IMMUNOLOGICAL STUDIES ON WHOLE-BODY HYPERTHERMIA TREATMENT FOR ADVANCED CANCER

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Why! The Fever Itself is Nature's Instrument

Thomas Sydenham

This thesis is dedicated to:-

My Parents
Dr. R. T. Pettigrew and the
Hyperthermia Patients

Abstract

There is renewed interest in the use of induced hyperthermia in the treatment of malignant disease. It has been proposed that the anti-tumour effects of hyperthermia are mediated by the immune system. The aim of this investigation was to determine whether whole-body hyperthermia (41.8^{o}C) affected the immune status of patients with advanced cancer. General immunocompetence has been monitored before, during, and after therapy, using several tests *in vitro*.

During treatment there was a stress-mediated leucocytosis, characterised by an increase in the concentration of phagocytically-active immature neutrophils, a T-lymphopenia, and a B-lymphocytosis, but no change in the total lymphocyte concentration. Lymphoid antibody-dependent cellular cytotoxicity, and phytohaemagglutinin-induced lymphocyte transformation, were slightly depressed by heating.

The day following treatment, the T lymphocyte concentration remained depressed, and there was a fall in the level of serum complement. Within 3-5 days of treatment, most parameters had returned to their pre-treatment values, and there was a marked increase in the T lymphocyte response to PHA, to 2-3 times the pre-hyperthermia level. This was no longer evident within a week of treatment. Hyperthermia therapy had no significant long-term effect on any of the indices of cellular, or humoral, immunocompetence that were monitored.

Heating lymphocyte suspensions *in vitro*, for 2½h at 42^{o}C, abolished PHA-induced RNA and DNA synthesis, and severely depressed their capacity to form spontaneous, normal and 'rapid' rosettes with unsensitised sheep erythrocytes. The ability of lymphoid cells to lyse antibody-coated tumour cells was also reduced by heating the

effector population *in vitro*. Granulocytes heated at 42°C were less efficient at phagocytosing low concentrations of latex particles. The depression of lymphocyte immune function occurred, to a lesser extent, when whole blood was heated *in vitro* before separation of the lymphocytes.

These results indicate that heat treatment, both *in vivo* and *in vitro*, depresses general immunocompetence. In the clinical situation the depression lasts for 24-48h following treatment, during which, thermoresistant, viable tumour cells may be seeded into the peripheral circulation, and become established as metastases.

CONTENTS

Foreword

Due to the diverse nature of the assays used in this work, the results are presented in the form of several papers, each of which deals with a different test. These are supplemented by general sections, containing information relevant to all of the assays.

PREFACE AND GENERAL INTRODUCTION

Preface

In recent years there has been an upsurge of interest in the treatment of cancer by raising the body temperature to 41-43^{o}C - whole-body hyperthermia. This form of therapy appears to damage tumour cells selectively, but it is likely to affect other organs and systems in the cancer patient.

Virtually nothing is known about the effects of systemic hyperthermia on the components of the immune system, although it is generally accepted that the immune response can modulate the course of malignant disease in both animals and man. An improvement in general immunocompetence of cancer patients correlates with a more favourable prognosis, and this has lead to the introduction of therapies specifically designed to potentiate immunity.

Conventional treatments, such as radiotherapy and chemotherapy, rely upon selective direct damage to the tumour cell, but also produce immunodepression as a side effect. As a result, the patient is deprived of what may be a highly-selective tumour destroying reaction.

The aim of this work was to determine whether whole-body hyperthermia treatment, for advanced malignant disease, affected the immunocompetence of the cancer patient, thereby either potentiating any tumour-directed immune response, or, producing the immuno-suppression associated with other forms of therapy.

Hyperthermia

A Definition

Therapeutic hyperthermia may be defined as the treatment of disease by the induction of fever (normally in the 40-43^{o}C range). Elevation of body temperature has been used as a form of therapy for a wide range of conditions, including gonorrhoea (Report, 1934), arthritis (Markson & Osborne, 1933), neurological diseases (Nelson, Jeffreys and McDowell, 1958) and advanced cancer (Pettigrew et al, 1974). Although many of these applications have been superseded by the introduction of simpler, and more effective, drug treatments, there is still considerable interest in the use of hyperthermia in the therapy of malignant disease (Symposia, 1975 & 1977). This interest has, as its basis, the evidence that cancer cells are selectively sensitive to temperatures between 40 and 45^{o}C (Lambert, 1912 ; Cavaliere et al, 1967).

The Clinical Development of Hyperthermia Therapy for Cancer

Evidence that hyperthermia has a beneficial effect on the status of the cancer patient stems from two main sources:-

i) The correlation observed between spontaneous tumour regressions and concomitant febrile infections.

ii) The clinical improvements that have been observed following systemic, regional, or localised heat treatments.

These observations are supported by studies using animals, and in vitro.

Spontaneous regressions and the introduction of toxin therapy

For more than a century there have been reports of spontaneous tumour regressions in patients who have had intercurrent pyrogenic

infections.

In 1866 Busch described the disappearance of a histologically-confirmed sarcoma of the face, in a patient who had suffered two attacks of erysipelas. Some years later, Bruns (1888) reported the complete regression of a number of melanomas in a terminal patient, following an attack of erysipelas, in which the body temperature exceeded 40°C for several days. In 1893 Coley reviewed 26 cases of erysipelas in cancer patients, and found that, following pyrexia, the tumour size decreased in the majority of cases, and nine patients were finally cured. Remissions of between 5 and 44 years were reported by Fowler (Unpublished observations) in a series of 33 patients with a variety of histologically-confirmed cancers, who had had concomitant, pyrogenic, infections.

In 1918 Rohdenberg reviewed the literature on spontaneous regressions of tumours, and concluded that 72 of the 166 carcinoma patients, and 19 of the 26 sarcoma patients, had had high fevers, heat applications, or severe infections. Everson and Cole (1966) accepted only one of these as a genuinely 'spontaneous' regression, however, they excluded all the pre-1900 cases. Nevertheless, in their own review of more than 1,000 claimed regressions, they mention fever, or infection, in some of the 176 cases which they consider to be adequately documented.

Towards the end of the nineteenth century, the link between fever and tumour regression was so well established that fevers started to be used as a form of therapy. The earliest treatments simply involved placing the cancer patient in close proximity to a case of erysipelas (Busch, 1869), but these soon 'progressed' to to the direct inoculation of bacteria (Fehleison, 1883), often with lethal results (Janicke & Neisser, 1884). One of the pioneers of

this form of treatment was the American physician Coley. He produced the complete disappearance of tumours by the injection of live erysipelas cultures, obtained from Koch in Berlin (Coley, 1893). He soon realised the dangers and difficulties in the use of these vaccines, and tried heat-sterilised or filtered cultures, but without great success. Coley was to spend many years of his life trying to discover and standardise a more effective product. This work formed the foundation of the dubious toxin therapies that are still in use today (Miller & Nicholson, 1971 ; Issels, 1971). Although Coley originally believed that he was injecting a toxin that killed the 'cancer-producing microbe', it is now believed that his success was, in some measure, due to the hyperpyrexia that the toxins produced. Nauts (1976) has extensively reviewed the literature on toxin and bacterial therapies, and has concluded that they were of greatest benefit when they produced marked febrile reactions. It is unlikely however that toxins cause large-scale tumour necrosis solely by the low level of hyperthermia that they produce (39-41^{o}C). Their action is probably multifactorial, involving both hyperpyrexia, and possibly, stimulation of the immune system. Potentiation of the host's immunity would give Coley's toxins a similar role to that of Bacillus Calmette Guerin (BCG) and Corynebacterium parvum.

Externally-applied heat

The elevation of body temperature, for therapeutic purposes, by the production of systemic pyrogenic infections, is fraught with difficulties. Any such treatment runs the risk of killing the patient directly by the infection, or toxin, employed, and is virtually impossible to control or standardise. It was therefore inevitable

that an externally-applied heat source would gradually replace natural, or induced, fever as a form of therapy.

Methods for direct heating can be conveniently divided into two categories: localised or regional, and whole-body. Both are increasingly being used in combination with a second, more conventional, form of treatment, such as radiotherapy or chemotherapy.

Regional and localised heating Localised hyperthermia is of greatest use in cases where the tumour is either external, or directly-accessible to the heat source e.g. melanomas and bladder cancers, although it has been used on tumours exposed by surgery (Crile, 1962).

In 1898 Westermark treated an inoperable, ulcerated, carcinoma of the cervix by passing hot water through a spiral metal tube inserted into the vagina, thus achieving a temperature of 42.5^{o}C, which resulted in complete healing. Good results were also achieved by Goetze (1932) who treated carcinoma of the penis by immersion in a hot bath.

Chemotherapy has been used in combination with local hyperthermia in the treatment of malignant gliomas of the brain, by Sutton (1971), who suggested that the procedure showed particular promise for early, localised, tumours. Combinations involving heat and radiation, or drugs, have been used since 1909 (De Keating-Hart) and are of greatest value where the hyperthermia is produced by heated perfusion, or irrigation, of the affected area. Often the drugs are administered in the heated perfusates, but heat alone has been used in a few instances. Cavaliere et al (1967) have used pre-warmed, heparinised, blood to perfuse limb tumours. In a survey of their first 22 patients they reported major complications, including burns, kidney failure and

shock; however, there was massive histological necrosis of the tumour in 8 patients, 6 of whom were still alive and well in 1975 (Cavaliere, Moricca & Caputo, 1976). Subsequently, the procedure was refined, and between October and December 1974, a further 111 patients were treated either by heat alone, or in combination with chemotherapy or surgery. They concluded that hyperthermia could be very effective, especially in cases of recurrent melanoma (Cavaliere et al, 1976). This view is also held by Stehlin et al (1975), who showed that the heating of perfusions, containing the drug Melphalan, may produce a 300 percent increase in survival rates, and eliminate the necessity for amputation. They have also treated soft-tissue sarcomas by the combination of heated perfusions, irradiation and excision, and report that this regime greatly reduced the need for radical amputation.

Promising results have been obtained by Cummins et al (1976) who have used local perfusions of the cerebral hemisphere to treat inoperable malignant gliomas of the brain.

Heated (42-45^{o}C) irrigations produced tumour necrosis in 60 percent of non-invasive transitional cell carcinomas of the urinary bladder, and superficial damage to more deeply-invasive tumours (Hall, Schade & Swinney, 1974). Heated bladder irrigations have been combined with radiotherapy (Cockett et al, 1967) and chemotherapy (Lunglmayr, 1973). Two complete regressions were reported by Woodhall et al (1960) who used hyperthermic, cytotoxic perfusions in the treatment of 20 head and neck cancers. Abdominal tumours have been treated by perfusions of alkylating agents at 38^{o}C, whilst cooling the surrounding area to 31^{o}C, but without remarkable results (Shingleton et al, 1962).

In spite of considerable technical difficulties (See p. 8), microwaves are now being used to produce localised heating, and are

currently being evaluated, in combination with radiotherapy, in the treatment of melanomas (Hahn, 1977 ; Johnson, 1977, Personal communications).

Local heating can only be of value in the treatment of large, recurrent, or single metastatic cancers, which are localised to an extremity or perfusable organ. In cases where the tumour is advanced, and has metastasised to other organs, whole-body hyperthermia may be of greater benefit.

Whole-body hyperthermia Whole-body heating has long been used in the treatment of non-malignant conditions, e.g. general paralysis of the insane, and resistant gonorrhoea. A variety of techniques has been employed to induce hyperthermia, but most fall into one of two groups:-

i) Direct heating by short-wave radiations.

ii) Immersion in a warmed medium e.g. water, wax, or air.

Infra-Red and Short-Wave Radiations. At its simplest, this technique uses high power lights as a heat source. Warren (1935) treated 32 advanced cases of cancer using five, 200 watt, carbon filament lamps to produce and maintain rectal temperatures of 41.5^{o}C. He reported an immediate improvement in the general condition of most of the patients, which lasted for up to 6 months, and indicated that there may be some synergism between whole-body hyperthermia and radiotherapy. More recently, Heckel (1976) has used infra-red lamps to produce temperatures up to 40^{o}C for several hours in un-narcotised patients, however, he has not yet published the clinical results.

Recently there has been increasing interest in the production of localised, or whole-body hyperthermia by microwaves and ultrasound (Symposium, 1975). Although the techniques for achieving controllable,

uniform heating are still under development, there have been some clinical applications (Brenner & Yerushalmi, 1975). The Tronado microwave machine, which basically consists of banks of encircling diathermy antennae, has been used to treat more than 300 patients in Australia. The controversial results have been disputed, and the machine was phased out in 1975 (Report, 1976).

Both ultrasound (Lele, 1976 ; Baker, 1976) and radio-frequency fields (Doss, 1976 ; Dickson and Shah, 1977) are under experimental evaluation for use in heat generation, however, there are problems to be surmounted (e.g. non-thermal damage to normal tissues, differential absorption and reflection, and difficulties of temperature monitoring due to interactions between the microwave field and metallic thermo-couples) before these methods can find wide clinical application.

Immersion Techniques. The earliest method of elevating the body temperature was the hot bath, whose beneficial effects have been known since the time of Hippocrates. Therapeutic hot water immersions have been used in the treatment of malignant disease by Von Ardenne, as part of his Selective Multiphase Cancer Therapy (Krebs-Mehrschritt-Therapie-Forschung) (Von Ardenne, 1972). Unanaesthetised patients were immersed in water at 40°C (while the head region was kept cooled to 20°C), for periods of 150 minutes, without complications. It is not clear from the literature, however, what clinical results have been obtained using this method. A similar technique has been documented by Suryanarayan (1966), who has produced oral temperatures of 44°C (whilst cooling the carotid arteries and head) in unanaesthetised patients, who were give infusions of cytotoxic drugs when they reached peak temperature. He reported rapid palliation of symptoms, but the cases were only followed-up for 60 days. Kirsch

and Schmidt (1966) have used immersions and local diathermy to treat 48 cancer patients, and produced only 5 cases of insignificant clinical improvement. Domisch and Koeppen (1968) have also used immersions to treat neoplastic disease, but have not detailed the clinical response to therapy.

Circulating hot air has been used by Edwards (1976) to produce and maintain temperatures of 41.8^{o}C for up to 5 hours in unanaesthetised patients. At present it is used to try to eradicate residual viable tumour, following surgery for lung cancer, and although still at an early stage, the results seem promising.

Each of these methods has its own particular advantages and disadvantages. Hot water baths can produce unpleasant side-effects such as skin damage (Giles, 1938), and Von Ardenne has reported pre-collapse, or collapse, states in elderly patients after only 120 minutes of 40^{o}C treatment. Hot air techniques seem to produce fewer complications, and the use of general anaesthesia has been found to increase both the patient's tolerance and reduce the incidence of jaundice (Edwards, 1976).

It is possible that a combination of methods may result in a safer, more controllable, procedure. It is known that retention of metabolic heat by insulation can raise the body temperature to over 40^{o}C within 6 hours (Giles, 1938). Therefore, by insulating the patient against the normal methods of heat loss, and, applying an additional external heat source, it should be possible to produce temperatures in excess of 40^{o}C fairly quickly,without the risk of burning or scalding. This was the approach adopted by Henderson and Pettigrew (1971), and subsequently modified by Pettigrew (1976). They produced temperatures greater than 41^{o}C by:-

i) Insulating the body against the loss of metabolic heat

by covering the patient with a layer of solidifying paraffin wax.

ii) Heating theinspired gases to prevent heat loss via the respiratory surfaces, and to provide a small additional heat source.

iii) Immersing the patient in molten paraffin wax. This acts as the primary heat source, and although it is initially at $50^{o}C$, the wax in contact with the patient cools to form the insulating layer. The surrounding molten wax gradually solidifies, at between 40 and $43^{o}C$, and as it does so, it releases its latent heat of solidification to the patient, thereby acting as a form of thermostat, keeping the temperature elevated, but still tolerable.

The clinical results from this form of hyperthermia therapy have been encouraging (Pettigrew et al, 1974 ; Pettigrew, 1976). More than 100 patients, ranging in age from 3 to 80, have been treated, either by heat alone or in combination with radiotherapy or chemotherapy (Pettigrew et al, 1977). Most patients received 2-4 treatments (each lasting 2-4 hours), however in one case, a patient was given 24 heating sessions. The results from 49 patients treated by hyperthermia alone are summarised in Table 1. In general, sarcomas of soft tissue origin showed the best response. Gastro-intestinal tumours also responded well, whereas breast cancers were less responsive to heat alone, but reacted better to a combination of hyperthermia and cytotoxic drugs. Pettigrew has concluded that the initial results from whole-body hyperthermia treatment of gastro-intestinal tumours justify further study, in the form of a controlled clinical trial.

This method for inducing hyperthermia has also been used by

TABLE I - RESULTS OF TREATMENT WITH HYPERTHERMIA ALONE.

TUMOUR TYPE	NO. OF PATIENTS TREATED	PREVIOUS TREATMENT			OBJECTIVE RESPONSES	SUBJECTIVE RESPONSE	NO RESPONSE	SURVIVAL FROM START OF THERMOTHERAPY (WEEKS)
		SURGERY	RADIOTHERAPY	CHEMOTHERAPY				
SARCOMA	8	6	4	2	3 + 1*	6	1	84, 52, 20, 8, 4, 2, 1, 0.14
GASTRIC CARCINOMA	5	5	0	1	3 + 1*	3	1	16, 16, 0.28, 12, 4+
CARCINOMA COLON	8	8	0	1	4	3	3	20, 8, 4, 0.71, 10, 13, 4, 4
MELANOMA	8	8	2	2	3	5	3	12, 12, 8, 8, 4, 4, 3, 2
CARCINOMA LUNG	4	0	3	0	2	3	1*	24, 20 4, 0
CARCINOMA BREAST	2	2	2	1	0	1	1	12, 12
OVARIAN CARCINOMA, TERATOMA TESTES	4	4	2	3	0	0	4	24, 16, 8, 4
NEURPBLASTOMA, NEPHROBLASTOMA	3	3	3	3	1 + 2*	1	0	24, 0.85, 0.28
MISCELLANEOUS	7	4	5	6	2	2	4	32, 24, 12, 0.57, 10, 24+, 6

* NECROPSY EVIDENCE OF RECENT TUMOUR NECROSIS.

TABLE 1

CLINICAL RESULTS FROM WHOLE-BODY HYPERTHERMIA TREATMENT FOR ADVANCED CANCER

From: Pettigrew (1976)

Levin (Levin, 1977 ; Blair & Levin, 1977), who has combined heat treatment with radiotherapy. Six patients with various advanced tumours experienced immediate pain relief and tumour shrinkage, within 4-5 days of treatment. The cancers grew again in all cases, however, it should be remembered that, at present, all the cases being treated by hyperthermia are in the terminal stages of their disease. Levin is currently conducting a controlled clinical trial of hyperthermia in the treatment of Stage IV carcinoma of the cervix.

In conclusion:-

i) Safe and effective methods for achieving hyperthermia by whole-body, or localised methods exist, and are being developed.

ii) Clinical results are still sketchy and anecdotal, but, taken in combination with experimental studies, indicate that this method may be of value in the treatment of human cancer, especially when used in conjunction with other forms of therapy.

Further investigation of the potential of hyperthermia is clearly warranted.

The Effects of Hyperthermia on the Cell

Introduction

Clinical applications of hyperthermia in the treatment of malignant disease have been parallelled by laboratory investigations into its mode of action. There have been reports on the inhibitory effects of heating *in vivo*, on both the growth and metastasis of animal tumours, as assessed by direct tumour measurement or autopsy evidence (Westermark, 1927 ; Crile, 1962 ; Overgaard & Overgaard, 1972 ; Thrall, Gillette & Bauman, 1973 ; Yerushalmi, 1976). However, since there are, at present, few good animal systems which can be used to investigate hyperthermia *in vivo*, studies have focused on the effects of heat on malignant and normal cell lines *in vitro*. The first of these experiments, more than 70 years ago, demonstrated the thermosensitivity of tumour cells by the decreased number of 'takes' resulting from the injection of heated cancer cells into animals (Loeb, 1903 ; Jensen, 1903). Inhibition of growth *in vitro* was reported, at about the same time, by Lambert (1912), in possibly the first experiment in which tumour cells were tissue cultured. Since then a large number of workers have confirmed this inhibitory effect using both cultured tumour cells (Levine & Robbins, 1969 ; Giovanella *et al*, 1973 ; Harisiadis *et al*, 1975), and on cells explanted from human tumours (Schrek, 1966 ; Giovanella, Stehlin & Morgan, 1976 ; Dickson & Suzangar, 1976).

It is important to emphasise that the sensitivity of tumour tissue to hyperthermia is relative. Cancer cells appear to be damaged selectively at temperatures above 41.5^{o}C, and there is a definite relationship between the temperature and the exposure time (Stevenson, 1919 ; Overgaard & Overgaard, 1972). At temperatures

above a critical level (usually about 40.5°C - depending upon the tumour) an increase of one Centigrade degree halves the time required to produce the same biological effect (Gerweck & Dewey, 1975), but also tends to reduce the selectivity of the heat damage. Therefore, at any temperature above the threshold value, there is likely to be a fine balance between tumour destruction and normal tissue damage. Clinical treatments, whilst aiming to optimise the former, are likely to produce varying degrees of the latter, especially during whole-body hyperthermia, in which normal cells and organs, with a range of thermal sensitivities, will be subjected to elevated temperatures.

A brief summary of the effects of heat on tumour cell structure and metabolism is given below. This is included because the nature of the selectivity of heat damage is not fully understood, and it is likely that the changes produced in tumour cells occur (but presumably to a lesser extent, or at higher temperatures) in other cell types, including those which mediate immune responses.

Mechanisms by which Heat Damages Tumour Cells

Effects on Cell Structure and Ultrastructure Characteristic changes in tumour cell structure, following hyperthermia *in vivo*, have been described by the Overgaards (Overgaard & Overgaard, 1972 & 1976). In general the cytoplasm and nuclei shrank and vacuoles appeared in the cytoplasm, which stained more intensely. Cells in culture may also show deformation after heating (Levine & Robbins, 1969 ; Selawry *et al*, 1957) and the surface topology may change (Overgaard, 1976).

At the ultrastructural level the changes are often more profound. Overgaard and Overgaard (1976) have observed an increase in the numbers

of lysosome-like bodies in the cytoplasm, following hyperthermia *in vivo*. The mitochondria were also affected, initially showing dilatation, and subsequently, inflation, and membrane rupture. In tissue cultured cells the effect on mitochondria seems to be less severe (Simard & Bernhard, 1967), but there have beer reports of increased lysosome numbers (Heine *et al*, 1971). The most sensitive cytoplasmic structure appears to be the polyribosome, which shows reversible disaggregation in response to heat (McCormick & Penman, 1969), both *in vitro* (Love, Soriano & Walsh, 1970), and *in vivo* (Overgaard, 1976).

Changes also occur in nuclear ultrastructure. The number of perichromatin granules increases (Heine *et al*, 1971) and chromosome aberrations may occur, but at low frequency (Dewey *et al*, 1971); the most sensitive structure seems to be the nucleolus. Changes in nucleolar structure following hyperthermia are striking. Chromatin and granular ribonucleoprotein are lost, and the nucleolar reticulum disappears (Simard & Bernhard, 1967). The whole nucleolus becomes morphologically and histologically amorphous (Love *et al*, 1970), consisting largely of fibrils (Heine *et al*, 1971). These changes are reversible if the cells are returned to normal temperatures, providing that the exposure time has not passed a critical limit.

Effects on Cell Metabolism

Respiration and glycolysis Temperatures above 40^{o}C, applied both *in vivo* (Muckle & DIckson, 1973) and *in vitro* (Cavaliere *et al*, 1967) have been found to inhibit selectively the respiration of tumour cells (Muckle & Dickson, 1971). This inhibition becomes irreversible if the duration of heating passes a critical time

(Mondovi et al, 1969a), and can be correlated with irreversible cell damage (Mondovi, 1976). This correlation has formed the basis of an assay in vitro for predicting the sensitivity of human solid tumours to hyperthermia (Dickson & Suzangar, 1976).

The effect of heat on glycolysis is less predictable. Inhibition has been reported for some tumours, e.g. the Yoshida rat tumour (Dickson & Ellis, 1974) and the Sprague Dawley breast tumour (Dickson & Shah, 1972); but not in a number of others (Cavaliere et al, 1967 ; Mondovi et al, 1969a). A reduction in anaerobic glycolysis has been described for human tumours heated in vitro (Dickson & Suzangar, 1976).

The time scales for the inhibition of both respiration and glycolysis of tumours suggest that these factors are not the primary target of hyperthermia (Dickson & Shah, 1972 ; Mondovi, 1976).

Nucleic acid and protein synthesis Nucleic acid and protein synthesis have been studied by measuring the uptake of specific, radiolabelled, precursors. Heating in vitro to 41^{o}C has been found to inhibit the uptake of these precursors by both tumour cells and rapidly-proliferating tissues (Wust et al, 1973). In contrast, Mondovi et al (1969b) have shown that uptake was only inhibited in tumour cells, in comparison to cells from regenerating liver. Other workers have also reported selective inhibition of synthesis, but are divided as to which pathway is the most sensitive. Mondovi et al (1969b) have found that DNA production was the most inhibited, whereas Dickson and Shah (1972) reported that RNA synthesis was the more severely affected. Mondovi (1976) has since stated that these discrepancies may be due to differences in experimental conditions, and in the rates of ribosomal RNA synthesis (See p.16).

In an attempt to discover which process is the primary target for heat damage, drugs have been used to block the individual synthetic pathways during heating. Drugs inhibiting DNA synthesis were found to increase the thermoresistance of a tumour cell line (Giovanella & Heidelberger, 1969 ; Palzer & Heidelberger, 1973a), indicating that the depression of DNA synthesis, which itself occurs as a response to heat, may possibly be thermoprotective. Dewey et al (1971) found that there was an additive, rather than synergistic, reaction between the drug BUdR and heat, indicating that the same structures were damaged by both. They concluded that this structure was chromatin - the DNA of which was damaged by the BUdR, and the associated protein, by the heat. In contrast, radiation acted synergistically with BUdR, and was of least effect during the heat-sensitive late S phase of the cell cycle. Respiration is also much less sensitive to radiation than glycolysis (Caputo & Giovanella, 1960). These differences between the effects of heat and radiation would argue against a common primary target of damage, and as radiation is believed to act directly upon DNA, it is unlikely that this is also the centre for heat killing. It is possible however, that DNA is indirectly affected by heat inactivation of some enzyme, or protein, associated with it (Loshek, Orr & Solominidis, 1976).

Compounds which inhibit RNA production have been shown to increase hyperthermic cell killing e.g. MPB and Cordycepin (Palzer & Heidelberger, 1973b) and Actinomycin D (Giovanella et al, 1970), indicating that RNA may be the primary target for heat damage. However, increasing the extracellular concentration of RNA precursor (uridine) can reduce the inhibition of uptake (Strom et al, 1973), which suggests that the damage might be occurring at

the cell membrane level. This is supported by experiments showing that membrane passive permeability (Strom et al, 1973) and trans-cellular migration (Strom et al, 1969) are both increased by elevated temperatures. Such changes could be responsible for the heat-induced inhibition of RNA synthesis, but, in contrast to drugs which increase membrane permeability, or inhibit nucleoside permeation, heat did not affect the ability of labelled uridine to be incorporated into RNA (Strom et al, 1973). It has now been shown that, although drugs which alter membrane permeability may have superficially the same effect as heat upon RNA synthesis, their mode of action is not identical; and the membrane changes that occur after hyperthermia are, in fact, secondary (Mondovi, 1976), and are therefore not responsible for the inhibition of RNA synthesis (Strom et al, 1975). This implies that RNA production may indeed be the primary target for heat damage. High-resolution autoradiography (Simard & Bernhard, 1967), cytochemistry (Love et al, 1970), and extraction techniques (Heine et al, 1971 ; Strom et al, 1975) have shown that there is a selective inhibition of nucleolar RNA by heat. The marked morphological changes that occur in the nucleolus of the heated cell would tend to support the theory that it is the centre of heat damage.

In the normal nucleolus, DNA is transcribed into nucleolar RNA, which eventually matures and becomes ribosomal RNA. As a step in this maturation process, a high molecular weight (45S) RNA is formed - pre-ribosomal RNA. This cleaves to form first 35S, and then 28S RNA. Morphologically the maturation of pre-ribosomal RNA can be correlated with a change from a fibrillar to a granular structure (Simard & Bernhard, 1967), the fibrillar component being the 45S molecule (Love et al, 1970).

Following hyperthermia, the granular component of the nucleolus disappears and the structure develops an homogenous appearance, consisting mainly of fibrils (Heine et al, 1971). Love et al (1970) have suggested that hyperthermia alters the configuration of the RNA, and the reduced synthesis may depress the production of the pre-ribosomal particles, or ribosomes, by the nucleolus. They state that the more mature, granular RNA is probably eliminated from the nucleolus, via the nuclear pores, to the cytoplasm, where it is seen as inclusions, and that this process is accentuated by heat. Simard and Bernhard (1967) have, however, shown that the granular material is not lost from the nucleolus, but is converted back into the fibrillar form, by unravelling. They believe that heating affects the hydrogen bonds of the nucleolar DNA (which may be more sensitive to heat than nuclear DNA), and maturing, pre-ribosomal RNA. This results in a severe depression of RNA synthesis and configurational changes in the already-synthesised RNA.

Normal RNA production is essential for the synthesis of late-replicating DNA (Mueller & Kajawara, 1965), and the progression into mitosis. It is possible that, by disrupting RNA production, hyperthermia dissociates this relationship, and thereby affects tumour cell replication, as suggested by Dickson and Shah (1972). This is probably an over-simplification, and it is more likely that there are a number of mechanisms involved in the process of thermal cell damage and killing.

Factors Affecting Selectivity of Heat Damage

As has been stated, many of the changes produced in tumour cells by elevated temperatures probably occur to a lesser extent in other types of cells. The extent to which this happens may be governed by

an inherent thermal sensitivity of the cell, but it is likely to be greatly influenced by the following factors:-

i) pH Several workers have reported that the thermosensitivity of tumour cells can be increased by reducing the intracellular, or extracellular pH (Overgaard, 1976 ; Kim, 1977 & Gerweck, 1977 Personal communications). Other groups have not found a pH effect (Dickson & Oswald, 1976).

ii) Oxygen Status Hypoxic cells are reported to be more thermosensitive than fully-oxygenated equivalents (Gerweck, Gillette & Dewey, 1974 ; Schulman & Hall, 1974 ; Thrall et al, 1975 ; Harisiadis et al, 1975). This is in marked contrast to the effect of oxygen status on radiation killing.

iii) Nutritional Status (probably related to (i) and (ii) above). Cells deprived of nutrients are more sensitive to hyperthermic killing (Hahn, 1974).

iv) Phase of the Cell Cycle There are two peaks of thermosensitivity during the cell cycle. The first occurs during mitosis (Rao & Engelberg, 1965 ; Gerweck & Dewey, 1976), and, in particular during metaphase (Sisken, Morasca & Kibby, 1965 ; Selawry, Goldstein & McCormick, 1957); and the second during S phase (Dewey et al, 1971), especially during late S and early G_2 (Palzer & Heidelberger 1973b ; Kase & Hahn, 1975). Rapidly-proliferating cells may also be more sensitive (Wust et al, 1973 ; Wust, 1976).

During whole-body hyperthermia treatment every system and organ in the body will be exposed to increased temperatures, and differences in the parameters listed above, which are insignificant at $37^{o}C$, may have

a critical role in determining the degree of thermal damage sustained by a particular cell or organ.

Hyperthermia is known to produce changes in, for example, the liver (Wills, Findlay & McManus, 1976) and alterations in serum protein and vitamin levels (Mendez et al, 1959), and pituitary/adrenal responses (Few & Worsley, 1975) are suggestive of effects on other normal tissue.

Little is known about the action of hyperthermia on the components of the immune system (See p. 38), in spite of the fact that it may have an important role in mediating hyperthermia therapy (Symposia, 1975 & 1977). In addition, if one accepts that tumours may be inhibited by an immune reponse, then it is of primary importance to ascertain whether whole-body hyperthermia therapy affects the immunocompetence of the patient.

Cancer and the Immune Response

Introduction

It is believed that the purpose of the immune system is to recognise, and eliminate, that which is 'not self'. This role may be described as one of surveillance (Thomas, 1959 ; Burnet, 1971), and, as such, can be used to explain defence against bacterial, fungal, and parasitic infections, and phenomena such as the rejection of foreign grafts. Whilst the process of antigen recognition is not fully understood, the nature of some of the immune responses to antigenicity have been elucidated.

The Immune Response to Antigens - A Brief Summary

The response to an antigen may involve many different facets of the immune system, however, it can be visualised as taking two forms, namely the humoral and the cell-mediated response. Although this division is now regarded as somewhat artificial, it is still a useful concept for the classification of immune responses.

Humoral Immunity The humoral response to antigens is mediated by immunoglobulins produced by B cells. These cells are lymphocytes which have originated in the bone marrow and have become immuno-competent, following processing by the Bursa of Fabricius in birds, or its equivalent in mammals (possibly gut-associated lymphoid tissues, and/or the haemapoietic tissues e.g. the cortex of the lymphoid follicles, foetal liver and spleen). It is believed that each B cell can make one (or a very limited number) particular antibody, which it bears at its surface as a receptor. When the cell contacts an antigen which complements this receptor, it divides and proliferates (clonal selection theory of antibody production).

The original receptors are shed and replaced. The lymphocyte then undergoes repeated mitoses, resulting, after several days, in the production of a mature clone of specific-antibody secreting plasma cells, and a number of memory cells. The latter respond more sensitively and rapidly to any re-appearance of that particular antigen.

The released antibody can act by precipitating antigen which is in solution, by binding with it to form insoluble complexes. If the antigen is bound to cells then these may be:-

i) Agglutinated - by the antibody cross-linking antigen on adjacent cells.

ii) Phagocytosed - after coating with antibody (opsonisation) and, in the case of immune adherence, complement, cells show an affinity for the receptors on polymorphs and macrophages.

iii) Lysed - antibodies on the cell surface may fix complement, thus activating the sequence leading to damage of the cell membrane and lysis. Alternatively, antibody-coated cells can be killed by K cells (See p.25)

In addition, both B lymphocytes and humoral antibody have an important role in the modulation of cell-mediated responses (Moller, 1972).

Cell-Mediated Immunity Cellular immunity may be mediated by a variety of cell types, but most is known about T cell responses.

The T cell is a lymphocyte which derives from stem cells in the bone marrow, and is processed by the thymus gland, before becoming immunocompetent (Davies, 1969). Mature T cells are usually long-lived and mobile, being found in the peripheral blood, paracortical areas of the lymph nodes and the white pulp of the spleen. They

are thought to carry surface receptors (IgT) for the antigen to which they have become sensitised by previous contact. The nature of these receptors is still in doubt, but it is possible that they are immunoglobulin M monomers, derived either from B lymphocytes, or possibly synthesised by the T cell itself (Burnet, 1976). Alternatively, they may be complexes of antibody and antigen (Roitt, 1974).

Once the antigen combines with the receptors, the cell is transformed from a small lymphocyte into a large lymphoblast, which proliferates to produce large numbers of specifically-sensitised cells which are able to kill any cells which bear that antigen. The killing process is independent of added complement and is believed to involve initial cell-to-cell contact (Perlmann & Holm, 1969), producing membrane damage, resulting in final osmotic lysis (Ferluga & Allison, 1974), although this mechanism is disputed (Sanderson, Hall & Thomas 1977).

Blastogenesis is usually accompanied by the synthesis, and release of soluble factors - lymphokines. It is possible that lymphokine production may occur independently of blast transformation (Spittler et al, 1970). These factors are identified by their action, and up to date more than 20 have been described (Pekarek and Krejci, 1974). Their functions, in the mediation of delayed hypersensitivity reactions, are diverse, ranging from inhibiting cell growth - lymphotoxins (Ruddle & Waksman, 1967) and virus multiplication - interferon (Wheelock, 1965), to the induction of blastogenesis in non-sensitised lymphoid cells - mitogenic factor (Kasakura and Lowenstein, 1965) and the inhibition of macrophage migration - MIF (Bloom & Bennet, 1966).

T cells also have a vital role in controlling immune responses. They act synergistically in the production of some antibodies, both

in vivo (Claman, Chaperon & Selner, 1968) and *in vitro* (Feldmann *et al*, 1974).

In complete contrast, T cells can have a suppressor effect on the immune response (Moller, 1975). In this role they have been found to abrogate, or diminish, both humoral (Droege, 1971 ; Herzenberg *et al*, 1973), and cell-mediated immunity (Hardin, Chused & Steinberg, 1973 ; Zembala & Asherson, 1973). The T cell population is therefore not as homogenous as was once thought, but is probably composed of various subpopulations of suppressor and helper cells, which may exert a stimulatory, or an inhibitory influence, depending upon the circumstances.

Macrophages Macrophages can show both specific and non-specific cytotoxicity. Specific killing is shown *in vitro* by cells which have been armed (Evans & Alexander, 1972) by:-

i) Incubating the macrophages in the supernatant from a culture of immune lymphoid cells and specific antigen - this supernatant contains a specific arming factor produced by the sensitised T cells (Ziegler, Lohmann-Matthes & Fischer, 1975).

ii) Incubating normal macrophages with immune lymphoid cells.

If these armed macrophages are now incubated with the specific antigen, they become activated (Evans & Alexander, 1972) and are then non-specifically cytotoxic. Activation is believed to occur *in vivo* (Hibbs, Lambert & Remington, 1972) where it appears that the activated macrophages are selectively cytotoxic for malignant cells (Droller & Remington, 1975)

Macrophages are also involved in the generation of helper T cells (Erb & Feldmann, 1975), potentiation of T cell responses (Gery & Waksman, 1972), and the presentation of antigen to lymphoid cells

(Unanue, 1972). Monocytes, which may be the precursors of peripheral blood macrophages in the human (Krikorian et al, 1975), have also been found to display cytotoxicity in vitro (Holtermann et al, 1974). Similar effects have been claimed for other cell classes (Pickaver et al, 1972).

Antibody-Dependent Cellular Cytotoxicty - ADCC Leucocytes can kill target cells in the presence of sub-lytic concentrations of antibody to the target cell. This effect was first described by Moller (1965), and is distinguished by the following:-

i) Mediation by non-immune effector cells.

ii) Lack of species barrier between target cell, antibody and effector cell.

iii) Requirement for sub-lytic concentrations of target cell-specific antibody (Perlmann, Perlmann & Wigzell, 1972).

iv) Lack of requirement for complement (Van Boxel et al, 1974).

The fact that ADCC can be demonstrated in syngeneic, allogeneic and xenogeneic systems has made it difficult to compare results from different laboratories, with the result that the nature of the effector cell is still in doubt. The cell preparations that have been shown to have lytic activity differ widely in source and purity, so that lysis may have been due to small numbers of contaminating cells. In fact, it has been found that virtually every type of leucocyte, including perhaps the T lymphocyte (Saal et al, 1977a ; Greenberg & Wolosin, 1977), which was originally excluded by Van Boxel et al (1972) and Perlmann et al (1972), amongst others, can display ADCC under the right conditions. This rather confusing situation has been clarified recently by the demonstration that the nature of the effector cell is largely determined by the type of target cell used

in the assay (MacDonald et al, 1975). The two most commonly used target are the erythrocyte and the continuous cell line.

Cytotoxicity against cell lines is usually shown by non-phagocytic mononuclear cells - K cells (MacDonald et al, 1975 ; Trinchieri et al, 1975 ; Peter et al, 1975). These cells may be lymphocytes (Zeijlemaker et al, 1975), as judged by their morphology (Kovithavongs et al, 1975), radiosensitivity and low density. Neutrophils and macrophages have also been found to show K cell activity by a number of workers (Gale & Zighelboim, 1974 ; Wardley, Rouse & Babuik, 1976 ; Ormerod & Jolley, 1976).

Human erythrocytes are lysed by both adherent, phagocytic, mononuclear cells and polymorphonuclear cells (MacDonald et al, 1975 ; Zeijlemaker et al, 1975 ; Trinchieri et al, 1975). However, Urbaniak (1976) has found that the adherent cells could be removed from a lymphoid effector cell population, without greatly affecting its cytotoxic activity. Sheep and chicken erythrocytes can be lysed by virtually any leucocyte population. The adherent cells are probably monocytes, macrophages and polymorphs (Inglis et al, 1975), whereas the non-adherent cells are of lymphoid origin, but probably not T or B cells (Calder et al, 1974).

The nature of the effector cell therefore seems to depend on the type of target cell used. The reason for this is not clear, although there have been several explanations:-

i) The size of the target cell is the critical factor (Papamichail & Temple, 1975).

ii) The distribution and fluidity of the antigen on the target cell surface, and the receptors on the K cell, determine the type of effector cell activated (Trinchieri et al, 1975).

iii) There is a difference between the Fc receptors on lymphocytic and monocytic effector cells (Larsson et al, 1975).

Whatever the mechanism involved, it is now apparent that antibody-dependent cellular cytotoxicity can be displayed by a number of cell types, making it one of the most interesting cell-mediated immune responses.

The Antigenicity of Tumour Cells

For a cell to elicit an immune response it must be antigenic to the host which bears it. Furthermore, any proposal that the immune response may play a role in the prevention, or control, of cancer depends on the existence of antigens which, although not absolutely specific to tumour cells, will initiate a selective, anti-tumour immune response. These antigens were first demonstrated at the turn of the century, when it was shown that animals in which tumours had spontaneously regressed, were resistant to challenge by tumour cells from the same line (Clowes & Baeslack,1905); however the animals used in these experiments were not syngeneic, and therefore the resistance observed may have been directed towards histocompatibility antigens, which are present on all tissues. When homozygous, inbred, strains became available this possibility could be eliminated. Using these strains, Gross (1943) was able to show that animals, in which transplanted, methylcholanthrene-induced tumours had regressed, were resistant to subsequent challenges with the same tumour. This work was confirmed by Foley (1953), who found that the destruction of a growing tumour, by ligation, conferred resistance to future challenges, by the same tumour growing in a

syngeneic animal.

It was still possible to attribute these findings to some residual heterozygosity in the animals, but this question was finally resolved by Prehn and Main (1957). They extended Foley's work by showing that their animals were truly homozygous, since they were able to accept skin grafts from donors of the same inbred line. In addition, they demonstrated that the immunity they observed was tumour-specific, since normal tissue, obtained from the animal used to provide the immunising tumour implant, could not confer immunity against the tumour, conversely, tumour implants from an isologous animal failed to immunise against skin grafts from the implant donor.

All these experiments involved challenges with tumour grown in an isologous host, but Klein et al (1960) showed that an animal may be immunised against its own tumour. They amputated the tumour-bearing limbs of mice and immunised the animals with irradiated cells from the cancer. They then reinjected the original host with its autochthonous tumour, and were able to show resistance.

These tumour-specific transplantation antigens - TSTAs - have been detected in a variety of experimental animal tumours, but have different characteristics, depending upon how the tumours were induced, for example, all chemically-induced tumours seem to be antigenically distinct (Old et al, 1962), whereas all the tumours produced by one virus, share a common antigen (Sjogren, 1965). This is undoubtedly an over-simplification, as chemically-produced tumours may share common, weaker, antigens (Prehn and Main, 1957 ; Reiner and Southam, 1967), and viral tumours may display individual antigens (Heppner & Pierce, 1969 ; Vaage, 1968). So-called spontaneous tumours are thought to contain relatively weak TSTAs (Symes, 1974 ; Cuarie, 1974), however Hewitt, Blake and Walder

(1976) have failed to demonstrate an immune response to 27 different spontaneous tumours, during the course of more than 20,000 transplants. In addition, they have noted that immunisation with irradiated tumour cells often increased susceptibility to challenge by the tumour. This work may be of considerable importance to the future of tumour immunology, as spontaneous animal tumours are thought to provide a good model for human cancers. However, in general, present evidence indicates that many animal tumours bear specific antigens that are capable of eliciting an immune response in the host.

The Immune Response to Human Tumours

The transplantation of malignant tumours between human subjects, besides being highly unethical, does not provide evidence for TSTAs, since humans are outbred, and will therefore reject almost any implant because of histocompatibility differences. This has resulted in the development of assays for detecting the immune response to human tumour tissue in vitro. The antigens which these tests detect may be shown to be tumour-specific by the inclusion of the appropriate normal controls in the test system; since these antigens may not be identical to those demonstrated by transplant techniques, they are known as Tumour-Associated Antigens - TAAs.

Humoral Immunity Anti-tumour antibodies have been discovered in the serum of cancer patients, using a variety of techniques. Indirect fluorescent antibody tests have shown that patients with Burkitt's lymphoma (Klein et al, 1967), malignant melanoma (Morton et al, 1968), osteogenic sarcoma (Mortom & Malmgren, 1968) and leukaemia (Dore et al, 1967) possess humoral antibody to their tumours. Their role in the cancer patient is not clear, although it has been shown that they may be cytotoxic in melanoma (Canevari et al, 1975) and breast cancer

(Baldwin et al, 1973), and their presence may correlate with delay in the recurrence of disease, following surgical procedures (Hudson et al, 1974).

It is generally accepted, however, that the immune response to tumours is largely cell mediated (Baldwin & Price, 1976 ; Report, 1975). The evidence for this has been extensively reviewed by the Hellstroms (1974) and Herberman (1974), but basically it centres around the demonstration that tumour-specific immunity could be transferred adoptively to a normal animal by the injection of cells, rather than serum, from an immune animal (Old et al, 1962).

Cellular Immunity Cell mediated immune responses to human tumours have been detected using a wide range of test systems (Monograph 1973) Measurements in vivo have been made, using neutralisation tests and skin testing. The former involve assessing the patient's hypersensitivity response to a sub-cutaneous injection of a mixture of autochthonous, viable tumour cells and blood leucocytes (Southam, 1967). Apart from being very doubtful ethically, it is virtually impossible to determine whether the absence of a tumour nodule is due to an immune response to the cells, or some secondary event.

Delayed cutaneous hypersensitivity reactions to a variety of non-vable tumour preparations have been described in various cancers. In these tests an extract of autochthonous, or allogeneic tumour of the same histological type, is injected intradermally, and the response compared to that obtained by the injection of various control extracts. Positive reactions have been noted in leukaemia (Oren & Herberman, 1971), Burkitt's lymphoma (Bluming et al, 1975), malignant melanoma (Holmes, Roth & Morton, 1975), breast cancer (Alford, Hollinshead & Herberman, 1973) and carcinoma of the colon (Hollinshead et al, 1972). These tests can produce

many problems, arising mainly from the lack of purity and sterility of the extracts, and the incidence of false positive reactions to the control tests (Burdick, Wells & Herberman, 1975). As a result, emphasis has been placed on the measurement *in vitro* of cell-mediated immunity.

The most commonly used of these assays is the microcytotoxicity test, in which lymphocytes from patients with tumours have been shown to kill, or inhibit the growth, or metabolism of, tumour cells of the same histological type (Pekarek & Krejci, 1974). Positive results have been reported in meningioma (Pees & Seidel, 1976), bladder carcinoma (O'Toole *et al*, 1973), lung tumours (Vose, Moore & Jack, 1975), melanomas (DeVries & Rumke, 1976) and renal cell carcinomas (Cole *et al*, 1976), amongst others. This test is particularly prone to problems (Herberman & Oldham, 1975 ; Baldwin, 1975 ; Devries, 1976). These range from those involving questions of technique and quantitation of results (Report, 1975), to very serious doubts about the nature and specificity of the response. Hellstrom *et al* (1971) suggested that human tumours of the same histological type, share common antigens, since lymphocytes from patients with a particular tumour were more reactive against tumours of that type than were lymphocytes from normals, or patients with other cancers. On this basis, allogeneic tumours and tumour cell lines of the same histological type, have been used in microcytotoxicity tests, however it now appears that lymphocytes from both normals, and cancer patients, can display activity against a range of tumour cell targets (Takasugi, Mickey & Terasaki, 1973 & 1974 ; Oldham *et al*, 1975). Recently, Brooks, Rees and Baldwin (1976) have suggested that the killing by normal lymphoid cells may be an artefact, due to modifications of the tissue culture medium by the effector cells. In general, there is

evidence that the cancer patient does show a positive reaction against his own tumour type (Hakala et al, 1974).

Another commonly used assay for cellular immunity is the leucocyte or macrophage migration inhibition test. If the patient's lymphocytes are sensitised to his tumour, then upon contact with tumour antigen, they will release a lymphokine that inhibits the otherwise normal migration of human leucocytes, or guinea pig macrophages, in vitro (Sinkovics, 1973). Inhibition has been shown in a variety of tumours (Elias & Elias, 1975a), however, the test is often difficult to interpret, since the results can be affected by the nature of the tumour extract (Jones & Turnbull, 1975 : Elias & Elias, 1975b), and the type of indicator cell (Armitstead & Gowland, 1975). Migration may also be inhibited by serum (Bice, Gruwell & Salvaggio, 1976), and tumour factors (Wolberg, 1974). Nevertheless, positive results have been obtained, and although the technique can show histogenic cross-reactions (Segal et al, 1972), it may correlate with clinical findings (Kjaer, 1976). A modification of this system, known as the monocyte spreading test, has been used to demonstrate tumour antigen in melanoma patients (Mazuran et al, 1976).

Lymphocyte blastogenesis assays, using either autochthonous or allogeneic tumour cells, or extracts, have been developed, (Stjernsward, 1974 ; Mavligit, Hersh & McBride, 1974). However, they show considerable cross-reactivity if tumour extracts are used (Dean et al, 1975), and it is almost impossible to determine whether a blastogenic response to whole cells is really a secondary reaction, rather than a primary, recognition, response.

An immune response to human tumours has also been detected by the leucocyte adherence inhibition test (Halliday, Maluish & Isbister, 1974 ; Grosser et al 1976). This test was thought to resemble the

leucocyte migration inhibition assay, in that a lymphokine was released by sensitised lymphocytes, in the presence of specific tumour antigen (Holt et al, 1975). It now seems that the test may depend upon circulating anti-tumour antibody, rather than lymphokines, and is therefore not strictly a cell-mediated response (Marti, Grosser & Thomson, 1976).

In summary, assays in vitro and in vivo, using human cells and humoral factors, taken together with comparable animal experiments, tend to indicate that the tumour-bearing host can mount an immune response to his tumour.

These experimental results are reinforced by clinical observations. The incidence of cancer in patients with primary immunodeficiency is about 10,000 times that in the general, age-matched, population (Gatti & Good, 1971); however, these figures should be treated with caution, since the majority of the tumours in these patients are lymphoreticular, and may be the result of the immune system failing to modulate the response to antigen (Prehn, 1976). It has also been found that tumours transplanted accidently into immunosuppressed kidney transplant patients, have regressed when the immunosuppression was discontinued (Gatti & Good, 1971). It is important to realise that the transplanted tumour was an allograft, and therefore the rejection is not really tumour-specific. The immune system may have a role in the many cases of spontaneous regression that have been reported (Everson & Cole, 1966), and also in the remissions occurring in Burkitt's lymphoma patients on minimal chemotherapy (Burkitt, 1967). It may be implicated in the regression of metastases, following the removal of the primary tumour, and in the waxing and waning of tumour deposits (Currie, 1974). Although there is no conclusive proof that there is a tumour-directed immune response

in the cancer patient, the body of evidence is now so large that it cannot be ignored.

How Tumours Evade the Immune Response

There are numerous theories as to how a tumour evades the destructive mechanisms of the immune system:-

i) 'Sneaking through'. Very small numbers of tumour cell may not activate an immune response, and can therefore become established (Bonmassar et al, 1974).

ii) Immunodepression prior to tumour development. There is very little evidence to confirm, or reject, this theory. Recent investigations tend to disprove the concept of immune surveillance against tumours (Moller, 1976) and this would argue against any immunodepression theory.

iii) Shedding of Tumour Antigen. Some tumours have been found to shed their TAAs under certain conditions (Currie and Alexander, 1974).

iv) Antigenic Modulation. Antigens which are subjected to a continuous immune response may disappear by a process that is known as modulation (Boyse and Old, 1969).

v) Inhibition and Blocking. Virtually all tests in vitro for cell-mediated immunity can be inhibited by the addition of serum from a tumour-bearing animal. It is believed that two mechanisms may be involved (Baldwin and Price, 1976):-

Blocking occurs at the level of the tumour cell and involves masking of tumour antigen, probably by antibodies, in the early stages of tumour growth, and later, by immune

complexes.

Inhibition involves the receptors of the sensitised lymphocytes being blocked by immune complexes from the tumour cell surface, and by free tumour antigen, which may be shed late in tumour development.

v) Stimulation of the Tumour by the Immune System. (Prehn, 1976)

At present, although it is generally accepted that there is an immune response to cancer, there is no convincing explanation of how tumours become established in the face of this response.

The Relationship between Tumour-Specific and Non-Specific Immunity

In many cases it is not possible to measure tumour directed responses, due to lack of either biopsy material, or suitable cell lines, or both. In this situation, tests of general immunocompetence are substituted, since there is evidence that these bear some relevance to tumour directed responses. This evidence stems from two main sources:-

i) Therapies which stimulate general immunocompetence have been shown to have an anti-tumour effect e.g. Bacillus Calmette Guerin (BCG), Corynebacterium parvum and Levamisole (Morales & Eidinger, 1976a; Lieberman, Epstein & Fudenberg, 1974 ; Mathe et al, 1973 ; Israel, 1973). Israel and Edelstein (1975) have used C. parvum to treat more than 400 patients with advanced cancer. They found that it produced a significant increase in survival in cases of bronchogenic carcinoma and advanced breast cancer. Although C. parvum is known to be a potent immunological stimulant, resembling BCG (Stiffel et al, 1970), its use

in cancer immunotherapy depends upon both inhibitory and stimulatory responses. Carter (1976) has indicated that most immunopotentiating agents are more effective when they come into direct contact with the tumour, suggesting that their mode of action is complex and multifactorial. Nevertheless their use is based upon the stimulation of general, and thereby, tumour-directed, immunity.

ii) A correlation has been shown between the course of malignant disease and various assays of general immunocompetence in vivo (Eilber & Morton, 1970) and in vitro (Silverman et al, 1976). The nature of these tests, and their value as monitors of tumour-directed immunity, and clinical prognosis, are discussed in greater detail later. The assays cannot discriminate between cause and effect when used to monitor the response to therapy e.g. does an increase in immunocompetence during treatment reflect direct potentiation of tumour-directed responses, by the therapy, or is it a result of changes in the tumour itself, which secondarily stimulate the immune response?

However, the fact that the results from these tests can be related to the course of malignant disease, validates their use in charting the effects of therapy on both general, and possibly, tumour-directed immune responses.

Principle behind Tests of General Immunocompetence

Tests of general immunocompetence are based on three procedures:-

i) Determining the concentration of immunologically-competent cells in the peripheral blood.

ii) Determining the ease of *in vivo* sensitisation to, and subsequent rechallenge with, a previously unencountered antigen.

iii) Monitoring changes in pre-existing sensitivity to commonly occurring antigens (including histocompatibility antigens) *in vivo* and *in vitro*.

The methodology of, and results from, the various individual tests are reviewed in later sections.

All forms of cancer therapy exploit a sensitivity which is peculiar to the tumour cell. Whole-body hyperthermia treatment depends mainly upon selective heat sensitivity. However, this sensitivity is relative, and other organs and systems in the body, including the immune system, will be affected by generalised heating.

If one accepts that the immune system has a role in cancer control, it is of primary importance to determine the effects of hyperthermia therapy on the lymphoreticular system. In the absence of empirical evidence, it has been suggested that hyperthermia may either stimulate, or depress, tumour-directed immunity. Much of this speculation is based upon the disappearance of metastases, or immunity to tumour rechallenge, in animals whose primary tumour has been destroyed by localised heating, or electro-coagulation (for reviews of early literature see:- Nauts, 1970 & 1976 ; Dickson, 1976 ; Dietzel, 1976).

Dickson and Muckle (1972) have proposed a role for the immune response in the destruction of metastases, observed following local hyperthermia treatment of the VX-2 carcinoma in the rabbit. Similar animals, treated by local radio-frequency heating, showed increased delayed hypersensitivity skin responses to Bovine Serum Albumin (BSA), Dinitrochlorobenzene (DNCB) and tumour extracts; and lymphocytes from these rabbits caused greater inhibition of tumour cells, than those from untreated animals. Local heating of normal rabbits had no effect on the response to DNCB, but reduced the anamnestic response to BSA (Dickson & Shah, 1977).

Immunopotentiation by local heating has also been reported by Szmigielski et al (1977a). They subjected rats bearing Guerin epitheliomas in the logarithmic phase of growth, to local microwave

heating (intra-tumour temperature - 43°C ; maximum body temperature - 38°C), in three 45 minute fractions, during the third and/or fifth week after tumour implantation. In some cases, heating was supplemented by the administration of immunopotentiators - intra-peritoneal Streptolysin-S, or intra-tumoral C. parvum, during the period of treatment. Heat alone, whilst producing no cures, caused inhibition of tumour growth; however, combination with potentiators resulted in the disappearance of 75% of the tumours, and the regression of the remainder. Following hyperthermia alone, there was a significant increase in non-specific immunocompetence, as measured by:-

i) Serum titre of BSA antibodies (BSA antibody synthesis is T cell dependent).

ii) Incorporation of radio-labelled uridine or leucine by non-adherent spleen cells, in short-term culture with phytohaemagglutinin (PHA) (Response to PHA is a T cell phenomenon).

iii) Serum lysozyme activity.

Tumour-directed immunity, as assessed by:-

i) Blastogenic response of non-adherent spleen cells to Mitomycin-C treated Guerin cells.

ii) Microcytotoxicity tests, using non-adherent spleen cells, or peritoneal macrophages, against ^{51}Cr-labelled Guerin cells.

was also elevated. The largest increases were evident after two, three-fraction treatments, which was the regime that produced the best anti-tumour response. Combination with immunopotentiators produced increases in all of the immunological parameters, but only the addition of Streptolysin-S resulted in any significant

improvement over hyperthermia alone. Szmigielski has suggested that the increases in both general, and tumour-directed, responses, following local hyperthermia, may be due to activation of the immune system, either by necrotic tumour, or, by a change in the antigenicity of heat-suppressed, but still-viable, cancer cells.

There is some experimental evidence to support the hypothesis that heat may alter the antigenicity of cells. Mondovi et al (1972) found that immunisation of mice with Ehrlich ascites cells that had been heated in vitro for 3 hours at 42.5°C, conferred greater resistance to challenge, than injection with radiation-inactivated cells. They concluded that heating resulted in increased immunogenicity of the tumour, possibly by unmasking, or modification, of antigens on the cell surface. Similar results have been reported by Bourdon and Halpern (1976), who found that immunisation with tumour cells heated at temperatures ranging from 46-52°C, resulted in subsequent resistance to tumour challenge, and that this resistance could be adoptively transferred by the injection of immune peritoneal or spleen cells, into syngeneic hosts.

As an explanation for these phenomena, Rosen (1976) has proposed that hyperthermia reverses the binding of a repressor molecule to the cancer cell genome. This results in the cell losing the ability to produce antigens randomly, and thereby escape elimination by the immune system. However, the alteration in immunogenicity, following heating, is not restricted to intact cells. Bhatti, Ablin and Guinan, (1976) found that leucocytes from patients with genito-urinary tumours were more reactive to heated (40°C for 1 hour), rather than unheated, allogeneic, specific and non-specific tumour antigen extracts, in a leucocyte adherence inhibition assay.

In the only study on tumour directed immunity in vitro, in humans

being treated by local hyperthermia, Stehlin et al (1975) found that the cytotoxicity of melanoma patients' serum and lymphocytes, towards autochthonous, cultured, tumour cells, increased following heated perfusions of their cancer. This increase was seen after each treatment, and seemed to correlate with the amount of tumour destroyed.

Modification of tumour immunogenicity by local heating, and stimulation of the immune system by tumour debris, may explain some of the responses to hyperthermia that have been reported in vivo, when the heat is restricted to the region of the tumour. If whole-body hyperthermia is used the differential effect is lost, in that cells of the immune system are also subjected to increased temperatures. Preliminary results indicate that this complicates the situation.

