EXPERIMENTAL STUDIES OF THE EFFECT OF CANCER CHEMOTHERAPY ON CELLULAR IMMUNITY AND ITS MODIFICATION

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SYNOPSIS

The experimental evidence for an immune response to cancer has been reviewed,and the effects of cytotoxic chemotherapy shown to affect nearly all of its aspects. Augmentation of the immune response (immunotherapy) has been discussed in the context of several important agents. It is argued that in advanced cancers the tumour burden is too great for significant benefit from immunotherapy. However in patients with 'minimal residual disease' there is little evidence for impairment of immune responses. When such patients receive adjuvant chemotherapy the number of residual cancer cells is further reduced, but the patient's immunological ability to complete their elimination is seriuosly impaired by the treatment. This situation may be a most suitable opportunity to achieve benefit from immunotherapy,and this study concerns attempts to identify means of achieving this.

The work is concentrated on one major arm of the immune response, both in normal rats and some in whom breast cancers were induced. T lymphocyte function was measured in rats by an in vivo (DTH response) and an in vitro (PHA blastogenesis) method. Clear depression was seen following one injection of cyclophosphamide or 5 fluorouracil (5FU) in both normal and tumour bearing animals, and this lasted for at least one month (PHA). The rebound overshoot phenomenon was observed in both groups following 5FU but not cyclophosphamide.

Levamisole did not improve the depression of in vitro T cell function produced by cyclophosphamide,but alleviated that following 5FU if administerd after a delay of 3 days. This effect was somewhat marginal but seen consistently in both normal and tumour bearing animals. The combination of glucan with either cytotoxic agent significantly worsened in vitro T cell function, even if the timing of each drug was varied. This observation is interpreted as a directly depressive effect of glucan on T cell function, revealed only in conjunction with cytotoxic therapy. A similar effect was also seen following C parvum ,but not thiabendazole.

The use of a small priming dose of either chemotherapeutic agent did not alleviate the immunosuppressive effect of a subsequently administered large dose. Wide variation of the priming delay for cyclophosphamide did not alter this conclusion. Similarly no benefit was gained either from the regular administration of cimetidine,or the timing of cytotoxic injections to opposing extremes of diurnal rhythms. The difficulties encountered in this field of research and questions for future study are discussed.

INTRODUCTION

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INTRODUCTION

Human suffering from cancer is not new.Nor are the efforts of doctors to study and treat its victims. With few exeptions,surgical treatment still offers the best chance of cure. In the Egyptian middle period (ca 1600 BC) medical texts were written describing cancerous growths and operations for a few of them. As early as 1600 AD Guilhelmus Fabricius Hildanus published a detailed description of the technique of total mastectomy including axillary dissection for nodes (Hill 1979). Inevitably the main development of operation for most tumours came with the advent of general anaesthesia (first major operation 1846). There has been little fundamental change in the surgical approach to cancer since the turn of the century. Subsequent clinical research has largely directed attention towards other methods of treatment ,in an attempt to augment the limited success of surgery.

Radiotherapy was the first hope. Although Xrays and radioactivity were only discovered between 1895 and 1898,the first report of their use in basal cell carcinoma was only one year later. Initial results with skin tumours stimulated an enthusiastic and crude application to a wide variety of cancers. Poor results in most of these nearly led to the method being abandoned altogether. From 1919 onwards,imaginative research by Regaud in Paris led to fractionation of doses and consequently safer and more impressive clinical results (Kaplan 1979).

Since then a steady improvement in equipment and application has led to an enormous role in modern treatment - curative as well as palliative. As an adjunct to surgery it has established a small but significant role.

In the early 1950's the efficacy of antibiotics in infectious diseases led to extravagant hopes for similar results with drugs and cancer. Although the use of arsenic in chronic myeloid leukaemia had been described in 1865,a more symbolic beginning was made in the late 1930's with several rather weakly effective agents- colchicine,urethane and benzene (Zubrod 1979). It is interesting that nearly all the first wave of more powerful drugs were devloped as by-products of other fields of research. They were recognised as potentially useful anticancer agents primarily through the availability of transplantable animal tumour models. For example nitrogen mustard was developed for chemical warfare and was the first powerful agent put to clinical use. Its immediate derivatives were busulphan chlorambucil and cyclophosphamide. These drugs were developed on theoretical grounds of tumour metabolism which were later found to be completely inappropriate. However 5 Fluorouracil was one of two drugs (the other 6-mercaptopurine) developed inductive reasoning - after a difference was noted in the metabolism of uracil by rat hepatoma compared to normal liver. Its clinical activity was confirmed one year later in 1958.Since then the sophistication of treatment regimes and their application have increased in parral-

lel.

Cure of rapidly growing tumours such as some lymphomas and leukaemias has now become quite possible with chemotherapy alone. Results with solid tumours have not been so encouraging even when used as an adjunct to surgery.Nephroblastoma offers an uncommonly good example that can be achieved from the combination of methods aimed at tumour eradication.

The first nephrectomy was performed by Kocher in 1878 and the first reported surgical cure was by Israel in 1894. By 1920 nephrectomy was widely used to treat this condition and postoperative radiotherapy was added around 1940 (Wolff 1975). In 1955 the use of a course of actinomycin D was first described,and Subsequently the National Wilms Tumour Study Group and other multi-centred organisations have initiated a number of trials to refine the indication and use of further agents and combinations. This has led to the improvements in prognosis illustrated overleaf:

Before	1920	Ø	survived 3 yrs	(likely cured)
After	1920	20%	"	(1)
	1945-	18%	"	(2)
	1950-	19%	"	
	1955-	48%	"	
	1960-	55%	"	
	1965-	67%	"	
	1970-	80%	"	
	1975-	86%	"	(3)

(1)=BMJ editorial 1976 (2)=Li et al 1975 (3)=adapted from d'Angio et al 1981

CANCER AND THE IMMUNE SYSTEM

The concept of immunity to cancer was first described by Clowes and Baestack in 1905. Using mouse tumours they found resistance to the growth of transplanted cancers in animals that had previously recovered spontaneously from a similar tumour. Unfortunately these experiments were poorly controlled, and designed before the significance of transplantation antigens was fully appreciated. However many subsequent animal studies have supported this concept,of which the experiments of Klein et al (1960) are a classic example. Sarcomas were induced in mice by methylcholanthrene and transplanted between inbred (isologous) animals. Resistance to tumour growth was seen in animals immunised by prior exposure to irradiated tumour,and in autochthonous hosts when reexposed to their original tumour. Resistance was shown as rejection of tumour innocula in normal animals ,or slowing of growth when it did occur. Transplanted tumour could be established in resistant animals,but this required 10 to 100 times the number of cells needed to establish growth in normal animals.

Sir Macfarlane Burnet has postulated that a fundamental purpose of the host's immune responses is to eliminate developing cell lines that are abnormal and potentially malignant. An animal's susceptibility to tumour growth and the rate of growth is increased by neonatal thymectomy,irradiation or anti-lymphocytic serum. In

fact many carcinogens are themselves immunosuppressive (Hersh et al 1972). Conversely,experimental stimulation of immunity by various methods has been associated with slowing of tumour growth or cure (Bansall et al 1978). There is a strikingly high rate of malignancy in patients with congenital immune deficiencies (Periman et al 1980),those intensively treated with radiation or chemotherapy (Rossner et al 1979) and long term transplantation survivors taking immunosuppressive drugs (Starzl 1970). Furthermore there are a few reported examples of cancers acquired from renal homograft recipients which have metastasised but regressed with removal of the primary and cessation of immunosuppressive treatment (Wilson and Penn 1975)

It has been widely assumed that the immune system also plays a role in the control and even cure of many cancers that do develop. It is now realised that such an action may take place not only by specific responses to tumour antigens but also non-specific cell killing. Tumour specific responses are directed towards tumour associated antigens (TAAs) which are abnormal glycoproteins and glycolipids usually recognised at the cell surface. A subset of these (tumour associated transplantation antigens - TATAs) are responsible for reactions to tumour cells when transplanted to other experimental isologous hosts (Woodruff 1979).

T lymphocytes are the most well established cell in-

volved in cell mediated immunity to cancer. Such tumour specific immunity has been demonstrated in certain cancer patients by delayed-type hypersensitivity (DTH) responses to the injection of tumour extracts. Roberts and Bathgate (1975) tested patients with breast cancer following mastectomy with a subcutaneously administered extract of their tumours,and found positive responses in 35% of cases. In vitro exposure to tumour antigens has been more widely investigated. This may cause the proliferation of lymphocytes which can be measured by the incorporation of radio-labelled substances. This effect can be difficult to demonstrate but is seen in a proportion of patients (Harris and Sinkowitz 1977).

Responses may be detected in a greater proportion of patients using tests for lymphokines secreted following exposure to tumour antigens. The adherence of leucocytes to glass decreases in the presence of antigens, and the degree of this phenomenon may be measured using the leucocyte adherence inhibition test (Halliday 1979). Several groups have reported that this response can be measured in at least 65% of patients with breast cancer following mastectomy, and appears to be specific to their individual tumour extracts (Grossner and Thomson 1975,Fujisawa et al 1976). Lymphokines inhibitory to the random migration of leukocytes in vitro have also demonstrated such tumour specific responses in the majority of patients with breast or colonic carcinoma (Jones 1976,House and Watt 1979). Direct lymphocyte cytotoxici-

ty of tumour cells is a difficult measurement but has been demonstrated in some cases (Herberman 1978).

The involvement of B cells in the host response to tumours has also been established - tumour specific antibodies and circulating immune complexes have been demonstrated by complement fixation or immunodiffusion (Harris and Sinkovics 1977,Good 1979). Specific antibody dependant cellular cytotoxicity (ADCC) of tumour cells has been measured in several groups of cancer patients. This test is not fully understood but probably also involves killer lymphocytes and perhaps macrophages in cell killing requiring specific antibody (Herberman 1978). Plain et al (1982) tested the serum of 125 breast cancer patients against 9 cultured breast cancer cell lines,and found activity to each in up to 79% of cases,a higher proportion than amongst normal controls or patients with other cancers.

Non-specific tumour killing is also thought to occurr and ascribed mainly to natural killer (NK) cells and macrophages. NK cells are present in both normal and cancer patients and may be shown to lyse tumour cells in vitro. A number of tumour cell lines particularly susceptible to this action have now been developed for assays of this function. Macrophages have a more complex role in tumour immunology. Fixed macrophages (the reticuloendothelial system) probably play an important part in non-specific killing and clearance of debris but this

is relatively difficult to investigate (Antikatzides and Saba 1977). Free macrophages (and monocytes) may be shown to have in vitro tumouricidal properties analagous to NK cells (Nelson 1981). Furthermore this can be increased by antigen specific reactions (which possibly involve lymphokines),and is termed 'Arming' (Evans and Alexander 1972). Receptors to immune complexes have been identified on the macrophage cell surface which may also lead to tumour specific activity (Rhodes 1975). In addition to these effector roles macrophages probably play an important early role by ingesting antigen and presenting it as the initial stimulus to lymphocytes capable of specific immunological responses (Carr 1978,Cline 1978).

Clearly these various effector aspects of the immune response to cancer must be considered to act as a whole,and cannot easily be stratified in importance. However at present the non-specific killing of monocytes and NK cells has only been convincingly demonstrated in vitro. Although subsequent study may show its relevance in vivo, this remains to be fully established. Tumour specific reponses imply a more clearly purposeful role and this has been established in animal studies. Of these the role of T cells has probably been more firmly established than that of B cells in both transplantation and tumour immunology,and has therefore been selected as the most important area for this study. Since the measurement of tumour specific responses is somewhat unpredictable and not always appropriate,many workers have used

more generally applicable methods which produce measurable results in all cases. Cell division in response to substances which are universally recognised to be strong stimuli (mitogens) have become one of the most established and precise in vitro methods (Ling and Kay 1975). The delayed-type hypersensitivity response following the application of certain substances to the skin is one of the earliest established in vivo tests ascribed to T cell function (Turk 1980). Therefore these two tests were chosen for this study.

It seems a paradox that patients die from cancers which they may be capable of immunologically rejecting. This is vividly illustrated by the experimental phenomenon of concomitant immunity. An animal may effectively resist challenge with tumour cells at a site distant from that where tumour continues to grow apparently unrestrained. A number of phenomena have been suggested to explain this problem.

First,it has become clear that there is a quantitative limit to the tumoricidal ability of the immune system. Even when fully effective it cannot contain a large or widespread cancer that is rapidly growing. In most animal experiments,a tumour innoculum of over 1-10 million cells is too large to be rejected by an immunised host (Hersh 1972). This corresponds to a solid tumour less than 1 cubic mm in size.

Second, the immune response may be activated but less effective than usual. Non-specific depression of immune responses is seen in cancer and several other disease states. This problem was first studied by skin testing of the DTH response - despite the incompleteness of this as a method of assessment (Johnson et al 1971,Roth et al 1975). Other immunological approaches have suggested that this phenomenon may also be seen in lymphocyte (Harris and Sinkovicz 1977) monocyte (Boetcher and Leonard 1974) and NK function (Steinhauer 1982). Although it may be seen in early cancer it is more commonly a feature of advanced disease (Cochran et al 1976),and largely caused by substances secreted by the tumour (Nelson 1980).

Specific depression of the immune response to the tumour itself is a seperate phenomenon, produced by blocking factors. These are tumour antigens,antibodies or complexes of both,any of which may interfere with the effectiveness of the cellular attack on target tumour cells. The incidence of these factors varies with different cancers and the type of test used to detect them,but they are common (Harris and Sinkovics 1977,Currie 1977). They probably play an important role in the early stages of tumour growth or recurrence,and they may cause local effects before becoming systemically active. It is becoming increasingly clear that many arms of the immune response are subject to biofeedback involving suppressor and perhaps helper cells. These former

are regulatory cells of various types (lymphocytes and macrophages) which may act locally or more probably from the thymus spleen or blood stream. Their immunological effects can be non-specific but are more probably antigen specific in most situations (Taussig 1980,Ting and Rodriguez 1980). It has been suggested that blocking factors may be shed by a tumour to reach such cells and stimulate them to suppress immune responses to it (Zimbala et al 1977). Alternatively it is theoretically possible that blocking of the immune response occurrs by saturation of immune cell surface receptors with free tumour antigens, preventing recognition of growing tumour (Kilpatrick and Fahey 1982).

Thirdly,some tumours may be more immunogenic than others. Considerable differences may be demonstrated even amongst closely related animal tumours (Evans 1978). In man however such differences are more difficult to prove. On occasions a tumour may develop 'immunoresistance'.This is a reduction or minor change of surface antigen expression which lowers the quantitative immunogenicity of subsequently produced cells (Castro 1977). There is also a theoretical possibility that whilst potentially immunogenic,a small tumour may be isolated in some anatomical way from the normal immune response.

In summary it is easier to show a valuable role for host immunity to cancer in animals than man. A variety

of methods have been discussed which demonstrate the immune response to cancer in man,but a number of factors have also been identified which may contribute to its failure.

DRUGS EFFECTIVE AGAINST CANCER

The last 30 years have seen a geometrical growth in the number of anticancer drugs undergoing clinical trial. Table 1 summarises the main classes of agent and the more commonly used of each. Discussion will be restricted to the two drugs of main relevance to this study.

Cyclophosphamide is effective against a wide variety of diseases,and probably the most extensively used anti-cancer agent today.It is inactive until converted by a liver microsomal oxidase system to aldophosphoramide,which attaches weakly to blood proteins (Brock and Hohorst 1967). This has some activity,but inside the tumour cell is metabolised to release acrolein and nor-nitrogen mustard,which are probably primarily responsible for the biological effects. In the liver,aldehyde oxidases convert aldophosphoramide to inactive metabolites excreted by the kidneys (Hill 1975). Liver conversion begins minutes after IV or IP injection,and the plasma half-life is a few hours.

Alkylating agents

- -bis(chlorethyl)amines-cyclophosphamide
  chlorambucil,melphalan
- -ethyleneimine derivatives-thioTEPA
- -alkyl sulphonates-busulfan
- -triazine derivatives-dacarbazine
- -nitroseureas-BCNU,CCNU
- -miscellaneous alkylator like-cisplatinum

Antimetabolites

- -folate antagonists-methotrexate
  Baker's antifol
- -purine antagonists-6mercaptopurine
  azathiaprine
- -pyrimidine antagonists-5fluorouracil
  cytosine arabinoside

Antibiotics

- -athracyclines-adriamycin
- -others-bleomycin,actinomycinD,mithramycin

Plant Alkaloids

- -vincristine,vinblastine

Hormones including adrenal steroids

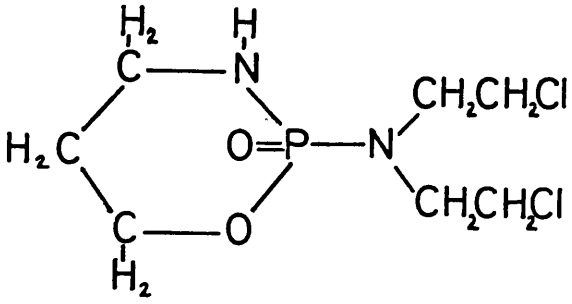
Others-procarbazine,L-asparaginase

Table 1. Classification of main anticancer drugs
(Dorr and Fritz 198Ø)

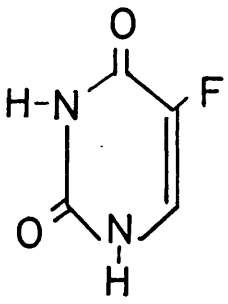
Alkylation occurrs when the highly electrophilic carbonium ion forms a covalent bond with cellular constituents.The most important target bound is the number 7 nitrogen atom in guanine. This causes destruction of the imidazole ring of guanine and miscoding in bonding with thymidine. This leads to abnormal crosslinking of DNA strands,and even destruction of some.There are also less important effects on mitochondrial RNA and other cellular systems

To a large extent these destructive effects occurr independantly of cell division,ie. they are phase non-specific. Therefore both resting and dividing cells are attacked.This is an advantage in the treatment of most human and particularly solid tumours,where there is a high proportion of cells in the reasting (GØ) phase. Because of the broad target action of alkylating agents,the cell-kill produced is related primarily to the total dosage employed. There is little or no advantage from divided or scheduled dosage systems.This is clearly shown with mouse L1210 leukaemia where maximal extension of lifespan after inocculation is produced by a single high dose of cyclophosphamide,rather than any divided regime even of greater total dose (in Hill 1975). As with all alkylating agents it is advantageous to use the maximum dose possible within the limit of toxicity to normal tissues.These are mainly haematopoietic, gastrointestinal and gonadal. Cyclophoshpamide is especially noted for producing marked depression of the immune response, and

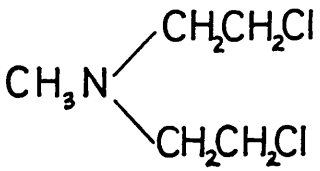
much early work was devoted to taking advantage of this in transplantation.



CYCLOPHOSPHAMIDE



5-FLUOROURACIL



NITROGEN MUSTARD

5-Fluorouracil

For more than 20 years 5-fluorouracil (5FU) has maintained its position as one of the most important cancer chemotherapeutic drugs available. It is inactive until converted intracellularly to its active form 5-fluorodeoxyuridilate.This competes irreversibly for the enzyme thymidylate synthetase,and blocks the synthesis of thymidine hence DNA (and RNA at high concentrations). 5FU itself is mainly catabolised in the liver by dihydrouracil dehydrogenase,but is also excreted in the urine. It is rapidly taken up in the body with a plasma half-life of 10 minutes after IV injection. However 5FU and its metabolites are found in tissues for very long periods, which may explain the prolonged effect of single doses sometimes seen. Unlike cyclophosphamide its gastrointestinal absorbtion may be erratic (Fraile et al 1980), but not sufficiently to preclude this as a possible route for treatment (Ansfield et al 1977).

The action of 5FU is phase specific (only dividing cells are susceptible) and then only during the synthetic (S) phase of replication.Consequently cell kill produced by it is not so critically related tototal dose,but is more sensitive to the proportion of cells in the synthetic (S) phase.Although this often implies advantage from combining it with other agents in a timed regime,it is also established as a useful agent on its own. It appears to be more effective in the treatment of endodermal

tumours (eg breast and gastrointestinal). The dose limiting toxic effect is nearly always haematopoietic,although minor gastrointestinal disturbances are common.

The Application of cytotoxic agents

In animals,induced or transplanted tumours usually have very high mitotic indices. Consequently they are more susceptible to cytotoxic agents than human tumours.Response rates vary of course (Faanes et al 1979), but it is quite possible to choose situations of 100% cure (Di Luzio et al 1977). This is very convenient for the study of other experimental manoevres (such as immunotherapy) since marked differences in response may then occurr.

The combination of several agents to produce a higher therapeutic index has become widespread in the last 15 years.This has several theoretical advantages. Greater effect may be gained by combining drugs which are individually active against the tumour concerned. They are selected with different sites of action in the cell cycle,so that the additive gain is maximal and a broader spectrum of tumour cells susceptible. By choosing agents with different toxicities a greater quantity of CT may be tolerated.This can be shown in animals- for example a more than additive effect of cyclophosphamide and 5FU in three mouse tumours (Mulder et al 1980).

In man,responses to CT are less pronounced even though combination regimes have largely replaced single agent use. Cure may be achieved in the haematopoietic and a few other tumours. For most others that are suit-

able for treatment ,two different approaches have emerged. In most cases there is a relatively large tumour burden and cure is unlikely,even if the death of several orders of cells ('log kills') is achieved. Therefore regression of tumour and palliation of symptoms is the only realistic objective. In other cases major initial reduction of the tumour bulk is possible by surgery and/or radiotherapy. There may then be a reasonable hope that after drug treatment the order of remaining cells will be small enough for the immune system to destroy. This 'adjuvant' approach to therapy offers hope for cure. Studies of palliative CT may help in the selection of suitable regimes,but their effectiveness in adjuvant therapy takes much longer to assess.

THE EFFECT OF TREATMENT MODALITIES ON THE IMMUNE RESPONSE

Three factors are of particular relevance to the surgical patient.

Surgery

It is generally accepted that surgery has a deleterious effect on host immunity. This is particularly disadvantagous since haematogenous metastases may theoretically be spread by operative manipulation, and permitted to seed because of compromised immunological rejection of them. Postoperative dpression of absolute T and B lymphocyte numbers has been clearly described by Miller et al (1976). Transient functional inhibition of T cells has also been demonstrated - as asessed by DTH responses (Meakins et al 1978), blastogenesis (Park et al 1971) and migration inhibition (Windle et al 1979, Cochran et al 1972). These effects last between 3 and 30 days according to individual patients and test used. Anaesthesic agents have also been shown to have a deleterious effect on cellular immunity (Bruce 1972).

Antibody dependant cellular cytotoxicity (K cell activity) has been shown to be depressed by surgery in some patients (McCredie et al 1979). More complicated responses to surgery are seen in the RES, and illustrate some of the difficulties of this type of investigation. Particulate tests of fixed RES phagocytic activity show a

depression following surgery, which may be accounted for by changes both in circulating opsonic proteins and cellular activity. However this is often followed by a transient stimulation of phagocytosis (Donovan 1967, Saba and Scovill 1975). Tests of peripheral blood monocyte function and numbers have been described which show increases following surgery, although these may not be seen in patients undergoing operations for cancer (Oladimeji et al 1982, Everson et al 1981).

It appears therefore that whilst the overall picture is one of depressed immune responses, individual measurements of these can vary with the test and timing employed.

Radiotherapy

Radiotherapy has been known to be immunosuppressive since the early days of transplantation when attempts were made to exploit this effect (Makinodan et al 1965). T and to a lesser extent B lymphocytes are extremely sensitive to ionising radiation. For example a postmastectomy course of irradiation with 4600 rads has been shown to produce marked reduction in the numbers of circulating T and B cells which may not return to pretreatment levels for many months (Petrini 1981). These effects are seen even when a localised field is irradiated, probably from exposure of cells in the blood circulating through that area. During a similar course of treatment Cosimi et al (1973) have shown a striking fall of T cell function as measured by DTH skin responses and in vitro blastogenesis. Both antibody production and suppressor cell activity show a moderate sensitivity to radiation (Markoe 1980,Hersh 1980). Macrophage and cytotoxic lymphocytes - NK cells appear to be only slightly affected by such treatments (Blomgren 1982,Markoe 1980).

Chemotherapy

The immunosuppressive properties of cytotoxic agents were first described by Hektoen and Corper in 1921. They found that rabbits and dogs exposed to mustard gas showed markedly reduced antibody titres in response to challenge with foreign red cells. The DTH response to various allergens was used in many early studies following this,and shown to be often depressed by these agents. It has subsequently become clear that this reaction is very sensitive to details of timing,which may in fact be manipulated to produce completely opposite effects (Turk 1964).

Almost all cytotoxic drugs have now been shown to be capable of deleterious effects on the immune system.Owing to the complexity of the immune response and methods used to assess it,there is considerable variation in observations for each drug. Table 2 summarises the known effect of some important drugs on certain fundamental immunological functions.

Antigen uptake and/or process	Antigen recognition and precursor cells	Blastogenesis	Proliferation	Antibody production	Effector reactions
ActinomycinD	Alkylating agents	Lasparaginase	ActinomycinD	CyclophoS	Steroids
Steroids	ALS	ActinomycinD	Cytosinearab		ALS
Cyclophos	Steroids	Cyclophos	Methotrexate		
X rays	X rays	5FU	Vinca alkaloids		
		6MP			

Table 2.sites of action of certain immunosuppressive agents on the immune response (Hersh 1973)

Both T and B cell counts in peripheral blood may be considerably reduced by pulses of combination CT (Harris et al 1976). Much in vitro work has described depression of lymphocyte blastogenesis to both T and B mitogens. This has been shown to occurr in groups of patients with various advanced malignancies receiving combinations of cytotoxic agents (eg Serrou and Dubois 1975, Harris et al 1973,Green and Borella 1973). It was not seen in one study of breast cancer patients (Webster et al) ,but this may have been due to imprecise timing of measurements in relation to therapy.

Several observers have noted effects from these treatments on the humoral immune response. Santos et al (1964) studied groups of patients with advanced cancers receiving 7 day courses of five different single agents. All drugs suppressed or abolished the antibody response to immunisation with foreign antigens. However no effect was seen on levels of ABO blood group antibodies.

Inhibition is also seen in the MPS. This was described with circulating monocytes following 6-MP using the skin window technique (Philips and Zweiman 1973). It has also been produced by in vitro incuabation of monocytes with various chemotherapeutic agents (Norris et al 1977). Effects on the fixed macrophage system have been studied mainly in animals. Depression of the clearance of colloidal carbon has been shown following the administration of cyclophosphamide in rats (Sharbaugh et al

1969) and nitrogen mustard in mice (Zschiesche 1970). Pisano et al (1972) confirmed this effect with some agents but found none at aLl if RES activity was assessed by the uptake of an RE test lipid emulsion. The uptake of radiolabelled aggregated human albumen has been used in patients with advanced cancer,and was depressed following combination chemotherapy (Margarey 1972). Ahlgren et al (1980) used this test in the rat and found no effect following cyclophosphamide and 5FU. However Zschiesche et al (1970) found that several cytotoxic agents produced inhibition of phagocytosis in mice,when measured by uptake of radiolabelled bacteria. These results suggest an overall pattern of depression, with considerable methodological variation.

