Indeed if cells with high avidity receptors for SRBC are removed from the lymphocyte population, greater than 95% of NK or K cell activity remains in the remaining lymphocytes (Herberman 1983a). It has further been demonstrated that only the low avidity E-receptor cells within the apparent T lymphocyte population are able to display K cell activity (West et al. 1978). These low avidity E-receptor cells are therefore probably a subpopulation of of the K or NK population, the Tg cells.

West et al. (1978) showed that these low avidity E-receptor K cells could be encouraged to form rosettes by incubation overnight at $4^{o}C$ together with SRBC, a finding in keeping with the earlier demonstration by Winchester et al. (1975) that lymphocytes which could bind aggregated IgG only, i.e. carrying Fc - IgG receptors, were also able to form E-rosettes if incubated at $4^{o}C$ overnight. Similarly, it has been shown here that the main requirement for rises in % T counts on

overnight incubation is that lymphocytes are incubated together with SRBC during that time, whereas lymphocytes incubated alone, prior to rosetting the following day show much lesser rises in % T counts. Indeed, the difference in % T counts obtained, comparing the two methods, results in a surprisingly constant figure in all groups (controls = 11%; benign breast disease = 9.4%; Stage I and II breast cancer = 12.6%; Stage III breast cancer = 11.1%; Stage IV remission = 12.1%) indicating perhaps a relatively fixed subpopulation of cells existing in both non-malignant and cancer populations which will form E-rosettes only on prolonged incubation at $4^{o}C$ in the presence of untreated SRBC. This population bears a strong numerical similarity to the number of Tg cells, which comprise 14% of the lymphocyte population.

However, there is also an increasing trend with increasing stage of breast cancer, significant in those with untreated metastases or disease progression despite treatment, for lymphocytes to form higher proportions of E-rosettes when rosetted on the following day, than when counts are performed immediately. This suggests a 'blocking' component present on the cell which dissociates from the lymphocyte surface following prolonged incubation in isotonic saline.

There thus appear to be two likely components to the increased E-rosetting manifest by lymphocytes incubated with SRBC for prolonged periods of time. One is the relatively constant formation of E-rosettes in all patient groups by cells with low avidity receptors for SRBC, these probably being Tg, KN/K cells. The second component, not seen in controls, relates to T lymphocyte receptor 'blocking', cancer patients' lymphocytes becoming 'unblocked' by dissociation of 'blocking' factor(s) into the medium following prolonged incubation in isotonic saline. This component was most obvious in patients with advancing metastatic disease,

indeed the group with which the 'blocking' phenomenon is most closely associated. Thus, when lymphocytes are incubated overnight with SRBC, the differences between non-malignant, malignant disease and the various stages of cancer, completely disappear as a result of these two components. This may explain some of the discrepancies in results between publications from various institutions and why some have found T lymphocyte counts unhelpful in assessing tumour burden.

There is considerable evidence that circulating immunecomplexes (CICs) are responsible for the 'blocking' phenomenon and, as such, a correlation between positive levels of CIC and depressed % T counts, might be expected. Assays of patients' sera, using the CIq deviation test, indicated that both mean CIC levels and numbers of patients with positive levels of CIC, increased with progressive disease stage as a general rule; suggesting a positive correlation between CICs and tumour burden, similar to the inverse correlation exhibited by % T lymphocyte counts. Similarly, like % T counts, mean CIC levels and numbers of patients positive for CIC did not change significantly following mastectomy for for early disease. However, unlike % T counts, CICs were apparently unaffected by beneficial hormone therapy in patients with Stage IV breast cancer. Moreover, when CIC levels were correlated with % T counts performed in the same patients and from the same blood samples, although there was an inverse relationship between the two, there was no significant correlation.

This may indicate that some other factor may inhibit E-rosetting. For instance, acute phase proteins, although poor markers of tumour load, occur in the serum of patients with breast cancer and in particular C-reactive protein has been measured in two-fifths of those with metastatic disease (Cooper & Stone 1979). C-reactive protein has been demonstrated to

inhibit E-rosetting (Mortensen et al. 1975); but Whitehead et al. (1977) have shown that this substance is not the cause of 'blocking' in carcinoma of the breast. Although E-rosette inhibition, in this instance, by substances other than CICs, cannot be excluded, it is still possible that the failure to demonstrate correlation between this putative 'blocking' factor and % T counts is due to inadequate measurement of CICs.

Some authors have found CICs when measured by assays which utilise complement binding, to be of little value as tumour markers (Krieger et al. 1983), lacking significant discrimination between cancer patients and normal people (Herberman et al. 1981). Indeed, it has been suggested that the use of a single assay technique is insufficient, and, that several different types of assay should be used for each individual sample if adequate analysis is to be obtained (Lambert et al. 1978; Theofilopoulos & Dixon 1979). This is because of the heterogeneity of IC in the circulation, not all of which may be related to the disease under study, and because each individual assay is susceptible to complicating factors. For instance, and in particular, the requirement by the CIq assay for complement fixation by CICs may not be met if the tumour burden is very great, in which case CICs formed in large antigen excess do not fix complement (C) to the same degree as CICs formed at conditions near equivalence (Poskitt & Poskitt 1979; Theofilopoulos & Dixon 1979) and, as such, may remain undetected or under-estimated. Similarly, CICs containing non-C activating antibodies like IgG_4 or those made with antibody which activates C preferentially via the alternative pathway such as IgE and IgA will not be measurable (Theofilopoulos & Dixon 1979). Falsely low values may also occur if CICs are already saturated with endogenous CIq (Jones & Orlans 1981). On the other hand CIC levels may appear disproportionately high because of CIq binding to contaminating

molecules like DNA, bacterial products and aggregated IgG (AHG), AHG formation being facilitated by heat inactivation and repeated freezing and thawing (Lambert et al. 1978; Theofilopoulos & Dixon 1979; Jones & Orlans 1981). However, in the assay employed in this particular work, serum samples were not heat inactivated and were stored continuously at -70^{o}C until use. CIC levels may also be falsely elevated, to a limited degree, by binding of C by monomeric IgG (Lambert et al. 1978).

Difficulties such as these, with assays which utilise CIq binding, may possibly explain the poor correlation between CIC levels and % T counts, and the apparent absence of change in serum CICs with therapeutic responses in metastatic breast cancer.

4.6. Summary

Abs T and % T counts are depressed even in early breast cancer but Abs L counts only depressed in advanced breast cancer.

Abs T and % T counts decrease with increasing extent of breast cancer and there is a similar trend with regard to Abs L counts.

Lymphocyte counts do not change following surgery in operable breast cancer.

Pre-operative lymphocyte counts are not predictive of outcome and show no correlation with other prognostic indicators.

Post-operative lymphocyte counts are not predictive of outcome nor indicative of residual disease.

Adjuvant tamoxifen in early breast cancer results in a trend to increased Abs L and Abs T counts but significantly raised % T counts, suggesting systemic suppresion of tumour with a secondary decrease in serum 'blocking' ability.

Hormone therapy in breast cancer results in a trend to Abs L count increases in patients whose disease burden decreases, and significant rises in Abs T and % T counts in patients benefiting from treatment. Counts do not increase in patients whose disease continues to progress, indicating that changes in lymphocyte counts are a secondary phenomenon to tumour response.

Changes in % T counts are relatively rapid and occur in patients in whom progression of disease ceases and predate objective evidence of response or of disease recurrence. Changes in absolute counts are slower and occur in patients only in whom there is actual disease regression. Absolute counts may therefore relate more to tumour bulk and % T counts more to tumour activity.

Overnight incubation of lymphocytes with SRBC results in increased % T counts as a consequence of a combination of low avidity E-receptor cell rosetting, possibly by K/NK cells, and dissociation of 'blocking' factor(s) from the T lymphocyte surface. This results in a loss of the usual discriminatory value of % T counts between the various stages of the cancer process.

CIC measured in patients' sera bears a positive correlation to tumour burden but does not have the discriminatory capacity of % T counts. Moreover, although patients with positive CIC values tend to have depressed % T counts this correlation was not significant, indicating perhaps that some other factor is responsible for E-rosette depression, or that faults with either one or other assay are responsible.

4.7. Conclusions

This work shows that absolute lymphocyte, absolute T lymphocyte and especially % T lymphocyte counts are depressed in breast cancer, even in its early stages. This depression becomes more pronounced with increasing tumour burden. Lymphocyte counts have no prognostic significance and are unrelated to other prognostic parameters prior to treatment and are unaffected by surgery in early cancers, but tend to rise in such patients when given systemic adjuvant hormone treatment.

Serum levels of IC increase with tumour load and bear an inverse but non-significant correlation to % T counts. CICs do not, however, have the same discriminatory powers in assessing treatment outcome in patients with metastatic disease.

Earlier assessment of response of advanced breast cancer to tamoxifen and other forms of endocrine manipulation can be achieved than has been previously possible, a trial period of at least 12 weeks normally being required, by studying changes in T cell and in particular % T cell counts; thereby permitting earlier evaluation of the patient on treatment and allowing a more rational selection of alternative treatment modalities at an earlier phase in the patient's illness.

However, for this to be effective, strict laboratory methodology must be adhered to, otherwise the discriminatory value may be lost.

Appendix 4.1 Pre-Treatment Benign Breast Disease

	Age	% T	Abs T	Abs L
JH	19	64	1.421	2.221
EG	25	64	1.728	2.700
KC	27	60	1.587	2.645
LB	27	53	1.189	2.244
DJ	27	55	1.294	2.352
CW	28	65	1.509	2.322
AM	30	66		
KM	26	68		
AM	29	61		
JH	23	63		
RB	38	60	1.386	2.310
DC	35	55	0.785	1.428
AE	37	62	1.927	3.108
CG	40	45	1.488	3.306
SJ	39	66	1.705	2.583
GJ	37	59	1.634	2.770
TP	40	63	0.765	1.215
SP	33	68		
EW	36	61	1.217	1.995
LW	33	63	0.802	1.273
EB	38	68		
AM	33	50	0.728	1.456
AH	40	54	1.461	2.705
JR	40	58		

(continued)

	Age	% T	Abs T	Abs L
NR	37	59		
JF	34	64		
JE	41	57	0.730	1.280
AH	43	61	2.651	4.346
JH	44	57	0.946	1.660
ML	41	63	1.764	2.800
DP	45	60	1.240	2.040
DP	43	53	0.758	1.431
DC	42	62		
DP	42	54	1.406	2.604
SH	43	63		
LY	41	64		
MB	43	51		
MH	44	59		
SW	43	59		
CB	41	64		
SM	43	59		
BW	46	70	0.819	1.117
DW	46	60	1.170	1.949
DB	50	64	1.293	2.020
MD	48	56		
GH	48	56		
IE	54	56	0.851	1.519
JR	51	68		
MB	62	58	0.651	1.122
ID	62	56	1.062	1.896

Appendix 4.2 Pre-Treatment Stage I and II Breast Cancer

	Age	% T	Abs T	Abs L
EC	48	60	1.540	2.567
PE	67	57	0.752	1.320
GF	60	64	3.003	4.692
VR	49	48	0.850	1.771
JR	49	53	0.859	1.620
JJ	43	48	0.814	1.696
PB	44	47	0.790	1.700
BR	46			2.649
JR	41			1.305
HL	42			2.325
VC	39	51	1.053	2.064
PJ	78	38		
MH	78	53	1.256	2.369
MM	74	66	0.835	1.265
VP	71	65	1.115	1.716
EL	62	42		
MR	47	44	0.891	2.025
ES	41	62	1.259	2.030
MT	48	50	0.651	1.302
BT	53	56	0.902	1.610
NV	49	52	1.215	2.336
MM	48	50	0.648	1.295

(continued)

	Age	% T	Abs T	Abs L
AB	38	48	1.056	2.200
EH	60	51	1.313	2.574
AJ	42	69	1.127	1.633
GJ	66	58	0.872	1.504
MS	40	49	0.459	0.936
ET	49	58	1.137	1.960
EJ	68	36	0.519	1.442
PT	52			1.846
EE	73	62	0.729	1.176
MJ	58	58	0.390	0.672
JL	53	69	1.377	1.995
AM	72	60	2.020	3.366
FP	87	21	0.208	0.990
EP	80	51	0.656	1.525
EJ	94	43	0.771	1.512

Appendix 4.3 Pre-Treatment Stage III Breast Cancer

	Age	% T	Abs T	Abs L
RF	79	42	0.670	1.596
IS	63			2.065
KD	62			1.466
AJ	42	45	0.990	2.200
KM	60	48	1.387	2.889
AL	66	43	0.624	1.452
EL	50	56	0.926	1.653
PM	41	55	1.320	2.400
MWJ	68	63	1.270	2.016
LH	59	45	0.630	1.596
DG	55	63	0.756	1.200
RJ	58	68	1.163	1.710
GL	80	50	1.003	2.006
ER	82	54	1.340	2.480
HS	84	64	1.299	2.030
FL	66	69	0.924	1.339
BW	74	44	0.832	1.890
ES	71	38	0.399	1.050
ED	71	50	0.899	1.798
OA	76	41	0.718	1.750
AM	53	49	0.600	1.224
IH	50	43	1.231	2.862

Appendix 4.4 Pre-Treatment Stage IV Breast Cancer

	Age	%T	Abs T	Abs L
GW	81	62	0.730	1.118
OJ	68	43		
CD	57	50	0.650	1.300
AB	38	42	1.053	2.508
VC	39	52	1.906	3.665
HT	65	30	0.139	0.462
EJ	63	50	0.320	0.639
EB	74	61	1.426	2.337
MJ	75	39	0.325	0.833
EM	74	62		
OJ	60	55	2.035	3.700
HD	61	54		
MJ	63	61	0.561	0.920
BH	54	58	1.336	2.304
WG	49	50		
MH	38	31	0.095	0.308
RB	42	35	1.021	2.916
VB	48	52	0.366	0.704
DB	37	51	0.685	1.344
FD	72	27	0.351	1.140
BD	50	59	0.504	0.855
LE	78	45	0.796	1.769
AL	76	48		

(continued)

	Age	%T	Abs T	Abs L
ML	31	51	0.968	1.898
BD	48	25	0.249	0.996
EE	49	46	1.228	2.669
MF	83	21	0.161	0.765
EH	60	52	0.478	0.920
WN	74	56		
AP	69	53	1.230	2.320
AC	75	56	1.434	2.596
WM	72	63	0.635	1.512
ET	74	42	0.635	1.512
EI	49	51	0.576	1.130
CL	40	47	1.167	2.484
RP	58	54	1.518	2.812
SJ	44	40	0.436	1.089
AM	45	42	0.318	0.756
LS	45	53	0.750	1.416
EE	49	32		
HT	74	42	0.578	1.375
AA	78	62	0.450	0.726
MM	39	66	0.314	0.476
MA	44	27	0.421	1.560
AK	44	51	0.592	1.160

Appendix 4.5

Stage I & II Breast Cancer Pre- & Post-operative Lymphocyte Counts

		Pre-operative			Post-operative		
	Age	% T	Abs T	Abs L	% T	Abs T	Abs L
EC	48	60	1.540	2.567	60	1.286	2.144
PE	67	57	0.752	1.320			
GF	60	64	3.003	4.692			
GG	60				61	1.757	2.880
BN	56	54	0.720	1.334			
VR	49	48	0.859	1.620	47	1.278	2.720
MR	60				64	0.977	1.527
JR	49				53		
MB	72				48	0.586	1.221
PB	44				55	1.478	2.688
EB	49				58	0.845	1.456
JJ	43	48	0.814	1.696			
PB	44	47	0.790	1.700			
VC	39	51	1.053	2.064			
PJ	78	38			41		
MH	78	53	1.256	2.369	59	1.265	2.144
MM	74	66	0.835	1.265	57	1.160	2.034
VP	71	65	1.115	1.716	53	0.952	1.796
PP	59				47	1.960	4.171
EL	62	42			40		
ES	72				50		

(continued)

		Pre-operative			Post-operative	
	Age	% T	Abs T	Abs L	% T	Abs T	Abs L
MR	47	44	0.891	2.025			
ES	41	62	1.259	2.030	46	1.278	2.556
MT	48	50	0.651	1.302	50	1.268	2.535
BT	53	56	0.902	1.610	53	1.093	2.062
NV	49	52	1.215	2.336			
MM	48	50	0.648	1.295			
AB	38	48	1.056	2.200			
PE	51				61	1.876	3.076
EH	60	51	1.313	2.574		1.307	2.376
AJ	42	69	1.127	1.633			
GJ	66	58	0.872	1.504			
MS	40	49	0.459	0.936	44	0.484	1.100
ET	49	58	1.137	1.960	58	1.347	2.322
EJ	68	36	0.519	1.442			
MT	53			1.846	39	0.587	1.504
BA	46			2.649			1.727
JR	41			1.305			
PT	52			1.846			

Appendix 4.6

Stage III Breast Cancer Pre- and Post-operative Lymphocyte Counts

		Pre-operative			Post-operative		
	Age	% T	Abs T	Abs L	% T	Abs T	Abs L
AJ	42	45	0.990	2.200			
AL	66	43	0.624	1.452	43	0.373	0.867
EL	50	56	0.926	1.653			
PM	41	55	1.320	2.400			
MWJ	68	63	1.270	2.016	66	1.030	1.560
EO	48				48	0.759	1.581
RF	79	42	0.670	1.596			
LH	59	45	0.630	1.400			
DM	65				41	0.780	2.052
IM	55				57	1.160	2.034
DG	55	63	0.756	1.200	52	0.755	1.425
KM	60	48	1.387	2.889	56	1.472	2.628
WD	63	39			41		
IS	63			2.065			
KD	62			1.466			

Appendix 4.7

Pre-operative Lymphocyte Counts Stage I, II & III Breast Cancer

Axillary Node Negative					Axillary Node Positive				
	Age	% T	Abs T	Abs L		Age	% T	Abs T	Abs L
MR	47	44	0.891	2.025	EC	48	60	1.540	2.567
AB	38	48	1.056	2.200	PE	67	57	0.752	1.320
EH	60	51	1.313	2.574	GF	60	64	3.003	4.692
AJ	42	69	1.127	1.633	BN	56	54	0.720	1.334
GJ	66	58	0.872	1.504	VR	49	48	0.850	1.771
MS	40	49	0.459	0.936	PB	44	47	0.790	1.700
ET	49	58	1.137	1.960	VC	39	51	1.053	2.064
MJ	58	58	0.390	0.672	PJ	78	38		
AJ	42	45			MH	78	53	1.256	2.369
KM	60	48	1.387	2.889	MM	74	66	0.835	1.265
PM	41	55	1.320	2.400	VP	71	65	1.115	1.716
MWJ	68	63	1.270	2.016	ES	41	62	1.259	2.030
IS	63			2.065	MT	48	50	0.651	1.302
KD	62			1.466	BT	53	56	0.902	1.610
					NV	49	52	1.215	2.336
					EJ	68	36	0.519	1.442
					EE	73	62	0.729	1.176
					JL	53	69	1.377	1.995
					AM	72	60	2.020	3.366
					RF	79	42	0.670	1.596
					AL	66	43	0.624	1.452
					WD	63	39		
					LH	59	45	0.630	1.596
					DG	55	63	0.756	1.200

Appendix 4.8

Pre-operative Lymphocyte Counts Stage I, II, III Breast Cancer

	Oestrogen Receptor Positive					Oestrogen Receptor Negative			
	Age	% T	Abs T	Abs L		Age	% T	Abs T	Abs L
EC	48	60	1.540	2.567	VR	49	48	0.850	1.771
PE	67	57	0.752	1.320	VC	39	51	1.053	2.064
GF	60	64	3.003	4.692	MR	47	44	0.891	2.025
PB	44	47	0.790	1.700	MT	48	50	0.651	1.302
MM	74	66	0.835	1.265	BT	53	56	0.902	1.610
ES	41	62	1.259	2.030	AB	38	48	1.056	2.200
NV	49	52	1.215	2.336	AJ	42	69	1.127	1.633
MM	48	50	0.648	1.295	MS	40	49	0.459	0.936
EH	60	51	1.313	2.574	ET	49	58	1.137	1.960
GJ	66	58	0.872	1.504	AM	72	60	2.020	3.366
MJ	58	58	0.390	0.672					
JL	53	69	1.377	1.995					
AL	66	43	0.624	1.452					
PM	41	55	1.320	2.400					
FL	66	69	0.924	1.339					
MWJ	68	63	1.270	2.016					
LH	59	45	0.630	1.596					
DG	55	63	0.756	1.200					

Appendix 4.9

Pre-operative Lymphocyte Counts Stage I, II, III Breast Cancer

	No Recurrence					Recurrence <Four Years			
	Age	% T	Abs T	Abs L		Age	% T	Abs T	Abs L
BN	56	54	0.720	1.334	ER	48	60	1.540	2.567
MH	78	53	1.256	2.369	VR	49	48	0.850	1.771
MM	74	66	0.835	1.265	PB	44	47	0.799	1.700
VP	71	65	1.115	1.716	VC	39	51	1.053	2.064
MR	47	44	0.891	2.025	NV	49	52	1.144	2.200
ES	41	62	1.259	2.030	EH	60	51	1.313	2.574
MT	48	50	0.651	1.302	AJ	42	69	1.127	1.633
BT	53	56	0.902	1.610	GJ	66	58	0.872	1.504
MS	40	49	0.459	0.936	MWJ	68	63	1.270	2.016
AJ	42	45	0.990	2.200	RF	79	42	0.670	1.596
AL	66	43	0.624	1.452	DG	55	63	0.756	1.200
EL	50	56	0.926	1.653					
PM	41	55	1.320	2.400					
LH	59	45	0.630	1.400					
MM	48	50	0.648	1.295					

Appendix 4.10

Post-operative Lymphocyte Counts Stage I, II, III Breast Cancer

	No Recurrence					Recurrence <4 Years			
	Age	% T	Abs T	Abs L		Age	% T	Abs T	Abs L
GG	60	61	1.757	2.880	EC	48	60	1.286	2.144
MR	60	64	0.977	1.527	VR	49	47	1.278	2.720
PB	44	55	1.478	2.688	MB	72	48	0.586	1.221
EB	49	58	0.845	1.456	ES	72	50	1.278	2.556
MH	78	59	1.265	2.144	PE	51	61	1.876	3.076
MM	74	57	1.160	2.034	MT	53	39	0.587	1.504
VP	71	53	0.952	1.796	MWJ	68	66	1.030	1.560
PP	59	47	1.960	4.171	RF	79	42		
ES	41	46			IM	55	57	1.160	2.034
MT	48	50	1.268	2.535	DG	55	52	0.755	1.425
BT	53	53	1.093	2.062					
MS	40	44	0.484	1.100					
AL	66	43	0.373	0.867					
EL	50	56							
PM	41	55							
LH	59	45							
DM	65	38	0.780	2.052					

Appendix 4.11

Stage II Breast Cancer - Postmenopausal Women

	Age	% T	Abs T	Abs L	ER status	Involved nodes	Recurrence <4 years	Adjuvant Tamoxifen
MB	72	48	0.586	1.221	-	0	nil	nil
MH	78	59	1.265	2.144	N/A	0	nil	nil
MM	74	57	1.160	2.034	+	0	nil	nil
VP	71	53	0.952	1.796	N/A	0	nil	nil
PP	59	47	1.960	4.171	N/A	0	nil	nil
ES	72	50	1.278	2.556	-	0	Brain 15/12	nil
BT	53	53	1.093	2.062	-	0	nil	nil
PE	51	61	1.876	3.076	+	2	Bone 33/12	nil
EH	60	55	1.307	2.376	N/A	3	Bone 12/12	nil
MT	53	39	0.587	1.504	-	1	Brain 30/12	nil
EE	73	65	1.013	1.558	N/A	0	nil	yes
CG	55	56	1.926	3.440	+	0	nil	yes
MJ	58	52	1.287	2.475	+	1	nil	yes
KK	67	49	0.558	1.139	+	3	nil	yes
JL	53	75	2.738	3.650	+	0	Multiple 28/12	yes
PM	63	69	1.604	2.325	N/A	0	nil	yes
AM	72	60	2.640	5.280	-	0	nil	yes
MR	68	59	1.224	2.074	N/A	0	nil	yes
MS	53	58	1.051	1.812	+	0	nil	yes
CS	66	63	1.023	1.624	+	0	nil	yes
RW	60	53	1.224	2.310	N/A	1	nil	yes
MJ	71	50	1.475	2.950	+	0	nil	yes