Dickson and Muckle (1972) have reported an increased incidence of metastases in rabbits with VX-2 tumours treated by whole-body heating. They attribute this to a combination of immune depression and more favourable metabolic conditions for the seeding of viable tumour cells. Advancement of the appearance of metastases, in mice bearing the Lewis lung carcinoma, following generalised hyperthermia, has also been described by Yerushalmi (1976), who cites immune depression as one of the possible causes.

Recent work by Dickson and Shah (1977) supports this idea. They have shown that VX-2-bearing rabbits treated by local radio-frequency heating, followed 8 days later by total-body hyperthermia, displayed poorer responses to BSA, DNCB, and tumour-extract skin testing, and a lower cure rate, than animals treated by local heating alone. A combination of local heating and simultaneous, unintentional systemic heating (to approximately 40^{o}C), in rats with Yoshida sarcomas implanted in the leg, resulted in enhanced tumour dissemination, if the tumour had passed a critical size (Dickson &

Ellis, 1974). Dickson believes that this indicated that the immune system was still functional at 40°C, but may not be able to cope once the tumour burden has reached a certain level, and become 'activated' by inadequate heating (Dickson, 1976).

In contrast, Luczak et al (1977) and Szmigielski et al (1977b) have found that general microwave hyperthermia of mice, following infection by Herpes or Vaccinia virus, inhibits the establishment of the virus, and can prevent it completely in 80% of the cases of Herpes virus infections. This may indicate direct stimulation of the immune system, however, heat administered before virus infection, was ineffective, suggesting that hyperthermia may be affecting the multiplication, or immunogenicity, of the virus particles. Work on tissue culture cells favours the former explanation.

Whole-body heating, in the absence of introduced antigen, in the form of tumour cells or viruses, has been reported to decrease the delayed hypersensitivity skin response to BSA, but not to DNCB (Dickson and Shah, 1977). Degenerative changes in the lymphoid tissue have been described, following whole-body heating of normal rats to 41°C (Williams & Galt, 1977), and are associated with a depression of general immunocompetence (Galt & Williams, 1976).

The results of heating animals in vivo correlate with several observations in vitro. Harris (1976) has found that mouse splenic T cells, heated for 45min at 43°C, virtually lost their capacity to lyse allogeneic mastocytoma cells, although still 70% viable by dye exclusion. Mendes, Saraiva and Santos (1975) have reported that human T lymphocytes no longer rosetted with sheep erythrocytes (an accepted marker for T cells) after heating in vitro for 1 hour at 45°C.

Little work has been done on the effects of heat in vivo on human immunocompetence. Temperatures of 38.8°C have recently been

reported to result in increased B cell, decreased T cell, and unchanged total lymphocyte levels in healthy volunteers (Bork-Wolwer, Buhring & Krippner, 1977). Only slight changes in these parameters were observed in healthy adults heated for 1 hour at 40^{o}C, although a significant increase in the lymphocyte response to PHA was observed (Fabricius et al, 1977).

While studies on normal subjects are of fundamental importance to our understanding of the mode of action of hyperthermia therapy, they cannot be applied directly to the clinical situation. Patients undergoing heat therapy are likely to have impaired immunocompetence due to their advanced disease, in addition, they will be subjected to higher temperatures for longer times, and will receive various medications during their treatment. These procedures are all likely to affect the immune system, but , at present, they are inherent in nearly all forms of whole-body hyperthermia therapy, and must be considered as an integral part of the treatment.

There is only one study of the immunocompetence of whole-body hyperthermia patients, and this mentions several of these factors.

DeHoratius et al (1977) have monitored various indices of general immunocompetence in patients with disseminated malignancy, treated by whole-body heating to 42^{o}C for 2 hours. Results from three patients have been reported, two of whom received concomitant chemotherapy.

They found an increase in leucocyte count, 15 hours after treatment, but no significant changes in the percentage, or total lymphocyte count. There was no significant change in the percentage or total number of T or B cells, although rapid-rosetting T cells rose significantly in numbers after treatment. Lymphocyte response to mitogens (PHA, Concanavalin A and Pokeweed Mitogen) showed no

general trends, although there were changes in individual patients. Antibody-dependent cellular cytotoxicity, mediated by lymphocytes against chicken erythrocytes, was unaffected by hyperthermia, whereas polymorph-mediated cytotoxicity was depressed in all three patients. There was a marked fall in the concentration of complement, as C'3, after the first treatment, and C'4, C3PA and CH50 components were also depleted. Levels of IgG, IgA and IgM were not persistantly affected by treatment.

They believe that whole-body heating may have a beneficial effect upon the immune balance between the patient and his tumour, however the study is only preliminary, and they emphasise the need for more work on this aspect of hyperthermia tretment.

The aim of this research was to try to determine whether the whole-body hyperthermia treatment, used by Pettigrew to treat cases of advanced malignant disease, produced any changes in the general immunocompetence of the patients; and, if so, whether these changes could be related to the clinical response to therapy.

PREFACE TO THE PAPERS ON IMMUNOCOMPETENCE

Preface to the Papers on Immunocompetence

Introduction

The studies on the immunocopetence of hyperthermia patients, and the parallel experimental work on the effects of heat *in vitro*, are presented in separate papers, each of which describes a particular assay. The factors governing both the choice of these assays, and the selection of hyperthermia patients, are detailed in this preface.

Selection of Patients for Immunocompetence Studies

Every patient given whole-body hyperthermia treatment during the period of this research project, was considered for inclusion in the immunocompetence studies. Patients were excluded from the study if:-

i) they were grossly leucopenic (fewer than 2×10^6 leucocytes per ml peripheral blood) or anaemic.

ii) they were difficult to bleed.

iii) they proved to be very immunosuppressed.

A record was kept of all previous forms of therapy, together with procedures likely to have a marked effect on the patient's immune status.

Selection of Normal Control Subjects

Normal control subjects were obtained from healthy volunteers amongst hospital staff, and donors at two sessions organised by the South East Regional Blood Transfusion Service. All were informed of the nature of the experiments for which their blood was required. None was taking medications, or was Australia Antigen positive. All the samples were taken by a physician, or qualified haematology technician, using venepuncture of the median cubital vein.

Non-Hyperthermia Patients

A few blood samples were taken from hospital patients with non-malignant conditions. These were obtained with the permission of the patient, and the consultant in charge of the case.

Immunity Studies

Since the patients treated by hyperthermia were suffering from a variety of tumours (See Table 2), it was not feasible to study their tumour-directed immunity, as this would have entailed, either establishing a large number of human tumour cell lines (a notoriously difficult procedure), or extracting a range of soluble tumour antigens. Although the latter was technically possible, it had to be ruled out, since the majority of tumours were not biopsied during treatment, and consequently, any changes in their antigenicity could not have been followed. As a result, a range of tests was used to monitor the effects of hyperthermia therapy on the patients' general immunocompetence.

Selection of Assays for Monitoring General Immunocompetence

The tests which were selected had to fulfil several criteria:-

i) As well as indicating general immune status, they should, where possible, reflect tumour-directed immunity.

ii) They should involve the minimum of discomfort to the patient.

iii) Micro-modifications of the tests should be available, to avoid the repeated withdrawal of large volumes of blood.

iv) The tests should not assume prior exposure to a specific antigen.

PATIENT	DIAGNOSIS	PREVIOUS TREATMENT			NO. OF HEAT TREATMENTS	CLINICAL RESPONSE
		SURGERY	RADIO-THERAPY	CHEMO-THERAPY		
A.S. ♂ 77	CARCINOMA OF THE STOMACH				1	NR
H.A. ♀ 54	HYPERNEPHROMA		√	√	3	?
P.N. ♂ 49	PILONIDAL SINUS	NON-TUMOUR PATIENT			3	?
M.D. ♀ 36	HYPERNEPHROMA	√		B.C.G.	4	?
L.K. ♂ 63	CARCINOMA OF THE CAECUM	√		√	4	NR
D.G. ♂ 63	FIBROSARCOMA IN LUMBAR SPINE AREA		√		3	R
I.W. ♀ 46	GASTRIC CARCINOMA WITH KRUKENBERG METASTASES			√	3	R
M.M. ♀ 56	CARCINOMA OF THE COLON			√	2	?
M.P. ♂ 60	HYPERNEPHROMA				2	?
T.M. ♂ 66	CARCINOMA OF THE BRONCHUS				5	?
J.C. ♂ 64	ADENOCARCINOMA OF THE PROSTATE		√		2	?
L.T. ♀ 73	PSEUDOMYXOMA				2	?
K.S. ♂ 35	ADENOCARCINOMA OF THE RECTUM	√		√	3 / 2	NR / NR
A.W. ♂ 23	ADENOCARCINOMA OF THE COLON	√			2	R
R.M. ♂ 65	CARCINOMA OF THE STOMACH			√	2	NR
W.M. ♂ 39	LEIOMYOSARCOMA OF SMALL BOWEL	√	√		1	NR
A.B. ♂ 51	ADENOCARCINOMA OF THE RECTUM	√			1	?
E.C. ♀ 51	LEIOMYOSARCOMA		√	√	2	R

TABLE 2

CLINICAL DETAILS OF HYPERTHERMIA PATIENTS STUDIED BY IMMUNOCOMPETENCE TESTING

R = Responsive NR = Non responsive ? = Unclassified

v) The test should not take longer than 4½h to set up. Hyperthermia treatments rarely last longer than 5h, and therefore a time limit has to be imposed on the establishment of any test, since it is not feasible to deal with pre-, end- and in-treatment blood samples simultaneously.

A number of assays were examined to determine their suitability (Table 3). On the basis of this assessment it was decided to use the following tests:-

i) Peripheral blood leucocyte and lymphocyte concentration.
ii) Peripheral blood T and B lymphocyte concentration.
iii) Blastogenic response of lymphocytes to Phytohaemagglutinin.
iv) Antibody-dependent cellular cytotoxicity testing.
v) Serum immunoglobulin and complement concentrations.

as these satisfied virtually all of the criteria. A more detailed analysis of the value of each of the selected tests is given in the introductory section to the paper on its use.

The remaining tests were rejected on the following, more detailed grounds:-

DNCB Sensitisation and Skin Testing This test involves topically sensitising the patient *in vivo* to a measured dose of a hapten - 2,4-dinitrochlorobenzene (DNCB). After 14 days, during which delayed hypersensitivity will have been developed, the subject is challenged with much smaller doses, and the ability to respond to these challenges is graded to give an overall hypersensitivity rating (Lee *et al*, 1975). This test has many advantages:-

i) Circulating antibodies do not develop with contact sensitisation, and so it is a good measure of cell-mediated

TEST	CRITERIA				
	REFLECT TUMOUR IMMUNITY (i)	MINIMUM PATIENT DISCOMFORT (ii)	MICRO-METHOD AVAILABLE (iii)	PRIOR EXPOSURE TO ANTIGEN NOT REQUIRED (iv)	TIME FACTOR (v)
1. LEUCOCYTE / LYMPHOCYTE COUNTS	√	√	√	√	√
2. T AND B LYMPHOCYTE COUNTS	√	√	√	√	√
3. BLASTOGENIC RESPONSE TO PHYTOHAEMAGGLUTININ	√	√	√	√	√
4. K CELL ACTIVITY	?	√	√	√	√
5. SERUM IMMUNOGLOBULIN & COMPLEMENT CONCENTRATIONS	X ?	√	√	√	√
6. DNCB SENSITISATION & SKIN-TESTING	√	X	√	√	√
7. RECALL ANTIGEN SKIN-TESTING	√	?	√	X	√
8. LYMPHOCYTE TRANSFER TEST	?	√	X	√	X
9. LEUCOCYTE MIGRATION / ADHERENCE INHIBITION TEST	?	√	√	X	√
10. MIXED LYMPHOCYTE CULTURES	?	√	√	√	X?

TABLE 3

ASSESSMENT OF THE SUITABILITY OF TESTS OF GENERAL IMMUNOCOMPETENCE FOR USE ON HYPERTHERMIA PATIENTS

See text for details

immunity (Waksman, 1960).

ii) Both the sensitising and challenging procedures are controlled by the investigator.

iii) 95% of normal subjects can be sensitised, thereby reducing the number of false negatives (Kligman & Epstein, 1959).

iv) It detects defects in both the afferent and efferent limbs of the immune system.

v) Changes in immunocompetence, detected by this test, have been shown to correlate with the patient's clinical course (Eilber, Nizze & Morton, 1975 ; Pinsky et al, 1974) and in certain tumours, with the stage of the disease (Huus et al , 1975 ; Eilber & Morton, 1970 ; Schellhammer et al 1976).

Although permission for its use was obtained from the Hospital Ethical Committee, this test was not used because:-

i) Patients were not always available to allow pre-treatment sensitisation and challenge.

ii) In a trial run, it produced a painful, irritant, reaction, soon after the application of the sensitising dose (Hartman, Hoedemaeker & Nater, 1976).

iii) It has been found not to correlate with prognosis, or response to therapy, in some cases (Mandel, 1976).

iv) It was not possible to obtain sufficient age-matched controls.

Skin Testing with Recall Antigens This technique tests the functional integrity of the efferent branch of the immune system, by exposing the patient to a battery of antigens (by intradermal injection) with which he should have had previous contact e.g. Candida, Tuberculin

P.P.D., Mumps etc.. Assumed prior sensitisation allows the test to be performed at short notice, however this assumption also leads to a number of false negative responses. Results tend to be very variable,for example, Eilber and Morton (1970) found that 45% of patients found to be free of cancer were still negative to all of the seven antigens which they tested. Although the assays have been found to correlate with some measures of cell-mediated immunity in vitro (Brugarolas et al, 1973), their use was ruled out because of the lack of control over the sensitisation procedure.

The Human-to-Mouse Lymphocyte Transfer Test This test, originally developed by Rees and Symes (1973), involves measuring the induration produced by the graft-versus-host reaction resulting from the injection of human lymphocytes into a mouse. Lymphocytes from patients with a variety of tumours have been found to produce a diminished reaction. The test was rejected because it requires large numbers of lymphocytes (up to $1x10^7$) from both controls and patients. Furthermore, the relationship between the induration produced, and indices of tumour-directed immunity, remains to be established.

Leucocyte Adherence/Migration Inhibition Both of these assays have produced interesting results when used with antigen extracted from autochthonous or allogeneic tumours (See p. 32). Much less information is available on their use in conjunction with other types of antigen. Migration inhibition by P.P.D. has been studied in patients with trophoblastic tumours (Walden et al, 1976), but with disappointing results. Again, lack of control over prior sensitisation to the antigen, makes these tests unsuitable for routine use.

Mixed Lymphocyte Cultures Mixed lymphocyte cultures are widely used in work on tissue typing for transplantation surgery. Test lymphocytes undergo blastogenesis in response to histocompatibility antigens on the surface of stimulator lymphocytes (pre-treated with irradiation or Mitomycin-C, to prevent blast transformation). This test represents a graft-versus-host reaction in vitro, and as such, is a more 'natural' assay of lymphocyte activity, than response to a powerful mitogen. In addition, lymphocytes from patients with advanced cancer have been reported to show depressed responding and stimulating capacity in an MLC assay, in comparison to cells from a healthy control and disease-free patients (Blomgren et al, 1977). Similarly, an increased incidence of negative responses, by patients with untreated Hodgkin's disease, has been described, but the results did not correlate with the patients' clinical stage or symptoms (Lang et al, 1974). It was proposed to include this test, using a lymphoblastoid cell line (ODY-1, kindly supplied by Dr. C.M. Steel, M.R.C. Clinical and Population Cytogenetics Unit, Edinburgh) as a source of stimulator lymphocytes; however, the relevance of the results to tumour-directed immunity, and the time factor involved in setting up the test, made it unsuitable for inclusion.

Experimental Design

Since patients were not accepted for hyperthermia treatment on a regular basis, it was important to obtain the maximum amount of information from each patient. This meant that the assays had to be used on material from patients, before their worth could be fully evaluated. As a result, the normal evaluation experiments were carried out during the periods when no patients were being treated. Although this situation is not ideal, it was adaptable to a clinically-

oriented research project, the activity of which was dependent on the availability of the clinical material.

The selected assays were developed on an initial group of eight patients, the results from these tests were then analysed. The tests were subsequently extended, modified, or discarded, on the basis of this analysis, taken together with the results from normal individuals, and experiments in vitro.

GENERAL MATERIALS AND METHODS

Index of Materials

This index applies only to materials used in immunocompetence assays, and not to those used in tests performed by hospital service departments.

Reagents and Glass/Plasticware

Agglutination Tubes, Dreyer, Neutral Glass, Townson & Mercer Ltd., Edinburgh.

Air/CO_2 Mixture, 95%/5%, British Oxygen Co. Ltd., Edinburgh.

Araldite 'Rapid', CIBA/GEIGY (UK) Ltd., Duxford.

Blood Agar Base Number 2, Oxoid Ltd., London.

Blood Smearing Slides, Asschem, Falkirk.

Buffer Powder, for staining procedures, pH 6.8, Difco Labs., West Molesey.

Crystal Violet, BDH Chemicals Ltd., Poole.

Crystamycin, Glaxo Labs., Greenford.

Decon 75 Concentrate, Decon Labs. Ltd., Brighton.

Dextran T500, Pharmacia (Great Britain) Ltd., London.

Diaminoethanetetra-acetic acid, di-sodium salt, EDTA, BDH Chemicals Ltd., Poole.

Dimethyl Sulphoxide, DMSO, Grade 1, Sigma London Chemical Co. Ltd., Kingston-upon-Thames.

Eagles Basal Medium, Powdered, Wellcome Reagents Ltd., Beckenham.

EDTA Blood Collection Tubes, 1ml, Pediatric, Teklab Medical Labs., Durham.

Eosin, Water soluble, Yellow Shade, BDH Chemicals Ltd., Poole.

Euparal Essence, GBI Labs. Ltd., Manchester.

Euparal 'Vert' Mountant, GBI Labs. Ltd., Manchester.

Ficoll 400, Pharmacia (Great Britain) Ltd., London.

Ficoll-Paque, Pharmacia (Great Britain) Ltd., London.

Foetal Calf Serum, Heat Inactivated, Gibco Bio-Cult Ltd., Glasgow.

Freund's Adjuvant, Complete, Difco Labs., West Molesey.

Fungizone, (Amphotericin B), 250μg/ml, Gibco Bio-Cult Ltd., Glasgow.

Giemsa Stain, Exogen Modified, Exogen Ltd., Glasgow.

Glutaraldehyde EM, 25% Aqueous solution, Taab Labs., Reading.

Glutamine, L-Glutamine powder, Flow Labs. Ltd., Irvine.

Haemacytometer, Improved-Neubauer, Cristalite, Gelman Hawksley Ltd., Lancing.

Ham's F10 Medium, Powdered, with L-Glutamine, without Sodium Bicarbonate, Flow Labs. Ltd., Irvine.

HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid), Buffer powder, Flow Labs. Ltd., Irvine.

Human Serum, Pooled, Normal, Heat Inactivated, South East Regional Blood Transfusion Service, Edinburgh.

Hydrometer, Range 40.0 to Proof, Peter Stevenson Ltd., Edinburgh.

Hypaque, Sodium Powder, (Sodium Diatrizoate), Winthrop Labs., Surbiton-upon-Thames.

Lasso Adhesive Tape, T.J. Smith & Nephew Ltd., Welwyn Garden City.

Latex, Bacto, 0.8l, Difco Labs., West Molesey.

Lunch Boxes, Plastic, A. & J. Beveridge Ltd., Edinburgh.

May-Grunwald Stain, (Gurr), Searle Diagnostic, High Wycombe.

Membrane Filters, 0.22μm pore size, Millipore (UK) Ltd., London.

Microtest Plates, Tissue Culture Grade, Sterile,

Flat Bottom, Sterilin Ltd., Teddington.

V-Bottom, Sterilin Ltd., Teddington.

U-Bottom, (Linbro), Flow Labs. Ltd., Irvine.

Nitrogen Bank Ampoules, Polypropylene, Sterile, Nunc UK Ltd.,Stafford.

Nitrogen Bank Ampoule Canes, Jencons Scientific Ltd., Hemel Hempstead.

Phenol Red, BDH Chemicals Ltd., Poole.

Phosphate Buffered Saline Tablets, Dulbecco 'A', Oxoid Ltd., London.

Phytohaemagglutinin, Reagent Grade, Wellcome Reagents Ltd., Beckenham.

POPOP, (Phenyl-oxazolyl-phenyl-oxazolyl-phenyl), Sigma London Chemical Co. Ltd., Kingston-upon-Thames.

PPO, (2,5-Diphenyloxazole), Koch-Light Labs. Ltd., Colnbrook.

Pyroneg, Diversey Ltd., Barnet

Rabbit Anti-Sheep Red Cell Serum, Wellcome Reagents Ltd., Beckenham.

Roux-Pattern Tissue Culture Flasks, Pyrex, Central Neck, Townson & Mercer Ltd., Edinburgh.

Saponin, White, Suitable for Haemolysis, BDH Chemicals Ltd., Poole.

Scintillation Vials, Low-Potassium Glass, Pathlab Supplies Ltd., Gillingham.

Serum Storage Tubes, 5ml Crystal Polystyrene + Screw Cap, Label-Printed, Sterile, Raven Scientific Ltd., Haverhill.

Silicon Rubber Bungs:-

For Roux Flasks - Size E23,

For Dreyer Agglutination Tubes - Size E5,

Esco (Rubber) Ltd., London.

Sodium Bicarbonate, 4.4% Sterile Aqueous Solution, with Phenol Red, Wellcome Reagents Ltd., Beckenham.

Sodium Cacodylate EM, Taab Labs., Reading.

Sodium Chromate ^{51} Cr, solution, B.P., 1mCi/ml, Radiochemical Centre, Amersham.

Specimen Stubs, Aluminium, Agar Aids, Bishop's Stortford.

Thymidine (2-^{14}C), 50mCi/mmol, Radiochemical Centre, Amersham.

Toluene, Scintillation Grade, BDH Chemicals Ltd., Poole.

Tris, Tris(Hydroxymethyl)-Aminomethane, Hopkin & Williams Ltd., Romford.

Tubes, Flat-Bottom + Lid, 2ml, Product Number: NA2, Sterile, Sterilin Ltd., Teddington.

Trypsin Solution, (x10), 2.5%, Gibco Bio-Cult Ltd., Glasgow.

Tylocine, 5,000µg/ml, Gibco Bio-Cult Ltd., Glasgow.

Universal Containers, Plastic, Sterile, Sterilin Ltd., Teddington.

Uridine, (2-^{14}C), 50mCi/mmol, Radiochemical Centre, Amersham.

Visking Dialysis Tubing, Size 1, (8/32"), Medicell International Ltd., London.

All other reagents were 'Analar' grade, and were obtained from BDH Chemicals Ltd., Poole.

Cells and Continuous Cell Lines

H.Ep.2 Human epidermoid carcinoma of the larynx was supplied as a monolayer culture by the Department of Bacteriology, University of Edinburgh.

KB Human epidermoid carcinoma of the mouth was purchased, as a monolayer culture, from Gibco Bio-Cult Ltd., Glasgow.

Sheep Erythrocytes were supplied by courtesy of Dr. C.M. Steel, M.R.C. Clinical & Population Cytogenetics Unit, Edinburgh.

Rhesus Monkey Erythrocytes were supplied by courtesy of Inveresk Research International, Musselburgh.

Apparatus and Equipment

Automatic Cell Harvesters (with accessories):-

Titertek Multiple Cell Culture Harvester*, Flow Labs. Ltd., Irvine.

Mash II Multiple Automatic Sample Harvester, Dynatech Labs. Ltd., Billingshurst.

AutoMash Multiple Automatic Sample Harvester, Dynatech Labs. Ltd., Billingshurst.

Titertek Supernatant Harvester, Flow Labs. Ltd., Irvine.

Automatic Pipettes:-

Fixed Volume - Eppendorf, V.A. Howe & Co. Ltd., London.

Variable Volume - Pipetman, Anachem Ltd., Hale.
Finnpipette, Buckley Membranes Ltd., Great Missenden.

Centrifuges:-

Refrigerated - MSE 'Mistral' 4L, MSE Scientific Instruments, Crawley.

Bench - MSE 'Minor', MSE Scientific Instruments, Crawley.

Cytocentrifuge** - Shandon Elliot Cytospin, Shandon Southern Instruments Ltd., Camberley.

Critical Point Drier, Model: E3000, Polaron Equipment Ltd., Watford.

Differential Cell Counter, Manual, Clay-Adams Inc., U.S.A.

Dispensor, for Scintillator, Model: Oxford Pipetter, Boehringer Corp. (London) Ltd., Lewes.

Electric Digital Thermometers, Model: Ivac 841, Loxley Medical Supplies Ltd., London.

Gamma Sample Counter***, Model: 80000, LKB Wallac, U.S.A.

Photomicroscopy:-

Microscope - Orthoplan Wide Field, Leitz Wetzlar, F.R.G.

Camera - Orthomat-W Automatic Microscope Camera, Leitz Wetzlar, F.R.G.

Films - Black and White - Panatomic X, Eastman Kodak Ltd., London.
Colour - Photomicrography Film 2483, Eastman Kodak Ltd.

Scanning Electron Microscope*, Model: Stereoscan 180, Cambridge Instrument Co. Ltd., Cambridge.

Scintillation Spectrometer***, Model: TriCarb 2425, Packard, U.S.A.

Sterile Cabinet, Model: Laminar Flow Work Station, Microflow Ltd., Aldershot.

Water Baths:-

37^{o}C Unstirred, Thermostatic Bath, Model: JB3, Grant Instruments (Cambridge) Ltd., Cambridge.

Variable:-

Static Bath, Model: 020, Griffin & George Ltd., Glasgow.

Tempunit Constant Temperature Bath Controller, Model: TU-8 Techne (Cambridge) Ltd., Duxford.

Constant Level Device, Techne (Cambridge) Ltd., Duxford.

Key

* Facilities provided by courtesy of the M.R.C. Clinical and Population Cytogenetics Unit, Edinburgh.

** Facilities provided by courtesy of Dr. Anne Ferguson, Gastro-Intestinal Unit, Department of Medicine, Western General Hospital, University of Edinburgh.

*** Facilities provided by courtesy of the Department of Medical Physics, Western General Hospital, University of Edinburgh.

Animals

Mature New Zealand White Rabbits (Age 8-10 months) were used for the production of antisera (See p.174). These animals were fed (SG1 Rabbit diet, Oxoid Ltd., London, and fresh meadow hay) and watered ad libitum.

Preface

This section describes procedures which are common to more than one of the assays. Methods peculiar to any particular test are described in the section relating to that test.

Washing Procedure

Unless stated otherwise, plasticware was of tissue culture grade and was discarded after use.

All glassware was discarded into a 10% v/v dilution of Chloros in tap water, and then tissue culture washed, according to the following procedure:-

i) Rinse thoroughly in running tap water.

ii) Soak overnight in a solution of Pyroneg (2.4g/l) in tap water.

iii) Rinse thoroughly in running tap water.

iv) Soak overnight in tap water.

v) Rinse in deionised water.

vi) Soak overnight in glass distilled water.

vii) Dry in hot air oven.

Pipette-Tip Washing Procedure

New disposable pipette tips were soaked overnight in distilled water, and hot air dried. They were then packed into glass beakers, which were sealed with steam-permeable paper, and autoclaved (See p.61)

Scintillation Vial Washing Procedure

Waste scintillator and glass fibre filter pads were discarded. The vials were rinsed 6 times in methanol (Reagent grade) and left

to soak overnight in a 20% v/v solution of Decon Concentrate in distilled water. After rinsing in tap water the vials were washed in accordance with the schedule given on p.60.

Sterilisation

Glassware and apparatus was either heat sterilised, at a temperature of 160°C for 2h, or autoclaved, at a pressure of 15lb/in^2 for 15min. Solutions were autoclaved or membrane filtered through a filter of 0.22μm pore size (Millipore (UK) Ltd.).

Water

All aqueous solutions were prepared in glass-distilled, deionised water, which had been autoclaved and stored at 4°C until required.

Storage

Solutions were stored at 4°C for the stated period, unless indicated otherwise.

Tissue Culture Media

All media contained 0.06mg benzylpenicillin (sodium) and 0.1mg streptomycin sulphate per litre. These antibiotics were added together, in the form of a solution of Crystamycin.

Bicarbonate-Buffered Ham's F10 Medium - F10B

Ham's F10 Medium, powdered	9.90g
L-Glutamine powder	0.15g
$NaHCO_3$	1.20g
Water	To 1 l

The pH of the medium was adjusted to 6.90 with 1M Hydrochloric acid. The solution was membrane filtered and stored for up to

1 month.

HEPES-Buffered Ham's F10 Medium - F10H

Ham's F10 Medium, powdered.....9.90g

L-Glutamine powder.....0.29g

$NaHCO_3$.....0.85g

HEPES powder.....4.76g

Water.....To 1 l

The pH of the medium was adjusted to 7.15 with 1M Sodium Hydroxide. The solution was membrane filtered, and stored for up to 1 month.

Eagles Basal Medium

Eagles Basal Medium, powdered.....9.50g

Fungizone, (250 g/ml).....1ml

Water.....950ml

The solution was membrane filtered and stored for up to 1 month.

Growth Medium

Eagles Basal Medium, as above,.....150ml

4.4% $NaHCO_3$ solution.....8.0ml

Foetal Calf Serum, heat inactivated.....17.5ml

This medium was prepared immediately before use.

Sterility

The sterility of tissue culture medium was routinely tested by plating out samples onto Blood Agar Base 2 plates. These were incubated for up to 72h at $37^{o}C$, and checked for signs of bacterial and fungal growth.

Salt Solutions

Phosphate-Buffered Saline - Dulbecco 'A' - PBS'A' One buffer tablet was dissolved in 100ml water and the solution autoclaved, and stored for up to 3 months.

Tris-Buffered Ammonium Chloride Solution 100ml Tris buffer solution (24.3g/l) was added to 900ml 0.83% w/v Ammonium Chloride solution. The pH was adjusted to 7.20 with 1M Hydrochloric acid. The solution was membrane filtered, and stored at room temperature for up to 6 months.

Alsever's Solution

Dextrose.....20.50g

Sodium Citrate (Dihydrate).....8.00g

Citric Acid (Monohydrate).....0.55g

NaCl.....4.20g

Water.....To 1 l

The ingredients were dissolved successively in the water, and the final solution autoclaved (pH 6.1). The solution was stored until required.

Density Gradient Solutions for Cell Separations

Solution A

Hypaque sodium.....33.9% w/v in water, allowing for the water content of the powder. Membrane filtered and stored, in the dark, for up to 6 months.

Solution B

Ficoll 400.....9% w/v in water. Membrane filtered and stored for up to 6 months.

Before sterilisation, 3.75ml Solution A was mixed with 9ml Solution B and the specific gravity checked, using a mini-hydrometer. If it

was between 1.0076 and 1.0078 the solutions were membrane filtered, and mixed in this proportion immediately before use.

Trypsinising Solution for Cell Subculture

Trypsin solution, 2.5% w/v.....0.4ml

EDTA solution, 2% w/v.....0.2ml

Phenol Red solution, 0.1% w/v.....0.1ml

PBS'A'.....9.3ml

The mixture was prepared immediately before use.

Reagent Solutions

The following solutions were prepared and stored as indicated:-

Acetic Acid (glacial) 2% v/v in water, tinged with Crystal Violet. Used non-sterile. Stored at room temperature.

Dextran T500 6% w/v in physiological saline (see below). Membrane filtered or autoclaved. Stored for up to 3 months.

Eosin 0.1% w/v in physiological saline (see below). Autoclaved. Stored in the dark, at room temperature, until required.

EDTA 2% w/v in water. Membrane filtered. Stored until required.

Glutamine 3% w/v L-Glutamine in water. Membrane filtered. Stored at -20^{o}C for up to 3 months.

HEPES 500mM solution in water (119.5g/l). Membrane filtered. Stored at room temperature until required.

Phenol Red 0.1% w/v in water. Membrane filtered. Stored until required.

Physiological Saline Single strength (0.9% w/v) and double strength (1.8% w/v). Autoclaved. Stored at room temperature.

until required.

Saponin 15% w/v in water. Autoclaved. Stored until required.

Scintillator

PPO.....5.00g

POPOP.....0.30g

Toluene.....1 l

The toluene used was scintillation grade. The solution was left stirring, on a magnetic stirrer, for at least 6 hours before use. Scintillator was dispensed from an automatic dispensor. It was reused after being filtered through Whatman's No.1 qualitative paper, and checked for background radioactivity. Exhausted scintillator was disposed of by the Department of Medical Physics, Western General Hospital, University of Edinburgh.

Blood Sample Collections

Blood was obtained by venepuncture of the median cubital vein, using a siliconised 21-G needle. In some cases a Butterfly-21 intermittent infusion set (Abbott Laboratories Ltd., Queenborough.) was used when more than 20ml blood was required.

Separation of Lymphocytes from Peripheral Blood

The method used was that of Boyum (1968) with several modifications.

The needle was removed from the collecting syringe, and the blood gently expelled into a sterile plastic universal container, or glass tube (in the case of volumes greater than 25ml). It was slowly stirred with sterile wooden orange sticks, until the clot had formed, and retracted onto the sticks, which were then removed and discarded. The defibrinated blood was transferred to another universal container and centrifuged at 1,000g for 15min. The serum was removed to a sterile container (for storage at -25^{o}C), and replaced by an equal, or greater, volume of F10B. The cell pellet was gently resuspended by inverting the bottle several times. 4-9ml of the blood mixture was carefully layered onto 7ml Ficoll-Hypaque, or Pharmacia Ficoll-Paque solution, in a glass universal container. This was done by letting the blood flow slowly down the walls of the container and onto the surface of the Ficoll mixture. The bottle was then centrifuged at 400g, at the blood/Ficoll interface, for 30min at 18^{o}C.

The supernatant medium and the white lymphocyte band were removed to a sterile centrifuge tube, using a Pasteur pipette. The cells were pelleted by centrifugation at 800g for 15min. The pellet was washed in 10ml F10B and recentrifuged for 10min.

Contaminating erythrocytes were removed by incubating the cells in 1ml Tris-buffered Ammonium Chloride solution, for 5min at 37°C. 9ml F10B was then added to the tube and the cells mixed by gentle pipetting before centrifugation at 800g for 7min. The cell pellet was resuspended in 10ml F10B, and recentrifuged for a further 7min. The cells were finally resuspended in the appropriate medium for the particular test, and the concentration of viable cells assessed by Eosin exclusion.

This method routinely produced approximately $1x10^6$ viable cells per ml. of normal blood. Viability was usually in excess of 95%. The yield of cells from cancer patients' blood was often lower, as reported by Greaves (1977) for various disease states.

The purity of the cell separation was determined by making cytocentrifuge preparations of $2\text{-}7x10^4$ cells in a total volume of 200-400µl medium. This suspension was centrifuged onto a very clean glass slide, at a speed of 1,500 rpm for 10min. This separation technique usually produced greater than 95% mononuclear cells, mainly small lymphocytes (Fig.1), when used on normal blood. The main contaminants were neutrophils and eosinophils.

Separation of Granulocytes from Peripheral Blood

The method used was that of Boyum (1974).

Granulocytes were isolated from the cell pellet remaining after the separation of lymphocytes from whole blood. Each cell pellet was resuspended in 10ml PBS'A' and transferred to a plastic universal container. The glass bottle was rinsed with a further 5ml PBS'A', which was added to the cell suspension. The cells were pelleted by centrifugation at 1,000g for 15min, and then resuspended in F10H supplemented with 10% v/v autochthonous serum,

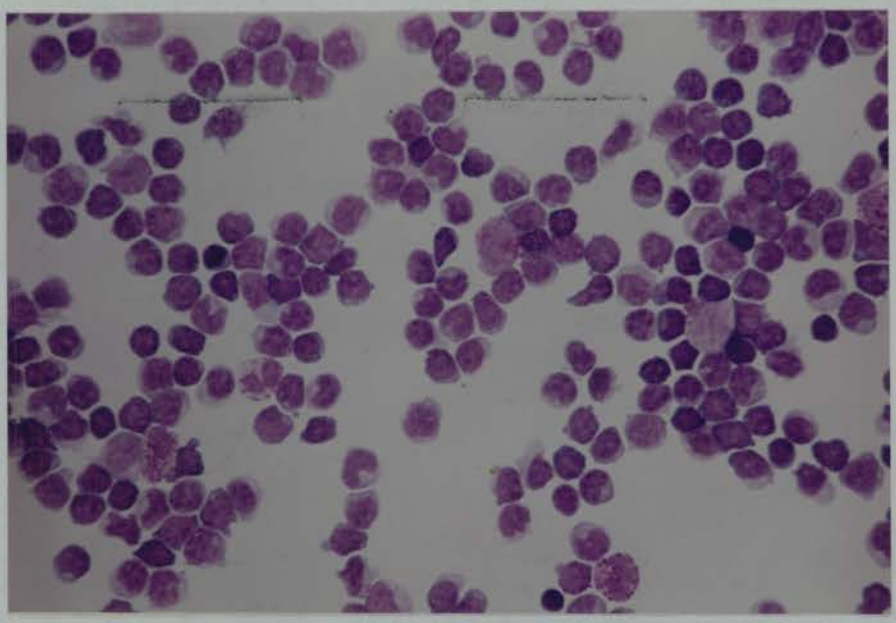


FIGURE 1

CYTOCENTRIFUGE PREPARATION OF FICOLL/HYPAQUE-SEPARATED HUMAN MONONUCLEAR LEUCOCYTES

May-Grunwald/Giemsa stained (Magnification x445)

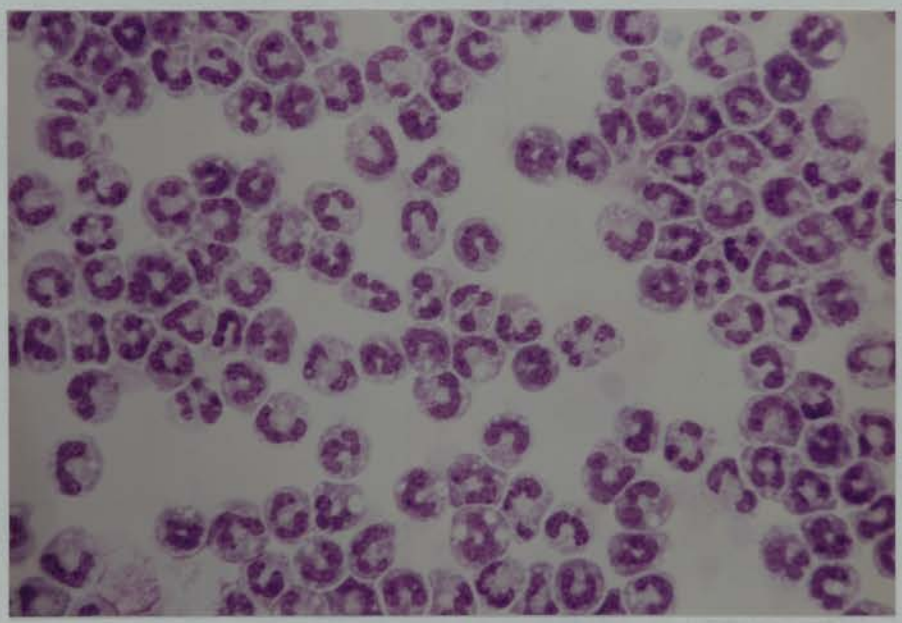


FIGURE 2

CYTOCENTRIFUGE PREPARATION OF GRANULOCYTES SEPARATED FROM HUMAN PERIPHERAL BLOOD

May-Grunwald/Giemsa stained (Magnification x445)

and the appropriate volume of 6% w/v Dextran T500 in saline, added, e.g. for 9ml whole blood, the cell pellet was resuspended in 4.5ml F10H + serum, and 1.35ml 6% Dextran added.

After vortex mixing, the suspension was transferred to a clean plastic universal container, care being taken to avoid trapping air bubbles in the mixture. The erythrocytes were then allowed to sediment for approximately 20min, when the granulocyte-rich supernatant was transferred to another plastic universal bottle. The cells were spun down at 800g for 7min, and the contaminating erythrocytes removed, either by Tris-buffered Ammonium Chloride treatment (See p. 67), or by resuspending the cell pellet in sterile water for 5sec, and then adding an equal volume of 1.8% w/v saline. The cells were recentrifuged for 7min, washed once in 15ml F10H + 10% v/v foetal calf, or autochthonous, serum, and resuspended in the appropriate medium for the assay.
The concentration was assessed by counting a dilution of the suspension in 2% acetic acid, this prevented the cells attaching to the haemacytometer during counting, as this hinders focusing. Viability of a second dilution, in medium, was determined by eosin exclusion.

This technique produced variable results, with regard to yields (up to $4x10^6$ granulocytes/ml whole blood), and purities (45-99% See Fig. 2), depending on the blood sample. Viability was routinely in excess of 98%.

Determination of Cell Concentrations and Viability

The concentration and viability of cell suspensions was determined in a haemacytometer, using the eosin exclusion method (Schrek, 1936).

Culture of Continuous Cell Lines

H.Ep.2 and KB human tumour cell lines (fuller details of which are give on p.174) were grown in monolayer culture in Roux flasks.

Both cell lines were cultured in Growth Medium. This was supplemented, in the case of the KB cell line, by a final 1% v/v concentration of Minimum Essential Medium Non-Essential Amino Acids. The media were gas buffered using an humidified 95%/5% Air/CO_2 mixture.

Tylocine solution was occasionally added to the medium to a final concentration of 1% v/v, to combat any suspected mycoplasma infection.

Subculturing and Passaging

Confluent cell monolayers were washed twice in 100ml PBS'A' and then incubated, at room temperature, with 10ml Trypsin solution (See p. 64), until the cells became detached from the glass. 10ml growth medium was added to each flask to inactivate the trypsin. The suspension of cells was transferred to a plastic universal container, and centrifuged at 800g for 5min. The cell pellet was resuspended in 2ml growth medium and gassed. A viable count was made of a 1/10 dilution of this suspension, and from this, $3x10^6$ cells were seeded into a Roux flask containing 100ml growth medium. The flask was gassed and incubated at 37^oC.

Fully-confluent monolayers could be maintained in a viable condition, for several days, by replacing the growth medium with bicarbonate-buffered Basal medium containing a 2% v/v concentration of serum - Maintenance Medium.

Preparation of Cell for Storage in Liquid Nitrogen

The possible loss of a continuous cell line was prevented by long-term storage of samples in liquid nitrogen. The cells were

harvested in the usual way, and adjusted to a concentration of 3.4×10^6/ml in growth medium. Dimethyl Sulphoxide was then slowly added to this suspension, with thorough mixing, to give a final concentration of 10% v/v. 3×10^6 cells, i.e. 1ml of the cell suspension, were seeded into Nitrogen bank ampoules, which were sealed and placed in an expanded-polystyrene rack, wedged in the neck of a vacuum flask, half-full of liquid nitrogen. The flask was kept closed for at least 3h, when the vials were rapidly transferred to the nitrogen bank, for long-term storage.

Recovery of Cells from the Nitrogen Bank

Vials were removed from the bank, and rapidly thawed in water at 37°C (taking precautions against the possible explosion of the vial). The contents of the ampoule were transferred to a universal bottle containing 20ml growth medium, and the cells pelleted by centrifugation at 800g for 5min. The cell pellet was resuspended in 10ml growth medium and recentrifuged. The cells were finally dispersed in 2-5ml medium and transferred to a plastic tissue culture flask, containing about 50ml growth medium, which was gassed, sealed, and incubated at 37°C.

Viability of cryopreserved cells was routinely greater than 90%, by eosin exclusion.

The Pettigrew Technique of Whole-Body Hyperthermia Therapy

This safe, controllable, technique has been used by Pettigrew to treat more than 100 patients in Edinburgh (Pettigrew, 1976), and is also in use in other centres (Mackenzie et al, 1976 ; Levin, 1977). The method is described in some detail since it was used by Pettigrew to treat all the patients studied as part of this research.

Selection of Patients All the patients treated by whole-body hyperthermia had previously received conventional forms of therapy e.g. surgery, radiotherapy or chemotherapy, with little or no success, and had reached the terminal stages of their disease. Although these patients were largely unselected as to tumour type, the majority had gastro-intestinal malignancies (Table 2).

Every patient gave informed consent to the nature and use of hyperthermia, and fully understood that the treatment offered no guarantee of success.

Hyperthermia Treatment The patient, who had been fasted overnight, was first narcotised (Thiopentone) and curarised (Curare). He was then intubated with a thermally-insulated endotracheal tube, and maintained on an oxygen/nitrous oxide mixture. An epidural catheter was inserted, and the patient transferred to a specially-constructed bath. A long-line intravenous catheter was inserted into the median cubital vein, and threaded into the superior vena cava. This was connected, via a three-way tap, to allow both the intravenous infusion of pre-warmed ($43^{o}C$) fluids, and the withdrawal of blood samples, including those used in the immunity studies.

A naso-gastric tube and urinary catheter were passed, and left on free continuous, drainage. Cardiovascular monitoring was by

FIGURE 3

WHOLE-BODY HYPERTHERMIA TREATMENT

Patient immersed in molten paraffin wax (50°C) at the start of heating

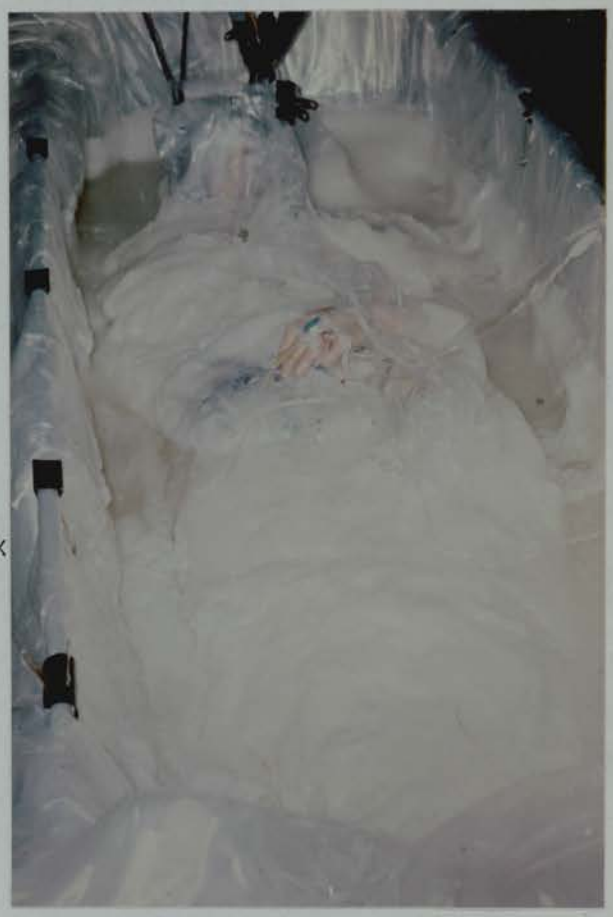


FIGURE 4

WHOLE-BODY HYPERTHERMIA TREATMENT

Patient at peak temperature (41.8°C) Molten wax has been removed, leaving the patient enclosed in a thermally-insulating carapace of solidifying wax

means of ECG chest electrodes, a thumb pulsometer and a blood-pressure cuff. The temperature was recorded using 5 thermometers:- 2 rectal, 2 oesophageal (at different levels), and one tympanic probe; the readout was both graphic and visual. The eyes and all possible pressure areas were padded, and an intravenous diuretic (Lasix) given. Ventilation was then maintained via a heat exchanger, which warmed the inspired gases to about 80°C, and so counteracted the heat losses via the respiratory surfaces. The plastic bag, containing the patient, was then heat sealed, and molten paraffin wax, at 50°C, was pumped into the bath, until the body was almost completely immersed (Fig. 3). During treatment anaesthesia was maintained by topping-up the epidural line with Lignocaine (Xylocaine), and by intravenous Operidine, as required. Further injections of diuretic were given if the urinary output was low.

The rectal temperature rose at between 3 and 6°C per hour, depending on body weight, until it reached 41.8°C, when the wax was siphoned off, leaving the patient in an insulating carapace of solidifying wax (Fig. 4). The rectal temperature continued to rise slowly, and at 41.9 - 42°C the bag was opened and the skin was exposed, to allow cooling by the evaporation of sweat. By varying the area of exposed skin, and the thickness of the insulating layer of wax, the temperature could be controlled to within 0.1°C of 41.8°C (Fig. 5).

At the end of the hyperthermia period - usually 2-5h - the patient was removed from the bath, and cooling was rapid (2-3°C/h). Patients were returned to the recovery room overnight, and were discharged to the ward, or home, the following day.

Treatments were given in fractions, which were standardised for different temperature/time equivalents by using the Centigrade

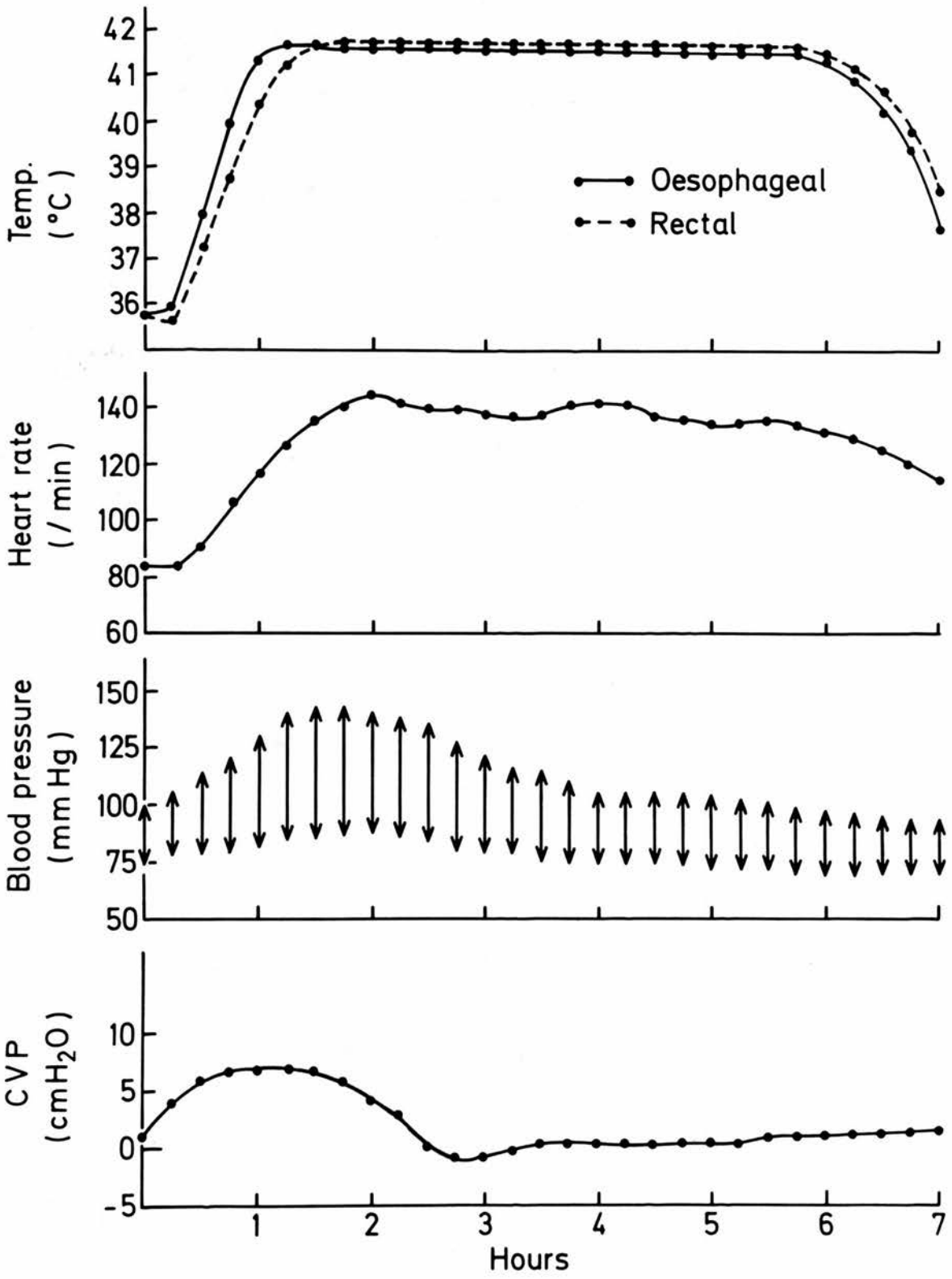


FIGURE 5

CHANGES IN BODY TEMPERATURE AND CARDIOVASCULAR PARAMETERS DURING WHOLE-BODY HYPERTHERMIA TREATMENT

From: Pettigrew (1976)

Degree Minute ($C^{o}M$). This is defined as the number of Centigrade degrees by which the rectal temperature exceeds $40^{o}C$, multiplied by the time over $40^{o}C$, in minutes. Most patients were treated in serially-increasing, weekly, fractions, starting at $300C^{o}M$ (Ludgate et al, 1976).

Assessment of the Clinical Response to Hyperthermia Therapy

The response to treatment was judged to be favourable if there was either pain relief, or weight gain, in addition to one of the following:-

i) Regression of tumour size on measurement.

ii) Radiological evidence of regression.

iii) Pathological evidence of necrosis in serial biopsy sections.

Safety and Complications of the Technique

The safety of this technique for inducing hyperthermia is demonstrated by the fact that over 300 sessions have produced remarkably few complications, especially since the introduction of epidural anaesthesia (Pettigrew, 1976). This has reduced the requirement for intravenous barbiturates and narcotics, and hence problems of liver detoxication. Approximately one third of the patients develop circum-oral Herpes simplex, but this only occurs after the first treatment. Pressure sores can be a problem if padding is insufficient. Lack of fractionation can result in consumption coagulopathy of the blood elements (Ludgate et al, 1976), and this may be a limiting factor in this form of therapy. The method is labour-intensive, requiring the constant attendance of a physician and nurse.

In conclusion, this technique still provides the safest, most controllable method for inducing whole-body hyperthermia.

PAPER A

THE EFFECTS OF WHOLE-BODY HYPERTHERMIA THERAPY ON THE TOTAL AND DIFFERENTIAL LEUCOCYTE COUNT

The Effects of Whole-Body Hyperthermia Therapy on the Total and Differential Leucocyte Count

Introduction

Leucocytes are the carriers of cell-mediated immune mechanisms. Meyer (1970) has stated that the lymphocyte level is an index of immune competence. This statement has been borne out by numerous experimental studies.

Zacharski and Linman (1971), in a survey of 7,850 patients, found that the most common factor associated with lymphopenia, was malignancy. Krant et al (1968) also noted a significantly lower lymphocyte count in the peripheral blood of patients with bronchogenic cancer, in comparison with age-matched normal controls.

Lymphocyte concentrations within the normal range, were found by King et al (1976), in lymphoma patients who had been successfully treated by chemotherapy, or radiotherapy, in spite of the fact that various other immune functions were impaired. In contrast, Cochran et al (1976) could find no significant difference between the total lymphocyte count, or percentage of lymphocytes, in the blood of cancer patients and controls. They concluded that these parameters 'had little or no place' in the purely immunological assessment of their patients. In the same study, they found no correlation between the lymphocyte count and the extent of the disease, whereas Morales and Eidinger (1976) found significantly lower counts in patients with metastases. They also noted that the number of lymphocytes declined with advancing disease. This observation had been made earlier by Krant et al (1968), who found that a smooth regression curve could be obtained by plotting the lymphocyte concentration against the time until

death. However they emphasised that there was nothing to indicate a causal relationship between the lymphopenia and clinical deterioration. Nonetheless, their results suggest that the total lymphocyte count could be used as an indicator of prognosis.

This has been confirmed by various workers. Papatestas and Kark (1974) showed that patients who had been free of metastases for 5 years, following mastectomy, had significantly higher lymphocyte concentrations than those who had subsequently developed metastases. A larger retrospective study was published in 1976 (Papatestas et al), which consolidated these observations, and suggested that low lymphocyte counts may be due to the presence of suppressor substances, possibly produced by the thymus.

Similar relationships between lymphocyte concentrations and clinical prognosis, have been described for a variety of tumours, e.g. breast (Fodor, 1976), bladder (Amin & Lich, 1974), colon (Kim & Papatestas, 1974) and advanced lung cancer (Wanebo et al, 1976). In a survey of nearly 600 patients, with different types of cancer, Riesco (1970) found a significant, positive, correlation between cancer curability and the total numbers of peripheral lymphocytes. This would suggest that the lymphocyte concentration could be used as an indication of the probable response to therapy, however, this is rarely the case.

Two of the most common forms of cancer treatment, namely radiotherapy and chemotherapy, are immunosuppressive, producing leucopenia and lymphopenia. As such, they can be likened to a double-edged sword, which attacks the tumour directly, but, by simultaneously depressing immunocompetence, leaves the patient vulnerable to both the re-establishment of his tumour, and to infectious diseases.

Induction of lymphopenia by radiotherapy has been reported in breast cancer (Jenkins et al, 1975 ; Glas et al, 1976 ; Blomgren et al, 1976), seminoma testis (Heier et al, 1975) and various other tumours (Byfield, Stratton & Cole, 1976 ; Raben et al, 1976 ; Stratton et al, 1975). However, one group (Braeman & Deeley, 1972), found that fractionated, radical radiotherapy for carcinoma of the bronchus, did not produce the fall in leucocyte numbers reported by other workers. In general, though, leucopenia usually results from both wide-field, and local radiotherapy (Stutz & Slawson, 1976), including, or excluding the thymus gland (Stratton et al, 1975).

Many of the chemotherapeutic drugs in use in cancer therapy are known to produce leucopenia as a side effect. This results mainly from their toxic effects on the rapidly-proliferating stem cells in the bone marrow. As a result, leucopenia is often a limiting factor in the effective treatment of cancer by either radiotherapy or chemotherapy. Immunotherapy with B.C.G. is still in its early stages, but reports indicate that it has relatively little effect on lymphocyte numbers (Bluming et al, 1972 ; Gutterman et al, 1973 ; Lui et al, 1975), and this may indicate that this is an effective cancer therapy without an associated vulnerable period of immunosuppression.

The purpose of this study was to determine whether the Pettigrew method for whole-body hyperthermia, produced changes in the total and differential concentration of peripheral blood leucocytes characteristic of other forms of cancer therapy, and whether these changes could be related to the patients' clinical status.

Methods

Total Leucocyte Count 1ml samples of peripheral blood from subjects under study, were collected in commercially-available plastic tubes containing EDTA. These were agitated for up to 1h on a blood cell suspension mixer. A 0.1ml sample was then transferred to a plastic tube containing 1.9ml 2% aqueous acetic acid. The pipette tip was rinsed several times in the acid solution to remove any residual blood. The tube was capped and mixed by inversion, before duplicate samples were counted in an improved-Neubauer haemacytometer.

The reproducibility of the counting and sampling techniques is shown in Table A1.

Differential Leucocyte Counts One drop of well-mixed, anti-coagulated (EDTA) peripheral blood was placed on a clean glass microscope slide, and smeared using a commercially-available blood-spreader slide. Smears were air-dried and stained using the following protocol:-

i) 'Analar' Methanol.....10min

ii) May-Grunwald Stain.....5min

iii) Giemsa Stain, diluted 1/10
in staining buffer pH6.8.....7min
Two changes

iv) Staining buffer pH6.8.....Rinse

Stained slides were air-dried and scanned under a x40 objective to assess the general appearance and quality of the smear. Differential counts were made using an oil-immersion x100 objective. Smears were longitudinally scanned, using the method described by Dacie and Lewis (1975). At least 200 leucocytes were classified per slide, although in patients with marked leucopenia

DILUTION NUMBER	COUNT NUMBER 1	2	3	4	5	MEAN OF 10 COUNTS OF 1 S.D.	MEAN OF MEANS ± 1 S.D.
SAMPLE A							
a	3.92	4.32	4.44	4.36	4.24		
	4.04	4.08	4.20	4.04	4.28		
MEAN	3.98	4.20	4.32	4.20	4.26	4.19 ± 0.17	4.19 ± 0.15
b	4.08						
	4.00						
MEAN	4.04						
c	3.96						
	4.00						
MEAN	3.98						
d	4.20						
	4.08						
MEAN	4.14						
e	3.96						
	3.88						
MEAN	3.92						
MEAN OF 10 COUNTS ± 1 S.D.	4.01 ± 0.09						
MEAN OF MEANS ± 1 S.D.	4.01 ± 0.08						
SAMPLE B	3.84	4.36	3.76	4.36	4.16		
	4.20	4.08	4.00	4.44	4.36		
MEAN	4.02	4.22	3.88	4.40	4.26	4.16 ± 0.23	4.14 ± 0.21

TABLE A1

REPRODUCIBILITY OF TOTAL LEUCOCYTE COUNTS

A and B are two samples of anticoagulated blood obtained from the same donor at the same time. For both samples replicate counts (1-5) were made on a 1/20 dilution of the blood in 2% acetic acid. Both chambers of the haemacytometer were used for each count, and a mean calculated. In addition, similar counts were made on five separate dilutions of Sample A (a-e) and means calculated.

