IMMUNOLOGICAL PARAMETERS AND THE USE OF IMMUNOTHERAPY IN OVARIAN CARCINOMA.

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DECLARATION

I certify that this Thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any University; and that to the best of my knowledge and belief, it does not contain any material previously published or written by another person except where due reference is made in the text.

M. E. Crowther

PREFACE

The work described in this Thesis is a continuation of that begun by Dr. L. Levin in the Williamson Laboratory, St. Bartholomew's Hospital, London. He and his co-workers showed that patients with ovarian cancer demonstrated lymphocyte responses to extracts of ovarian tumours, which were specific and related to the Stage of the disease. Subsequently, an Immunotherapy programme was initiated, which closely followed the protocol used by Dr. R. Powles, in the Acute Myeloid Leukaemia Trial, then being conducted at St. Bartholomew's Hospital.

This Thesis describes the results of that initial Trial, as well as a new properly controlled and randomized Trial being carried out in conjunction with the MRC Ovarian Cancer Chemotherapy Trial. The procedure is shown to be safe, possibly efficacious in prolonging life, and definitely worthy of further study. In vitro monitoring of patients shows that immunological parameters are boosted by immunotherapy with BCG and tumour cells, but its effects would not seem to be mediated through an increase in specific anti-tumour lymphocyte responses, but rather through a "boosting" of bone marrow, thereby allowing greater amounts of chemotherapy to be given.

The study of specific blastogenic responses to tumour extracts was not conclusive, and suggested that such responses were non-specific and unrelated to tumour load. Control subjects showed responses that were very significantly related to parity, and it was postulated that foetal sensitization of the mother's lymphocytes might explain the lower incidence of parous women with ovarian cancer, compared with those who are nulliparous.

The effects of chemotherapy on various immunological parameters were also studied, and led to rather paradoxical results. It was also suggested that although relapse patients had significantly decreased numbers of T-cells, as assessed by in vitro E-Rosetting, this did not represent a true decrease but the presence of serum factors

which prevented lymphocytes from binding to SRBC.

The study of in vitro serum effects was inconclusive but the measurement of immune complexes showed very significant relationships to the Stage of disease and presence of recurrence post-operatively. The measurement of ectopically produced trophoblast-specific hormones in ovarian cancer serum was also deemed to be of limited use.

The historical background to this work has been described in some length, but I think this is justified to give perspective to an area which is not only controversial in the field of Gynaecology, but also in the much wider world of Oncology.

I wish to gratefully acknowledge the helpful discussions and kind cooperation of a number of people: the Library staff at St. Bartholomew's, statisticians Miss M. Leighton and Mrs. J. Wadsworth, the Nursing Staff of Pitcairn Ward and Sr. P. Walker, my co-worker Mr. T. A. Poulton, my supervisor Professor C. N. Hudson, and my husband G. H. Kirkpatrick. Finally, one should not forget the courage of these patients, nearly all of whom are now dead, in submitting themselves to an experimental form of treatment, and who uncomplainingly had great demands made on their time for the in vitro monitoring described herein.

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CHAPTER 1. THE CONCEPT OF IMMUNOSURVEILLANCE.

Since last century the relationship between various immunological phenomena and tumour regression has been recognised (COLEY 1896), but it was not until in-bred animals became available that experiments involving tumour sensitization and rejection could implicate specific tumour antigens rather than tissue histo-incompatibility. Such tumour rejection has been demonstrated in many murine tumours induced by viruses (OLD & BOYSE 1965), carcinogens (GROSS 1943; KLEIN 1966) and those arising spontaneously (HAMMOND et al, 1967). More recently specific immune responses to postulated tumour antigens have been investigated in human tumours (see Chapter 3).

BURNET's (1971) Theory of Immunosurveillance briefly states that random somatic mutation of cells occurs in vertebrates and results in a neoplastic cell line with antigenic determinants new ("non-self") to the host. Evidence for this concept of immunological recognition and rejection of these cells came from a number of clinical indications in human malignancy that host resistance occurred, as well as experimental manipulations of the immune system in animals.

1.1 INDIRECT EVIDENCE FOR IMMUNOSURVEILLANCE.

(i) Spontaneous regression of tumours. For many years it has been observed that there may be complete disappearance of a malignancy in the absence of any adequate treatment. The capricious nature of malignant melanoma with regressions and re-appearances of metastases is a well-known example (BODENHAM 1968). The definitive article by EVERSON (1964) sited 130 cases, mostly nephroblastoma, choriocarcinoma, neuroblastoma and malignant melanoma.

(ii) Regression of metastases after surgical removal of the primary tumour. This was well documented in EVERSON's (1964) study, and more recently by COLE (1976).

(iii) Recurrence after a prolonged interval. Again, this is

recognised as part of the behaviour of malignant melanoma, but renal carcinoma may give rise to metastases 20 years or more after the original nephrectomy (DONALDSON et al, 1976).

(iv) Increased incidence of post-mortem in situ-carcinoma compared with clinical frequency. Post-mortem examination of the thyroid (Lancet Editorial 1964), prostate (ASHLEY 1965) and infant adrenals (BECKWITH & PERRIN 1963) has shown a much higher incidence of in-situ carcinoma than that of the general population.

(v) Lymphocyte infiltration of tumours correlated with prognosis. This has been demonstrated for a wide range of human tumours including breast (BLACK et al, 1955), stomach (WATANABE et al, 1976), lung (IOACHIM et al, 1976) and ovary (BARBER et al, 1975). However, there have been conflicting reports (UNDERWOOD 1974) and evidence that the lymphocytes in tumours may be immunologically unresponsive (see Chapter 4). Certainly in animal studies, regressing Moloney sarcomas are associated with a dense mononuclear infiltrate of T-cells and macrophages, compared with the progressive lesions (RUSSELL et al, 1976).

(vi) Response to chemotherapy. Both Burkitt's Lymphoma (BURKITT 1967) and choriocarcinoma (MATHÉ et al, 1964) may dramatically regress after a single dose of chemotherapy suggesting that some immunological mechanism apart from drug cytotoxicity is involved.

1.2 DIRECT EVIDENCE FOR IMMUNOSURVEILLANCE.

(i) Poor auto-transplantability of tumours. Large numbers of malignant cells are known to leave tumours in the venous blood supply yet often do not form metastases (GRIFFITHS et al, 1973). Further, auto-transplantation of tumour cells appears to take only in about 20% of patients, and biopsy shows tumour cells surrounded by a dense inflammatory reaction (HOWARD 1963).

(ii) Age and increased cancer incidence. There is a higher incidence of cancer at the extremes of life when

there is poor immune responsiveness (STUTMAN 1974). Studies in aging mice show diminished antibody responses, loss of thymic helper cells and an increase in suppressor cell activity (GOIDL et al, 1976).

(iii) Congenital immunodeficiency and cancer. In both animals and humans with auto-immune diseases, such as Bruton's type agammaglobulinaemia or Wiscott-Aldrich Syndrome, there is an increase in reticulo-endothelial malignancy (KERSEY et al, 1973). Conversely, the incidence of allergic diseases in patients with cancer appears to be significantly lower than in controls (MACKAY 1966). It is difficult to know whether the genetic defect causing the immunodeficiency state might also increase host susceptibility to malignant transformation.

(iv) Iatrogenic Immunosuppression and cancer. There appears to be a 100-fold increase in the incidence of de novo malignancies compared with the general population, in organ transplant patients (PENN 1970). The average time of development in the original series of 432 patients, followed for up to 11 years, was 28 months, and most common were epithelial tumours of the skin and lip, squamous cell carcinoma of the cervix (KAY et al, 1970) and the very rare reticulum cell sarcoma of the brain (PENN 1974).

Data on patients who have inadvertently received organs from donors with cancer show that 36% developed cancer of similar histology, which could be detected in kidneys removed shortly after transplant (MUIZNIEKS et al, 1968), and which could cause death from metastases and rapid growth (PETERS & STUARD 1978). Dramatic regression of such tumours once immunosuppressive therapy is stopped has been reported (WILSON et al, 1968).

There are increasing reports of acute myeloid leukaemia in patients receiving chronic Cyclophosphamide for medical conditions such as renal disease (ROBERTS & BELL, 1976); and an increased frequency of second tumours in patients treated with cytotoxic drugs for primary malig-

nancy (ARSENAU et al, 1972). It is not known whether this arises from a direct carcinogenic effect of such drugs (SIEBER 1975), the immunosuppression they cause, or the possibility that they potentiate the effects of environmental carcinogens such as radiation, tobacco and viruses (STEVENS 1973). Since 1970 over 20 cases of acute myeloid leukaemia following alkylating therapy have been reported in patients with ovarian cancer (KAPADIA & KRAUSE 1978).

In animal studies the use of anti-lymphocyte serum or immunosuppressive drugs can lead to an increase in the rate of development and incidence of induced tumours, as can neonatal thymectomy (TRAININ & LINKER-ISRAELI 1971).

Despite these observations, there are a number of discrepant results, especially in animal experiments, which indicate that T-cell cytotoxicity may not be the first line of detection and defence. Nude mice (if they can be kept alive from the hazards of infection) do not have an increased incidence of malignancy (RYGAARD & POLVSEN 1974), and immunologically privileged sites such as the cornea and hamster cheek pouch, whilst ideal for tumour culture in vitro, likewise have no remarkable incidence of de novo malignancy (SCHWARTZ 1975; MÖLLER & MÖLLER 1976). Lethal tumours in normal animals can be produced by the transplantation of very few cells, indicating that generalized immunosuppression is not an essential feature in the aetiology of malignant disease, or may have only a limited and specific effect (ALEXANDER 1970). Furthermore, neonatal thymectomy may actually decrease the incidence of murine mammary carcinoma and thymus reconstitution restores normal susceptibility to this tumour (YUNIS et al, 1969).

It has been suggested that immunostimulation and not immunosuppression may be the factor resulting in malignancy (PREHN & LAPPÉ 1971), and it may certainly be overstimulation of the immune system by a foreign graft which leads to the high incidence of reticulo-endothelial malignancies in transplant patients (the increase in

cervical and basal cell carcinoma being a fortuitous consequence of the closer medical attention such patients receive). It is well known that continuous antigenic stimulation can lead to reticulo-endothelial malignancies (GLEICHMAN et al, 1975), and animals which have survived a graft-versus-host reaction to experimental bone marrow transplants have a high incidence of malignancy (WALFORD & HILDEMANN 1965). It has been proposed that such lymphocytic stimulation leads to derepression of a ubiquitous viral genome, which induces an oncogenic virus (SCHWARTZ 1975). It is interesting to note in this regard that many years ago PULVERTAFT (1965) commented on the likeness between transformed lymphocytes and Burkitt lymphoma cells, and suggested that chronic reticulo-endothelial stimulation from malaria might be involved in the aetiology of the disease. Likewise, Dilantin-induced lymphocyte transformation has been associated with the occurrence of a malignant lymphocytic lymphoma from a previously benign lesion (LAPES et al, 1976).

Despite attempts to abandon the concept of immuno-surveillance (SCHWARTZ 1975; MÖLLER & MÖLLER 1976), ALEXANDER (1977) has attempted to reconcile the conflicting evidence by considering separately the genesis of a tumour from its subsequent biological behaviour. Certainly the first line of defence against infection is through primitive phagocytic cells; it is the second process, requiring specific memory and highly selected cells, which results in the production of antibody-producing and cytotoxic cells. The analogy with tumour cells, foreign to the host, can be made; initial surveillance may work in the absence of T-cells, but established groups of cells released systematically cannot be counteracted by macrophages and require specific T-cells for their destruction. This would explain why removal of T-cells from animals facilitates distant metastases. Macrophages appear to react selectively against tumour cells but not normal ones (MANSELL et al, 1975), and certainly viz à viz contact suppression of tumours with BCG, the macrophage is vital

since such suppression will occur in nude mice (PIMM & BALDWIN 1975) but not if silica-abrogation of macrophage activity has been carried out (HOPPER et al, 1976).

Clearly, other factors such as genetic control of the immune response also play a part in the control of neoplastic cells, and there appears to be some relationship between tumour susceptibility and one's HL-A status (DELLON et al, 1975; OLIVER et al, 1977). Certain families are also known to have a predilection for particular carcinomas, especially those of the breast, ovary and colon, and LYNCH et al (1975) HL-A typed 115 members of a family and found that 21 of the 22 individuals with cancer had the A2-A12 haplotype.

Hence the concept of immunosurveillance holds good with certain modifications, and once primitive defence mechanisms can no longer maintain the host free of malignant cells, more specific anti-tumour mechanisms are then operative. Aspects of some of these phenomena, especially in relation to ovarian cancer, are the basis of this thesis. Chapter 2 will describe the materials and methods, and statistical analysis of data used throughout. Chapter 3 discusses evidence for tumour-associated antigens and the immunological responses they invoke. Cellular immunodeficiency, with particular reference to iatrogenically induced immunosuppression during chemotherapy, is the subject of Chapter 4 and Chapter 5 discusses methods of tumour escape with particular reference to serum factors such as immune complexes.

As a result of earlier in vitro work on lymphocyte responses in patients with ovarian cancer (LEVIN 1976), an Immunotherapy trial was begun in 1973, and the results and criticisms of that unrandomised trial are presented in Chapter 6 along with details of a current, prospective, randomized trial being conducted within the framework of the Medical Research Council trial into oral chemotherapy of ovarian cancer. Chapter 7 describes the in vitro

immunological monitoring performed during cycles of immunotherapy with BCG and/or tumour cells. The final Chapter 8, in discussing hormonal immunosuppression in cancer, provides evidence that the measurement of ectopic placental hormone production in ovarian cancer is not helpful.

CHAPTER 2. MATERIALS AND METHODS.

ABBREVIATIONS USED IN THIS THESIS:

A.S.	Autologous serum
AFP	Alpha-fetoprotein
ALP	Alkaline phosphatase
BCG	Bacillus Calmette-Guérin
^{o}C	Centigrade degrees
CEA	Carcinoembryonic antigen
CME	Cell membrane extract of ovarian tumour
CMI	Cell mediated immunity
cpm	Counts per minute
Con A	Concanavalin A
DHR	Delayed hypersensitivity reaction
DNA	Deoxyribonucleic acid
E-RFC	E-Rosette Forming Cells (T_a= active component, T_t= total component)
FCS	Foetal calf serum
g	Centrifugal force
G	Gram
Human ABS	Human AB Rh+ serum
HCG	Human chorionic gonadotropin
HPL	Human placental lactogen
^{125}IUDR	^{125}I-Iododeoxyuridine
PBS	Phosphate Buffered Saline
PHA	Phytohaemagglutinin
PPD	Purified protein derivative of Tuberculin
SI	Stimulation Index = $\frac{\text{cpm stimulated cells}}{\text{cpm unstimulated cells}}$
S-RBC	Sheep red blood cells
SP_1	Pregnancy specific $beta_1$-glycoprotein
TCA	Trichloroacetic Acid

REAGENTS USED IN THIS THESIS:

BCG	Vaccine BP for percutaneous use; Glaxo.
Con A	Pharmacia Chemicals, Sweden.
FCS	Virus and Mycoplasma screened; Gibco Biocult.
Ficoll-Triosil	(S.G. 1.077g/ml.) Nyegaard & Co., Oslo.
Folin & Ciocalteu's Phenol Reagent;	BDH Chemicals.
Heparin	1000U/ml; Weddel Pharmaceuticals, Ltd.
Human ABS	This was kindly donated by the Blood Transfusion Service, as required.
^{125}IUDR	Radiochemical Centre, Amersham.
Medium 199 with Hanks' Salts and L-glutamine;	Gibco Biocult.
Mitomycin C	Sigma Chemical Co., USA
PPD	Supplied in powder form by H.M.G. Department of Agriculture and Fisheries.
Penicillin	5000U/ml in combination with Streptomycin 5000mcg/ml; Sigma Chemical Co.
RA Latex Test containing strongly positive and negative	serum controls; Hyland Labs.
S-RBC	25% solution in Alsever's; Gibco Biocult.

2.1 PREPARATION OF CELL MEMBRANE EXTRACTS.

(i) Ovarian Tumour Cell Membrane Extract. A modification of the method by OREN & HERBERMAN (1971) was used. Fresh tumour specimens, removed at surgery and kept in sterile containers, were minced manually with scissors in hypotonic saline, in approximately 5G. quantities. This suspension was homogenised at high speed for 30 seconds using an Ultra-Turrax TP1 homogeniser, and placed in liquid Nitrogen for at least 1 hour. The suspension was then thawed out in order to further disrupt the cell membranes, and 5mls. of sterile distilled water were added to cause further osmotic disruption. This was then centrifuged at 3000g for 1 hour, and the supernatant saved and kept at -20°C. 1ml. was kept for bacteriological culture and Folin-Lowry Protein Estimation.

(ii) Foetal Cell Membrane Extracts. Foetuses of 16-26 weeks gestation were obtained fresh from the delivery rooms and were either the results of spontaneous still-births or prostaglandin therapeutic abortions. They were dissected under sterile conditions by a medically qualified person and the appropriate tissues removed for preparation of extract as above. Tissues were not pooled.

(iii) Protein Estimation of Cell Membrane Extracts. The Folin-Lowry Method (LOWRY et al, 1951) was used as follows:

(a) 20µ l.of sample to be measured was made up to 2ml. in N saline.

(b) Reagents were as such:

Sample A: 0.5ml. 1% Cupric sulphate
0.5ml. 2% Sodium potassium tartrate
10ml. 11% Sodium carbonate

Sample B: 4ml. water with 4ml. Folin Reagent

(c) 0.4ml. Sample A into each sample tube. Wait 15 minutes.

(d) 0.4ml. Sample B into each tube. Wait 15 minutes.

(e) Read each tube at an optical density of 760mm.

A standard curve was obtained and subsequent readings of samples were taken from this.

2.2 PREPARATION OF SERUM.

10ml. of clotted blood in a sterile container was left at room temperature for 1 hour and then left overnight at 4°C. This was then centrifuged at 3000g for 15 mins. at 4°C and the supernatant serum was gently aspirated and stored in aliquots at -20°C. It was heat inactivated as required by suspension in water at 56°C for 30 mins.

2.3 PREPARATION OF LYMPHOCYTES.

(i) Mononuclear Cell Purification. The method of BØYUM (1968) was used. 20-50ml. of blood were obtained by venepuncture into sterile Universal containers with Heparin (20U/ml. blood) added. As soon as possible this was layered onto Ficoll-Triosil, but in cases where blood was obtained from distant hospitals a lapse of 1-2 hours could occasionally occur. On no occasion was blood left overnight as not only was the subsequent yield of lymphocytes poor, but the risk of infection was increased.
10ml. of blood was gently layered onto 10ml. Ficoll-Triosil in a sterile Universal container, using a large syringe with a Kwill Tip attached. The Universals were then centrifuged at 400g at 18°C for 40 mins. Four layers were seen to have formed; a large pellet of predominantly red blood cells, a layer above it of Ficoll-Triosil contaminated with clumps of platelets and cells (in allergic people, often clumps of eosinophils); on top of this a thin, usually well-defined layer of mononuclear cells; above this the top serum layer. This top layer was discarded and the interface of mononuclear cells removed and placed in sterile Universal containers which were then filled with Medium 199 such that the cells were always diluted in a 2:1 ratio at least. These were centrifuged at 400g at 4°C for 15 mins. The resulting cell pellet was washed in Medium 199 twice more and on each occasion centrifuged at 150g at 4°C for 10mins. The final pellet was diluted in 3ml. of Medium 199 and 50µl. of this removed for counting on the haemocytometer.

(ii) Trypan Blue Stain. Trypan Blue exclusion was used to

assess cell viability for both lymphocytes and tumour cells. Fresh stain using 0.4% Trypan Blue was used thus:

(a) 0.4ml. PBS
(b) 50µl. Trypan Blue
(c) 50µl. Cell aliquot

Cells were counted in the haemocytometer by counting the total number of dye excluding cells in 16 large squares, and using the following formula:

No. of cells in 16 squares x no. of mls. in which cells suspended = total no. of cells x 10^5

(iii) Viability. For mononuclear cells this was always 98-100%.

(iv) Lymphocyte yield. In healthy control subjects the yield was at least 1 million lymphocytes per ml. of blood taken. In cancer patients in relapse the yield was invariably less then this.

(v) Cell populations. Normal control subjects usually gave a "clean" population of mononuclear cells, whereas relapse patients often gave a cell population contaminated with much debris. This consisted chiefly of platelet clumps, and the cells themselves were often surrounded by platelets.

To determine the number of monocytes in the final cell suspension, two methods were used:

(a) May-Grunwald Giemsa stain of a cell smear
(b) the cell suspension was left on the haemocytometer for half an hour and the number of cells which had transformed and were clearly sticking to the glass were counted.

Comparable results were achieved with these two crude techniques and gave results of between 15-23% incidence of contamination. No attempt was made to remove these cells as their presence is required in the blastogenic assay (HERSH & HARRIS, 1968). Similar results of macrophage contamination have recently been reported by CURRIE et al,1978).

(vi) May-Grunwald Giemsa Stain. As follows:

(a) cell suspension air dried on slide after smearing and methanol fixed for 10 seconds.

(b) May-Grunwald Stain 100% added to slide for 1 min.
(c) Dilute to 50% with water and leave 2 mins.
(d) Drain. Add Giemsa 1:4 for 3-4 mins.
(e) Wash off excess stain, dry and mount.

2.4 THE BLASTOGENIC ASSAY.

The lymphocyte suspension was adjusted to $1x10^6$ cells /ml. of Medium 199 containing 12.5% serum (generally Human ABS but sometimes A.S. or FCS - see below). $1x10^6$ cells were placed in a Falcon Tube in the presence of antigen, mitogen or N saline for unstimulated cultures. 100µg. ovarian or fetal cell membrane extract were used; 10µg. PPD; and 25µg. Con.A unless otherwise stated. Each experiment was performed in duplicate and more usually triplicate.

The cultures were incubated for 6 days at 37^oC in 5% CO_2 and humidified air, and for the last 6 hours 1µCi of ^{125}IUDR was added to each tube. At the end of this time, the cells were washed in Medium 199 twice, by spinning at 150g at 4^oC for 10 mins. The final wash was done in cold 5% TCA, and spun at 3000g for 15 mins. and the ^{125}IUDR incorporation into the acid insoluble fraction was then measured in a Wilj gamma Counter, and expressed as counts per minute.

The Stimulation Index was used as well as cpm. and cpm difference (between stimulated and unstimulated cultures) in statistical analysis. A cpm. difference of 500 cpm was considered positive.

(i) Serum used in the Assay. Originally FCS was used as the source of nutrient serum in the assay, and was batch-tested from several different sources to discover the best sample. Table 2.1, Fig. 2.1 represent the results in 2 control patients of different PPD responses in different samples of FCS. However it soon became apparent that FCS had a stimulatory effect on lymphocytes (see Chapter 5) and so all assay systems were changed to use Human AB Serum.

(ii) ^{125}IUDR Incorporation. ^{125}IUDR is a gamma-emitting thymidine analogue and so is incorporated into the cell

DNA. It is not spontaneously released from DNA and re-utilization following cell death (should this occur) is negligible (HUGHES et al, 1964). Table 2.2, Fig. 2.2 gives the results of an experiment designed to see what amount of ^{125}IUDR could be added to cells before obvious toxicity occurred. Table 2.3, Fig. 2.3 correlates length of exposure to ^{125}IUDR with its incorporation into the cells. From these experiments it was decided to use 1μCi ^{125}IUDR per $1x10^6$ lymphocytes and pulse for 6 hours before harvesting. Clearly by 24 hours some degree of toxicity and cell death has occurred with release of isotope; and on some occasions 8 hours would have been impossible to manage.

(iii) Dose response curves for mitogens and antigens. Dose response curves for ovarian tumour CME had previously been studied (LEVIN, 1976). Table 2.4, Fig. 2.4 confirms his results that 100μg. of extract was an optimum amount in the Blastogenic Assay. Dose response curves for PPD and Con. A are shown in Table 2.5, Fig. 2.5. Thereafter, they were used in concentrations of 10μg (PPD) and 20μg (Con A) per $1x10^6$ lymphocytes unless otherwise stated.

(iv) Length of incubation of culture. Cultures containing mitogens were cultured for 3 days, and those with antigens for 6 days (CME and PPD). Tables 2.6, 2.7 and Fig. 2.6, 2.7 give results for the pertinent studies.

2.5 THE E-ROSETTE ASSAY.

A modification of the method of JONDAL et al (1972) was used. SRBC were stored in Alsever's solution and were obtained fresh monthly. Before use they were washed three times in PBS and adjusted to a 1% solution in 50% FCS. Lymphocytes were made up as $4x10^6$ /ml. and were mixed with an equal volume (1ml.) of the SRBC. This was incubated at 37°C for 10 mins. and then centrifuged at 200g at 4°C for 5 mins. For the active rosettes (T_a), duplicate tubes were assayed immediately; for the total rosettes (T_t), duplicate tubes were assayed after an overnight incubation at 4°C. The supernatant was gently pipetted off and the pellet with

a small amount of supernatant remaining was resuspended. One drop of this was added to a Trypan Blue stain and read under the haemocytometer. 200 viable cells were counted and all lymphocytes binding 3 or more SRBC were considered positive. Clumps of cells were disregarded. On this basis the %E-RFC could be calculated. The total number of such cells could be calculated from a knowledge of the % of lymphocytes in a routine blood smear of the patient:

Total no. E-RFC = %E-RFC x % lymphocytes x WCC

In some cases, smears were taken and there was a good correlation between results from such counting and those from the haemocytometer count.

(i) Temperature of incubation. Table 2.8, Fig. 2.8 confirms that the optimal temperature of incubation between the SRBC and lymphocytes is 37°C.

(ii) Length of incubation for Total E-RFC. Table 2.9, Fig. 2.9 indicates that the optimal times for assessing the T_t value are between 4 and 24 hours after 4°C incubation.

(iii) The use of FCS to create more stable rosettes. A comparison between PBS and 50% FCS indicates that its inclusion in the SRBC solution allows stronger binding of the SRBC to the lymphocytes (Table 2.9, Fig. 2.9).

2.6 SEPARATION OF LYMPHOCYTE SUB-POPULATIONS FOR THE BLASTOGENIC ASSAY.

A modification of the technique of WYBRAN et al (1974) was used. Lymphocytes, prepared as above, were adjusted to a final population of 5×10^6 /ml. These were incubated with an equal volume of washed SRBC in a 1% solution in PBS, for 10 mins. at 37°C, spun at 200g for 5 mins at 4°C and left overnight at 4°C. The following day the pellets were gently resuspended, combined into one volume, and layered onto Ficoll-Triosil to be centrifuged at 400g at 18°C for 40 mins. This gave two cell populations - an interface of T-depleted cells and a pellet of T-enriched cells at the bottom of the Universal. Both populations were washed in the usual manner to purify them. The rosettes were dis-

sociated by agitation at 37°C and a T-cell suspension free of SRBC was obtained by re-layering this cell population onto Ficoll-Triosil and centrifuging at 400g for 15 mins. prior to routine washing. The cells in each group were used in the blastogenic assay in a concentration of $1x10^6$ /ml. and therefore cannot be compared with the routine blastogenic assay consisting of all lymphocytes at $1x10^6$ /ml. Representative cell suspensions were taken from each population and rosetted in the normal way to see the degree of contamination with other cells.

(i) Cell yield. Losses were about 50% and usually affected the T-depleted population of cells more than the T-enriched population.

(ii) Contamination. The degree of contamination with other cells in both populations was of the order of 8-14%.

2.7 THE SUPPRESSOR CELL ASSAY.

A modification of the method of SHOU et al (1976) was used. Lymphocytes were obtained from the two subjects to be studied and purified in the usual manner, to a final concentration in Medium 199 and 12.5% human ABS. Those from the "stimulator" were prepared 48 hours ahead of those from the "responder". In control experiments both groups of cells were from normal, healthy individuals. In all other experiments the "stimulator" cells (i.e. those in which a population of Suppressor Cells is induced) were from ovarian cancer patients.

(i) Con.A treatment of lymphocytes. Lymphocytes in a concentration of $3x10^6$ /ml. were cultured in the presence of Con A (20µg /10^6 cells) at 37°C in a humidified incubator for 48 hours. At the end of this time they were washed three times in Medium 199 and resuspended at a final concentration of $1x10^7$ /ml. At the same time, an equal volume of control cells, not treated with Con A, were incubated in the same manner in the presence of N Saline.

(ii) Mitomycin C treatment of Stimulating Cells. Both groups of stimulating cells were then incubated at 37°C for 30 mins. in the presence of Mit. C (50µg /10^6 cells). Both populations were then washed three times in Medium 199 and made up to a final concentration of $1x10^6$ /ml.

(iii) Responding Cells. These cells were adjusted to a final concentration of $1x10^6$ /ml.

(iv) One-way Mixed Lymphocyte Culture. An 0.5ml. aliquot of stimulating Mitomycin C-treated cells was mixed with an equal volume of responding cells to give a final concentration of $1x10^6$ /ml. These cells were then cultured as for the Blastogenic Assay and in some experiments the effect of the stimulating cells on antigen or mitogen-induced blastogenesis in the responding cells was assessed. The antigens in these cases were CME (ovarian tumour) and PPD; the mitogen was Con A. The % of blastogenic stimulation in the responding cells which was inhibited by the Con A induced-suppressor cells in the "stimulating" cell population, was calculated as follows:

$$\% \text{ Inhibition} = 1 - \{(C_m^s - C^s) / (C_m - C)\} \times 100$$

where C_m^s = cpm of responding cells in the presence of antigen plus Con A, Mit. C treated stimulating cells (i.e. suppressor cells)

C^s = cpm of responding cells plus suppressor cells

C_m = cpm of responding cells in the presence of antigen plus Mit. C treated stimulating cells

C = cpm of responding cells plus Mit. C treated stimulating cells.

(v) Mit. C Control cells. To show that the Mit. C treatment of cells still inhibited the DNA incorporation of ^{125}IUDR in the presence of Con.A, control cultures were set up of the stimulating cells with and without Con A as a mitogenic agent. There was little further incorporation of ^{125}IUDR into the cells.

Table 2.10 gives the results of a Suppressor Cell Assay between a normal control and a cancer patient.

TABLE 2.1 BLASTOGENIC RESPONSES TO 10µg PPD USING DIFFERENT SOURCES OF FCS IN THE CULTURE MEDIUM: $1x10^6$ LYMPHOCYTES / ml.

		Patient 1			Patient 2		
FCS Sources		O	L17	U6	K97	L17	L27
cpm	Nil	10794	8870	7158	7866	7784	6806
	PPD	51920	39448	32186	36596	33180	47596

TABLE 2.2 INCORPORATION OF ^{125}IUDR AFTER 6 HOURS AS PRODUCT OF CONCENTRATION USED: $5x10^5$ CELLS.

Conc^n. µCi. ^{125}IUDR	0.25	0.5	1	2	4	6
cpm	434	826	1048	1952	4248	5158

TABLE 2.3 INCORPORATION OF ^{125}IUDR INTO STIMULATED (PPD) AND UNSTIMULATED LYMPHOCYTES AS PRODUCT OF DURATION OF INCUBATION: $1x10^6$ CELLS /ml.

Time (hours)		2	4	6	8	24
cpm	Nil	789	1299	1164	1308	1317
	PPD	17331	38973	47742	61473	39462

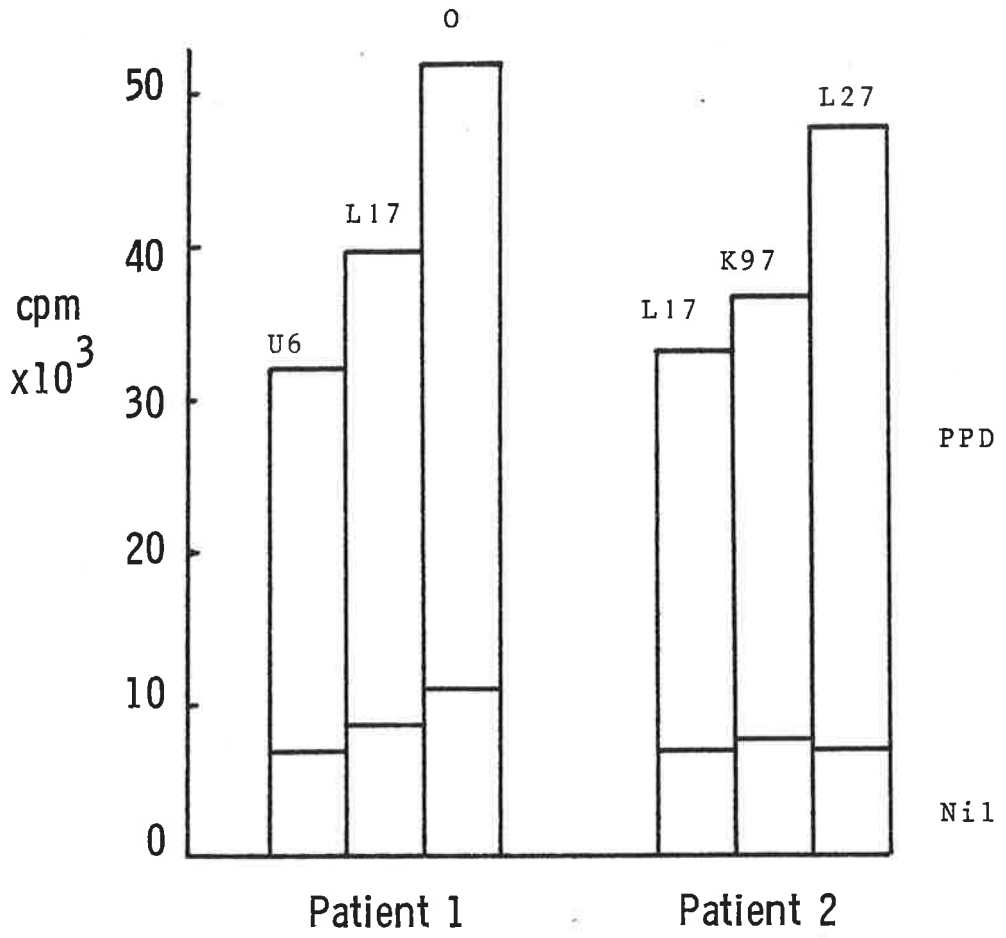


FIGURE 2.1 BLASTOGENIC RESPONSES TO 10µg PPD USING DIFFERENT SOURCES OF FCS IN THE CULTURE MEDIUM

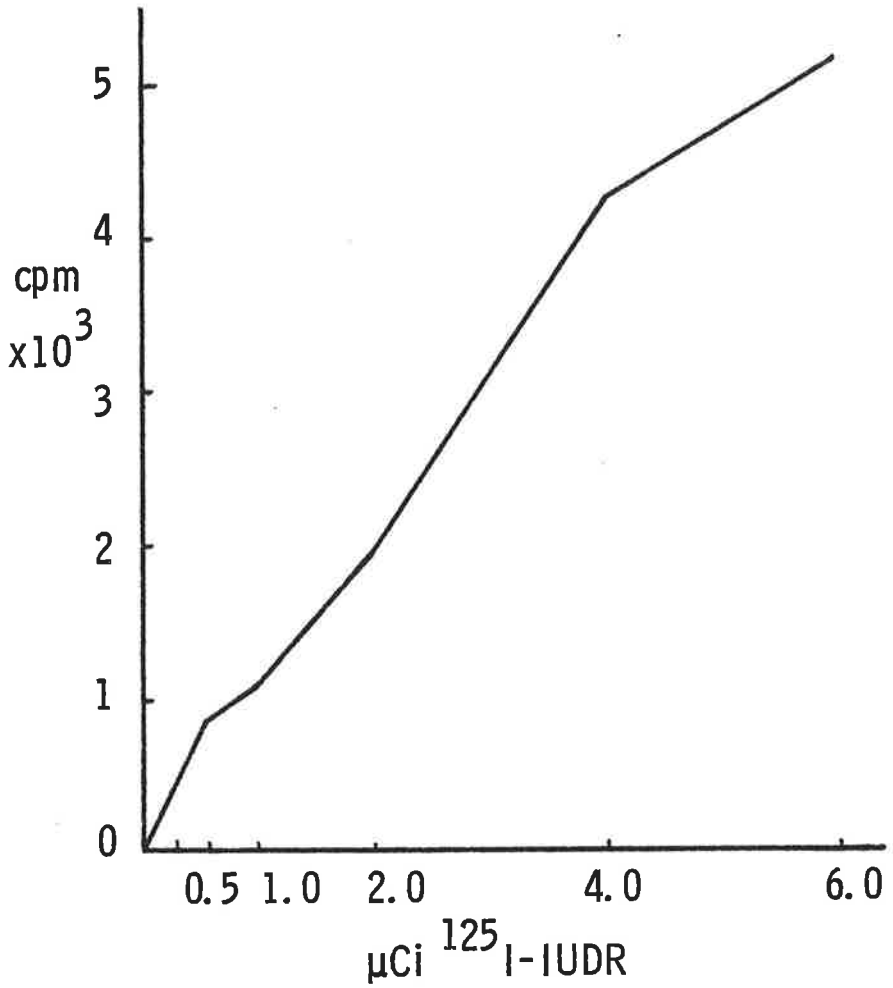


FIGURE 2.2 INCORPORATION OF ^{125}I-IUDR AS PRODUCT OF CONCENTRATION USED

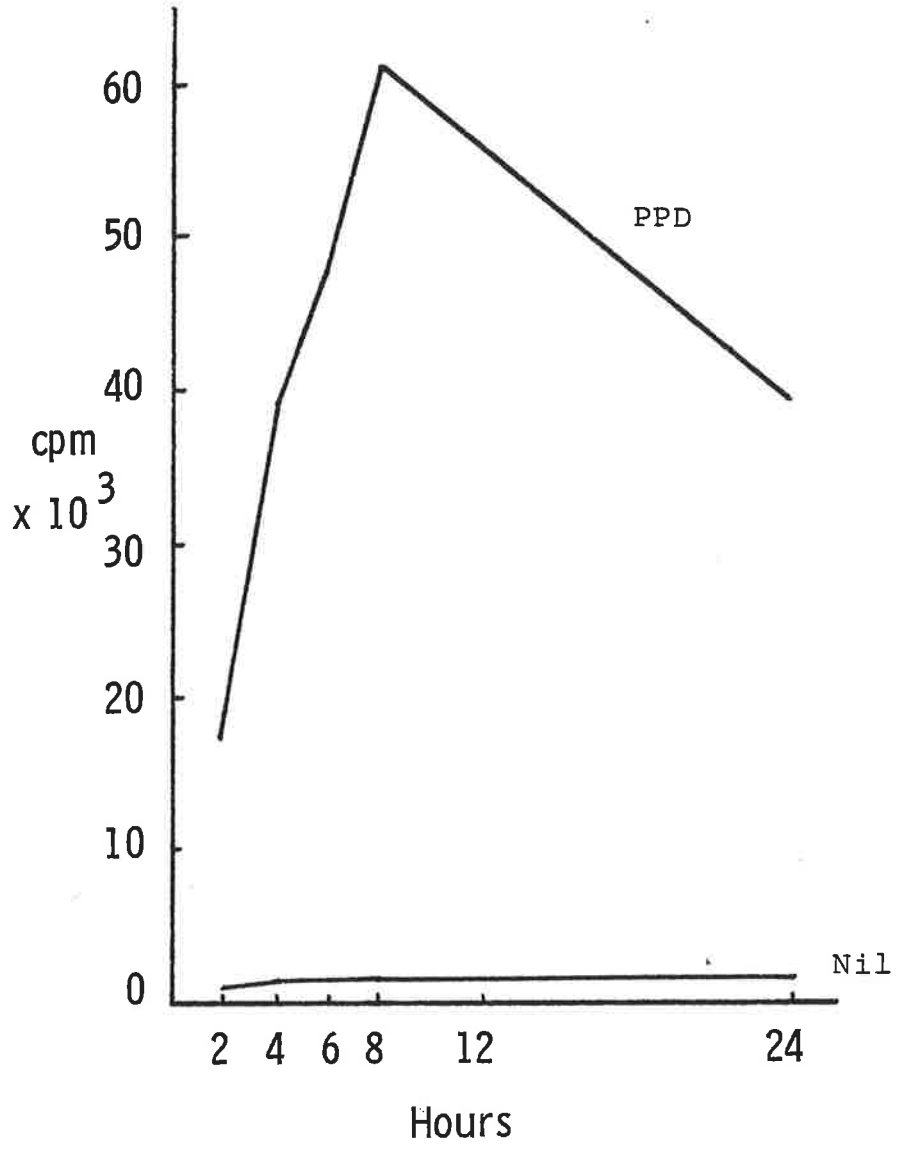


FIGURE 2.3 INCORPORATION OF ^{125}I-IUDR AS PRODUCT OF DURATION OF INCUBATION

TABLE 2.4 DOSE RESPONSE CURVE FOR OVARIAN TUMOUR CELL MEMBRANE EXTRACT: $1x10^6$ CELLS /ml.

Conc^n. µg/ml. CME	Nil	10	20	50	100	200
cpm	21734	17004	24972	24519	26202	17754

TABLE 2.5 DOSE RESPONSE CURVES FOR CON A AND PPD: $1x10^6$ CELLS /ml. & $5x10^5$ /ml.

Conc^n. µg/ml. PPD	Nil	2.5	5	10	20	40
cpm	1164	32991	40455	47742	58317	69990
Conc^n. µg/ml. CON A		1	10	20	40	
cpm		10581	46971	56343	51186	

TABLE 2.6 BLASTOGENIC RESPONSES TO 20µg CON A AS PRODUCT OF LENGTH OF INCUBATION: $1x10^6$ CELLS.

Day	1	2	3	4	5	6
cpm (Pt. A)	948	44763	46173	56043	25275	
cpm (Pt. B)	837	29352	50922	51693	39411	26190

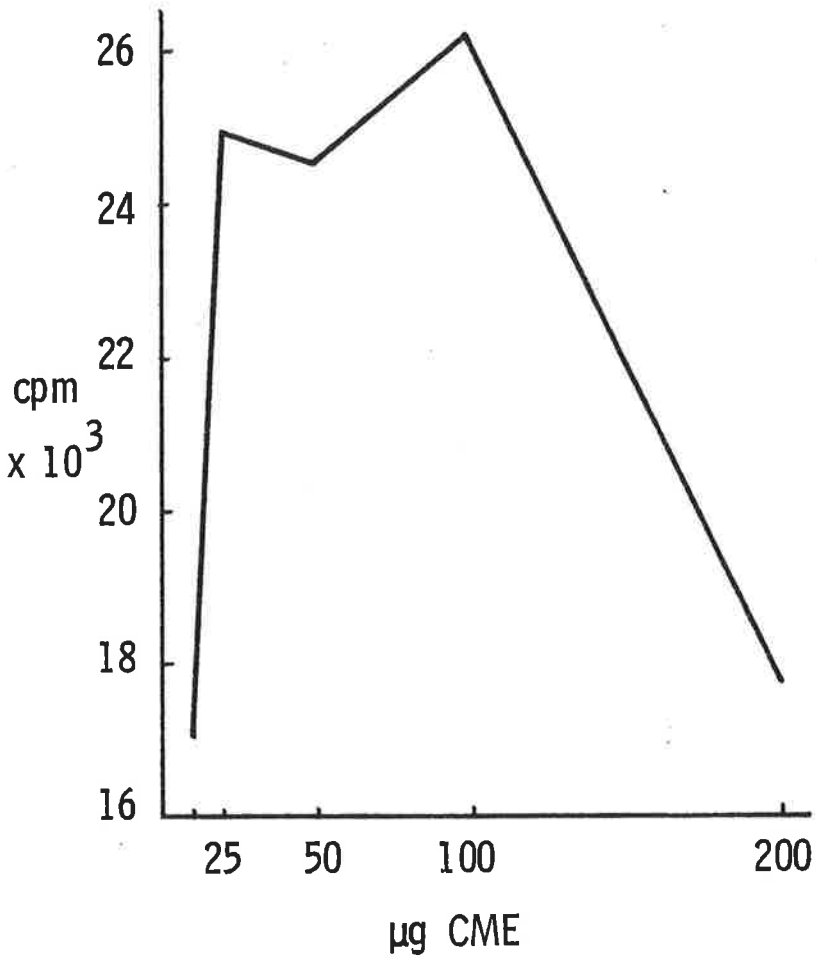


FIGURE 2.4 DOSE RESPONSE CURVE FOR OVARIAN TUMOUR CME

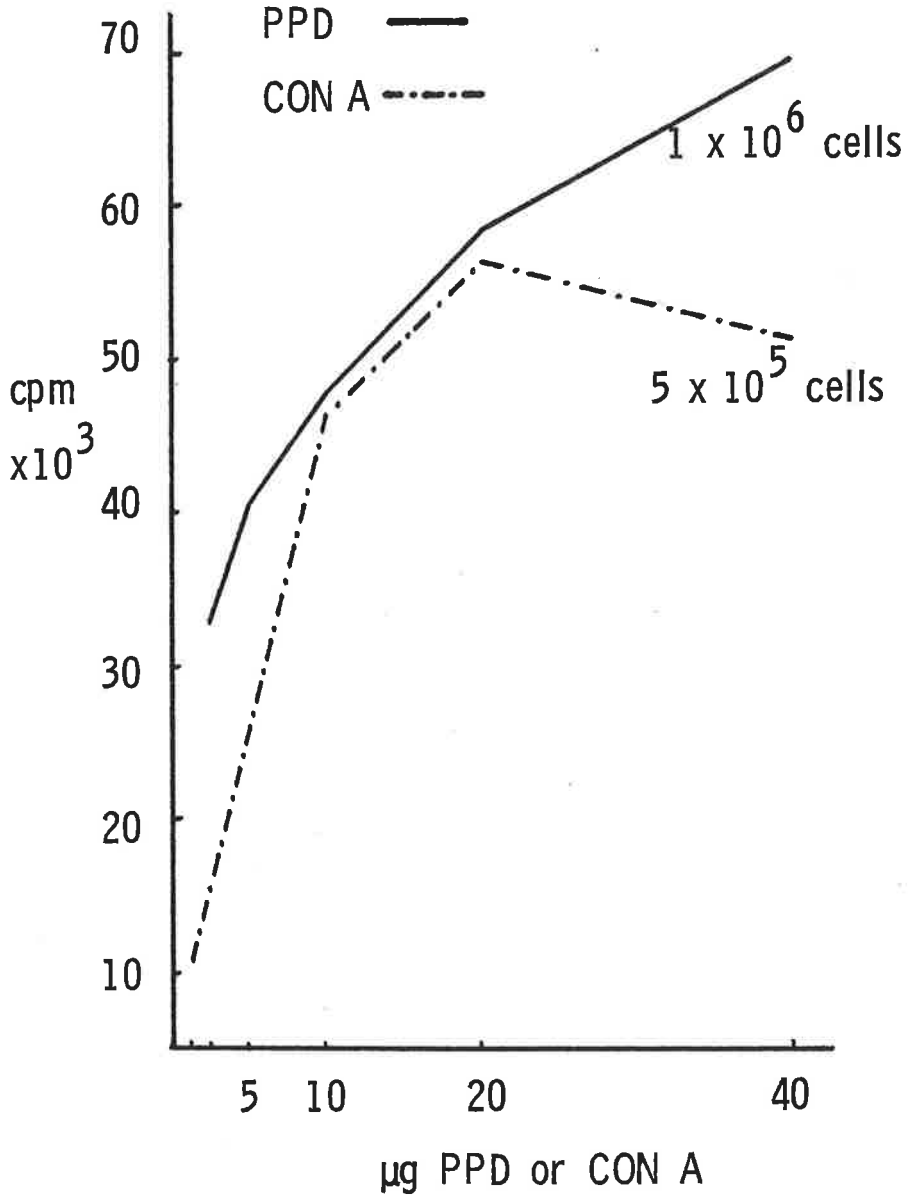


FIGURE 2.5 DOSE RESPONSE CURVE FOR CON A AND PPD

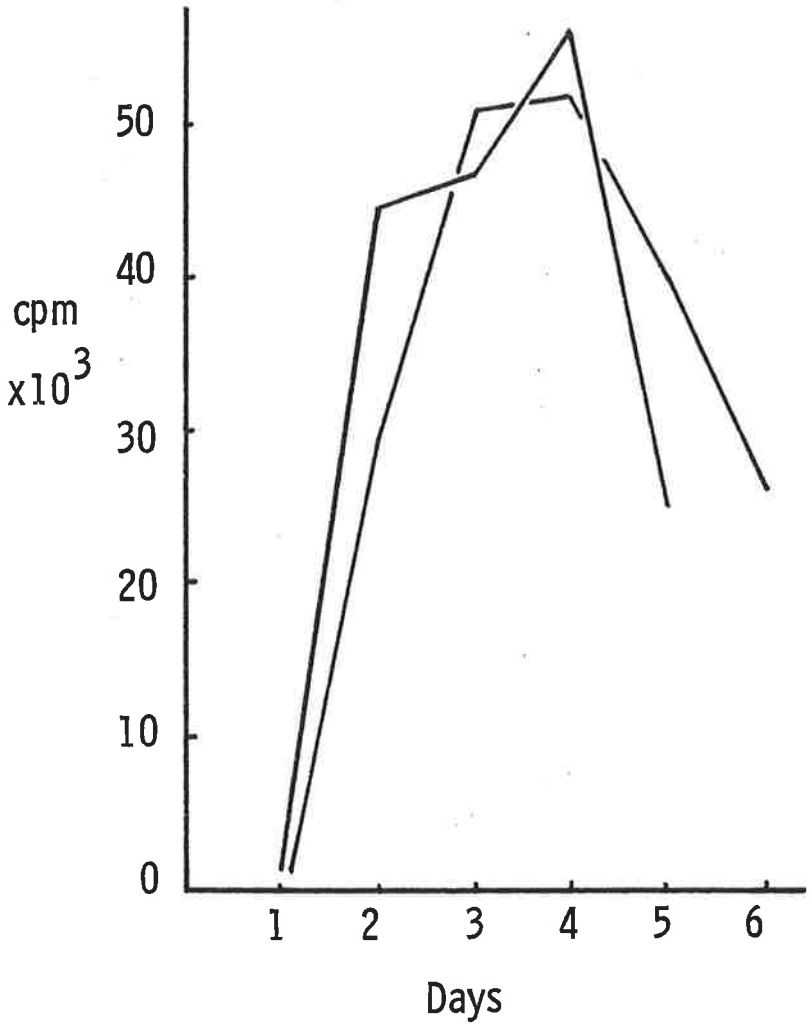


FIGURE 2.6 BLASTOGENIC RESPONSES TO 20µg CON A AS PRODUCT OF LENGTH OF INCUBATION

TABLE 2.7 BLASTOGENIC RESPONSES TO 10µg PPD AS PRODUCT OF LENGTH OF INCUBATION: $1x10^6$ CELLS /ml.

Day	1	2	3	4	5	6	7	8
cpm	390	1101	3324	9306	15306	16275	19527	18558

TABLE 2.8 %E-RFC AS PRODUCT OF TEMPERATURE OF INCUBATION.

Temperature ^{o}C	4^{o}	21^{o}	30^{o}	37^{o}
%E-RFC	57	58	48	64

TABLE 2.9 %E-RFC AS PRODUCT OF LENGTH OF INCUBATION AND INCLUSION OF FCS IN CULTURE MEDIUM.

Time (hours)	0	1	4	24	48
PBS	16	23	40	17	5
FCS	36	49	57	51	46

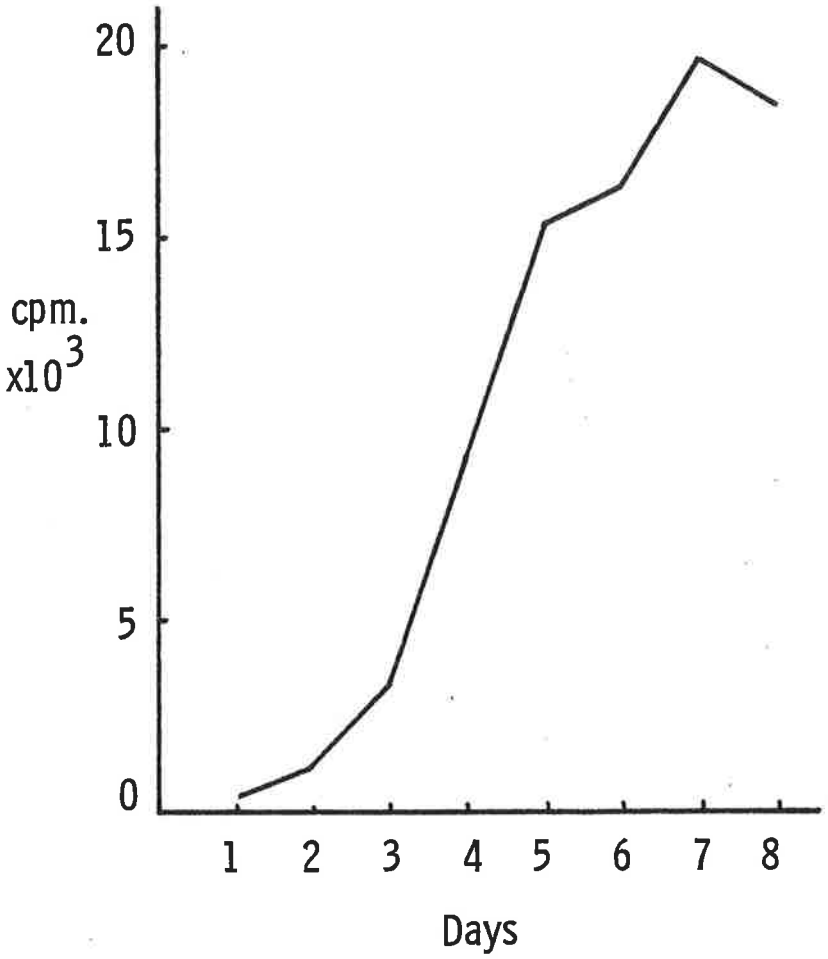


FIGURE 2.7 BLASTOGENIC RESPONSES TO 10µg PPD AS PRODUCT OF LENGTH OF INCUBATION

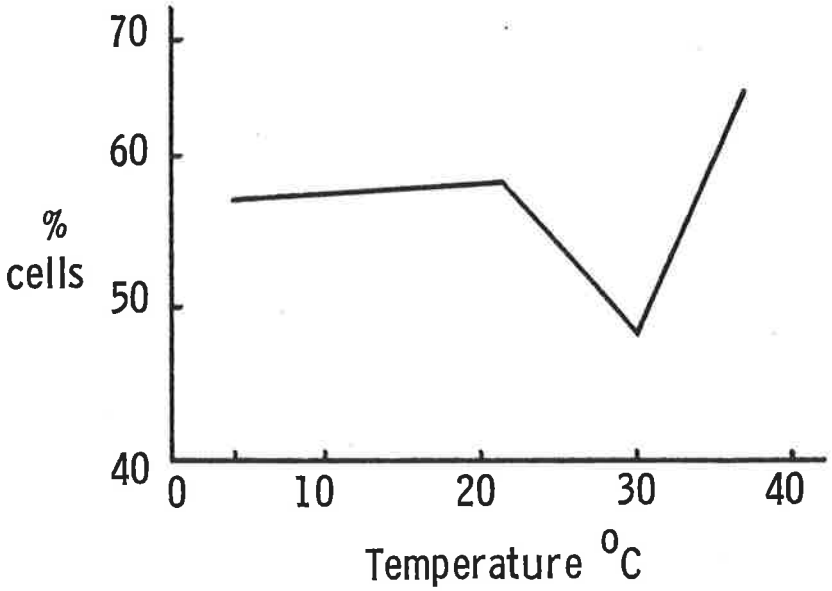


FIGURE 2.8 %E-RFC AS PRODUCT OF TEMPERATURE OF INCUBATION

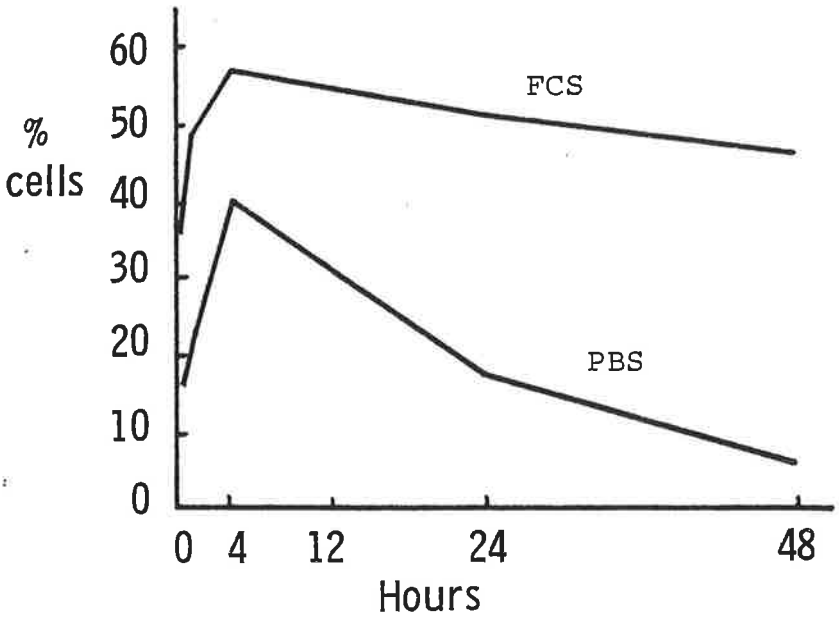


FIGURE 2.9 %E-RFC AS PRODUCT OF FCS IN CULTURE MEDIUM

TABLE 2.10 RESULTS OF A SUPPRESSOR CELL ASSAY BETWEEN A NORMAL CONTROL (RESPONDING CELLS) AND A PATIENT WITH OVARIAN CANCER (STIMULATING CELLS IN WHICH A POPULATION OF SUPPRESSOR CELLS IS INDUCED WITH CON A TREATMENT)

BLASTOGENIC RESPONSES OF CONTROL RESPONDER CELLS BY THEMSELVES: $5x10^5$ CELLS /ml.

	cpm.	cpm diff.
Nil	1827	
PPD	39471	37644
CME	2304	477
Con A	106044	104217

BLASTOGENIC RESPONSES OF CONTROL RESPONDER CELLS WITH MIT.C-TREATED STIMULATING CELLS FROM CANCER PATIENT: $5x10^5$ CELLS OF EACH GROUP.

	cpm	cpm diff.
Nil	12366	
PPD	51756	39390
CME	18186	5820
Con A	127287	114921

BLASTOGENIC RESPONSES OF CONTROL RESPONDER CELLS WITH CON A, MIT.C-TREATED CELLS (i.e. SUPPRESSOR CELLS) FROM CANCER PATIENT: $5x10^5$ CELLS OF EACH GROUP.

	cpm	cpm diff.
Nil	756	
PPD	7794	7038
CME	3786	3030
Con A	53094	52338

% Blastogenic "suppression" for (e.g.) PPD responses:

$= \{1 - (7038/39390)\} \times 100\% = 82\%$

MITOMYCIN C- TREATED CELLS:

Nil values: Mit.C only 366 cpm

Mit.C, Con A 426 cpm

2.8 DISAGGREGATION AND CRYOPRESERVATION OF TUMOUR CELLS FOR IMMUNOTHERAPY.

Fresh and sterile ovarian neoplasms were brought from the operating theatre and prepared in a sterile tissue-culture room. They were minced manually with a pair of scissors, in Medium 199 containing Penicillin and Streptomycin. 20G portions of minced tissue were then placed in large glass centrifuge tubes and gently homogenised at slow speed for 15 seconds, using an Ultra-Turrax homogeniser with a TP 18/2 shaft. This tissue was then passed through a sterile muslin filter to give a predominantly single-cell suspension with occasional clumps of 3-5 tumour cells. This filtrate was centrifuged at 200g for 10 mins., the supernatant discarded and the pellet resuspended in a small amount of Medium 199. A 50µl. aliquot was removed for a cell count in Trypan Blue.

Mucinous tumours were not used as the mucin led to cell trapping. Enzymatic disaggregation was not used since antigenic changes may occur (STUHLMILLER & SIEGLER, 1977). A viability of 30-50% in the cell count was considered good and suspensions with a viability less than 20% were not used for immunotherapy.

The suspension was adjusted to a concentration of $1x10^7$ viable cells /ml. with the final volume containing 5% Dimethylsulphoxide. This cryopreservative agent was added slowly to the cell mixture, and 2ml. aliquots were then put into plastic screw-top vials. The procedure was done as quickly as possible and the tumour cells kept at 4^oC in the event of any delay. Serum was not used with the cells.

The ampoules were placed in a Planar Liquid Nitrogen Freezer at room temperature and frozen at -1^oC per minute to -30^oC when the temperature was held for 10 mins. (POWLES et al, 1973). The cells were then rapidly frozen to -60^oC and stored in a liquid nitrogen container at -196^oC. Of each batch prepared, one ampoule was sent to Microbiology for culture and if positive the cells were discarded.

10mls. of serum from the donor patient were sent to Virology for Australian Antigen testing.

2.9 IMMUNOTHERAPY.

(i) Tumour cells. One ampoule per patient was rapidly thawed in a 37°C water-bath, then diluted slowly over 3 mins. with 10mls. Medium 199 in a Universal container. The cells were then washed twice by spinning at 200g for 10 mins., and the final volume for resuspension was 0.4 ml per ampoule. Previous experiments (LEVIN, 1976) have shown these to be the optimal conditions for cell preservation. Viability was maintained as assessed by Trypan Blue counting and the ability of the cells to be cultured in vitro (see Fig. 2.10).
Prior to use for immunotherapy the cells were irradiated with 10,000 rads over 5 mins. , using a 4MeV Linear Accelerator in the Department of Radiotherapy. Cells could not be cultured in vitro following this procedure.

(ii) BCG. Glaxo strain lyophilized BCG was used. One ampoule was resuspended in 1 ml. N saline and then diluted to 40 mls. For each patient 0.1 ml. BCG was then added to 0.4 ml. tumour cells.

(iii) Vaccination. The BCG and tumour cells in mixture were injected intradermally into 4 adjacent sites over the deltoid region or the upper thigh. The arms or legs were used alternately for monthly innoculations. Chemotherapy was given 2 weeks after the immunotherapy, and innoculations were continued for 2 years where possible.

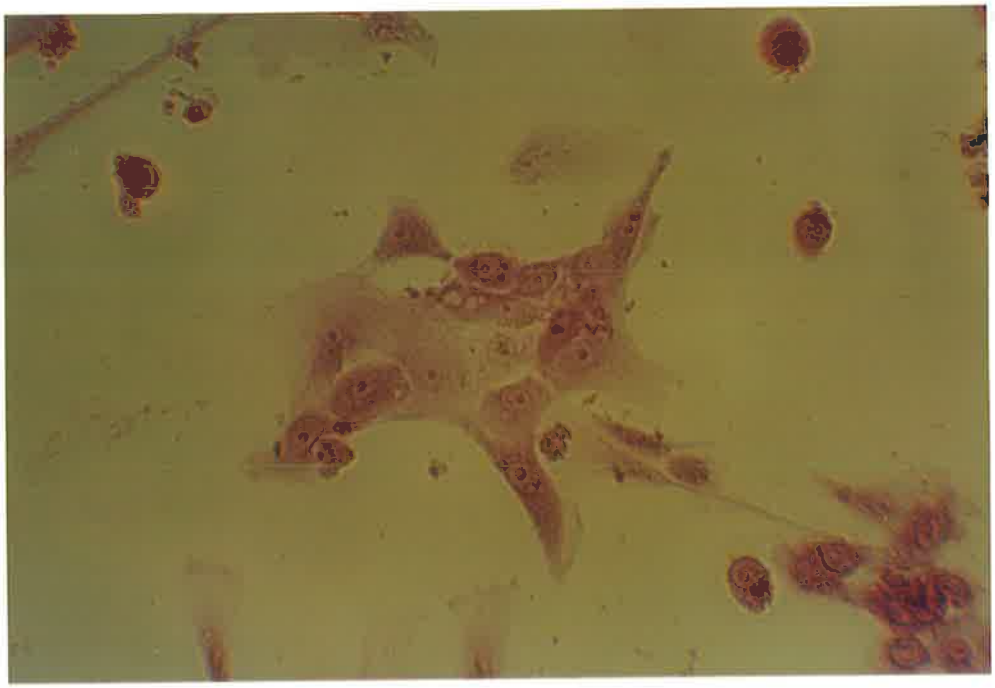


FIG. 2.10 IN VITRO TISSUE CULTURE OF TUMOUR CELLS PREVIOUSLY CRYOPRESERVED.

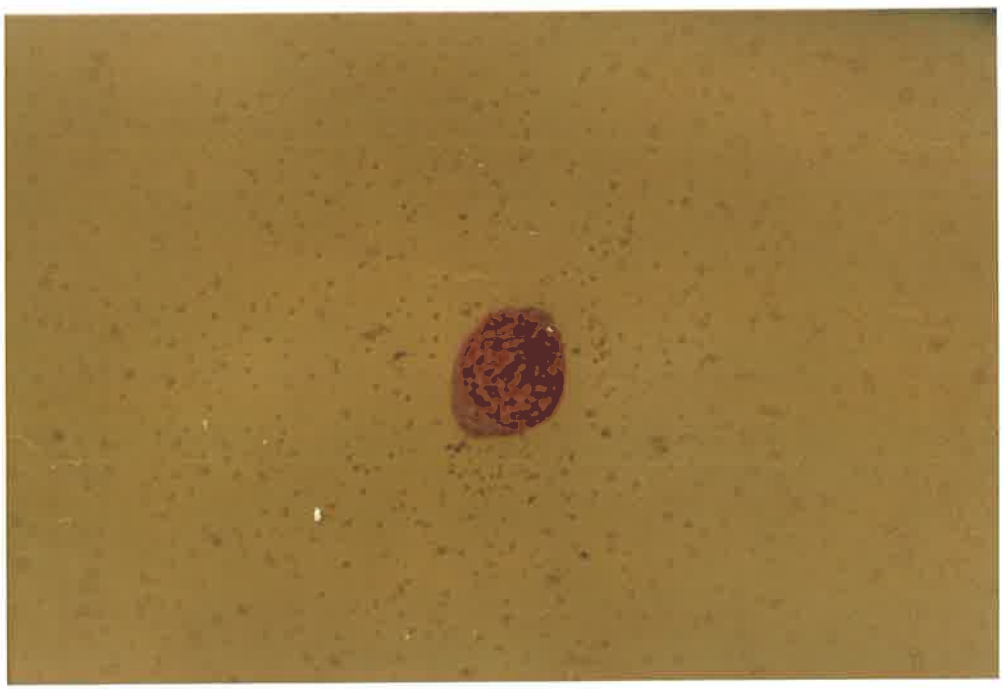


FIG. 2.11 TRANSFORMED LYMPHOCYTE FROM CULTURE STIMULATED WITH OVARIAN TUMOUR CME.

CHAPTER 3. TUMOUR-ASSOCIATED ANTIGENS IN EXPERIMENTAL ANIMAL AND HUMAN TUMOURS.

Analysis of animal tumour antigens indicated that tumours induced by carcinogens or radiation had tumour antigens specific for each individual tumour, even within the same animal (LAW 1970), whereas tumours induced by oncogenic viruses had antigens specific for that virus (KLEIN 1966). Antigenic strength is variable, and indeed some tumours have no demonstrable antigens (BALDWIN 1973). Likewise, in tissue culture, it appears that cells may lose their antigenicity with increasing passages (MOORE et al, 1973). Isolation and characterization is difficult (ANDERSON et al, 1974), but most are thought to be macromolecules on the cell surface which closely resemble their normal HL-A (or H-2) counterparts i.e. glycoproteins with a MW of about 50,000 daltons (BOWEN & BALDWIN 1975). Recent studies in breast cancer have however suggested that the antigen may be altered F(ab) fragments of the pazient's own immunoglobulins and that "tumour-specific" antibody detected in patient serum is anti-F(ab) or auto-antibody (HUMPHREY et al, 1977).

In vitro studies in the human rely on the demonstration of specific immune responses to such putative antigens i.e. sensitized lymphocytes and/or immune serum. In antibody studies the source of the antigen is often a water-soluble or 3M KCl extract from the tumour (ROTH et al, 1976b) and the serum is often xenogeneic, raised in rabbits. Interpretation of results may therefore be ambiguous since immunization can occur against CEA, normal tissue antigens in excess in the tumour, HL-A antigens or even blood group antigens, and proper control studies must therefore be done. Patient serum may be used as the source of antigen (as with CEA and AFP - see Chapter 8), and hypothetically the kidneys and plasmapheresis products can be used as a source of immune complexes from which the antigen can be purified. It may also be obtained from tumour cells grown in culture (BYSTRYN 1976; STUHLMILLER & SEIGLER, 1977). All such attempts at

purification are important because they aim to provide a source of antigen for immunodiagnostic tests.

3.1 THE ONCO-FETAL RELATIONSHIP.

In both animal and human tumours, tumour-associated antigens appear to be similar to antigens expressed on normal cells during various phases of fetal development. STONEHILL & BENDICH (1970) have proposed that "retrogenetic expression" of normally repressed genes occurs during oncogenesis. By showing that tumour cells react with serum or lymphoid cells from multiparous female donors, or anti-fetus antiserum, it is argued that the donors are sensitized to fetal antigens during embryogenesis (BALDWIN et al, 1974). In animals, immunotherapy with embryonic antigens (irradiated fetal cells) will protect against the development of virally-induced tumours (TING et al, 1972; COGGIN et al, 1971). It has also been shown that the RNA from fetal liver cells and rat hepatoma cells is similar, but both are different from that in adult rat liver cells (GONANO et al, 1973).

In humans, AFP and CEA are the two most characterised onco-fetal antigens. However, there is evidence that other tumours bear such antigens (HARRIS et al, 1971; KLAVINS et al, 1971). Amniotic fluid as well as fetal tissues themselves have been used to raise specific anti-sera to tumour extracts (BERG et al, 1976). Cancer patients display cellular immune responses against fetal cells (FOSSATI et al, 1973) and TROUILLAS (1971) has shown that glioblastoma and astrocytoma cells contain macromolecules also found in fetal brain. GOLD (1967) early on showed that anti-CEA antibodies were present in the serum of pregnant and post-partum women. It has also been shown that patients with cancer and pregnant women contain antibodies to human and murine fetal liver cells, again suggesting that the fetal antigen causing sensitization during pregnancy is again re-expressed during neoplasia (SALINAS et al, 1978).

Two areas of research have opened as a result of these experiments. The first is the use of patient serum as a source of antigen for immunodiagnostic RIA's, now routinely used for CEA and AFP, although there are difficulties in interpretation (ALEXANDER 1972; LAURENCE & NEVILLE 1972). The second is more hypothetical and relates to the epidemiological evidence that pregnancy may have a protective effect in some neoplasms. Sex differences in cancer are well known (SILVERBERG & HOLLEB 1970); there is an excess of colonic tumours in nuns (FRAUMENI et al, 1969) and parous women have better survival rates that non-parous women in malignant melanoma (HERSEY et al, 1977); and less have ovarian cancer (BERAL et al, 1978). Since the incidence, at least of malignant melanoma, is the same for men as for women, immunization against fetal antigens does not appear to prevent the development of tumours, but may play some role in controlling their dissemination. It has been postulated that immunization to fetal antigens permits accelerated immune responses on re-exposure to onco-fetal antigens on malignant cells.

Finally, it must be stressed that there is still some doubt as to whether such antigens are related to organ and tissue-specific antigens in the body (MARTIN & MARTIN 1975). CASPARY & FIELD (1971) have suggested that there is a ubiquitous antigen common to all tumours (Cancer Basic Protein), which appears rapidly on the surface of both adult normal and fetal cells when they are cultured under abnormal conditions, and cross-reacts with a brain tissue antigen (encephalitogenic factor).

3.2 THE STUDY OF TUMOUR-ASSOCIATED IMMUNITY IN HUMANS.

There are many ethical constraints to in vivo experimentation in humans, and since syngeneic systems can not be used as in animals, in vitro systems aim to demonstrate cross-reactivity between autologous and allogeneic tumours in the responses which they elicit.

(i) In vivo studies. The use of the DHR to intradermal

injections of autologous tumour cells or extract is a simple means of assessing both the host's sensitization to the antigenic stimulus and ability to respond (HERBERMAN 1974). However such tests are difficult to quantitate and there are theoretical hazards to the inoculation of neoplastic tissue. Responses to crude extracts of tumour may also reflect bacterial (STEWART 1969) or viral (HOLLINSHEAD et al, 1973) contamination, and it should be shown that responses are tumour-specific and not tissue-specific.