Appendix 4.12

Stage IV Breast Cancer

Disseminated Disease					vs	Locoregional Disease			
	Age	Pre RxT	Pre Rx Abs T	Pre Rx Abs L		Age	Pre RxT	Pre Rx Abs T	Pre Rx Abs L
RB	42	35	1.021	2.916	EE	49	46	1.228	2.669
VB	48	52	0.366	0.704	MF	83	21	0.161	0.765
DB	37	51	0.685	1.344	EH	60	52	0.478	0.920
FD	72	27	0.351	1.140	WN	74	56		
BD	50	59	0.504	0.855	AP	69	53	1.230	2.320
LE	78	45	0.796	1.769	AC	75	56	1.434	2.596
AL	76	48			ET	74	42	0.635	1.512
ML	31	51	0.968	1.898	OJ	60	55	2.035	3.700
BD	48	25	0.249	0.996	HD	61	54		
WM	72	63			MJ	63	61	0.561	0.920
GW	81	62	0.730	1.118	BH	54	58	1.336	2.304
OJ	68	43			WG	49	50		
CD	57	50	0.650	1.300					
AB	38	42	1.053	2.508					
VC	39	52	1.906	3.665					
HT	65	30	0.139	0.462					
EJ	63	50	0.320	0.639					
EB	74	61	1.426	2.337					

(continued)

	Disseminated Disease				vs	Locoregional Disease			
	Age	Pre RxT	Pre Rx Abs T	Pre Rx Abs L		Age	Pre RxT	Pre Rx Abs T	Pre Rx Abs L
MJ	75	39	0.325	0.833					
EM	74	62	1.480	2.387					
MH	38	31	0.095	0.308					
EI	49	51	0.576	1.130					
CL	40	47	1.167	2.484					
RP	58	54	1.518	2.812					
SJ	44	40	0.436	1.089					
AM	45	42	0.318	0.756					
LS	45	53	0.750	1.416					
EE	49	32							

Appendix 4.13

Stage IV Breast Cancer - Hormone Therapy - Responders

	Age	Pre Rx % T	Pre Rx Abs T	Pre Rx Abs L	Post Rx 6/52 % T	Post Rx 6/52 Abs T	Post Rx 6/52 Abs L	Post Rx Mean % T	Post Rx Mean Abs T	Post Rx Mean Abs L	Prerecurrence % T	Prerecurrence Abs T	Prerecurrence Abs L	Recurrence % T	Recurrence Abs T	Recurrence Abs L	Median Delay to New Disease Months
	P.R.																
EB	53							49	1.110	2.265				39			15
VB	48	52	0.366	0.704	68	0.486	0.714										10
LE	78	45	0.796	1.769				59	1.353	2.293	43	1.481	3.444				19
AL	76	48			61			61	1.147	1.881							16
WN	74	56			59	1.081	1.833	56	0.963	1.720	48	0.778	1.620	47	0.691	1.470	12
AP	69	53	1.230	2.320				68	1.520	2.236							43
AC	75	56	1.434	2.596	69	1.942	2.814										13
OJ	68	43			58	1.315	2.268				47	0.513	1.092				6
CD	57	50	0.650	1.300	57	0.830	1.456	54	0.814	1.508							30
WG	49	50			52	1.096	2.107										12
WG	49							55	1.742	3.168	50			52	1.096	2.107	27
SJ	44	40	0.436	1.089				53	1.420	2.680							17
	NC/NA																
FD	72	27	0.351	1.140	64	0.451	0.705	55	0.716	1.302	41	0.597	1.456	50	0.767	1.534	10
MF	83	21	0.161	0.765				58	0.282	0.486							47
PP	61				52	1.104	2.124	50	1.333	2.665							37
WM	72	63			63			57	1.358	2.382							68
ET	74	42	0.635	1.512	60	0.893	1.488	62	0.493	0.795	51			47	0.553	1.176	7
NL	59							55						46	0.505	1.098	12
MB	51							41	0.638	1.556							21
MJ	49							57	1.078	1.891							10

Appendix 4.13 (continued)

	Age	Pre Rx			Post Rx 6/52			Post Rx Mean			Prerecurrence			Recurrence			Median Delay to New Disease Months
		% T	Abs T	Abs L	% T	Abs T	Abs L	% T	Abs T	Abs L	% T	Abs T	Abs L	% T	Abs T	Abs L	
EM	74	62	1.480	2.387	60	0.729	1.215	55	1.108	2.015				45	1.765	3.922	14
HD	61	54			57	0.445	0.780	58	0.394	0.680	44	0.563	1.280				6
MJ	63	61	0.561	0.920	56	0.202	0.360	52	0.560	1.078							44
RP	58	54	1.518	2.812				52	1.482	2.850				50	1.500	3.000	17
LS	45	53	0.750	1.416	56	0.926	1.800	56	1.288	2.300	47			38	0.728	1.917	10

Appendix 4.13 (continued)

Stage IV Breast Cancer - Hormone Therapy - Progression

	Age	Pre Rx			Post Rx 6/52			Post Rx Mean		
		% T	Abs T	Abs L	% T	Abs T	Abs L	% T	Abs T	Abs L
RB	42	35	1.021	2.916	43	0.741	1.722			
DB	37	51	0.685	1.344	30	0.547	1.824	49	0.885	1.806
BD	50	59	0.504	0.855	59	0.597	1.012	58	0.773	1.333
GW	39				50	1.029	2.058			
ML	31	51	0.968	1.898	38	0.481	1.266	34	0.839	2.468
BD	48	25	0.249	0.996						
EE	49	46	1.228	2.669	48	1.112	2.317			
ED	56				37	0.408	1.102			
AM	45				42	0.318	0.756			
EH	60	52	0.478	0.920	53	0.420	0.792			
GW	81	62	0.730	1.118	65	0.351	0.540			
MP	63							35	0.373	1.065
ES	71				57	0.804	1.410			
AB	38	42	1.053	2.508	50	1.332	2.664	36	1.094	3.040
VC	39	52	1.906	3.665						
HT	65	30	0.139	0.462						
EJ	63	50	0.320	0.639	50					
EB	74	61	1.426	2.337	55	0.469	0.852			
MJ	70				34	0.592	1.740			
MJ	75	39	0.325	0.833						
OJ	60	55	2.035	3.700						
BH	54	58	1.336	2.304	54	1.053	1.950			

Appendix 4.13 (continued)

	Age	Pre Rx			Post Rx 6/52			Post Rx Mean		
		% T	Abs T	Abs L	% T	Abs T	Abs L	% T	Abs T	Abs L
MD	57							37	0.686	1.855
MH	38	31	0.095	0.308						
CS	36				44	1.245	2.829			
EI	49	51	0.576	1.130				30	0.454	1.512
CL	40	47	1.167	2.484						
AM	45	42	0.318	0.756	37	0.559	1.512			
EE	49	32						46	1.228	2.669
IM	58				40					

Appendix 4.14 Variation in Rosetting of Lymphocytes

	Age	Day 1 Counts % T	Day 2 (New Rosette Counts) % T	Day 2 (Delayed Counts) % T	Day 2 (Small Lymphocyte Pellets) % T
CONTROLS					
GR	38	59	64	79	
AP	36	64	64	64	
NP	38	58	57	65	
KR	38	61	56	71	
DW	44	61	55	74	
SH	44	67	67	82	
RL	34	71	72	83	
MO	42	66	68	78	
SD	28	68	70	76	
BENIGN BREAST DISEASE					
LB	27	53	50	66	
MB	62	58	59	66	72
DC	35	55	63	62	65
IE	54	56	63	67	58
JE	41	57	55	71	58
OG	29	64	60	74	
CG	40	45	34	54	28
DJ	27	55	60	63	58
GJ	37	59	52	57	
ML	41	63	64	72	69
DP	43	53	60	79	68
DP	42	54	68	58	
CW	28	65	65	70	
EW	33	61	65	81	
LW	33	63	58	72	62
AE	37	59	61	75	

Appendix 4.14 (continued)

	Age	Day 1 Counts % T	Day 2 (New Rosette Counts) % T	Day 2 (Delayed Counts) % T	Day 2 (Small Lymphocyte Pellets) % T
STAGE I & II Pre Rx					
EC	48	60		76	
PE	67	57	50	75	
PE	67	61		79	
GF	60	64	40	80	
BN	56	64	56	69	59
MR	47	52	63	77	
MR	47	64		70	
AB	38	48	53	79	
PB	44	47	42	67	
VC	39	51		66	
EH	60	51		62	
MH	78	53	53	55	
MH	78	54		60	
AJ	42	69	74	71	
GJ	66	58		71	
SJ	66	58		64	
MM	72	66		76	
MS	40	49		58	
ES	41	62	60	76	60
MT	48	50	50	65	
BT	53	56		68	
ET	49	58	58	69	68
NV	49	52	41	64	25
MR	50	42	38	64	29
STAGE I & II Post Rx					
VR	49	47	65	78	
VR	49	57	68	78	
GG	60	61	63	77	54
MR	60	62		61	
MR	60	65		72	
FP	87	43	58	60	57
FP	87	50	55	72	
FP	87	50		76	

Appendix 4.14 (continued)

	Age	Day 1 Counts % T	Day 2 (New Rosette Counts) % T	Day 2 (Delayed Counts) % T	Day 2 (Small Lymphocyte Pellets) % T
MB	72	50	64	80	
MB	72	45	45	64	
PB	44	49	58	52	45
PB	44	60	60	67	
EB	49	58	73	74	
PG	51	64	63	82	59
PE	51	57		78	
EH	60	43		59	
MH	78	59		71	
MH	78	55		73	
VP	71	50	55	69	
VP	71	50	63	67	
PP	59	47	40	69	
ES	72	50	39	54	39
MS	40	44		72	
MS	40	41		70	
GS	41	47		66	
ES	41	44		71	
MT	48	50		67	
BJ	53	56	59	66	
GT	49	55		78	
GT	49	61		73	
MT	53	33	42	61	
MT	53	43	59	57	
NV	49	62	65	69	
EE	73	65		76	
MJ	58	46	55	55	42
PM	63	69	69	71	
PM	63	64		80	
MR	68	59	67	62	
CS	66	63	64	75	76
MS	53	68		67	
MS	53	63		74	
RW	60	53		86	

Appendix 4.1[4] (continued)

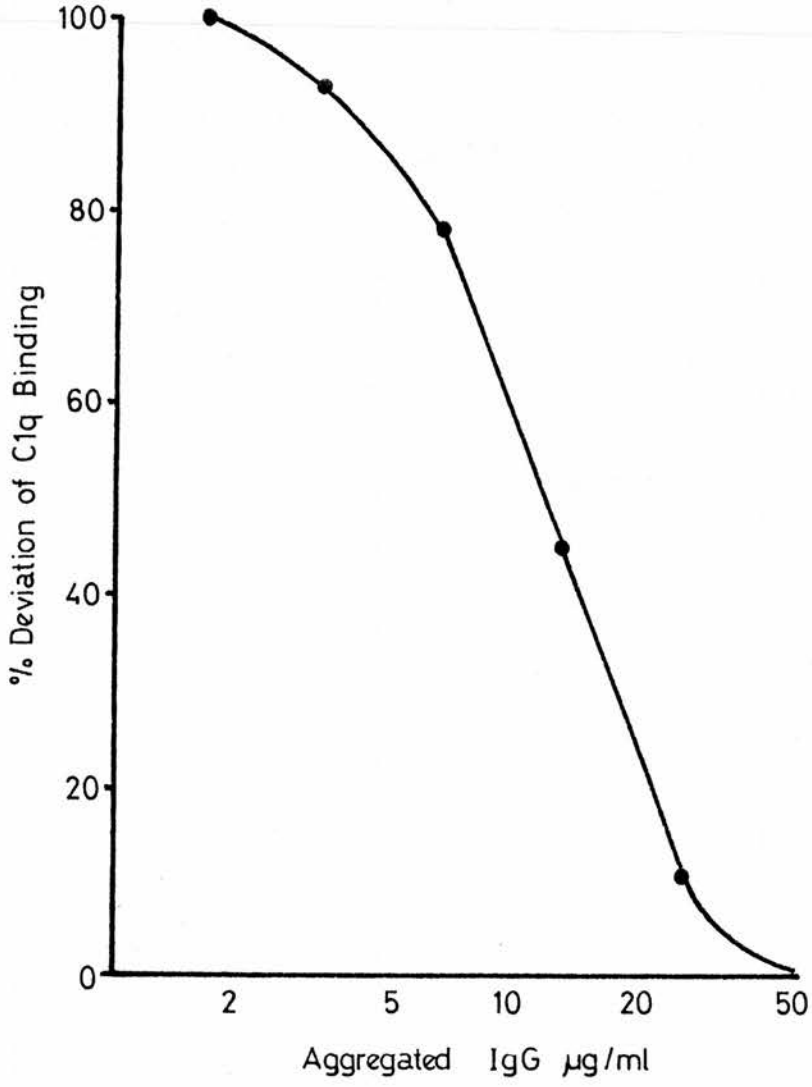
	Age	Day 1 Counts % T	Day 2 (New Rosette Counts) % T	Day 2 (Delayed Counts) % T	Day 2 (Small Lymphocyte Pellets) % T
KK	67	51		72	
KK	67	47		62	
MJ	71	50	57	68	
FP	87	68		64	
BT	53	49	49	76	
MT	53	37	61	56	50
STAGE III					
AL	66	43	32	39	27
EL	50	56	65	69	
PM	41	55	65	73	
MWJ	68	61	57	76	75
GL	80	50	50	59	
AM	53	49	60	72	
ED	71	50		72	
LH	59	45		65	
DG	55	62		70	
AL	66	43	62	74	
AL	66	54		74	
MWJ	68	68	65	83	
MWJ	68	61		85	
STAGE IV New/Progressive					
VB	48	52	61	74	
VB	48	51	54	76	
FD	72	46		62	
LE	78	43	57	69	
LD	57	50		70	
JJ	56	53		62	
ML	31	37		54	
ML	31	38	40	63	
EM	74	45		62	
MO	57	32	44	68	20
MP	63	35	54	70	
RT	50	28	28	63	
TC	39	52		61	
AB	38	42		68	
EJ	63	50		71	

Appendix 4.14 (continued)

	Age	Day 1 Counts % T	Day 2 (New Rosette Counts) % T	Day 2 (Delayed Counts) % T	Day 2 (Small Lymphocyte Pellets) % T
AC	75	56		72	
ED	56	37	50	68	
MF	83	21	56	52	51
EH	60	53	50	80	
WN	74	47		59	
MW	76	61	57	72	
STAGE IV Responders					
AA	78	56		62	
MB	51	39	51	69	
EB	53	43	48	76	
LE	78	62		69	
LE	78	66		81	
WG	49	55	50	62	
NL	58	55	56	60	
AL	76	61		73	
WM	72	56	57	69	
RP	59	52	65	85	
RP	59	47	60	74	
HT	74	51	54	61	43
PP	71	50		82	
MF	83	58	50	66	
MJ	63	56		75	
MJ	63	46	52	57	
WN	74	59		78	
WN	74	56	62	59	60
WN	74	48		60	
WN	74	52		71	

Appendix 4.15

Reference Curve for Soluble C1q Binding Antigen-Antibody Complexes



Chapter 5

Chapter 5

5. Lymphocyte Oxygen Consumption in Patients with Cancer

5.1. Introduction

Considerable evidence exists that immune complexes (ICs) are the putative 'blocking' factors which exist in cancer and are responsible for the immune depression seen in autoimmune and some other disease states. It has been shown, in the preceding chapter, that CICs measured by the CIq deviation test, appear to be, in general terms, directly related to the extent of breast cancer tumour load. However, as previously discussed, this particular method of measuring ICs is neither sufficiently sensitive nor specific and most other IC assay techniques have been similarly disappointing (reviews by Lambert et al. 1978; Theofilopoulos & Dixon 1979; Jones & Orlans 1981).

ICs have been shown to increase the uptake of oxygen (O_2) by human blood (Strauss & Stetson 1960), to activate polymorphonuclear leucocytes (PMN) measured by O_2 uptake (Rossi et al. 1970) and heat production (Monti et al. 1980) and to sensitise (Uhr 1965) and stimulate lymphocytes, measured by DNA activity (Moller 1969; Bloch-Shtacher et al. 1968; Lundgren et al. 1968a; Soderberg & Coons 1978), and cell-mediated cytotoxicity (Lundgren et al. 1968a). This stimulation is possibly exerted through binding to cell receptors for Fc-Ig, complement (C) (reviewed by Theofilopoulos & Dixon 1979) or possibly specific antigen binding sites (Theofilopoulos & Dixon 1979; Kontiainen 1975; Kontiainen & Mitchison 1975). The effect of ICs on immune cells to which they adhere is therefore to change their behaviour and, it follows, their metabolic activity. Indeed, neutrophil aerobic

metabolism has been used as a means of detecting such complexes (Clarke et al. 1978).

Peripheral blood lymphocytes have a predominantly oxidative metabolism with low or absent glycolysis (Barron & Harrop 1929; Kemper 1939; Rauch et al. 1961; Elves 1972) but are capable of small amounts of aerobic glycolysis and increased glycolysis under anaerobic conditions (Elves 1972). Even the lymphocyte's requirement for carbohydrate during the 'lethal hit' stage is independent of carbohydrate's capacity to act as an energy source (MacLennan & Golstein 1978). Therefore, it would seem reasonable to propose that metabolic changes occurring in the lymphocyte will be reflected by changes in cell O_2 utilisation. For instance, PHA lymphocyte stimulation, suggested to use aerobic oxidation as an energy source (Becker & Henson 1973) for transformation, has been demonstrated in equine lymphocytes to cause increased O_2 uptake by these cells, using polarographic measurement (Pachman 1967).

It will be demonstrated in this chapter, that like CIC, lymphocyte O_2 utilisation increases with tumour burden and that this is not a feature of the lymphocyte per se, but rather a property of the serum from which the lymphocyte has been separated. Evidence will be presented which suggests that the serum factor(s) which stimulates increased lymphocyte O_2 utilisation shows some of the features necessary for IC-mediated lymphocyte activation; that sera from patients with immune complex associated disease, are also able to increase O_2 utilisation of control lymphocytes, and further that a positive correlation exists between CIC and lymphocyte O_2 uptake; all implying that the phenomenon is an IC-mediated one.

5.2. Methods

The Clark electrode [Clark et al. 1953] [Fig. 5.1]

This apparatus permits polarographic measurement of the O_2 tension of solutions. Briefly, it comprises a silver anode and platinum cathode connected by a potassium chloride bridge, enclosed in an epoxy block, except for the exposed end of the cathode which is separated from the external environment by a thin (25u) teflon membrane which allows O_2 to diffuse into the probe but effectively isolates the sensor elements from contamination by the external environment. When a polarising voltage is applied across the probe all O_2 within the probe is consumed (reduced) at the platinum cathode

$$O_2 + 2e + 2H^+ \rightleftharpoons H_2O_2$$

$$H_2O_2 + 2e + 2H^+ \rightleftharpoons 2H_2O$$

and the resultant reduction current measured at the non-polarizable reference silver anode. Both voltage and O_2 tension affect the current but within a certain voltage range, -0.65 to -0.8 volts, known as the 'current-voltage plateau' (Fig. 5.2), the current - O_2 tension relationship is essentially independent of the voltage. Thus, at any voltage within this range, any change in current is a reflection of changing O_2 tension. The reduction current will accordingly flow in direct stochiometric relation to the rate of O_2 consumption. O_2 then diffuses through the teflon membrane at a rate proportional to the O_2 tension outside the probe, since O_2 tension within the probe is effectively zero.

This system satisfies the criteria essential for measurement of O_2 tension by polarographic means, namely, a definite O_2 tension

Fig. 5.1

CLARK OXYGEN ELECTRODE

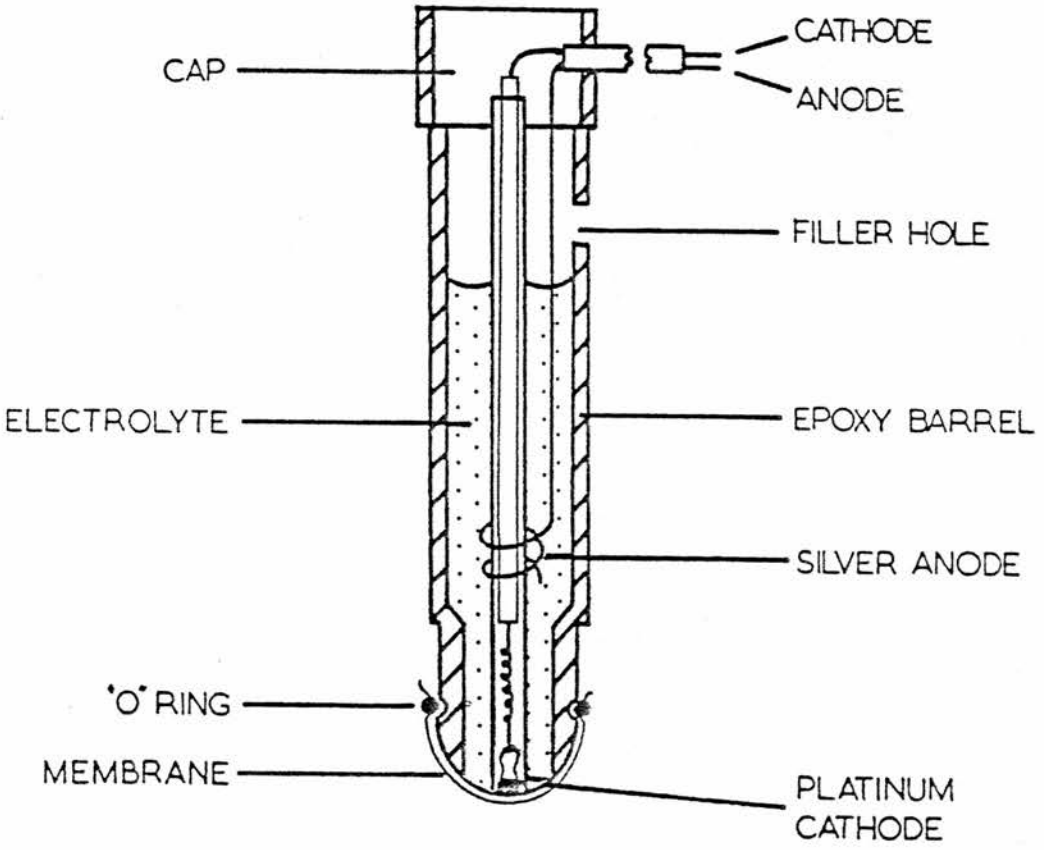
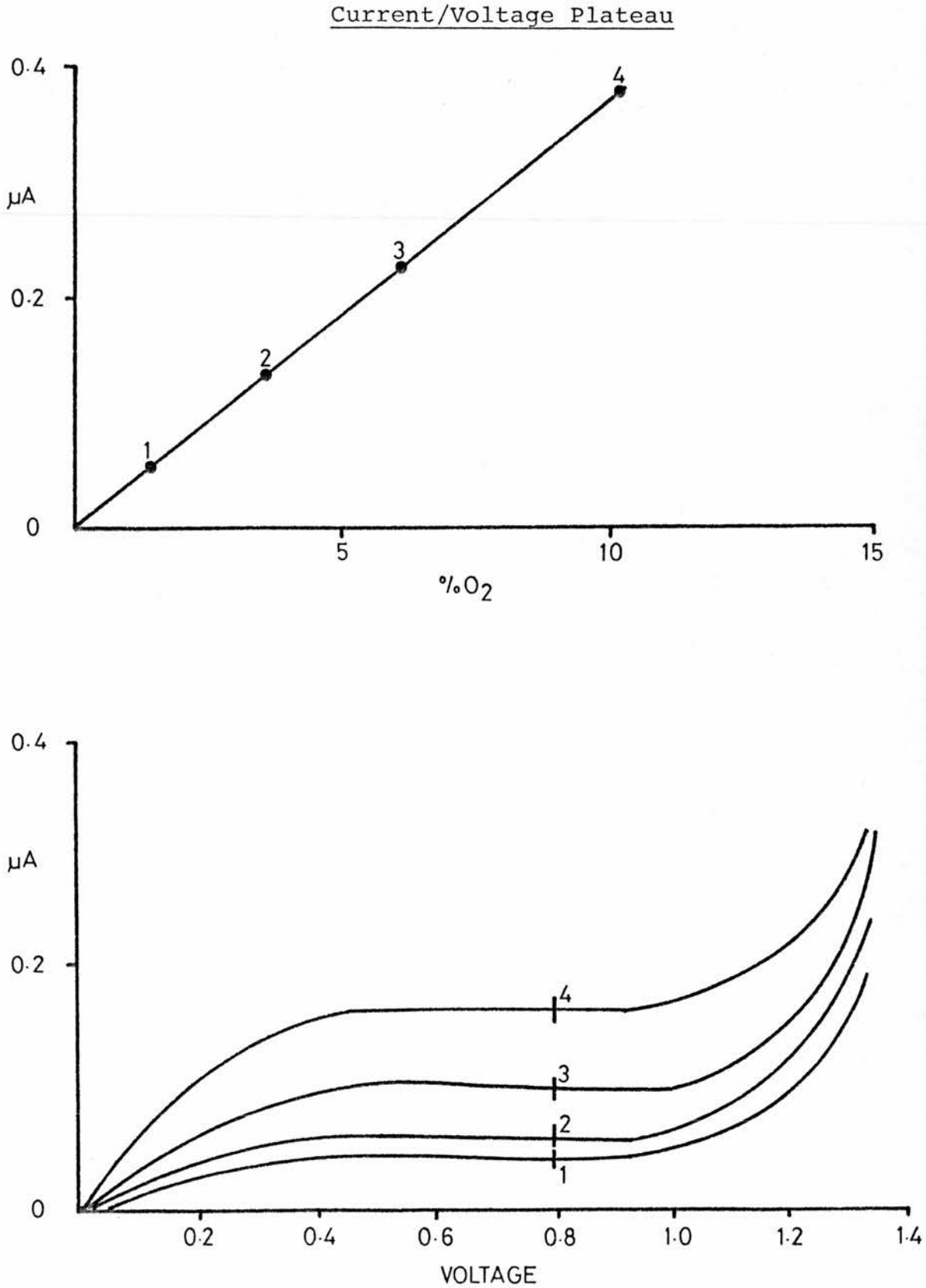