Harris et al (1976) summarise early experimental work showing a 'hierarchy of resistance' to cytotoxic drug effect. Immune responses are generally lost in the following order:

1 - newly acquired delayed hypersensitivity

2 - primary humoral immune response

3 - secondary humoral immune response

4 - established delayed hypersensitivity

In general,short courses of agents given in high dosage act predominantly on the cell-mediated immune response. They may not affect overall levels of immunoglobulins or even the humoral response to antigen. Thus for a few days after a pulse of treatment marked depression of T cell function can be seen using in vitro mitogen responses (Serrou and Dubois 1975). Conversely,prolonged administration of lower drug doses is more deleterious to antibody responses than cell-mediated immunity (which may not change at all - Hersh et al 1973). Long-term maintainance treatment for childhood leukaemia may be associated with little (Sen et al 1973) or no (Jones et al 1971,Borrella et al 1971) change of T cell functions. However the children in these studies show clear impairment of humoral resoponses ,with particular suppression of IgG antibody synthesis.

The thiopurines (azathiaprine and 6-MP) are the most potent agents in this respect.Consequently they have found little use in cancer treatment,but widespread application in transplantation. When given in normal doses, they are capable of supressing antibody production to both new and recall antigens, as well as inhibiting cell-mediated immunity and the mononuclear phase of the inflammatory response (Harris and Sincovics 1977).

The immunosuppressive effect of 5FU has not been thoroughly studied.In animals somewhat variable effects have been reported (Sterzl 1961,Mitchell and DeConti

1970).In man however it has been shown more clearly to produce immunosuppression,albeit in only a few groups of patients with advanced disease receiving fairly high doses. Nordman et al (1978) found in vitro lymphocyte responses to PHA and PPD increasingly depressed at one and three months after the onset of therapy.There was no change of T and B cell percentages and immunoglobulin levels. Mitchell and DeConti (1970) found a reduction of both primary and secondary humoral responses to tetanus toxoid and Salmonella antigens. They also describe marked inhibition of DTH but their protocol for this was pinadequate for satisfactory interpretation.

The immunological effects of cyclophosphamide are considerable but complicated. Impairment of T cell function can be seen using in vitro PHA responses, and to a lesser extent DTH and skin graft rejection (Winklestein et al 1973, Milton et al 1975). Primary and secondary antibody responses are also inhibited (Haskell 1977, Berenbaum and Brown 1964). In general at lower doses its action is primarily on B cells, but T cells are also affected at higher doses . Under experimental conditions it may be manoevred to selectively inhibit suppressor cells (Polak and Turk 1974).It is not clear what role this action plays in man,especially in the autoimmune diseases. In one study of patients taking low doses (50-75 mgs/day) for rheumatoid arthritis no immunological effects were detected by in vitro blastogenesis,DTH responses and circulating immunoglobulin levels (Curtis et

al 1973). Nevertheless,when used in doses for the treatment of cancer it remains one of the most immunosuppressive drugs available (Santos 1964).

The importance of drug induced immune depression is more difficult to assess. Certainly suppressed patients are more susceptible to infection of all types,including bacterial and fungal organisms relatively non-pathogenic to normal patients (Warnock and Richardson 1982). The relative contributions of drug-induced granulopoenia and impaired activity is difficult to discriminate, but there seems to be little doubt that both contribute to a major degree. This constitutes a real problem for all patients receiving chemotherapy, and is the main cause of death amongst those treated for the haematological malignancies and transplantation recipients (Bodey 1975, Hersh et al 1973).

Losses in resistance to cancer may be more important but are usually overshadowed by tumoricidal effects. Two experiments have been reported that relate to this question. In one (Habs et al 1981),normal rats receiving regular CMF CT at several different doses developed malignant tumours several times more often than controls. The carcinogenic effect of this treatment may be related to ineffective immune surveillance. In the second experiment the effect of 5FU treatment on the transplantable mammary adenocarcinoma of mice was studied (Suhrland et al 1972). Tumour growth was reduced by doses of 15

mg/kg,but enhanced by the subtherapeutic dose of 1 mg/kg. Suppression of the humoral antibody response to bovine gammaglobulin was produced by 5FU to the same extent by the low as the high dose.Therefore an impairment of the antibody related immune responses to tumour may have accounted for enhancement of its growth at low dosage of 5FU.

In conclusion,despite considerable variation in published studies nearly all cancer chemotherapeutic agents have been found to produce immune depression. This appears to occurr in all arms of the immune response so far studied. Whilst this is a clear danger with regard to resistance against infection, its implications for tumour control are potentially greater but more difficult to asess.

MODIFICATION OF THE IMMUNE RESPONSE

Specific immunotherapy is fairly laborious in most forms and has not proved as useful as once hoped (Castro 1976). Since cytotoxic drugs cause a non-specific depression of immunity,attention will be restricted to a few immunotherapeutic agents which have been tested in this respect.

BCG

This attenuated strain of Mycobacterium bovis was developed in 1908 by Calmette and Guerrin,by the addition of bile to routine TB culture medium (then maintained for 13 years!).It was first used by injection into tumour masses alone or in combination with tumour derived antigens. A number of reports demonstrate some response in a proportion of patients (Laucius et al 1974). However there have been no trials to show that this approach is as good as or better than other treatments, such as surgery (Spitler 1980).

BCG has been widely used as a non-specific systemic immune stimulant - alone or in combination with CT. In animals under suitable conditions,it may be shown to augment the effect of cytotoxic drugs and prolong survival (Purnell et al 1979, Mathe et al 1978). In humans its role is not so clear,and there are conflicting reports even with regard to one disease. Critical differences

are said to exist between preparations,dosage regimes and routes of administration which may explain some contradictory results.For example a difference in dose administered may alter a beneficial effect to a harmful one (Laucius et al 1974).

When used alone in advanced disease,marginal or no differences have been reported in measures of host immunity such as DTH skin responses, lymphocyte mitogen responses and circulating antibody levels (Pacheco-Rupil et al 1980, O'Connell et al 1979). Hersh at al (1981) have reported significant improvement in monocyte function with prolonged BCG treatment in a mixed group of cancer patients. Several uncontrolled series have suggested some clinical benefit from its use alone or in combination with chemotherapy, particularly in malignant melanoma (Gutterman et al 1974), breast cancer (Hortobagyi et al 1979) and colonic cancer (Mavligit et al 1975). However these have not been so clearly confirmed by a number of controlled clinical trials. No clear benefit has been reported in trials of a mixed group of cases of advanced cancers (O'Connell et al 1979) in colorectal cancer (Richards et al 1979) or in breast cancer (Muss et al 1981). More encouraging results have been claimed in a small of group patients with stage III ovarian cancer in conjunction with repeated tumour specific immunisation and CT,but these findings remain to be fully established (Hudson et al 1976).

As adjuvant therapy in 'minimal residual disease' with or without other treatment, there are only slightly more encouraging results. For example in malignant melanoma a number of early reports of the use of BCG alone following potentially curative surgery in combination with chemotherapy showed favourable results compared to historical controls (Gutterman et al 1974, Gutterman et al 1976). These have not been clearly confirmed by several more recent and carefully controlled studies,and it is even possible that the disease is accelerated in a few subgroups (Spitler 1980). Early enthusiasm also arose from studies of its use in acute myeloblastic and lymphoblastic leukaemia in remission, where it has been given both with or without CT (Gutterman et al 1974). However subsequent controlled trials have only found such effects to be marginal, though it may have a place as an alternative to maintainance chemotherapy (Vogler 1980). In early breast cancer beneficial effects have been reported with oestrogen receptor positive tumours (Hubay et al 1980).

Levamisole

This synthetic agent was established as an anti-helminthic treatment in 1966,and 5 years later its immunological potential was first described by Renoux and Renoux. A wealth of animal studies have established its effect in vivo,when given in appropriate dosage (Sampson et al 1977). It has no direct tumoricidal action,and its immunological benefits are essentially restricted to the improvement of depressed functions (Symoens and Rosenthal 1977).Its main action appears to be on T cells - as shown in vitro (Padarathsingh et al 1978) and in vivo (Griswold and Walz 1978).It also has a restorative action on circulating macrophage function as measured by several in vitro tests (Nathanson et al 1978,Fisher and Gebhardt 1978).

In a variety of animal tumour models it has been shown to augment the benefits of appropriate CT (Miura et al 1980, Chirigos et al 1975,Fisher and Gebhardt 1978). For example,Chirigos et al (1975) compared the effects of BCNU and levamisole on transplantable murine MCAS-10 leukaemia. Treatment with BCNU led to 32% survival (from none), and this rose to 90% when appropriately combined with levamisole- which was ineffective alone. This was interpreted as an additive tumour-kill by the less depressed host immune system.

In advanced human cancers there is broad agreement

that levamisole improves in vitro T cell function (Conesa et al 1979,Amery and Gough 1981). Delayed hypersensitivity responses are improved to a lesser and more variable extent (Wilkins et al 1977,Hirshaut et al 1980). Nathanson et al (1978) found improvement in monocyte function in patients with bladder cancer following levamisole. These effects have been translated into some benefit in the majority of clinical studies reported.In advanced breast cancer significant improvement of disease free interval and survival have been described when levamisole is combined with CT (Stephens et al 1978, Hortobagyi et al 1979) or radiotherapy (Rojas et al 1976). No benefit in conjunction with CT was seen in two studies of colorectal cancer (Bancewicz et al 1980,Bedikian et al 1978), although improvement has been noted in gastric cancer (Miuwa and Orita 1978). Slight benefit which is not always statistically significant has been noted in other studies of advanced tumours of skin, head and neck and bladder (Smith 1978,Amery and Gough 1981).

There is a surprising paucity of information on the use of levamisole as adjuvant therapy to potentially cured cancers. Following levamisole treatment in stage C colorectal cancer an improvement in survival was seen 3 years after surgery (in Amery and Gough 1981). Marginal benefits were suggested in squamous cancer of the head and neck (Wanebo et al 1978) and leukaemia in remission (in Amery and Verhaegen 1978),but none after surgery for early melanoma (Spittler and Sagebiel 1980). In one

study of early breast cancer patients treated by mastectomy and radiotherapy,the administration of levamisole for one year was associated with a significantly more rapid recurrence rate (Danish Breast Group 1980). However in operable lung cancer a marginal advantage was gained by patients taking levamisole alone for 2 years,and this was clearly significant if only those under 70 kg were considered (Amery 1978).

The restorative action of levamisole implies that only a weak effect may be expected if it is given alone,since most patients with early cancer are not seriously immunologically depressed (Cochran et al 1976). However it may well have a greater role when given to these patients in conjunction to other immunosuppressive treatments such as CT and radiotherapy. Results of further trials may clarify this question.

C parvum and Glucan

These two agents are reputed to act primarily through the mononuclear phagocytic system (MPS) and will be summarised together. Corynebacterium parvum is used as a heat-killed suspension of bacteria,and is effective when administered by a number of routes (Israel and Edelstein 1975). Glucan is a polysaccharide extracted from Saccharomyces cerevisae, and commonly injected intravenously or into a tumour locally (Proctor and Yamamura 1978). Both produce marked hepatosplenomegaly and increased phagocytosis of particles from the circulation (Castro 1974,DiLuzio et al 1978). C parvum causes some depression of T cell function but B cell stimulation (Scott 1974).Conversely glucan is reported to produce T cell stimulation and not to affect B cells (Hamuro et al 1978,Kitagawa 1975). The MPS effects of these two agents are considered to be primarily responsible for their immunological actions.

In animals significant reduction of tumour growth rates and number of metastases may be demonstrated with the use of these agents (Sadler and Castro 1976,DiLuzio et al 1978,Karrer et al 1979,Gatenby 1980).They are generally most effective when administration begins before or synchronous to a small tumour inocculum. For example glucan has been shown to have more than additive benefit to cyclophosphamide in two animal tumours by DiLuzio et al (1978). Rats were innoculated with transplantable

acute myeloid leukaemia cells and by 11 days had all died. If treated with either cyclophosphamide or glucan 10% or 40% were alive after 2 weeks,but this rose to 92% if both were combined.

There is little conclusive work with either agent in man. Intralesional administration of either leads to an invasion of macrophages and frequent partial regression (Proctor and Yamamura 1978,Goodnight and Morton 1980). Israel and Edelstein claim (1975) to have used parenteral C parvum in over 600 patients without serious side-effects. It has been added to combination CT regimes in advanced cases with conflicting results (Goodnight and Morton 1980). There are now further controlled clinical trials in progress which should help clarify the usefulness of these agents.

In summary,the impact of immunotherapy on clinical practice has been remarkably slight. Very few measurements of immunity have been made during these treatments but improvement has been noted in some. Proper clinical trials have negated many beneficial claims for BCG, shown marginal effects from levamisole and barely commenced for C parvum and glucan. These agents have not been extensively tried in the adjuvant setting,with the exception of levamisole which may confer slight benefit.

Conclusions

Immune depression is a general feature of most chemotherapeutic regimes. Consequently resistance is reduced to infectious diseases, and theoretically also to cancer. This has been confirmed in a few experimental situations,but in patients is generally overshadowed by the tumoricidal benefit of CT. Potential may therefore exist for extra benefit by restoration or augmentation of the immune response. This is a particularly appealling objective since the log-kill nature of cytotoxic drug action can greatly reduce but not eliminate all tumour cells,and the immune system might complete this.

It had been hoped to begin the study by examining the effects of CT for early cancer on the immune system in man using a wide ranging immunological profile. Considerable effort was spent establishing a number of tests for this purpose. Stage II breast cancer patients were chosen for study since it was felt they were likely to be minimally or not at all immunesuppressed by their tumours. This would enable the effects of CT to be studied most precisely, since there should be minimal intrusion from the immunological effects of widespread cancer. However the reluctance of local radiotherapists to use adjuvant chemotherapy in these patients, and their adherence to a 3 way trial of such regimes when used led to inadequate numbers for the study.

An experimental study was undertaken to assess the use of certain immunotherapeutic manoevres in conjunction with chemotherapy. Most experiments were conducted in normal animals since they most closely resembled patients receiving adjuvant chemotherapy. Also it was felt important to examine conclusions reached from these experiments in tumour bearing animals. It may be expected that there would be specific antitumour immune responses active in these animals,and that there may also be general immune suppressive effects. The rat was chosen so that a relatively large number of animals could be available,yet each able to lose a small quantity of blood regularly for immunological studies. This quantity necessarily limited the number of tests possible. It has been argued that the T lymphocyte is fundamental to the response to cancer,and attention was concentrated on this cell using one in vitro and one in vivo technique to asess its function. Two common anti-cancer drugs with different modes of action were chosen ,and their effect on the immune system studied. A model was set up of immune depression produced by these,and various attempts made to alleviate this. Some of these were selected for testing in tumour bearing animals. The immunotherapeutic manoevres studied vary in claims for their potential benefit, and will be introduced in each section.

METHODS

CONTENTS

Rats

Sprague Dawley rats were used throughout.Animals weighing 200-250 grams were found to be of sufficient size to give blood regularly without deleterious consequences. For the induction of breast cancer they were selected between 50 and 55 days of age. During this time the breast buds are developing and most susceptible to carcinogens. After preliminary and methodological studies,each experiment consisted of groups of 20 animals. Half of these served as simultaneous controls for the other half - usually both received chemotherapy and one an additional agent or manoevre. In some experiments only the mitogen response was monitored, but in the tumour bearing animals and other selected groups the DTH response was also measured.

Induction of Breast Cancer

N-nitrosomethylurea (NMU) was chosen as a carcinogen following the favourable report of Gullino et al (1975). 500 mgs of NMU was dissolved in 1.5 mls dimethyl formamide, which was slowly mixed with 11 mls of peanut oil,to give a dose of 20 mgs in 0.5 mls. This was administered to the conscious rat by gavage. Groups of 20 rats were given carcinogen and these were fully isolated for two weeks to protect animal attendants. Tumours developed between 3 and 10 months later,with a median delay of 7 months. An illustration is given at the beginning of the

appendix of a tumour being removed, and the histology of several tumours.

Rats were examined weekly and transferred to different cages when a tumour was detected. Each week they were first sensitised to oxazolone, then randomised between two treatment regimes being compared. When an adequate total had accumulated for one study, subsequent animals were entered into the next comparison etc. Tumours were measured weekly under anaesthesia;the greatest length and that perpendicular to it were multiplied to produce a 'size'.

Drugs

Cyclophosphamide (WB Pharmaceuticals) was dissolved in saline.It was usually used in a dose of 8mg/kg or 40mg/kg. 5 fluorouracil (Roche) was used as supplied at a concentration of 50mgs/ml and used at 60mg/kg except in priming experiments. Levamisole (ICI) was diluted in saline and used at 5mgs/kg. All these drugs were given intraperitoneally at volumes around 0.3ml. Glucan was a kind gift from Prof DiLuzio (Tennessee).It was used at 10 mgs/kg intravenously. C parvum (BA 3935/A Wellcome labs Beckenham) contained 7mgs/ml of heat-killed bacteria and a dose of 1ml was given intravenously to each rat. The activity of these two agents was confirmed by one experiment in which postmortems showed hepatosplenomegaly of at least threefold in each animal. Thiabendazole (Merke Sharpe and Dohme ltd Herts) was given once at 5mgs/kg intraperitoneally in 40% alcahol,and cimetidine (Smith Klein and French) intraperitoneally twice daily for two weeks at 2mgs/rat. Purified phytohaemagglutinin (PHA - Burroughs Wellcome and Co. Beckenham UK) was used,in a dose of 2 µgms per well unless otherwise stated (making 10 µgm PHA/ml diluted blood). Thymidine (The Radiochemical Centre,Amersham UK) was used,tritium labelled at 24 mCi/mmol specific activity. Unless otherwise stated the dose used was 0.5 µCi or 0.0055 µgms per well. Oxazolone (4 ethoxymethylene-2 phenyl oxazolone) was obtained from BDH chemicals and used in two doses as discussed below. NMU was obtained from Sigma chemicals (St.Louis).

MITOGEN RESPONSE

The method used was a modification of the whole blood technique described by Han and Pauley (1972).

Cultures Under ether anaesthesia 0.4 mls of blood was collected from the tail vein into tubes containing 50 units of heparin. Blood was diluted by 1:10 with tissue culture medium. Gibco culture medium 199 was used with Earle's salts and 2.2 gm/l sodium bicarbonate,to which 20,000 units of penicillin and 20 mgs of streptomycin was added. This suspension was pipetted in 200 µl aliquots into a microculture plate - illustrated overleaf.Mitogen was added to 12 wells leaving 4 control wells per rat. These plates were incubated at 37 C in a 5% carbon dioxide atmosphere. After 24 hours tritium-labelled thymidine was added, followed by a further 24 hours incubation. The labelled nuclei were then harvested by a Skatron A.S. filter harvester,and each filter disc counted by liquid scintigraphy using a LKB 81000 beta counter.

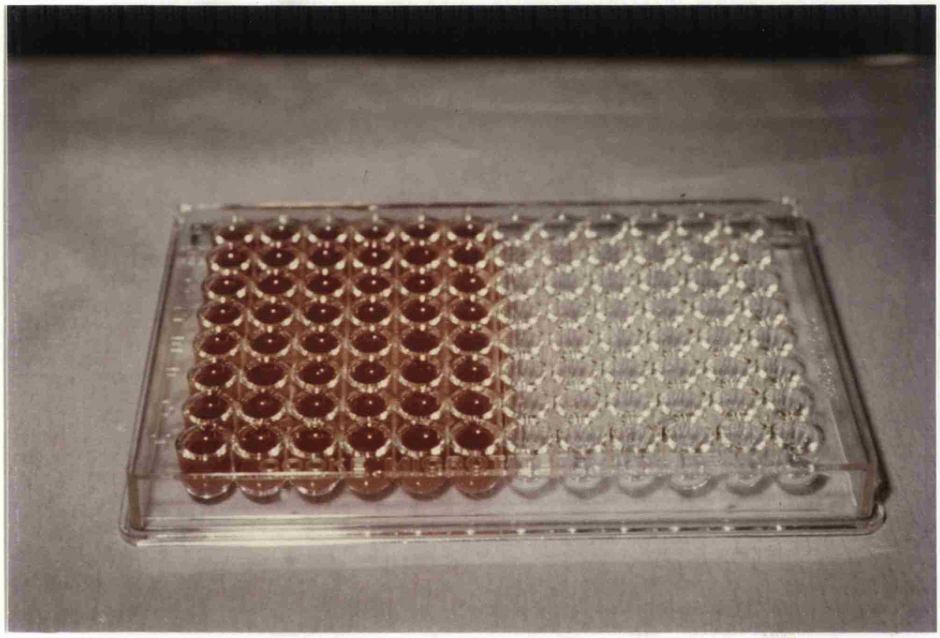


Fig 1 Multiwell tissue culture plate for PHA studies

Results This procedure gave 12 stimulated results per rat per day,and the mean of these 12 was calculated. The results of the four unstimulated wells was used to monitor satisfactory technique and the absence of infection. Only the stimulated result was used in calculations rather than a 'stimulation index' (mean PHA wells/mean control wells) as advocated by some (Han and Pauly 1971).The former method involves only one order of biological error,the latter may multiply two. Furthermore since essentially all experiments involved simultaneous controls ,comparison between 'absolute' values was not required.

Calculations For simplicity,results were expressed in disintegrations per minute (dpm) in methodology experiments. For all other experiments the log(ten) function of these values were used,so that the natural tendency of this data to 'skew' was removed (Dei and Urbano 1977). In order to minimise the effects of scatter within groups the values for individual rats on each experimental day were compared as ratios to control (pre-treatment) values for individual rats thus producing a 'log ratio' index of change:

example:	(rat 1)	Day0	cf	Day3
	absolute value	59352 dpm		10698 dpm
	logged value	4.773		4.029

log ratio=4.029/4.773

=0.8441

The tables for each experiment may be found in the Appendix. Individual results are given as measured (in dpm). The means and standard deviations of each day's results are given at the bottom of each table,as are those of individual log ratios calculated for each day. Statistical comparison between groups of rats were made using the Students t test on log ratios values,and is printed with each table. All important experiments are represented graphically in the appropriate results chapter. The table corresponding to each graph is indicated on it and also in the text. It is important to realise that the scale of each graph varies so that their vertical axes are of uniform height. This is done for clarity,and because meaningful comparisons of absolute values between different experiments are better avoided considering the variation inherent in such data.

Technical Studies

Plastic vs glass Blood was collected from the tail into small tubes,and a measured volume subsequently pipetted into medium.Table 1a shows the mean of three experiments using glass or plastic tubes for the original collection. Clearly it was essential to use plastic for satisfactory results at all doses of PHA.

Time before dilution Blood was left at room temperature in these heparinised plastic tubes for varying lengths of time before diluting with culture medium. Fig 2a shows the deterioration of resulting counts with time. It seemed advisable not to wait more than 10 minutes before diluting blood with medium. This clearly contrasts to human blood,which can be left for at least a day provided that it is at room temperature (Farrant et al 1980).

Time in medium When dealing with larger numbers of rats it was convenient to delay plating out diluted blood as long as possible,in order to collect the maximum number of samples.Fig 2b shows the effect of various delays under different situations. Delay up to 2 hours appeared to be unimportant if the mixture was incubated at 37 C during that time. However after 1 hour at room temperature the consequent counts are significantly lower ($p<0.001$).

Whether to cover plates Blood diluted in medium was pipetted in Ø.2 ml aliquots into individual wells of the microtitre plate. After the addition of PHA these were incubated and covered loosely by their plastic lids. Table 1b shows three experiments where the same blood was also distributed in plates sealed additionally with a fitted piece of wide celotape. It was quite clearly essential to cover the plates in this way. Carbon dioxide in the atmosphere of the incubator should have ensured the maintainance of a satisfactory pH by the medium buffer even when uncovered. It is possible that dehydration explained this phenomenon.

Times of incubation Fig 3a shows the effect of seperately varying the incubation periods before and after labelling.Maximum counts were obtained after 24 hours of both periods. This was therefore adopted for all experiments. It is a shorter period than for optimal human responses (several days) ,and this may be explained by the in vitro life of the cultured rat lymphocyte being half of that for man (Farrant et al 198Ø).

Dose response Table 3b shows the mitogenic effect of different doses of PHA. There was an inadequate supply of blood to permit the simultaneous use of several PHA doses. Therefore Ø.2 mgs/ml of PHA was chosen as the most economical dose producing an adequate response. This is essentially similar to most other studies (Hall and Gordon 1976).

