Corynebacterium Parvum treatment in normal and tumour bearing hosts

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Contents

Abbreviations used in this thesis

C. Parvum	Corynebacterium Parvum
C. Granulosum	Corynebacterium Granulosum
M.P.S.	Mononuclear Phagocyte System
R.E.S.	Reticuloendothelial System
I.V.	Intravenous
I.P.	Intraperitoneal
T.S.A.	Tumour specific antigen
T.A.A.	Tumour associated antigen
C.E.A.	Carcino embryonic antigen
B.C.G.	Bacille Calmette Guerin
M.E.R.	Methanol extractable residue
A.L.S.	Anti lymphocytic serum
D.M.H.	Dimethylhydrazine dihydrochloride
C.N.S.	Central Nervous System
P.H.A.	Phytohaemagglutinin
Con. A.	Concanavalin A.
S.M.A.F.	Specific macrophage arming factor
D.A.B.	Dulbecco solution A & B
F.C.S.	Foetal calf serum
A.C.T.	Trizma ammonium chloride
P.E.C.	Peritoneal exudate cells
H & E	Haematoxylyn and Eosin
D.P.X.	Distrene dibutylphthalate xylene

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Tables

Figures

Illustrations

Plan of Experimental and Clinical Work

Experimental

Wistar and WAG rats 150-250 G

A. Selection of the dose of C. Parvum used in subsequent experiments. By assessing the effect of I.V. and I.P. C. Parvum in graduated doses in terms of the resulting splenomegaly.

B. Studies on effluent gut lymph

Surgical excision of mesenteric lymph nodes —(at least 6 weeks later)→ Cannulation of thoracic duct

Groups studied		Lymph analysis for
No other treatment	5 days drainage of lymph	1. Hourly cell output
C. Parvum 0.7 mg I.V. 1-4 weeks before cannulation		2. Hourly output of esterase positive cells
rats bearing early colorectal cancer		3. Hourly output of phagocytic cells
rats with colorectal cancer given C. Parvum 0.7 mg I.V.		

C. The effect of C. Parvum on cells in the peripheral blood.

D. The effect of C. Parvum on cells in the peritoneal cavity and in splenic macerates.

E. The histological changes induced by C. Parvum.

Clinical

A. A clinical trial of specific immunotherapy using C. Parvum for its adjuvant effect and injecting this intradermally with irradiated autologous tumour cells in patients with treated lung cancer.

B. A clinical trial of serial infusions of I.V. C. Parvum in treated colorectal cancer patients aimed to non-specifically stimulate the immune system against residues of cancer.

Summary

Corynebacterium Parvum is an anaerobic diphtherioid bacterium which suppresses the rate of development and spread of a wide variety of transplantable rodent tumours. It has also been suggested that the bacterium might be of therapeutic benefit to humans suffering from cancer. The experimental work of this thesis discusses some of the effects of C. Parvum on mononuclear phagocyte cells both in normal rats, and rats bearing carcinogen induced cancers of the colon. Particular attention has been paid to this class of cells since it is considered that they are the mediators of the anti tumour effects of the bacterium. In addition, the results of two clinical trials reporting the results of C. Parvum in the treatment of post operative human cancers are presented.

Experimental Work

The dose of C. Parvum used in the experimental work was calculated from preliminary experiments. C. Parvum was given to adult female Wistar Rats by a variety of routes at different dose rates. Stimulation of the reticuloendothelial system was assessed by measurements of the resulting splenomegaly. It was considered that the optimum dose calculated by this work would also cause stimulation of mononuclear phagocytes.

An experimental rat model has been described at the Sir William Dunn School of Pathology in Oxford which enables the continuous collection of effluent gut lymph containing mononuclear phagocytes. It is argued that these cells represent tissue mononuclear phagocytes and lymph collected in this way enables a dynamic assessment of cell traffic in the gut wall.

A dose of C. Parvum which caused marked reticuloendothelial stimulation had no measured effect on these gut associated mononuclear cells nor on cells showing the capacity to ingest antibody coated sheep red blood cells. These studies were repeated in rats bearing small colonic cancers induced by dimethylhydrazine. Once again the dose of C. Parvum which was given had no effect on gut associated mononuclear cells or phagocytes. However, the numbers of phagocytic cells were markedly suppressed in all of the tumour bearing rats, whether or not they had been treated with C. Parvum.

Since the dose of C. Parvum caused splenomegaly but had no effects on mononuclear phagocytes in effluent gut lymph, other effects of this treatment were studied.

The dose of C. Parvum which was used caused:

1. Blood changes : an immediate lymphocytopaenia with a concomittant granulocytosis in peripheral blood. Subsequently the granulocyte level returned to normal levels but the lymphocytes showed a small, but significant increase above the original value by two weeks after treatment. Monocyte numbers in the peripheral blood were not affected.

2. Peritoneal exudates and splenic macerates : esterase positive and phagocytic cells obtained from both of these sources showed a fall one week after treatment followed by a rise by two weeks.