Counts are shown as leucocytes/ml peripheral blood $\times 10^{-6}$

SLIDE CODE	NEUTROPHILS %	LYMPHOCYTES %	MONOCYTES %	EOSINOPHILS %	BASOPHILS %
A	59.0	34.0	4.5	2.0	0.5
	57.0	33.5	4.0	4.0	1.5
	59.5	33.5	5.5	1.0	0.5
	56.5	37.0	5.5	0.0	1.0
	61.0	33.0	4.0	1.5	0.5
MEAN ± 1 S.D.	58.6 ± 1.8	34.2 ± 1.6	4.7 ± 0.8	1.7 ± 1.5	0.8 ± 0.4
B	57.0	37.0	4.0	1.5	0.5

TABLE A2

REPRODUCIBILITY OF DIFFERENTIAL LEUCOCYTE COUNTS OF 200 CELLS

Slides A and B were prepared from the same sample of normal human blood. Slide A was counted 5 times to determine the reproducibility of cell classification. Slide B was counted once to indicate the reproducibility of the blood smearing technique.

the number was occasionally reduced to 100. The criteria used for classification were those of Diggs, Sturm and Bell (1973).

The reproducibility of the technique is shown in Table A2.

Phagocytosis Assay

Materials

Bacto Latex 0.81μ - dialysed in Visking tubing, for 4 days against four changes of glass-distilled water, and stored at $4^{o}C$, until required.

Tubes - 2ml capacity, flat bottomed tubes, code NA2, with cap. Supplied sterile. Sterilin Ltd., Teddington.

Method

Isolated polymorphonuclear leucocytes were resuspended in a few ml of F1OH, and the concentration determined by counting a dilution of the suspension in 2% v/v acetic acid (to prevent cells attaching to the haemacytometer during counting and thereby hindering focusing). The viability of a second dilution of cells in medium, was determined by dye exclusion.

Tubes for phagocytosis assays were prepared as follows:-

i) Viable polymorphs.....$2.5x10^{6}$ cells

ii) Autochthonous serum.....0.1ml

iii) Dialysed latex diluted 1/25 or 1/50 in F1OH 0.1ml

iv) F1OH.....to 1ml

The tubes were capped, briefly vortex mixed, and incubated for 30min in a $37^{o}C$ water bath. Each tube was gently shaken and replaced for a further 30min. The cells were centrifuged at 800g for 5min, and the cell pellet carefully resuspended in 1ml F1OH supplemented with 20% v/v foetal calf serum. 10-20μl of the cell suspension was transferred to a tube containing 0.4ml medium

and this suspension was cytocentrifuged at 1,500-1,700rpm for 10min. The preparation was air-dried, and May-Grunwald/Giemsa stained. Stained preparations were mounted in Euparal 'Vert' and examined using an oil immersion x100 objective. Cells were classified into three groups, namely, those containing:-
i) no latex, ii) 1-5 particles, iii) 6+ particles (Fig. A1).

Classification was sometimes facilitated by the use of phase contrast, or dark-field illumination.

Serum Cortisol Measurements

The concentration of cortisol in serum samples obtained from hyperthermia patients during and after treatment, was measured by the Department of Clinical Chemistry, Western General Hospital, Edinburgh, using the method described by Mattingly (1962).

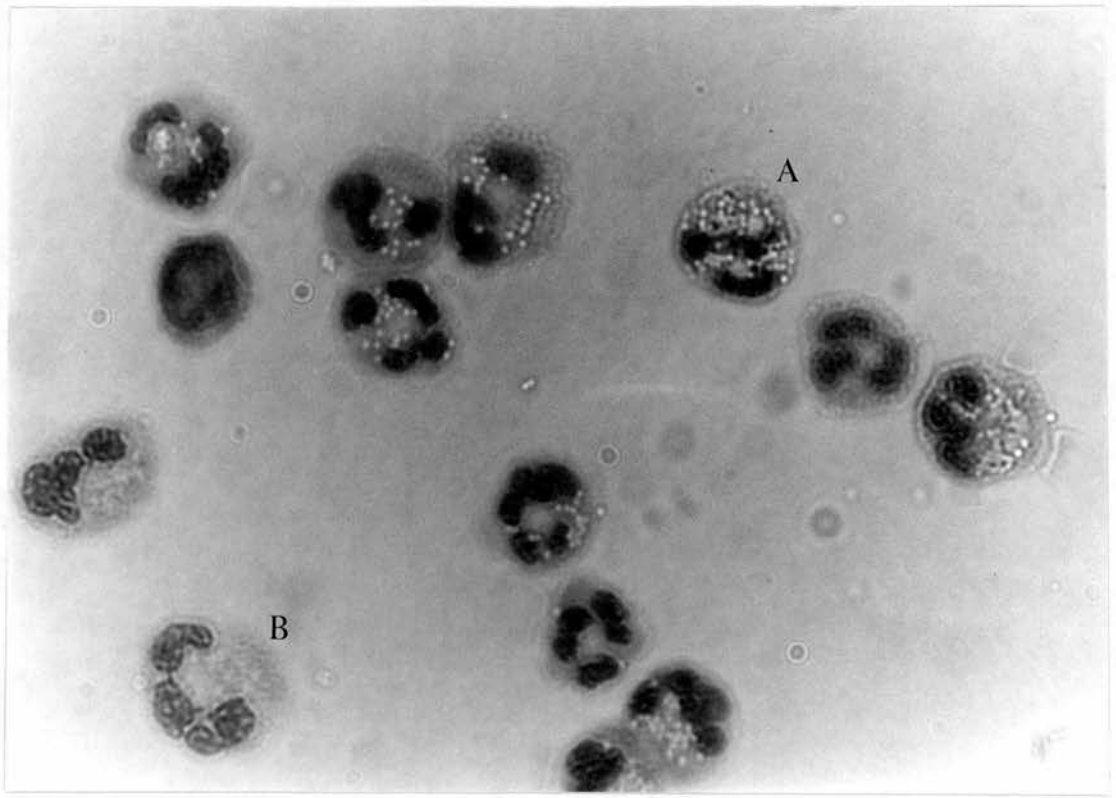


FIGURE A1a

CYTOCENTRIFUGE PREPARATION OF PERIPHERAL BLOOD GRANULOCYTES SHOWING PHAGOCYTOSED LATEX PARTICLES
A = Cell containing numerous particles
B = Cell apparently containing no latex
May-Grunwald/Giemsa stained (Magnification x1,490)

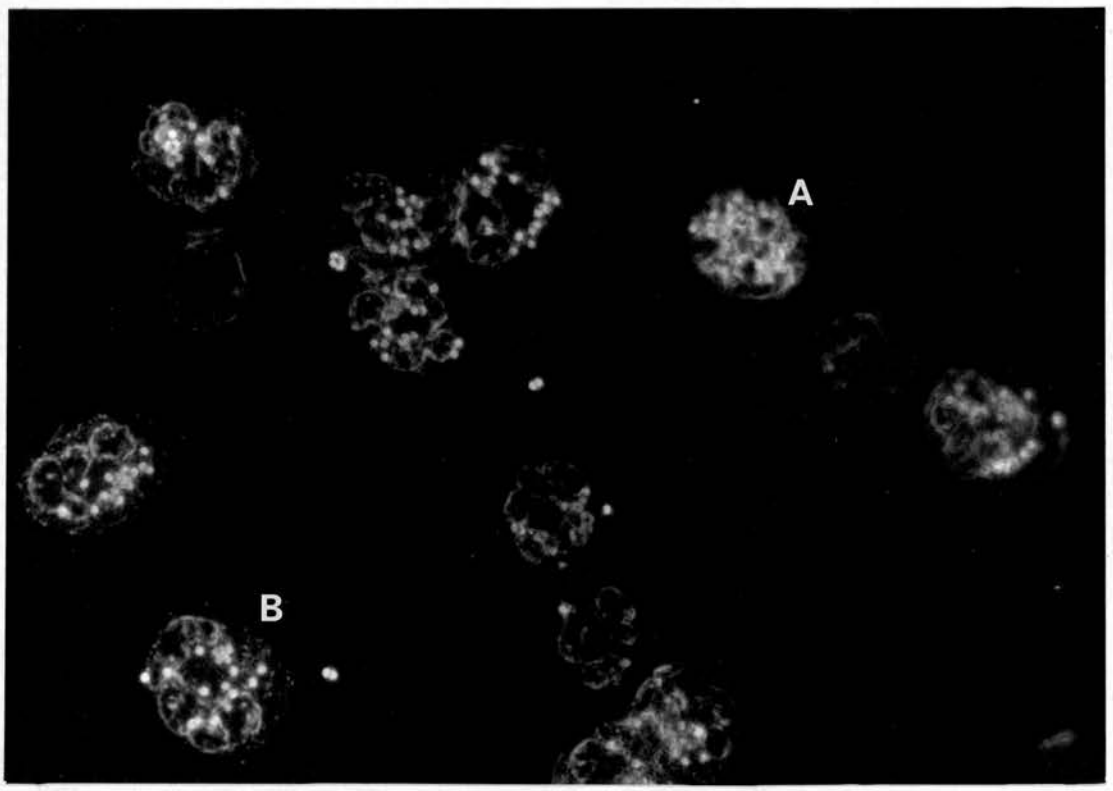


FIGURE A1b

CYTOCENTRIFUGE PREPARATION OF PERIPHERAL BLOOD GRANULOCYTES SHOWING PHAGOCYTOSED LATEX PARTICLES
Same field as in Figure A1a under dark field illumination
Cell B is shown to contain phagocytosed latex particles

Results

Total and Differential Leucocyte Concentration prior to Hyperthermia

Prior to hyperthermia the total and differential leucocyte concentration of 18 patients (12 ♂, mean age 53.8 years, range 23-77 years) were compared with those of a group of 35 normal controls (19 ♂, mean age 32.1 years, range 17-55 years), and with two published series of normal values (Wintrobe, 1961 ; Dacie & Lewis, 1975) (Table A 3). Statistical analysis was performed using the Student's t test, and p values of less than 0.05 were considered significant.

The patients had significantly higher ($p<0.001$) total leucocyte concentrations than the control group, but were still within the published normal ranges. This elevation was primarily due to a significant ($p<0.001$) increase in the proportion of neutrophils, the concentration of which was approximately double that of the controls, and at the upper limit of the published normal ranges. This was accompanied by a significant ($p<0.01$) reduction of lymphocyte concentration in the patients, to a level at the lower end of the normal range. Eosinophil and basophil counts also tended to be lower in the hyperthermia patients ($p<0.1$).

Total and Differential Leucocyte Concentration during and after Hyperthermia

The effect of hyperthermia treatment on total and differential leucocyte counts was studied by taking blood samples at regular intervals during, and following, treatment. Results from some typical patients are shown in Table A 4 and Figures A 2 and A 3. Statistical analysis was performed on the concentrations (before and after logarithmic transformation to correct for any possible skew distribution of the data) using the Student's t test

SUBJECT	SOURCE	NUMBER	TOTAL LEUCOCYTE CONC^{N} ($\times 10^{6}$/ml BLOOD)	DIFFERENTIAL LEUCOCYTE COUNT (%)				
				NEUTROPHILS	LYMPHOCYTES	MONOCYTES	EOSINOPHILS	BASOPHILS
HYPERTHERMIA PATIENTS	-	18	7.81*	73.4	20.4	4.4	1.4	0.1
NORMALS	-	35	5.57	56.1	35.5	5.0	2.7	0.8
	WINTROBE (1961)	-	5.0 - 10.0	58 - 70	25 - 33	3 - 7	1 - 3	0 - 0.75
	DACIE & LEWIS (1975)	-	4.0 - 11.0	40 - 75	20 - 45	2 - 10	1 - 6	< 1.0

TABLE A3a

TOTAL AND DIFFERENTIAL LEUCOCYTE COUNTS IN THE PERIPHERAL BLOOD OF NORMAL CONTROLS AND OF ADVANCED CANCER PATIENTS PRIOR TO WHOLE-BODY HYPERTHERMIA TREATMENT

Two published ranges of normal values are shown. * Statistically significant by Student's t test.

SUBJECT	SOURCE	NUMBER	TOTAL LEUCOCYTE CONC^N (x 10^6/ml BLOOD)	DIFFERENTIAL LEUCOCYTE CONC^N (x 10^6/ml)				
				NEUTROPHILS	LYMPHOCYTES	MONOCYTES	EOSINOPHILS	BASOPHILS
HYPERTHERMIA PATIENTS	-	18	7.81*	6.03*	1.49*	0.36	0.09	0.01
NORMALS	-	35	5.57	3.19	1.93	0.27	0.15	0.04
	WINTROBE (1961)	-	5.0 - 10.0	3.3 - 6.2	1.5 - 3.0	0.29 - 0.50	0.05 - 0.25	0.02 - 0.05
	DACIE & LEWIS (1975)	-	4.0 - 11.0	2 - 7.5	1.5 - 4.0	0.2 - 0.08	0.04 - 0.4	0.10

TABLE A3b

TOTAL AND DIFFERENTIAL LEUCOCYTE CONCENTRATIONS IN THE PERIPHERAL BLOOD OF NORMAL CONTROLS AND OF ADVANCED CANCER PATIENTS PRIOR TO WHOLE-BODY HYPERTHERMIA TREATMENT

Two published ranges of normal values are shown. * Statistically significant by Student's t test

for paired samples. p values of less than 0.05 were considered significant.

The following response occurred in most of the patients studied. Following pre-medication, but prior to heating, there was a fall in the leucocyte count, characterised by a decrease in the neutrophil concentration, but little change in lymphocyte numbers. This fall continued during active heating, the minimum leucocyte count occurring when the oesophageal temperature was 41.4-41.9^{o}C (i.e. the temperature at which the wax was removed). This leucopenia was then followed by a substantial leucocytosis, the magnitude of which varied from patient to patient. The rise in leucocyte numbers usually started during the maximum temperature phase of treatment, but continued after the patients had been removed from the bath, and allowed to cool. At this stage, during the first treatment, the mean leucocyte count reached 125% of the pre-heating value; by the following morning it had dropped to 116%, and, in most cases, had returned to the pre-treatment value within a further 2 days. This pattern was repeated during subsequent treatments (Figures A 2 and A 4), although the magnitude of the leucocytosis generally increased during each treatment. This may be due either directly, or indirectly, to the increasing fraction of hyperthermia given on each occasion, or to the increased likelihood of registering the peak of leucocytosis during the longer second and third treatments.

The changes in leucocyte count during hyperthermia can be largely explained by variations in the neutrophil concentration. During the period of leucopenia, and neutropenia, there was in some cases, a slight lymphocytosis. This was followed by the leucocytosis and neutrophilia, which was initially accompanied by

FIRST TREATMENT				
(11 PATIENTS)	PRE	END	1 DAY POST	3-5 DAYS POST
TOTAL LEUCOCYTES	100	125.5	116.5	116.1
NEUTROPHILS	100	142.0	151.3*	129.1
LYMPHOCYTES	100	101.6	75.5	86.5

SECOND TREATMENT				
(7 PATIENTS)	PRE	END	1 DAY POST	3 DAYS POST
TOTAL LEUCOCYTES	100	158.9	125.5	102.4
NEUTROPHILS	100	182.3	145.8*	113.9
LYMPHOCYTES	100	123.2	92.3	81.6

THIRD TREATMENT				
(4 PATIENTS)	PRE	END	1 DAY POST	3 DAYS POST
TOTAL LEUCOCYTES	100	185.0	147.5	95.3
NEUTROPHILS	100	213.1*	191.8	102.9
LYMPHOCYTES	100	145.2	69.6	89.6

TABLE A4

EFFECT OF HYPERTHERMIA TREATMENTS ON THE TOTAL AND DIFFERENTIAL CONCENTRATION OF PERIPHERAL BLOOD LEUCOCYTES

Pretreatment concentration = 100
* Statistically significant by Student's t test on logarithm-transformed data

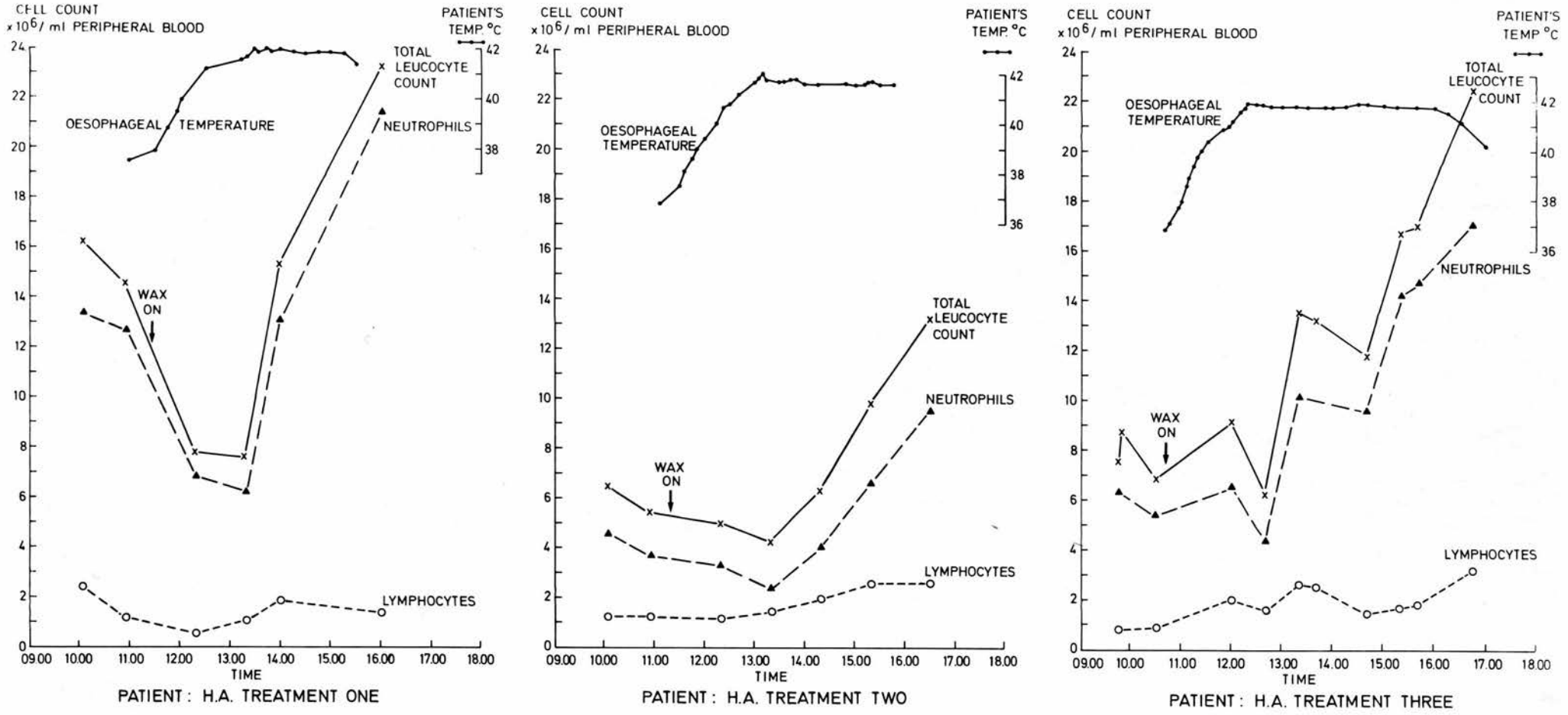


FIGURE A2

TOTAL AND DIFFERENTIAL PERIPHERAL BLOOD LEUCOCYTE CONCENTRATION IN ONE PATIENT DURING THREE SUCCESSIVE HYPERTHERMIA TREATMENTS

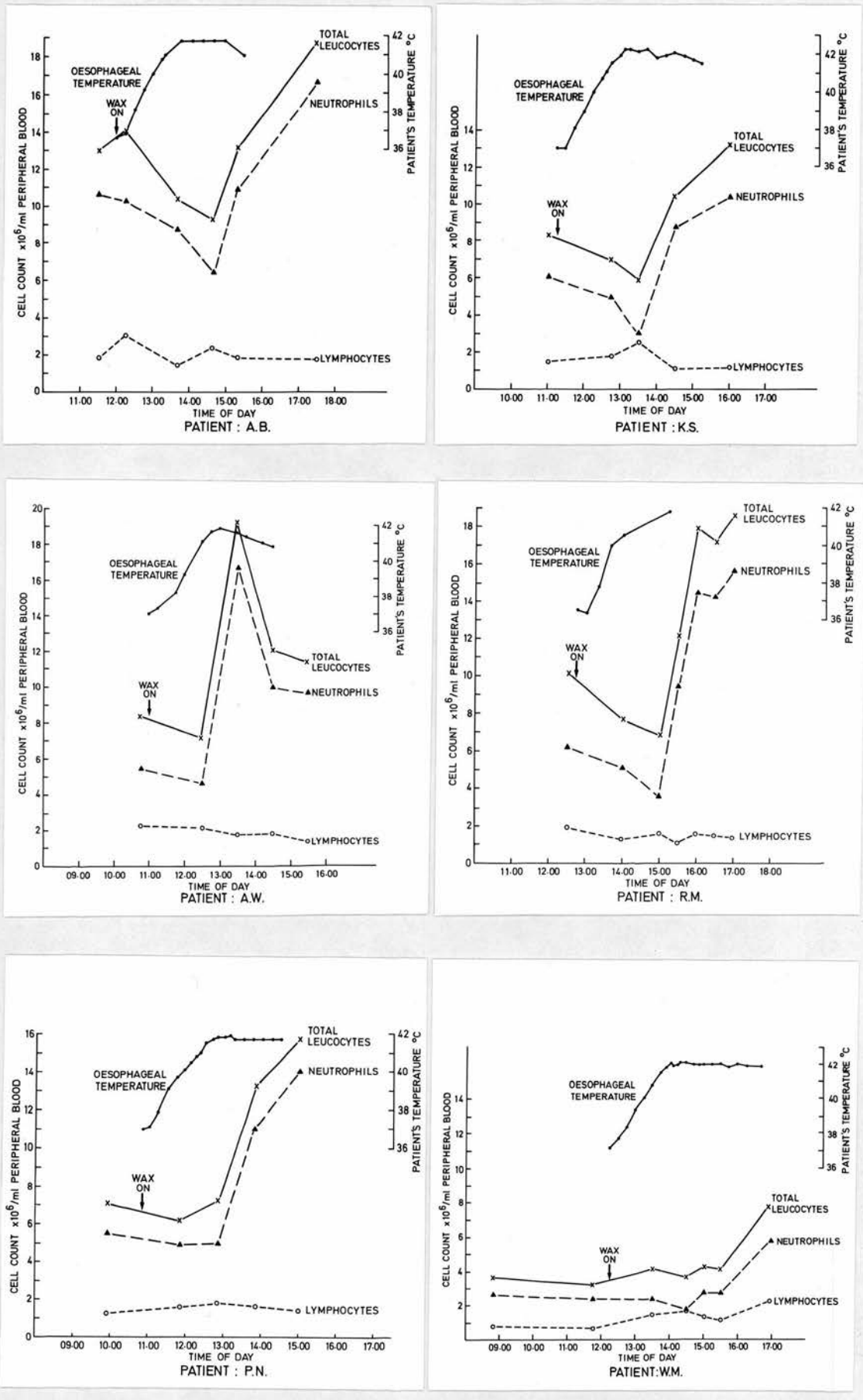


FIGURE A3

TOTAL AND DIFFERENTIAL PERIPHERAL BLOOD LEUCOCYTE COUNTS IN SIX PATIENTS DURING WHOLE-BODY HYPERTHERMIA TREATMENT

a lymphopenia, and subsequently, by a return to the pre-hyperthermia lymphocyte concentration. During subsequent treatments, in which there was a substantial neutrophilia, there was often a slight lymphocytosis towards the end of treatment. The peak in neutrophil concentration usually occurred within 48h of treatment, with a return to the pre-hyperthermia concentration within 3 days. There was a tendency for the lymphocyte count to be slightly depressed following treatment, although this was only statistically significant ($p<0.01$) after the first session.

Over a complete course of hyperthermia treatments, there was no significant difference between the initial and final total and differential leucocyte count.(Figure A4)

Serum Cortisol Concentrations during and after Hyperthermia The concentration of serum cortisol was measured in 5 patients, during and after their first treatment.

At the end of hyperthermia the concentration had risen to 169.0% of the pre-heating level (Range 100.0-328.2%).. The following day it had fallen to 118.2%, and 2-3 days later stood at 103.4%. None of these changes was statistically significant, by the Student's t test for paired samples.

Discussion

In this study, the total and differential leucocyte concentration in hyperthermia patients, prior to treatment, was compared to that in a control group which was younger, and contained a lower proportion of males. Olbrich (1947) however, could find no difference in the peripheral leucocyte count of the elderly, and that in younger adults. This is supported by the finding that

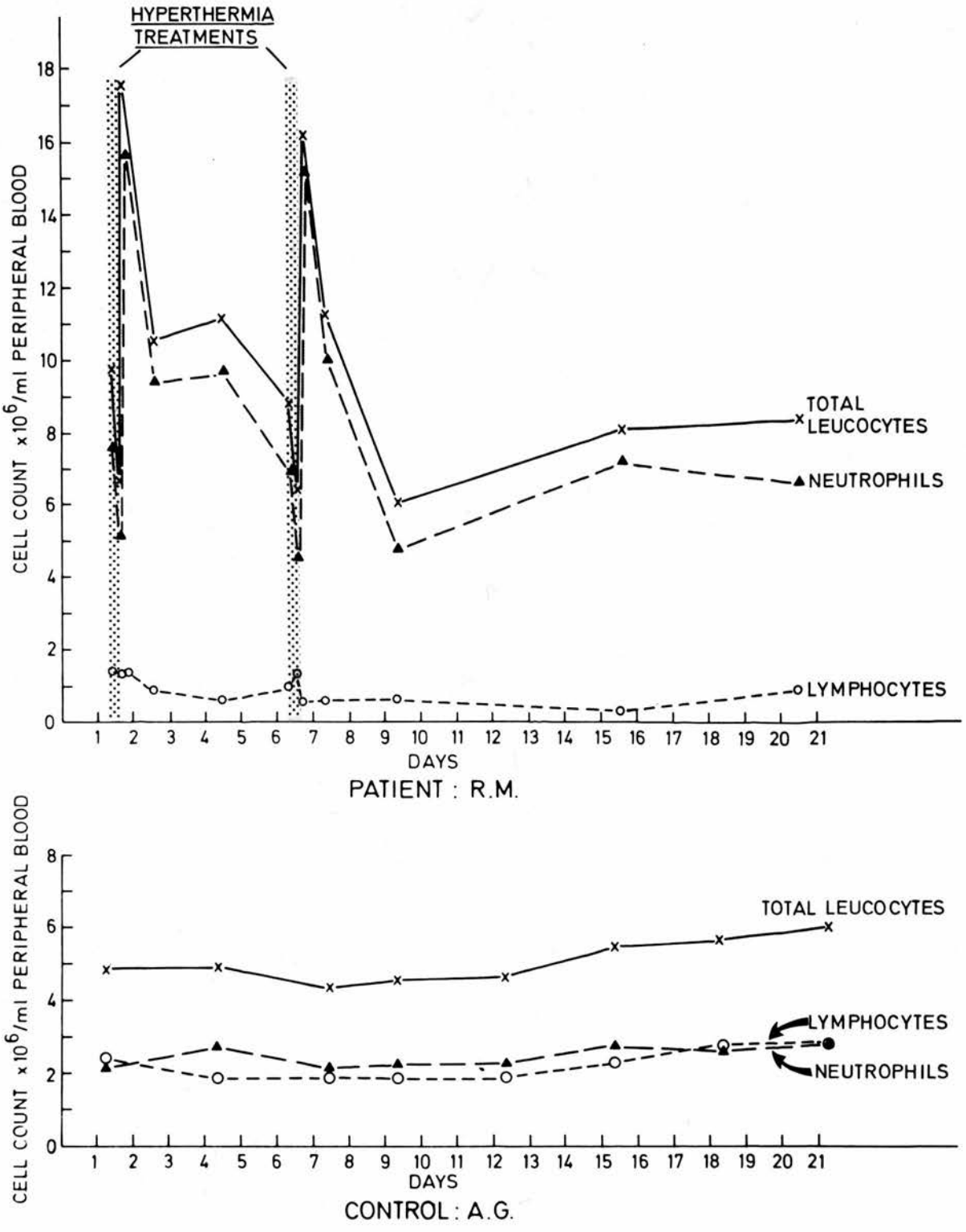
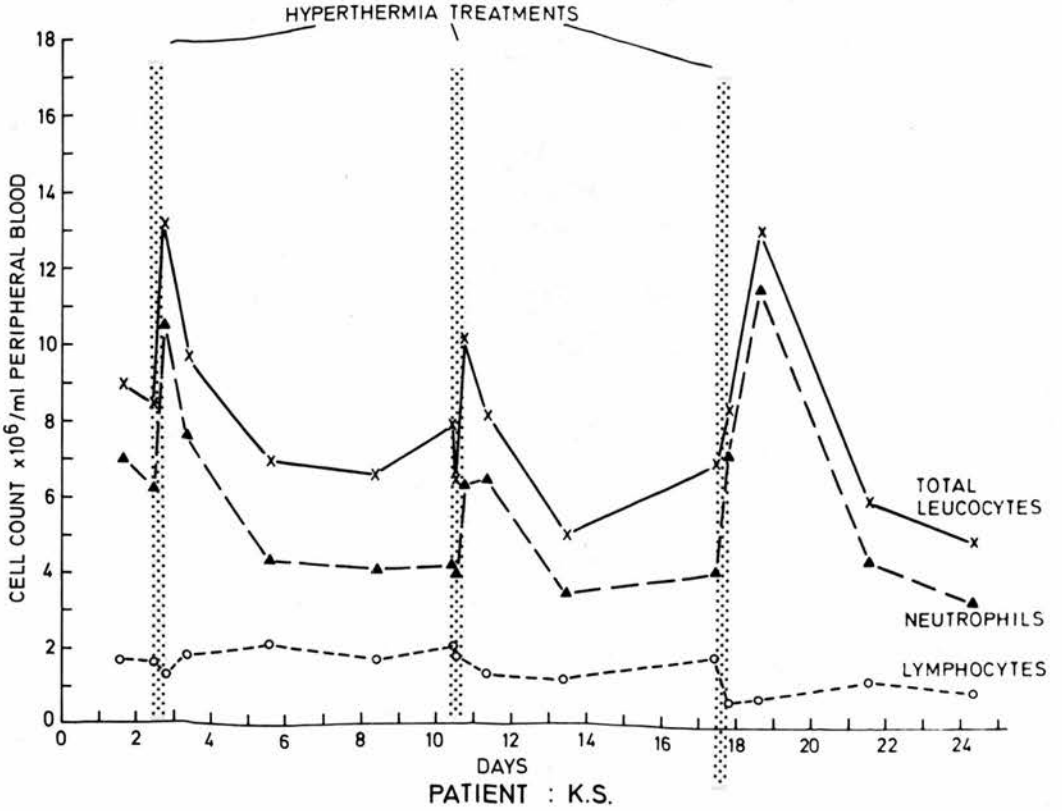
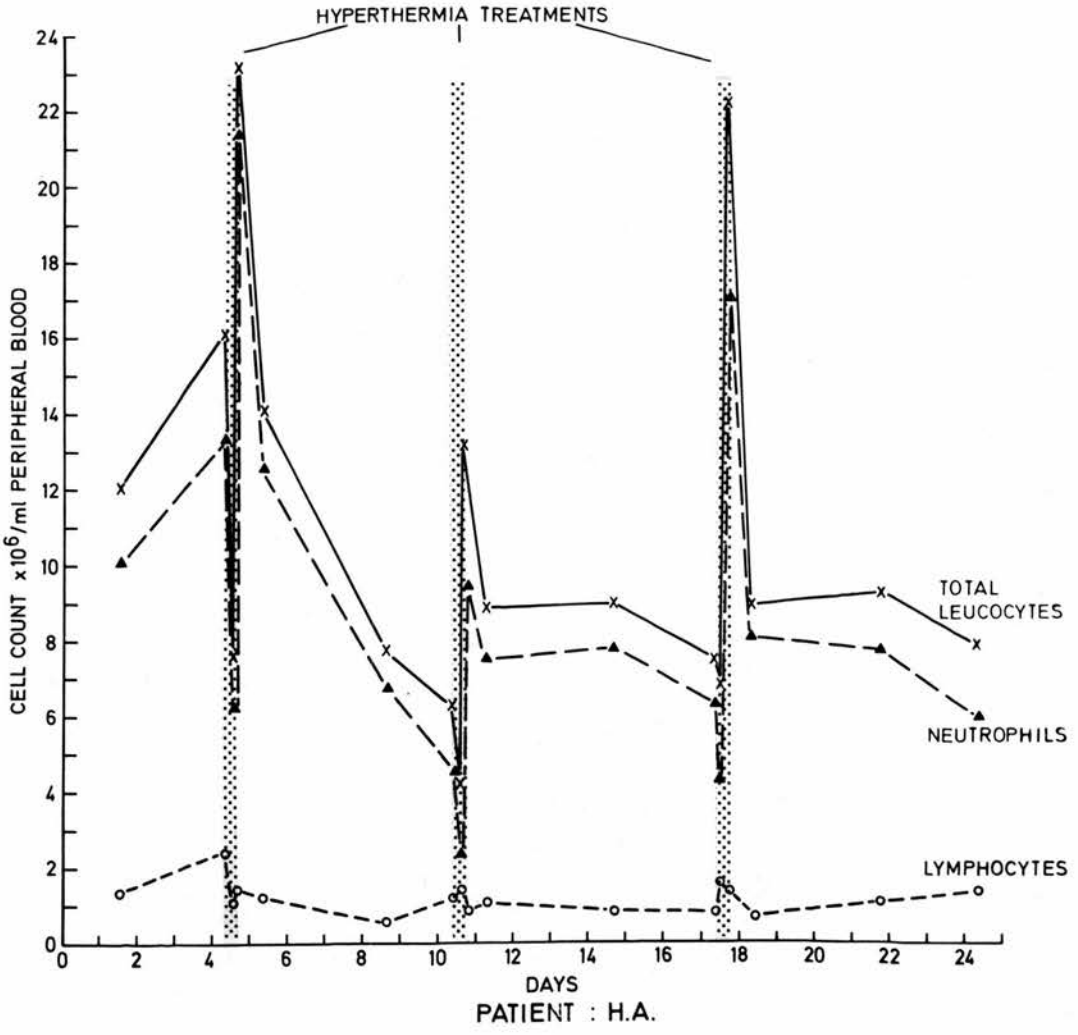


FIGURE A4a

EFFECT OF SUCCESSIVE HYPERTHERMIA TREATMENTS ON THE TOTAL AND DIFFERENTIAL CONCENTRATION OF PERIPHERAL BLOOD LEUCOCYTES

The lower graph shows the concentrations in a normal control sampled over the same time period





FIGURES A4b & A4c

EFFECT OF SUCCESSIVE HYPERTHERMIA TREATMENTS ON THE TOTAL AND DIFFERENTIAL CONCENTRATION OF PERIPHERAL BLOOD LEUCOCYTES

subdivision of the control group, used in this study, into two sub-populations, with significantly different mean ages (24.2 years, and 40.5 years), did not produce a significant difference in the leucocyte count between the two groups. Sex differences probably do not account for these results, since they do not have a significant effect on the total leucocyte count, except that the count in women above 50 years of age is less than that in men (Dacie & Lewis, 1975). It is known that leucocyte counts can be affected by exercise (Wintrobe, 1961), climate and environment (Woodliff et al, 1972), season and time of day (Dacie & Lewis 1975), pain and emotional disorders, and possibly, ingestion of food (Wintrobe, 1961), although Holm and Palmblad (1976) found that acute energy deprivation (involving 11 days starvation) produced no significant changes in the lymphocyte or monocyte concentrations. Furthermore, the validity of the results obtained by comparing the patient and control groups, is reinforced by reference to the published normal ranges. Whilst the controls are within these ranges, the neutrophil and lymphocyte concentrations in the hyperthermia patients tend to be at their limits. It is therefore concluded that the increased peripheral leucocyte concentration in hyperthermia patients, prior to treatment, is a reflection of their poor clinical condition.

The leucocytosis in these patients is due to an increase in the percentage, and concentration, of neutrophils. The reason for this granulocytosis is not clear. It may be a response to a secondary infection, or a reaction to the neoplasm (De Gruchy, 1964). Wintrobe (1961) has indicated that cancers which involve the liver and gastro-intestinal tract, can produce a leucocytosis. This explanation would seem applicable to these patients, the

majority of whom had G.I. tumours, with liver metastases.

Lymphopenia in cancer patients has been reported previously by Zacharski and Linman (1971) and Krant et al (1968), but was not seen by Cochran et al (1976). In many cases, lymphopenia was defined as a lymphocyte concentration below $1.5x10^6$ (Riesco, 1970), or $1.0x10^6$ (Amin & Lich, 1974) cells/ml peripheral blood. On this basis, just over half (62.5%) of the hyperthermia patients were lymphopenic at the $1.5x10^6$ lymphocytes/ml level, and 18.8% at the $1.0x10^6$ lymphocytes/ml level - an incidence comparable to the 16% reported by Zacharski and Linman (1971). However, the degree of lymphopenia is somewhat masked by the high leucocyte count in the patient group, which compensated for their low percentage (20.4%) of lymphocytes.

The reason for this lymphopenia is not fully understood. It is unlikely to be the result of previous chemotherapy and radiotherapy, since these are known to produce an associated leucopenia. Adrenalin is known to cause a two-phase increase in the leucocyte count. During the second stage there is a granulocytosis and an accompanying lymphopenia. It is possible that the increase in adrenocortical activity, reported in pre-terminal cancer patients (Werk, Sholiton & Marnell, 1963), may result in the abnormalities in the differential leucocyte concentrations observed in these patients. Alternative explanations, which do not account for the granulocytosis, are those of depressed immunocompetence in these subjects, resulting in the development of a tumour, or, the presence of a substance which suppresses lymphocyte proliferation (Papatestas et al, 1976).

In this study there was no clear relationship between the pre-treatment lymphocyte concentration and the response to therapy,

as judged by survival time following treatment. However, the number of patients was too small, and the follow-up too short to exclude such a relationship.

The marked leucocytosis occurring during hyperthermia treatment has been described previously (Simon, 1935 ; Beisel, Goldman & Joy , 1968 ; Cohen & Warren, 1935), and has been seen in cases of heat stroke (Shibolet, Lancaster & Danon, 1976). The preceeding leucopenia is not documented by these authors.

Variations in blood volume, during heating, are unlikely to account for these fluctuations in the leucocyte concentration. Fluid losses were minimised by intravenous infusions, and measurement of haematocrit and haemoglobin concentrations indicated no appreciable haemoconcentration or dilution (Pettigrew et al, 1974). In addition, falls in the numbers of one type of leucocyte were often accompanied by increases in that of another.

It is not likely that anaesthesia is responsible for the leucocytosis, since the previous studies, conducted on unanaesthetised patients, produced similar results. Although slight increases in leucocyte numbers have been described in volunteers undergoing anaesthesia alone, it is generally accepted that the haematological changes previously attributed to anaesthetics, are due to stress (Cullen & Van Belle, 1975). Lecky et al (1974) reported similar changes to those in hyperthermia patients (i.e. neutrophilia, but no change in lymphocyte concentrations), in volunteers exposed to N_2O/O_2 d-tubocurarin anaesthesia. They attributed these to an increase in the levels of serum cortisol which were indicative of a stress response.

A number of different effects may be classified as stress responses, but all involve a disruption in the balance of

catecholamine, ACTH and cortisol production. Depending on the nature of the disruption, the effect on the leucocyte concentration can differ widely (Bruce & Wingard 1971). The type of hyperthermia treatment used in this study is known to produce an initial fall in cortisol concentration (Ludgate et al, Unpublished observations), followed by an increase during active heating. This is accompanied by an earlier fall and rise in the ACTH concentration (Ludgate et al, Unpublished observations). These changes could account for the leucocytosis, which is initiated during the rise phases of both ACTH and cortisol. The leucopenia seen before active heating is unlikely to be due to a change in the cortisol concentration, which is not thought to be affected by muscle relaxants, or pre-medications (Hammond et al, 1958). It is possible, however, that these agents have a similar effect to that of barbiturates, which are known to produce an immediate neutropenia, with a slight lymphocytosis, similar to that seen in these patients.

The relationship between leucocyte concentration and the level of stress, as measured by serum cortisol concentrations, is reinforced by the follow-up studies. These indicated that, whilst initially elevated, both parameters returned to the pre-treatment level within 3-4 days. Suryanarayan (1966) described a post-heating leucopenia, consisting of neutropenia and lymphocytosis, lasting for 15 days, in patients treated by hyperthermia and concomitant chemotherapy. This has not been seen, either in this study, or in that of Cohen and Warren (1935).

In summary, hyperthermia treatment produces a marked granulocytosis in most patients. Lymphocyte concentrations are usually unaffected. These changes may be due to a stress response, involving increased serum cortisol levels, rather than to anaesthesia.

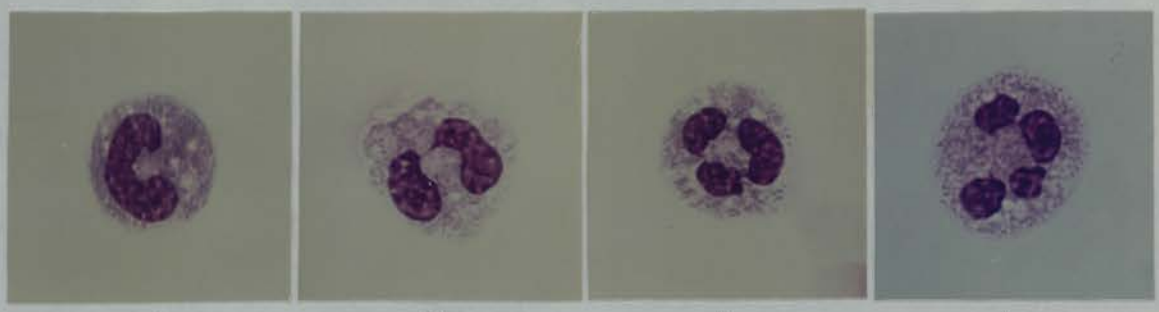
Pre-treatment peripheral leucocyte concentrations return within 3-4 days, indicating that hyperthermia does not produce the long-term leucopenia associated with radiotherapy and chemotherapy.

Studies on the Granulocytosis occurring during Hyperthermia Treatment The granulocytosis occurring during, or immediately following, treatment, may be produced by the release of neutrophils from capillary beds, due to heat-induced vasodilatation and a hyperaemic circulation. Alternatively, hyperthermia may, directly or indirectly, stimulate the production of granulocytes. In the latter case there should be a marked shift in the age distribution of the cells towards juvenile forms.

To find out which effect was predominant, the age distribution of the neutrophils was determined by assessing the proportion of peripheral blood granulocytes with 2, 3, and 4 or more nuclear lobes, before and after treatment. Classification was based on the system developed by Arneth (Wintrobe, 1961), in which segmentation of the nucleus can be related to cell maturation (Figure A 5). 400 neutrophils from stained, peripheral blood films were classified for each time point.

With such a large influx of granulocytes into the peripheral bloodstream, it was also of interest to determine whether there was an associated increase in phagocytosis. If the neutrophilia was due to the release of morphologically immature forms, it was possible that these may, nonetheless, have some of the functions of mature cells. Phagocytosis assays were carried out on granulocytes isolated from peripheral blood before and after treatment. A limited follow-up was possible.

MATURATION



1 2 3 4

FIGURE A5

NUCLEAR LOBE FORMS USED TO DETERMINE THE AGE-DISTRIBUTION OF PERIPHERAL BLOOD GRANULOCYTES

Cytocentrifuge preparations, May-Grunwald/Giemsa stained (Magnification x1045)

PATIENT	LOBES PER NUCLEUS			
	1	2	3	≥ 4
K.S.				
1ST TREATMENT	432	171	106	103
2ND TREATMENT	643	205	89	83
3RD TREATMENT	670	201	82	81
A.W.				
1ST TREATMENT	307	133	97	128
2ND TREATMENT	347	234	121	38
I.W.	774	280	190	95
H.A.	941	148	334	158
D.G.	799	281	149	92

PRE-TREATMENT VALUE FOR ALL CATEGORIES = 100

TABLE A5

EFFECT OF HYPERTHERMIA TREATMENT ON THE CONCENTRATION OF PERIPHERAL BLOOD GRANULOCYTES SHOWING A PARTICULAR NUCLEAR LOBE FORM

200 cells were classified from Pre & End-treatment peripheral blood smears

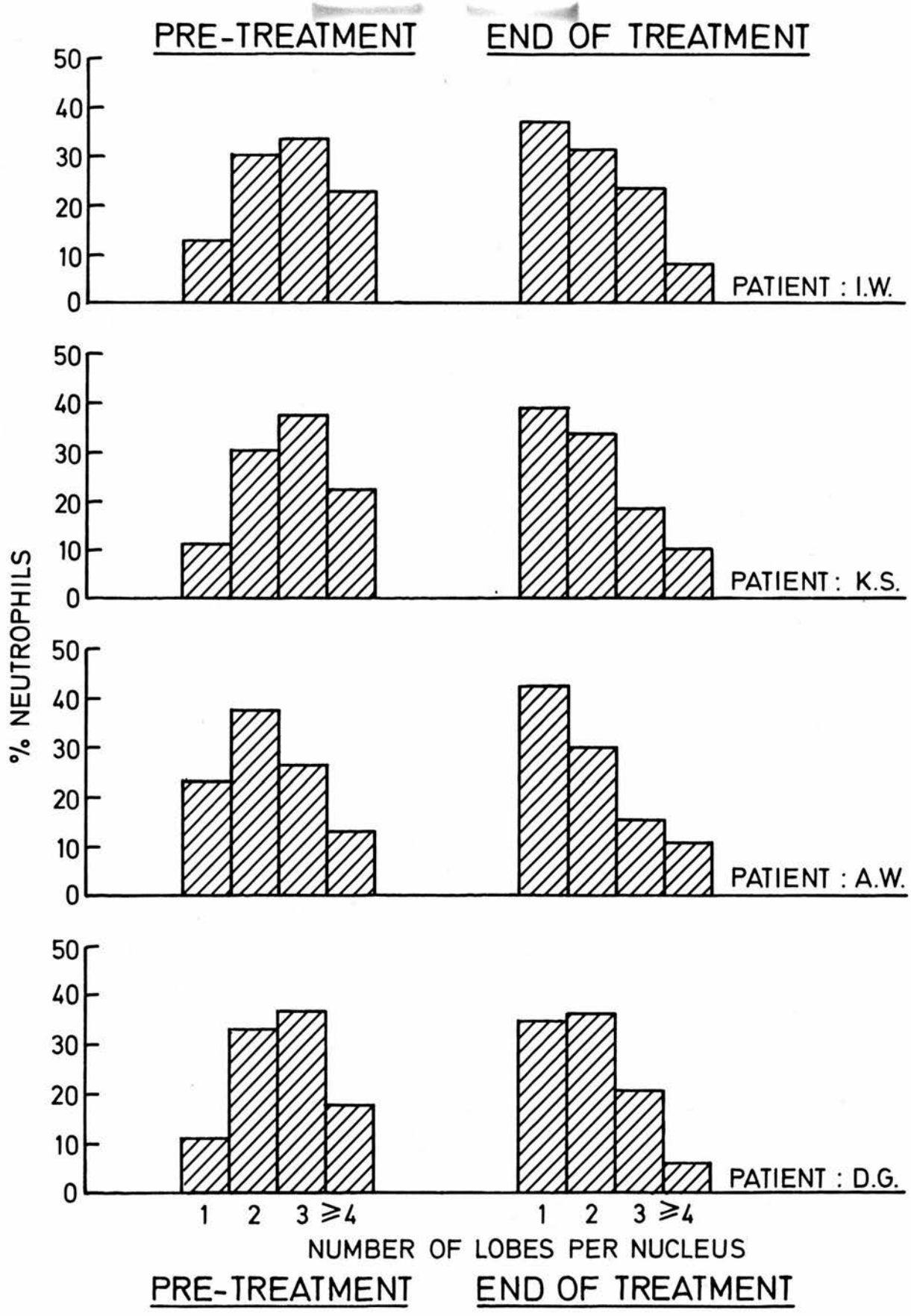


FIGURE A6

EFFECT OF HYPERTHERMIA TREATMENT ON THE AGE-DISTRIBUTION OF PERIPHERAL BLOOD NEUTROPHILS

Increasing age of the cells is indicated by segmentation of the nucleus. See Fig.A5

Prior to hyperthermia, the majority of neutrophils were 2 or 3-lobed forms, however, at the end of treatment, there was a marked increase in the proportion of less mature (1 and 2 lobed forms) in the peripheral blood (Figure A 6). The possibility of selective destruction of the older forms during heating, was excluded by determining the concentration of each of the age groups before, and after heating. The results (Table A 5) indicate that the mature granulocytes (4 lobed forms) were largely unaffected, or may even increase in numbers, during hyperthermia.

The results of the phagocytosis studies (expressed as concentrations of phagocytic neutrophils/ml peripheral blood) are shown in Figure A 7.

Where there was an appreciable neutrophilia during hyperthermia, there was an accompanying rise in the number of cells capable of phagocytosing latex particles (Patients: L.K., M.D., and M.W.), indicating that the immature forms were functional in this respect. In one patient (T.M.), who received radiotherapy between hyperthermia treatments, leucopenia developed during the first four heating sessions (Figure A 7), but there was a slight leucocytosis during the fifth. This is reflected in the changes in the concentration of phagocytic neutrophils, which fell during the first four treatments, but rose during the fifth. In these, and similar sessions in which there was no massive granulocytosis (so that essentially the same granulocyte population was studied before and after heating) the <u>percentage</u> of neutrophils capable of latex phagocytosis did not change, indicating that the treatment did not affect the process of phagocytosis.

This was confirmed <u>in vitro</u>, by carrying out the incubation of cells and latex at $42^{o}C$, in parallel with the normal test at $37^{o}C$.

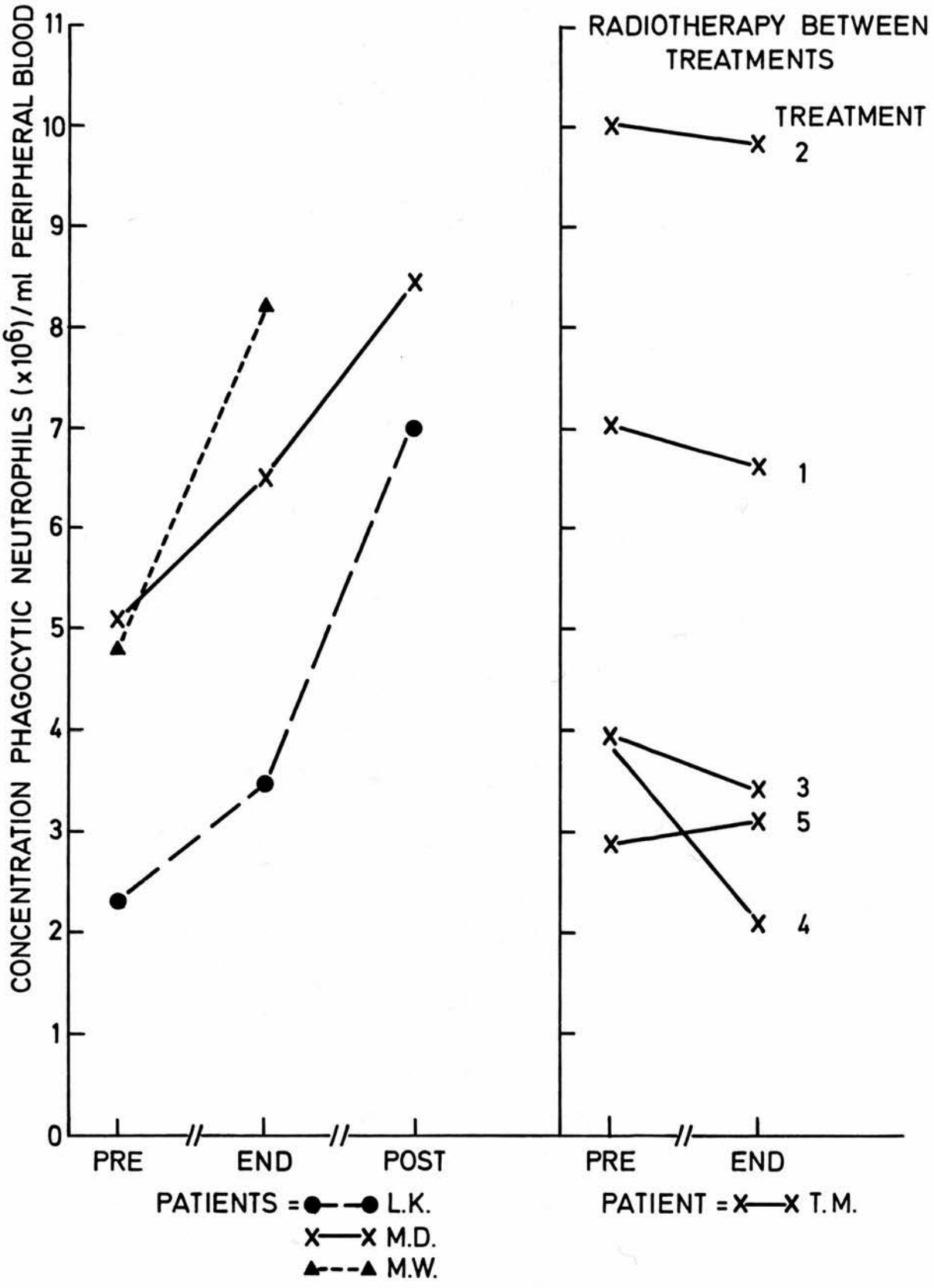


FIGURE A7

EFFECT OF HYPERTHERMIA TREATMENT ON THE CONCENTRATION OF ACTIVELY-PHAGOCYTIC NEUTROPHILS IN THE PERIPHERAL BLOOD

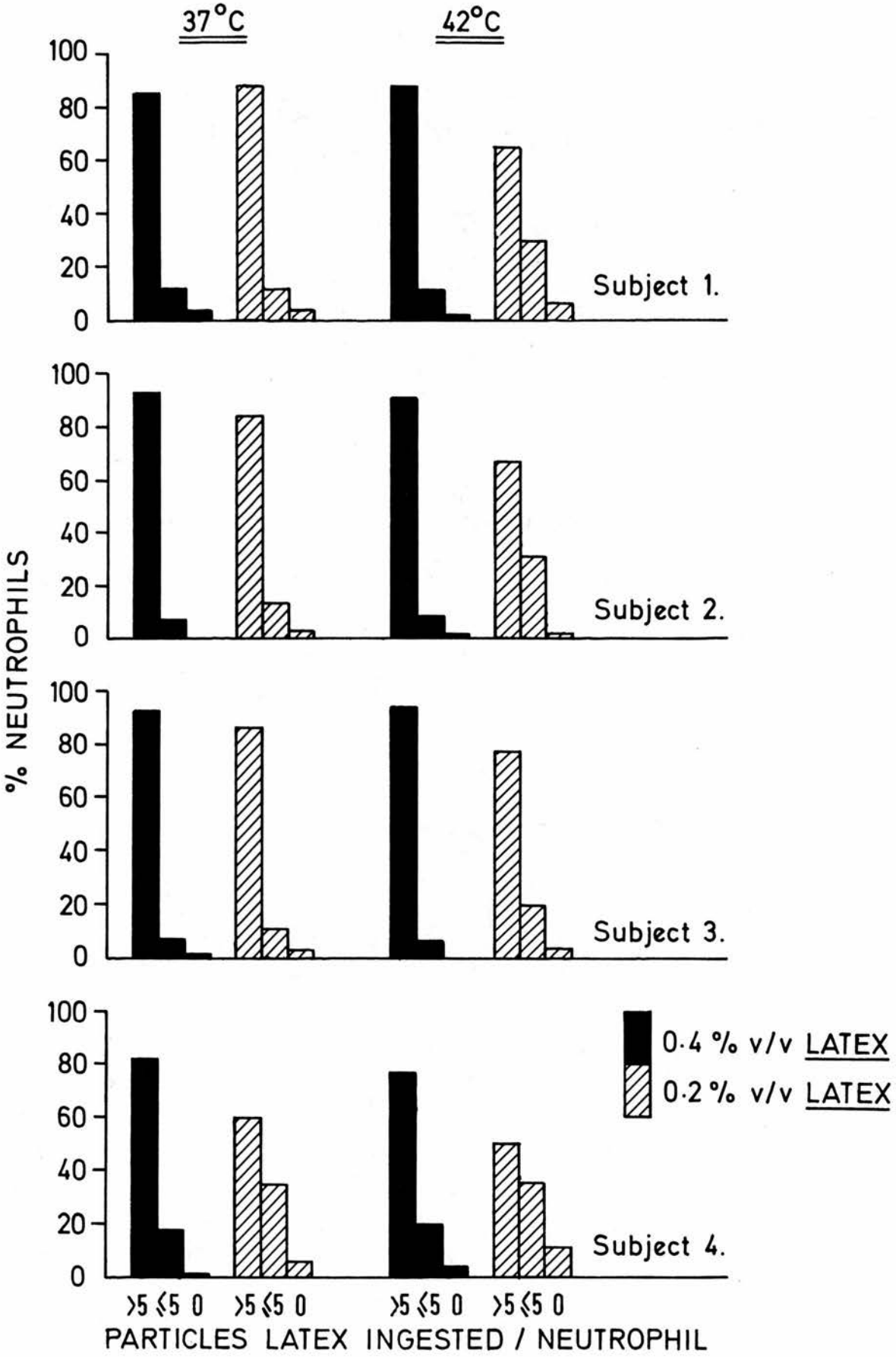


FIGURE A8

EFFECT OF DIFFERENT INCUBATION TEMPERATURES AND LATEX CONCENTRATIONS ON THE EFFICIENCY OF PHAGOCYTOSIS BY NEUTROPHIL GRANULOCYTES

See text for details

The results are shown in Figure A 8. At 37°C there was little difference between the percentage of cells capable of phagocytosis when a high (0.4% v/v), or a low (0.2%v/v) concentration of latex was used. At 42°C the total number of cells phagocytosing latex at high concentrations was similar to that seen at 37°C, and there is little difference in the 'efficiency' (as measured by the number of particles ingested). However, at a low latex concentration there was an increase in the proportion of neutrophils ingesting fewer than 5 particles, at the expense of those phagocytosing more than five particles.

Discussion

The finding that the granulocytosis was primarily due to an influx of immature neutrophils into the bloodstream is supported by the work of Cohen and Warren (1935). Simon (1935) and Beisel et al (1968) both reported a granulocytosis during heating in vivo, but indicated that the neutrophilia was due to an increase in the number of mature cells. It is likely that a combination of the two effects occurs during hyperthermia. Athens et al (1961) have indicated that in the human, only 47% of granulocytes are in circulation at any one time. The remainder form the so-called marginal pool, which is in rapid equilibrium with the circulating pool. The distribution of cells between these two pools can be altered by exercise, injection of adrenalin etc., and it is probable that hyperthermia also increases the size of the circulating fraction. This may be combined with stimulation of neutrophil production (which is normally 8.5×10^8 cells/kg body weight/day (Dancey et al, 1976)), either directly by heat, or indirectly, possibly by release of tissue autolysates (Lawrence et al, 1950)

from neoplastic, or normal tissue.