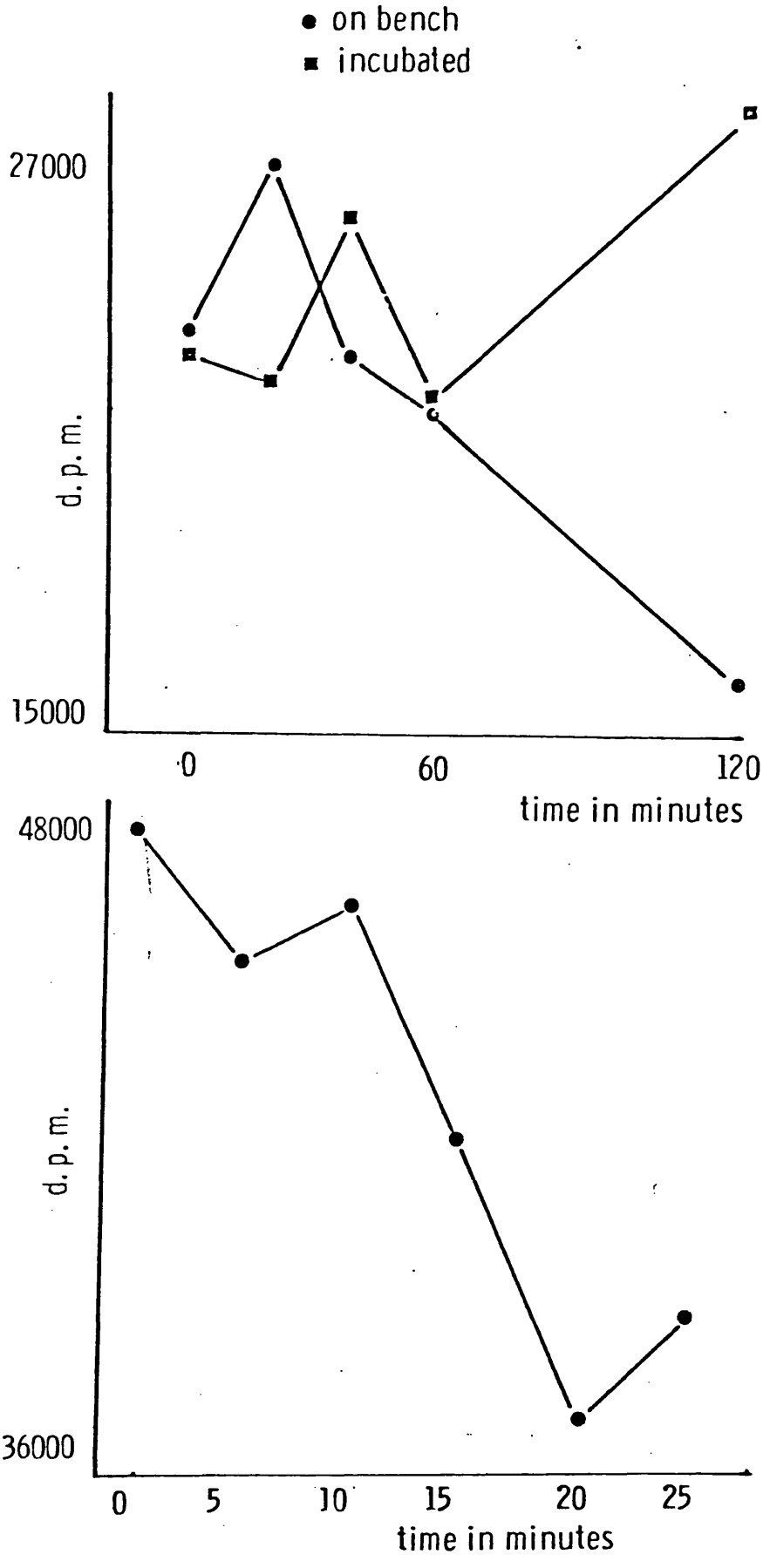


FIG 2 THE EFFECT OF DELAY IN MEDIUM AND BEFORE DILUTION IN MEDIUM ON THE SUBSEQUENT MITOGEN RESPONSE

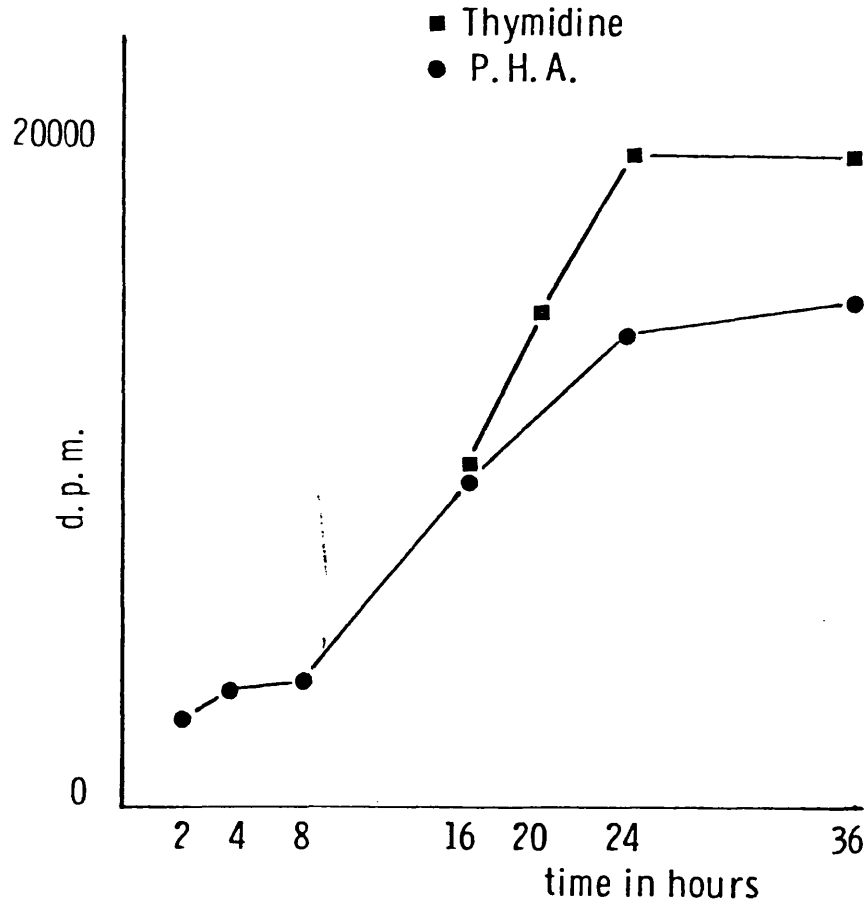


FIG 3 THE EFFECT OF VARIOUS INCUBATION PERIODS ON THE MITOGEN RESPONSE

DELAYED-TYPE HYPERSENSITIVITY

The method used was a modification of that described by Pownall et al (1979). For sensitisation,the abdomen of each rat was shaved under general anaesthesia and painted with a 10% solution of oxazolone in acetone and ethanol (3 to 2 parts by volume). Care was taken to prevent sensitisation of the observer and gloves always worn. A week later the thickness of both ears was measured using a special micrometer illustrated overleaf. This was preferred to a standard rotary micrometer which traumatised the ear by shear,causing a scab and consequent error. Care was taken to measure the same part of each ear. The thinnest site was then identified,and usually proved to be the peak at the apex of the ear or slightly posterior to it. This was achieved by allowing 1 mm of ear to protrude inside the micrometer faces. Multiple measurements were then made until a consistent reading was achieved.

Ear thickness being measured with the spring micrometer

Animals were challenged with a solution of 220 mgs/ml oxazolone in acetone and olive oil (4 and 1 part by volume). This produced an even distribution of oxazolone over the ear,with rapid drying and no dripping. Four drops were applied to one ear,and four drops of solvent to the other. 24 hours later the thickness of both ears was measured and the increase attributable to oxazolone calculated. An example of this simple calculation is given below:

	Right ear	Left ear
thickness before oxazolone	470 μ	435 μ
" after "	485 μ	675 μ
difference	015 μ	240 μ
increase due to oxazolone		225 μ

Tabulated results are given for each experiment in the appendix ,with all measurements expressed in microns. Statistical calculations were made between simultaneous paired groups wherever possible.These were usually the same animals partaking in the PHA experiments.

Technical points

Challenge timing Table 4 shows that 7 days are required before a full response may be elicited. Longer delays up to 15 days do not clearly affect the response further. This confirms a well established time for the DTH reaction (Turk 1980).

Challenge dose It was found that two drops of challenge solution was often inadequate to cover the experimental area of the ear.More than 4 drops exceeded its capacity and led to inflammation of the adjacent side of the head,or dripping and consequent error. 3 and 4 drops produced similar responses without ulceration ,therefore 4 was assumed as a standard challenge.

Measurement timing Full response was found at 24 and 48 hours,and reduction in swelling began after 48 hours (Table 5). 24 hours was chosen as a satisfactory time for measurement,and this fitted conveniently into protocol to preclude the need for further anaesthesia.

Diurnal variation There are marked differences in the DTH response measured at various times of day,and results may be changed by up to threefold. Therefore it is important to use simultaneous controls wherever possible, and to time challenges near 10 a.m. when responses are maximal ('acrophase'). It is not important at what time sensitisation occurrs (Pownall et al 1979).

RESULTS

CONTENTS

CHAPTER 1

ESTABLISHMENT OF THE MODEL

Table 6 shows the results of PHA stimulation expressed as disintegrations per minute for 10 control rats. They received 0.3 mls saline I.P. and blood was taken sequentially over 14 days. It is clear that although values remain relatively similar there is significant daily variation over the month. Therefore all subsequent experiments included a simultaneous control group to allow for this potential error.

There is a wide variation in the dose of cyclophosphamide used in published animal studies. Many have used tumouricidal doses which are toxic to animals, and it was felt that the massive immunesuppression from such doses might mask a potentially interesting immunotherapeutic effect. Therefore the effect on the PHA response of several doses was determined, and these are shown in Tables 7 and 8. From these 8 mgs/kg was chosen since it produced clear depression,which was not so marked as to preclude alleviation by other manoevres. An alternative dose of 40 mgs/kg was also used in some experiments since this may be more comparable to human use. Freireich et al (1966) showed that drug doses are best compared between species as a function of surface area. Repeated human use is recommended at 30-600 mgs/m per day,so that a median dose corresponds to 40 mgs/kg in the rat.

There are limited guidelines for the use of 5FU in the rat. The recommeded dose for humans is 5-15 mg/kg per day, which corresponds to 70 mgs/kg in the rat. In

an experiment with increasing doses of 5FU no immunosuppression was observed until 60 mgs/kg was used (Table 9). Therefore this was selected as a suitable dose for the purposes of this study.

Figure 4 (Tables 6,7,9) shows a comparison of rats given one injection of these two drugs to a control group. For more accurate comparison results are expressed as the mean of individual log ratios, as explained in the previous methods section. It can be seen that roughtly comparable depression of the PHA response was produced by both drugs. This was maximal at 1-3 days.

Seven days after the injection of 5FU values return to near control levels or higher. They then fall back towards the initially depressed levels,from which they recover to a variable degree. This is the 'rebound overshoot' effect which has previously been described in cancer patients following combination chemotherapy (Serrou and Dubois 1975). It was occasionally seen to a lesser and more inconsistent degree after cyclophosphamide.

From two to four weeks values are always significantly below pre-treatment levels. Tables 11 and 12 show the results of two experiments where small groups of animals were bled on no day other than control and the 14th or 28th days. Results were depressed in these rats to a

degree similar to that of normal experimental groups. This suggests that the late depression was not primarily due to the effects of repeated anaesthesia and blood loss.

This depression was not consistently related to overall changes in peripheral white blood cell counts (Tables 13 and 14). Therefore it seems probable that it primarily represents an effect of the agents on lymphocyte function.

Fig 5 (Table 15,26,31) shows the effect of each agent when given alone to groups of tumour bearing animals compared to untreated tumour bearing controls. Following CT similar patterns are seen in the PHA response to those described in normal animals (above). Again the rebound overshoot is only clearly seen after 5FU.

Almost all experiments involving the DTH response concerned the use of cyclophosphamide. Table 16 shows that cyclophosphamide 40 mgs/kg produces significant depression of the oxazolone DTH response. However there is a wide variation of responses, so that groups of 10 animals may not necessarily be adequate to show differences which are statistically significant.

Table 17 shows the progress of tumour 'sizes' during the month following therapy for each experimental group.

Since there was considerable variation in sizes between animals, changes are also shown as individual percentage change to pretreatment values (the mean of 2 weeks readings). None of these regimens significantly influenced the slow growth of these tumours. Although certain rats appeared to respond to CT by tumour shrinkage,this was also observed in a few untreated animals. Numbers do not appear to be adequate to comment on the effect of CT, but this was not a primary purpose of the study.

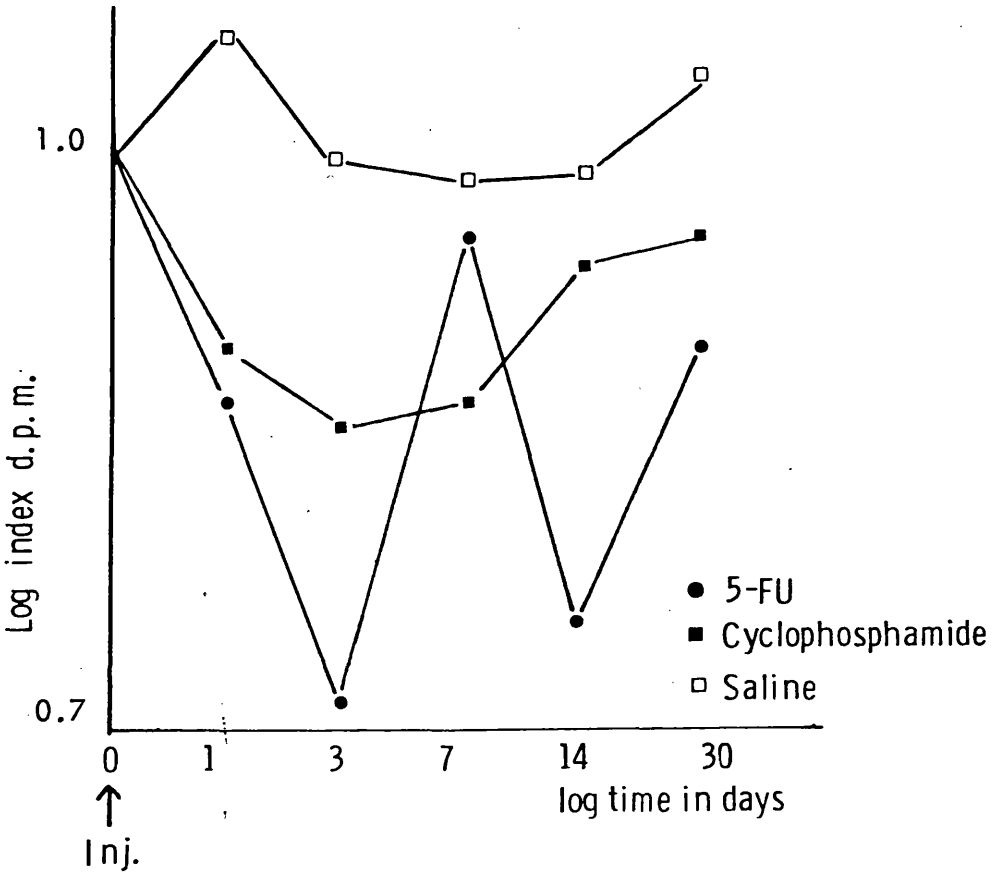


FIG 4 THE EFFECT OF ONE INJECTION OF CYCLO (8 mgs/kg) OR 5FU (60 mgs/kg) COMPARED TO SALINE CONTROLS

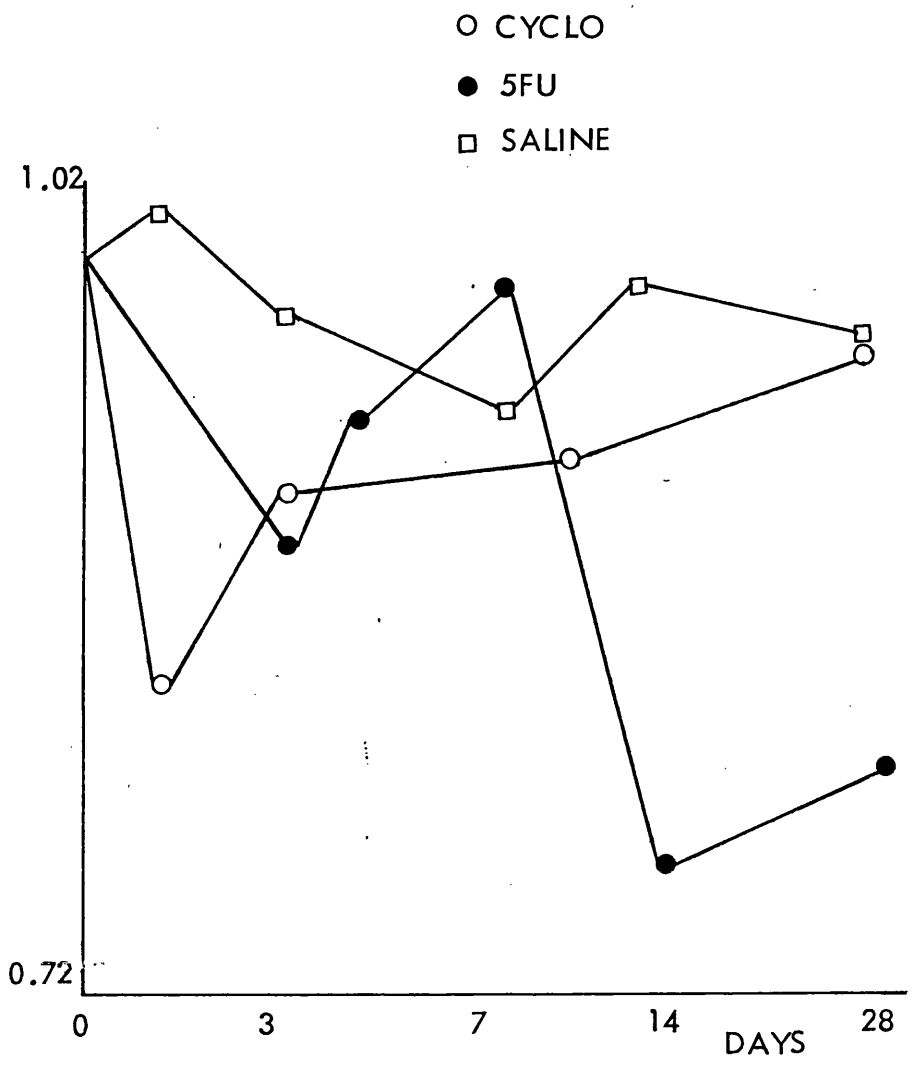


FIG 5 THE EFFECT IN TUMOUR BEARING ANIMALS OF ONE INJECTION OF CYCLO (8 mgs/kg) OR5FU (60 mgs/kg) COMPARED TO SALINE (CONTROLS)

Summary

Immune depression is shown in this rat model following one injection of either 5FU or cyclophosphamide. This was demonstrated in both normal and tumour bearing animals. The in vitro test showed a depression lasting for at least one month after injection. The rebound overshoot phenomenon was clearly seen after 5FU. Neither of these changes is primarily related to changes in absolute cell numbers. The in vivo test shows impairment of response during the first 24 hours after cyclophosphamide in normal animals.

CHAPTER 2

LEVAMISOLE

When given alone levamisole had no effect on PHA responses over one month compared to a control group receiving saline (Tables 18 and 19). This is consistent with its essentially 'restorative' action (Symoens and Rosenthal 1977). However when it was given with 5FU or cyclophosphamide 8 mgs/kg no significant influence was seen on subsequent immunesuppression (Figs 6 and 7,Tables 2Ø and 21).

In other experiments 3 days was allowed before the administration of levamisole,in order to study its activity in established immunesuppression. Figs 8 and 9 (Tables 22 and 23) show no influence of levamisole on the depression produced by cyclophosphamide 8 mgs/kg or 4Ø mgs/kg given 3 days before. The numbered study dates are counted from the injection of levamisole. A restrained but clear rebound overshoot can be seen at both these doses ('day 3' is 6 days after cyclophosphamide). A more clear benefit was seen when levamisole was given 3 days after one injection of 5FU (fig 1Ø,Table 24). Counts were consistently higher in the group receiving levamisole, although the difference to controls was only statistically significant on day 1. This experiment was repeated and the same results obtained. Both groups of resutls were combined to provide those given in Table 24.

This observation was tested using the DTH response. 3 groups of animals received either saline,5FU or 5FU followed by levamisole after 3 days when they were chal-

lenged with oxazolone. No clear differences were seen in the DTH responses following this between each group (Table 25).

This protocol was followed in tumour bearing animals and an identical pattern seen (Fig 11,Table 26). The group receiving levamisole maintained consistently higher responses although these never reached statistical significance.

Conclusions

1) Levamisole does not influene depression of PHA responses when combined with an injection of cyclophosphamide or 5FU.

2) A small but consistent improvement of PHA responses was seen following 5FU,if 3 days elapsed before the administration of levamisole. This phenomenon occurred in both normal and tumour bearing animals. No similar effect was not seen in the DTH response.

CHAPTER 3

RES-ACTIVE AGENTS

Glucan C parvum and Thiabendazole

These agents have been combined with chemotherapy in many animal experiments because of their stimulating action on the reticulo-endothelial system. Therefore it is interesting to see from Fig 12 (Table 27) that the PHA response following cyclophosphamide (8 mgs/kg) is markedly depressed in normal rats by the addition of glucan. This effect was also seen at the higher dose of 40 mgs/kg (Fig 13 Table 28), and with 5FU (Fig 14,Table 29). However it was not seen on day 3 in either cyclophosphamide group, or on day 7 following 5FU (the peak of the rebound overshoot).

The DTH response was assessed in two groups of normal rats receiving 40 mgs/kg of cyclophosphamide. Half of these were also given glucan IV at the same time. It can be seen from Table 30 that this did not influence the magnitude of their subsequent DTH responses, which was slightly greater in the groups receiving glucan.

A similar pattern was seen in tumour bearing animals. Figure 15 (Table 31) shows that the PHA response was depressed by the addition of glucan to cyclophosphamide (40 mgs/kg). Although this effect only reached statistical significance on days 7 and 14,the pattern was clear throughout. The DTH response following cyclophosphamide (40 mgs/kg) was not influenced by glucan in these animals (Table 30).

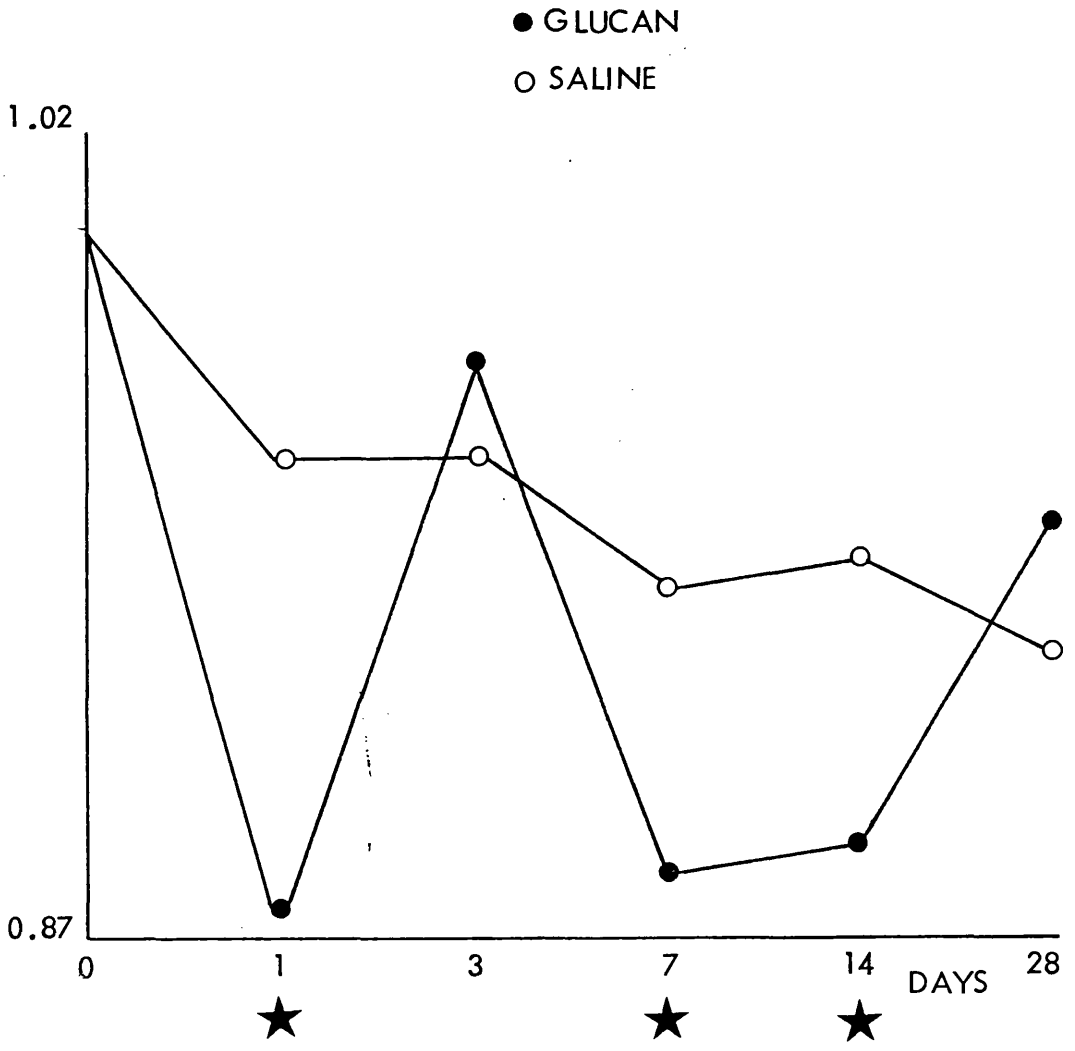


FIG 12 (TABLE 27) THE EFFECT OF GLUCAN (10 mgs/kg) COMPARED TO SALINE (CONTROLS) WHEN ADMINISTERED WITH CYCLO (8 mgs/kg)
★ $P < 0.01$

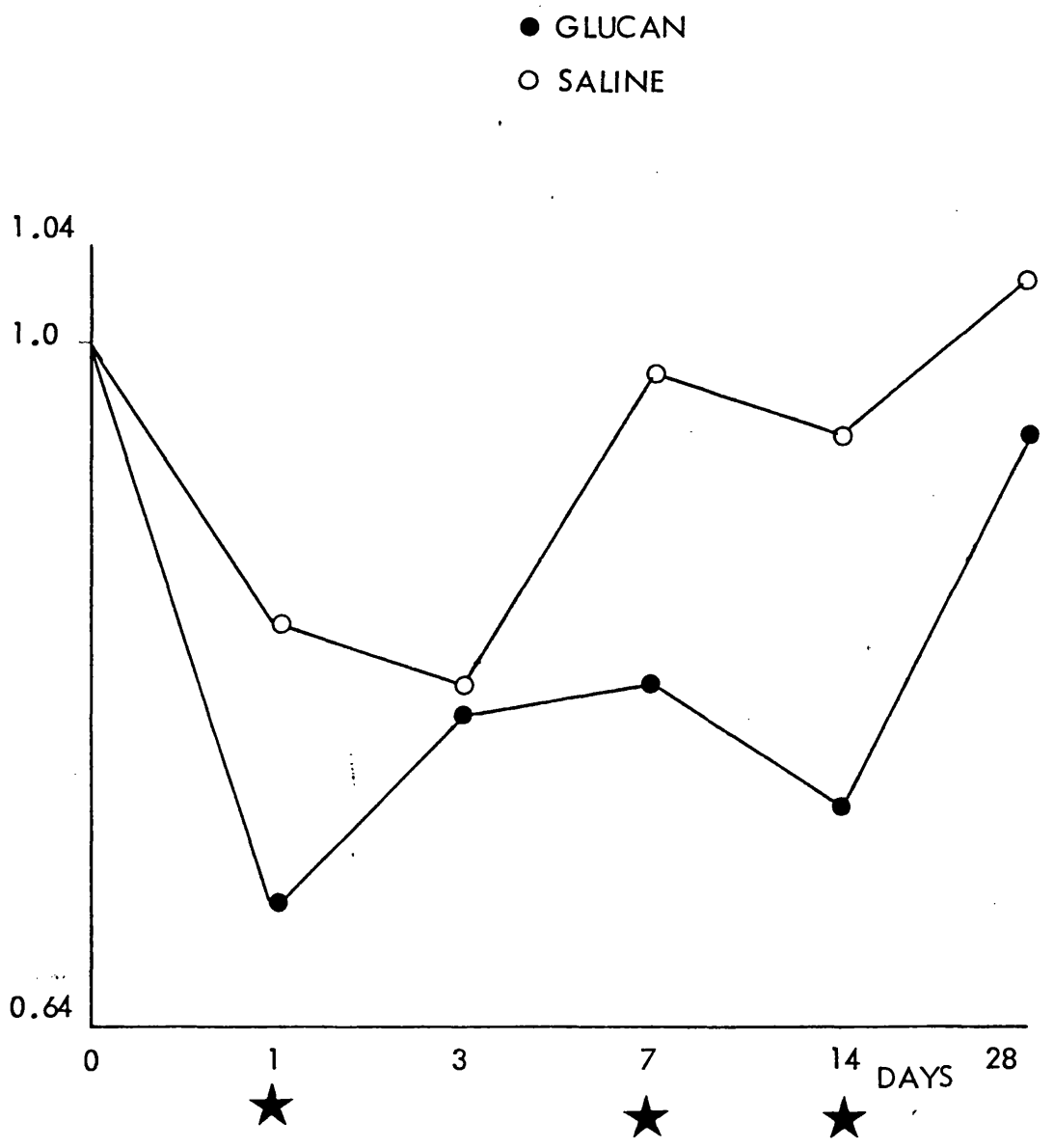


FIG 13 (TABLE 28) THE EFFECT OF GLUCAN (10 mgs/kg) COMPARED TO SALINE (CONTROLS) WHEN ADMINISTERED WITH CYCLO (40 mgs/kg)
★ $P < 0.01$