3. Histological changes : there was a marked increase in the area of the white pulp, and probably also the red pulp of the spleen. Both the spleen and caecal lymph nodes showed histological evidence of stimulation of the reticuloendothelial system. It had been hoped to identify

tissue macrophages using acid phosphatase, β glucuronidase and non specific esterase staining techniques. However, it was not possible to assess post treatment changes by this method.

Clinical

Two clinical trials are reported describing the use of C. Parvum in the adjuvant treatment of two common human cancers. In the first of these, the bacterium was given in a small intradermal dose along with an inoculum of autologous irradiated tumour cells to post operative patients after resection of Stage 1 and 2 lung cancer. In this trial an attempt was made to stimulate specific immunity against residual tumour cells. In the second trial C. Parvum was given by serial intravenous injections to patients who had undergone resection of Dukes stage B & C colorectal cancers. This approach attempted to non specifically stimulate the reticuloendothelial system with the aim of increasing the destruction of residual tumour cells by "activated" mononuclear phagocytes.

In each trial nearly 100 patients were included with an equal distribution between the treatment and the control groups. It was apparent that the median times to recurrence or death were not influenced by the treatment in either trial.

Considerable enthusiasm for the use of bacteria in the treatment of human cancers was generated in the early 1970's. This followed reports of improved remission rates notably after the use of B.C.G. in the treatment of acute lymphoblastic leukaemia and malignant melanoma.

Carefully constructed clinical trials have failed to support the continued use of this kind of treatment. Both the experimental and clinical work described in this thesis suggest that immune stimulation mediated by bacteria will not provide an easy answer to the treatment of human malignant disease.

Aims of the study

Corynebacterium Parvum (C. Parvum) has been shown to stimulate the reticuloendothelial system and to suppress the growth of a variety of tumours in experimental animals. Reports of benefit from the systemic use of the bacterium in patients suffering from advanced cancer encouraged clinical trials. For example two such trials have been carried out in the Oxford Region, on patients who have undergone surgical excision of lung and colon cancers.

C. Parvum given systemically has an anti-tumour action which is considered to be mediated by cells of the mononuclear phagocyte system. (M.P.S.) This theory has been studied in normal rats and in rats bearing dimethylhydrazine induced colonic cancers.

Experimental Work

1. The principal aim of the study has been to assess whether systemic C. Parvum influences M.P.S. cells in effluent gut lymph. These cells may reflect the state of M.P.S. cells within the gut wall. This study has been performed on both normal rats and rats bearing dimethylhydrazine induced colonic cancers.

2. The effect of C. Parvum on M.P.S. cells in the blood, peritoneal exudates, the spleen and in tissue sections has been assessed.

3. As a preliminary to this work, a safe dose of the bacterium which reliably stimulates the reticuloendothelial system was established.

Clinical Trials with C. Parvum

Two clinical trials are reported.

1. Lung Cancer: Lung cancer patients who had undergone surgical clearance of Stage I and II disease were randomised in the operating theatre to treatment or no treatment groups. In those patients drawing treatment a tumour cell suspension was prepared and frozen. During convalescence 2×10^7 of these cells were irradiated with 10000 rads and injected intradermally in the thigh along with 50 µg of C. Parvum.

2. Colorectal Cancer: Selected patients who had undergone surgical clearance of Dukes B & C colorectal cancer were randomised to treatment or no treatment groups. Patients drawing treatment commenced a series of intravenous infusions of C. Parvum within 30 days of surgery after giving informed consent.

The capacity of C. Parvum to stimulate the reticuloendothelial system.

The ability of a large group of myobacteriae and propionibacteriae to stimulate the reticuloendothelial system (RES) has been widely investigated. (1) C. Parvum is one of a group of anaerobic bacteria which are members of the propionibacterium class. An "activated" RES, capable of increased tumour cell destruction could be of therapeutic benefit.

The clinical hope has been to use C. Parvum as a treatment to stimulate the host's anti-tumour defences but it seems important to survey the evidence that a real stimulation of the RES has taken place.

Bacteriology

C. Parvum is one of a large group of preferential anaerobic bacteria (some 600 being described by Prévot) which appear to quickly lose their pathogenicity on being cultured. Some of these bacteria can produce a fatal malignant disease of the RES both in experimental animal models and in humans. (1) In the animal models their injection causes lethal stimulation of the RES with the liver, lung, spleen and kidney showing increased numbers of histiocytes, lymphocytes and macrophages. Prévot has a theory that recurrent anaerobic corynebacterial septicaemias occur in humans and eventually lead to the development of a malignant reticulosis. He also describes corynebacteriae being found in lymph nodes of patients with Whipples disease and claims that this is another

manifestation of over stimulation of the lymphatic system.

The increase in phagocytes caused by C. Parvum is paralleled by an increase in splenic and liver mass.

The most obvious result from the systemic use of C. Parvum is very marked hepatosplenomegaly and this forms the starting point for examining RES stimulation.