The results from the phagocytosis experiments indicate that heat may depress the 'capturing efficiency' of the neutrophils, whilst not affecting their ability to ingest latex. The fact that this phenomenon is apparent at low particle densities could be of relevance in vivo, where small amounts of tumour debris may be in circulation after hyperthermia. In addition, bacteria and viruses are less liable to be 'captured' by heated cells, which may leave the patient open to infection. It is likely however, that this effect is amply counteracted by the greatly increased numbers of phagocytic cells, which are in circulation after treatment.

The reason for the depression of phagocytosis at 42^{o}C is not clear. Anaesthesia has been reported to inhibit phagocytosis, Nitro-blue Tetrazolium reduction (Cullen, Hume & Chretien, 1975), and neutrophil chemotaxis (Stanley et al, 1976). However, in the former case, the inhibition was minimal, and it is likely that many of the effects attributed to anaesthetics may have been due to stress, and an associated elevation of serum steroid levels (Duncan & Cullen, 1976); although Brote and Stendahl (1975) found essentially normal phagocytic function after surgical trauma, but some depression in burns patients. A direct effect of elevated temperature on phagocytosis has been reported by Mandell (1975), where temperatures above 40^{o}C inhibited the ingestion of bacteria by human neutrophils in vitro. It is therefore possible that heat directly affects the cell, or, alternatively, may degrade the opsonins required for phagocytosis. Douglas (1970) has stated that neutrophils require complement to accomplish efficient phagocytosis, and this study has indicated that complement concentrations are depressed by hyperthermia treatment (See p.168). It is possible

therefore, that low levels of complement, whilst not inhibiting at high latex densities, become critical when combined with a decrease in the latex concentration.

It remains to be established whether this slight depression in phagocytic ability of individual cells, when balanced against the increase in the total number of cells capable of phagocytosis, is of relevance in the clinical situation.

Effect of Hyperthermia Treatment on Granulocyte Morphology As part of a later study (See p.130) the effect of hyperthermia treatment in vivo, on granulocyte morphology was assessed by scanning electron microscopy (See p.107). In order to compare similar cell populations, granulocytes were isolated before, and after, a treatment during which there was only a slight leucocytosis (Patient: T.M. ; Treatment 5 - Figure A 7).

Prior to hyperthermia the majority of cells were amoeboid in shape, with relatively few surface ridges, or villi (Figure A 9). In contrast, granulocytes separated from end-of-treatment blood were spherical, and the surface was folded and villous (Figure A 10).

Discusssion

These findings are in agreement with those of Lichtman et al (1976), who noted a rounding-up of granulocytes kept in suspension at $4^{o}C$. They also described changes in the cell surface, similar to those reported in this study, and indicated that this may be due to the folding, or rucking, of the surface membrane, when the amoeboid processes are retracted. Their study correlated the amoeboid form with granulocyte integrity, and normal functional capacity, and this is partly supported by the work on hyperthermia

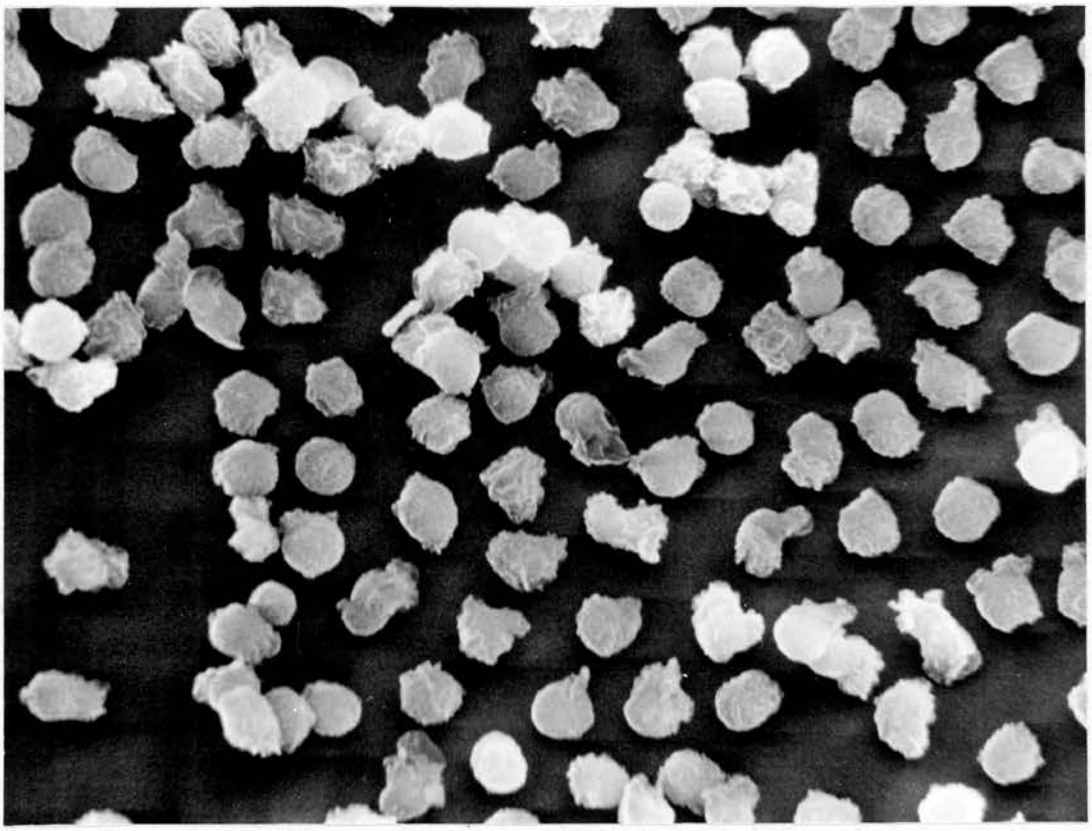


Figure A9a

Scanning Electron Micrograph of Peripheral Blood Granulocytes prior to Hyperthermia Treatment (x 1,100)

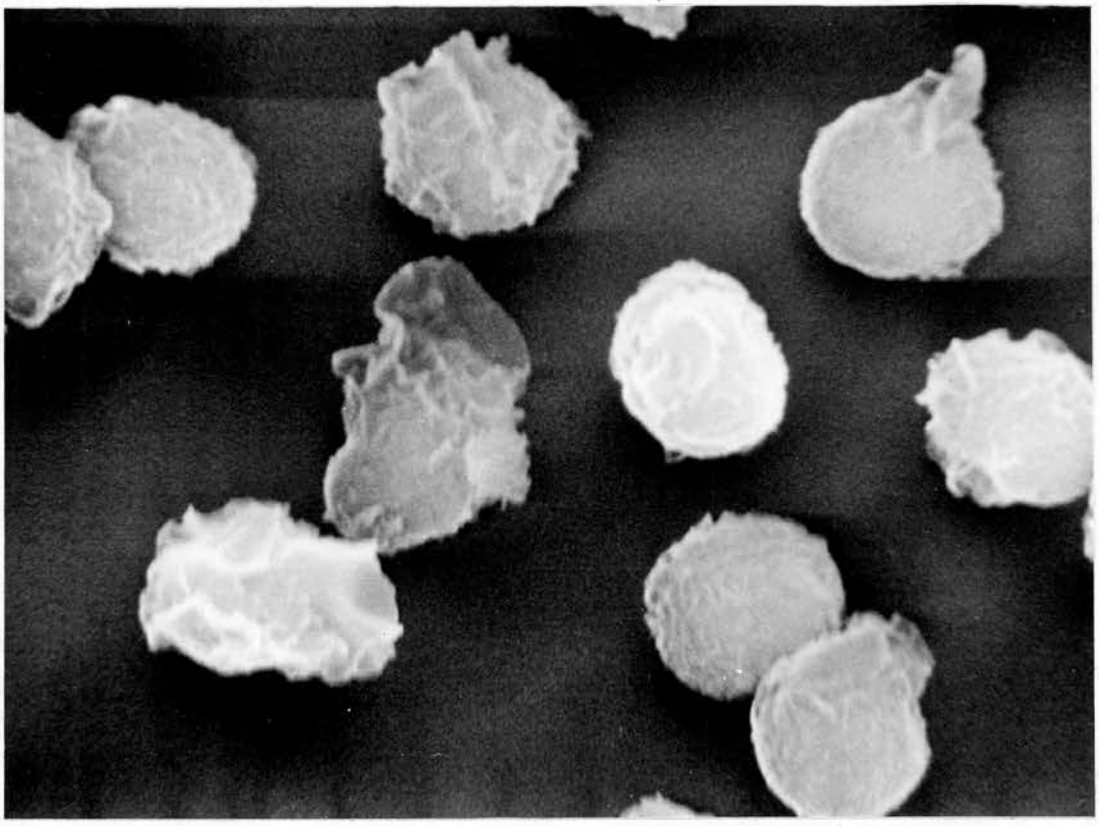


Figure A9b

Scanning Electron Micrograph of Peripheral Blood Granulocytes prior to Hyperthermia Treatment (x 3,600)

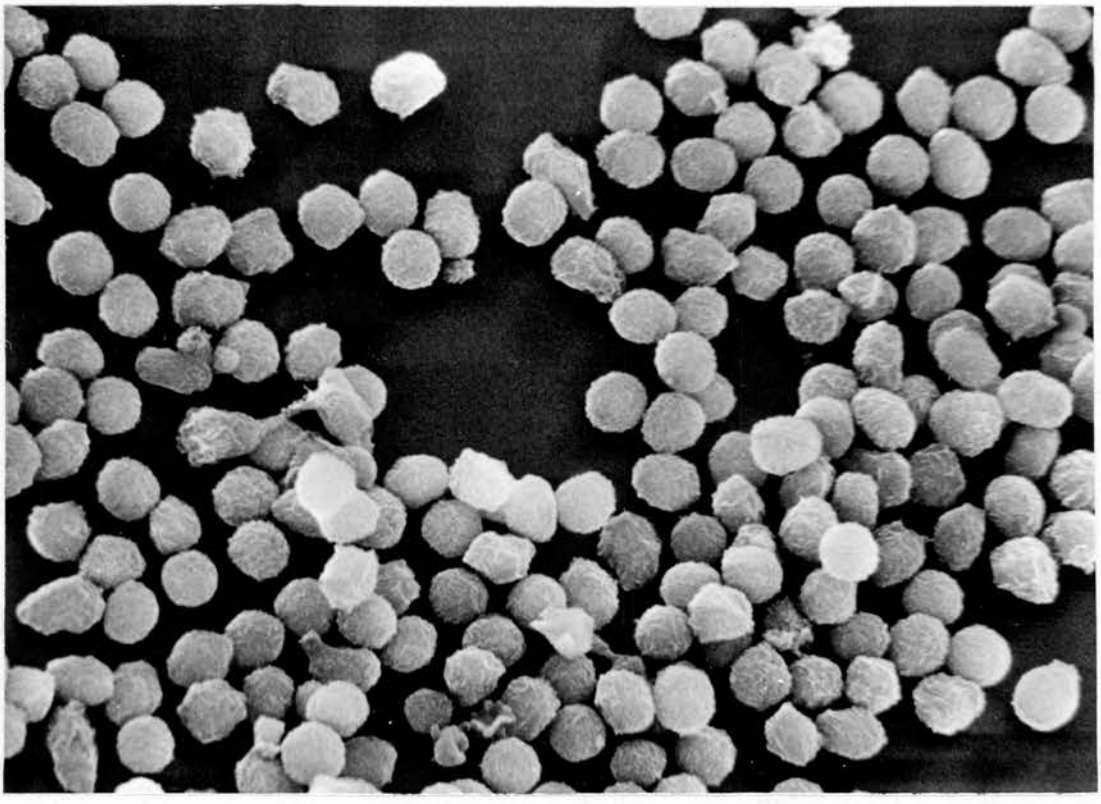


Figure A10a

Scanning Electron Micrograph of Peripheral Blood Granulocytes at the end of Hyperthermia Treatment (x 1,100)

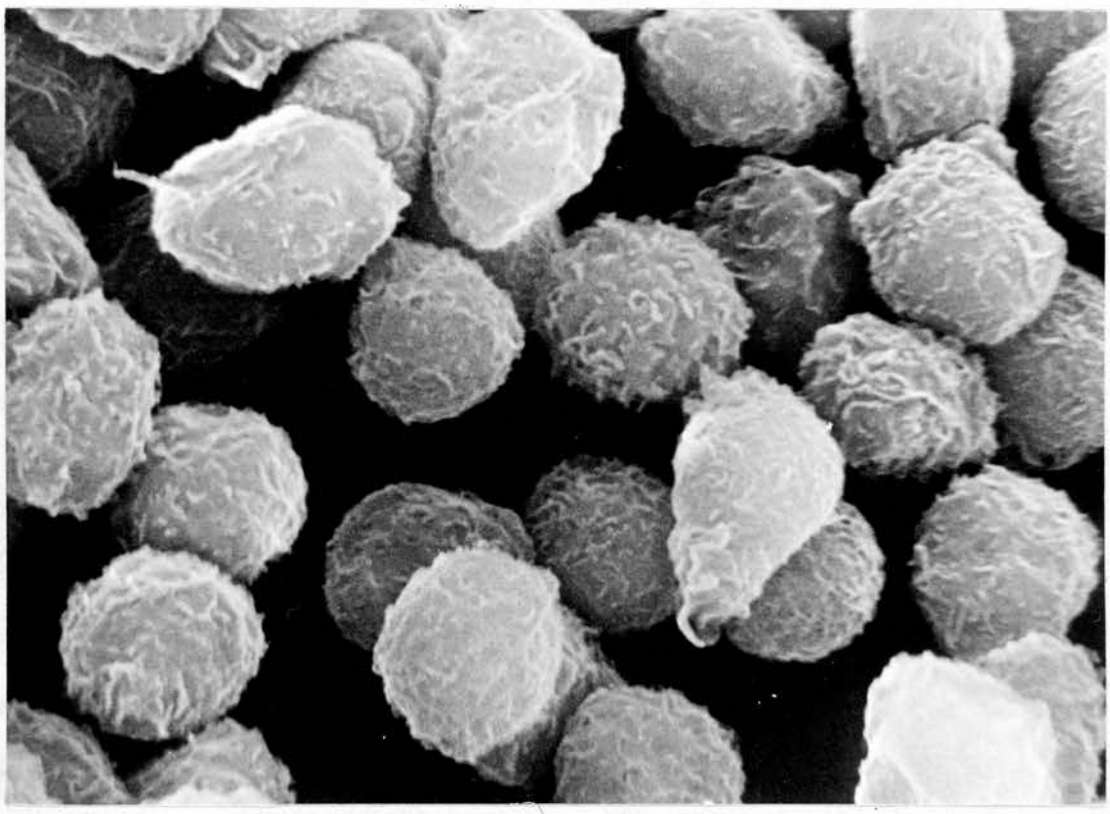


Figure A10b

Scanning Electron Micrograph of Peripheral Blood Granulocytes at the end of Hyperthermia Treatment (x 3,600)

patients. It is possible that the reduction in latex 'capturing efficiency' at 42°C is due to the withdrawal of the cell processes at this temperature, so that the chance of contact between the cell and the latex particles is reduced.

In summary, although this finding of a change in granulocyte morphology during hyperthermia treatment, is based on a single observation, it may be of value in explaining the effect of elevated temperatures on the 'capturing efficiency' of these cells.

PAPER B

THE EFFECTS OF WHOLE-BODY HYPERTHERMIA THERAPY ON THE CONCENTRATION OF PERIPHERAL BLOOD T AND B LYMPHOCYTES

The Effects of Whole-Body Hyperthermia Therapy on the Concentration of Peripheral Blood T and B Lymphocytes

Introduction

Mammalian and avian lymphocytes can be grouped into various subclasses, the more important of which include T and B cells.

T Lymphocytes T lymphocytes are responsible for cell-mediated immune responses, and, as such, are potentially of great importance in the host's defence against, and response to, malignant disease.

The percentage of human lymphocytes bearing T cell markers has been reported to be between 1% (Bach et al, 1969) and 82% (Bentwich et al, 1973), however much of this variation is due to technical differences in the assay method (See p. 97). It is generally accepted that the normal range is 50-80% (Mendes et al, 1973 ; Steel, Evans & Smith, 1974a ; Robinson & Lertratanakul, 1975 ; Holm et al, 1975), but it may be affected by age (Teasdale et al, 1976), strenuous exercise (Steel, Evans & Smith, 1974b) etc..

Depression of T lymphocyte levels has been reported in various clinical conditions e.g. sarcoidosis (Cummiskey, McLaughlin & Keelan, 1976), lepromatous leprosy (Dwyer, Bullock & Fields, 1973), various immunodeficiency diseases (Wybran et al, 1973), and in patients who are immunosuppressed, following heart (Khalaf et al, 1976) or kidney, transplants, or who are on haemodialysis (Sengar, Rashid & Harris, 1975).

There has also been considerable interest in measuring the relative (%) and absolute numbers (concentration) of T cells in the blood of patients with malignant disease. Much of this work has concentrated on cancers of lymphoid cells and tissues (Bobrove et al, 1975 : Esber et al, 1976), including the classification of the cell types involved

in various leukaemias (Mathe & Belpomme, 1974). A number of investigations have been carried out on patients with non-lymphoid tumours. The results from some of the more recent studies are shown in Table B1.

Although technical variations strictly invalidate direct comparisons between the results obtained by different workers (Holland, Browne & Thornes, 1975), several trends are apparent:-

i) The relative and absolute numbers of T lymphocytes are depressed in cancer patients, in comparison to normal control subjects.

ii) In many cases, the degree of depression can be related to the extent of disease, such that progression of the tumour correlates with a decline in T cell numbers.

iii) In several cases, a correlation has been reported between the T lymphocyte count and the prognosis, response to treatment etc.

It is the combination of these observations which makes the monitoring of T cell levels valuable in the assessment of the immunocompetence of cancer patients. However this assay is purely quantitative, and gives no indication of the functional integrity of the cells. It is therefore of greater value when used in association with a test of T cell activity, such as response to Phytohaemagglutinin (See Paper C).

There have been several reports that assays which do not detect the maximum number of T cells, give results which correlate more closely with the patient's clinical status and prognosis (Wybran & Fudenberg, 1973 ; Cochran et al, 1976 ; Herberman, 1976). These tests are believed to detect a subpopulation of 'active' or 'early-rosetting' cells (Fudenberg, Wybran & Robbins, 1975). This subgroup may be synonymous with the T-effector cell population,

REFERENCE	CANCER	NO.	CONTROLS	NO.	ASSAY	RESULT	COMMENT
BRUGAROLAS et al (1973)	LUNG CANCER	21	NORMAL	?	E ROSETTING 2h @ 4°C	% T CELLS? NORMALS > CANCER PATIENTS; BREAST CANCER > LUNG CANCER	-
WYBRAN & FUDENBERG (1973)	VARIOUS SOLID - C.L.L. -	104 11	NORMAL AGE, SEX & RACE STATED	100	E ROSETTING IMMED. RESUSP^{n}	% T CELLS GENERALLY NORMALS > CANCER PATIENTS	SOME CORRELATION WITH EXTENT OF DISEASE & CLINICAL STATUS
ANTHONY et al (1975)	BRONCHOGENIC CANCER (MALE)	31	NORMAL AGE RANGE STATED	11	E ROSETTING 1½-4h IN ICE	% T CELLS GENERALLY NORMALS > CANCER PATIENTS	A LOW % AT DIAGNOSIS CORRELATED WITH SHORTER SURVIVAL, ESPECIALLY IN SQUAMOUS CARCINOMA PATIENTS. NO CORREL BTWN. SURVIVAL & ABSOLUTE COUNT.
CATALONA et al (1975)	GENITO-URINARY	52	NORMAL (AGE > 50)	?	E ROSETTING 60' @ 4°C	ABSOLUTE T CELL NOS. NORMALS > 42% CANCER PATIENTS	-
DELLON, POTVIN & CHRETIEN (1975)	BRONCHOGENIC CANCER (VARIOUS STAGES)	112	NORMAL AGE RANGE STATED RACE MATCHED	237	E ROSETTING 60' @ R.T.	ABSOLUTE T CELL NOS. NORMALS > CANCER PATIENTS	RESULTS DEPENDED ON TUMOUR TYPE, BUT CORRELATED WITH EXTENT OF TUMOUR & CLINICAL COURSE USEFUL IN PROGNOSIS OF METASTASES
FRANCHI et al (1975)	LUNG RECTUM COLON	20 4 1	NORMAL AGE RANGE STATED	20	E ROSETTING 1-2h IN ICE	% T CELLS NORMALS ≥ CANCER PATIENTS	NO STATISTICAL ANALYSIS
GROSS et al (1975)	LUNG CANCER	29	PULMONARY DISEASE NORMAL AGE & SEX MATCHED	24 12	E ROSETTING IMMED. RESUSP^{n}	% T CELLS NORMALS > BENIGN > CANCER PATIENTS	SOME CORRELATION WITH TUMOUR SIZE
HOLLAND, BROWNE & THORNES (1975)	VARIOUS (ADVANCED)	14	YOUNG MIDDLE AGED	22 21	E ROSETTING 18h @ 4°C	% T CELLS YOUNG NORMALS > OLDER NORMALS > CANCER PATIENTS	-
KOZINER, COSIMI & BLOCH (1975)	MELANOMA FOLLOWING RESECTION OF 1ry	25	NORMALS	38	E ROSETTING INCUB^{n} @ 4°C	% T CELLS NORMALS > STAGE I, STAGE II CANCER	-
BABUSIKOVA et al (1976)	MELANOMA	64	NORMAL	33	E ROSETTING INCUB^{n} @ 4°C	% T CELLS NORMALS > CANCER PATIENTS	% T CELLS CORRELATED WITH STAGE OF DISEASE AND POST-OP PROGNOSIS

TABLE B1

REVIEW OF PREVIOUS STUDIES ON THE NUMBER OF T LYMPHOCYTES IN THE PERIPHERAL BLOOD OF CANCER PATIENTS

REFERENCE	CANCER	NO.	CONTROLS	NO.	ASSAY	RESULT	COMMENT
COCHRAN et al (1976)	MELANOMA BREAST CANCER	52 59	NORMAL HOSPITALISED BENIGN BREAST DISEASE AGE RANGE STATED	51 41	E ROSETTING 'OPTIMAL' CONDITIONS 'SUB-OPTIMAL' CONDITIONS	% T CELLS N.S.D. CANCER PATIENTS > NORMALS	NO CORRELATION WITH STATE OF DISEASE EXCEPT NUMBER OF 'SUPER' - ROSETTES WAS GREATER IN METASTATIC DISEASE
ELHILALI et al (1976)	UROLOGICAL CANCER	103	YOUNG NORMALS AGE-MATCHED NORMALS	41 47	E ROSETTING O/N @ 4°C	% T CELLS AGE MATCHED NORMALS > LOCALISED CANCER > METASTATIC CANCER	-
GOH & REDDY (1976)	VARIOUS (STATED)	54	NORMAL AGES STATED	33	E ROSETTING O/N INCUBATION	RELATIVE & ABSOLUTE T CELL NOS. NORMALS > CANCER PATIENTS	-
HERBERMAN (1976)	VARIOUS	?	NORMAL	?	E ROSETTING 'SUB-OPTIMAL' CONDITIONS	% T CELLS NORMALS > CANCER PATIENTS	NO CLEAR CORRELATION WITH EXTENT OF DISEASE SOME CORRELATION WITH SUCCESS OF THERAPY.
KELLER et al (1976)	BREAST CANCER	47	BENIGN LESIONS MATCHED NORMALS	36 33	E ROSETTING 60' @ 4°C	% T CELLS NORMALS > BENIGN > CANCER PATIENTS	-
MORALES & FIDINGER (1976)	RENAL CANCER	18	-	-	E ROSETTING 1-2h IN ICE	ABSOLUTE T CELL NOS. LOCALISED CANCER > METASTATIC CANCER	CORRELATION WITH CLINICAL STATUS & RESPONSE TO THERAPY
SILVERMAN et al (1976)	MELANOMA	42	NORMAL AGE MATCHED	41	E ROSETTING 60' @ 24°C	RELATIVE & ABSOLUTE T CELL NOS. N.S.D. SEE COMMENT	% T CELLS STAGE I STAGE II TUMOUR > TUMOURS ABSOLUTE T CELLS STAGE I STAGE II > STAGE III TUMOURS
WANEBO et al (1976)	LUNG CANCER	70	NORMALS	67	E ROSETTING 18h @ 4°C	ABSOLUTE T CELL NOS. NORMALS = STAGE I > STAGE III CANCER	CONCENTRATION OF T CELLS DEPRESSED IN 44% WITH STAGE III CANCER
WHITEHEAD et al (1976)	BREAST CANCER	71	HEALTHY BENIGN BREAST DIS. AGE MATCHED	15 14	E ROSETTING 1½-4h IN ICE	% T CELLS CONTROLS > ALL STAGES OF CANCER EXCEPT LOCALLY - ADVANCED	N.S.D. BETWEEN PRE TREAT & DISEASE - FREE POST TREATMENT
HEIER et al (1977)	VARIOUS (STATED) (- SOLID TUMOURS	62 20)	NORMAL	37	E ROSETTING O/N @ 4°C	RELATIVE & ABSOLUTE T CELL NOS. ↓ IN ALL BUT LYMPHOSARCOMAS SEE COMMENTS	% OF T CELLS REMAINED NORMAL WHILE ABSOLUTE COUNT WAS DEPRESSED BY VARYING DEGREES

TABLE B1 Cont.

since the number of 'active' T cells has been found to correlate with delayed cutaneous hypersensitivity to microbial antigens, in the human (Felsberg, Edelman & Gilman, 1976 ; Felsberg & Edelman, 1977).

It is likely, however, that the T lymphocyte population, in both animals and humans, is composed of a number of sub-groups (Kaplan & Uzgiris, 1976, Cantor et al, 1975), differing in both function and activity (Sasaki et al, 1975). At present, the significance of numerical changes in these groups is not understood and the majority of studies have therefore concentrated upon monitoring variations in the population, as a whole.

Methodology Human T lymphocytes are usually detected by their ability to form non-immune rosettes with unsensitised sheep erythrocytes (Coombs et al, 1970 ; Brain, Gordon & Willetts, 1970 ; Lay et al, 1971). Evidence for the thymus-dependence of the rosette-forming cells was based upon their:-

i) lack of surface immunoglobulin (Jondal, Holm & Wigzell, 1972)

ii) localisation in thymus-dependent areas of the tissues (Silveira, Mendes & Tolnai, 1972).

iii) possession of a thymus-dependent antigen - HTL - Human Thymus-Lymphoid Tissue Antigen (Yata, Tsukioto & Tachibana, 1973).

The nature of the receptor involved in the binding of sheep erythrocytes is not fully understood. Treatment of either lymphocytes, or erythrocytes, with Neuraminidase results in an increase in the number of rosettes formed (Galili & Schlesinger, 1974) ; probably by the reduction of surface electronegativity, by the cleavage of Sialic

Acid residues from lipid chains (Small, 1975 ; Sandilands et al, 1975). Alternatively, the enzyme may unmask previously-hidden receptors, as it is known to alter the antigenicity of cells (Sedlacek, Seiler & Schwick, 1977). These experiments do not however indicate the nature of the receptor. It is known that heating lymphocytes to 45°C for 1h removes the receptors, and that rosetting is restored by incubating the cells in human serum (Mendes et al, 1975). This suggests that the receptor is labile, but that it can be rebound to the cell. This 'fluidity', and the weak bonding between the red cells and lymphocytes - consisting of only limited areas of membrane contact (Tonietti et al, 1975) - have probably contributed to the large variations in estimates of T cell numbers in normal subjects. The technique for rosetting is simple (See p.103), enumeration being performed on wet preparations in a haemacytometer, although permanent preparations can be used (Scafer et al, 1975), and various automated counting techniques have been reported (Vandenbark, Burger & Vetto, 1976 ; Teodorescu, Mayer & Dray, 1975). However, this apparently straightforward test may be affected by numerous variables e.g. method used to separate the lymphocytes (Holm et al, 1975), pre-incubation of the cells (Roszman, Brooks & Muse, 1977), source and age of sheep erythrocytes (Steel et al, 1974a), centrifugation speed and rough handling (Anthony et al, 1975), ratio of red cells to lymphocytes (Chisholm & Tubergen, 1976), temperature of incubation (Galili & Schlesinger, 1975), serum concentration (Steel et al, 1974a) etc. (Reviewed by Browne & Greaves, 1974 ; Mendes et al, 1973 ; WHO Report, 1974). It is therefore important, when making serial measurements, e.g. in monitoring the response to cancer therapy, to adopt strictly standardised techniques, in order to minimise the effect of these variables(WHO Report, 1974).

Alternative methods for the detection of T lymphocytes have been described (Browne & Greaves, 1974), but the simplicity and rapidity of the rosetting technique, makes it the most popular method. In addition, Small (1975) has stated that 'the surface areas involved in rosette formation are also involved in other T cell functions' and that 'the SRBC receptor may be of general importance for T cell functions'.

The rosetting assay can also be of value in detecting various subpopulations e.g. morula-form rosettes may be formed by activated T cells i.e. T-blast cells (Huang et al, 1976 ; Yu, 1976). Another marker for these cells is thought to be rosette formation under 'sub-optimal' conditions, or with autologous erythrocytes (Sheldon & Holborrow, 1975 ; Sandilands et al, 1975).

In conclusion, rosette formation with sheep erythrocytes provides a useful marker for human T lymphocytes, and, under special conditions, for their subpopulations. It can also give valuable information on cellular immunocompetence, provided that carefully-standardised conditions are used.

B Lymphocytes B cells mediate humoral immune reactions (See p.21) and, as such, are probably of lesser importance in the response to cancer.

Changes in the relative and absolute numbers of B cells have been reported in response to heart transplants (Khalaf et al, 1976), and in various immunodeficiency diseases (Seligmann, Preud'Homme & Brouet, 1973). Some studies have been carried out on patients with cancer (especially lymphoma and melanoma). Results from some of the more recent of these are shown in Table B2.

The reports of both elevated and depressed B cell concentrations

REFERENCE	PATIENTS	NO.	CONTROLS	NO.	MARKER	RESULT PATIENT/ CONTROL	COMMENT
BOBROVE et al (1975)	UNTREATED HODGKIN'S DISEASE	42	HEALTHY	?	S - Ig	↓d	IN ABOUT ½ OF PATIENTS ↓ RELATED TO SYMPTOMS
FRANCHI et al (1975)	RESPIRATORY - & DIGESTIVE TRACT - TUMOURS	20 5	HEALTHY	20	EAC S - Ig Agg. - Ig	↔ OR ↓d	NO DIFFERENCE BETWEEN LYMPHOCYTES IN LYMPH NODES & IN PERIPHERAL BLOOD
KOZINER, COSIMI & BLOCH (1975)	MALIGNANT MELANOMA	25	HEALTHY	38	S - Ig	↓d	IN STAGES I & II
BABUŠÍKOVÁ et al (1976)	MALIGNANT MELANOMA	64	NOT STATED	33	EAC	↑d	↑ BECOMES SIGNIFICANT WITH ADVANCING DISEASE
ELHILALI et al (1976)	UROLOGICAL TUMOURS	103	HEALTHY YOUNG - AGE - MATCHED -	 41 47	EA	↑d	↑ d COMPARED TO AGE - MATCHED CONTROLS. ↑ d FURTHER IN METASTATIC DISEASE
GOH & REDDY (1976)	VARIOUS PROGRESSING & INOPERABLE TUMOURS	54	HEALTHY	33	S - Ig	N.S.D. SEE COMMENTS	N.S.D. IN % B CELLS ABSOLUTE COUNT ↓d IN CANCER PATIENTS, & WAS ↓d BY RADIATION THERAPY
KING et al (1976)	LYMPHOMAS IN COMPLETE REMISSION	17	HEALTHY	?	S - Ig EAC	↑d SEE COMMENTS	↑ NOT SIGNIFICANT, EXCEPT INCREASED EAC IN PATIENTS TREATED BY RADIATION THERAPY
HEIER et al (1977)	LYMPHOMAS - VARIOUS SOLID TUMOURS-	42 20	HEALTHY	37	S - Ig	N.S.D.	RANGE WIDER IN PATIENTS THAN IN CONTROLS

TABLE B2

REVIEW OF PREVIOUS STUDIES ON THE NUMBER OF B LYMPHOCYTES IN THE PERIPHERAL BLOOD OF CANCER PATIENTS

in these patients, probably reflects the heterogeneity of the B cell population (See below).

Methodology T lymphocytes can be identified by their exclusive ability to form rosettes with unsensitised sheep erythrocytes. In contrast, the recognised markers for B cells, namely, surface immunoglobulin and receptors for complement and the Fc portion of immunoglobulin, are shared by other leucocytes (Table B3).

Although T cells have been shown to possess surface immunoglobulin (S-Ig) under certain circumstances (Marchalonis, Atwell & Cone, 1972), it is difficult to detect, and as a consequence, this marker has been used to identify B lymphocytes, which are known to produce and secrete immunoglobulin. However, it is now realised that there are two distinct populations of S-Ig-bearing lymphocytes in human peripheral blood - L cells and B cells. In the former group the immunoglobulin is not membrane-incorporated, but is bound to the cell surface by receptors (Lobo, Westervelt & Horwitz, 1975), in addition, these cells possess high-affinity, trypsin-resistant Fc receptors (Lobo & Horwitz, 1975), and therefore probably constitute a separate cell population (Horwitz & Lobo, 1975).

Receptors for complement have been used to enumerate B lymphocytes, by rosette formation with erythrocytes coated with antibody and complement (WHO Report, 1974). These receptors are shared by monocytes. Simultaneous detection of S-Ig and complement receptors has resulted in the discovery of four subpopulations (Winterleitner, Rotter & Knapp, 1977), and the conclusion that the cells bearing S-Ig are not identical with those possessing complement receptors. This is supported by the work of Holm et al, (1975) and Gilbert, Grezes and Ropartz (1974).

MARKER	B CELLS	L CELLS	MONOCYTES	T CELLS
SURFACE IMMUNOGLOBULIN	+ (MEMBRANE-INCORPORATED)	+ (LABILE)	+ (CYTOPHILIC) (IgG)	-/+
F_c RECEPTORS, DETECTED BY:- i) BINDING OF AGGREGATED IMMUNOGLOBULIN	+/-	+	+	-
ii) ROSETTE FORMATION WITH ERYTHROCYTES COATED WITH HUMAN ANTI-ERYTHROCYTE IMMUNOGLOBULIN	-	+	+	-
iii) ROSETTE FORMATION WITH ERYTHROCYTES COATED WITH XENOGENEIC ANTI-ERYTHROCYTE IMMUNOGLOBULIN	+	?	+	+ (ISOLATED REPORT)
COMPLEMENT RECEPTORS	+	-/+?	+	-
ROSETTE FORMATION WITH MOUSE ERYTHROCYTES	+	-?	-	-
ROSETTE FORMATION WITH SHEEP ERYTHROCYTES	-	-	-	+
BINDING OF EPSTEIN-BARR VIRUS	+	?	-	-

TABLE B3

DISTRIBUTION OF SURFACE MARKERS ON HUMAN MONONUCLEAR LEUCOCYTES

The use of the Fc receptor as a B cell marker is complicated by the various results that have been obtained using different detection techniques. The receptor detected by the binding of aggregated-IgG may be distinct from that responsible for rosette formation with erythrocytes coated with human antiserum (Buckley, 1976). Additionally. it has been found that B cell form rosettes with rabbit anti-ox coated ox erythrocytes, but not with human red cells sensitised with Ripley IgG (Lobo & Horwitz, 1976 ; Hallberg, Gurner & Coombs, 1973), suggesting that different receptors may be involved in the recognition of human and xenogeneic Fc portions.

Techniques have been described which may specifically detect B lymphocytes. These include the binding of Epstein-Barr virus (WHO Report, 1974), and rosette formation with unsensitised mouse erythrocytes (Gupta, Good & Siegal, 1976), although the latter may be a property of a subset of B cells (Bertoglio et al, 1977). Methods have also been described for the simultaneous enumeration of B and T cells (Robinson & Letratanakul, 1975 ; Brain et al, 1976).

The main problem in any study on B lymphocytes, therefore, is the exact nature of the cell population involved. The simple division of the human lymphocyte population into two subgroups is now revealed as an over-simplification. The original markers for B cells are possessed by a number of cell types, and it is not clear to what extent each of these subclasses is involved in the mediation of humoral immunity. The use of several markers may be advisable, and has been described by several groups (Franchi et al, 1975 ; Holm et al, 1975), but it may complicate the situation by detecting the various subgroups (Winterleitner et al, 1977). An alternative method has been documented by Eby et al (1975), which detects cells

that are actively secreting immunoglobulin, by the formation of haemolytic plaques (by the lysis of erythrocytes sensitised to human immunoglobulin). This technique is not however a strictly quantitative measure of B cells, since it cannot detect those which are not producing immunoglobulin at the time of assay.

At present, therefore, any enumeration of B lymphocyte numbers, whilst of value in the assessment of general immunocompetence, is of limited worth, since the exact nature and function of the cell types which comprise this population, remains to be established.

The aim of this study was to determine the effect of whole-body hyperthermia treatment on the numbers of peripheral blood T and B lymphocytes, as detected by commonly-accepted markers.

Materials and Methods

T Lymphocyte Counts T lymphocytes were enumerated using the spontaneous sheep erythrocyte rosette test. The method used was a modification of that of Steel et al (1974a).

Isolated lymphocytes were suspended in F10H supplemented with 20% v/v heat inactivated foetal calf serum, at a concentration of $2\text{-}4\times10^6$ viable cells/ml.

Sheep red blood cells (from a specified animal - originally SC1, and later 764 - both of which gave comparable results) were supplied in Alsever's solution and stored, pelleted, at 4^oC for up to 14 days. Immediately before use, 0.1ml of pelleted cells was resuspended in 10ml PBS'A' and centrifuged at 800g for 5min. The cell pellet was resuspended in 10ml PBS'A' to give a 1% suspension, and used immediately.

Equal volumes (30-100µl) of erythrocyte and lymphocyte suspensions were mixed in round-bottomed glass (50x8mm), or plastic (Titertek transfer vials) tubes. The cells were pelleted by centrifugation at 200g for 5min, and incubated overnight at 4^oC.

The majority of the supernatant medium was removed and replaced by an equal volume of 0.1% eosin in saline. The cells were resuspended by very gently rocking the tubes. A sample was removed with a wide-bore Pasteur pipette, to a mirrored improved-Neubauer haemacytometer, fitted with a thin coverslip. The cells were left to settle for 3-4min, and examined using a x40 objective. Viable lymphocytes with 3 or more adherent erythrocytes (Fig. B1) were classified as rosettes. The percentage of rosette-forming cells in the preparation was calculated from a count of at least 200 lymphocytes.

Routinely, the counts obtained by different observers, or from

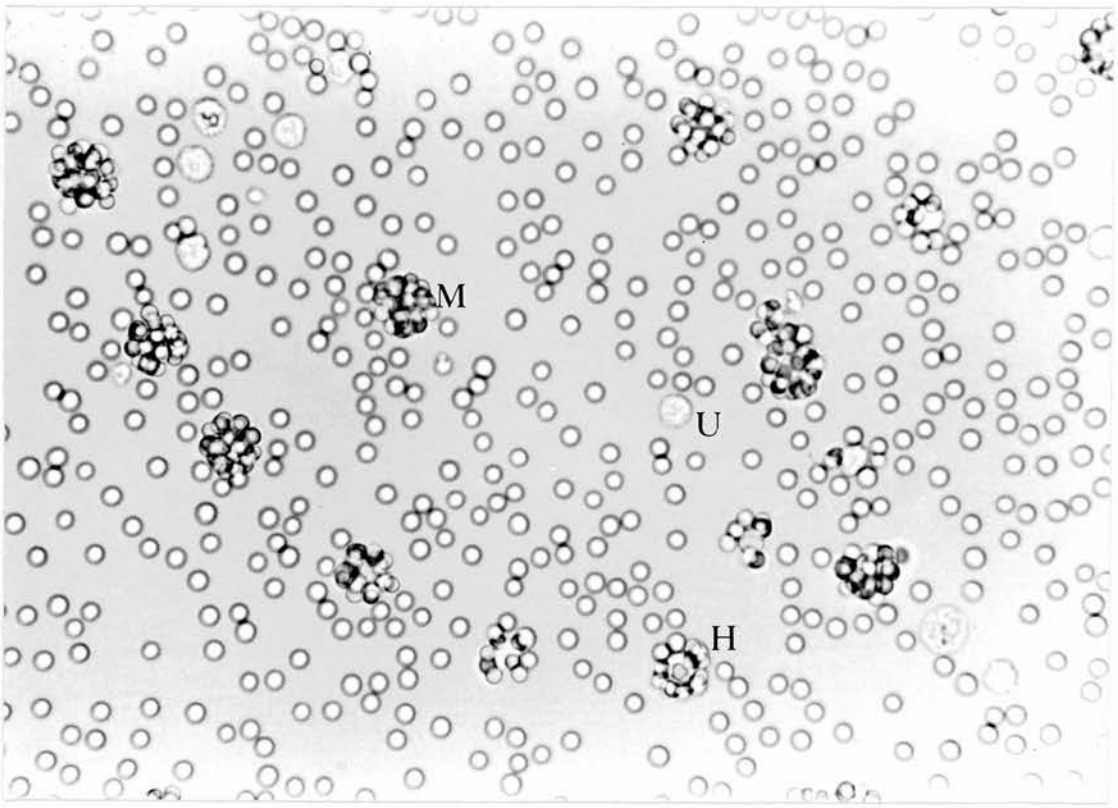


FIGURE B1
SPONTANEOUS ROSETTE FORMATION BETWEEN HUMAN LYMPHOCYTES AND UNSENSITISED SHEEP ERYTHROCYTES
M = Morula form of rosette H = Halo form U = Unrosetted lymphocyte
(Magnification x595)

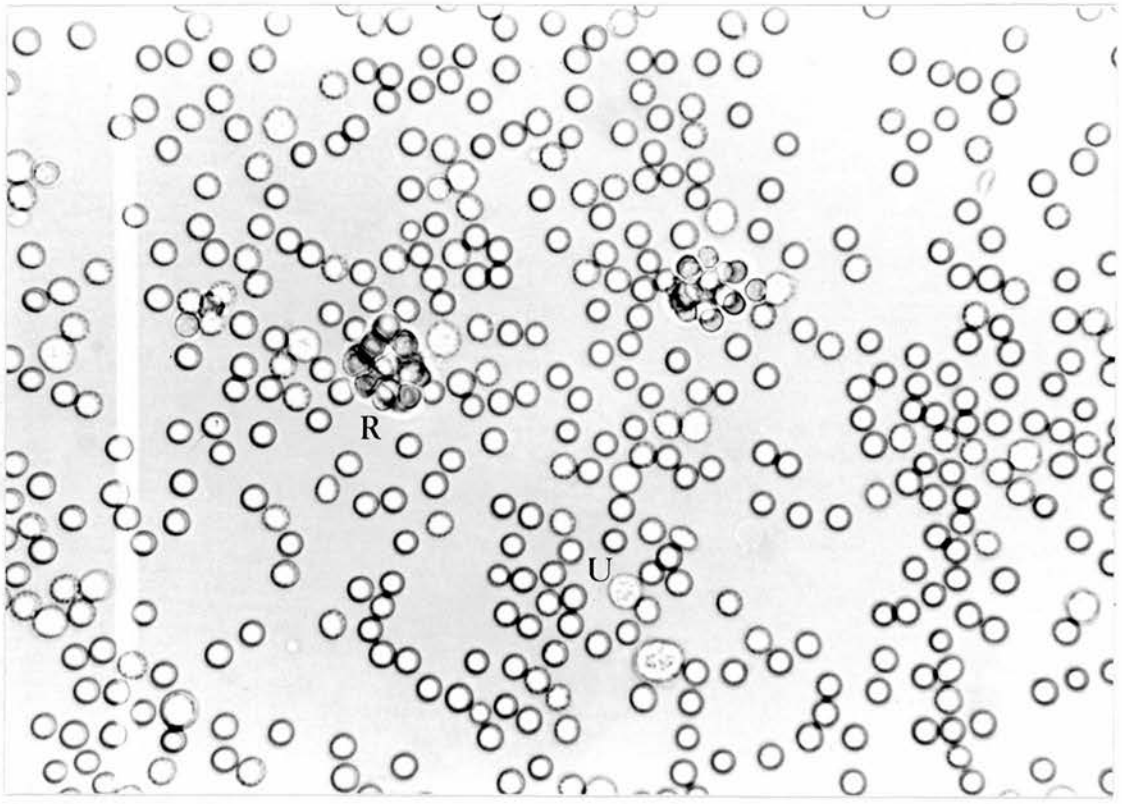


FIGURE B2

SPONTANEOUS ROSETTE FORMATION BETWEEN HUMAN LYMPHOCYTES AND UNSENSITISED RHESUS MONKEY ERYTHROCYTES
R = Rosetted lymphocyte U = Unrosetted lymphocyte
(Magnification x595)

two lymphocyte preparations from the same blood sample, did not differ by more than 5%. Replicate counts made by one observer, on triplicate tubes, usually varied by less than 4%.

'Active' or 'Rapid-Rosetting' T Lymphocyte Counts 'Active' T lymphocytes were enumerated using the same technique as for total T lymphocytes, except that the cell pellets were resuspended immediately after centrifugation at 200g. Reproducibility of the technique was the same as for total T lymphocytes.

Rosette Formation with Rhesus Monkey Erythrocytes (See p.119) The procedure followed was the same as that for total T rosetting with sheep erythrocytes, except that the cell pellets were incubated overnight at room temperature. Rapid-rosetting with rhesus red cells was exactly as for sheep cells. Criteria for classification of rosettes (Fig. B2) were as given above.

Conditions under which Lymphocytes were Heated in vitro Unless stated otherwise, lymphocytes were heated at a concentration of $4x10^6$/ml in F10H supplemented with 20% v/v heat inactivated foetal calf serum. Cells were washed once, in an equal volume of the same medium at room temperature, before use.

B Lymphocyte Counts The number of B lymphocytes in a lymphocyte preparation was calculated from the number of cells forming rosettes with sheep erythrocytes which had been coated with antibody and complement. This technique detects the complement receptor on B cells. The method used was a modification of that described by Stjernsward et al (1972).

Freshly-separated lymphocytes were suspended at a concentration of $2\text{-}4x10^6$ viable cells/ml in F10H supplemented with 20% v/v heat-inactivated foetal calf serum.

Sheep erythrocytes were collected and stored as for T lymphocyte rosetting. 0.1ml of pelleted cells was resuspended in 10ml PBS'A' and centrifuged at 800g for 5min. The cell pellet was resuspended in 4ml of a 1/2,000 dilution of heat inactivated, rabbit anti-sheep red cell serum (Batch K9804 was used throughout the study, and had been titrated by erythroagglutination - the dilution used was double the end-point concentration) in PBS'A'. The suspension was incubated at 37°C for 30min, and the cells pelleted at 800g for 5min, and washed in 3x10ml PBS'A' (centrifugations at 800g for 5min). The cells were then resuspended in 2ml PBS'A' and divided into two aliquots. One sample (EA) was pelleted and stored at 4°C until required. 1ml of a 1/80 dilution (double the end-point for the lysis of antibody-coated erythrocytes) of fresh human serum (obtained from the same donor - Group A, Rh +ve, - throughout the study) in PBS'A' was added to the second tube, which was incubated at 37°C for 30min. The cells (EAC) were then washed three times in PBS'A', and the cell pellet resuspended in 5ml buffer. Before use, the antibody-coated red cells (EA) were brought to room temperature, and 4ml PBS'A' added , to give a final 1% suspension.

For each test the following mixtures were prepared in round-bottomed glass tubes (50x8mm) :-

50µl lymphocyte suspension +

a) 50µl 1% suspension of uncoated sheep red cells E Control

b) 50µl 1% suspension sheep red cells coated with antibody EA Control

c) 50µl 1% suspension sheep red cells coated with antibody and human complement EAC Test

Immediately after mixing the suspensions were centrifuged at 200g for 5min, and incubated in a 37°C water bath for 20min.

The majority of the supernatant medium was removed and replaced by an equal volume of 0.1% v/v eosin in saline. The cells were resuspended by vigorous vortex mixing. A drop of the suspension was placed in a mirrored haemacytometer, under a thin coverslip. The cells were classified as rosettes using the same criteria as for T cell rosetting. The E control repeatedly showed no rosettes, and was eventually abandoned. The EA control occasionally showed 1-5% rosettes.

The reproducibility was similar to that for T cell rosetting.

Scanning Electron Microscopy (See p.130)

Materials

Solution A

1.43% w/v Sodium Cacodylate in boiled, cooled, glass-distilled water.

Solution B

Solution A.....500ml

Sucrose.....28.5g

Fixative

Solution B.....45ml

Glutaraldehyde (25% aqueous).....5ml

Cacodylate Wash

Solution A.....500ml

Sucrose.....51.3g

Method

Cells for examination by scanning electron microscopy (SEM), were resuspended in tissue culture medium at a high concentration e.g. greater than $1x10^7$/ml. Using a wide-bore Pasteur pipette,

5-10 drops of the cell suspension were put into 10ml fixative at 37°C. This suspension was incubated for 1h at 37°C, and then transferred to a 4°C refrigerator, at least overnight, before further processing.

Supernatant fixative was removed, and the cells suspended in 10ml cacodylate wash, at room temperature, for 30min. The cells were then pelleted by centrifugation at 800g for 10min, and then transferred successively through 30min incubations in cacodylate wash : distilled water (1:1), distilled water, and 25%, 50%, 75%, 80%, 95%, 100% (two changes) v/v acetone in distilled water. The final cell pellet was resuspended in approximately double its own volume of 100% acetone, layered onto glass coverslips, under absolute acetone, and dried in a critical point drier. The coverslips were mounted onto aluminium specimen stubs with Araldite Rapid, gold coated in a sputter coater, and stored under vacuum in a dessicator.

Preparations were examined, and photographed, on a Cambridge Stereoscan (Model: 180) scanning electron microscope.

Results

T Lymphocyte Concentration prior to Hyperthermia The pre-treatment concentration of peripheral blood T lymphocytes in 14 hyperthermia patients (8♂, Mean age 52.6 years, Range 23-75 years) was compared with that in 31 normal controls (17♂, Mean age 31.0 years, Range 17-55 years). Statistical analysis was performed using the Student's t test. Although the mean level in patients was slightly lower (1.16×10^6 T cells/ml against 1.34×10^6/ml for normals), the difference was not statistically significant. There was also no significant difference in the percentage of E-rosetting lymphocytes in the two groups.

T Lymphocyte Concentration during and after Hyperthermia Treatment The response of T lymphocytes to hyperthermia treatment was measured in 14 patients (9♂, Mean age 52.6 years, Range 23-75 years) (Tables B 4 and B 5). Statistical analysis was performed on the concentrations (before and after logarithmic transformation, to correct for any possible skew distribution of the data), using the Student's t test for paired samples. p values of less than 0.05 were considered significant.

Prior to treatment the T cell concentration varied widely between patients, however, during heating, it fell significantly ($p < 0.01$) in 78% of cases, to approximately 75% of the pre-treatment level. (The figures presented in this section were obtained after omitting patients H.A. and E.C. from the analysis - the reason for this will be described later). This decrease was apparent in both the original and log-transformed data. Within 24h of hyperthermia, the mean T cell concentration had increased to 86% of the pre-treatment level, and was no longer significantly depressed.

PATIENT	FIRST TREATMENT					SECOND TREATMENT					THIRD TREATMENT				
	PRE 1	END 1	1d POST	3-5d POST	7-9d POST	PRE 2	END 2	1d POST	3-5d POST	7-9d POST	PRE 3	END 3	1d POST	3-5d POST	7-9d POST
M.D.	0.83	0.81	0.97	-	-										
M.M.	1.50	0.98	0.68	0.41	-										
W.M.	0.59	0.79	0.59	0.89	-										
A.B.	1.44	0.78	0.93	1.69	1.35										
A.S.	0.94	0.52	0.49	1.09	0.97										
L.T.	0.82	0.52	-	0.82	-	1.44	0.50	-	0.95	-					
A.W.	3.23	2.19	3.34	-	2.31	1.50	1.14	1.50	1.41	-					
R.M.	1.20	1.00	0.80	-	0.94	0.94	0.50	0.52	0.62	0.36					
L.K.	0.72	0.29	0.62	0.63							0.65	0.51			
P.N.						1.11	0.84	1.23	1.24	1.37	1.37	0.89	1.35	1.33	1.08
K.S.	1.03	0.54	1.40	1.34	-	1.34	0.99	1.19	0.85	1.48	1.48	0.56	0.64	1.04	0.74
T.M.	0.75	0,82	RADIOTHERAPY			0.34	0.19	RADIOTHERAPY			0.36	0.62	RADIOTHERAPY		
H.A.	1.92	1.00	0.99	0.46	-	0.97	2.33	0.89	0.61	0.82	0.82	2.06	0.55	0.76	1.06
E.C.	0.15	0.29	0.06	0.03	0.14	0.14	0.40	0.27	-	-					
	($\times 10^6$/ml PERIPHERAL BLOOD)														

TABLE B4

EFFECT OF HYPERTHERMIA TREATMENT ON THE CONCENTRATION OF PERIPHERAL BLOOD T LYMPHOCYTES

	FIRST TREATMENT					SECOND TREATMENT					THIRD TREATMENT				
PATIENT	PRE 1	END 1	1d POST	3-5d POST	7-9d POST	PRE 2	END 2	1d POST	3-5d POST	7-9d POST	PRE 3	END 3	1d POST	3-5d POST	7-9d POST
M.D.	100	97.59	116.87	-	-										
M.M.	100	65.33	45.33	27.33	-										
W.M.	100	133.90	100.00	150.90	-										
A.B.	100	54.17	64.58	117.36	93.75										
A.S.	100	55.32	52.13	115.96	103.19										
L.T.	100	63.41	B/T	99.53	-	100	34.72	-	65.97	-					
A.W.	100	67.80	103.41	-	71.52	100	76.00	100.00	94.00	-					
R.M.	100	83.30	66.67	-	80.91	100	53.19	55.31	65.95	38.29					
L.K.	100	40.28	86.10	87.50	-	-	-	-	-	-	100	78.46			
P.N.	-	-	-	-	-	100	75.67	110.81	111.71	123.42	100	64.96	98.54	97.08	79.02
K.S.	100	54.43	135.92	130.10	-	100	73.88	88.88	63.43	110.44	100	37.83	43.24	70.27	50.00
T.M.	100	109.33	RADIOTHERAPY			100	55.88	RADIOTHERAPY			100	172.20	RADIOTHERAPY		
H.A.	100	52.08	51.56	23.96	-	100	240.20	91.75	62.88	84.53	100	251.20	67.07	92.68	129.26
E.C.	100	193.30	40.00	20.00	93.33	100	285.71	192.85	-	-					
MEAN (ALL PATIENTS)	100	82.33*	78.41*	85.85	88.54	100	111.91	106.60	77.32*	89.17	100	120.93	69.62	86.68	86.09
MEAN (ALL PATIENTS - HA & EC)	100	74.99*	85.67	104.10	87.34	100	61.56*	88.75	80.21	90.72	100	88.36	70.89	83.67	64.51

TABLE B5

EFFECT OF HYPERTHERMIA TREATMENT ON THE CONCENTRATION OF T LYMPHOCYTES EXPRESSED AS A PERCENTAGE OF THE PRE-TREATMENT VALUE

* Statistically significant by Student's t test on paired, log-transformed, values. B/T = Blood transfusion.

There was a further rise, in most patients, during the following 2-4 days, which resulted in a net increase in the concentration over the pre-treatment value. Any such 'peak' was no longer apparent by 7-9 days after hyperthermia, when the concentration had fallen to 87% of the pre-heating value.

Similar decreases in T cell concentration, during heating, were seen in the smaller number of patients receiving second and third treatments (Figure B 3), however, they were only statistically significant ($p < 0.02$) during the second treatment. The return to pre-treatment values was again seen within 24h, but there was no indication of a 3 day 'peak' in numbers.

While most patients followed this trend, there were some marked exceptions. The T cell concentration rose during heating in several patients (notably H.A. and E.C.). This was due to an atypical increase in the concentration of peripheral blood lymphocytes, rather than in the percentage of E-rosette forming cells. Similar increases were seen during subsequent treatments of these patients. The following day both H.A. and E.C. showed falls in T cell numbers (which, in some cases, were very substantial) often to below the pre-treatment level, with recovery within 7-9 days. Due to their persistantly atypical responses, statistical analysis was performed both omitting, and including, these patients. Their inclusion masks the fall in T cell concentration during the second and third treatment, but indicates that there may be some slight lymphopenia persisting after hyperthermia. This is supported by the data from the other patients, but is not statistically significant.

Only one (E.C.) of the two omitted patients showed atypical clinical responses. She developed a leucopenia, with a relative

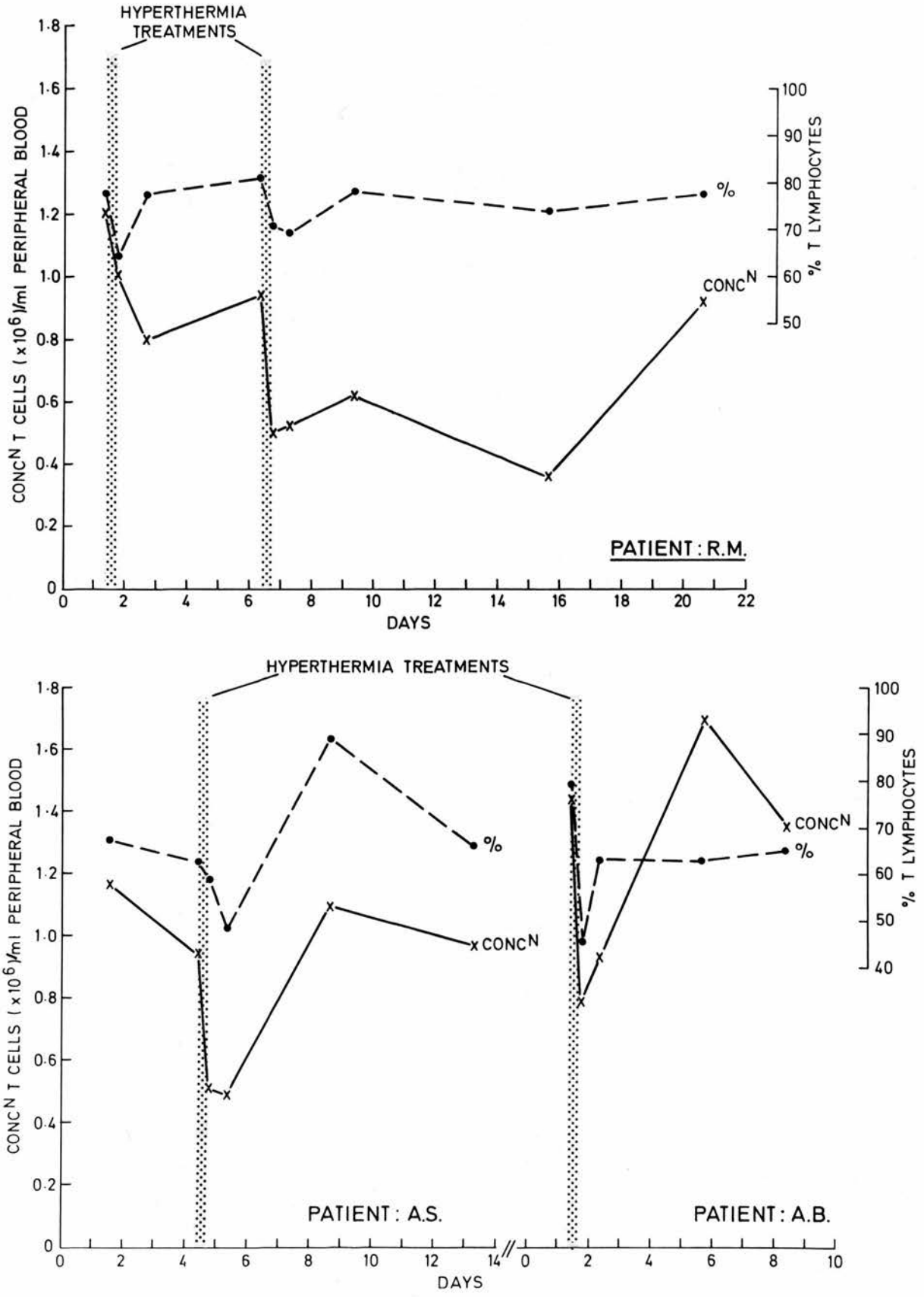


FIGURE B3

EFFECT OF SUCCESSIVE HYPERTHERMIA TREATMENTS ON THE PERCENTAGE AND CONCENTRATION OF PERIPHERAL BLOOD T LYMPHOCYTES IN THREE PATIENTS

and absolute lymphocytosis, during her first treatment. During the second treatment, the lymphocytosis was associated with an increase in the total leucocyte concentration. This patient died of a pulmonary embolism four days after her second hyperthermia fraction, and, on post mortem, there was evidence of massive tumour necrosis. This was taken as indicative of a positive response to therapy. The only other clinically responsive patient studied, using this assay, was A.W.. In contrast to E.C., he displayed the 'typical' pattern of T lymphopenia during hyperthermia, but it is interesting to note that he had by far the highest pre-treatment T cell concentration, although this was rapidly depressed by hyperthermia therapy (Table B 4).