Responses to tumour extracts have been seen in a wide range of human tumours (HUGHES & LYTTON 1964; BLUMING et al, 1971), and inability to respond can be correlated with advancing disease (FASS et al, 1970). However, reactions to normal and benign tumour tissues have also been shown (BLUMING et al, 1972; ALFORD et al, 1973).

There is a close correlation between in vivo DHR's and blastogenic responses of lymphocytes from the same patient to his tumour extract (MAVLIGIT et al, 1973). Responses to allogeneic extracts have been noted but reactions to autologous extracts are usually stronger and occur more frequently (ALFORD et al, 1973; WELLS et al, 1973). Demonstrations to fetal tissue extracts have also been reported suggesting an onco-fetal element to the antigen (WELLS et al, 1973; HOLLINSHEAD et al, 1970).

In ovarian cancer CHATTERJEE et al (1975) found no DHR responses in any of 11 patients tested with autologous tumour extract. On the other hand, LEVIN et al (1976 b) found positive responses in 4/6 patients in remission and 0/7 patients in relapse.

(ii) In vitro responses.

(A) Evidence for humoral responses to tumour antigens. The presence in cancer serum of antibodies which will bind to tumour cells has been demonstrated with a number of techniques including immunofluorescence, complement-mediated cytotoxicity and complement fixation (LEWIS et al, 1969; SETHI & HIRSHAUT 1976).

GOLD (1967) showed that 70% of patients with gastro-

intestinal cancer that was not metastatic had antibody to purified CEA. Since then, other tumours have been shown to react with cancer serum (see Table 3.1). ALEXANDER & HALL (1970) have postulated that anti-tumour antibody controls blood-borne metastases,by showing that fibrosarcomas in rats do not give rise to metastases unless antibody is removed. This may be analogous to the situation in malignant melanoma (LEWIS et al, 1969) and osteosarcoma (EILBER & MORTON 1970 b) where antibody disappears with metastatic disease.

Anti-tumour antibody has been found in the serum of relatives of patients with soft-tissue sarcomas (EILBER & MORTON 1970 b) suggesting a viral aetiology in these tumours. Its presence in normal serum may reflect occult or pre-malignant conditions (SPARKS et al, 1976) or infection with a virus which produces antibody in most people but malignancy in only a few e.g. the Epstein-Barr virus (KLEIN 1975). However, in cancer serum, ARPELS & SOUTHAM (1969) have found antibody related to uraemia, the giving of cytotoxic drugs and prolonged storage at -20^{o}C. To further complicate matters, IRIE et al (1974) have shown that normal and cancer serum contains natural antibody to FCS-derived antigens on tumour cells in tissue culture (see Chapter 5).

Hence caution must be used when invoking specific anti-tumour effects to antibodies that can be reacted in vitro against tumour cells.In so far as they may be involved in tumour immunity, such antibodies may not act directly on tumour cells but regulate the expression of cell-mediated responses to the tumour (CANTRELL & KALISS 1974).

(B) Evidence for cell-mediated responses to tumour antigens. Three test systems are generally used (see Table 3.2):

(1) Leucocyte Migration Inhibition Assay. The addition of tumour antigen to the leucocytes of a sensitized person inhibits their migration from capillary tubes, by the production of inhibitory lymphokines. There is good correlation between this in vitro test and the DHR in vivo (ROSENBERG & DAVID 1970), and it has been used successfully in patients with various tumours (COCHRAN et al, 1974; DEAN et al, 1977; HALLIDAY et al, 1975; KJAER & BENDIXEN 1976).

TABLE 3.1 REPRESENTATIVE STUDIES ON ANTI-TUMOUR ANTIBODY IN HUMAN TUMOURS.

TUMOUR	REFERENCE	METHODOLOGY	NO. +	COMMENTS
Leukaemia	FINK et al, 1965	Im	16/34	Higher levels in relapse; X-reactivity with lymphoma, myeloma, Hodgkin's Disease and serum from neonate of mother with AML
Colonic Ca.	GOLD, 1967	Hem.	30/43	In non-metastatic Ca. and pregnancy serum
Neuroblastoma	HELLSTRÖM et al, 1968 b	CMC & CI	4/7	Serum from some mothers also positive
Melanoma	LEWIS et al, 1969	CMC & Im	24/68 14/36	Allogeneic reactions, negative in metastatic disease; ?2 antibodies present
Melanoma	MORTON et al, 1970 a	CF & Im	42/63	18% controls positive; low results in metastatic disease
Squamous Ca skin	NAIRN et al, 1971 a	Im & CMC	5/9 3/7	No allogeneic reactivity
Ovary	DORSETT et al, 1975	Im	7/7	Extracted from immune complexes from effusion and specific reactivity with tumour cells
Cervix	CHIANG et al, 1976	Im	176 tests	Titres also found in some husbands ? viral aetiology
Astrocytoma	PHILLIPS et al, 1976	CMC	14/21	19% controls also positive
Sarcoma	SETHI & HIRSHAUT, 1976	CF	16/19	Titres increased post-op; titres in 40% family members ans also other cancers
Breast	SPARKS et al, 1976	CF	22/56	Titres fell with recurrence; also in 47% patients with sarcomas and 13% controls

ABBREVIATIONS: Im = Immunofluorescence CI = Colony Inhibition CF = Complement Fixation
CMC = Complement mediated cytotoxicity Hem = Hemagglutination

TABLE 3.2 REPRESENTATIVE STUDIES ON CELL-MEDIATED RESPONSES TO HUMAN TUMOUR ANTIGEN(S).

TUMOUR	REFERENCE	METHODOLOGY	NO. +	COMMENTS
Various	HELLSTRÖM et al, 1968 a	CI	27/32	Autologous and allogeneic inhibition specific for tumour histology; normal lymphocytes -.
Neuroblastoma	HELLSTRÖM et al, 1968 b	CI	13/20	2 mothers had lymphocytes cytotoxic for their children's and other children's tumour cells; cancer lymphocytes had no effect on fibroblasts.
Leukaemia	FRIDMAN & KOURILSKY, 1969	MCR with blasts	6/9	Autologous system, therefore reactivity not due to HL-A differences.
Bladder	BUBENIK et al, 1970	LMC	8/8	Autologous and allogeneic cytotoxicity, specific for bladder; normal lymphocytes -.
Hypernephroma	STJERNSWÄRD et al, 1970	MCR with tumour cells	3/6	Autologous and allogeneic reactivity; no response to normal renal cells.
Melanoma	JEHN et al, 1970	LB to cyst fluid	7/7	Allogeneic responses weaker than autologous. Normal lymphocytes -.
Squamous Ca skin	NAIRN et al, 1971 a	LMC	6/8	Cancer lymphocytes had no effect on normal epidermoid cells or fibroblasts; time-lapse cinephotomicrography showed lymphocytes attacking tumour cells within 24 hours.
Colonic Ca.	NAIRN et al, 1971 b	LMC	8/24	Allogeneic responses weaker than autologous. Peripheral but not regional node lymphocytes were cytotoxic.
Sarcoma	VANKY et al, 1971	MCR with tumour cells	13/26	Autologous system using cryopreserved and fresh tumour cells. No response to benign fibroma cells.
Melanoma	CURRIE et al, 1971	LMC	3/22	No reaction to allogeneic melanoma or other tumour cells. Normal lymphocytes -.
Colonic Ca	LEJTENYI et al, 1971	LB to CEA	0/21	Responses to CEA not significantly diff. from those in controls and pregnancy.
Leukaemia	GUTTERMAN et al, 1972	LB	25/35	Autologous system. Correlation between strength of response and prognosis.
Various	MAVLIGIT et al, 1973	LB	10/24	5 of these patients also had + DHR; 8% controls also responded.
Sarcoma	COHEN et al, 1973 a	LMC	14/18	Autologous and allogeneic responses. No effect of lymphocytes on autol. fibroblasts.
Breast	COCHRAN et al, 1974	LMI	74/138	Autologous and allogeneic responses, fewer in metastatic disease; 15% controls +.
Melanoma	HALLIDAY et al, 1975	LAI	22/24	Responses persisted in 6/8 patients treated successfully. Normal lymphocytes -.
Cervix	CHIANG et al, 1976	LMI	28/28	+ responses also in 5/5 patients with Ca-in-situ; other tumours and controls -.
Endometrial	CHEN et al, 1976	LMI	8/11	+ responses also in 2/5 patients with endometrial hyperplasia; other gynae. tumours -.
Breast	McCOY et al, 1976	LMI	56%	Multiple antigens postulated; correlation with Stage. 11% controls reacted.
Renal	KJAER & BENDIXEN, 1976	LMI	50%	Allogeneic and autologous reactivity only in localized disease; responses also to fetal renal cells but not normal adult kidney.
Breast	DEAN et al, 1977	LMI	12/34	Allogeneic and autologous responses specific for breast; no effect with normal tissue.
Colonic Ca	JUBERT et al, 1977	MCR with tumour cells	9/35	Peripheral and mesenteric node lymphocytes responded; correlation with Stage.
Leukaemia	COCKS et al, 1977	MCR with	6/15	Responses to blasts as well as remission lymphocytes.

ABBREVIATIONS: LB = Lymphocyte blastogenesis
MCR = Mixed cell reaction
CI = Colony inhibition
LMC = Lymphocyte mediated cytotoxicity
LMI = Leucocyte migration inhibition
LAI = Leucocyte adherence inhibition.

In an extensive analysis of women with breast cancer, McCOY et al (1976) found significant inhibition of leucocytes to tumour extracts in 56% of the women with cancer compared with 11% of the controls. However, he pointed out a number of difficulties with the assay. There were several instances where a patient's leucocytes were inhibited by one tumour extract and not another which had been shown to be active in the assay; this suggested that multiple common breast antigens were involved in the reactivity. There was a correlation with the Stage of the disease, the greater reactivity coming from patients with localized disease.

(2) Lymphocyte blastogenesis. Cultured blood lymphocytes undergo morphologic transformation to lymphoblasts in the presence of non-specific mitogens such as Con A or PHA (HIRSCHORN et al, 1963) or specific antigens to which they have been previously sensitized, such as PPD (COULSON & CHALMERS, 1967). Transformed lymphocytes may be assessed visually on smears (cells are larger, more sluggish, develop a pronounced nucleolus and increase their cytoplasm prior to mitosis - ELVES et al, 1963), or objectively by the increased uptake of a radiolabelled substance compared with unstimulated control lymphocytes.

The blastogenic assay was first used to assess histocompatibility antigens in a mixed leucocyte culture, where the stimulating lymphocytes were treated with Mitomycin C to prevent DNA replication (BACH & VOYNOW, 1966). Likewise, in assessing sensitization to autologous and allogeneic tumour antigens a variety of sources of antigen have been used, including whole tumour cells (POWLES et al, 1971; GUTTERMAN et al, 1972), membrane extracts (SAVEL 1969) and solubilized extracts (HSU & COOPERBAND 1971).

There is now increasing recognition of the many limitations of this assay. Firstly, it may not correlate with other tests of immune function, perhaps because of difficulties in the purification of the tumour antigen. For example, CEA which has been shown to evoke a DHR in vivo (HOLLINSHEAD et al, 1970) is non-stimulatory to autologous lymphocytes in the assay (LEJTENYI et al, 1971).

Secondly, the specificity of the response is question-

able. McCOY et al (1976) compared the leucocyte migration inhibition assay and the blastogenic assay in women with breast cancer (vide supra). With the blastogenic assay they obtained positive responses in 4/26 breast cancer patients and 12/30 normal controls to allogeneic breast tumour extract. Overall, normal controls responded better to allogeneic extracts than cancer patients, and the authors commented on such unreliable results with the use of allogeneic extracts. It is known that in vitro stimulation may occur to soluble HL-A antigens, bacteria and viruses (VIZA et al, 1968). MAVLIGIT et al (1974 a) have found significant responses to autologous tumour cells in 19/29 patients with a variety of tumours, but no response to normal tissue cells. However, the lymphocytes from normal donors responded vigorously and more often to stimulation with allogeneic tumour cells and 24/27 had significant responses, presumably to HL-A antigens. The authors also noted the clear relationship between the degree of stimulation in cancer patients and the clinical state of their disease. 12/16 patients with localized disease had positive responses compared with 7/13 patients with disseminated disease, in whom the degree of stimulation was markedly lower.

Thirdly, the requirement for nutritional serum adds another variable to the assay. VANKY et al (1975 b) showed that blastogenic responses to autologous tumour extract occurred in 65/197 patients with a variety of tumours, but when incubated in autologous serum lymphocyte responses were inhibited in 31/37 cases. This is discussed further in Chapter 5, along with evidence that the use of FCS leads to stimulation which may make results spurious. Suffice it to say that suspensions of tumour cells themselves may contain immunoglobulin which inhibits blastogenesis (VANKY et al, 1975 a).

Fourthly, the 5-7 day incubation may interfere with the reproducability of the results because of variations in survival of cultured lymphocytes, and also allows for primary sensitization to HL-A antigens to occur. In order to overcome this, ROTH et al (1976a) have designed a rapid assay which assesses the protein synthesis of stimulated lymphocytes by the incorporation of labelled ^{3}H-leucine, and

requires only 20 hours incubation in serum-free medium. 5/15 patients tested had positive responses to 3M KCl tumour extracts, and 50% of allogeneic extracts tested were stimulatory.

Fifthly, the time at which the lymphocytes are tested is vitally important. Both chemotherapy and radiotherapy decrease the blastogenic responses to secondary recall antigens (CHEEMA & HERSH, 1971). COCKS et al (1977) have shown in patients with AML that after chemotherapy there are remarkable day to day fluctuations in the responsiveness of their lymphocytes, for up to as much as 18 weeks after the last dose of chemotherapy. Surgery also depresses lymphocyte reactivity (KUMAR & TAYLOR, 1974; ROTH et al, 1974; SLADE et al, 1975). Furthermore, responses to tumour antigens may very well decrease in patients who remain free from clinical disease for at least 2 years (COCHRAN et al, 1976; O'TOOLE et al, 1972) although HELLSTRÖM et al (1971a) found that 11/12 patients, tumour-free, still had lymphocyte-mediated anti-tumour immunity after 2 years.

Lack of response in lymphocytes thought to be sensitized may be caused by a number of factors: alteration in the antigenicity of the tumour extract during preparation or preservation (DEAN et al, 1977) have found that hypotonic extracts are better than 3M KCl extracts), lack of sufficient antigen to stimulate the cells, suppression of responses by the antigen, or inhibitory factors in the tumour preparation (BONNARD et al, 1976), or a genuine inability of the lymphocytes to respond. The methodology for lymphocyte separation is also important, and it has been shown that heparin may suppress lymphocyte responses (GYTE & WATKINS, 1978). Other cell constituents such as macrophages are also required for blastogenesis to occur, and in cancer impaired processing of antigen by these cells may occur (HERSH & HARRIS 1968).

In spite of the criticisms of the assay, it has been used as the basis for the immunological investigations in this thesis. It is a simple technique, gives reproducable results in our hands, and can be used to look at responses not only to tumour extracts but also to mitogens and secondary recall antigens at the same time. It is also of

use when investigating in vivo alterations in lymphocyte behaviour during chemotherapy, radiotherapy or immunotherapy. Furthermore, the requirement for serum can be used to advantage when assessing "serum factors" which may stimulate or repress blastogenic responses (see Chapters 4 and 5).

(3) Lymphocyte mediated cytotoxicity against tumour cells.

The incubation of lymphocytes from sensitized persons with autologous or allogeneic tumour cells can lead to lysis which can be assessed visually by direct cell count or inhibition of colony formation (HELLSTRÖM et al, 1971 a) or the loss of incorporated radioactive compounds (COHEN et al, 1973 a).

The early work by the HELLSTRÖM'S stressed the specificity of this cytotoxicity, in that it was not apparent against normal tissue cells, and conversely there was no cytotoxic action against tumour cells by normal lymphocytes. Since then, many reports have appeared indicating that there may be a lack of specificity in that the cells of patients with both cancer and benign diseases of the same organ may be equally cytotoxic for tumour cells, and normal donor lymphocytes certainly display cytotoxicity (TAKASUGI et al, 1973; PIERCE & DE VALD, 1975; PETER et al, 1975; PERLMANN et al, 1977). GILLESPIE & BARTH (1978) tested the lymphocytes of 40 malignant melanoma patients and 58 control subjects against various tumour lines; melanoma lymphocytes were cytotoxic in 67% of the tests against melanoma cells and 56% of the tests against non-melanoma cells. Normal lymphocytes were cytotoxic in 25% of the tests against melanoma cells and 29% of the tests against other tumour cells. Hence the predominant response of the melanoma lymphocytes was non-specific cytotoxicity. Other workers have also found that responses are not affected by the disease status of the patient (JEEJEBHOY 1975).

It is unlikely that this cytotoxicity is directed against HL-A antigens since it can be found in male donors who have never had blood transfusions. It may be directed towards normal tissue antigens; HELLSTRÖM et al (1973) showed that the lymphocytes of healthy Negroes were cytotoxic

to melanoma tumour cells. It is equally feasible that it is directed against unidentified viral antigens on the tumour cells, which are often cultured cell lines and may have undergone antigenic change. Methodology for lymphocyte separation clearly has an effect on the degree of cytotoxicity seen in normal lymphocytes (PIERCE & DE VALD, 1975; GYTE & WATKINS, 1978). It is interesting to note that in their original study, HELLSTRÖM et al (1971a) did find that 27/527 control lymphocyte suspensions were toxic to both normal and tumour cells, but they excluded this data from their final calculations.

PERLMANN et al (1977) have suggested that both disease-related cytotoxicity and "spontaneous" cytotoxicity against unrelated target cells are caused by lymphocytes with F(c) receptors for IgG, since cytotoxicity is abolished by the removal of these cells (O'TOOLE et al, 1973). This is antibody-dependent K-cell-mediated cytotoxicity as opposed to T-cell-mediated cytotoxicity, which requires that effector cells and target cells be identical in regard to some of their major histocompatibility antigens (MÖLLER & MÖLLER, 1976). It must therefore be stressed, that as long as allogeneic systems are used this is likely to escape detection.

Other workers have also shown that the removal of B- and K-cells from lymphocyte suspensions may lead to a loss of cytotoxicity, although this tends to be more noticeable in allogeneic rather than autologous situations (BUKOWSKI et al, 1976; PERLMANN et al, 1977). DE VRIES et al (1974) have also shown that target cells from melanoma cell lines were more susceptible to cytotoxicity from normal donor lymphocytes than short-term-cultured cells, where the cytotoxicity from melanoma lymphocytes was greater than from normal lymphocytes. Again, cytotoxicity was contained in the non-T cells, suggesting that these cells if synthesizing antibody could be complexed with target-cell antigens, and so lead to cytolysis i.e. antibody-mediated cytotoxicity.

In contrast with these reports, WYBRAN et al (1974) have shown that T-enriched cell suspensions have greater cytotoxicity against tumour cells that the T-cell-depleted fractions, and that significant killing activity was achieved with very few cells. This indicated that in

unseparated blood there might be inhibitory factors to decrease the activity of T-cells, a suggestion re-iterated by others (TISMAN & HERBERT, 1977) as a means by which the immune system prevents over-stimulation.

3.3 IMMUNOLOGICAL STUDIES IN OVARIAN CANCER.

Early work in ovarian cancer indicated the presence of tumour-associated antigens, demonstrated by antigen-antibody reactions on agar gel diffusion plates, immuno-electrophoresis and immunofluorescence, when extensively absorbed anti-tumour antisera were reacted against extracts of ovarian epithelial tumours, or cyst fluid; it appeared that these antigens were not present in normal ovary, non-epithelial ovarian tumours, or other malignancies including those of Müllerian derivative (LEVI et al, 1969; LEVI 1971; BHATTACHARYA & BARLOW, 1973, a & b). LEVI et al (1969) also showed that their anti-tumour antiserum was cytotoxic for ovarian tumour cells in culture and this could be neutralized by prior absorption of the antiserum with tumour lyophilisate, but not normal ovary.

Table 3.3 is a list of major studies in the identification of ovarian cancer antigens, most suggesting that the antigen is of high MW. It must again be stressed that xenogeneic antiserum has been used in most of these studies, and may therefore have resulted in antiserum formed to phase-specific antigens present in normal and tumour tissues, e.g. CEA.

There is some evidence that the antigen is thermo-stable (BURTON et al, 1976); BHATTACHARYA & BARLOW (1973 b) found that heating mucin samples to 80^{o}C for 1 hour to decrease their viscosity did not alter their antigenicity. There is also evidence that the antigen may be a sub-species of CEA (KNAUF & URBACH, 1978), and these authors have developed a RIA to detect levels greater than 1.9ng/ml, which are considered pathological. It is interesting to note that McNIEL et al (1969) found that anti-serum produced in rabbits to mucin from mucinous cysts

would react with both normal and neoplastic tissue extracts, as well as colonic cancer extracts, but not those from other mucin-producing tumours such as breast, lung and stomach. Extensive further investigation by BHATTACHARYA & BARLOW (1978) has produced at least 2 antigens that are absent from normal Müllerian-derived tissues. One is found only in mucinous and serous carcinomas and has been used in a RIA to follow out-patient progress. The other antigen is found in about 90% of all ovarian and gynaecological tumours tested, and sometimes in breast, pancreas and colonic tumours, as well as in 80% of the pre-operative serum of ovarian cancer patients.

DORSETT et al (1975) used an immunofluorescence technique to show large amounts of antibody on the surface of tumour cells from pleural effusions; purification showed that it reacted with autologous tumour cells but not normal ovarian or other malignant cells (including those known to produce large amounts of CEA). This specificity has been confirmed by others (HILL et al, 1978), although yet other workers have been unable to detect such anti-bodies (BARLOW & BHATTACHARYA, 1975; GERBER et al, 1977). The latter study did however find a high incidence of auto-antibodies in the patients. As might be expected, mucinous tumours contain large amount of the secretory component of IgA (GARCIA et al, 1977).

Studies of cell-mediated immunity to tumour cells or extract are presented in Table 3.4 The original work by HELLSTRÖM et al (1968) showed that ovarian tumour cells could be inhibited from growing by both autologous and allogeneic lymphocytes from women with ovarian cancer. DI SAIA et al (1971) confirmed this and presented photographs of lymphocytes, some of which were blasts, clustered around tumour cells. The tumour cell killing began in 24 hours, although normal lymphocytes were also cytotoxic but only after 96 hours of incubation. Lymphocytes from pelvic lymph nodes were inert. He subsequently showed

TABLE 3.3 MAJOR STUDIES ON THE IDENTIFICATION OF OVARIAN TUMOUR-ASSOCIATED ANTIGENS.

REFERENCE	METHODOLOGY	MW	COMMENTS
LEVI et al, 1969	XS (rabbit) to tissue extracts; ID; IE.	200,000	4 precipitin lines obtained, 2 tumour specific & 1 cross-reacting with normal ovarian tissue.
BHATTACHARYA & BARLOW, 1973	XS (rabbit) to tissue extracts; ID; IE; IF.	High; ?muco-protein	Precipitin lines to ovarian tumour but not normal ovary or other normal tissues; very extensive absorptions against normal tissues.
GALL et al, 1973	XS (rabbit) to tissue extracts; ID.	?	Confirmatory report, no fractionation analysis.
KALASHNIKOV et al, 1976	ID	? ?α-globulin	Antigen also found in fetus, neonatal serum & tumour extracts, but not normal ovary or adult serum.
KNAUF & URBACH, 1976	XS (rabbit) to tissue extracts; SDS gel IE; fractionation.	10^4-10^5	4 different antigens, one tumour-specific, and one in normal tissue.
BURTON et al, 1977	XS (rabbit) to tissue extracts; ID.	38-50,000	5 distinct antigens, all found in normal tissue as well. Thermostable at 100°C.
GERBER et al, 1977	XS (rabbit); IF; fractionation.	High	Extensive absorptions removed cross-reactivity with normal and other neoplastic tissue.
BHATTACHARYA & BARLOW, 1978	XS (rabbit); ID; IE; IF.	High	2 antigens, one specific for ovarian epithelial tumours, the other common to breast, pancreas, colon and gynae. tumours. RIA developed.
KNAUF & URBACH, 1978			Fewer antigenic determinants than CEA but ?sub-species. RIA developed.

ABBREVIATIONS: XS = xenogeneic serum; IF, IE, ID = Immuno-fluorescence, -electrophoresis, -diffusion

TABLE 3.4 MAJOR STUDIES ON CELL-MEDIATED IMMUNITY IN OVARIAN CANCER.

REFERENCE	METHOD	NO. +	COMMENTS
DI SAIA et al, 1971	LMC	8/8	Cytotoxicity specific, but shown in normal lymphocytes after 96 hours; photographs of blast cells surrounding tumour cells.
CHEN et al, 1973	LMI	5/8	Inhibition to autologous & allogeneic tumour extracts; normal lymphocytes & those from other tumours inert.
KITSCHKE & KREBS, 1973	LB	7/11	Cross-reactivity between breast cancer and ovarian cancer lymphocyte responses.
CHATTERJEE et al, 1975	LB	0/11	No responses in vivo or in vitro to tumour extract; normal PHA responses.
LEVIN et al, 1975	LB	32/44	Significant difference in responses between cancer patients & controls found only in remission patients; cross-reactivity with normal ovary and fetal ovary extracts.
MELNICK & BARBER, 1975	LMI	6/7	Specific for ovarian tumour, normal lymphocytes inert.
MITCHELL & KOHORN, 1976	LMC	14/24	Allogeneic system, normal lymphocytes inert. No correlation with stage of disease, and chemotherapy did not affect responses.
FAIFERMAN et al, 1977	LMI	11/17	Specific for ovarian tumours, normal lymphocytes inert.

ABBREVIATIONS: LMC = Lymphocyte-mediated Cytotoxicity LB = Lymphocyte Blastogenesis
LMI = Lymphocyte Migration Inhibition

cytotoxicity against fetal gut cells, although CEA could not be detected (DI SAIA 1975). KITSCHKE & KREBS (1973) demonstrated cross-reactivity between mammary and ovarian tumour antigens, and noted that 64% of ovarian cancer patients tested responded to ovarian tumour extract, as did 30% of the breast cancer patients.

CHATTERJEE et al (1975) were unable to demonstrate cell-mediated immunity in 11 patients with ovarian cancer, using blastogenesis to autologous and allogeneic tumour extracts. Since the lymphocytes of all patients responded normally to PHA and autologous serum was not inhibitory to these responses, the authors concluded that lymphocyte abnormality could not explain their negative results. On the other hand, LEVIN et al (1975) used the blastogenic assay and found that ovarian cancer patients in remission had very definite responses to allogeneic and autologous tumour extracts, and these responses were significantly greater than those of controls or patients in relapse. Cancer patients, but not controls, also responded to extracts of fetal ovary as well as normal ovary, suggesting that antigenic determinants were not tumour-specific and might very well be onco-fetal. In a subsequent study it was shown that patient survival correlated with the ability to respond to tumour extract in vitro (LEVIN et al, 1976 a) FAIFERMAN et al (1977) have used the leucocyte migration inhibition assay to show very definite specificity of the response, and also found that 3M KCl tumour extracts were more effective than either saline or deoxycholate acid extracts. It appears that the lymphocyte cytotoxicity against tumour cells may be mediated by non-T cells (SAKSELA et al, 1974).

3.4 EXPERIMENTAL DESIGN: CELLULAR RESPONSES IN PATIENTS WITH OVARIAN CARCINOMA AND RELATIONSHIP TO PARITY.

The aim of the following experiments was:

(i) to assess the degree to which the lymphocytes from women with ovarian cancer might respond in vitro to tumour extracts,

(ii) to see whether the lymphocytes from pregnant women

would respond in vitro to ovarian tumour extract as well as fetal extracts, to investigate onco-fetal sensitization, (iii) to see whether in vitro responses to tumour extract occurred predominantly in the T or B cell populations.

MATERIALS & METHODS:

Patient groups were:

20 healthy, normal controls, non-pregnant and matched for age with the cancer patients wherever possible

25 patients with relapsing ovarian cancer as assessed by new effusions or increasing metastatic deposits

11 patients with "static disease" or technically in remission as assessed by palpation or ultrasonic scanning

15 women with other cancers - breast (1), bowel (3), endometrial (4), uterine sarcoma (4), malignant melanoma (1), retroperitoneal sarcoma (1), cervix (1)

16 pregnant women from all trimesters of pregnancy, 11 primiparous and 5 multiparous.

Preparation of extracts: as previously described, for both fetal and tumour tissue.

Preparation of lymphocytes: as previously described. In the assays using lymphocyte sub-populations, 10^6 cells /ml. were set up in the blastogenic assay, and the results compared with those of the unseparated cells.

No patients receiving immunotherapy were included in this study, and no cancer patients had received chemotherapy or radiotherapy for at least one month prior to the time of testing.

RESULTS:

Blastogenic responses to tumour extracts are illustrated in Fig. 3.1 A difference in cpm between unstimulated and stimulated cells of 500 cpm is considered positive (see Table 3.5). Such responses occurred in all groups tested and there were no differences in these responses which were statistically significant. The median cpm difference for control patients was 84 (range -2548 to 4095) compared with 177 for remission patients (-714 to 2412), 33 for relapse

patients (-600 to 3405), 627 for pregnant women (-1290 to 6702) and 542 for patients with other cancers (-692 to 8406). In the control patients there was a significant difference in the responses of parous and nulliparous women ($p<0.02$).

The lymphocytes of pregnant women were able to respond to all extracts of fetal tissue as well as ovarian tumour extract. There were no significant differences in the degree to which these extracts caused stimulation except for the fetal liver extract which caused significant suppression of responses ($p<0.001$), see Fig. 3.2, Table 3.6

The responses of cell sub-populations cultured with tumour extract are illustrated in Fig 3.3 (see Table 3.7). Of the 13 patients in this study, 10 were in relapse. The median cpm difference for the unseparated lymphocytes was -48 (range -1740 to 9518), compared with 1035 for the T-enriched population (-10,398 to 6822) and 19 for the T-depleted population (-3102 to 1167). The differences between these values were not significantly different.

DISCUSSION:

The present study shows that the lymphocytes of normal, healthy female controls, pregnant women and patients with ovarian and other cancers can at times be stimulated in vitro by extracts of ovarian tumours. 4 remission patients (37%), 8 relapse patients (32%), 8 women with other cancers (53%), 4 controls (20%) and 9 pregnant women (56%) had blastogenic responses of more than 500 cpm above their control values.

A number of points can be made from these results:

(i) relapse and remission patients had the same incidence of stimulation (vide infra)

(ii) the data do not preclude the possibility that the responses are to an antigen shared by non-malignant cells (i.e. not ovarian-tumour specific). The reactivity of patients with other cancers, especially of the female genital tract (Müllerian derived) and breast indicates some degree of antigenic cross-reactivity which may have its origins in the embryological development of these tissues, and indeed

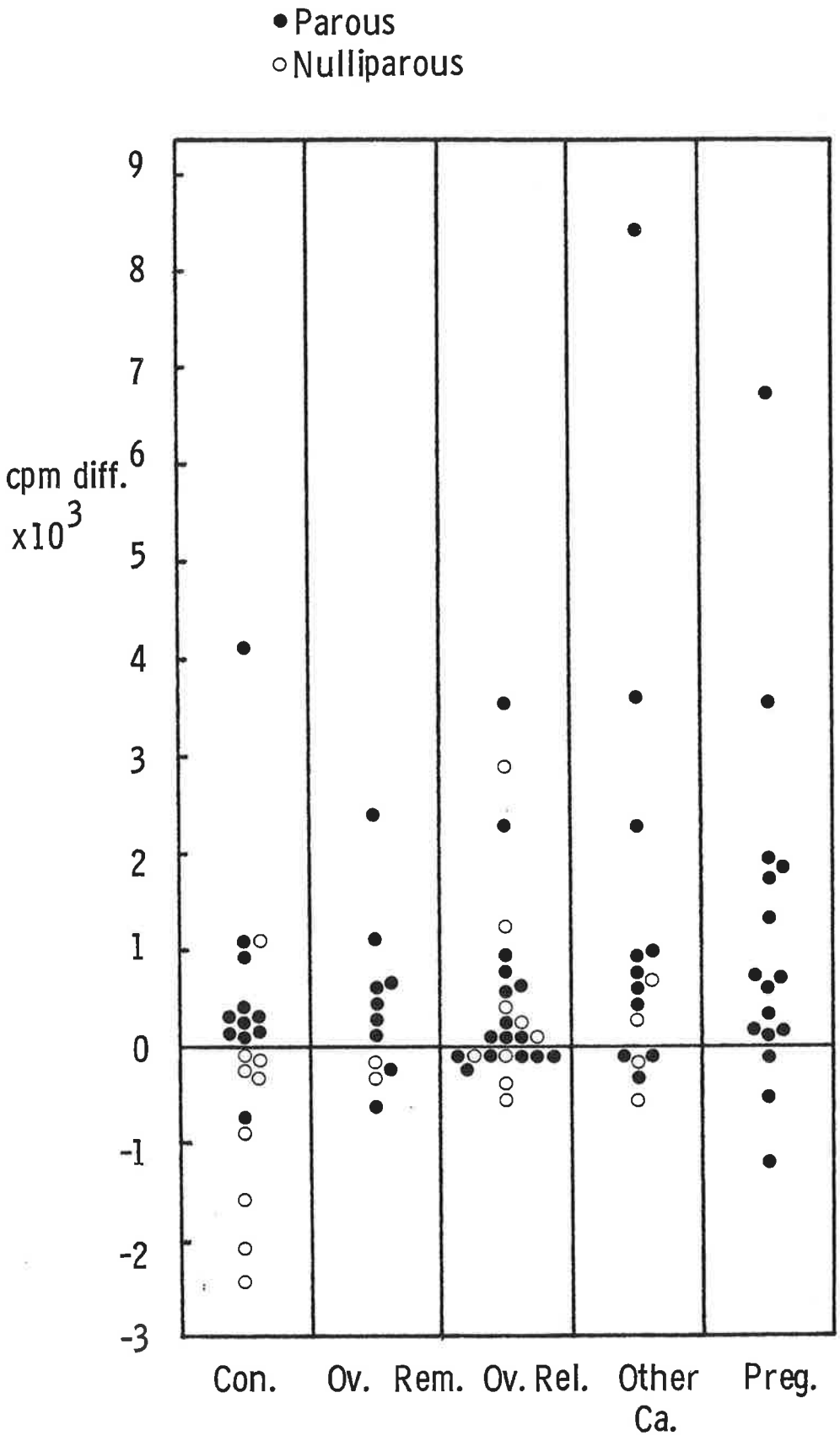


FIGURE 3.1 BLASTOGENIC RESPONSES OF CONTROLS, PREGNANT WOMEN, AND CANCER PATIENTS TO 100µg OVARIAN TUMOUR CME

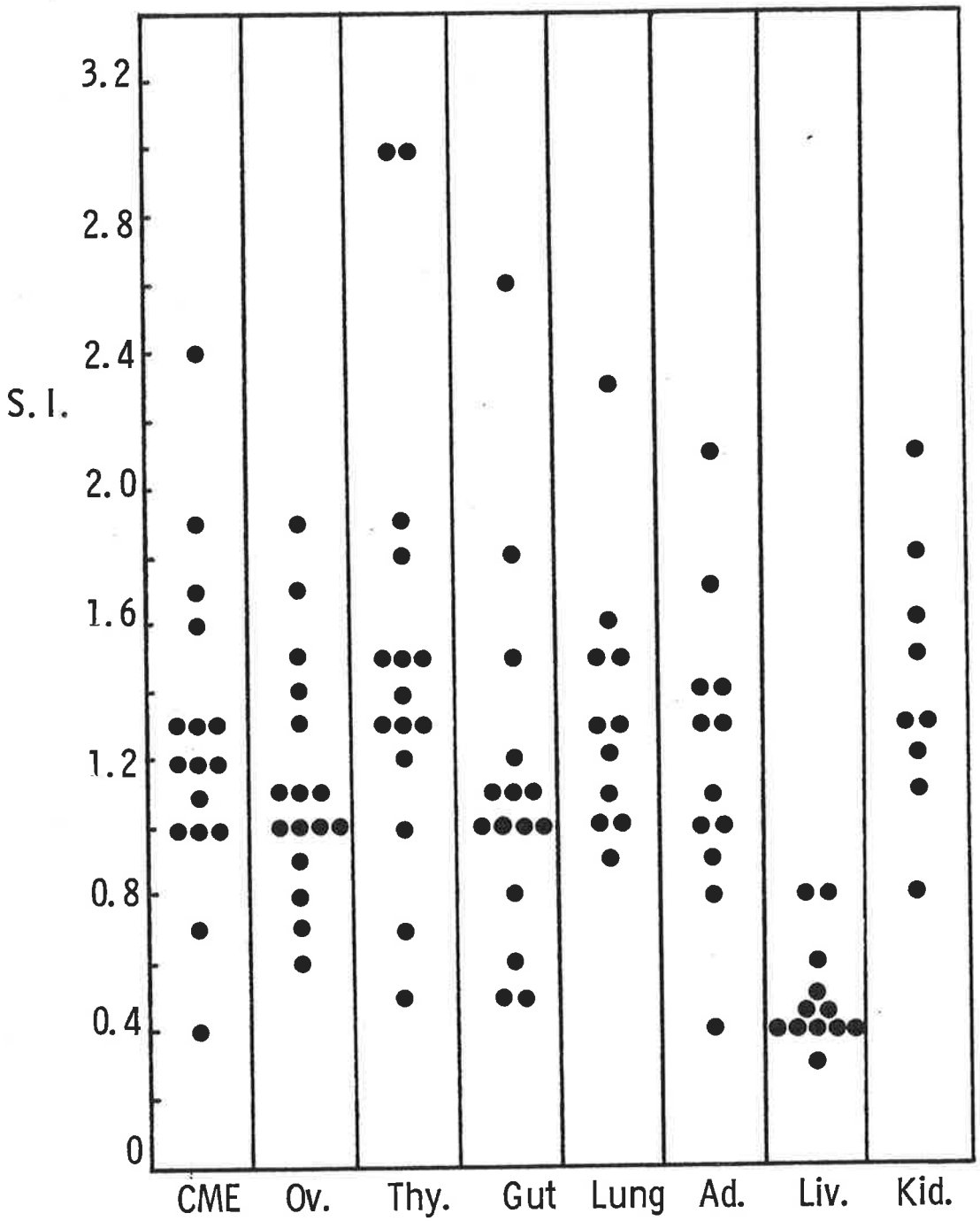


FIGURE 3.2 BLASTOGENIC RESPONSES OF PREGNANT WOMEN TO 100µg VARIOUS FOETAL TISSUE EXTRACTS AND OVARIAN TUMOUR CME

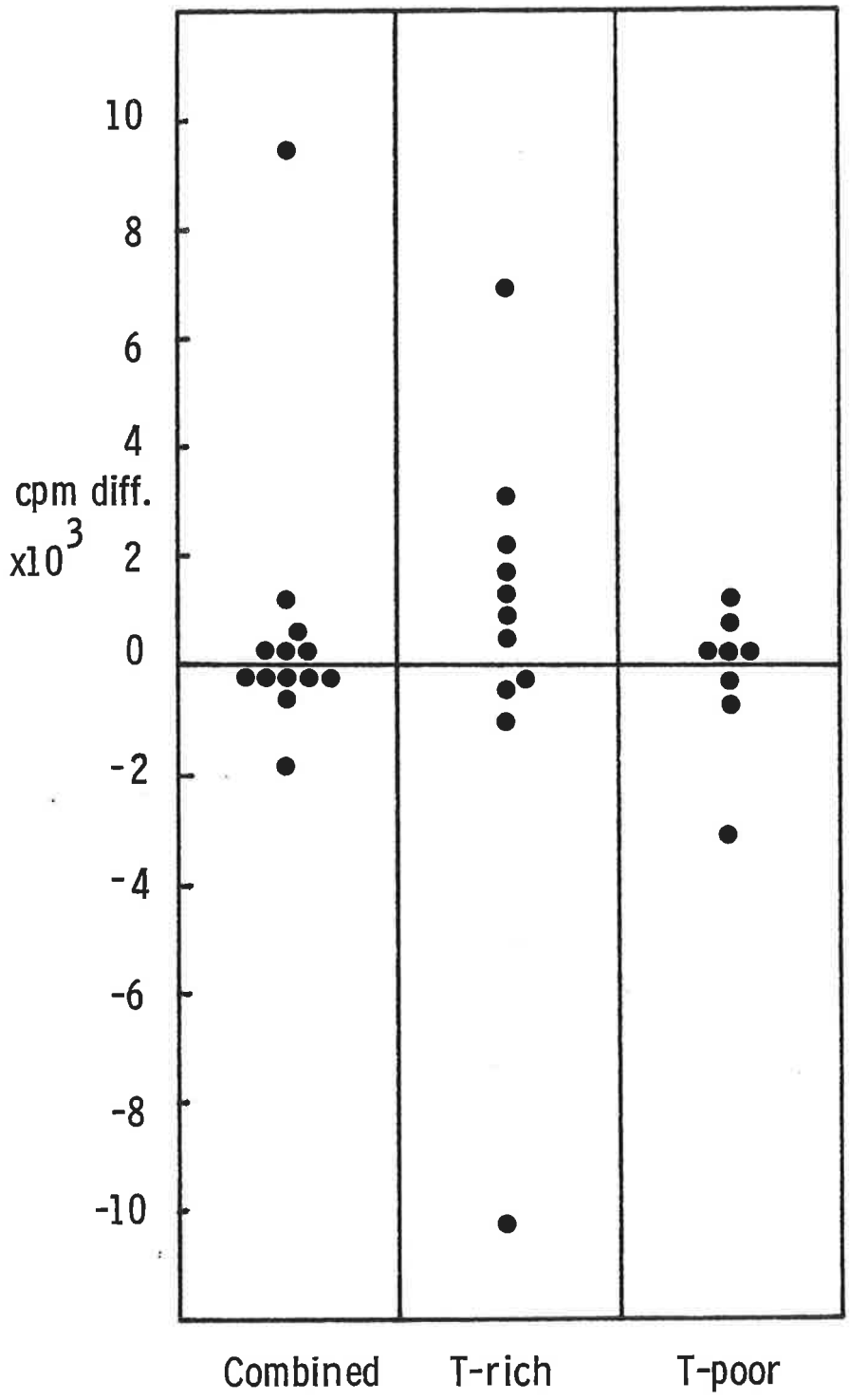


FIGURE 3.3 BLASTOGENIC RESPONSES OF CELL POPULATIONS TO 100µg OVARIAN TUMOUR CME

confirms the findings of others (McNIEL et al, 1969; KITSCHKE & KREBS, 1973; CHEN et al, 1976; BHATTACHARYA & BARLOW, 1978). Whether these are truly onco-fetal tumour-associated antigens or normal tissue antigens in higher concentration in neoplastic tissue has not been clarified. It is known that normal ovarian tissue has antigens which cross-react with those from normal breast and Fallopian tube (BAILEY et al, 1972), and women with ovarian cancer have been shown to respond in vitro to extracts of normal ovary (LEVIN et al, 1975).

(iii) Lymphocytes from 5 control patients (25%) were markedly inhibited by tumour extract and this phenomenon was not seen to the same extent in any of the other patient groups. 4 of these women had never had children. Of the 4 control patients who were stimulated by tumour extract, 3 had had children and the fourth was a laboratory worker who handled ovarian tumours frequently and cultured them. These predominantly positive responses in parous controls, and absence of such responses in all but one of the nulliparous controls, were a significant feature ($p<0.02$). They suggest that the responses of normal lymphocytes to ovarian tumour extract are not non-specific (PERLMANN et al, 1977), but relate very strongly to previous sensitization during pregnancy.

(iv) the lymphocytes of pregnant women responded to tumour extracts as well as fetal extracts, although in no significantly greater degree. This and the previous point suggests firstly, that there may be an onco-fetal nature to the stimulating antigen in the tumour extract. Secondly, the sensitization of parous women to fetal ovarian antigens may explain why they have a lower incidence of ovarian cancer than nulliparous women (BERAL et al, 1978). In the cancer patients in the study, all but 2 in remission were parous, and this distinction was lost in those with relapsing disease (see Fig. 3.1).

The suppression of lymphocyte responses by fetal liver extracts is presumably caused by AFP which is known to be immunosuppressive (GLOBERSON et al, 1975; MURGITA 1976).

(v) in spite of the finding that there are no significant differences between cellular populations in their response to tumour extracts, the median value of 1035 cpm difference for the T-enriched cells, was much higher than the values

for either the unseparated cells or the T-depleted cells. This suggests that the recognition and sensitization to tumour antigen is contained in the T-cells, despite conflicting reports to the contrary (WYBRAN et al, 1974; O'TOOLE et al, 1973; PERLMANN et al, 1977).

This study was unable to find any statistically significant difference in the responses of the various patient groups. Others have also claimed no evidence of in vitro responsiveness to ovarian tumour extract (CHATTERJEE et al, 1975), although in that study 4 of 6 patients with cancer, and 3 of 4 controls had responses to tumour extract greater than 500 cpm above unstimulated cell cultures. It is, however, difficult to explain these results in the light of a very extensive study showing significant differences in blastogenic responses between normal controls and remission ovarian cancer patients, but not those in relapse (LEVIN et al, 1975). There are three possible reasons for the differences in results between the two studies.

The first relates to patient selectivity. Only a small number of "remission" patients were included in the present study, and all except 2 patients had Stage III disease (F.I.G.O. Classification). Non-haematological tumours are very difficult to define in terms of disease progression, and although objective parameters of tumour growth were used in this study, some of these patients may very well have had slowly progressive disease. As well as more remission patients in the LEVIN (1975) study, none of their relapse patients had had chemotherapy or radiotherapy for at least two months. Most of the patients in the present study were on pulsed monthly chemotherapy, generally with Cyclophosphamide, Adriamycin and Cis-Platinum. It is known that chemotherapy has differential effects on lymphocyte sub-populations (TURK & POULTER, 1972), and that the ability of lymphocytes to rebound after chemotherapy is related to prognosis (CHEEMA & HERSH, 1971). Furthermore, chemotherapy can decrease "blocking factors" in the serum of patients with relapsing disease (SINKOVICS et al, 1972), and can increase tumour-cell

killing by antibody (SEGERLING et al, 1975). It has previously been shown that these patients have blocking factors of immune complexes in their serum (POULTON et al, 1978), and therefore the responsiveness of the lymphocytes seen in these women may represent reversal of suppressive serum factors by successful chemotherapy.

Secondly, the use of crude tumour extracts may give rise to stimulation by a wide variety of substances other than the putative tumour antigen. It is unlikely to result from viral or bacterial contamination of the extracts, since generalized stimulation is not demonstrated, and in fact obvious suppression of responses occurs in some cases. Responses are also unlikely to be to HL-A antigens since not all lymphocytes are stimulated in control patients, and furthermore patients with ovarian cancer have demonstrated responses to both autologous and allogeneic extracts (Fig. 3.4, Table 3.8). It should be noted that the technique for preparation of the tumour extract varied slightly from that of LEVIN et al (1975), in that the supernatant from the solubilized tumour was not ultra-centrifuged. It is therefore feasible that the stimulation seen resulted from such substances as normal tissue antigens which were present in greater concentrations in necrosing tissue (MITCHELL, 1973). This may be similar to the Cancer Basic Protein found also on normal and fetal cells (CASPARY & FIELD, 1971). However, it seems unlikely as it has been shown that the use of purified fractions of chromatographed crude tumour extract leads to very significant lymphocyte responses in ovarian cancer patients. These responses are specific and are not found in control donor lymphocytes (POULTON, TA - unpublished results).

Finally, one must consider whether the blastogenic assay is an appropriate means of assessing tumour antigen-icity. This is unlikely in view of the distinctive responses mentioned above using the assay. It may be however, that only autologous extracts should be used in the assay, in spite of demonstrations of cross-reactivity with other similar tumours (McCOY et al, 1976).

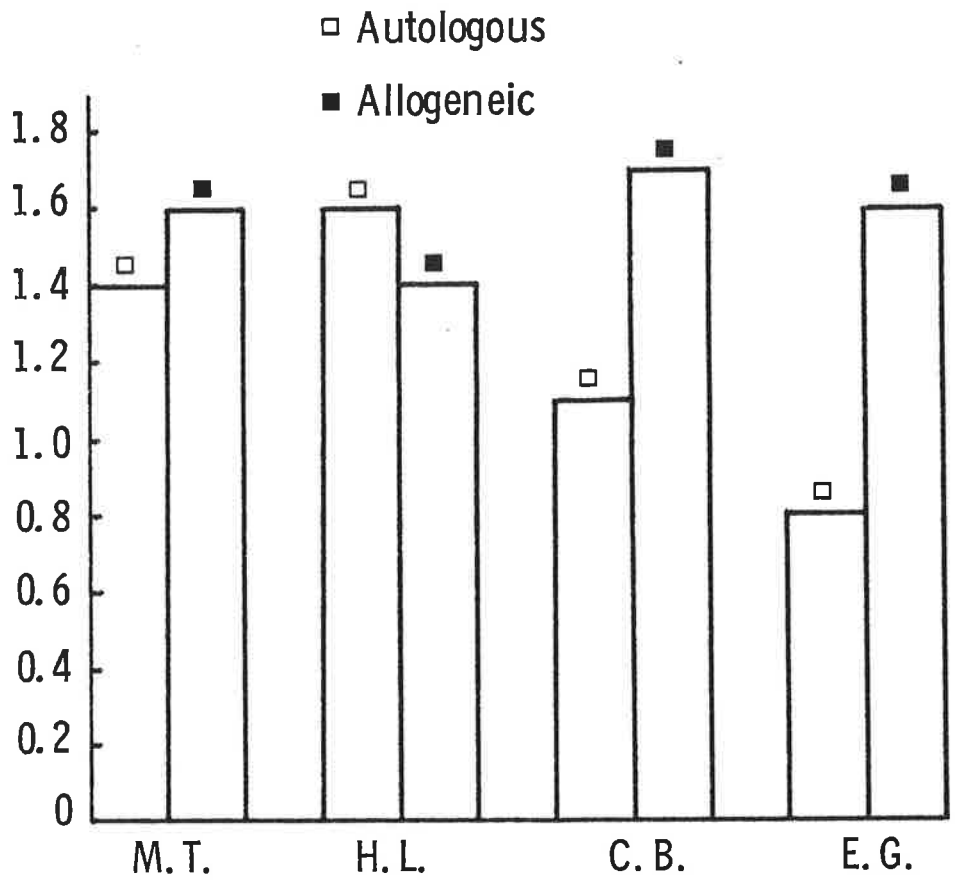


FIGURE 3.4 BLASTOGENIC RESPONSES OF CANCER PATIENTS TO 100µg AUTOLOGOUS OR ALLOGENEIC OVARIAN TUMOUR CME

TABLE 3.8

Patient	Nil	AUTOLOGOUS cpm	cpm Δ	S.I.	ALLOGENEIC cpm	cpm Δ	S.I.
M.T.	6002	8460	2458	1.4	9598	3596	1.6
H.L.	1044	1626	582	1.6	1467	423	1.4
C.B.	894	936	42	1.1	1482	588	1.7
E.G.	3972	3310	-662	0.8	6384	2412	1.6

Whatever the antigenic determinants, they are weak since the blastogenic responses which they induce are mild. It has been shown that small tumours tend to have high antigenicity compared with metastasising tumours (KJAER & BENDIXEN 1976), and this may explain why the responses seen in this study are much less than those seen in studies with patients who have localized breast cancer or malignant melanoma (SAVEL 1969; MAVLIGIT et al, 1973).

This Chapter concludes therefore that patients with ovarian cancer have lymphocyte responses to extracts of ovarian tumours, but this is probably a non-specific phenomenon as similar responses are also seen in normal, healthy control subjects, where they relate very significantly to parity. Although, the relationship is lost in women with disseminated ovarian cancer, nevertheless it is postulated that it may be parity which protects women from ovarian cancer by immunological mechanisms (e.g. fetal sensitization), as yet not fully investigated.

TABLE 3.5 BLASTOGENIC RESPONSES OF CONTROLS, OVARIAN CANCER PATIENTS AND WOMEN WITH OTHER CANCERS TO 100µg OVARIAN TUMOUR EXTRACT.

Patient code		Nil	cpm	cpm diff.	S.I.
CONTROLS					
B (NP)		7434	4886	-2548	0.7
M (NP)		4281	2181	-2100	0.5
W (NP)		9538	7922	-1616	0.8
X (NP)		4413	3429	- 984	0.8
P (P)		8214	7320	- 894	0.9
P (NP)		3381	3000	- 381	0.9
W (NP)		4404	4108	- 296	0.9
D (NP)		4458	4342	- 116	1.0
D (NP)		4170	4089	- 81	1.0
K (P)		4328	4378	50	1.0
L (P)		772	890	118	1.2
H (P)		4048	4174	126	1.0
B (P)		879	1059	180	1.2
W (P)		2960	3200	240	1.1
X (P)		2079	2475	396	1.2
G (P)		1232	1632	400	1.3
S (P)		2085	3066	981	1.5
H (P)		2874	3944	1070	1.4
C (NP)		1164	2259	1095	1.9
D (P)		10254	14349	4095	1.4
REMISSION					
M (P)		3498	2784	- 714	0.8
T (NP)		3276	2880	- 396	0.9
H (P)		1050	771	- 279	0.7
R (NP)		2745	2478	- 267	0.9
W (P)		555	561	6	1.0
M (P)		10938	10761	177	1.0
F (P)		5138	5540	402	1.1
S (P)		2199	2811	612	1.3
G (P)		2985	4035	1050	1.4
G (P)		3972	6384	2412	1.6
OTHER CANCERS					
R (NP)	Ret.Sa.	3298	2606	- 692	0.8
L (P)	M.M.	4362	4064	- 298	0.9
C (P)	Endom.	2048	1890	- 158	0.9
W (NP)	Ut.Sa.	1982	1833	- 149	0.9
T (P)	Ut.Sa.	528	519	- 9	1.0
R (NP)	Endom.	1046	1320	274	1.3
S (P)	Ut.Sa.	1952	2318	366	1.2
P (P)	Col.	2158	2700	542	1.3
W (NP)	Ut.Sa.	2792	3472	680	1.2
B (P)	Col.	1950	2685	735	1.4
F (P)	Col.	1886	2788	902	1.5
B (P)	Endom.	10814	11748	934	1.1
C (P)	Cervix	6596	8937	2341	1.4
M (P)	Breast	5442	9034	3592	1.7
C (P)	Endom.	4564	12970	8406	2.8

Patient Code	Nil	cpm	cpm diff.	S.I.
RELAPSE				
B (NP)	1599	999	- 600	0.6
B (NP)	2980	2538	- 442	0.9
H (P)	5142	5042	- 100	1.0
W (NP)	588	498	- 90	0.8
C (P)	722	638	- 84	0.9
R (P)	5294	5212	- 82	1.0
B (P)	680	606	- 74	0.9
C (P)	456	390	- 66	0.9
B (NP)	2818	2770	- 48	1.0
M (P)	618	570	- 48	0.9
W (P)	3114	3132	18	1.0
R (NP)	846	873	27	1.0
H (P)	1686	1719	33	1.0
D (P)	1302	1384	82	1.1
H (P)	4424	4598	174	1.0
W (NP)	2484	2679	195	1.1
F (NP)	554	822	268	1.5
M (P)	812	1404	592	1.7
D (P)	1244	1852	608	1.5
W (P)	2769	3540	771	1.3
F (P)	2577	3477	900	1.3
F (NP)	1144	2478	1334	2.2
V (P)	2894	5272	2378	1.8
J (NP)	6788	9630	2842	1.4
M (P)	4143	7548	3405	1.8

Median values:

Controls = 84 (-2548 to 4095)

Remission = 177 (-714 to 2412)

Other Cancers = 542 (-692 to 8406)

Relapse = 33 (-600 to 3405)

Mann Whitney U Test: no sig. diff.

ABBREVIATIONS: P = Parous NP = Nulliparous

Difference in responses of parous and nulliparous control patients: $U = (9 \times 11) + \frac{(9 \times 10)}{2} - 59 = 85$

$U' = 14 \quad p < 0.02$

TABLE 3.6 BLASTOGENIC RESPONSES OF PREGNANT WOMEN TO 100µg VARIOUS FETAL TISSUE EXTRACTS AND OVARIAN TUMOUR CELL MEMBRANE EXTRACTS.

Patient	Nil Result	CME	Ov.	Thy.	Gut	Lung	Adr.	Liv.	Kid.
PRIMIPS				Results expressed thus:					
T (2)	2900	3562	2850	cpm stimulated cells					
		662	-50	cpm difference					
		1.2	1.0	S.I.					
G (3)	1749	1206	1284	1299	912	1566	783	516	1362
		-543	-465	-450	-837	-183	-966	-1233	-387
		0.7	0.7	0.7	0.5	0.9	0.4	0.3	0.8
B (3)	2316	1026	1290	1155	1239				
		-1290	-1026	-1161	-1077				
		0.4	0.6	0.5	0.5				
C (2)	2643	3336	2748	3297	1656				
		693	105	654	-987				
		1.3	1.0	1.2	0.6				
C (2)	2964	3555	2490	4296	3006	3282	4110	1632	
		591	-474	1332	42	318	1146	-1332	
		1.2	0.8	1.4	1.0	1.1	1.4	0.6	
M (2)	5082	8643	7161	7638	5007	8139	5481	1893	6858
		3561	2079	2556	-75	3057	399	-3189	1776
		1.7	1.4	1.5	1.0	1.6	1.1	0.4	1.3
C (2)	2625	3021	2643	3510	2883	3426	3483	939	
		396	18	885	258	801	858	-1686	
		1.2	1.0	1.3	1.1	1.3	1.3	0.4	
W (2)	2319	3645	2970	4308	2451	3039	2985	924	3792
		1326	651	1989	132	720	666	-1395	1473
		1.6	1.3	1.9	1.1	1.3	1.3	0.4	1.6
T (2)	2132	3999	3591	6438	3228	3147	4494	1620	2805
		1863	1455	4302	1092	1011	2358	-516	669
		1.9	1.7	3.0	1.5	1.5	2.1	0.8	1.3
T (2)	4938	11640	9210	15036	12924	11358	8493	1818	10191
		6702	4272	10098	7986	6420	3555	-3120	5253
		2.4	1.9	3.0	2.6	2.3	1.7	0.4	2.1
B (3)	2310	2241	2487	2937	2298	2295	2277	834	2721
		-69	177	627	-12	-15	-33	-1476	411
		1.0	1.1	1.3	1.0	1.0	1.0	0.4	1.2

ABBREVIATIONS:

CME = Ovarian Tumour Cell Membrane Extract

Ov = Fetal ovary extract

Thy = Thymus; Adr = Adrenal; Liv = Liver; Kid = Kidney.

Number in parentheses = Trimester of pregnancy.

TABLE 3.6 (Contd.)

Patient	Nil Result	CME	Ov.	Thy.	Gut	Lung	Adr.	Liv.	Kid.
MULTIPS.									
J (3)	1014	1026	867	1473	843	1059	792	378	1086
		12	-147	459	-171	45	-222	-636	72
		1.0	0.9	1.5	0.8	1.0	0.8	0.4	1.1
H (3)	777	858	816	1200	906	1140	789	630	1161
		81	39	423	129	363	12	-147	384
		1.1	1.1	1.5	1.2	1.5	1.0	0.8	1.5
L (3)	1203	1224	1359	1611	1347	1479	1116	438	2115
		21	156	408	144	276	-87	-765	912
		1.0	1.1	1.3	1.1	1.2	0.9	0.4	1.8
P (3)	5802	7596	5943	5982	5853				
		1794	141	180	51				
		1.3	1.0	1.0	1.0				
E (2)	6123	8118	9033	10875	10806		8340	3132	
		1995	2910	4752	4683		2217	-2991	
		1.3	1.5	1.8	1.8		1.4	0.5	
Median values for S.I. :		1.2	1.1	1.4	1.0	1.3	1.2	0.4	1.3

Mann Whitney U Test: no significant difference between these values for S.I. except for the significantly lower values with fetal Liver Extract ($p < 0.001$)

Median value of responses to tumour cell membrane extract : 627 cpm (range -1290 to 6702). No significant difference when compared with normal controls and other cancer patients.

TABLE 3.7 BLASTOGENIC RESPONSES OF CELL POPULATIONS TO 100µg OVARIAN TUMOUR CELL MEMBRANE EXTRACT

Patient	Unseparated			T-enriched			T-depleted		
§ = relapse	Nil	cpm	cpm Δ	Nil	cpm	cpm Δ	Nil	cpm	cpm Δ
W §	10380	19898	9518	17128	19172	2044	1740	1770	30
W	588	498	-90	6258	8134	1876	1203	-	-
R	2745	2478	-267	6318	13140	6822	11085	-	-
R §	1046	1320	274	9880	8800	-1080	2244	3066	822
W	555	561	6	4314	4910	596	1734	-	-
T §	528	480	-48	852	-	-	1790	981	-817
M §	812	1404	592	3616	5256	1640	3351	3360	9
C §	456	390	-66	2268	1701	-567	1725	1464	-261
L §	1514	1630	116	2178	3213	1035	1088	1116	28
W §	11781	13140	1359	19718	9320	-10398	1065	-	-
B §	2818	2770	-48	-	-	-	1779	2946	1167
R §	5158	3441	-1740	6474	5850	-624	23304	-	-
M §	618	570	-48	8576	11746	3170	6894	3972	-3102
Median values cpm Δ:			-48			1035			19

Mann Whitney U Test:

Unseparated vs. T-enriched	U = 58, n.s.
Unseparated vs. T-depleted	U = 49, n.s.
T-enriched vs. T-depleted	U = 30, n.s.

CHAPTER 4. TUMOUR ESCAPE FROM IMMUNOLOGICAL DESTRUCTION: CELLULAR DEFECTS.

In spite of immune responses directed against tumours, they are often inadequate to eradicate malignancy, more especially its metastatic spread. Table 4.1 presents a list of hypotheses as to why this may occur. This Chapter concentrates on cellular defects, and the following Chapter discusses serum factors which may block immunological responses in vivo.

Numerous animal experiments have demonstrated that the presence of a tumour or even tumour-inducing virus stimulates specific cellular responses. If normal mouse lymphocytes are exposed in vitro to syngeneic tumour cells, blastogenesis occurs; but when the same stimulus is applied to the lymph node cells from a tumour-bearing mouse, no additional stimulation is seen above the already high back-ground activity. Following surgery, this activity drops back to normal (McKHANN & JAGARLAMOODY 1971). There is clearly a gentle balance between the stimulating antigenic presence of tumour cells and the specific cellular responses they evoke (COGGIN et al, 1974). GERSHON et al (1968) have shown that early removal of a sensitizing tumour leads to a low degree of immunity and meatastases, whereas animals carrying tumours for a longer time develop a high degree of immunity and no metastases.

Such experimentation in humans is of course impossible; in animals, ALEXANDER et al (1969) have shown that although the draining lymph nodes are stimulated, they do not function properly. In humans the evidence is conflicting, and while some workers have found non-reactive lymphocytes in local nodes (DI SAIA et al, 1971; NAIRN et al, 1971 b; MAVLIGIT et al, 1974 b), others have demonstrated distinct activity to both non-specific mitogens as well as tumour-associated antigens (FISHER et al, 1972; JUBERT et al, 1977). Indeed this was the rationale for CRILE's (1965) assertion that lymph nodes be preserved in Stage I breast cancer. It is not known whether local contamination

TABLE 4.1 PROPOSED MECHANISMS BY WHICH TUMOURS ESCAPE DESTRUCTION.

I. Special Properties of Tumour Cell Antigens:

(i) weak or non-immunogenic (e.g. if normal tissue components)

(ii) masked - sialic acid residues

II. Cellular Defects:

(i) non-specific - related to old age, stress or malnutrition

- iatrogenically produced with radio- and chemotherapy

(ii) specific - genetic immunodeficiency

- paralysis of action by tumour "products"

- decreased numbers of cells (e.g. T cells)

- production of Suppressor Cells

- true specific tolerance of host to tumour (rare)

III. Inhibitory Serum Factors:

(i) immunosuppressive hormones (e.g. ACTH, HCG)

(ii) immunosuppressive foetal antigens (CEA, AFP)

(iii) non-specific globulins and acute-phase proteins (e.g. in uraemia)

(iv) tumour antigen excess paralysing local lymph nodes and lymphocytes

(v) antibody which may be "enhancing" or "blocking" and so prevent tumour cell lysis

(vi) immune complexes of antigen and antibody

with tumour cells abrogates lymphocyte reactivity, or whether abnormalities in lymph nodes result from a pre-existing biological deficit which encourages metastases there. Since there tend to be clear anatomical patterns to metastatic spread, the former suggestion seems more likely.

4.1 CELLULAR DEFECTS.

(i) In vivo responses to DNCB and secondary recall antigens.

Cutaneous anergy has been seen in many patients with reticulo-endothelial malignancies (AISENBERG 1962; HERSH et al, 1971; MAGRATH 1974), as well as solid tumours (see Table 4.2). EILBER & MORTON (1970 a) found that following DNCB sensitization only 60% of pre-operative patients responded compared with 95% of a control group. Further, 93% of the patients who failed to respond were inoperable or developed recurrence within 6 months. Responses to a battery of skin antigens were less clear.

There appears to be a correlation between the degree of anergy and tumour burden (KOPERSZTYCH et al, 1976; WANEBO et al, 1976); for example, a third of breast cancer patients (early stages) retain optimal immunocompetence compared with only one tenth of those with metastatic disease (STEIN et al, 1976). Reactivity is also related to prognosis (EILBER & MORTON 1970 a; ANTHONY et al, 1974), although this has been disputed by others (WANEBO et al, 1976; PRITCHARD et al, 1978).

(ii) In vitro responses to non-specific mitogens and secondary recall antigens.

Largely investigated by comparing blastogenic responses to PHA, Con A, PWM or PPD between cancer patients and normal controls, reactivity appears to be decreased in patients with reticulo-endothelial as well as solid neoplasms (see Table 4.2). This has not been confirmed by all workers however (SUTHERLAND et al, 1971; COCHRAN et al, 1976), and NELSON (1969) even noted that the lymphocytes from cancer patients demonstrated greater responses to PHA than those from control patients. Some of these differences may in part relate to culture conditions, including both the nature of the culture serum (see Chapter

5) and the concentrations of mitogens used, since concentrations of PHA which stimulate one population of cells may be toxic for another (DUCOS et al, 1970; SAMPLE et al, 1971). It is difficult to interpret these results and even more difficult to assess their usefulness as prognostic tests. PRITCHARD et al (1978), in the only large prospective study of its kind, have shown that some immunological parameters are depressed in patients with melanoma, and some not, and furthermore there is no correlation with the clinical stage. They stress that in any case it is notoriously difficult to assess the Stage of disease accurately, and conclude that such crude tests have no prognostic importance.

There are a number of possible explanations for the depressed cell mediated immunity seen in some patients:

(i) there may be a genuine decrease in the number of T-cells able to respond to the sensitizing agent. GOLDING et al (1977) have shown that the in vitro responsiveness of unstimulated lymphocytes from cancer patients is significantly higher than that of control cells, suggesting in vivo activation of peripheral lymphocytes. It has been postulated that a subpopulation of T-cells becomes committed to tumour antigen, and so results in an effective depletion of circulating lymphocytes responsive to other mitogens and antigens (MATCHETT et al, 1973). This seems unlikely, since only about 2% of circulating lymphocytes are committed to respond to a particular antigen (CROWTHER et al, 1969). Sub-populations of T-cells have been shown to be decreased in cancer patients (WYBRAN & FUDENBERG 1973) (vide infra), and since such cells may differ in their responsiveness to PHA and Con A (STOBO & PAUL 1973), this may partly explain the conflicting experimental results.

(ii) iatrogenic immunosuppression may be reflected in results showing depressed CMI and it is important to know whether the patient has received chemo- or radio-therapy, especially as the latter may depress CMI for many years after the event (STJERNSWÄRD et al, 1972).

(iii) serum factors, which are known to inhibit in vitro

TABLE 4.2 REPRESENTATIVE STUDIES OF IMMUNOCOMPETENCE IN PATIENTS WITH CANCER.

TUMOUR	REFERENCE	PARAMETERS	NO. PTS.	COMMENTS
Hodgkin's Disease	AISENBERG, 1962	DNCB	37	100% patients with active disease anergic compared with 1/15 patients with inactive disease
Burkitt's Lymphoma	MAGRATH 1974	STA	51	Sig. impairment related to clinical Stage and correlated with survival and length of remission
AML	HERSH et al, 1971	STA LB to PHA, KLH, SL.	25	Sig. impairment related to ability to respond to chemotherapy - 80% patients who were immunocompetent went into remission after treatment
Gynae.	KHOO & MACKAY, 1974	DNCB & STA	178 ovary 39	All patient groups had sig. anergy related to spread of disease and response to therapy
Ovary	LEVIN et al, 1976 b	LB to PHA, PPD	25	No sig. anergy but serum effects noted in relapse lymphocytes cultured in autologous serum
Cervix	LEVY et al, 1978	DNCB & STA LB to PHA WCC, T/B count	42	STA & LB sig. decreased in cancer and related to Stage of disease. Normal cell counts in localized disease. Correlation between PHA anergy and T-cell count in metastatic disease.
Breast	STEIN et al, 1976	DNCB & STA LB to PHA WCC, T count	255	STA & LB sig. reduced in all cancer groups, related to Stage. Cell counts not sig. different from controls although 33% cancer pts. with value below lowest control value
Breast	WANEBO et al, 1976	DNCB & STA LB to PHA, Con A PWM, STA WCC, T/B count Serum Ig & C'	134	No impairment to STA, LB to mitogens not impaired, but responses to STA sig. reduced. B count sig. decreased, but not other cells. "High risk" patients paradoxically had higher LB responses to PHA than "low risk" patients
Breast & Melanoma	COCHRAN et al, 1976	STA LB to PHA, PPD WCC, T count Ig levels	111	Sig. reduced responses to STA; PHA responses higher in cancer patients than controls, and "active" rosettes sig. increased. IgG and IgA sig. increased
Melanoma	PRITCHARD et al, 1978	DNCB & STA LB to PHA, PWM and Con A T/B count	262	Response to STA only sig. decreased in patients with Stage IV disease. LB sig. reduced. T & B counts same as controls
Various	EILBER & MORTON 1970 a	DNCB & STA	83	Sig. anergy related to Stage and subsequent prognosis
Various	GOLUB et al, 1974	DNCB & STA LB to PHA, PWM, Con A & MLC	52	Impaired responses to STA but not sig. DNCB non-responders had very low responses in the MLC
Various	LEE et al, 1975	DNCB & STA WCC	183	Low WCC related to progression, and DNCB responses correlated with normal WCC and slow rate of progress

ABBREVIATIONS:
- DNCB = Dinitrochlorobenzene skin sensitizing agent
- STA = Skin test antigens e.g. Tuberculin, Candida, Streptokinase-Streptodornase
- LB = Lymphocyte blastogenesis - to Phytohemagglutinin (PHA), Concanavalin A (Con A), Poke-weed Mitogen (PWM), Keyhole Limpet Haemocyanin (KLH), Streptolysin (SL), Mixed Lymphocyte Cultures (MLC)
- WCC = White cell count, including T and B counts
- Ig = Immunoglobulin; C' = Complement

lymphocyte responsiveness (GATTI et al, 1971) may be coating the cells. Extensive washing or incubation of cells prior to testing should remove such factors (see Chapter 5).

(iv) there may be a genuine intrinsic defect in the lymphocytes of people with cancer. CERCEK et al (1974) have shown that changes in the lymphocyte cytoplasmic matrix occur in patients with progressive disease and may be responsible for decreased responses to mitogens.

(v) there may be abnormalities in other cells which are involved in lymphocyte recognition and proliferation to antigenic stimulation e.g. macrophages (BOETCHER & LEONARD, 1974).

(vi) there may be spontaneous in vitro production by cancer lynphocytes of an inhibitor of blastogenesis. GOLDING et al (1977) have shown that the supernatants of Hodgkin's Disease lymphocytes contain a lymphokine which inhibits the capacitiy of normal lymphocytes to respond to PHA.

(vii) there may be an excess of suppressor cells in patients with cancer (vide infra) (KIRCHNER 1978).

4.2 DIMINISHED NUMBERS OF T-CELLS AND LYMPHOCYTES.

Many reports suggest that decreased CMI is caused by decreased numbers of circulating peripheral blood lymphocytes. LEE et al (1975) correlated tumour progression with lymphocytopenia and anergy to DNCB in 183 patients. Similarly, PAPATESTAS & KARK (1974) showed that the lymphocyte counts between Stage I and IV breast cancer were significantly different. Other workers have correlated prognosis with total lymphocyte count (EILBER & MORTON 1970 a; RIESCO 1970).