Fig. 5.2


Current/Voltage Plateau
0·4
μA
0·2
0
1
2
3
4
5
10
15
%O2
0·4
μA
0·2
0
1
2
3
4
0·2
0·4
0·6
0·8
1·0
1·2
1·4
VOLTAGE

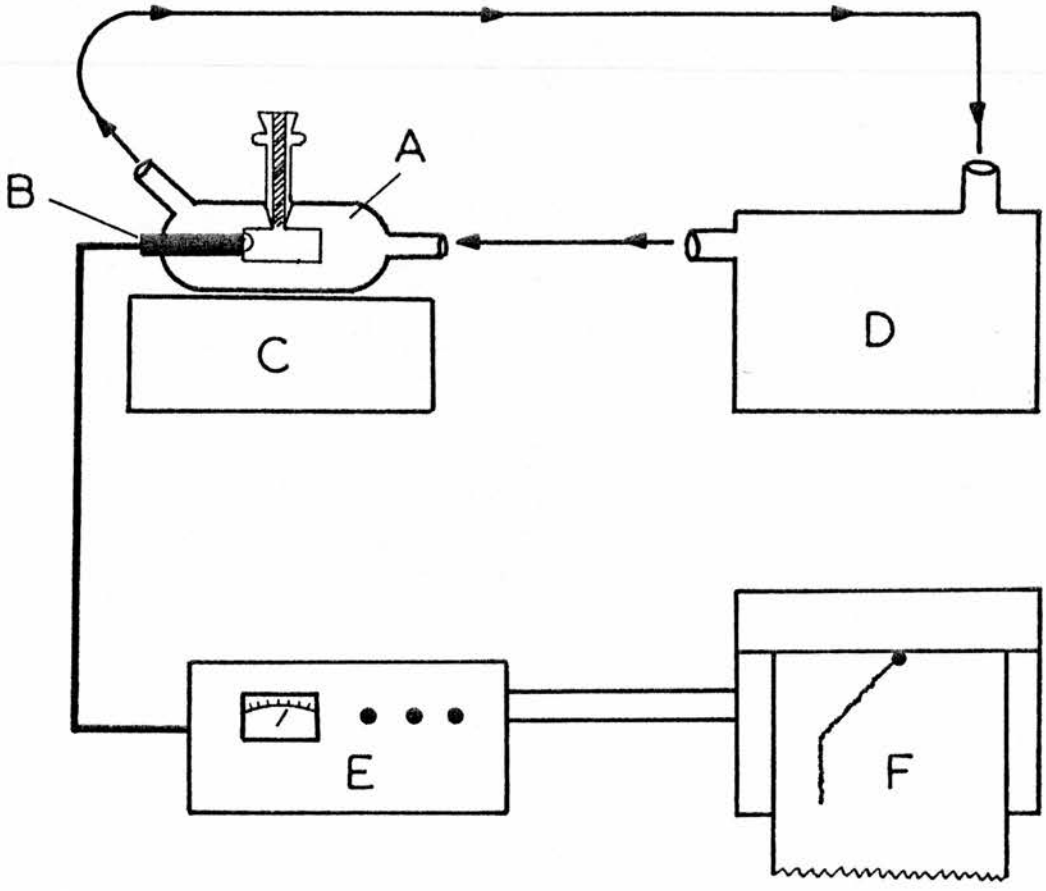
at the platinum electrode surface (preferably zero) and a constant diffusion zone between the sample to be measured and the platinum electrode surface. It takes approximately 30 secs for steady state conditions to develop, after which current flows through the probe proportional to the external O_2 tension but the probe is capable of giving 90% of final value readings by 10 secs.

The polarographic circuit is completed by a recorder to measure reduction current in the Gilson 5/6 oxygraph apparatus (Gilson Medical Electronics, Middleton, USA) (Figs. 5.3 & 5.4). The Clark electrode in this system is pushed through an opening in a water jacketed reaction chamber (capacity 1.5ml) (Fig. 5.5) such that the membrane only is in contact with the contents of the chamber. Temperature changes which may affect cell metabolic rate, O_2 saturation of the solution and teflon membrane permeability are prevented by maintaining a flow of water at $37\pm^{1}/_{2}^{o}C$ from a circulating water bath through the jacket surrounding the reaction chamber. Stasis of the reaction chamber contents, encouraging localised temperature variation and a static layer of cells adjacent to the membrane, is avoided by the use of a magnetic stirring flea, care being taken that its rotation is not sufficiently agitated to damage the probe membrane or cells in the solution. The contents of the chamber may be excluded from the outside environment by a capillary bore stopper which permits venting of the chamber thereby equalising internal and external pressures, and reduces diffusion of O_2 into the chamber by presenting a very small gas-liquid interface and any O_2 that does pass across this must first diffuse through the long narrow liquid column before entering the chamber.

Calculation of the amount of O_2 used by cells in a solution

Fig. 5.3

Diagram of Gilson Oxygraph



A = 1.5ml reaction vessel surrounded by a water-jacket with capillary bore stopper

B = Clark electrode

C = Magnetic stirrer

D = Circulating water bath

E = Polariser and amplifier

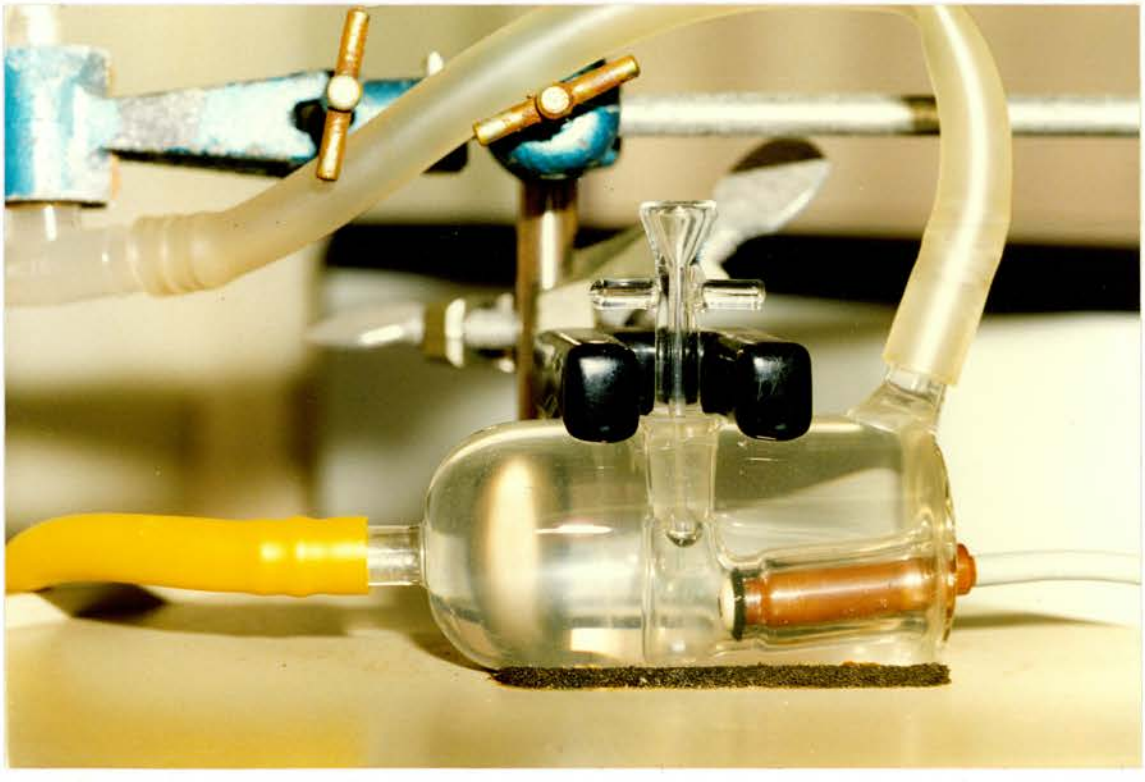
F = Pen recorder

Fig. 5.4

Gilson 5/6 Oxygraph

Fig. 5.5

Clark Electrode in Water Jacketed Reaction Vessel



requires prior knowledge of the normal saturation of O_2 in that solution at 37°C and the atmospheric pressure. However, the latter was found to influence results very little and its use in the calculations was quickly discarded. Prior to the introduction of the capillary bore stopper the recorder can be calibrated to read 100% saturation thus enabling changes in recorder pen deflection to be read directly as a percentage.

The advantages of this polarographic circuit are that the system is very sensitive permitting measurement of O_2 consumption by small numbers of cells, the response is very rapid avoiding long periods of equilibration time required in manometric techniques; there is no contamination by the external environment; and the probe itself does not consume large amounts of O_2 (8×10^{-11} gms/sec/uA). One disadvantage of the apparatus is that O_2 saturation is measured in a closed system and it is necessary to perform readings over short periods of time to avoid development of severe anaerobic conditions. Another problem is silver anode contamination due to the formation of silver chloride on the anode after a few weeks of use. This results in a non-linear response when calibrating the probe against solutions of differing known O_2 saturations (Fig. 5.6) and can be removed by treating the probe with 15% ammonium hydroxide solution for 10 mins. (Le Fevre 1969). This was done once a week.

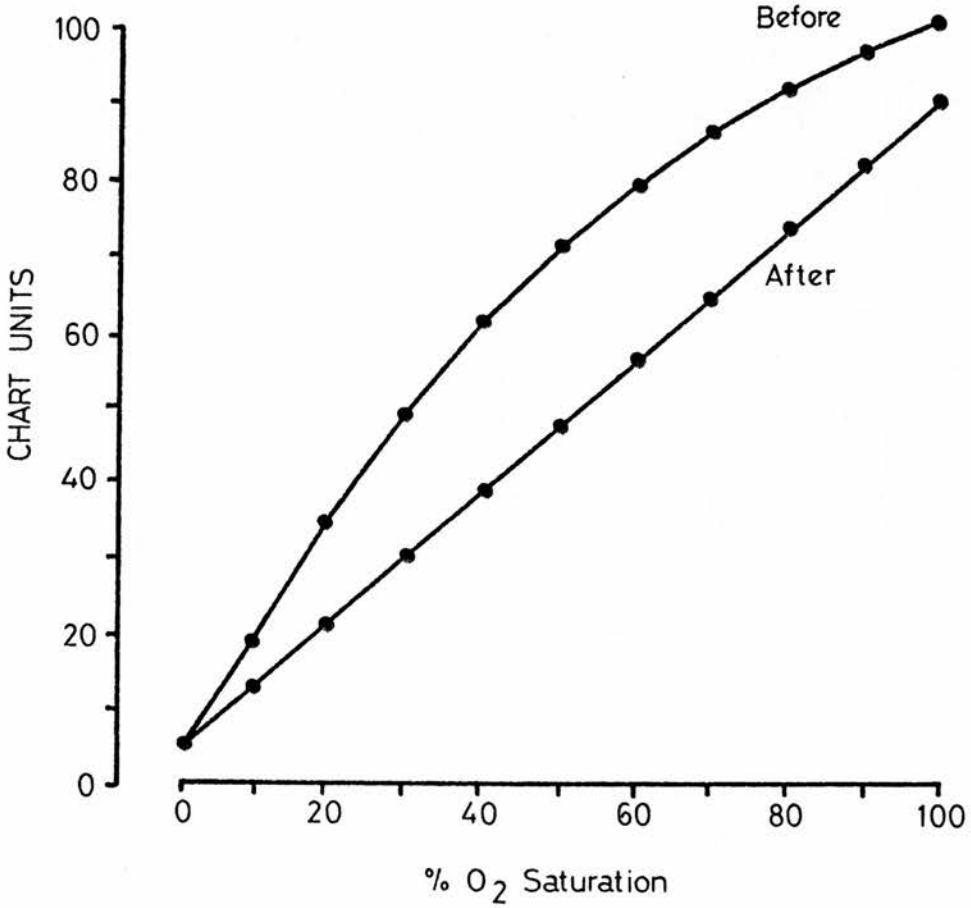
5.2.2. Separation of lymphocytes

Polymorphonuclear leucocytes (PMNs) use similar (Barron & Harrop 1929; Hedeskov & Esmann 1966) or slightly less (Rauch et al. 1961; Evans & Blore 1973) O_2 than lymphocytes under resting conditions and monocytes rather less O_2 than neutrophils (see later). Platelets use approximately one

Fig. 5.6

Treatment of Silver Electrode Contamination

With Ammonium Hydroxide



eleventh to one eighteenth as much O_2 as lymphocytes on a one to one basis (Altman & Dittmer 1968; Muenzer et al. 1975). O_2 utilisation by erythrocytes is very low (Altman & Dittmer 1968) but these cells interfere with O_2 tension measurements within the oxygraph chamber by dissociation of oxyhaemoglobin and diffusion of O_2 into the medium as the O_2 tension drops through use by other cells under assay. These contaminating cells must therefore be excluded as best possible.

Twenty to 30ml of blood was taken by venepuncture, placed in a sterile container and immediately defibrinated using a sterile glass rod, by stirring gently for approximately 5 mins to get rid of platelets. The blood was placed over sterile ficoll-hypaque in aliquots of 7ml blood to 3ml ficoll-hypaque and the mononuclear cells separated, washed and pelleted as described in Chapter 4. The pellet was resuspended vigorously in 0.5ml of distilled sterile water for 20 to 25 secs to lyse erythrocytes and then flooded with 8mls of sterile isotonic saline and centrifuged for 5 mins at 400g. This pellet was left in isotonic saline and stored at $4^{o}C$ until use. This did not affect viability within the 2 to 3 hours for which the cells were, at most, stored prior to testing. The fact that centrifugation and hypotonic lysis did not affect either viability or ability of lymphocytes to consume O_2 is in accord with the findings of Pachman (1967) following centrifugation separation of lymphocytes and hypotonic lysis of red cells. Granulocyte contamination of the lymphocyte sample was assessed morphologically and ranged from $2.74 \pm 1.38\%$ in controls to $6.31 \pm 3.84\%$ in patients with metastatic breast cancer, similar to that of lymphocyte pellets from heparinised blood (Chapter 4).

5.2.3. Separation of polymorphonuclear leucocytes (PMN)

Ten ml of venous blood was mixed with Dextran 110 in isotonic saline (Fisons Pharmaceutical Division, UK) in a ratio of 2 parts blood to 1 part Dextran and allowed to sediment for 1 hr. The leucocyte-rich plasma was layered onto 3ml ficoll-hypaque and centrifuged at 400g for 30 mins. The supernatant was removed and the residual pellet washed once at 400g for 5 mins in isotonic saline. Red cells were removed by hypotonic lysis as previously described, leaving a resultant population of PMN of 98% purity. Viability of cells was checked by Trypan blue exclusion and found always to be greater than 95%.

5.2.4. Separation of monocytes

Twenty ml of venous blood was separated over ficoll-hypaque as described in Chapter 4 and the mononuclear band removed and washed once in isotonic saline. The monocytes were then purified using a discontinuous hyperosmolar percoll gradient. (This discontinuous gradient is formed by layering three differing specific gravity solutions of percoll on each other. Stock solution of percoll comprises 9ml percoll to 1ml x 10 Eagle's MEM. Percoll of SG 1.074 is made by adding 1.734ml of Eagle's MEM to 2.266ml stock percoll. Percoll of SG 1.066 is made by adding 2ml Eagle's MEM to 2ml stock percoll. Percoll SG 1.057 is made by adding 2.304ml of Eagle's MEM to 1.696 stock percoll.) The mononuclear cell pellet was resuspended in 2ml. percoll of SG 1.074 onto which was carefully layered 2ml of percoll SG 1.066 followed by a layer of 2ml percoll SG 1.057. This was centrifuged at 2,200g for 90 mins. The monocytes were recovered from interface I between percoll of SG 1.057 and SG 1.066, and washed twice at 400g in isotonic saline. Viability was checked by Trypan blue exclusion and found to be greater than 95%.

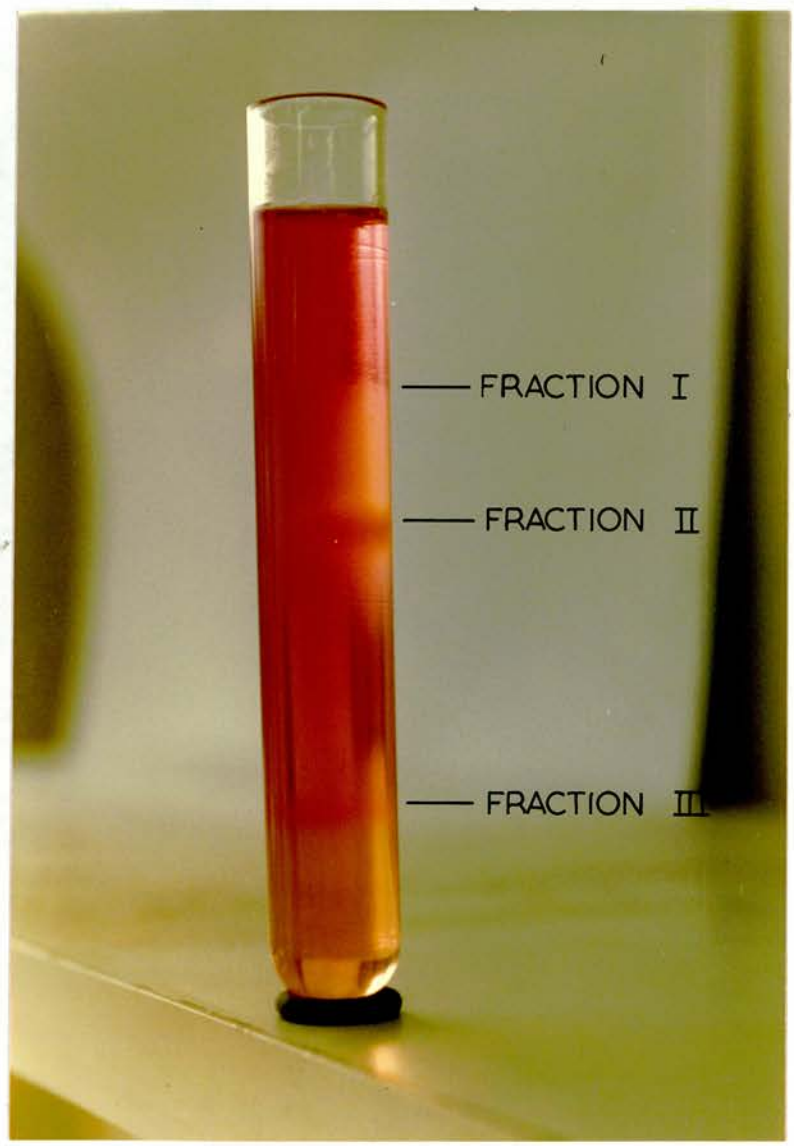
5.2.5. Separation of lymphoyctes into T-rich and B-rich fractions

Twenty to 30ml of venous blood to which had been added 150mg carbonyl iron was mixed on a blood wheel at 37°C for 30 mins. The blood was placed in 10ml sterile tubes over a magnet and allowed to sediment. The monocyte depleted plasma was then layered over ficoll-hypaque and the mononuclear band harvested in the usual manner and washed once in isotonic saline. Enrichment of T and B cell rich subpopulations was achieved by discontinuous percoll gradient (Gutierrez et al. 1979). Percoll 100% was made by mixing 1 part x 10 phosphate buffered saline (PBS) with 9 parts percoll which was progressively diluted in normal PBS to obtain solutions containing 70, 60, 50 and 40% of percoll. The cell pellet was resuspended in 100% percoll and 2ml of each percoll solution, in order of decreasing concentration, layered over this with great care. This was centrifuged at 2,200g for 10 mins. Cells remaining in the interface between 40 and 50% percoll concentration, 'fraction I', were B cells. Cells between 50 and 60%, 'fraction II' and between 60 and 70%, 'fraction III', were T lymphocytes (Fig. 5.7).

Identification of purity of the T cell rich population was performed by E-rosetting as described in Chapter 4. Identification of purity of the B cell rich population was performed using immunofluorescence staining for surface membrane immunoglobulin (SMIg). Essentially an aliquot of 10^6 B-rich lymphocytes was resuspended in 1ml acetate buffered saline and incubated at 37°C for 30 mins. The cells were washed twice in PBS and resuspended in 5ml Eagle's MEM at 37°C for 1 hr to remove cytophilic antibodies. The cells were then centrifuged at 400g for 5 mins and the pellet resuspended in 1ml of 0.02% sodium azide in PBS and 50ul anti IgG fluorescent conjugated rabbit antiserum (Becton Dickinson, Middlesex, UK) added to this, mixed and allowed to stand on crushed ice for 30 mins. Five mls Eagle's MEM

Fig. 5.7

Percoll Separation of Lymphocyte Subsets



was then added, mixed and centrifuged at 400g for 5 mins to remove unbound conjugates. The supernatant was discarded and the cells washed twice in Eagle's MEM. The cell pellet was mixed in 50ul Eagle's MEM, placed on a slide and covered with a coverslip which was sealed with nail varnish and examined under a fluorescent microscope and a % count performed.

5.2.6. Medium

The medium used in all experiments was Eagle's MEM.

Ten mls Eagle's MEM containing Earle's salts and L-glutamine without sodium bicarbonate, 10 x concentration (Gibco Europe UK) was added to 90mls double distilled sterile water, 100ul streptomycin, 50ul crystapen and buffered to pH 7.4 with 7% sodium bicarbonate. Protein supplements were not used in the medium as they may cause an unpredictable loss of sensitivity of the electrode and were felt unnecessary for the short period of incubation.