In most cases, a course of treatment did not substantially affect the concentration of peripheral blood T lymphocytes, and there was no apparent correlation between any changes, and the response to treatment, or survival time.

Discussion

In contrast to previous studies (Table B 1), there was no evidence of depressed T cell numbers in these cancer patients, prior to hyperthermia, in comparison with the normal control group. This was probably not due to the difference in the mean ages of these groups. Teasdale et al (1976) reported a fall in the percentage of rosetting cells with increasing age, and as the patient group in this study was significantly older than the controls, this should, if anything, reduce the slight difference in T cell numbers between the two groups. Alexopoulos and Babitis (1976) also note a progressive decline in the T cell concentration with increasing age, due to a reduction in lymphocyte numbers in

elderly subjects. Again this should further reduce the small difference between the cancer patients and normal control group in this study.

It is, however, notable that, whereas T cell concentrations below 1.0×10^6/ml peripheral blood were seen in only 19% of normal subjects, they occurred in 50% of the patients. Data available for four of the seven T-lymphopenic patients, indicated that they tended to have reduced survival times following treatment (three survived a mean of 22 days, however, the fourth survived for over 200 days) in comparison to the remainder (data is available for five patients, mean survival time 150 days, range 11-485 days). It is possible that a longer study may reveal a more widespread depression of T lymphocyte numbers in these subjects, similar to that previously reported in cancer patients. However, since all of the hyperthermia cases had received other forms of treatment, their increased incidence of T lymphopenia may not be attributable to cancer, but rather to the residual effects of previous therapy. This is supported by studies which have shown that radiotherapy produces a marked T lymphopenia (Stefani, Kerman & Abbate, 1976 ; Raben et al, 1976 ; Anderson & Warner, 1976) which may last for up to a year (Stjernsward et al, 1972). In this study, one patient (T.M.) had had recent radiotherapy, and this may have been reflected by his continued T lymphopenia throughout the investigation (Table B4). It is likely that similar changes may occur following surgery, chemotherapy, anaesthesia and medications, and few studies that report T lymphopenia in cancer patients, have taken account of this.

In summary, the investigation on pre-hyperthermia cancer patients, indicated that, as a group, they had normal relative and absolute T cell numbers, but that there was an increased incidence

of T lymphopenia. The reason for this is not clear. In addition, those patients with decreased T lymphocyte counts tended to have reduced survival times, in comparison with the remainder.

The fall in the peripheral T cell count during hyperthermia is due largely to a decrease in the percentage of rosetting cells, since the total lymphocyte concentration is unaffected by treatment (See p. 82). A similar phenomenon has been reported in healthy subjects subjected to temperatures of 38.8^{o}C (Bork-Wolwer et al, 1977). In contrast, Fabricius et al (1977) described only slight changes in T cell numbers during 1h of 40^{o}C hyperthermia. Neither group found changes in the total lymphocyte count, and Fabricius et al indicated that there was no change in the peripheral leucocyte concentration, during and after heating. In the only other study on cancer patients, DeHoratius et al (1977) found no statistically significant changes in the proportion, or total number, of T cells, following 2h at 42^{o}C, in three patients. Nonetheless, in the two treatments they studied in which cytotoxic drugs were not given, the concentration of T cells fell to 53% and 63% of the pre-treatment values. This was mainly due to a fall in the total lymphocyte count, although the percentage of E-rosetting cells also fell by 20% in one case. In the two treatments in which chemotherapy was given, the T cell concentration rose, but there were slight falls in the percentage of rosette forming cells. However the small number of patients studied, makes this report difficult to evaluate.

The results from this study, taken together with those from other workers, suggest that T lymphocyte concentrations are depressed by whole-body hyperthermia. This is probably not due to the effects of anaesthesia, since the normal subjects treated by sub-41^{o}C heating

were unanaesthetised. It is possible that the changes may be due to a stress reaction, similar to that affecting the total leucocyte concentration (See p. 87). It is known, for example, that cortisol levels affect T cell function (Tavadia et al, 1975 ; Zeman et al, 1972), and Steel et al (1974) have reported that the ratio of T to B lymphocytes is decreased following strenuous exercise. This may be due to elevated steroid levels, and they concluded that the majority of rapidly-mobilised cells were B lymphocytes. Their data, however, indicated that the decrease in T cell numbers was only relative, their concentration remaining largely unaffected. In contrast, the results from the hyperthermia patients indicated a decrease in both the percentage and concentration of T cells. In addition, in the patients, the lymphocyte concentrations did not change significantly during heating, whereas the drop in E-rosetting cells, described by Steel, was accompanied by a marked lymphocytosis. These observations would argue against a steroid-mediated depression of T cell concentration. The stability of the total lymphocyte concentration during hyperthermia also suggests that the changes are not due simply to haemodilution. Whilst the medications given during treatment may contribute to the fall, it was concluded that the most likely cause was the increased body temperature.

Experiments involving heating in vitro, were carried out to test this hypothesis (See p. 116).

Concentration of 'Active' or 'Rapid-Rosetting' T Lymphocytes before and after Hyperthermia Treatment The concentration of peripheral blood 'rapid-rosetting' T cells has been reported to correlate more closely with clinical status, and prognosis, that that of total T

lymphocyte concentration (Wybran & Fudenberg, 1973 ; Cochran et al, 1976). This assay was carried out on two patients (L.T. and T.M.) towards the end of this study.

Prior to treatment, the percentage of rapid-rosetting cells was 70.8% and 49,3% in T.M. and L.T. respectively. In a group of five, unmatched, normal controls, the mean percentage (± 1 S.D.) was 41.7% (± 11.2).

T.M. was given radiotherapy between hyperthermia fractions, and so the test was performed only at the beginning and end of each heating session. During each of four fractions, the active T cell concentration fell to 54-92% of the pre-treatment value. This was associated with a fall in the percentage of rapidly-rosetting cells to between 53% and 70% of the pre-treatment percentage. During the full course of treatments, there was a progressive decline in both the percentage, and concentration of these cells, probably reflecting the effects of the radiotherapy. There was a sudden marked increase in the percentage of rapidly-rosetting cells between the fourth and fifth treatments, and examination of the separated lymphocytes, showed a very high granulocyte contamination (33%). This emphasises the need to determine the separation purity for each test, as it appears that, in this assay, it is easy to confuse immune adherence, prior to phagocytosis, with rapid-rosette formation.

The second patient, (L.T.) showed falls in active T cell concentration during both treatments, to 74% and 66% of the pre-treatment values. This depression was still apparent the following day, and was deepened by a blood transfusion, but within 5 days, the concentration had increased to 151% of the pre-hyperthermia level. This was due to an increase in both the total lymphocyte

concentration, and the percentage of rosetting cells. Following the second treatment, the resulting active-T lymphopenia was still evident four days later. As these are recent findings, the clinical response of the patients to treatment, has yet to be determined, and so it is not possible to relate these results to prognosis.

Discussion

The fall in concentration of rapid-rosetting cells is at variance with the findings of DeHoratius et al (1977), who reported significant increases in both the percentage, and the number of the cells during hyperthermia. They liken this increase to that in cancer patients responding to treatment with transfer factor (Levin, Byers & Fudenberg, 1975). They do not, however, indicate the purity of their end-of-treatment lymphocyte suspensions. This is a most important parameter in this assay, since these cell populations tend to contain higher proportions of granulocytes, which may be erroneously classified as rapid-rosette-forming cells.

In the absence of literature on the effects of radiotherapy, anaesthesia etc. on rapid-rosetting lymphocytes, it was felt that the changes seen in these patients, since they were similar to those occurring in the total T cell population, were probably primarily the result of the increased body temperature. This was examined in a series of experiments in vitro.

Effect of Heating Blood in vitro on the Percentage of T Lymphocytes

The effect of heating whole blood in vitro at 42°C on the percentage of E-rosette-forming lymphocytes is shown in Figure B4. In every case, the percentage was depressed by heating at 42°C, but unaffected, or slightly increased, in blood kept at 37°C. The effect of the same heat dose, on different blood samples, varied widely, but the

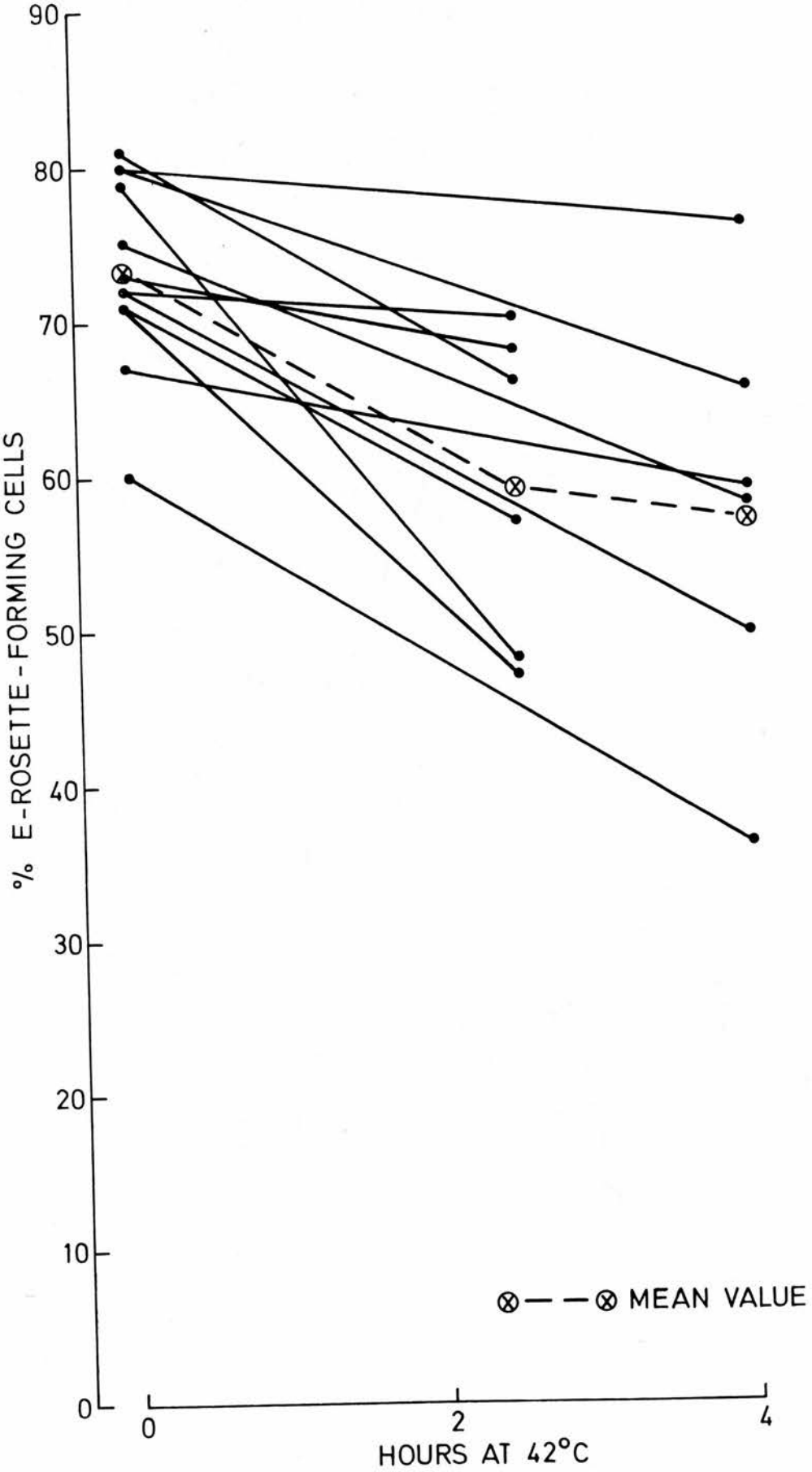


FIGURE B4

EFFECT OF HEATING WHOLE BLOOD IN VITRO ON THE E-ROSETTE FORMING CAPACITY OF THE CONSTITUENT LYMPHOCYTES

Lymphocytes were separated from heated blood and rosetted overnight at 4°C. See text for details.

magnitude of the decrease was, in general, related to the duration of heating.

Blood heated at 39°C, or at 40°C, for 2½h, showed an intermediate depression of the percentage of E-rosetting cells. The viability of lymphocytes from heated blood was routinely greater than 95%, by dye exclusion.

To determine to what extent the fall in T lymphocyte concentrations seen *in vivo*, was due to raised body temperature, rather than to medications etc., an *in vitro/in vivo* experiment was set up.

Blood was taken from a patient (K.S.) immediately before treatment, defibrinated, and split into three aliquots. One sample was used at once to determine pre-hyperthermia T cell concentration. The remaining samples were sealed in plastic tubes, one of which was incubated in a water bath at 37°C, and the other was inserted into the patient's rectum. The blood in this tube was exposed to the elevated body temperature, but not to the drugs etc., used during treatment. At the end of hyperthermia, an end-of-treatment blood sample was taken, and used to determine the post-treatment T cell concentration. The percentage of rosetting cells in the rectal, and control tubes was also measured.

Prior to treatment 50.8% of lymphocytes formed E-rosettes, this fell to 31.5% during hyperthermia treatment. Pre-treatment blood kept at 37°C contained 55.7% rosetting cells, but blood from the rectal tube contained only 26.4% T lymphocytes. The viability of all the separated cell suspensions was greater than 95%.

Effect of Heating Isolated Lymphocytes in vitro on the Percentage of T cells The effect of heating a suspension of normal lymphocytes *in vitro* at 42°C (See p. 104) on the percentage of E-rosetting

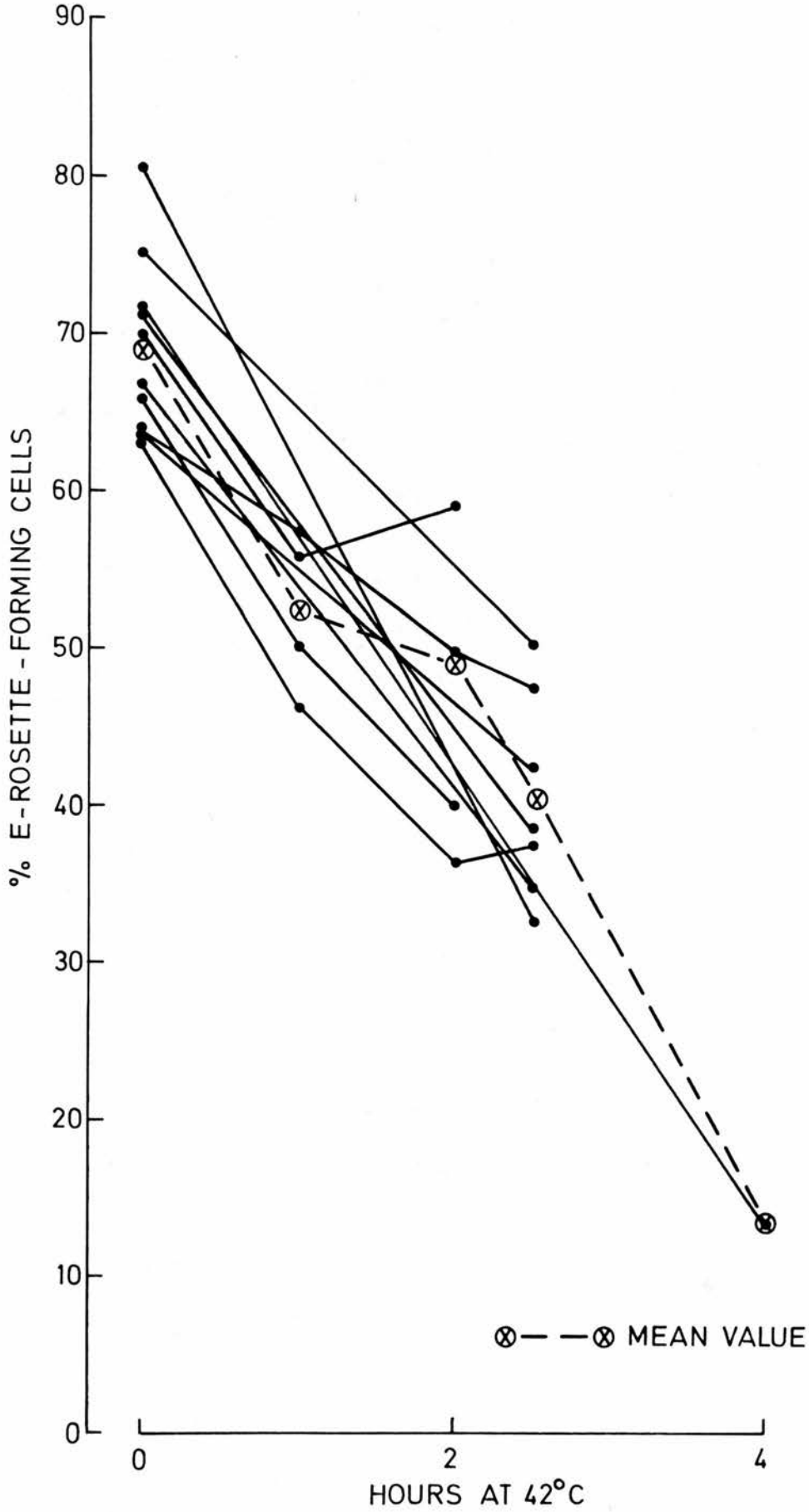


FIGURE B5

EFFECT OF HEATING LYMPHOCYTE SUSPENSIONS IN VITRO ON THEIR E-ROSETTE FORMING CAPACITY
Heated lymphocytes were washed once and rosetted overnight at 4°C. See text for details.

cells is shown in Figure B 5 . Heating at 42°C resulted in a marked decrease in rosetting lymphocytes (in the absence of any fall in cell viability, as detected by dye exclusion), whilst there was no change in the control suspensions, incubated at 37°C. The depression was directly related to the heat dose, and was similar in magnitude for lymphocytes from various donors. It could not be produced by incubating cells for 2½h at 37°C in medium which had previously been heated at 42°C.

Typical results, from a more detailed study of shorter-term incubations at 42°C, are shown in Figure B 6 . The decrease in the percentage of E-rosetting cells was still apparent, however, in general, the percentage did not fall below 30% during 150min at 42°C. There was also evidence of cycling of the percentage about this value. It is unlikely that this is an artefact due to technical variations in setting up the tests, as all the conditions were rigorously standardised, and there is no evidence of cycling in the control suspension incubated at 37°C. The phenomenon was more marked in some lymphocyte suspensions than in others.

These results all point to the heat sensitivity of the T cell, which was more apparent when isolated lymphocytes were heated in medium + serum, rather than in whole blood. Cells heated in suspension, for 2½h at 42°C, and then incubated in autochthonous serum for 1h at 37°C, showed no increase in rosetting capacity. Lymphocytes incubated for 15min in medium + foetal calf serum, at 37°C or 4°C, following heating at 42°C for 15-150min, showed some evidence of recovery of rosetting ability, as indicated by a 0.5-15.5% increase in the percentage of rosette-forming cells. However, this recovery was not always observed, and in some cases, post-42°C incubation resulted in a fall (ranging from 0.5-7.0%) in

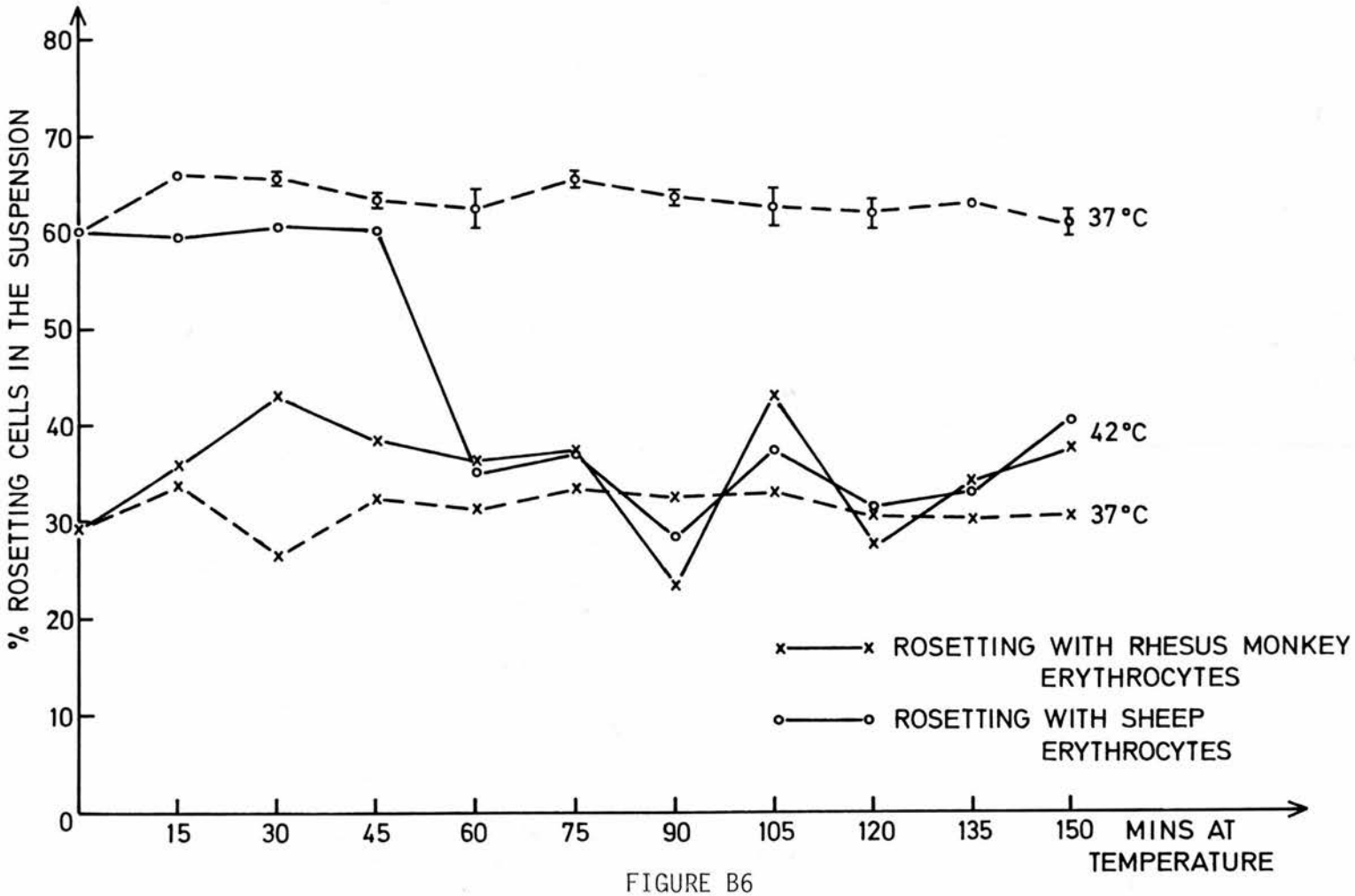


FIGURE B6

EFFECT OF PRE-HEATING ISOLATED LYMPHOCYTES IN VITRO ON THEIR ABILITY TO FORM SPONTANEOUS ROSETTES WITH SHEEP OR RHESUS MONKEY ERYTHROCYTES

Points shown are mean of triplicate samples. The standard deviations shown are typical of those for all points. Lymphocytes were pre-incubated at the temperatures shown and rosetted overnight. See text for details

the percentage of E-rosetting cells. Where recovery was apparent, its magnitude could not be related to the heat dose which the cells had received.

There was some indication that heating isolated lymphocytes in suspension in autochthonous serum, rather than medium, had a less deleterious effect, as indicated by an increased percentage of rosetting cells e.g. lymphocytes heated in autochthonous serum for 2½h at 42°C contained 53% rosette-forming cells, whereas the same lymphocytes, heated in medium + foetal calf serum, contained only 33% rosetting cells - the suspension kept at 37°C contained 69% rosetting cells.

Rosetting between human lymphocytes and rhesus monkey erythrocytes (EM-rosette formation) has been reported to be a T cell phenomenon (Lohrmann & Novikovs, 1974). In contrast to the E-rosette assay, it is believed to be unaffected by the temperature of incubation during rosetting (Lohrmann & Novikovs, 1974 ; Galili & Schlesinger, 1975). This system was investigated to determine whether it was affected by pre-heating of the lymphocyte suspension.

The percentage of cell forming EM rosettes (33.3%-67.5%) was consistently below that forming E-rosettes. However, in marked contrast to the results with sheep erythrocytes, EM rosette-formation was not affected by the pre-incubation of the lymphocytes at either 42°C, or 37°C (Figure B 6). There was some evidence of cyclic variation of the percentage of rosetting cells around the control value.

In parallel experiments, the percentage of E-rosetting cells in a lymphocyte suspension, decreased during heating at 42°C, until

it reached a level similar to the percentage forming EM rosettes. The E and EM rosetting populations then cycled synchronously (Figure B 6).

In an attempt to identify the EM-rosetting lymphocytes, purified T and 'B' cell populations were used. These were produced by centrifuging a suspension of lymphocytes, which had been rosetted with sheep erythrocytes for 3-4h at $4^{o}C$, on a Ficoll-Hypaque gradient. The denser, rosetted T lymphocytes were collected as the cell pellet, and the adherent sheep erythrocytes lysed with Tris-buffered Ammonium Chloride. The unrosetted 'B' lymphocytes were retained at the Ficoll-Hypaque interface and were removed and washed in medium, before use. This purification process produced populations containing 1.75-14.50% E-rosetting (B enriched) and 80.5-81.2% E-rosetting (T enriched) cells. The number of EM-rosetting lymphocytes, however, was similar in both populations e.g. 58.5% in a 'B'-enriched fraction (containing 1.75% E-rosetting lymphocytes) and 56.5% in a T-enriched fraction (containing 80.5% E-rosetting lymphocytes). This indicated that EM rosette formation was not restricted to either T or B lymphocytes.

Effect of Heating Isolated Lymphocytes in vitro on the Percentage of Rapid-Rosetting T cells The effect of heating whole-blood in vitro on the proportion of rapid-rosetting lymphocytes, was not determined.

Heating isolated lymphocyte suspensions had a similar effect to that described for total E-rosetting, namely, a substantial depression , within 2h at $42^{o}C$. The effect was more severe than that seen with E-rosetting, in that fewer than 10% of lymphocytes formed rapid-rosettes after heating for 2h. Pre-incubation of the lymphocytes at $37^{o}C$, for up to $2\frac{1}{2}$h, has no effect on rapid rosette

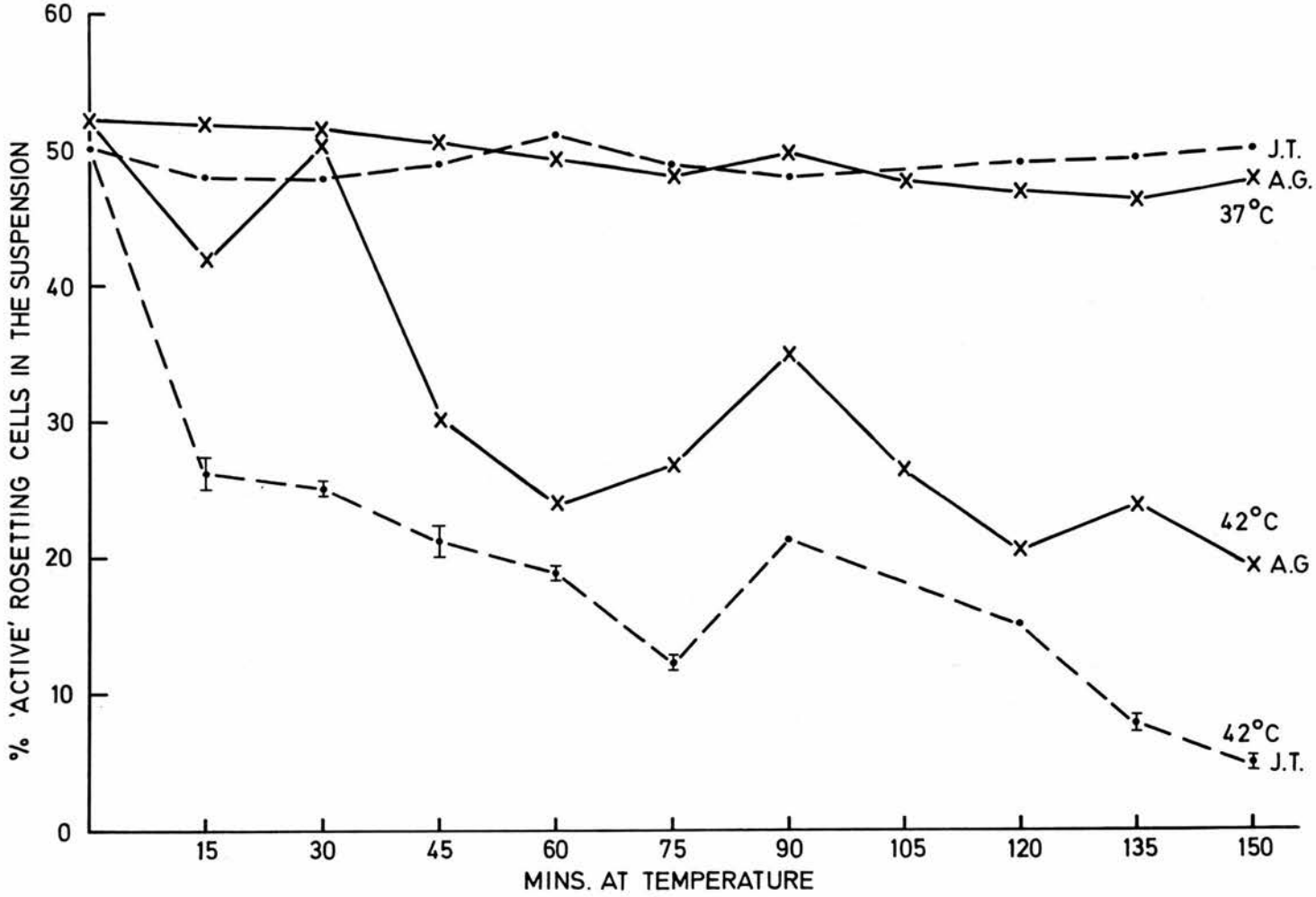


FIGURE B7

EFFECT OF PRE-HEATING ISOLATED LYMPHOCYTES IN VITRO ON THEIR ABILITY TO FORM "RAPID" ROSETTES WITH UNSENSITISED SHEEP ERYTHROCYTES

Lymphocytes from 2 subjects (J.T. & A.G.) were pre-incubated at the temperatures shown, rosetted, & scored immediately. Points shown are mean of triplicate samples. The standard deviations shown are typical of those for all points.

formation (Figure B 7).

Parallel experiments in which rapid and E-rosetting tests were carried out on the same heated lymphocyte suspension, confirmed the increased heat sensitivity of the rapid-rosetting cells e.g. after 2h at 42°C the E-rosetting population fell to 76% of the pre-heating value, whereas the rapid-rosetting population had declined to 32%.

If heated (15-150min at 42°C) lymphocytes were incubated for 15min at 37°C or 4°C, in medium + foetal calf serum, there was a variable effect on their rosetting ability. This ranged from a 15% increase in the percentage of rosetting cells, to a 16% decline. No clear trend to these changes could be discerned.

Rapid-rosetting with rhesus monkey erythrocytes was examined in two experiments. In both cases, the proportion of rosetting cells was not affected by the pre-incubation of the lymphocytes at either 37°C or 42°C. Experiments involving parallel rapid - rosetting with sheep and monkey erythrocytes, using the same lymphocyte suspension, produced similar results to those tests with overnight rosette formation (as shown in Figure B 6), i.e. a decline in the proportion of rapid-rosetting cells to a level similar to that forming rapid-EM rosettes (~20%). There was also evidence of cycling around this value.

Discussion

These results indicate that T lymphocytes are sensitive to temperatures of 42°C *in vitro*, as indicated by their decreased ability to form rosettes with unsensitised sheep erythrocytes. Since this marker may be an indicator of T cell function (Small, 1975), it is likely that heat also depresses T cell mediated cytolysis, and indeed, this has been reported by Harris (1976).

The temperature sensitivity of the incubation procedure during rosette formation has been noted previously, but reports on the effect of pre-heating the lymphocytes, are less common. Coombs et al (1970), and Mendes et al (1975) found that heating normal lymphocytes for 1h at 45°C, had little effect on their viability (as assessed by dye exclusion), but abolished E-rosette formation. Neither study included a control suspension kept at 37°C. Roszman, Brooks and Muse (1977) reported that pre-treatment of the lymphocytes by incubation at 37°C for 30min, in medium (± foetal calf serum) significantly decreased their rosetting ability. However, longer incubations resulted in a return to normal levels.

In this study, pre-incubation of lymphocytes at 37°C consistently had no effect on the percentage of E-rosette, or rapid-rosette, -forming cells. The results are generally in agreement with those of Coombs et al and Mendes et al, although abolition of rosetting was not seen, even after heating lymphocytes in whole-blood at 42°C for 4h.

There is some indication that the thermosensitivity of the cells could be increased by using conditions which progressively departed from those pertaining during hyperthermia in vivo. Heating of the 'same' blood sample in vivo and in vitro, in the rectal tube experiment, showed that isolation from the peripheral circulation, during hyperthermia treatment, resulted in a larger fall in the T cell percentage, than that seen in vivo. In addition, heating isolated lymphocytes in suspension, depressed rosetting capacity more than heating cells in whole blood. This suggests that lymphocytes in blood are either 'buffered' against, or can recover from, thermal effects. The results from this study favour the former explanation, since heating at 42°C had less effect

on lymphocytes in autochthonous serum, than in medium + foetal calf serum. Also, pre-heated cells showed no recovery of rosetting capacity following incubation in autochthonous serum at 37°C, although there was some slight increase in rosette formation, after incubation in medium + foetal calf serum, at 37°C and at 4°C, suggesting that the damage is not completely irreversible. This shows some similarity to the results obtained by Roszman et al, who found that the decrease in rosetting capacity produced by pre-incubating lymphocytes at 37°C, in medium + foetal calf serum, was not seen if human autologous, or autochthonous, serum was added to the medium.

The nature of the cyclic variation in the proportion of rosetting cells, during heating, is not understood. Conditions were chosen which minimised technical variation during replicate assays, however, these cannot be totally excluded. It is possible that the increases in rosetting represent resynthesis, or recovery, of E receptors following thermally-induced deformation, but it is difficult to explain why this should occur while the cells are still at elevated temperatures. If the variations are due to receptor resynthesis, it is possible that the process can occur at 42°C, but becomes increasingly inhibited during prolonged exposure.

Any theory on receptor resynthesis must be balanced by the observation that the process was not apparent when cells were incubated in medium at 37°C or 4°C for 15min, after heat exposure, Mendes et al have reported a return of rosetting capacity in heated (1h at 45°C) lymphocytes incubated in human serum, and have postulated that E receptors are readsorbed onto the cell surface. If this is the case then the dilution of cells, during the post heating incubation (resulting from the addition of the cell suspension to an equal volume of medium at the appropriate temperature) in

these experiments, may have resulted in a reduction in the concentration of shed receptors, and an associated reduction in the efficiency of readsorption. This would not explain the lack of recovery of rosetting capacity seen after heated ($2\frac{1}{2}$h at 42^{o}C) cells were incubated in autochthonous serum (1h at 37^{o}C), although it is conceivable that the effects of this size of heat dose, are irreversible.

There is no real evidence from Mendes' work that the receptors are shed from the cell surface during heating, it is possible that they are deformed by the heat, so that rosette formation is no longer possible, and that the recovery is due to the reversal of this process, rather than to the readsorption of complete receptors. This could explain the lack of recovery seen in these experiments, where the conditions may not have been suitable for the receptors to recover within the 15min allowed. It is also likely that the severity of receptor deformation is related to the heat dose, so that the lack of recovery, seen when heated lymphocytes were incubated in autochthonous serum for 1h, may have been due to irreversible damage to the surface receptors, during $2\frac{1}{2}$h heating.

Although the results indicate that the effects of heat on E-rosetting and rapid-rosetting cells, are broadly similar, it would appear that the latter are more thermosensitive, in that the decrease in their rosetting capacity was greater (usually to less than 10% rosetting cells). Assuming that rapid-rosette formation detects a subpopulation of T cells, then it is possible to explain much of the decrease in E-rosette formation, during heating, to a selective effect on the rapid-rosetting fraction. If this is indeed the case, it may be of clinical importance, since changes in the concentration of these cells are believed to be of value in predicting prognosis etc.

The tests involving rosette formation with rhesus monkey erythrocytes, are difficult to interpret. The results indicate that this phenomenon is not an exclusive marker for T cells, but possibly detects another lymphocyte subpopulation. This is at variance with the findings of Lohrmann and Novikovs, who indicated that the EM receptor was present in an accessible form only on T cells. However they did demonstrate EM-rosette formation with Vibrio cholerae neuraminidase-treated human B lymphocytes. Pellegrino, Ferrone and Theofilopoulos (1975) found that rosette formation between human lymphocytes and erythrocytes from a different species of monkey (the Japanese ape, Macaca speciosa) was a marker for B cells. These studies may be open to a different interpretation, as indicated by this study. Certainly some EM-rosetting cells are T lymphocytes by the criterion of E-rosette formation, and will therefore have thermostable EM-receptors; but it is not clear whether these same cells are those responsible for the apparently thermoresistant tail of the E-rosetting population. The fact that the E-rosetting population declines during heating, to a proportion similar to that forming heat-stable EM rosettes, indicates that the same cells may be involved, and that rosette formation with rhesus monkey red cells may be an indication of thermoresistance.

In summary, heating whole blood, or isolated lymphocytes, in vitro, results in a decline in the proportion of T cells, as detected by both E-rosette and rapid-rosette formation. The effect is more severe on lymphocyte suspensions, indicating some thermoprotection by whole blood. Rapid-rosette-forming T cells have greater heat sensitivity than the E-rosetting population as a whole, suggesting that the T lymphocyte population may be composed

of various subpopulations, differing in their thermal tolerance. This is reinforced by the heat stability of EM-rosette-forming lymphocytes. These cells are neither exclusively T or B lymphocytes, by conventionally-accepted markers, and may represent a thermo-resistant third population, whose clinical significance remains to be established.

B Lymphocyte Concentration prior to Hyperthermia B cell concentrations in 7 patients (3 ♂, Mean age 55.0 years, Age range 35-75 years), prior to hyperthermia treatment, were compared with those of 10 normal controls (2♂, Mean age 32.2 years, Range 20-55 years).

The mean concentration of B lymphocytes in the patients was 2.97×10^5 cells/ml peripheral blood (S.D., 2.97) and the mean percentage of EAC-rosetting cells was 19.8 (S.D., 12.6). In the control group, the mean concentration was 3.33×10^5 cells/ml ($\pm$1.61), and the mean percentage was 17.0 ($\pm$5.99).

None of these differences was statistically significant by the Student's t test.

B Lymphocyte Concentration during and after Hyperthermia Treatment

B lymphocyte concentrations in the peripheral blood, were monitored in the same 7 patients, during and after treatment. The results are shown in Tables B 6 and B 7. Statistical analysis was performed as for the data on T cell concentrations.

During hyperthermia the concentration of B cells increased in the majority of patients. Although this increase was often substantial, it was only statistically significant ($p < 0.02$ -log. data) the second treatment. In general, there was a return to the pre-treatment level within 24h, with some indication of an undershoot after the third treatment. The concentration remained essentially unchanged (with respect to the pre-hyperthermia level) 3-9 days following heating. This pattern was repeated during subsequent treatments (Figure B 8)

Effect of Heating Blood in vitro on the Percentage of B Lymphocytes

In four experiments the effect of heating lymphocytes in whole blood

PATIENT	FIRST TREATMENT					SECOND TREATMENT					THIRD TREATMENT				
	PRE 1	END 1	1d POST	3-5d POST	7-9d POST	PRE 2	END 2	1d POST	3-5d POST	7-9d POST	PRE 3	END 3	1d POST	3-5d POST	7-9d POST
M.D.	1.58	0.91	1.12	-	-										
L.K.	1.16	2.26	1.72	2.17	-										
M.M.	9.07	4.46	3.08	1.69	-										
A.B.	4.44	9.44	4.14	9.14	6.68										
A.S.	1.93	2.21	3.86	2.29	2.59										
E.C.	0.38	0.46	0.36	0.12	0.43	0.43	1.95	0.96	-	-					
H.A.	2.26	6.82	0.24	1.55	3.48	3.48	12.14	3.81	2.18	3.05	3.05	15.24	1.85	0.47	4.84
K.S.						2.73	4.23	-	-	-					
P.N.						2.41	4.58	2.26	2.71	3.82	3.82	4.70	2.26	2.98	1.26
	(x 10^5/ml PERIPHERAL BLOOD)														

TABLE B6

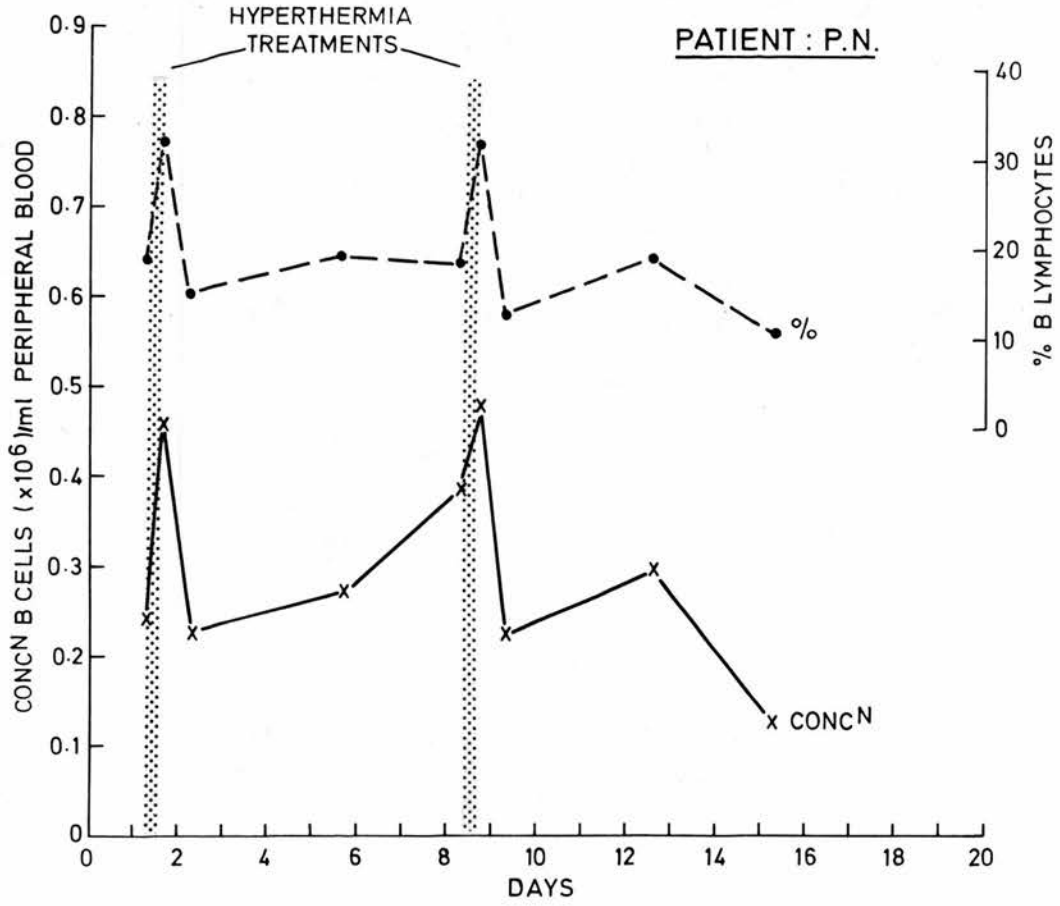
EFFECT OF HYPERTHERMIA TREATMENT ON THE CONCENTRATION OF PERIPHERAL BLOOD B LYMPHOCYTES

	FIRST TREATMENT					SECOND TREATMENT					THIRD TREATMENT				
PATIENT	PRE 1	END 1	1d POST	3-5d POST	7-9d POST	PRE 2	END 2	1d POST	3-5d POST	7-9d POST	PRE 3	END 3	1d POST	3-5d POST	7-9d POST
M.D.	100	57.59	70.89	-	-										
L.K.	100	194.83	148.28	187.07	-										
M.M.	100	49.17	33.96	18.63	-										
A.B.	100	262.61	93.24	205.86	150.45										
A.S.	100	114.51	200.00	118.65	134.20										
E.C.	100	121.05	94.74	31.58	113.16	100	453.49	223.26	-	-					
H.A.	100	301.78	12.43	68.58	153.98	100	348.85	109.48	62.64	87.64	100	499.67	60.66	154.09	158.69
K.S.						100	154.95	-	-	-					
P.N.						100	190.04	93.78	112.45	158.5	100	123.00	59.16	78.00	32.98
MEAN (ALL PATIENTS)	100	150.22	93.36	105.61	137.95*	100	286.83*	142.17	87.54	123.07	100	311.33	59.91*	116.04	95.83

TABLE B7

EFFECT OF HYPERTHERMIA TREATMENT ON THE CONCENTRATION OF PERIPHERAL BLOOD B LYMPHOCYTES EXPRESSED AS A PERCENTAGE OF THE PRE-TREATMENT CONCENTRATION

* Statistically significant by Student's t test on paired, log-transformed, values.



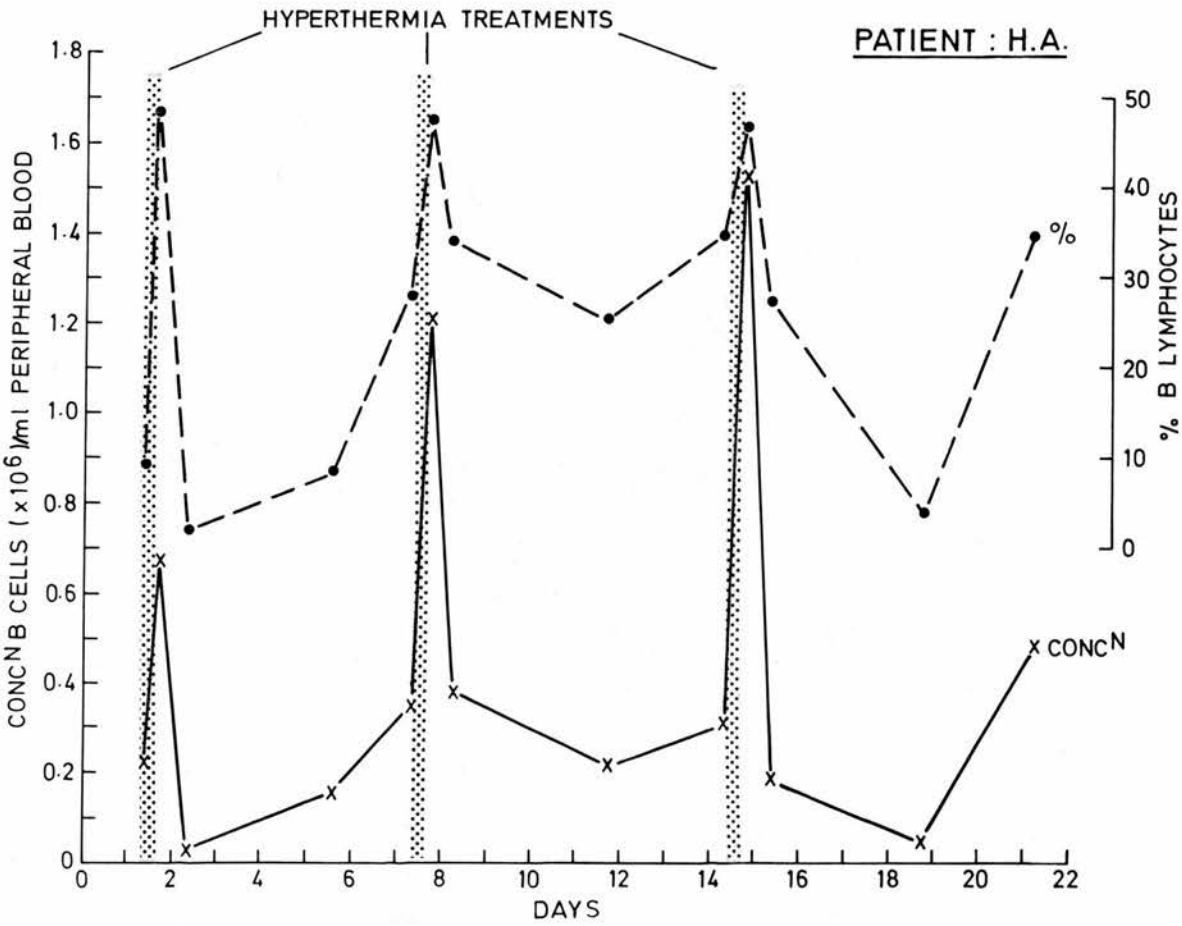


FIGURE B8

EFFECT OF SUCCESSIVE HYPERTHERMIA TREATMENTS ON THE PERCENTAGE AND CONCENTRATION OF PERIPHERAL BLOOD B LYMPHOCYTES IN TWO PATIENTS

on their EAC-rosetting capacity was examined. In two cases there was no significant change in the percentage of rosetting cells after 2½h heating at 42°C. In the remining two experiments, the same heating conditions, resulted in the reduction of the percentage of rosette-forming cells by one half.

Discussion

Although the study on the pre-hyperthermia B cell concentration was small, the findings are in general agreement with those of Heier et al (1977). This showed no significant difference in B cell numbers between cancer patients and normals, but indicated that the range of the concentrations was wider in the patient group.

The results obtained during treatment, indicate that hyperthermia therapy produces a substantial B lymphocytosis, similar to that seen in sub-40°C heating, in normal subjects (Bork-Wolwer et al, 1977). DeHoratius et al (1977), did not observe an elevation of B cell concentration, although all of their patients showed an increase in the percentage of EAC-rosetting cells, during 2h of 42°C hyperthermia.

It is possible that the increase seen in hyperthermia patients is similar to the exercise-induced increase, reported by Steel et al (1974), which occurred during an associated lymphocytosis. In most of the patients, however, there was no increase in the concentration of peripheral blood lymphocytes during hyperthermia, and the changes seen would have to be at the expense of the T lymphocyte population, as reported by Bork-Wolwer.

It is felt that a more likely explanantion of these changes, is variation in the purity of the lymphocyte separation. The percentage of EAC-rosetting cells, in most patients, was low prior to

treatment, and could be greatly increased by the mistaken inclusion of a few non-B cell rosettes. This was very likely to occur at the end of treatment, because the neutrophilia makes it difficult to separate a >97% pure lymphocyte population. The contaminating polymorphs and monocytes have complement receptors, and will therefore, form rosettes with EAC cells, thus falsely increasing the B cell percentage, and hence the concentration. In addition, in some cases, erythrocytes coated with antibody alone, also formed rosettes, indicating the presence of cells with Fc receptors, and also complicating the B cell count.

The results from the experiments in which whole blood was heated in vitro, were also confusing, but probably indicate a slight depression of B cell rosetting by heat. In an attempt to overcome the problems with this assay, an immunofluorescence technique, for detecting surface immunoglobulin, was used. This procedure proved to be too lengthy, however, for use on samples from the hyperthermia patients, and was therefore discontinued.

In summary, the concentration of B lymphocytes is increased by hyperthermia treatment, and returns to the pre-heating level the folllowing day. It is not certain, however, that this increase is real, since the assays used, could be heavily biased by the presence of a small number of contaminating monocytes or neutrophils.

Effect of Heating in vitro on Lymphocyte Morphology The effect of heat in vitro on the surface morphology of lymphocytes was assessed by scanning electron microscopy.

Cells were heated at 42°C for 2½h, either in whole blood, or as an isolated suspension in tissue culture medium. Lymphocytes heated in blood were separated using the standard technique, and processed for SEM examination, as described in the Methods (See p.107). Heated lymphocyte suspensions were centrifuged at room temperature (800g for 5min) and resuspended in less than 1ml of the supernatant medium, prior to fixation at 37°C. Control lymphocytes were obtained from unheated blood samples, or suspensions kept at 37°C.

Lymphocytes from control preparations were uniformly spherical in shape, with a mean diameter of 4.4μm. The surface of most cells was covered with short, blunt microvilli, the number of which varied widely from cell to cell (Figure B9).

Following heating, either in blood, or in suspension, there were no marked changes in the size or appearance of the lymphocytes. There was an indication that heated cells were somewhat less microvillous, the surface membrane appearing 'rucked' or folded (Figure B10). In spite of this, some highly-microvillous forms were still seen in the heated suspensions.

Discussion

Temperature-induced changes in leucocyte morphology, as studied by SEM, have been reported previously (Lichtman et al, 1976). However, in most cases, they have been reported to occur at temperatures outside the 30-42°C range. The results from this study are in agreement with those of Lin, Wallach and Tsai (1973), who found that there were few changes in lymphocyte surface morphology between 37°C and 44°C. Above

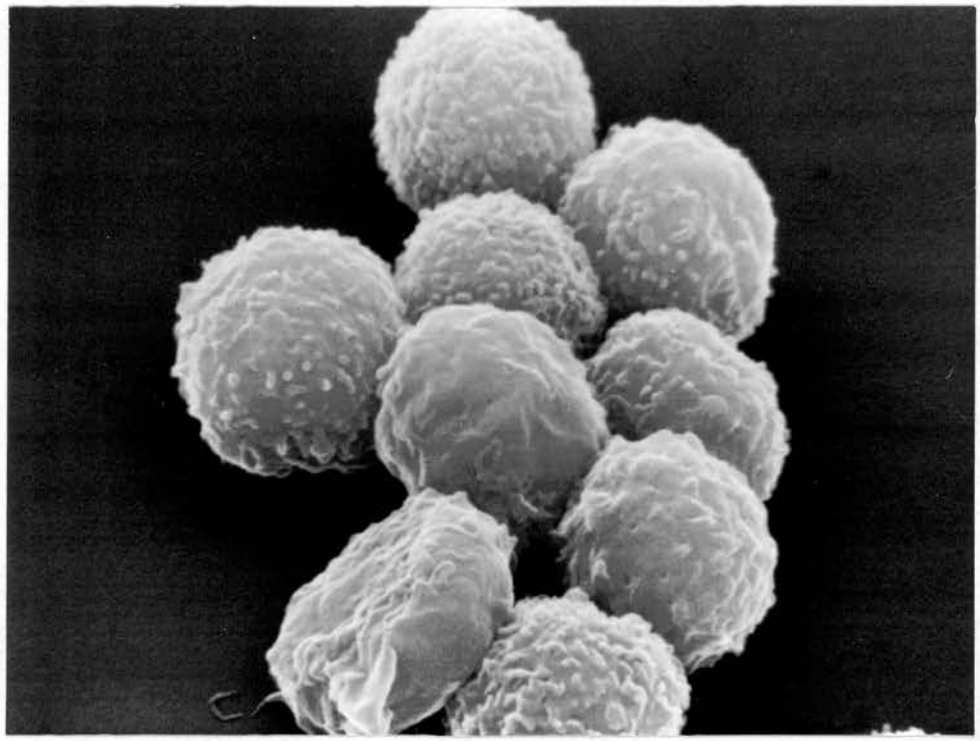


FIGURE B9

SCANNING ELECTRON MICROGRAPH OF PERIPHERAL BLOOD LYMPHOCYTES INCUBATED IN VITRO FOR 2½h AT 37°C
(Magnification x7,300)

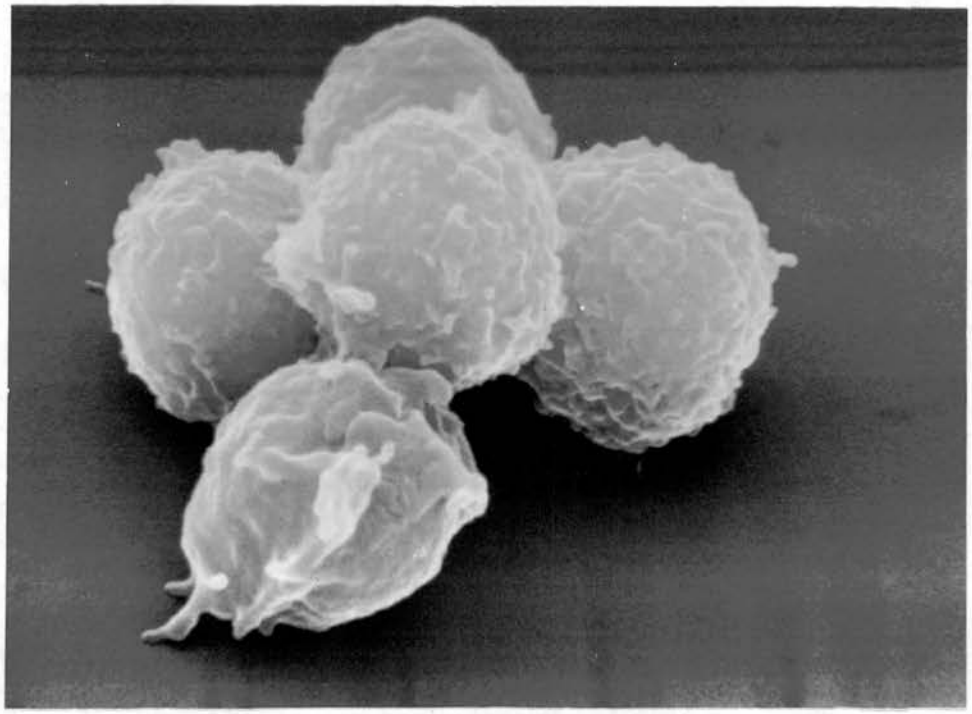


FIGURE B10

SCANNING ELECTRON MICROGRAPH OF PERIPHERAL BLOOD LYMPHOCYTES INCUBATED IN VITRO FOR 2½h AT 42°C
(Magnification x8,700)

44°C, the alterations which they observed showed a similar trend to those produced by heating isolated cells at 42°C for 2½h, namely, a decrease in the number of microvilli. This occurred very rapidly (within 2min) at 47°C, and was much more severe than that seen in this study. These changes were attributed to alterations in the membrane proteins, and may reflect a change in the activity of the cells. Polliack et al (1976) noted an increase in the number of surface villi on mouse thymic lymphocytes, which had been activated by incubation with Concanavalin A; a decrease in their number may therefore indicate a reduction in the functional integrity of lymphocytes. However Polliack reported that non-microvillous forms predominated in the circulation, and that these were probably T-lymphocytes, in contrast, in this study, the majority of unheated lymphocytes possessed surface villi. This is in agreement with the work of Firket and Schaaf-LaFontaine (1976), who believed that the position of the cells in the blood or tissues, during the hours prior to fixation, considerably influenced their surface morphology, which could not therefore be used to distinguish between T and B-lymphocytes. In this study, the same cell population was examined before and after heating, and so the changes cannot be explained on this basis. It is known that the method of fixation and preparation can affect the appearance of the cell surface, and although this may be partly responsible for the changes seen here, it is felt that there was some small direct effect of heat on lymphocyte morphology in this study. A reduction in the numbers of surface villi has been described during lymphocyte culture, but it is less severe if PHA is added to the culture medium (Firket & Schaaf-LaFontaine, 1976). The tendency for lymphocytes to have fewer surface structures after heating at 42°C for 2½h, may therefore be related to the reduction in their capacity

to form E rosettes (See p120) and to respond to PHA (See p.154).