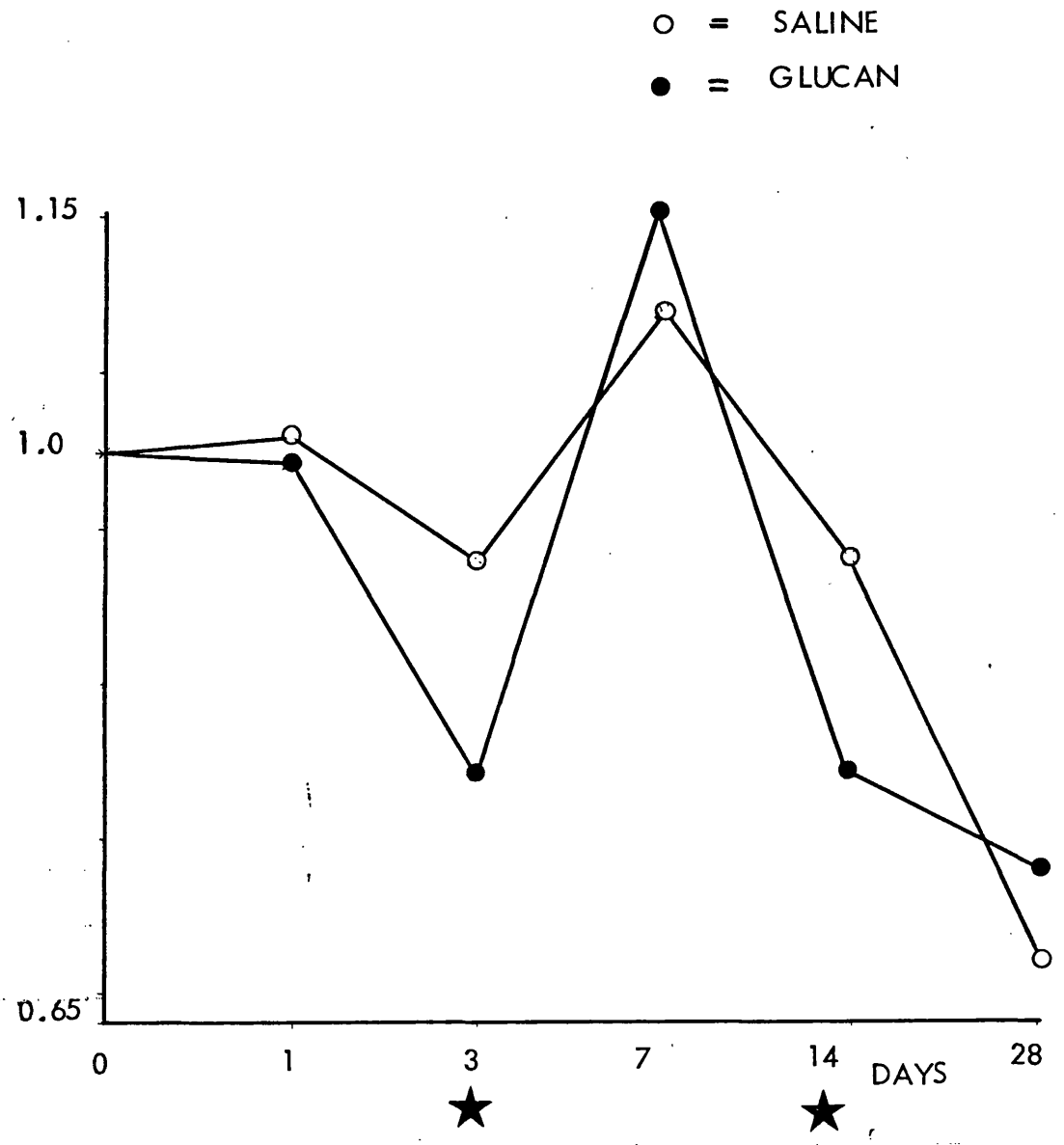


FIG 14 (TABLE 29) THE EFFECT OF GLUCAN (10 mgs/kg) COMPARED TO SALINE (CONTROLS) WHEN ADMINISTERED WITH 5FU (60 mgs/kg)
★ $P < 0.05$

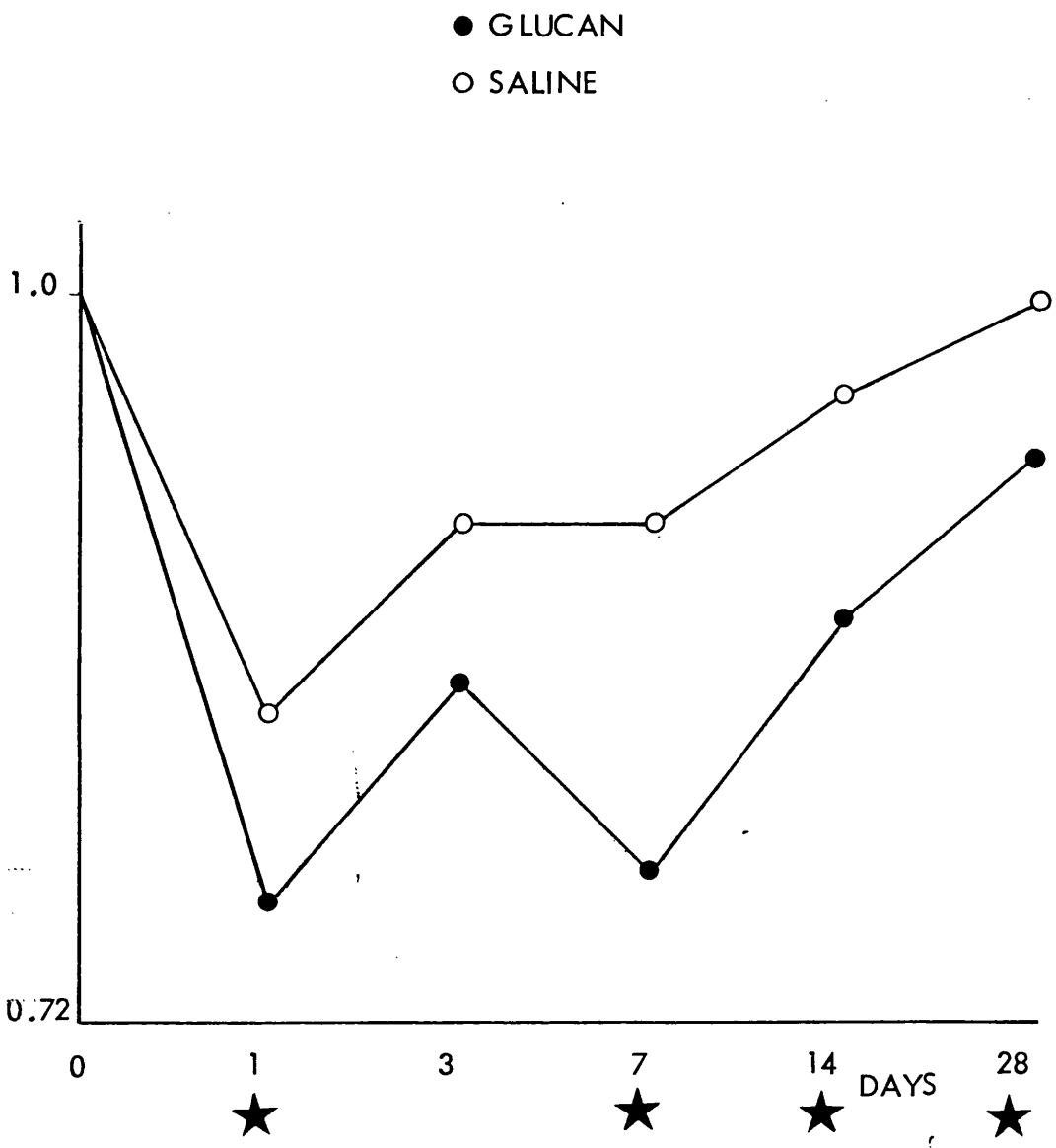


FIG 15 (TABLE 31) THE EFFECT IN TUMOUR BEARING ANIMALS OF GLUCAN (10 mgs/kg) COMPARED TO SALINE (CONTROLS) WHEN ADMINISTERED WITH CYCLO (40 mgs/kg) ★ $P<0.01$

In order to investigate the role of timing on this effect, one group of rats were given glucan 3 days before cyclophosphamide (8 mgs/kg) (Fig 16,Table 32). Significant suppression of the PHA response was produced on days 3 and 7,compared to controls receiving cyclophosphamide alone. In this experiment a difference in mean control values (45382 to 62468) may have masked a greater depressive effect occurring on days 1,14 and 28. In a second experiment the administration of glucan was delayed until 3 days after cyclophosphamide and some depression was again seen (Fig 17,Table 33). This experiment was combined with a third group of rats who also received levamisole (Table 33c). Consequently each subgroup consisted of only 7 animals,and this smaller number led to greater variation which may explain the surprising result on day 7. It seems reasonable to conclude that neither of these alterations in the timing of glucan administration prevented its negative effects. The combination of levamisole with glucan also failed to influence this phenomenon (Table 33c).

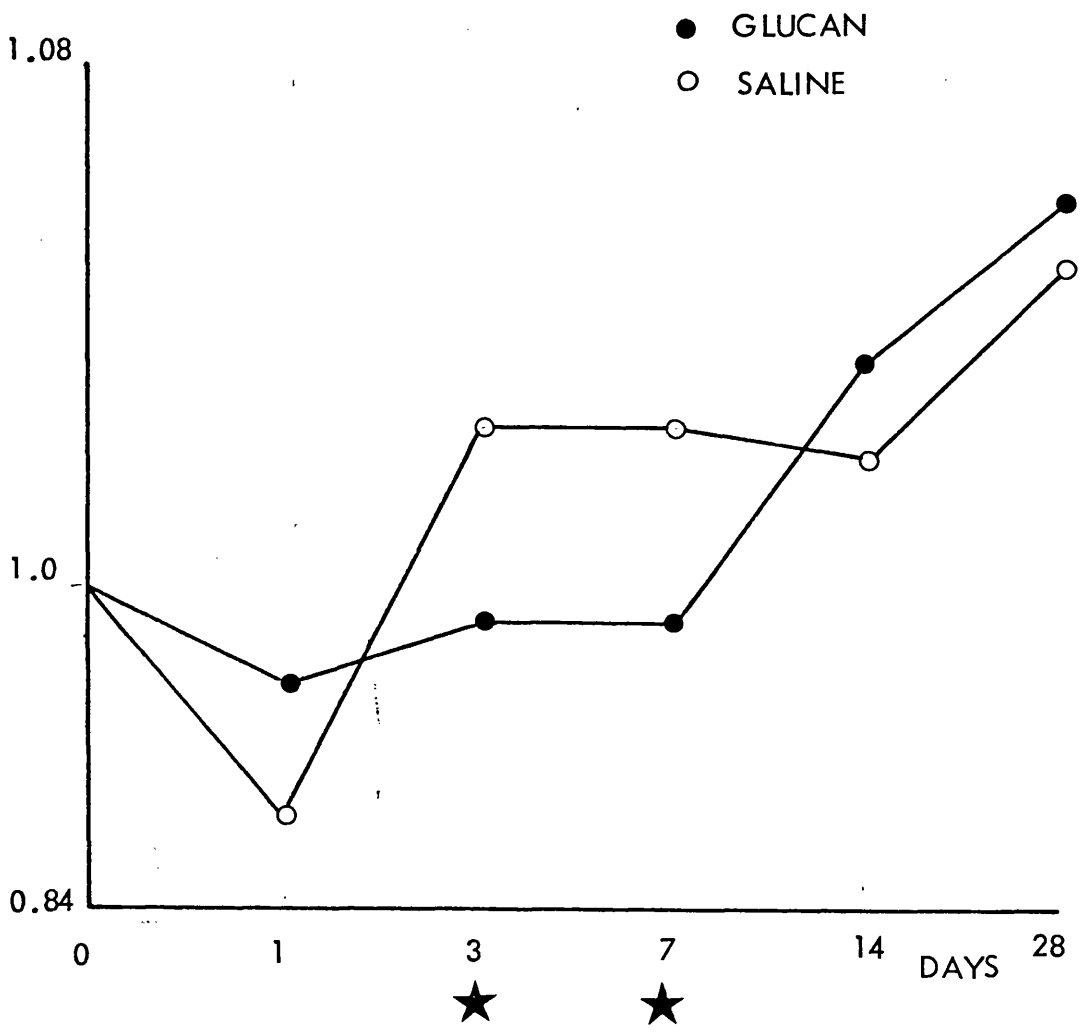


FIG 16 (TABLE 32) THE EFFECT OF GLUCAN (10 mgs/kg) COMPARED TO SALINE (CONTROLS) WHEN ADMINISTERED 3 DAYS BEFORE CYCLO (8 mgs/kg) ★ $P<0.05$

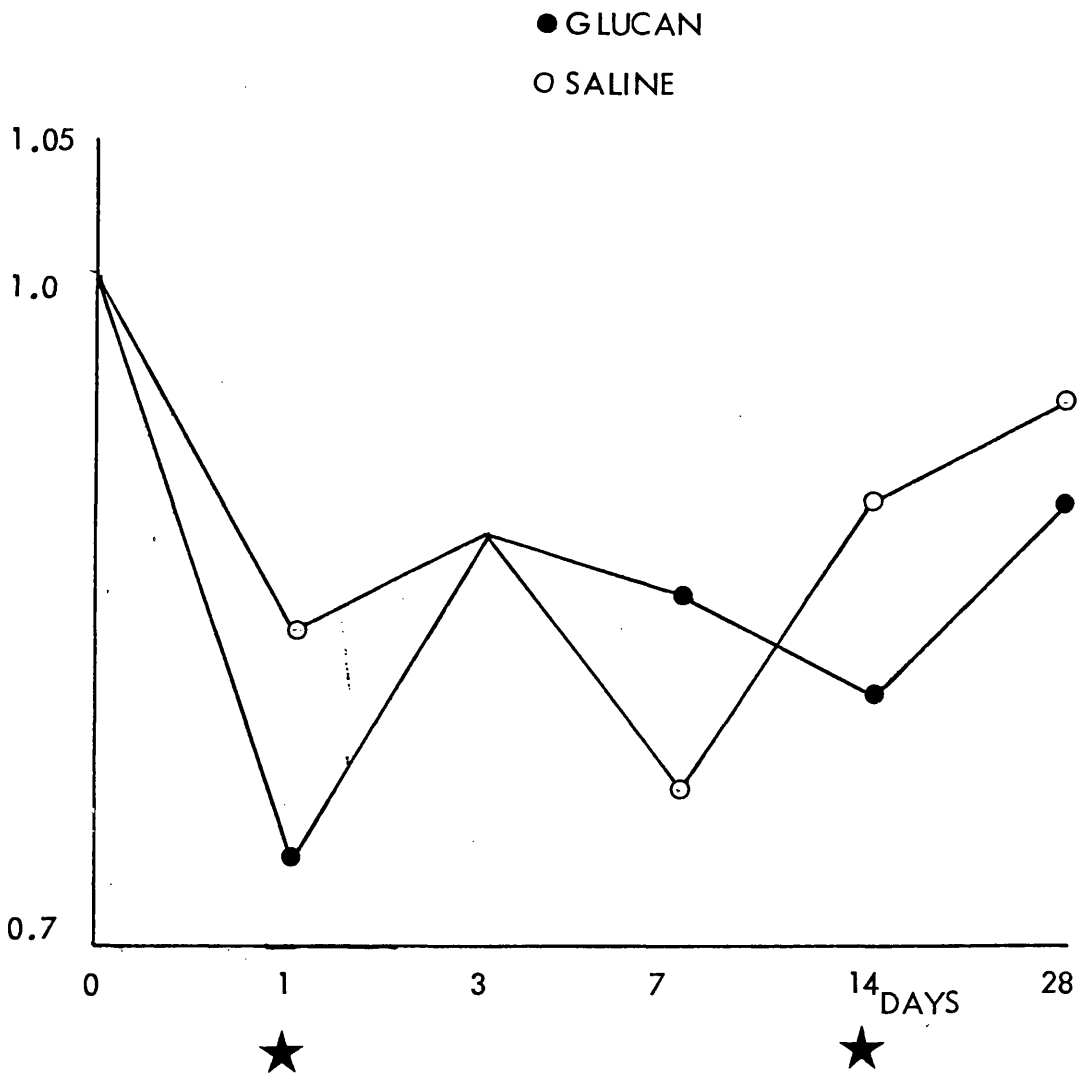


FIG 17 (TABLE 33) THE EFFECT OF GLUCAN (10 mgs/kg) COMPARED TO SALINE (CONTROLS) WHEN ADMINISTERED 3 DAYS AFTER CYCLO (8 mgs/kg)
★ $P<0.05$

The mechanism of this depressive effect was studied in one group of animals who reveived glucan alone. They were compared to a control group receiving normal saline (IV) to ascertain whether the depression was an artefact of the method. There was no significant difference between subsequent PHA responses from either group (Fig 18,Table 34). Secondly white blood counts were followed over four weeks in groups given either cyclophosphamide alone or in combination with glucan (Table 13). Glucan did not influence the marrow depression caused by cyclophosphamide, and in fact was associated with marginal protection. This suggests that its effect on the PHA response was not mediated by an alteration in circulating lymphocyte numbers, and implies a funcional change.

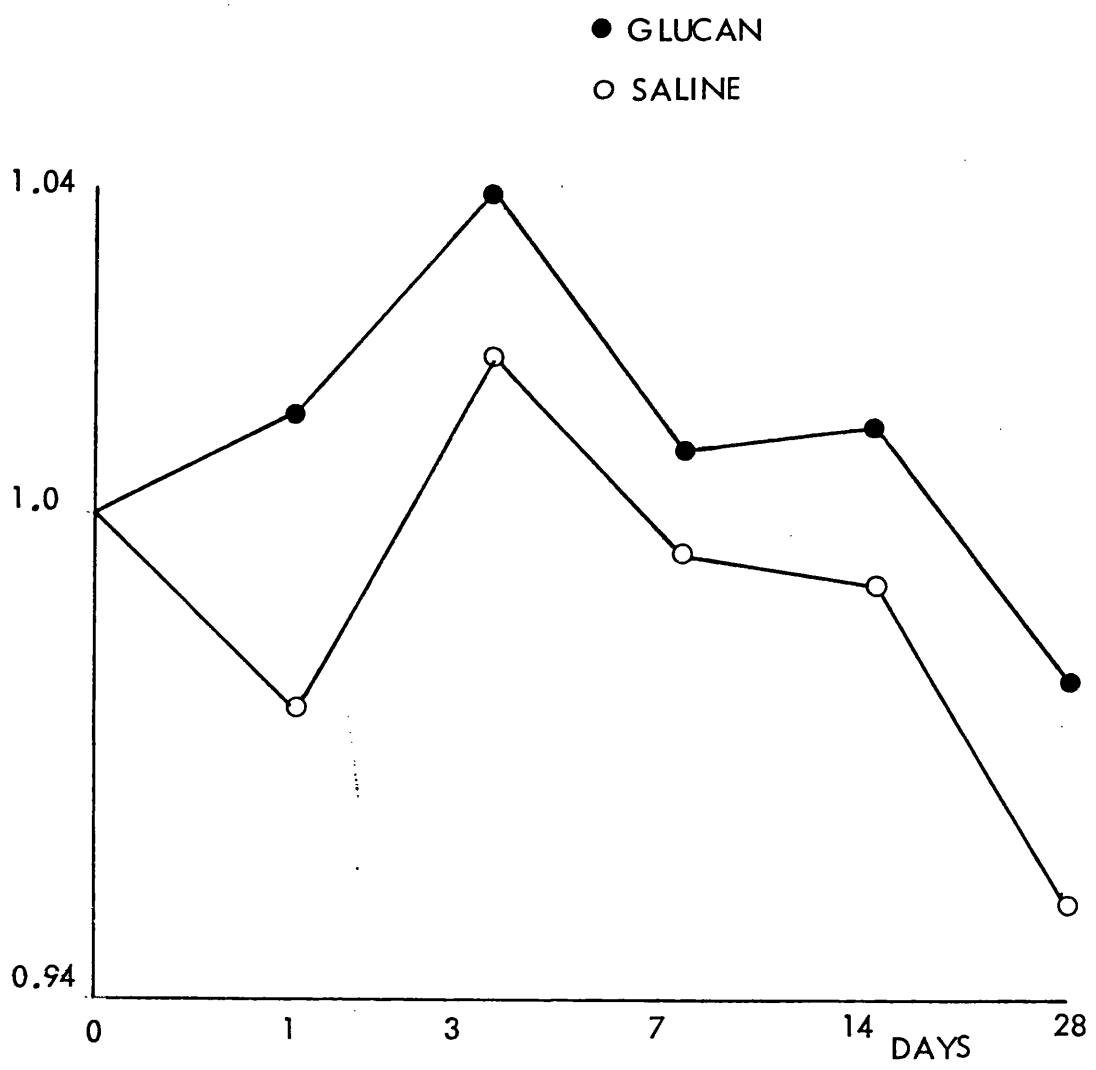


FIG 18 (TABLE 34) THE EFFECT OF GLUCAN (10 mgs/kg) COMPARED TO SALINE (CONTROLS)

For completeness and comparison,these experiments were briefly repeated with C parvum. Confirmation of the established depressive effect of C parvum alone is seen in Fig 19 (Table 35). However when given with cyclophosphamide 8 mgs/kg it produced a dramatic depression of subsequent PHA responses (Fig 2Ø,Table 36).

A third macrophage stimulating agent was tested - thiabendazole.This was given 3 days after 5FU and no effect at all was seen on the subsequent PHA responses (Fig 21,Table 37).

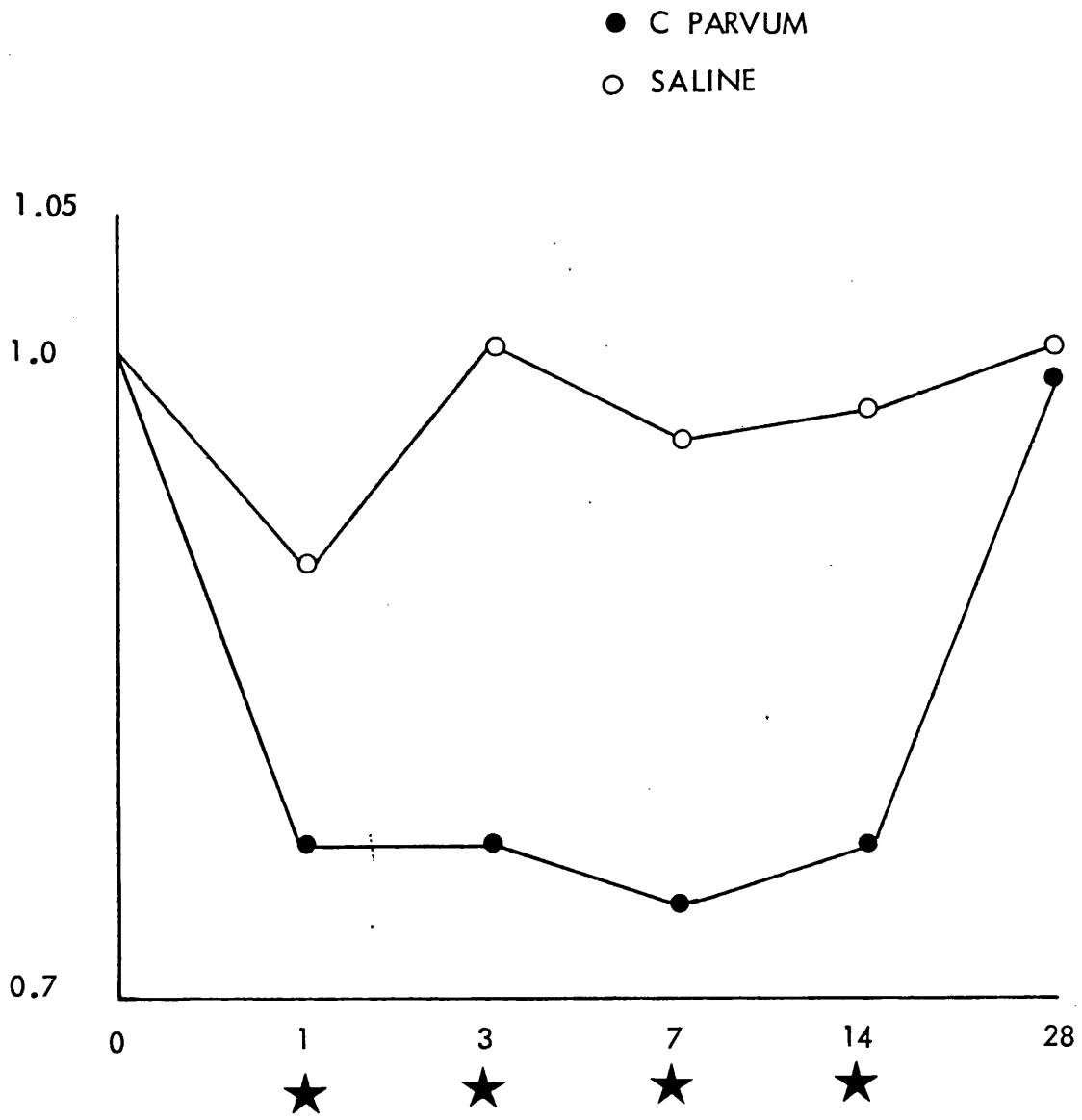


FIG 19 (TABLE 35) THE EFFECT OF C.PARVUM ALONE (1 ml) COMPARED TO SALINE (CONTROLS) ★ $P < 0.01$

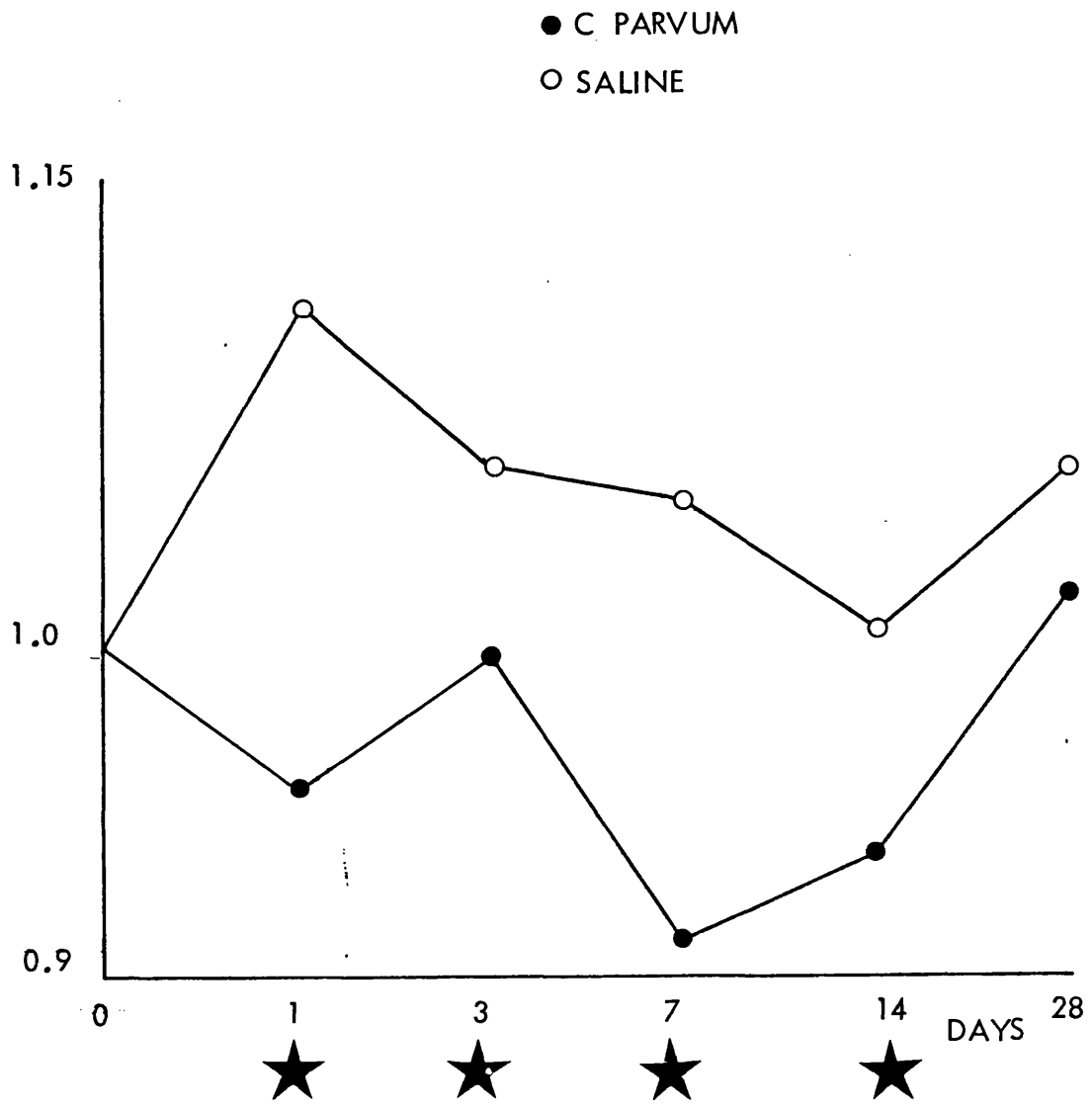


FIG 20 (TABLE 36) THE EFFECT OF C.PARVUM (1 ml) COMPARED TO SALINE (CONTROLS) WHEN ADMINISTERED WITH CYCLO (8 mgs/kg)
★ $P < 0.001$