The capacity to divide lymphocytes into sub-populations of T, B, and Null cells has meant that it is now possible to assess these proportions in human disease states, and to follow the effects of various treatments such as radio- and chemotherapy on individual responses. In most species the B-cell is identified by the presence of surface immunoglobulin as well as immune complexes with complement (C3) (BIANCO et al, 1970). BRAIN et al (1970)

first showed that a high percentage of human lymphoid cells would form spontaneous rosettes on incubation with sheep RBC, and proved that the phenomenon was not due to antibody, since the lymphocytes from umbilical cord blood and from women with no detectable anti-sheep agglutinin in their blood still rosetted. Elegant work on fetal lymphocytes indicated that the rosetting cells were thymus-derived with spill-over into the peripheral blood by the 20th. week of gestation (WYBRAN & FUDENBERG, 1971). Since then a profusion of methodologies leading to vastly differing results has occurred (see Table 4.3).

The human B-cell is detected by incubation of lymphocytes with sensitized SRBC in the presence of complement (EAC-rosette). On the other hand, the T-cell is detected by incubating lymphocytes with unsensitized SRBC and then centrifuging slowly to bring the cells into close contact. Resuspended cells are then counted immediately ("active" Erythrocyte-Rosette forming cell - T_a - representing cells with the highest affinity receptors for SRBC - WYBRAN & FUDENBERG 1976), or after a minimum period of 1 hour at 4°C ("total" E-RFC - T_t). The longer this incubation period, the less likely is one to find subtle differences in the levels of T-cells in cancer patients (WHITEHEAD et al, 1978).

Rosetting is an active cellular process and is affected by a number of agents which alter cell membranes and the receptor sites, or affect intra-cellular physiology (see Table 4.4). Furthermore, changes in methodology give very significantly different results. For example, incubation of lymphocytes with SRBC in FCS rather than PBS gives a higher value of E-RFC (BRAIN et al, 1970; WHITEHEAD et al, 1976), and the latter study showed that the reduced levels of E-RFC in cancer patients approached those of normal in the presence of FCS. Optimal temperature of incubation is between 4°C and 24°C and not higher than room temperature (MENDES et al, 1973). E- and EAC-rosettes are mutually exclusive if EAC-rosetting is performed at 37°C to prevent

TABLE 4.3 CLASSIC STUDIES ON E-ROSETTE FORMATION OF T-CELLS.

REFERENCE	%E-RFC	METHODOLOGY
BRAIN et al, 1970	9-31	Procedures all at room temp. Counted immediately after spinning. Rosette = 5 or more RBC / cell. Better results in serum than saline.
JONDAL et al, 1972	52-81	Incubated at 37°C. Counted after 1-2 hours on ice. Rosette = 3 or more RBC / cell.
LAY et al, 1972	20-40	Incubated at 37°C. Counted after 1 hour on ice. Rosette = 3 or more RBC / cell.
PAPAMICHAEL et al, 1972	29	Kept at 4°C for 30 mins. only. Rosette = 3 or more RBC / cell.
CHAPEL, 1973	73	At room temp. In FCS. Counted immediately after spinning. Cells washed 6 times. Rosette = 3.
MENDES et al, 1973	30.4±8.8 55.4±9.4	At room temp., then ice for 1 hour. Incubated at 20°C for 1 hour, then on ice for 1 hour. Rosette = 3.
WORTIS et al, 1973	21-62 (36.7)	as LAY et al, 1972.
WYBRAN & FUDENBERG, 1973	28.4±6.5	Lymphocytes incubated at 37°C for 1 hour prior to contact with SRBC. Procedures at room temp. Counted immediately after spinning. Rosette = 3 or more RBC / cell.
ANTHONY et al, 1975 a	61-87	as JONDAL et al, 1972; except that all carried out in N saline.

TABLE 4.4 AGENTS WHICH ALTER THE %E-RFC.

REFERENCE	AGENT	EFFECT ON E-RFC	HOW EFFECT IS MEDIATED
BRAIN & GORDON, 1971	Sodium cyanide	Abolished	Kills cells
	Methotrexate	Abolished	Kills cells
	Antilymphocyte serum	Abolished	Masks receptors on cell surface
JONDAL et al, 1972	Trypsin	Abolished	Irreversibly removes surface receptors
	Iodoacetate	Abolished	Inhibits glycolysis
CHAPEL 1973	Papain	Increased	Removes "masking" receptors on cells - reversible
	Trypsin	Decreased	Irreversibly removes surface receptors
	Phospholipase	Decreased	Reversibly removes surface receptors
WORTIS et al, 1973	Anti-T serum	Abolished	Kills cells
	Anti-B serum	No effect	
GALILI & SCHLESINGER 1974	Neuraminidase	Increased	Removes sialic acid from cell surface and so decreases negative charge on cells
HOLLAND et al, 1975	Brinase	Increased	Proteolytic agent which unmasks SRBC binding sites and thus rosettes stronger and tighter
PANG et al, 1976	EDTA	Abolished	Chelates calcium; rosetting restored on addition of $CaCl_2$
WHITEHEAD et al, 1976	Papain	Increased	Phenomenon reversed by incubating in "cancer" serum but not "normal" serum
NÉKAM et al, 1977	Transfer Factor	Increased	Effect on T_a but not T_t

E-rosetting from occurring. A recent report however suggests that at 29°C a "high-affinity" rosette forming cell can be quantitated, and this test method may be a more sensitive means of assessing differences between cancer and normal subjects, than incubation at 4°C (QUAN & BURTIN 1978).

Rough handling and rapid centrifugation decrease the number of rosettes (ANTHONY et al, 1975 a) and extensive washing increases the number of rosettes, presumably by removing "masking factors" in normal or cancer serum. Neuraminidase and proteolytic enzymes increase numbers substantially (GALILI & SCHLESINGER 1974). All accounts stress the importance of carefully standardizing the technique used in any particular laboratory, and the fruitlessness of comparison between different workers' results, where identical techniques have not been used.

The nature of the receptor on the T-cell is unknown, but calcium appears to act as a bridge between the two cells and this is inhibited by chelating agents (PANG et al, 1976). Antigen recognition is not involved and the phenomenon is recognized in other species e.g. pig T-cells bind with rabbit RBC, dog T-cells bind with human RBC (WYBRAN & FUDENBERG 1976).

(i) Human non-malignant disease states and E-RFC. Absent or low levels of rosetting cells are found in systemic lupus erythematosus, congenital hypoplasia of the thymus, Wiskott-Aldrich Syndrome, and chronic mucocutaneous candidiasis (WYBRAN et al, 1973), and this would seem relevant when considering the known association of these diseases with cancer. Defective rosetting is found in viral URTI's and the low levels of T_a return to normal within 5 days of remission of symptoms (WYBRAN & FUDENBERG 1973). The persistence of defective rosetting in viral hepatitis A and B is associated with the subsequent development of chronic active hepatitis (CHISARI et al, 1977). Bacterial infections and severe illnesses such as myocardial infarction do not appear to affect the levels of E-RFC.

(ii) Human Cancer and E-RFC. Numerous studies have shown abnormal levels of both T_a and T_t populations (see Table 4.5), which appear to be related to the Stage of disease and precede the appearance of metastases (WYBRAN & FUDENBERG 1973). This fall in E-RFC may be associated with an increase in B-cells and Null cells (WHITEHEAD et al, 1976; ANTHONY et al, 1975 a), and it has been suggested that Null cells represent T-cells which fail to rosette because of damage, immaturity or blocking of receptors by immune complexes. WHITEHEAD et al (1976) and RUGARLI et al (1975) have both demonstrated that "factors" on the surface of T-cells can be removed with extensive washing or incubation in papain, so increasing significantly the numbers of E-RFC. This can be reversed by incubation in cancer serum.

Correlations between in vivo DHR and in vitro assays and the numbers of E-RFC have been made (FELSBURG et al, 1976; KERMAN et al, 1976). PETER et al (1975) found that patients with a low %E-RFC had low reactivity in cell-mediated cytotoxicity against melanoma cells. It is always important to assess both the %E-RFC as well as the absolute numbers, since one value and not the other may be reduced (OLKOWSKI & WILKINS 1975).

The "active" component (T_a) is also decreased in malignancy and may be a better in vitro correlate of patient immunocompetence that the "total" E-RFC value (WYBRAN & FUDENBERG 1973; HOROWITZ et al, 1975; KERMAN et al, 1976). Although their immunological role is not defined, these cells are thought to be functionally active T-cells, since their % is high in human tonsils, the mixed lymphocyte culture (GERGELY et al, 1976), and in PHA blastogenesis where their responsiveness is higher than that of other T-cells (SASAKI et al, 1975).

(iii) Surgery and E-RFC. Although many animal experiments show that depressed CMI is restored after adequate surgery, the human situation is more complex. Several reports indicate that surgery may decrease lymphocyte counts, T-cell counts, and the ability of cancer patients to initiate immune responses (PARK et al, 1971; ROTH et al, 1974; ANTHONY et al, 1975 b; MILLER et al, 1976). It is also

TABLE 4.5 REPRESENTATIVE STUDIES ON % AND TOTAL NUMBERS OF E-RFC IN HUMAN CANCER.

TUMOUR	REFERENCE	NO.PTS.	%E-RFC Con.	%E-RFC Cancer.	COMMENTS
Various	WYBRAN & FUDENBERG, 1973	104	28	12	Patients with metastatic disease had sig. reduced levels T_a, whereas remission patients had same values as controls.
Various	HOLLAND et al, 1975	14	61	36	Sig. reduced levels. Brinase treatment restored values to normal.
Various	KERMAN et al, 1976	102	26	29	No difference in $\%T_a$ between controls and cancer patients, but absolute numbers sig. reduced.
Bladder and Prostate	CATALONA et al, 1974	46	73	61	Sig. reduced %E-RFC and also absolute cell count.
Bronchogenic	ANTHONY et al, 1975 a	31	77	29-89	66% patients had %E-RFC less than 1 s.d. from the mean value of normals, and 50% dead within 3 months.
Bronchogenic	ROBERTS et al, 1977	68	66	66	No. sig. difference, but sig. lymphopenia in patients with metastases.
Melanoma	PETER et al, 1975	21	53	37	Sig. reduction correlated with poor reactivity in tests of cell-mediated immunity.
Breast	STEIN et al, 1976	255	56	45	No sig. diff. although 33% patients had levels below lowest control value. Sig. lymphopenia in post-op patients and those with metastases.
Breast	WHITEHEAD et al, 1976	71	55	44	Only Stage III patients had levels which were not sig. reduced, suggesting ? different biological behaviour of tumour. Papain treatment restored values to normal.
Breast	TEASDALE et al, 1977	100	54	48	Subsequent study found sig. reduction in all Stages, together with sig. lymphopenia.
Head & Neck	OLKOWSKI & WILKINS, 1975	46	75	51	Sig. reduction in both % and absolute numbers of E-RFC, although many patients had normal %E-RFC and reduced absolute count only.
Head & Neck	DEEGAN & COULTHARD, 1977	26	68	57	Sig. reduced in advanced but not local disease; absolute count not sig. different from controls.
Hodgkin's Disease	FUKS et al, 1976	87	65	51	%E-RFC sig. reduced but absolute counts same as controls (although 39% total lymphocyte values more than 2 s.d. below mean for normals).
Cervix SCC	LEVY et al, 1978	42	75	70	%E-RFC same as controls, but absolute count sig. reduced in patients with metastatic disease.

known that anaesthesia can cause depressed CMI, especially to tumour antigens (DUNCAN et al, 1977), and this may in part explain the observation by some that surgery can have a detrimental effect and result in rapid growth of residual tumour.

(iv) Radiotherapy and E-RFC. Radiotherapy causes a consistent and prolonged lymphopenia, accounted for mostly by T-cell depression. STJERNSWÄRD et al (1972) recorded a profound decrease in %E-RFC in women given parasternal radiotherapy for breast cancer, with PHA responses decreased by up to 20% of their pre-radiotherapy values. Even up to 10 years post-radiotherapy, responses to mitogens and T-cell counts may be reduced (FUKS et al, 1976; ROMAGNINI et al, 1976). This is disturbing in its implications, and is highlighted by 2 reports of an increased mortality and incidence of visceral metastases in patients treated with radiotherapy for breast cancer (BRUCE 1971) and renal cancer (PEELING et al, 1969).

KOHORN et al (1978) found that radiotherapy in women with ovarian cancer depressed T-cells markedly but left B-cells unaffected. Recovery occurred and there was no consistent decrease in CMI as assessed by lymphocyte mediated cytotoxicity against tumour cells. A recent report now suggests that in patients with non-Hodgkin's Lymphoma who responded to radiotherapy, increased mitogenic responses occurred and the low %E-RFC pre-radiotherapy rose to normal levels afterwards. It is noteworthy that ANTHONY et al (1975 b) found post-radiotherapy lymphocytes difficult to resuspend, and the rosettes easily disrupted.

(v) Chemotherapy and E-RFC. Cytotoxic agents depress both primary antibody responses (HERSH et al, 1966; 1971) and cellular responses in vivo (AL-SARRAF et al, 1970) and in vitro (HERSH & OPPENHEIM 1967). However, the effects are complicated and may be quite paradoxical. For example, BORELLA & WEBSTER (1971) showed that a combination of drugs in Acute Lymphocytic Leukaemia which depressed antibody responses did not effect in vitro lymphocyte responses to PHA. This continuous chemotherapy had no effect even

after 29 months, whereas intensive chemotherapy abolished PHA responses after 3 days.

However, further work now suggests that intermittent chemotherapy, while causing some cell death and depletion, does not cause alterations in the functional capabilities of the remaining cells, whereas continuous chemotherapy does (HERSH et al, 1973; HUNNINGHAKE & FAUCI 1977). Multiple agent chemotherapy causes a more profound depression in "active" E-RFC and B-cells than single agent chemotherapy, and this takes effect within 48 hours of being administered. The decrease may persist for some time (EZDINLI et al, 1978).

It appears that there may be some circumstances in which chemotherapy is immunologically advantageous. For example, Cyclophosphamide in normal animals leads to a temporary decrease in immunoblasts which drain from the lymph nodes of antigenic sites; yet in animals, where a state of immune tolerance has been induced, Cyclophosphamide reverses it with a 4-fold proliferation of T-cells in the draining lymph nodes. This reversal of immunological unresponsiveness derives from the drug's ability to act preferentially on B-cells and short-lived cells with a rapid turn-over in lymphoid tissue (POULTER & TURK 1972; POLAK & TURK 1974), as well as destroying suppressor cells. Most chemotherapeutic agents appear to have differential effects on T and B-cells (OHNUMA et al, 1978) and these effects must be considered when interpreting in vitro results of immune function. For example, under some circumstances Cyclophosphamide minimally effects mitogen responses, but markedly suppresses antigen-induced lymphocyte proliferation in vitro (BALOW et al, 1975).

Animal studies suggest synergism between inherent anti-tumour immunity of the host and chemotherapy which controls the tumour burden (STOLFI et al, 1974). Likewise, in human malignancy there seems to be a relationship between those whose immune system recovers from immunosuppressive chemotherapy and those who stay in remission from AML for a prolonged period (HERSH et al, 1971). Similarly, in patients with solid tumours recovery of immunological

responses with significant overshoot above pre-treatment levels is associated with regression and may be used as a prognostic indicator (CHEEMA & HERSH 1971). Rebound to tumour antigens is also associated with good prognosis, even though chemotherapy causes decreased responses, not only by immunosuppression but also by decreasing the production of tumour antigen (NATHANSON et al, 1971).

(vi) Immunotherapy and E-RFC. The administration of Transfer Factor to patients with immunodeficiency diseases leads to an appearance of cellular immunity and a significant increase in the number of "active" E-RFC (WYBRAN et al, 1973). In cancer, the administration of Thymic Factor (NÉKAM et al, 1977) and BCG (WYBRAN & FUDENBERG 1976) also causes an increase in T_a. It is thought that BCG releases tumour antigens or enzymes which directly activate T-cells, since there is an increase not only in T_a but also other immunological parameters (LIEBERMAN et al, 1975; ANTHONY et al, 1975 b). This is discussed further in Chapter 7.

4.3 SUPPRESSOR CELLS AND THEIR ROLE IN HUMAN CANCER.

It is now well established that immune responses, both antibody production and cell-mediated proliferation, are controlled by a population of suppressor cells (GERSHON 1974). In both animals and humans, suppressor cells can be induced in vitro by a number of immunological stimuli, including non-specific mitogenic stimulation (RICH & RICH 1975; SHOU et al, 1976). Such activated suppressor cells can then be shown, in vitro, to inhibit lymphocyte responses of other cells to antigenic stimuli.

Suppressor cells are implicated in the pathogenesis of a number of human non-malignant diseases, including certain fungal diseases and Common Variable Hypogammaglobulinaemia where they are present in excessive numbers (WALDMANN et al, 1974); and Systemic Lupus Erythematosus where their absence leads to auto-immune complications (HOROWITZ et al, 1977).

There is now evidence in animal tumours that excess suppressor cells are associated with immunosuppression (ROTTER & TRAININ 1975). GASCOYNE et al (1978) have shown that immunosuppression in tumour-bearing mice was associated with suppressor cells, and these disappeared on resection of the tumour. They were also associated with the production of a suppressive serum factor, which likewise disappeared as immunocompetence returned. A number of human tumours have also been investigated in relation to excessive suppressing cell populations, including Hodgkin's Disease (TWOMEY et al, 1975), multiple myeloma (BRODER et al, 1975), breast cancer (BLÖMGREN et al, 1976) and patients with a variety of solid tumours (ZEMBALA et al, 1977). The latter two studies suggested that suppressor cells might be a macrophage subpopulation, although in some patients inhibitory T-cells were also found.

Hence, work in this area is still elementary, and it should be stressed that none of the papers reviewed so far have measured the suppression of specific anti-tumour immune responses, but only non-specific responses to mitogens or secondary-recall antigens.

4.4 EXPERIMENTAL DESIGN: IMMUNOCOMPETENCE IN PATIENTS WITH OVARIAN CANCER AND ITS RELATIONSHIP TO SUB-POPULATIONS OF LYMPHOCYTES, FOLLOWING SURGERY, CHEMOTHERAPY AND IMMUNOTHERAPY.

The aim of the following experiments was :

(i) to assess %E-RFC, both "active" (T_a) and "total" (T_t) populations, and their absolute numbers in the peripheral blood of patients with ovarian cancer

(ii) to assess cell mediated immunity in these patients using in vitro blastogenesis to PPD

(iii) to examine the effects of chemotherapy, surgery and immunotherapy on the levels of E-RFC

(iv) to use T-cell rosetting as a means of separating cells into T-enriched and T-depleted populations, in order to examine their individual responses

(iv) to examine the incidence of suppressor cells in these patients.

MATERIALS AND METHODS:

Patient groups were: (a) Blastogenic responses to PPD:

21 normal, healthy, non-pregnant controls, age matched where possible with the cancer patients, and obtained mostly from the Gynaecology Clinic in St. Bartholomew's Hospital, where most where awaiting elective surgery for menorrhagia or prolapse

9 patients with ovarian cancer in remission or with "static disease"

25 patients with relapsing ovarian cancer

15 patients with other tumours that were associated with minimal tumour burden e.g. Stage I endometrial carcinoma

(b) %E-RFC, T_a and T_t; absolute numbers of T-cells

15 normal controls

21 patients with minimal tumour load i.e. in remission, with "static disease" or immediately post-operatively when at least 75% of the tumour had been removed

29 patients with large tumour load - 13 patients who were pre-operative and 16 who were inoperable, or were post-operative when a biopsy only was possible; all patients were examined prior to chemotherapy or radiotherapy

11 patients who had been receiving immunotherapy for at least 3 months; 9 of these had active disease at the time of study.

Blastogenic Assay: as previously described, lymphocytes in a concentration of $1x10^6$/ml. were stimulated with 10µg PPD.

%E-RFC: at the same time as the blastogenic assay was done, $2x10^6$ lymphocytes were removed and rosetted in 4 different tubes as previously described. Duplicate tubes were counted immediately for "active" rosettes and "total" rosettes were counted after overnight incubation at 4^oC.

Suppressor Cell Assay: lymphocytes from 4 patients were tested. One of these patients had Stage II disease, another static disease, and the remaining two had progressive disease not responding to chemotherapy. Suppressor cells were induced by stimulation with Con A, and the same donor (normal) lymphocytes were used on each occasion to assess

the degree of suppression in the Mixed Lymphocyte Reaction. Blastogenesis with the donor lymphocytes on each occasion showed that there was minimal week-to-week variation of their responsiveness.

The Mann Whitney U Test for non-parametric populations was used in all statistical analysis.

RESULTS:

The absolute lymphocyte counts for the patient groups are illustrated in Fig. 4.1 The median value for control subjects was 1940 (range 364 to 2920) compared with 1012 for relapse patients (range 160 to 3294), 1558 for patients with minimal tumour load (116 to 2849) and 1147 for patients receiving immunotherapy (189 to 3213). Although the value for relapse patients was less than that of controls or remission patients, this difference was not statistically significant. However, patients receiving immunotherapy had significantly less lymphocytes than controls ($p < 0.05$). (see Table 4.6)

Fig. 4.2 illustrates the % T_a and T_t. The median value for control subjects was 15% T_a / 59% T_t compared with 9% / 41% for patients with relapsing disease, 19% / 48% for remission patients and those with minimal tumour load, and 17% / 45% for immunotherapy patients. The significance of these values is summarised in Table 4.6 Briefly, patients with a large tumour load had significantly less $\%T_a$ and $\%T_t$ than controls or remission patients. Patients in remission had similar $\%T_a$ as control subjects, but their $\%T_t$ was significantly less. Although immunotherapy patients, 9 of whom were in relapse, had $\%T_t$ similar to other relapse patients, their $\%T_a$ was significantly higher and similar to values for control subjects. There was no difference in results when patients with a large tumour load were further analysed in relation to whether they were pre-operative, or post-operative and awaiting further treatment.

The absolute numbers of T_a and T_t are represented in Fig. 4.3 The median values for control subjects were

234 T_a / 1113 T_t, compared with 90 / 407 for patients with relapsing disease, 287 / 734 for patients with minimal tumour load, and 166 / 487 for patients on immunotherapy. Hence patients with large tumour burden had significantly less total numbers of T_a and T_t than controls and patients on immunotherapy also had significantly less T_t than the controls. Patients with relapsing disease also had significantly less T_a than patients in remission.

Fig. 4.4 illustrates blastogenic responses to PPD. There is no significant difference in the responsiveness of lymphocytes between the various groups (see Table 4.7). However, it is worth noting that 72% of the patients with large tumour load had responses less than the median value for the control group. In unstimulated cultures of lymphocytes, the sponteneous incorporation of ^{125}IUDR is significantly less in cancer patients with progressive disease than in the controls ($p < 0.01$; see Fig. 4.5).

Fig. 4.6 and Table 4.8 confirm that the responsiveness to PPD is a property predominantly of the T-cell population. The T-depleted cell population showed responses which were significantly less than those in either the unseparated or T-enriched populations ($p < 0.05$). In the unstimulated cultures, the T-enriched population had a significantly greater spontaneous incorporation of ^{125}IUDR than the unseparated population of lymphocytes ($p < 0.02$), suggesting that other cells may maintain a suppressive action on T cells in their natural state (Fig. 4.7).

A correlation between responses to PPD, tumour extract and %E-RFC was calculated on 27 of the 29 patients with progressive disease. The correlation was negative.

Surgery had variable effects on lymphocyte function, but the general trend was for all in vitro parameters to be elevated in the week immediately post-operatively and then to settle to levels, which were generally higher than those pre-operatively (Fig. 4.8, Table 4.9). It appeared that this general increase in lymphocyte reactivity, including

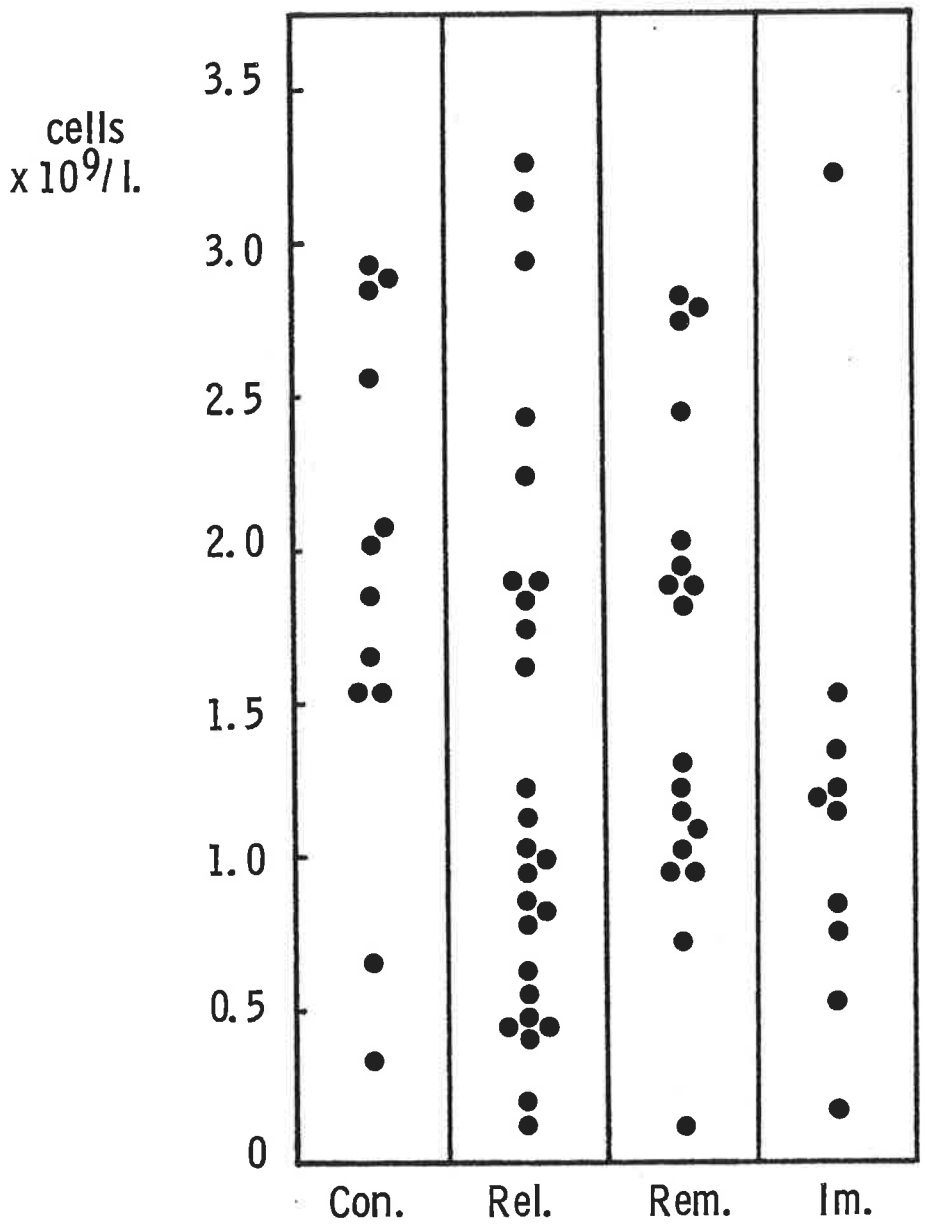


FIGURE 4.1 ABSOLUTE LYMPHOCYTE COUNTS IN CONTROLS, CANCER PATIENTS AND IMMUNOTHERAPY PATIENTS

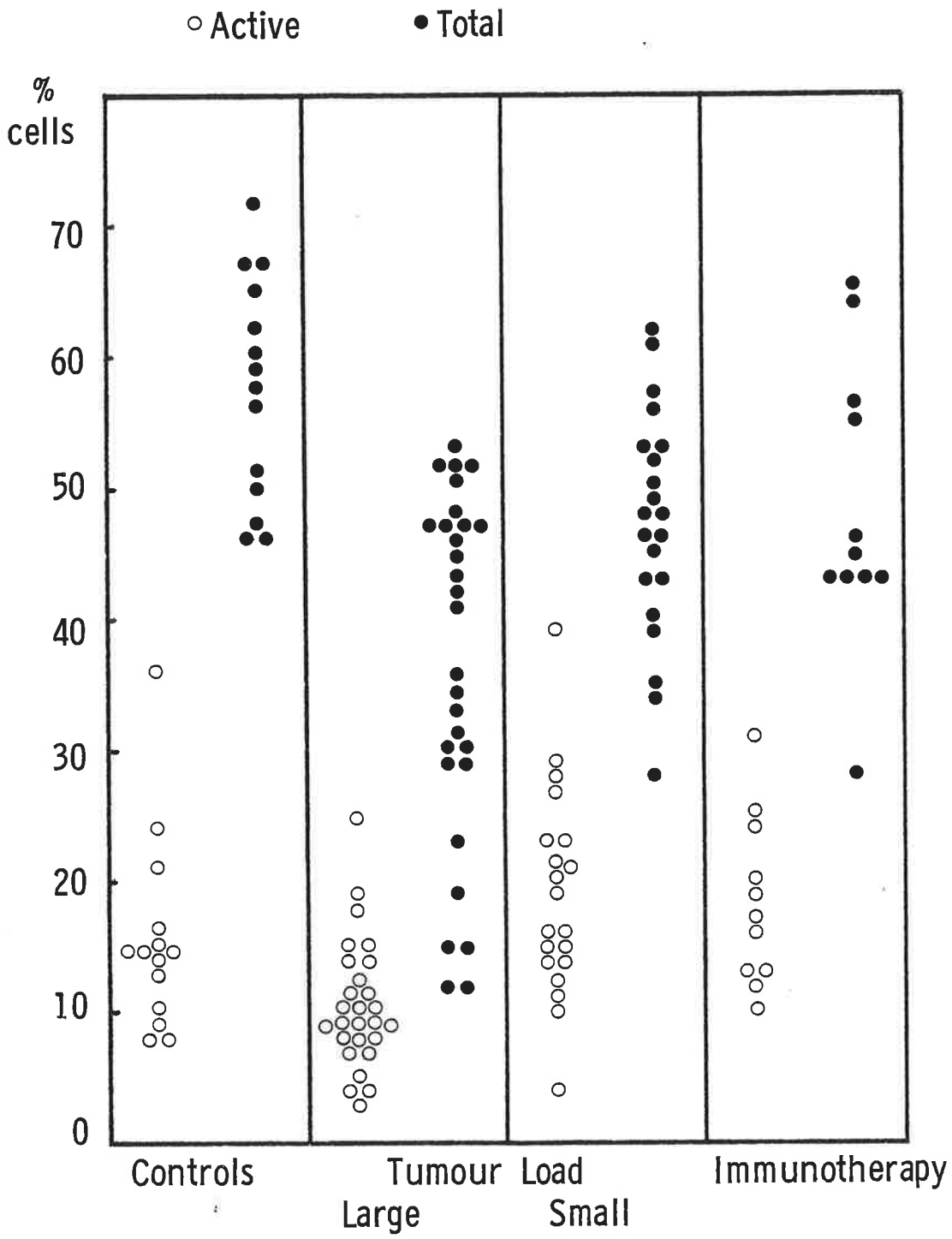


FIGURE 4.2 % ACTIVE AND TOTAL E-RFC IN CONTROLS, CANCER PATIENTS AND IMMUNOTHERAPY PATIENTS

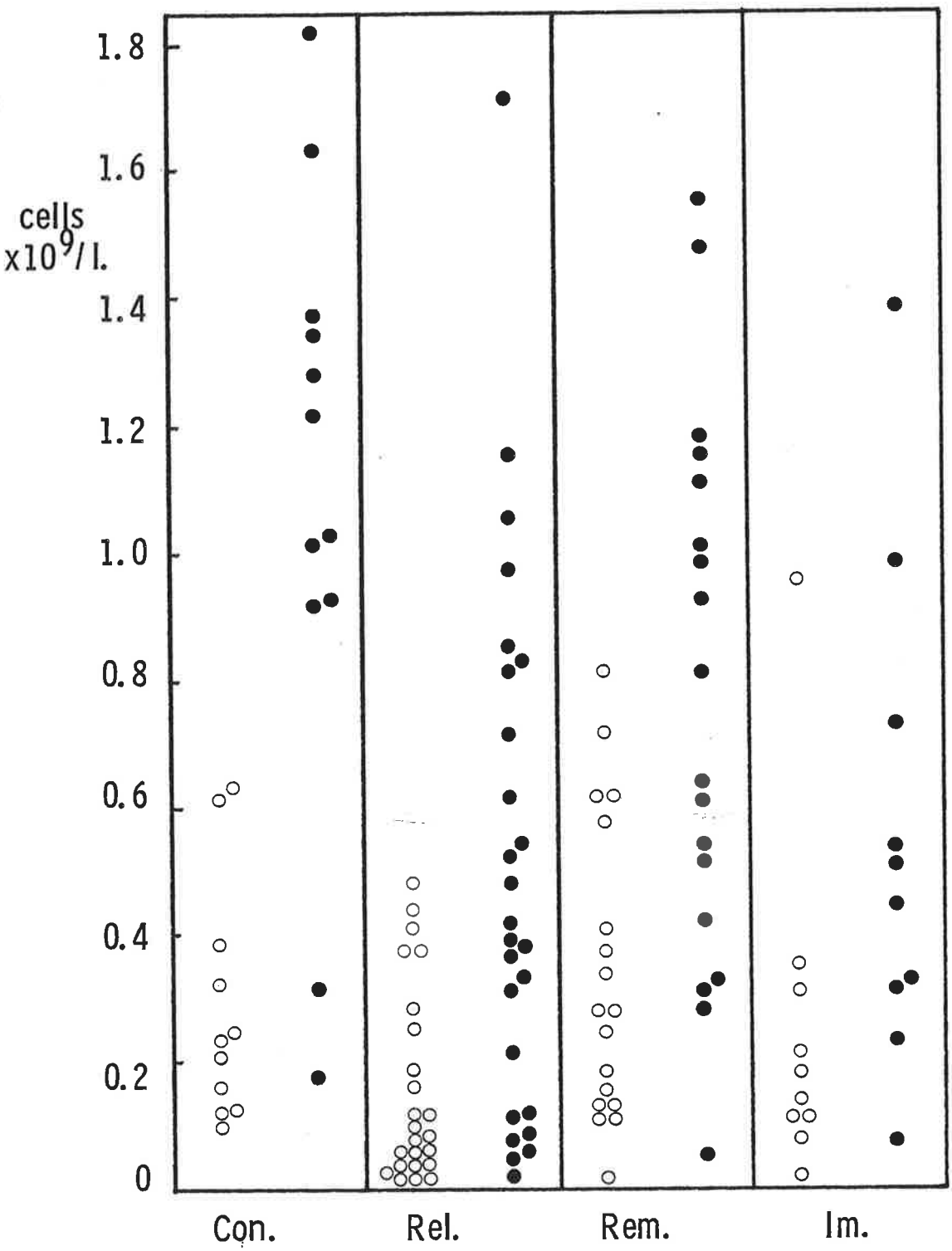


FIGURE 4.3 ABSOLUTE NUMBERS OF ACTIVE AND TOTAL E-RFC IN CONTROLS, CANCER PATIENTS AND IMMUNOTHERAPY PATIENTS

○ Active E-RFC ● Total E-RFC

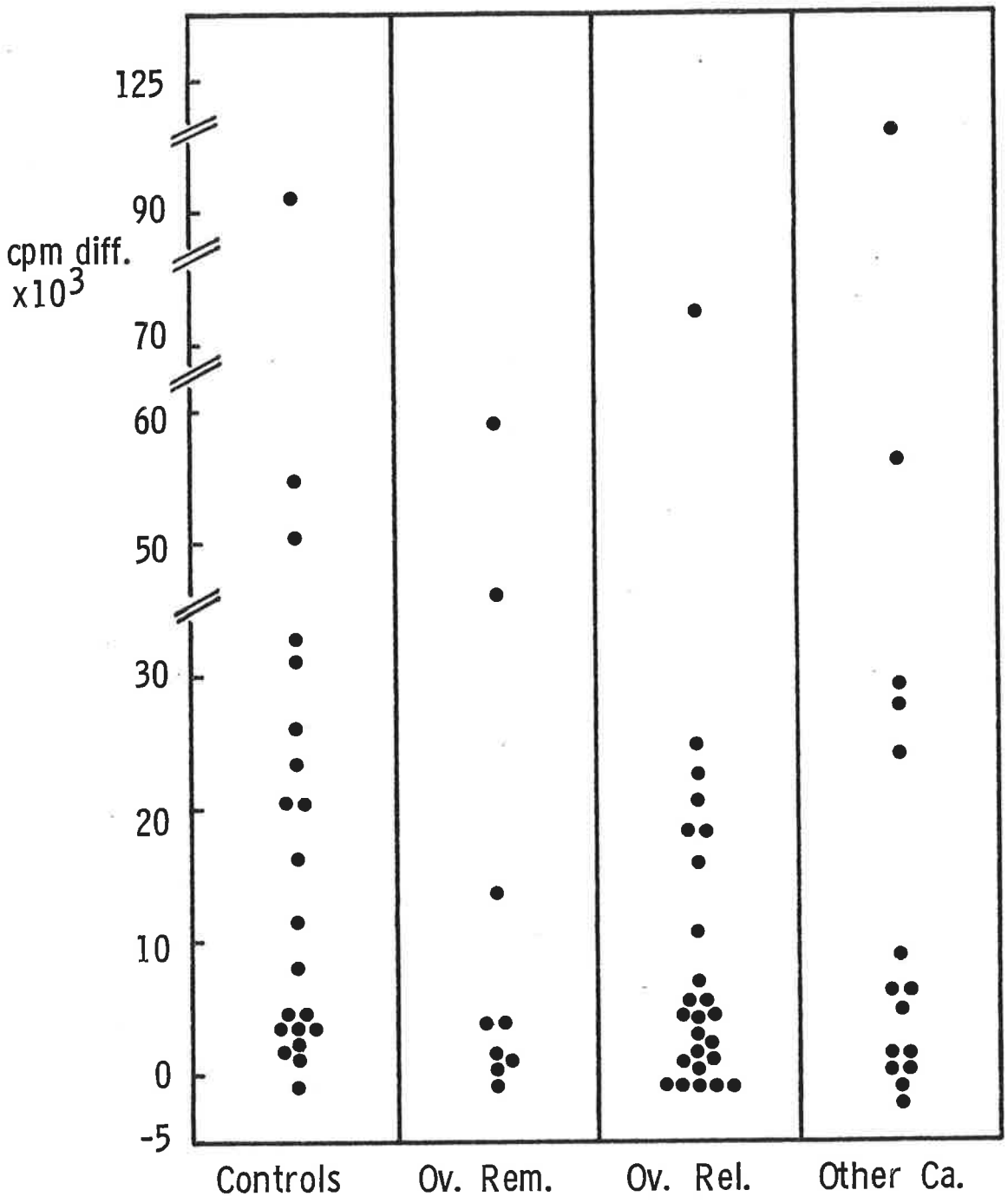


FIGURE 4.4 BLASTOGENIC RESPONSES TO 10µg PPD IN CONTROLS AND PATIENTS WITH OVARIAN AND OTHER CANCERS

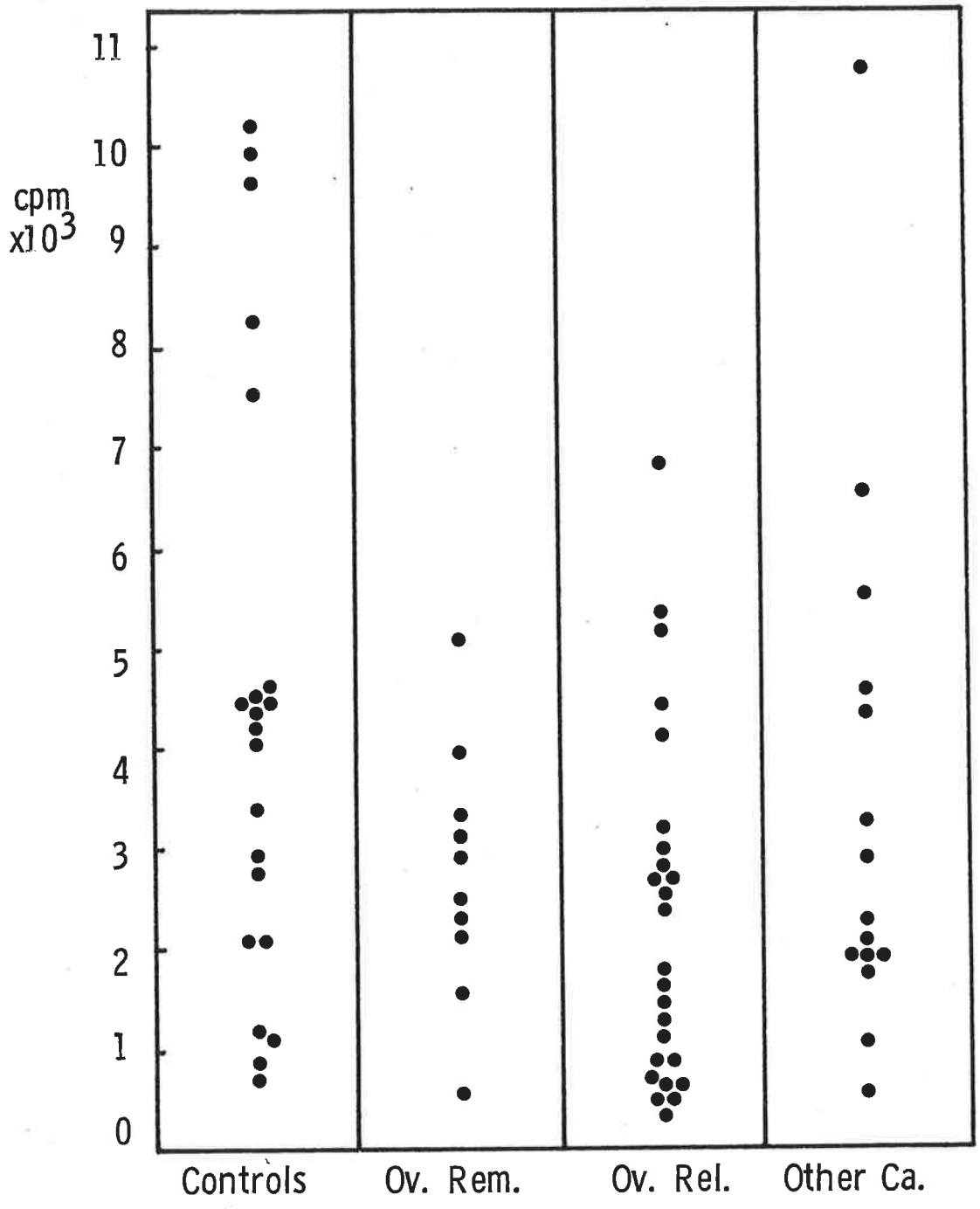


FIGURE 4.5 INCORPORATION OF ^{125}IUDR IN UNSTIMULATED LYMPHOCYTES FROM CONTROLS AND PATIENTS WITH OVARIAN AND OTHER CANCERS

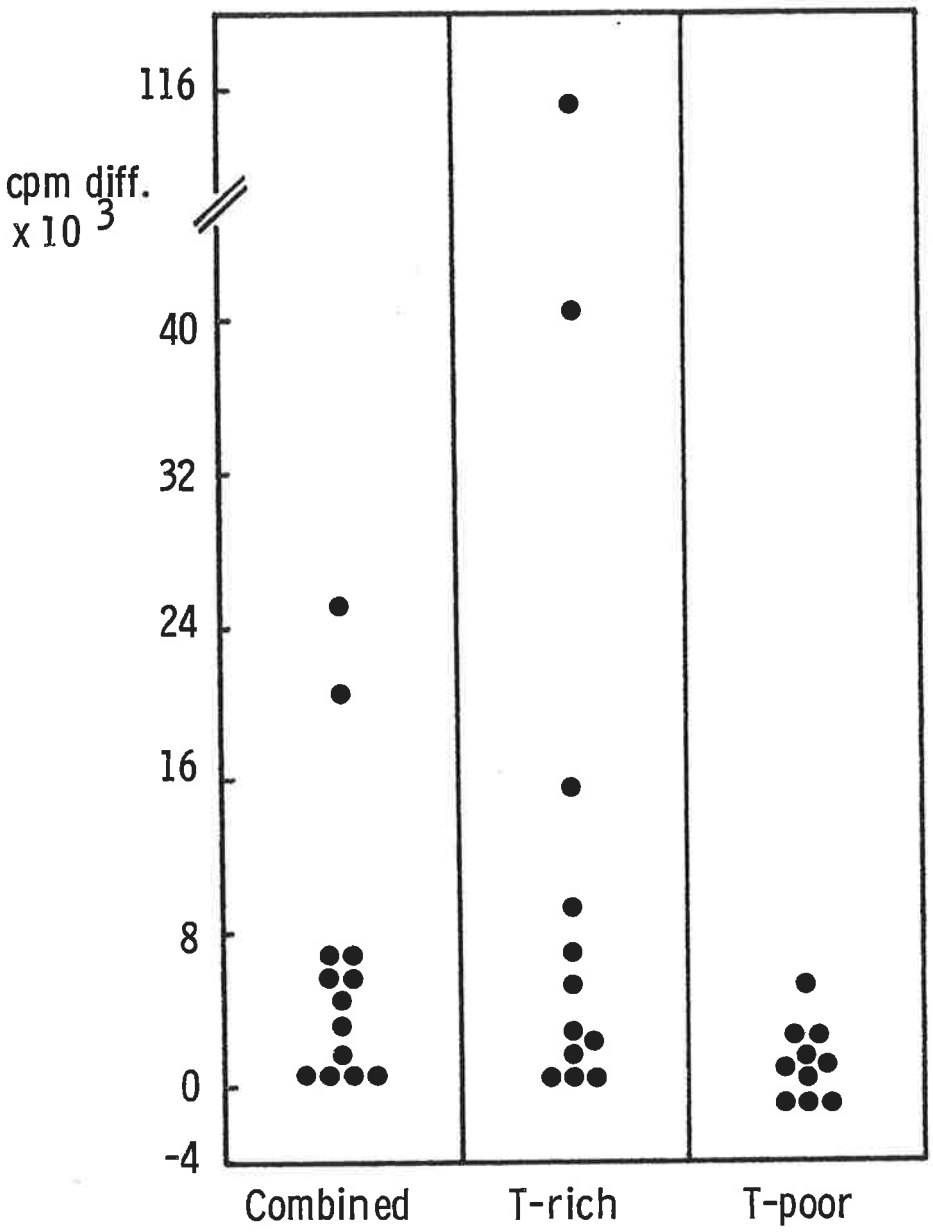


FIGURE 4.6 BLASTOGENIC RESPONSES OF CELL SUB-POPULATIONS IN OVARIAN CANCER PATIENTS TO 10µg PPD

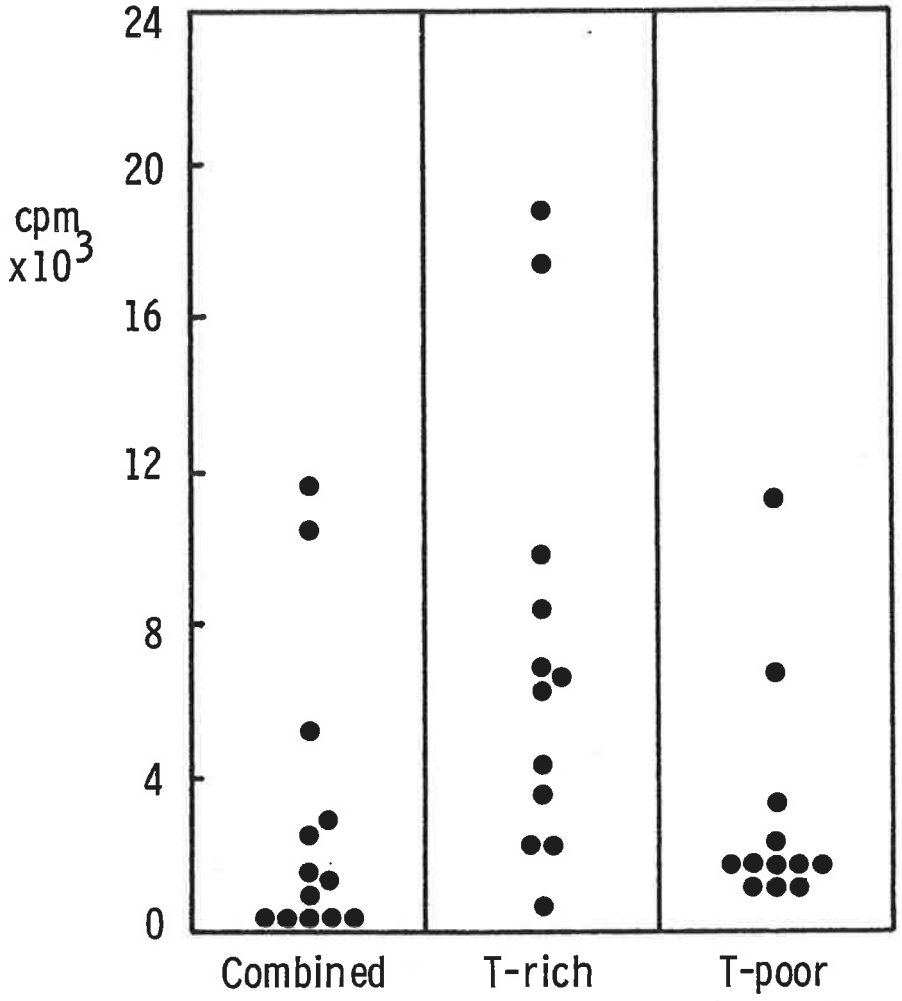


FIGURE 4.7 INCORPORATION OF ^{125}IUDR IN UNSTIMULATED LYMPHOCYTE SUB-POPULATIONS OF PATIENTS WITH OVARIAN CANCER

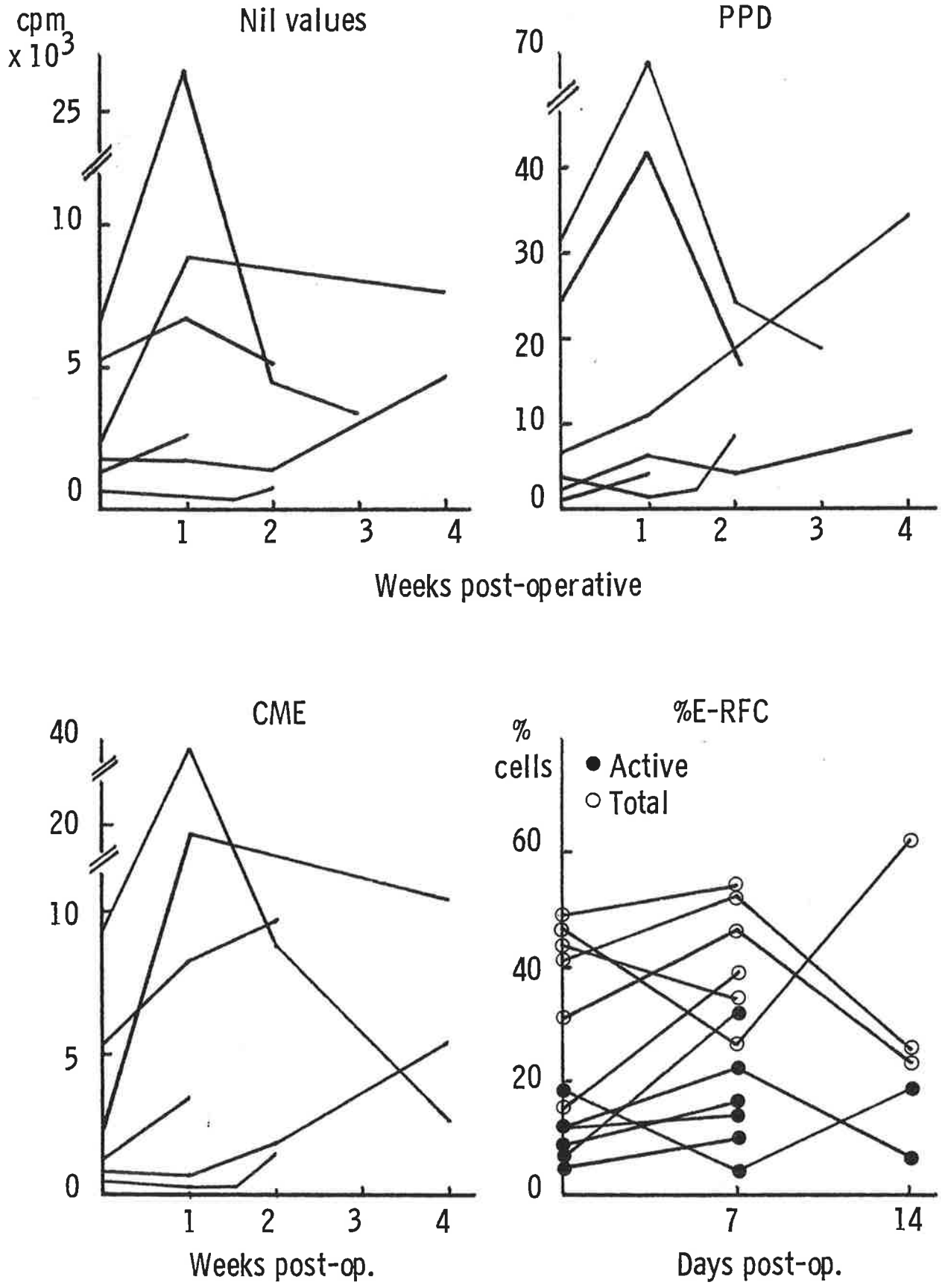


FIGURE 4.8 EFFECT OF SURGERY ON IN VITRO BLASTOGENIC RESPONSES AND %E-RFC

spontaneous incorporation of ^{125}IUDR into unstimulated cells, might be related to the increased levels of T_a and T_t.

Figs. 4.9, 4.10, 4.11, 4.13 (Table 4.10) illustrate the effects of chemotherapy on various parameters of lymphocyte function. In the three patients who were on pulsed chemotherapy, there were initial rises in the % of T_a and T_t in the face of falling total white cell and lymphocyte counts. When suppression was manifest, usually at Days 7 to 10, this was followed by a period of recovery with rebound by Day 14. Figure 4.14 illustrates the mean values of these parameters in 6 patients, 3 of whom were receiving immunotherapy (see Chapter 7). Blastogenic responses similarly recovered after immunosuppression, usually with rebound. It is of interest to note that the patient on continuous Cyclophosphamide (Fig. 4.9) showed progressively diminished %E-RFC and blastogenic responses, in the face of chronic immunosuppression.

In the one study of lymphocyte sub-populations (Fig. 4.12, Table 4.10 Appendix), the T-depleted cells (mainly B cells) were not affected by chemotherapy, whereas the T-cell population suffered an initial depletion in its ability to respond in vitro, followed by a rapid recovery.

Although only 4 patients were studied for suppressor cells, it is interesting to see that the 2 patients in relapse showed very marked induction of suppressor activity, whereas of the 2 patients with static disease, one showed slight suppression and the other showed suppression only of Con A responses (Fig. 4.15, Table 4.11).

DISCUSSION:

Numerous investigations have shown that the immunological responsiveness in vivo and in vitro of cancer patients is depressed, and that the degree of depression correlates with the extent of disease and tumour load. In this Chapter a number of immunological parameters in both cancer patients and normal controls have been examined, and also the effects of iatrogenic immunosuppression with chemotherapy on these functions.

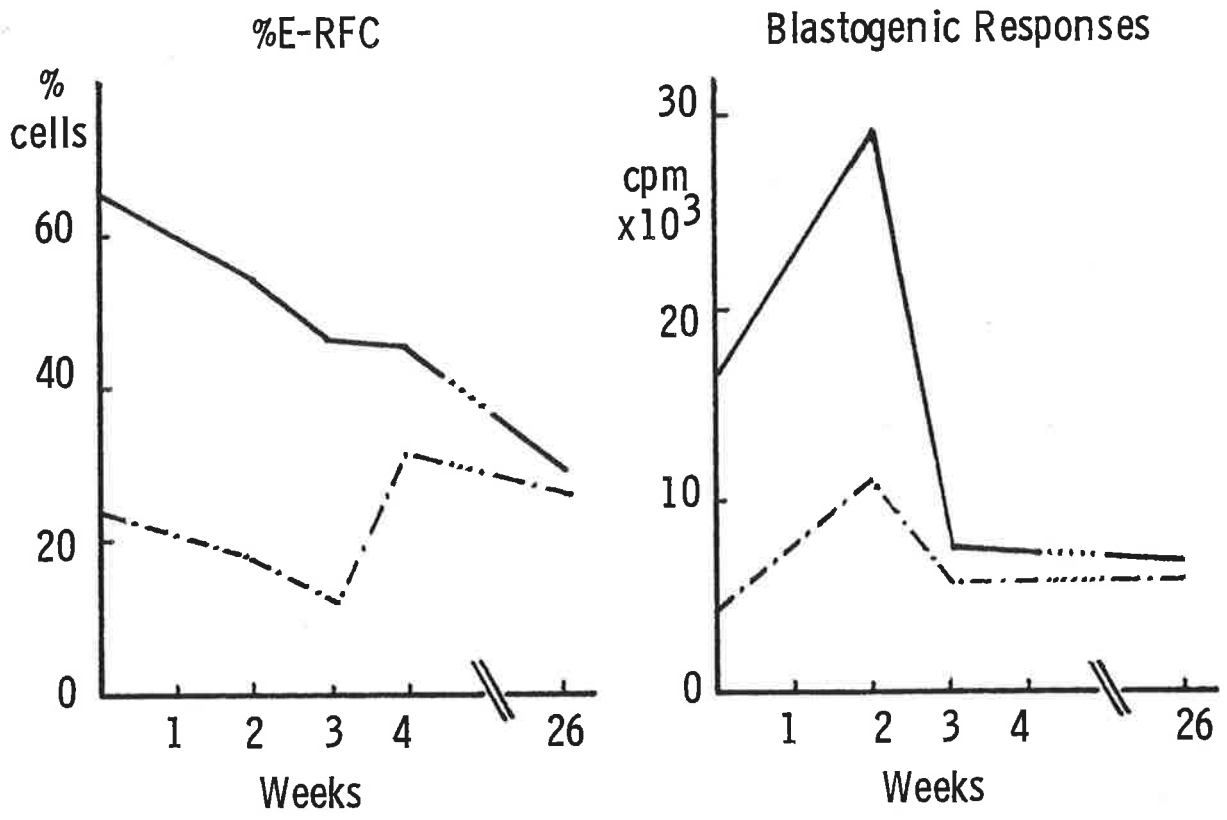


FIGURE 4.9 EFFECT OF CHEMOTHERAPY ON VARIOUS PARAMETERS OF LYMPHOCYTE FUNCTION (Patient S.G.)

CHEMOTHERAPY : Continuous oral Cyclophosphamide 100mg. die

IN THE FOLLOWING FIGURES :

—— %E-RFC (Total); WCC; Total T-cell count; Blastogenic Responses to PPD

–·–· %E-RFC (Active); % lymphocytes; Active T-cell counts; Blastogenic Responses to Ovarian Tumour CME

····· Total lymphocyte count

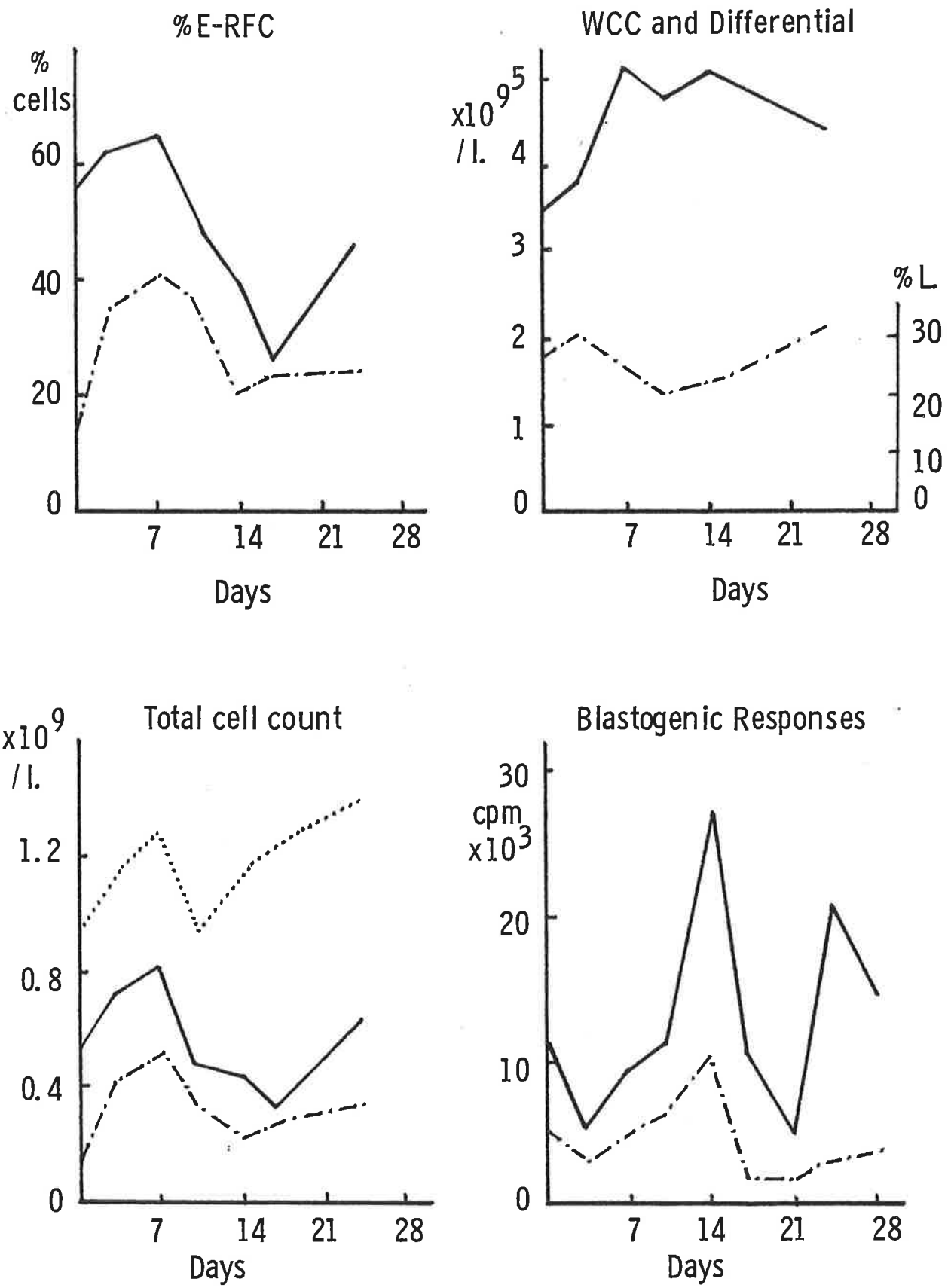


FIGURE 4.10 EFFECT OF CHEMOTHERAPY ON VARIOUS PARAMETERS OF LYMPHOCYTE FUNCTION (Patient D.W.)

CHEMOTHERAPY : Oral Cyclophosphamide, Hexamethylmelamine, and Methotrexate for 14 days

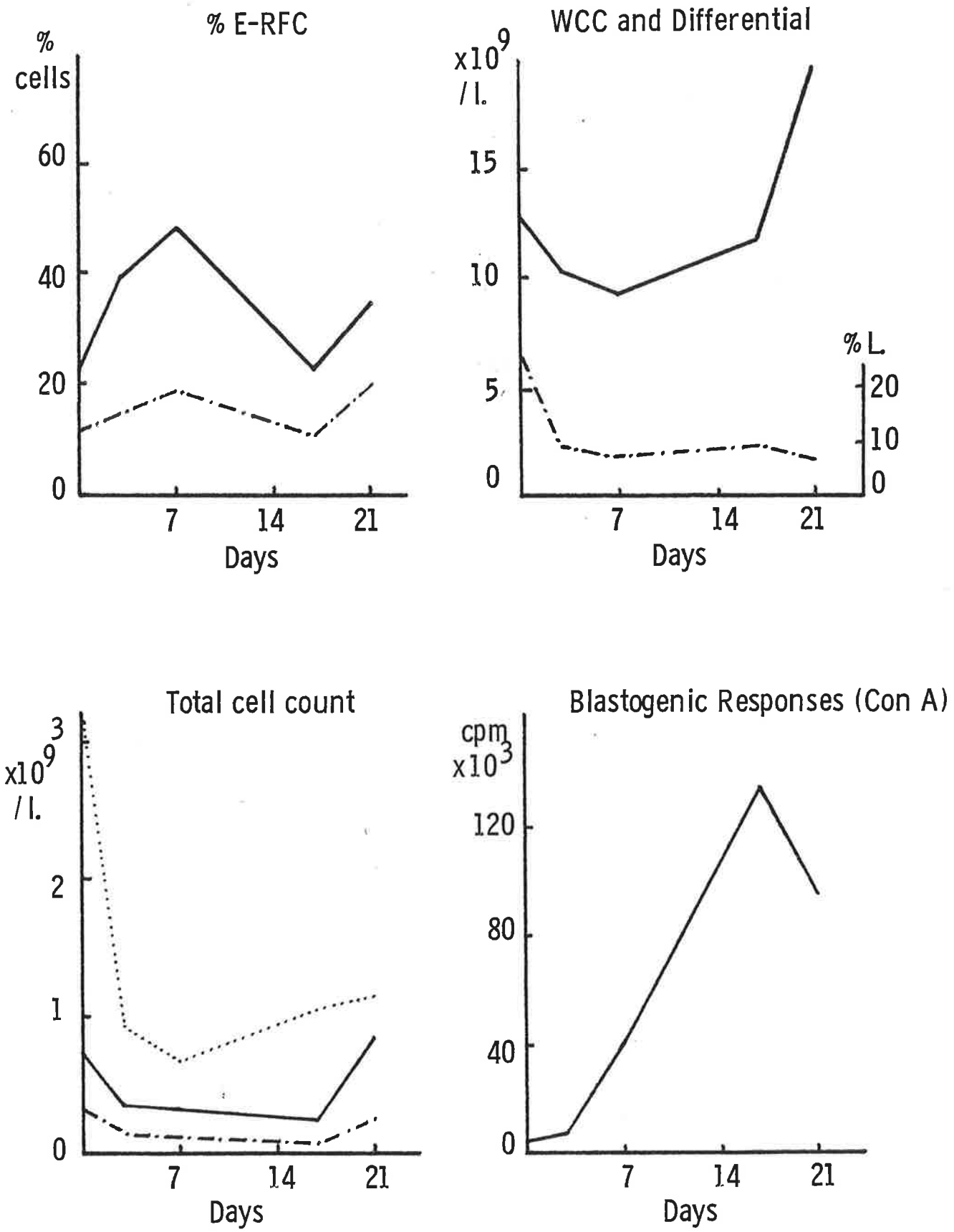


FIGURE 4.11 EFFECT OF CHEMOTHERAPY ON VARIOUS PARAMETERS OF LYMPHOCYTE FUNCTION (Patient R.M.)

CHEMOTHERAPY : Adriamycin and Cyclophosphamide I/V on Day 1

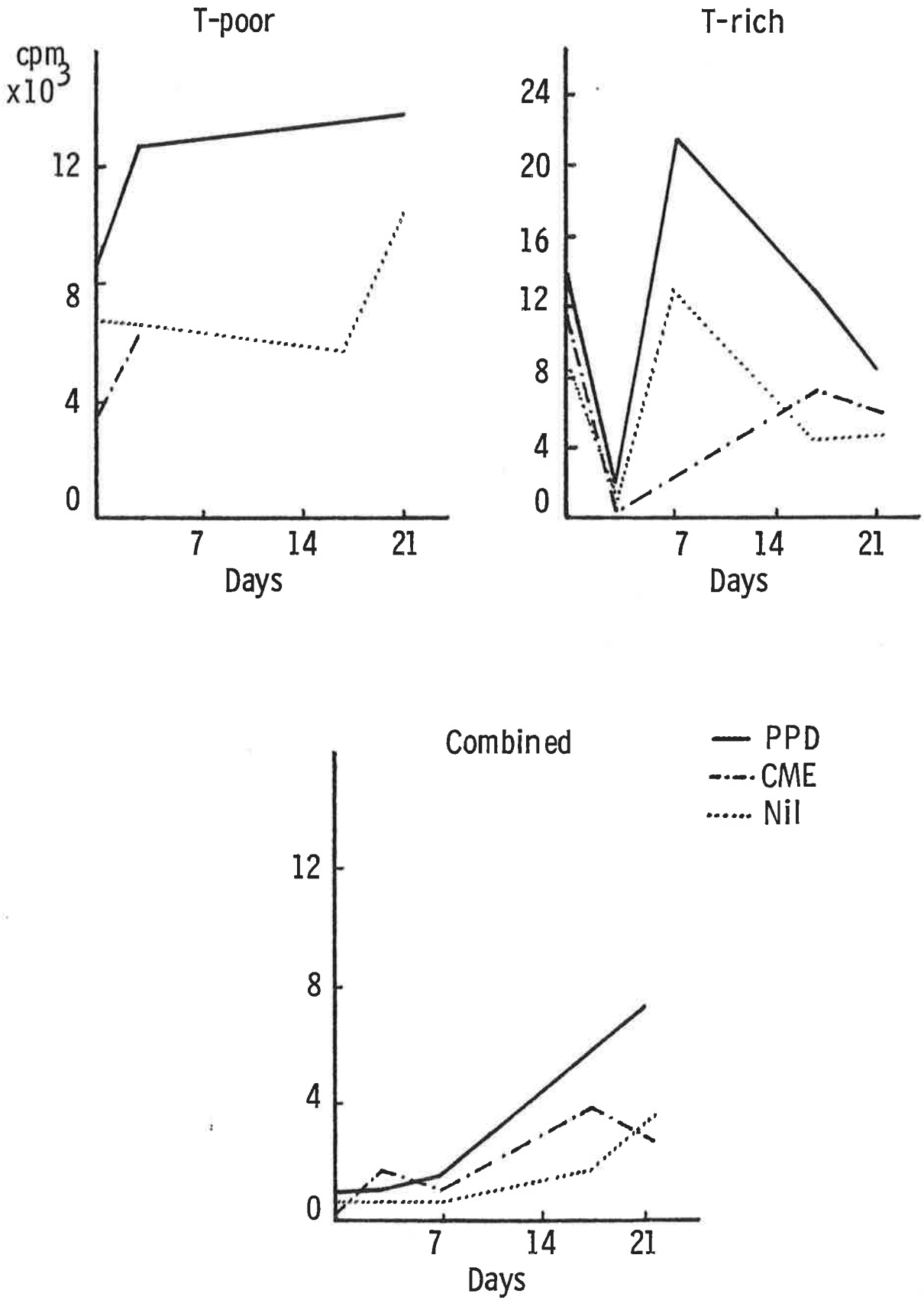


FIGURE 4.12 BLASTOGENIC RESPONSES OF LYMPHOCYTE SUB-POPULATIONS FOLLOWING CHEMOTHERAPY (Patient R.M.)