PAPER C

THE EFFECTS OF WHOLE-BODY HYPERTHERMIA THERAPY ON THE LYMPHOCYTE RESPONSE TO PHYTOHAEMAGGLUTININ

The Effects of Whole-Body Hyperthermia Therapy on the Lymphocyte Response to Phytohaemagglutinin

Introduction

Contact between a T lymphocyte and an antigen, to which it has become specifically-sensitised, results in the transformation of the cell into a proliferating lymphoblast. This transformation process is associated with morphological changes, resulting in a large cell with basophilic and pyroninophilic cytoplasm, virtually no rough endoplasmic reticulum, abundant free ribosomes and prominant nucleoli. Mitotic figures are often seen, and there is active incorporation of DNA precursors. The cells are highly motile, moving from the lymph into the peripheral bloodstream, and then into the tissues, however, there is no evidence that they are attracted to the target antigen (Roitt, 1974 ; Currie, 1974).

Lymphoblasts are the main effectors of cell-mediated immune responses. They are able to lyse target cells bearing the antigen to which they are sensitised. This has been demonstrated by tests *in vitro*, in which tumour cells were killed by lymphocytes from patients bearing cancers of the same histogenesis. It is believed that the effector cells had become sensitised to the tumour-associated antigen(s) *in vivo*, however there is, at present, no clear evidence for tumour cell killing occurring *in vivo*, possibly due to the abrogation of cytotoxicity by serum blocking factors, e.g. antigen-antibody complexes, an effect which has been described *in vitro*.

Due largely to methodological problems, and doubts concerning the relevance of tumour cell cytotoxicity tests to the situation *in vivo*, other methods have been developed to assay T lymphocyte

activity. The commonest of these is the phytohaemagglutinin transformation test.

Phytohaemagglutinin is the generic name for the aqueous extracts of the seeds of certain plant species, especially the bean Phaseolus vulgaris. Its powerful erythrocyte-agglutinating properties made it useful in the separation of leucocytes from whole blood (Li & Osgood, 1949), however, in 1960 Nowell found that it induced mitosis of lymphocytes in culture (Fig. C1). The gross morphological and biochemical changes that it produced e.g. induction of increased DNA synthesis (Cooper, Barkhan & Hale, 1961) were suggestive of the action of an antigen upon a previously-sensitised cell (Elves, Roath & Israels, 1963). It was proposed that PHA acted through the normal immunological pathways of lymphocyte stimulation, but by-passed the early antigen-recognition stages (Coulson & Chalmers, 1964).

In 1966 Mills showed that the transformation of lymphocytes by an antigen in vitro could be correlated with the delayed hypersensitivity reaction to that antigen in vivo. This suggested that the reaction, in vitro, of a patient's lymphocytes to a non-specific mitogen, such as PHA, may be indicative of the subject's cellular immunocompetence. This has proved to be the case (Oppenheim, 1968).

The value of PHA tests in the assessment of cellular immune status was reinforced when it was demonstrated that the mitogen acted primarily upon the T lymphocyte population (Janossy & Greaves, 1971 & 1972). These observations were supported by the finding of a diminished, or absent, PHA response in patients with congenic thymic aplasia - DiGeorge's syndrome (Lischner, Punnet & DiGeorge, 1967). It now seems that this may be an over-simplification. Phillips and Roitt (1963) found that 30% of blast cells in PHA

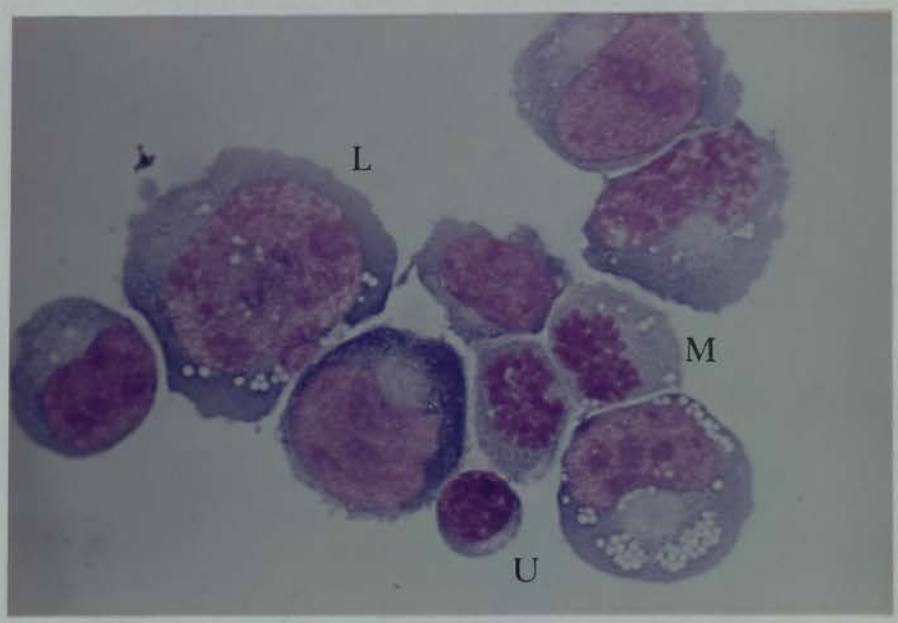


FIGURE C1

CYTOCENTRIFUGE PREPARATION OF HUMAN LYMPHOBLASTS

Blast cells were produced by incubating peripheral blood lymphocytes with PHA for three days in vitro
U = Untransformed lymphocyte L = Lymphoblast
M = Lymphoblast in mitosis
May-Grunwald/Giemsa stained (Magnification: x1,100)

cultures of normal peripheral blood lymphocytes, bore surface immunoglobulin. They demonstrated that this was not a membrane component of T cells, and was not passively adsorbed from B lymphocytes during the culture period. They concluded that human B cells were also able to respond to PHA in conventional cultures. This was later confirmed by Chess, MacDermott and Schlossman (1974), who found that more than 90% of B lymphoblasts bore surface immunoglobulin, whereas blasts derived from T cells were devoid of immunoglobulin. The action of PHA is therefore not wholly restricted to T lymphocytes (although it is possible that contaminating T cells may be responsible for the responses noted in purified B lymphocyte populations (Han & Dadey, 1977)).

The situation has been further complicated by the observations of Potter and Moore (1975) which indicated that pure T cell populations responded less well to PHA than those containing a proportion of B lymphocytes, or non-lymphoid cells. The degree of contamination could have a critical effect upon the results (Potter & Moore, 1977). In addition, Yachnin (1972) has shown that blastogenesis may be affected by the presence of autologous erythrocytes, and platelets, in the culture. These findings, combined with numerous different techniques, and technical problems, make the critical evaluation of this test rather difficult. Nevertheless it remains the commonest method of monitoring cellular immunocompetence.

PHA tests have been used to study the influence of ageing (Weksler & Hutterotes, 1974), renal failure (Hosking, Fitgerald & Simons, 1971 ; Oppenheim, Blaese & Waldmann, 1970) etc. upon the immune system, and to investigate various immunodeficiency states. Perhaps the most extensive application however, has been in the study of the effects of malignant disease upon immuno-

competence. There is a large amount of literature on this subject, but some of the more recent results are summarised in Table C1.

Differences in technique, make these studies difficult to compare, but there are several general conclusions which can be drawn:-

i) The PHA response of fractionated peripheral blood lymphocytes is usually depressed in cancer patients, in comparison with both normal controls and patients with benign disease.

ii) This impairment appears to be due to some defect in the lymphocyte, but is often associated with serum blocking factors.

iii) These blocking factors may also inhibit the response of normal lymphocytes to PHA.

iv) In many cases a correlation has been found between the response to PHA and the patient's clinical status, prognosis, response to therapy etc..

In spite of the encouraging relationship between the lymphocyte response in vitro and clinical status, the results from PHA tests do not allow the distinction between cause and effect. In an animal system, it has been found that the PHA response of rat peripheral blood lymphocytes was progressively diminished by the growth of a tumour, probably due to a factor produced by the cancer and released into the serum, which then bound to the lymphocytes (Schumm & Billmire, 1976 ; Schumm, Billmire & Morris, 1976). A similar phenomenon has been described in a mouse system (Gillette & Boone, 1975). However, any increase in the response of a patient's lymphocytes, following hyperthermia therapy, may indicate, either, direct action of the treatment on cell responsiveness, removal of factors previously blocking the response, or improvement of the

REFERENCE	CANCER	NO.	CONTROL	NO.	CONTROL AGE	SERUM	PHA RESPONSE PATIENT/CONTROL	COMMENTS
DUCOS et al (1970)	LUNG	27	HEALTHY	45	NOT STATED	NOT STATED	↓	EMPHASISES NEED FOR SUB-OPTIMAL CONC^{n}S OF PHA
GARRIOCH, GOOD & GATTI (1970)	VARIOUS (NON-LYMPHOID)	46	NOT STATED	28	AGE MATCHED	NOT STATED	↓	DEGREE OF DEPRESSION MAY BE RELATED TO EXTENT OF DISEASE
GATTI (1971)	VARIOUS	104	NOT STATED	104	AGE MATCHED	VARIOUS	↓ IN OVER 2/3	SERUM BLOCKING FACTORS IN 16/21 PRESENCE NOT RELATED TO TYPE OF THERAPY
WHITTAKER, REES & CLARK (1971)	BREAST	30	BENIGN BREAST DISEASE	24	PATIENTS > CONT.	AUTOLOGOUS	↓	DEPRESSION IS STAGE RELATED. SERUM BLOCKING FACTORS
HAGEN, FROLAND & WEBERG (1972)	VARIOUS - ADVANCED NON-METASTATIC - UTERINE CERVIX - INVASIVE - NON INVASIVE	5 5 10	HEALTHY HEALTHY	- 9	AGE MATCHED SAME RANGE	FCS FCS FCS	↓ N.S.D. N.S.D.	IMPAIRMENT OCCURS IN ADVANCED CANCER N.S.D. IN ACTIVITY BETWEEN INVASIVE AND NON-INVASIVE NON-METASTATIC TUMOURS
BRUGAROLAS et al (1973)	LUNG - UNTREATED	21	NO CONTROLS	-	-	WHOLE BLOOD	-	APPROX. CORRELATION WITH EXTENT OF DISEASE
SUCIU - FOCA et al (1973)	VARIOUS (NON-LYMPHOID)	60	HEALTHY	81	SAME RANGE	AUTOLOGOUS & POOLED NORMAL	↓	ACTIVITY IN AUTOLOGOUS SERUM RELATED TO EXTENT OF DISEASE. DEGREE OF SERUM BLOCKING ↑d WITH ADVANCING DISEASE
GOLUB, O'CONNELL & MORTON (1974)	VARIOUS (MAJORITY WITH MALIG. MELANOMAS) VARIOUS STAGES	52 29	HEALTHY	NS	SAME RANGE	HUMAN AB	↓ SEE COMMENTS	ONLY SIGNIFICANT IMPAIRMENT WAS IN MELANOMA PATIENTS. SARCOMAS & CARCINOMAS WERE NORMAL
LANG et al (1974)	HODGKIN'S DISEASE UNTREATED	32	HEALTHY	40	NOT STATED	HUMAN AB	↓	NO CORRELATION WITH STAGE OF DISEASE
LEHANE & LANE (1974)	LUNG (ADVANCED) HEAD & NECK (ADVANCED)	10 20	HEALTHY	6	NOT STATED	FCS	↓ ESPECIALLY IN IN LUNG	NO CORRELATION WITH RESPONSE TO SUBSEQUENT CHEMOTHERAPY OR STAGE OF DISEASE

TABLE C1

REVIEW OF PREVIOUS STUDIES ON PHA-INDUCED LYMPHOCYTE TRANSFORMATION IN CANCER PATIENTS

REFERENCE	CANCER	NO.	CONTROL	NO.	CONTROL AGE	SERUM	PHA RESPONSE PATIENT/CONTROL	COMMENTS
MEKORI, SHER & ROBINSON (1974)	VARIOUS - NON LYMPHOID	103	HEALTHY	46	SAME RANGE	FCS	↓SEE COMMENTS	RESPONSE ↓d IN ADV. DISEASE. RESPONSE RELATED TO PROGNOSIS, BUT FEW STATISTICALLY - SIGNIGICANT RESULTS
ROBERTS & JONES-WILLIAMS (1974)	BREAST	29	MINOR SURGERY PATIENTS	9	NOT STATED	WHOLE BLOOD + FCS	N.S.D.	NORMAL RESPONSE TO PHA IN ALL STAGES
TWOMEY, CATALONA & CHRETIEN (1974)	VARIOUS - PRE-OPERATIVE - POST-OPERATIVE	200 100	CANCER-FREE HEALTHY	400	AGES STATED	POOLED HUMAN AB + NORMAL & CANCER SERA	SEE COMMENTS	CURED SQUAMOUS CARCINOMAS ↓d, CURED SARCOMAS, MELANOMAS & ADENOCARCINOMAS ↑d COMPARED TO PRE-OP & NORMALS. BLOCKING FACTORS IN SERUM FROM PRE-OP SQUAMOUS CARCINOMAS & SARCOMAS AND CURED SQUAMOUS CARCINOMAS. ↓d RESPONSES IN CURED PATIENTS INDICATE RESIDUAL TUMOUR?
BLOMGREN, WASSERMAN & GLAS (1975)	BREAST - LOCAL - METASTATIC	86 31	BENIGN BREAST DISEASE	85	AGE MATCHED	AUTOLOGOUS & NORMAL	STUDIED RESPONSE OF NORMAL CELLS IN VARIOUS SERA	N.S.D. IN EFFECT OF SERA FROM LOCAL OR METASTATIC DISEASE OR CONTROLS ON LYMPHOCYTE ACTIVITY. NO RAPID CHANGES IN BLOCKING EFFECT AFTER MASTECTOMY OR LOCAL RADIOTHERAPY.
BRAEMAN & DEELEY (1975)	LUNG BEFORE/AFTER RADIOTHERAPY	120	-	-	-	-	↓	PRE-TREATMENT RESPONSE NOT CORRELATED TO PROGNOSIS. FALL DURING TREATMENT RELATED TO SURVIVAL.
CATALONA et al (1975)	GENITO-URINARY	50	HEALTHY	284	AGE MATCHED	POOLED HUMAN AB	↓IN 42%	
DE GAST et al (1975)	MALIGNANT MELANOMA VARIOUS STAGES	61	HEALTHY	56	AGE CORRECTION USED	AUTOLOGOUS	↓SEE COMMENTS	ONLY IN ADVANCED CASES. IN REMAINDER NO CORRELATION BETWEEN ACTIVITY AND STAGE OR PROGRESSION. BLOCKING FACTORS IN 1/20
FRANCHI et al (1975)	LUNG RECTUM, COLON	20 5	HEALTHY	20	SAME RANGE	NOT STATED	↓ IN LUNG CANCER	-
KAPLAN et al (1975)	COLORECTAL - LOCALISED - METASTATIC	7 11	HEALTHY DISEASED	5 13	AGES STATED	NORMAL, OR CANCER + FCS	N.S.D.	NO SIGNIFICANT EFFECT OF DIFFERENT SERA
LUI et al (1975)	MALIGNANT MELANOMA	35	HEALTHY	32	NOT STATED	WHOLE BLOOD CULTURES	↓	ONLY ↓d PRE-TERMINALLY USING OPTIMAL DOSES OF PHA. WITH SUB-OPTIMAL DOSES RELATED TO EXTENT OF DISEASE

TABLE C1 Cont.

REFERENCE	CANCER	NO.	CONTROL	NO.	CONTROL AGE	SERUM	PHA RESPONSE PATIENT/CONTROL	COMMENTS
REDDY, GOH & POULTER (1975)	VARIOUS UNTREATED RADIATION TREATED	16 15	HEALTHY HEALTHY	16 16	AGES STATED AGES STATED	FCS FCS	N.S.D. ↓	
WHITEHEAD et al (1975)	BREAST	119	BENIGN BREAST DISEASE	-	NORMALS < PATIENTS	FCS	↓	SUB-OPTIMAL DOSES OF PHA OF VALUE IN PROGNOSIS
BICE, HEINS & SALVAGGIO (1976)	VARIOUS	31	HEALTHY	20	AGES STATED	AUTOLOGOUS	↓	SERUM HAD ↓ING OR ENHANCING EFFECT. ABNORMAL MITOGEN RESPONSES ASSOCIATED WITH INHIBITORY PLASMAS
BOLTON et al (1976)	BREAST	176	BENIGN BREAST DISEASE	51	AGE EFFECTS CONSIDERED	-	N.S.D.	FINDINGS COMPLICATED BY AGE DIFFERENCES. NO EFFECT OF STAGE OF DISEASE
COCHRAN et al (1976)	MALIGNANT MELANOMA BREAST	52 59	MINOR DISEASE BENIGN BREAST DISEASE	51 60	AGE STATED	NOT STATED	↑ BUT N.S.	NO CORRELATION WITH STAGE OF DISEASE
ELHILALI et al (1976)	GENITO-URINARY	103	HEALTHY - YOUNG - AGE MATCHED	41 47	NON-MATCHED AGE MATCHED	HUMAN AB	↓ IN GENERAL	NOTED EFFECTS OF AGE AND OTHER VARIABLES
SILVERMAN et al (1976)	MELANOMA - UNTREATED	42	HEALTHY	41	AGE MATCHED	POOLED HUMAN AB	↓	IMPAIRMENT RELATED TO TUMOUR BURDEN
SIMMLER et al (1976)	MALIGNANT MELANOMA SARCOMAS	28 17	HEALTHY	80	NOT STATED	HUMAN AB	N.S.D.	NO CHANGE AFTER THERAPY
SIMÓ - CAMPS et al (1976)	VARIOUS NON-LYMPHOID	121	HEALTHY BENIGN TUMOURS	50 11	EXCLUDED IF OVER 80	HUMAN AB	↓	CORRELATION WITH STAGE OF DISEASE, PROGNOSIS, SURVIVAL & RESPONSE TO THERAPY
WANEBO et al (1976)	LUNG	154	BENIGN THORACIC LESIONS HEALTHY	20 109	AGE STATED	POOLED NORMAL	↓	CORRELATION WITH SURVIVAL FOR PATIENTS WITH MORE ADVANCED DISEASE
WATKINS (1976)	VARIOUS (NON-LYMPHOID)	30	NOT STATED	-	NOT STATED	AUTOLOGOUS	↓ IN 2/3 PRE-OP	THOSE WITH NORMAL PRE-OP ACTIVITY HAD IMPROVED PROGNOSIS

TABLE C1 Cont.

patient's general health, following reduction of the tumour burden. In addition, it is not possible to tell whether the low response in cancer patients is associated with, or secondary to, the establishment of their disease. However, it is the correlation with clinical status, and the lack of a suitable alternative assay, that has sustained the use of PHA transformation tests in the assessment of cellular immunocompetence in cancer patients.

Methodology There are a number of methods of studying lymphocyte response to PHA.. Probably the simplest technique is to add the mitogen directly to defibrinated, or anti-coagulated, whole blood. Blast transformation can then be assessed, after first lysing the erythrocytes in the cultures, by a differential count of transformed and untransformed cells, in a stained smear (Roberts & Jones-Williams, 1974). Alternatively, the cultures can be pulsed with radioactively-labelled RNA or DNA precursors (Hall & Gordon, 1976 ; Eskola et al, 1975). The uptake of the precursor is measured by radioactive counting of either cell fragments (resulting from the hypotonic lysis techniques used in automatic cell harvesters), or, solubilised DNA (extracted from the cells by precipitation with Trichloracetic acid). The advantage of the whole blood method lies mainly in the ease and rapidity with which the cultures can be established. It requires very small volumes of blood, e.g. 2ml (Buimovici-Klein et al, 1976), a factor that can be of great importance in the clinical situation (Pauly, 1976). However, the serum of cancer patients often contains factors which may block the response, these will be present in whole blood cultures, making it difficult to determine whether an impaired response is due to a cellular, rather than humoral, defect.

Buimovici-Klein et al (1976), using a modification of the method developed by Park and Good (1972), have attempted to overcome this problem by replacing autochthonous serum by human AB plasma. It is likely that more extensive washing procedures are required to remove both serum-associated blocking factors, and/or the immunosuppressive effects of anaesthesia, noted by Espanol, Todd and Soothill (1974).

Kjaer (1976) has found that washing leucocytes lead to an increase in their migration index, and that this could also be achieved by using Ficoll Hypaque-separated cells, suggesting that the separation procedure may remove blocking factors, and give a clearer indication of lymphocyte responsiveness. The use of separated cells also allows the investigator to control the number of potentially-responsive cells in the culture, whereas their concentration in whole blood may vary during the period of repeated sampling, and this may affect the accuracy of the results (Hall & Gordon, 1976). In addition, the elimination of erythrocytes from the cultures, obviates the problems of erythroagglutination by PHA, and of red cell lysis during prolonged culture. The former can cause problems when automatic cell harvesters are used, and the latter is likely to result in the release of unwanted factors into the culture medium. Due to these complications, the majority of workers have used either unpurified (Lehane & Lane, 1974), or purified leucocyte populations.

The purification process usually involves the removal of granulocytes, e.g. by isopyknic centrifugation of the blood on a Ficoll-Hypaque gradient (Boyum, 1968), and/or phagocytic mononuclear cells, by incubation with carbonyl iron (Catalona et al, 1975). Glass-adherent cells have also been removed by some workers (Webel, Briggs & Ritts, 1975). In most cases transformation is assessed

by the uptake of the radioactively-labelled DNA precursor - Tritiated Thymidine, although ^{14}C Thymidine can also be used (Wanebo et al, 1976). Uptake can be quantitated by autoradiography (Whittaker, Rees & Clark, 1971) or scintillation counting, following either DNA precipitation (with (Potter and Moore, 1975), or, without solubilisation (Bader et al, 1976)) or automatic cell harvesting (Simmler et al, 1976). Blastogenesis can also be measured by flow cytofluorometry (Braunstein et al, 1976 ; Darzynkiewicz et al, 1976), and visual (Simo-Camps et al, 1976) or automated counting (Stewart & Ingram, 1967 ; Stewart, Cramer & Steward, 1975).

Due to these variations in technique, and the results which they produce, the need for the standardisation of conditions has been emphasised (Yamamura, 1973 ; Fitzgerald, 1971). In addition, the requirement for large volumes of blood (usually 15-20ml), for most of the methods, has lead to the development of micro-assays, for use in repeated testing (Webel, Briggs & Ritts, 1975 ; Pees & Pappas , 1975 ; Penhale et al, 1974). The reduction in cell numbers has probably been taken to its limit in the method developed by Steel and Creasey (1976), in which the cultures contains as few as $1x10^4$ lymphocytes, although 100µl volumes containing $2.5x10^4$ cells were routinely used.

The aim of this study was to determine the effects of whole-body hyperthermia treatment on the response of the patient's peripheral blood lymphocytes to PHA, at various times during their period of therapy.

Materials and Methods

Three different assay systems were used to measure the PHA response of lymphocytes.

Materials

Pooled Normal Human Serum Serum was isolated from the peripheral blood of 10 healthy, untransfused, young males (5 Group O, 4 Group A and 1 Group B or AB). It was decomplemented by heating at 56°C for 30min, centrifuged, and Seitz filtered, before storage at -25°C.

Culture Medium Prepared immediately before use.

Ham's F10H.....7.50ml

L-Glutamine, 3% v/v, aqueous.....0.05ml

HEPES solution, 500mM.....0.20ml

Pooled Normal Human Serum.....2.00ml

Isotope For isolated lymphocyte cultures: 2-^{14}C thymidine, (specific activity 50μCi/mmol) diluted to 5μCi/ml in F10H, and stored at -20°C until required.

For whole blood cultures: (Methyl-^{3}H) thymidine, (specific activity 2Ci/mmol), diluted to 12.5μCi/ml in F10H, and stored at 4°C until required.

Phytohaemagglutinin The same batch of Reagent Grade PHA (K0763, Wellcome Reagents Ltd.) was used throughout the study.

Methods

i) Using Isolated Lymphocytes Isolated lymphocytes were suspended in freshly-prepared culture medium, at a concentration of $5x10^5$ viable cells/ml. 50μl cell suspension was added to an equal volume of double the required concentration of PHA, diluted in

culture medium, in a Dreyer agglutination tube. Control tubes contained no PHA. All the tests were performed in triplicate, or quadruplicate. The tubes were sealed with silicone rubber bungs, and incubated in racks in a 37°C incubator for 3, 4, and 5 days. 17h before the termination of incubation, 0.1μCi 2-^{14}C thymidine (20μl) was added to each tube.

Thymidine uptake was terminated either by transferring the tubes to a 4°C refrigerator, or by harvesting the cell immediately. Cells were harvested onto glass fibre mats using a Skatron Automatic Cell Harvester (the principle behind automatic cell harvesters is shown in Fig C2). The mats were thoroughly washed with water, to remove any unbound isotope, and dried overnight at 37°C. Each pad was then transferred to a glass scintillation vial, to which was added 10ml scintillator. The vials were counted for 10min each, using the ^{14}C window of a liquid scintillation spectrophotometer. Results are expressed as $\log_{10}$ radioactive counts/min.

ii) Modified Method for Isolated Lymphocytes (modified from Steel and Creasey (1976)). 50μl samples of lymphocyte suspension, at a concentration of 5×10^5 viable cells/ml in culture medium, were seeded into the flat-bottomed wells of a microtiter plate containing triplicate, serial dilutions of double the required concentration of PHA in culture medium. Cells were added to the lowest concentrations of PHA first, to avoid the accidental transfer of mitogen. The block of test wells was surrounded by a double row of wells, each of which contained 100μl distilled water, to help to maintain a stable environment within the plate (See p.145) The lids of the plates were sealed in place with Lasso tape, and the cells incubated at 37°C in a fan-assisted incubator (See p.145)

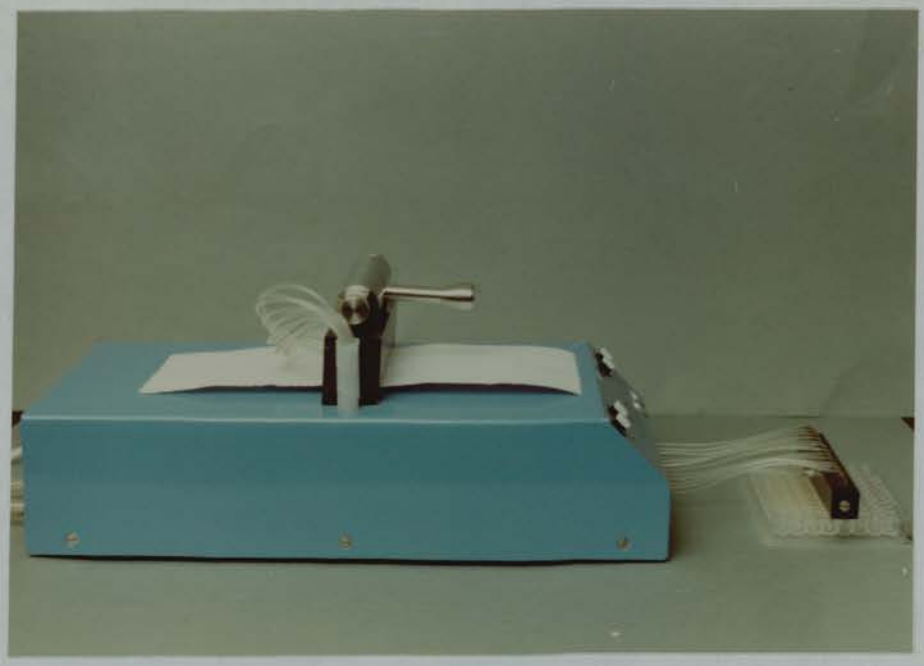


FIGURE C2a

SIDE VIEW OF THE 'AUTOMASH' AUTOMATIC CELL HARVESTER

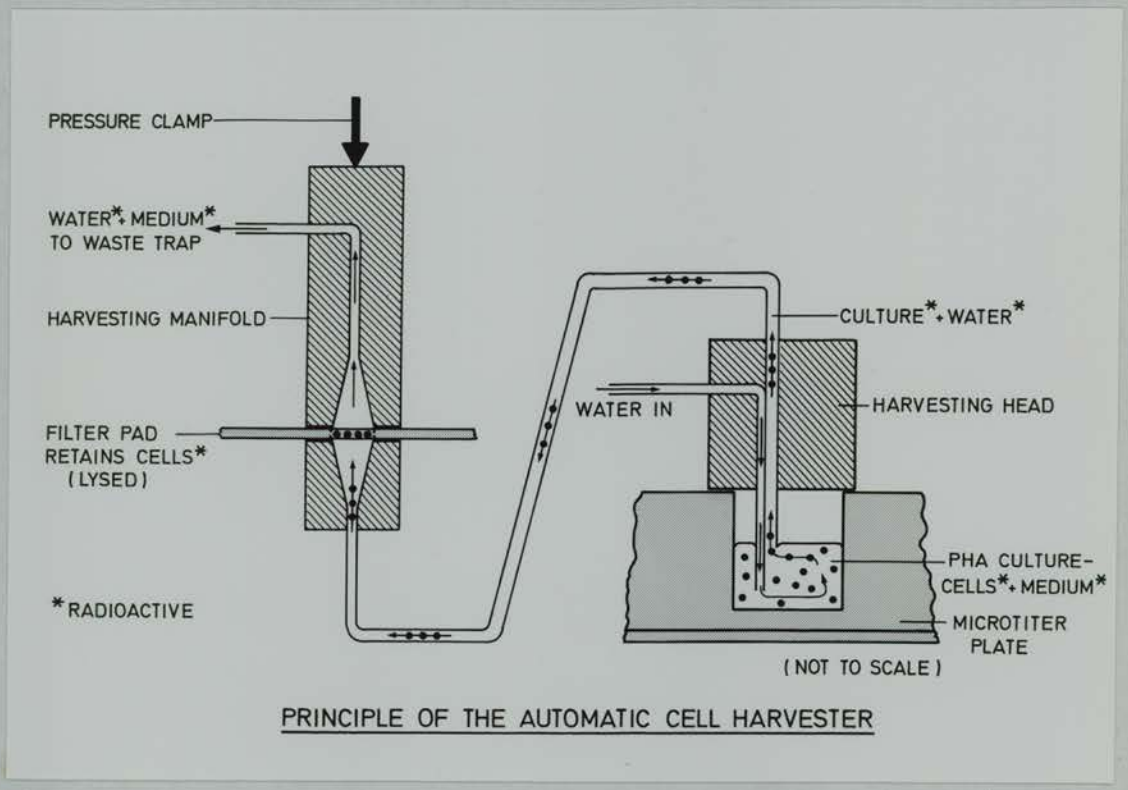


FIGURE C2b

DIAGRAMATIC SECTION THROUGH AN AUTOMATIC CELL HARVESTER

Radioactivelly-labelled cells are collected onto filter pads which are dried, placed in scintillator, and counted in a liquid scintillation spectrophotometer

for either 3 or 4 days. 0.1μCi 2-^{14}C thymidine (20μl) was added to each well (starting again at the control cultures) 24h before the end of incubation. The cells were harvested and counted as for Method i.

iii) Whole Blood Method 25μl samples of well-mixed whole blood were added to 100μl dilutions of PHA in F10H, to give a final volume of 125μl at the required PHA concentration. Blood was added by reverse-pipetting, as this was found to give more reproducible volumes. The plates were sealed and incubated as for Method ii. 24h before the end of incubation, 0.25μCi (Methyl-^{3}H) thymidine (20μl) was added to each well. The cells were harvested and counted as for Method i.

Heating of Lymphocyte Suspensions in vitro Lymphocyte suspensions were heated in vitro under the same conditions shown on p.104.

Measurement of RNA synthesis Cultures were established using Method (ii). 2-^{14}C uridine was diluted as indicated for 2-^{14}C thymidine (See p.141). 1 day cultures had 0.1μCi isotope added immediately after establishment. Cultures harvested after 2-4days had isotope added 24h before the end of incubation. All cultures were harvested as indicated for Method (ii).

Presentation of Results Results are shown as the $\log_{10}$ of radioactive counts per minute plotted against:

i) the treatment time scale, for Method (i)

ii) the PHA concentration, for Methods (ii) and (iii).

Results

Effect of Hyperthermia Treatment on the Response of Peripheral Blood Blood Lymphocytes to PHA as studied using Method (i) - (incubation in glass tubes) This technique was used to study lymphocyte transformation in 6 patients (A.B., E.C., W.M., H.A., P.N. and A.S., 4♂s, Mean age 46.5 years, Range 35-51 years). Blood was taken immediately before and after each treatment session, the following day, and on alternate days, until the next treatment, or until the patient was discharged. The results from individual patients are shown, since the range of responsiveness to PHA was so large that it was not practicable to take the mean values.

In general, during heating, there was a fall in the response to a single concentration of PHA (1%). The following day the response increased, to return to, or exceed the pre-treatment level. This trend was sustained, so that by 3-5 days after hyperthermia, lymphocyte blastogenesis was markedly increased, often to more than double the pre-treatment level (Figures C3 and C4). Over the following 3-4 days there was a fall in lymphocyte responsiveness, so that within 7 days of treatment there was a return to the level seen prior to hyperthermia. This general pattern was common to all the patients studied, and was seen after each treatment (Figure C4). It was not due to variations in the PHA culture conditions, as was shown by including lymphocytes from a normal control studied over the same period (Figure C3). Cells cultured for periods ranging from 2-5 days all showed the 3-5 day peak in lymphocyte activity, with the maximum response usually occurring following a 3 day incubation with PHA. The use of autochthonous serum had little effect on the general pattern of lymphocyte activity, but the responses were always lower than those with pooled normal serum,

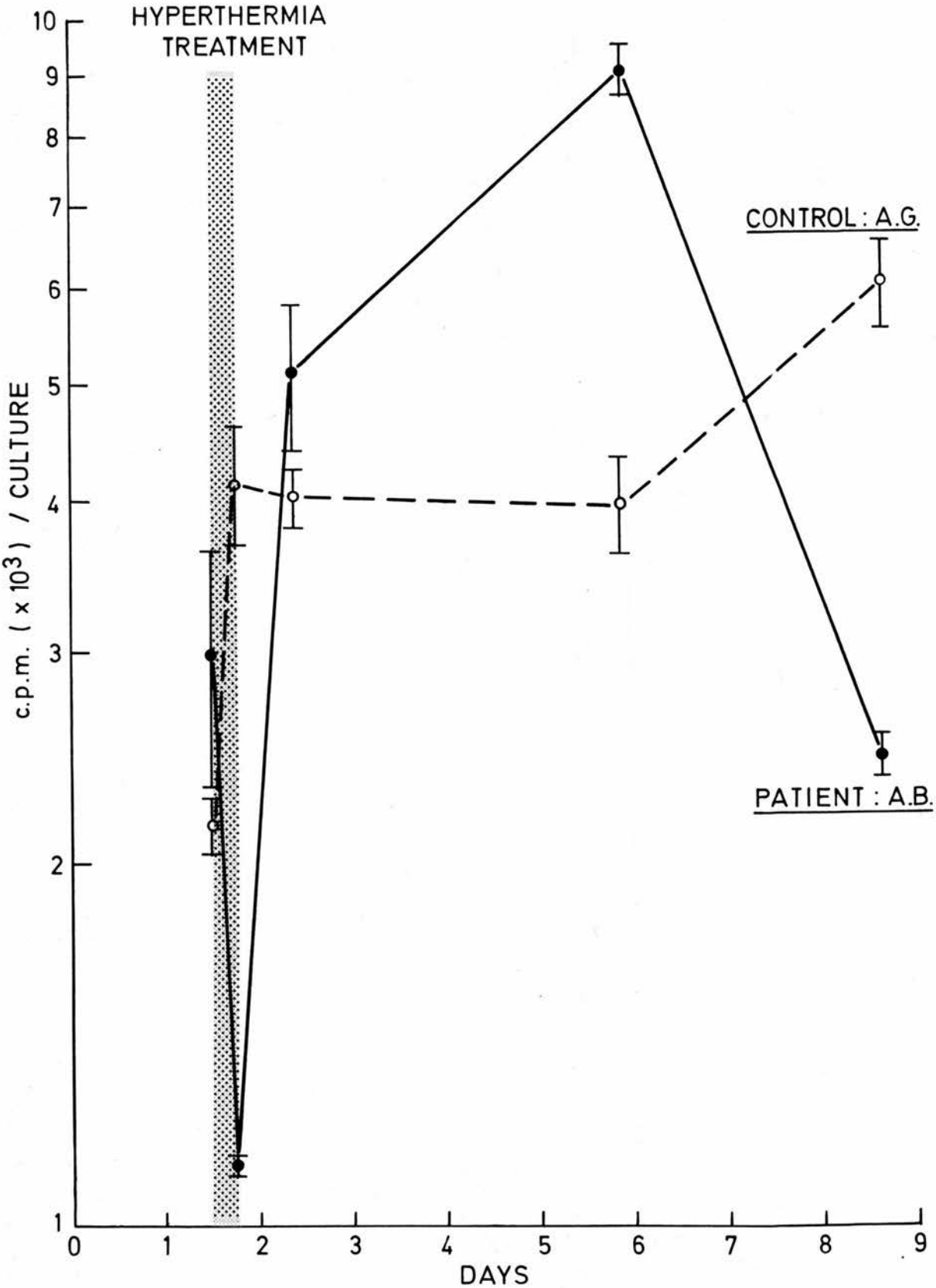
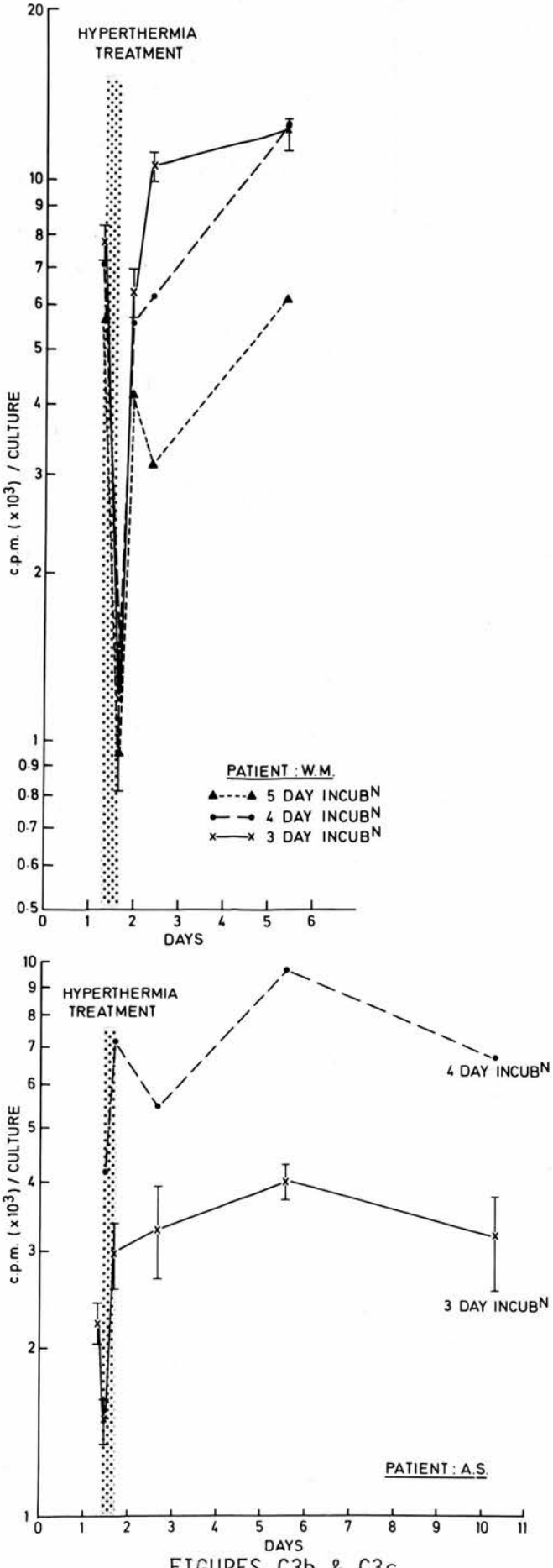


FIGURE C3a

EFFECT OF A SINGLE HYPERTHERMIA TREATMENT ON THE PHA RESPONSE OF PERIPHERAL BLOOD LYMPHOCYTES

The broken line shows the response of lymphocytes from a normal control tested simultaneously
Cultures were set up using Method (i). PHA concentration: 1% ; Incubation time: 3 days
Points shown are the mean of quadruplicate samples ± 1 S.D.



FIGURES C3b & C3c

EFFECT OF A SINGLE HYPERTHERMIA TREATMENT ON THE PHA RESPONSE OF PERIPHERAL BLOOD LYMPHOCYTES
Cultures were set up using Method (i)
PHA concentration: 1% ; Incubation time: As shown
Points shown are mean of quadruplicate samples
Standard deviations are shown for the 4-day points

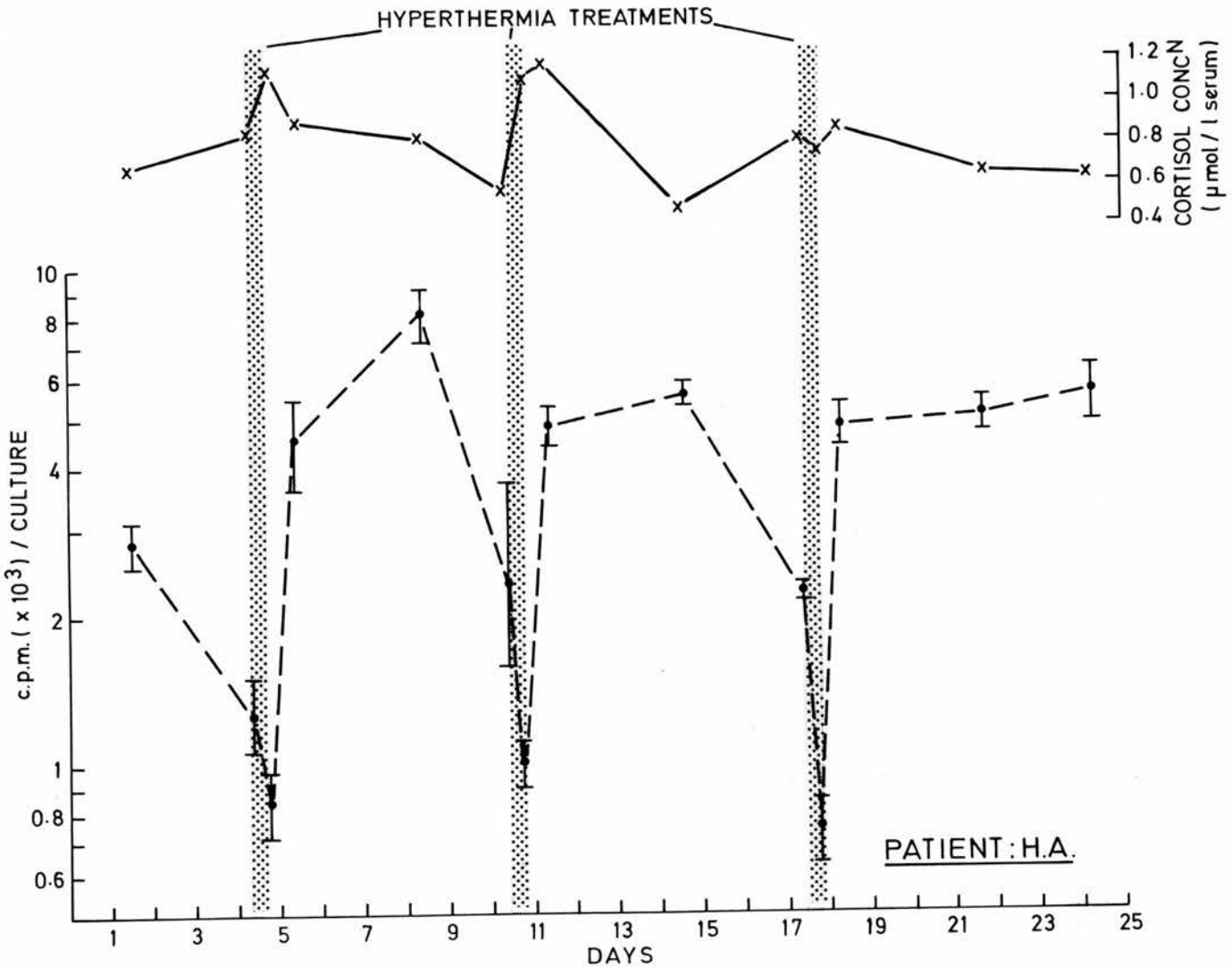


FIGURE C4

EFFECT OF SUCCESSIVE HYPERTHERMIA TREATMENTS ON THE PHA RESPONSE OF PERIPHERAL BLOOD LYMPHOCYTES

Cultures were set up using Method (i). PHA concentration: 1% ; Incubation time: 3 days
Serum cortisol concentrations were measured on the blood used for the separation of lymphocytes

indicating the possible presence of a blocking factor(s) in the patients' serum. Results from one patient (A.B.) (not shown) indicated that the degree of blocking was not altered during the week following hyperthermia. This factor(s) in the patients' serum also inhibited the PHA response of lymphocytes from a normal control.

Changes in the concentration of plasma cortisol have been reported to affect the response of lymphocytes to PHA (Tavadia et al, 1975). In the hyperthermia patients there was no statistically significant correlation between the serum cortisol concentration and the PHA response, although the peak in response was usually associated with a slight fall (usually less than 0.2μmol/l) in the cortisol concentration (Figure C4).

It was felt that the standard deviation on triplicate, or quadruplicate, cultures was rather large (usually greater than 10% of the mean), using this technique. In addition, the method required large numbers of tubes, which made PHA dose/response testing impracticable. The tubes themselves were not uniform, in that the bottoms varied widely in shape, resulting in different cell densities during incubation. In an attempt to solve these difficulties, Method (ii) was introduced.

Effect of Hyperthermia Treatment on the Response of Peripheral Blood Lymphocytes to PHA as studied using Method (ii) - (incubation in Microtiter plates)

Methodology This technique produced smaller standard deviations (usually less than 7% of the mean) than Method (i), but was extremely difficult to establish. The use of sealed microtiter plates resulted in condensation forming on the inside of the lids. During prolonged incubation this became so bad that the medium in some wells became concentrated due to the evaporation of water, and cells in others were lysed by droplets of condensation falling into the culture. Tests

eventually showed that this was due to the use of a static convection incubator, and although condensation could be considerably reduced by stacking the plates, or by wrapping them in aluminium foil, it could be eliminated only by using a fan incubator. Even so, during incubation, the medium in the outer row of wells became more concentrated, resulting in a gradient of PHA response across the plate. This was prevented by surrounding each block of test wells by a double row of buffering wells containing distilled water. Using these conditions it was possible to obtain, from each plate, good dose/response curves with small standard deviations. At this stage, the test was used to study the responses of the hyperthermia patients.

During the intervals between hyperthermia treatments, the range of responsiveness in normal controls, and the influence of a number of variables on the test system were examined.

A standard dose/response curve (Figure C5) was constructed from the dose/response curves of 17 normal individuals obtained after a 4 day incubation with PHA. The result is shown as the mean of the responses at each concentration $\pm$ 1 standard deviation. Although the number of controls was small, there was no correlation between the age or sex, and the magnitude of the PHA response, however, the range of the results was so wide that the time course of the PHA response was examined. In the standard system the maximum response generally occurred with a 4-5 day incubation with PHA, however, increasing the density of the cells in culture (by tilting the plate, or changing the well shape) resulted in an earlier peak (day 3-4).

PHA tests established using different lymphocyte separations from the same blood sample, occasionally showed wide variation. However, this was also shown when identical plates were set up using the same batch of cells. Examination of the kinetics of the response

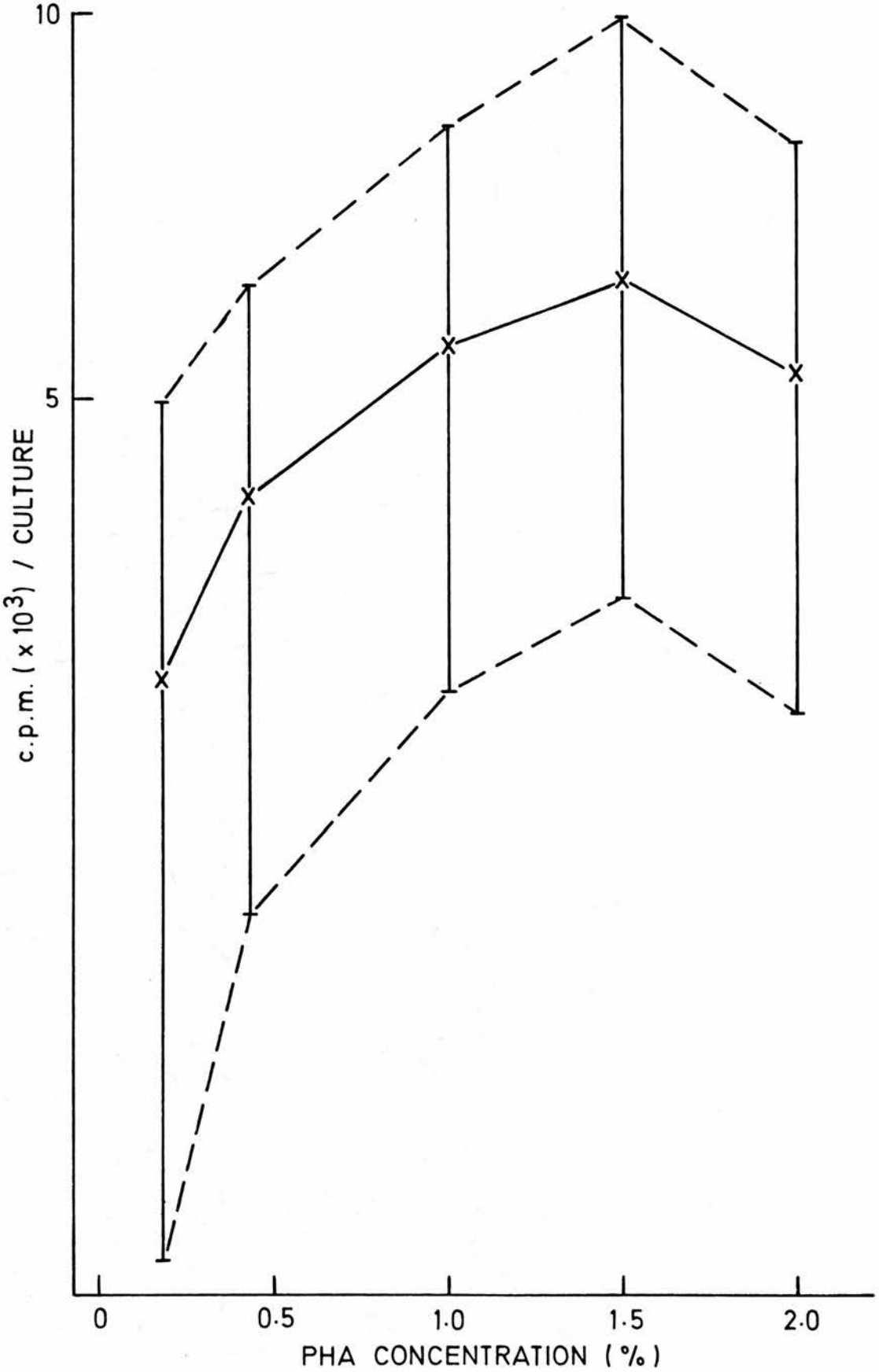


FIGURE C5

STANDARD PHA DOSE/RESPONSE CURVE FOR 17 NORMAL CONTROL SUBJECTS

The dose/response curve was established from 17 dose/response curves set up for normal individuals, using Method (ii). Points shown for each PHA concentration are the average of 17 mean values ± 1 S.D.. See text for details.

indicated that these differences were due to variation in the time course for the stimulation in parallel cultures, such that lymphocytes in both plates reached a similar peak of response, but did so at different times. By harvesting the cultures at one time point a false impression of the reproducibility of the tests could be obtained.

The situation was complicated by the results from an experiment in which PHA tests on a single normal control were set up at regular intervals over a 3 week period (the maximum time of hospitalisation for a hyperthermia patient). These indicated that response measured at a single time point (4 days) could remain stable (in this case 5,000 cpm) over this period. However, the same individual sampled at various intervals over 2 years, had shown responses ranging from below 2,000cpm to above 10,000cpm, although the average response was around 5,000cpm. Repeated samples, taken every day, showed little variation. Therefore, the results from any plate are probably influenced by:-

i) the microenvironment within the plate, which affects the time course of the PHA response, and,

ii) the spontaneous variation in the activity of the cells themselves.

These factors have been borne in mind in interpreting the results from the hyperthermia patients, and the experiments on heat in vitro. Significance has only been attached to either gross changes in PHA response, or to those which showed a consistent pattern.

Response of Lymphocytes to PHA prior to Hyperthermia Treatment Prior to hyperthermia the dose/response curves of all the patients studied were within the normal range.

Response of Lymphocytes to PHA following Hyperthermia Treatment This

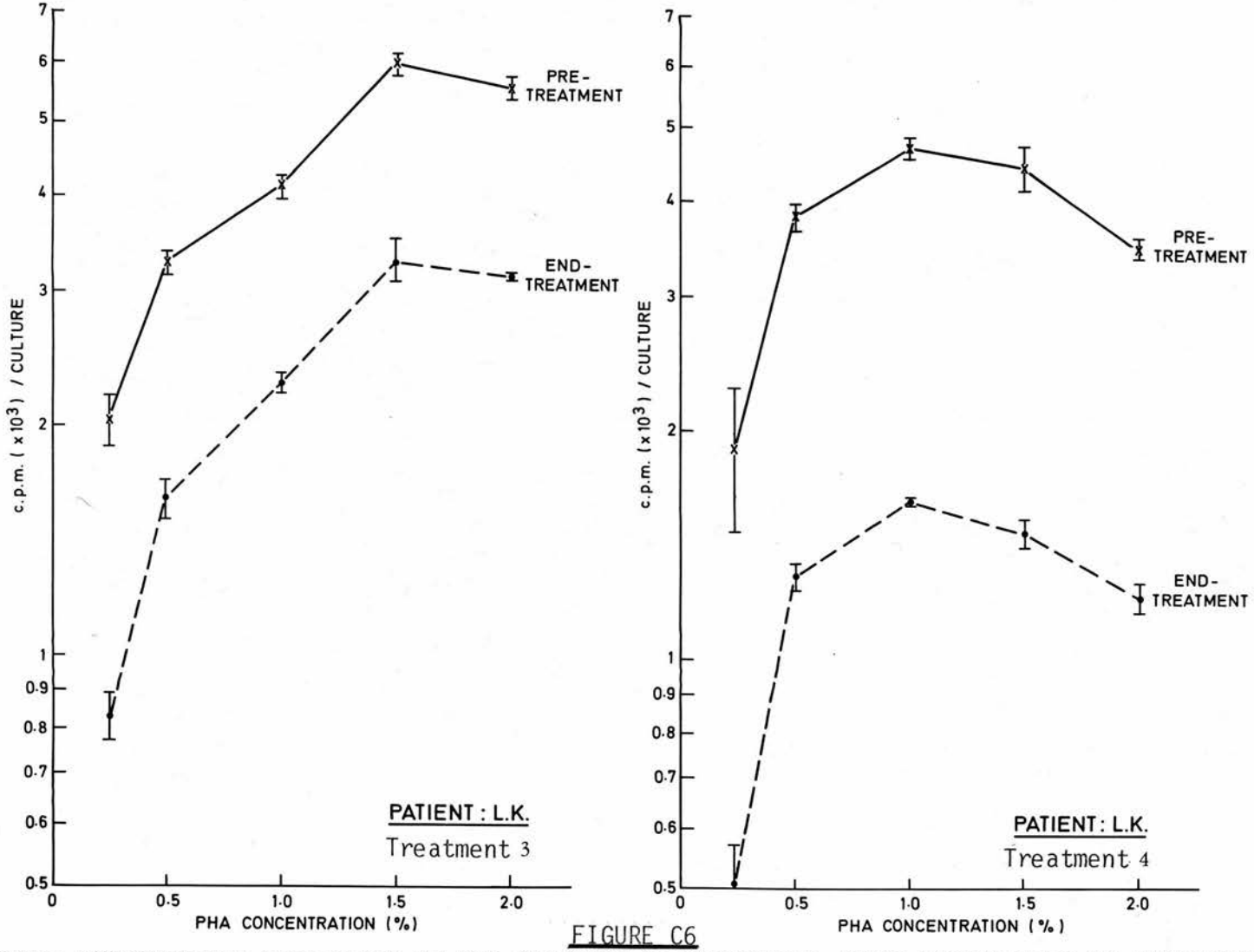


FIGURE C6

EFFECT OF SINGLE HYPERTHERMIA TREATMENTS ON THE RESPONSE OF PERIPHERAL BLOOD LYMPHOCYTES TO PHYTOHAEMAGGLUTININ
Cultures were set up using Method (ii). Incubation time: 3 days. Points shown are mean of triplicate samples ± 1S.D.

method was used to study lymphocyte transformation in 5 patients (L.K., M.M., K.S., T.M. and L.T., 3♂s, Mean age 58.6 years, Range 35-73 years).

During heating there was a decrease in response in 4 patients (for example L.K. - Figure C6), and an increase in the remaining one (M.M. - Figure C7). The changes were often slight, and were evident at all concentrations of PHA. Responses in autochthonous serum were depressed in comparison to those in pooled normal serum (Figure C7), as with Method (i). A satisfactory follow-up was only possible with 2 patients (M.M. and L.T.). M.M. showed a steadily increasing response during the period following hyperthermia (Figure C7) Results from this patient indicated a slight increase in serum blocking accompanied the post-heating elevation of lymphocyte transformation. Results from L.T, obtained using autochthonous serum in the cultures, showed low levels of lymphocyte activity throughout the study, but were indicative of a post-treatment increase in responsiveness.

To determine to what extent the changes in lymphocyte activity were due to raised body temperature, rather than drugs etc., an in vitro/in vivo experiment was set up.