5.2.7. Calculation of solubility coefficient of O_2 in Eagle's MEM

The O_2 concentration of fully air saturated Eagle's MEM was calculated using an adaptation of Bruhn's modification of the Winkler technique described by Golterman (1969). This is described in Appendix 5.6. The value for Eagle's MEM was 4.24ul O_2/ml of medium at atmospheric pressure and 37^{o}C.

5.2.8. Measurement of lymphocyte O_2 consumption

A flask of Eagle's MEM in a water bath at 37^{o}C was air saturated by slowly bubbling filtered preheated air through it at 37^{o}C. This medium was used to set the full scale deflection (FSD) on the pen

recorder at the 100% value. The baseline was set by using the zero current setting on the oxygraph and could be checked by depleting the O_2 content of the medium with sodium sulphite.

The pellet of lymphoyctes, following removal of the isotonic saline supernatant, was resuspended in 2ml of air saturated Eagle's MEM and allowed to equilibrate for 10 mins in the water bath at 37°C. An aliquot was taken for cell count and viability assay by Trypan blue exclusion test. Viability was never less than 95% and all cells including PMNs and monocytes were counted, no special calculations being made to exclude their potential effect. It should be noted that later experiments showed PMNs and monocytes were not affected by serum in the same way as lymphocytes, and that there was little variation in the contaminating numbers of PMNs and monocytes between different patient groups sufficient to have affected results. 1.5ml of the cell suspension was then placed in the reaction chamber, allowed a further 5 mins equilibration with respect to temperature, following which, the capillary bore stopper was inserted, and the percentage drop in O_2 tension of the medium measured over the ensuing 15 mins. This percentage drop reflected the percentage of O_2 in the chamber consumed by the cells together with the small amounts of O_2 normally used by the electrode itself. The electrode's consumption was calculated at the beginning of each day, by measuring the percentage drop in O_2 tension of Eagle's MEM without cells in the presence of the Clark electrode over a 15 min period of time. By subtracting the percentage drop caused by the electrode alone from that caused by the electrode plus cell suspension, the percentage drop in O_2 tension caused by the cells alone was calculated. With knowledge of the amount of O_2 dissolved in 1ml of Eagle's MEM at 37°C at atmospheric pressure and the number

of cells in the chamber, the absolute volume of O_2 consumed per million cells per unit of time could be calculated. Lymphocyte O_2 consumption was then expressed in ul/million cells/sec.

Because of the 'crowding effect' on lymphocytes, described by others using manometric methods, occurring after half an hour from the beginning of measurements (Baron & Harrop 1929; Hedeskov & Esmann 1966), a series of readings of O_2 consumption were plotted for different concentrations of lymphocytes, monocytes and PMNs (Fig. 5.8; Appendix 5.7). These indicated that the crowding effect, shown as a reduction in O_2 uptake per cell when cell concentrations are high, and thought to be a result of reduced availability of O_2 (Hedeskov & Esmann 1966), was not a feature of experiments utilising the oxygraph system. This may have been a consequence of the short periods over which experiments were carried out, or the efficient equilibration of O_2 tension throughout the medium by the magnetic stirrer. However, in order to prevent any doubt, aliquots of lymphocytes were kept as often as possible between 2 to 3 x 10^6 cells/ml of medium.

5.2.9. Measurement of PMN and monocyte O_2 consumption

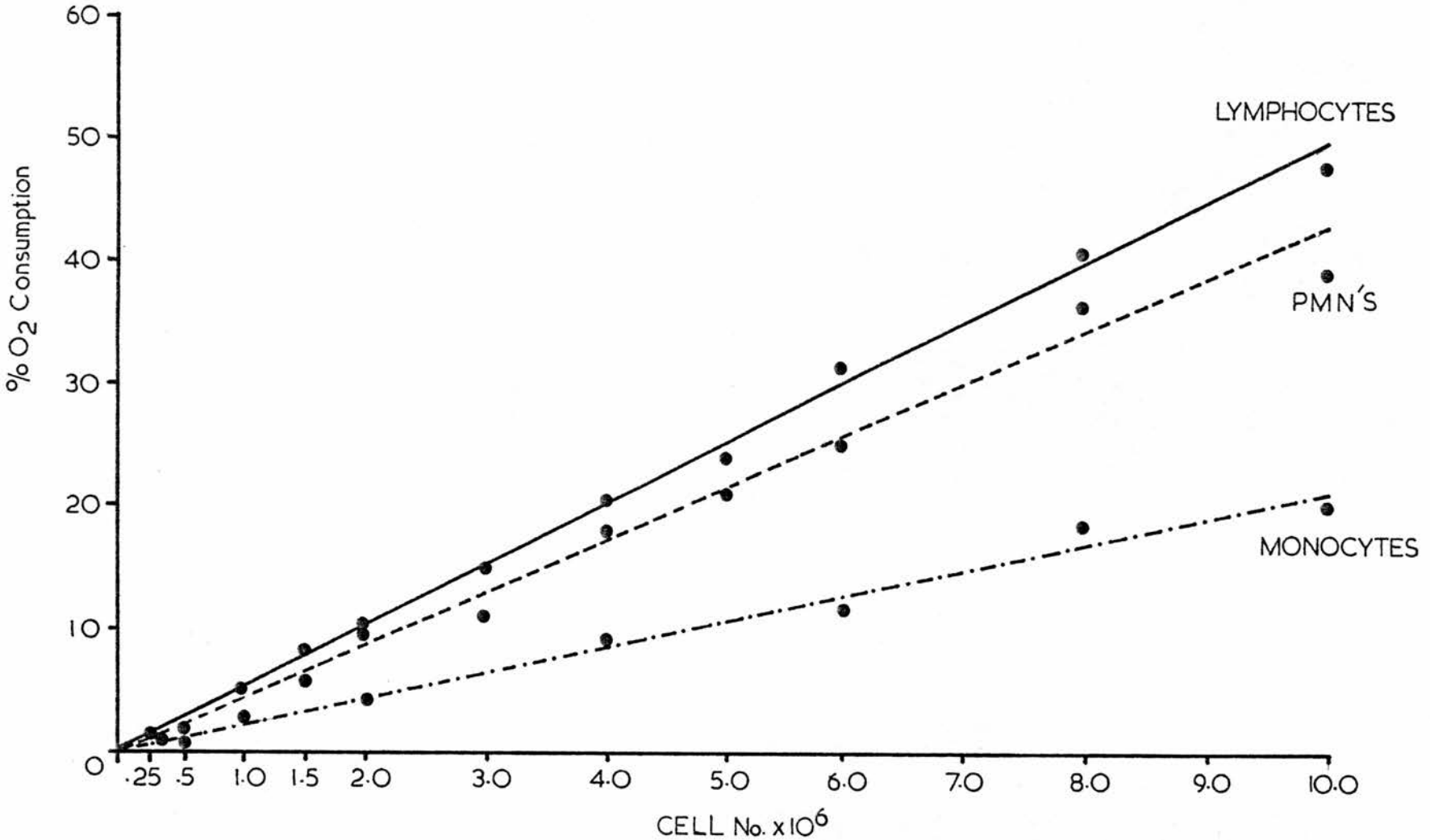
These cells following separation were incubated and treated in identical fashion to lymphocytes prior to assay of O_2 consumption.

5.2.10 Papain treatment

Lymphocytes or other cells separated and washed as previously described, were incubated with 0.6mg/ml of papain (twice crystallised, 11 units/mg, Sigma, London) in 2.5mmol/l cysteine hydrochloride in Earle's

Fig. 5.8

% O_2 Consumption vs Cell Concentration



balanced salt solution containing 5% normal human serum for 1 hr at 37°C (Chapel 1973; Whitehead et al. 1976) or at 4°C. The cells were then washed once at 400g for 5 mins and resuspended in 2mls of air-saturated Eagle's MEM and incubated at 37°C for 10 mins prior to numbers, cell viability and O_2 consumption being calculated; or in autologous or allogeneic serum depending on the experiment being performed.

5.2.11. Serum incubation

All cells were first treated with papain as described and following washing were resuspended in either autologous or allogeneic serum and incubated at 37°C for a further 1 hr. The cells were then washed once at 400g, resuspended in 2ml of air-saturated Eagle's MEM and incubated at 37°C for 10 mins prior to numbers, cell viability and O_2 consumption being calculated.

5.2.12. Separation and storage of serum

Ten ml of venous blood was withdrawn by venepuncture from a peripheral vein and placed in a sterile container containing no anti-coagulant. The blood was allowed to clot at room temperature and left standing for 2 to 3 hrs following which it was centrifuged at 500g for 20 mins. The serum was removed aseptically and either used immediately or stored at -70°C. Sera for experiments on patients with immune complex related diseases however were stored at -20°C and used within 3 months.

5.2.13. Serum dilution

Sera stored at -70°C for less than 1 month from 3 patients

with breast cancer and 2 with advanced colonic cancers were thawed and diluted to varying concentrations with isotonic saline. Donor papain treated control lymphocytes were incubated in these various sera at 37°C for 1 hr, washed once and incubated in Eagle's MEM for a further 10 mins prior to calculation of cell numbers, viability and lymphocyte O_2 consumption.

5.2.14. Heat inactivation of serum

Blood was withdrawn by venepuncture and lymphocytes and sera separated in the usual manner from 5 controls, 3 patients with breast cancer and 2 with advanced bowel malignancies. Lymphocytes from each subject were divided into 4 aliquots, one of which was used to assess lymphocyte O_2 consumption of the patients' cells and the remainder treated with papain. Sera were split into 3 aliquots, 2 of which were heated to 56°C for 30 mins. The remaining papain treated lymphocytes were incubated for 1 hr in a) autologous serum; b) autologous heat inactivated serum; c) autologous heat inactivated serum to which mixed control donor serum (200ul per 1800ul heat inactivated serum), as a complement source, was added. The cells were then washed, incubated in Eagle's MEM and their O_2 consumption assessed.

5.2.15. Sera and plasmapheresis

One patient with polymyositis, 2 with myaesthenia gravis, 1 with polyneuropathy, 1 with Guillain-Barre syndrome, 1 with autoimmune pancytopaenia and myositis, and 2 with Waldenstrom's macroglobulinaemia were examined (Appendix 5.17).

Plasmapheresis was performed by Mr. R. Nelson and Mr. R. Williams, and members of the Department of Haematology, Singleton Hospital, Swansea,

as part of the proscribed treatment by the patients' consultants. The apparatus used was the 'Progress BT 790 A' blood cell separator manufactured in Italy by 'Dideco' which can be used for plasma, platelet and leucopheresis by means of centrifugation in a semi-continuous mode. The separator requires the use of a sterile disposable circuit which includes a 'harness' attached to the patient's circulation through a 'Venflon' intravenous cannula in a peripheral vein, and a 'bowl', where the extracorporeal circulation of the patient will develop. This circulation takes place in a semi-continuous mode, because the machine operates at different phases; drawing a certain volume of blood from the patient, based on the volume of the 'bowl' used and the patient's haematocrit, and dividing it into its components. In the case of plasmapheresis the plasma was collected into a waste bag and the red cells together with a volume of 4.5% albumin and/or isotonic saline, equivalent to the volume of plasma removed at each cycle, was retransfused into the patients. By repetition of the operation it was possible to collect significant volumes of plasma from each patient, usually 2 to 3 litres at each procedure. At the beginning of the procedure each patient was given a loading dose of heparin (2,000 units/m^2). Heparinised isotonic saline (25,000 units/500ml) was used to anticoagulate the blood, prior to entry to the centrifuge 'bowl', through a pump which delivered a constant volume of one part heparinised saline to eight parts blood irrespective of the blood flow rate.

In each patient 10ml of blood for serum separation was removed from the venepuncture cannula prior to heparinisation. The cell separator was then connected to the patient's circulation through the intravenous cannula and the procedure commenced by giving the appropriate loading dose of heparin. After the required number of cycles had been performed a 10ml sample of the plasma was collected aseptically from the waste bag

and placed in a sterile container. The cell separator was disconnected from the patient and between 1 and 2 hrs later, 10mls blood for serum separation was removed from the cannula. The two samples of sera 'before' and 'after' plasmapheresis were also placed in sterile containers and, together with the plasma from the machine, stored at -20°C until the day of testing. They were then transported packed in ice in polystyrene boxes and were used within 4 hrs of removal from the freezer.

Papain treated control lymphocytes were incubated in all 3 samples at 37°C for 1 hr, washed and assessed for their O_2 consumption in the usual manner.

5.2.16. Statistical analysis

The significance of the difference in mean lymphocyte and other cell O_2 uptake values between groups and within groups was determined using the two-tailed Mann-Whitney U test.

Tables

Tables show the mean $\pm$ standard deviation of lymphocyte O_2 consumption.

Post-therapeutic values of O_2 consumption shown in the tables and appendices are the mean of all post-treatment results derived later than six weeks after the start of treatment in any single phase of the disease, unless otherwise stated.

5.3. Results

5.3.1. The effect of age, sex and disease status of patients and controls on lymphocyte O_2 consumption:

Lymphocyte O_2 consumption was assessed in 13 males and 31 females awaiting inguinal hernia repair or cholecystectomy; 75 women with benign breast disease; 27 with Stage I and II breast cancer; 24 with Stage III breast cancer and 25 with Stage IV breast cancer, prior to treatment (Appendices 5.1, 5.2, 5.8, 5.9, 5.10, 5.11; Table 5.1)

Age: Lymphocyte O_2 consumption was not affected by age. There was very little variation between different age groups in 31 female controls (Table 5.2). Moreover, when age was correlated with lymphocyte O_2 consumption within this group of women and 75 women with benign breast disease the correlation coefficients (r) were r=0.046 and r=0.062 respectively, indicating that there is no relationship between age and lymphocyte O_2 consumption.

Sex: Thirteen male controls had comparable lymphocyte O_2 consumption to the female controls indicating that sex of the patient does not influence this (Table 5.3).

Disease status: Women with benign breast disease tended to have greater lymphocyte O_2 consumption than female controls but this did not quite reach significance (p=0.06). Patients with malignant breast disease had significantly greater lymphocyte O_2 consumption than either controls or patients with benign breast disease ($p<0.001$) (Table 5.3; Fig. 5.9). This rise in lymphocyte O_2 consumption in those with breast

Table 5.1

Patients and Controls who had Pre-Treatment Assessment of Lymphocyte O_2 Consumption

Treatment	Male Controls	Female Controls	Benign Breast Disease	Breast Cancer I & II	Breast Cancer III	Breast Cancer IV
Surgery	n/a	n/a	75	21	15	0
Surgery & Tamoxifen	0	0	0	1	0	0
Hormone Therapy	0	0	0	5	5	25
Chemotherapy	0	0	0	0	4	0
Total	13	31	75	27	24	25

Table 5.2

Female Controls. Lymphocyte O_2 Consumption for Varying Age Groups ($\mu l \times 10^{-5}/10^6$ cells/sec)

Age Range	Controls	Lymphocyte O_2 Consumption
<30 yrs	7	6.20 ± 0.86
31 - 40 yrs	6	6.01 ± 0.78
41 - 50 yrs	8	6.21 ± 0.80
51 - 60 yrs	5	6.38 ± 0.71
60+ yrs	5	6.13 ± 0.23

cancer was also stage related but the difference was only significant between Stage I and II cancer and Stage IV disease ($p<0.05$).

5.3.2. The effect of surgery in operable breast cancer on lymphocyte O_2 consumption (Appendix 5.8).

Lymphocyte O_2 consumption values were available in 21 patients pre-operatively and 22 patients at least 6 weeks post-operatively with Stage I and II disease, and in 15 patients pre-operatively and 10 patients at least 6 weeks post-operatively with Stage III breast cancer. There was no significant difference between pre- and post-operative values (Table 5.4).

5.3.3. The relationship between prognostic factors in operable breast cancer and lymphocyte O_2 consumption (Appendix 5.8).

Insufficient detailed data were available on primary tumour grading but there was adequate data on oestrogen receptors and axillary node histology to permit comparison with the lymphocyte O_2 consumption of these patients (Table 5.5). Neither pre- nor post-operative lymphocyte O_2 consumption bore any relationship to ER or axillary node status. Furthermore, there was no significant difference in lymphocyte O_2 consumption, either pre-operatively or post-operatively, between patients who developed disease recurrence in less than 4 yrs from surgery and those who remained disease free (Table 5.5).

5.3.4. The effect of hormone therapy in primary locoregional disease on lymphocyte O_2 consumption (Appendix 5.7).

Six patients with Stage I and II disease had their lymphocyte O_2 consumption assessed; 5 prior to tamoxifen, 2 during PR on tamoxifen

Table 5.3

Lymphocyte O_2 Consumption in Controls, Benign Breast Disease and Varying Stages of Malignant Breast Disease Prior to Treatment

($\mu l \times 10^{-5}/10^6$ cells/sec)

Patients	Age	Lymphocyte O_2 Consumption	Mann-Whitney U Test
Control male (13)	41±15	6.38±0.63	
Control female (31)	44±16	6.18±0.70	vs male controls Z=0.78; NS
Benign breast disease (75)	42±15	6.69±1.23	vs control Z=1.84; p=0.06
Stage I & II breast cancer (27)	57±17	8.89±1.30	vs control Z=5.92; p<0.001 vs benign breast disease Z=5.78; p<0.001
Stage III breast cancer (24)	64±15	9.54±1.89	vs I & II Z=0.93; NS
Stage IV breast cancer (25)	55±13	9.74±1.76	vs I & II Z=2.0; p<0.05

Table 5.4

Pre- and Post-Operative Lymphocyte O_2 Consumption in Stage I, II, and III Breast Cancer

	Pre-Operative Lymphocyte O_2 Consumption	Post-Operative Lymphocyte O_2 Consumption	Mann-Whitney U Test
Stage I & II	8.97±1.35 (n=21)	8.39±1.67 (n=22)	Z=1.34; NS
Stage III	9.11±1.86 (n=15)	8.62±0.54 (n=10)	U=67.5; NS

($\mu l \times 10^{-5}/10^{-6}$cells/sec)

Table 5.5

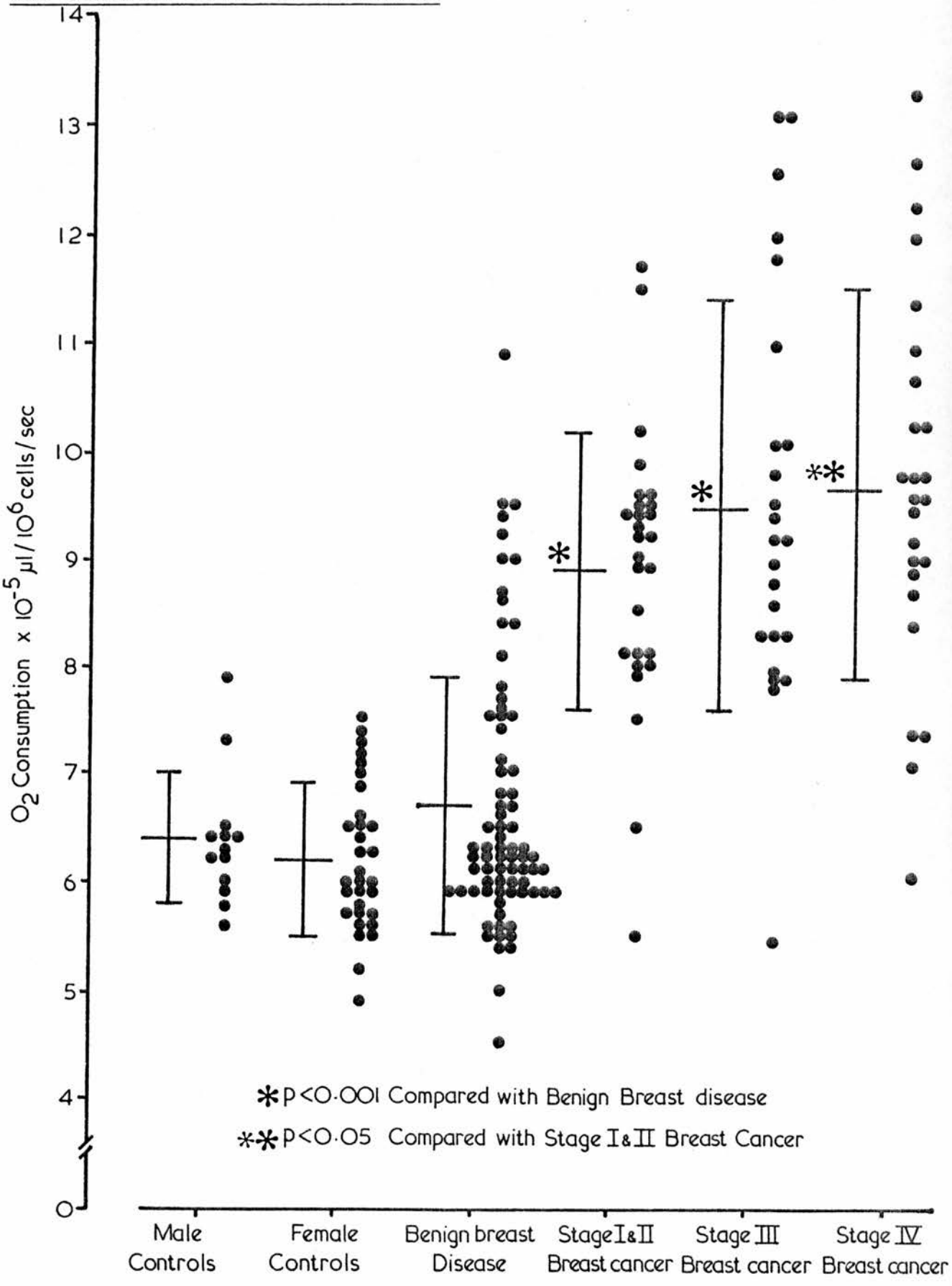
Pre- and Post-Operative O_2 Utilisation in Stage I, II and III Breast Cancer Compared with Prognostic Factors and Recurrence Within 4 Years from Treatment

($\mu l \times 10^{-5}/10^{-6}$ cells/sec)

	Lymphocyte O_2 Consumption	
	Pre-Operative	Post-Operative
ER +ve	9.15±1.16 (n=10)	8.45±0.96 (n=12)
ER -ve	8.83±2.17 (n=5)	8.56±1.75 (n=8)
Node +ve	9.21±1.08 (n=13)	8.56±0.66 (n=10)
Node -ve	8.90±1.65 (n=18)	8.91±1.75 (n=14)
No recurrence <4 yrs	9.17±1.21 (n=7)	8.77±1.62 (n=13)
Recurrence <4 yrs	9.49±2.12 (n=9)	8.64±1.12 (n=10)

Fig. 5.9

Lymphocyte O_2 Consumption in Various Stages of Breast Disease and Controls Prior to Treatment



and 1 during progression of disease. Eight patients with Stage III breast cancer were similarly assessed; 5 prior to tamoxifen, 1 during PR on tamoxifen, 2 during NC on tamoxifen and 4 during progression of disease.

There was no difference between the lymphocyte O_2 consumption of patients prior to treatment and those with progressive disease (Table 5.6; Fig. 5.10) but patients benefiting from tamoxifen had significantly reduced lymphocyte O_2 consumption compared with pre-treatment and progressive disease values ($p<0.02$).

5.3.5. The effect of hormone therapy in Stage IV breast cancer on lymphocyte O_2 consumption (Appendix 5.10).

Data on lymphocyte O_2 consumption were available in 42 patients with Stage IV disease, 36 of whom were treated with tamoxifen, 5 with oophorectomy and 1 with androgens. Twenty-two patients were tested prior to treatment; 18 during progression of disease, 7 during PR, 2 during NC and 2 who were not assessable using UICC criteria but had no symptomatic or clinical evidence of disease progression for at least 6 months.

There was no difference between lymphocyte O_2 consumption in patients prior to treatment and in those in whom disease continued to progress or following failure of remission (Table 5.7; Fig. 5.11). Patients in whom disease was affected in a beneficial manner by treatment showed significantly decreased lymphocyte O_2 consumption compared with pre-treatment and progressive disease patients ($p<0.001$) and even patients with Stage III disease ($p<0.005$) and Stage I and II breast cancer ($p<0.01$).