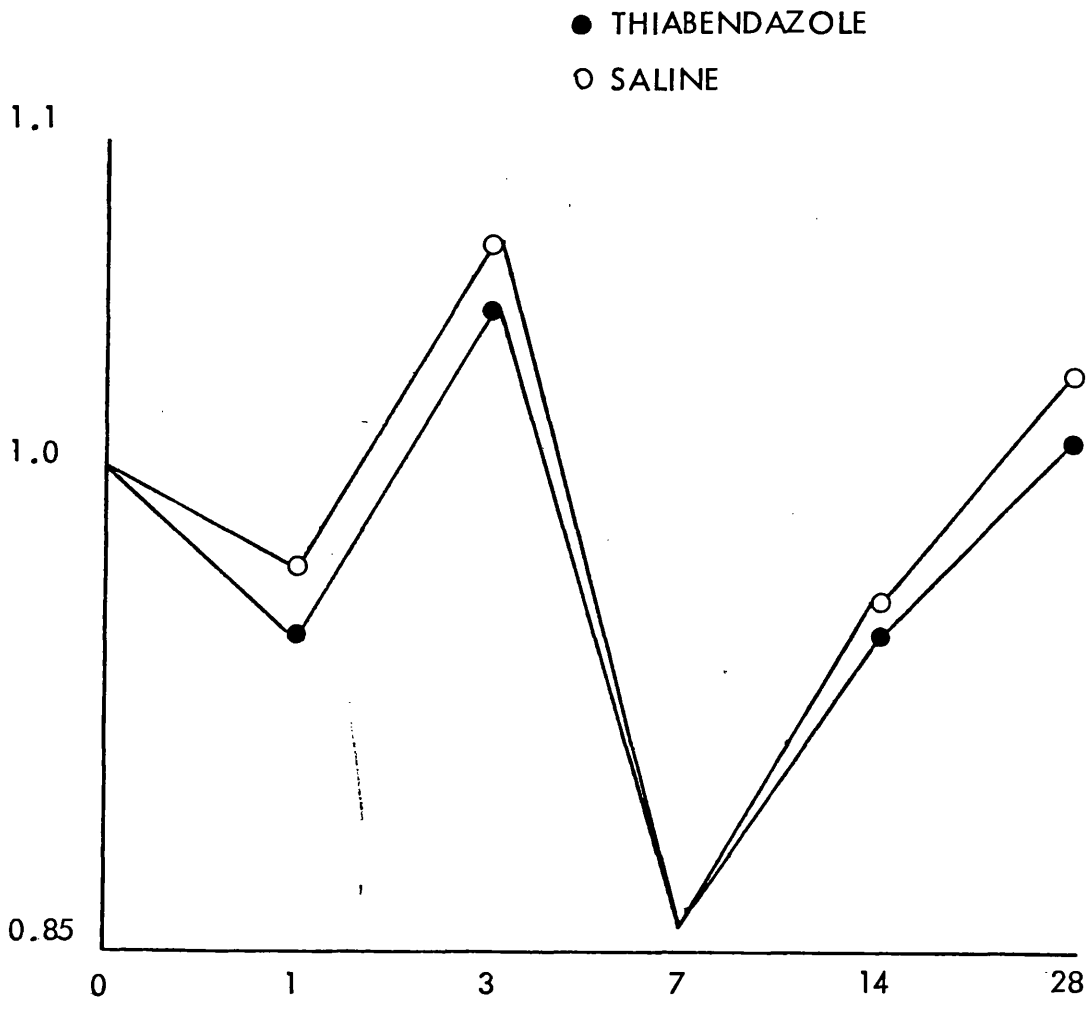


FIG 21 (TABLE 37) THE EFFECT OF THIABENDAZOLE (5 mgs/kg) COMPARED TO SALINE (CONTROLS) WHEN ADMINISTERED 3 DAYS AFTER 5FU (60 mgs/kg)

Conclusions

1) Glucan further depresses the in vitro T cell response,when given in conjunction with the chemotherapeutic agents tested. This occurrs in both normal and tumour bearing animals. However no effect of glucan was seen in the DTH responses of either group.

2) The depression of PHA responses is not prevented by a few days variation in the timing of its administration. It is not influenced by levamisole.

3) This effect is also seen with C parvum but not with thiabendazole.

CHAPTER 4

OTHER MANOEVRES

Priming

Recent reports have suggested that the haematopoietic and gastrointestinal effects of major chemotherapy may be reduced by the prior administration of a small 'priming' dose of one chemotherapeutic agent. This concept has so far been applied only to very high dose chemotherapy regimens in man. There are few established guidelines to the choice of agents, dosage and timing. In their original description Hedley et al (1978) used a priming dose of cyclophosphamide 7 days before a large dose of melphalan, in 7 patients with advanced malignant melanoma.

Therefore a small dose of cyclophosphamide (4 mgs/kg) was given to one group of rats five days before the main dose of 40 mgs/kg. A control group of animals received first an equal volume of saline, then the same dose of cyclophosphamide. Blood was taken for testing immediately before each injection, and at the usual intervals following the main dose. The priming dose produced no alleviation of the depression caused by the main dose of cyclophosphamide (Fig 22,Table 38). The DTH response was tested from the day of the larger dose, and was not affected by prior priming (Table 39).

A second experiment tested the concept using 5FU. The protocol was identical with a dose of 15 mgs/kg used to prime, five days before a main dose of 60 mgs/kg. No

difference was seen in subsequent PHA responses between these rats and their controls (Fig 23,Table 40).

In a third experiment alterations in the time delay were assessed. Groups of 10 animals received a priming dose of cyclophosphamide 4 mgs/kg at intervals of up to 14 days before the main dose of 40 mgs/kg, which they all received on the same day. Table 41 shows the results of the PHA responses measured over the subsequent month. These can be compared as absolute values,or as ratios both to pretreatment or to prepriming values. Whichever method is used,no benefit was found from priming at any of the time delays,when compared to controls. The only statistically significant difference between groups is depression in those primed at five days,which was not seen in the first experiment and may simply represent random data variation.

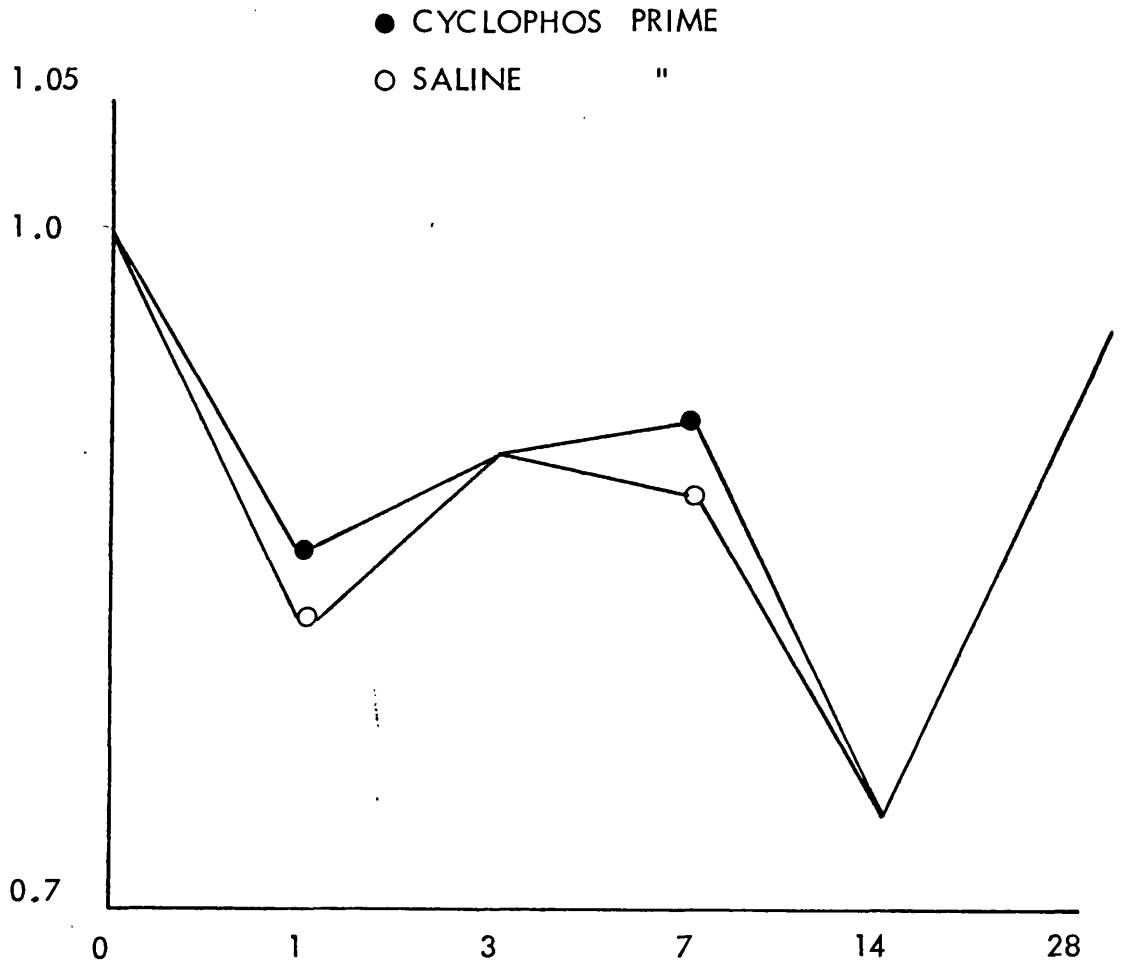


FIG 22 (TABLE 38) THE EFFECT OF A CYCLO (4 mgs/kg) PRIME COMPARED TO SALINE (CONTROLS) WHEN ADMINISTERED 5 DAYS BEFORE A SECOND INJECTION OF CYCLO (40 mgs/kg)

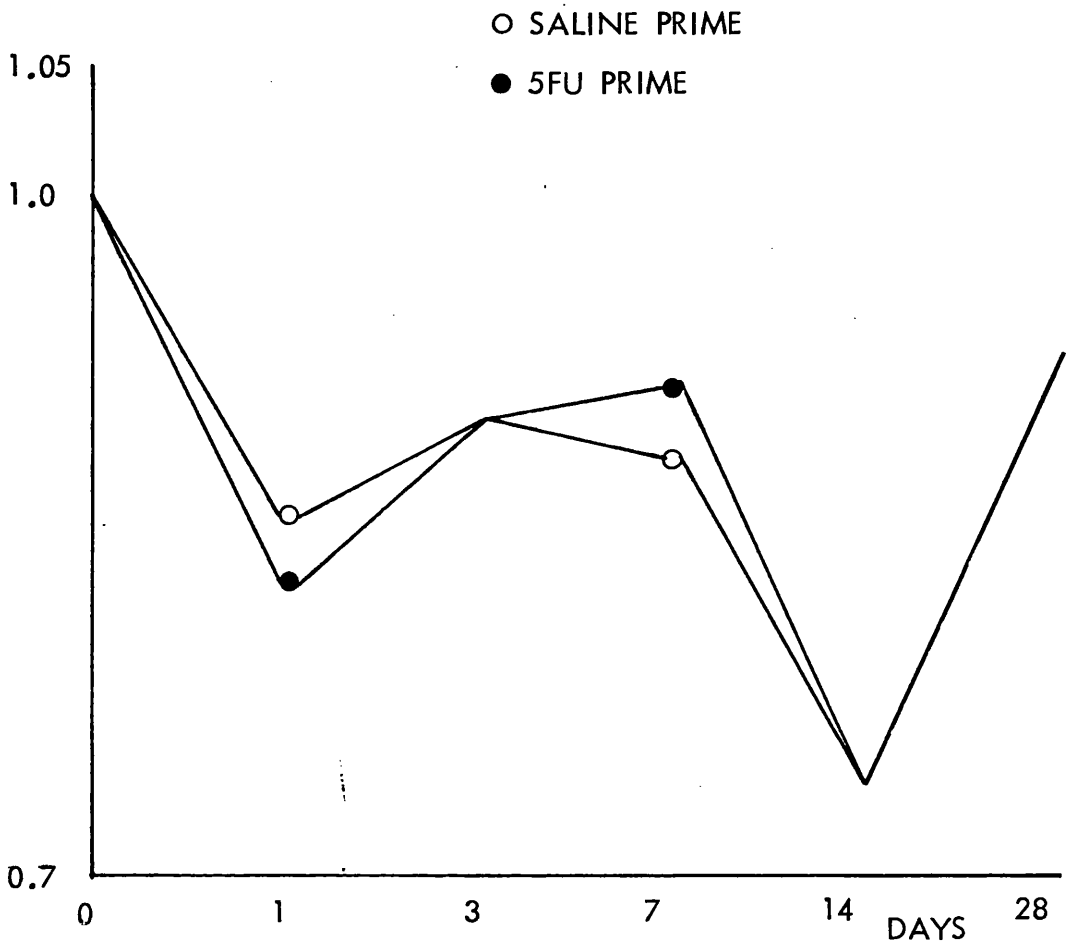


FIG 23 (TABLE 40) THE EFFECT OF A 5FU (15 mgs/kg) PRIME COMPARED TO SALINE (CONTROLS) WHEN ADMINISTERED 5 DAYS BEFORE A SECOND INJECTION OF 5FU (60 mgs/kg)

Cimetidine

Recent animal experiments with tumour transplantation have claimed that the administration of cimetidine can lead to some retardation of tumour growth and metastasis (Gifford et al 1981, Osband et al 1981). It is proposed that this effect occurrs by an immunological action of cimetidine. Therefore the effect of this drug was tested in two groups of rats following one dose of 5FU.Three days later one group received cimetidine by IP injection twice daily for two weeks. This was felt to be the most reliable route of administration with best control of dosage. A delay was included in the protocol in case stimulation from cimetidine led to increased toxicity from 5FU,and because such timing appeared beneficial with levamisole.

The administration of cimetidine led to no beneficial effect on the subsequent PHA responses. These were in fact consistently lower in that group, by a difference which was never statistically significant (Fig 24,Table 42).

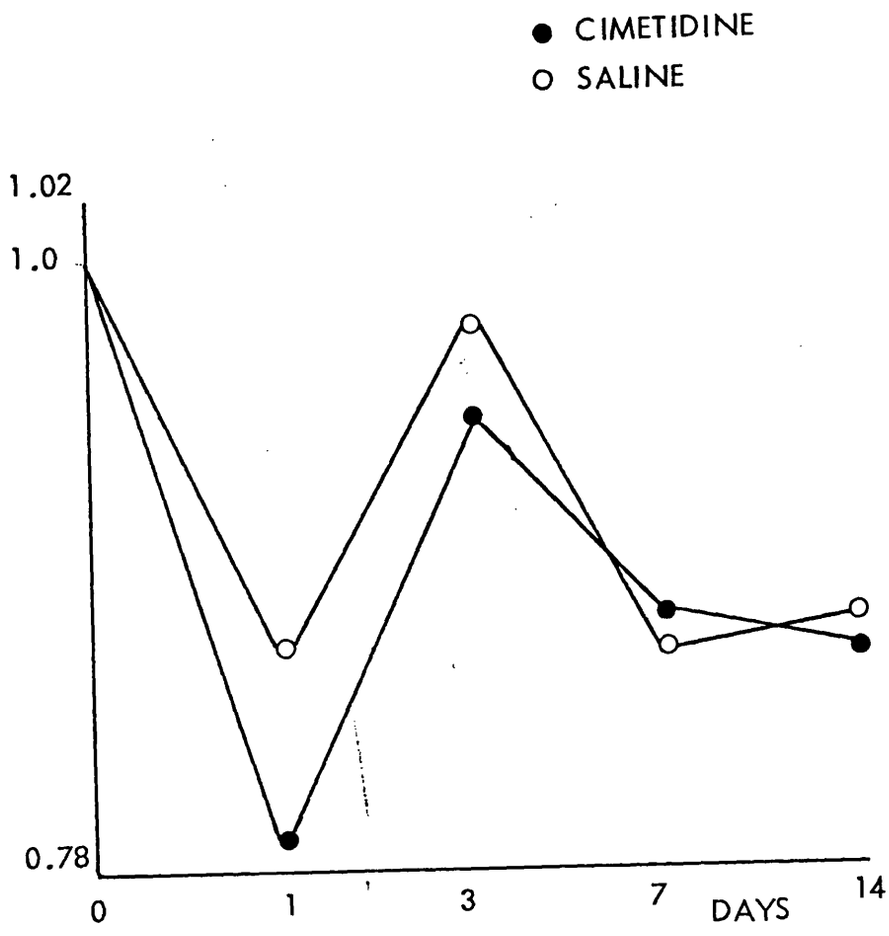


FIG 24 (TABLE 42) THE EFFECT OF CIMETIDINE (2 mgs daily) COMPARED TO SALINE (CONTROLS) WHEN ADMINISTERED 3 DAYS AFTER 5FU (60 mgs/kg)

Diurnal Rhythm

Using transplantable tumours Hallberg et al (1980) have suggested that markedly higher response rates of rats to CT may be seen when administered in early dark phase of their diurnal cycle. This phenomenon was investigated in an experiment for which two groups of animals were prepared by identical and regular daily lighting routines for 3 weeks. Clearly it was essential for each to be tested at the same time of their days to eliminate differences between them due to diurnal variation, and for tests to be performed simulatneously on each group to eliminate experimental variation. Therefore the animals were conditioned to the same light cycles, and injections of cyclophosphamide 40 mgs/kg were given to one group at 10 a.m. (2 hours after light phase),and the other at 10 p.m. (2 hours after dark phase). Measurements began with the next morning as day 0.

Fig 25 (Table 43) shows that no difference was seen in the depression of PHA responses by either group. The DTH response was also similar in both groups (Table 44).

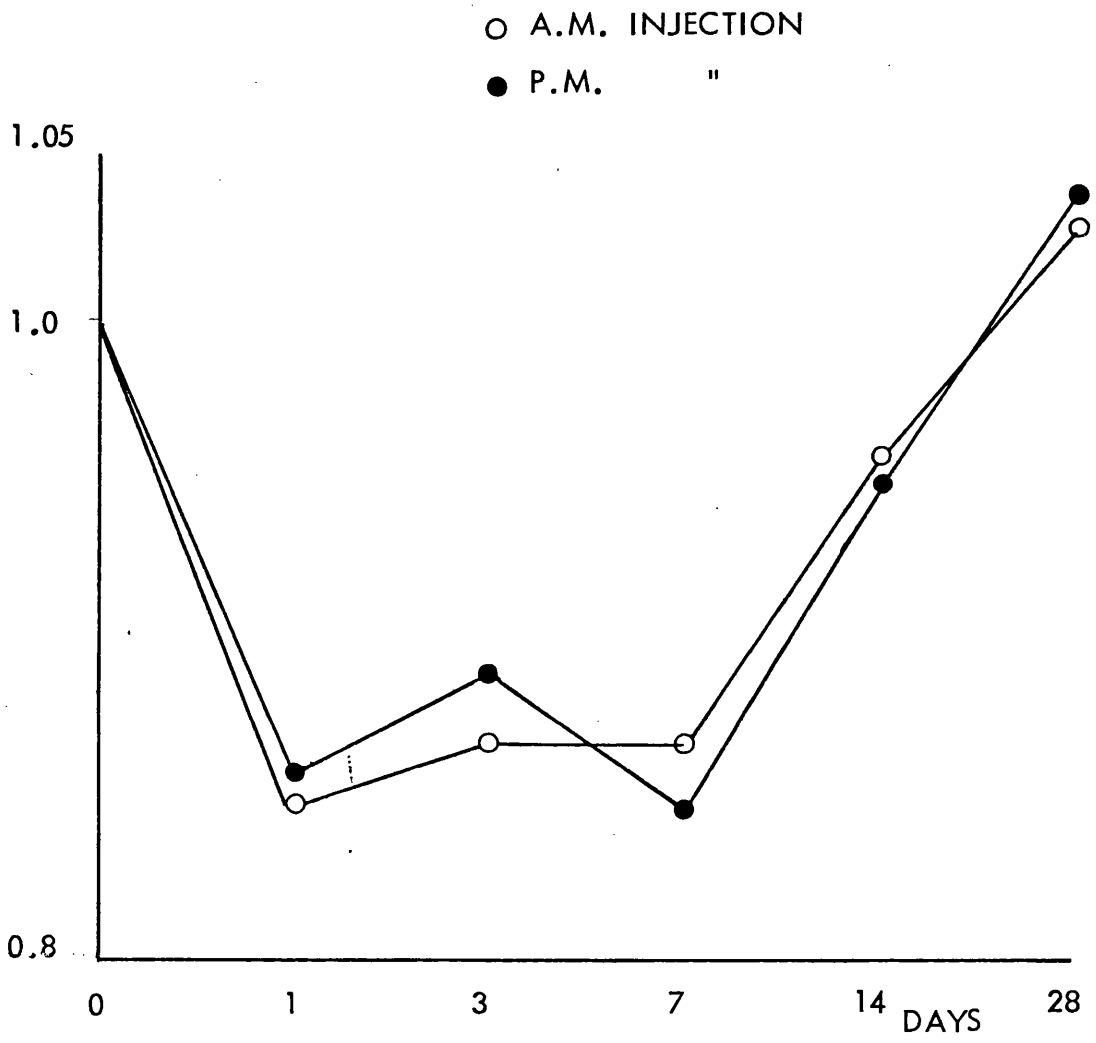


FIG 25 (TABLE 43) THE EFFECT OF ONE INJECTION OF CYCLO (40 mgs/kg) WHEN ADMINISTERED AT OPPOSING PHASES OF THE LIGHT CYCLE

Conclusions

1) Priming before large doses of cyclophosphamide or 5FU does not afford immunological protection as assessed by the tests used. No effect was seen from variation of the priming interval for cyclophosphamide.

2) Regular administration of cimetidine did not alleviate immune depression following one injection of 5FU.

3) The timing of administration of cyclophosphamide at different phases of animals' diurnal rhythms did not affect subsequent immune depression.

DISCUSSION

CONTENTS

Introduction

This study has confirmed other animal work showing marked immune depression following cancer chemotherapy. This phenomenon has also been described in man and has been discussed. It appears to be an inevitable consequence of the systemic use of such treatments, and this is generally required to attack the potentially widespread nature of spread in most cancers.

There have been several different approaches to the use of these agents which may not cause such immunological effects ,but none of these is likely to affect the general problem. The local application of cytotoxic agents is only suitable for a few tumours (such as bladder cancer and malignant ascites), and is rarely curative. Regional perfusion of drugs by arterial injection has generally failed to prove of clear benefit (Cline and Haskell 1980). An interesting exception is regional cytotoxic perfusion of the liver,principally in cases of colonic cancer. This has been shown to be capable of some significant local action both in treatment and prevention of metastases (Taylor 1981,Reed et al 1981), but the somewhat marginal overall gains obtained have not yet led to its clinical acceptance. The effect is probably due to high local cytotoxic concentrations,but systemic immune depression may well be minimised by this technique and therefore contribute.

Finally a very brief course of systemic CT may have a place in certain situations. In a randomised series of over one thousand women with breast cancer, Nissen-Meyer et al (1978) tested the effect of a 5 day course of cyclophosphamide following mastectomy. This produced a 10% improvement in recurrence free and surviving patients compared to untreated controls. This treatment is now undergoing further clinical trial,but appears to offer comparable benefit to many aggressive and protracted regimens of adjuvant CT for breast cancer. There are a number of possible explanations for this but the minimising of immunosuppression may well be an important factor.

In general there seems no doubt that systemic chemotherapy can effectively kill several orders of cancer cells and benefit the patient, despite simultaneously causing significant depression of the immune system. Furthermore several large clinical trials have established that the survival of women with early breast cancer after simple mastectomy is not influenced by a postoperative course of radiotherapy (Cancer Research Campaign working party 1980,Lythgoe and Palmer 1982). However this must involve major systemic immunosuppression from radiation without apparrent clinical detriment. What relevance therefore is the immune response compared to other treatments like chemotherapy? This question has recently been taken further and the value of the immune response to human cancer under any circumstance has recently been questioned.