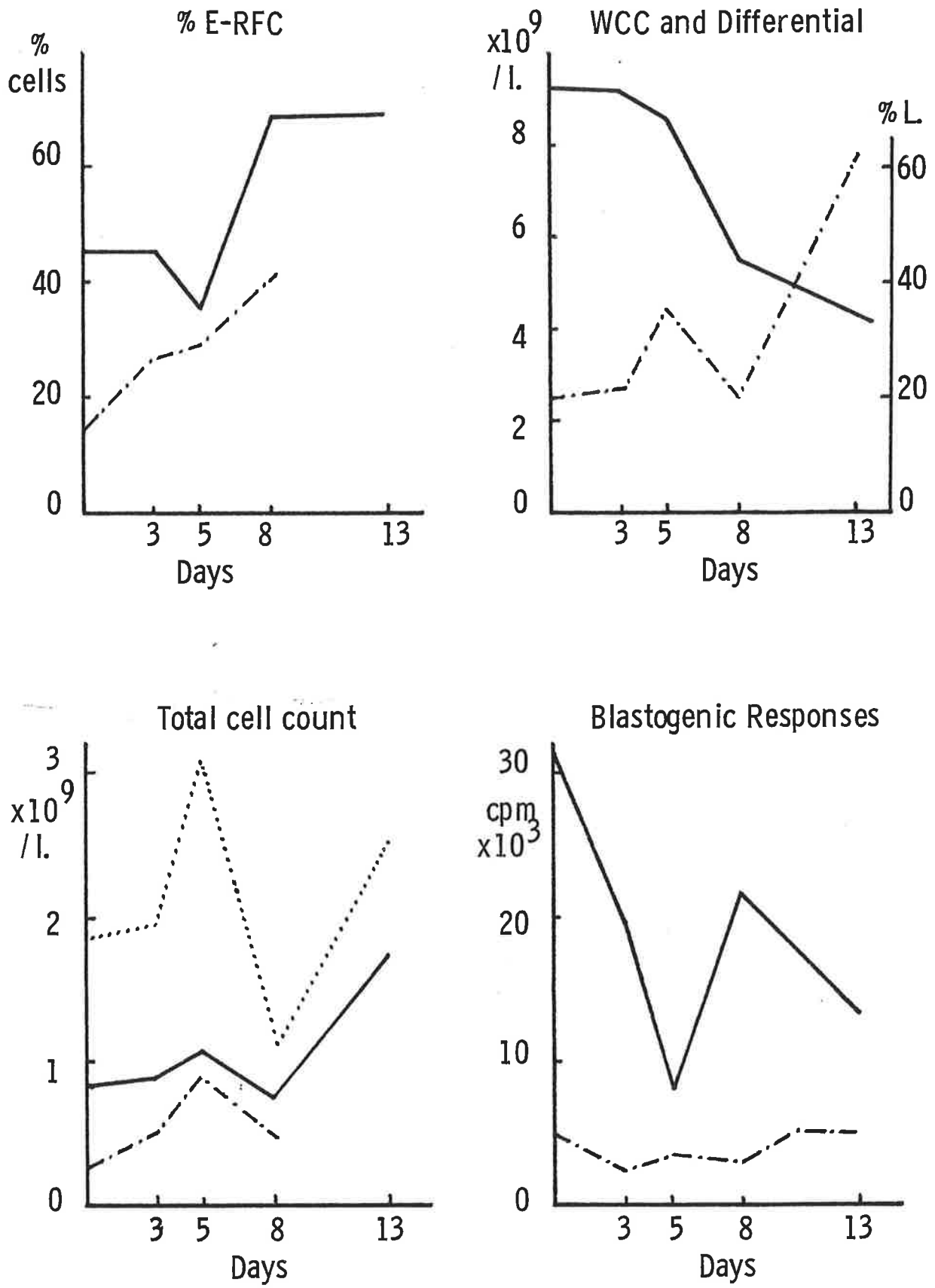


FIGURE 4.13 EFFECT OF CHEMOTHERAPY ON VARIOUS PARAMETERS OF LYMPHOCYTE FUNCTION (Patient I.W.)

CHEMOTHERAPY : Adriamycin and Methotrexate Infusion over 5 days

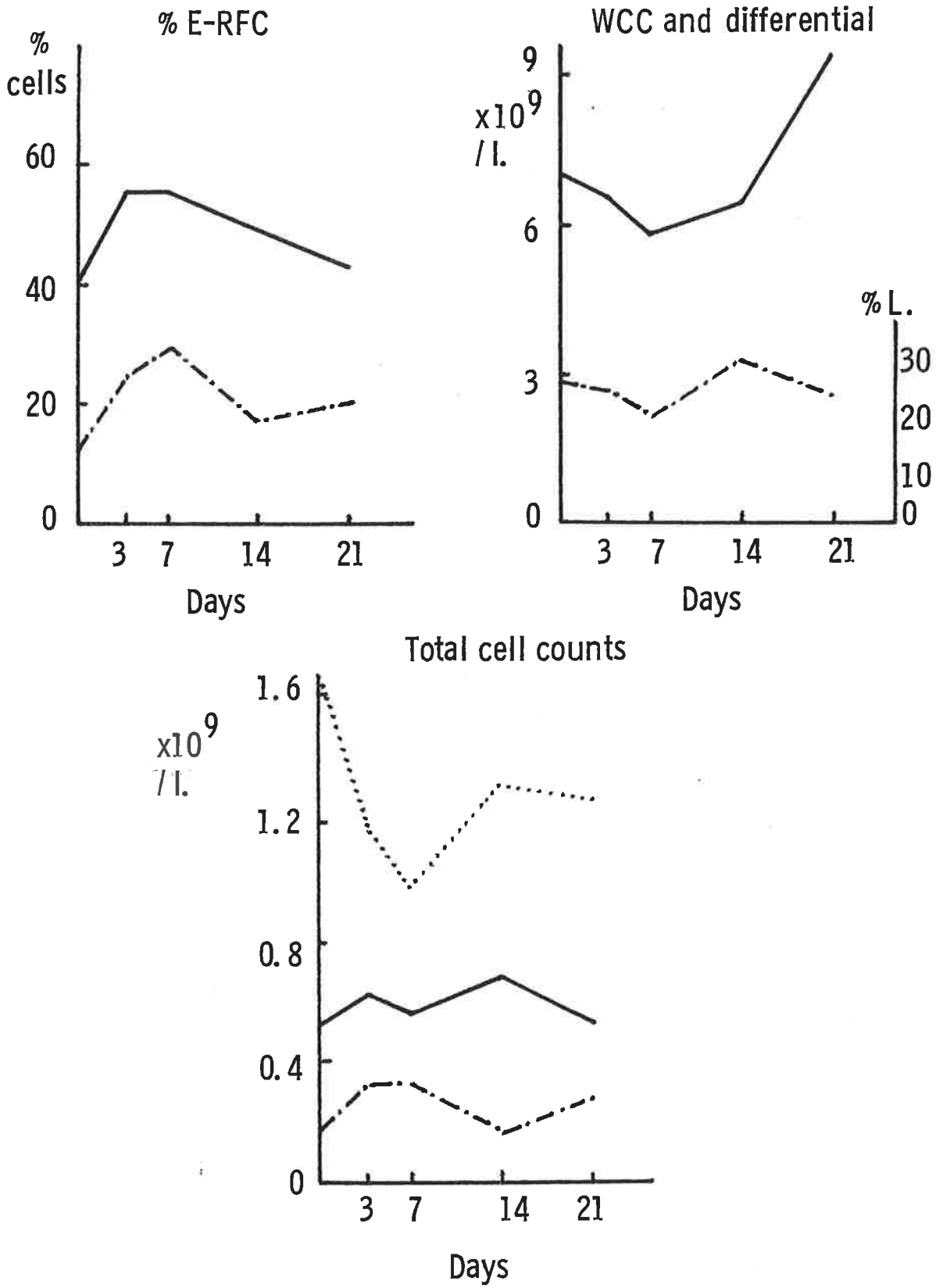


FIGURE 4.14 MEAN VALUES OF LYMPHOCYTE PARAMETERS DURING CHEMOTHERAPY IN SIX PATIENTS (THREE RECEIVING IMMUNOTHERAPY)

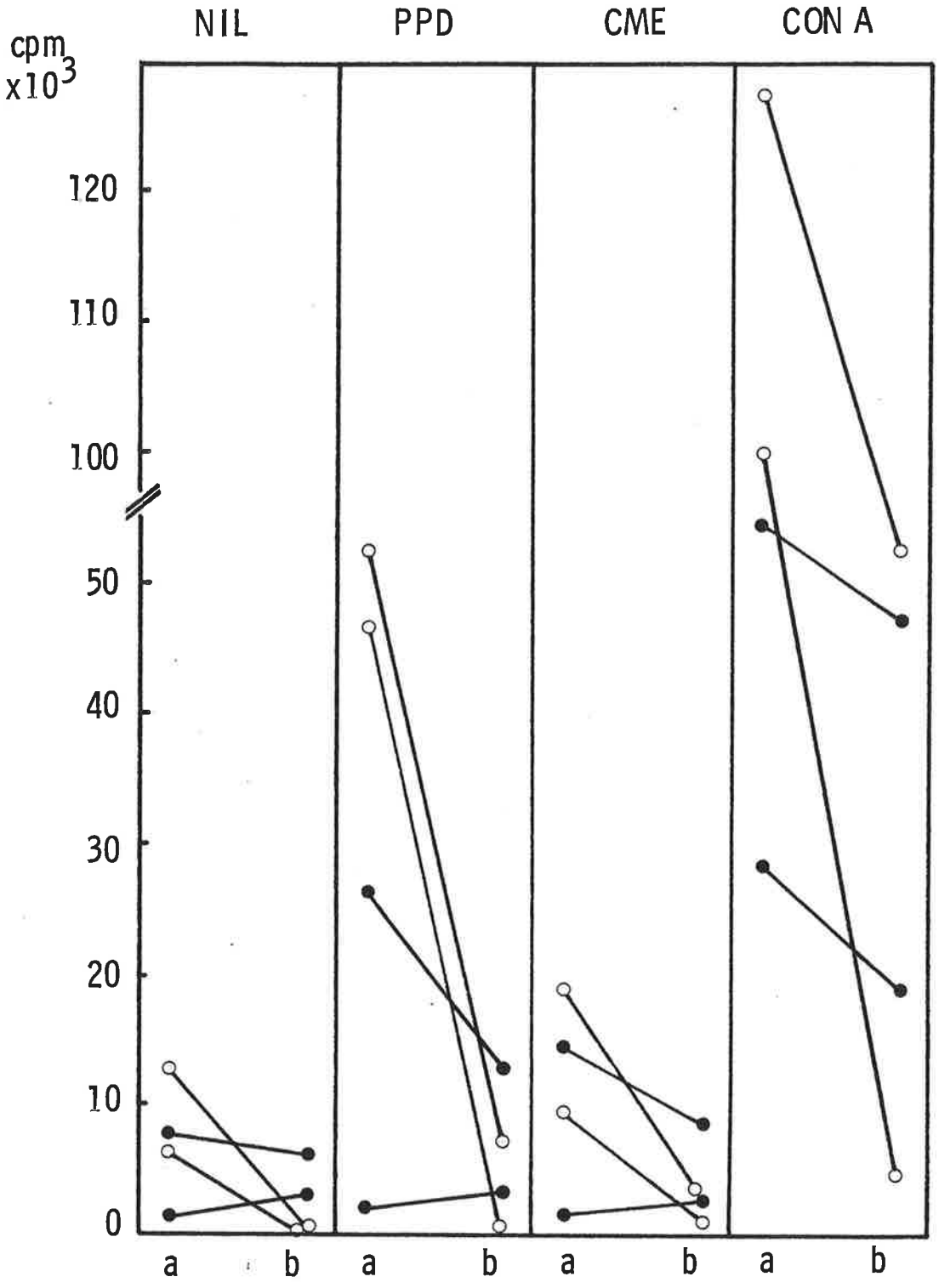


FIGURE 4.15 SUPPRESSION OF BLASTOGENIC RESPONSES OF NORMAL LYMPHOCYTES TO PPD, OVARIAN TUMOUR CME AND CON A BY CO-CULTURE WITH INDUCED SUPPRESSOR CELLS FROM CANCER PATIENTS

● Patients in remission

○ Patients in relapse

a: Cancer lymphocytes in MLC

b: Con A-treated lymphocytes

The spontaneous E-rosette test is a useful means of assessing the T-cell population in peripheral blood. However, even in healthy controls there appears to be a wide range of values between 9% and 90% with an average of about 65% (see Table 4.3), and a decline in values is seen with age (WHITEHEAD et al, 1978). Furthermore, the test is extremely sensitive to alterations in methodology such as length of incubation (WHITEHEAD et al, 1978), temperature of incubation (MENDES et al, 1973; QUAN & BURTIN, 1978), the use of FCS rather than PBS (BRAIN et al, 1970; WHITEHEAD et al, 1976), extensive washing (ANTHONY et al, 1975 a; RUGARLI et al, 1975), and the use of agents such as Neuraminidase (GALILI & SCHLESINGER, 1974), Brinase (HOLLAND et al, 1975), Papain (CHAPEL, 1973; WHITEHEAD et al, 1976), all of which presumably unveil receptor sites on the surface membrane and so increase the binding of SRBC. The conflicting reports on levels of E-RFC in patients with cancer may partly be explained on the basis of differences in methodology.

In this Chapter it has been shown that patients with ovarian cancer in relapse have significantly less $\%T_a$ and T_t than normal controls or patients with static disease. The absolute numbers of these cells are also significantly decreased. However, the study does not clarify whether there is a true decrease in the number of E-RFC in these patients, or whether the presence of serum factors in relapse patients prevents adequate binding of the SRBC to the lymphocytes. It has been shown that the incubation of lymphocytes in serum from cancer patients leads to a decrease in the %E-RFC (RUGARLI et al, 1975; FUKS et al, 1976; WHITEHEAD et al, 1976), and that the use of proteolytic enzymes such as papain or Brinase restores these levels to those of normal controls (vide supra). Similarly in patients with acute hepatitis, it has been shown that the decreased levels of E-RFC are caused by serum factors which result from tissue necrosis (CHISARI et al, 1977. We have found that Ficoll-Triosil preparations of lymphocytes from patients with relapsing disease are often "dirty" and heavily contaminated with platelets, which can

be seen stuck to the lymphocytes. It is known that human platelets aggregate in the presence of immune complexes (PENTTINEN et al, 1969), and such complexes have been demonstrated in patients with ovarian cancer (POULTON et al, 1978). It may therefore be that the decreased %E-RFC seen in these patients is non-specific and does not reflect a true decrease in the numbers of circulating T-cells.

The finding that relapse patients had no significant impairment in their ability to respond to PPD compared with control subjects supports the idea that there is not a true decrease in the numbers of circulating T-cells. We did not assess PHA or Con A responses, since mitogen stimulation does not reflect any known in vivo phenomenon, and some reports have shown that the differences between cancer and normal lymphocytes reflect only the concentration of PHA used (DUCOS et al, 1970; SAMPLE et al, 1971). It should be noted however that in this study the range of PPD responses in all groups was very wide, and that 72% of patients with relapsing disease had PPD responses less than the median value for the control group. The finding that there is not an obvious depression of cell-mediated immunity in these patients does not refute the postulate that cancer patients may have an intrinsic defect in their lymphocytes. Indeed, in the present study, the spontaneous incorporation of ^{125}IUDR was significantly less in the patients with relapsing disease. Others have observed abnormal protein synthesis in cancer lymphocytes, even when the %E-RFC was the same as normal controls (WHITCOMB & PARKER, 1977).

It is postulated in this Chapter that the lowered in vitro reactivity of cancer lymphocytes may be due to the presence of suppressor cells in these patients. In all 4 patients studied in this regard, lymphocytes could be induced which caused marked suppression of the MLC reaction, and this was most noticeable in the two patients with progressive disease. Separation of cell populations also showed that the T-enriched population had a significantly higher spontaneous incorporation of ^{125}IUDR than either the T-depleted cells or the unseparated population of lympho-

cytes, suggesting that in the latter case their reactivity was suppressed. This phenomenon was not observed when cells were stimulated with PPD. This is not inconsistent with other reports of depressed CMI since only 2% of lymphocytes are probably involved in antigen recognition (CROWTHER et al, 1969) and there is likely to be a margin of reserve and cross-reactivity with other sensitizing antigens, so that small levels of depression are not detected.

A surprising feature of the serial determinations of E-RFC in the patients undergoing chemotherapy was that the $\%T_a$ and T_t increased initially in the face of the expected fall in white cell count and lymphocyte count. However, since 5 of the 6 patients tested were receiving Cyclophosphamide, this may reflect the known differential effect on lymphocytes which this drug has, partly preserving T-cells at the expense of B-cells (TURK & POULTER, 1972). This result was not confirmed in the one study of lymphocyte sub-populations in a patient undergoing chemotherapy; however, the inclusion of Adriamycin in the drug regimen may have abrogated the differential effects of the Cyclophosphamide. In the serial determinations, all values of %E-RFC had returned to normal or greater by 14 days, and this confirms the results of others that rebound phenomena occur, especially in patients who are responding successfully to their chemotherapy (CHEEMA & HERSH, 1971). Conversely, blocking factors in the sera, such as immune complexes, might disappear under the influence of chemotherapy (SINCOVIKS et al, 1972), although it is difficult to see how this might happen within 48 hours after receiving the drug, so that %E-RFC were increased accordingly.

Surgery likewise increased the $\%T_a$ and T_t and also caused an initial increase in blastogenic responses to PPD and ovarian tumour CME. Indeed the unstimulated lymphocytes showed increased incorporation of ^{125}IUDR, which suggests increased reactivity in vivo. This contrasts with the findings of others that lymphocytopenia follows surgery (PARK et al, 1971; HOLT et al, 1972), and that the proportion of T-cells also decreases (ANTHONY et al, 1975 a; MILLER et al, 1976). However, WEESE et al (1977) have

reported that cancer patients with decreased E-RFC pre-operatively show a return to normal values within 3 weeks of surgery.

It is interesting to note that patients receiving immunotherapy, 9 of whom were in relapse at the time of testing, showed evidence of lymphopenia like the other cancer patients with relapsing disease. The absolute numbers of both T_a and T_t as well as the $\%T_t$ were all significantly decreased compared with normal control subjects, and were not different from values for other relapse patients. However, the $\%T_a$ was significantly increased to control values. A similar increase in the $\%T_a$ has been noted by others (LIEBERMAN et al, 1975; WYBRAN & FUDENBERG, 1976), and stresses the importance of calculating both % and absolute numbers of cells, since one value and not the other may be abnormal (OLKOWSKI & WILKINS, 1975).

Hence, the findings in this Chapter suggest that patients with progressive disease, and therefore a large tumour burden, have a degree of lymphocytopenia compared with normal controls, although this is not statistically significant. This lymphopenia does not appear to cause a significant inability of the lymphocytes to respond to secondary recall antigens such as PPD, although the metabolism of these cells in their unstimulated state appears to be suppressed. This is more likely to be caused by the presence of suppressor cells rather than an intrinsic defect. Chemotherapy and surgery both cause an initial increase in the proportion of T_a and T_t even though in the case of chemotherapy there is always a drug-induced lymphopenia. Immunotherapy is unable to restore the lymphopenia of patients in relapse to normal values, but does cause a significant increase in $\%T_a$ to normal levels. There is however, no correlation between this increase and the increased blastogenic responses to antigens seen in immunotherapy patients (see Chapter 7). It is postulated that the decrease in %E-RFC seen in cancer patients with relapsing disease, is due to serum blocking factors, rather than a reflection of a true decrease in circulating T-cells.

TABLE 4.6 %E-RFC (BOTH T_a AND T_t), ABSOLUTE NUMBERS OF E-RFC AND TOTAL LYMPHOCYTE COUNTS IN NORMAL CONTROLS, OVARIAN CANCER PATIENTS WITH LARGE TUMOUR BURDEN AND OVARIAN CANCER PATIENTS WITH MINIMAL TUMOUR BURDEN.

Patient code	$\%T_a$	$\%T_t$	WCC	% Lymph.	Abs. Lymph.	Abs. T_a	Abs. T_t
PATIENTS WITH RELAPSING DISEASE							
PRE-OPERATIVE							
H	-	47	6.0	14	840	-	395
W	8	12	8.0	2	160	12	19
R	10	34	6.4	29	1856	186	622
L	8	41	6.0	15	900	56	369
W	18	46	6.3	36	2268	408	1050
J	14	47	15.6	-	-	-	-
C	7	15	10.3	5	515	36	77
B	4	52	8.0	10	800	32	416
C	8	30	7.8	-	-	-	-
B	7	30	21.4	2	428	30	125
R	9	42	-	-	-	-	-
F	25	48	8.6	20	1720	430	826
M	10	33	10.9	27	2943	294	971
INOPERABLE (INCLUDING BIOPSY ONLY), PRE-THERAPY							
H	-	53	15.2	4	608	-	322
H	15	52	12.2	27	3294	484	1703
W	14	45	9.1	20	1820	255	819
F	9	19	10.9	4	436	39	83
W	19	36	4.5	19	855	162	304
M	9	47	5.2	9	468	42	221
D	11	43	8.5	13	1105	116	470
R	15	47	6.5	38	2470	378	1161
B	4	29	2.5	8	200	8	58
W	9	51	7.6	14	1064	96	543
P	9	12	6.0	16	960	82	113
B	5	51	6.7	25	1675	84	854

TABLE 4.6 (Contd.)

Patient code	$\%T_a$	$\%T_t$	WCC	% Lymph.	Abs. Lymph.	Abs. T_a	Abs. T_t
INOPERABLE (Contd.)							
F	3	29	4.0	46	1840	55	534
T	10	31	10.7	12	1284	128	398
M	12	23	12.6	25	3150	378	725
I	11	15	5.0	9	450	50	68
Range	3 - 25	12 - 53			160 - 3294	8 - 484	19 - 1703
Median	9	41			1012	90	407
PATIENTS WITH MINIMAL TUMOUR LOAD							
H	29	53	9.0	31	2790	801	1478
G	23	49	-	-	-	-	-
L	16	52	9.0	13	1170	187	608
W	19	61	8.1	23	1863	345	1133
D	15	34	5.9	16	944	137	321
B	4	43	-	-	-	-	-
T	12	43	6.6	18	1188	143	511
C	10	39	-	-	-	-	-
D	22	50	14.4	9	1296	285	648
C	31	46	11.3	18	2034	631	936
J	20	35	7.7	37	2849	570	997
R	23	57	7.6	36	2736	629	1560
R	27	40	8.9	12	1068	288	427
B	11	28	6.6	16	1056	116	296
G	39	62	6.1	31	1891	737	1172
T	21	53	4.9	39	1911	401	1013
M	28	48	5.8	2	116	32	56
W	14	45	9.1	20	1820	255	819
W	14	56	3.5	27	945	130	527
W	16	48	5.9	12	708	113	336
R	15	47	6.5	38	2470	378	1161

TABLE 4.6 (Contd.)

Patient code	%T_a	%T_t	WCC	% Lymph.	Abs. Lymph.	Abs. T_a	Abs. T_t
PATIENTS WITH MINIMAL TUMOUR LOAD (Contd.)							
Range	4 - 39	28 - 62			116 - 2849	32 - 801	56 - 1560
Median	19	48			1558	287	734
CONTROL SUBJECTS							
	36	51	5.2	7	364	131	186
	-	67	6.8	40	2720	-	1817
	8	65	4.9	32	1568	125	1019
	9	72	5.5	34	1870	168	1346
	15	46	13.3	5	665	100	306
	10	46	6.7	30	2010	201	925
	13	-	-	-	-	-	-
	15	60	6.5	24	1560	234	936
	15	67	-	-	-	-	-
	15	62	3.5	47	1645	247	1020
	14	59	8.7	32	2784	390	1643
	8	56	-	-	-	-	-
	16	58	7.7	27	2079	333	1206
	24	50	12.3	21	2583	620	1292
	21	47	7.3	40	2920	613	1372
Range	8 - 36	46 - 72			364 - 2920	100 - 620	186 - 1817
Median	15	59			1940	234	1113

TABLE 4.6 (Contd.)

Patient code	$\%T_a$	$\%T_t$	WCC	% Lymph.	Abs. Lymph.	Abs. T_a	Abs. T_t
IMMUNOTHERAPY PATIENTS		† = Relapse					
S	25	55	3.9	34	1326	328	729
C †	13	56	4.9	17	833	106	462
G †	24	65	4.8	32	1536	364	994
G †	20	64	-	-	-	-	-
L †	31	43	5.7	13	741	227	321
H †	16	46	4.6	11	506	82	233
T †	12	43	6.6	18	1188	143	511
N †	19	43	6.3	3	189	36	81
P	17	28	6.5	17	1105	188	309
W †	13	43	11.9	27	3213	964	1382
D †	10	45	4.3	28	1204	120	536
Range	10 - 31	28 - 65			189 - 3213	36 - 964	81 - 1382
Median	17	45			1147	166	487

Pt. group	No.	Abs.L.	p	$\%T_a$	p	$\%T_t$	p	Abs.T_a	p	Abs.T_t	p
Controls	14	1940	-	15	-	59	-	234	-	1113	-
Relapse	27	1012	n.s.	9	p<0.03	41	p<0.0002	90	p<0.025	407	p<0.001
Remission	21	1558	n.s.	19	n.s.	48	p<0.003	287	n.s.	734	n.s.
Immunoth.	11	1147	p<0.05	17	n.s.	45	p<0.01	166	n.s.	487	p<0.05

Note also: Remission vs. Immunotherapy - n.s. for either $\%T_a$ or $\%T_t$

Relapse vs. Immunotherapy - $\%T_a$ p<0.001; $\%T_t$ n.s.

Relapse vs. Remission - $\%T_a$ p<0.001; $\%T_t$ p<0.01; Abs. T_a p<0.01; Abs. T_t n.s.

TABLE 4.7 BLASTOGENIC RESPONSES OF CONTROLS, OVARIAN CANCER PATIENTS AND WOMEN WITH OTHER CANCERS TO 10µg PPD.

Patient code	Nil	cpm	cpm diff.	S.I.
CONTROLS				
V	9993	8976	-1017	0.9
S	2085	2106	21	1.0
L	772	1428	656	1.9
B	879	2829	1950	3.2
G	1231	4442	3210	3.6
M	4281	7605	3324	1.8
X	2079	5706	3627	2.7
H	4048	7726	3678	1.9
K	4328	8906	4578	2.1
X	4413	12831	8418	2.9
D	4170	15690	11520	3.8
P	3381	19008	15627	5.6
W	2960	23134	20174	7.8
D	10254	30489	20235	3.0
W	4404	27840	23436	6.3
W	9538	35400	25862	3.7
C	1164	32991	31827	28.3
B	7434	39986	32552	5.4
H	2874	53330	50456	18.6
D	4458	60026	55568	13.5
P	8214	99952	91738	12.2
REMISSION				
H	1050	891	-159	0.8
F	5138	6052	914	1.2
W	555	1995	1440	3.6
G	3972	5566	1594	1.4
R	2745	5838	3093	2.1
S	2199	5376	3177	2.4
G	2985	16812	13827	5.6
J	2388	38157	35769	16.0
T	3276	61314	58038	18.7
OTHER CANCERS				
R	3298	2352	-946	0.7
W	1982	1972	-10	1.0
B	1950	2154	204	1.1
S	1952	2256	304	1.2
T	528	1266	738	2.4
P	2158	3604	1446	1.7
R	1046	6104	5058	5.8
C	2048	7810	5762	3.8
L	4362	10264	5902	2.4
F	1886	9298	7412	4.9
M	5442	29240	23798	5.4
C	4564	31674	27110	6.9
W	2792	31734	28942	11.3
B	10814	67068	56254	6.2
C	6596	131908	125312	20.0

Patient code	Nil	cpm	cpm diff.	S.I.
RELAPSE				
H	1686	1476	-210	0.9
C	722	698	-24	1.0
B	2980	2958	-22	1.0
F	2577	2556	-21	1.0
D	1302	1292	-10	1.0
M	618	657	39	1.1
M	812	1112	300	1.4
W	588	1078	490	1.8
B	1599	2145	546	1.3
F	554	1233	679	2.2
B	680	3318	2638	4.9
W	2484	6123	3639	2.5
H	4424	8850	4426	2.0
D	1244	5826	4582	4.7
C	456	5050	4594	11.1
B	2818	7832	5014	2.8
R	5294	10710	5416	2.0
W	3114	13941	10827	4.5
R	846	16878	16032	19.9
F	1144	18832	17688	16.5
M	4143	21852	17709	5.3
H	5142	25714	20572	4.0
V	2894	25680	22786	8.9
J	6788	31646	24858	4.7
W	2769	76098	73329	27.5

		NIL VALUES		PPD VALUES	
Pt. group	No.	cpm diff. median/range	p	cpm diff. median/range	p
Controls	21	4170 (772 to 10254)	-	11520 (-1017 to 91738)	-
Relapse	25	1686 (456 to 6788)	p<0.01	4426 (-210 to 73329)	n.s.
Remission	9	2985 (555 to 10938)	n.s.	3093 (-159 to 58038)	n.s.
Other Ca.	15	2158 (528 to 10814)	n.s.	5762 (-946 to 125312)	n.s.

TABLE 4.8 BLASTOGENIC RESPONSES OF CELL POPULATIONS TO 10μg PPD.

Patient	Unseparated		T-enriched		T-depleted	
	Nil	cpm	Nil	cpm	Nil	cpm
W (Rel)	10380	16818 6438	17128	132700 115572	1740	7310 5570
W (Rem)	558	1078 490	6258	7230 972	1203	2301 1098
R (Rem)	2745	5838 3093	6318	7164 846	11085	13896 2811
R (Rel)	1046	6104 5058	9880	10658 778	2244	2568 324
W (Rem)	555	1995 1440	4314	8004 3690	1734	- -
T (Rel)	528	1266 738	852	1032 180	1790	1438 -360
M (Rel)	812	1112 300	3616	6542 2926	3351	4968 1617
C (Rel)	456	5050 4594	2268	42597 40329	1725	4617 2892
L (Rel)	1514	22360 20846	2178	11578 9400	1088	1084 -4
W (Rel)	11781	37191 25410	19718	35642 15924	1065	- -
B (Rel)	2818	7832 5014	-	- -	1779	1536 -243
R (Rel)	5158	11532 6351	6474	13924 7450	23304	- -
M (Rel)	618	657 39	8576	13998 5422	6894	8655 1761

Median values for cpm:	Nil		PPD	
Unseparated:	1046	(456 to 11781)	4594	(39 to 25410)
T-enriched:	6288	(852 to 19718)	4556	(180 to 115572)
T-depleted:	1779	(1065 to 23304)	1357	(-360 to 5570)

Tests of significance:	Nil		PPD	p
Unseparated:	1046	-	4596	-
T-enriched:	6288	U = 33; p < 0.02	4556	U = 64; n.s.
T-depleted:	1779	U = 54; n.s.	1357	U = 35; n.s.
T-rich vs. T-poor:		U = 43; n.s.		U = 28; p < 0.05

TABLE 4.9 EFFECT OF SURGERY ON IN VITRO BLASTOGENIC RESPONSES TO TUMOUR CME AND PPD, AND %E-RFC.

		PRE-OP.			1 WEEK POST-OP.			2 WEEKS POST-OP.			3 WKS.	4 WKS.
Patient		cpm	$\%T_a$	$\%T_t$	cpm	$\%T_a$	$\%T_t$	cpm	$\%T_a$	$\%T_t$	cpm	cpm
B	Nil	680	5	41	508	10	52	894	-	25	-	-
	PPD	3318			950			8822				
	CME	606			438			1482				
D	Nil	1302	11	43	2596	14	34	-	-	-	-	-
	PPD	1292			4130							
	CME	1384			3354							
B	Nil	1599	18	46	1885	4	26	1450	19	62	-	4708
	PPD	2145			5577			4344				9174
	CME	999			840			1506				5260
W	Nil	2484	7	15	8718	32	39	-	-	-	-	7602
	PPD	6123			10428							33522
	CME	2679			18666							10242
H	Nil	5152	9	47	6984	16	53	4844	-	-	-	-
	PPD	25714			42861			17894				
	CME	5042			8055			9588				
J	Nil	6788	11	30	25092	22	46	4458	6	24	3194	-
	PPD	31646			67770			24112			19108	
	CME	9630			37104			8680			2498	

Note also Patient 1 (B): results at 10 days post-op: Nil 496
PPD 2840
CME 620

TABLE 4.10 EFFECT OF CHEMOTHERAPY ON VARIOUS PARAMETERS OF LYMPHOCYTE FUNCTION.

Pt.	Day	cpm		%T_a	%T_t	WCC	%L	Abs. L.	Abs.T_a.	Abs.T_t.
SG (4.9)	0	Nil	2985	24	65	4.8	32	1536	364	994
		PPD	16812							
		CME	4035							
	14	Nil	10878	18	54	-	-	-	-	-
		PPD	29232							
		CME	11601							
	21	Nil	4374	12	46	-	-	-	-	-
		PPD	7764							
		CME	5259							
	28		-	31	45	-	-	-	-	-
	(wks) 26	Nil	3864	26	29	4.4	15	720	187	209
		PPD	6686							
		CME	5604							
DW (4.10)	0	Nil	4716	14	56	3.5	27	945	130	527
		PPD	10608							
		CME	4968							
	3	Nil	2319	35	63	3.8	30	1140	402	718
		PPD	5664							
		CME	2643							
	7	Nil	4245	40	65	5.1	25	1275	504	822
		PPD	9375							
		CME	4431							
	10	Nil	5025	36	51	4.8	20	960	344	490
		PPD	11397							
		CME	6957							
	14	Nil	9420	20	39	5.1	22	1122	228	435
		PPD	26769							
		CME	9849							

TABLE 4.10 (Contd.)

Pt.	Day	cpm		$\%T_a$	$\%T_t$	WCC	%L	Abs. L.	Abs.T_a.	Abs.T_t.
DW (contd)	17	Nil	2229	23	26	4.9	25	1225	276	322
		PPD	10323							
		CME	1899							
	21	Nil	2988	-	-	-	-	-	-	-
		PPD	4425							
		CME	1809							
	24	Nil	3669	24	45	4.5	31	1395	339	628
		PPD	20487							
		CME	3507							
	28	Nil	3096	-	-	-	-	-	-	-
		PPD	14244							
		CME	3826							
RM (4.11)	0	Nil	618	12	23	12.6	25	3150	378	725
		PPD	657							
		CME	570							
		Con A	5436							
	3	Nil	630	15	40	10.3	9	927	140	371
		PPD	789							
		CME	1620							
		Con A	8880							
	7	Nil	678	19	49	9.3	7	651	124	319
		PPD	1230							
		CME	786							
		Con A	40368							
	17	Nil	1838	11	23	11.8	9	1062	117	244
		PPD	5964							
		CME	3782							
		Con A	132672							

TABLE 4.10 (Contd.)

Pt.	Day	cpm		$\%T_a$	$\%T_t$	WCC	%L	Abs. L.	Abs.T_a.	Abs.T_t.
RM (contd)	21	Nil	3070	20	35	19.4	6	1164	232	407
		PPD	7090							
		CME	3062							
		Con A	94254	(for cell sub-population results, see Appendix to this Table)						
IW (4.13)	0	Nil	2792	14	45	9.1	20	1820	255	819
		PPD	31734							
		CME	4370							
	3	Nil	2253	26	45	9.1	21	1911	485	850
		PPD	19809							
		CME	2157							
	5	Nil	3422	29	35	8.6	35	3010	867	1062
		PPD	8068							
		CME	3522							
	8	Nil	5496	40	69	5.5	20	1100	438	756
		PPD	21939							
		CME	3078							
	13	Nil	4954	-	69	4.2	60	2520	-	1736
		PPD	12560							
		CME	4522							

TABLE 4.10 (Contd). MEAN VALUES OF LYMPHOCYTE PARAMETERS DURING CHEMOTHERAPY IN 6 PATIENTS 3 RECEIVING IMMUNOTHERAPY (see Chapter 7)

Day	$\%T_a$	$\%T_t$	WCC	%L	Abs. L.	Abs. T_a	Abs T_t
0	13 (2-20)	40 (11-64)	7.0 (3.5-12.6)	24 (20-27)	1637 (945-3150)	193 (20-378)	515 (139-819)
3	24 (6-36)	55 (40-66)	6.5 (2.8-10.3)	22 (9-30)	1191 (784-1911)	326 (140-485)	607 (371-850)
7	29 (11-40)	55 (43-69)	5.9 (3.8-9.3)	18 (9-25)	999 (741-1275)	325 (140-504)	556 (321-822)
14	17 (11-24)	49 (23-69)	6.4 (3.1-11.8)	27 (8-60)	1313 (688-2520)	184 (83-339)	690 (244-1736)
21+	20 (13-25)	43 (26-55)	9.2 (4.5-19.4)	21 (6-34)	1276 (1164-1395)	264 (158-339)	519 (311-729

TABLE 4.10 (Appendix) BLASTOGENIC RESPONSES OF LYMPHOCYTE SUB-POPULATIONS FOLLOWING CHEMOTHERAPY.

Day	cpm	Unsep.	T-rich	T-poor
0	Nil	618	8576	6894
	PPD	657	13998	8655
	CME	570	11746	3792
	Con A	5436	42075	-
3	Nil	630	810	6534
	PPD	789	1251	12726
	CME	1620	567	6322
	Con A	8880	-	156646
7	Nil	678	13044	-
	PPD	1230	21213	-
	CME	786	-	-
	Con A	40368	-	-
17	Nil	1838	4202	5871
	PPD	5964	13203	-
	CME	3782	6696	-
	Con A	132672	143991	-
21	Nil	3070	4647	10341
	PPD	7090	8184	13734
	CME	3062	5434	-
	Con A	94254	157930	123414

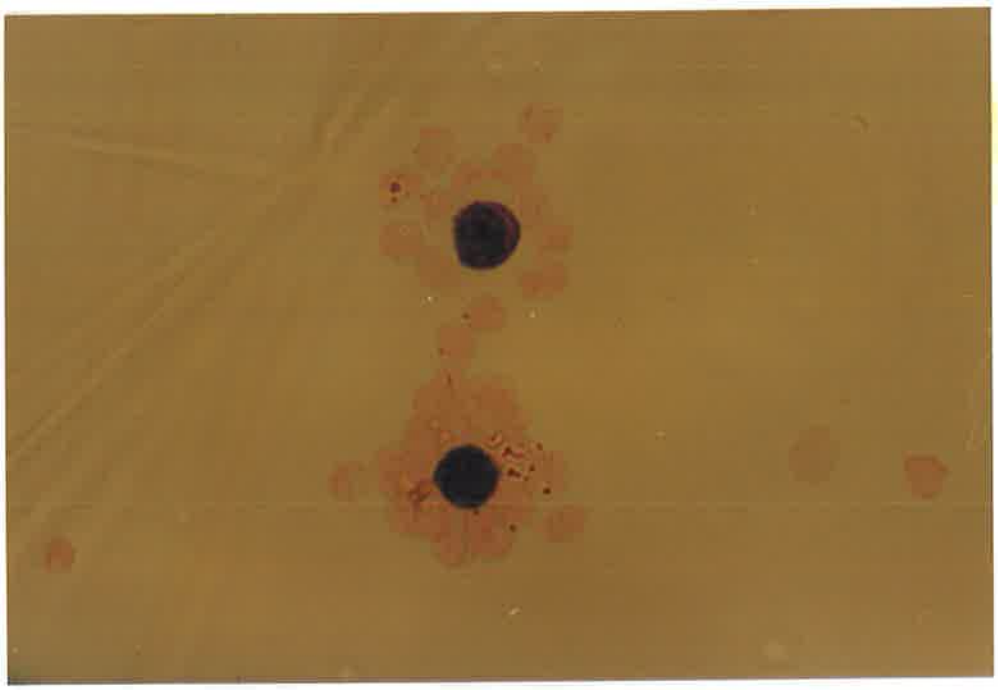


FIG. 4.16 E-ROSETTE FORMING CELL (GIEMSA STAIN).

TABLE 4.11 SUPPRESSION OF BLASTOGENIC RESPONSES OF NORMAL DONOR LYMPHOCYTES TO PPD, OVARIAN TUMOUR CME, AND CON A BY CO-CULTURE WITH INDUCED SUPPRESSOR CELLS FROM OVARIAN CANCER PATIENTS.

cpm	Mixed Cell Reaction	(cpm diff.)	MLC + Suppressor Cells	(cpm Δ)	% suppression
PATIENT 1 - REMISSION (EJ)					
Nil	1406		2864		
PPD	2174	768	3176	312	-
CME	1984	578	2464	-400	-
Con A	28188	26782	19044	16180	40
PATIENT 2 - REMISSION (VD)					
Nil	7905		6450		
PPD	26130	18225	13401	6951	62
CME	14406	6501	8775	2325	64
Con A	54504	46599	47991	41541	11
PATIENT 3 - RELAPSE (RM)					
Nil	12366		756		
PPD	51756	39390	7794	7038	82
CME	18186	5820	3786	3030	48
Con A	127287	114921	53094	52338	54
PATIENT 4 - RELAPSE (RC)					
Nil	6603		555		
PPD	46443	39840	705	150	100
CME	9225	2622	2097	1542	41
Con A	95604	89001	5133	478	100

Suppressor Cells induced by 48 hour stimulation with Con A, followed by Mitomycin C treatment before co-culture with normal donor lymphocytes. The same donor lymphocytes were used in each experiment.

CHAPTER 5. SERUM EFFECTS ON LYMPHOCYTE CULTURES AND IMMUNE COMPLEXES IN OVARIAN CANCER.

The previous Chapters have discussed both the stimulation of antibody and cellular mecahnisms in response to tumour growth. In spite of these mechanisms tumours continue to grow and this Chapter discusses the evidence for serum factors which may inhibit specific anti-tumour mechanisms. Table 4.1, which is a modification of that expressed by COGGIN et al (1974), suggests three possible mechanisms which may be involved: tumour antigen excess which paralyses sensitized effector cells; immunoglobulin which may either enhance tumour growth or "block" effector cells; and immune complexes of tumour antibody and antigen which have "blocking" effects on lymphocytes.

5.1 EVIDENCE FOR INHIBITORY SERUM FACTORS.

(i) Tumour Antigen. All the evidence to date suggests that tumour antigens are weak and may in many cases be non-immunogenic (ALEXANDER 1970). Following from this, ALEXANDER (1974) has since suggested that spontaneous shedding of tumour antigen determines the growth pattern of the tumour, and in situations where there is a build-up of tumour antigen, there is also rapid tumour growth. This effect is mediated by paralysis of regional nodes and sensitized effector cells (CURRIE & BASHAM, 1972). In their study, the cytotoxicity against tumour cells by lymphocytes from cancer patients was significantly increased or restored after extensive washing of the lymphocytes. It could then be abrogated by re-incubation in the patient's serum, and this was a specific phenomenon unaffected by either normal serum or that from a patient with a different type of tumour. The degree of serum inhibition was related to the tumour burden. It has also been shown that tumour antigen can tolerize both T and B cells (NOSSAL 1974), as well as inhibiting T-cell effector function (PLATA & LEVY, 1974).

(ii) "Blocking" and "Enhancing" Antibody. Early immunotherapy models in animals revealed a phenomenon whereby the passive transfer of immune serum from an immunized mouse to

a tumour-bearing mouse resulted in enhanced tumour growth (KALISS 1958). Further work confirmed these findings and suggested that immunoglobulins, presumably anti-tumour antibody, was the responsible factor (SNELL et al, 1960).

CHARD et al (1968) subsequently showed that enzymatic digestion of IgG to remove the F(c) portion converted it from a cytotoxic to an enhancing antibody. LINSCOTT (1970) explained this on the basis of the density of antigenic sites on the tumour cell surface; tumour cells with a low antigenic density when bound by antibody were "blocked" from complement-mediated lysis. Cells with a high antigenic density, on the other hand, could be destroyed. "Enhancing" antibody as such probably only has relevance for immunotherapeutic situations where iatrogenic stimulation of anti-tumour antibody may disturb the balance between antigen and antibody.

The theory of "blocking" antibody derived from early animal experiments which showed that in vitro lymphocyte cytotoxicity against tumour cells could be inhibited by incubation in serum from the tumour-bearing host (HELLSTRÖM & HELLSTRÖM, 1969). This was thought to be a 7S non-complement-binding antibody which shielded tumour cells from lymphocyte cytotoxicity. In mice too, the "blocking" factors in serum appear to be restricted to IgG (RAN & WITZ, 1972). However, the picture was complicated by the finding that not only was there "blocking" antibody, but there was also "unblocking" antibody; that is, serum from tumour-free hosts, or animals with regressing tumours, could abrogate the inhibitory in vitro effects of "blocking" serum (BANSAL & SJÖGREN, 1971; HELLSTRÖM et al, 1971 b).

In spite of this, elegant in vitro experiments have shown that highly malignant and undifferentiated human tumour cells are associated with surface immunoglobulin that inhibits lymphocyte responsiveness to the cells (VÁNKY et al, 1975 a).

(iii) Immune complexes. It is difficult to discuss anti-tumour antibody per se as the sole "blocking" factor, since much work has shown that in many cases of rapidly growing

tumour isolated antibody can not be found (BALDWIN et al, 1973; THOMSON et al, 1973). This corresponds in humans to the situation of undetectable antibody in states of relapse and its rapid re-appearance post-operatively (LEWIS et al, 1969; MORTON et al, 1970 a).

Numerous animal experiments have now confirmed that it is probably complexes of tumour antibody and antigen which either bind to target tumour cells or the sensitized lymphocytes and so prevent tumour cell lysis (SJÖGREN et al, 1971). In their study these authors dissociated their "blocking" factors into 2 fractions with molecular weights of 10-100,000 and over 100,000. Neither fraction alone could block in vitro cytotoxicity, but when recombined in a ratio of 1:1 did so. In patients with malignant melanoma (STEELE et al, 1975) and children with neuroblastoma (JOSE & SKVARIL, 1974) it appears that the antibody part of the complex is IgG, and in animal tumour cells receptor sites for antigen-antibody complexes have been demonstrated, with a greater affinity than to either component alone (WOOD et al, 1975).

The presence of immune complexes is established in diseases such as systemic lupus erythematosus, chronic nephritis and the normal state of pregnancy (Lancet Editorial, 1977). In certain human neoplasms these complexes have also been identified, including neuroblastoma (JOSE & SESHADRI 1974), leukaemia (CARPENTIER et al, 1977), lung (IOACHIM et al, 1976), breast (HOFFKEN et al, 1977) and ovarian cancer (DORSETT et al, 1975; POULTON et al, 1978). In most cases however, it has not been possible to formally establish that the complexes consist of specific anti-tumour antibody and tumour antigen.

5.2 MEASUREMENT OF IN VITRO INHIBITION OF LYMPHOCYTE RESPONSES BY SERUM FACTORS.

Many experiments have shown that lymphocyte responses (generally to mitogens such as PHA) of both cancer and normal lymphocytes are impaired in the presence of cancer-patient serum (see Table 5.1), although some workers have found no such inhibition (GOLOB et al, 1969), and at least one report has found increased responses of normal lympho-

TABLE 5.1 REPRESENTATIVE STUDIES OF SERUM EFFECTS ON LYMPHOCYTE BLASTOGENESIS TO PHA.

TUMOUR	REFERENCE	LYMPHOCYTE SOURCE	COMMENTS
Various	SILK, 1967 b	Normal	Cancer serum sig. decreased responses compared with autologous serum.
Various	GOLOB et al, 1969	Normal	Cancer serum had no effect.
Various	AL-SARRAF et al, 1971	Normal Benign dis. Cancer	All cells had decreased responses in allogeneic serum compared with autologous serum. No sig. differences between results of cancer & normal cells in autol. serum.
Various	SAMPLE et al, 1971	Normal Cancer	Responses in autol. serum no different from those in ABS. Normal cells in cancer serum had sig. lower responses than in ABS. PHA dose-response assessment important.
Various	STEWARD, 1973	Cancer	Sig. decreased responses in autol. serum to PPD.
Colonic	GUILLOU et al, 1973	Normal Cancer	Cancer pts. had higher responses in ABS than in autol. serum, but in normal cells no differences. Cancer serum depressed normal lymphocytes.
Colonic	McILLMURRAY et al, 1973	Normal	Sig. higher responses in autol. serum than allogeneic serum, but cancer serum led to increased responses.
Breast	WHITTAKER et al, 1971	Normal Cancer	Cancer serum depressed normal cells, and cancer cells had sig. lower responses in autol. serum than ABS.
Breast	WHITEHEAD et al, 1974	Normal Cancer	Various sera used and all normal cells & cancer cells were inhibited by allogeneic sera.
Hodgkin's Disease	ROMAGNANI et al, 1976	Normal Cancer	Cancer cells not inhibited in autologous sera, and cancer serum had no effect on normal cells.

cytes when incubated in cancer serum (McILLMURRAY et al, 1973).

Such experiments are fraught with difficulties in both management and interpretation. Firstly, PHA as an indicator of lymphocyte reactivity may not be useful for several reasons. It reflects no known physiological in vivo mechanisms, and also the in vitro concentration of PHA may have a great effect on the final results (SAMPLE et al, 1971). Since either T or B lymphocytes can respond to PHA (CHESS et al, 1974) its use gives no useful information on the cell populations involved. Further confusion results from the demonstration that allogeneic plasma, regardless of its source, may inhibit the responses of either cancer or normal lymphocytes to PHA (AL-SARRAF et al, 1971; FISHER et al, 1972). Such non-specific cytotoxicity may come from anti-HLA antibodies, especially if the source of normal allogeneic serum is parous women (CEPPELLINI et al, 1971). Finally, PHA itself may bind to serum substances such as α_2-macroglobulin, and lead to spurious results suggesting inhibition (AMLOT & UNGER, 1976).

Secondly, if there is evidence of reduced reactivity in cancer lymphocytes when incubated in autologous serum, it is then mandatory to differentiate between a true suppressive serum effect and an underlying cellular defect. This requires as a standard of comparison the use of normal control lymphocytes incubated in autologous serum, and compared with those incubated in homologous control serum (e.g. pooled ABS), and the demonstration that there are no differences in the responses. However, this must be most rigorous, as it is known that infection (THOMAS et al, 1968) or drug ingestion (QUASTEL & KAPLAN 1968) may cause decreased reactivity. In the study of GOLOB et al (1969), the lymphocyte reactivity of normal lymphocytes in serum from "control" patients with benign diseases, was lower than in sera from cancer patients.

Thirdly, it is most important that only human serum be

used for purposes of comparison, and not foetal calf serum which is known to stimulate blastogenesis (vide infra).

Fourthly, while it may be tempting to attribute any demonstration of inhibited responses to specific factors in the cancer serum, a number of other phenomena should be taken into account including uraemia (SILK 1967a) previous chemotherapy (SINKOVIKS et al, 1972), surgery (PARK et al, 1971) and immunotherapy (CURRIE 1973), all of which may alter both lymphocyte reactivity and serum factors.

In an extensive study of 31 patients with benign and malignant breast tumours, and where the lymphocytes were incubated in autologous, human ABS, pooled advanced breast cancer, pooled advanced colonic cancer and FCS, the authors found that autologous serum gave the best stimulation to PHA in all control patients and all but one of the cancer patients (WHITEHEAD et al, 1974). Allogeneic sera all inhibited responses to much the same degree, suggesting that this was the cause rather than a specific factor in breast cancer sera.

Apart from the difficulties mentioned with the use of PHA, it would seem more relevant to assess the effects of cancer serum on specific tumour-associated lymphocyte responses (Table 5.2) Many reports have suggested that the presence of serum inhibition is tumour-specific (CURRIE & BASHAM, 1972; VÁNKY et al, 1973; YONEMOTO et al, 1978). However, results are variable and not always consistent even for the same patient. VÁNKY et al (1973) found that autologous inhibitory sera did not always inhibit in the allogeneic situation, yet they concluded that even so inhibition was due to specific factors, because tumour cells were also inhibited from stimulating blastogenesis when incubated in the serum. Subsequent experiments (VÁNKY et al, 1975 b) showed that allogeneic serum inhibited blastogenesis to tumour cells far more than autologous serum. They then, very rightly, questioned whether their previous demonstration of anti-tumour activity in FCS was valid, since it might not reflect the in vivo situation.

TABLE 5.2 REPRESENTATIVE STUDIES OF SERUM EFFECTS ON ANTI-TUMOUR MECHANISMS IN VITRO.

TUMOUR	REFERENCE	METHODOLOGY	COMMENTS
Ovary	HALBRECHT & KOMLOS, 1971	Direct cytotoxicity of tumour cells	Cancer sera only had cytotoxic effect after patient had been treated with Thiotepa.
Ovary	MITCHELL & KOHORN, 1976	LMC	Inhibitory serum preceded clinical detection of recurrence and disappearance then associated with increasing tumour.
Ovary Bladder	SAKSELA et al, 1976	LMC	Sig. inhibition in autologous serum, and also by incubating lymphocytes in Rheumatoid Factor.
Sarcoma	COHEN et al, 1973 b	LMC	Sig. inhibition in sera from cancer-bearing pts.and very little when pt. tumour-free. Specific for sarcoma.
Sarcoma	VÁNKY et al, 1973	Blastogenesis to tumour cells	Sig. inhibition in autologous and allogeneic sera, and specific for sarcoma. 15% sera from ordinary normal donors were also inhibitory.
AML	GUTTERMAN et al, 1972	Blastogenesis to blast cells	Inhibition in autologous serum, but was tumour-specific since PHA responses were unaffected. Facilitation seen in some patients.
Breast Melanoma	COCHRAN et al, 1976	LMI	Sig. inhibition in autologous serum in 35% cases, but no relationship to stage of disease.
Breast	YONEMOTO et al, 1978	LAI	Sig. inhibition in autol. and allogeneic cancer serum, specific for breast. Direct correlation between stage and degree of blocking, which disappeared after surgery.

ABBREVIATIONS: LMC = Lymphocyte-mediated cytotoxicity LMI = Leucocyte migration inhibition
LAI = Leucocyte adherence inhibition

Serum inhibition in ovarian carcinoma has been assessed by a number of workers. HALBRECHT & KOMLOS (1971) showed that the sera from patients with relapsing disease had no cytotoxic effect on primary cultures of tumour cells, but after treatment of the patient with Thiotepa there was marked cytotoxicity. "Blocking" factors have been related to clinical relapse (MITCHELL & KOHORN 1976) and used in a prognostic test (UEDA et al, 1978). DORSETT et al (1975) showed that the antibody derived from immune complexes in ascites is tumour-specific and appears to be IgG. SAKSELA et al (1974; 1976) have on the other hand suggested that serum blocking effects in ovarian cancer are non-specific, and autologous serum has the same effect as serum containing Rheumatoid Factor or artificially made immune complexes.

There are two rather important implications resulting from these in vitro experiments. The first is that regardless of whether the serum factors are specific or non-specific, plasmapheresis should be helpful to cancer patients. A number of reports suggest that this may be so. In melanoma patients, in vitro tests following plasmapheresis have shown enhanced antibody-dependent cytotoxicity against tumour cells (HERSEY et al, 1976). Similarly, the E-Rosette forming capacity of lymphocytes after plasmapheresis is increased (BROWNE et al, 1976), and this would support the thesis that the decreased %E-RFC in cancer reflects serum factors binding to receptor sites and not a true depletion. ISRAEL et al (1977) have reported objective clinical responses in 8 of 23 patients with disseminated disease before any other treatment was instituted. 6 patients had enhanced skin DHR's to secondary-recall antigens.

The second implication is that Rheumatoid Factor or anti-globulins may be involved in the clearance of tumour immune complexes from the system, and may represent "unblocking" factors (vide supra). It has been shown that although untreated cancer patients do not have a higher incidence of Rheumatoid Factor than the general population, about 85% of those with breast or lung cancer become sero-positive after radio- or chemotherapy (TWOMEY et al, 1978), and it has been

postulated that tissue injury causes the release of cell-bound immunoglobulin which forms complexes that stimulate Rheumatoid Factor production (JOHNSON & FALK, 1976). TWOMEY et al (1978) demonstrated in vitro that RF positive plasma combined with normal plasma caused complement-mediated cytotoxicity of malignant melanoma cells, whereas neither serum alone could do this. Although the significance of this is unknown, the authors suggest that this may be a mechanism after successful therapy whereby tumour cells are killed.

5.3 EXPERIMENTAL DESIGN: THE INVESTIGATION IN VITRO OF VARIOUS SERA ON BLASTOGENIC RESPONSES OF LYMPHOCYTES, AND THE CORRELATION OF IMMUNE COMPLEX LEVELS WITH CLINICAL CONDITION IN PATIENTS WITH OVARIAN CANCER.

AIMS:

(i) to examine the effects of FCS in the culture medium on both cancer and normal lymphocytes

(ii) to examine the effects of autologous and allogeneic relapse serum on cancer lymphocytes, and to compare incubation in human ABS with these results

(iii) to examine the effects of allogeneic relapse cancer serum on normal lymphocytes, compared with ABS

(iv) to measure the levels of immune complexes in patients with ovarian cancer and correlate serial determinations with the clinical condition of the patient.

MATERIALS & METHODS:

Patient groups:

(i) FCS studies: 9 patients with relapsing ovarian cancer
8 normal controls

(ii) Allogeneic and autologous cancer serum studies:
11 patients with relapsing ovarian cancer

(iii) Allogeneic relapse cancer serum on normal lymphocytes:
8 normal controls

(iv) Immune complexes:
24 patients with relapsing ovarian cancer
14 patients in clearly established remission
15 normal controls awaiting elective surgery
6 patients with benign cystic ovarian tumours

Blastogenesis: as previously described, in the presence of 12.5% heat inactivated (56°C for 20 mins) test serum, all tests being run concurrently for purposes of comparison. When stimulated cultures were required, this was done in the presence of PPD, ovarian tumour CME or Con A. Lymphocyte sub-populations were prepared by the E-Rosette techniques previously described.

Immune complex estimations: These were performed by Mr. T.A.Poulton, Williamson Laboratory, St. Bartholomew's Hospital in collaboration with Drs. L.J.Nineham and F.C.Hay at the Dept. of Immunology, Middlesex Hospital Medical School, London (POULTON et al, 1978). A solution of 12% Polyethylene glycol (PEG) 6000 in veronal/EDTA buffer was added to test sera to achieve a final concentration of 2% PEG. After 16 hours at 4°C, the tubes were spun at 9000g for 1 minute, washed in 2% PEG and the precipitates were re-solubilized in barbitone-buffered saline. The IgM and IgG content were quantitated on Mancini plates by single radial immunodiffusion in agar containing IgG antibody. The quantity of immune complex present was expressed as µg/ml IgG precipitated.

Statistical analysis: The Wilcoxon Signed Rank Test was used to analyse differences between paired comparisons of test serum. The Response Index (RI) was calculated as:

cpm unstimulated cells in FCS / cpm unstim. cells in ABS

RESULTS:

The unstimulated cultures of 16 of 17 patients tested showed increased ^{125}IUDR incorporation in FCS compared with ABS, and this was significant for both normal controls ($p < 0.02$) and cancer patients ($p < 0.01$) (see Table 5.3, Fig. 5.1). For all patients the RI ranged from 0.9 - 4.3 . The Stimulation Index (SI) was used in all calculations involving stimulated cultures (Table 5.4, Fig. 5.2). In all cells stimulated with PPD, there were significantly higher responses in ABS than FCS ($p < 0.01$); further analysis indicated that control lymphocytes showed no differences, but cancer lymphocytes showed very significant differences

in these two sera ($p < 0.02$). Blastogenic activity in cells stimulated with ovarian tumour CME was not affected by the serum used in either of the patient groups. Table 5.5, Fig. 5.3 shows that the peak of the blastogenic activity occurs at day 6 or thereafter, and that it is the T-enriched cell population which is predominantly stimulated by FCS (Table 5.6).

The responses of control lymphocytes in both autologous and human ABS were not significantly different (Table 5.7). This acted as the control for the cancer lymphocytes in both autologous and allogeneic serum. Table 5.8, Fig. 5.4 shows that there was a significant reduction in ^{125}IUDR incorporation in unstimulated cancer lymphocytes ($p < 0.01$), cells stimulated by PPD ($p = 0.05$) and those stimulated by ovarian tumour CME ($p = 0.02$) when incubated in ABS compared with autologous serum.

Normal control lymphocyte responses were significantly depressed in unstimulated cultures ($p < 0.02$) and those stimulated with PPD ($p < 0.05$) when cultured in allogeneic cancer relapse serum compared with ABS (Table 5.9, Fig. 5.5). When cancer lymphocytes were cultured in allogeneic relapse serum compared with ABS, their unstimulated counts were significantly depressed ($p < 0.01$) as were their responses to ovarian tumour CME ($p < 0.05$). However, responses to PPD were seemingly unaffected (Table 5.10, Fig. 5.6).

Levels of IgG-containing complexes were significantly elevated in patients with relapsing disease (mean 83.3 µg/ml) compared with those in remission (mean 22.2 µg/ml; $p < 0.001$). Levels in remission patients were not significantly different from those with benign disease or the control group (mean 25.1 µg/ml). (Fig. 5.7). Sequential analyses from the time of surgery are illustrated in 6 patients (Figs. 5.8, 5.9, 5.10). There is an obvious correlation between tumour load and activity, as represented in the abdominal diagrams, and levels of complexes, which appeared to increase before the clinical detection of

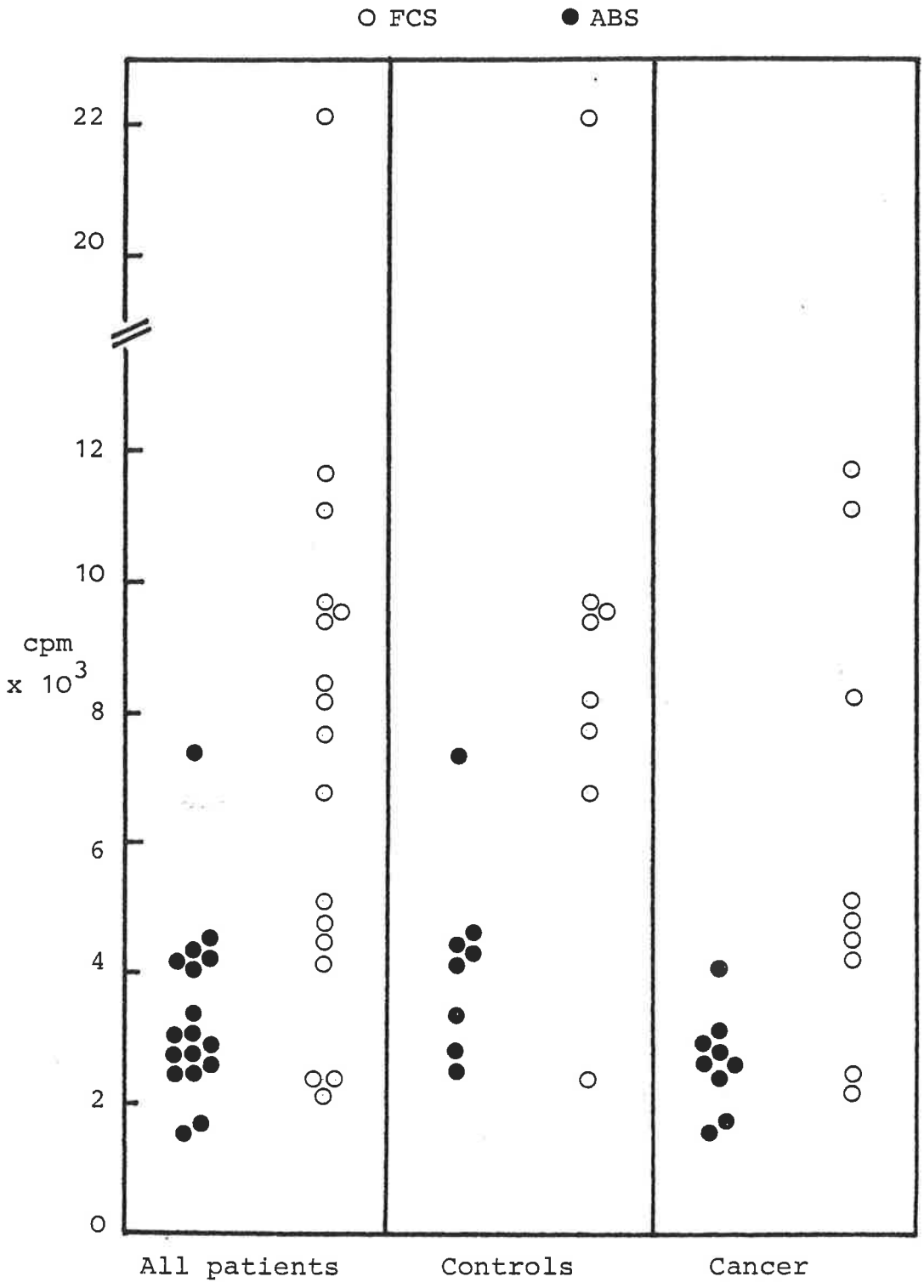


FIG. 5.1 BLASTOGENIC RESPONSES OF UNSTIMULATED LYMPHOCYTES CULTURED IN FCS AND ABS.

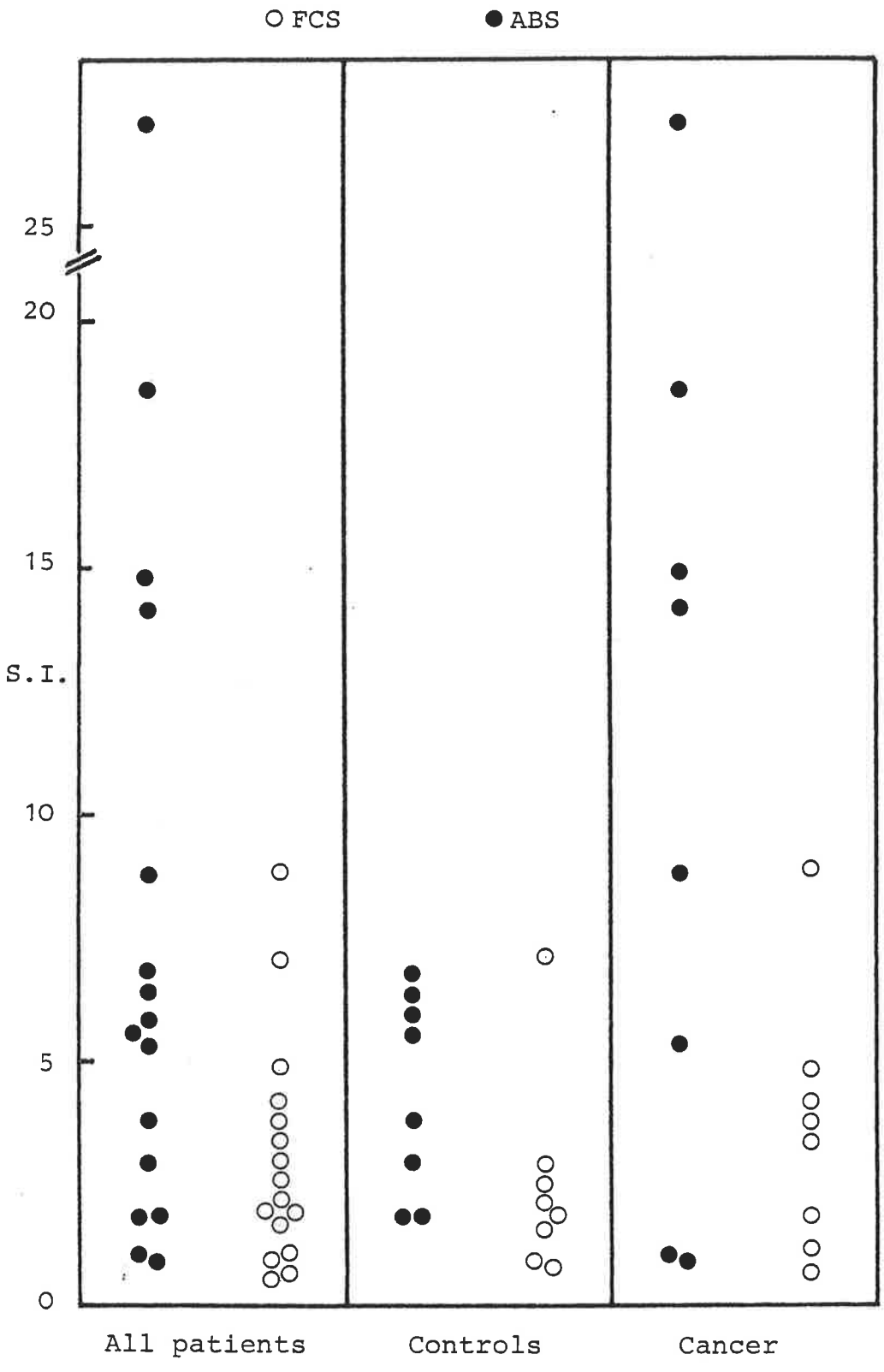


FIG. 5.2 BLASTOGENIC RESPONSES IN FCS AND ABS OF LYMPHOCYTES STIMULATED WITH PPD.

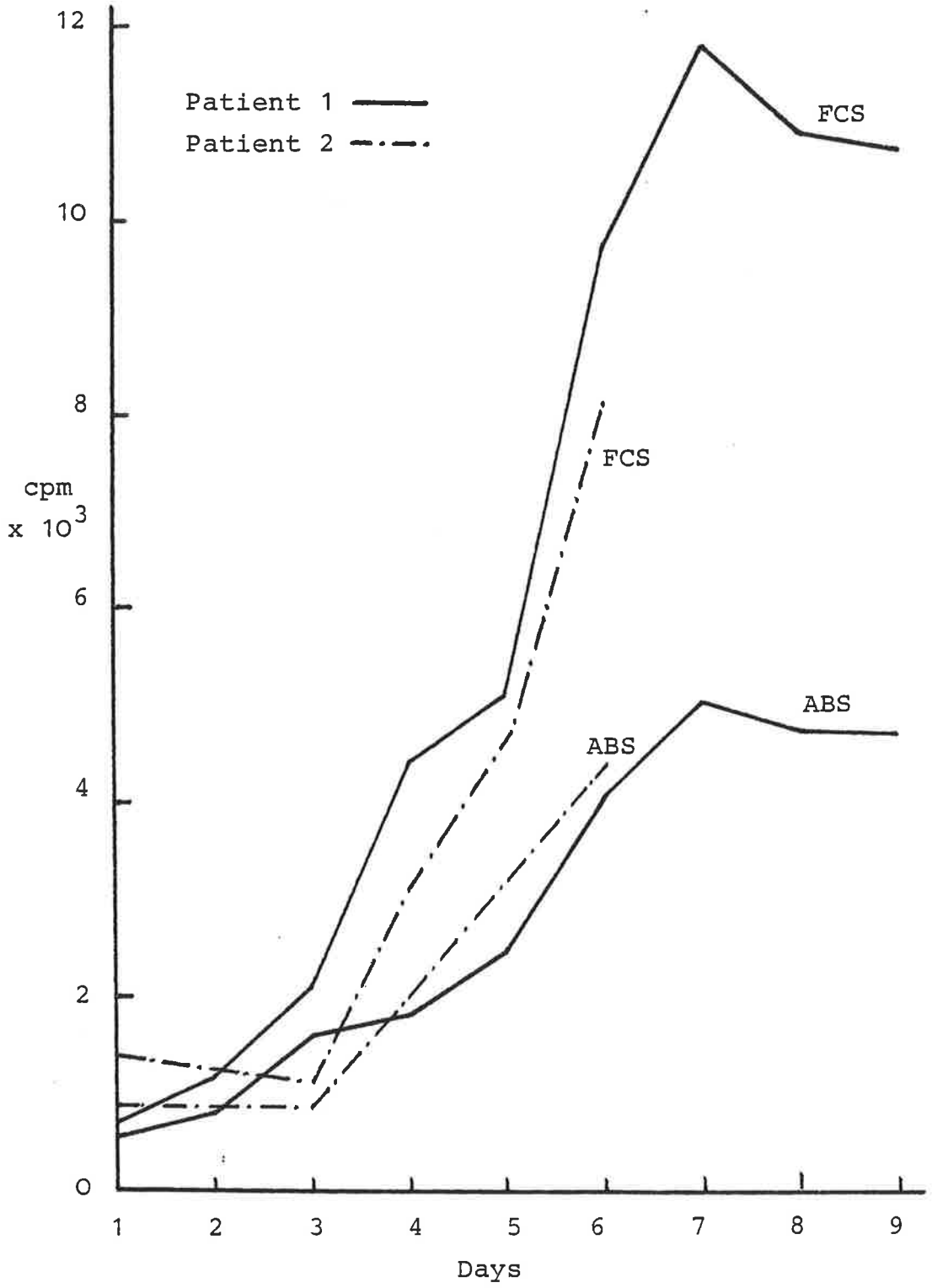


FIG. 5.3 KINETICS OF FCS-INDUCED BLASTOGENESIS IN UNSTIMULATED LYMPHOCYTES.