Five patients with primary locoregional breast cancer and 12 with Stage IV disease treated by endocrine manipulation had their lymphocyte O_2 consumption tested in more than one phase of their illness. The

Table 5.6

Primary Locoregional Breast Cancer Treated with Tamoxifen

Change in Lymphocyte O_2 Consumption with Response

($\mu l \times 10^{-5}/10^6$ cells/sec)

Patients	Lymphocyte O_2 Consumption	Mann-Whitney U Test	
Pre Rx (10)	9.80±2.10		
Progression (5)	10.01±1.75	vs Pre Rx	U=24.0; NS
PR/NC/NA (5)	6.22±1.10	vs Pre Rx	U=2.0; p<0.02
		vs Prog.	U=0; p<0.01

Table 5.7

Stage IV Breast Cancer Treated with Hormone Therapy.

Change in Lymphocyte O_2 Utilisation with Response.

($\mu l \times 10^{-5}/10^6$ cells/sec)

Patients	Lymphocyte O_2 Utilisation	Mann-Whitney U Test	
Pre Rx (25)	9.74±1.76		
Progression (18)	9.67±2.02	vs Pre Rx	Z=0.07; NS
PR/NC/NA (11)	7.87±1.42	vs Pre Rx	Z=3.04; p<0.002
		vs Prog.	U=44.5; p<0.02

Fig. 5.10

Lymphocyte O_2 Consumption Related to Response of Patients with Primary Locoregional Breast Cancer Treated with Tamoxifen

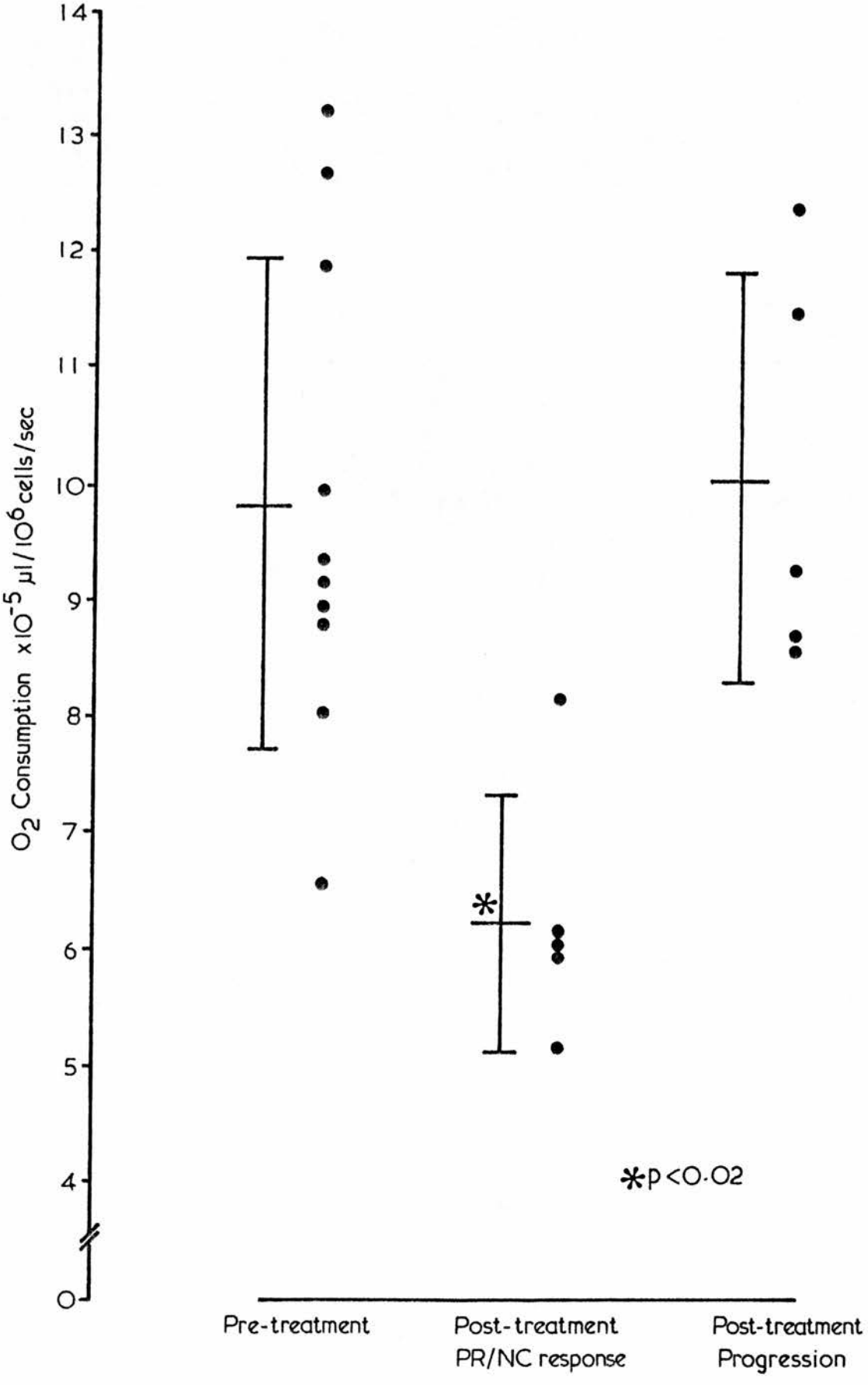
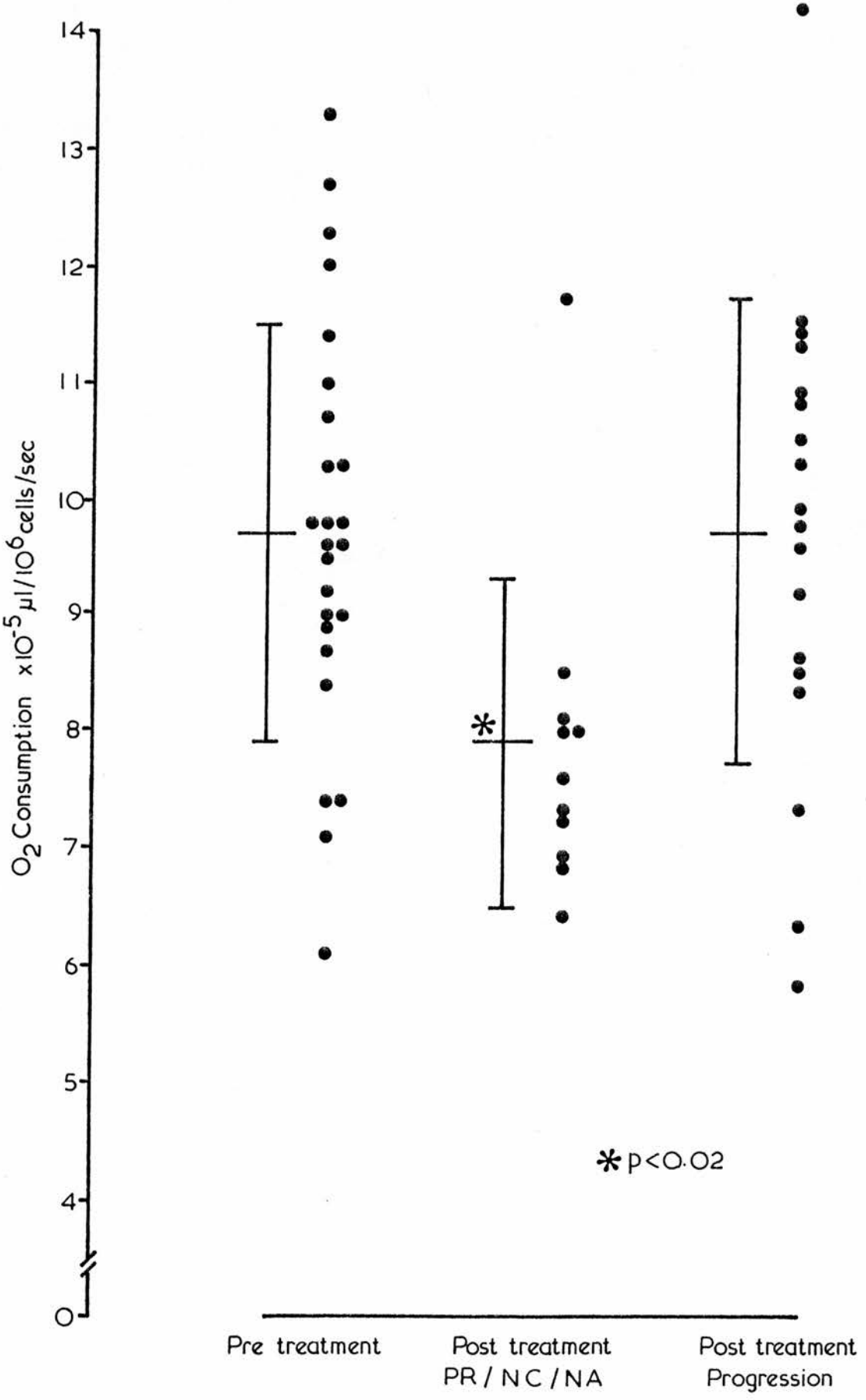


Fig. 5.11

Lymphocyte O_2 Consumption Related to Response of Patients with Stage IV Breast Cancer Treated by Hormonal Manipulation



changes in lymphocyte O_2 consumption with respect to treatment outcome are shown in Fig. 5.12. Moreover, it was noted in 11 patients with various stages of breast cancer treated by hormone manipulation who were tested between 6 and 8 weeks from the start of therapy that patients who derived benefit had decreased lymphocyte O_2 consumption compared with their pre-treatment values and with that of patients in whom disease continued to progress (Table 5.8).

5.3.6. The effect of other cancers on lymphocyte O_2 consumption (Appendix 5.12).

Several patients with other types of cancer were also investigated with regard to their lymphocyte O_2 consumption. Six had carcinoma of the stomach, 2 carcinoma of the pancreas, 3 malignant melanoma, 1 carcinoid tumour, 1 carcinoma of the lung, 1 ovarian cancer and 13 had colorectal carcinoma. One patient with colorectal cancer was staged Dukes' A, 3 Dukes' B, 4 Dukes' C and 5 Dukes' D.

The majority of these cancer patients had lymphocyte O_2 consumption values at least as great as that of patients with disseminated breast cancer with the exception of those with Dukes' A and B colorectal carcinoma in whom lymphocyte O_2 consumption was lower (Table 5.9). Indeed, there was a stage related trend for lymphocyte O_2 consumption to increase with progression of colorectal cancer, the difference between localised node negative disease and disseminated cancer being significant ($p<0.02$).

5.3.7. The effect of cancer on O_2 consumption of B and T lymphocyte subpopulations (Appendix 5.13).

Table 5.8

Lymphocyte O_2 Consumption in Patients Tested Between 6 and 8 Weeks from the Start of Hormone Therapy

Patient	Stage	Response	Pre Rx	Post Rx 6 to 8 weeks
EW	II	PR	9.32	6.99
FL	III	NC	13.12	6.14
VB	IV	NC	9.20	7.20
CD	IV	PR	11.99	7.75
LS	IV	N/A	12.70	8.29
EP	II	Prog.	9.91	9.22
ED	III	Prog.	-	8.60
GL	III	Prog.	11.75	13.68
GJ	IV	Prog.	-	9.90
AM	IV	Prog.	8.90	8.99
CS	IV	Prog.	-	9.60

Table 5.9

Various Cancers. Lymphocyte O_2 Consumption Prior to Treatment

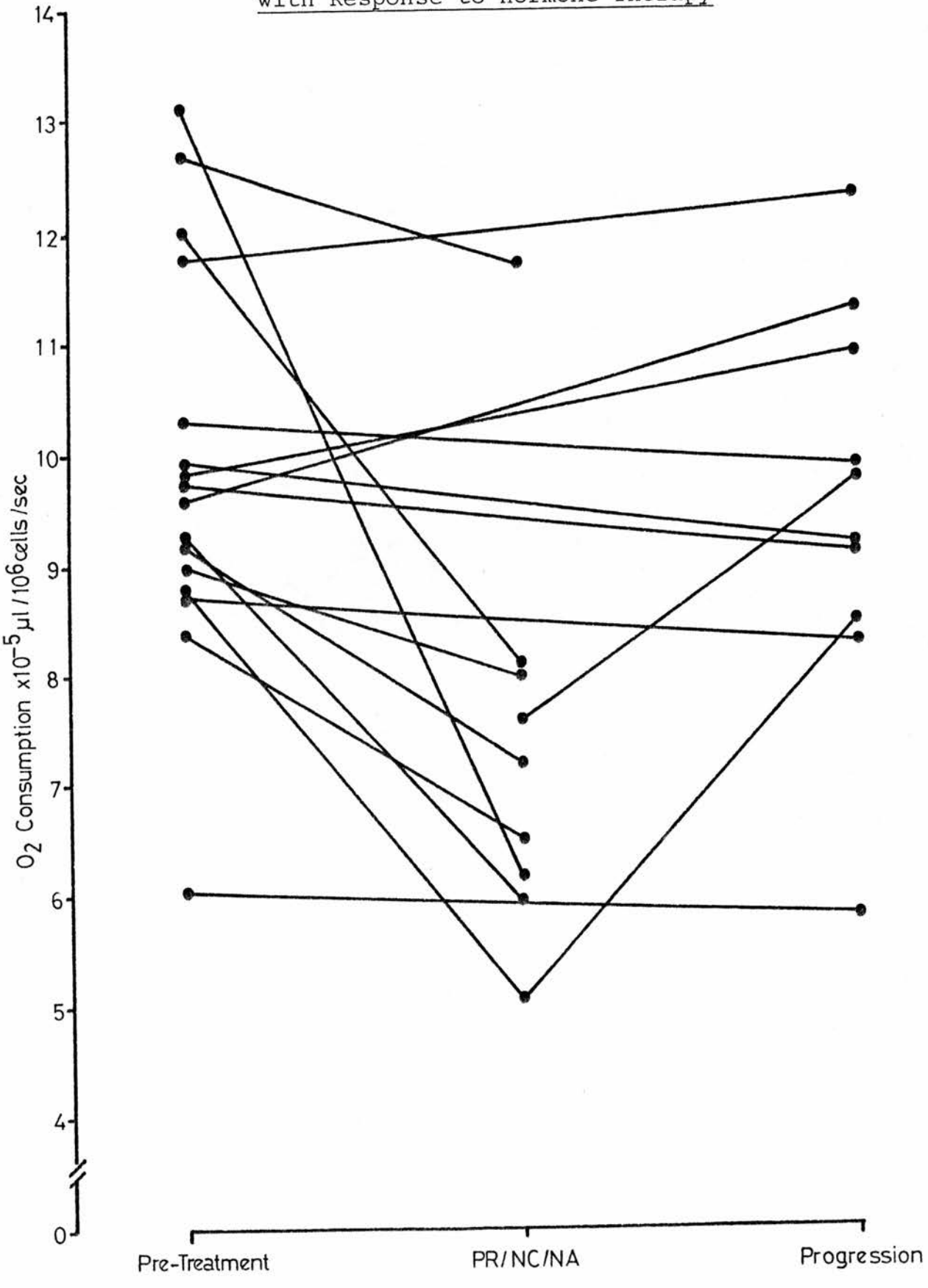
($\mu l \times 10^{-5}/10^6$ cells/sec)

Organ	Stage	Lymphocyte O_2 Utilisation
Colorectal	Dukes' A & B	7.94±1.27 (n=4)
Colorectal	Dukes' C	9.23±2.35 (n=4)
Colorectal	Dukes' D	11.57±2.03* (n=5)
Stomach	Locally Advanced	10.53 (n=1)
Stomach	Disseminated	10.62±1.54 (n=5)
Malignant melanoma	Disseminated	11.17±1.53 (n=3)
Ovary	Disseminated	14.21

* Comparison of Dukes' D with Dukes' A & B colorectal cancer ($U=0$, $p<0.02$) Mann-Whitney U Test

Fig. 5.12

Stages II, III & IV Breast Cancer. Lymphocyte O_2 Consumption with Response to Hormone Therapy



Separation of lymphocytes into T-rich and B-rich subpopulations showed no difference in separation properties of control and cancer lymphocytes (Table 5.10). The T cell rich fraction from control patients however, consumed significantly more O_2 than did B cells ($p<0.001$). The T cell rich fraction from cancer patients consumed more O_2 than the B-rich cells ($p<0.001$) and also more than the T-rich population from controls ($p<0.001$). There was no difference between the O_2 consumption of B-rich lymphocyte populations from cancer patients and controls.

5.3.8. To show that increased lymphocyte O_2 consumption in cancer patients is an effect of a serum factor(s) (Appendix 5.14).

It has previously been shown that incubation of lymphocytes from patients with cancer, with papain, results in a loss of 'blocking' factor(s) from the cell surface and that this factor(s) can be replaced by subsequent incubation in cancer serum (Whitehead et al. 1976; Whitehead et al. 1977), or indeed that lymphocytes from healthy individuals may become 'blocked' by incubation in cancer serum (Whitehead et al. 1977; Shukla et al. 1979). Experiments were performed to see if a similar factor(s) was responsible for stimulated lymphocyte O_2 consumption.

Lymphocytes from 15 healthy controls and 19 cancer patients were separated and incubated with papain at 37°C and lymphocytes from 4 controls and 10 cancer patients incubated with papain at 4°C for 1 hr, washed and incubated in Eagle's MEM prior to assessment of lymphocyte O_2 consumption. Papain treated lymphocytes from 11 controls and 15 cancer patients were also incubated in their autologous sera at 37°C for 1 hr. Eight cancer patients also had their papain treated lymphocytes

Table 5.10

O_2 Consumption of Unseparated Lymphocytes and B Rich and T Rich Subpopulations from Patients with Cancer and Controls

Purity of Cell Populations	O_2 Consumption	Mann-Whitney U Test
Unseparated control	5.94±0.27 (n=11)	
B rich control (77.1±4.1%)	4.56±0.43 (n=11)	
T rich control (79.9±3.5%)	6.14±0.17 (n=11)	vs B control U=0; p<0.001
Unseparated cancer	9.71±1.35 (n=15)	
B rich cancer (77.8±2.5%)	4.93±0.28 (n=15)	vs B control U=0; p<0.001
T rich cancer (79.9±2.3%)	10.06±1.32 (n=15)	vs T control U=0; p<0.001 vs B cancer U=0; p<0.001

incubated in allogeneic individual control freshly prepared sera for 1 hr and 10 of the controls had their papain treated lymphocytes similarly incubated in individual sera from cancer patients which was either freshly prepared or had been stored at -70°C for less than 3 months. Lymphocytes incubated in allogeneic or autologous sera were then washed and incubated in Eagle's MEM prior to assessment of lymphocyte O_2 consumption.

Papain treatment of lymphocytes at 37°C had no effect on control lymphocyte O_2 consumption but following incubation of these lymphocytes in cancer sera their O_2 consumption rose significantly ($p<0.001$) (Table 5.11). Papain treatment at 37°C of lymphocytes from cancer patients had a significant effect ($p<0.001$) on their O_2 consumption, decreasing this to control values (Table 5.12). This fall in O_2 consumption was maintained following incubation in allogeneic control sera but reversed by reincubation in autologous cancer sera.

Papain treatment at 4°C had no significant effect on either control or cancer patients' lymphocytes, indicating that its effect on lymphocyte O_2 consumption was most likely to be mediated through its enzyme activity (Table 5.11; Table 5.12).

5.3.9. The effect of cancer sera on other white blood cells

(Appendix 5.15).

Lymphocytes, PMNs and monocytes were isolated from peripheral venous heparinised blood of 5 healthy controls as earlier described, treated with papain at 37°C and each cell type divided into aliquots, one of which was incubated in autologous serum and the other, in allogeneic cancer serum previously prepared from a patient with breast cancer and stored at -70°C for less than 3 months. Following incubation and

Table 5.11

Papain Treated Control Lymphocytes Incubated with Autologous Serum and Allogeneic Cancer Serum

($\mu l \times 10^{-5}/10^6$ cells/sec)

		Lymphocyte O_2 Consumption
Lymphocytes	(15)	6.30±0.57
Lymphocytes (Papain 37°C)	(15)	6.04±1.05
Lymphocytes + (Papain 4°C)	(4)	6.47±0.42
Lymphocytes (Papain 37°C) + autologous serum	(11)	6.14±0.74
Lymphocytes (Papain 37°C) + cancer sera	(10)	10.01±0.97*

* Compared with all other values U=0; $p<0.002$

Mann-Whitney U Test

Table 5.12

Papain Treated Cancer Lymphocytes Incubated with Autologous Serum and Allogeneic Control Serum

($\mu l \times 10^{-5}/10^6$ cells/sec)

	Lymphocyte O_2 Utilisation	Mann-Whitney U Test
Lymphocytes (19)	9.82±2.18	
Lymphocytes (19) (Papain 37°C)	6.24±1.20	vs L (U=17; p<0.002) vs Papain 4°C (U=7;p<0.002) vs Papain + autol serum (U=12; p<0.002) vs Papain + allogen control serum (NS)
Lymphocytes (10) (Papain 4°C)	9.80±1.72	
Lymphocytes (12) (Papain 37°C) +autologous serum	10.46±1.95	
Lymphocytes (8) (Papain 37°C) +allogeneic control serum	6.93±0.71	vs L (U=8.5; p<0.002) vs Papain 37°C (U=51; NS) vs Papain 4°C (U=3; p<0.002) vs Papain +autol serum (U=8; p<0.002)

washing the O_2 consumption of each cell type in its own and allogeneic cancer serum was assessed.

Lymphocytes tended to use slightly more O_2 than PMNs and significantly more than monocytes when incubated in autologous serum ($p<0.01$). Lymphocytes from all 5 controls were stimulated to consume more O_2 by incubation in cancer serum ($p<0.01$) but this stimulatory effect was completely absent in PMNs and monocytes (Table 5.13).

5.3.10 The effect of storage of cancer sera on lymphocyte O_2 consumption

Six patients with advanced cancers had aliquots of serum stored at $-70^{o}C$ for at least 3 months. Papain treated lymphocytes from 1 control were incubated in these aliquots at various time intervals in the usual fashion. There was no significant decrease in the ability of cancer sera to stimulate control lymphocytes at 3 months. Three of these patients were similarly examined following storage of serum for 7 months. These sera retained their stimulatory capacity (Table 5.14).

5.3.11. The effect of cancer serum dilution on lymphocyte O_2 consumption

The effect of cancer on O_2 consumption of papain treated control lymphocytes was completely abrogated by dilution of the sera to 50% concentration (Table 5.15).

5.3.12 The effect of heat inactivation of cancer serum on lymphocyte O_2 consumption (Appendix 5.16).

Neither heat inactivation of control serum nor the addition of complement to heat inactivated control serum changed the O_2

Table 5.13

Oxygen Consumption of Control Lymphocytes, PMN and Monocytes in Autologous and Cancer Sera

		Lymphocyte O_2 Consumption
Lymphocytes + autologous serum	(5)	5.64±0.36
Lymphocytes + cancer serum	(5)	8.23±0.48*
PMN + autologous serum	(5)	4.98±1.10
PMN + cancer serum	(5)	4.65±1.06
Monocytes + autologous serum	(5)	3.75±0.69*
Monocytes + cancer serum	(5)	3.30±0.88

* Comparison with lymphocytes + autologous serum (U=0; $p<0.01$) Mann-Whitney U Test

Table 5.14

Sera from Cancer Patients stored at -70°C, Effect on Papain Treated Control Lymphocytes

($\mu l \times 10^{-5}/10^6$ cells/sec)

Serum	Lymphocyte O_2 Consumption				
	0 months	3 months	4 months	5 months	7 months
ER	9.70	9.55	8.99 9.40	9.33	-
ME	10.88	-	-	9.81	9.79
BW	10.10	9.79 9.87	-	-	9.81
IM	8.92	-	-	-	8.47
JE	12.37	11.33	-	-	-
AA	9.53	10.28	-	-	-

Table 5.15

Papain Treated Control Lymphocytes in Cancer Sera at Varying Concentrations by Dilution with Isotonic Saline

Serum Concentrations	Lymphocyte O_2 consumption donor cells with each patient's serum					
	AA	BW	ME	IM	AH	Mean±SD
100%	11.24	10.10	10.88	9.09	9.93	10.25±0.84
90%	10.28	8.81	9.79	8.92	8.65	9.29±0.71
80%	11.88	8.66	10.85	7.84	9.93	9.83±1.63
70%	8.28	8.95	7.34	8.30	6.64	7.90±0.91
60%	7.13	6.85	7.72	6.71	6.18	6.92±0.57
50%	5.93	6.24	7.60	6.53	6.53	6.57±0.63
Lymphocyte O_2 consumption donor cells in autologous serum						
	6.12	6.39	6.84	6.09	6.44	6.38±0.30

consumption of autologous papain treated control lymphocytes incubated in such sera. On the other hand, heat inactivation of serum from cancer patients resulted in a drop in the lymphocyte O_2 consumption of their autologous papain treated lymphocytes to control levels ($p<0.01$). The addition of complement brought about a return to previous values of lymphocyte O_2 consumption associated with cancer sera (Table 5.16).