Such doubts have arisen from two main areas. First the results of clinical trials of immunotherapy are remarkably disappointing,as has been discussed. Furthermore even in the most successful animal models of immunotherapy a clear benefit can be lost by small changes of tumour dosage and protocol. Although these problems might represent the weakness of immunotherapeutic agents in current use,they have led to more fundamental question about their worth.

Secondly a wide gap has opened between the implications of animal experiments and the realities of human cancer. Animals offer a more complete and scientifically satisfactory medium of study, and have therefore been the focus of much recent cancer research. Almost all such work has involved tumours induced with chemicals or viruses,but these are usually highly immunogenic compared to spontaneously arising tumours which are generally not (Klein 1980). Consequently it is not clear how artificial are modern developments in the understanding of cancer immunology,most of which are based on such models.

This question is further complicated by a few studies which suggest that demonstrable immune responses do not necessarily confer benefit. Valid protection can be seen against many induced animal tumours, but some grow apparently unimpeded (Klein 1980). Furthermore a few examples of facilitation of tumour growth by antitumour immunity have been mentioned (Prehn 1978). Conversely it

may be argued that if immunological surveillance occurs,any cancer which becomes established may represent a relatively non-immunogenic variety which has escaped. Fortunately there is no confirmation of these concepts in man. Furthermore evidence has already been outlined which shows that a number of specific immunological responses to cancer are demonstrable in man. Therefore it seems reasonable to conclude that there is a potentially valuable immune response to cancer in man,but that its value remains to be clearly proved and may vary between individuals.

What can be said for immunotherapy if tumour immunology itself is regarded as unpredictable and of unproven benefit. Certainly clear clinical benefits from immunotherapy would establish its value beyond doubt. Most attempts at immunotherapy have been not been tumour specific but aimed at the general restoration of inadequacies in the immune response. Three contrasting views for the place of such therapy have been proposed.

First,that such an approach should be most applicable to patients with advanced disease since they are most likely to be immunologically depressed. However it is probably unrealistic to expect a significant impact from the immune system on such a large tumour burden. Furthermore spread of such disease may imply the development of certain means by which the cancer has escaped normal immunological reactions.

Consequently the second view is that immunotherapy should be aimed at patients with a manageable number of malignant cells,such as after potentially curative surgery ('minimal residual disease'). However such immunological profiles as are currently available do not suggest that these patients are failing immunologically. If their immune response is compromised at a more detailed level by mechanisms such as blocking factors or altered suppressor cell activity,it may prove more appropriate to identify and tackle these individually.

Third,immune stimulation may be most effective when there is a small number of residual tumour cells but the host's immune responses are depressed by treatment. This situation will only apply with 'Minimum Residual Disease' when relatively normal anti-tumour responses may be depressed by surgery or adjuvant therapy. Adjuvant chemotherapy may offer the most suitable treatment to demonstrate potential, since its effect is to reduce the number of residual cancer cells by several orders and also to depress the immune response markedly.

Since there are now many doubts about the value of immunotherapy it is important to test its worth where there is most chance for gain. It is also esential to begin by developing such therapy through animal models followed by human study to measure its immunological benefit. This has all too frequently been omitted in many clinical trials which have shown no benefit from regimes

which were never clearly established to be immunotherapeutic. For these reasons this study was undertaken.

It may be considered simplistic to have studied T cell function alone since increasing complexity is constantly revealed in the details of each arm of the immune response and their interactions. However limitation is necessarily imposed on animal experimentation by the size of blood samples possible and the complexity of each test. T cell function probably remains one of the most important aspects of the immune response to cancer,and it seemed more important to study one facet of this thoroughly. Developments suggested by research into different immunological functions could be tested in this context, and any encouraging possibilities from this passed on for assessment in others. Only in this way can a composite picture be constructed of the full effects of immunotherapeutic manoevres on the immune response. A number of different approaches to restore this function have been used. A few were based on tentative or speculative ideas which have not proved rewarding,but progress can only come from such attempts. It was considered important to concentrate primarily on the effects of chemotherapy on the normal immune response,since this appears to be the situation of most patients receiving adjuvant chemotherapy.

It also seemed appropriate to examine these effects in tumour bearing animals.In this situation the immune

response may be significantly altered. There is no published information regarding specific immune responses to the tumour chosen, but their existence is very probable since they have been described in such a wide variety of chemically induced animal tumours (Klein 1980). This tumour was chosen because its behaviour was reported to be most similar to human breast cancer,including the development of metastases which is most unusual for induced breast cancers (Gullino 1975). However the author has not as yet found metastases in animals followed for up to twelve months after mastectomy,and this is consistent with another report (Wilson personal communication 1980). Nevertheless the model proved quite satisfactory for the purposes of this study.

A single bolus of CT was used since the object of the study was primarily immunological and this aspect could therefore be studied more precisely. It was considered unimportant that these regimens produced no clear influence on tumour size (although there was a trend towards reduction after one week). Drug regimens capable of producing objective response or cure in animals nearly always involve more toxic doses administered daily for 5-7 days. Clearly immunotherapeutic manoevres should also be tested in this context, but only after they have been developed and proven. The cytotoxic drugs used were chosen because they are probably the most important representatives of the two principle types of agents in common use, both in single and combined regimes.

Methods

The response to PHA has been widely used as a test of T cell lymphocyte function. Whilst its mechanism is undoubtedly complex and involves other cells (Hollter and Jarrett 1978,Rosenstreich and Misel 1978), it is still accepted to primarily reflect this aspect of the immune response. It is open to the criticism of all in vitro tests that it does not represent a process which may in fact occurr in vivo, although the whole blood technique used offers a somewhat less artifial mode. The test is often performed on seperated white cells,but whole blood methods are well recognised and equally reflect states of altered immunity (Hall and Gordon 1976,Han and Pauly 1971). Whole blood techniques require much smaller quantities of blood so that repeated samples can be taken from one animal. It may also be argued that conditions of maximal cell stimulation do not bear any relation to in vivo responses nevertheless there must be intrinsic changes to explain differences observed and these are born out by the clinical associations where they are noted.

It has been suggested that several doses of PHA should be employed simultaneously in order to reveal subtle changes of response patterns at suboptimal levels of stimulation (Whitehead et al 1975). This approach does not detract from the use of maximal PHA stimulation - which has been most widely used - but offers a means to

identifying a few extra differences. Since it was not possible to repeatedly obtain sufficient blood for such an approach, it was felt that maximal stimulation would most clearly show the greatest proportion of differences seen at one concentration.

The test proved technically satisfactory since a fifty-fold increase of 3H-thymidine incorporation was measured in PHA stimulated wells compared to control. On any given day values for individual rats were usually very consistent.However there was considerable day to day variation. As a result it proved essential to plan all experiments as comparisons to simultaneous control groups of animals, in order to eliminate this error. The calculation of results has been explained and although complicated seems to be the most logical and precise. It led to data showing marked and consistent changes of response following chemotherapy,which implied that the method was a sensitive measurement of T cell function.

The delayed-type hypersensitivity (DTH) test did not prove so precise. Measurements made in vivo are potentially more important than those made in vitro, but are notoriously difficult to reproduce consistently and interpret. The DTH response was chosen because it has well been established as a method of functional assessment of T cells activity and represents a clear response to antigenic stimulus. Oxazolone has been one of the most commonly used agents for this. The use of ear measurement

offers an appealing method of quantitating the response which other techniques do not provide.

A marked response appeared following the aural application,which usually led to at least a 50% increase in ear thickness. The swelling produced was significantly reduced following one injection of cyclophosphamide,and this was almost exclusively used in the experiments involving this test. However there was a wide variation in the magnitude of the response, and subsequently no clear differences were seen between any of the paired experimental groups. There are two possible explanations for this. First,the test may be too insensitive to measure small but significant effects. The use of simultaneous controls throughout all groups excluded some variables (such as a diurnal rhythm),and great care was taken to ensure continuity of observer for all parts of each experiment. However despite considerable efforts some variation was inevitable from technical factors such as ear thickness, dosage actually received and sites and pressures of measurement. The use of groups of at least 10 rats should have led to a more equal distribution of these variables between groups so that overall comparisons could be made. The second possible explanation is that no significant immunotherapeutic benefit was obtained from any of the manoevres employed. These two tests must be interpreted together,but the wide distribution seen in the DTH response implies that a small effect could not be detected by this test,and that no large ef-

fects can have occurred.

One disadvantage of this test is that repeated measurements cannot be made on one animal within the experimental period. Furthermore maximal depression of responses are seen in the first 24 hours (Turk 1980), which may not be the only time period of interest. A variety of studies have previously been described which involve the use of other tests suggesting that the immune effects of chemotherapy last longer than one day. This type of problem exists for most in vivo tests of T cell function,and supports the value of in vitro testing.

The Model

Measurements of PHA stimulation show that in the model used pronounced immune-depressive effects are seen following one bolus of chemotherapy in doses equivalent to those used in patients. These effects last for at least a month and appear to be unrelated to circulating white cell numbers. This implies that they are indeed cytotoxic effects on functional cells,an observation confirmed to some extents by the reduced DTH response measured following cyclophosphamide administration.

The rebound overshoot phenomenon (ROP) has been clearly seen following 5FU in both normal and tumour-bearing animals. It has previously been observed in humans following combination CT and correlated with a favourable prognosis (Serrou and Dubois 1978). Although this might only be an epiphenomenon,it represents a worthwhile focus of attention for immunotherapy. The above authors have proposed that it represents immunological release from blocking by antibodies (or immune complexes) which have been inhibited by CT. This is contradicted by its clear occurrence in this study in normal rats to an extent which equals that seen in tumour bearing animals. It is interesting that it has been observed weakly and not at all following cyclophosphamide,but clearly and consistently after 5FU. This is a cycle specific agent whereas cyclophosphamide is relatively non-specific and toxic to cells in nearly all phases. These observations

suggest that the ROP may represent an alignment of lymphocyte cell phases by chemotherapy administered. Its occurrence in patients with better progoses may therefore reflect their more healthy immune system. This deserves claricatión.

MANIPULATIONS

Levamisole

The effect of levamisole was disappointing. When given in conjunction with 5FU or cyclophosphamide it did not influence the ensuing depression of PHA responses. However there was a consistently beneficial effect on PHA responses if 3 days elapsed before its administration following 5FU,but not cyclophosphamide. This was seen in both normal and cancer rats. However the effect barely reached statistical significance. Such improvement was not seen in the DTH response but neither was there a clear reduction of this at that time (4 days later).

These results are consistent with many reports of a restorative effect of levamisole on depressed T cell function, without apparent stimulation when given alone. Perhaps they are also consistent with the marginal nature of clinical benefits that accrue from its use in conjunction with CT. The effect of timing of administration is interesting and underlines the need for studies of this type,in which immunological benefit is precisely measured to define optimum conditions before trials of immunotherapy in patients. Nearly all clinical trials of levamisole have involved its commencement at the same time as CT,which may not be desirable. This point could be elucidated by study of a relatively small number of patients. Nevertheless a crude quantitative estimate from

these experiments might indicate that such refinements will not dramatically alter the usefulness of levamisole in this situation.

Glucan

The addition of glucan markedly increased depression of the PHA response following the administration of either cytotoxic agent. It did not influence the DTH response following cyclophosphamide. This has not previously been described,and in fact there are few reports of the effect of glucan on T cell functions. One (Kitagawa et al 1975) describes alleviation of T cell depression in tumour bearing mice by lentinan alone (a branched beta-glucan).

It is possible that the observed effect was an artefact of the method, perhaps through stimulation of monocyte function. Both an increase and a decrease of monocyte numbers has been shown to reduce 3Hthymidine incorporation in PHA response assays (Rosenstreich and Misel 1978,Hollister and Jarrett 1978). However these require alterations of several orders of monocyte numbers,and glucan has not been shown to produce such effects. Furthermore the PHA response was not affected in animals receiving glucan alone. The antitumour activity of glucan has been attributed to its stimulatory effect on the size and phagocytic ability of the RES (DiLuzio et al

1978). Thiabendazole has also been established as an agent capable of stimulating the RES (Lundy and Lovett 1978) but it did not influence immune depression following 5FU. This observation supports the contention that the observed effects of glucan are not due to any action on the MPS.

It is likely therefore that this phenomenon represents a synergistic effect of glucan with CT on lymphocyte function ,analagous to that seen to an even greater degree with C parvum. However in normal animals this property of glucan appears to be significant only when combined with CT. It is speculative whether this implies an enhancing of sensitivity to the lymphcytotoxic effects of CT. Alterations in timing of administration reduced the phenomenon but did not prevent its occurrence,and combination with levamisole did not affect it at all. This is unfortunate since it must represent an immunological loss to be weighed against potential gains in the design of chemoimmunotherapy trial protocols involving this agent. Certainly this aspect of its action must be monitored if it is put to serious clinical investigation.

Priming

Priming is a recent development in the clinical application of high dose chemotherapy. When a small dose of one agent is given several days before the main re-

gime, a significant reduction in toxicity to the marrow and gastrointestinal tract may be obtained (Hedley et al 1978). This may even permit the use of higher doses than otherwise possible. So far the regime or priming agent has not appeared to be important, although a cyclophosphamide prime has been most frequently employed (Dalton personal communication 1981). Preliminary results in mice suggest that tumour cells do not also benefit from such protection (Miller et al 1978). The mechanism of its effect is not understood,but it has been assumed that the priming dose orientates cells to at a relatively less vulnerable stage of their cycle,when the main treatment is given. The immunological effects of such a program have not been assessed.

In this study both agents were tested in priming protocols which were as clearly analogous as possible to previous reports. In a third experiment the effect of wide variations of the priming delay was investigated. Considerable effort was devoted to this project since its clinical application appeared simple,and the absence of guidelines required the testing of several protocols in detail. However no immunological benefit at all was seen from priming in any form tested. Whilst it is possible that such benefit might be be seen in another aspect of the immune response,this seems improbable. Further study of humans receiving such therapy might confirm this.

Cimetidine

Two recent reports (Gifford et al 1981,Osband et al 1981) have shown that long term administration of cimetidine to mice after tumour innoculation can prolong survival and reduce the growth and metastasis rates. This appears from slightly tenuous in vitro testing of splenic lymphocytes to be associated with blockade of suppressor cells.

In this study the depression of PHA responses following 5FU was not affected by the administration of cimetidine. The drug was given by intraperitoneal injection in order to produce a reliable daily dosage equivalent to human use. In the published reports on its effectiveness it was mixed with drinking water in a slightly unclear quantity. Its excretion is primarily renal,and any hepatic inactivation during this experiment may also be expected to have occurred following oral administration. Therefore it seems most improbable that the lack of effect observed in these experiments was related to its route of administration.

These results must be distinguished from the mouse experiments reported by Gifford et al (1981), where benefit from cimetidine was seen from apparent enhancement of the immune response in the absence of any other therapy. Furthermore it is possible that in the tumour-bearing state,suppressor cell populations are more active than in

normal animals. It is not clear what (if any) role suppressor cells play in the immune depression following CT,and whether they offer a realistic mechanism to influence such depression. Nor is it clear to what extent the in vitro PHA test reflects their function. Furthermore the effects of CT on lymphocytes may be too overwhelming to be influenced by suppressor cell activity,and the current study is consistent with this explanation. Further study of this interesting question requires the availability of satisfactory tests of suppressor cell activity which are not yet easily available. Since cimetidine appears to be a safe drug in common clinical use, it seems reasonable to study its effects on immunological parameters in patients treated with it under various circumstances.

Diurnal Rhythms

It is well established that many immunological and other bodily parameters vary in a diurnal pattern (Tavadia et al 1975). Early studies of these rhythms and the timing of administration of chemotherapeutic agents showed a potential to influence mortality from very toxic regimes (Kuhl 1973,Cardoso et al 1978). Subsequently non-fatal doses have been used in experiments with transplanted tumours. These have shown progressive changes in response to chemotherapy as the time of administration is varied over 24 hours. The difference between maximal

and minimal cure rates was 68% and 8% in one study when administration was in early dark and light phases respectively (Hallberg et al 1980,Scheving et al 1980). One report of a small clinical trial of timing has suggested a similar trend in human cancer (Focan 1979). There is no clear explanation of these patterns,and the associated immunological consequences have not been assessed.

It is difficult to design experiments concerning this question which employ simultaneous controls,without error from these being measured at different phases of their daily rhythms whenever studied. Therefore the protocol described was devised so that benefit from the (more likely) evening timing might become apparrent despite a slightly shorter interval from initiation of drug induced depression. The opposite arrangement might have suggested an effect for which the interpretation would be uncertain. Had any difference appeared a larger experiment dividing times round the clock would have been appropriate,but none occurred.

These studies do not support an immunological mechanism for the enhanced effects of chemotherapy in early dark phase. Although other aspects of the immune response may be responsible,it is unlikely since T cell function is one of the parameters with a more clearly identified rhythmicity (Tavadia et al 1975). A diurnal pattern to tumour growth has been discerned in several animal studies (Badran et al 1965,Echave-Llanos 1970),and

also observed in a few human cancers (in Focan 1979). This has followed most normal bodily parameters and is maximal in early light phase ('acrophase'). This does not provide an obvious explanation of enhanced sensitivity at the least active time of tumour growth.

It is theoretically possible that altered rates of drug conversion and elimination result in a longer exposure of tumour to the active agents,but this is not supported by the established reduction of systemic toxicity at the same time. Alternatively it is conceivable that the time taken for drug conversion and actual intracellular effect is about 12 hours.However there is little factual support for this. Further study of this potentially beneficial phenomenon in man is clearly justified, both to establish its validity and mechanism.

CONCLUSIONS

The value of the immune response to cancer in man remains to be proven. Immunotherapy is an important means of investigating this issue and its combination with adjuvant chemotherapy is one of the pricipal areas of interest. A useful model has been developed to test potential immunotherapeutic manoevres with regard to T lymphocyte function. A number of potentially useful approaches have been shown to be of little value. This illustrates the need for careful development of any method intended to provide immunological protection from the effects of CT,and the value of such testing before premature clinical trials. Research should continue into this important field in both animals and man to establish areas of immunological gain to be put to clinical trial.

APPENDIX

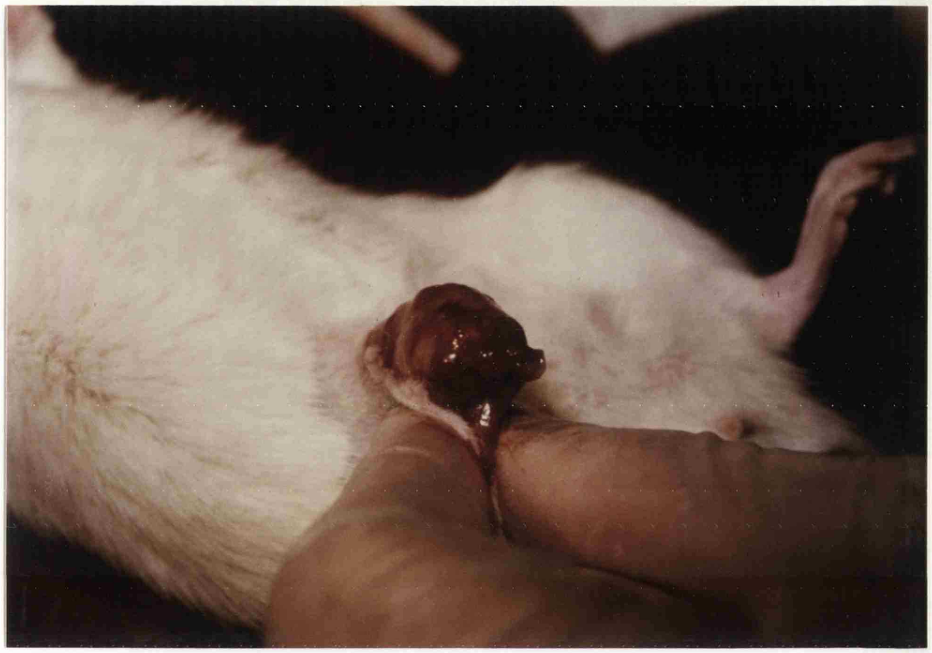


Fig 43 A breast tumour being removed

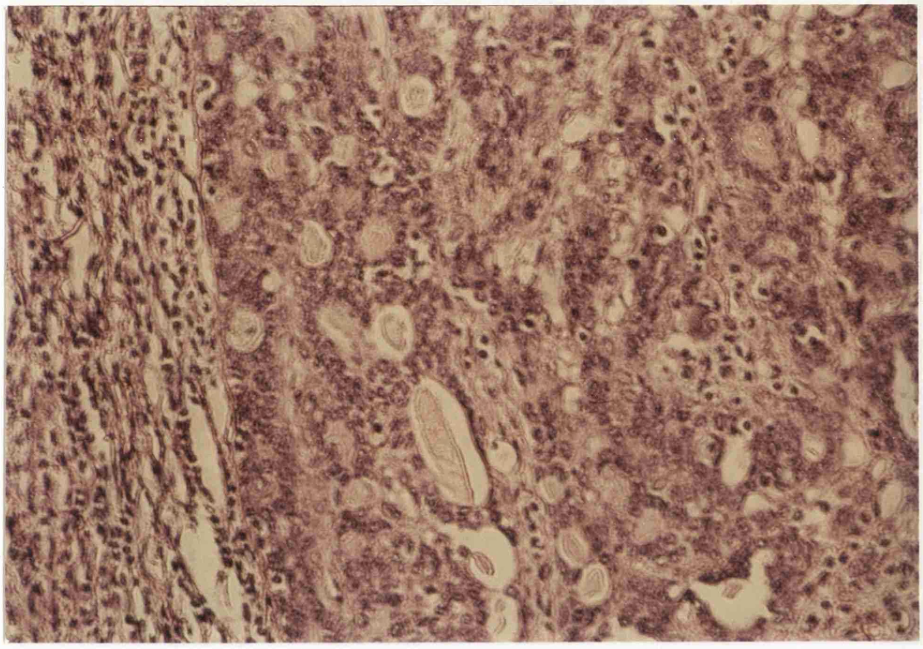


Fig 44 Histology of a relatively well differentiated breast tumour

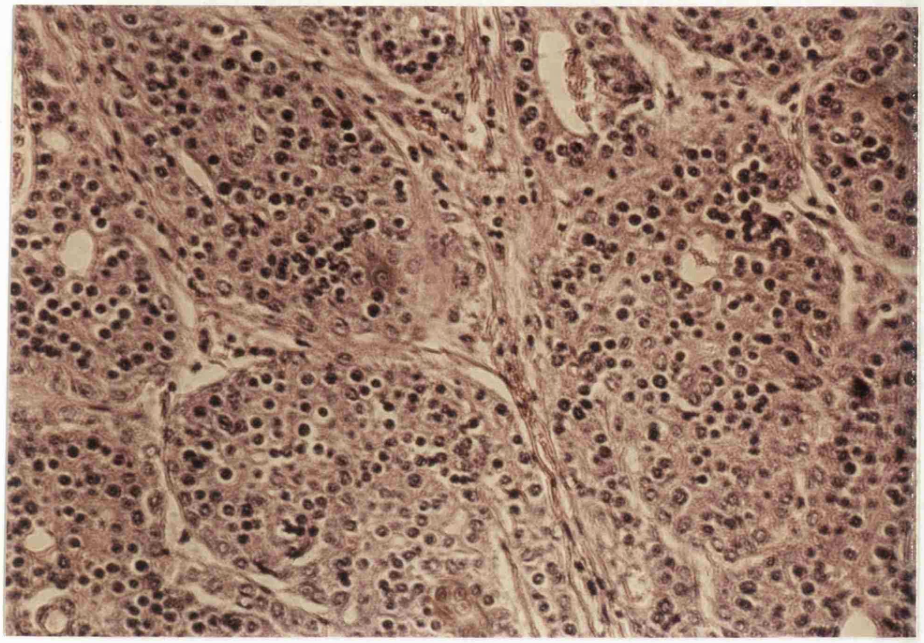


Fig 45 Histology of a breast tumour of intermediate differentiation

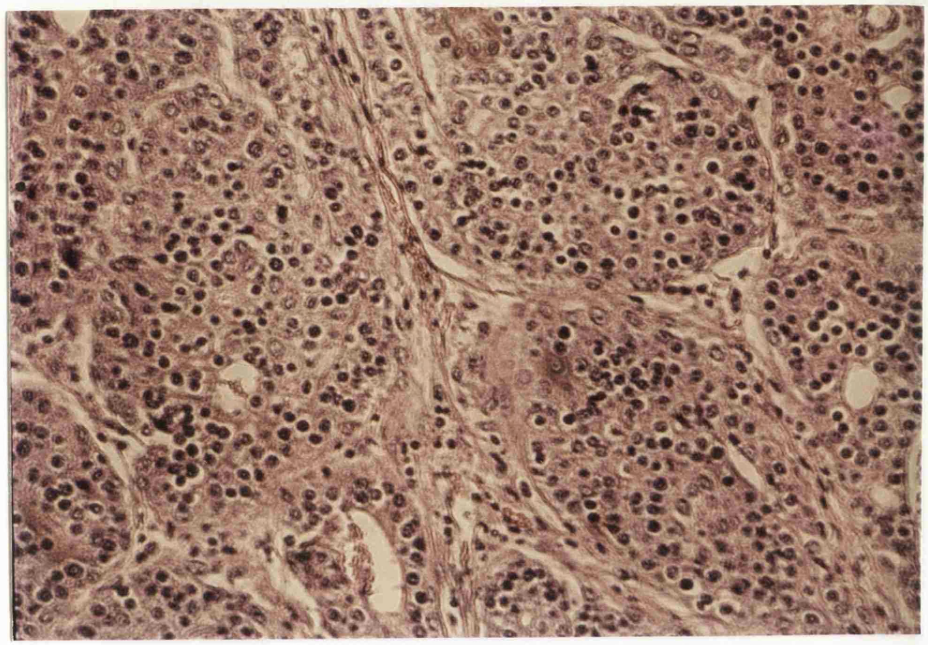


Fig 46 Histology of a breast tumour of intermediate differentiation

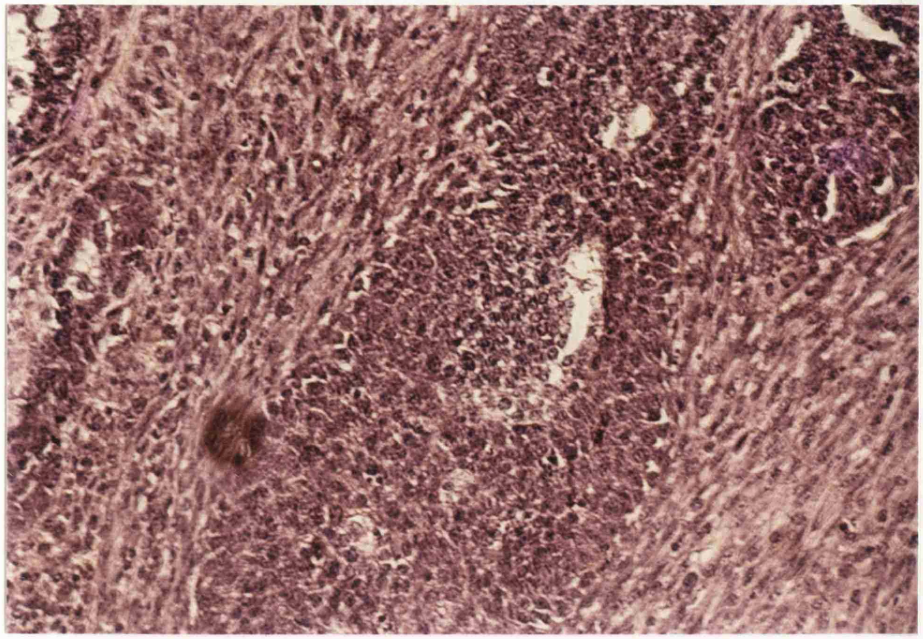


Fig 47 Histology of a relatively undifferentiated breast tumour

TABLE 1a

THE EFFECT OF COLLECTING BLOOD IN PLASTIC OR GLASS

Dose PHA per well	Plastic	S.D.	Glass	S.D.
1 µgm	28228	(5198)	2230	(216)
2	45014	(3249)	4609	(565)
3	54552	(849)	5306	(393)
4	44294	(3131)	4618	(339)