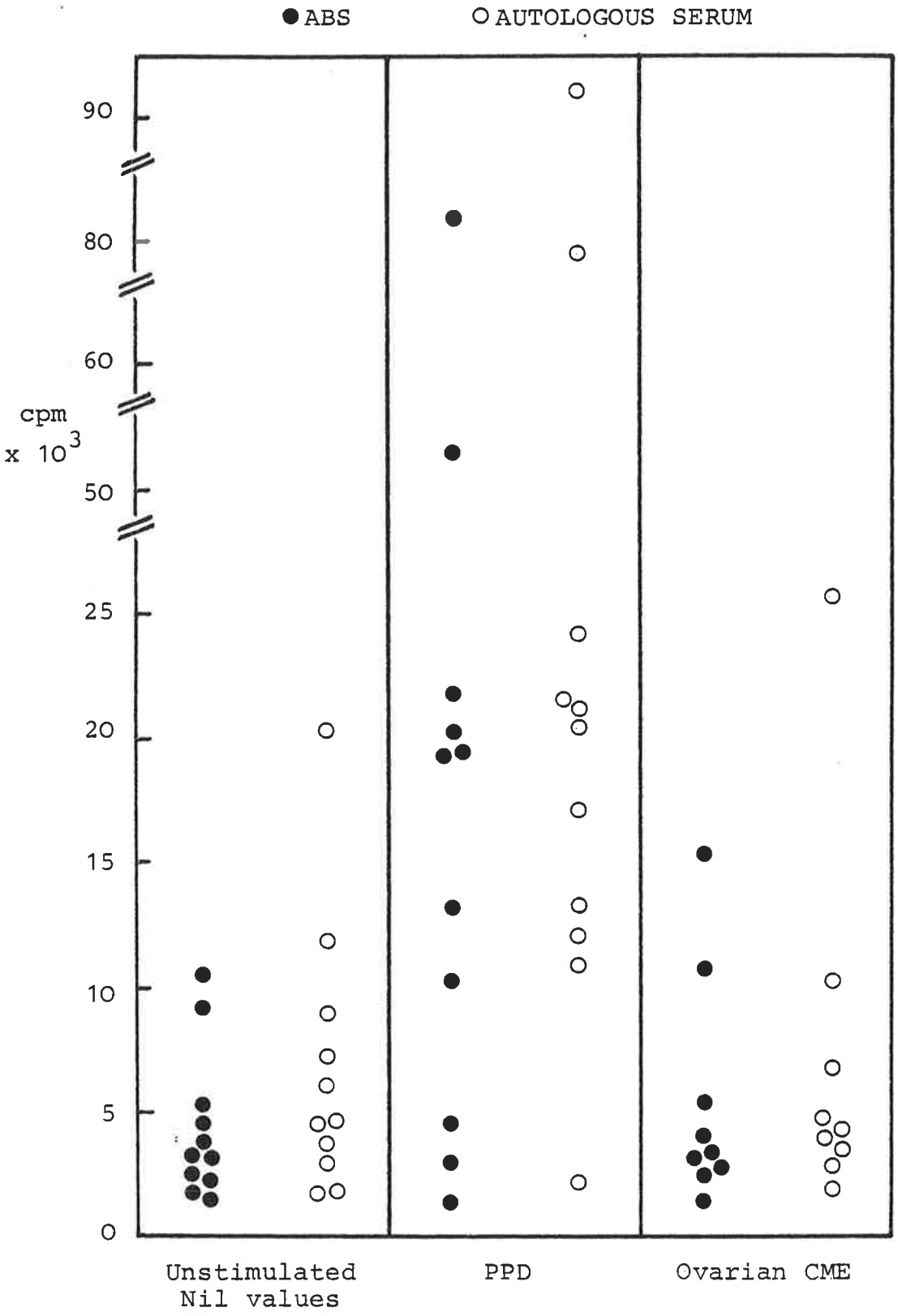


FIG. 5.4 BLASTOGENIC RESPONSES IN AUTOLOGOUS RELAPSE SERUM AND ABS OF CANCER LYMPHOCYTES TO PPD AND OVARIAN TUMOUR CME

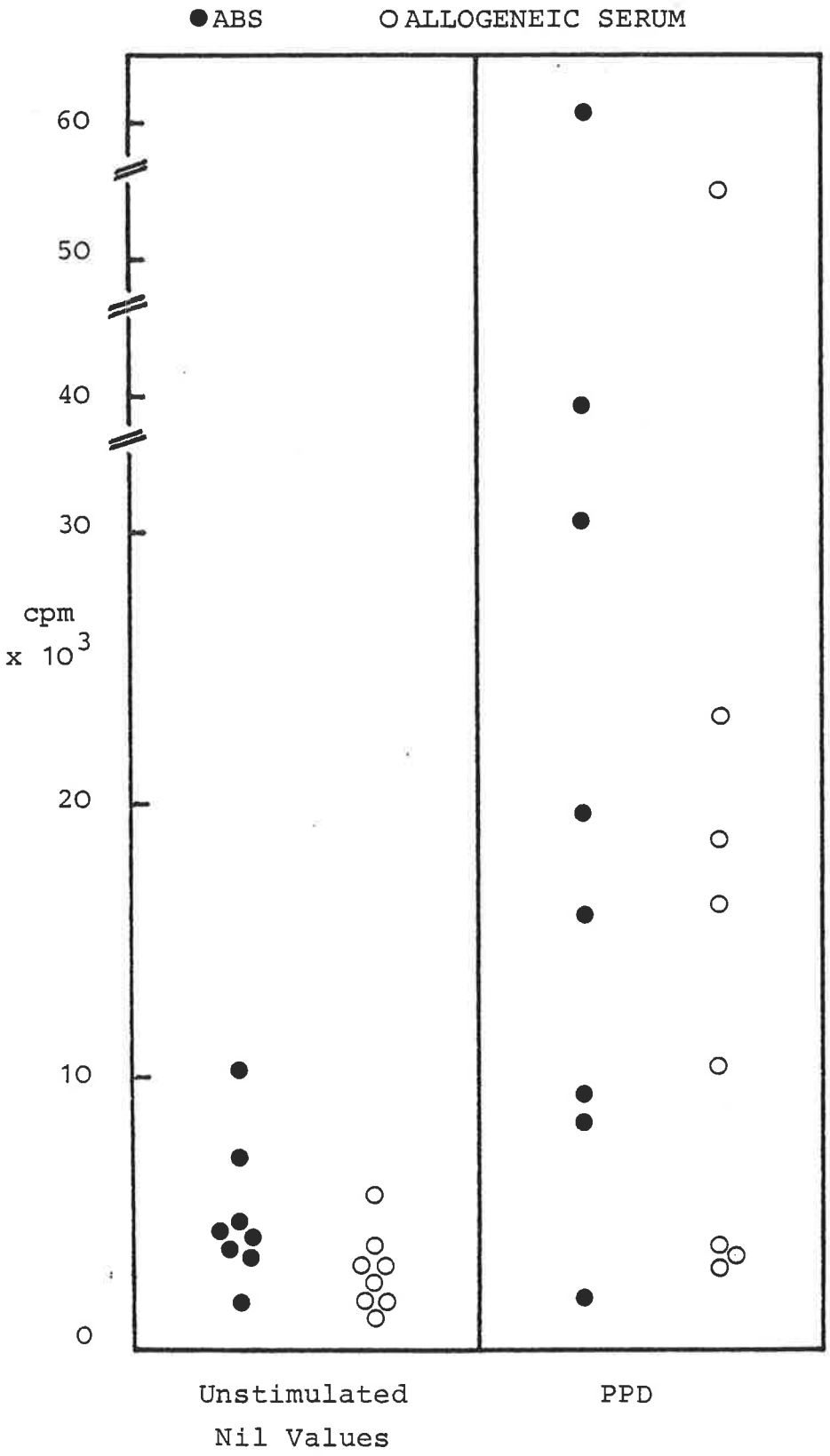


FIG. 5.5 BLASTOGENIC RESPONSES IN CANCER RELAPSE SERUM AND ABS OF NORMAL LYMPHOCYTES TO PPD.

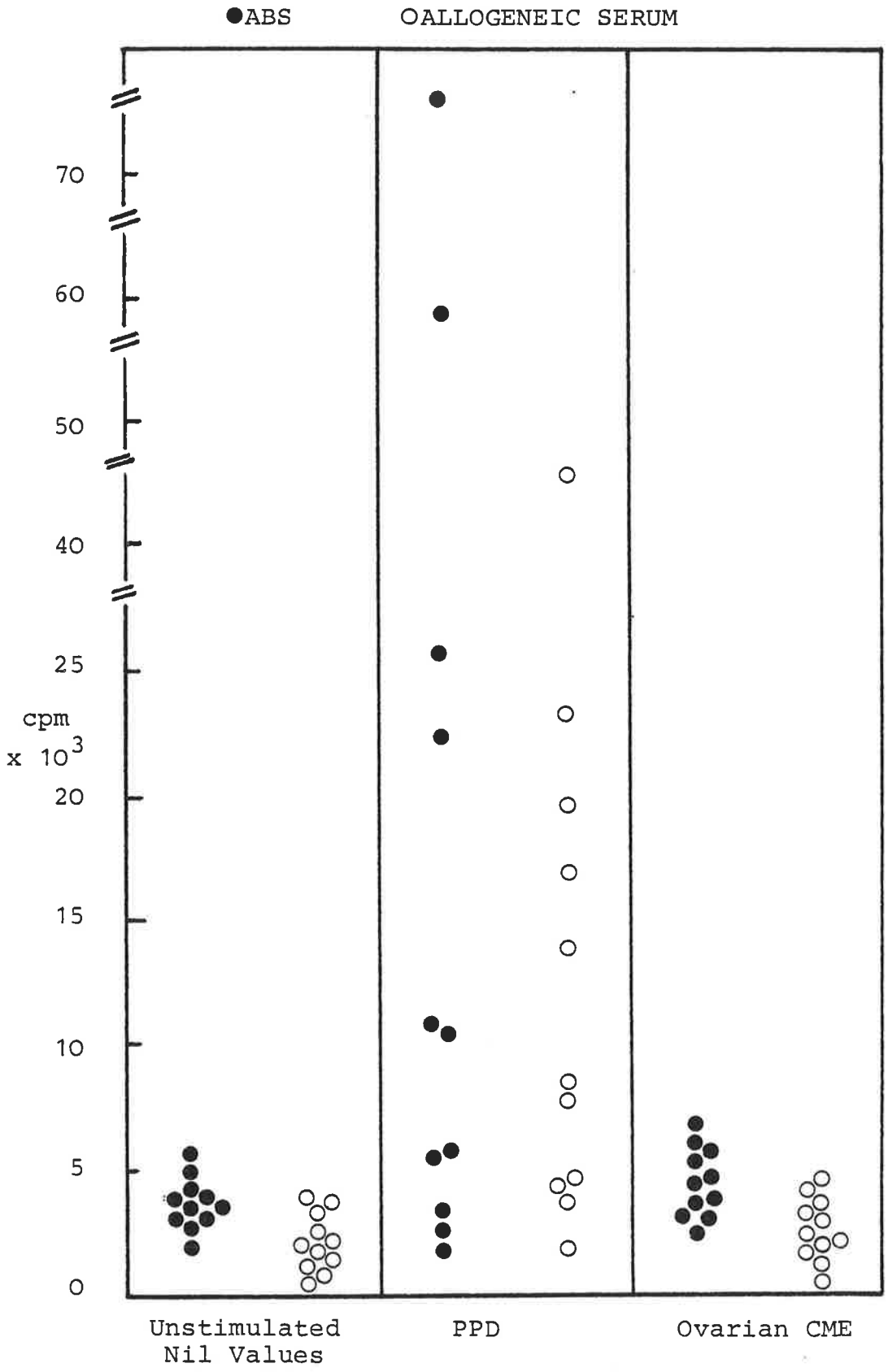


FIG 5.6 BLASTOGENIC RESPONSES IN ALLOGENEIC RELAPSE SERUM AND ABS OF CANCER LYMPHOCYTES TO PPD AND OVARIAN TUMOUR CME

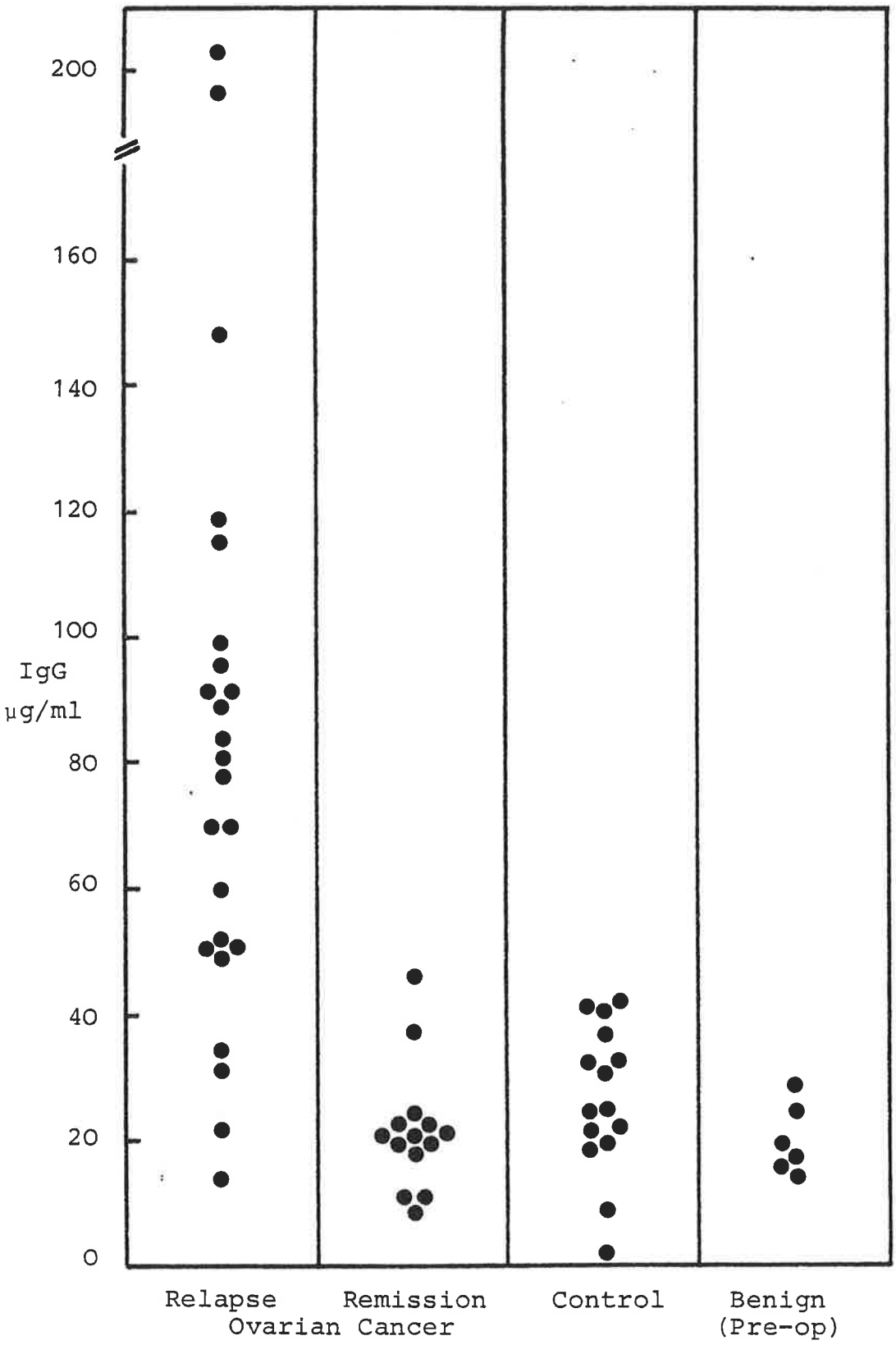
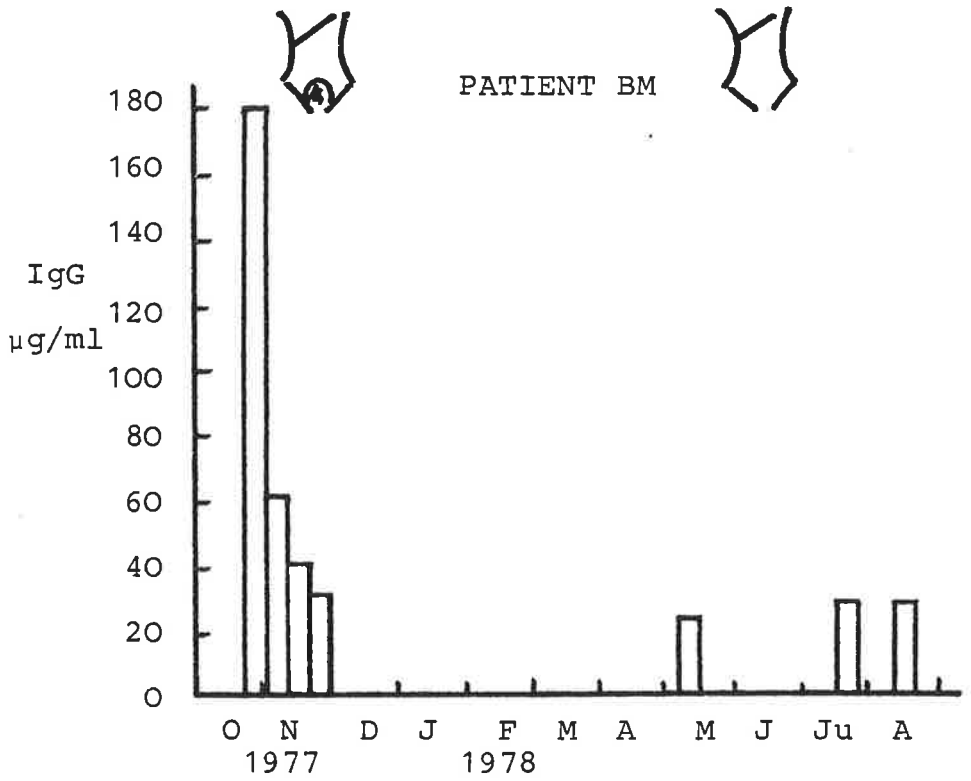
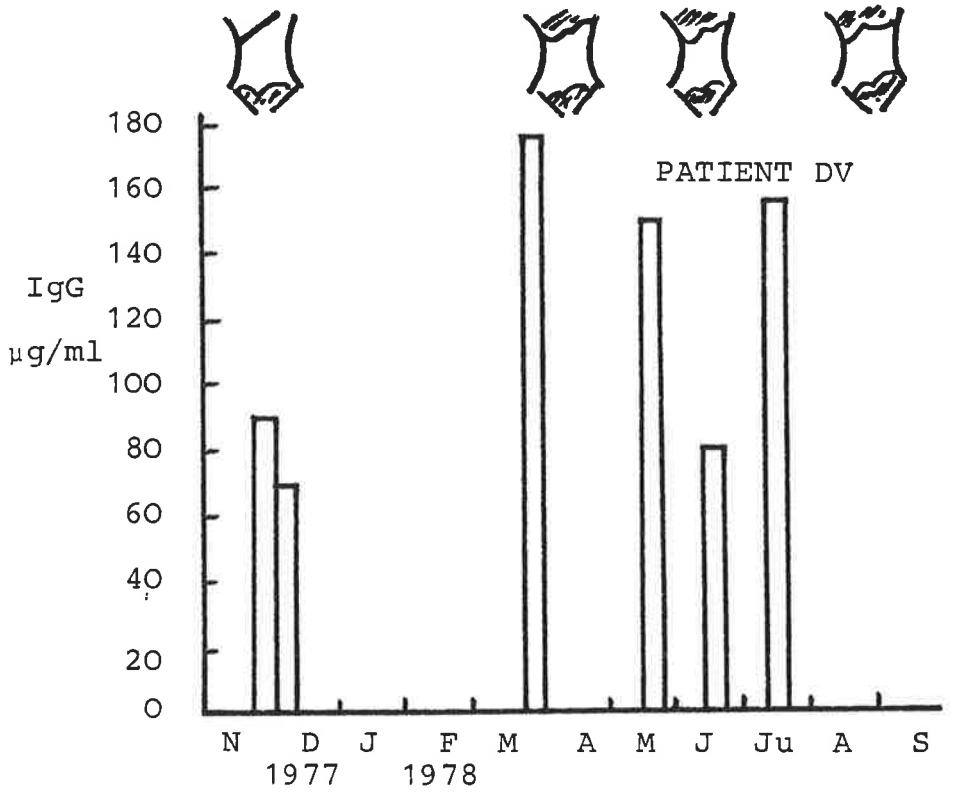


FIG. 5.7 SERUM IMMUNE COMPLEXES IN PATIENTS WITH OVARIAN CANCER AND CONTROLS ASSAYED BY PEG PRECIPITATION.

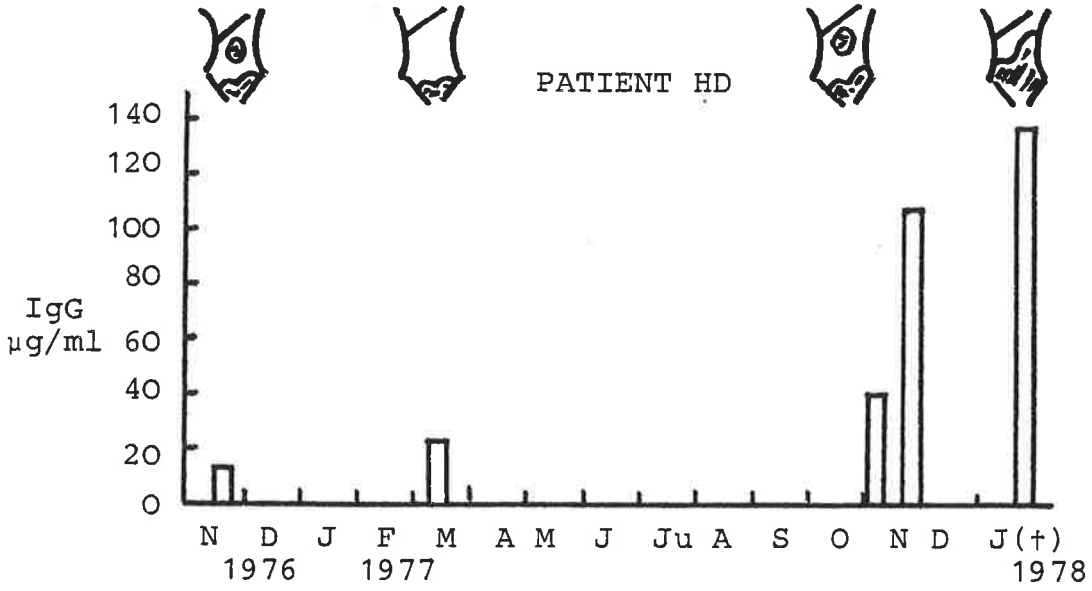


STAGE I ENDOMETRIAL CARCINOMA. NO EVIDENCE OF RECURRENCE.

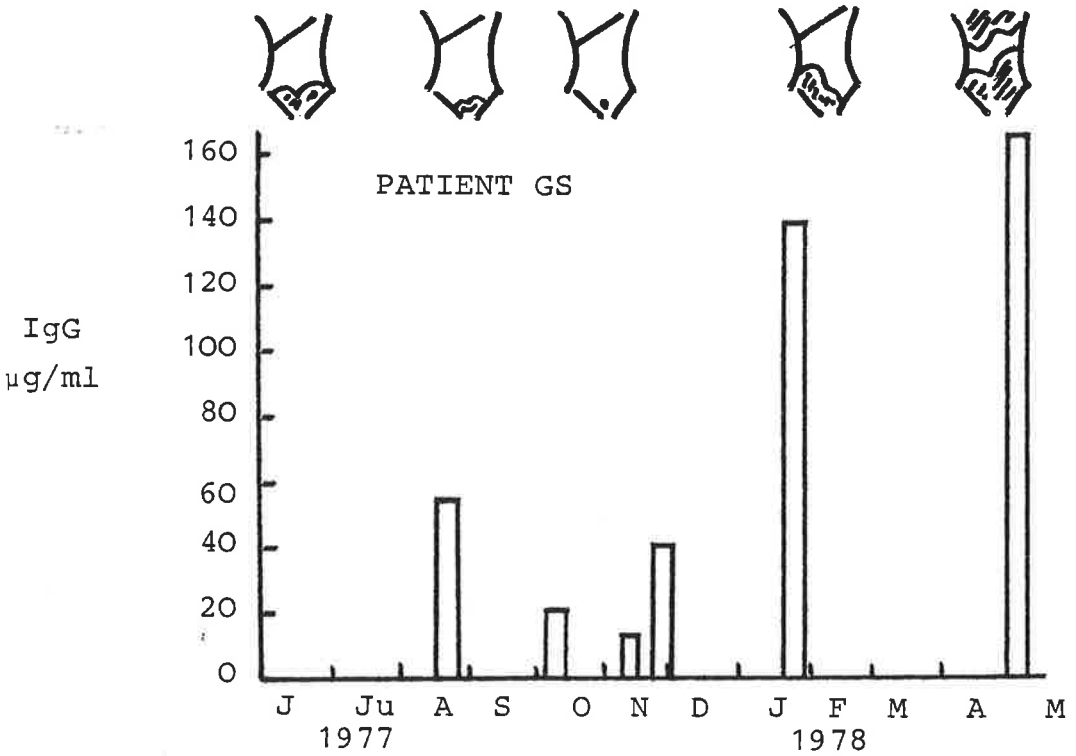


STAGE III OVARIAN CARCINOMA REMOVED INCOMPLETELY. INITIAL GOOD RESPONSE TO CYCLOPHOSPHAMIDE. LIVER METASTASES TREATED WITH ADRIAMYCIN BUT MINIMAL RESPONSE.

FIG. 5.8 SERIAL ESTIMATIONS OF IMMUNE COMPLEXES.

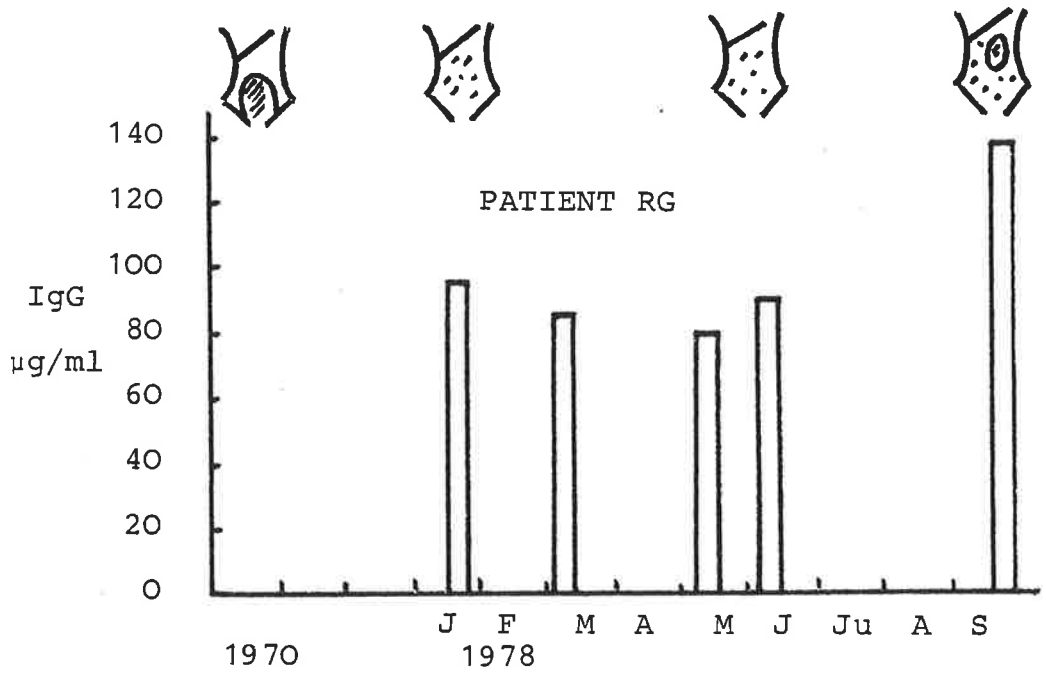


STAGE III OVARIAN CARCINOMA INCOMPLETELY REMOVED.
UNBILICAL METASTASIS FOLLOWED BY ABDOMINAL RECURRENCE.

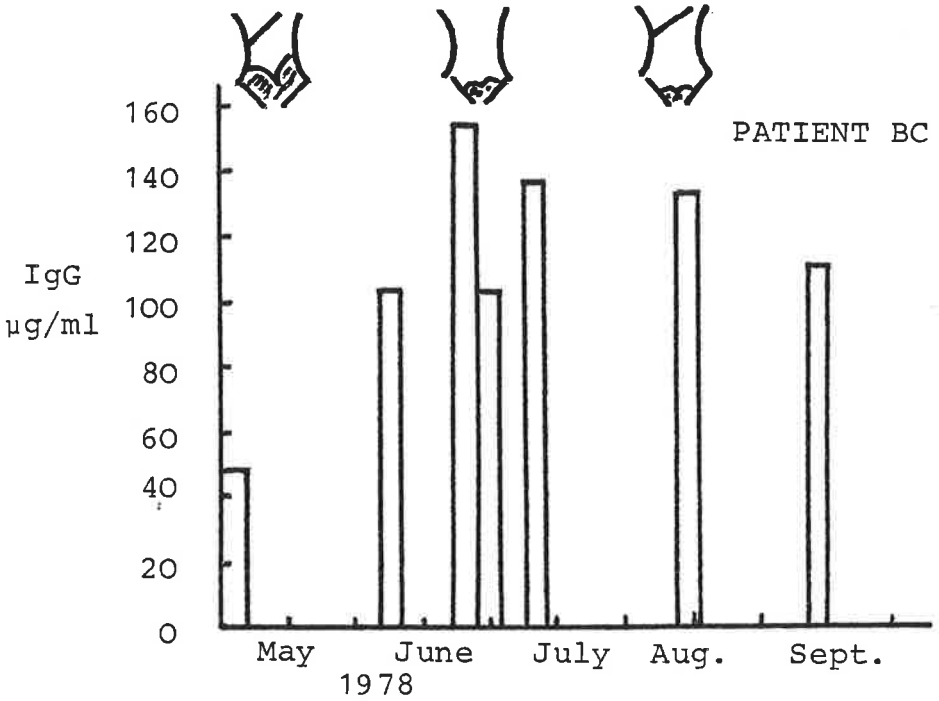


STAGE III OVARIAN CARCINOMA INCOMPLETELY REMOVED.
INITIAL GOOD RESPONSE TO CYCLOPHOSPHAMIDE. ABDOMINAL AND HEPATIC METASTASES DID NOT RESPOND TO ADRIAMYCIN.

FIG. 5.9 SERIAL ESTIMATIONS OF IMMUNE COMPLEXES.



STAGE I MUCINOUS TUMOUR REMOVED WITH SPILLAGE AT OPERATION FOLLOWED BY INTRAPERITONEAL GOLD. CARCINOMATOSIS PERITONEI HELD BY CHEMOTHERAPY BUT EVENTUALLY BOWEL METASTASES.



STAGE III OVARIAN CARCINOMA INCOMPLETELY REMOVED INITIAL GOOD RESPONSE TO CYCLOPHOSPHAMIDE FOLLOWED BY RECURRENT ASCITES.

FIG. 5.10 SERIAL ESTIMATIONS OF IMMUNE COMPLEXES.

relapse (usually with ultra-sonic scanning or gross appearance of effusions). Patient BM is the only patient who did not have ovarian cancer; she had Stage I endometrial carcinoma.

DISCUSSION:

The results of cells cultured in FCS show that it has a distinct blastogenic effect on unstimulated cultures, leading to ^{125}IUDR incorporation of up to 4 times that seen in cells cultured in ABS. In contrast, PPD responses (especially in cancer patients) were consistently higher in ABS than in FCS. This finding supports the observation of JOHNSON & RUSSEL (1965), that more cells were transformed by PHA when cultured in autologous serum rather than FCS, although the use of FCS resulted in greater blastogenic transformation of unstimulated cultures. This latter increase could therefore not be accounted for on the basis of better nutrition in FCS.

The peak of the blastogenic activity in FCS-cultured lymphocytes occurs at 6-7 days, suggesting that it is caused by an antigenic component in the serum rather than a mitogenic effect. This concurs with the findings of ZIELSKE & GOLUB (1976), although they proposed that the phenomenon was a mitogenic one affecting predominantly T-cells. However, the peak of mitogenic stimulation normally occurs at 3 days (see Fig 2.6, Table 2.6) and would be expected to give rise to a very much greater blastogenic transformation than that observed in FCS, where the degree of stimulation in comparison with ABS is consistent with values normally obtained for secondary recall antigens. Our work also confirms that it is the T-enriched cell population which responds to the FCS, further supporting the idea of an antigenic rather than a mitogenic stimulus. It has been shown that FCS has Thymosin-like activity (BYROM et al, 1977), and the stimulation of mouse spleen cells by FCS appears to be caused by the fraction containing transferrin (VOGT et al, 1969). However, it can affect B-cells, and in nude mice, B lymphocytes increase their IgM production under its influence (MELCHERS & ANDERSSON, 1974).

The effects of FCS are however not simply restricted to the stimulation of lymphocytes. A number of studies have examined the differences in tumour-associated immune responses evoked by FCS, in both human and animal systems. IRIE et al (1974) showed that human tumour cells cultured in FCS acquired a new surface antigen, which they termed "heterologous membrane antigen". It was found that both normal controls and cancer patients possessed a normal antibody to this antigen, as assessed by the immune adherence test, and the ability of sera to react with tumour cells disappeared if they were cultured in ABS. It was subsequently shown that FCS-cultured melanoma cells were more susceptible to lymphocyte cytotoxicity from both normal and cancer lymphocytes, when compared with ABS-grown cells (SULIT et al, 1976). Similarly EMBLETON & IYPE (1978) have shown antigenic alterations in rat liver cells cultured in FCS.

All these results therefore should caution on the use of FCS in in vitro systems, since comparative results may be quite significantly altered when cells are cultured in the presence of FCS. Certainly, in this laboratory, all systems were thereafter changed to the use of ABS. À propos of serum analysis, one is forced to conclude that while it examines the phenomenology of immune alterations, it does not appear to be useful either as a diagnostic or prognostic tool. Certainly the results in this study show that the responses of cancer lymphocytes cultured in autologous serum are significantly higher than those cultured in ABS, yet the same sera used allogeneically give rise to significantly lower responses in both the cancer lymphocytes and normal lymphocytes. This paradoxical finding is difficult to explain, but it would seem that the depression is not due to anti-HLA antibodies, since responses of normal lymphocytes cultured in autologous serum were not significantly different from those in allogeneic ABS. Furthermore, the ABS was taken from a single, male Rh + donor.

Such paradoxical results concur with the findings of

several workers that incubation in allogeneic serum may be the cause of non-specific depression in cancer lymphocytes (AL-SARRAF et al, 1971; McILLMURRAY et al, 1973; WHITEHEAD et al, 1974), but is not unfortunately re-inforced by our further finding that cancer lymphocytes cultured in allogeneic relapse sera had significantly lower values than in ABS. One can conclude therefore, that all serum studies are difficult to interpret since a wide range of disease states may result in serum "inhibitory" factors (GATTI 1971), and this means that attempts to compare effects of sera from normal and cancer patients on lymphocyte responses may not always be conclusive, since many unrelated factors can influence the results.

This study does not determine whether there is a correlation between the levels of immune complexes and the degree of immune suppression, but clearly this is an area worthy of investigation. The significantly elevated levels of immune complexes in patients with relapsing disease compared with those in remission, strongly suggests that these may in some way be involved in the inability of the host to overcome the tumour activity. Even more striking are the clinical studies which generally show an increase in immune complex levels before clinical detection of recurrence or new metastases. Clearly this would seem to be an area for further research, both for the preparation of a prognostic/diagnostic test, as well as determination and identification of the ?tumour-associated components within the complex.

TABLE 5.3 BLASTOGENIC ACTIVITY OF UNSTIMULATED LYMPHOCYTES CULTURED IN FCS AND ABS.

cpm ABS	cpm FCS	R.I.	(FCS-ABS) cpm diff.	Rank
CONTROLS:				
2572	6806	2.6	4234	4
4413	9438	2.1	5025	5
2826	2466	0.9	-360	-1
4048	7706	1.9	3658	2
7434	22116	2.9	14682	8
3381	9702	2.9	6321	7
4170	9684	2.3	5514	6
4404	8290	1.9	3886	3
Wilcoxon Signed Rank Test: T = -1; p < 0.02				
CANCER:				
1581	2253	1.4	672	1
2894	4864	1.7	1970	6
2769	11781	4.3	9012	9
2985	11034	3.7	8049	8
1650	2445	1.5	795	2
2520	8304	3.3	5784	7
4144	5062	1.2	918	3
2577	4509	1.7	1932	5
3276	4392	1.3	1116	4
Wilcoxon Signed Rank Test: T = 0; p < 0.01				

TABLE 5.4 BLASTOGENIC ACTIVITY OF LYMPHOCYTES STIMULATED WITH PPD AND OVARIAN TUMOUR CME IN FCS AND ABS.

	cpm FCS	S.I.	cpm ABS	S.I.	(AB-FCS) SI diff.	Rank
CONTROLS:					(PPD results only)	
Nil	6806		2572			
PPD	47596	7.0	16872	6.6	-0.4	-2.5
CME	-	-	-	-		
Nil	9438		4413			
PPD	24756	2.6	12831	2.9	0.3	1
CME	8631	0.9	3429	0.8		
Nil	2466		2826			
PPD	5751	2.3	5481	1.9	-0.4	-2.5
Nil	7706		4048			
PPD	7802	1.0	7726	1.9	0.9	4
CME	6802	0.9	4174	1.0		
Nil	22116		7434			
PPD	38468	1.7	39986	5.4	3.7	7
Nil	9702		3381			
PPD	28296	2.9	19008	5.6	2.7	6
CME	10149	1.0	3000	0.9		
Nil	9684		4170			
PPD	18717	1.9	15690	3.8	1.9	5
CME	13170	1.4	4089	1.0		
Nil	8290		4404			
PPD	7824	0.9	27840	6.3	5.4	8
Median Values:						
	PPD	2.1		4.6	T = 5,	n.s.
	CME	1.0		0.9	T = 2,	n.s.
CANCER PTS:						
Nil	2253		1581			
PPD	19809	8.8	23366	14.8	6.0	5
Nil	4864		2894			
PPD	22848	4.7	25680	8.9		
CME	6506	1.3	5272	1.8	4.2	4
Nil	11781		2769			
PPD	37191	3.2	76098	27.5	24.3	8
CME	13140	1.1	3540	1.3		
Nil	2445		1650			
PPD	2892	1.2	1734	1.1	-0.1	-1
CME	2322	0.9	1641	1.0		

TABLE 5.4 (contd)

	cpm FCS	S.I.	cpm ABS	S.I.	(AB-FCS) SI diff.	Rank
Nil	8304		2520			
PPD	15615	1.9	13344	5.3	3.4	3
CME	11613	1.4	3222	1.3		
Nil	5062		4144			
PPD	20644	4.1	58378	14.1	10.0	6
CME	4830	1.0	5430	1.3		
Nil	4509		2577			
PPD	3642	0.8	2556	1.0	0.2	2
CME	3465	0.8	3477	1.3		
Nil	4392		3276			
PPD	15402	3.5	61314	18.7	15.2	7
CME	6384	1.5	2880	0.9		

Median Values:

PPD	3.4	11.5	T = -1, p < 0.02
CME	1.1	1.3	T = 8.5, n.s.

If all PPD results are ranked together, T = 10, p < 0.01

TABLE 5.5 KINETICS OF BLASTOGENESIS IN UNSTIMULATED LYMPHOCYTES CULTURED IN FCS.

Day	Patient 1. FCS	Patient 1. ABS	Patient 2. FCS	Patient 2. ABS
1	645	564	1422	972
2	1158	648	-	-
3	2010	1506	1122	834
4	4407	1749	3021	1968
5	5076	2652	4668	3273
6	9684	4170	8290	4404
7	11772	5064	-	-
8	10974	4818	-	-
9	10812	4869	-	-

TABLE 5.6 RESPONSES OF CELL SUB-POPULATIONS TO CULTURE IN FCS AND ABS.

Patient 1		T-depleted	T-enriched
ABS	Nil	3618	3332
	PPD	33555	17044
FCS	Nil	1563	6942
	PPD	8004	11520
Patient 2			
ABS	Nil	9993	10686
	PPD	8976	13754
FCS	Nil	9906	22562
	PPD	10092	17764

TABLE 5.7 BLASTOGENIC RESPONSES OF NORMAL LYMPHOCYTES IN AUTOLOGOUS SERUM AND ABS.

Unstimulated Values				PPD Values			
Autol.	ABS	cpm diff.	Rank	Autol.	ABS	cpm diff.	Rank
4020	4458	-438	-2	66150	60026	6124	4
3808	4328	-520	-3	5094	8906	-3812	-3
12250	4048	8202	6	10602	7726	2876	2
8680	7434	1246	5	32036	39986	-7950	-5
3177	4413	-1236	-4	23952	12831	11121	6
6004	5739	265	1	8182	10446	-2264	-1
T = -9, n.s.				T = -9, n.s.			

TABLE 5.8 BLASTOGENIC RESPONSES IN AUTOLOGOUS RELAPSE SERUM AND ABS OF CANCER LYMPHOCYTES TO PPD AND OVARIAN TUMOUR CME.

Nil Values				PPD VALUES			
Autol.	ABS	cpm diff.	Rank	Autol.	ABS	cpm diff.	Rank
11574	10293	1281	5	76929	82029	-5100	-7
20400	9074	11326	11	92236	53502	38734	11
2814	2980	-166	-1	11522	2958	8564	10
8834	5294	3540	10	12222	10710	1512	2
6612	3194	3418	9	22260	19108	3152	6
7461	4143	3318	8	24753	21852	2901	5
1938	1620	318	3	13305	4890	8415	9
4545	3669	876	4	22653	20487	2166	3
1950	1650	300	2	2154	1734	420	1
4797	2268	2529	7	17154	19647	-2493	-4
4008	2520	1488	6	20730	13344	7386	8

Tumour CME Values			
Autol.	ABS	cpm diff.	Rank
11013	11298	-285	-2
25256	15194	10062	9
2796	2538	258	1
5944	5212	732	4
1953	2763	-810	-5
4629	3507	1122	7
2685	1641	1044	6
4104	3591	513	3
4410	3222	1188	8

Wilcoxon Signed Rank Test:

Nil Values	T = -1,	$p < 0.01$
PPD	T = -11,	$p < 0.05$
CME	T = -1,	$p < 0.02$

TABLE 5.9 BLASTOGENIC RESPONSES IN CANCER RELAPSE SERUM AND ABS OF NORMAL LYMPHOCYTES TO PPD.

Nil Values				PPD Values			
ABS	Ca. serum	cpm diff.	Rank	ABS	Ca.	cpm diff.	Rank
2085	2559	-474	-1	2106	3186	-1080	-2
10254	3210	7044	8	30489	19128	11361	7
4458	3282	1176	3	60026	55665	4361	3
4328	2758	1570	4	8906	3888	5018	5
4048	2124	1924	5	7726	3260	4466	4
7434	6358	1076	2	39986	23680	16306	8
3381	1251	2130	6	19008	10554	8454	6
4170	1914	2256	7	15690	16620	-930	-1

T = -1, $p < 0.02$ T = -3, $p < 0.05$

TABLE 5.10 BLASTOGENIC RESPONSES IN ALLOGENEIC CANCER RELAPSE SERUM AND ABS OF CANCER LYMPHOCYTES TO PPD AND CME.

Nil Values		cpm		PPD Values			
ABS	Ca. allog.	diff.	Rank	ABS	Ca.	cpm diff.	Rank
5294	3380	1914	7	10710	4724	5986	6
2980	2046	934	5	2958	8414	-5456	-4
2268	1557	711	3	1964	17070	-15106	-9
2862	3351	-489	-2	5241	8178	-2937	-2
2577	2292	285	1	2556	2286	270	1
3738	3009	729	4	22522	19161	3361	3
2769	1611	1158	6	76098	23268	52830	11
2894	876	2018	10	25680	4212	21468	10
4144	2196	1948	8	58378	46668	11710	8
4716	2544	2172	11	10608	4980	5628	5
3972	1962	2010	9	5566	13184	-7618	-7

T = -2, $p < 0.01$ T = -22, n.s.

TABLE 5.10 (contd.)

ABS	Ovarian Tumour CME Values Ca.	cpm diff.	Rank
5212	3034	2178	6
2538	2358	180	1
3591	1275	2316	7
2691	3177	-486	-3
3477	1950	1527	5
4120	4884	-764	-4
3540	3897	-357	-2
5272	957	4315	10
6858	2092	4766	11
4968	2382	2586	8
6384	3358	3026	9

$T = -9, \quad p < 0.05$

CHAPTER 6. IMMUNOTHERAPY AND ITS USE IN THE TREATMENT OF OVARIAN CANCER.

The ultimate goal of tumour immunology is seen as the ability to successfully immunise patients with cancer against their own tumours, the rationale for such immune manipulation being the demonstration of animal and human tumour cell antigenicity (see Chapter 3). This Chapter will discuss (a) animal experiments and models for immunotherapy, (b) human immunotherapy trials, and (c) the results of an immunotherapy trial in women with ovarian cancer. The following Chapter will examine the monitoring of immunological parameters in women receiving immunotherapy.

The following definitions for methods of immunotherapy will be used in this Chapter:

"Passive" - the use of xenogeneic or allogeneic anti-tumour sera

"Non-specific" - the stimulation of an immune response by the use of materials not antigenically related to the tumour (e.g. BCG, C.Parvum)

"Adoptive" - the transfer of immunity from one individual to another using specifically immune lymphoid cells or material from such cells which is capable of transferring specific immunological information to the recipient lymphocytes

"Specific" - immunization with tumour cells or antigenic products directed specifically towards the tumour or others sharing the same antigens.

Table 6.1 is an outline of immunotherapeutic methods adapted from a Table given by PIROFSKY (1978). It should be pointed out that the use of rabbit anti-human-plasmacytoma serum (BEAULIEU et al, 1976), murine anti-plasma cell serum and immunosuppression of T-cells with agents such as Oxisuran, mentioned in this Table, are still at this stage purely experimental. The use of plasmapheresis has previously been discussed (see Chapter 5).

In the immunotherapeutic approach to human cancer, it

TABLE 6.1 METHODS OF IMMUNOTHERAPY.

IMMUNOINHIBITORY METHODS.

I. Suppression of sub-populations of cells:
   - (i) anti-B lymphocyte serum
   - (ii) anti-plasma cell serum
   - (iii) inhibition of T-helper cells with Oxisuran

II. Neutralization or elimination of blocking activity:
   - (i) plasmapheresis
   - (ii) infusion of normal IgG to restrict production of anti-tumour antibody by feedback mechanism
   - (iii) disulphide reduction of IgM to inactivate it (e.g. with Penicillamine)

IMMUNOSTIMULATORY METHODS.

I. Adoptive Immunotherapy with immunocompetent cells:
   - (i) transplant of allogeneic immunocompetent cells (non-sensitized) to immunosuppressed host
   - (ii) transplant of allogeneic HL-A compatible cells sensitized against tumour cells
   - (iii) in vitro activation of autologous cells which are then re-infused into the host

II. Active Specific Immunotherapy by stimulation of effector cells:
   - (i) purified tumour antigens
   - (ii) non-viable or irradiated allogeneic or autologous tumour cells
   - (iii) modified tumour cells or antigen

III. Passive Immunotherapy with effector material:
   - (i) infusion of lymphokines
   - (ii) infusion of xenogeneic or allogeneic antibody with anti-HLA and anti-tumour activity, and which may be coupled to a chemotherapeutic agent
   - (iii) infusion of sensitized xenogeneic or allogeneic RNA
   - (iv) infusion of Transfer Factor

IV. Non-specific Stimulation:
   - (i) DNCB, BCG, C.Parvum, viral vaccines
   - (ii) Levamisole
   - (iii) Lectin stimulation of T cells

would appear that the following principles are most important (McKHANN & GUNNARSSON, 1974):

(i) minimal tumour burden should be present. It was shown in a murine L1210 leukaemia model (MATHÉ et al, 1969 a) that the effect of immunotherapy with BCG depended upon the number of tumour cells grafted into the animal. In control mice, 10^2 tumour cells gave a mortality of 60% and 10^3 cells a 100% mortality; if BCG was given after a graft of 10^5 cells it was effective in preventing tumour growth, but it was ineffective if 10^6 or more tumour cells were grafted. In the human situation it has been shown that patients with localized disease and small tumour burden have better cell mediated immunity than patients with disseminated disease (see Chapter 4); and since, 35% of solid tumours can now be cured with surgery, or a temporary remission obtained with chemotherapy or radiotherapy (AMERICAN CANCER SOCIETY, 1970), such primary treatment should be given before immunotherapy is attempted.

(ii) the host should be immunologically competent or become so after immunotherapy. In guinea pigs, the use of anti-lymphocyte serum abrogates the effects of BCG and tumour cells in preventing the development of diethylnitrosamine-induced hepatomas (HANNA et al, 1973). EILBER & MORTON (1970) have reported that 93% of patients with malignant melanoma who failed to react to DNCB were inoperable or developed recurrences within 6 months (see Chapter 4), and that BCG does not eradicate intradermal metastases in anergic patients (MORTON et al, 1970 b).

(iii) the timing of immunotherapy is vital especially if chemotherapy is given concurrently, so that the immunosuppressive effects of the drugs do not abrogate the immunostimulation of the immunotherapy. CURRIE & BAGSHAWE (1970) showed that chemotherapy with Cyclophosphamide given prior to C.Parvum immunotherapy in a murine fibrosarcoma model, led to an increased anti-tumour effect, but the time interval between the two was important.

(iv) sites of immunization with active immunotherapy are important. Since lymph nodes draining tumour sites may

be "paralysed" from releasing sensitised lymphoblasts into the circulation (ALEXANDER & HALL, 1970), active immunotherapy should be administered in lymphatic drainage areas which are free of tumour.

(v) evidence should be shown of augmentation of the immune system, with minimal production of anti-body which may cause enhancement (vide infra).

(vi) the tumour must be of proven antigenicity by in vivo or in vitro experimentation, and antigenic cross-reactivity between autologous and allogeneic tumour cells should be shown. There is a wealth of experimental data showing that active immunotherapy will protect only against a tumour of identical antigenicity (DELORME & ALEXANDER, 1964; MIHICH, 1969). Furthermore, processes such as cryopreservation must be shown not to alter tumour antigenicity (POWLES et al, 1973).

(vii) the process must be shown to be safe. The production of enhancing antibody is a risk (KALISS, 1958) and autoinoculation with viable tumour cells may lead to progressively growing tumours (HOWARD, 1963). It is a salutary experience to read the report of fatal homotransplantation of malignant melanoma from a daughter to her 80 year old mother (SCANLON et al, 1965). 22 days after the transplant of a piece of tumour less than 0.5 cm in size, biopsy at the site showed melanoma, and in spite of wide excision, the women died of disseminated melanoma 451 days after the transplant. Such experimentation has only confirmed the scepticism that many feel towards immunotherapy.

(viii) the route of administration is as important as the site. The skin is known to give stronger and more lasting immune responses to antigenic stimulation (KRONMAN et al, 1970) and work done on regional lymph nodes (ALEXANDER & HALL, 1970) suggests that immunotherapy will only be successful if nodes draining the site are intact.

6.1 METHODS OF IMMUNOTHERAPY.

(i) Prophylactic Immunotherapy. In animal experiments the

sensitisation of the host prior to innoculation with viable tumour cells leads to rejection of those cells. In humans, this would only be feasible against a causative agent such as an oncogenic virus. Non-specific prophylactic immunization with BCG in school children was reported to have resulted in a lower incidence of leukaemia (23.54 per 100,000 compared with 55.87 in the non-immunized group) (DAVIGNON et al, 1970; ROSENTHAL et al, 1972). However, objections to the methods used in these studies have been raised (HOOVER, 1976) and further studies have not substantiated these findings (SNIDER et al, 1978).

(ii) Passive Immunotherapy. Although it is thought that cell mediated immunity is more effective in tumour graft rejection than humoral immunity, the use of specifically immune serum has resulted in tumour regression in some cases. GORER & AMOS (1956) showed that immune serum from CBA mice sensitized to E1-4 leukaemia prevented tumour takes in C57BL mice, even when the tumour cells were injected 2 days prior to the serum. BANSAL & SJÖGREN (1971) showed that immune serum to polyoma virus-induced tumours, not only caused tumour regression in syngeneic rats in vivo, but in vitro could reverse the blocking of immune cytotoxicity by autologous serum from rats with growing tumours. They postulated that such serum works by unblocking antibody or antigen-antibody immune complexes, or else masks antigenic sites on tumour cells and so prevents immune complexes inhibiting cellular responses.

In humans the use of immune serum has not generally been successful. Last century there were reports of regressions and amelioration of pain after injection of dog or horse anti-human-tumour serum, but the risks of serum sickness were great (CURRIE, 1972). MURRAY (1958) treated 233 patients with a variety of metastatic tumours with globulins from horses immunized to those tumours. Most patients tolerated this well and palliation was obvious in many cases, especially in patients with mediastinal obstruction and intracranial metastases. In more recent years, attempts have been made to attach a tumour specific and therefore localizing antibody to a chemotherapeutic agent

(LEVY et al, 1975; EVERALL et al, 1977).

The rationale behind this treatment is the finding of tumour-specific antibody in the serum of cancer patients (see Chapter 3), and the hope that sera from cured patients with high titres of antibody could be used in other patients. There are a number of theoretical risks, some of which have been demonstrated in animal models, and not the least disturbing of which is enhancement of tumour growth (KALISS, 1958; PIERCE, 1971), by small amounts of immune serum (see Chapter 5). The serological specificity of such serum may also be a complication in conditions such as leukaemia, where it may have an anti-lymphocyte effect and so lead to facilitation of tumour growth (MOTTA 1971).

(iii) Adoptive Immunotherapy. There are two aspects to this form of immunotherapy: the specific transfer of information ("transfer factor", RNA) from immune to non-immune lymphocytes, and the exploitation of the non-specific graft-versus-host (GVH) reaction of healthy allogeneic lymphocytes.

DELORME & ALEXANDER (1964) were able to induce regressions of carcinogen-induced fibrosarcomas in 57% of rats with lymphoid cells from other rats which had been immunized with a tumour biopsy. Thoracic duct lymphocytes collected 5-8 days after immunization had to be given repeatedly, and isogeneic as well as allogeneic cells worked, suggesting that the GVH reaction was not involved. This was an effect which was tumour specific and ALEXANDER et al (1967) subsequently isolated RNA from lymphocytes sensitized to the tumour, and showed that injection of this RNA into the rats led to temporary tumour regression. It was postulated that lymphocytes released from nodes in response to antigen stimulation are not primarily antibody producers, but messenger cells, migrating through the lymphoid system and initiating local responses. The antigen is processed by macrophages and either stimulates or links to the RNA to stimulate the production of immune lympho-blasts. To test this, the lymph was drained off from locally stimulated nodes in sheep and no circulating anti-

body could thereafter be detected; the cells were then injected into a chimaeric twin and antibody production occurred.

This work has been further investigated by PILCH and his co-workers who have shown that immunity can be transferred in vitro from sensitized lymphocytes to normal spleen cells (RAMMING & PILCH, 1971); and also that xenogeneic "immune" RNA from sheep or guinea pigs immunized with human tumour cells can induce marked cytotoxicity in normal lymphocytes against human tumour target cells (KERN et al, 1976). Interestingly, lymphocytes incubated with RNA from animals immunized only with complete Freund's adjuvant showed no increased cytotoxicity, but immunization with normal skin fibroblasts (autologous to the immunizing tumour) did lead to increased cytotoxicity. This suggested that the immune responses were directed against normal transplantation antigens rather than tumour-specific antigens.

The other aspect of adoptive immunotherapy, the GVH reaction, has been investigated extensively (MATHÉ et al, 1965). In animals with acute leukaemia, the acute GVH reaction induced by a bone marrow transplantation, can destroy leukaemia cells before irreparable damage occurs to the host; Cyclophosphamide in small doses can modify the reaction and not alter the anti-leukaemia effect (BORANIC & TONCOVIC, 1971).

In human studies the effect of adoptive immunotherapy has not been convincing. NADLER & MOORE (1969) grafted pieces of malignant tissue between pairs of patients with the same histological diagnosis for melanoma, breast, colonic carcinoma and sarcomas. When rejection occurred, they began leucocyte transfusions of "sensitized" lymphocytes between the patients and reported some beneficial effects in at least 23 of 118 patients. Similar attempts by others have not led to dramatic improvements (JEWELL et al, 1976). In a recent report, the use of autologous lymphocytes in 17 patients with recurrent glioblastomas appears to have led to a sustained clinical improvement in 8 patients. Lymphocytes were obtained by leucopheresis and after washing were re-injected through the craniotomy site into the tumour bed (YOUNG et al, 1977). Mitogen testing

in vitro showed no acute changes in lymphocyte function after leucopheresis.

Sub-cellular fractions have also been used in the treatment of human cancer. Transfer factor, a dialyzable product of lysed peripheral blood leucocytes, with a MW of 10,000 daltons approx.,has been used to treat a number of immunological deficiency diseases as well as cancer (LO BUGLIO & NEIDHART, 1974). Cross-immunization of transfer factor from paired patients has been reported in malignant melanoma (BRANDES et al, 1971) and OETTGEN et al (1974) have used Transfer Factor from the leucocytes of women over 45 years to treat women with breast cancer, presuming that such women would have immunity to breast cancer. "Immune" RNA made from sensitized sheep lymphocytes has been given safely to 34 patients with various solid tumours and led to stability or improvement in 12 (PILCH et al, 1976). Its advantages over other forms of adoptive immunotherapy are that serum with its risks is not given, nor cells which may lead to sensitization of the host to foreign histo-compatibility antigens or GVH reactions, and RNA itself is not a strong antigen.

The re-infusion of autologous lymphocytes activated in vitro for 48 hours with PHA has led to regression of pulmonary metastases in 8 of 10 patients; however, the other metastases continued to grow. This followed animal work showing that mitogen activated lymphocytes were cytotoxic to target cells in vitro, and that in vivo lymphoblasts released from lymph nodes stimulated with antigen, localized in the lungs and gut (FRENSTER & ROGOWAY,1970). Work by others has not been convincing (AUST et al, 1970; CHEEMA & HERSH, 1972).

The GVH reaction may be exploited as a successful form of immunotherapy in leukaemia where the marrow transplant also serves the other purpose of repopulating the leukaemia cells following chemotherapy or radiotherapy. Mortality is high, but patients otherwise refractory to treatment have benefited (THOMAS 1978).

(iv) Active Non-specific Immunotherapy. Since last century the association between infection and enhancement of host immunity has been observed and one of the earliest attempts at immunotherapy followed the observation by a physician (Coley) that an inoperable sarcoma regressed for 7 years following 2 attacks of erysipelas in the patient (COLEY 1896). Following this, Coley treated patients with recurrent cancer with "Coley's Toxin", a mixture of living or heat-killed Streptococcus and Bacillus prodigiosus, given intravenously or intra-lesionally. The impressive regressions that he reported have not been seen by others (NAUTS et al, 1953) but in more recent times a rationale for intrapleural immunotherapy in lung cancer has followed the observation that patients who suffer from post-operative empyema survive longer than patients without this complication (TAKITA 1970; RUCKDESCHEL et al, 1972).

A number of bacterial substances have been used as well as synthetic ribonucleotides and plant products. The commonest bacterial adjuvant is undoubtedly Bacillus Calmette-Guérin (BCG). OLD et al (1961) studied the effect of pre-treatment with BCG on the growth of a large number of antigenically distinct, chemically induced murine fibrosarcomas. BCG inhibited the growth of 45%, had no effect on 40% and possibly facilitated growth in 10% of the lines studied. Further, administration during the latent period after innoculation with oncogenic virus prevented the appearance of tumours. WEISS et al (1961) found that prior injection of living BCG, phenol-killed BCG or the methanol extracted residue (MER) were all equally effective in retarding the development of a transplanted murine tumour, or inhibiting metastases, or (in those animals who developed massive tumours) prolonged survival. A more disquieting note comes from others who have found tumour enhancement following BCG administration (WEPSIC et al,1976; PIESSINS et al, 1970).

Corynebacterium Parvum has been shown to inhibit significantly the growth of mammary carcinomas and sarcomas in mice, following inoculation of tumour cells (WOODRUFF &

BOAK, 1966). Double-stranded synthetic ribonucleotide, poly-inosinic polycytidylic acid (Poly-IC) is effective in inhibiting murine tumours and may work by inducing the production of interferon which inhibits both chemically and virally induced tumours (LEVY 1970). Polysaccharides such as Pachyman, derived from Glucan, and Lentinan, also lead to tumour regression but are effete in the presence of anti-lymphocytic serum (CHIHARA et al, 1970). The potent anti-helminthic drug, Levamisole (TRIPODI et al, 1973), enhances cellular immunity and has led to a significant decrease in recurrence and death in patients with bronchogenic carcinoma (AMERY 1976).

In human immunotherapy programmes the rationale for using stimulants of the reticulo-endothelial system is sound in patients whose immune defences may be impaired. BCG is most commonly used as an adjuvant with tumour cells, but in the first definitive article on its use in acute lymphoblastic leukaemia (MATHÉ et al, 1969 b), the authors showed that BCG was as effective as a mixture of BCG with allogeneic tumour cells or cells alone. The Medical Research Council (MRC) Trial was unable to repeat these results (MRC 1971), but MATHÉ (1971) has pointed out that the two regimens are very different in that Mathé's group gave more intensive chemotherapy prior to immunotherapy, CNS irradiation, splenectomy and used a different strain of BCG (vide infra). In both malignant melanoma patients (GUTTERMAN et al, 1973 a) and those with colonic carcinoma (MAVLIGIT et al,1976b) there have been significant increases in survival and disease-free interval post-operatively, when BCG is given repeatedly. McKNEALLY et al (1976) have shown in a randomized controlled trial of lung cancer patients that a single dose of 10^7 viable Tice-strain BCG organisms into the intra-pleural space post-operatively, can significantly increase survival in Stage I but has no effect in Stage III or IV disease. Of 17 patients with Stage I disease, none had recurrence or death (follow-up period longer than a year) compared with 9 of 22 recurrences in the control group, 5 of whom died. This has not been confirmed by others (ILES et al, 1978), although again a different strain of BCG (Glaxo) was used.

C.Parvum has prolonged survival in patients with both breast and bronchogenic carcinoma (ISRAEL et al, 1975), although at least one study suggests that intravenous administration leads to temporary but marked immunosuppression (MINTON et al, 1976). Other vaccines containing Mumps virus (ASADA 1974), vaccinia virus (HUNTER-CRAIG et al, 1970), and Bordetella pertussis (GUYER & CROWTHER, 1969) have all been used with varying success.

The intralesional administration of BCG in patients with malignant melanoma has been shown to lead to regression in 90% of treated lesions and 17% of untreated lesions (MORTON et al, 1974); histologically, there was an intense lymphocytic and monocytic infiltration into the lesions, suggesting that the BCG exerted its toxic effect through the DHR which formed. In animals, Con. A intralesionally causes regression of polyoma-induced tumours (SHOHAM et al, 1970). In humans with skin metastases and non-invasive vulvar neoplasia, intralesional DNCB has been used with some success (STJERNSWÄRD & LEVIN, 1971; WEINTRAUB & LAGASSE, 1973; GUTHRIE & WAY, 1975).

(v) Active Specific Immunotherapy. In human trials it is especially difficult to discuss this form of immunotherapy without including the effects of a non-specific adjuvant, generally BCG, added to the specific component which may be tumour cells or a vaccine of sub-cellular fractions. The rationale for this form of immunotherapy derives from numerous animal experiments showing resistance to transplantable tumours by animals pre-sensitized to the tumour through prior inoculation of tumour cells.

Living tumour cells, given intradermally in numbers insufficient to cause progressive growth, are generally the most effective immunogens in animal experiments (MORTON et al, 1970 a & b). Such immunotherapy can have a curative as well as preventive role. MATHÉ et al (1969a) showed that the injection of 10^7 irradiated L1210 leukaemia cells given 24 hours after a graft of L1210 cells prevented tumour takes, whereas BCG at this time was ineffective; both together, however, were more effective than cells alone, suggesting a potentiating and additive effect. These results were con-

firmed by PARR (1972) who showed that irradiated cells and BCG were more effective in preventing tumour takes than BCG alone; furthermore, the effectiveness of the therapy depended on the immunogenicity of the tumour, having no effect in weakly immunogenic and strongly metastasising tumours. Irradiated autologous tumour cells, given to rats with benzpyrene-induced fibrosarcomas, have been shown to increase the tumour inhibitory effects of radiotherapy (HADDOW & ALEXANDER, 1964). In fact, such results were used as the rationale for an immunotherapy trial in patients with glioblastoma multiforme (BLOOM et al, 1973), but the results showed no benefits.

The number of tumour cells inoculated is also important (ZBAR et al, 1971; VANWICK et al, 1971). In a study of mice with fibrosarcomas, the inoculation of 10^4 viable tumour cells provided complete protection from metastases, whereas 5×10^4 cells led to death in all mice. It has furthermore been suggested that frozen and thawed cells, or those which have been irradiated, are not as effective as viable cells (WEPSIC et al, 1970; BALDWIN & PIMM, 1973). This lack of immunotherapeutic effectiveness is presumably due to the loss of normal transplantation antigens and tumour antigens, which has been demonstrated in polyoma-virus induced tumour cells subject to irradiation (McKHANN, 1964). In the clinical situation, however, POWLES (1974) found that patients with acute myeloid leukaemia did not do better when given viable tumour cells rather than irradiated cells. It should be noted that although viability may not be altered significantly by cryopreservation, immunogenicity may be decreased (BARTLETT et al, 1977).

Many attempts have been made to increase the antigenicity of the tumour cells by modifying them in a number of ways; substances used include sulphydryl alkylating agents such as iodoacetate, which stabilise the antigenic determinants of proteins (APFFEL et al, 1966; PRAGER et al, 1971); Con. A which aggregates tumour antigens and may alter electrostatic charges on the cell surface (ENKER & JACOBITZ, 1976; TAKITA & BRUGAROLAS, 1973); aldehyde (BERTOLINI et al, 1976); human gamma-globulin coupled to tumour cells with bis-diazobenzidine (CZAJKOWSKI et al,

1966); and neuraminidase which liberates sialic acid moieties from the cell surface, so unmasking antigens and allowing detection by the host immune system (BAGSHAWE & CURRIE, 1968; SIMMONS et al, 1971). Another way in which tumour cell modification may work is by altering the organ distribution of the inoculated cells. WEISS et al (1974) have shown that in normal mice, neuraminidase treated fibrosarcoma cells were localised in the liver, whereas in mice which had previously been sensitized to the tumour, the inoculating cells were localised in the lymph nodes and spleen.

Despite the profusion of animal experiments, there are conflicting results. Many experiments suggest that tumour cells per se may have no immunotherapeutic effect unless in close contact with an adjuvant such as BCG. ZBAR et al (1971) found that inoculation with tumour cells resulted in progressive growth of guinea pig hepatomas, whereas intradermal BCG with or without tumour cells led to prevention of tumour growth. BCG and the tumour cells had to be in close contact, since inoculation at different sites was ineffective. This has been confirmed by others (BALDWIN & PIMM, 1971). SIMMONS et al (1971) used neuraminidase treated tumour cells with viable BCG against a murine fibrosarcoma, and when these were injected separately only 1 of 14 animals had tumour regression, compared with 15 of 42 when cells and BCG were inoculated together.

In human malignancies, forms of active immunotherapy have been used since the early part of this century, usually with some form of autologous cell suspension. Many of these attempts were bizarre, but regressions were reported and it is interesting to note that even tumour enhancement was reported (RISLEY 1911). In an extensive series, GRAHAM & GRAHAM (1959; 1962) reported on the immunization of 232 women with gynaecological tumours, with autologous vaccine and sometimes Freund's adjuvant. 13% of these patients were alive at 2 years compared with 6% of the 139 controls. As would be expected, there were severe local reactions. Other workers have used a combination of Freund's adjuvant and tumour extracts with no untoward

effects (FINNEY et al, 1960), but not striking success.

Table 6.2 summarises the major human immunotherapy studies since MATHÉ (1969) presented his definitive paper. Most of these studies use a combination of BCG and allogeneic or autologous irradiated tumour cells, so that it is difficult to interpret the results in the light of pure anti-tumour immunity, and not non-specific immunostimulation. Many of the patients were also receiving chemotherapy (vide infra).

6.2 THE NATURE OF IMMUNOPOTENTIATION WITH BCG.

Mycobacteria have been used for many years as immunopotentiating agents, since it was first shown that guinea pigs with tuberculosis produced greatly elevated titres of haemolysin in response to stimulation with sheep RBC inoculation (LEWIS & LOOMIS, 1924). DIENES (1930) then showed that common antigens such as egg white provoked a tuberculin type of hypersensitivity when introduced into the site of a tuberculous infection. The use of BCG with Freund's adjuvant (FREUND & McDERMOTT, 1942) potentiates both general and specific immune responses, and also amplifies reticuloendothelial function by causing hyperplasia and hyperactivity in the cellular components (MACKANESS 1964). This explains phenomena associated with its use such as increased antibody production (HALPERN et al, 1958), accelerated homograft rejection (VITALE & ALLEGRETTI, 1963), and enhancement of resistance to grafted and chemically induced tumours (OLD 1961). This latter finding provides the rationale for its use as an immunotherapeutic agent in cancer. BCG therefore has the capacity to (a) augment immune responses to various antigens unrelated to it, and (b) activate host reticuloendothelial function.

The quantitative and morphological aspects of lymphoproliferation in response to BCG depend very much on the strain used, its viability, virulence, immunogenicity and the number of organisms given (MACKANESS et al, 1973). Following inoculation with live BCG, blast cells rapidly appear in the draining lymph nodes and these are predominantly thymus-dependent. Dead bacteria, on the other hand, cause

TABLE 6.2 REPRESENTATIVE IMMUNOTHERAPY STUDIES IN HUMAN MALIGNANCY.

Bladder	MORALES et al, 1976	9	BCG into bladder	U/Ur	Sig. decrease in number of recurrences.
Gynae-various	GRAHAM & GRAHAM, 1962	232	Freund's adj.& & tumour mix	U/Ur	Of 48 ovarian Ca. pts. receiving I/T, 30% alive at 30 months compared with 13% controls.
Ovarian	HUDSON et al 1976	10	BCG & cells	U/Ur	2 year survival for I/T group 60 % compared with 12% for controls.
Gynae-various	PATTILLO, 1976	45	BCG & "antigen"	U/Ur	Inadequate analysis but results promising.
Ovary	HUMPHREY et al, 1977	32	Tumour vaccine	C/R	Sig. increase in survival (25% at 2 years) compared with controls (0%). I/T alone had no effect.
Gynae-various	MANGAN et al, 1977	13	BCG intra-lymphatic	U/Ur	All pts. with advanced disease, and possible prolonged survival. Severe complications.
Ovarian	KALPAKTSOGLOU et al, 1978	21	Oral BCG	U/Ur	Inadequate analysis.

ABBREVIATIONS:

I/T = Immunotherapy

C/R = Controlled, randomized

U = Uncontrolled, or historical retrospective controls used

Ur = Unrandomized

no response unless suspended in oil, when they cause a massive accumulation of plasma cells in the nodes i.e. an antibody response. Virulent strains (e.g. Pasteur) lead to a rapid blastogenic response in vitro which is not sustained because of lymph node destruction, unlike less virulent strains (e.g. Tice) where the response is greater but sustained. Lyophilized strains (e.g. Glaxo) contain many dead bacteria and antigenic débris, and cause an initial antibody response followed by the response seen with fresh viable BCG. This has particular relevance when discussing immune responses during immunotherapy (see next Chapter).

In studies in mice where BCG is given with another antigen (e.g. sheep RBC), the best augmented response is achieved when BCG is injected about 12 days before the other antigen (MILLER et al, 1973); furthermore, both antigens must impinge on the same draining lymph nodes. The cellular response to BCG thus primes the lymphoid tissue for an enhanced response to the other antigenic stimulus, and this is mediated through T-cells.

Tumour regression in response to BCG involves a number of steps, and is often variable. There must be specific immunity to BCG. Guinea pigs treated with anti-lymphocytic serum are not protected from tumour challenge by BCG (HANNA et al, 1973). In contrast with these results, PIMM & BALDWIN (1976) have found that general immunosuppression does not abrogate the effects of BCG, which are also apparent in athymic "nude" mice. Silica-induced host macrophage depletion however, does abrogate the BCG effects (HOPPER et al, 1976). Again, in contrast to previous experimental work described above, HAWRYLKO & MACKANESS (1973 a & b) have shown that the best immunotherapy for weakly immunogenic Methyl-cholanthrene induced murine fibrosarcomas, uses a schedule where the irradiated tumour cells are given 10 days after the BCG.

As well as activating T-cells, BCG also stimulates macrophages in a number of ways, causing a change in the lysosomal content (COLLINS & MACKANESS, 1970), stimulating enzymes which catalyse the metabolism of polycyclic hydro-

carbon carcinogens (BAST et al, 1973) - this explains why BCG can delay the appearance of, and decrease the incidence of, carcinogen-induced tumours - and activating cytotoxic mechanisms against tumour cells to which the animal has not been sensitized (GERMAIN et al, 1975). Macrophage activation may also explain how BCG decreases the incidence of metastases, and it has been shown that transplanted tumours in BCG-treated mice have less tumour cell clumps in the venous effluent from the tumours, and the cells which do escape are associated with macrophages (LIOTTA et al, 1976).