5.3.13. The effect of serum from patients with immune complex associated disease on lymphocyte O_2 consumption and the effects of plasmapheresis on this (Appendix 5.17).

Fifteen experiments were performed in 8 patients. Pre-plasmapheresis sera stimulated papain treated control lymphocytes to consume more O_2, in amounts equivalent to that seen in patients with disseminated malignant disease. Post-plasmapheresis sera although still capable of stimulating an increase in control lymphocyte O_2 consumption, did so to a significantly lesser degree ($p<0.002$) (Table 5.17). Plasma from the cell separator had a stimulatory capacity greater even than that of pre-plasmapheresis serum ($p<0.02$).

It was noticeable that in all 4 patients who underwent plasmapheresis on more than one occasion that the pre-plasmapheresis serum's ability to stimulate lymphocyte O_2 consumption had fully returned by the time of the next treatment, i.e. between 3 and 4 weeks later (Appendix 5.17).

5.3.14. Correlation between lymphocyte O_2 consumption and % T lymphocyte depression and CICs

Lymphocyte O_2 consumption was correlated with % T lymphocyte

Table 5.16

The Effect of Heat Inactivation of Serum and Complement on the Lymphocyte O_2 Consumption of Autologous Papain Treated Lymphocytes

	Lymphocytes	Papain treated lymphocytes in autologous serum	Papain treated lymphocytes in heat inactivated autologous serum	Papain treated lymphocytes in heat inactivated autologous serum plus complement
Controls (5)	6.22±0.30	5.68±0.26	5.98±1.10	5.62±0.57
Cancers (5)	9.72±1.44	9.28±0.88	6.27±0.64*	9.39±0.91

*Comparison with autologous serum and heat inactivated autologous serum plus complement (U=0; $p<0.01$)

Table 5.17

Effects of Serum Pre- and Post-Plasmapheresis and Separated Plasma from Patients with Autoimmune Disease on Allogeneic Control Lymphocytes

($\mu l \times 10^{-5}/10^{-6}$ cells/sec)

		Lymphocyte O_2 Consumption	Mann-Whitney U Test	
Control lymphocytes plus pre-plasmapheresis allogeneic serum	(15)	12.06±2.79		
Control lymphocytes plus post-plasmapheresis allogeneic serum	(15)	8.30±1.96	vs pre-pheresis	U=13.0; $p<0.02$
Control lymphocytes plus separated allogeneic plasma	(15)	15.74±4.30	vs pre-pheresis	U=51.5; $p<0.002$

counts from the same blood specimens of 125 controls and patients with benign and malignant breast disease (Appendix 5.18). The correlation coefficient was r-0.409, showing a significant ($p<0.001$) correlation between increased lymphocyte O_2 consumption and depressed % T lymphocyte counts (Fig. 5.13).

Lymphocyte O_2 consumption was correlated with CIC values of sera from the same blood specimens of 75 controls and patients with benign and malignant breast disease (Appendix 5.19). The correlation coefficient was r=0.399, showing significant correlation ($p<0.005$) between increased lymphocyte O_2 consumption and raised levels of CICs (Fig. 5.14).

Fig. 5.13

Correlation Between O_2 Consumption and Percentage T Lymphocytes in Breast Cancer, Benign Breast Disease and Controls

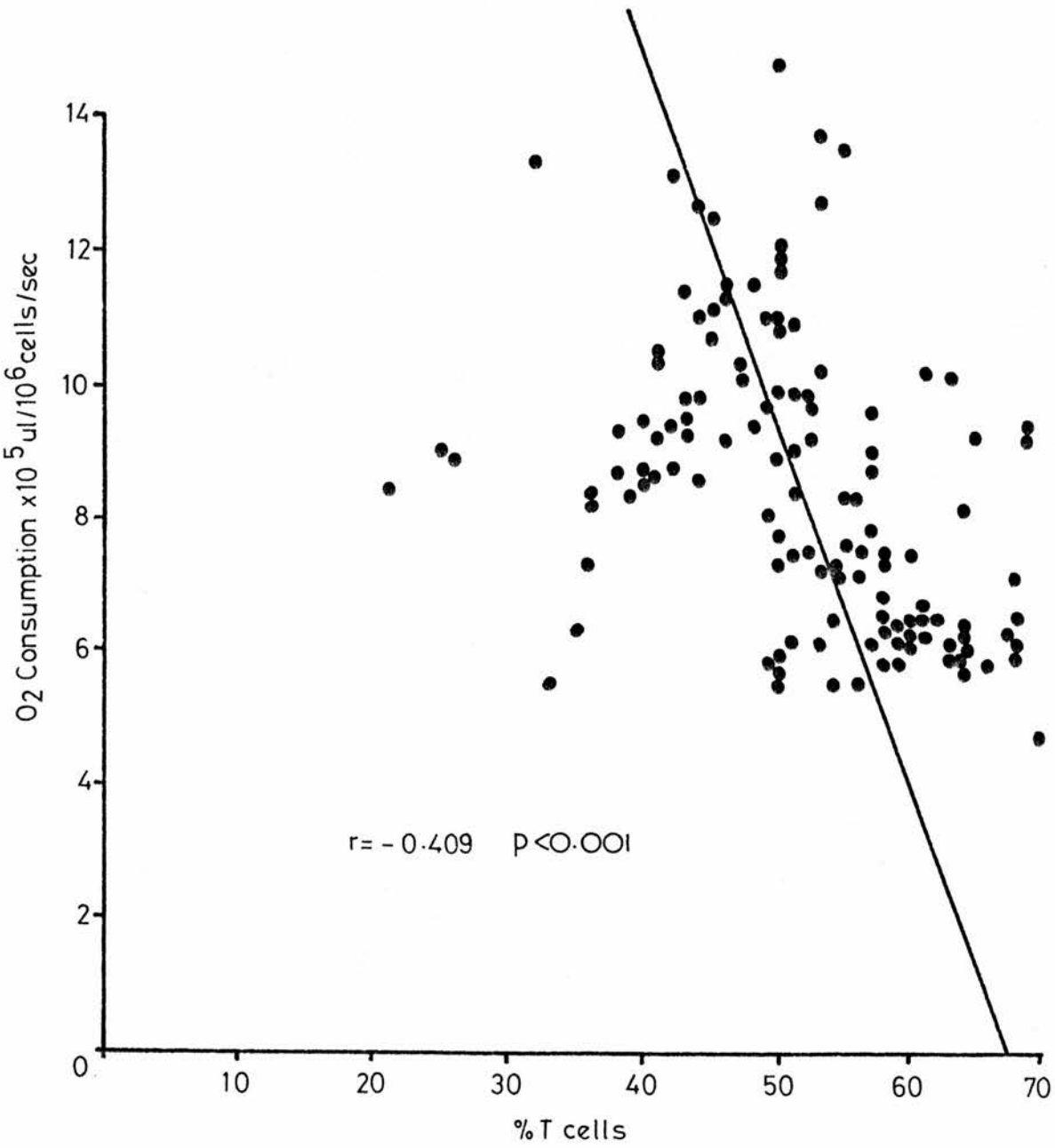
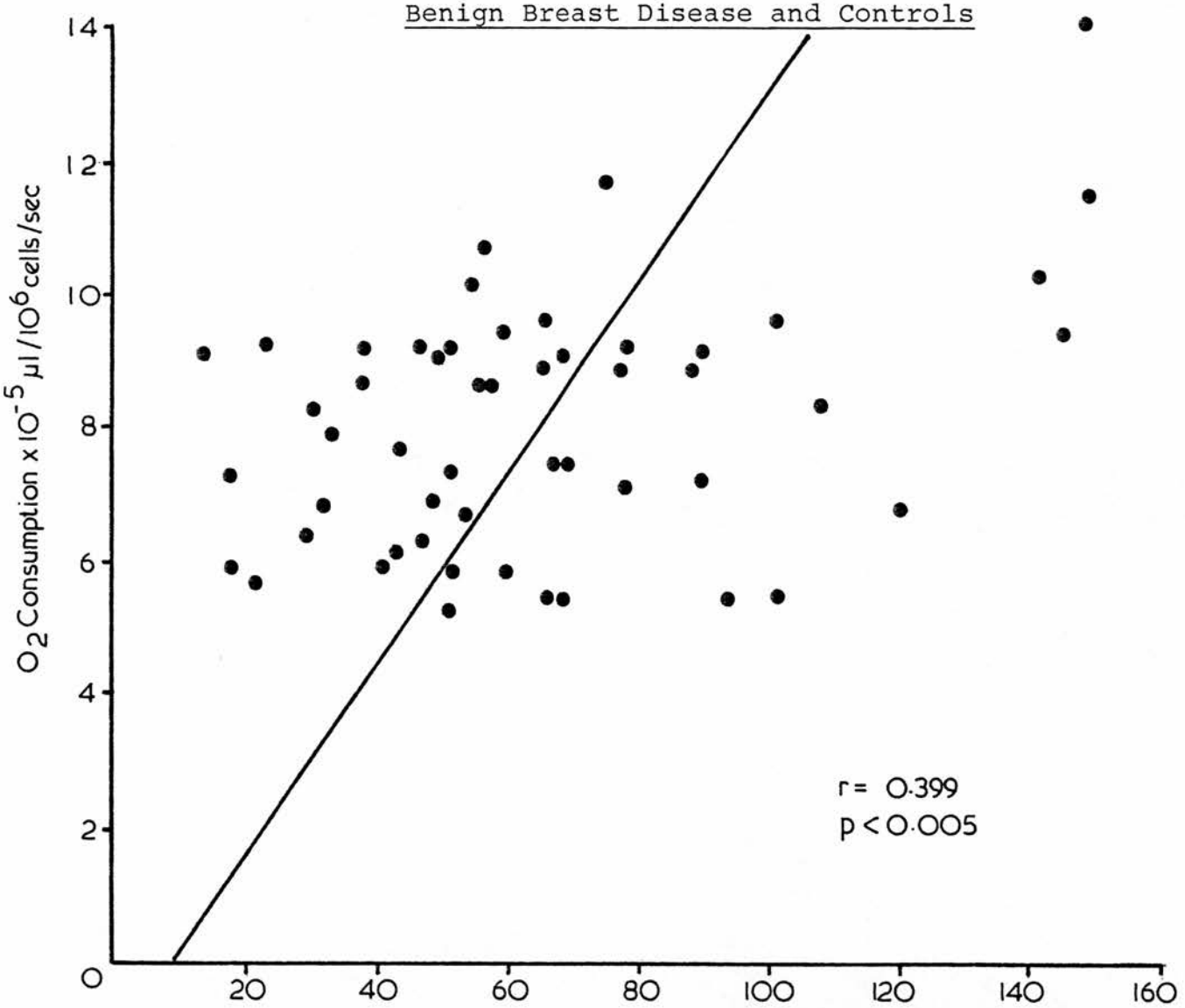


Fig. 5.14

Correlation Between O_2 Consumption and Circulating Immune Complexes in Breast Cancer, Benign Breast Disease and Controls



5.4. Discussion

Neither age nor sex of the individual affected lymphocyte O_2 consumption but disease state did. Women with benign breast disease tended to have higher lymphocyte O_2 consumption than healthy female controls and it is of interest to note that mean CIC levels in women with benign breast disease have been reported to be twice that of controls (Day et al. 1982); that PHA responses and skin sensitivity to recall antigens tend to be more depressed in women with benign breast disease than controls (Mandeville et al. 1982), and % T counts in those with benign breast disease when compared with controls tend to be lower (Keller et al. 1976; see also Chapter 4, Table 4.27). Women with breast cancer had significantly increased lymphocyte O_2 consumption compared both with controls and patients with benign breast disease, this increase tending to be more pronounced with greater disease load and indeed the difference was significant between early localised disease and metastatic disease. Other types of advanced tumour displayed increased lymphocyte O_2 consumption but early colorectal cancers, although showing this tendency, had significantly lesser values. Colorectal tumours which had disseminated to liver, rather than only to nodes, or which were restricted to local invasion of surrounding organs, showed the highest lymphocyte O_2 consumption indicating perhaps that such tumours, thought to facilitate their metastatic potential by an increased ability to shed membrane molecules, produce greater amounts of substances which stimulate lymphocyte O_2 consumption. Unlike early breast cancer, colorectal tumours when treated in the early stages of disease, have a reasonably high incidence of cure implying that these particular cancers are commonly unaccompanied

by occult metastases. As such they would be expected to be comparatively stable in terms of surface membrane 'shedding' and this may be reflected by the lower lymphocyte O_2 consumption seen in these patients.

Lymphocyte O_2 consumption was unaffected by surgery and bore no relationship to any of the prognostic factors studied and was not predictive of later tumour recurrence occurring within a 4 year period from treatment. These findings are remarkably similar to those reported and discussed earlier on % T lymphocytes in breast cancer and it therefore appears that lymphocyte O_2 consumption has no place as a prognostic indicator, although admittedly the numbers of patients tested in each group were small and the follow-up period of 4 years, taken as a cut-off point, is rather short for the study of patients with early breast cancer. It may be that prolonged follow-up (and the increased numbers presently under study) will show significant differences.

Systemic hormonal treatment was shown to affect lymphocyte O_2 consumption both in early and advanced breast cancer, when beneficial to the patient, reducing this significantly compared with pre-treatement or progressing disease values. This effect, like that seen with the change in % T lymphocyte counts, was noticeable by 6 to 8 weeks after the initiation of endocrine manipulation. Similarly, 2 women who, following initial benefit, developed further progression of disease, displayed increased lymphocyte O_2 consumption compared with their response values, at the time of this event.

Chapel (1973) demonstrated that treatment of control human lymphocytes with papain at $37^{o}C$ removed a serum factor, present on a proportion of SRBC receptors, from these without damaging the receptor itself and that this factor could be reattached following incubation of

lymphocytes in normal serum. Serum factor(s) present in cancer patients, as previously noted, is also removable from a proportion of SRBC receptors and this is reversible by incubation in cancer sera. Similar experiments were performed to investigate if such a factor(s) was responsible for the increased O_2 consumption of lymphocytes from cancer patients. These showed that papain, working enzymatically, was able to remove the factor(s) responsible for the increase in O_2 consumption from the surface of lymphocytes from cancer individuals. Subsequent incubation of such lymphocytes in control serum had no effect, but incubation in cancer serum resulted in return of the increase in lymphocyte O_2 consumption. Papain treatment of control lymphocytes did not affect their O_2 consumption, nor did subsequent incubation in autologous serum, but incubation of these cells in cancer serum resulted in an increase in their O_2 consumption to levels comparable with that of lymphocytes from cancer patients. It therefore appears that this increased O_2 consumption, exhibited by lymphocytes from cancer patients, is not a property of the lymphocyte itself but rather a feature of a factor(s) present in the cancer serum, which bears remarkable similarity in terms of behaviour following enzyme treatment and serum incubation, to the 'blocking factor' of cancer sera. However, the 'blocking' effect can be induced by simple incubation of control lymphocytes in cancer sera (Whittaker et al. 1971a; Gatti 1971; Suciu-Foca et al. 1973; Whitehead et al. 1977; Shukla et al. 1979), whereas it was impossible to demonstrate stimulated O_2 consumption in control lymphocytes unless these were pre-treated with papain.

This phenomenon also appears, like that of E-rosette inhibition (Whitehead et al. 1977), to be independent of blood groups and HLA

activity, in that papain treated lymphocytes incubated in allogeneic control serum were not stimulated to consume more O_2. One reason for this may be that papain also removes the HLA antigen from the lymphocyte surface and this takes at least 6 hrs to be replaced by the cell (Chapel 1973). However, it has also been reported that even heterologous rabbit serum will not stimulate human lymphocyte proliferation in the absence of ICs (Soderberg & Coons 1978). Allogeneic serum stimulation therefore does not appear to complicate this system and stimulated O_2 consumption is dependent on the presence of tumour or other disease states (see later) in the serum donor.

Storage of sera at -20°C or -70°C did not affect the capacity of immune complex related disease or cancer sera to stimulate papain treated control lymphocytes over a period of several months. The factor(s) responsible therefore appears to be as stable as those contained in 'inhibitory' sera of tumour bearers, which retain their 'blocking' capacity for as long as one year following storage at -20°C (Gatti 1971), or CIC which maintain their integrity for up to 2 years when stored at -20°C (Jones & Orlans 1981).

When cancer serum was diluted by 50% its capacity to stimulate papain treated control lymphocytes to consume more O_2 was abrogated. This is reminiscent of the loss of the inhibitory effect of cancer serum to inhibit E-rosetting when diluted to one fourth (Whitehead et al. 1977) and of the loss of the stimulatory capacity exerted by ICs on lymphocyte transformation when serum is diluted to 30% (Bloch-Shtacher et al. 1968; Soderberg & Coons 1978).

Heat inactivation of cancer serum abrogated its stimulatory effect on lymphocyte O_2 consumption but this was reversed by the

addition of complement to the system indicating firstly, that the stimulating factor(s) is resistant to heating to 56°C for 30 mins, a property which it holds in common with the inhibitory factor(s) in cancer sera (Gatti 1971; Whittaker et al. 1971b), and secondly, that the serum factor(s) is dependent on complement to induce stimulation. IC stimulation of lymphocyte DNA synthesis was demonstrated to be complement independent by Moller (1969) but he was unable to exclude the presence of residual complement on the cell membrane following lymphocyte washing. Others (Bloch-Shtacher et al. 1968; Soderberg & Coons 1978) have shown that IC induced lymphocyte transformation is virtually completely complement dependent. Indeed, this is hardly surprising as it has been clearly demonstrated that ICs cannot sensitize lymphocytes unless first complexed with complement (Uhr 1965; Uhr & Phillips 1966). Once such complexes become attached to monocytes, macrophages, PMNs and B lymphocytes, these cells are activated (Theofilopoulous & Dixon 1979; Cooper 1987) triggering release of biologically active substances and inducing phagocytosis and cell regulation. Some T lymphocytes and NK or K cells also have C receptors (Ross & Hedof 1985) although binding of ICs may not necessarily be to these, as lymphocytes have antigen receptors on their surfaces (Kontiainen 1975; Kontiainen & Mitchison 1975) and some, Fc-Ig receptors which are mainly for IgG (reviewed Theofilopoulos & Dixon 1979). Complement may be necessary in instances of IC binding to such receptors by permitting increased aggregation of solubilised ICs (Maurer & Talmage 1953) through classical pathway activation and by such aggregation, facilitate adherence to the receptors; or it may act in some, as yet, undefined way.

The sera from patients with IC related diseases exerted a stimulatory effect on control papain treated lymphocyte O_2 consumption similar to that of sera from individuals with advanced cancer. Following removal of

plasma by plasmapheresis, this stimulatory capacity was still present in the sera but significantly reduced. The plasma had the greatest capacity to increase control lymphocyte O_2 consumption suggesting that a factor(s) responsible for this had been removed from the host by plasmapheresis and remained concentrated in the removed plasma. Factor(s) responsible for increased lymphocyte O_2 consumption therefore also appears to be present in patients with IC related diseases. Such patients also carry inhibitory or 'blocking' factors (Gatti 1971) in their sera. Furthermore, it has been demonstrated that plasmapheresis will remove 'blocking' factors from patients with advanced malignancies affecting E-rosetting (Browne et al. 1976), macrophage migratory capacity (Samak et al. 1981) and MLC reactions (Retsas et al. 1981). Taken together with the fact that there was significant correlation between depressed % T counts and increased lymphocyte O_2 consumption in controls and patients with benign and malignant breast disease in this study; these facts imply that the factor(s) causing increased lymphocyte O_2 consumption and 'blocking' is one and the same, and further because it occurs in IC related disease groups that it may be CIC. However, other factors which can suppress lymphocyte activity such as acute phase proteins (Mortensen 1975; Cooper & Stone 1979) are also present in the serum of both patients with autoimmune disease and malignancy. Indeed, plasmapheresis has been demonstrated to remove both acute phase proteins and CIC from the serum of cancer patients for short periods of time (Samak et al. 1981; MacDonald et al. 1981; Salinas et al. 1981; Cupissol et al. 1981), resembling the short lived effect of removal of the stimulator of lymphocyte O_2 consumption by plasmapheresis. The CICs removed were of medium size (MacDonald et al. 1981; Salinas et al. 1981) and the antibody moiety, mainly IgG (Cupissol et al. 1981; Samak et al. 1981), that is, having the properties

usually associated with the CICs implicated in 'blocking' (Theofilopoulos & Dixon 1979). Some evidence, perhaps indicating the more important place of CIC over acute phase proteins, as an inhibitory factor in cancer, was produced by Samak et al. (1981) who demonstrated a significant drop in IgG, suggested to be incorporated in CIC, only in patients with advanced cancer who responded clinically to plasmapheresis. Furthermore, Whitehead et al. (1977) were unable to demonstrate any correlation between C-reactive protein and E-rosette inhibition in breast cancer. Also, this acute phase protein occurs in only 42% of patients with metastatic breast disease (reviewed by Cooper & Stone 1979) whereas the majority of patients with such dissemination have significantly increased lymphocyte O_2 consumption. Acute phase proteins, such as C-reactive protein, by themselves are therefore unlikely to be the sole, if at all, stimulator of increased lymphocyte O_2 consumption. Furthermore, there was a highly significant correlation between CIC levels and lymphocyte O_2 consumption in blood specimens from controls and patients with benign and malignant disease, implying that CIC may be responsible for the phenomenon.

Monocytes and PMNs were demonstrated to consume rather less O_2 than lymphocytes under basal conditions and their O_2 consumption remained unaffected by cancer sera. However, both these cells derive much of their energy from anaerobic metabolism and demonstrate a high degree of glycolysis even under aerobic conditions and only during the process of phagocytosis will these cells utilise O_2 in larger quantities (Cline 1975). It would therefore be expected that cancer serum, although possibly exerting some immunological effect on these cells, would not increase their O_2 consumption. The population of B-rich lymphocytes from both controls and cancer patients consumed equal quantities of O_2 and significantly less

O_2 than the T-rich population from controls; but the T-rich population from patients with cancer had significantly higher O_2 consumption than that from controls. These facts indicate that the factor(s) in cancer serum, responsible for stimulating O_2 consumption does not affect the B lymphocyte rich population and exerts its effect on the T lymphocyte population only. This is reminiscent of the 'blocking' effect exerted by cancer sera on T lymphocytes; but not on B lymphocytes, as demonstrated by their normal EAC-rosetting (Whitehead et al. 1977; Shafir et al. 1980) and PWM stimulation (Shafir et al. 1980).

5.5. Summary

Lymphocyte O_2 consumption is increased in patients with breast cancer and other cancers and this is a stage related phenomenon. It is unaffected by surgery, bears no relationship to the prognostic factors studied, and is not predictive of later tumour recurrence. Systemic hormonal treatment in both early and metastatic breast cancer, significantly reduces elevated lymphocyte O_2 consumption in patients benefiting from treatment, and this effect can be seen between 6 and 8 weeks prior to clinical evidence of benefit.

The factor(s) responsible for the stimulation of lymphocyte O_2 consumption can be removed from the cell and is present in the serum of cancer patients and those with IC related diseases. This factor(s) is heat stable at 56°C and can be stored at -20°C for several months without diminishing its potency but loses its stimulatory capacity on dilution. The factor can be removed from the serum by plasmapheresis but only for 3 weeks at most and appears to be independent of HLA and blood group activity.