TABLE 1b

THE EFFECT OF COVERING PLATES DURING INCUBATION

Expt no.	Covered	S.D	Uncovered	S.D.
1	36325	(1955)	2383	(388)
2	32075	(9143)	8333	(2415)
3	35111	(2243)	3918	(1449)

TABLE 2a

THE EFFECT OF VARYING DELAY BEFORE DILUTION OF BLOOD

Delay	cpm	S.D.
Ø mins	5Ø219	(6675)
5	47150	(9096)
1Ø	48506	(8657)
15	43181	(7599)
2Ø	36801	(6884)
25	38882	(8391)

TABLE 2b

THE EFFECT OF VARYING DELAY AFTER DILUTION

delay-Room temp capped (mins)		37 C capped		37 C not capped	
15	23445 (3562)	23596	(4Ø33	21973	(4476)
3Ø	26766 (5423)	22872	(52Ø3)	21651	(3815)
45	22971 (4Ø52)	22124	(3258)	24621	(433Ø)
6Ø	21632 (3337)	19210	(3266)	21366	(513Ø)
12Ø	15814 (1183)	23471	(5269)	26295	(2597)

TABLE 3a

EFFECT OF DIFFERENT INCUBATION PERIODS

Time	varying before labelling with 24 hours after		varying after labelling with 24 hours before	
16 hours	7758	(1331)	34873	(5554)
20	12721	(2558)	31635	(6087)
25	27058	(1813)	42918	(7283)
30	16876	(1903)	33676	(4777)

TABLE 3b

PHA DOSE RESPONSE

Dose per well	cpm	S.D.
1 µgm	33433	(3639)
2	49974	(5274)
3	49941	(7803)
4	39809	(8718)
5	38308	(8264)

TABLE 4

CHALLENGE TIMING

Time after sensitisation

	5days	7days	9days	11days	13days	15days
	130	270	250	100	60	180
	90	210	130	120	140	150
	70	150	80	200	290	180
	60	140	160	30	130	160
	180	150	80	90	70	190
Means	90	170	140	110	130	170

TABLE 5

DTH RESPONSE - DELAY BEFORE MEASUREMENT

	Hours from challenge		
	24hrs	48hrs	72hrs
	120	90	80
	170	150	120
	0	150	110
	150	70	75
	180	170	135
	145	30	75
	250	280	300
	210	150	130
	230	350	280
	100	80	90
	195	215	195
	220	120	90
	70	90	10
	205	295	285
Means	159	160	141

TABLE 6

SALINE ALONE

Rat	day0	day1	day3	day7	day14	day28
1	40963	115878	48696	55510	29158	38292
2	31985	63876	49323	38075	23804	38181
3	61103	109282	68588	37178	34643	43895
4	28458	76042	88406	60576	23386	56849
5	19512	61323	33759	24422	22961	29584
6	33526	76073	32459	59994	28923	52527
7	23707	87135	44939	57476	41765	70932
8	18533	84121	52831	30003	32902	43415
9	17117	66510	31006	25447	12026	36825
10	12394	89447	53381	51420	39976	56003
11	79184	47529	55809	50654	64306	38827
12	101876	48963	63808	60249	73248	43181
13	60568	37059	76007	91935	38851	36090
14	37955	33351	58087	65282	69751	29834
15	61361	33374	54003	50149	45368	29648
Means	41883	68664	54073	50558	38738	42939
S.D.	25790	25923	15886	17640	17971	11690
Mean log ratio		1.06	1.039	1.031	1.00	1.02
S.D.		0.086	0.054	0.054	0.050	0.071

TABLE 7

THE EFFECT OF DIFFERENT DOSES OF CYCLO

Dose/kg	Day0	Day1	Day3	Day7	Day14
8 mgs	26618	10538	29974	33459	30598
	39497	3199	33808	40761	44429
	41325	4888	27159	65699	49584
	46285	21278	24670	58679	58752
	14980	5656	20936	35686	51952
Means	33741	9111	27309	46857	47063
15 mgs	14708	6146	12748	35467	15397
	47647	6785	27000	-	-
	23008	3654	31247	17664	-
	34559	4049	39722	29804	21618
	28669	6665	41778	32525	24830
Means	29718	5459	39499	28865	20615
30 mgs	11234	4286	6224	16951	14801
	13405	2566	12591	21589	12103
	20121	3134	11937	27378	18542
	36459	8010	25662	37196	18673
Means	20305	4499	14104	25779	16030

TABLE 8

THE EFFECT OF CYCLOPHOSPHAMIDE 4 mgs/kg

Rat	Day0	Day1	Day3	Day7	Day14
1	89143	94726	97104	102896	62016
2	54295	36640	41288	14402	15097
3	42613	68057	80962	34712	33500
4	49909	-	35029	25501	11358
5	27692	31781	43396	25739	15210
6	45916	-	53573	53068	25540
7	44624	-	81789	37063	75908
8	69113	-	42414	51346	68716
9	39984	-	52187	21221	24377
Means	51447	57801	58638	40661	36858
S.D.	18008	29402	22179	26715	25150

TABLE 9

THE EFFECT OF DIFFERENT DOSES OF 5FU

Dose/kg	day0	day1	day3	day7	day14	day28
15 mgs	66019	108025	69387	54106	25782	-
	54935	72264	61982	31390	23848	39218
	99232	108343	117923	51154	64964	70355
	43441	72353	41190	25341	39323	25472
Means	65907	90246	72621	40498	38479	45015
30 mgs	19994	51116	47293	31253	18209	37895
	20036	22530	51615	25194	24590	46776
	28729	64493	40151	19215	28860	34461
	26978	37329	52308	36410	33780	59103
	22540	21446	50344	18572	34412	41242
	22465	45530	46740	40943	35991	10720
	46776	48838	45006	35188	24629	29934
	38138	66395	73735	40626	31615	51267
	21523	40796	68890	36727	23627	50823
	23480	70316	68396	48171	69187	63310
Means	27066	46879	54448	33230	32490	42553
60 mgs	individual figures in Table 20a					
Means	20194	10575	1542	20881	3633	6593

TABLE 11

5FU AND MINIMAL BLOOD LOSS

Rat	Day0	Day14	Day28
1	62465	26460	-
2	57644	15869	-
3	52714	15707	-
4	61142	28491	-
5	81305	18440	-
6	55894	-	31402
7	67249	-	30395
8	70863	-	42137
9	55649	-	38539
10	71096	-	59389
Means	63602	20993	40372

TABLE 12

CYCLO AND MINIMAL BLOOD LOSS

Rat	Day0	Day14	Day28
1	45829	30017	-
2	31159	34796	-
3	12345	10473	-
4	57707	28141	-
5	39928	28560	-
6	58713	-	24886
7	71985	-	32695
8	49152	-	20851
9	61812	-	45851
10	42823	-	39501
Means	47146	26397	32757

TABLE 13

PERIPHERAL WBC COUNTS FOLLOWING CYCLOPHOSPHAMIDE AND GLUCAN

Day	Cyclo only	Cyclo and glucan
Ø	11.16 (2.4Ø)	9.66 (2.Ø9)
1	6.9Ø (2.4Ø)*	6.89 (3.37)
3	2.Ø6 (3.25)**	3.25 (1.46)**
9	8.Ø9 (3.41)	8.37 (1.85)
14	8.53 (3.18)	9.19 (2.12)
28	7.46 (2.1Ø)	8.77 (1.Ø9)

Results given as total WBC (giemsa) for groups of 1Ø rats with S.D. in parenthesis **p<Ø.Ø1 *p<Ø.Ø5 to day Ø values

TABLE 14

WBC FOLLOWING 5FU

Days	count	s.d.
Ø	11.35	(1.41)
1	11.27	(2.81)
3	7.73	(1.87)**
7	9.31	(3.32)
14	14.5	(3.6Ø)*
28	8.67	(2.57)**

Results given as total WBC (giemsa) for groups of 1Ø rats, with S.D. inparenthesis

**p<Ø.Ø1 **p<Ø.Ø5 to day Ø

TABLE 15

CANCER RATS - SALINE ALONE

Rat	day0	day1	day3	day7	day14	day28
1	89844	44383	46088	81928	-	59760
2	62224	-	45070	27196	24209	39102
3	21509	20587	5918	-	-	-
4	49648	46112	61809	41698	87275	53888
5	35564	32998	23676	39162	36348	-
6	32021	21256	28997	32776	34074	42713
7	10216	28511	-	-	-	-
8	39879	21926	13887	22478	19418	24520
9	53313	44564	40329	46956	51161	54425
10	42281	63825	33628	-	-	-
11	72949	45233	27542	35662	27182	38649
Mean	46313	36940	32694	40982	39952	44722
S.D.	22833	14200	16437	18300	23257	12144
Mean log ratio		1.028	0.952	0.973	0.974	0.979
S.D.		0.084	0.046	0.032	0.054	0.032

TABLE 16

DTH RESPONSE - THE EFFECT OF CYCLO 40 mgs/kg

	Controls	Cyclo
	300	190
	920	30
	400	70
	590	220
	200	260
	200	300
	245	175
	135	165
	205	150
	40	140
		0
		160
Means	323	155
S.D.	252	88

p for difference = 0.02 (Mann Whitney U test)

0.05 (Students t test)

TABLE 17

THE EFFECT OF REGIMES ON TUMOUR SIZES

(expressed in sq mm)

	week0	week1	week2	week3	week4
Controls	150	182	61	52	101
as % change		130	64	51	100
5FU alone	373	284	622	418	210
as % change		98	124	119	210
5FU+levamisole	248	232	119	152	137
as % change		103	77	98	126
Cyclo alone	301	319	288	(340)	(341)
as % change		112	104	(132)	(132)
Cyclo+glucan	194	222	267	(392)	(282)
as % change		117	147	182	191
All groups	275	249	282	296	221
as % change		109	106	117	137

Bracketed figures are means of less than 5 animals

TABLE 18

SALINE ONLY CONTROLS FOR LEVAMISOLE

Rat	day0	day1	day3	day7	day14	day28
1	33145	59883	58585	52255	50919	49023
2	29730	96477	52309	49764	35033	39062
3	11027	36745	12745	12912	15740	19132
4	23402	64799	39856	15934	41491	43260
5	22213	36359	23870	10782	28344	31862
6	70515	51319	43868	22524	18081	35617
7	58310	51989	36002	12094	41863	43033
8	20391	20069	13358	7436	12143	19455
9	39547	60527	22454	24666	31420	31448
10	16003	17604	48484	20183	13981	23051
Means	32428	49577	35153	22855	28902	33494
S.D.	18966	23330	16281	15794	13537	10447
Mean log ratio		1.046	1.017	0.963	0.994	1.014
S.D.		0.046	0.057	0.068	0.054	0.041

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TABLE 19

LEVAMISOLE ONLY

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	85478	91977	110740	62930	75351	71884
2	35214	46165	49920	23265	37467	41831
3	52045	42634	33844	22147	46843	54209
4	63350	83025	86906	46722	70623	67399
5	23886	44626	91881	36075	47801	42878
6	40402	44892	34110	23809	41582	35118
7	49002	54164	39378	36891	33481	33010
8	52491	64744	44533	37622	81215	40699
9	73199	89047	52268	38014	50296	45068
10	97849	95362	49280	57590	54690	44231
Means	57292	65664	59286	38507	53935	47633
S.D.	22942	21960	27090	13959	16416	12974
Mean log ratio		1.015	1.004	0.966	0.999	0.988
S.D.		0.021	0.055	0.031	0.036	0.036
cf controls p=		NS	NS	NS	NS	NS

TABLE 20a (Fig 6)

5FU AND LEVAMISOLE TOGETHER - CONTROLS

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	15612	6122	879	34728	1701	4798
2	18351	15419	-	-	-	-
3	18479	7969	1083	19005	4093	16568
4	21695	16943	938	14150	3295	8342
5	11585	1405	775	4512	1577	2244
6	14450	7004	884	23979	6296	2572
7	-	4148	1002	13267	812	795
8	36024	26214	4391	39176	1503	7538
9	23269	12611	1128	27364	10841	13601
10	31560	12102	2871	-	-	-
11	10913	6383	1471	11751	2579	2883
Means	20194	10575	1542	20881	3633	6593
S.D.	7245	6986	470	16045	8358	4725
Mean log ratio		0.923	0.730	1.003	0.824	0.884
S.D.		0.059	0.042	0.056	0.075	0.060

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TABLE 20b (Fig 6)

5FU AND LEVAMISOLE TOGETHER

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	27473	7953	2037	10037	28723	5641
2	28723	22706	2505	36769	3122	16611
3	37573	14145	2873	41067	12456	14099
4	24432	21735	2282	48318	5694	13136
5	16444	23563	1744	7726	13623	8110
6	31640	17582	1662	36567	11971	16370
7	19110	6627	2725	19115	7738	6124
Means	26485	16330	2261	28514	11904	11442
S.D.	7245	6986	470	16045	8358	4725
Mean log ratio		0.946	0.759	0.991	0.904	0.912
S.D.		0.058	0.026	0.059	0.074	0.037
cf controls p=		NS	NS	NS	NS	NS

TABLE 21a (Fig 7)

CYCLO 8mgs/kg AND LEVAMISOLE TOGETHER - CONTROLS

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	69539	33127	28544	42702	44143	25505
2	57417	42261	21117	24468	22356	23332
3	45237	20208	14540	21259	34272	19089
4	48471	19594	8766	23005	32791	31665
5	61359	34343	10580	21154	23236	27420
6	36937	19433	17432	25093	29541	9196
7	25179	5893	7820	10074	9602	12295
8	38130	16614	39058	35877	28931	19854
9	37376	15500	22713	46637	21646	43008
10	20045	7386	5276	14463	18748	18353
11	31613	27015	38037	31015	26822	31230
12	48280	27808	47317	32580	34641	48342
13	26780	11591	48328	21174	26686	19431
B14	24416	27296	43320	28260	19404	39034
15	39782	13654	23843	18326	24912	56274
16	34548	18042	28758	28132	-	-
17	56995	30339	59216	36132	49538	39571
18	36397	23650	4867	9874	21804	30526
19	50622	23983	45630	28651	30970	50132
20	47754	20402	41495	27829	-	-
Means	41844	21907	27833	26335	27780	30237
S.D.	13302	9193	16590	9675	9349	13427
Mean log ratio		0.934	0.945	0.961	0.965	0.932
S.D.		0.033	0.073	0.038	0.028	0.050

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TABLE 21b (Fig 7)

CYCLO 8mgs/kg AND LEVAMISOLE TOGETHER

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	52178	21232	38198	59632	28325	28681
2	31878	2935	14702	18214	14638	11322
3	44869	21697	22811	23814	27298	30669
4	56508	11226	29531	45705	26359	33732
5	53573	19107	20778	35381	21649	33457
6	22631	-	21687	20175	10737	16307
7	32998	12533	22863	30008	31157	29758
8	25327	5696	20051	27415	20816	20676
9	25293	2580	15626	30529	23137	15749
10	19087	11852	17969	13006	11499	14868
11	23770	25340	21852	21762	23923	18844
12	39878	21008	39390	40437	44728	48982
13	41216	18546	34048	14669	25538	39546
14	37985	20563	26174	23324	30991	22469
15	60705	29721	37429	30525	30027	49274
16	46657	32329	37719	23532	31646	44339
17	32046	14064	43654	42779	20739	47737
18	30164	16642	53299	26153	27019	18060
19	46787	41447	54474	26620	29242	43570
20	31541	12268	32659	35619	21579	20976
Means	37755	17559	30246	29465	25052	29451
S.D.	12211	9791	11794	11325	7709	12586
Mean log ratio		0.913	0.977	0.975	0.961	0.972
S.D.		0.060	0.035	0.034	0.028	0.030
cf controls p=		NS	NS	NS	NS	NS

TABLE 22a (Fig 8)

CYCLO 8mgs/kg DELAY SALINE- CONTROLS FOR LEVAMISOLE

(Days from saline)

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	28692	23766	43056	31472	25802	23892
2	30134	10036	34402	37635	24070	17266
3	41039	19407	41775	32015	20925	19662
4	32148	16695	22036	19958	17896	21689
5	27378	16698	28830	14007	12740	18754
6	35311	8832	59119	51782	30848	13201
7	41028	16178	39137	47252	29344	18655
8	16005	15258	19860	20742	14250	8734
9	23739	18148	32458	35640	17932	18001
10	40172	28678	47973	37895	22338	29261
11	106793	58995	72695	37885	45557	52058
12	41445	22956	28891	15562	33735	38539
13	39066	29893	34957	35019	20948	28020
14	43216	31026	54086	30602	36525	36018
15	90277	39489	37607	35004	43031	33590
16	95198	46140	49000	35116	38120	30270
17	80542	36319	47209	42552	31435	52676
18	100421	27759	47329	23552	20824	28435
19	81399	42279	42903	27988	30957	23564
20	121993	28448	38757	31847	39839	36062
Means	55800	26850	41104	32176	27856	27417
S.D.	32524	12863	12519	9820	9573	11661
Mean log ratio		0.937	0.984	0.961	0.946	0.942
S.D.		0.036	0.042	0.054	0.034	0.034

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TABLE 22b (Fig 8)

CYCLO 8mgs/kg DELAY LEVAMISOLE

(Days from levamisole)

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	36952	26881	35150	45143	25622	16714
2	23947	6882	20378	13767	19351	20827
3	24454	8779	13013	22254	20146	30845
4	9093	3670	2098	4746	5079	4935
5	21377	6618	13878	21550	16492	14577
6	14245	10888	19723	12306	16714	4294
7	23284	29777	25455	26971	34130	36039
8	33750	11396	31936	17150	29936	17080
9	17479	13102	15432	5074	24600	34218
10	10663	4143	13526	6006	27218	28642
11	82657	40143	45090	31985	33032	29522
12	34430	28510	29519	11516	25352	25808
13	58060	30545	39552	26628	21249	24189
14	59970	28835	43177	41851	26604	36189
15	70222	43183	42034	22694	30235	40517
16	97751	54302	42253	18466	20720	33648
17	67995	34495	34052	21634	46593	36169
18	63858	47171	42327	33659	20012	21176
19	110852	60108	62872	34380	33660	62601
Means	45318	25759	30077	21988	25092	27263
S.D.	30569	17719	15086	11816	8818	13461
Mean log ratio		0.939	0.966	0.938	0.962	0.961
S.D.		0.039	0.043	0.047	0.054	0.061
cf controls p=		NS	NS	NS	NS	NS

TABLE 23a (Fig 9)

CYCLO 40 mgs/kg DELAY SALINE - CONTROLS FOR LEVAMISOLE

(days from saline)

Rat	day0	day1	day3	day7	day14	day28
1	19896	1560	10628	13478	12383	35896
2	30366	2751	5978	7072	5932	22063
3	26638	4253	24277	11716	11930	22560
4	13933	1967	14200	11346	13682	28873
B5	43992	1530	11613	12367	9158	21183
6	29411	3533	9182	7047	14928	29126
7	44497	3271	12894	10224	12028	31534
8	41801	2737	15660	4204	11497	33874
9	57957	1889	7455	5826	8700	27137
10	71726	1395	13835	55132	14341	30101
means	38022	2489	12572	13841	11458	28235
S.D.	17627	975	5129	14833	2792	5025
Mean log ratios		0.744	0.899	0.887	0.894	0.982
S.D.		0.055	0.060	0.071	0.055	0.052

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TABLE 23b (Fig 9)

CYCLO 40mgs/kg DELAY LEVAMISOLE

(days from levamisole)

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	44560	4644	19385	16888	15382	21226
2	50875	3684	16819	10363	16936	38567
3	45535	1173	15133	5997	3983	18434
4	73409	2205	24852	5553	4618	21936
5	58013	3420	26410	15319	21665	31441
6	40602	2102	29773	7838	8305	44857
7	53035	1448	24634	17470	21733	46512
8	39511	2089	24447	7011	7818	21717
9	42223	7467	6851	18653	16930	39649
10	41138	2520	13390	10436	7198	31455
Means	48890	3075	20169	11553	12457	31579
S.D.	10524	1871	7086	5078	6824	10454
Mean log ratio		0.732	0.913	0.860	0.860	0.957
S.D.		0.054	0.039	0.047	0.60	0.038
cf controls p=		NS	NS	NS	NS	NS

TABLE 24a (Fig 10)

5FU DELAY SALINE - CONTROLS FOR LEVAMISOLE

(days from saline)

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	7081	1351	311	12105	14211	11592
2	13829	10294	42816	3399	15562	36609
3	10992	4001	7626	8680	15009	18686
4	21592	2001	19518	24217	12991	19973
5	7154	1563	26920	9628	1048	6287
6	44630	3797	34552	39315	20909	18767
7	32278	10442	26112	33691	21914	13030
8	23990	2241	38253	14555	9157	29492
9	21121	6132	32552	17295	7591	35208
10	20173	3726	16122	27552	8684	13986
11	88310	7884	37373	8389	10361	25628
12	43670	6667	54829	22520	31884	26804
13	46486	16137	86536	8387	27294	16158
14	112639	29557	139185	9933	6181	6146
15	81443	17671	144137	27589	36491	28849
16	61566	1674	73400	14030	22495	22612
17	36032	10239	67509	4262	21683	24217
18	34816	6189	B71192	23603	32678	17481
19	53767	8108	95213	10094	19853	25315
20	58961	14289	63393	22564	29186	17809
Means	41027	8198	56077	17090	21513	24532
S.D.	28593	7014	37601	10033	12852	9605
Mean log ratio		0.837	1.036	0.927	0.952	0.974
S.D.		0.062	0.066	0.086	0.060	0.084

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TABLE 24b (Fig 10)

5FU DELAY LEVAMISOLE

(days from levamisole)

Rat	day0	day1	day3	day7	day14	day28
1	9753	30579	31236	22769	17624	41547
2	26630	24007	23635	19848	29640	34459
3	25827	4907	70544	35836	14966	42900
4	8760	10899	17755	20151	8434	17836
5	8382	1119	30054	26601	13558	14981
6	12765	1198	29808	16814	5775	8822
7	11055	674	11815	51647	7109	9168
8	17197	9085	30547	40627	9756	26437
9	14855	6091	37093	27172	17051	32406
10B	12067	2408	21931	12551	8772	15388
11	85600	33831	161093	14433	22164	28076
12	49829	28813	103738	17989	30452	23298
13	61562	46029	102364	3297	17604	37457
14	61067	17093	79829	12620	30621	23226
15	38839	16949	43960	6981	23023	17533
16	53199	7121	98112	17688	25537	27873
17	40868	36906	82463	6301	23665	24051
18	35896	6722	73155	5988	23400	30039
19	36501	2976	62019	24150	-	-
20	35952	14365	75439	14173	28555	25399
Means	32330	15089	59330	19882	18827	25310
S.D.	21658	13718	38360	12192	8339	9878
Mean log ratios		0.892	1.06	0.963	0.963	0.996
S.D.		0.105	0.038	0.121	0.049	0.069
cf controls p=		0.05	0.12	NS	NS	NS

TABLE 25

THE EFFECT OF 5FU DELAY LEVAMISOLE

	Controls	5FU alone	5FU+levamisole
	160	130	220
	150	230	40
	130	330	380
	220	210	260
	140	200	260
	190	220	300
	260	210	320
	280	190	180
	170	210	130
	140	210	420
Means	184	214	251
S.D.	(53)	(49)	(114)

TABLE 26a (Fig 11)

5FU DELAY SALINE - CANCER RATS

- CONTROLS FOR LEVAMISOLE

Rat	day0	day1	day3	day7	day14	day28
1	1996	48553	44594	2315	-	-
2	22716	15270	5491	-	-	-
3	53031	15037	74684	2419	-	-
4	-	30974	29721	-	-	-
5	-	21601	39070	6623	-	-
6	49721	-	84794	155291	9297	-
7	-	18214	26379	6919	-	-
8	-	10253	18174	2380	-	-
9	8930	30872	74399	11357	3814	34484
10	17851	18790	93428	1398	6781	24533
11	19170	29121	51813	1361	6607	38133
12	3110	1713	2743	2080	10818	23725
13	5170	10351	11743	6812	1667	9651
14	20584	18309	43905	1598	5765	7676
Means	44026	25275	47960	4547	5909	25609
S.D.	23209	20616	41107	3478	3090	11563
Mean log ratio		0.936	0.984	0.768	0.801	0.936
S.D.		0.106	0.122	0.063	0.098	0.097

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TABLE 26b (Fig 11)

5FU DELAY LEVAMISOLE - CANCER RATS

Rat	day0	day1	day3	day7	day14	day28
1	13599	9055	111040	2606	1726	13920
2	39137	29324	11714	7633	17720	18101
3	30268	12366	15323	-	-	-
4	17295	20583	4003	2761	-	-
5	6140	16683	5945	-	-	-
6	3749	15087	1962	-	-	-
7	28311	57527	118823	66692	2780	41180
8	3251	13212	11593	3153	2098	13414
9	26703	7369	3932	4634	-	-
10	11663	12213	26421	3937	13858	18980
Means	35247	19342	31076	13059	7636	21119
S.D.	19458	14797	44805	23712	7576	11482
Mean log ratio		0.941	0.922	0.845	0.837	0.977
S.D.		0.078	0.155	0.113	0.103	0.057
p value		NS	NS	NS	NS	NS

TABLE 27a (Fig 12)

CYCLO 8mgs/kg AND SALINE - CONTROLS FOR GLUCAN

Rat	Day	Day1	Day3	Day7	Day14	Day28
1	59692	43881	33237	36990	35922	29951
2	30860	21810	27967	25731	40435	12242
3	43670	35082	30253	29612	24294	16205
4	36792	29514	43603	34627	25615	11989
5	56914	39152	40533	19821	50127	14132
6	39379	33910	33200	49546	44750	35892
7	30627	13487	12596	26765	13085	15112
8	51781	39694	29758	37167	25538	33898
9	61245	37185	34617	47068	28448	15321
10	68581	55026	23458	16610	52020	9071
11	35902	18729	25891	11911	12920	13610
12	36657	9823	13312	14213	16978	10369
13	54528	33592	22735	16078	9411	9624
14	33812	30763	25894	17640	14413	15856
15	24180	25245	42378	10831	26307	12547
16	21527	13521	15631	11513	11712	22377
17	18249	15885	11600	11696	7726	20579
18	27869	20736	30768	12947	11952	24843
19	26159	9347	20562	7271	7815	21021
20	43971	27896	19163	12858	13141	11739
Means	40120	27714	26858	22545	23630	17819
S.D.	14460	12318	9627	12698	14281	7950
Mean log ratio		0.960	0.962	0.938	0.939	0.922
S.D.		0.032	0.039	0.044	0.047	0.055