Blood was taken from a patient (K.S.) immediately before treatment, defibrinated, and split into three aliquots. One sample was used at once to set up a PHA test. The remaining samples were sealed into plastic tubes, one of which was incubated in a water bath at 37°C, and the other was inserted into the patient's rectum. The blood in this tube was exposed to the elevated body temperature, but not to the drugs etc. used during treatment. At the end of hyperthermia an end-of-treatment blood sample was taken, and used for establishing PHA cultures, alongside those from the blood incubated in the tubes. The results are shown in Figure C8. Lymphocytes from blood kept at

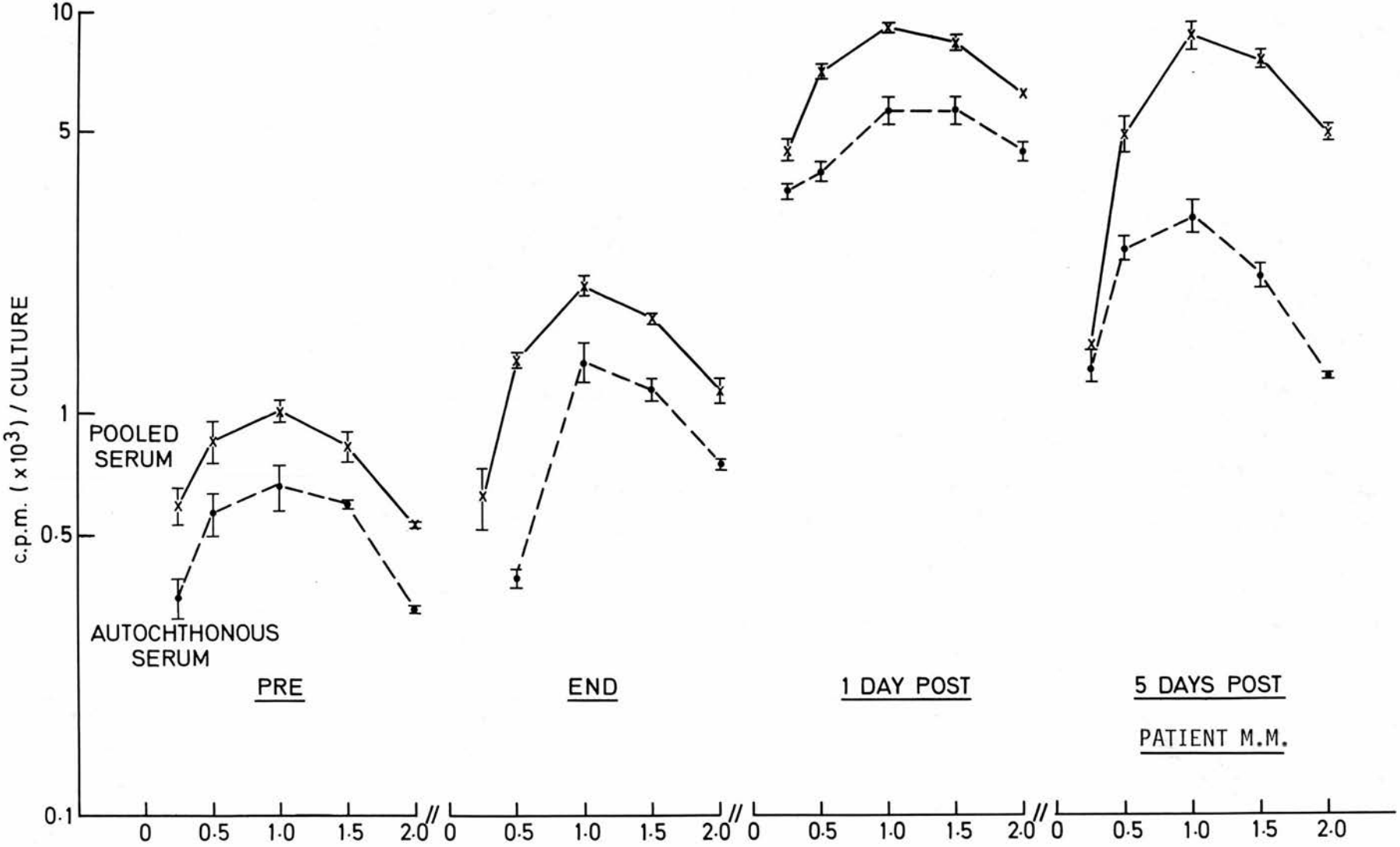


FIGURE C7

EFFECT OF A SINGLE HYPERTHERMIA TREATMENT ON THE RESPONSE OF PERIPHERAL BLOOD LYMPHOCYTES TO PHYTOHAEMAGGLUTININ
Cultures were set up using Method (ii). Incubation time: 4 days. Points shown are mean of triplicate samples ± 1 S.D.

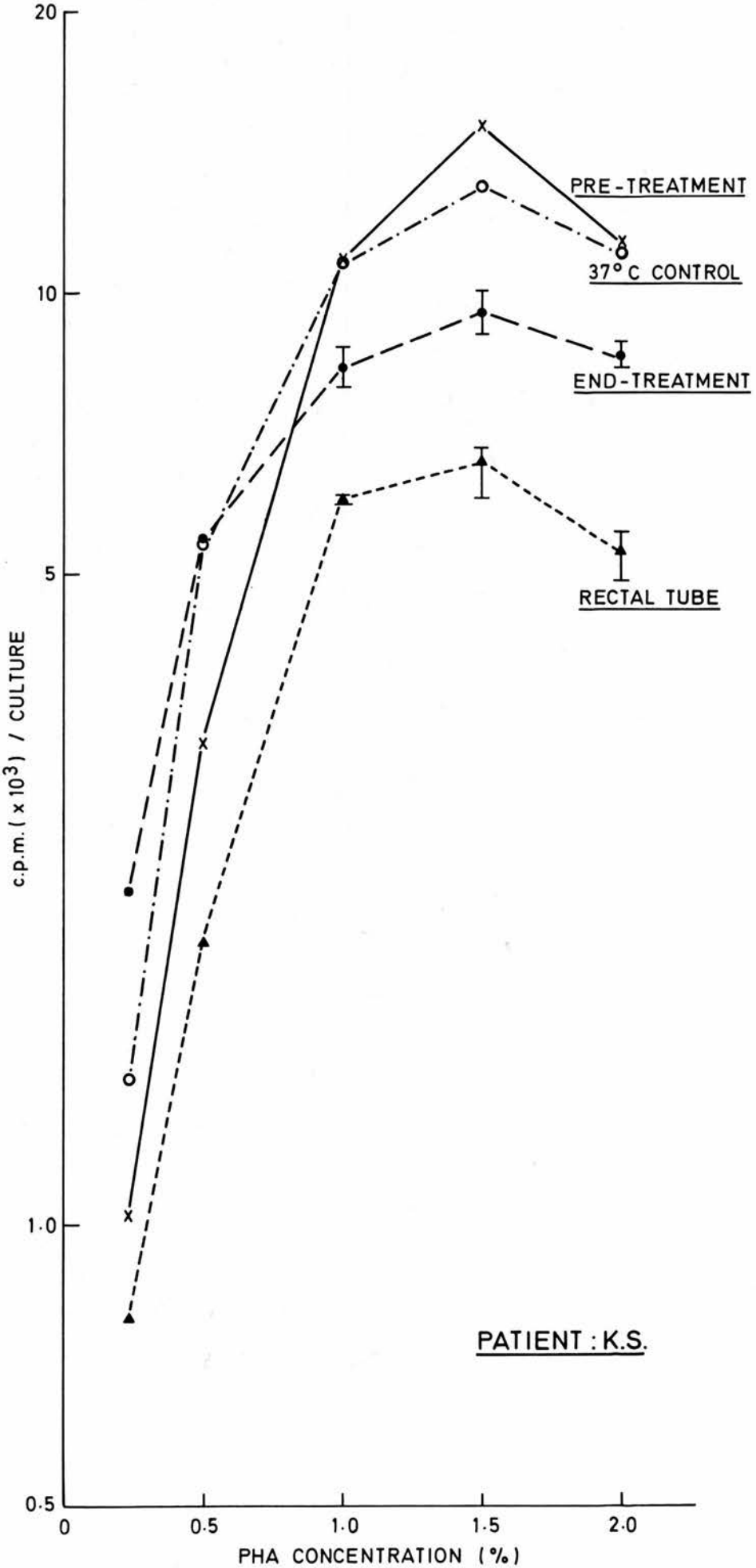


FIGURE C8

EFFECT OF IN VITRO/IN VIVO HYPERTHERMIA ON THE RESPONSE OF LYMPHOCYTES TO PHYTOHAEMAGGLUTININ
See text for details

$37^{o}C$ showed similar PHA responsiveness to those from unheated pre-treatment blood. During hyperthermia *in vivo* there was a fall in PHA response, which was also apparent, but to a greater extent, in the lymphocytes isolated from the blood, in the rectal tube.

Discussion

Method (i) The range of PHA response in normal controls was not measured using this method, and so the pre-hyperthermia levels of lymphocyte activity cannot be related to any standard. This was because the technique was established using blood samples from hyperthermia patients, and was subsequently modified into Method (ii) before use on control samples.

The fall in response seen during hyperthermia treatment may be due to a number of factors e.g. anaesthetics and drugs. Depression of PHA-induced lymphocyte transformation has been attributed to anaesthesia (Espanol, Todd & Soothill, 1974), but studies on normal volunteers (Lecky *et al*, 1974), and healthy surgical patients (Kanto, Vapaavuori and Viljanen, 1974), and *in vitro* (Saito *et al*, 1975 ; Bruce, 1976) have failed to show any effect by anaesthesia alone. It is now believed that the fall may result from trauma during surgical procedures (Cullen & Van Belle, 1975 ; Riddle & Berenbaum, 1967 ; Kent & Geist, 1975) and may be related to rises in the concentration of plasma cortisol.

The large rise in serum cortisol concentrations during hyperthermia treatment, may be partially responsible for the depression of PHA response in the patients, however increased cortisol levels have been found to be associated with both enhanced (Tavadia *et al*, 1975) and depressed (Zeman *et al*, 1972) lymphocyte transformation. Berenbaum, Cope and Bundick (1976) have found that a suppressive effect of

incubating lymphocytes with cortisol *in vitro* was removed, or even reversed, by washing the cells prior to culture with PHA, but that this was not the case *in vivo*. They indicate that the depression seen in patients post-operatively cannot be readily explained solely by increases in the blood cortisol concentration, and suggest that prostaglandins may also be involved. The study on hyperthermia patients suggest only a very tenuous association between lymphocyte transformation and cortisol concentrations, and it is felt that the magnitude of the change in the PHA response, during heating, is not sufficient to indicate any single major suppressive factor, but rather points to a combination of influences. It may possibly due to the fall in the percentage of T lymphocytes which occurs during treatment, although this would not explain the peak in reponse seen after 3-4 days, when the percentage of these cells is usually similar to that seen prior to hyperthermia. These results agree with those of Potter and Moore (1977) which suggested that there was not a simple, direct relationship between the PHA response and the number of T lymphocytes in the culture.

The rise in response occurring the day after hyperthermia, probably reflect the release from the suppressive effects of the treatment. The significance of the peak occurring at 3-5 days is not understood. It is unlikely that it is due to the small fall in cortisol concentration which occurs at this time. Spontaneous lymphocyte activity (i.e. thymidine uptake in the absence of PHA) showed some increase during this period, but, since the radioactive counts from these cultures were so close to the background level, it is difficult to assess the relative importance of this change. It is possible that the increase is an overshoot of the recovery phase after treatment, similar to that seen following successful chemotherapy (Cheema & Hersh, 1971), however, it may represent a stimulation of lymphocyte activity, either

by changes in the immunogenicity of the heated tumour, or by the release of antigenic tumour debris into the bloodstream. If so, these changes must occur irrespective of the clinical response to hyperthermia, since this peak was seen in both responsive, and non-responsive patients. The return to pre-treatment levels of lymphocyte activity would indicate the physical removal (e.g. by phagocytosis) or blocking (e.g. by serum factors) of any antigenic stimulus, or alternatively, a recovery from the post-treatment overshoot. The indications are that there are no great changes in the degree of serum blocking during the post-treatment period, and the phagocytosis studies (see p.88) indicated that any tumour debris would be rapidly removed from the bloodstream soon after hyperthermia. It is believed that macrophages are involved in the presentation of antigen to lymphoid cells (Unanue, 1972), and so it is possible that phagocytosed tumour debris might first be processed to a more immunogenic form, resulting in the delayed increase in lymphocyte activity; and it is then removed from the system within a week of hyperthermia treatment. This explanantion is purely hypothetical, since tumour debris was not seen in either the blood, or the cytoplasm of polymorphs, following hyperthermia. However, it is possible that the antigen may be shed in a sub-microscopic, or soluble form.

There are only two other studies on the effects of whole-body hyperthermia on the PHA response in humans. Lymphocyte activity was increased in normal subjects following 1h of heating at 40^{o}C (Fabricius et al, 1977). In their study on cancer patients, DeHoratius et al (1977) found no change in the PHA response in two patients. In a third, lymphocyte activity increased markedly during one treatment, but decreased during a subsequent session. As with the previous study, there was no post-heating follow-up.

Method (ii) The results obtained from hyperthermia patients, by the use of Method (ii), were comparable to those from Method (i), in that the changes during heating were usually small, and tended towards a fall in responsiveness. A satisfactory post-heating follow-up was only possible for two of the five patients. The results from one of these showed the 3 day increase in PHA response seen in the previous patients; and the results from the second indicated a similar pattern. No difference between the effects at various PHA concentrations could be discerned, although the number of patients was too small to evaluate the worth of sub-optimal doses of PHA in predicting clinical prognosis (See Introduction, Table C1). Most responses were within the normal range throughout treatment, except in the case of the patient receiving radiotherapy (T.M.), where they were depressed during the whole period of the investigation.

The results from the experiment in which blood was heated in a rectal tube indicated that heat alone was affecting the PHA responsiveness of lymphocytes, and to a greater extent than when combined with drugs etc., during hyperthermia in vivo.

Further data to confirm this finding were obtained by heating lymphocytes, either in whole blood, or as a separated suspension, in vitro.

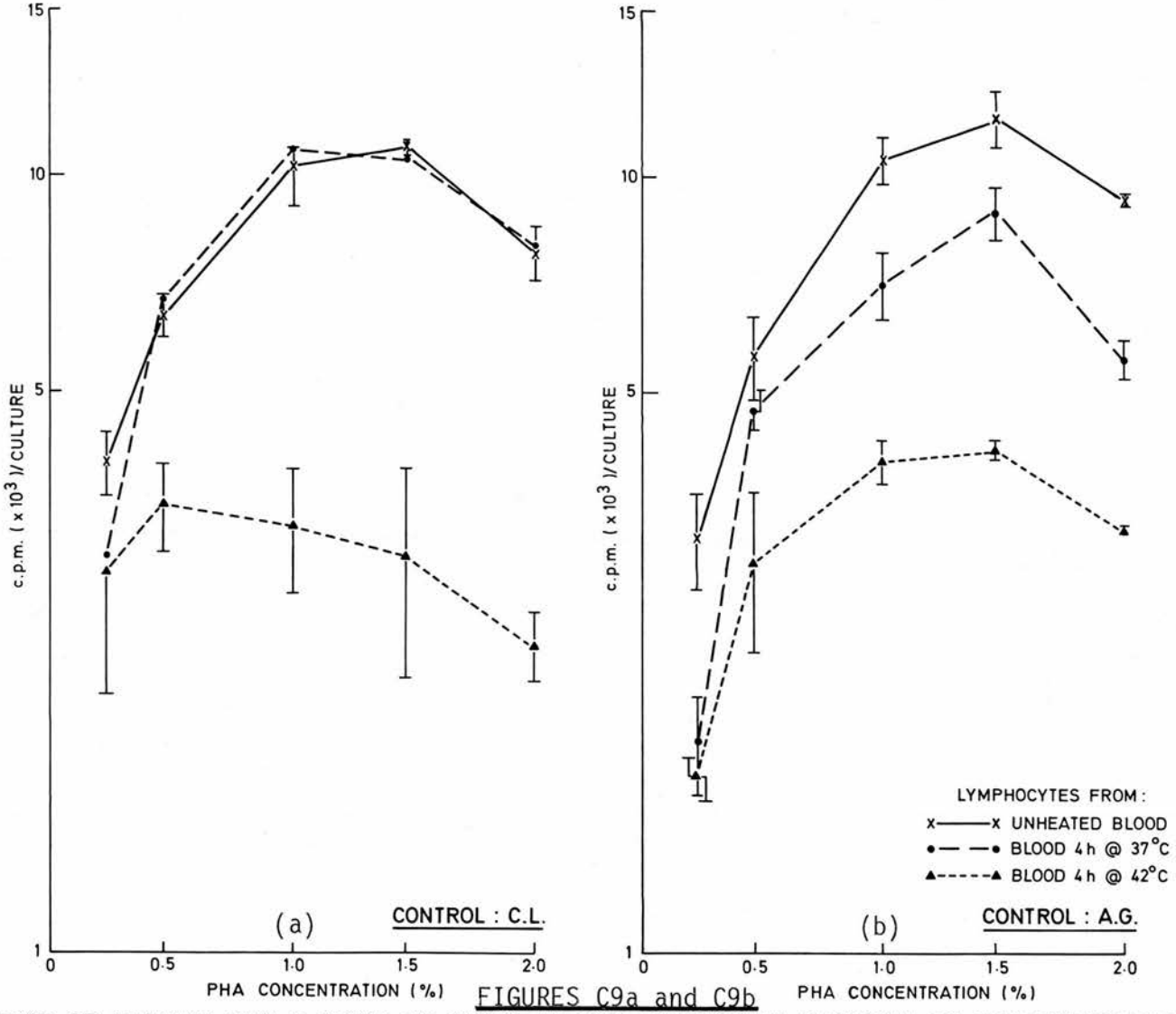
The Effects of Heat in vitro on the Response of Lymphocytes to PHA

The effect of heating Whole Blood in vitro The effect of heating blood in vitro on the PHA responsiveness of lymphocytes subsequently isolated from the blood, is shown in Figures C9a and b.

Heating at 42°C for periods up to 4h, resulted in a depression of PHA response in two cases. Incubation at 37°C had either no effect, or produced only a slight depression (Figure C9b). In two experiments, temperatures of 42°C resulted in an unchanged, or slightly increased, response. The effect of heating blood from hyperthermia patients in vitro, was studied in two subjects (M.M. and L.K.) in six experiments. The results indicated that a temperature of 42°C could either enhance (Figure C10b-blood taken prior to hyperthermia treatment) or depress (Figure C10a-blood taken at the end of hyperthermia treatment) the PHA response of different blood samples from the same patient, possibly indicating a change in the thermal tolerance of the cells during the course of treatment. However, the results obtained by heating the pre-hyperthermia treatment blood in vitro, were similar, in most cases, to those produced by the hyperthermia treatment itself, e.g. an increase in PHA response seen in M.M., following heating in vitro (Figure C10b), was seen during hyperthermia in vivo (Figure C7).

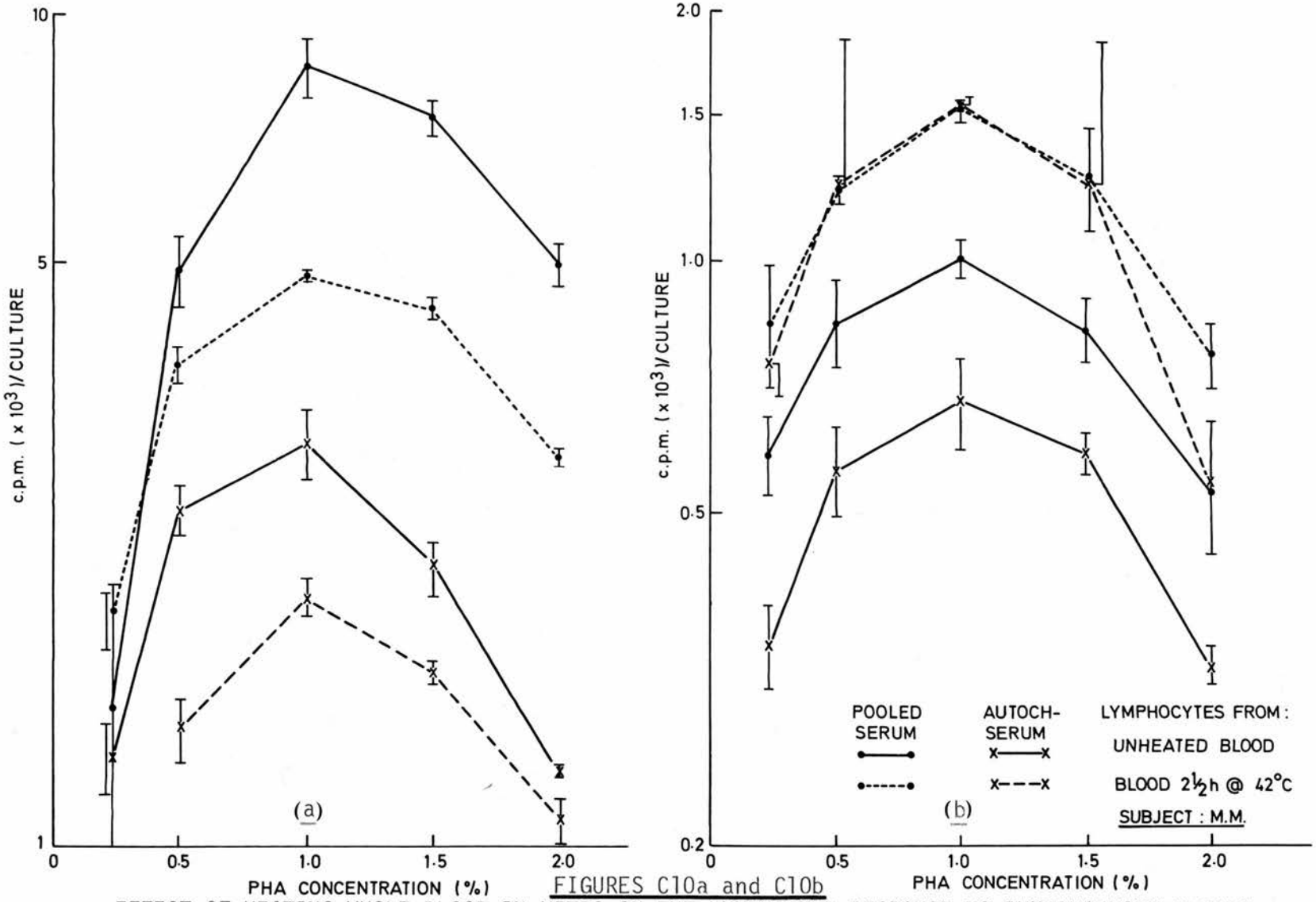
The effects of heat in vitro were usually similar at all concentrations of PHA, and in the case of cancer patients, were not affected by the use of pooled normal, or autochthonous serum in the cultures. In general, the degree of serum blocking was not affected by heating in vitro, although changes were occasionally seen (Figure C10b).

It was possible that lymphocytes from blood incubated at 42°C were being selectively affected in some way, during the separation procedure.



FIGURES C9a and C9b

EFFECT OF HEATING WHOLE BLOOD IN VITRO ON THE LYMPHOCYTE RESPONSE TO PHYTOHAEMAGGLUTININ
Lymphocytes were separated from heated blood and set up in culture using Method (ii)
Incubation time: 3 days. Points shown are the mean of triplicate samples ± 1S.D.



FIGURES C10a and C10b

EFFECT OF HEATING WHOLE BLOOD IN VITRO ON THE LYMPHOCYTE RESPONSE TO PHYTOHAEMAGGLUTININ

Lymphocytes were separated from heated blood and set up in culture using Method (ii)
Incubation time: 4 days. Points shown are the mean of triplicate samples ± 1S.D.

Since lymphocyte separation usually took 2-3h, there was the possibility that cells were recovering from the effects of heat during this period. To eliminate the delay between heating the blood and establishing the cultures, whole blood PHA tests were used. In these experiments, whole blood was put into culture with PHA within 5min of heating *in vitro*. The results from cultures established from 33 samples, taken at 15min intervals over 2½h periods, in three experiments, consistently showed no significant difference between the PHA response of blood kept at 37°C or 42°C (Figure C11). There was considerable variation between aliquots taken every 15min from the same blood sample, but the fluctuations were similar in both tests and controls, and were probably due to differences in the response kinetics between the different microtiter plates (See p. 145). It was difficult to assess the effect of the incubation process on lymphocyte transformation, because of these fluctuations, however the general trend was towards a fall in PHA response over a 2½h period at either 37°C or 42°C.

The Effect of heating Isolated Lymphocytes in vitro Lymphocytes, isolated by the standard method, and heated at 42°C for 2½-4h, showed virtually no response to PHA, although they were initially viable by dye exclusion. Control suspensions incubated at 37°C displayed a normal, or more frequently, a slightly depressed response, in comparison to cells from unheated blood. Over a 2½h period at 42°C the pH of the medium in which the cells were heated, fell by less than 0.1, to pH 7.10.

The inhibition seen at 42°C could not be reversed by incubating the heated normal lymphocytes in autochthonous serum for 1h at 37°C. Cells heated at 42°C *in* autochthonous serum tended to show slightly increased responses, compared to those incubated in tissue culture medium containing foetal calf serum, but the activity was still markedly

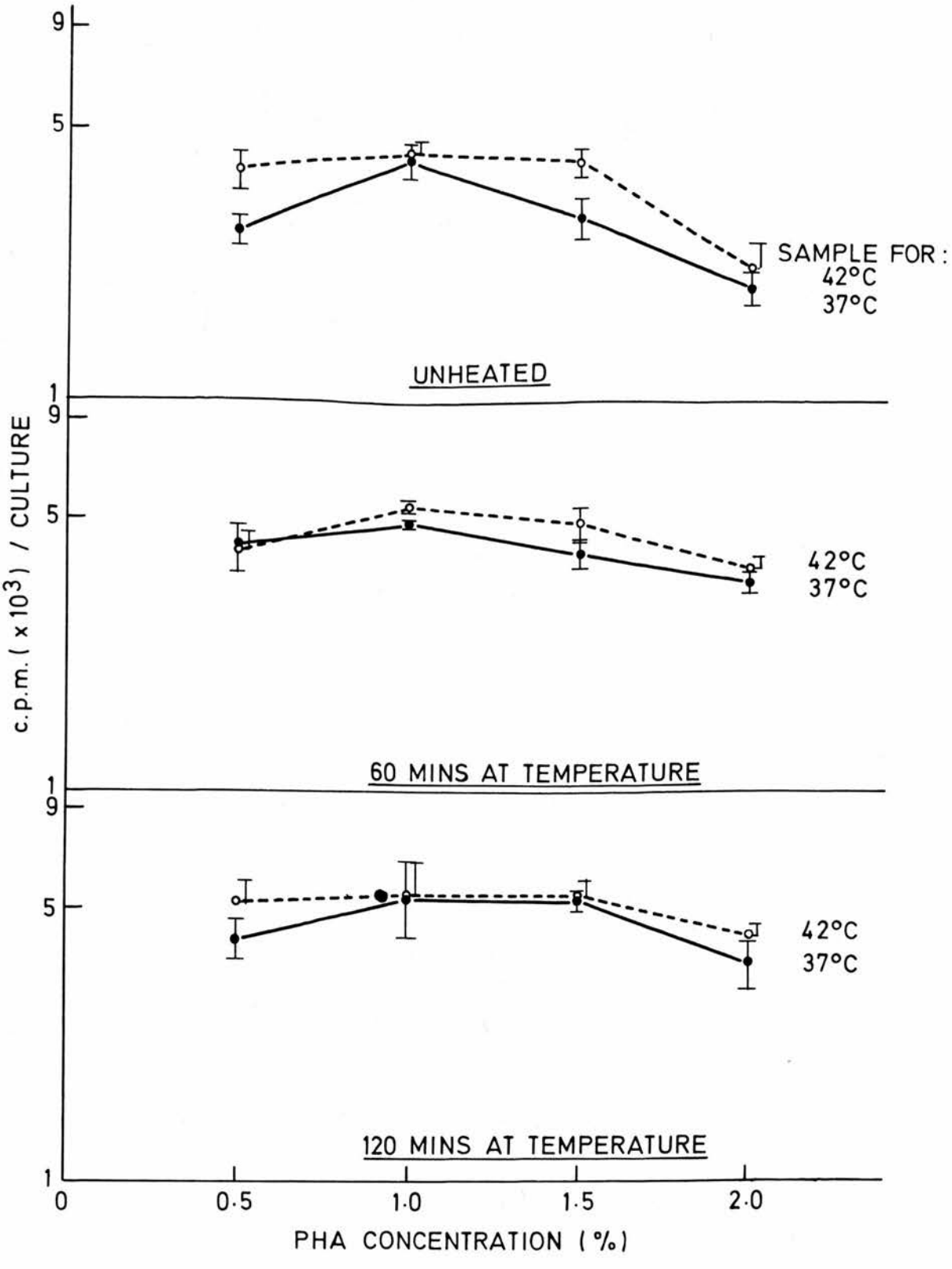


FIGURE C 11

EFFECT OF HEATING IN VITRO ON THE PHA RESPONSE OF WHOLE BLOOD

A sample of defibrinated normal blood was divided into two aliquots and incubated at the temperatures shown. Samples were taken at the times indicated, and used to set up PHA dose/response curves, using Method (iii).
Points shown are the mean of triplicate samples $\pm$ 1 S.D.

lower than that of control lymphocytes maintained at 37°C in serum. In the latter case, incubation in serum at 37°C always resulted in slightly higher responses than incubation in medium supplemented with foetal calf serum.

These results were confirmed by examination of May-Grunwald/Giemsa-stained cytocentrifuge preparations of PHA cultures of heated cells. Blast transformation was not seen in cultures of cells that had been pre-incubated in medium at 42°C, whereas a few colonies of lymphoblasts were observed in the cultures established from lymphocytes that had been incubated in autochthonous serum at 42°C. In standard cultures of unheated cells, the majority of cells in the preparation were lymphoblasts.

In an attempt to determine whether the thermal damage to isolated lymphocytes was evident immediately after heating, or became manifest during the subsequent incubation with PHA, the cultures were pulsed with ^{14}C-Uridine (See p. 143) to measure RNA synthesis. Since production of RNA precedes that of DNA, it was hoped that the uptake of the precursor by control lymphocytes, would be large enough, during short incubations (1-3 days), to determine at what point the damage to heated cells occurred.

This proved to be the case. The results are shown in Figure C12. They indicate that there is a marked inhibition of RNA synthesis within 24h of 42°C treatment (24h is the pulse time of the isotope). Although uridine uptake was higher in heated cells incubated with PHA for 48 and 72h, the increases were not significant, compared to those seen in the control lymphocyte suspension, pre-incubated at 37°C.

Discussion

The results from the experiments in which heat was applied in

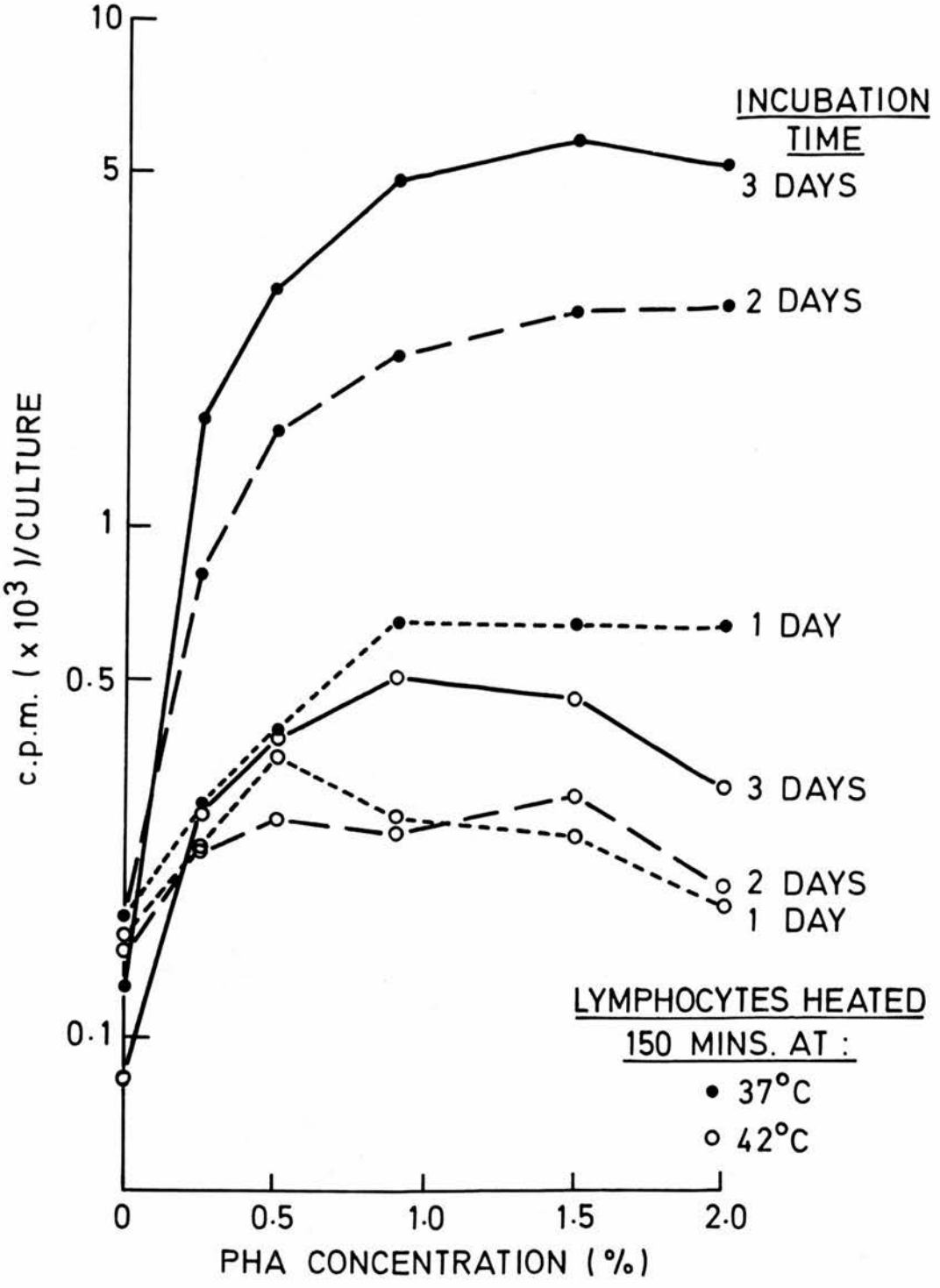


FIGURE C 12

EFFECT OF HEATING ISOLATED LYMPHOCYTES IN VITRO ON THEIR PHA-INDUCED RNA SYNTHESIS

Isolated normal lymphocytes were heated under the conditions shown, and used to set up PHA dose/response curves, using Method (iii). Cultures were pulsed with 0.1μCi ^{14}C-Uridine 24h before harvesting.

Points shown are the mean of triplicate samples.

vitro indicate that the effect becomes more severe the further one departs from the conditions *in vivo*. Parallel experiments in which blood was heated *in vitro* and *in vivo* suggested that the results were related, in that changes in the response, although they were often small, were usually in the same direction e.g. increased or decreased in both cases. However it is not clear to what extent the alterations produced by heating the blood *in vitro* were due to increased temperature, rather than to changes in the oxygen content of the medium, pH etc. all of which are known to affect the thermosensitivity of tumour cells. The one parameter that was monitored, pH, showed little change during the standard 2½h incubation period, when isolated cells were heated in medium.

The experiments in which whole blood was cultured with PHA are difficult to interpret because of the variation in response between replicate plates. In every case there was no significant difference between the results from blood pre-heated at 42°C or 37°C. This may indicate that cells are protected from, or can recover from, thermal effects, provided that they are kept in whole blood. The depression of responsiveness seen after hyperthermia *in vivo*, may be due to changes in serum steroid concentrations etc., which will not occur during heating *in vitro*. The hypothesis of thermoprotection may explain the other results, in that there was usually a difference in the PHA responsiveness of lymphocytes isolated from heated and control blood samples, although the difference varied in magnitude and direction depending upon the sample. This may be due to different thermosensitivities of the blood samples, combined with recovery from heat damage during the lymphocyte separation procedure. In contrast, the PHA response of isolated lymphocytes was virtually abrogated by pre-heating the cells at 42°C in medium, but was slightly less affected by heating the cells in serum. This also suggests that the components in whole

blood may be thermoprotective, although serum alone cannot prevent heat damage to lymphocytes, nor can it reverse the damage once it has occurred, since incubation of heated cells in serum did not reconstitute their PHA responsiveness. It would appear that the damage produced by 2½h incubation at 42°C is irreversible, since the cells also showed no recovery during the 3-5 day incubation with PHA. This was confirmed by the uridine uptake studies, which indicated that PHA-stimulated RNA synthesis by heated lymphocytes, was rapidly, severely, and irreversibly depressed.

As is the case with hyperthermic damage to tumour cells, the mechanism of this depression is not fully understood. The results from this study indicate that 2½h heating at 42°C (300C°M) is sufficient to virtually abolish RNA and DNA synthesis in lymphocyte suspensions, resulting in the 'death' of the cells within a few days. It is difficult to define cell death for lymphocytes. The cells which did not respond to PHA stimulation were more than 95% viable by eosin exclusion, when set up in culture. It is known that dye exclusion is not a good criterion for viablility in heat-treated cells (Harris, 1966), but the other commonly used criteria - isotope uptake and release, and colony formation - are affected by heating in vitro, or are unsuitable for use with lymphocytes. A morphological technique (Schrek, 1964 and 1966) was examined, but was unsuccessful. It is therefore possible that the heated lymphocytes were essentially dead when set up in culture.

In summary, heat applied either in vivo, during hyperthermia treatment, or in vitro, resulted in a depression of lymphocyte activity, as measured by blastogenic response to PHA. In vivo the depression was slight, and normal, or slightly increased responsiveness was seen the day after treatment. There was a marked peak in lymphocyte activity

within a further 2-3 days, followed by a return to the pre-treatment level within a week. This pattern of response was not directly related to the serum cortisol concentration, but may be associated with changes in the level of some other corticosteroid. Pre-heating at 42°C *in vitro* had no significant effect on the PHA response of whole blood, and lymphocytes separated from heated blood showed variable responses. An irreversible, almost complete inhibition of both uridine and thymidine uptake could be produced by heating isolated lymphocytes in tissue culture medium at 42°C for 2½h. These differences may be due to either thermoprotection of lymphocytes by the other components in whole nlood (for which there is some evidence from experiments *in vitro*), or to changes in the factors affecting the heat sensitivity of the cells. This study emphasises the need for a more reliable criterion of the viability of heated lymphocytes, and indicates that the results of studies *in vitro* are not directly applicable to the situation *in vivo*.

PAPER D

THE EFFECTS OF WHOLE-BODY HYPERTHERMIA THERAPY ON THE CONCENTRATION OF SERUM IMMUNOGLOBULINS AND COMPLEMENT

The Effects of Whole-Body Hyperthermia Therapy on the Concentration of Serum Immunoglobulins and Complement

Introduction

Although it is believed that the immune response to neoplastic disease is largely cell-mediated, there is evidence that the humoral system is involved. Anti-tumour antibodies have been found in the serum of patients with various types of cancer, e.g. malignant melanoma, osteogenic sarcoma and leukaemias (See p. 29). Their purpose is not fully understood, although humoral cytotoxicity against the appropriate tumour type, has been demonstrated in the serum of some cancer patients.

Work by Finney, Byers and Wilson (1960) indicated that the cancer patient is able to mount a humoral response to his own tumour. They noted a rise in the circulating antitumour antibody titre, following the intra-muscular injection of autochthonous tumour cells, homogenised in Freund's adjuvant. Other studies have shown that, in contrast to many indices of cellular immunocompetence, humoral antibody synthesis is not greatly impaired in malignant disease (Southam, 1968). For example, Krant et al (1968) found that patients with bronchogenic cancer, had normal primary and secondary humoral responses, when immunised with plague and diphtheria vaccines, respectively. In contrast, Lytton, Hughes and Fulthorpe (1967) found that cancer patients showed an impaired response to primary immunisation with tetanus toxoid, and this occurred irrespective of age, or general condition of the patient. In addition, the degree of impairment could be related to the survival time. Similar studies, this time on the primary humoral response, were conducted by Lee, Rowley and Mackay (1970). They found that patients with active, non-

lymphomatous cancers displayed an impaired antibody-producing response to immunisation with monomeric flagellin from Salmonella adelaide, in comparison to sick, non-cancer, control subjects. The degree of impairment could be related to the survival time. Those patients 'cured' by surgery and/or radiotherapy showed a return to near-normal levels of antibody response. This suggests that monitoring of humoral immunity may provide some useful indication of the patient's response to therapy.

Hobbs (1970) has stated that 'serum immunoglobulin patterns offer a crude window through which to view some immunological events, but can be useful in certain contexts'. Nonetheless, because of their ease of quantitation, serum immunoglobulin concentrations are widely used as an indication of general humoral immunocompetence in cancer patients. The results from some of these studies are summarised in Table D1.

Many of the contradictory results may be explained by the nature of the control population with which the patients were compared. Roberts used patients with benign disease, whereas Dostalova's control group was disease-free. In addition, a number of other factors are known to affect immunoglobulin levels, e.g. age (Teasdale, Newcombe and Hughes, 1976), sex (Rhodes et al, 1969) and environment (Cohen et al, 1961), and must be taken into account when interpreting these studies.

Several general conclusions can be drawn however:-

i) IgM concentrations are not greatly affected by malignant disease.

ii) IgG levels tend to be increased.

iii) IgA concentrations are often elevated, especially in those tumours affecting organs with mucous membranes.

REFERENCE	TUMOUR TYPE	IgG		IgA	IgM	COMMENTS
KRANT et al (1968)	BRONCHUS	N.S.D.		N.S.D.	N.S.D.	IgA ↑d IN LONG-SURVIVING PATIENTS
SOUTHAM (1968)	NON - LYMPHOMATOUS CANCERS	N.S.D.		N.S.D.	N.S.D.	
HUGHES (1971)	SKIN	↑		↑	N.S.D.	IN ♂s
	LUNGS	↑		↑	N.S.D.	IN ♂s
	MOUTH	N.S.D.		↑	N.S.D.	IgA/IgG ↑d
	GUT	N.S.D.		↑	N.S.D.	
	UTERUS	N.S.D.		↑	N.S.D.	
	OVARY	N.S.D.		N.S.D.	↓	
	SARCOMAS	N.S.D.		N.S.D.	↑	IN ♂s
	MELANOMAS	N.S.D.		N.S.D.	↑	IN ♀s
LEHANE & LANE (1974)	LUNG	N.S.D.		↑	N.S.D.	
	HEAD & NECK	N.S.D.		↑	N.S.D.	
DOSTÁLOVÁ et al (1975)	VARIOUS:- BREAST BRONCHUS LYMPHOMA LARYNX BLADDER KIDNEY (GRAWITZ)	GENERAL INCREASE - ESPECIALLY OF IgG & IgA IN KIDNEY BLADDER & BRONCHUS PATIENTS			N.S.D.	
ROBERTS, BATHGATE & STEVENSON (1975)	BREAST	↓		↑	N.S.D.	↓d IgG NOT CONCOMITANT WITH ↑d IgA
BOLTON et al (1976)	BREAST	N.S.D.		N.S.D.	N.S.D.	IgA ↑d IN ADVANCED CASES - N.S.D. IF AGE DIFFS. CONSIDERED
COCHRAN et al (1976)	MELANOMA	↑		↑	N.S.D.	
	BREAST	↑		↑	N.S.D.	
MUNZAROVA TRNKA & MALIR (1977)	BREAST	SEE COMMENTS		↑	-	MOST IgG LEVELS WITHIN NORMAL RANGE IgD ↓
N.S.D. = NO SIGNIFICANT DIFFERENCE FROM CONTROL VALUES						

TABLE D1

REVIEW OF PREVIOUS STUDIES ON SERUM IMMUNOGLOBULIN CONCENTRATIONS IN CANCER PATIENTS

This may reflect local antigenic stimulation of the IgA-secreting cells associated with these areas.

In some cases it has been shown that the levels of serum immunoglobulin can be related to the stage of disease, and prognosis. Meyer, Mackler and Beck (1973), found that women with increased IgA concentrations, prior to mastectomy and radiotherapy, had an improved prognosis. Roberts, Bathgate and Stevenson (1975) however indicated that immunoglobulin levels were not affected by mastectomy or hormone therapy, and showed no correlation with prognosis. A similar conclusion was reached by Cochran et al (1976) and Krant et al, (1968), whereas Dostalova et al (1976) found that the level of IgG correlated with the stage of disease. Munzarova et al (1977) showed that patients with advanced breast cancer had significantly increased IgA, in comparison to those with localised tumours, although the levels were elevated at all stages of the disease. Krant et al (1968) also suggested that moderate elevation of IgA, in longer surviving patients with bronchogenic cancer, merited further investigation.

Although not measured as part of this study, IgE may have an important role in tumour immunity. The serum concentrations of IgE are within the normal range in cancer patients, but there is evidence from both animals and man (Bartholomaeus & Keast, 1972 ; Grace & Kondo 1958) for the existence of tumour-specific IgE (or IgE-like) antibodies in cancer-bearing hosts. There may also be a relationship between IgE levels and T cell function. For a more detailed review, the reader is referred to the article by Rosenbaum and Dwyer (1977).

In summary, the results from measurements of serum immunoglobulin concentrations are often contradictory, and their full value, as both a monitor of humoral immunocompetence, and a guide to clinical prognosis, remains to be established.

There are very few studies on the concentration of serum complement components in cancer patients. In a survey of 200 cases of malignant disease, Verhaegen et al, (1976) found significantly higher levels of the CH'50, C'3, C'4 and C'1q components, in comparison to 90 healthy control subjects. Similar increases, especially of C'8 and C'9, were reported in 15 out of 21 patients with squamous carcinoma of the lung, by McKenzie, Colsky and Hetrick (1967). Southam (1967), however, found that levels of the second component were normal in cancer patients. Cochran et al (1976) have described various abnormalities of the complement system in 52% of the cancer patients that they examined. These included elevated concentrations of the various components - but they could find no significant difference between the levels in patients with primary, as opposed to metastatic, disease. Verhaegen et al (1976), in contrast, found a stage-linked increase in complement concentrations such that patients in remission has almost normal levels, whereas those with advancing disease showed progressively higher concentrations, until the terminal phases of their illness. Treatment by chemotherapy or radiotherapy resulted in the lowering of the levels, suggesting that serial measurements could provide information on the state of the disease. Lehane and Lane (1974) also found that advanced cancer patients had increased C'3 concentrations, which could be related to the future response to chemotherapy.

In summary, many patients with malignant disease have increased concentrations of serum complement. This may be a response to the elevated demand for complement, due to an increase in the levels of antigen-antibody complexes, formed between soluble tumour antigen and serum antibodies. There is also some indication that the concentration of complement, measured as the C'3 component, may

be related to the clinical status of the cancer patient.

The aim of this investigation was to determine the effects of whole-body hyperthermia therapy on the concentrations of serum IgG, IgA, IgM and C'3, with a view to correlating any observed changes with the clinical response to therapy.

Materials and Method

Measurement of the concentration of serum immunoglobulins (IgG, IgA, IgM) and complement (as C'3) was carried out by the Central Microbiology Laboratories, Western General Hospital, Edinburgh, using the radial immunodiffusion technique (Mancini, Carbonara and Heremans, 1965).

Materials

Radial immunodiffusion plates for IgG, IgA, IgM and complement as C'3 (BIC/BIA) estimations, control and reference sera were all obtained from the Oxford Diffu-Gen Division of Searle Dignostic, High Wycombe.

Method

Serum samples for immunoglobulin and complement estimations were stored at -25^{o}C until tested. They were then thawed and 5µl samples pipetted into the appropriate wells on the immunodiffusion plate. Standard and control sera were run for each test. The plates were incubated at room temperature for 18h after the addition of the final sample.

The diameter of the precipitin ring was measured using a Hoechst diverging-line scale, as this was found to give the most consistent results. A standard curve was plotted on 2-cycle semi-logarithmic graph paper, using the values obtained from the reference sera. The concentration of immunoglobulin, or complement, in the test serum, expressed as mg/dl, was determined from the standard curve.

Results

Serum Immunoglobulin and Complement Concentrations prior to Hyperthermia Prior to hyperthermia treatment, the concentration of serum IgA, IgG, IgM and complement (C'3), was measured in 8 patients (Table D 2).

Before therapy, 7 of the 8 patients had IgG concentrations within the normal range, whereas IgA levels were markedly elevated in 4 cases. The majority of patients had essentially normal concentrations of IgM, with the notable exception of the two cases of leiomyosarcoma (W.M. and E.C.), who had slightly depressed levels.

Complement (C'3) concentrations were increased in 62% of the patients, but within the normal range in the remainder.

Serum Immunoglobulin and Complement Concentrations during and after Hyperthermia Treatment Serum immunoglobulin and complement concentrations were monitored in the same 8 patients, over their course of treatment. Statistical analysis was performed, using the Student's t test for paired samples. p values of less than 0.05 were considered significant.

The effects of the first hyperthermia treatment are summarised in Table D 2. Measurements on individual patients often showed large changes in the concentrations of the various immunoglobulin classes, and, although these were not statistically significant, and did not show any clear trend, there was a tendency for IgM levels to increase, following hyperthermia. It is unlikely that the changes which occurred during treatment, were due to haemoconcentration, or dilution, since an increase in one class of immunoglobulin was sometimes accompanied by a fall in concentration of another class.

Similar changes in immunoglobulin levels were seen following the

TEST	PATIENT								MEAN ± 1 S.D.	
	KS	RM	AW	AB	WM	EC	AS	HA		
IgG										
PRE	1150	890	1580	1425	1450	580	1300	950	1166	339
END	782	800	1580	1550	1100	700	1150	950	1077	339
POST 1	1150	1250	1580	1425	980	660	1250	820	1139	306
POST 2	1200	890	1580	1550	700	600	1250	1100	1109	362
	NORMAL RANGE :- 700 - 1900 mg/dl									
IgA										
PRE	515	320	310	600	590	215	570	400	440	148
END	440	230	310	600	360	320	600	400	408	134
POST 1	515	450	410	450	425	290	540	400	435	76
POST 2	470	320	410	480	360	320	780	390	441	150
	NORMAL RANGE :- 90 - 450 mg/dl									
IgM										
PRE	62	45	192	210	38	52	115	107	103	67
END	62	40	170	210	52	75	180	120	114	66
POST 1	115	87	192	200	47	68	92	120	115	55
POST 2	80	87	285	170	45	63	92	130	130	78
	NORMAL RANGE :-♂s 45 - 180, ♀s 55 - 220 mg/dl									
C'3										
PRE	135	130	157	104	180	95	92	190	135	38
END	157	100	130	113	145	N/D	97	160	129	26
POST 1	145	86	130	104	140	95	100	117	115	22
POST 2	140	86	130	100	N/D	94	137	125	116	22
	NORMAL RANGE :- 55 - 120 mg/dl (♂>♀)									

TABLE D2

EFFECT OF THE FIRST HYPERTHERMIA TREATMENT ON THE CONCENTRATION OF SERUM IMMUNOGLOBULINS (IgG, IgA & IgM) AND COMPLEMENT (C'3)

All values are in mg/dl N/D = Not done
Post 1 samples were taken the day following treatment
Post 2 samples were taken 3-4 days following treatment

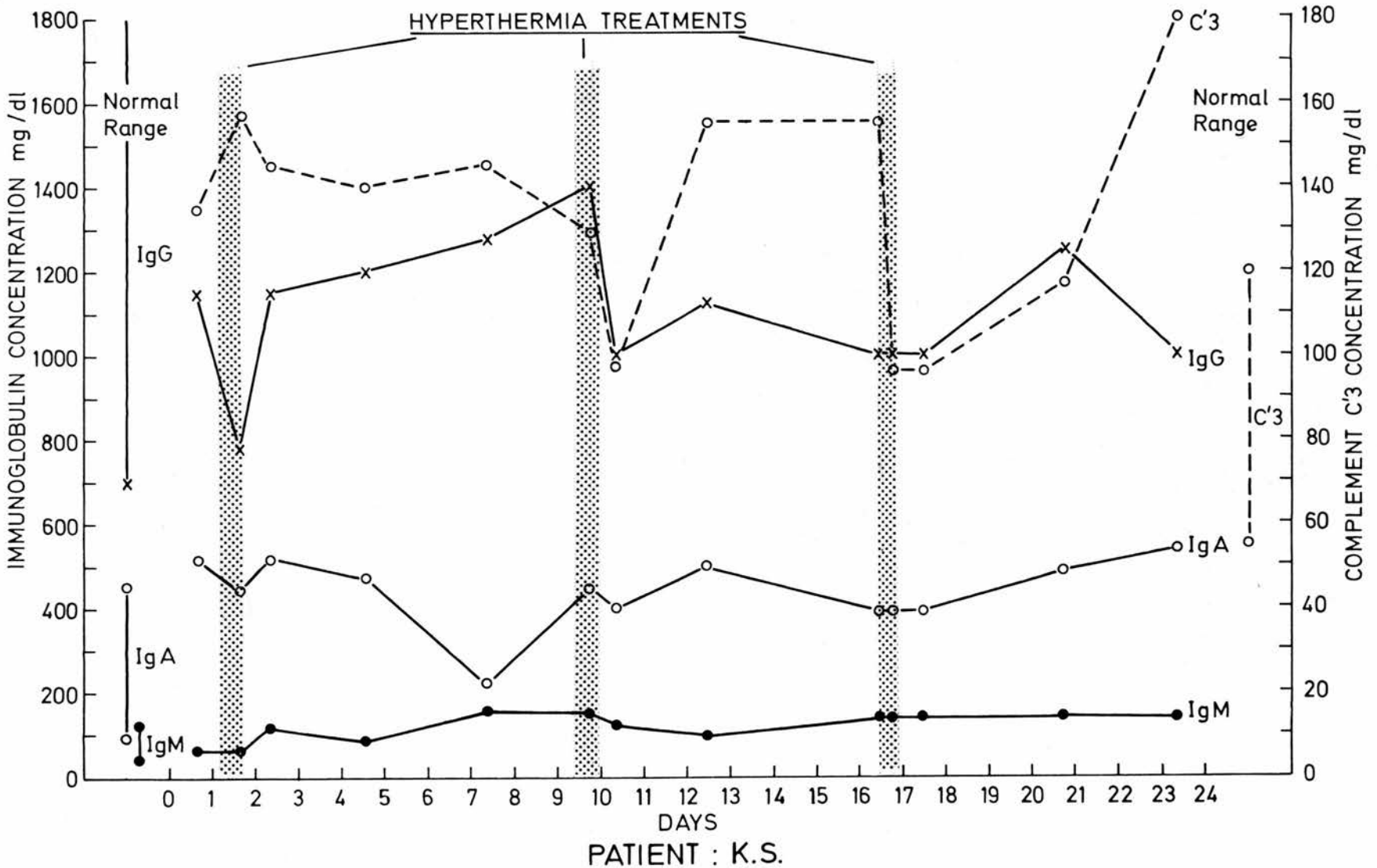


FIGURE D1a

EFFECT OF HYPERTHERMIA TREATMENTS ON THE CONCENTRATION OF SERUM IMMUNOGLOBULINS & COMPLEMENT (C'3)

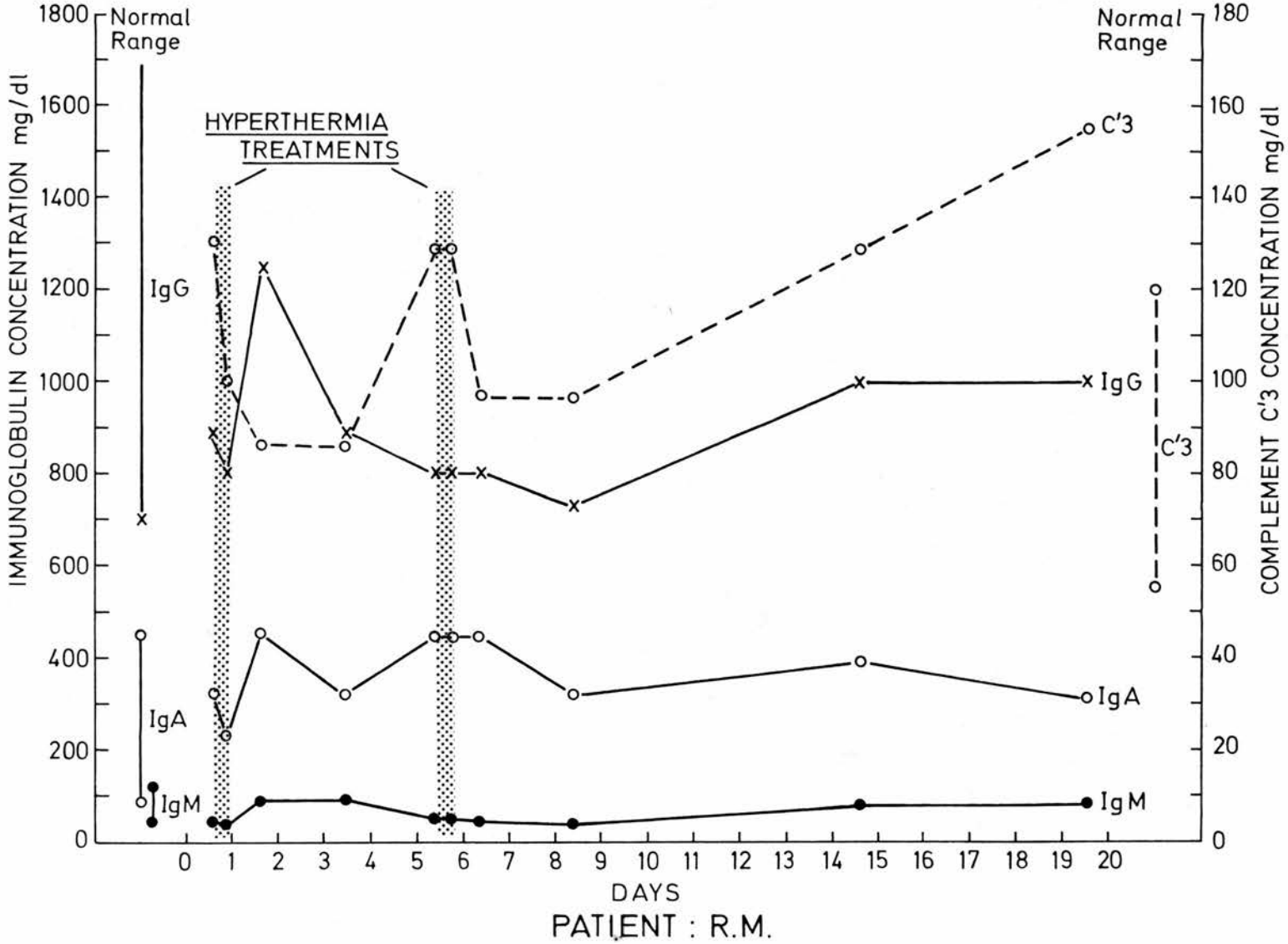


FIGURE D1b

EFFECT OF HYPERTHERMIA TREATMENT ON THE CONCENTRATION OF SERUM IMMUNOGLOBULIN & COMPLEMENT (C'3)

second and subsequent treatments (Figures D1a and D1b), and again, no clear trend could be discerned.

There was no significant change in the concentration of C'3 during hyperthermia, however, the levels fell to 88% of the pre-treatment value the following day ($p < 0.1$). The depression was transitory, with a return to the pre-hyperthermia level within a further 2 or 3 days. This pattern was usually repeated during subsequent treatments. (Figures D1a and D1b).

Discussion

The pattern of an isolated increase in serum IgA concentrations, prior to treatment, has been noted previously in cancer patients (Hughes, 1971). All the hyperthermia patients in whom this was detected, had gastro-intestinal malignancies. The association between G.I. disease and elevated IgA concentrations may be explained by the origin of this class of immunoglobulin. It is synthesised, as a 7S monomer, by the laminae propriae which underlie the mucous membranes throughout the G.I. tract. This monomer is stabilised, and protected from proteolysis, by combination with a secretory piece, which is produced by the epithelial cells. The pool of 7S IgA can enter the bloodstream, either by the lymphatics, or, directly, via the portal veins (Hobbs, 1970), and thus, diseases which involve an antigenic stimulation of the G.I. tract e.g. Crohn's disease and cancer (either directly, or via secondary infection) may result, not only in increased concentrations of IgA in mucous secretions, but also in the serum.

The finding of elevated C'3 concentrations in hyperthermia patients, prior to treatment, is in agreement with the study by Verhaegen *et al* (1976), who reported significantly higher levels in

cancer patients. This increase was linked to the stage of disease, in that the highest values were found in cases of metastatic cancer. They also indicated that the concentrations fell pre-terminally. It is interesting to note that the 3 hyperthermia patients with essentially normal levels of C'3, had a mean survival time of only 15 days (Range 9-25 days), following the final treatment, in contrast to the 184 days (Range 71-485 days) for the remaining patients; indicating that they were indeed in the pre-terminal stages of their disease.

Following hyperthermia, DeHoratius et al (1977) also found that there were no persistant changes in immunoglobulin concentrations. In contrast, Mendez et al (1959) reported a fall in gamma globulin levels in healthy subjects, after 2h 39°C-40°C hyperthermia. They did not, however, distinguish between the subclasses. In general, therefore, it would appear that whole-body hyperthermia treatment has no marked effect on the concentrations of serum IgG, IgA or IgM, although it may be necessary to carry out a longer-term follow-up to confirm this.