It appears that BCG may also antagonise the myelo-suppressive effects of chemotherapy (MITCHELL et al, 1973).This is again through the activating effects on macrophages and T-cells; the latter are known to produce factors which stimulate the proliferation and differentiation of haemo-poietic stem cells (CERNY 1974). This is particularly important in leukaemia where a change in the balance between malignant cells and activated normal marrow cells may be integral to the success of the immunotherapeutic process.

Augmented responses to tumour antigens have also been noticed in humans treated with BCG (ROTH et al, 1975). This may be related to shared antigens which have been found to exist between Mycobacteria and neoplastic cells, both in animal (MINDEN et al, 1974) and human cells (BUCANA & HANNA, 1974). Furthermore, it seems that effective responses to tumour antigens may well depend upon pre-existing states of sensitization to micro-organisms (BARDANA et al, 1973).

6.3 CHEMO-IMMUNOTHERAPY.

On purely empirical grounds, one would expect chemo-therapy to be of benefit in decreasing the tumour burden, although theoretically its immunosuppressive effects could abrogate any stimulatory effects of immunotherapy. Certainly, in animal studies where combined chemo-immunotherapy is used, the timing of the two components in relation to each other is important (CURRIE & BAGSHAWE 1970; PEARSON et al, 1973).

GLYNN et al (1969) showed that a combination of Cyclophosphamide and immune lymphocytes eradicated Moloney-virus induced murine lymphomas in a significant number of animals than either agent alone. FEFER (1969) found that Cyclophosphamide and immune cells led to complete cures in 60% of mice with Moloney-induced sarcomas, whereas Cyclophosphamide alone caused rapidly progressive tumours if given before they were palpable, and immune cells alone had no effect on tumour growth. It was postulated that the immunotherapy retarded tumour growth until immunosuppression from the Cyclophosphamide had ended; the host now being immunocompetent, it was able to handle what tumour burden remained.

It has previously been mentioned that chemotherapeutic agents may have a differential effect on the immune system, in that B-cells may be selectively depressed and T-cells relatively increased (see Chapter 4). Clearly, if immune responses may lead to either enhancement or rejection of tumour, this is an important area for investigation (MOTT 1973). When chemotherapeutic effects are shown to be tumour-specific, it may be that they allow better host cytotoxicity by decreasing the level of circulating tumour antigen (through tumour cell killing), circulating antibody (through the B-cell effect),and therefore the level of circulating immune complexes. Mice immunized with 10^6 Methylcholanthrene-induced fibrosarcoma cells, which were not excised when palpable, but treated with Cyclophosphamide only, were partly resistant to challenge 6 days later with 10^4 viable tumour cells. The tumour incidence and growth rates were significantly less than in mice where the immunizing tumour had been excised prior to challenge. This effect was tumour-specific (STEEL et al, 1974).

There have, however, been conflicting reports in animal studies on the benefits conferred by chemotherapy. MATHÉ et al (1970) suggested that when mice were treated with Cyclophosphamide before immunotherapy, BCG alone could induce cures. Subsequently, he reported (MATHÉ et al, 1975) that BCG given <u>before</u> Cyclophosphamide potentiated the

immunosuppressive effects of the drug.

Most human immunotherapy trials have incorporated the use of chemotherapeutic agents, although it is interesting to note that in the extensive acute myeloid leukaemia trials of POWLES and co-workers (1974), patients who received BCG and tumour cells only, did as well as those patients receiving immunotherapy and maintenance chemotherapy (median survivals of 83 and 78 weeks respectively). In an immunotherapy trial for colorectal carcinoma (MAVLIGIT et al, 1976 b) with patients randomised to have either BCG or BCG and 5-fluorouracil, there was no significant difference in the disease-free interval from operation for these two groups. However, actuarial analysis suggests that the survival of the chemo-immunotherapy might be significantly longer for the BCG only group. Paradoxically, also, chemotherapy was effective regardless of the number of tumour-involved lymph nodes at operation, whereas BCG alone was effective among patients with more than 6 involved lymph nodes, and ineffective in those with less than 5 nodes involved. The authors suggest that as well as a maximum tumour burden beyond which immunotherapy in ineffective, there may also be a minimum level required, since optimal amounts of tumour antigen may be necessary for the immunopotentiating effects of BCG.

It is doubtful, for ethical reasons, that chemotherapy will be deliberately excluded from trials where it has been shown to be of use in prolonging survival and inducing tumour reduction, and clearly more research on the appropriate timing between the two forms of treatment is required. Already, it appears that one benefit of immunotherapy with BCG is that by its stimulation of haemopoietic stem cells, stronger and more frequent doses of chemotherapy can be given (POUILLART et al, 1975).

6.4 IMMUNOTHERAPY TRIALS IN HUMAN CANCER.

(i) Immunotherapy trials in Gynaecological Cancer. The earliest large examination of the effects of immunotherapy

was conducted by GRAHAM & GRAHAM (1959; 1962). 114 patients (22 with ovarian carcinoma) with otherwise untreatable malignancy, were injected intradermally with Freund's adjuvant and tumour cells or tumour extract. At 7 months, 14 of 107 were living with no evidence of disease. The biggest problem was severe ulceration at the vaccination site, although it was noted that those who did not suffer from this tended to do worse than the others. Vaginal smears showed infiltration with histiocytes in 25% of the patients, and of these 76% survived at least 7 months. In 2 patients receiving cellular suspensions, tumour implants occurred. In their subsequent report (1962), they compared the results of 232 treated patients with 139 "controls". 48 patients had ovarian cancer, and of these 30% treated with immunotherapy were still alive at 30 months, compared with 13% of the controls. The authors concluded that this was a safe procedure, but its use could not be recommended since it failed to alter the course of the disease with sufficient regularity, and clearly had no effect on widely disseminated disease.

More recently, 45 patients with varying tumours have been treated with Tice strain BCG and autologous or allogeneic tumour cells (PATTILLO 1976). Of 10 patients with ovarian cancer, 50% were dead, and only 1 disease-free at the time of evaluation (length of follow-up is not established in the report). HUMPHREY et al (1977) reported on 32 women with Stage III and IV ovarian cancer who were randomised to receive chemotherapy alone with Melphalan, immunotherapy alone with tumour homogenate, or a combination. There was a significant increase in the survival of the chemo-immuno-therapy group; of 18 patients, 47% were alive at 1 year, 25% at 2 years, and 20% at 4 years. In both of the other groups, no patients were alive at 2 years. KALAPAKTSOGLOU et al (1978) have treated 21 patients (7 Stage I and II, 14 Stage III and IV) with a complicated schedule of agents to stimulate humoral immunity (tetanus, diphtheria, mumps and influenza vaccinations), followed by oral BCG and continuous Cyclophosphamide. The authors give no adequate analysis of survival other than to say that all cases treated

with hysterectomy are still alive (no length of follow-up is however given) and that Stage IV results are "unsatisfactory".

Local immunotherapy with DNCB for vulval atypia and non-invasive neoplasia (WEINTRAUB & LAGASSE, 1971) and vaginal adenocarcinoma (GUTHRIE & WAY, 1975) has been used with success.

(ii) Immunotherapy trials in Non-gynaecological Cancer.

In spite of a plethora of immunotherapy trials, very few are in the nature of randomized, controlled studies, thereby avoiding the criticism levelled at actuarial studies in non-randomized trials which use historical controls (PETO & GALTON, 1975; VAN DER BRENK & HEWITT, 1977).

The first important immunotherapy trial reported was in acute lymphoblastic leukaemia (MATHÉ et al, 1969 b) in which 10 patients were randomized as controls, 8 to have BCG only, 5 to have irradiated allogeneic cells only, and 7 to have BCG and cells. All patients received very intensive chemotherapy prior to immunotherapy and by 130 days after stopping chemotherapy all controls had relapsed, whereas only 9 of 20 immunotherapy patients had relapsed. There was no significant difference between the three immunotherapy groups in terms of remission length.

The MRC Trial into which 191 patients were entered (52 to receive BCG only, 52 to receive Methotrexate maintenance chemotherapy only, and 18 to receive no treatment) did not confirm these results, and found that the median length of remission was greatest in those on chemotherapy only (MRC 1971). The postulated reasons for this have previously been mentioned; apart from more aggressive treatment, the French workers also used the very virulent Pasteur strain of BCG compared with the British Glaxo lyophilized strain.

POWLES et al (1973) reported on their first large acute myeloid leukaemia trial, which was begun on the basis of studies in vitro showing that the frequent inoculation of 10^8 - 10^9 blast cells were needed to increase the patients' blastogenic responses to tumour cells (POWLES et al,

1971; GUTTERMAN et al, 1973c). Patients presenting to St. Bartholomew's Hospital with AML were allocated on an alternate basis (i.e. not randomized) to receive immunotherapy or not following chemotherapy-induced remission. Their first report showed that immunotherapy had a significantly beneficial effect on both the duration of remission and the length of survival. Actuarial survival analysis suggested a median duration of 303 days for the chemotherapy groups compared with 545 days for the immunotherapy group. Experimental variations in the use of immunotherapy were all accomodated in this trial, and further analysis suggested that patients receiving immunotherapy only did as well as patients also receiving maintenance chemotherapy, and that viable cells were no more beneficial than irradiated cells POWLES 1974). Despite animal experimentation suggesting that BCG and tumour cells should be in close contact (vide supra), patients received BCG and cells in different limbs. A further study (POWLES et al,1977a) indicated that patients who had received four extra inoculations of BCG mixed with tumour cells as well as their usual immunotherapy had longer remissions than other patients. The most recent paper from these workers (POWLES et al, 1977 b) has the advantage of being able to compare the actual results with the actuarial predictions of 1973. Median duration of survival is 270 days for those on chemotherapy only, and 510 days for those having immunotherapy as well. There is no significant difference between the two groups in length of first remission, but the median survival after relapse was 75 days as opposed to 165 days in the immunotherapy group, a highly significant difference. The importance of this paper is that it is the first to show that immunotherapy does not alter the shape of the survival curve by leading to a sub-population of patients who become long-term survivors. In other words, all patients ultimately die of their disease, even though immunotherapy increases the median length of survival (i.e. shifts the survival curve to the right). It seems unlikely that this prolongation of life is due to an increased immune response by the host to tumour-associated antigens and therefore an increased ability to

contain malignant cells, but rather to a stimulation of the bone marrow allowing higher doses of chemotherapy to be given (WOLMARK et al, 1974; DIMITROV et al, 1975).

HARRIS et al (1978) have obtained similar results and have shown that immunotherapy prolongs the first remission as well as facilitating re-induction with chemotherapy, and lengthening the post-relapse survival. Interestingly, the average time between relapse and death was significantly longer in those receiving immunotherapy only, compared with those receiving chemo-immunotherapy.

In malignant melanoma numerous trials (most of them uncontrolled) have suggested that immunotherapy might be useful. CURRIE (1973) described the loss of serum inhibitory effects in patients receiving BCG and irradiated autologous cells. In an uncontrolled trial of 30 patients (CURRIE & McELWAIN, 1975), all with disseminated malignant melanoma, objective regression of tumour occurred in 57% (compared with a value of 20% normally obtained with chemotherapy alone). Different donor cells were used each time in order to minimise any effects from high titres of anti-HLA antibody to the same donor. Sadly, such enthusiasm for immunotherapy was not confirmed by a controlled, randomized trial which these same authors discontinued for ethical reasons (HEDLEY et al, 1978). Patients with Stage IIB melanoma were randomized to receive monthly BCG or BCG with irradiated allogeneic cells. The median disease-free interval before relapse was 5 months in the 16 patients receiving BCG and cells, and 8 months in the 12 patients receiving BCG alone. Since no improvement had been shown, the trial was discontinued, but more disconcerting was the observation that 7 of 11 of those who relapsed did so initially in the lymphatic drainage area of the tumour, while all those who relapsed on BCG did so at a different and distant site. Stage IIB melanoma nearly always relapses at distant sites, hence active specific immunotherapy had in some way changed the biological behaviour of the tumour. It was postulated that the BCG and tumour cells had acted as a focus for trapping specifically sensitized effector cells, and had thus depleted other sites (e.g. local drain-

age areas of excised lymph nodes); or that antigenic overload, with its "paralysing" effects may have occurred (VAAGE 1973).

Again, in spite of promising results in uncontrolled trials (GUTTERMAN et al, 1973a; GRANT et al, 1974), properly randomized controlled trials have failed to find any significant differences between those given immunotherapy and those not (NEWLANDS et al, 1976; PINSKY 1976). McILLMURRAY et al (1977) also stopped a clinical trial of 15 patients with Stage IIB melanoma who had been given one inoculation with BCG and irradiated autologous cells 2 weeks postoperatively. The recurrence rates were the same for the immunotherapy patients as for controls, but the death rate was strikingly different at 1 year (57% for the immunotherapy patients and 0% for the controls). The recurrences in the immunotherapy patients occurred early and were rapidly fatal.

In other malignancies such as breast cancer (GUTTERMAN et al, 1976; SPARKS et al, 1976), colorectal cancer (MAVLIGIT et al, 1976b) and a variety of solid tumours (GERNER & MOORE, 1976; TOWNSEND et al, 1976) results tend to indicate that immunotherapy is beneficial. However, many of these preliminary reports relate to trials which were not controlled, and subsequent controlled studies may very well not confirm these impressions.

6.5 COMPLICATIONS FROM IMMUNOTHERAPY.

The use of immune serum may cause immunological enhancement of tumour growth, as has been shown in animal experiments (KALISS 1958; SNELL 1960). Presumably antibody blocks the cellular mechanisms involved in target cell cytotoxicity, by coating the tumour cells (SNELL et al, 1960). In animal systems antibody can also be eluted from the surface of neoplastic cells and this will block cell-mediated immunity against the cells (RAN & WITZ, 1972) (see Chapter 5).

The risk of graft-versus-host reaction is very real in the adoptive immunotherapeutic regimens, when immunocompetent allogeneic cells are used. In leukaemia, the use of marrow transplants has highlighted this, but the

reaction may be exploited and controlled (vide supra).

The injection of antigenic tissue in animal models may lead to enhanced tumour growth through "antigenic overload" (VAAGE 1973; HAWRYLKO et al, 1973 b). It has been postulated that desensitization occurs and the antigen causes temporary depression of existing hypersensitivity by binding to sensitized cells and preventing them from further interactions (McCLUSKEY et al, 1963; WEPSIC et al, 1971). This has been used to explain enhanced tumour growth in patients with malignant melanoma, treated with immunotherapy (HEDLEY et al, 1978). Since few human trials use viable tumour cells, the risks of tumour deposits at the inoculation site are small. There are, however, still reports of the transfer of serum hepatitis (SPARKS et al, 1976).

The most important complications now seen relate to BCG toxicity. There does not appear to be an increase in the incidence of auto-immune diseases from the repeated inoculations of antigen (DIXON et al, 1961), but clearly this is a possibility where there is prolonged overstimulation of the reticuloendothelial system. There are the actual visible effects of BCG which have been reported in many cases; severe ulceration at the immunotherapy sites, fevers and rigors usually of short duration, local lymphadenitis (GRANT et al, 1974; GERNER & MOORE1976).

Intralesional BCG is associated with far more risks than intradermal BCG (SPARKS et al, 1973). These include osteomyelitis (FOUCARD & HJELMSTEDT, 1971), disseminated BCG infection (WATANABE et al, 1969) and anaphylaxis and death (McKHANN et al, 1975). Hepatic dysfunction with the formation of non-caseating granulomata is a particularly common problem (HUNT et al, 1973) and leads to abnormal liver function studies and lesions detectable on ultra-sound scans. It can be treated successfully with Isoniazid. Of interest is a recent report describing significant impairment of in vitro cellular immunity as assessed by lymphocyte blastogenesis in 5 of 6 patients with disseminated BCG infection; which the authors suggest may abrogate the beneficial effects of BCG (ROSENBERG et al, 1978).

It has been shown in animal studies that BCG may cause

tumour enhancement (PIESSENS et al, 1970; WEPSIC et al, 1976); it may be that antigenic similarity between the tumour and BCG (MINDEN et al, 1974) leads to the induction of enhancing antibody, or that stimulation of the reticulo-endothelial system leads to an alteration in the balance of T and B-cells. In human malignancy such enhancement has been suggested (GERNER & MOORE 1976; McILLMURRAY et al, 1977). LEVY et al (1972) noted that BCG inoculation into a melanoma lesion in a man with previously cytotoxic lymphocytes, led to a rapid clinical deterioration and the appearance of factors in his serum which blocked the tumour-specific cytotoxicity. More recently it has been shown that large amounts of BCG can induce T-suppressor lymphocytes (MATHÉ 1976).

It has also been suggested that BCG may potentiate cerebral metastases in patients with lymphoma. ZEIGLER & MAGRATH (1973) found no differences in survival between their immunotherapy patients and controls with Burkitt's lymphoma, but a significant number of immunotherapy patients developed cerebral metastases. This has been reported by others (SNYDER et al, 1977).

It therefore appears that with BCG the dangers of toxicity may be lessened by intradermal administration rather than intralesional, and by great care in its administration to anergic patients. The risks of tumour enhancement will become apparent in individual cases, and emphasises the need for adequate in vitro immunological analysis to be performed on all patients receiving immunotherapy.

6.6 EXPERIMENTAL DESIGN: A STUDY OF ACTIVE SPECIFIC IMMUNOTHERAPY IN WOMEN WITH OVARIAN CARCINOMA.

The experimental work performed by Dr. L. Levin (LEVIN 1976) provided the scientific rationale for a programme of active specific immunotherapy in women with ovarian cancer. The protocol used was based on that in use for the Acute Myeloid Leukaemia Trial being conducted at St. Bartholomew's Hospital (POWLES 1974), namely the monthly administration of irradiated allogeneic tumour cells and BCG. Two studies will be described in this section. The first was a pilot

study with one objective only - to test the safety of the procedure and to see if there was any evidence for its effectiveness in prolonging survival. The results of that trial, uncontrolled and retrospective, were published in 1976 (HUDSON et al, 1976), and were subject to the justifiable criticism given to uncontrolled studies.

Following the promising results of this initial study, a prospective, randomized and controlled trial was begun. All the patients are derived from the current Medical Research Council Trial for chemotherapy in ovarian cancer (see Appendix), and suitable patients were then randomized into the Immunotherapy Trial (criteria below). On the advice of Dr. R. Peto, Department of the Regius Professor of Medicine, Radcliffe Infirmary, Oxford, the ratio of patients receiving immunotherapy to the control patients is 2:1. This is because the controls in the Immunotherapy Trial can then be compared with all other controls from the MRC Trial. Should there be a significant difference in survival in the Immunotherapy controls, this would indicate that one reason the Immunotherapy patients themselves may do better is because recurrent disease is detected earlier and treated more aggressively with a change in chemotherapy.

Meanwhile, patients not eligible for the MRC Trial have still been offered immunotherapy. Consequently, the first trial which originally described results in 10 patients, now gives data on 15. In comparison, only 7 patients have so far been randomized to receive immunotherapy in the MRC Trial.

TRIAL I: UNRANDOMIZED AND RETROSPECTIVE.

Patients: criteria for entry are:

(i) Patients have primary surface epithelial ovarian adenocarcinoma, excluding low-grade malignancy

(ii) Patients have Stage III and IV disease (FIGO Classification) with incomplete initial surgery or recurrent disease

(iii) Patients must show disease control by chemotherapy at 3 months following surgery. Unlike leukaemia there is no fine criterion of relapse and the

term "disease stasis" covers the situation where there is no crude evidence of progressive disease as assessed by palpation or ultrasonic scan, and no recrudescent malignant effusions.

So far, 15 patients have been entered into the Pilot Study. Clinical staging was as follows:

Stage III - 9 patients
Stage IV - 5 patients
Recurrent disease - one patient with recurrence in the pelvis 27 months after a total abdominal hysterectomy and bilateral salpingo-oophorectomy for Stage IIB disease.

The Clinical details of these patients are presented in Table 6.3.

Historical controls (25) were obtained by matching as far as possible for age, histology, Stage and the above criteria; all patients were obtained through an ongoing epidemiological study in the North East Thames Region, under the guidance of Professor C.N.Hudson (now Professor of Obstetrics & Gynaecology, Westmead Hospital, University of Sydney, Australia). Chemotherapy regimens were therefore not standardized.

Chemo-immunotherapy: the tumour cells and BCG were mixed as previously described (Chapter 2) and injected into 4 separate sites in the arms or legs, at monthly intervals. Allogeneic cells were always used, and when possible each patient was given a different donor supply of cells on each occasion. In this way it was hoped to avoid the hazards of large amounts of anti-HLA antibody forming, if the same batch of cells were always used.

Chemotherapy was given as a bolus dose two weeks after the immunotherapy. This consisted of Cyclophosphamide 1G orally over three days in those patients who had responded to an initial 5G intravenous induction course with Cyclophosphamide post-operatively. However, it was not possible in this trial to standardize chemotherapy, and consequently many patients had been on combinations of agents, including Vincristine, Procarbazine, Thiotepa, and

TABLE 6.3 CLINICAL DETAILS OF 15 PATIENTS IN UNRANDOMIZED TRIAL I.

CODE/STAGE	SURGERY	HISTOLOGY /DIFF.	CHEMO.	NO.I/T COURSES (REACTION)	CLINICAL CONDITION	(MONTHS) PRE-DEATH RELAPSE	(MONTHS) TOTAL SURVIVAL
BF / III	BSO	S.Pap. /poor	Cyclo	15	Bowel obstruction from malignant adhesions. Pulm. embolus.	2	20
MW / III	BSO & TAH	Endom. /well	Norethis.	20 (4+)	Pelvic recurrence controlled with Norethisterone. Excellent health.	-	alive (79)
JP / IV	BSO, Oment.	S.Pap. /poor	Cyclo & Adr. RXT	8	No response to RXT, recurrent ascites & pleural effusions.	5	13
WN / III	Biopsy only, 1 year later BSO & STAH	S.Pap. /mod.	Vcr, Procarb, CHM. RXT after BSO. Cyclo	24 (4+)	Pre-terminal haematuria related to RXT and Cyclo. Post-mortem shows no increase in tumour state.	2	46
MB / III	TAH, BSO & Oment.	S.Pap. /poor	Thiotepa RXT to inguin. nodes. Adr. & Cyclo Cis-Plat.	20 (3+)	7 months after initial surgery, inguinal nodes involved. Pre-terminal surgery to malignant gastro-colic fistula & abscess.	5	23
DH / III	Biopsy only Pre-terminal I-TA & removal mass from ant. abd. wall	S.Pap. /mod.	CHM RXT to sinus Treo.	20 (4+)	In excellent health until I/T ends. Umbilical malignant sinus responds to RXT. Pre-terminal bowel obstruction.	3	40
AH / III	Bilat. ooph.	Endom. /mod.	Cyclo RXT to spine	20 (3+)	Spinal metastasis responds to RXT. Pre-terminal bowel obstr. and ileal resection. Septicaemia.	3	22

TABLE 6.3 (contd).

CODE/STAGE	SURGERY	HISTOLOGY /DIFF.	CHEMO.	NO.I/T COURSES (REACTION)	CLINICAL CONDITION	(MONTHS) PRE-DEATH RELAPSE	(MONTHS) TOTAL SURVIVAL
BP / IV	BSO & TAH (Cx. node)	S.Pap /poor	Thiotepa & Vcr; Adr & Cyclo. Pelvic RXT	11 (2+)	No response to RXT, recurrent pleural effusions. Supra-clavic. node fluctuated in size with each I/T. Post-mortem showed node tumour-free & sclerosed compared with other malignant nodes.	7	20
HL / III	BSO (rad) & STAH	Undiff.	Cyclo Adr. & Cis-Plat.	24 (3+)	Rapid relapse once I/T ends with liver mets. and pleural effusions.	3	32
MT / III	STAH, BSO & Oment.	S.Pap. /poor	Cyclo. Caesium to cervical met. Adr. & Cis-Plat.	24 (4+)	2nd. look laparoscopy at 21m. showed no increased growth. Rapid relapse once I/T ends with liver mets. and recurrent ascites.	5	31
SB / IV	TAH & BSO	Undiff.	Adr. & Cyclo RXT to rib & pelvis. Cis-Plat.	14 (1+)	Initial good response to chemo. then symptoms from various mets. in lungs, liver, ribs, vault.	9	16
MW / III	BSO & TAH	Undiff.	RXT, Cyclo & Adr. Treo.	7 (2+)	Recurrent ascites contained with Treosulphan.	5	25
IC / IV	Bilat ooph. (breast & axill. nodes) 16 m. later I-TA.	Endom. /mod.	Cyclo & Adr. Cis-Plat.	14 (1+) after ITA (2+)	Initial good response to chemo, then ITA for bowel obstruction. Skin and breast mets. responded to Cis-Plat, then pre-terminal liver mets. and loculated ascites.	3	29

TABLE 6.3 (contd).

CODE/STAGE	SURGERY	HISTOLOGY /DIFF.	CHEMO.	NO.I/T COURSES (REACTION)	CLINICAL CONDITION	(MONTHS) PRE-DEATH RELAPSE	(MONTHS) TOTAL SURVIVAL
MG / IV	Biopsy & Oment. 7 m. later BSO.	S.Pap. /poor	Adr, Cyclo, & Cis-Plat. Melphalan Pelvic RXT	7 (2+)	Excellent initial response to chemo, with 2nd. look laparotomy for BSO. Pelvic recurrence not responsive to RXT, recurrent effusions.	7	23
NS / III	Biopsy. 1 yr. later BSO & TAH	S.Pap. /poor	Cyclo	22 (4+)	Excellent initial response to chemo, with 2nd. look laparotomy 1 yr. later. Numerous biopsies of liver, diaphragm and pelvis were negative for tumour cells.	-	alive (36)

ABBREVIATIONS:

BSO = Bilateral salpingo-oophorectomy Oment. = Omentectomy
(S)TAH = (Sub)Total abdominal hysterectomy RXT = Radiotherapy
I-TA = Ileo-transverse anastomosis Rad. = Radical oophorectomy
CHM = Chlorambucil Treo = Treosulphan Vcr = Vincristine
Pro = Procarbazine Adr = Adriamycin Cyclo = Cyclophosphamide
Cis-Plat = Cis-Platinum
Diff = Tumour differentiation - well, moderate or poor.

NOTE: These clinical details are to the end of Dec. 1978. However, at the time of writing (June 1979), the two patients who are classified as "alive", are still so with no evidence of tumour recurrence.

Adriamycin. All patients who relapsed while on immunotherapy were changed to a standard protocol of monthly intravenous Adriamycin 40mg/m^2 (up to total of 500mg/m^2), Cis-Platinum 20mg/m^2 (depending on renal function and audiometry), and Cyclophosphamide 500mg/m^2 if they had not previously received this drug.

RESULTS:

Tables 6.4 and 6.5 present the statistical analysis of patients in immunotherapy and control groups, à propos of survival from the time of operation. They are constructed by the method of MANTEL (1966), and a conservative χ^2 approximation to the conditional log rank test (PETO & PIKE, 1973) is used to compare month to month survival over the period of time (48 months since operation). In the immunotherapy group all the patients except 1 have been followed for the 4 years, compared with all patients in the control group. Table 6.6 shows that there is little difference between the two groups in terms of their tumour histology and degree of differentiation.

Figs. 6.1 and 6.2 illustrate the clinical progress of all patients in this trial. Fig. 6.3 is the actuarial survival curve, calculated to 48 months , and shows that the median survival in the immunotherapy group is 24 months compared with 12 months in the control group. At 24 months, the actuarial survival calculated for the immunotherapy group is 54% compared with 12% for the control group (an actual rather than actuarial analysis for the control patients, since all are dead at the time of calculation). This difference is significant ($\chi^2 = 8.79$; $p < 0.01$) at 48 months (9% compared with 4% for controls).

Side effects have been minimal; sometimes a "flu-like" syndrome with slight fever, malaise and local tenderness following the first course and lasting about 24 hours. No BCG granulomata, systemic infection, serum hepatitis or tumour deposits at the site of inoculation have been seen. Three patients have suffered from hyperimmunization to BCG

FIGURE 6.1 SURVIVAL CHART FOR THE RETROSPECTIVE HISTORICAL CONTROLS
(25 Patients)

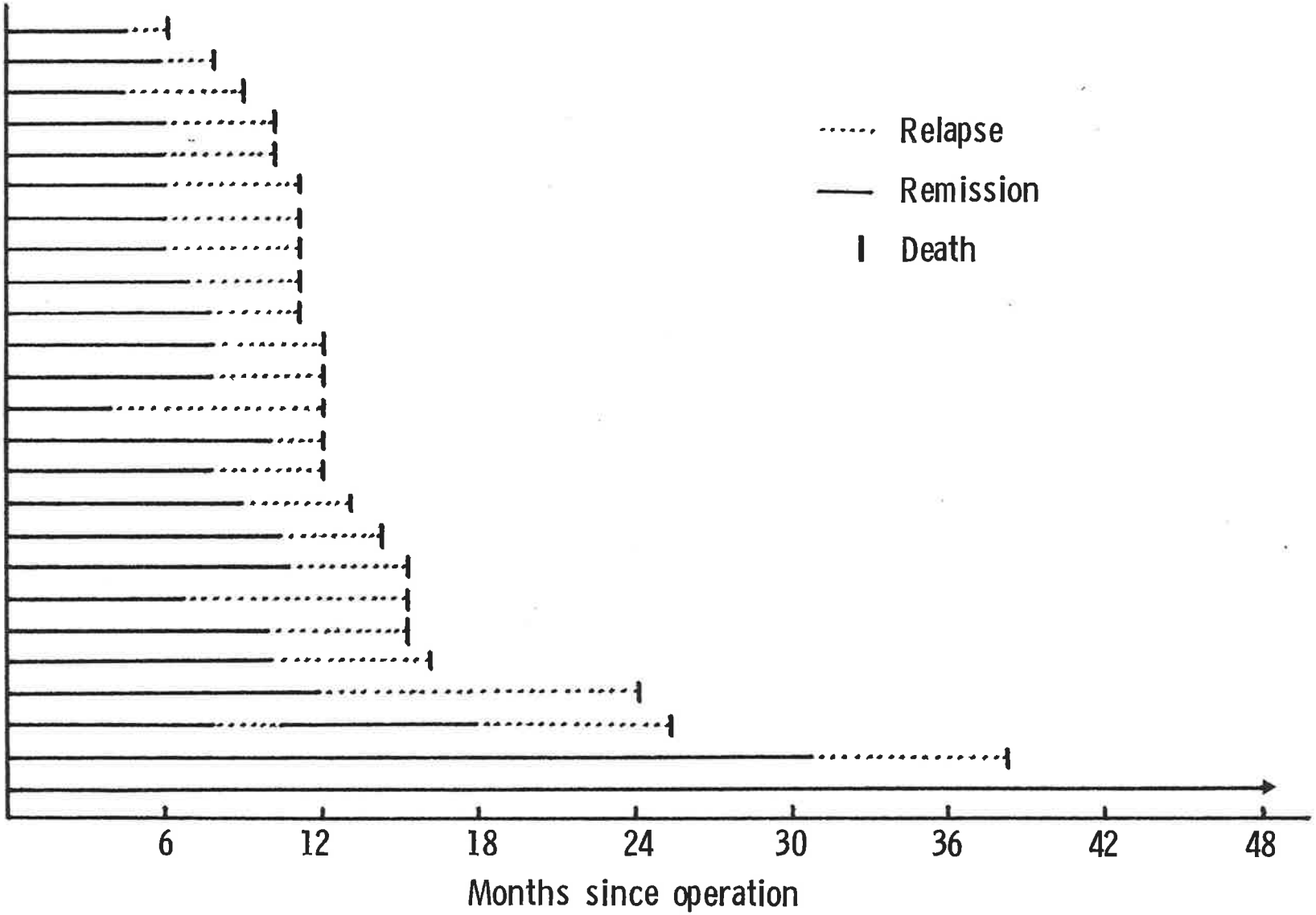


FIGURE 6.2 SURVIVAL CHART FOR THE UNRANDOMIZED IMMUNOTHERAPY GROUP
(15 Patients)

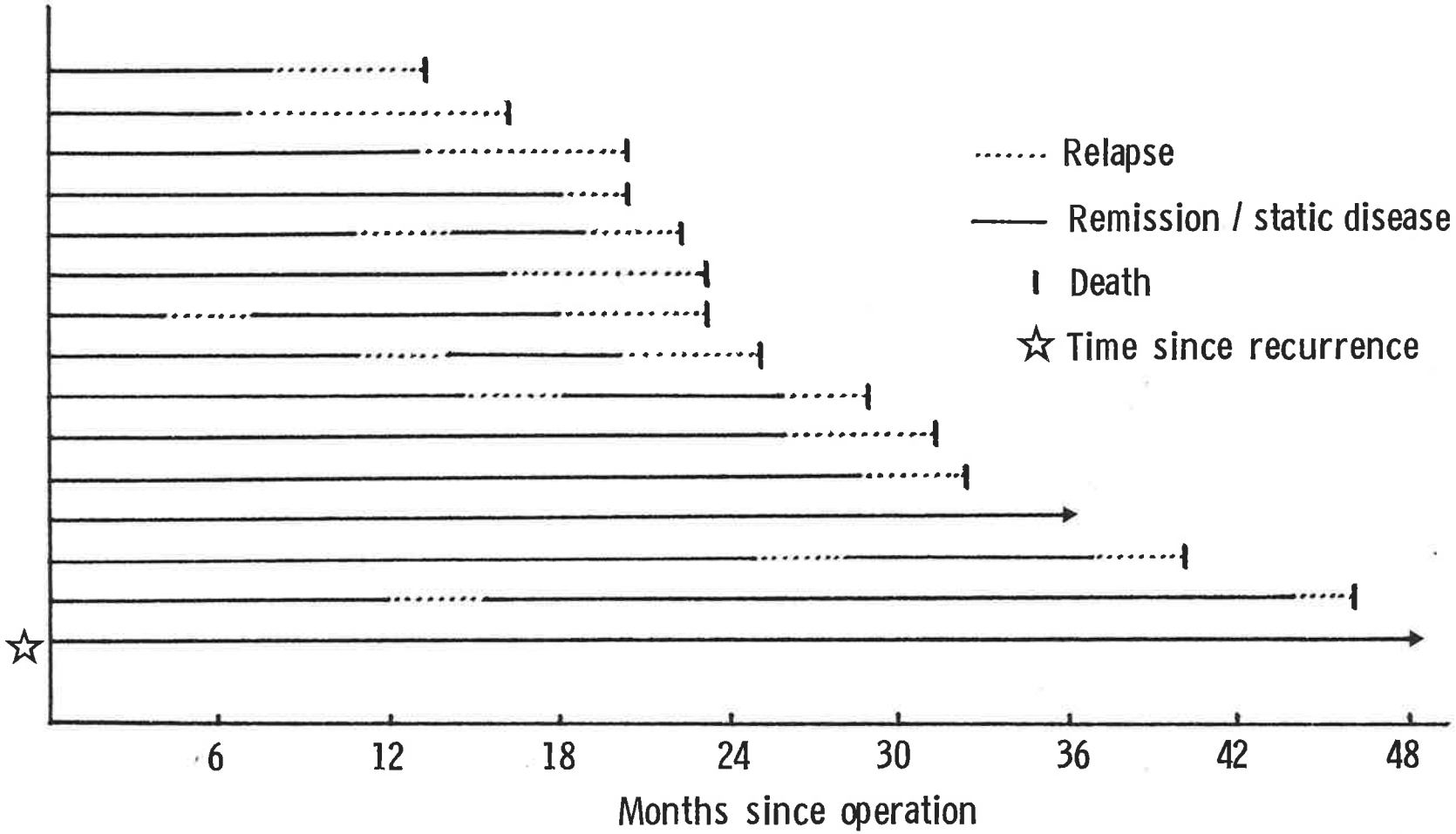


FIGURE 6.3 PERCENTAGE SURVIVAL FOR IMMUNOTHERAPY AND CONTROLS TO 4 YEARS

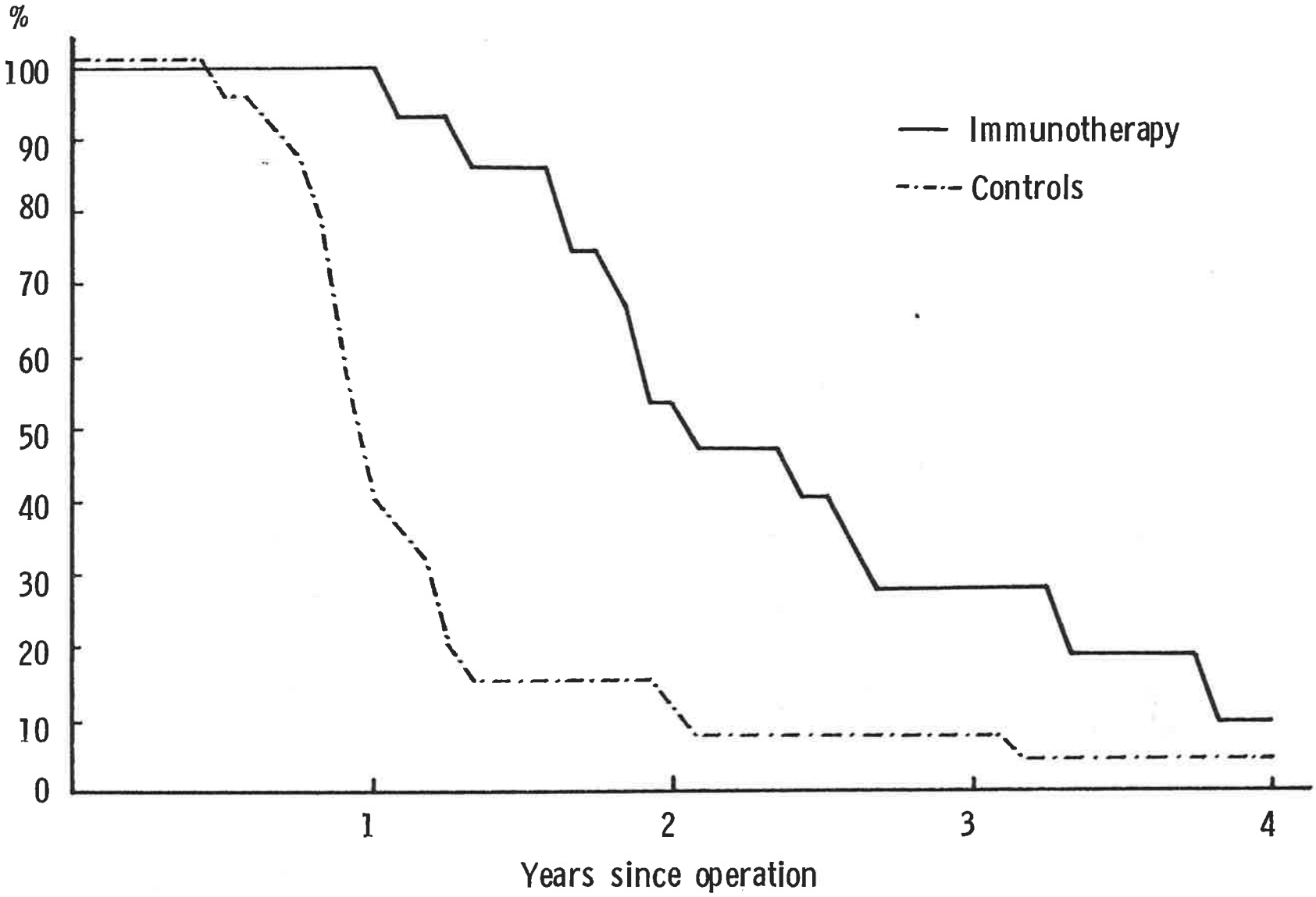


TABLE 6.4 ACTUARIAL ANALYSIS OF SURVIVAL IN 15 IMMUNOTHERAPY AND 25 NON-IMMUNOTHERAPY PATIENTS.

Time from Operation	No. of deaths (dx)		No. lost to follow-up (Wx)		No. at risk of dying (nx)		Probability of dying (dx/nx)		Prob. of not dying (1-dx/nx)		% alive	
	I/T	C	I/T	C	I/T	C	I/T	C	I/T	C	I/T	C
0 - 1					15	25	0	0	1	1	100	100
1 - 2					15	25	0	0	1	1	100	100
2 - 3					15	25	0	0	1	1	100	100
3 - 4					15	25	0	0	1	1	100	100
4 - 5					15	25	0	0	1	1	100	100
5 - 6		1			15	25	0	0.04	1	0.96	100	96
6 - 7					15	24	0	0	1	1	100	96
7 - 8		1			15	24	0	0.04	1	0.96	100	92
8 - 9		1			15	23	0	0.04	1	0.96	100	88
9 - 10		2			15	22	0	0.09	1	0.91	100	80
10- 11		5			15	20	0	0.25	1	0.75	100	60
11- 12		5			15	15	0	0.33	1	0.67	100	40
12- 13	1	1			15	10	0.07	0.10	0.93	0.90	93	36
13- 14		1			14	9	0	0.11	1	0.89	93	32
14- 15		3			14	8	0	0.38	1	0.62	93	20
15- 16	1	1			14	5	0.07	0.20	0.93	0.80	86	16
16- 17					13	4	0	0	1	1	86	16
17- 18					13	4	0	0	1	1	86	16
18- 19					13	4	0	0	1	1	86	16
19- 20	2				13	4	0.15	0	0.85	1	74	16
20- 21					11	4	0	0	1	1	74	16
21- 22	1				11	4	0.09	0	0.91	1	67	16
22- 23	2				10	4	0.20	0	0.80	1	54	16
23- 24		1			8	4	0	0.25	1	0.75	54	12
24- 25	1	1			8	3	0.13	0.33	0.87	0.67	47	8
25- 26					7	2	0	0	1	1	47	8
26- 27					7	2	0	0	1	1	47	8
27- 28					7	2	0	0	1	1	47	8
28- 29	1				7	2	0.14	0	0.86	1	40	8
29- 30					6	2	0	0	1	1	40	8
30- 31	1				6	2	0.17	0	0.83	1	34	8
31- 32	1				5	2	0.20	0	0.80	1	27	8
32- 33					4	2	0	0	1	1	27	8
33- 34					4	2	0	0	1	1	27	8
34- 35					4	2	0	0	1	1	27	8
35- 36					4	2	0	0	1	1	27	8
36- 37			1		3	2	0	0	1	1	27	8
37- 38		1			3	2	0	0.50	1	0.50	27	4
38- 39					3	1	0	0	1	1	27	4
39- 40	1				3	1	0.33	0	0.67	1	18	4
40- 41					2	1	0	0	1	1	18	4
41- 42					2	1	0	0	1	1	18	4
42- 43					2	1	0	0	1	1	18	4
43- 44					2	1	0	0	1	1	18	4
44- 45					2	1	0	0	1	1	18	4
45- 46	1				2	1	0.50	0	0.50	1	9	4
46- 47					1	1	0	0	1	1	9	4
47- 48					1	1	0	0	1	1	9	4

TABLE 6.5 ACTUARIAL ANALYSIS OF SURVIVAL IN 15 IMMUNOTHERAPY AND 25 NON-IMMUNOTHERAPY PATIENTS: CONSERVATIVE χ^2 APPROXIMATION.

Time from Operation	CONTROL No. at risk	CONTROL Obs.†	CONTROL Exp.†	IMMUNOTHERAPY No. at risk	IMMUNOTHERAPY Obs.†	IMMUNOTHERAPY Exp.†	TOTAL No. at risk	TOTAL Obs.†
0 - 1	25			15			40	
1 - 2	25			15			40	
2 - 3	25			15			40	
3 - 4	25			15			40	
4 - 5	25			15			40	
5 - 6	25	1	0.63	15		0.37	40	1
6 - 7	24			15			39	
7 - 8	24	1	0.62	15		0.38	39	1
8 - 9	23	1	0.61	15		0.39	38	1
9 - 10	22	2	1.18	15		0.82	37	2
10 - 11	20	5	2.86	15		2.14	35	5
11 - 12	15	5	2.50	15		2.50	30	5
12 - 13	10	1	0.80	15	1	1.20	25	2
13 - 14	9	1	0.39	14		0.61	23	1
14 - 15	8	3	1.09	14		1.91	22	3
15 - 16	5	1	0.53	14	1	1.47	19	2
16 - 17	4			13			17	
17 - 18	4			13			17	
18 - 19	4			13			17	
19 - 20	4		0.47	13	2	1.53	17	2
20 - 21	4			11			15	
21 - 22	4		0.27	11	1	0.73	15	1
22 - 23	4		0.57	10	2	1.43	14	2
23 - 24	4	1	0.33	8		0.67	12	1
24 - 25	3	1	0.54	8	1	1.46	11	2
25 - 26	2			7			9	
26 - 27	2			7			9	
27 - 28	2			7			9	
28 - 29	2		0.22	7	1	0.78	9	1
29 - 30	2			6			8	
30 - 31	2		0.25	6	1	0.75	8	1
31 - 32	2		0.29	5	1	0.71	7	1
32 - 33	2			4			6	
33 - 34	2			4			6	
34 - 35	2			4			6	
35 - 36	2			4			6	
36 - 37	2			3			5	
37 - 38	2	1	0.40	3		0.60	5	1
38 - 39	1			3			4	
39 - 40	1		0.25	3	1	0.75	4	1
40 - 41	1			2			3	
41 - 42	1			2			3	
42 - 43	1			2			3	
43 - 44	1			2			3	
44 - 45	1			2			3	
45 - 46	1		0.33	2	1	0.67	3	1
46 - 47	1			1			2	
47 - 48	1			1			2	
		24	15.13		13	21.87		37

$\chi^2 = (24 - 15.13)^2/15.13 + (13 - 21.87)^2/21.87 = 5.2 + 3.59 = 8.79$ $p < 0.01$

TABLE 6.6 HISTOLOGY AND DEGREE OF DIFFERENTIATION OF OVARIAN TUMOURS IN IMMUNOTHERAPY PATIENTS AND RETROSPECTIVE HISTORICAL CONTROLS IN TRIAL I.

Histology		Serous	Mucinous	Endometrioid	Undiff.
I/T	(15)	9	-	3	3
Controls	(25)	18	2	-	5

Differentiation		Good	Moderate	Poor	Undiff.
I/T	(15)	1	4	7	3
Controls	(25)	2	10	8	5

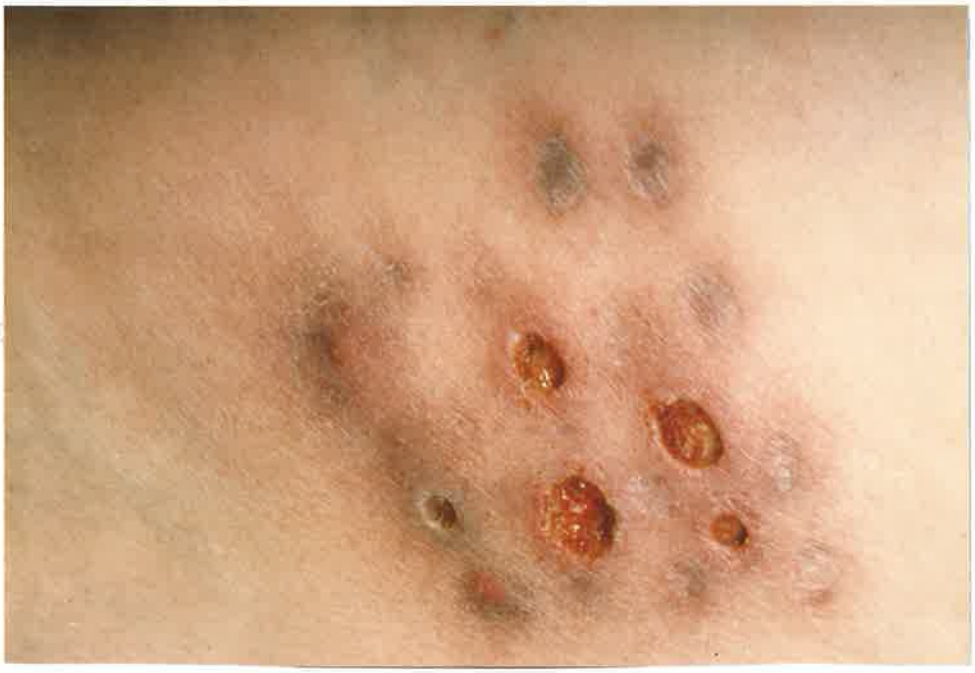


FIG. 6.4 TYPICAL IMMUNOTHERAPY LESIONS.

with painful, ulcerating lesions which took some months to heal. In such cases, the BCG was deleted from the mixture for a few cycles, and then re-introduced at a lower concentration. Bacteriological analysis of the purulent material in these lesions did not find Mycobacteria.

Early immunotherapy sites resemble ordinary BCG inoculation sites with ulceration and gradual healing to form a small scar. In patients in remission, further courses would lead to flaring of previous sites, and cells alone had the capacity to cause this phenomenon. As patients passed into relapse, this ability was lost, and the BCG lesions became weaker. In 2 patients, initial lesions were weak until second-look laparotomy with further removal of tumour; following this, their immunotherapy sites responded aggressively and the previous lesions developed flaring.

Local lymphadenopathy sometimes occurs in the axillary, cervical or inguinal nodes, with enlargement and regression in a cyclical fashion. Biopsy to exclude malignant deposits should of course be performed in any patient where this phenomenon appears suspicious.

TRIAL II: RANDOMIZED AND PROSPECTIVE.

Patients: criteria for entry are the same as in the previous trial except:

(i) Patients are all receiving standard MRC chemotherapy (see Appendix) i.e. either pulsed oral Methotrexate, Hexamethylmelamine and Cyclophosphamide; or continuous oral Cyclophosphamide

(ii) Patients must show disease "stasis" by this chemotherapy at 16 weeks after operation

Thus far, 11 patients have been randomized into the trial, 7 to receive immunotherapy and 4 as controls. Appropriate clinical details are given in Table 6.7.

TABLE 6.7 CLINICAL DETAILS OF 7 IMMUNOTHERAPY AND 4 CONTROL PATIENTS IN THE CONTROLLED, RANDOMIZED TRIAL II.

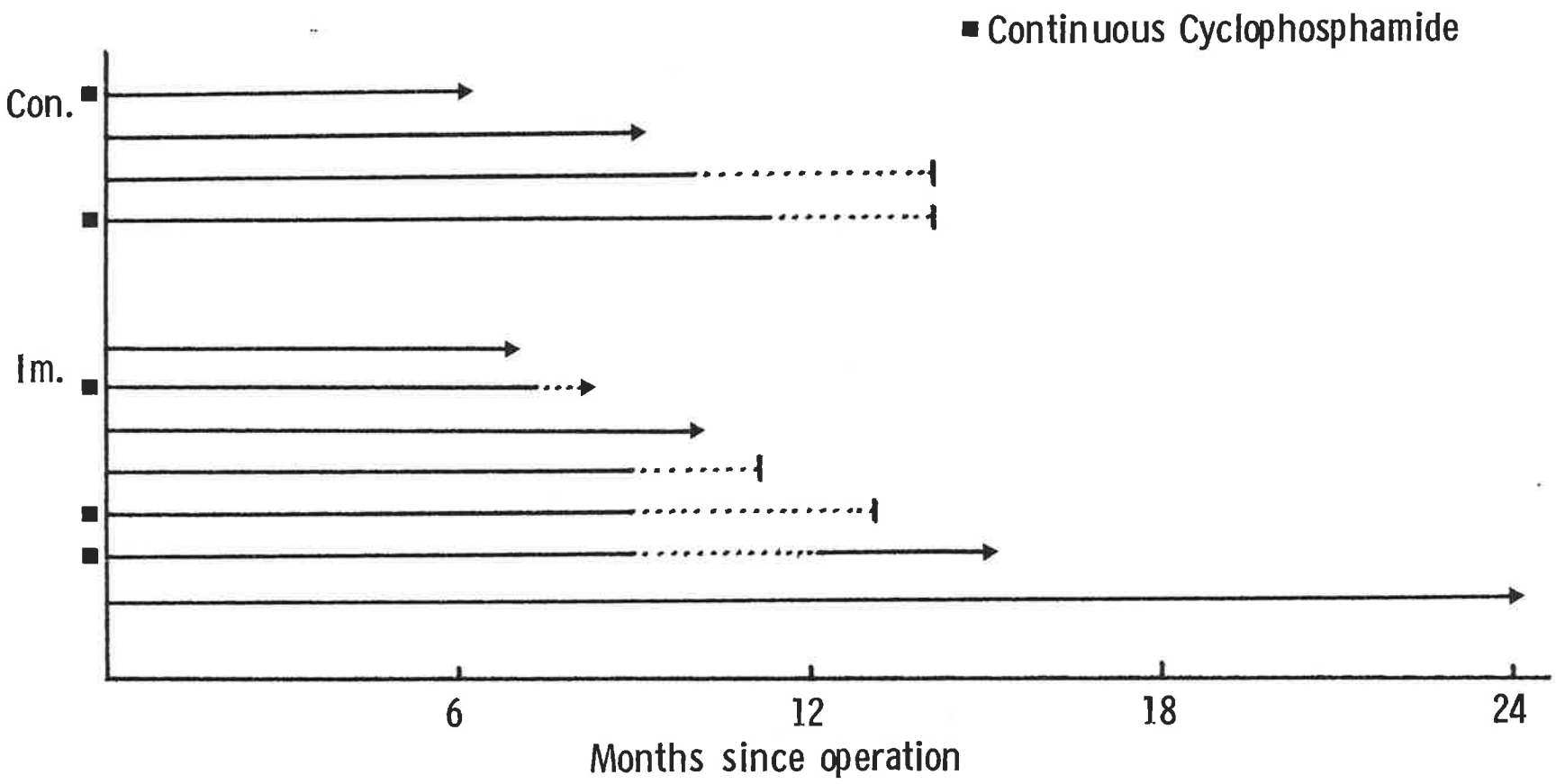
CODE/STAGE	SURGERY	HISTOLOGY /DIFF.	CHEMO.	NO.I/T COURSES (REACTION)	CLINICAL CONDITION	(MONTHS) PRE-DEATH RELAPSE	(MONTHS) TOTAL SURVIVAL
CONTROL PTS.							
DW / III	Biopsy only	S.Pap. /poor	Triple Adr, Cyclo & Cis-Plat.	-	Initial excellent response to chemo, then recurrent ascites.	4	14
BP / III	Biopsy	S.Pap. /well	Cont. Cyclo	-	Initial excellent response to chemo, then recurrent ascites.	4	14
MH / III	Oment & IT-A.	S.Pap. /poor	Cont. Cyclo	-	Initial excellent response to chemo.	-	alive (9)
JH / III	Bilat.ooph.	S.Pap. /mod.	Cont. Cyclo	-	Initial excellent response to chemo, ?involvement bladder base.	-	alive (6)
I/T PTS.							
SG / III	Bilat.ooph. & Oment.	S.Pap. /poor	Cont. Cyclo Adr. & Cis-Pl.	8 (2+)	Initial excellent response to chemo, then liver & bone mets.	4	13
LP / IV	Biopsy (liv.mets)	S.Pap. /poor	Triple	12 (3+)	2 yrs. after initial surgery, CAT scan shows ?pannus around L kidney, but no liver mets.	-	alive (24)
VD / IV	Bilat.ooph. & Oment. (clav. node)	S.Pap. /poor	Cont. Cyclo Adr & Cis-Pl.	10 (2+)	Apparent liver mets. 7 m. after surgery responding well to change in chemo.	?	alive (13)

TABLE 6.7 (contd).

CODE/STAGE	SURGERY	HISTOLOGY /DIFF.	CHEMO.	NO.I/T COURSES (REACTION)	CLINICAL CONDITION	(MONTHS) PRE-DEATH RELAPSE	TOTAL SURVIVAL
I/T PTS. (contd.)							
RF / III	Bilat.ooph.	S.Pap. /poor	Triple	1 (1+)	Tumour just held by chemo. Pre-terminal bowel obstn. treated with ileo-caecal anast.	2	11
CB / III	Bilat. ooph. & Oment.	S.Pap. /mod.	Cont Cyclo	3 (2+)	Initial good response to chemo.	-	alive (8)
AG / IV	BSO, TAH & & Oment. (clav. node)	S.Pap. /poor	Triple	5 (2+)	Initial good response to chemo.	-	alive (9)
WC / III	Biopsy	Undiff.	Triple	1 (2+)	Initial excellent response to chemo.	-	alive (7)

NOTE: These details are to the end of Dec. 1978. However, at the time of writing (June 1979), LP is still alive with no evidence of recurrence; VD is still alive but in relapse; CB died from uraemia with ascites 1 month later; AG went into relapse 2 months later (recurrent supra-clav. node treated with RXT) and is still alive; WC is still alive with no evidence of recurrence.

FIGURE 6.5 SURVIVAL CHART FOR PROSPECTIVE RANDOMIZED IMMUNOTHERAPY AND CONTROL PATIENTS



RESULTS:

Too few patients have been entered for the statistical analysis at this stage to be meaningful. However, 2 immunotherapy patients have survived beyond the longest follow-up time (14 months) for the control group, and while there is insufficient evidence that the immunotherapy group are doing better, they are certainly not doing any worse than the control group.

DISCUSSION:

Adjuvant immunotherapy with or without chemotherapy has been shown to be of use in prolonging survival in many animal models and in human cancer trials. BCG has a non-specific immunostimulatory effect on the haemopoietic system, whereas the rationale for the inclusion of tumour cells rests on the demonstration of tumour-associated immune responses in patients with ovarian cancer. That both BCG and the tumour cells have these immunostimulatory effects will be demonstrated in the next Chapter.

The addition of chemotherapy must be timed carefully so that its immunosuppressive effects do not abrogate the stimulatory effects of the immunotherapy. It is well established that high dose pulsed chemotherapy leads to immunosuppression with recovery and rebound in 8 - 10 days (CHEEMA & HERSH, 1971), and for this reason immunotherapy and chemotherapy were administered 2 weeks apart from each other.

The data in Study I has been analysed using actuarial survival tables and show a significant difference in survival ($p < 0.01$) between the immunotherapy and control groups at 48 months following operation. Clinical details (Figs. 6.1 & 6.2) also suggest that as well as prolonging survival, immunotherapy may facilitate the achievement of a second "remission" for patients with recurrent or relapsing disease, a phenomenon noted by others (HARRIS et al, 1978). It is worth pointing out that in no patients were tumour "regressions" as such noted, since all the patients had

visible tumour remaining after surgery, generally in the form of peritoneal seedlings rather than measurable tumour deposits. For this reason, the term "disease stasis" is used to denote the absence of gross, progressive disease as detected by palpation, ultrasonic scanning or the appearance of new effusions. Occult progress may very well be occurring, but there is no way to detect this short of a second-look laparoscopy or laparotomy.

Although the use of actuarial tables has been criticised, it may be the only adequate means of predicting median survival in a situation where new information is continually being added. This can be demonstrated with a simple comparison; in March 1977, calculations based on 10 immunotherapy patients and 25 retrospective controls predicted that the median survival for the immunotherapy group would be 23 months compared with 12 months for the controls. By January 1st 1979 (the point to which all follow-up is taken) 15 immunotherapy patients have now been followed for at least 24 months, and hence the % survival at 24 months is no longer a prediction but an actual result (54%) and the median survival is indeed 24 months.

The use of retrospective, historical controls has also been criticised as being far from satisfactory, since there is no standardisation in terms of original surgery, subsequent chemotherapy or follow-up and detection of new lesions. One reason that the immunotherapy patients may have done better in this study is that they were seen more often than the controls, and so aggressive treatment with changed chemotherapy could be carried out early in the detection of recurrence. In the randomized trial all patients were seen monthly.

The survival curve (Fig. 6.3) to 48 months shows that the actual shape is not altered, but that the curve is shifted by a few months to the right of the graph. This confirms the findings of POWLES et al (1977 b) as well as numerous other workers, that immunotherapy does not

apparently lead to a sub-population of long-term survivors, but simply delays death by a number of months. Ultimately all patients succumb, and it is interesting to note that 3 patients in Study I relapsed within a few months of ceasing their immunotherapy after 2 years of inoculations. Ways in which immunotherapy may prolong survival are discussed in the following Chapter.

No untoward side-effects were seen in any of the patients, and the most serious problem was hyperimmunization in 3 patients. Ulcerating lesions healed within a few months when BCG was deleted from the mixture, and no swabs from the lesions showed the presence of acid-fast bacilli.

It therefore appears that immunotherapy is a safe procedure which may be efficacious in prolonging survival in women with advanced ovarian cancer. The significant differences in survival between the immunotherapy and control groups of Study I can not be wholly attributed to the immunotherapy, and it will therefore be crucial to see the outcome of the prospective, randomized trial currently being conducted. Furthermore, analysis of the chemotherapy sub-populations in this trial is vital, and may very well show significant differences between those on continuous chemotherapy (does this completely abrogate the effects of immunotherapy?) and those on pulsed chemotherapy. To date, too few patients have been entered for adequate statistical analysis.

CHAPTER 7. MONITORING OF CELLULAR RESPONSES DURING IMMUNOTHERAPY.

This Chapter reviews attempts to monitor immunological parameters in patients receiving immunotherapy. In so doing, it attempts to realize one of the aims of immunotherapy, namely the production of immunostimulation. However, reports are conflicting as to the immunological benefits of immunotherapy, and clearly there is a dichotomy between the immunological effects (many workers have in fact shown no effect on immunocompetence) and the clinical effects (amelioration of pain, increased survival, and tumour shrinkage). This suggests therefore that although augmentation of immunocompetence may occur it is not the sine qua non for the achievement of some beneficial effect from immunotherapy.

Cell mediated responses following immunization have been extensively studied (PERLMANN & HOLM, 1969; BIESECKER 1973). During the primary response to antigens which elicit a cell-mediated response, T-cells proliferate in the lymph nodes draining the antigenic site, and blast cells appear in the efferent lymph within 3 days (HALL et al, 1967). However, about 50% of the lymphocytes in the node have come from the general blood pool, since local irradiation to the nodes may result in a 50% decrease in the lymphocyte output yet antigenic stimulation will result in a still normal immune response (HALL & MORRIS, 1964). These T-cells survive and proliferate as memory cells and then re-enter the circulating pool of long-lived lymphocytes (CRADDOCK et al, 1971). It has in fact been calculated that the life of one of these cells may be more than 500 days (NORMAN et al, 1965).

There are two important implications relating to the formation of these long-lived memory cells. Firstly, re-exposure to the antigen provokes an immune response of longer duration, which is marked by a shorter latent period than the original primary response. These memory T-cells undergo blastogenesis and proliferate with cyclical variations that have been observed for murine tumour antigens

(SIMPSON & BEVERLEY, 1972). Secondly, it is likely that such long-lived cells may escape depletion by agents such as sub-lethal radiation or a short course of chemotherapy (MILLER & COLE, 1967), and this may explain why whole-body irradiation leads to severe and prolonged lymphocyte suppression compared with local irradiation (NOWELL 1965).

BCG antigenic stimulation results in a typical cell-mediated immune response. In mice, blast cells appear in the T-dependent areas of the lymph node within 2 days and these cells reach a maximum of proliferation and activity within 14 days (MACKANESS et al, 1973). As mentioned in Chapter 6, such responses depend on the dosage, virulence and viability of the BCG organisms injected. BCG also facilitates the recruitment of T-cells to respond more vigorously to a second unrelated antigen, e.g. sheep RBC, probably via a soluble mediator released from the T-cells in response to the first (MAILLARD & BLOOM 1972; RUBIN & COONS, 1972; MILLER et al, 1973). Certainly, the role of the T-cell is vital, since the effects of BCG in animals can be abrogated by the use of anti-theta serum (RAY et al, 1977).

As well as increased production of activated T-cells, BCG may act to inhibit the blocking of immune responses by immune complexes, via activation of the reticulo-endothelial system (MACKANESS et al, 1974 a). To test this hypothesis, "blocking" serum from mice immunized with SRBC was given intravenously to mice at the time of in vivo sensitization to SRBC. In normal mice, it could completely block the cell-mediated response but in BCG-treated mice it had no effect (MACKANESS et al, 1974 b). Hence the immune response is less subject to feedback inhibition, and immunity, both cellular and humoral,becomes enhanced in BCG-infected mice.

In animals immunological responses to multiple injections of tumour cells have been assessed. SONIS et al (1975) investigated the effect of repeated injections of viable EL-4 lymphoma cells on a number of immunological parameters in mice. In the 3 assay systems investigated (cell-mediated

cytotoxicity, antibody-dependent cellular cytotoxicity, and complement-dependent cytotoxicity) the nature of the response was similar i.e. detectable cytotoxicity by about the 6th day after immunization, reaching a peak by 14 days, dropping to a low by 3 weeks, and then continuing in a cyclical manner unrelated to recurrent immunizations at 10 day intervals. Presumably these fluctuations are due to the interplay of feedback mechanisms that modulate the original response.

In vitro a number of techniques have been used to assess the alteration of lymphocyte responsiveness following immunization in vivo, including blastogenic responses to secondary recall antigens, mitogens, tumour-associated antigens, and cell-mediated cytotoxicity against tumour cells (CURRIE et al, 1971). In vivo skin DHR's to PPD, tumour vaccine, other secondary recall antigens and DNCB have been used (see Table 7.1) Such monitoring is particularly important in those cases where it is thought that tumour enhancement may be occurring in response to immunotherapy (LEVY et al, 1972). It is known too that certain immunotherapeutic agents such as C.Parvum may be immunosuppressive (KIRCHNER et al, 1975; MINTON et al, 1976). Furthermore, we have already discussed the paradoxical results that chemo-immunotherapy may on occasion lead to additive immunological effects (MACKANESS et al, 1974 b). These workers showed that mice given Cyclophosphamide 10 days after BCG, and then immunized with sheep RBC, produced a persistent and high level of immune reactivity to the SRBC which was much greater than the responses in the presence of either agent alone. This was presumably caused by the interference of Cyclophosphamide with B-cell production of antibody, thereby interfering with the feedback mechanism of T-cell blocking by antibody and immune complexes (LAGRANGE et al, 1974). However, MATHÉ et al (1975) have shown that such effects depend very much on the time of administration of the chemotherapy; in a L1210 leukaemia murine model, BCG given before Cyclophosphamide led to accelerated tumour growth, whereas if given 5 days after the Cyclophosphamide there was an increase in the dose of chemotherapy which could

be given. In man BCG has been shown to increase the immunosuppression of the antibody response following Cyclophosphamide, Methotrexate and 5-fluorouracil; however there was no effect on cell-mediated immune responses (SPARKS et al, 1975). Such potential inhibition of the immune response may under some circumstances of course be advantageous, for example in the blocking of immune complexes and suppressor cells (POLAK & TURK, 1974).

More sophisticated methods of identifying sub-populations of lymphocytes and other cells need to be established so that monitoring may identify patients in whom immunotherapy is clearly not beneficial. EMBLETON (1976) has shown that immunotherapy with tumour cell membranes in rats with MCA-induced sarcomas has no effect, and that in vitro there is a lack of cell-mediated cytotoxicity, the presence of serum blocking factors and also the induction of suppressor lymphocytes. In patients with a variety of tumours, VETTO et al (1975) have shown that those who initially respond and then subsequently relapse undergo changes in their lymphocytes and serum factors which are unlike those seen in patients who never responded at all (vide infra). Clearly therefore, it is only by careful monitoring in the human situation that we can begin to understand the ways in which immunotherapy may modify the immune system in the tumour-bearing host.

7.1 IMMUNOLOGICAL PARAMETERS MONITORED IN HUMAN CANCER.

(i) Effects on Bone Marrow. It has previously been mentioned that BCG as a stimulant of the reticulo-endothelial system may lead to proliferation of cell sub-populations, and indeed this may allow the giving of larger quantities of chemotherapeutic agents. POUILLART et al (1975) have demonstrated that both animals and humans receiving chemotherapy and BCG show a significantly earlier restoration of leucocytes than controls. In mice BCG leads to a significant increase in splenic macrophages and the production of a lymphocyte-activating factor which stimulates

TABLE 7.1 REPRESENTATIVE STUDIES OF IN VIVO AND IN VITRO MONITORING DURING IMMUNOTHERAPY.

TUMOUR	REFERENCE	PARAMETERS	COMMENTS
Melanoma	IKONOPOSIV et al, 1970	CMC; IF	Tumour specific antibodies increased transiently if more than 10^7 cells injected.
AML, ALL	POWLES et al, 1971	MLC	Increased stimulation of patients' lymphocytes by their blast cells following autoimmunization; transient.
Melanoma	CURRIE et al, 1971	LMC	Increased cytotoxicity in 5/12 patients, whereas negative results pre-immunotherapy.
Sarcoma	MORTON 1971	CMC; DHR to tumour cells	Both parameters increased in 7/9 patients after immunotherapy with BCG and autologous tumour cells.
AML	GUTTERMAN et al, 1973 c	LB	LB to blast cells and PHA increased in 6/9 patients, after autoimmunization with non-irradiated cells.
Lymphoma	SOKAL 1973	DHR; MCR	DHR to PPD and tumour cells and LB to tumour cells increased.
Melanoma	CHESS et al, 1973	DHR; LB; Ig & C'3	DHR to PPD reverted to positive in all 4 patients; LB increased to PPD but not PHA; Ig levels increased.
Melanoma	GUTTERMAN et al, 1973 b	DHR; LB; WCC; Ig	DHR to PPD increased significantly in remission patients; LB to PPD and mitogens did not change; no alteration in WCC, monocyte count or Immunoglobulin levels.
Melanoma	CURRIE 1973	LMC	Serum inhibitory effects fell within 1 week of immunotherapy.
Melanoma	GRANT et al, 1974	DHR; LB; Ig; LMC; LMI	DHR to PPD reverted to positive in 7/10 patients. All other parameters generally increased.
Lung	TAKITA et al, 1974	LB; LMI	LB increased to PPD and PHA, and LMI converted to positive in one patient.
Melanoma	BERKELHAMMER et al, 1975	LMC	Increased non-specific reactivity to melanoma and other tumour cells in 6/8 patients.
ALL	EKERT et al, 1975	DHR; LB; IF; %E-RFC; bone marrow smear	DHR converted to positive in 10/12 patients. LB increased to PHA and leukaemia cell antigen. %E-RFC increased from 35% to 50% and smears showed increased lymphoid cells. Tumour-associated antibody increased even after BCG stopped.
Melanoma	MAVLIGIT et al, 1976 a	DNCB	No difference between controls and immunotherapy patients treated with BCG.
Melanoma	GERNER & MOORE, 1976	DHR	Positive conversion to PPD in 26/45 patients with flaring in previous sites.
Gynae.	PATTILLO 1976	LB	Increased to PHA, Con A and PWM.
Melanoma	GOLUB et al, 1977	DHR; LB; MLC; %E-RFC	DNCB and PPD responses not useful. LB increased sig. to PPD but not other antigens or mitogens. %E-RFC unaltered. Patients given tumour cells had increased responses in MLC.
Lung	MANKIEWICZ et al, 1977	DHR; LB; Macrophage activation	Responses to PPD converted in 3/8 patients. LB increased to PPD, PHA and tumour antigen. Macrophage and lymphocyte clusters in patients given tumour cells.
Melanoma	EMBLETON et al, 1978	IF; CMC; LB; LMC; LMI;	No obvious differences between vaccinated and non-vaccinated groups.

ABBREVIATIONS: IF = Immunofluorescence for anti-tumour antibody CMC = Complement mediated cytotox.
LB = Lymphocyte blastogenesis LMC = Lymphocyte mediated cytotoxicity
MLC = Mixed Lymphocyte Culture MCR = Mixed Cell Reaction
LMI = Leucocyte Migration Inhibition DHR = Delayed Hypersensitivity Reaction
DNCB = Dinitrochlorobenzene Ig = Immunoglobulin C'3 = Complement.

T-cells (MITCHELL et al, 1973). In patients with bronchogenic carcinoma receiving tumour cell vaccine and BCG, macrophage activation with clusters of lymphocytes around mature macrophages and tumour cells in culture has been demonstrated (MANKIEWICZ et al, 1977). In fact, the greatest number of clusters in vitro was seen in cell cultures stimulated with tumour-associated antigen.

Increased numbers of white cells have been found in some studies (PENDERGAST et al, 1971; EKERT et al, 1975; ANTHONY et al, 1975). PENDERGAST et al (1971) showed that rapidly progressive disease was associated with an average decrease of 67% in the peripheral lymphocyte count; patients who responded objectively to immunotherapy had a 40% increase in this count. On the other hand many other studies have shown no such effects (GUTTERMAN et al, 1973 b; GOLUB et al, 1977; EMBLETON et al, 1978).