Only the T-rich subpopulation of lymphocytes can be stimulated to increased consumption of O_2 and this reaction is complement dependent. Lymphocytes must first be treated with papain digestion for stimulation of O_2 consumption to occur *in vitro*.

There is a high degree of correlation between lymphocyte O_2 consumption and both CIC levels and depressed % T lymphocyte counts in blood samples taken from controls and patients with benign and malignant breast disease.

5.6. Conclusions

Lymphocyte O_2 consumption is increased in breast cancer, even in its early stages, and this stimulated consumption becomes more pronounced with increasing tumour burden. Lymphocyte O_2 consumption is unaffected by local treatment of breast cancer, indicating perhaps the high proportion of women with metastatic disease at the outset, but stimulated levels are diminished significantly in patients responding to systemic therapy and this effect predates objective clinical evidence of benefit.

This phenomenon may therefore be used to evaluate patients' responses to hormonotherapy at an earlier phase of their illness.

Appendix 5.1

Controls % T, O_2 Utilisation, CICs

	Age	Sex	% T	O2 Consumption	CICs	Disease
EC	73	F	-	5.86	-	osteoarthritis
SD	26	F	68	-	57	nil
			-	7.21	90	
CD	43	F	-	5.65	-	gallstone obstructive jaudice
EG	25	F	-	7.05	-	appendicitis
MG	59	F	-	6.02	-	nil
AG	45	F	-	5.60	-	nil
SH	43	F	61	-	-	nil
			67	-	66	nil
DH	54	F	-	6.53	-	nil
AJ	50	F	-	4.96	-	nil
PC	38	F	-	5.69	-	duodenal ulcer
NR	31	F	-	5.17	-	duodenal ulcer
LT	35	F	-	5.52	-	duodenal ulcer
KO	42	F	-	7.03	-	nil
MO	42	F	-	7.34	18	nil
			66	-	46	nil
JE	36	F	-	7.35	-	nil
KM	63	F	-	6.45	-	nil
IW	37	F	-	5.90	-	nil
RP	55	F	-	5.60	-	nil
ET	58	F	-	7.50	-	nil
GR	38	F	50	-	108	nil
			59	-	125	nil

Appendix 5.1 (continued)

	Age	Sex	% T	O2 Consumption	CIC	Disease
JJ	38	F	-	6.42	-	nil
EH	50	F	60	6.47	-	gallstones
SJ	49	F	68	6.04	-	gallstones
AM	50	F	-	6.62	-	gallstones
WS	71	F	53	6.09	-	gallstones
OT	70	F	54	6.25	-	gallstones
ET	68	F	50	5.99	-	gallstones
JG	56	F	61	6.25	-	gallstones
GS	20	F	-	5.69	-	nil
AG	18	F	-	5.85	-	nil
LJ	20	F	-	4.90	-	nil
EE	20	F	-	6.89	-	nil
AG	28	F	-	5.78	-	nil
SC	29	M	-	-	30	nil
JD	48	M	-	6.19	-	oesophagitis
KD	22	M	-	6.45	-	nil
DG	32	M	-	5.88	19	nil
			67	-	38	nil
MH	34	M	-	5.88	-	nil
DM	23	M	-	-	30	nil
			-	6.39	-	nil
			58	-	39	nil

Appendix 5.1 (continued)

	Age	Sex	% T	O2 Consumption	CICs	Disease
AP	34	M	63	-	-	nil
			61	6.17	64	nil
FP	40	M	-	7.94	-	non-specific abdominal pain
KR	36	M	61	-	30	nil
JT	37	M	60	-	-	nil
			-	5.64	50	nil
ES	34	M	-	-	25	nil
DW	38	M	61	-	37	nil
HY	31	M	74	-	30	nil
RL	30	M	71	-	30	nil
			-	6.00	28	nil
GB	72	M	51	6.42	-	hernia
GF	58	M	60	6.39	-	hernia
RH	60	M	58	5.81	-	hernia
CV	40	M	58	7.33	-	hernia

Appendix 5.2

Benign Breast Disease

	Age	Meno	% T	O2 Consumption	CICs	Post-Treatment
SA	23	Pre	-	9.35	-	-
LB	27	Pre	53	7.13	-	-
CB	41	Pre	-	8.62	-	-
GB	35	Pre	-	8.39	10	-
MB	62	Post	58	-	54	-
MB	62	Post	57	-	45	Yes
JB	38	Pre	-	6.68	-	-
RB	38	Pre	60	6.11	43	-
CB	22	Pre	-	7.50	-	-
SB	45	Pre	61	-	20	-
DC	35	Pre	55	-	45	-
DC	35	Pre	45	-	42	Yes
GC	25	Pre	-	6.13	-	-
CD	21	Pre	-	5.51	-	-
JD	27	Post	-	6.63	-	-
PD	34	Pre	-	8.39	-	-
ID	62	Post	56	7.54	-	-
AE	37	Pre	62	-	30	-
AE	37	Pre	59	-	44	Yes
IE	54	Post	56	-	30	-

Appendix 5.2 (continued)

	Age	Meno	% T	O2 Consumption	CICs	Post-treatment
IE	54	Post	50	-	31	Yes
JE	41	Pre	57	-	43	-
AG	25	Pre	64	-	38	-
LG	29	Pre	-	8.66	-	-
JG	52	Post	-	9.00	-	-
CG	40	Pre	45	-	21	-
AG	45	Pre	-	5.60	-	-
AH	43	Pre	61	-	20	-
WH	39	Pre	-	5.49	94	-
JH	46	Pre	-	10.85	-	-
JH	32	Pre	-	6.25	-	-
SH	34	Pre	-	5.88	-	-
CH	36	Pre	-	6.04	-	-
JH	44	Pre	57	-	-	-
MI	40	Pre	-	6.25	-	-
SJ	39	Pre	66	-	47	-
AJ	30	Pre	-	4.96	-	-
DJ	27	Pre	55	-	19	-
DJ	45	Pre	-	9.50	-	-
GJ	37	Pre	59	-	30	-
GJ	37	Pre	-	-	46	Yes
ML	41	Pre	63	-	30	-
GL	24	Pre	-	7.56	-	-
BM	36	Pre	-	5.91	41	-

Appendix 5.2 (continued)

	Age	Meno	% T	O_2 Consumption	CICs	Post-treatment	
AM	33	Pre	50	5.73	22	-	
LM	19	Pre	-	5.60	-	-	
KO	42	Pre	-	7.03	-	-	
CP	34	Pre	-	-	44	-	
DP	45	Pre	57	-	-	-	
TP	40	Pre	63	-	29	-	
DP	43	Pre	53	-	36	-	
DP	42	Pre	54	-	42	-	
ER	68	Post	50	6.68	-	-	
SR	32	Pre	-	6.25	-	-	
MS	29	Pre	-	7.66	-	-	
PS	40	Pre	-	5.90	-	-	
BW	37	Pre	-	4.47	23	-	
BW	37	Pre	50	-	49	Yes	
CW	28	Pre	65	9.18	45	-	
CW	28	Pre	68	-	96	Yes	
RW	82	Post	-	7.83	-	-	
BW	46	Pre	70	-	62	-	
EW	37	Pre	61	7.4	52	-	
JW	27	Pre	-	8.13	-	-	
LW	33	Pre	63	-	30	-	
LW	31	Pre	-	5.92	-	-	
MW	47	Pre	-	5.85	52	-	
MW	47	Pre	-	6.91	32	-	

Appendix 5.2 (continued)

	Age	Meno	% T	O2 Consumption	CICs	Post-Treatment
CB	41	Pre	64	6.10	-	-
EB	38	Pre	68	5.63	-	-
MB	80	Post	51	6.12	-	-
DC	42	Pre	62	6.16	-	-
JF	60	Post	64	6.53	-	-
MH	44	Pre	59	5.88	-	-
JH	23	Pre	63	6.02	-	-
GH	48	Peri	56	5.51	-	-
SH	42	Pre	63	5.92	-	-
MI	46	Pre	-	-	46	-
AM	30	Pre	66	5.97	-	-
KM	26	Pre	68	5.94	-	-
SP	33	Pre	68	6.46	-	-
JR	40	Pre	58	6.18	-	-
NR	38	Pre	59	6.06	-	-
JR	51	Peri	51	8.38	-	-
GT	59	Post	58	7.45	-	-
SW	43	Pre	59	6.38	-	-
AM	29	Pre	61	6.77	-	-
SH	43	Pre	63	5.92	-	-
VH	42	Pre	-	5.44	-	-
LY	41	Pre	64	6.20	-	-
DW	46	Pre	60	6.12	-	-
AH	40	Pre	54	5.44	-	-
MD	48	Pre	58	6.31	-	-
JH	19	Pre	64	6.16	-	-

Appendix 5.2 (continued)

	Age	Meno	% T	O2 Consumption	CICs	Post-Treatment
JH	21	Pre	-	6.24	-	-
PT	52	Peri	-	6.08	-	-
LF	55	Post	-	6.01	-	-
JM	79	Post	-	6.52	-	-
JM	58	Post	-	5.79	-	-
DB	51	Post	-	5.90	-	-
JM	59	Post	-	6.23	-	-
IJ	57	Post	-	6.30	-	-
MW	73	Post	-	6.80	-	-
LA	68	Post	-	7.00	-	-
DS	19	Pre	-	-	44	-
LW	31	Pre	-	-	29	-

Appendix 5.3

Stage I & II Breast Cancer CICs (combined with % T)

		Pre-operative		Post-operative	
	Age	% T	CIC	% T	CIC
EE	73			65	83
CG	55			55	30
MJ	58			46	30
MJ	71			50	80
JL	53	69	79	75	86
PM	63			69	140
				-	120
CS	66			63	33
RW	60			53	94
MB	72			50	70
				45	57
WB	42		66		
PB	44			49	30
				60	44
AB	38	48	30		
EB	49			58	30
PB	44	47	42		
VC	39	51	33		
PE	51			57	34
PG	67	57	30		
GF	60	64	70		
NG	55			-	84
GG	60			61	30

Appendix 5.3 (continued)

		Pre-operative		Post-operative	
	Age	% T	CIC	% T	CIC
EH	60	51	78	55	88
MH	78	53	30	59	30
AJ	42	69	60		
GJ	66	58	54		
MM	74	66	44	57	60
BN	56	54	30		
NP	71	65	84	55	51
				50	88
VR	49			47	38
MR	60			62	48
				65	30
ES	72			50	30
				-	19
MS	40	49	30	44	30
ES	41	62	37	47	30
					39
				45	29
MT	48	50	50	50	78
				49	90
				56	88
GT	49	58	30	58	18
NV	49	52	58	62	35
				37	30
				33	37
				34	42
				53	45

Appendix 5.4

Stage III Breast Cancer CICs (combined with % T)

Surgery	Age	Pre Rx % T	Pre Rx CIC	Post-Op % T	Post-Op CIC
DG	55	63	55	-	-
LH	59	45	18	-	-
AJ	42	45	28	-	33
AL	66	43	30	54	30
EL	50	56	22	-	-
PM	41	55	31	-	-
EO	48	-	46	-	-
AR	86	-	102	-	-
MWJ	68	63	49	-	-
IM	55	-	-	57	38

Tamoxifen	Age	Pre Rx % T	Pre Rx CIC	PR/NC/NA % T	PR/NC/NA CIC	Prog. % T	Prog. CIC
ED	71	50	41	-	-	-	78
GL	80	50	76	-	-	-	-
JP	73	-	-	64	74	-	-

Inoperable	Age	Pre Rx % T	Pre Rx CIC
BD	48	-	69
IH	50	43	24
IM	53	49	71
DM	45	-	70

Appendix 5.5

Stage IV Breast Cancer CICs (combined with % T)

	Age	Pre Rx		PR/NC/NA		Progression	
		% T	CIC	% T	CIC	%T	CIC
GB	33	-	30	-	-	-	-
RT	60	28	51	-	-	-	-
MW	77	61	71	-	-	-	-
AA	78	-	-	56	80	-	-
MB	51	-	-	39	76	-	-
EB	53	-	-	33	68	-	-
VB	48	52	47	-	-	-	-
AB	38	42	58	-	-	50	117
AC	75	56	78	69	86	-	-
VC	38	42	58	-	-	-	-
ED	56	-	-	-	-	37	118
FD	72	-	-	-	-	46	150
BD	48	25	86	-	-	-	-
LE	78	-	-	64	61	43	63
MF	83	21	108	58	121	-	-
EH	60	52	66	-	-	53	86
MJ	63	-	-	46	93	-	-
GJ	39	-	51	-	-	-	-
EJ	63	50	183	-	-	-	-
JJ	56	-	-	-	-	53	34

Appendix 5.5 (continued)

	Age	Pre Rx		PR/NC/NA		Progression	
		% T	CIC	% T	CIC	% T	CIC
MJ	49	-	-	-	-	57	148
OJ	60	55	78	-	-	-	-
NL	58	-	102	-	-	-	-
AL	76	-	-	61	57	-	-
ML	31	-	-	-	-	37	62
ML	31	-	-	-	-	38	52
WM	72	-	-	56	80	-	-
WN	74	-	-	59	66	-	-
WN	74	-	-	56	43	-	-
AP	69	53	30	68	100	-	-
PP	61	-	61	52	55	-	-
PP	61	-	-	50	112	-	-
RP	58	-	-	52	65	-	-
MP	63	-	-	-	-	35	47
ER	65	-	141	-	-	-	-
HT	74	-	-	-	-	47	100
WW	64	-	66	-	-	-	-
EN	45	-	-	-	-	-	150
EM	74	-	-	52	38	-	-

Appendix 5.6

Calculation of Solubility Coefficient of O_2 in Eagle's MEM

The O_2 concentration of fully air saturated Eagle's MEM was calculated using a modification of Bruhn's modification of the Winkler technique (described by Golterman 1969). The principle behind this titrimetric calculation of the O_2 content of a solution is that O_2 combines with manganese hydroxide ($Mn[OH]_2$) forming higher hydroxides which on subsequent acid fixation in the presence of iodide (I^-), liberate iodine (I_2) in an amount equivalent to the original dissolved O_2 content of the sample. The I_2 is then determined by titration with sodium thiosulphate ($Na_2S_2O_3$). Nitrite (NO_2) interference is eliminated by the use of sodium azide (NaN_3).

Ten ml of solution to be tested was drawn into a syringe, ensuring that no air bubbles were trapped in the syringe and placed in a water bath at 37°C after the sample had been air saturated using a Charles Austin pump, the air being preheated in a coil at 37°C and allowed to equilibrate for 10 mins. 0.1ml of manganese sulphate ($MnSO_4 5H_2O$) and 0.1ml of alkaline iodide-azide solution was added to the test sample using a fine gauge needle and thoroughly mixed and the resultant precipitate allowed to settle. Mixing was repeated and the precipitate again allowed to settle leaving a completely clear supernatant. 0.2ml of phosphoric acid [H_3PO_4 (s.g. 1.75)] was then added and mixed thoroughly, the precipitate dissolving almost instantaneously. The solution was thoroughly mixed again before measurement and placed in a 50ml conical flask and the iodine (I_2) titrated against sodium thiosulphate ($Na_2S_2O_3$).

Starch solution, 0.2ml, used as an indicator was added towards the end of

the titration.

The amount of dissolved O_2 was calculated from the formula.

$$O_2 ug/ml = \frac{\text{ml of titrant B} \times N \times 8 \times 1000}{\text{ml of flask} - 0.2ml}$$

Where B = $Na_2S_2O_3$

N = Normality of $Na_2S_2O_3$

Allowance is made in the formula for the slight displacement of sample by 0.1ml $MnSO_4 5H_2O$ and 0.1ml of alkaline-azide solution.

To convert ug/ml to ul/ml at 37°C, because:

lug/ml = 0.7ul/ml at 0°C, equate:

$$\frac{ug/ml \times 0.7 \times 273}{273 + 37^{\circ}C} \quad ul/ml$$

Results

Date	N/O_2/ml solution Eagle's MEM	NaCl
19.5.81	4.24	5.02
16.9.81	4.28	-
	4.20	-
2.10.81	4.24	4.98
	4.20	5.00
9.10.81	4.28	4.91
	4.30	5.03
	4.23	5.06
4.11.81	4.23	4.96
	4.21	4.90
	4.24 ±0.04	4.98 ±0.06

Appendix 5.7

Cells O_2 Consumption Plotted Against Cell Concentration

Cell Concentration $x10^6/ml$	% Drop in O_2 Tension of Medium		
	Lymphocytes %	Polymorphonuclear Leucocytes %	Monocytes %
0.25	1	1	-
0.5	2	1.5	0.5
1.0	5	3	-
1.5	8	5.5	-
2.0	9.5	10	4
3.0	15	11	-
4.0	20.5	18	9
5.0	24	21	-
6.0	31.5	25	11.5
8.0	41	37	18.5
10.0	48	39	20
Correlation coefficient	r = 0.998	r = 0.975	r = 0.990

Lymphocytes, PMN and monocytes from one control.

The experiment was run over several days.

Appendix 5.8

Stage I, II and III Breast Cancer Surgery

Lymphocyte O_2 Consumption

	Age	Pre Rx	Post Rx	ER status	Axillary Node no.	Recurrence <4yrs
SURGERY I & II						
MB	72	-	10.11	NP	0	NSR
WB	42	5.48	9.72	-	0	Local 21/12
OB	58	9.59	-	+	0	NSR
EB	49	-	8.84	+	0	NSR
BR	40	9.22	9.33	-	1	Bone 15/12
NG	55	-	7.79	+	0	NSR
MH	54	9.49	-	NP	NP	NP
EH	60	8.98	8.84	+	3	Bone 12/12
MH	78	-	9.42	NP	0	NSR
AH	54	-	10.07	+	0	NP
AJ	42	9.42	8.73	-	1	Multiple 43/12
JJ	43	11.48	-	-	4+	Multiple 12/12
GJ	66	9.71	-	+	3	Local 29/12
MM	48	11.68	6.99	+	0	NSR
AM	47	8.54	-	-	0	NSR
BM	40	8.91	-	NP	0	NSR
PP	59	-	11.75	NP	0	NSR
MR	47	-	8.31	-	3	NSR
JR	49	10.18	6.59	NP	NP	NP
ES	72	-	5.80	-	0	Brain 15/12
MS	40	-	7.12	-	1	NSR
ES	41	-	6.78	+	0	NSR
GS	53	8.11	-	+	0	NSR
MT	48	-	11.62	-	0	NSR
MT	72	8.08	-	+	0	NP
MT	53	-	7.88	-	1	Brain 30/12
HL	42	7.96	-	NP	1	too early
EL	62	9.38	8.44	NP	0	too early
OD	51	-	6.24	NP	0	too early
PJ	78	9.35	8.20	NP	0	too early
DR	42	7.88	-	NP	0	too early
SR	38	9.45	-	NP	0	too early
EJ	68	8.06	-	NP	0	too early
JR	41	7.48	-	NP	0	too early
PT	52	-	6.08	NP	0	too early

Appendix 5.8 (continued)

	Age	Pre Rx	Post Rx	ER status	Axillary Node no.	Recurrence <4yrs
SURGERY AND ADJUVANT TAMOXIFEN						
JL	53	9.20	-	+	0	Multiple 28/12
SURGERY III						
RF	79	13.06	-	NP	0	Local 12/12
DG	55	10.06	9.46	+	0	Lungs 43/12
JF	37	7.96	8.72	+	0	Local 7/12
DM	65	-	8.95	+	1	NSR
PM	41	8.34	8.55	+	2	NSR
EO	48	9.01	7.73	+	0	NSR
AR	86	5.51	-	NP	NP	NP
KW	61	-	9.23	NP	1	Lungs 45/12
IM	55	-	8.66	+	2	Lungs 37/12
DE	78	12.04	-	NP	NP	too early
PS	63	7.88	-	NP	2	too early
KM	60	9.44	8.10	NP	1	too early
WD	63	8.25	8.04	NP	0	too early
FS	74	7.88	-	NP	0	too early
BW	40	9.46	-	NP	12	too early
IA	74	8.24	-	NP	1	too early
KD	62	8.56	-	NP	1	too early
GW	82	10.96	8.75	NP	4+	too early

NP = not performed

Appendix 5.9

Stage I, II & III Breast Cancer
Hormonotherapy: Lymphocyte O_2 Consumption

Stage I & II

	Age	Pre Rx	PR/NC	Progression
MF	80		8.05(PR)	
MM	86	6.47		
MM	82	8.88		
EP	80	9.91		9.22
EW	84	9.32	5.96(PR)	
FM	85	7.98		

Stage III

FL	66	13.12	6.14(NC)	
ED	71			8.60
GL	80	11.75		12.32
JP	73		5.91(PR)	
ES	71	8.81	5.06(NC)	8.49
BW	75	12.61		
OA	76	9.18		
GR	82			11.42

Appendix 5.10

Stage IV Breast Cancer - Hormone Therapy
Lymphocyte O_2 Consumption

	Age	Pre Rx	PR/NC/NA	Prog.
EB	68	7.43	6.40(PR)	
MH	38	13.30		
JJ	61	11.37		
AA	60	12.26		
MA	44	8.94		
VB	48	9.20	7.20(NC)	
AB	38	8.73		8.29
AC	75	7.09		
VC	38	7.43		
EC	48	11.02		
MD	74	10.68		
BD	48	9.04		
CD	57	11.99	8.09(PR)	
MF	83	8.40	6.84(PR)	
MJ	63	9.00		
GJ	39	6.06		5.84
SJ	44	9.51		
EJ	63	10.31		9.90
NL	58	9.64		11.31
AM	45	9.80		9.18
ER	65	10.28		
LS	45	12.70	11.72(N/A)	
MW	46	9.79		
WW	64	9.61		
EH	60	9.84		10.91
FD	72	-		11.45
LE	78	-		8.57
PE	73	-		10.50
WG	49	-	7.63(PR)	9.84
ML	77	-	-	7.30
IM	58	-	-	8.47
WN	74	-	-	10.26
MP	63	-		6.25

Appendix 5.10 (continued)

	Age	Pre Rx	PR/NC/NA	Prog.
ER	85	-	-	11.42
CS	36	-	-	9.61
HT	74	-	-	10.76
EN	45	-	-	14.16
MJ	63	-	8.03(PR)	-
GM	53	-	6.88(PR)	-
PP	61	-	7.34(PR)	-
MB	51	-	7.97(NC)	-
EM	74	-	8.47(N/A)	-

Appendix 5.11

Stage III & IV Breast Cancer

Chemotherapy: Lymphocyte O_2 Consumption

	Age	Pre Rx
Stage III		
BD	48	9.70
IH	50	9.23
DM	45	10.11
FC	72	7.83
Stage IV		
EB	68	7.43
MH	38	13.30
JJ	61	11.37

Appendix 5.12

Lymphocyte O_2 Consumption in Tumours Other Than Breast Cancer

	Age	Grade	Lymphocyte O_2 Utilisation
AL	52	Dukes' A	7.84
MR	58	B	9.53
HW	71	B	6.42
WA	78	B	7.96
MR	58	C	6.18
WE	69	C	8.60
IS	57	C	10.93
BD	47	C	11.22
ME	55	D	13.99 (liver)
BW	79	D	11.48 (liver)
WE	63	D	11.85 (liver)
AG	55	D	15.29 (liver)
TC	53	D	9.97 (local invasion)
Stomach/Lower Oesophagus			
RW	54	Local	10.53
EB	58	Disseminated	11.48 (local invasion)
MM	60	Disseminated	8.81 (local invasion)
AW	74	Disseminated	10.36 (liver)
PC	69	Disseminated	9.58 (liver)
RN	62	Disseminated	12.85 (liver)

Appendix 5.12 (continued)

	Age	Grade	Lymphocyte O_2 Utilisation
Pancreas			
CM	74	Disseminated	11.04 (local invasion)
CC	70	Disseminated	10.04 (liver)
Ovary			
ES	74	Disseminated	14.21
Lung			
MR	55	Disseminated	10.58
Carcinoid			
JH	65	Disseminated	11.06
Malignant Melanoma			
FA	63	Disseminated	9.45
PL	28	Disseminated	11.69
JE	49	Disseminated	12.37