There was also no significant change in the concentration of C'3 during heating, however the levels fell to 88% of the pre-treatment value within 24h. A similar decrease in serum C'3 was noted by DeHoratius et al, in 2 patients receiving whole-body hyperthermia alone, and a third,given concomitant chemotherapy. This could be correlated with a decline in total haemolytic complement activity in two of the cases. Failure to detect a significant fall in the complement components C3PA and CH50, involved in the alternative pathway, indicated that the classical pathway was primarily affected by heat treatment. It is not clear whether the fall, seen in both studies, is due to partial thermal degradation of complement (which can be inactivated by heating to 56°C), or, to the selective

consumption of the various components, possibly by an increase in the numbers of tumour antigen-antibody complexes, which may be formed following hyperthermia.

These changes in complement concentrations may prove to be of considerable interest. Schutte et al (1975) have indicated that the components may act as acute phase reactants, the levels of which could be related to various immune parameters, the extent of disease, and tumour stage in cancer patients (Chretien, 1977 - Personal Communication). If this proves to be the case, a fall in C'3 concentration following hyperthermia, may be indicative of an enhanced immune status, and/or tumour destruction. It is notable that in the 3 pre-terminal patients, the C'3 levels were not depressed 24h after treatment, which could indicate that the treatment was without effect. One of these cases (E.C.) received a second hyperthermia session, during which the concentration of complement fell by 25%. This patient died 4 days later, and, upon post mortem, there was evidence of massive tumour necrosis. It should be noted, however, that a non-cancer patient (P.N.), treated with three sessions of hyperthermia, showed a fall in C'3 within 24h of one of the sessions (although the concentration actually increased during hyperthermia), which may suggest that, in the absence of a tumour, there is a direct effect of heat on complement.

In summary therefore, whilst the number of patients studied was small, there was some indication that concentrations of serum C'3 may be of value in charting the clinical response to hyperthermia treatment.

PAPER E

THE EFFECTS OF WHOLE-BODY HYPERTHERMIA THERAPY ON ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

The Effects of Whole-Body Hyperthermia Therapy on Antibody-Dependent Cellular Cytotoxicity

Introduction

Since its discovery in 1965 by Moller, the cytotoxic effect of non-sensitised effector cells upon target cells coated with specific antibody, has been under intensive investigation. This has largely centred around the nature of the lytic mechanism, and the identity of the effector cell. It is now accepted that the latter is determined by the type of target cell used in the assay (Nelson et al, 1976), and that more than one class of leucocyte can display antibody-dependent cellular cytotoxic activity.

Due to these technical and methodological difficulties, reports on clinical applications of the ADCC test are rather rare. The majority have concentrated upon investigations in patients with immune disorders, e.g. Hashimoto's thyroiditis (Calder et al, 1973), systemic lupus erythematosus (Scheinberg & Cathcart, 1976) and immunodeficiency diseases (Rachelefsky et al, 1975). Depressed cytotoxicity has been reported in kidney transplant patients (DuPont et al, 1977) and infectious asthmatics (Flaherty et al, 1977).

One of the earliest studies in cases of malignant disease, was carried out by Blaese, Rosenberg and Wunderlich (1973), who found that the ability of lymphoid cells to lyse HL-A-coated lymphocytes, was not impaired in patients with cancer, in comparison to a single normal control subject. Similar results were obtained by Peter, Knoop and Kalden (1976), in a study of 14 melanoma patients, and 13 healthy controls. They found that cytotoxicity in the cancer patients was slightly lower, but that

this depression was not statistically significant. However both of these investigations were carried out on relatively small numbers of patients, and a larger study (44 patients with various tumours), conducted by Ting and Terasaki (1974), found that ADCC was significantly lower in patients with cancer, than in normal and 'sick' controls. They also showed that this could not be correlated with reduced antibody production in these subjects, but was due to a deficiency of K cells (See p. 160) in cancer patients. In addition, the degree of impairment could not be related to the stage of disease, or age.

Similar impaired responses have been described in patients with acute myeloid leukaemia, by Hersey et al (1973). They proposed that this failure could be due to serum blocking factors, e.g. antigen-antibody complexes, or K cell depletion, assuming that these patients produce antibodies to autologous myelocytes. Nonetheless, they suggested that the assay may be of use in charting the response to immunotherapy, especially in giving early warning of relapse.

The belief that K cell activity can be blocked by serum factors, has received some support recently. Matthews and Whitehead (1976) found that sera from 39% of breast cancer patients (and 8% of healthy controls) inhibited lysis. Partial characterisation of the blocking factor(s) suggested that it was not an IgG-containing complex. Serum inhibition has also been reported in animal systems (Matthews et al, 1976), where it was found to be independent of the stage of tumour growth. In contrast, Calder, Irvine and Ghaffer (1975) found that spleen cells from tumour-bearing mice were actually more active in a K cell system, than those from control animals. They also noted that normal lymph node cells had virtually no ADCC activity, whereas

cells from nodes draining the tumour were active, suggesting that K cells may be present in the tumour mass. A similar situation may exist in the human, since O'Toole (1977) has found that lymphocytes from lymph nodes are inactive in a K cell assay. As yet, there are no comparable studies on cancer patients.

In summary, studies on antibody-dependent cellular cytotoxicity in cancer patients are still in their early stages. There are indications that, in the human, K cell activity is essentially normal or slightly depressed, probably by immune complexes in the serum. These blocking factors may be affected by therapeutic procedures (Vose and Moudgil, 1976). In the animal, the presence of a tumour can lead to increased ADCC, either due to elevated K cell numbers and/or activity, although blocking factors may be present.

The purpose of this investigation was to determine the effect of hyperthermia in vivo on the K cell activity of the patient, and to try to relate any changes to alterations in clinical status.

Materials and Methods

Materials

Medium The medium used in all the assays was sodium bicarbonate-buffered Eagles Basal Medium, supplemented with 10% v/v heat inactivated, foetal calf serum, (+1% v/v non-essential amino acids, for tests using KB cells).

Target Cells The cells used as targets in these tests were grown in serially subcultured monolayers (See p.69)

They were:-

i) H.Ep.2 Established from a human epidermoid carcinoma of the larynx (Moore, Sabachewsky & Toolan, 1955). Used between passages 375 and 420

ii) KB Established from a human epidermoid carcinoma of the mouth (Eagle, 1955). Used between passages 386 and 412.

Unless stated otherwise, the cells used in all the tests were obtained from confluent monolayers.

Antiserum Antisera to both cell lines was prepared using the same protocol (given below). Two mature New Zealand White rabbits were used for each line. (The H.Ep.2 antiserum was prepared and titrated by Dr. M.A. Scragg, Teaching and Research Centre, Western General Hospital, University of Edinburgh).

Production and Titration of Antiserum. Prior to the injection of cells, 12ml blood was collected from each animal by venepuncture of the marginal ear vein. The serum was separated (See p.175), membrane filtered, and stored at -20^{o}C.

Day 0 Tumour cells were harvested from two Roux flasks, using standard procedure (See p. 69) and washed in 2x20ml PBS'A'. The cell pellet was resuspended in 1ml PBS'A' counted, and the

concentration adjusted with PBS'A' to $4x10^7$ viable cells/ml. 0.5ml of this suspension was emulsified with an equal volume of Complete Freund's Adjuvant, by repeated aspiration through a 21 guage needle. The emulsion was stored overnight at 4^oC, re-emulsified, and injected intra-muscularly into the right hind leg of the rabbit.

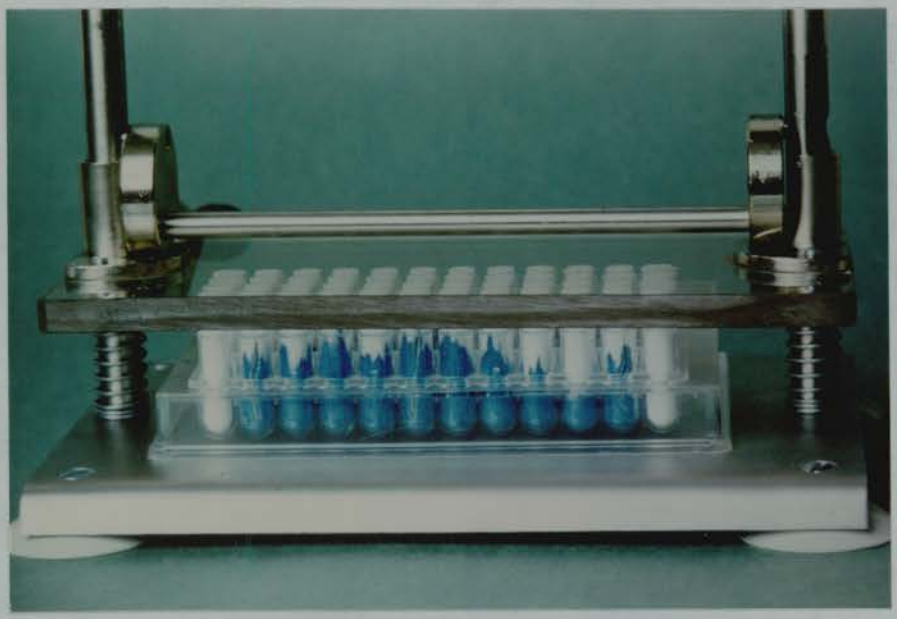
Day 11 1ml suspension, containing $1x10^7$ washed tumour cells, emulsified in an equal volume of Complete Adjuvant, was injected i.m. into the left hind leg of the same animal.

Day 23 Approximately 12ml blood was obtained from each animal by venepuncture of the marginal ear vein. The blood was collected in a sterile glass universal container, and allowed to clot for 1h at 37^oC. The clot was ringed and left to retract at 4^oC overnight. The serum was removed to a glass centrifuge tube and spun at 4,000g for 20min. The supernatant fraction was removed, membrane filtered, heat inactivated at 56^oC for 30min, and stored at -20^oC.

Normal and immune rabbit serum was titrated against the appropriate cell line to determine the concentration of antibody. The method used was complement-dependent lysis of radioactively-labelled target cells.

Cells of the appropriate cell line were harvested from a confluent monolayer, and $1x10^7$ viable cells, suspended in tissue culture medium, were incubated with 100μCi ^{51}Cr (sodium chromate) in a total volume of 1ml, for 1h at 37^oC. Extracellular label was removed by washing the cells in 6x10ml medium (centrifugations at 800g for 5min). The final cell pellet was resuspended in 3ml medium and the concentration of viable cells determined by dye exclusion. The labelling procedure did not affect the viability of the cells, which usually remained greater than 97%. Immediately before use, the concentration was adjusted to $3x10^5$/ml in medium.

1



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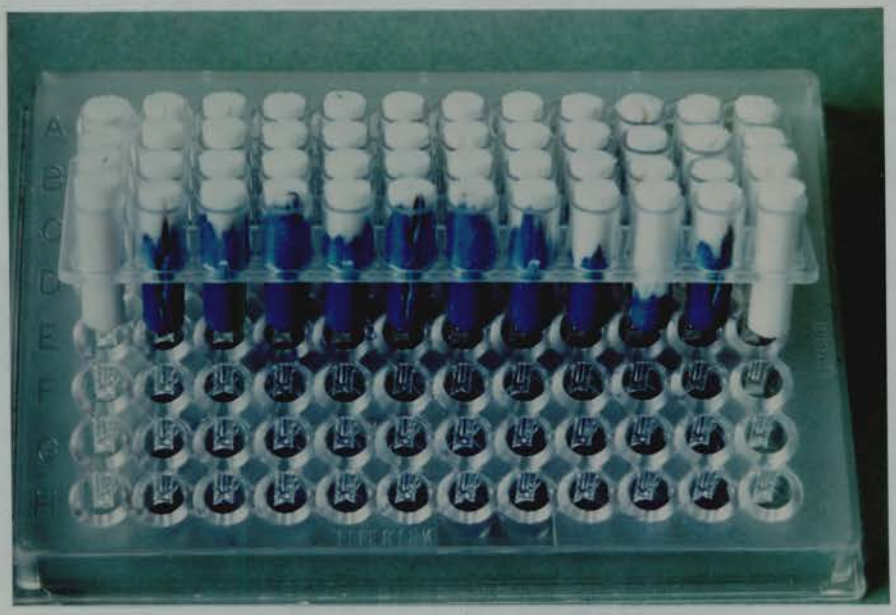


3

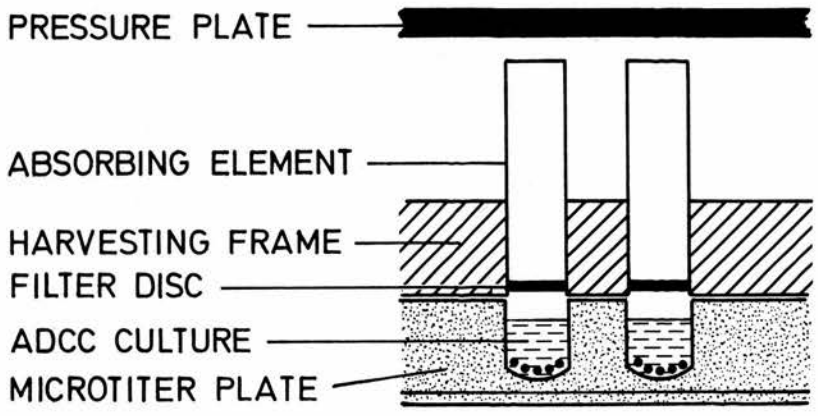
FIGURE E1

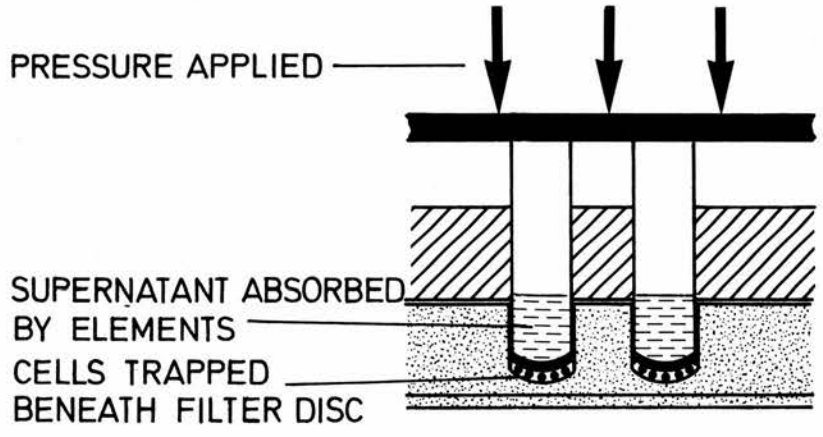
PRINCIPLE OF THE AUTOMATIC SUPERNATANT HARVESTER

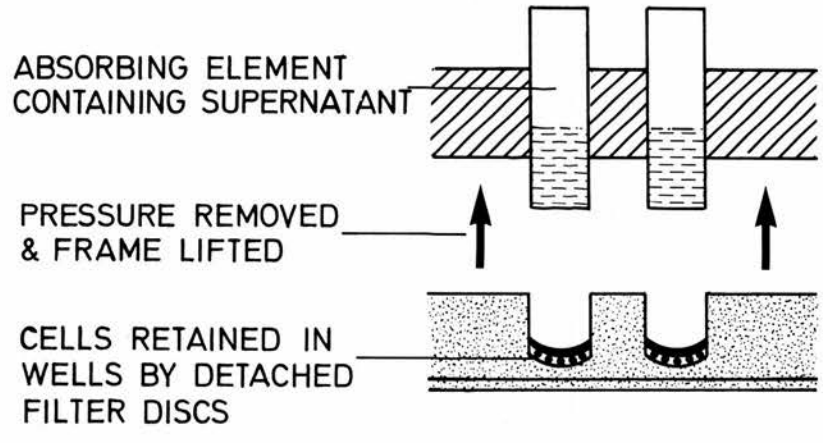
1) Microtiter plate and harvesting frame positioned beneath press.

2) Press activated, radioactive supernatant taken up by absorbing elements.

3) Press released and harvesting frame raised. Cells are retained in the wells by the detached filter discs.


PRESSURE PLATE
ABSORBING ELEMENT
HARVESTING FRAME
FILTER DISC
ADCC CULTURE
MICROTITER PLATE


PRESSURE APPLIED
SUPERNATANT ABSORBED
BY ELEMENTS
CELLS TRAPPED
BENEATH FILTER DISC


ABSORBING ELEMENT
CONTAINING SUPERNATANT
PRESSURE REMOVED
& FRAME LIFTED
CELLS RETAINED IN
WELLS BY DETACHED
FILTER DISCS

Fresh guinea pig serum was used as the source of complement. Guinea pig blood was collected by cardiac puncture and allowed to clot. The clot was ringed and left to retract for approximately 1h. The serum was removed and centrifuged at 4,000g for 20min. The supernatant fraction was removed, diluted 1 part in 3 in medium, and used immediately.

Freshly-thawed normal and immune serum were diluted in medium to give final concentrations of 33%, 3.3% and 0.33% v/v.

The plan of the test is shown in Table E1. Each row of the table represents the contents of one U-bottomed well in a microtiter plate. All the tests and controls were performed in triplicate. The suspensions and dilutions were seeded, in the order shown, into the wells of a plate standing in a plastic lunch box, through which was passed an humidified 95%/5% Air/CO_2 mixture. The completed plate was then placed in a second lunch box, containing sterile swabs moistened with distilled water, to maintain the humidity of the atmosphere during incubation. This box was gassed and the lid sealed in place with Lasso tape. It was incubated for 20h at 37°C.

At the end of incubation the supernatants were collected from each well, using a Titertek Supernatant Harvester (the principle of which is shown in Fig. E1). The absorption elements, containing the supernatants, were transferred to plastic counting vials, which were counted on a Wallac gamma sample counter for 2min, using the ^{51}Cr window. The following labelling controls were also included, in triplicate:-

Total Radioactive Count 50µl samples of the target cell suspension.

150µl samples of the following suspensions were seeded into the wells of a microtiter plate, at the start of incubation, and

				NORMAL SERUM			IMMUNE SERUM		
TEST	CELLS	MED.	COMP.	33% v/v	3.3% v/v	0.33% v/v	33% v/v	3.3% v/v	0.33% v/v
CONTROL ONE	√	√√	–	–	–	–	–	–	–
CONTROL TWO	√	√	√	–	–	–	–	–	–
CONTROL THREE	√	√	–	√	–	–	–	–	–
	√	√	–	–	√	–	–	–	–
	√	√	–	–	–	√	–	–	–
	√	√	–	–	–	–	√	–	–
	√	√	–	–	–	–	–	√	–
	√	√	–	–	–	–	–	–	√
TEST	√	–	√	√	–	–	–	–	–
	√	–	√	–	√	–	–	–	–
	√	–	√	–	–	√	–	–	–
	√	–	√	–	–	–	√	–	–
	√	–	√	–	–	–	–	√	–
	√	–	√	–	–	–	–	–	√

TABLE E1

EXPERIMENTAL DESIGN FOR THE TITRATION OF RABBIT ANTI-KB SERUM BY COMPLEMENT-DEPENDENT LYSIS

See text for full explanation of table and titration experiments

Key:- Cells = ^{51}Cr-labelled KB cells at 3×10^5/ml
Med. = Eagles Growth Medium
Comp. = Fresh guinea pig serum diluted 1/3 in medium
Serum = Normal or immune serum diluted in medium as shown

√ = 50µl : - = No addition

TEST	- COMPLEMENT	+ COMPLEMENT	% SPECIFIC RELEASE
NORMAL SERUM			
10^{-1} FINAL DILN.	1353.0	1302.0	-0.97
10^{-2} FINAL DILN.	1180.7	1261.3	1.48
10^{-3} FINAL DILN.	1111.0	1284.0	3.14
IMMUNE SERUM			
10^{-1} FINAL DILN.	2409.0	6522.7	97.50
10^{-2} FINAL DILN.	1137.5	1532.3	7.19
10^{-3} FINAL DILN.	1144.3	1317.3	3.16
CONTROLS	TOTAL RADIOACTIVE COUNT	8074.7	
	TOTAL RELEASABLE LABEL	6628.0	

TABLE E2

RESULTS FROM THE TITRATION OF KB ANTISERUM BY COMPLEMENT-DEPENDENT LYSIS

Figures shown in columns 2 and 3 are radioactive counts / 2min and the mean of triplicate samples.
Percent specific release was calculated as shown in the Methods section See text for details.

harvested immediately:-

Supernatant Count 50µl target cell suspension + 100µl medium.

Total Releasable Label 50µl target cell suspension, heated for 30min at $60^{o}C$, + 100µl medium.

Isotope release, due to the presence of antibody, was calculated as:-

$$\% \text{ Specific Release} = \frac{C - B}{A - B} \times 100$$

Where:- A = Total Releasable Label count

B = Count from (Target cells + Test Serum + Medium)

C = Count from (Target cells + Test Serum + Complement)

Results from a typical experiment are shown in Table E2, and indicate that the end-point for this serum lies at a concentration between 10^{-2} and 10^{-3}. Serum was used in ADCC tests at a concentration tenfold below the end-point.

Method for ADCC Tests

Three slightly different assay systems were used, all of which gave comparable results.

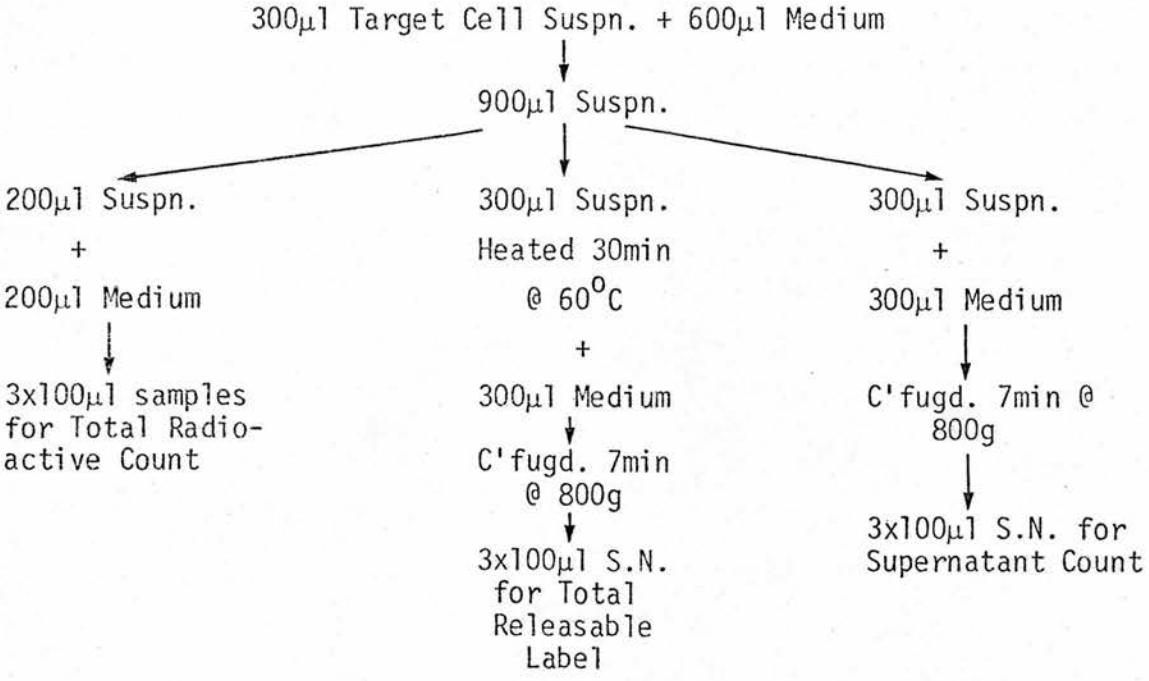
i) Target cells were labelled with ^{51}Cr (sodium chromate) as for the complement dependent lysis experiments (See p.175), and were adjusted to a concentration of $3x10^{5}$ viable cells/ml in tissue culture medium, immediately before use. The cell suspension was kept gassed throughout, to prevent fluctuations in pH.

Lymphocytes and/or polymorphonuclear cells were separated from peripheral blood using the standard techniques (See p. 66 and 67). The cells were counted and resuspended in medium at the required concentration.

Freshly-thawed normal and immune rabbit sera were diluted to triple the final required concentration in medium.

The test was carried out in 2ml flat-bottomed tubes (Sterilin) standing in a rack, in a gassed, humidified plastic lunch box. All the tubes were initially seeded with 100µl target cell suspension. Control tubes then received 100µl of the dilution of normal serum, 100µl of diluted immune serum was added to the test tubes. 100µl of the appropriate effector cell concentration was then added to both tests and controls. Blank tubes, containing target cells, serum and medium only, were set up to determine the rate of leaching of ^{51}Cr from the labelled cells. All the tubes were set up in triplicate. The lid of the lunch box was sealed with Lasso tape and the box incubated at 37°C. After 20h 300µl PBS'A' was added to each tube. The cells were pelleted by centrifugation at 800g for 7min, and 100µl of the supernatant transferred to plastic, gamma counting vials. All the samples, including the labelling controls, were counted for 5min each, using the ^{51}Cr channel on an automatic gamma counter.

Labelling controls were set up at the start of the incubation period, using the following flow chart:-



All the radioactive counts are expressed, in the results, as the mean of the triplicate. For each experiment the following values were calculated:-

i) Cell Bound Count = Total Radioactive Count - Supernatant Count

ii) % Total Releasable Label =

$$\frac{\text{Total Releasable Label Count - Supernatant Count}}{\text{Cell Bound Count}} \times 100$$

iii) % Specific Release =

$$\frac{\text{Test Count - Control Count}}{\text{Total Releasable Label Count - Control Count}} \times 100$$

where:-

Test Count = Count from Target cells + Effector cells + immune serum

Control Count = Count from Target cells + Effector cells + normal serum

ii) Target cells, effector cells and serum dilutions were prepared as for Method i. The volume of each addition was reduced to 50µl, thereby cutting the cell requirement by one half. The tests were carried out in the V-bottomed wells of a microtiter plate, which was incubated, without a lid, in a gassed, humidified lunch box. 20h later, 50µl samples of the supernatant were carefully withdrawn from each well, and transferred to a gamma counting vial. Samples were counted as for Method i.

Labelling controls were performed in triplicate.

Total Radioactive Count = 50µl Target Cell Suspension

Supernatant Count = 50µl sample of supernatant from:

50µl Target Cell Suspension, diluted 1/3 in medium and centrifuged at 800g for 7min.

Total Releasable Label Count = 50µl sample of supernatant from 50µl Target Cell Suspension, heated at 60°C for 30min, diluted 1/3 in medium, and centrifuged at 800g for 7min.

Results were calculated as for Method i.

iii) Method iii was a modification of Method ii, which allowed the supernatants to be collected using an automatic harvester. Cells were incubated in round-bottomed wells of a microtiter plate for 20h. The supernatants were sampled using a Titertek Automatic Supernatant Harvester. The absorption elements, containing the collected supernatants, were placed in gamma counting vials, and counted as for Method i.

Labelling controls were set up as described in the section on the titration of antisera (See p.176)

Results were calculated as for Methods i and ii.

Results

In the assay system used in this study, lymphocytes were found to be the primary effector cells. The low levels of cytotoxicity (usually less than 20% specific release) produced by a granulocyte population, could be largely explained by contamination of the cell suspension by small numbers of lymphoid cells. Lymphocytes from normal controls routinely produced 80%-90% cytotoxicity during 20h incubation with the target cells.

Antibody-Dependent Cellular Cytotoxicity before and after Hyperthermia Treatment The antibody-dependent cytotoxic effect of lymphoid cells, on the H.Ep.2 cell line, was studied in 5 patients (D.G., J.C., M.P., M.M. and I.W.). The results are shown in Figures E 2 and E 3.

Prior to treatment, 3 patients showed levels of cytotoxicity comparable to those in normal subjects (i.e. greater than 80% specific release). Cytotoxicity was markedly depressed (less than 25% specific release) in one patient (J.C.), and slightly lower (64%) in the remaining case (M.P.).

During the first hyperthermia treatment, 2 patients (J.C. and M.P.) showed an increase in K cell activity to 160% and 120% of the pre-treatment values, respectively. In contrast, in a third patient, cytotoxicity fell to 80% of the pre-treatment level, following hyperthermia. All of these changes represent alterations in the percent specific release values, of between 13% and 17%, which were not statistically significant. Within 24h of treatment, the specific release in one patient (M.P.), returned to within 4% of the pre-hyperthermia value.

Two patients (I.W. and M.M.) were studied during their second treatment. In both cases there was a slight fall in K cell activity

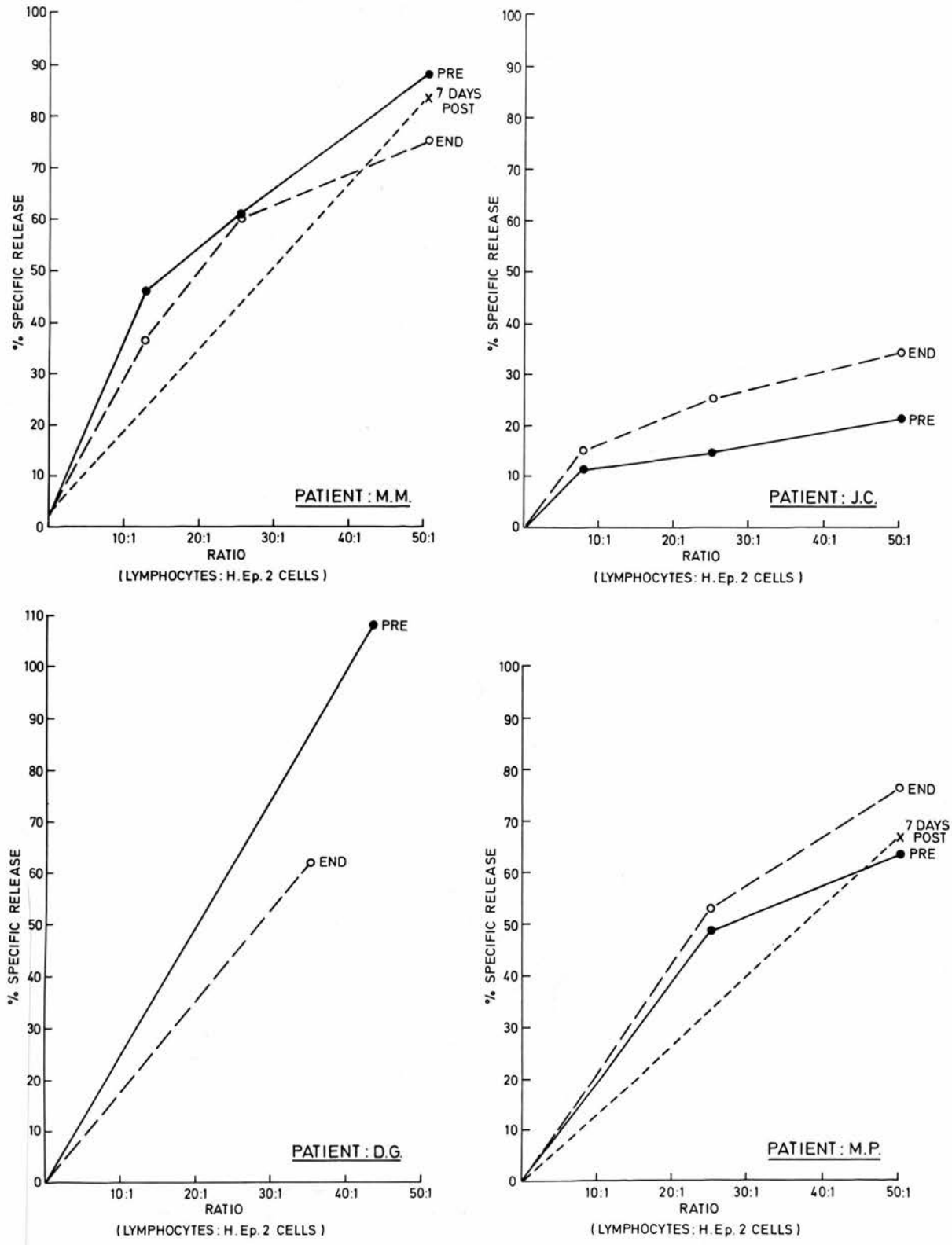


FIGURE E2

EFFECT OF SINGLE HYPERTHERMIA TREATMENTS ON THE K CELL ACTIVITY OF LYMPHOCYTES AGAINST H.Ep.2 TUMOUR CELLS IN VITRO

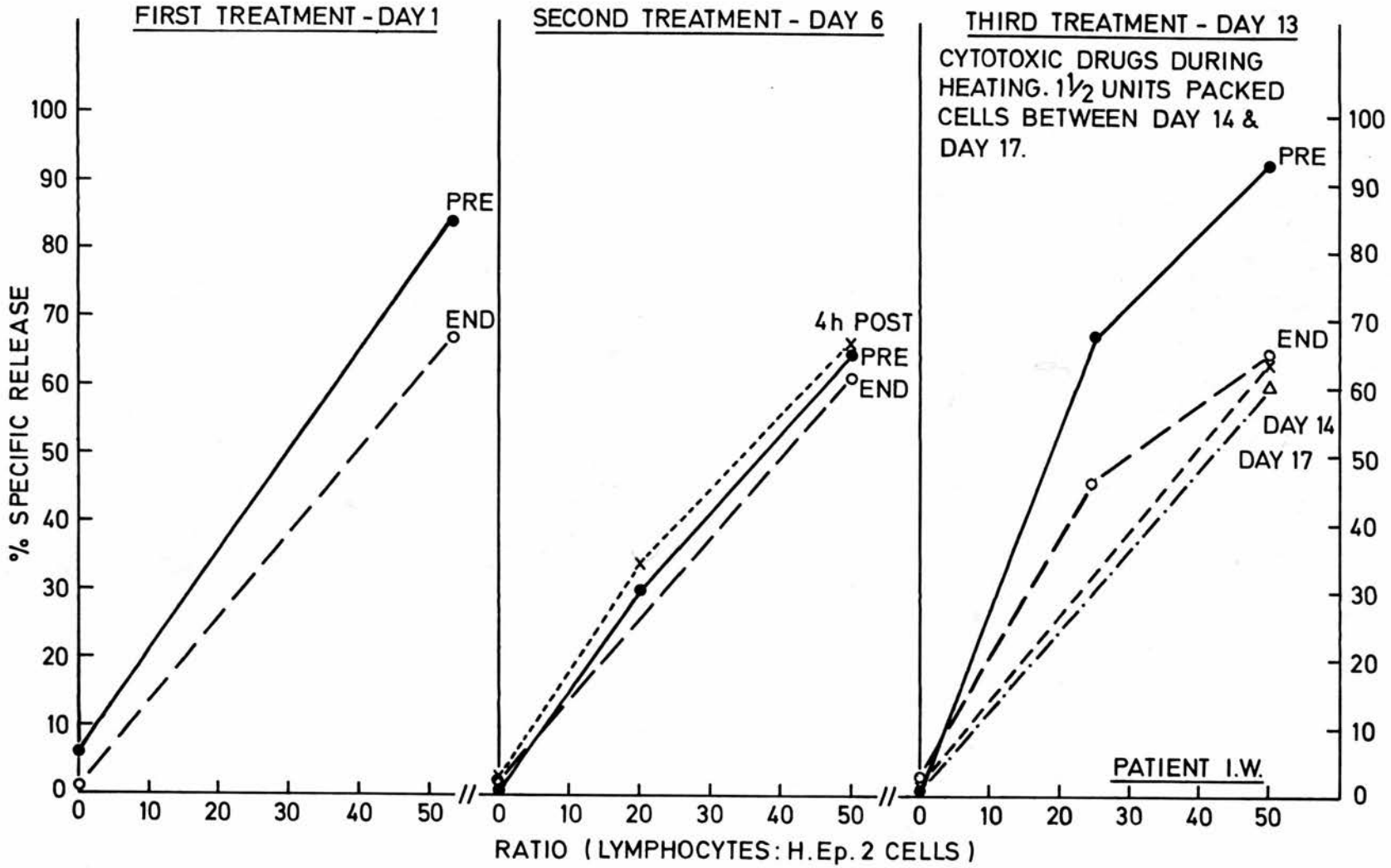


FIGURE E3

EFFECT OF SUCCESSIVE HYPERTHERMIA TREATMENTS ON THE K CELL ACTIVITY OF LYMPHOCYTES AGAINST H.Ep.2 TUMOUR CELLS

(to 95% and 86% of the pre-treatment level, respectively). Pre-hyperthermia levels of cytotoxicity were shown by lymphoid cells from blood taken 4h (Patient I.W., Figure E 3) and 7 days (Patient M.M., Figure E 2) after treatment.

The effects of a third treatment were studied in patients D.G. and I.W.. Although the results indicated that there was a marked fall in cytotoxicity during hyperthermia, the results are not definitive, since one of the patients (I.W.) was given cytotoxic drugs during heating, and it was not possible to obtain sufficient cells from the second, to allow directly comparable ratios of effector cells : target cells to be used before and after hyper-thermia.

It was possible to conduct a limited follow-up on patient I.W.. This indicated that cytotoxicity remained depressed (Figure E 3) the day after treatment, and was virtually unchanged 3 days later, in spite of a blood transfusion of 1½ units of packed cells, between the tests.

The effects of a course of hyperthermia treatments on antibody-dependent cytotoxicity, in a single patient are shown in Figure E 3. Each treatment produced a small fall in H.Ep.2 cell killing. Following the first hyperthermia fraction (158C^{O}M), cytotoxicity fell to 80% of the pre-treatment value, and remained depressed for 6 days. The second fraction (204C^{O}M) had virtually no effect on K cell activity, however, within a weeek, there was an increase in cytotoxicity (represented by an increase of over 30% in the percent specific release), to above the pre-treatment value. A third treatment (202C^{O}M), with concomitant gastric heating and chemotherapy (5 Fluorouracil and Vincristine), resulted in the largest decrease

in cytotoxicity (from 92.5% to 62.5% specific release), which was still evident the following day, and 3 days later. Although each treatment produced a fall in K cell activity of varying magnitude, and apparently unrelated to the size of the fraction, it is notable that the specific release values fell, in each case, to between 60% and 65%.

Effect of Heating Lymphoid Effector Cells in vitro on their K Cell Activity Three experiments were conducted to determine the effects of heat in vitro on ADCC by lymphoid cells. In all cases, the control effector cells were unheated.

In two experiments, heating the lymphocytes in whole blood at $42^{o}C$ for 2½h, resulted in a fall in the specific release to 59.8% and 29.1% of the value seen in unheated cells, at a 25:1 and 50:1 effector:target cell ratio, respectively.

Heating isolated lymphocytes for 2½h at $42^{o}C$ (at a concentration of $7x10^{6}$ cells/ml in Eagles Basal Medium + 20% v/v Foetal Calf Serum), resulted in a fall in cytotoxicity to 33.9% of that produced by unheated cells, at a 50:1 effector:target cell ratio. The viability of the lymphocytes was not affected by the heating procedure.

Discussion

The results from patients, suggest that ADCC is only marginally affected by hyperthermia treatment, but, on balance, tends to be depressed. A depression of cytotoxicity has been reported in surgical patients post-operatively (Vose & Moudgil, 1976), and in anaesthetised animals (Vose & Kimber, 1977), where it was attributed to the use of the anaesthetic agents, rather than to stress. This would not, however, explain the small increases in K cell activity seen in some of the hyperthermia patients. In those cases where an increase was seen (J.C.

and M.P.) it was small, and the pre-treatment level of cytotoxicity was below that usually found in normals. In the patients with normal pre-hyperthermia K cell activity, it is not clear why the cytotoxicity concistently remained above 60-70%. It may be related to the size of the heat fraction that these subjects received, although this varied from patient to patient, or might indicate the presence of thermo-resistant cells. This is not, however, supported by the results involving heating *in vitro*, which indicated that ADCC could be markedly inhibited by heating the effector cells in whole blood, or as a suspension in tissue culture medium.

These results are in agreement with those reported by DeHoratius *et al* (1977), who found a non-significant decline in lymphoid cell-mediated ADCC, and a significant fall in polymorph-mediated ADCC against erythrocyte targets, following hyperthermia therapy for 2h at 42°C. The results from the *in vitro* study were supported by those of Romano and Shore (1977), who found that heating a suspension of mononuclear cells at 46°C for 10min, abolished its ability to kill antibody-coated, herpes virus-infected cells. They indicated that this inactivation could be reversed by incubating the cells for 24h at 37°C, and that the recovery was dependent on RNA and protein synthesis (Shore *et al*, 1977). If this is indeed the case, then recovery should not occur with the heating conditions which were used in this study (2½h at 42°C), since these have been shown to abolish RNA synthesis in T lymphocytes (see p. 155), which have now been shown to be capable of K cell activity (Saal *et al* 1977a and 1977b). There was some indication of rapid recovery of ADCC, after hyperthermia treatment, in three of the patients studied, but the changes are too small to be definite.

The mechanism by which cytotoxicity was depressed by heating the

effector cells *in vitro*, has not been studied. Shore *et al* (1977) have proposed that the inhibition of ADCC by elevated temperatures, is mediated by heat-labile proteins at, or near, the effector cell surface, and that these may be enzymes or cytoskeletal structures.

In summary, hyperthermia therapy usually resulted in a slight fall in antibody-dependent cellular cytotoxicity mediated by lymphoid cells, against tumour cell targets. The depression was largest during a treatment in which cytotoxic drugs were given. Heating of the effector cells, in whole blood, or as isolated cells, *in vitro*, produced a substantial inhibition of their cytotoxic potential, greater than that seen *in vivo*.

GENERAL SUMMARY AND CONCLUSIONS

General Summary and Conclusions

A number of tests have been used to determine the effects of whole-body hyperthermia therapy, and heat *in vitro*, on immunocompetent cells.

The results from advanced cancer patients indicate that this form of treatment produced an initial stress-mediated leucocytosis, resulting from an increase in the concentration of actively-phagocytic peripheral blood neutrophils. This was accompanied by a T-lymphopenia and B-lymphocytosis, but no significant changes in the total lymphocyte concentration. There was also a tendency for antibody-dependent cellular cytotoxicity, and PHA-induced lymphocyte transformation, to be slightly depressed during heating.

Within 16-18h of treatment, the T-lymphopenia became more severe, and the concentration of serum complement (as C'3) was also depressed. Most parameters returned to their pre-treatment values within 3-5 days, at which time there was an increase in T lymphocyte activity, as measured by response to PHA; this declined to the pre-hyperthermia level, within a week of treatment. A course of hyperthermia treatments had no significant overall effect on the parameters studied.

These results indicate that cancer patients may be temporarily immunosuppressed, for periods up to 3 days, following whole-body hyperthermia treatment. This could be of clinical importance, especially during the hours immediately after therapy. It is possible that, with inadequate heating, still-viable tumour cells may be seeded into a hyperaemic circulation at this time. In the absence of a full immune response these may become established as metastases, and this may explain the increased incidence of tumour dissemination, in animal models, following generalised hyperthermia (Dickson & Muckle, 1972).

Depression of general immunocompetence has also been described in tumour-bearing rabbits treated by generalised, as opposed to local hyperthermia (Dickson & Shah, 1977). This would suggest that elevation of body temperatures depresses the activity of immunocompetent cells, and this is supported by the results from the experiments, in this study, in which lymphocytes and granulocytes were heated in vitro. During local tumour hyperthermia very few immunocompetent cells will be subjected to increased temperatures, and so there is not likely to be any marked inhibition of the patient's immune status. In addition, antigenic material may be shed from the heated tumour into the blood-stream, and elicit a renewed immune response, explaining the potentiation of immunity by local heating described by Dickson and Shah (1977) and Szmigielski et al (1977a). Although changes in antigenicity of the tumour are also likely to occur during whole-body treatment, the antigenic stimulus would be acting on a depressed immune response.

It is possible that the depression of lymphoid cell function may be partially counteracted by the increase in the concentration of actively-phagocytic neutrophils which occurs towards the end of treatment, and persists the following day. Although these cells were found to be inactive in the ADCC assay system that was used in this study, they may be capable of tumour cell killing in vivo.

It is interesting to note that the pattern of general immunological responses following hyperthermia treatment, is similar to that reported by Minton et al (1976) after the intravenous administration of Corynebacterium parvum, which is believed to stimulate general, and thereby tumour-directed, immunity. This would suggest either that

i) C. parvum does not potentiate immunity

ii) The potentiation by C. parvum is not detected by these assays

iii) The changes are associated with the fever produced by C. parvum.

It is felt that this latter explanation is the most likely, since Minton indicated that most of the patients experienced fevers, ranging from 37.8°C - 40.0°C, following the administration of C. parvum.

The experiments in which heat was applied in vitro, indicate that elevated temperatures markedy depress the indices of general immunocompetence, that were monitored in vivo. This inhibition became more severe as the conditions progressively departed from those in vivo. It is possible, therefore, that this increase in thermosensitivity is due to changes in the factors affecting the heat tolerance of cells e.g. pH, oxygen tension etc.. A purely heat-mediated depression of immunological function is probably also involved, and has been reported by other workers (Shore et al, 1977).

This study is limited by two factors. Firstly, as with all in vitro assays, there is always uncertainty as to how closely the results correlate with measurements of tumour-directed immunity in vivo, and with clinical prognosis. No correlation has been found in this small group of patients, in which there were few clear-cut responses to therapy. The finding that the changes in the parameters have been common to most patients, indicates that they may be inherent in this form of treatment.

Secondly, it is not known how the immunogenicity of a tumour is affected by hyperthermia treatment. Elevated temperatures have been shown to affect cell membranes etc., and it is likely that the antigenicity of the cancer cell (and perhaps that of normal cells) may change during a course of heat treatments (Mondovi et al, 1972). Antigenic, necrotic tumour tissue may also be released into the bloodstream during hyperthermia. The tests used in this study, do not

take account of this, e.g. a fall in the T lymphocyte concentration, following treatment, may be associated with the sequestration of sensitised T cells by a more-immunogenic tumour, rather than with direct depression of the cells by heat.

The effect of hyperthermia treatment on the immune status of the cancer patient is therefore the resultant of the effect on the immunocompetent cells, and on the immunogenicity of the tumour. The indications, from previous work, are that the latter is increased by elevated temperatures, whilst immune function tends to be depressed, as shown by this study. Although the change in tumour antigenicity may eventually become masked by serum factors etc., it is possible that it persists longer than the general immunodepression (i.e. 2-3 days) which could mean that the overall effect of whole-body hyperthermia treatment, may, in fact, be benificial, as regards the tumour-directed immune response.

In any clinical study it is difficult to evaluate the influence of factors ancillary to the treatment under investigation (e.g. drugs, anaesthetics etc.), on the immune status of the patient. Since these procedures are, at present, inherent in most clinical hyperthermia treatments, it will be necessary to develop a good animal model in order to isolate the effects of heat on the immunocompetence of the tumour-bearing host. This has presented many problems, since the temperatures used in clinical treatments are poorly tolerated by laboratory animals, because of their different thermoregulatory mechanisms. In spite of this, animal models have been described, and used, in preliminary studies on immunocompetence (Williams & Galt, 1977).

Future work should be directed towards monitoring tumour-directed immunity in these systems, since they permit the use of

procedures which are not ethical in the clinical situation, e.g. tumour transplantation. The use of syngeneic systems also allows more rigid control experiments to be performed, which should aid in distinguishing between the effects of heat and anaesthetics, drugs etc.. Results from these investigations could then be applied to the study of tumour-directed immunity in patients undergoing whole-body hyperthermia treatment for a specific type of tumour.

APPENDIX

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DECLARATION

I declare that this thesis has been composed by myself, and that, unless stated otherwise, I have carried out all the work described herein.

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
B cell	Bursa (or equivalent) - derived lymphocyte
BCG	Bacillus Calmette Guerin
BSA	Bovine serum albumin
BUdR	Bromodeoxyuridine
^{o}C	Degree Centigrade
Ci	Curie
$C^{o}M$	Centigrade degree minute
cpm	Radioactive counts per minute
DNA	Deoxyribonucleic acid
DNCB	2,4-Dinitrochlorobenzene
E	Sheep erythrocyte
EA	Antibody-coated sheep erythrocyte
EAC	Antibody and complement-coated sheep erythrocyte
ECG	Electrocardiogram
EM	Rhesus monkey erythrocyte
F10B	Bicarbonate-buffered Ham's F10 medium
F10H	HEPES-buffered Ham's F10 medium
Fc	Crystallisable fraction of immunoglobulin
g	Gramme
g.	Gravitational constant
h	Hour
HEPES	(N-2-Hydroxyethylpiperazine-N'-ethanesulphonic acid)
HL-A	Human leucocyte antigen
Ig	Immunoglobulin
l	Litre
M	Molar

m	Milli (10^{-3})
mm	Millimetre
MIF	Macrophage migration inhibition factor
min	Minute
MLC	Mixed leucocyte culture
PBS'A'	Dulbecco 'A' Phosphate-buffered saline
PHA	Phytohaemagglutinin
P.P.D.	Purified protein derivative of Tuberculin
Rh	Rhesus blood grouping
RNA	Ribonucleic acid
rpm	Revolutions per minute
S	Svedberg sedimentation unit
sec	Second
SEM	Scanning electron microscopy
S-Ig	Surface-bound immunoglobulin
SRBC	Sheep red blood cell
T cell	Thymus-derived lymphocyte
TAA	Tumour-associated antigen
TSTA	Tumour-specific transplantation antigen
μ	Micro (10^{-6})
v/v	Volume per volume
w/v	Weight per volume

PRELIMINARY STUDIES ON THE IMMUNOCOMPETENCE OF PATIENTS UNDERGOING WHOLE BODY HYPERTHERMIA THERAPY FOR ADVANCED MALIGNANCY.

A. P. GEE, R. T. PETTIGREW, A. N. SMITH and A. E. WILLIAMS, *Teaching and Research Centre and Departments of Anaesthetics and Clinical Surgery, Western General Hospital, Edinburgh.*

The general immune status of 8 patients with advanced gastrointestinal or genito-urinary cancer has been monitored prior to, during and following whole body hyperthermia. During treatment the absolute number of T cells in peripheral blood increased in patients with responsive tumours (5) and decreased significantly in the remainder (3). In both groups T cell activity, as measured by response to PHA, decreased at the end of heating but increased the following day, reaching a peak within a further 3 days and returning to the pre-treament level within a week. Changes also occurred in numbers of circulating B cells and in serum immunoglobulin and complement levels, but these did not show any clear trend. Although work is still at an early stage, these results indicate that hyperthermia therapy may induce a small but favourable change in the responsive patient's immune status at a critical time.

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PRELIMINARY STUDIES ON THE EFFECT OF WHOLE-BODY HYPERTHERMIA ON THE IMMUNE RESPONSE OF CANCER PATIENTS AND EXPERIMENTAL ANIMALS

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The effect of whole-body hyperthermia (41–42 deg.) on the general immunocompetence of patients with advanced malignancy and of normal rats is being studied. In both humans and rats, hyperthermia causes a lymphocytopenia followed by a granulocytosis with a return to control values within four days. However, patients with tumours responding to hyperthermia show an increase in absolute numbers of circulating T cells. The lymphocyte response to PHA is depressed after hyperthermia in both rats and humans. At three days, the response of human lymphocytes is above pre-treatment levels, but is still partially depressed in rat lymphocytes. In anaesthetized control rats, a less severe and shorter lasting lymphocytopenia is produced. PHA responsiveness is not depressed. During the granulocytosis in patients there is an increase in the number of actively phagocytosing cells. Antibody-dependent cytotoxicity of both lymphocytes and granulocytes is severely depressed by heating *in vivo* or *in vitro*.

The results of this pilot study suggest that the possible effects of hyperthermia on the immune response should be considered when assessing the clinical application of this mode of therapy.

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The Effects of Whole-Body Hyperthermia Therapy on the General Immunocompetence of the Advanced Cancer Patient

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Introduction

The immune system can modulate the course of cancer in animals and man (1,2). Many workers have suggested that the anti-tumour effects of hyperthermia are mediated by an immune response (3,4,5). Heat may stimulate cells of the lymphoreticular system and/or change the immunogenicity of the tumour, however little experimental evidence exists.

The aim of our work was to determine whether whole-body hyperthermia used by Pettigrew (6) in terminal cancer, affected the patient's immunocompetence. Several *in vitro* assays were used to monitor the general immune status before, during and after treatment. 19 patients with various advanced cancers (mainly gastro-intestinal) have so far been studied. In addition, the effect of heating *in vitro* on the function of isolated lymphocytes has been studied.

Whole-Body Hyperthermia

The duration of hyperthermia treatments varied slightly depending on the fraction given and the patient, but one session usually involved heating at 41.8°C for 2-4hr.

Total and Differential Peripheral Blood Leucocyte Counts During heating there was an initial leucopenia, followed by a substantial leucocytosis, resulting in a mean increase in the white cell count of 24% during the first treatment and over 60% during subsequent treatments. This was due primarily to an increase in the numbers of immature neutrophils in the peripheral blood. These cells could phagocytose latex particles; hence the concentration of actively-phagocytic cells may more than double following heating, resulting in an increased capacity to remove tumour debris that may result from hyperthermia treatment

The concentration of lymphoid cells decreased by *ca.* 25% during the first treatment and remained depressed during the following 24hr. Within 3-4 days the concentration had returned to the pre-treatment value. In the smaller group of patients receiving a second treatment, the mean mononuclear cell count increased slightly during heating, but then decreased to *ca.* 85% of the pre-heating value at 24hr., and then remained low at 3-4 days after treatment. This longer term depression occurred after a third treatment.

The changes during heating are characteristic of a stress response and have been noted in induced fever therapies (7,8). Over a full course of hyperthermia sessions the total and differential leucocyte count was not significantly altered.

Concentration of Peripheral Blood T and B Lymphocytes The concentration of T and B lymphocytes was determined by E (9) and EAC (10) rosetting techniques on isolated lymphocytes prepared by Ficoll-Hypaque centrifugation (11).

In the majority of patients, during heating, there was a significant fall in the T cell concentration to ca. 70% of the pre-treatment level, followed by a slow recovery over the following 3-4 days This pattern was repeated during the second and third treatments. In three patients the pattern was completely reversed. T cell concentrations increased during heating and subsequently declined sharply and remained depressed 3-4 days later. These patients displayed other abnormal clinical and immunological responses to hyperthermia.

B lymphocytes changed rather erratically in response to treatment. The percentage of EAC-rosetting cells tended to increase by ca. 50% in most patients, however no clear trend could be seen.

Serum Immunoglobulin and Complement Levels Concentration of serum immunoglobulins (IgG, IgA and IgM) and complement (as C'3) were measured by radial immunodiffusion (12). All three subclasses showed large variations during and after hyperthermia (Range 48-193% of the pre-heating value), but no general patterns were apparent. A small, but significant, decrease in the concentration of C'3 occurred the day after treatment; however, normal values had been regained by 3-4 days post-treatment.

Response of Lymphocytes to Phytohaemagglutinin (PHA) Phytohaemagglutinin acts mainly upon T lymphocytes (13) and hence the blastogenic response to PHA may be used to assess T cell activity. A modification of Steel and Creasey's method was used (14).

In virtually all the patients there was a slight decrease in the PHA response during heating. Within 24hr. the values had returned to, or exceeded, the pre-treatment level, and subsequently increased to a marked peak within a further 2-3 days. (2-3 fold the pre-heating level). This was followed by a decline to the pre-hyperthermia value within a week of treatment. This pattern of response followed each heating session (Fig.1), and occurred whether autochthonous or pooled normal serum was used in the cultures. Lymphocyte responses were lower if the patient's own serum was used, indicating a possible blocking factor(s). The extent of blocking was not significantly altered by hyperthermia treatment. The clinical significance of this pattern of PHA responsiveness is not clear but could not be related to plasma cortisol levels, which affect lymphocyte activity (15). The peak in response may however be partially due to release from the suppressive efffects of the anaesthesia and/or drugs used during hyperthermia treatment.

Antibody-Dependent Cellular Cytotoxicity The cytotoxic effect of peripheral blood lymphocytes on a ^{51}Chromium-labelled human tumour cell line (H.Ep.2), in the presence of anti-H.Ep.2 antiserum, was measured at the beginning and end of hyperthermia.

During heating there was usually a slight decrease in cytotoxicity (to about 80-95% of the pre-heating level) followed by recovery before the next treatment. In one case where cytotoxic drugs were given during heating, the suppression was more marked (30% decrease in cytotoxicity).

Effects of Heating in vitro

Concentration of 'Total' and 'Active' Lymphocytes in a Suspension

T cells in a suspension of lymphocytes heated at 42°C for up to 3hr. were monitored using three methods:-

i) Rosetting with sheep erythrocytes for 18hr. at 4°C - total T cells (9)
ii) Rosetting with sheep erythrocytes for 5min. at 20°C - 'active' T cells (16)
iii) Rosetting with rhesus monkey erythrocytes for 18hr. at 20°C EM rosettes (17)

'Active' T cells were studied because their concentration in the peripheral blood has been reported to correlate more closely with the clinical status and prognosis of the cancer patient (18). EM rosettes are of interest because the incubation temperature used during rosette formation is less critical than with sheep erythrocyte rosetting tests (17).

During heating the number of total and active T cells decreased but a control suspension kept at 37°C was unaffected. Although the trend was a decrease in T cells, the proportion of rosetting cells varied cyclically during incubation at 42°C. In addition, if the cells that had been heated at 42°C were subsequently incubated at 37°C or 4°C for 15min., the number of rosetting cells increased. These findings suggest that, although erythrocyte receptors on T cells are destroyed by heating at 42°C, they can be resynthesised.

In contrast to the results with sheep erythrocytes, the proportion of lymphocytes rosetting with monkey erythrocytes (although smaller 20-40%) was little affected by heating at 42°C. In parallel experiments the percentage of total T cells in the suspension decreased during heating, until it reached a level similar to the percentage rosetting with monkey erythrocytes. The total and EM-rosetting populations then cycled synchronously (Fig.2). These results indicate that there may be two types of erythrocyte receptor on T cells:-

i) Thermolabile - detected by 'active' T cell rosetting but also as a subpopulation during total T cell rosetting.
ii) Thermoresistant - detected by EM rosetting but again present as a subpopulation during total T cell rosetting.

As it is believed that 'active' T cell numbers correlate more closely with clinical parameters, the possible thermosensitivity of this cell type could be of concern to the clinician using hyperthermia treatment.

Lymphocyte Response to PHA Isolated lymphocytes heated at 42°C for 2½-4hr. failed to respond to PHA, although initially viable by dye exclusion. PHA-stimulated RNA synthesis was also virtually abolished by pre-heating the cell suspension. Lymphocytes heated in whole blood were less susceptible. Preliminary results from whole-blood cultures showed no significant difference between the PHA response of blood incubated at 42°C and that kept at 37°C, for periods up to 2½hr..

Antibody-Dependent Cellular Cytotoxicity Heating of the lymphoid effector cell population, either in whole blood, or as an isolated, suspension, for 2-2½hr., resulted in a substantial reduction in its cytotoxic potential to about 40% of the pre-heating value. This decrease was much greater than that seen *in vivo*.

Conclusion

Whole-body hyperthermia treatment appears to cause a slight, short-lived, general immunosuppression, particularly of cellular immune responses. Most parameters return to their pre-treatment levels within 3-4 days, although T lymphocyte activity reaches a marked peak at this time. Heat applied *in vitro* produces a more severe depression of immune function.

These results contrast with similar studies on chemotherapy and radiotherapy (19,20), which produce marked, long-term immunosuppression. However, the temporary depression of immunity resulting from hyperthermia treatment may be of critical importance. It is possible that, with inadequate heating, viable tumour cells may be seeded into a hyperemic circulation, and, in the absence of a normal immune response, become established as metastases (21).

This initial study has concentrated on indices of general immunocompetence, and may not necessarily relate directly to tumour-directed immunity. Also possible changes in the immunogenicity of the tumour, resulting from hyperthermia treatment, have not been investigated. These parameters, which are of primary interest to the tumour immunologist, are currently under study using an animal model (22).

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Addendum

Matthews, N. and Whitehead, R.H. (1976) Inhibition of K cell by human breast cancer sera. British Journal of Cancer 34, 635.