In populations of E-RFC, the $\%T_a$ has been shown to rapidly increase following intralesional BCG (WYBRAN et al, 1973); and this appears to be associated with histological evidence of tumour invasion by macrophages and lymphocytes (MORTON et al, 1970b). A similar increase in T_a is found after the administration of Transfer Factor and is associated with an increase in the patient's cell-mediated cytotoxicity against tumour cells. There is also a direct correlation between the increase in T_a from low to normal and those patients who respond to immunotherapy (LEVIN et al, 1975).

(ii) <u>Effects on in vivo Delayed Hypersensitivity Reactions to PPD and other secondary recall antigens and tumour cells</u>.

It would be expected that the regular inoculation of BCG would lead to positive PPD responses in patients previously negative, and this is generally the case. Many studies have shown this conversion in a large proportion of patients (GERNER & MOORE, 1976; GRANT et al, 1974; ZIEGLER & MAGRATH 1973). There seems indeed to be a good correlation between the patients' immunocompetence at the beginning of immunotherapy and whether they subsequently respond to it (MORTON et al, 1970 a&b), and SOKAL (1973) has shown in patients with Hodgkin's Disease Stage IV that

those who developed a strong PPD reaction following immunotherapy lived at least one year, whereas 7 of 9 patients who failed to convert died within a year of vaccination. GUTTERMAN et al (1975 b) disagree with this hypothesis and describe 2 patients with melanoma, anergic and still in remission.

DNCB has been suggested as a more useful prognostic indicator than PPD (MORTON et al, 1970; GERNER & MOORE 1976; GOLUB et al, 1977), although MAVLIGIT et al (1976 a) have shown no significant differences in the sensitization of malignant melanoma patients receiving BCG compared with those who are not.

Responses to secondary recall antigens such as Mumps, Candida etc. tend to be variable and equivocal (TAKITA et al, 1974) although again there are reports of significant potentiation of such responses (ZIEGLER & MAGRATH 1973; GERNER & MOORE 1976). One reason may be that the antigens of BCG are similar to those of a number of common bacteria (MINDEN et al, 1972).

Clearly, the demonstration that immunotherapy increases cell-mediated responses to tumour associated antigens provides a rationale for its use. Again, there are conflicting reports. POWLES et al (1971) found on no occasion any DHR following autoimmunization of leukaemia patients with their tumour cells, even though in vitro testing showed increased blastogenic responses. In contrast, others (MORTON 1971; SOKAL et al, 1972) have reported such enhanced skin reactions. Of particular interest is the report of GERNER & MOORE (1976) that 16 of 20 patients developed DHR's to tumour cells during immunotherapy with BCG and tumour cells. 9 of 45 patients developed "flares" in previous immunotherapy sites following subsequent inoculations and 1 patient developed a flare at the site of an uninjected metastasis. This melanotic lesion completely regressed and biopsy showed no tumour cells but a dense mononuclear infiltrate. "Flares" in immunotherapy sites also followed the injection of Varidase, suggesting cross-reactivity between microbial and tumour antigens.

(iii) Effects on Blastogenic Responses of Lymphocytes to Various Stimuli.

As with the in vivo studies, there are conflicting reports. POWLES et al (1971) showed that autoimmunization of leukaemic patients with their blast cells led to specific and non-specific effects on their lymphocytes. Not only was there a transitory increase in their response to leukaemic cells in a mixed cell reaction, but there was also an increased response to normal allogeneic lymphocytes. It was suggested that immunization had increased the proportion of lymphocytes capable of responding to the leukaemic blast cells, but it was not known if immunotherapy had changed the patients' serum in such a way that blastogenic stimulation was more effective (all cultures were done in autologous serum).

In malignant melanoma, autoimmunization of patients with their irradiated tumour cells led to an increased cytotoxicity of their lymphocytes against tumour cells (CURRIE et al, 1971), but only in the autologous situation.

Increased blastogenesis to tumour associated antigens has been reported by many other workers (GRANT et al, 1974; EKERT et al, 1975; ROTH et al, 1975; MANKIEWICZ et al, 1977), lasting sometimes for as much as 17 weeks without further stimulation (SOKAL 1973). Other reports however have been negative (GUTTERMAN et al, 1973 a & b; EMBLETON et al, 1978), and BERKELHAMMER et al (1975) have suggested that if increased lymphocyte reactivity occurs it may be non-specific.

Most reports indicate that blastogenic responses to PPD are, as expected, increased (CHESS et al, 1973; TAKITA et al, 1974; GOLUB et al, 1977). There are conflicting reports on increased responsiveness to mitogens, with as many negative reports (CHESS et al, 1973; GOLUB et al, 1977; EMBLETON et al, 1978) as positive (TAKITA et al, 1974; EKERT et al, 1975; MANKIEWICZ et al, 1977). Certainly, in animals, BCG leads to increased responses to PHA and PPD in vitro, and increased DNCB responses in vivo (COHEN et al, 1974).

The Mixed Lymphocyte Reaction has also been used as

an in vitro means of assessing lymphocyte blastogenesis. Whereas CHESS et al (1973) found no increased responsiveness, GOLUB et al (1977) found this to be the only parameter to be augmented by immunotherapy, and postulated that this was due to shared histocompatibility antigens between the tumour cells used in the immunotherapy, and the pool of normal lymphocytes used as stimulator cells in the assay.

In view of the large number of negative reports it has been suggested that BCG may not augment the proliferative capacity of immunocompetent cells as such, but simply stimulate the macrophage system to handle tumour antigens more efficiently. This might explain why mitogen responses (which do not rely on antigenic recognition) may not be altered, but antigenic responses to PPD, tumour associated antigens and other secondary recall antigens are augmented.

(iv) Effects on Complement, Antibody Production and "Serum Factors".

Early reports (IKONOPISOV et al, 1970) demonstrated an increased amount of tumour-specific cytotoxic antibody after autoimmunization of melanoma patients with their cells; this was transient, disappeared within 2 weeks, and depended on the number of cells injected, less than 10^7 giving no response. They postulated that such antibody might be cytotoxic for blood-borne metastases rather than the actual tumour, although they noted that immunotherapy did not seem to alter the clinical course of their patients.

CURRIE et al (1971) reported variable antibody responses as detected by immunofluorescence in melanoma patients following immunotherapy, although there was no correlation between this and lymphocyte mediated cytotoxicity against tumour cells. In a subsequent report (CURRIE 1973) he showed that immunotherapy led to the rapid disappearance of "serum blocking" factors, although the phenomenon was transient and lasted for only 2-3 weeks. HUGHES et al (1970) demonstrated a marked post-immunotherapy rise in tumour-associated antibody in 2 melanoma patients, and suggested that tumour cells might undergo antigenic modulation in the face of an immune attack. He reported a case where 40

melanoma lesions regressed in a woman following immunotherapy, but new lesions then developed in other sites. Immunofluorescent studies detected 2 tumour-specific antibodies against the old and new lesions respectively.

Transient increases in tumour-associated antibody have also been reported for soft-tissue sarcomas (MORTON 1971) and children with leukaemia (EKERT et al, 1975).

VETTO et al (1975) found that patients who responded to immunotherapy had stable or increased responses to PHA in vitro, compared with low responses in clinically deteriorating patients. However, these low responses could be reversed by extensive washing of their lymphocytes. On the other hand, in patients who had originally responded to immunotherapy and then relapsed, this manoevre could not restore lymphocyte blastogenesis, indicating that perhaps not a blocking factor but a defect at the cellular level was causing this phenomenon. They suggested that "immune clone consumption" had occurred as a result of the stimulation induced by the immunotherapy. MILLER et al (1976) showed that in patients who responded to immunotherapy, there was a rapid decrease in serum blocking effects and even "potentiation" of lymphocyte mediated cytotoxicity against their tumour cells. 3 patients became clinically free of disease and showed an increase in their serum immunoglobulins.

Changes in complement (C'3) and serum immunoglobulins (generally IgG) have been reported by some workers (GRANT et al, 1974; CHESS et al, 1973), although there seems to be no consistent pattern to the changes. Finally, antibody titres to BCG have been determined by WILE et al (1977), and show, as expected a rapid increase following immunotherapy with BCG and tumour cells. The titres remained high in patients who remained free of recurrence, but in those who relapsed, titres disappeared preceding detection by up to 5 months. It was concluded that antibody titres to BCG were a better indicator of the patient's immune response to immunotherapy than the DHR to PPD.

It should be noted that a recent report suggests that the detection of antibody is not related to tumour-antibody, but anti-HLA antibodies, especially in situations where allogeneic cells are injected into the patient (GALE

& MacLENNAN 1977); furthermore the detection of serum blocking factors in patients following immunotherapy may indicate enhancement (LEVY et al, 1972; CURRIE 1973).

7.2 EXPERIMENTAL DESIGN: THE MONITORING OF PATIENTS WITH OVARIAN CANCER AFTER IMMUNOTHERAPY WITH BCG AND/OR IRRADIATED ALLOGENEIC TUMOUR CELLS.

The aim of the following experiments was to assess:

(i) lymphocyte counts pre- and during immunotherapy after a period of at least 3 months

(ii) the % T_a and T_t and their total numbers; in this case the immunotherapy evaluations were compared with a population of pre-operative patients and normal controls (see Chapter 4)

(iii) blastogenic responses to PPD and ovarian tumour CME during individual immunotherapy cycles

(iv) the effect of immunotherapy serum from different parts of the cycle on responses of normal donor lymphocytes, compared with the same responses in ABS

(v) Rheumatoid Factor in Immunotherapy serum.

MATERIALS AND METHODS:

Patient groups were:

lymphocyte counts - 16 patients
E-RFC counts - 11 patients, 9 of whom had relapsing disease
skin test responses - 18 patients
blastogenic responses - 6 patients in detail
serum effects - sera from 4 patients
Rheumatoid Factor - 85 samples from 9 patients

Blastogenic Assay: 50-60 mls. of heparinised blood were taken twice weekly for the 4 weeks of an immunotherapy cycle. For ethical reasons only patients who were thought to have static disease were investigated as it was felt that patients with active disease might not be able to compensate satisfactorily for the blood loss. In retrospect, however, 2 patients of the 6 were seen to have recurring disease at the time of study. Lymphocytes were prepared as previously described and E-RFC were assessed simultaneously.

Serum: 10 mls. clotted blood was taken at the same time and the serum stored at -20°C until further use.
Rheumatoid Factor: a commercial kit was used.
Serum effects: the sera from various stages of each immunotherapy cycle were set up in a concentration of 12.5% with normal lymphocytes, and the results compared with standard responses in ABS done at the same time. The effect on blastogenesis to PPD, Con A and ovarian tumour CME was assessed.

RESULTS AND DISCUSSION:

Table 7.2 gives the values of lymphocyte counts in 16 patients before they started immunotherapy and at least 3 months after they had been receiving it. In most cases the latter blood count was done at the time of the next immunotherapy course i.e. 2 weeks after chemotherapy had been given. In most cases this consisted of 1G Cyclophosphamide orally over 3 days, although some patients were receiving intravenous chemotherapy with Adriamycin, Cis-Platinum and Cyclophosphamide. 2 patients were not receiving chemotherapy, 2 were on continuous Cyclophosphamide and the third was on the MRC Trial chemotherapy of Methotrexate, Hexamethylmelamine and Cyclophosphamide.

A straight statistical comparison of these two values is spurious and in fact shows a significant decrease in lymphocyte counts during immunotherapy ($p < 0.02$). However, when patients are divided into groups on the basis of their clinical state at the time of testing, it can be seen that the trend in patients with static disease is for immunotherapy to maintain if not increase their total lymphocyte count; in patients in relapse this trend is not seen and immunotherapy had no effect in maintaining lymphocyte levels. It is worth noting that in patients on continuous Cyclophosphamide there was a marked decrease in their lymphocyte counts even though they were in clinical remission.

Fig. 4.2 illustrates the values of %E-RFC (T_a and T_t) and Table 4.6 details their statistical significance. $\%T_a$ values are restored from low to normal and are significantly

greater then the pre-operative (large tumour burden) values ($p < 0.001$), but the $\%T_t$ is not significantly altered from the pre-operative values. In fact, these % values were not significantly different from those of patients in remission or with minimal tumour burden.

Table 7.3 indicates the values of blastogenic responses of lymphocytes to PPD and ovarian tumour CME in these patients at the time of E-RFC assessment. There is no correlation between the Stimulation Index and the % of either T_a or T_t (Fig. 7.1)

Table 7.4 gives the %E-RFC during individual immunotherapy cycles in 3 patients. There is no well-defined pattern, but levels tend to fall after chemotherapy and are rapidly restored within 1 week. In each case there is a clear association between this rise and the increased blastogenesis seen following chemotherapy (Figs. 7.2, 7.3 & 7.4).

The aggressiveness of the patients' skin responses to BCG at the time of immunotherapy has been correlated with ultimate survival (Fig. 7.5). It must be stressed that such assessment is quite subjective but derives from the observations that patients who have clinically static disease tend to have far more aggressive responses than patients with relapsing disease, and that should relapse occur in patients it is usually accompanied by progressively weakened responses. The following criteria are taken into account - erythema, itchiness, pustular formation, time taken to heal, ability to "flare" with subsequent immunizations.

A number of clinical phenomena are worth describing:

Patient WN: 7 courses of immunotherapy with BCG and tumour cells had been given to this woman with inoperable tumour when she developed bowel obstruction. At laparotomy, it was possible to perform radical surgery and remove the uterus and ovaries. Her immunotherapy lesions prior to this time had been weak. Following surgery, aggressive skin responses occurred and the previous sites developed "flares" around them, consisting of erythema and pruritis.

Patient DH: This woman became hyperimmunized to BCG and

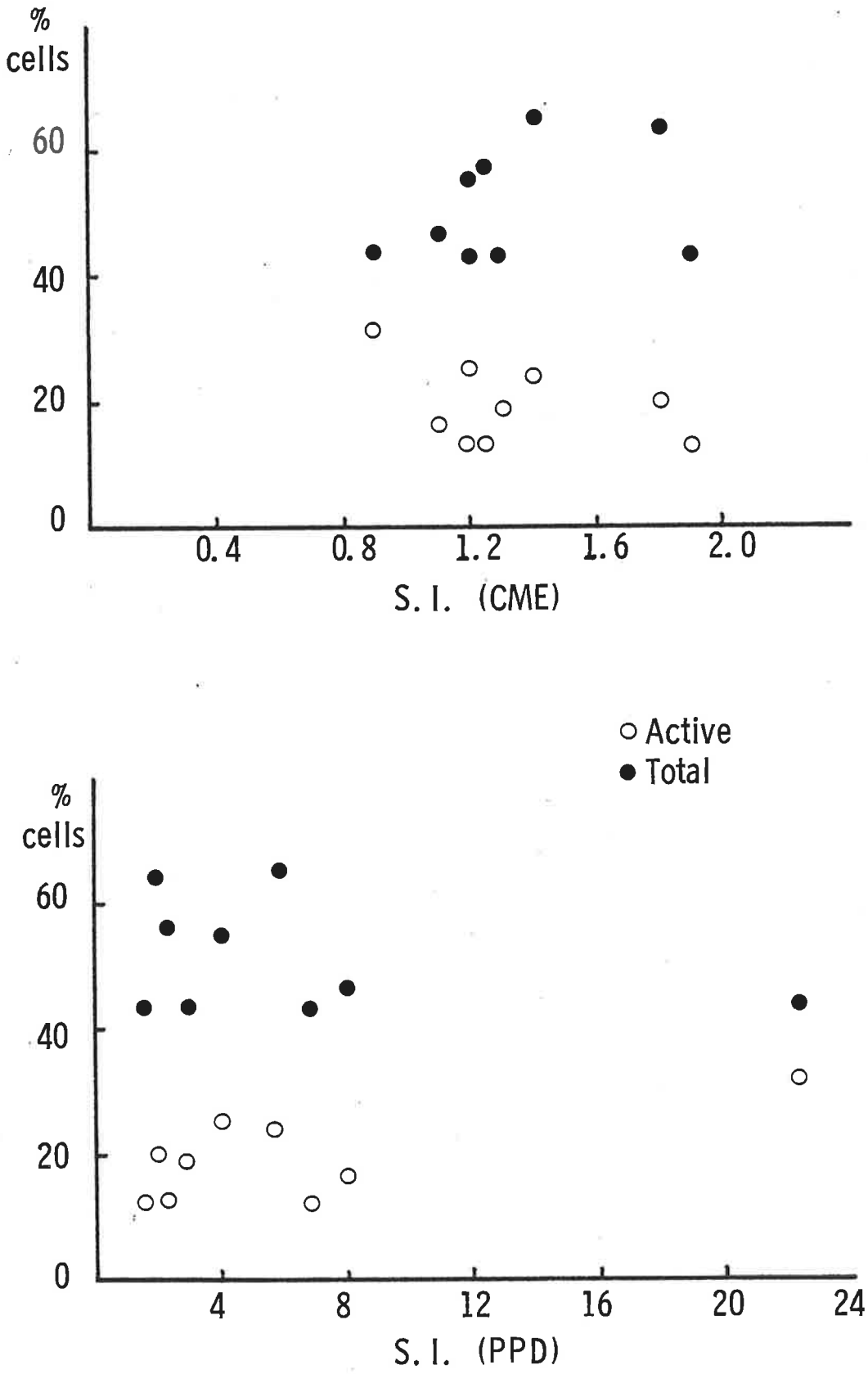


FIGURE 7.1 CORRELATION BETWEEN %E-RFC AND BLASTOGENIC RESPONSES TO PPD AND OVARIAN TUMOUR CME

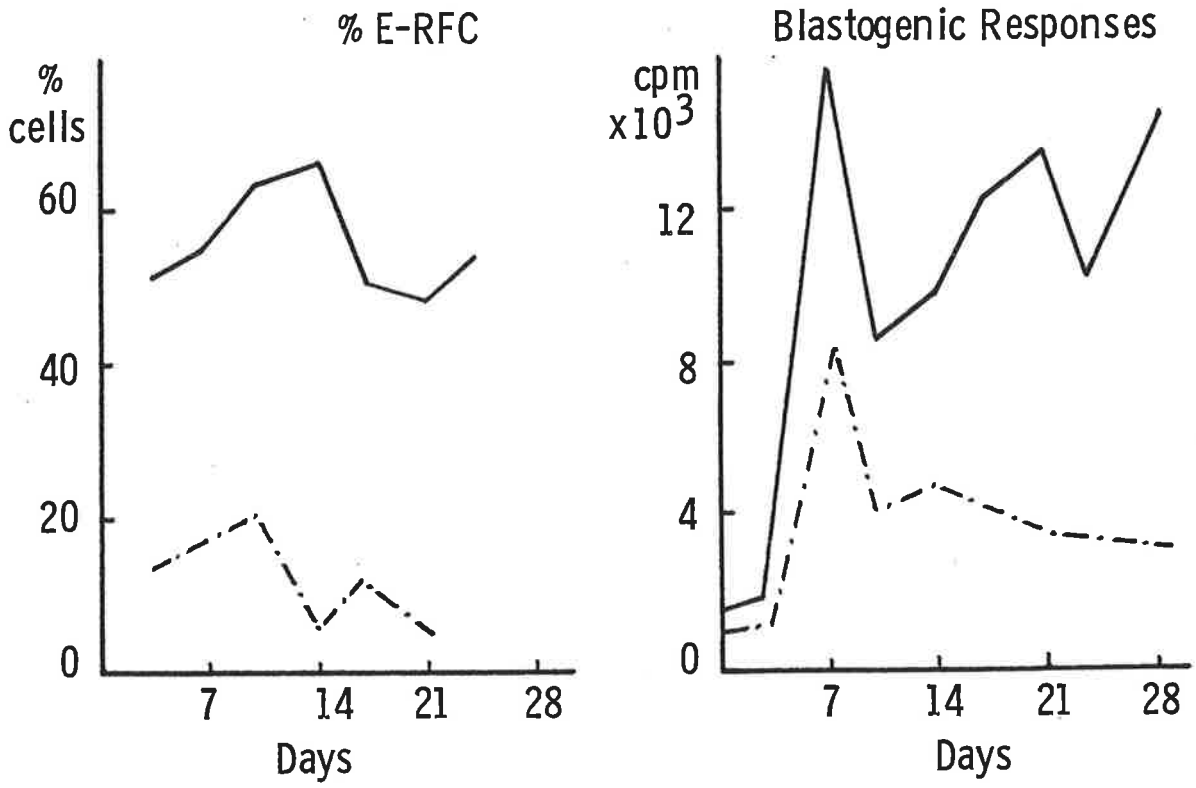


FIGURE 7.2 EFFECT OF IMMUNOTHERAPY ON VARIOUS PARAMETERS OF LYMPHOCYTE FUNCTION (Patient M.G.)

CHEMOTHERAPY : Melphalan orally Days 10-15

IN THE FOLLOWING FIGURES :

—— %E-RFC (Total); WCC; Total T-cell count; Blastogenic responses to PPD

-·-·- %E-RFC (Active); % lymphocytes; Active T-cell count; Blastogenic responses to Ovarian Tumour CME

····· Total lymphocyte count

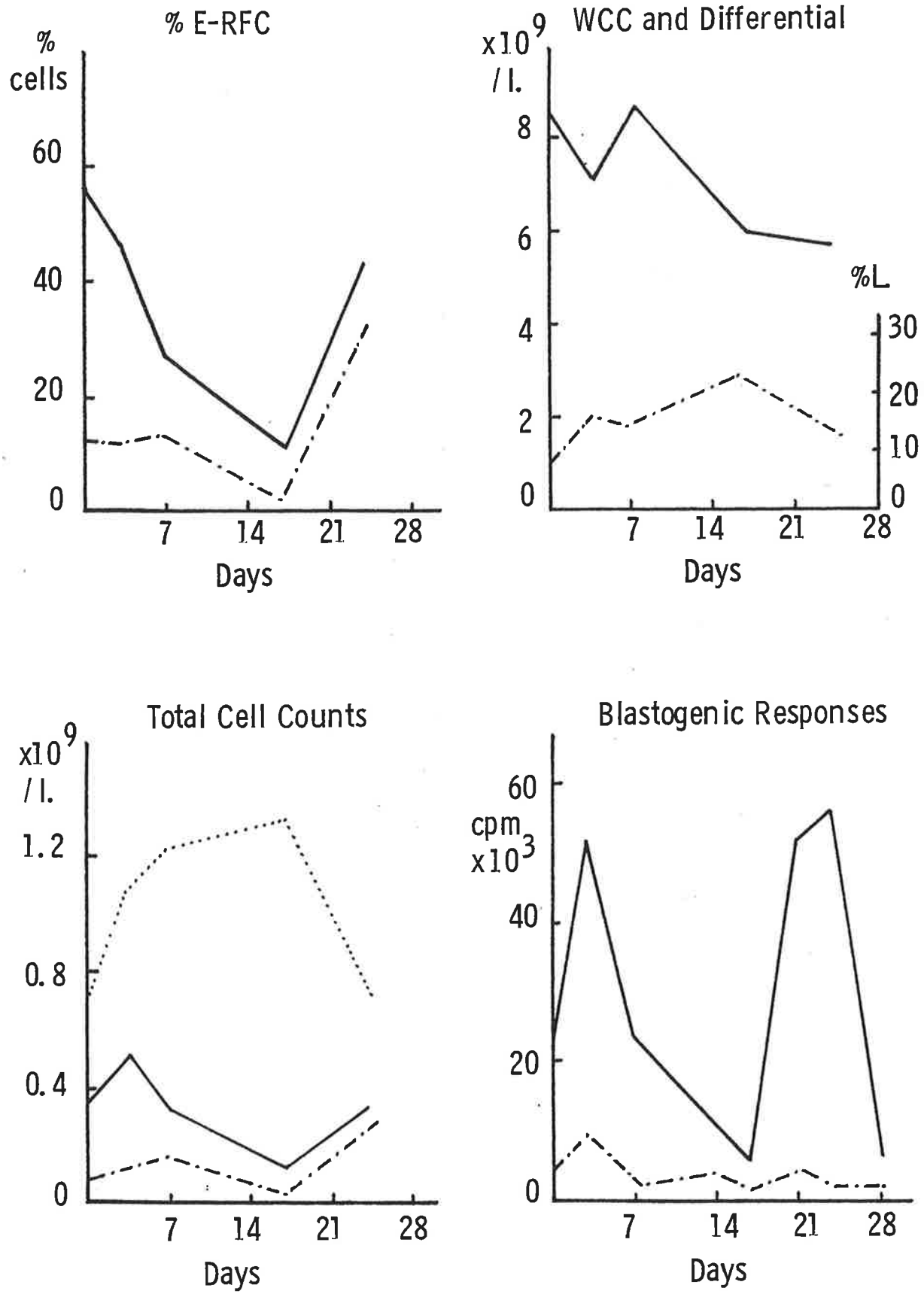


FIGURE 7.3 EFFECT OF IMMUNOTHERAPY ON VARIOUS PARAMETERS OF LYMPHOCYTE FUNCTION (Patient H. L.)

CHEMOTHERAPY: Oral Cyclophosphamide 1G Days 16-18

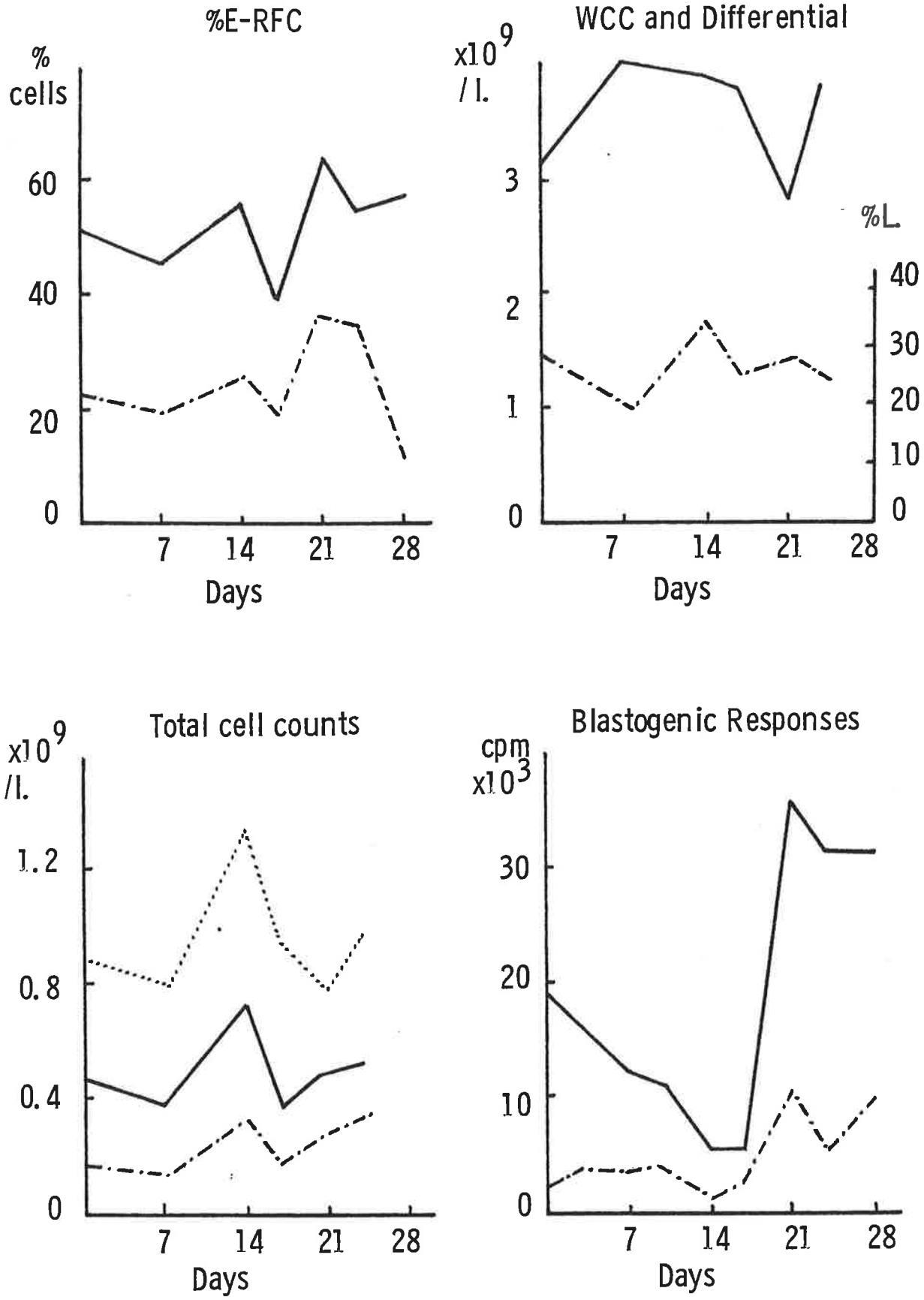


FIGURE 7.4 EFFECT OF IMMUNOTHERAPY ON VARIOUS PARAMETERS OF LYMPHOCYTE FUNCTION (Patient N. S.)

CHEMOTHERAPY: Oral Cyclophosphamide 1G. Days 16-18

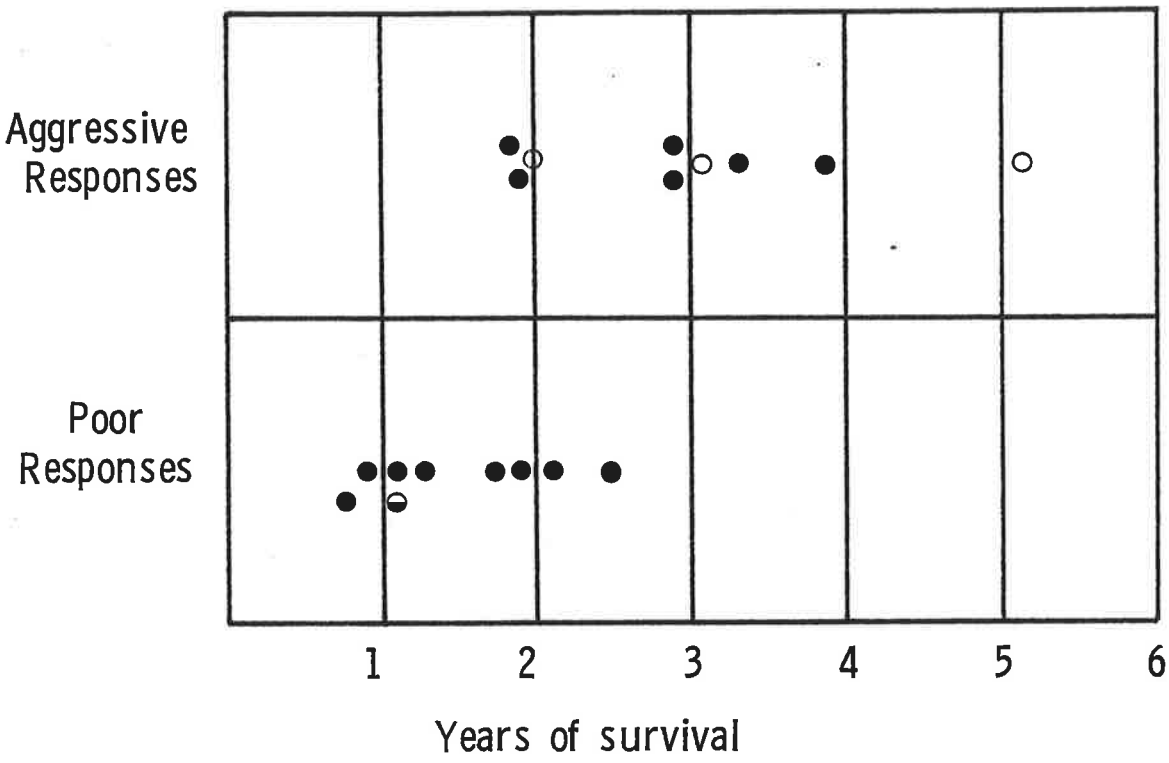


FIGURE 7.5 CORRELATION BETWEEN SURVIVAL AND STRENGTH OF SKIN RESPONSES TO IMMUNOTHERAPY

● Dead
○ Alive
◒ Alive with relapsing disease

had painful ulcerations which took some time to heal. During this time, she was given irradiated tumour cells only and on two occasions this led to "flares" in previous immunotherapy sites, suggesting some cross-reactivity between tumour antigen and BCG antigens.

Patient NS: As with the previous patient, this woman became hyperimmunized to BCG and for the time that she was given tumour cells only, developed "flares" in previous BCG sites.

The twice-weekly blastogenic responses of patients' lymphocytes to PPD and ovarian tumour CME will be discussed separately:

Patient NS: This woman originally had a biopsy only for Stage III disease, and underwent dramatic regression of disease in response to Cyclophosphamide. A second-look laparotomy for the removal of uterus and ovaries was performed, at which time numerous biopsies of the peritoneal cavity, diaphragm, and liver showed no evidence of viable tumour cells. Her first course of immunotherapy was with BCG and tumour cells. Lymphocyte responses over that month (Fig. 7.6) indicate that the base-line counts (i.e. unstimulated cells represented as Nil counts) alter in a manner consistent with in vivo activation of cells by BCG i.e. the responses reflect an increase in circulating immunoblasts. In vitro this is represented by an increase in the ^{125}IUDR uptake into the cells immediately following immunotherapy, a decrease which occurs following chemotherapy, and a restoration as part of the normal rebound phenomenon which occurs after chemotherapy (CHEEMA & HERSH 1971). If one looks at the cpm difference however, between cells stimulated with PPD or CME and control cells, there is little difference and certainly no discernible pattern (lower part of the graph).

The next month (Fig. 7.7) shows a marked change in this pattern. There is an initial boost to PPD following immunotherapy, presumably accounted for by the blast cells which appear in local nodes in response to BCG immunization (secondary response - MACKANESS et al, 1973). This is then

followed by a spontaneous fall which may reflect lymph node sequestration of sensitized lymphoblasts in other sites (FROST & LANCE 1974) or the natural tendency for such responses to be self-limiting. Immediately following chemotherapy there is a paradoxical and rapidly rebounding rise and finally a fall. This is difficult to explain but may be related to the differential action of Cyclophosphamide on B cells, so inhibiting the normal feedback mechanisms against immune stimulation.

In the third course with BCG only (Fig. 7.8) a different pattern of responses is obtained following chemotherapy. However, responses to tumour CME are stimulated suggesting that there may be antigenic cross-reactivity between tumour cells and BCG, and also demonstrating that BCG does indeed prime lymphocytes for enhanced responses to other antigenic stimuli (MILLER et al, 1973). It can also be clearly seen that the peak of the PPD response has gradually increased from a cpm diff. of 3000 in the first course to nearly 30,000 by the third. It is difficult to explain why there should now be a prolonged state of immunosuppression following the Cyclophosphamide, but this may indicate a time when BCG is truly causing a potentiation of chemotherapeutic immunosuppression (MATHÉ et al, 1975; SPARKS et al, 1975).

When tumour cells only are given in a later course, there are gradually falling responses of lymphocytes to PPD, and then a dramatic rise following chemotherapy (Fig. 7.9). The initial falling levels are difficult to explain; perhaps there is lymph node sequestration of immunoblasts specifically sensitized to tumour antigens. This would explain why there is no discernible increase in blastogenic responses to CME until after the chemotherapy which is presumably accompanied by a release of sensitized cells.

Patient HL: This woman originally had a sub-total hysterectomy and bilateral oophorectomy for Stage III disease, and when this immunotherapy cycle was done she was on monthly oral Cyclophosphamide and was considered to have static disease. Fig. 7.10 shows the blastogenic responses

to PPD and her own tumour CME following BCG only. In this case, whilst there is an increase in responses following chemotherapy, it is not as great as the initial boost following the immunotherapy; there is no response to CME. Six months later the responses are nearly double those obtained previously (Fig. 7.11) and this follows immunotherapy with tumour cells only. Again there is no response to CME but PPD responses follow a fairly established pattern with a post-chemotherapy boost. During this study, the patient's lymphocytes were also incubated in "remission" serum (from a patient in clinical remission) and "relapse" serum (from a patient in relapse, not uraemic or jaundiced). It is interesting to note that the initial pre-immunotherapy boost (about 44,000 cpm diff.) is unaffected by either serum. The results in remission serum are similar to those in ABS except that the post-chemotherapy rise is about half the level of that in ABS (Fig. 7.12). However, in relapse serum, there is no post-chemotherapy responsiveness of the lymphocytes (Fig. 7.13).

Patient MG: This patient, originally inoperable with Stage IV disease, underwent second-look laparotomy for removal of the ovaries after a dramatic response to Adriamycin and Cyclophosphamide. When this cycle was done, she was on monthly Melphalan (orally over 5 days) and in retrospect clinical re-activation of disease was occurring. The degree of lymphocyte responsiveness is much less than that seen normally in remission patients (about 12,000 cpm diff.). Immunotherapy with BCG and tumour cells gives rise to increased responses to both PPD and tumour CME (Fig.7.14). Melphalan does not affect this, and the responses continue to rise quite significantly.

Patient SG: This patient had a bilateral oophorectomy for Stage III disease, with significant reduction of the tumour. She was randomized to have continuous Cyclophosphamide as part of the MRC Chemotherapy Trial, and achieved an excellent result with obvious shrinkage of deposits in the Pouch of Douglas. The first course of immunotherapy with cells alone gives rise to a marked increase in responses to CME (Fig.7.15)

although the PPD responses are fairly indifferent. This may represent dampening of the effect by chronic chemotherapy, or the transient nature of responses stimulated by tumour cells to other antigens. When BCG alone is given (Fig. 7.16) there is a response to both CME and PPD. Recurrent disease became apparent during this course, and chemotherapy was stopped on Day 14. Thereafter fluctuations in the responses appear to settle.

Patient MT: This patient, treated with removal of uterus (sub-total) and ovaries for Stage III disease, had completed two years' of immunotherapy when this cycle was done. A month after immunotherapy was stopped signs of recurrent disease were apparent. Immunotherapy was with autologous tumour cells (Fig. 7.17) and fluctuations in responses to both allogeneic and autologous CME are apparent. PPD responses are minimally affected by Cyclophosphamide.

Patient DH: This elderly patient, treated by surgical removal of tumour for Stage III disease, had completed nearly two years' of immunotherapy when this cycle was done. She was not (by her own refusal) on chemotherapy, although previously she had been on small doses of Chlorambucil. Following immunotherapy with BCG and tumour cells, there is a small rise in PPD responses followed by the usual fall (? lymph node sequestration) and another spontaneous rise to a peak by 14 days (Fig. 7.18). It is interesting to note the spontaneous fall by 28 days.

Table 7.5 details the actual results and also compares the Day O values with the peak values (generally Day 14). Fig. 7.19 indicates that the responsiveness of lymphocytes, unstimulated ($p < 0.05$), to PPD ($p = 0.02$), and to CME ($p < 0.01$) are all significantly increased during the cycle.

Figs. 7.20 & 7.21 compare the blastogenic responses of normal lymphocytes in immunotherapy sera with the actual responses in the patients at the time the sera were taken. There is no clear-cut pattern to the responses i.e. when there is stimulation of lymphocyte responses in the immunotherapy

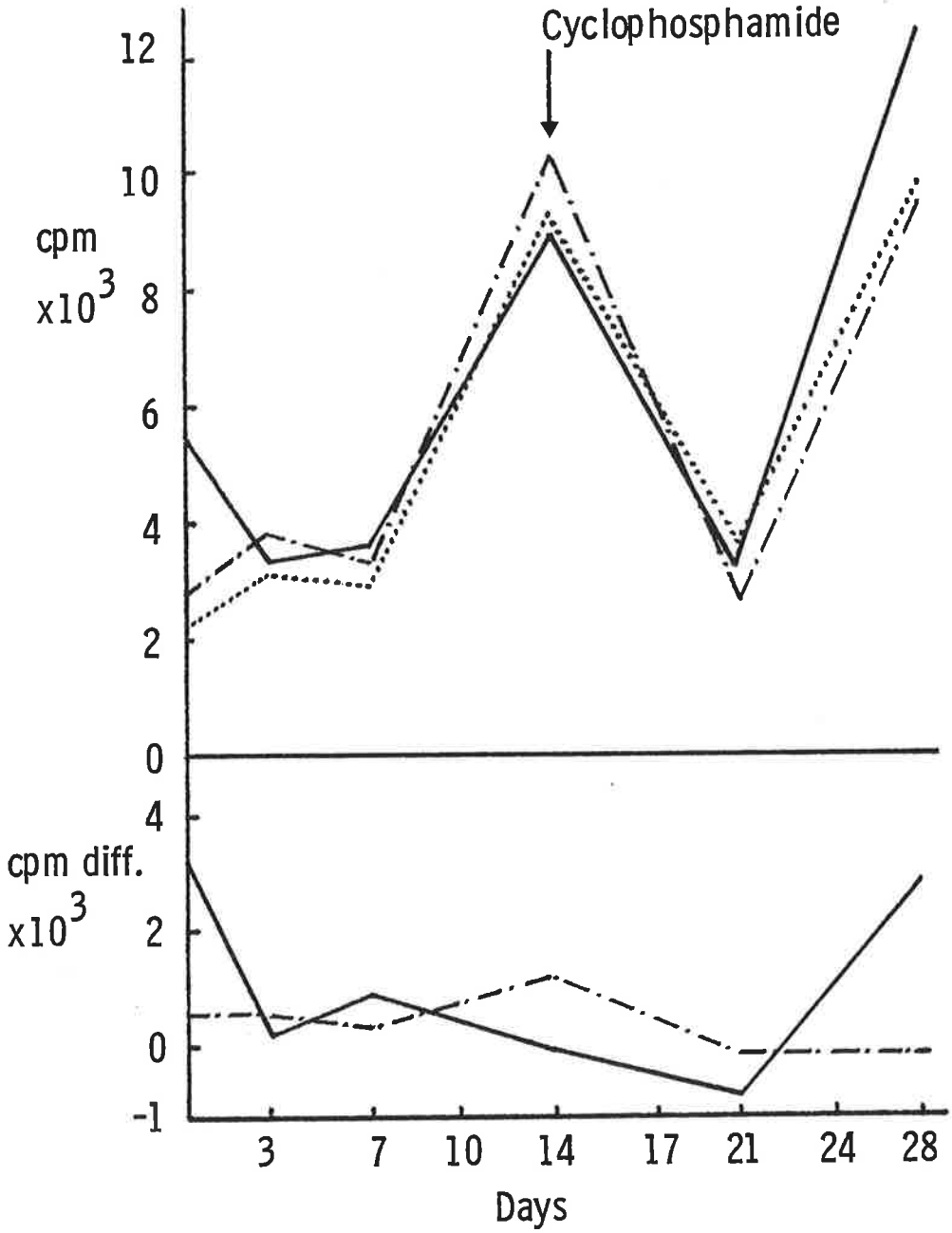


FIGURE 7.6 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH BCG AND CELLS 1st. COURSE (Patient N. S.)

..... Nil — PPD -·-·- CME

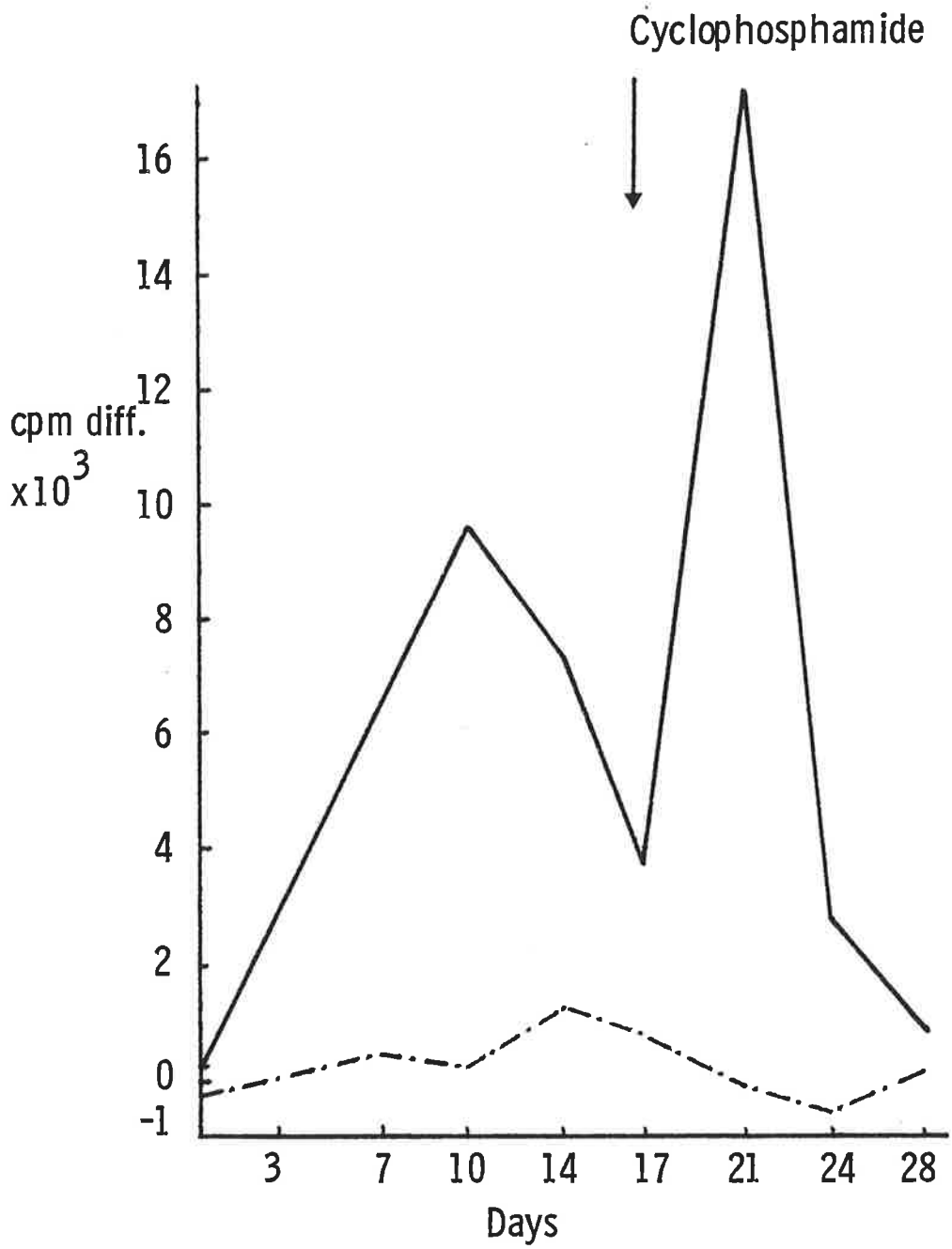


FIGURE 7.7 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH BCG AND CELLS 2nd COURSE (Patient N. S.)

—— PPD -·—·- CME

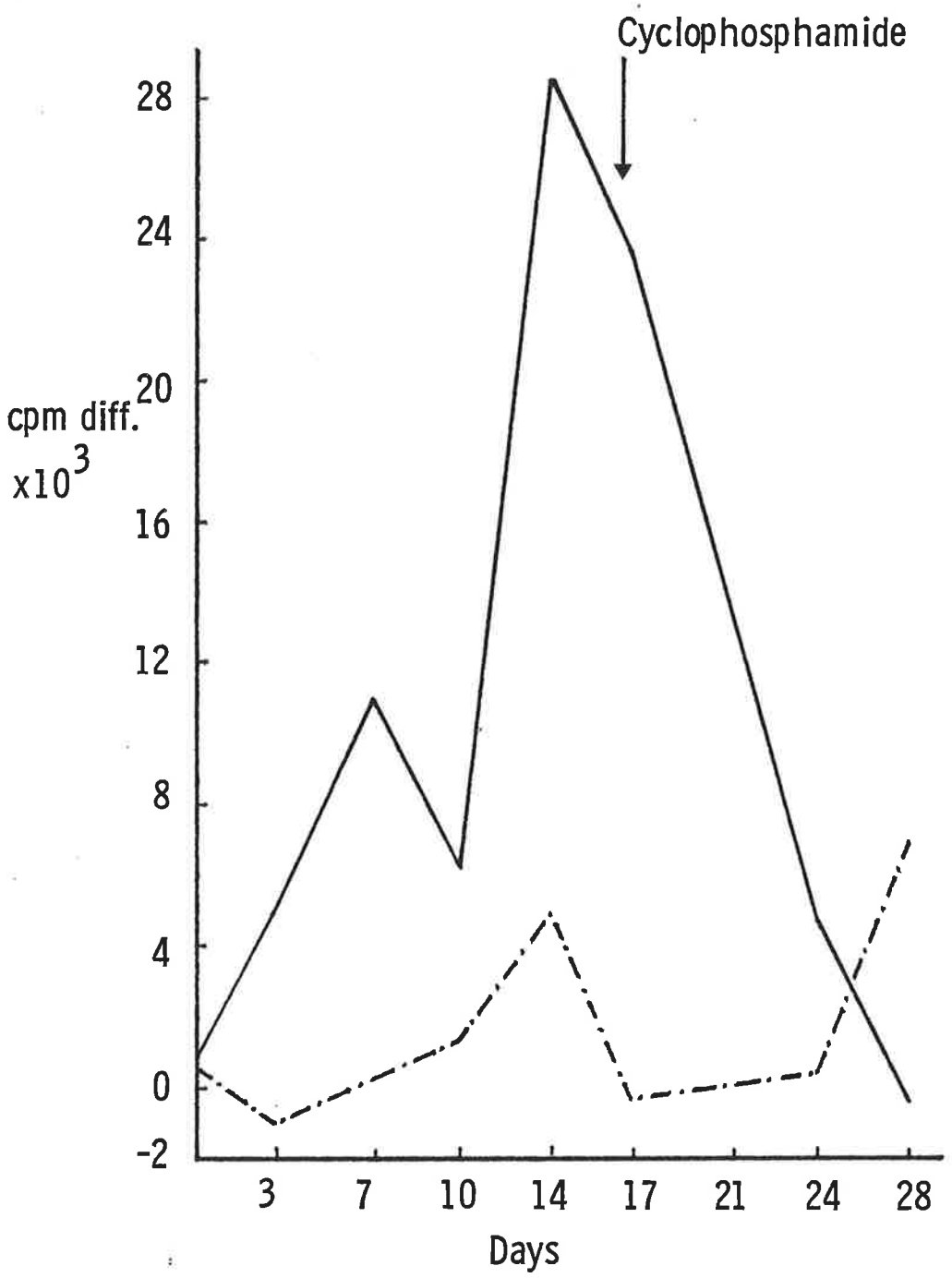


FIGURE 7.8 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH BCG ONLY 3rd. COURSE (Patient N. S.)

— PPD -·-·- CME

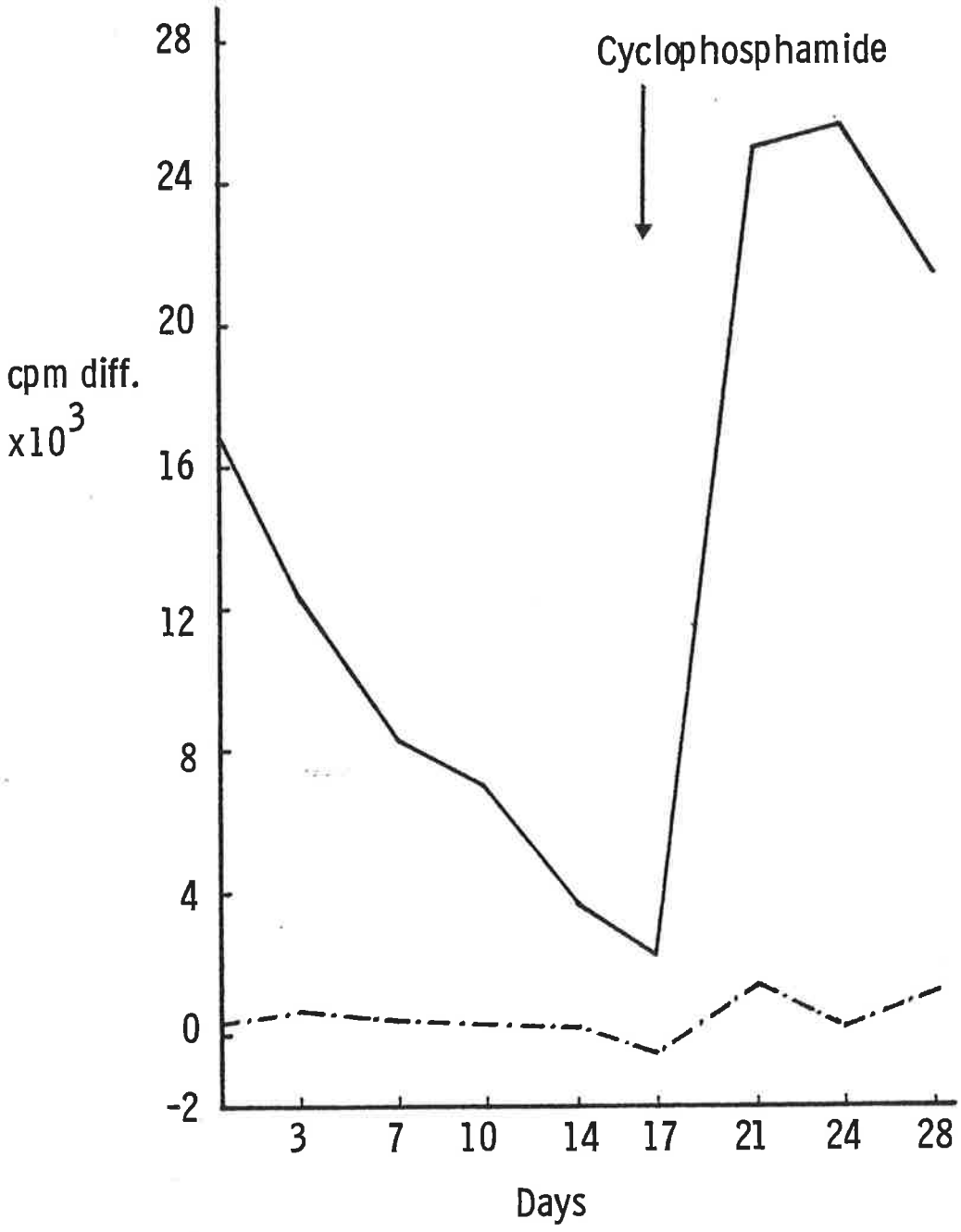


FIGURE 7.9 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH CELLS ONLY 5th. COURSE (Patient N. S.)

—— PPD -·-·- CME

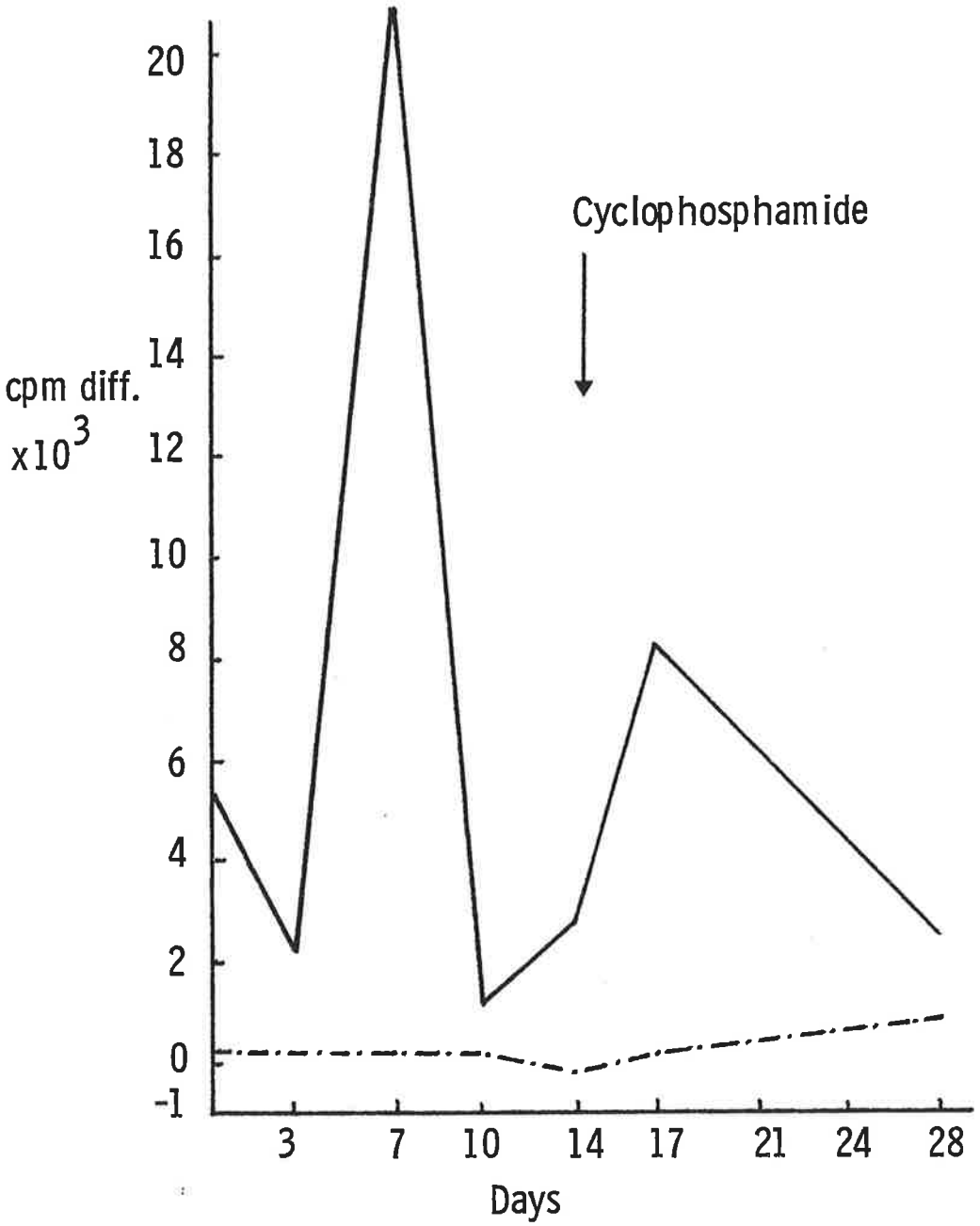


FIGURE 7.10 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH BCG ONLY 18th. COURSE (Patient H. L.)

—— PPD —·—· CME

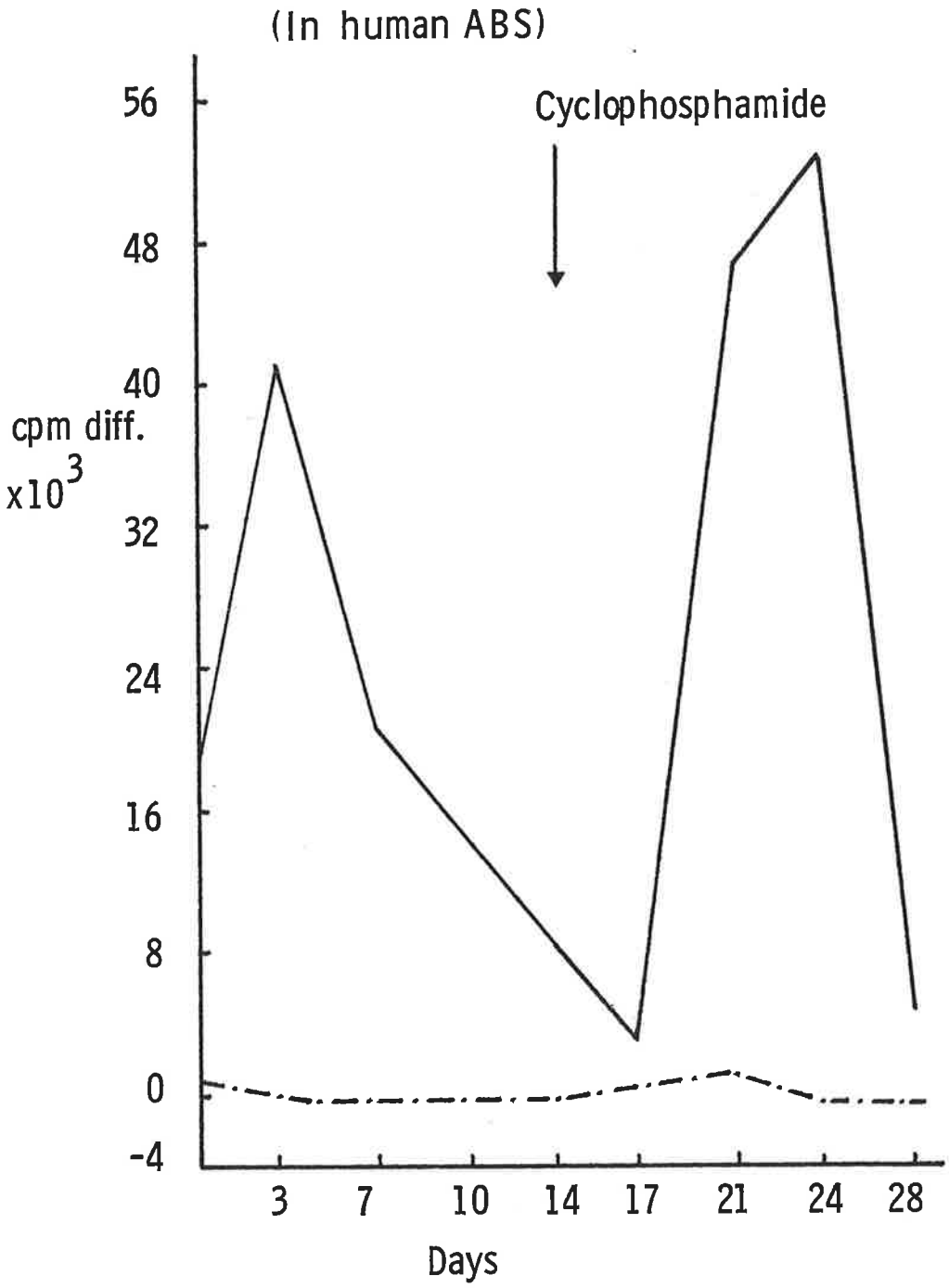


FIGURE 7.11 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH CELLS ONLY 24th. COURSE (Patient H. L.)

— PPD -·-·- CME

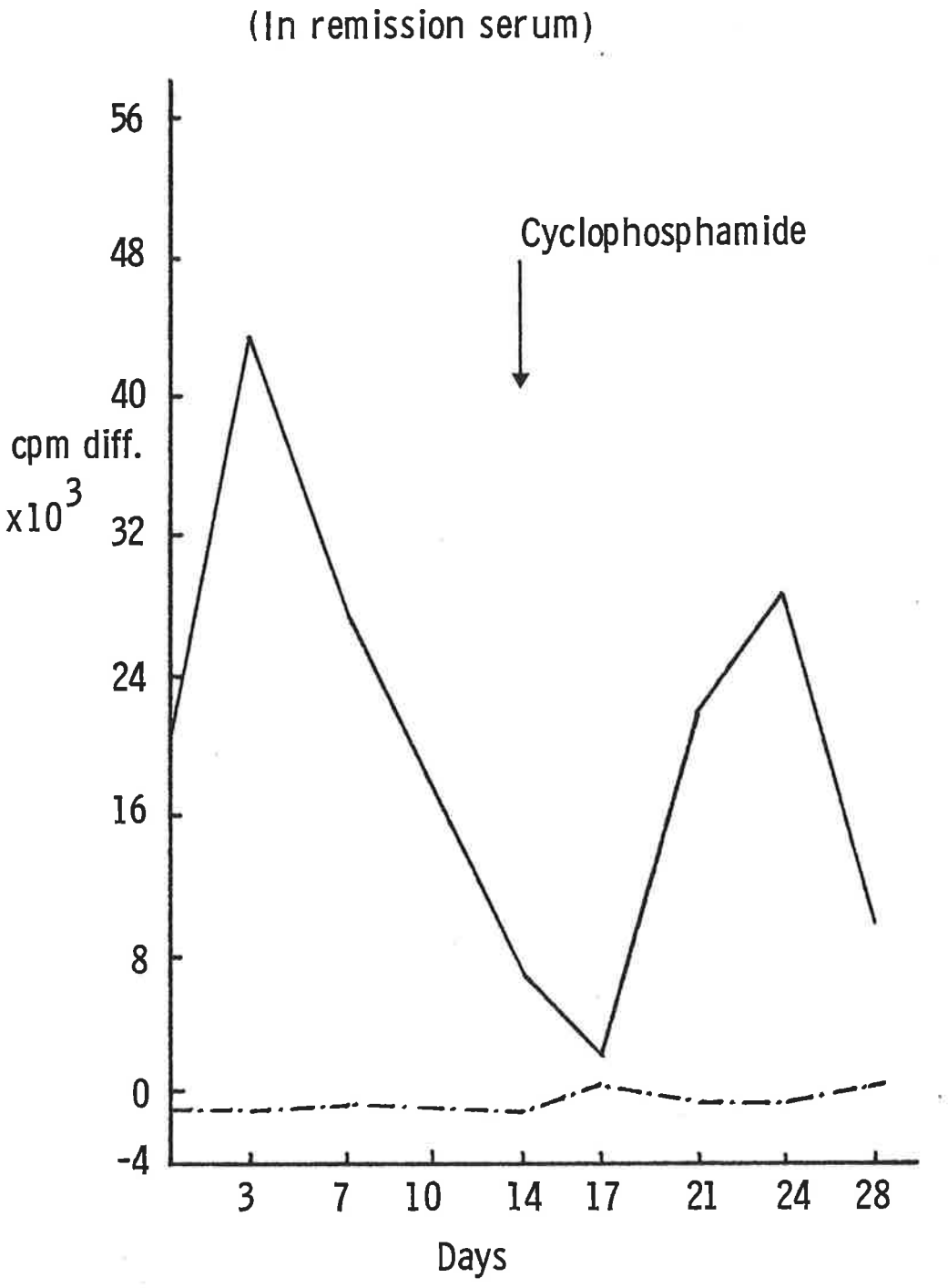


FIGURE 7.12 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH CELLS ONLY 24th. COURSE (Patient H. L.)

—— PPD —·—· CME

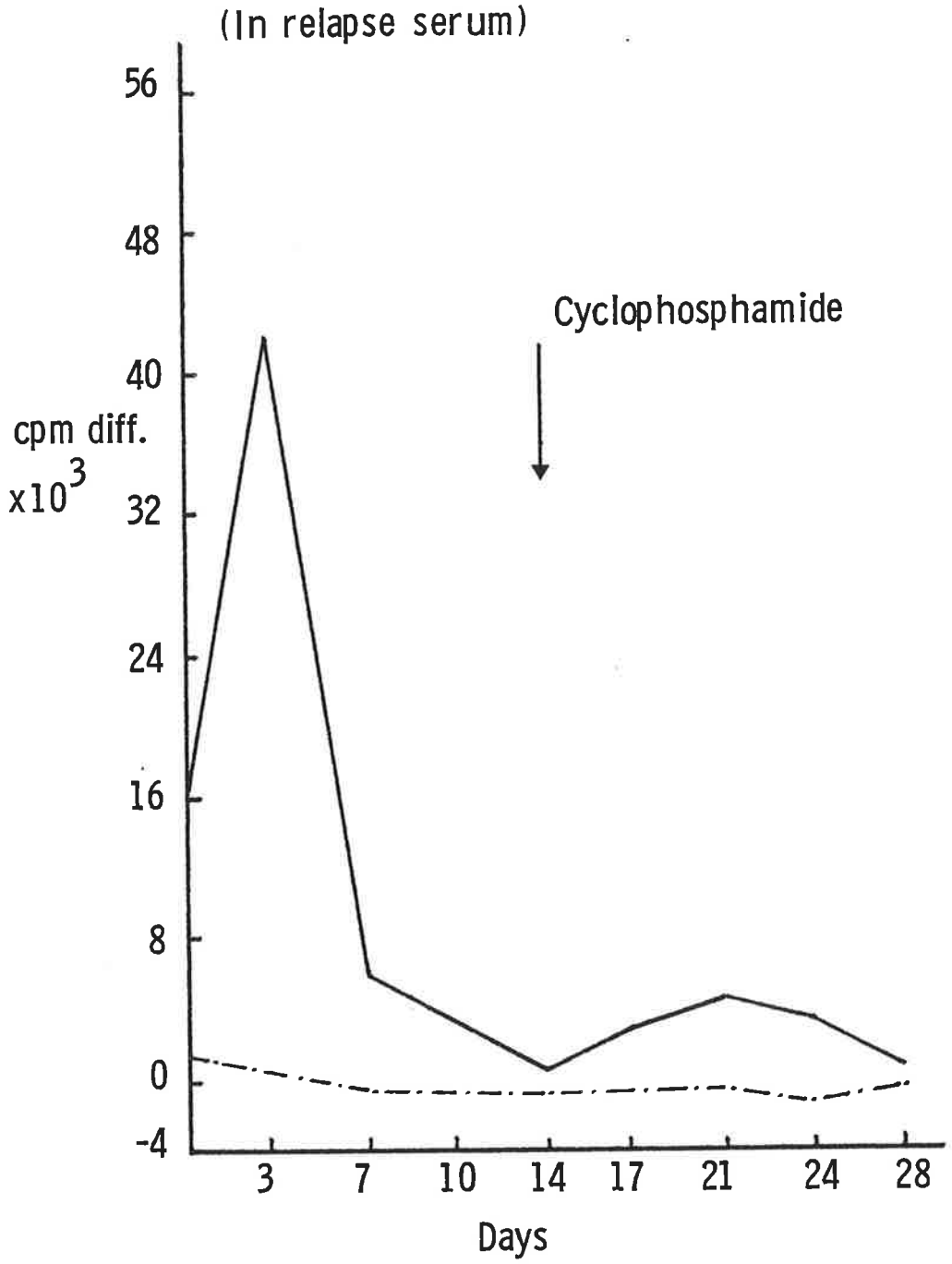


FIGURE 7.13 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH CELLS ONLY 24th. COURSE (Patient H. L.)

— PPD -·-·- CME

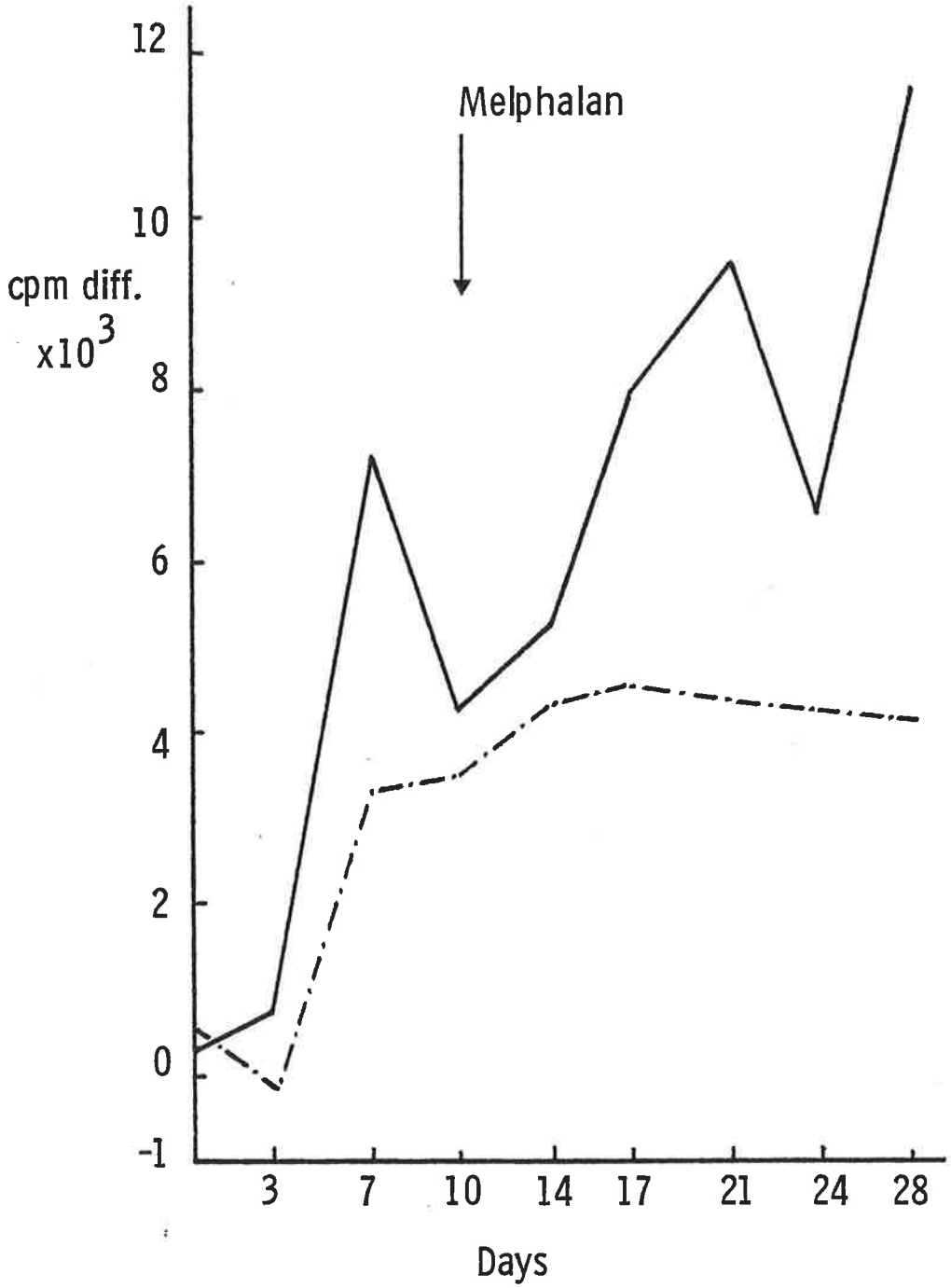


FIGURE 7.14 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH BCG AND CELLS 5th. COURSE (Patient M. G.)