Appendix 5.13

Separation of T-rich and B-rich Lymphocyte Populations

	Purification		Oxygen Consumption		
	% T	% B	T-rich	B-rich	Unseparated
CONTROLS					
DG	83	79	6.14	4.89	6.08
DB	80	81	6.34	4.26	5.98
MW	81	77	5.98	4.01	5.36
PJ	85	83	6.28	4.66	6.14
SB	78	74	6.00	4.04	5.78
FH	84	72	5.90	4.00	5.44
JJ	76	78	6.31	5.00	6.30
JS	80	69	6.00	5.08	6.09
FB	73	80	5.96	4.34	5.66
SB	79	79	6.34	5.00	6.22
MT	80	76	6.28	4.86	6.00
BREAST CANCER					
HW	81	80	9.95	5.08	9.14
FP	79	81	11.12	4.96	10.98
MT	78	76	8.76	5.20	8.89
HT	77	77	7.98	4.84	7.64
RH	82	80	9.14	4.79	8.90
CK	79	79	10.00	5.00	9.00
LH	78	76	9.26	5.02	9.14
KB	80	74	11.08	4.88	10.83
CL	80	80	9.22	4.69	9.00

Appendix 5.13 (continued)

	Purification		Oxygen Consumption		
	% T	% B	T-rich	B-rich	Unseparated
COLORECTAL CANCER					
MA	81	79	11.72	5.00	11.00
AS	84	81	12.14	4.97	11.98
FB	78	79	10.07	4.88	9.24
HC	76	74	9.28	5.24	10.02
JB	83	75	8.88	4.11	7.94
CW	82	76	12.28	5.24	11.99

Appendix 5.14 Treatment of Lymphocytes with Papain: Lymphocyte O_2 Consumption

	Age	Lymphocytes	Lymphocytes Rx Papain 37°C	Lymphocytes Rx Papain 37°C plus autologous serum	Lymphocytes Rx Papain 37°C plus allogeneic cancer serum	Lymphocytes Rx Papain 4°C
CONTROLS						
KO	42	7.03	4.30	-	-	-
AG	45	5.60	6.73	4.90	10.55	-
DH	54	6.53	6.78	6.71	8.11	6.04
EG	25	7.05	6.77	-	-	-
ET	58	7.50	7.65	7.76	11.23	-
CD	43	5.65	6.02	6.50	9.40	-
RP	55	5.60	6.87	5.80	8.99	-
MG	59	6.02	6.71	5.80	9.79	-
KM	63	6.45	6.46	5.78	9.87	-
EC	73	5.86	6.23	6.23	10.41	-
RL	30	5.99	4.12	-	10.88	-
AP	35	6.63	5.42	5.98	-	7.03
DM	25	6.39	4.43	6.32	-	6.28
JD	48	6.19	6.51	6.66	-	6.51
DG	32	6.03	6.31	-	10.85	-

Appendix 5.14 (continued)

		Age	Lymphocytes	Lymphocytes Rx Papain 37°C	Lymphocytes Rx Papain 37°C plus autologous serum	Lymphocytes Rx Papain 37°C plus allogeneic control serum	Lymphocytes Rx Papain 4°C
CANCERS							
WE	colon CA(D)	63	11.85	8.70	12.13	-	11.52
ME	colon CA (D)	55	13.99	5.22	11.75	7.83	-
BW	colon CA (D)	55	11.48	7.34	12.05	7.58	-
WA	colon CA(B)	78	7.96	6.35	10.40	-	10.98
MR	colon CA(B)	58	9.53	6.75	10.52	-	10.52
HW	colon CA(B)	73	6.42	4.05	5.88	-	6.84
WE	colon CA(C)	69	8.60	6.80	8.87	-	8.50
MM	stomach CA(D)	60	8.81	5.63	9.20	-	-
PC	stomach CA(D)	69	9.58	6.57	9.40	6.78	-
JE	melanoma IV	49	12.37	4.97	13.05	-	12.46
ES	ovary IV	74	14.21	6.74	13.51	5.98	-
MT	breast II	72	8.08	5.34	-	-	-
DG	breast III	55	10.49	7.71	11.00	7.80	10.67
AJ	breast II	42	9.40	5.69	9.10	-	9.50
PM	breast III	41	8.39	6.82	-	6.50	8.67
EO	breast III	48	7.73	3.98	-	-	-
IM	breast IV	40	8.47	6.13	9.09	6.42	8.30
FM	breast II	85	7.98	6.61	-	6.58	-
ER	breast IV	85	11.42	7.23	10.96	-	-

Appendix 5.15

Control Lymphocytes, PMN and Monocytes

O_2 Consumption in Autologous and Allogeneic Cancer Serum

	Lymphocytes	Lymphocytes + cancer serum	PMN	PMN + cancer serum	M	M + cancer serum
DG	5.64	8.84	6.51	6.27	4.62	4.14
PC	5.69	8.28	5.71	4.91	2.86	2.90
RL	6.17	8.43	4.41	4.36	3.31	3.11
NR	5.17	8.03	4.46	4.31	4.13	4.20
LT	5.52	7.56	3.82	3.38	3.81	2.13

Appendix 5.16

Controls, Cancers and Complement

	Lymphocytes	Lymphocytes in autologous serum	Lymphocytes in autologous serum at 56°C	Lymphocytes in autologous serum at 56°C + complement
CANCERS				
WD Stage III breast	8.25	8.72	5.98	9.00
JM Stage II breast	8.20	8.08	5.38	7.98
VH Stage III breast	9.99	10.26	6.76	9.84
JH Carcinoid dissem.	11.06	9.46	7.00	9.88
MB Colonic CA (B)	11.12	9.86	6.24	10.26
CONTROLS				
MD Benign breast disease	6.31	5.78	4.90	5.14
EH Gallstones	6.47	6.00	6.00	6.34
RH Right inguinal hernia	5.81	5.74	5.28	5.00
JH Benign breast disease	6.02	5.60	7.76	5.60
DJ Benign breast disease	6.49	5.76	5.98	6.00

($\mu l \times 10^{-5} / 10^{6}$cells/sec)

Appendix 5.17

The Effects of Plasmapheresis on Lymphocyte O_2 Consumption

	Age	Diagnosis	Date	Control Lymphocytes in allogeneic serum pre-plasmapheresis	Control Lymphocytes in allogeneic plasma from blood cell separator	Control Lymphocytes in allogeneic serum post-plasmapheresis
EJ	45	Polymyositis	10/12/84	12.93	16.45	8.23
EJ	45	Polymyositis	21/12/84	11.75	18.80	9.40
EJ	45	Polymyositis	9/5/85	14.10	17.63	9.40
EJ	45	Polymyositis	23/1/85	11.75	18.80	7.05
MH	38	Polyneuropathy	14/1/85	9.40	10.56	8.23
IH	44	Myaesthenia gravis	11/1/85	16.45	21.15	10.56
IH	44	Myaesthenia gravis	14/12/84	17.63	22.33	11.75
AN	40	Myaesthenia gravis	20/12/85	12.93	19.98	10.56
TB	60	Guillain Barre	18/12/84	10.56	16.45	5.88
BD	58	Autoimmune pancytopaenia and myositis	3/12/84	9.40	11.75	5.88
BD	58	" " " "	7/1/85	8.23	12.93	7.05
WE	60	Waldenstrom's	5/12/84	9.40	7.05	7.05
WE	60	Waldenstrom's	19/12/84	10.56	11.95	5.86
WE	60	Waldenstrom's	2/1/85	10.58	14.10	7.05
EJ	65	Waldenstrom's	4/12/84	15.28	16.54	10.56

Appendix 5.18

Lymphocyte O_2 Consumption and % T Counts

	Age	O_2 Uptake	% T Count
GB	72	6.42	51
GF	58	6.39	60
RH	60	5.81	58
CV	40	7.33	58
EH	50	6.47	60
SJ	49	6.04	68
WG	71	6.09	53
OT	70	6.25	54
ET	68	5.99	50
JG	56	6.25	61
CB	41	6.10	64
EB	38	5.63	68
MB	80	6.12	51
DC	42	6.16	62
JC	60	6.53	64
MH	44	5.88	59
JH	23	6.02	63
GH	48	5.51	56
SH	42	5.92	63
AM	30	5.97	66
KM	26	5.94	68
SP	33	6.46	68
JR	40	6.18	58
NR	38	6.06	59
JR	51	8.38	51
GT	59	7.45	58
SW	43	6.38	59
AM	29	6.77	61
SH	43	5.92	63
LY	41	6.20	64
DN	46	6.12	60

Appendix 5.18 (continued)

	Age	O_2 Uptake	% T Count
AH	40	5.44	54
MD	48	6.31	58
JH	19	6.16	64
LB	27	7.13	53
RB	38	6.11	60
ID	62	7.54	56
AM	33	5.73	50
ER	68	6.68	50
CW	28	9.18	65
EL	62	9.38	42
EL	62	8.44	40
PJ	78	9.35	38
SR	38	9.45	43
EL	68	8.06	36
MB	72	10.68	45
AB	38	8.73	42
AB	38	8.29	36
VC	39	7.43	52
VC	39	11.02	50
EH	60	8.98	51
EH	60	9.84	44
AJ	42	9.42	69
JJ	43	11.48	48
MM	48	11.68	50
JR	49	10.18	53
MS	40	7.34	36
JL	53	9.20	69
JL	53	8.93	50
MF	80	8.05	64
EP	80	9.91	51
EP	80	9.22	46
EW	84	9.67	49
KM	60	9.44	48

Appendix 5.18 (continued)

	Age	O_2 Uptake	% T Count
WD	63	8.25	39
RF	79	13.06	42
DG	55	10.06	63
DG	55	10.88	51
PM	41	8.34	55
IM	55	8.66	57
IM	55	8.47	40
GL	82	10.96	44
IH	50	9.23	43
BD	48	9.04	25
FL	66	6.14	57
ED	71	8.60	44
GL	80	11.75	50
GL	80	13.68	53
JP	73	7.09	68
JP	73	4.72	71
GS	71	8.81	38
BW	75	12.61	44
OA	76	9.18	41
MA	44	8.94	27
EB	53	5.46	33
EB	53	7.34	54
VB	48	9.20	52
VB	48	7.20	68
AC	75	7.09	56
EC	48	11.02	50
FD	72	11.45	46
CD	57	11.99	50
CD	57	7.75	57
CD	57	7.12	54
MF	83	8.40	21
MF	83	6.84	58
WG	49	7.63	55

Appendix 5.18 (continued)

	Age	O_2 Uptake	% T Count
WG	49	5.46	50
WG	49	9.84	52
MJ	63	7.96	49
GJ	39	5.84	50
SJ	44	9.51	40
EJ	63	10.31	41
EJ	63	9.90	50
MJ	49	9.56	57
NL	58	11.31	46
AM	45	8.99	57
IM	58	8.47	40
WN	74	10.26	47
WN	74	10.11	61
PP	61	7.34	50
LS	45	12.70	53
LS	45	12.49	45
LS	45	8.29	56
LS	45	13.72	56
EH	60	9.84	44
EM	74	9.92	51
MP	63	6.25	35
HT	74	10.13	47
HT	74	10.76	50
MH	38	13.30	31
JJ	61	11.37	42
MS	24	14.80	50
RW	54	10.53	41
JH	65	11.06	44

Appendix 5.19

O_2 Consumption and Immune Complexes

	Age	O_2 Uptake	Immune Complexes
DG	32	5.88	19
DM	25	6.39	30
SD	26	7.21	90
MO	42	7.34	18
RB	38	6.11	43
WH	39	5.49	94
BM	36	5.91	41
AM	33	5.73	22
EW	37	7.40	52
MW	47	5.85	52
MW	47	6.91	32
MB	72	10.68	57
WB	42	5.48	66
AB	39	8.73	58
VC	39	7.43	68
EH	60	8.98	78
EH	60	8.84	88
AJ	42	9.42	60
MT	53	7.88	34
JL	53	9.20	79
EP	80	9.91	14
EW	84	9.32	39
EW	84	6.99	49
DG	55	10.06	55
PM	41	8.34	31
EO	48	9.01	46
AR	86	5.51	102
IM	55	8.66	38
BD	48	9.70	69
IH	50	9.23	24
BD	48	9.04	86
GL	80	11.75	76
EB	53	5.46	69
VB	48	9.20	47
VB	48	8.81	66

Appendix 5.19 (continued)

	Age	O_2 Uptake	Immune Complexes
AB	38	8.73	58
AC	75	7.09	78
VC	38	7.43	68
FD	72	11.45	150
MF	83	8.40	108
MF	83	6.84	121
WG	49	7.63	44
GJ	39	6.79	54
GJ	39	5.32	51
MJ	49	9.56	148
GH	39	5.84	60
NL	58	9.64	102
ER	65	10.28	141
MP	63	6.25	47
WW	64	9.61	66
EN	60	14.16	150

Chapter 6

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6. General Discussion

Evidence indicates that spontaenous cancers, such as carcinoma of the breast, do not develop as a consequence of impaired immune surveillance, but rather as a result of exposure to carcinogens. Such tumours tend to be poorly immunogenic. Tests of immune function are however, affected adversely in these tumour bearing hosts and the extent to which immunity is impaired is generally a reflection of tumour load, indicating that depressed immune competence is secondary to the presence of cancer. There is strong evidence that the 'blocking' factors responsible for this phenomenon are CICs, formed by the combination of cell breakdown or membrane products of rapidly dividing cells, and antibody to these (non-tumour specific) antigens.

This immune depression may be harmful to the patient in terms of reduced resistance to bacterial, viral and fungal diseases or, possibly, as a result of immune complex deposition although the latter is rare. Are there other ways in which ICs may be harmful to the cancer patient?

Moller (1969) suggested that IC formation was perhaps beneficial in that the antibody manufactured by a few sensitised lymphocytes at the tumour site would combine with locally produced antigenic tumour material to form ICs. They in turn perhaps stimulate uncommitted cells into a cytotoxically active state only in the region of the tumour, that is, restricting the cytotoxic reaction to the site of antigen production. Indeed T lymphocyte activity is stimulated and ADCC activity inhibited under conditions of antigen excess (reviewed by Theofilopoulos & Dixon 1979). However, in vivo, such enhancement of T lymphocytes disappears to be replaced by inhibition when antigen excess changes to

antibody excess (Weigle 1975). Furthermore, ADCC activity is stimulated in antibody excess (Theofilopoulos & Dixon 1979). Thus, it is possible that different lymphocyte subsets are stimulated depending on the molar ratio of ICs.

Inhibition of T cell induced activity is probably effected through increased suppressor cell activity. Suppressor cells comprise mainly cells of the monocyte/macrophage system and of the T lymphocyte population (Herzenberg et al. 1973; Gershon et al. 1974; Moretta et al. 1977; Reinherz et al. 1980; Yamagashi et al. 1980; Herberman & Ortaldo 1981; Herberman 1983b) and are thought to exert their effect on interleukin - 2 - dependent cells (T, NK, LAK) through release of a factor which specifically blocks the action of interleukin - 2 (Daya et al. 1987). Such suppressor cell activity appears to be part of the normal apparatus for the induction of peripheral tolerance (Theofilopoulos 1987) and there is evidence that suppressor cell activity is continuously occurring in normal people (Vento et al. 1987). The reason for this is that there is continual recognition by immune cells of self antigens, the success of this being dependent on the immunogenicity of the antigen (Cunningham 1975). Self antigens most immunogenic are those normally sequestered, such as intracellular materials and cell membrane surface molecules expressed during cell death. Cancer patients with their persistent antigenaemia would be expected to display continuous immune reactivity to these antigens. However, constant stimulation, even with a foreign antigen, ultimately results in its being treated like 'self' (Gras & Dalmau 1966) probably through suppressor cell activity (Cunningham 1975).

That ICs are able to stimulate suppressor cell activity has been amply demonstrated (Gershon et al. 1974; Stout et al. 1976; Moretta et al. 1977). The Fc receptor portion of the complex is mandatory for stimulation

of suppressor or activity, but not for the 'blocking' effect exerted on other cells (Chan & Sinclair 1973), and it may be that only cells with Fc-Ig receptors are able to exert this activity. It would be expected that cancer patients with their persistent antigenaemia would have high levels of ICs in their blood and indeed, as has been previously discussed, this tends to be the case. It follows that suppressor cell activity in such patients would also be increased and this has been demonstrated (Toge et al. 1983; Braun et al. 1983; Kanayama et al. 1985). This activity tends to be greatest in areas of higher blood concentrations of ICs (Kanayama et al. 1985), that is closest to the tumour. For instance, cell cytotoxicity in animals and man is most markedly depressed within tumours and becomes less so within regional lymph nodes the further away from the tumour they are (Currie & Gage 1973; Nind et al. 1973; Hoon et al. 1987a). This effect is secondary to increasing suppressor cell activity with increasing proximity to the tumour (Kanayama et al. 1985; Hoon et al. 1987b) and such suppressor cell activity becomes more apparent the greater the aggression displayed by the tumour (Kanayama et al. 1985). Furthermore, as disease load increases tumour cytotoxicity diminishes (Currie & Gage 1973; Kanayama et al. 1985) and suppressor cell activity increases (Kanayama et al. 1985) in the peripheral blood. This spreading anergy may be explained by suppressor cell activation first in the vicinity of the tumour and its regional lymphoid system and then generally as the initial local production of ICs by the tumour becomes widespread, through release into the host circulation, as tumour load increases. Does this suppressor cell activity have any consequences in human tumour bearers?

Whilst accepting that the impaired immunity plays no part in the development of spontaneous cancers there is evidence that such tumours

may, to some extent, be under the control of the immune system. Indeed, it has been shown that a weak immune response may increase the ability of tumours to grow locally and metastasize (Prehn 1976) and further, that this may be an effect of suppressor cell activity (reviewed by Murasko & Prehn 1983). Furthermore, it has been demonstrated that cancer patients given additive immunosuppressive therapies have a worse prognosis than patients treated with less immunosuppressive regimes. For instance, patients with inoperable Stage III breast cancer treated with a combination of radiotherapy and chemotherapy, as part of a controlled study, had significantly poorer survival than those given radiotherapy alone (Paterson & Webster 1986). Patients with various spontaneous tumours if transfused with whole blood, which has apparent immunosuppressive properties in that it may help to suppress allograft rejection (Woodruff & van Rood 1983), have earlier recurrence of their tumours and poorer survival than patients given no blood or packed cells only (Blumberg et al. 1986; Hamblin 1986). It has been suggested that a factor present in the plasma is capable of 'turning on' suppressor cells (Woodruff & van Rood 1983). Tumour autotransplant and even allotransplant (dependent on HLA activity) rejection, has been demonstrated to be slower and sometimes incomplete in patients with advanced cancers and this is most significant in those with poorest CMI (Southam et al. 1965; Southam et al. 1966). These phenomena and others, discussed in Chapter 2, such as dormancy, the incidence of subclinical tumours, spontaneous tumour regression, variation in tumour growth rate between individuals with similar histological tumours, the beneficial effects of immune reactions histologically and the high incidence of failure of circulating tumour cells to seed, imply that the immune system may play a role in the control of spontaneous cancers once they have developed. Immune suppresssion by IC stimulated suppressor cells may perhaps aid

the growth and increase the metastatic potential of these tumours.

Thus, it may be proposed that CICs may act detrimentally on the cancer patient firstly through 'blocking' thereby preventing antigen recognition and activation of cells cytotoxic to tumour cells and secondly, by suppressor cell stimulation and suppression of any induced immune response. It has been demonstrated in this work that patients with breast cancer exhibit 'blocking' as manifested by depressed % T counts, and lymphocyte stimulation, displayed by increased respiration. There is no proof at the present that the lymphocytes so stimulated are suppressor cells and this must wait until the T lymphocyte subsets are examined in this context. There is evidence however, that the same factor(s) is responsible for both 'blocking' and stimulation of T lymphocytes.

There are many similarities between the serum factor(s) responsible for inducing increased O_2 consumption in lymphocytes and 'blocking' factor(s) of cancer. Both appear to exert their effect on T cells, are removed from the lymphocyte surface by papain digestion and reattached by incubation in cancer sera after as little as an hour. They appear to be independent of HLA and blood group activity; unaffected by storage at low temperature or incubation at $56^{o}C$ but lose their effect on dilution and are removed by plasmapheresis. Furthermore, neither affects B lymphocytes in an obvious manner, but both have been shown to be dependent on disease status and tumour load or activity, reflecting responses to systemic therapy within 6 to 8 weeks from the start of treatment. Finally, there is a strong correlation between depressed % T counts and increased lymphocyte O_2 consumption, indicating that the factor(s) responsible for both phenomena may be one and the same. There is one point of obvious difference however, in that it is impossible to demonstrate

stimulation of O_2 consumption in control lymphocytes incubated in cancer sera, unless they are first treated with papain.

CICs also have much in common with the serum factor(s) which stimulates lymphocyte O_2 consumption. Whilst recognising that heat inactivation causes difficulties in measuring CICs, due to aggregation of IgG, it does not affect the CICs themselves. Similarly, heat inactivation has no effect on the factor(s) responsible for lymphocyte O_2 consumption stimulation. The factor(s) is however, complement dependent and like CIC cannot exert its stimulatory effect on lymphocytes in the absence of complement. Both are also present in patients with immune complex related diseases and removed by plasmapheresis but reattain previous levels within 3 weeks and both reflect tumour burden. Finally, there is significant correlation between CIC levels and lymphocyte O_2 consumption.

It seems reasonable to suggest therefore, that CICs are responsible both for 'blocking' of T lymphocytes and their increased O_2 consumption. This may appear rather anomalous at first sight, but there are similarities between substances which 'block' immunological responses and those which stimulate these responses. Specific antigen on its own (but not non-specific antigen) can 'block' at the lymphocyte surface (Baldwin et al. 1973c; Baldwin et al. 1974; Gorczynski et al. 1975) and has been demonstrated to stimulate sensitised lymphocytes to synthesise DNA (Lundgren et al. 1968a; Moller 1969) or exhibit cytotoxicity (Lundgren et al. 1968a) whereas non-specific antigen does not have this capability (Lundgren et al. 1968a; Bloch-Shtacher et al. 1968; Moller 1969). Antibody, by itself, is similarly incapable of 'blocking' at the lymphocyte surface (Baldwin et al. 1973c; Baldwin et al. 1974; Gorczynski et al. 1975) and cannot stimulate lymphocytes unless specifically sensitised against them (Lundgren et al.

1968a; Bloch-Shtacher et al. 1968; Moller 1969). ICs however, are capable of 'blocking' at the lymphocyte (as well as at the tumour) surface (Sjogren et al. 1971; Hattler & Soehnlen 1974; Baldwin et al. 1972; Baldwin et al. 1973c; Baldwin et al. 1974; Gorczynski et al. 1975; Tanaka et al. 1979) in a non-immunologically specific fashion provided antibody and antigen are in the correct ratio. Both sensitised and non-specific ICs can also stimulate lymphocytes into their active state (Lundgren et al. 1968a; Bloch-Shtacher et al. 1968; Moller 1969; Soderberg & Coons 1978) when near equivalence in slight antigen excess, i.e. the 'right mix' required for exhibition of the 'blocking' phenomenon (Baldwin 1974).

Thus, it appears that substances which display 'blocking' ability are also those which are stimulatory to the lymphocyte. This is not surprising when it is considered that lymphocytes stimulated to transformation or cytotoxicity by ICs have been shown to be refractory to other stimuli, for instance, the non-specific stimulation of PHA on the cell (Lundgren et al. 1968a; Lundgren et al. 1968b). This suggests that lymphocytes triggered by one process become 'blocked' to certain other triggering reactions, perhaps by exerting suppression of the different membrane receptors responsible for stimulation by other agents.

This work suggests that the effects exerted on T lymphocytes, probably by CICs, appear to reflect tumour activity and may be utilised to permit earlier evaluation of hormone treatment responses in breast cancer. Neither % T counts nor measurement of lymphocyte O_2 consumption seem prone to the difficulties associated with assays of CICs, and both are simple to perform and can be assessed on the day of the patient's visit to the clinic. Furthermore, lymphocyte O_2 consumption may be an index of suppressor cell activity, although this has yet to be proved, and used in this way might allow us further insight into the complex

balancing mechanisms of the immune system in the patient with cancer.

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