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TABLE 27b (Fig 12)

CYCLO 8 mgs/kg AND GLUCAN

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	68997	25361	30647	31407	14106	32005
2	52779	3744	38802	26517	28322	17548
3	64591	12823	45062	12208	23970	38700
4	58528	20518	19597	24680	36955	38497
5	48850	8321	29690	14637	31438	45915
6	60300	10482	47409	9888	23616	38567
7	27259	13641	35347	16816	21877	29285
8	73936	20253	39006	4989	21823	29638
9	39792	17384	49094	12076	16615	20598
10	58258	9959	35502	16441	7706	44571
11	26820	4660	17915	7153	6889	8835
12	33336	10491	14803	9523	6459	18360
13	19864	6929	29749	5839	4079	12396
14	28143	13019	21192	9796	4101	16697
15	14636	1779	16542	16651	7903	9520
16	10291	4390	14457	2124	2135	4877
17	22252	15528	27057	4098	4716	15926
18	20517	8116	14191	3214	6608	8169
Means	40508	11522	29226	12670	14962	23895
S.D.	20393	6455	11747	8313	10857	13416
Mean log ratio		0.876	0.976	0.881	0.889	0.946
S.D.		0.054	0.041	0.058	0.053	0.031
cf controls p=		<0.0001	NS	0.002	0.004	0.11

TABLE 28a (Fig 13)

CYCLO 40 mgs/kg AND SALINE - CONTROLS FOR GLUCAN

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	27847	6118	1546	18285	6680	26681
2	52081	16924	7429	30747	17255	38483
3	41395	3172	9303	23498	-	37186
4	10394	2955	1811	25209	11767	40740
5	23329	4492	6557	25300	13757	31419
6	38434	7674	4093	29743	13317	26112
7	25989	8251	3960	21585	18321	42729
8	46774	6593	9361	28605	23195	48554
9	18557	7129	3145	13981	20744	29259
10	28865	4541	8331	18928	10910	24833
Means	31367	6784	5553	23588	15105	34600
S.D.	13052	4005	3006	5437	5208	8090
Mean log ratio		0.848	0.823	0.980	0.937	1.018
S.D.		0.044	0.048	0.044	0.053	0.054

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TABLE 28b (Fig 13)

CYCLO 40 mgs/kg AND GLUCAN

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	39495	1335	5123	3109	1135	21763
2	69107	1754	1994	11652	2937	25417
3	32477	2222	9069	7346	1443	19505
4	23171	1728	1335	3323	1651	12156
5	64013	1406	2270	8598	21021	24772
6	30084	1624	6800	10296	3803	30878
7	31678	1952	7509	4519	2083	19111
8	27334	1780	6305	6644	2782	21760
9	33454	1243	3060	5940	1618	16095
10	29291	2119	5088	1807	2741	19754
Means	38010	1716	4855	6323	4121	21121
S.D.	15670	325	2610	3225	5995	5185
Mean log ratio		0.710	0.795	0.822	0.751	0.948
S.D.		0.034	0.074	0.049	0.066	0.028
cf controls p=		<0.0001	NS	<0.0001	<0.0001	0.003

TABLE 29a (Fig 14)

5FU AND SALINE - CONTROLS FOR GLUCAN

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	23362	22680	31661	41978	14865	482
2	22936	44617	7895	63140	1048	656
3	14282	13204	1920	18977	6206	620
4	16254	10681	7205	52561	5567	712
5	18081	9824	4579	32022	39710	569
6	20781	15705	1939	50071	11289	566
7	27695	20636	15582	26230	17438	842
8	6994	17646	14973	65947	3858	673
9	9247	18017	6116	48556	26916	1778
10	37468	21670	36917	67528	13889	525
Means	19710	19468	12879	46701	14079	742
S.D.	8939	9877	12264	16829	11786	378
Mean log ratio		1.002	0.923	1.094	0.938	0.679
S.D.		0.059	0.102	0.078	0.114	0.065

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TABLE 29b (Fig 14)

5FU AND GLUCAN

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	11665	12390	1312	38213	3644	975
2	14448	16574	2308	52928	618	1016
3	13671	4090	746	39108	717	1162
4	23036	17074	993	38174	703	1054
5	16669	13022	3072	47600	4717	
6	9033	24101	4307	53126	1240	-
7	11281	19062	3233	47203	1713	-
8	9175	5626	988	63806	3780	-
9	17793	8529	1762	83578	675	-
10	12001	16280	1937	68073	11898	-
Means	13877	13675	2065	53181	2970	1041
S.D.	4322	6233	1164	14806	3487	73
Mean log ratio		0.991	0.789	1.143	0.791	0.720
S.D.		0.067	0.072	0.048	0.120	0.032
cf controls p=		NS	0.004	0.104	0.01	<0.001

TABLE 30

THE EFFECT OF CYCLO 40mgs/kg AND GLUCAN

(on the DTH response)

	normals		cancer rats	
Rat	Cy	+glucan	Cy	+glucan
1	260	240	80	40
2	230	170	50	85
3	200	350	130	110
4	200	280	100	150
5	210	220	80	185
6	50	190	160	370
7	70	320	170	240
8	280	240	170	250
9	200	210	440	175
10	220	110		
Means	192	233	153	178
S.D.	75	71	116	99

TABLE 31a (Fig 15)

CYCLO 8 mgs/kg and SALINE - CANCER RATS

- CONTROLS FOR GLUCAN

Rat	day0	day1	day3	day7	day14	day28
1	25187	9753	8465	10148	25275	18647
2	39999	6707	4218	6855	15680	22885
3	45728	6409	12074	18464	25238	-
4	43382	1768	3487	4183	9263	-
5	46180	15870	29509	23332	29753	31589
6	53042	24086	20797	27197	34332	32885
7	1548	1626	20016	2074	3143	5341
8	54864	713	5432	33321	26301	20998
9	23660	5002	6149	9467	13993	40288
Means	37066	7992	12239	15005	20331	24662
S.D.	17179	7661	9156	11014	17832	11144
Mean log ratio		0.837	0.911	0.913	0.956	0.996
S.D.		0.123	0.176	0.073	0.066	0.087

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TABLE 31b (Fig 15)

CYCLO 8mgs/kg AND GLUCAN - CANCER RATS

Rat	day0	day1	day3	day7	day14	day28
1	48952	4542	33604	2811	22374	40956
2	35434	1405	7020	2981	3301	13350
3	74498	15559	10725	5508	18121	23628
4	29738	8532	10361	3616	10441	-
5	33129	6923	8200	3548	10567	-
6	9563	807	1454	1737	2883	-
7	32354	761	1725	3004	5327	-
8	27377	2904	8555	3998	13165	16183
9	44648	1904	10012	3647	9681	22451
Means	37299	4815	10184	3427	10651	23314
S.D.	17832	4874	9442	1023	6550	10750
Mean log ratio		0.768	0.851	0.779	0.872	0.934
S.D.		0.083	0.069	0.025	0.049	0.034
cf controls p=		0.18	0.37	<0.0001	0.008	0.13

TABLE 32a (Fig 16)

SALINE DELAY CYCLO 8mgs/kg - CONTROLS FOR GLUCAN

(days from cyclo)

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	79874	8616	41206	32729	43175	66318
2	61725	7806	39752	39579	21832	65239
3	62069	19944	52892	42027	50779	48302
4	93317	10716	27795	30628	43424	70121
5	71662	28422	63347	46899	71359	60494
6	62946	17474	28929	29686	24300	36161
7	51161	11940	30724	32108	20448	51137
8	40097	6674	31102	36109	25375	50955
9	39795	8003	23875	23136	23137	44165
10	62035	23646	40503	39401	38032	74537
Means	62468	14324	38013	35230	36186	56743
S.D.	16609	7597	12339	6941	16455	12434
Mean log ratio		0.858	0.954	0.950	0.946	0.992
S.D.		0.043	0.028	0.025	0.030	0.022

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TABLE 32b (Fig 16)

GLUCAN DELAY CYCLO 8 mgs/kg

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	56998	30144	24597	13956	26697	13348
2	45901	11915	6186	7611	25498	32184
3	43321	19794	12505	22993	24593	103587
4	63228	19783	36018	24527	41598	30115
5	71938	31842	37479	28442	50257	68274
6	27997	9664	16203	15384	31762	58152
7	34764	6129	3894	9718	31226	67238
8	37912	5108	14838	16238	25944	44964
9	44956	20582	25240	21070	37451	33789
10	26808	4136	12165	7898	22799	57752
Means	45382	15910	18913	16784	31783	56743
S.D.	14829	10079	11584	7273	8831	25744
Mean log ratio		0.885	0.903	0.903	0.969	1.004
S.D.		0.049	0.057	0.035	0.024	0.071
cf controls p=		NS	0.02	0.003	0.7	NS

TABLE 33a (Fig 17)

CYCLO 8 mgs/kg DELAY SALINE - CONTROLS FOR GLUCAN

(days from saline)

1	150487	23195	49364	13604	50167	72967
2	91363	17461	21430	1529	14230	42286
3	75196	6908	10638	4954	17680	10419
4	114915	47611	24213	15516	44784	53134
5	24774	3183	15936	2657	17432	36789
6	88251	18477	29966	11134	23768	63620
Means	90831	19473	25258	8232	28010	46536
S.D.	41842	15709	13562	5951	15484	22143
Mean log ratio		0.845	0.889	0.771	0.898	0.940
S.D.		0.050	0.044	0 068	0.044	0.070

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TABLE 33b (Fig 17)

CYCLO 8 mgs/kg DELAY GLUCAN

(no levamisole)

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	64587	3202	22791	19951	-	8116
2	92748	29642	32744	9612	29160	40573
3	78798	8521	30811	19670	-	4257
4	115702	2953	23109	26545	-	41652
5	52820	2995	11433	14598	10684	44344
6	71022	1626	8801	12470	4265	45297
7	72263	1895	16578	9435	5691	40866
Means	78277	7262	20895	16040	12450	32158
S.D.	20553	10135	9144	6306	11475	17862
Mean log ratio		0.741	0.876	0.856	0.818	0.898
S.D.		0.085	0.037	0.035	0.070	0.089
cf controls p=		0.02	NS	0.03	0.11	NS

TABLE 33c

CYCLO 8 mgs/kg DELAY GLUCAN AND LEVAMISOLE

(part of 2 previous expts)

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	52463	3354	19097	26537	9374	46791
2	82784	2371	15566	15915	13340	53279
3	71890	1605	13358	15383	16128	53988
4	56539	1286	14247	9718	3992	39201
5	66924	1475	16252	16745	5342	55167
6	72982	2583	25223	20269	5065	41463
7	87488	2981	34697	2876	40439	67393
Means	70153	2236	19777	15349	13383	51040
S.D.	12779	798	7688	7516	12765	9558
Mean log ratio		0.687	0.883	0.850	0.824	0.972
S.D.		0.034	0.028	0.073	0.065	0.014
cf controls p=		NS	NS	NS	NS	NS

TABLE 34a (Fig 18)

SALINE ALONE - CONTROLS FOR GLUCAN

(received only saline)

Rat	day0	day1	day3	day7	day14	day28
1	42094	33139	76985	63076	49287	37527
2	62629	50863	88583	51035	29668	31655
3	56718	51538	94512	50727	45086	23529
4	74233	51634	-	-	-	-
5	80198	50380	81080	54323	46721	32008
6	59488	44641	77404	-	61492	-
7	34365	43997	54887	-	49854	24497
8	37995	70901	83841	-	74489	36520
9	30649	21685	28740	-	35087	54899
10	66284	34613	94314	-	48611	18551
Means	54465	45339	75594	48922	32398	40984
S.D.	17273	13388	21226	5759	13203	11232
Mean log ratio		0.984	1.034	0.994	0.996	0.959
S.D.		0.034	0.026	0.031	0.041	0.055

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TABLE 34b (Fig 18)

GLUCAN ALONE

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	79184	47529	55809	50654	64306	38827
2	101876	48963	63808	60249	73248	43181
3	60568	37059	76007	91935	38851	36090
4	37955	33351	58087	65282	69751	29834
5	61361	33374	54003	50149	45368	29648
6	42094	33139	76985	63076	49287	37527
7	62629	50863	88583	51035	29668	31655
8	56718	51538	94512	50727	45086	23529
9	74233	51634	-	-	-	-
10	80198	50380	81080	54323	46721	32008
11	59488	44641	77404	-	61492	-
12	34365	43997	54887	-	49854	24497
13	37995	70901	83841	-	74489	36520
14	30649	21685	28740	-	35087	54899
15	66284	34613	94314	-	48611	18551
Means	59040	43578	70576	59714	52273	33597
S.D.	19977	11771	18719	13414	14179	9593
Mean log ratio		0.975	1.020	0.996	0.993	0.954
S.D.		0.032	0.034	0.037	0.039	0.044
cf controls p=		NS	NS	NS	NS	NS

TABLE 35a (Fig 19)

SALINE ALONE - CONTROLS FOR C parvum

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	40963	115878	48696	55510	29158	38292
2	31985	63876	49323	38075	23804	38181
3	61103	109282	68588	37178	34643	43895
4	28458	76042	88406	60576	23386	56849
5	19512	61323	33759	24422	22961	29584
6	33526	76073	32459	59994	28923	52527
7	23707	87135	44939	57476	41765	70932
8	18533	84121	52831	30003	32902	43415
9	17117	66510	31006	25447	12026	36825
10	12394	89447	53381	51420	39976	56003
Means	29077	30121	14586	16425	16425	35172
S.D.	11051	8513	11711	9832	6901	9581
Mean log ratio		1.114	1.061	1.048	1.007	1.057
S.D.		0.047	0.050	0.051	0.056	0.056

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TABLE 35b (Fig 19)

C parvum ALONE

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	51211	20698	42615	26007	10716	39625
2	15664	18935	12411	3827	9998	29616
3	28382	4095	24763	19620	4178	23757
4	24683	24068	44086	20701	23445	41334
5	46183	38336	48129	32146	17031	32828
6	24550	18608	30202	8369	18456	24896
7	24549	19013	27280	12982	19110	38263
8	22694	24263	19427	5227	13290	31525
9	23180	21618	20555	3512	20950	54703
10	29670	15156	31745	13468	27076	-
Means	29077	20479	30121	14586	16425	35172
S.D.	11051	8513	11711	9832	6901	9581
Mean log ratio		0.961	1.002	0.912	0.940	1.023
S.D.		0.061	0.024	0.057	0.062	0.042
cf controls p=		<0.0001	0.005	<0.0001	0.02	0.15

TABLE 36a (Fig 20)

CYCLO 8mgs/kg and SALINE - CONTROLS FOR C parvum

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	49321	18618	73310	22134	26461	62854
2	48663	13930	48403	89320	18865	60602
3	50342	10597	58432	35132	31003	25358
4	28948	9434	26924	16660	13383	76800
5	52038	16786	55576	26940	40881	72553
6	78492	8422	53665	25814	42135	69528
7	33998	18060	41294	16139	35532	45304
8	81149	6526	37818	26699	34045	36832
9	27208	22434	27220	22663	22000	14070
10	44579	32002	45365	28333	61557	39471
Means	49474	15681	46801	30983	32586	50337
S.D.	18360	7688	14362	21244	13821	21316
Mean log ratio		0.891	0.997	0.951	0.959	0.998
S.D.		0.066	0.029	0.045	0.038	0.053

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TABLE 36b (Fig 20)

CYCLO 8mgs/kg AND C parvum

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	76560	6842	2760	4782	2294	60689
2	80775	4337	1891	3521	3235	62244
3	70502	18620	11522	5041	6333	60177
4	82838	11109	3756	8171	7133	51421
5	111746	6664	15294	2913	9709	104423
6	52952	3756	3294	4050	3571	49340
7	60252	3505	9017	2064	8702	26936
8	62211	2125	5666	12362	11659	66434
9	75898	3524	10112	2122	6703	86847
10	80180	8943	4806	2459	3442	50140
Means	75391	6942	6811	4748	6278	61865
S.D.	16197	4961	4442	3240	3127	21032
Mean log ratio		0.771	0.769	0.741	0.769	0.979
S.D.		0.054	0.059	0.056	0.049	0.026
cf controls p=		for all groups		<0.00001		NS

TABLE 37a (Fig 21)

5FU DELAY SALINE - CONTROLS FOR THIABENDAZOLE

(days from saline)

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	34673	73423	74386	1528	39074	70648
2	29780	41197	87816	7010	30456	48071
3	33257	17153	64405	21818	24034	58012
4	29494	18308	55757	12291	20147	35636
5	44371	27767	72908	8810	21675	49259
6	28265	25105	85760	18956	32619	58467
7	40716	54990	83597	6004	23353	46319
8	39015	23915	58398	16891	27623	45841
9	40721	20002	86495	1938	14803	39842
10	35108	11641	75459	14848	7079	43290
Means	35540	31350	74498	11009	24086	49539
S.D.	5503	19499	11769	7063	9115	10273
Mean log ratio		0.975	1.071	0.862	0.956	1.031
S.D.		0.054	0.022	0.094	0.052	0.026

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TABLE 37b (Fig 21)

5FU DELAY THIABENDAZOLE

(days from thiabendazole)

Rat	Day0	Day1	Day3	Day7	Day14	Day28
1	28204	57991	85130	3010	26419	25760
2	27386	18025	56807	10285	27767	43889
3	33665	18773	26244	6930	22298	46211
4	40309	71589	74848	5513	24897	41014
5	41225	15164	49907	14580	34985	45559
6	29318	10261	83834	2702	24693	49724
7	50227	9404	72655	15148	5574	33879
8	52858	19874	59092	13604	13627	36153
9	38152	52176	66944	15760	31341	50352
10	36040	16398	70842	15905	18409	51712
Means	37738	28966	64630	10344	23001	42425
S.D.	8768	22571	17588	5365	8610	8305
Mean log ratio		0.954	1.050	0.862	0.947	1.012
S.D.		0.074	0.041	0.056	0.064	0.031
cf controls p=		NS	NS	NS	NS	NS

TABLE 38a (Fig 22)

CYCLO PRIMING EXPT - CONTROLS

Rat	day-5	day0	day1	day3	day7	day14	day28
1	29359	37158	20419	41952	19308	46755	44249
2	37507	26160	2942	14369	7727	26686	24825
3	56911	19730	28942	39669	28263	22609	58140
4	72190	71165	12742	43849	45015	38773	74926
5	43046	54621	8064	31659	14344	29922	58068
6	73371	40148	6313	21224	9086	39936	46655
7	48907	35463	5597	19723	8354	21413	30564
8	73266	58425	6184	-	-	-	-
Means	54320	42859	11400	30349	18871	32299	64000
S.D.	17362	17280	8947	11951	13681	9650	15933
Mean log ratios			0.839	0.949	0.893	0.958	1.014
S.D.			0.076	0.049	0.060	0.043	0.003

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TABLE 38b (Fig 22)

CYCLO PRIMING EXPT - PRIMED ANIMALS

Rat	day-5	day0	day1	day3	day7	day14	day28
1	45778	33504	5969	9559	15628	28221	14722
2	73372	51444	22853	25103	15694	38114	38377
3	34312	51417	8819	37361	20515	36825	16353
4	43422	31972	10103	23674	15056	16830	36159
5	57142	39990	13664	37639	31325	36832	33680
6	41347	43987	13931	26202	19701	24525	51997
7	63055	53187	6301	-	-	-	-
8	52790	57062	-	27192	13331	31161	33600
9	88587	64711	669	12552	9420	22108	-
Means	55534	47475	10289	24910	17584	29327	32127
S.D.	17215	10935	6674	10099	6551	7798	12952
Mean log ratios			0.821	0.925	0.896	0.944	0.956
S.D.			0.108	0.059	0.049	0.038	0.011
cf controls p=			NS	NS	NS	NS	NS

TABLE 39

PRIMING EXPTS

	5FU		CYCLO	
Rat	control	primed	control	primed
1	150	80	190	145
2	190	260	90	25
3	240	170	200	150
4	340	170	60	60
5	250	390	135	60
6	240	330	35	40
7	200	270	95	205
8	150	170	75	70
9	270	160		
10	290	200		
Means	232	220	110	94
S.D.	61	92	60	64

TABLE 40a (Fig 23)

5FU PRIMING EXPT - CONTROLS

Rat	day-5	day0	day1	day3	day7	day14	day28
1	74460	59183	19428	33990	53591	22469	12221
2	116609	65056	8819	22076	27911	13280	40279
3	65484	44091	42789	57703	42587	2796	7952
4	59731	31737	11775	61521	11094	2536	17753
5	62256	22631	13166	32922	44668	1823	24202
6	82442	24539	13505	9307	24579	1946	17916
7	105044	46037	31051	36441	18556	5799	29157
8	81432	20773	9810	19655	18516	3235	16117
9	93257	58337	19073	3855	16361	1601	7874
10	116067	46094	24885	38223	7730	8071	11281
Means	85678	41848	19430	31569	26559	6356	18475
S.D.	21335	16217	10794	18713	15448	6753	10234
Mean log ratios			0.861	0.894	0.885	0.736	0.879
S.D.			0.048	0.084	0.065	0.074	0.019

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TABLE 40b (Fig 23)

5FU PRIMING EXPT

Rat	day-5	day0	day1	day3	day7	day14	day28
1	97145	16862	23677	39036	20209	11372	14322
2	77971	27997	17948	76945	46772	12786	12795
3	65930	27721	26101	25494	22484	6191	8588
4	77238	30988	1915	2213	15862	1138	15293
5	87213	57465	1949	63340	27372	2777	8784
6	94973	39252	22779	52912	31856	4783	11917
7	93811	54540	13216	23119	18849	6675	17102
8	85751	18946	10059	4782	56412	1520	16585
9	82870	29095	17153	28992	34755	1947	7094
10	45898	33821	13207	60607	55507	5591	15845
Means	80880	33669	14800	37744	33008	5478	12796
S.D.	15518	13449	8461	25245	15087	4012	5731
Mean log ratios		0.826	0.900	0.739	0.924	0.894	
S.D.		0.087	0.108	0.052	0.076	0.062	
cf controls p=		NS	NS	NS	NS	NS	

TABLE 41

PRIMING DELAY EXPT

(variable delay period=n days)

n	day-n	day0	day1	day3	day7	day14	day28
0	64398	64398	11609	26140	20904	26690	33081
		(1.0)	(.833)	.918	.902	.919	.928
1	64442	54317	12812	29474	18689	28604	10354
		.987	.850	.913	.885	.948	.924
3	40695	39562	6075	16312	18425	18957	11682
		.995	.811	.878	.923	.904	.926
5	56617	32535**	7945*	11241**	9681**	5419*	12047
		.950	.806	.788	.839	.731	.915
9	76358	55220	15750	25458	15090	25533	8732
		.977	.861	.907	.859	.912	.937
14	62019	72512	23547	46736	17857	30208	15009
		1.02	.915	.974	.879	.938	.967

Animals received cyclophosphamide 40 mgs/kg,after 4 mgs/kg prime given after different intervals (n days). Results are the means of 10 rats, expressed in cpm with log ratios to pretreatment values below. *p<0.05 **p<0.01 to controls (0 delay)

TABLE 42a (Fig 24)

5FU DELAY SALINE - CONTROLS FOR CIMETIDINE

(days from saline)

Rat	Day0	Day1	Day3	Day7	Day14
1	33800	5097	24915	4544	24332
2	50977	10541	55597	4407	10054
3	42904	14139	70741	7769	5270
4	51214	16486	56054	6473	24205
5	73773	15587	52517	20846	23944
6	80648	14909	81759	2438	34296
7	19616	1883	8327	6829	1944
8	53071	13636	28271	31605	6467
9	54811	34199	33765	13998	-
10	23865	1138	5653	19731	5728
Means	48468	12762	41760	11864	15138
S.D.	19536	9462	25609	9464	11578
Mean log ratio		0.847	0.967	0.851	0.868
S.D.		0.073	0.054	0.086	0.068

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TABLE 42b (Fig 24)

5FU DELAY CIMETIDINE

(days from cimetidine)

Rat	Day0	Day1	Day3	Day7	Day14
1	26525	3037	21503	7538	2229
2	27277	2952	3677	5073	2317
3	71217	18280	63820	5605	17836
4	58310	32502	33431	17246	23131
5	30904	8916	18448	9245	18032
6	49098	2340	37211	15495	13818
7	48121	1363	20997	44216	3406
8	65450	13391	112976	16996	18341
9	46992	1711	21757	1723	-
10	75091	1059	15776	17742	14240
Means	49899	8555	34960	14088	12594
S.D.	17741	10257	31792	12083	7928
Mean log ratio		0.783	0.941	0.858	0.848
S.D.		0.107	0.069	0.080	0.073
cf controls p=		NS	NS	NS	NS

TABLE 43a (Fig 25)

DIURNAL RHYTHM EXPT

(10a.m.cyclo 40mgs/kg)

Rat	day0	day1	day3	day7	day14	day28
1	50177	9097	14608	3941	9016	45151
2	57542	2952	18884	21400	40685	68467
3	51662	19770	17361	13187	28699	81760
4	69674	22541	5411	24511	70750	108046
5	44514	13339	23990	36650	22912	89481
6	83455	33636	25356	44860	32978	70136
7	18804	1720	1924	2457	17281	31571
8	31301	5290	9650	5695	39435	49866
9	71392	13830	16951	10954	61649	87510
Means	53169	13575	14904	18184	35934	70221
S.D.	20267	10389	7938	14943	20036	24427
Mean log ratios		0.856	0.887	0.847	0.948	1.039
S.D.		0.040	0.093	0.083	0.060	0.041

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TABLE 43b (Fig 25)

DIURNAL RHYTHM EXPT

(p.m.cyclo 40mgs/kg)

Rat	day0	day1	day3	day7	day14	day28
1	57657	9106	15628	17620	23110	73125
2	123739	9097	1902	15521	23084	-
3	85079	19752	36415	26230	37972	67421
4	58045	7798	9741	4691	10954	52771
5	57647	16005	28834	22688	39407	69168
6	39198	12844	16948	1740	39149	67576
7	61726	17308	18888	24641	41524	94663
8	48336	8445	22360	13799	46235	88239
9	34970	13120	20605	17200	32206	93384
10	37480	8225	23816	2319	44783	104728
Means	60388	12170	19514	14645	33842	79008
S.D.	26688	4312	9592	9026	11382	16894
Mean log ratio		0.856	0.887	0.847	0.948	1.039
S.D.		0.040	0.093	0.083	0.059	0.041
cf controls p=		NS	NS	NS	NS	NS

TABLE 44

DIURNAL RHYTHM EXPT

Rats	cyclo given previous a.m.	p.m.
1	340	370
2	280	270
3	240	80
4	260	250
5	270	40
6	370	210
7	300	370
8	270	470
9	-	170
10	-	230
Means	291	246
S.D.	44	133

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