—— PPD —·—· CME

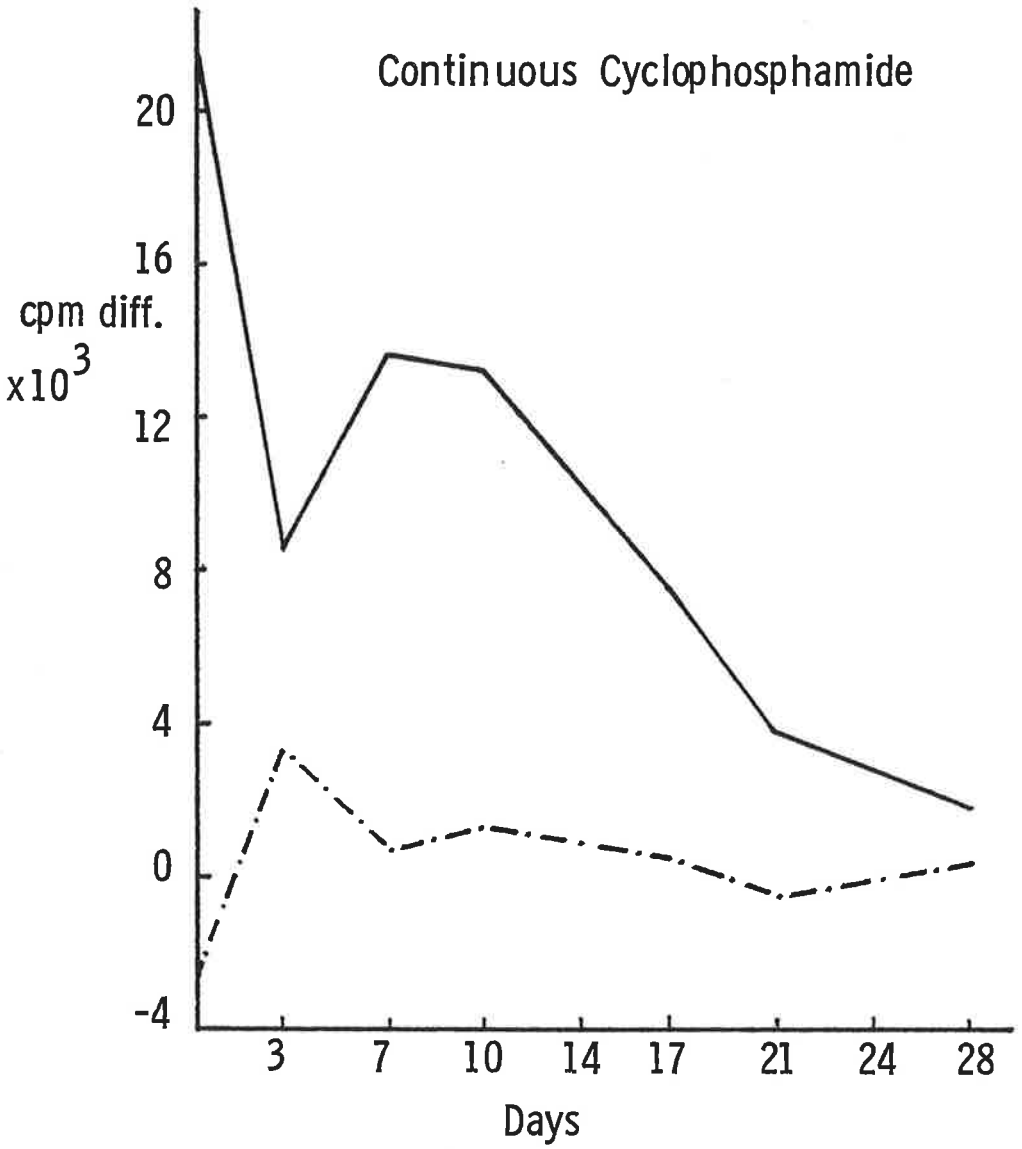


FIGURE 7.15 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH CELLS ONLY 1st. COURSE (Patient S.G.)

— PPD -·-·- CME

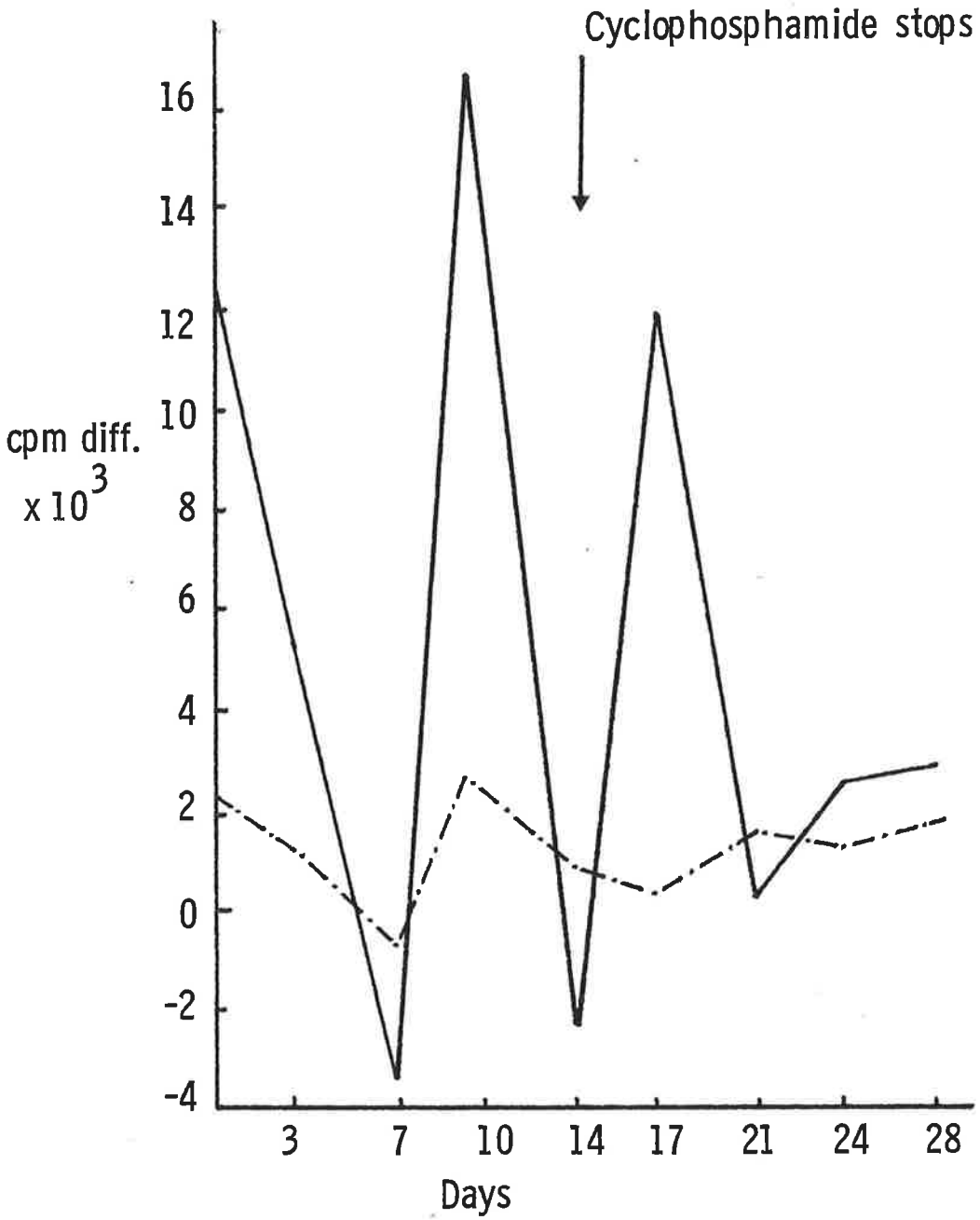


FIGURE 7.16 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH BCG ONLY 3rd. COURSE (Patient S.G.)

— PPD -·-·- CME

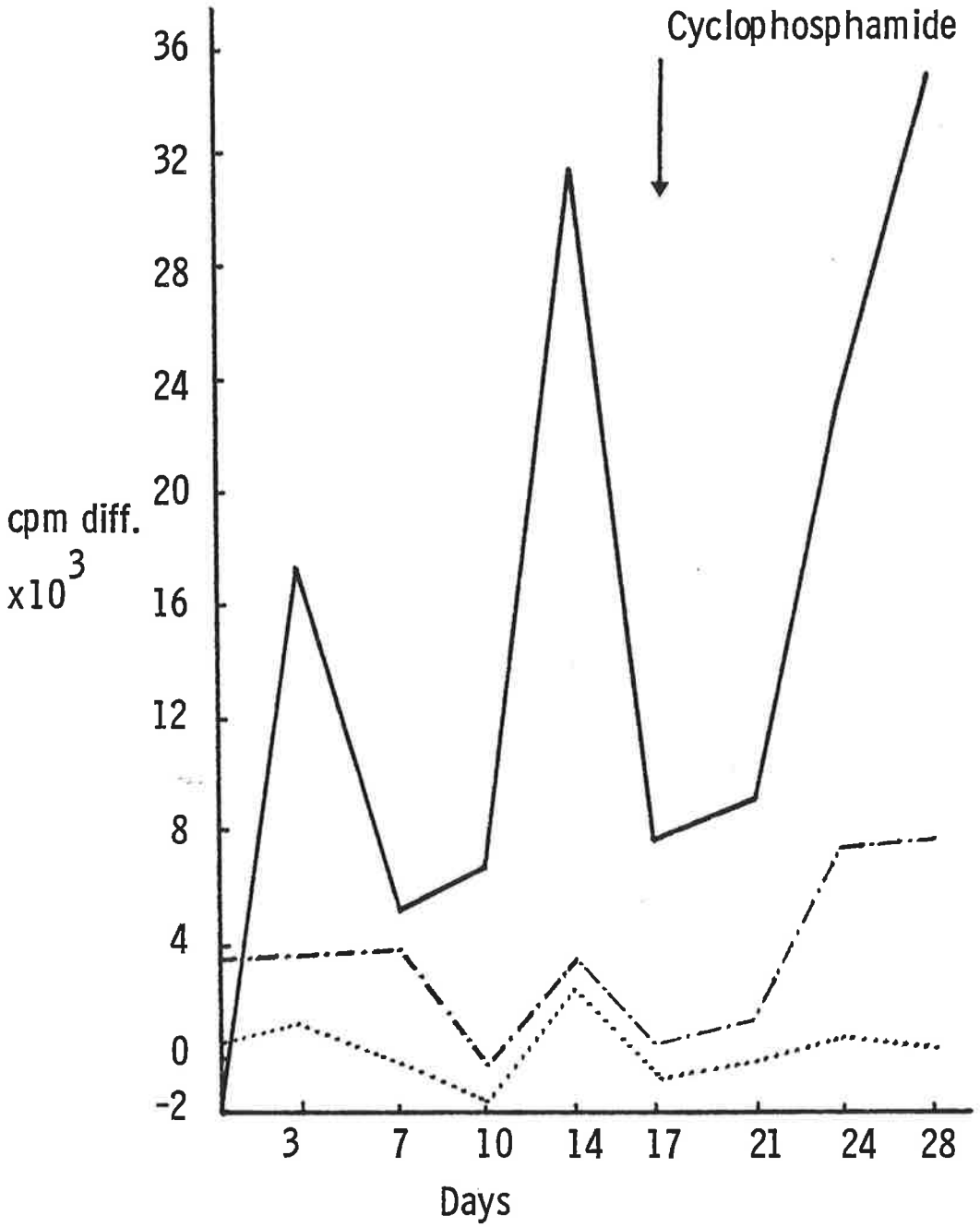


FIGURE 7.17 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH AUTOLOGOUS CELLS 24th. COURSE (Patient M.T.)

— PPD ····· Autologous CME -·-·-Allogeneic CME

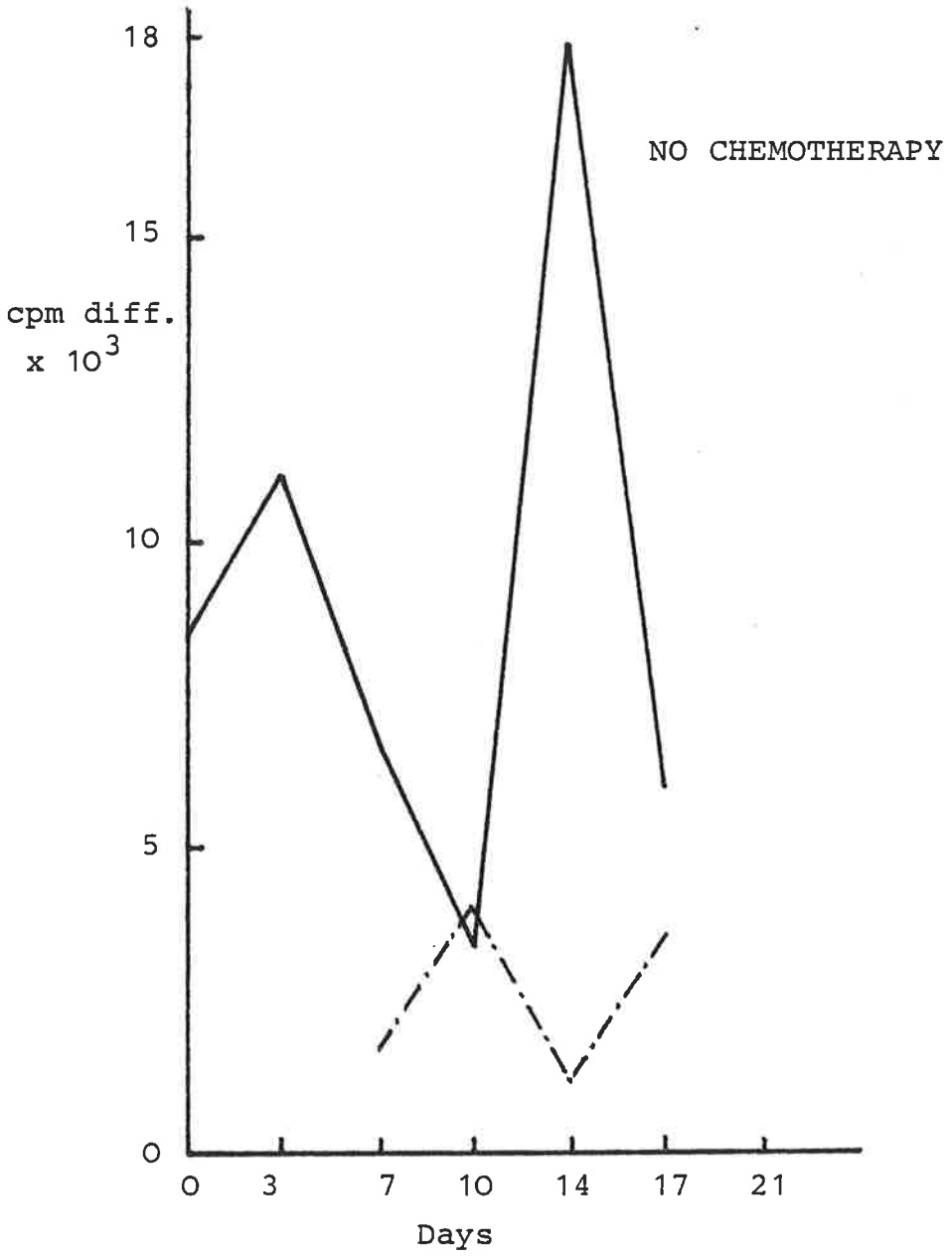


FIGURE 7.18 SERIAL BLASTOGENIC RESPONSES DURING IMMUNOTHERAPY WITH BCG AND CELLS 20th. COURSE (Patient D.H.)

——— PPD —·—· CME

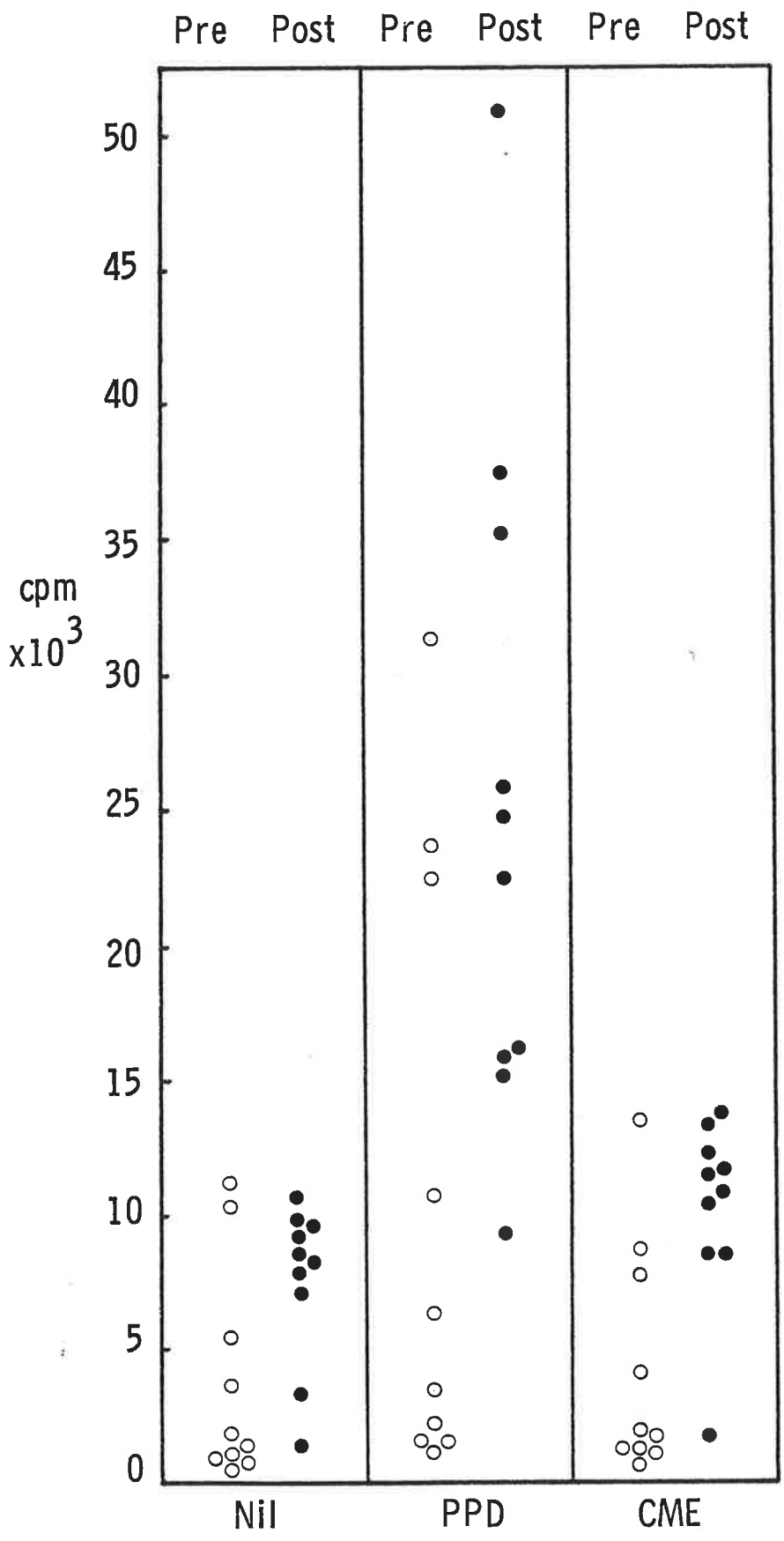


FIGURE 7.19 COMPARISON OF PRE- AND POST-IMMUNOTHERAPY BLASTOGENIC RESPONSES TO PPD AND OVARIAN TUMOUR CME

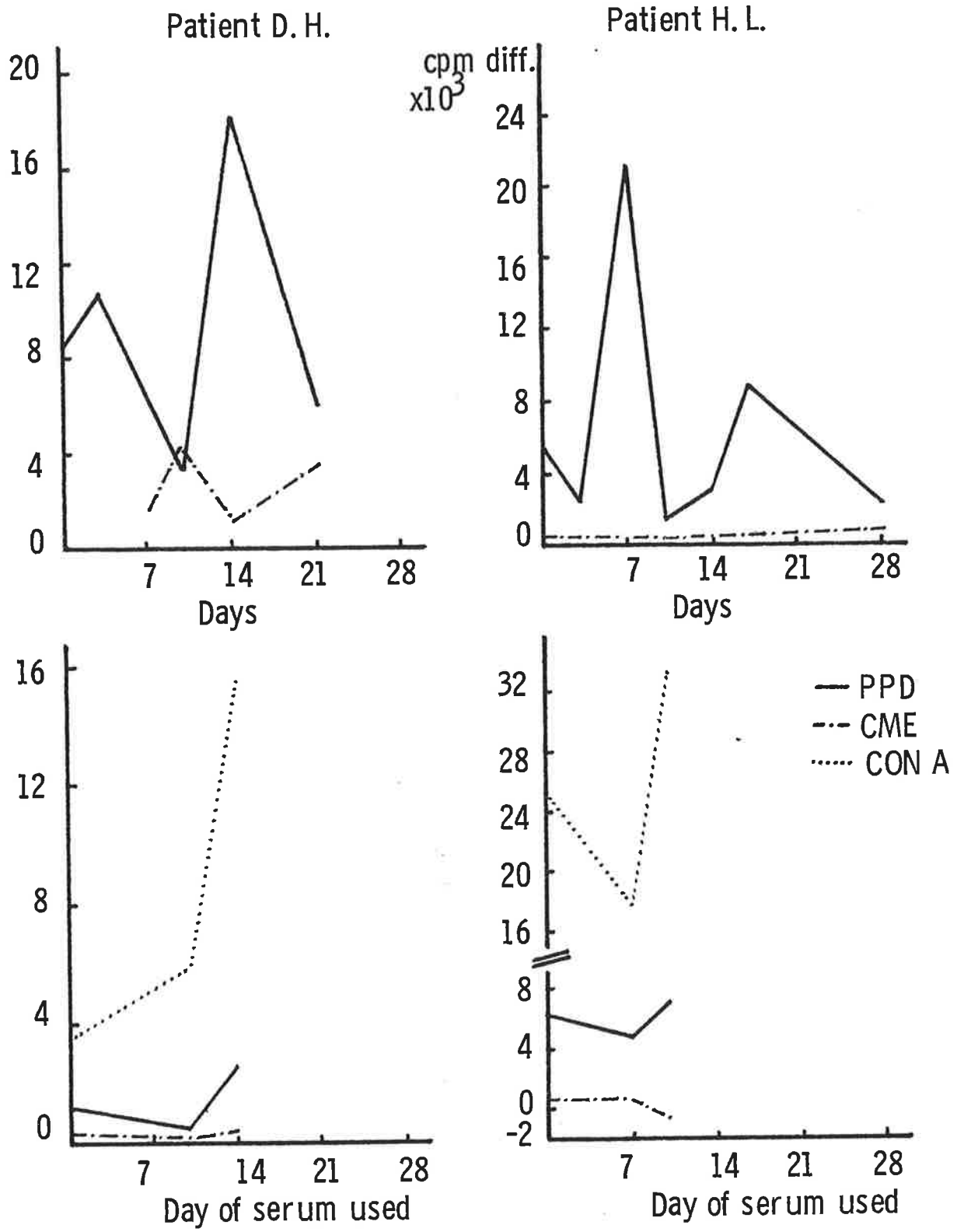


FIGURE 7.20 EFFECT OF IMMUNOTHERAPY SERUM ON BLASTOGENIC RESPONSES OF NORMAL LYMPHOCYTES

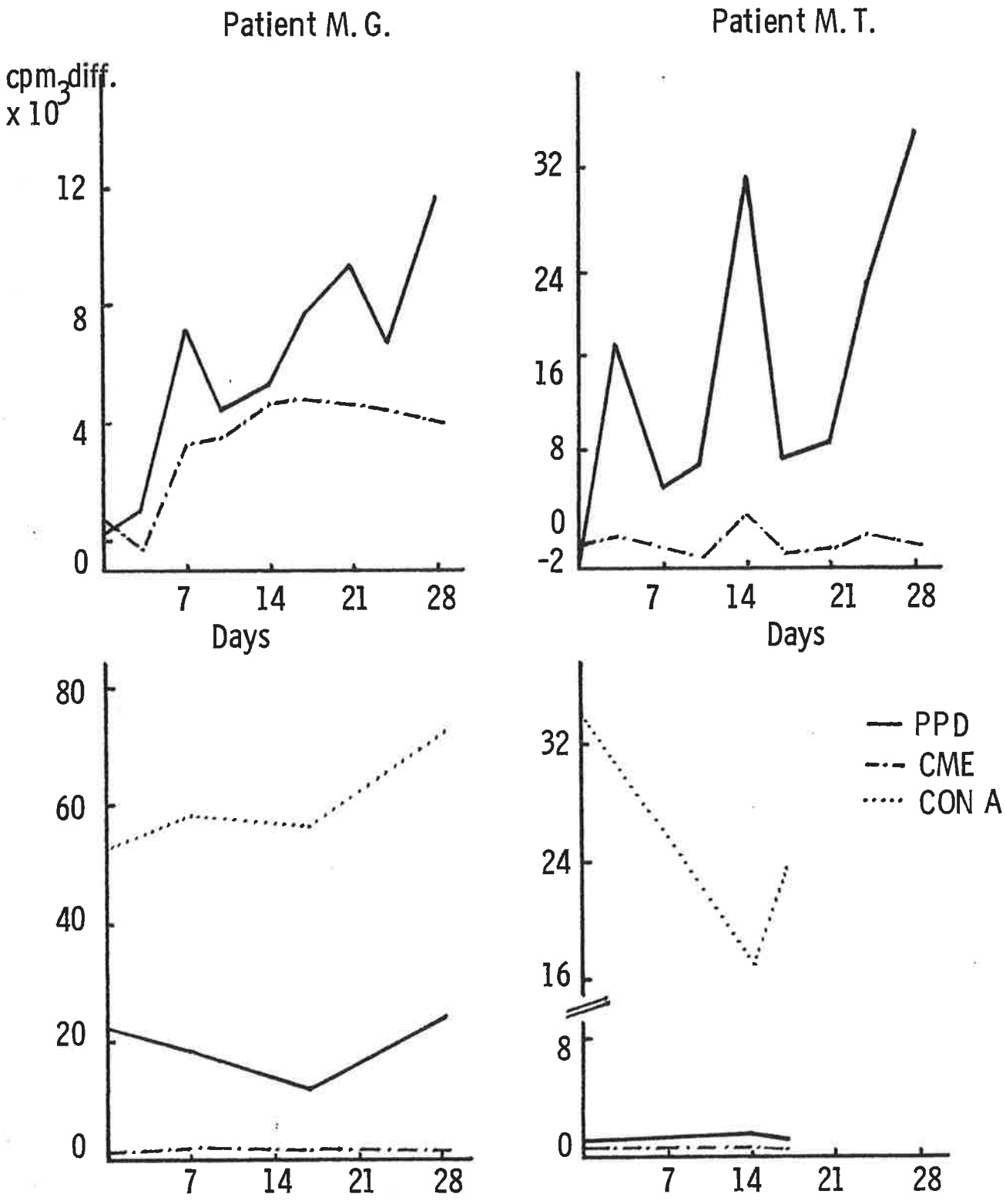


FIGURE 7.21 EFFECT OF IMMUNOTHERAPY SERUM ON BLASTOGENIC RESPONSES OF NORMAL LYMPHOCYTES

patient, the serum at this point does not necessarily cause stimulation of the normal lymphocytes and vice versa.

Patient DH: Responses in immunotherapy sera are all lower than in ABS(Table 7.7, Fig. 7.20). However, the Day 14 serum has a stimulatory effect on the lymphocyte responses to PPD and Con A when compared with Day O serum. This effect corresponds to potentiation of immune responses at Day 14 in the immunotherapy patient.

Patient HL: Again, the cpm of unstimulated control lymphocytes is lower in immunotherapy sera than in ABS, although PPD responses are higher. Mitogenic respones are also lower in ABS. Day 7 serum appeared to have an inhibitory effect compared with Day O or Day 14 sera on PPD and Con A responses (Fig. 7.20). This in no way corresponds to the immunotherapy pattern, where at Day 10 there is a marked drop in lymphocyte responses after a peak at Day 7.

Patient MG: Although nil counts are again lower in immunotherapy serum than ABS, PPD responses are greater in it, as are the Con A responses. The PPD pattern does not follow the immunotherapy pattern of responses, although the Con A values tend to increase accordingly (Fig. 7.21).

Patient MT: There is a marked suppression of PPD responses in all immunotherapy sera from this patient, and the reduced Con A value at Day 14 is the opposite to the peak in the patient's immunotherapy cycle at this time (Fig. 7.21).

The serum from 9 patients was assessed for Rheumatoid Factor (see Chapter 5). Of 85 samples from various parts of the immunotherapy cycle, only 5 samples were positive, and all weakly so. These samples occurred in 2 patients (DH and NS) both of whom were hyperimmunized to BCG.

CONCLUSIONS:

This Chapter confirms the findings of others that immunotherapy alters immunological parameters, however transiently. There is a tendency for the total number of lymphocytes to be increased in remission patients, whereas

relapse patients show gradually falling counts.It may be that immunotherapy does indeed protect the bone marrow from the effects of chemotherapy, and although anecdotal it was apparent that patients on immunotherapy tolerated their intravenous chemotherapy better than their counterparts not receiving immunotherapy. It appears however that this effect may only last as long as the tumour burden is not too great, and once the patient has active disease, then immunotherapy no longer has a therapeutic effect in maintaining lymphocyte counts in the face of chemotherapeutic immunosuppression. It also suggests that immunotherapy may be pointless in the face of chronic immunosuppression, and this view would be validated if the present MRC Trial shows that patients on the Triple chemotherapy do significantly better than those on chronic Cyclophosphamide.

The %E-RFC changes such that the $\%T_a$ is restored to control values, but the $\%T_t$ is still significantly reduced. Both values are similar to those in patients with static disease. Total cell counts are however, much lower than control values. This restoration of the active component of the T-cell population has been noticed by others (WYBRAN et al, 1973; LEVIN et al, 1975; ANTHONY et al, 1978).

In individual immunotherapy cycles there are not clearly defined patterns in the alteration of the % of T_a and T_t cells, but each pattern tends to follow the pattern of blastogenic responses for that patient, with a fall and rapid rebound after chemotherapy. However, there is no statistical correlation between the strength of the blastogenic responses and the T-cell values.

In immunotherapy cycles a number of consistent trends may be discussed. Firstly, the responsiveness to PPD in vitro increases with subsequent courses of immunotherapy and is often less in relapse patients than those with static disease. This may follow closely the in vivo decrease in skin responses to BCG. Secondly, there is often an initial decrease in the level of lymphocyte responses following immunotherapy, suggesting a specific decrease in the number

of sensitized lymphocytes; this may be because of lymph node sequestration of lymphoblasts. Thirdly, both PPD and CME responses are stimulated after BCG and cells. The rapid rise in PPD responses immediately after immunotherapy is similar to the response seen in animals given lyophilized BCG i.e. an initial antibody response (MACKANESS et al, 1973). Antigenic cross-reactivity between tumour cells and BCG is suggested by the ability of each to stimulate responses to the other, when given singly (BUCANA & HANNA 1974; MINDEN et al, 1974). Progressively greater responses to PPD occur with subsequent courses; responses to CME are always slight in comparison. This suggests that in these patients immunotherapy does not work by increasing the body's ability to recognise and respond to tumour-associated antigens.

In vivo there seems to be an association between the aggressiveness of the skin response to immunotherapy and survival. Cross-reactivity between these antigens is again suggested by the ability of tumour cells to cause flaring in old BCG lesions. Patients with active disease tend to get progressively weaker responses.

Changes in serum factors during immunotherapy do occur (VETTO et al, 1975) but their nature is obscure. The experiments described here are quite inconclusive and do not explain or even elucidate the nature of serum changes during immunotherapy, other to show that some form of change does occur. One postulate might be that immune complexes of anti-BCG and anti-tumour antibody form following immunotherapy and either suppress or stimulate the immune responsiveness of lymphocytes in vitro. However, the occurrence of Rheumatoid Factor positive serum in this series was slight.

The relationship between immunotherapy and chemotherapy is a delicate one. It was surprising to find that blastogenic responses continued to rise after chemotherapy, and relates to both the effect of Cyclophosphamide (and

perhaps other chemotherapeutic agents) on lymphocyte sub-populations and suppressor cells (POLAK & TURK 1974), as well as the BCG-"priming" effect on the bone marrow (WOLMARK et al, 1974; DIMITROV et al, 1975). However, at other times there is prolonged immunosuppression following chemotherapy and this may represent the undesirable effect of BCG in potentiating immunsuppression sometimes, depending on when it is given in relation to the chemotherapy (MATHÉ et al, 1975; SPARKS et al, 1975). In this context it is interesting to note that lymphocytes cultured in "relapse" serum were prevented from showing an in vitro increase in blastogenesis after chemotherapy. It may be that in relapse patients, immunotherapy does not protect against the effects of chemotherapy, and the immunosuppression which follows is even more detrimental to the patient. Since it was not felt to be ethical to assay patients in clear relapse (as opposed to initial evidence of re-activating disease), it has not been possible to test the above hypothesis.

Monitoring of immunological parameters is most essential in immunotherapy patients, not only to indicate that the procedure is safe, but to ascertain whether it is having a stimulatory effect. How it actually works to either prolong survival or improve the quality of life of the patient with cancer (ANTHONY et al, 1978) is not clear. The subtleties of interaction between chemotherapy and immuno-therapy also need to be more extensively investigated. The monitoring described here shows that immunopotentiation does occur following inoculation with BCG and/or tumour cells, but clearly the influence of chemotherapy on these effects is most important. In this regard, it will be interesting to see whether patients on continuous Cyclophosphamide in the current Trial do worse than patients on pulsed chemo-therapy.

TABLE 7.2 LYMPHOCYTE COUNTS PRE- AND DURING IMMUNOTHERAPY.

	Pre-I/T Values			During I/T Values		
Pt.	WCC	% L.	Total L.	WCC	% L.	Total L.
PATIENTS IN REMISSION, test done 2 weeks after chemotherapy						
HL	8.8	45	3960	6.3	27	1701
MT	10.3	5	515	5.6	20	1120
WN	5.6	8	448	5.7	15	855
BP	11.5	3	345	3.9	29	1131
NS	3.1	29	899	4.4	34	1496
Medians			515			1131
PATIENTS IN REMISSION, test done during continuous Cyclo.						
SG	4.8	32	1536	4.4	15	660
CB	9.4	15	1410	5.3	6	318
PATIENT IN REMISSION, 2 weeks after MRC Triple Chemotherapy						
LP	4.6	34	1564	6.5	17	1105
PATIENTS IN RELAPSE, patients on no chemotherapy						
DH	3.8	17	646	4.3	29	1247
IW	12.1	26	3146	11.2	38	4256
PATIENTS IN RELAPSE, test done 2 weeks after chemotherapy						
SB	13.3	7	931	6.9	9	621
IC	10.5	31	3255	4.9	17	833
AH	9.4	13	1222	3.6	17	612
VD	7.8	17	1326	5.3	28	1484
MG	9.2	9	828	3.2	25	800
RF	7.3	20	1460	3.9	6	234
Medians			1274			816

9 of the 16 values in this table are decreased during immunotherapy, and on rank correlation, $p < 0.02$

Medians (in toto)	1274	980

TABLE 7.3 BLASTOGENIC RESPONSES TO PPD AND OVARIAN TUMOUR CME CORRELATED WITH %E-RFC.

Pt.		cpm.	S.I.	$\%T_a$	$\%T_t$
NS	Nil	1305		25	55
	PPD	5166	4.0		
	CME	1551	1.2		
IC§	Nil	1219		13	56
	PPD	2562	2.1		
	CME	1498	1.2		
SG§	Nil	2985		24	65
	PPD	16812	5.6		
	CME	1050	1.4		
MG§	Nil	4248		20	64
	PPD	8664	2.0		
	CME	7635	1.8		
HL§	Nil	2454		31	43
	PPD	54792	22.3		
	CME	2235	0.9		
DH§	Nil	10293		16	46
	PPD	82029	8.0		
	CME	11298	1.1		
MT§	Nil	5140		12	43
	PPD	33946	6.6		
	CME	6060	1.2		
WN§	Nil	1671		19	43
	PPD	4854	2.9		
	CME	2139	1.3		
LP	-	-	-	17	28
IW§	Nil	10380		13	43
	PPD	16818	1.6		
	CME	19898	1.9		
VD§	-	-	-	10	45

§ = Relapse at time of testing

Correlation - none.

TABLE 7.4 EFFECT OF IMMUNOTHERAPY ON VARIOUS PARAMETERS OF LYMPHOCYTE FUNCTION.

Pt.	Day	cpm		$\%T_a$	$\%T_t$	WCC	%L	Abs. L.	Abs.T_a.	Abs.T_t.
MG (7.2)	0	Nil	1092	-	-	-	-			
		PPD	1527							
		CME	1545							
	3	Nil	1287	14	51	-	-			
		PPD	1902							
		CME	1242							
	7	Nil	3304	17	55	-	-			
		PPD	15615							
		CME	11613							
	10	Nil	4248	20	64	-	-			
		PPD	8664							
		CME	7635							
	14	Nil	4866	6	66	-	-			
		PPD	9987							
		CME	9656							
	17	Nil	4341	11	51	-	-			
		PPD	12168							
		CME	9177							
	21	Nil	3771	5	49	-	-			
		PPD	13278							
		CME	8514							
	24	Nil	3582	-	54	-	-			
		PPD	10257							
		CME	8301							
	28	Nil	3147	-	-	-	-			
		PPD	14500							
		CME	7696							

TABLE 7.4 (contd.)

Pt.	Day	cpm		$\%T_a$	$\%T_t$	WCC	%L	Abs. L.	Abs.T_a.	Abs.T_t.
HL (7.3)	0	Nil	3738	12	56	8.6	8	688	83	385
		PPD	22522							
		CME	4120							
	3	Nil	9732	12	46	7.2	15	1080	133	500
		PPD	50847							
		CME	8781							
	7	Nil	3210	13	26	8.7	14	1218	158	311
		PPD	23349							
		CME	2577							
	14	Nil	3537	-	-	-	-			
		PPD	11607							
		CME	2904							
	17	Nil	2397	2	11	6.0	22	1320	20	139
		PPD	5439							
		CME	2337							
	21	Nil	4137	-	-	-	-			
		PPD	50562							
		CME	4530							
	24	Nil	2454	31	43	5.7	13	741	227	321
		PPD	54792							
		CME	2235							
	28	Nil	2136	-	-	-	-			
		PPD	7113							
		CME	1632							
NS (7.4)	0	Nil	2188	22	51	3.1	29	899	198	456
		PPD	18826							
		CME	2214							
	3	Nil	3896	-	-	-	-			
		PPD	16247							
		CME	4860							

TABLE 7.4 (contd.)

Pt.	Day	cpm		$\%T_a$	$\%T_t$	WCC	%L	Abs. L.	Abs.T_a.	Abs.T_t.
NS (contd)	7	Nil	3888	19	45	4.0	20	800	148	362
		PPD	12412							
		CME	4780							
	10	Nil	4320	-	-	-	-			
		PPD	11178							
		CME	4221							
	14	Nil	1305	25	55	3.9	34	1326	328	729
		PPD	5166							
		CME	1551							
	17	Nil	2523	19	39	3.8	25	950	182	366
		PPD	5283							
		CME	2358							
	21	Nil	10668	36	63	2.8	28	784	278	490
		PPD	35148							
		CME	12279							
	24	Nil	5700	34	54	3.8	25	950	318	508
		PPD	31206							
		CME	5697							
	28	Nil	9786	11	56	-	-			
		PPD	31287							
		CME	10926							

TABLE 7.5 PRE- AND PEAK-IMMUNOTHERAPY BLASTOGENIC RESPONSES TO PPD AND OVARIAN TUMOUR CME.

Peak	Day O	cpm Δ	Peak	Day O	cpm Δ	Peak	Day O	cpm Δ
	NIL			PPD			CME	
9294	1281	8013	9258	1266	7992	10400	1377	9023
8583	1374	7209	25716	1587	24129	8475	1326	7149
7950	1281	6669	37596	2178	35418	13395	1431	11964
10668	2188	8488	35148	10826	24322	12279	2214	10065
1474	843	631	22632	6195	16437	1879	1314	565
9732	3738	5994	50847	22522	28325	8781	4120	4661
3304	1092	2212	15615	1527	14088	11613	1545	10068
7188	10239	-3051	15837	31317	-15444	10515	7932	2583
8326	11260	-2934	24980	23502	1478	11292	13416	-2124
9398	5392	4006	15016	3560	11456	13472	8726	4746

cpm Δ = cpm difference

Wilcoxon Signed Rank Test:

T = -7, $p < 0.05$ T = -5, $p = 0.02$ T = -2, $p < 0.01$

TABLE 7.6 BLASTOGENIC RESPONSES THROUGHOUT IMMUNOTHERAPY CYCLES TO PPD AND OVARIAN TUMOUR CME.

Pt.	Day: 0	3	7	10	14	17	21	24	28
NS (Fig. 7.6):	cpm values								
Nil	2199	3152	2784	-	9294	-	3372	-	9801
PPD	5376	3201	3675		9258		2604		12510
CME	2811	3750	3219		10400		3171		9633
(Fig. 7.7)									
Nil	1374	-	1197	6855	3178	4239	8583	2512	1281
PPD	1587		1383	16488	10612	8040	25716	5322	2178
CME	1326		1422	6987	4478	5220	8475	1956	1431
(Fig. 7.8)									
Nil	1281	4059	5112	3872	7950	9782	-	11685	10438
PPD	2178	9039	15588	10030	37596	33634		15993	9992
CME	1431	2634	5292	5808	13395	8980		12201	17586
(Fig. 7.9)									
Nil	2188	3896	3888	4320	1305	2523	10668	5700	9786
PPD	18826	16247	12412	11178	5166	5283	35148	31206	31287
CME	2214	4860	4780	4221	1551	2358	12279	5697	10926
HL (Fig. 7.10)									
Nil	843	450	1674	476	1080	1032	-	-	1044
PPD	6195	2607	22632	1857	3795	9362			3459
CME	1314	543	1879	711	951	1364			1626
(Fig. 7.11)									
Nil	3738	9732	3210	-	3537	2397	4137	2454	2136
PPD	22522	50847	23349		11607	5439	50562	54792	7113
CME	4120	8781	2577		2904	2337	4530	2235	1632

TABLE 7.6 (contd.)

Pt.	Day: 0	3	7	10	14	17	21	24	28
HL (Fig. 7.12)									
Nil	4815	6438	2913	-	3306	1596	3417	1986	1737
PPD	25581	49725	30171		10290	3996	26916	30462	11829
CME	3669	5787	2445		2805	1611	3228	1944	1857
(Fig. 7.13)									
Nil	3009	3012	3081	-	2724	1446	2571	2007	1662
PPD	19161	46674	8751		3171	4689	6930	5946	1977
CME	4884	3729	2436		2469	-	2160	1683	1509
MG (Fig. 7.14)									
Nil	1092	1287	8304	4248	4866	4341	3771	3582	3147
PPD	1527	1902	15615	8664	9987	12168	13278	10257	14500
CME	1545	1242	11613	7635	9656	9177	8514	8301	7696
SG (Fig. 7.15)									
Nil	10239	7188	6820	8373	-	5582	4332	-	2488
PPD	31317	15873	20364	21699		13266	8076		4394
CME	7932	10515	6874	9468		5998	3723		2722
(Fig. 7.16)									
Nil	11260	6088	6676	8326	6230	4990	6542	6036	3864
PPD	23502	11368	3560	24980	4080	16924	6758	8450	6686
CME	13416	7318	5832	11292	7088	5432	8030	7214	5604
MT (Fig. 7.17)									
Nil	5392	2268	9398	3382	6002	7968	4558	6470	2728
PPD	3560	19647	15016	10100	37396	15921	13382	29368	37018
Al.CME	8726	-	13472	3020	9598	8188	5738	13131	9572
Au.CME	6060	3591	8970	1491	8460	6693	4455	8361	3020

TABLE 7.6 (contd).

Pt.	Day: 0	3	7	10	14	17	21	24	28
DH (Fig. 7.18)									
Nil	1576	1734	3312	3816	2070	-	3006	-	-
PPD	10112	12480	9848	7254	20061		8994		
CME	-	-	4902	7617	3252		6507		

Note: these figures represent cpm. value, whereas the graphs are drawn from the cpm. difference.

TABLE 7.7 BLASTOGENIC RESPONSES OF NORMAL LYMPHOCYTES TO PPD, OVARIAN TUMOUR CME, AND CON A WHEN INCUBATED IN IMMUNOTHERAPY SERA TAKEN FROM DIFFERENT TIMES OF THE CYCLE.

cpm in ABS	
Nil	2079
PPD	5706
CME	2475
Con A	58665

cpm in Immunotherapy sera

Pt. DH:	Day 0	Day 10	Day 14
Nil	1056	1029	1044
PPD	2079	1482	3639
CME	1170	1122	1176
Con A	4626	6855	16461

Pt. HL:	Day 0	Day 7	Day 10
Nil	1488	1344	2541
PPD	8016	6387	9456
CME	1590	1377	2142
Con A	26838	18390	35907

Pt. MG	Day 0	Day 7	Day 17	Day 28
Nil	918	867	1074	1161
PPD	22731	18795	13140	25101
CME	1284	1605	1500	1704
Con A	53457	59694	57159	73536

Pt. MT:	Day 0	Day 14	Day 17
Nil	870	876	1086
PPD	1842	2124	2019
CME	903	1128	1194
Con A	35103	17538	24504

CHAPTER 8. ECTOPIC HORMONE SYNTHESIS AND TUMOUR MARKERS IN HUMAN CANCER.

In recent years much attention has focused on the ectopic production by human tumours of various hormones, enzymes and fetal antigens. Substances such as Adrenocorticotropic hormone (ACTH) and Parathyroid hormone (PTH), because of their potent clinical effects, were among the first substances to be investigated and many of the criteria used to establish such secretion have been set out in an excellent review by REES (1975). Abnormal hormone levels may not always indicate ectopic production, and clearly this is a lot easier to show with placental hormones when found in men or non-pregnant women, than with substances such as ACTH which may be coincidentally present with Cushing's Disease, or may be produced as part of the stress of malignancy.

Such hormones may also be demonstrated in tumour tissues as well as serum, by radioimmunoassay (RIA) or direct immunofluorescent studies. In vivo secretion of the hormone may be possible (TASHJIAN et al, 1973), but is difficult and depends on the successful cloning of the malignant cells.

The true incidence of this phenomenon is unknown since ectopic hormone production may be undetected if it does not lead to a clinical effect, or the appropriate feedback mechanisms may work for some time and so hide its presence, as with ACTH. It has been suggested that there is a clear association between various hormones and a particular histological type of tumour as with ACTH secretion from oat-cell tumours of the lung (OMENN & WILKINS, 1970). However, the finding is inconstant.

The interest in all these substances lies in the hope that they may be exploited as tumour markers. There are three main areas of clinical application:

(i) as a screen for cancer in high-risk populations e.g. smokers

(ii) to aid in the differential diagnosis of neoplastic disorders

(iii) to monitor the efficacy of treatment and the development of recurrence; this is particularly important where minimal residual disease following surgery can not be easily followed clinically, as in carcinoma of the ovary

There is as yet no substance which fulfils all these hopes, and with the exception of human chorionic gonadotropin (HCG) in choriocarcinoma and mono-clonal gamma-globulins in myelomatosis, there are no clinical indicators which correlate with tumour mass. Strictly speaking, of course, HCG is not ectopically produced in this situation. But the point to be made is that attempts to use these substances for screening purposes seem to have failed, either because their production is a fortuitous finding in individual cases (as with ACTH), or because they are also produced in non-neoplastic states (as is CEA).

The most plausible theory to explain this phenomenon is the "Derepression Hypothesis" (HOBBS & MILLER 1966) which argues that all cells are totipotential and carry genetic material in a latent and repressed state once they have differentiated. The malignant process results in derepression of this genetic material with resultant production of substances not normally associated functionally with that cell. The weakness of this theory is that the ectopic hormones produced should be identical with that produced normally, and this is not always the case (ROOF et al, 1971). However, it is endorsed by the finding in humans of the Regan isoenzyme of alkaline phosphatase (ALP) in patients with alcoholic cirrhosis, ulcerative colitis, and familial polyposis, all diseases with a known cancer diathesis (NATHANSON & FISHMAN 1971).

This Chapter is mainly concerned with placental hormone production by tumours, but other substances such as enzymes and fetal proteins are included for completeness.

8.1 BIOCHEMICAL MARKERS.

Many biochemical markers, including ferritin, acute phase reactant proteins, polyamines and prostaglandins have been studied in cancer. In one large study of women with breast cancer (COOMBES et al, 1977), a number of parameters were elevated in more than 50% of women with metastases, and it was suggested that these tests should be used prognostically. Lactic dehydrogenase (LDH) might be expected to be elevated in malignant tissue with increased glycolytic activity, although it has been found elevated in pre-invasive cervical carcinoma (LATNER et al, 1966). In ovarian cancer the reports are conflicting (AWAIS 1973; SCHWARTZ et al, 1975).

8.2 ONCO-FETAL MARKERS.

(i) Carcinoembryonic Antigen (CEA).

This glycoprotein was first described by GOLD & FREEDMAN (1965) after they had concentrated it from 12 colonic carcinomas. An immunologically identical substance was found in the fetal gut, liver and pancreas between 2 to 6 months of gestation. Further investigation suggests that CEA is a family of related glycoproteins with a wide range of molecular weights and variable structure; presumably it is this which accounts for its molecular heterogeneity (ALPERT 1978).

It is not thought to be antigenic either in vitro (LEJTENYI et al, 1971) or in vivo (HOLLINSHEAD et al, 1970), although GOLD (1967) has demonstrated antibodies to CEA in patients with gastro-intestinal carcinoma and pregnant women. Presumably purification may alter the "antigenicity" of the molecule.

It is now established that CEA is neither endodermal- nor tumour-specific. It has been detected by RIA in the serum of patients with a wide variety of tumours (LO GERFO et al, 1971; LAURENCE et al, 1972; REYNOSO et al, 1972). It is also present in meconium, colonic polyps, normal adult faeces, a wide range of inflammatory disorders including diverticulitis and Crohn's Disease, smokers

and normal serum (MOORE et al, 1971; CHU et al, 1972).

LAURENCE & NEVILLE (1972) claim that levels in excess of 40ng/ml are diagnostic of malignancy, and this therefore limits its usefulness as a diagnostic tool since many benign conditions are associated with levels between 2.5 and 40ng /ml, yet many workers take the upper limit of normal as 2.5ng/ml. It has been most commonly used as an adjunct to monitor therapy in gastro-intestinal cancer, but may be more useful in urothelial carcinoma to screen at-risk populations in the chemical industry, since the number of false positives is very low (HALL et al, 1972).

In gynaecological malignancy CEA estimations have not generally been successful either diagnostically or as a post-surgical aid to diagnose recurrence (see Table 8.1). However, KHOO & MACKAY (1976) found good correlations between CEA levels and response to therapy in 109 women with various gynaecological malignancies. It has been suggested that CEA may be more elevated in women with mucinous ovarian tumours, and can therefore be used diagnostically in these cases (MARCHAND et al, 1975; RUTANEN et al, 1978). Most other investigators would agree that there is a limited place for it in follow-up, and it may be more useful in cases of localized disease (e.g. endometrial carcinoma) than ovarian carcinoma which is usually advanced at the time of diagnosis (VAN NAGELL et al, 1975).

(ii) Alpha feto-protein (AFP).

ABELEV et al (1963) first described an abnormal protein synthesized by chemically induced hepatoma in mice which was antigenically similar to an $alpha_1$-globulin in neonatal and embryonic mouse serum, but absent from adult mouse serum. TATARINOV (1965) then described its presence in the serum of patients with primary hepatoma as well as the human fetus.

Like CEA, it has been found in normal serum (RUOSLAHTI & SEPPÄLÄ 1971). Apart from the hepatoma where increased levels may be present in up to 80% of patients, AFP has also been detected in gastric and prostatic carcinoma (MEHLMAN et al, 1971) and ovarian adenocarcinoma (SEPPÄLÄ

et al, 1975). Its main use in gynaecology is in the follow-up of patients treated for endodermal sinus tumour, where its presence is pathognomonic of the tumour (WILKINSON et al, 1973).

It has been suggested that it allow tumours to escape immunological destruction because in vitro it has an immuno-suppressive effect and its administration to animals will stimulate tumour growth (TOMASI et al, 1975).

8.3 TROPHOBLAST-SPECIFIC HORMONES.

The value of pregnancy-specific hormones is that they are not found in normal non-pregnant subjects and can therefore be exploited as "tumour-markers" early in any neoplastic disease where they are present. They are also not subject to the normal feed-back mechanisms that exist for other hormones in the body and so shield early detection. Furthermore, unlike CEA and AFP, they are not present in inflammatory conditions. Originally they were measured in biological assays, but since the advent of RIA's they can now be measured in minute quantities in plasma, urine, tissue extract, cerebro-spinal fluid and tissue culture medium in vitro, and can also be detected by immuno-histochemical stains on fixed tissues.

It was argued teleologically that the production of pregnancy-specific hormones allowed tumour escape from immunological mecahnisms, since in vitro such hormones are immunosuppressive (HORNE et al, 1976); however, recent work suggests that these in vitro results may be artefactual (BEAN et al, 1977).

(i) Human chorionic gonadotropin (HCG).

This glycoprotein which is similar biologically to luteinizing hormone, can now be measured in a RIA where there is insignificant cross-reaction with the latter hormone (VAITUKAITIS et al, 1972). Its production in human malignancy is variable, as is that of its two sub-units. VAITUKAITIS (1973) has found a disproportionate production of the sub-units in trophoblastic tumours, and it has been possible to

clone three individual cell lines from a bronchogenic carcinoma, which produce HCG and its sub-units respectively (TASHJIAN et al, 1973). Isolated production of the α-sub-unit has been described in ovarian carcinoma (FRANCHIMONT et al, 1972), gastric and bronchogenic tumours (ROSEN & WEINTRAUB 1974), and isolated production of the β-sub-unit has been seen in a variety of tumours (WEINTRAUB & ROSEN 1973) and in vitro from a cervical carcinoma cell line (PATTILLO et al, 1977).

The value of measuring HCG has of course long been recognized in choriocarcinoma and hydatidiform mole (LI 1971), where it can also be found in CSF up to 20 weeks before clinical detection of cerebral metastases (BAGSHAWE & HARLAND 1976). In solid, non-trophoblastic tumours the situation is not as clear. For many years the association of gynecomastia, tumour (usually bronchogenic) and urinary gonadotropins has been known (FUSCO & ROSEN 1966), and attempts to show that these tumours resemble choriocarcinoma have been unsuccessful. ROSEN et al (1975) examined 1319 patients with a variety of solid tumours, and found HCG in 12%, the highest incidence being in ovarian carcinoma (42%), although 5/12 represents a very small number. Significant amount of HCG have also been found in breast and gastric tumours (BRAUNSTEIN et al, 1973), and may correlate with post-surgical eradication of tumour by encouraging falls (GAILANI et al, 1976).

In gynaecological malignancy few large studies have been undertaken (see Table 8.2), and most workers have concluded that measurements are not helpful prognostically or as an aid to assess the efficacy of treatment (SAMAAN et al, 1976; STONE et al, 1977). In vitro, it has been shown that patients with detectable HCG levels may demonstrate lymphocyte sensitization to it (WASS et al, 1977).

Recently, however, HCG has been detected in normal pooled human pituitary tissue and urine from men (CHEN et al, 1976) and normal liver and colonic tissue (YOSHIMOTO et al, 1977). It also appears that some bacteria from cancer patients, but not normal patients, synthesize HCG (ACEVEDO et al, 1978), and it is postulated that this represents

an exchange of "de-repressed" genetic material from mammalian to bacterial cells.

(ii) Human Placental Lactogen (HPL).

In trophoblastic tumours, HPL is elevated and it has been proposed that a low HPL/HCG ratio may help to distinguish neoplastic from normal trophoblast during pregnancy (SAMAAN et al, 1966). It is also found in a wide variety of non-trophoblastic tumours (WEINTRAUB & ROSEN 1971), and in breast cancer, tumour cells which stained for one or more of HPL, HCG or PSβG (vide infra) were associated with a decreased survival time of the patient (HORNE et al, 1976).

In ovarian carcinoma, SAMAAN et al (1976) found HPL in 76% of women with epithelial tumours, and very few patients showed a good correlation between the plasma levels and clinical course. The secretion of HPL appears therefore to be less reliable than that of HCG, and is not easily exploitable as a tumour marker.

(iii) Placental Alkaline Phosphatase (Regan Isoenzyme ALP).

These ubiquitous isoenzymes are organ and tissue specific, and the placental isoenzyme is identical to that first found in a man (called Regan) with bronchogenic carcinoma (FISHMAN et al, 1968). It has a high incidence in many other solid tumours, including ovarian carcinoma (STOLBACH et al, 1969) where its presence in pleural effusions may be helpful diagnostically if cytology is unhelpful. Histochemical staining has also revealed it in ovarian tumour cells (SASAKI & FISHMAN 1973).

(iv) Pregnancy-specific β_1-glycoprotein (PSβG).

Known by a variety of other names (see Table 8.3), this glycoprotein first described by TATARINOV & MASYUKEVICH (1970) has since been purified by BOHN (1971). Its immunological identity has not as yet been revealed.

It is elevated in the serum of women with trophoblastic tumours (SEARLE et al, 1978) although levels are much

TABLE 8.1 MAJOR STUDIES OF CARCINOEMBRYONIC ANTIGEN PRODUCTION BY OVARIAN TUMOURS.

REFERENCE	NO. POS.	COMMENTS
BARRELET & MACH, 1975	31/63	Elevated levels were deemed to be useful prognostically.
DI SAIA et al, 1975	18/44	Highest levels in advanced disease.
SEPPÄLÄ et al, 1975	15/53	CEA also detected in ascites.
VAN NAGELL et al, 1975	10/11	11% controls and 18% patients with benign disease had levels greater than 2.5ng/ml.
KHOO & MACKAY, 1976	67%	90% patients with Stage IV disease had elevated levels, and deemed useful prognostically.
LEVIN et al, 1976	7/23	Good correlation with survival only when patients in relapse.
SAMAAN et al, 1976	28/51 (epith) 6/14 (germ)	Of limited value as tumour marker, and no correlation with Stage, histology or clinical state of patient.
STONE et al, 1977	9/26	Of limited value.
RUTANEN et al, 1978	6/30	Immunoperoxidase staining negative in all cases of malignant tumours, but positive for benign mucinous cysts.

TABLE 8.2 MAJOR STUDIES OF HCG PRODUCTION BY GYNAECOLOGICAL TUMOURS (NON-GERM CELL).

REFERENCE	NO. POS.	COMMENTS.
FRANCHIMONT et al, 1972	1/1	Assay for α-subunit
BRAUNSTEIN et al, 1973	0/6	
ROSEN et al, 1975	5/12	
SAMAAN et al, 1976	21/51	Measurements not clinically useful.
STONE et al, 1977	13/26	Measurements not clinically useful.
RUTANEN & SEPPÄLÄ, 1978	49/276	No correlation with disease spread.

lower than HCG and parallel changes in response to treatment.

In solid tumours, it has been found in about 15% of patients studied (TATARINOV & SOKOLOV 1977), including those with ovarian cancer. SEARLE et al (1978) found elevated levels in 2/15 (12%) women with ovarian cancer, but their study also found an unexplained occurrence in 13% of normal control subjects. The authors concluded that it was of no use in the detection and monitoring of either trophoblastic or non-trophoblastic tumours. HORNE et al (1976) on the other hand, suggested that it might be a useful prognostic indicator in women with breast cancer.

(v) Pregnancy-associated α-macroglobulin (PAM).

In 522 patients with various malignancies, 21 of whom had ovarian cancer, this glycoprotein was elevated in all but 2 (STIMSON 1975). There appears to be a good correlation between high levels and metastatic spread, and some workers have used PAM levels as a good indicator of tumour recurrence even before it is clinically detectable (ANDERSON et al, 1976; COOMBES et al, 1977).

In vitro studies show that PAM contains sialic acid, a substance implicated in the immunological inertia of trophoblast; it also has an immunosuppressive effect in vitro on T-lymphocytes in culture (STIMSON 1976).

8.4 EXPERIMENTAL DESIGN: A STUDY OF TROPHOBLASTIC HORMONES IN OVARIAN CANCER.

The purpose of this study was to measure circulating levels of HCG, HPL and PSβG in patients with ovarian cancer, in order to evaluate their usefulness in the management of the disease.

MATERIALS AND METHODS:

Patient groups were:

37 patients with primary epithelial ovarian carcinoma

100 normal non-pregnant female subjects as a control group.

The histological features of the tumours are presented in Table 8.4. The Stage was as follows: I - 3 patients; II - 4; III - 21; IV - 8; unclassified - 1.

The Radioimmunoassays: 146 plasma samples were taken from women at different times during their clinical course and were stored at -20°C for up to 2 years prior to the assay. Circulating levels of HCG, HPL and PSβG were measured using specific RIA's, and were performed by Dr. J.G.Grudzinskas, Department of Reproductive Physiology, St. Bartholomew's Hospital, London. Purified PSβG (Lot 8VE) was supplied by Dr. H. Bohn, Behringwerke AG, Marburg, West Germany, and the antiserum was obtained from Dako Immunoglobulins, Denmark (A131, Lot 116). The sensitivity limit of the assay was 10µg/l. Highly purified, intact HCG, β-subunit of HCG, and antiserum to the β-subunit (R485/6) were supplied by Ortho Diagnostics Inc., Raritan, USA. The β-subunit was used for labelling with ^{125}Iodine and specific antisera showed 0.10% cross-reactivity with human pituitary luteinizing hormone. The standard used in the assay was intact HCG and the sensitivity limit was 1µg/l (equivalent to 10mIU of HCG). Purified HPL was obtained from Nutritional Biochemicals Co., USA and the sensitivity limit of the assay was 0.5µg/l. The intra-assay variation was 3% and the inter-assay variation was 10-15%.

RESULTS:

In normal subjects circulating levels of HCG, HPL and PSβG were not detected using the assay. Elevated levels of PSβG were present in 5 patients (13.5%, range 10.5 to 15.5µg/l), HCG in 2 patients (5.4%, range 1.2 to 15.5µg/l), and HPL in 8 patients (21.6%, range 0.6 to 1.1µg/l).

Table 8.4 gives clinical details of all patients to the end of April 1978. At that time 9 of 10 patients with detectable levels of one or more of these hormones had died, compared with 10 of 27 patients without such levels who had died. Table 8.5 gives details of disease activity

in all patients with elevated levels, and indicates which hormones were present and the time to death from testing. None of the patients in this group had elevated levels in any other serum sample tested.

DISCUSSION:

Elevated levels of these hormones greater than the limits of sensitivity of the assay were not detected in normal controls. This observation is discordant with the findings of SEARLE et al (1978), who found that 13% of 94 controls had detectable levels of PSβG. In this latter study no data to exclude non-specific inhibition was presented.

The detection of HCG in only 5.4% of these patients is much less than that reported by comparable studies, and similarly detectable HPL in 21.6% of these women is lower than other studies (ROSEN et al, 1975; HORNE et al, 1976). Detectable PSβG in 13.5% of subjects is consistent with other reports but is based on a larger number of subjects than previously investigated (TATARINOV & SOKOLOV, 1977; SEARLE et al, 1978). The lower levels of HCG and HPL may be due to patient selection or assay technique variation.

This study confirms that there is no relationship between the occurrence of these placental proteins and the Stage, histology or course of the disease. A total of 24 women had active disease at the time of sampling and only 9 of these had elevated levels. There was no consistent pattern found when the survival of patients with and without hormonal levels was compared. Furthermore, as the circulating levels of these hormones are close to the sensitivity of the assay, the interpretation of the results is difficult. It appears, however, that the measurement of these placental proteins is of very limited value in the management of women with ovarian cancer. This confirms the findings of others in relation to women with gynaecological malignancies (STONE et al, 1977; RUTANEN & SEPPÄLÄ, 1978).

TABLE 8.4 DETAILS OF HISTOLOGY AND SURVIVAL OF ALL PATIENTS IN THE STUDY.

Histology	Stage	Hormone	Death	Survival from lap. (months)
Mucinous	I			8
Mucinous	I			13
Endometrioid	I			65
Mucinous	IIA			26
Mucinous	IIA			45
Mucinous	IIB	Yes	Yes	5
Endometrioid	IIB			25
Mucinous	III		Yes	2
Serous	III		Yes	3
Endometrioid	III		Yes	3
Serous	III	Yes	Yes	3
Serous	III		Yes	6
Undifferentiated	III		Yes	6
Serous	III		Yes	9
Serous	III			10
Serous	III		Yes	10
Mucinous	III			11
Mucinous	III			20
Serous	III			27
Serous	III	Yes		27
Endometrioid	III			30
Undifferentiated	III	Yes	Yes	32
Serous	III		Yes	40
Serous	III			41
Serous	III			42
Serous	III			45
Endometrioid	III	Yes	Yes	53
Endometrioid	III			83
Endometrioid	IV		Yes	4
Serous	IV	Yes	Yes	4
Serous	IV		Yes	6
Serous	IV	Yes	Yes	6
Serous	IV			13
Undifferentiated	IV	Yes	Yes	15
Undifferentiated	IV	Yes	Yes	23
Endometrioid	IV	Yes	Yes	29
Serous	Unclassified			14

Undifferentiated	4 patients.
Serous papillary	17
Mucinous	8
Endometrioid	8

TABLE 8.3 SYNONYMS OF PREGNANCY β_1-GLYCOPROTEIN.

Schwangerschafts spezifische protein 1	SP_1
Pregnancy specific β_1-glycoprotein	PSβG
Pregnancy associated plasma protein-C	PAPP-C
Trophoblast specific β_1-glycoprotein	TβG
β_1-glycoprotein	$\beta_1 SP_1$

TABLE 8.5 CLINICAL DETAILS OF PATIENTS WITH DETECTABLE LEVELS OF TROPHOBLAST SPECIFIC HORMONES.

Pt. &	Histology	Stage	Disease Activity	Hormones HCG	HPL	PSβG	Survival from test.
AH	Mucinous	II	Yes	+	+		2 months
AG	Serous	III	Yes		+	+	3
HL	Undiff.	III	Yes		+		6
DH	Endom.	III	Yes		+	+	8
NS	Serous	III	No[¶]	+			Disease free
SB	Undiff.	IV	Yes		+		3
JH	Serous	IV	Yes		+	+	3
SM	Serous	IV	Yes		+	+	6
IC	Endom.	IV	Yes		+		12
MG	Undiff.	IV	Yes			+	13

[¶] This patient had a second-look laparotomy 1 year after original biopsy and treatment with Cyclophosphamide. At this time a bilateral salpingo-oophorectomy and hysterectomy was performed and numerous biopsies of diaphragm, liver and peritoneal surface were negative for malignant cells.

APPENDIX.

MEDICAL RESEARCH COUNCIL: OVARIAN CANCER CHEMOTHERAPY FIRST STUDY (IL), "LATE" CANCER PROTOCOL. (Revised December 1976).

1. OBJECTIVE.

To determine the value of two different chemotherapy regimes in patients with advanced ovarian cancer. Change in tumour size, duration and quality of survival will be assessed.

2. SELECTION.

(a) Clinical: Patients with primary ovarian cancer will be selected who have been found at laparotomy to have disease which is incurable by operation and is extending into the upper abdomen and beyond (FIGO Classification Stages III and IV).

Exclusions - patients with:

i. malignant pleural effusion as the only sign of extra-pelvic disease
ii. isolated omental, supra-clavicular or inguinal node secondary the only sign of extra-pelvic disease
iii. known prior or concurrent administration of other forms of non-surgical anti-cancer therapy
iv. other cancer, previous or coincident
v. bone marrow involvement (leucoerythroblastic anaemia with thrombocytopenia less than 100,000/µl)
vi. impaired renal function - serum creatinine > 150µ.mol/l (1.5 mgms%), blood urea > 8µ.mol/l (50 mgms%)
vii. impaired liver function, serum bilirubin > 30µ.mol/l (2 mgm%), alkaline phosphatase > 15 K.A.U., lactic dehydrogenase and serum glutamic oxaloacetic transaminase > 50 I.U./l

Patients with recurrent ovarian cancer after primary surgery may be included if prior chemotherapy or radiotherapy has not been given.

(b) Pathological: all common forms of primary epithelial cancer as listed under the World Health Organisation Classification would be included in the study:

A. Serous Tumours - Malignant
   a. adenocarcinoma, papillary adenocarcinoma and papillary cystadenocarcinoma

b. surface papillary carcinoma

c. malignant adenofibroma and cystadenofibroma

B. Mucinous Tumours - Malignant

a. adenocarcinoma and cystadenocarcinoma

b. malignant adenofibroma and cystadenofibroma

C. Endometrioid Tumours - Malignant

a. adenocarcinoma

b. adenoacanthoma

c. malignant adenofibroma and cystadenofibroma

D. Clear Cell (Mesonephroid) Tumours - Malignant carcinoma and adenocarcinoma

F. Mixed Epithelial Tumours - Malignant

G. Undifferentiated Carcinoma

Exclusions:

Epithelial tumours of borderline malignancy or low potential malignancy

germ cell tumours, including malignant teratomata

Brenner and mixed mesodermal tumours

sex cord-stromal tumours such as granulosa cell tumours

Sertoli Leydig cell tumours

suspected secondary cancers

sarcoma, carcino-sarcoma

The initial primary diagnosis upon which the patient would be admitted to the study would be that of the local pathologist in the district where the operative treatment has been undertaken. It is appreciated that frequently biopsy material only will be available, but where possible sections from four blocks would be desirable. Sections would subsequently be reviewed by the Pathology Panel to ensure uniformity.

3. ENTRY PROCEDURE.

On receipt of the routine histology report the clinician may enter the patient into the study.....by random allocation to arm (i) or (ii). Treatment should be commenced within 2-4 weeks of surgery and should always be preceded by a chest X-ray and full blood count.

4. SCHEDULES.

Arm (i) Continuous Cyclophosphamide 100 mg daily by mouth, for as long as clinical control is obtained and as

far as haematological effects permit.

Arm (ii) Cyclophosphamide 200mg daily by mouth for days 1-14
Hexamethylmelamine 200mg daily orally for days 1-14
Methotrexate 50mg by mouth for days 1 and 8
No treatment days 15-28.

Patients who have received less than 2 cycles of treatment will be excluded from the statistical evaluation although follow-up information will still be collected.

A decision to interrupt treatment must be taken by the clinician concerned on an individual basis. Rate of fall of blood count may influence this decision, but the following guide-lines are suggested:

Haemoglobin less than 8.0g/dl (8.0g per 100ml)
Platelets less than 100 x 10^9/l (100,000 per c.mm)
White cell count less than 2 x 10^9/l (2,000 per c.mm)

At the end of one year clinicians may wish to consider whether a further laparotomy is desirable in any surviving cases.

5. CRITERIA FOR ASSESSMENT.

(a) measurable change in tumour bulk:
  i. clinical or radiological measurement
  ii. ultrasonic scanning
  iii. isotope scanning, or other techniques
(b) rate of development of:
  i. ascites
  ii. pleural effusion
(c) general health: Grades:
  1. at work or normal activity
  2. full activity but not at work
  3. out and about but activity restricted
  4. confined to home or hospital
  5. bedridden.
(d) new disease apparent
(e) survival time up to 5 years maximum
(f) death (necropsy findings)

6. DOCUMENTATION.

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