1. Hepatosplenomegaly: The observed increase in weight of liver and spleen after C. Parvum in experimental animals is dose related (2 & 3) and peaks about two weeks after the injection. This increase is only noted after inoculation which enables the bacterium to gain systemic exposure, that is, by the intravenous or intraperitoneal routes. After subcutaneous or intradermal inoculation local lymph nodes undergo hypertrophy but no systemic organomegaly occurs. That the enlargement of the liver is associated with an increased clearance of foreign particles by phagocytosis was shown in the classical work of Halpern. (2) He also showed that the increased rate of clearance of colloidal carbon was dose related. Histological sections of livers from these animals showed increased numbers of carbon containing phagocytes and lympho-histiocytic granulomata. The spleen showed marked hyperplasia of both the red and white pulps, an increase in histiocytes and lymphocytes, but carbon containing macrophages were limited to the red pulp. These appearances reverted back to normal a few weeks after injection of C. Parvum. C. Parvum induced organomegaly depends on direct interaction between the bacterium and the organ concerned (4) and is also partly dependent on lymphocyte trapping. (5)

Experiments using radiolabelled C. Parvum show that it becomes distributed principally in the spleen, liver and lungs. Smaller amounts are initially found in the blood, kidney, thymus and bone marrow but disappear rapidly. These observations remain the same with a variety of radiolabels. (6, 7 & 8) Autoradiographic and fluorescence studies show that the C. Parvum is localised within mononuclear cells. (8)

2. Phagocytosis and Fc receptor bearing cells of cellular homogenates (9): The work in this field appears to have been limited to an analysis of the macrophage content of tumour preparations. A spontaneously arising mouse mammary adenocarcinoma was transplanted syngeneically. Systemic C. Parvum caused marked splenomegaly and partly inhibited tumour growth. There was no increase in Fc receptor bearing cells nor of carbon ingesting cells within the tumour. A relative increase in the proportion of Fc receptor bearing cells over phagocytes was observed. It was suggested that this is due to the presence of increased numbers of non phagocytic monocytes which may represent the effector cell population. (9) C. Parvum has been observed to exert an appreciable anti-tumour effect without influencing Fc receptor cell concentration in a transplantable methyl cholanthrene induced mouse fibrosarcoma. (9, 10 & 11)

3. Phagocytosis of Radiolabelled particles: Radioactive emissions from various tissues some time after the intravenous (I.V.) inoculation of radiolabelled red blood cells has been studied in rats. This enables an estimate of the phagocytic capacity of each tissue to be made. (12)

The radioactive emission rate from the liver was considerably increased after C. Parvum. Although the spleen showed little such increase in the early stages there was an increase by day ten post C. Parvum. The emission rate correlated well with the disappearance of injected colloidal carbon from the blood, hitherto the standard in vivo test for assessing stimulation of phagocytosis. By calculating the organ emission rate/ blood emission rate a figure is obtained for the uptake ratio. In this study the splenic uptake ratio exceeded that of the liver by day ten after C. Parvum.

4. ^{3}H thymidine uptake: Experiments involving the tritiated thymidine uptake of liver cells in sublethally irradiated rats showed that C. Parvum not only induces an influx of new sinusoid lining (Kupffer) cells from an extra hepatic source, but also stimulated proliferation of the pre-existing cell population. (13)

5. Analysis of tissue and serum enzyme concentrations: Cellular suspensions obtained from chickens both by tracheal lavage and whole lung homogenisation were analysed. It was found that after intravenous C. Parvum the suspensions contained an increase in acid phosphatase, β - D - glucuronidase, β - D galactosidase and phospholipase A. This increase was due both to greater numbers of cells containing the enzyme and also to an increase in enzyme concentration per cell. (14)

The incubation of macrophages with anaerobic corynebacteriae for an hour caused a great increase in the concentration of acid phosphatase, β - D glucuronidase and β - D galactosidase found after lysis of these cells. A high percentage of these enzymes were also found free in the supernatant.

In addition to analysing the concentration of these hydrolases in the acute phase their levels were measured in tissue macrophages at various time intervals up to three weeks after IV and intraperitoneal (IP) injection of various anaerobic coryneforms. (15) This work showed that the enzyme content per cell gradually increases. It was suggested that C. Parvum both stimulates synthesis of new enzyme and also the release of pre-formed enzymes. This would explain the increased measurements found in both the acute and chronic groups.

Wilkinson suggests that the anaerobic coryneforms have cell wall molecules with an affinity for macrophage microfilaments and which stimulate the discharge of lysozomal enzymes. A subsequent period of protein synthesis and division ensures the appearance of a population of stimulated macrophages containing high enzyme concentrations. (16)

Increased levels of serum lysozyme were found in mice being treated with C. Parvum. These animals harboured isotransplanted methyl cholanthrene induced fibrosarcomas or spontaneous mammary carcinomas. The increased lysozyme levels were not found to be of any value as prognostic indicators. (17)

A chemotactic factor has been observed in both cultures and culture filtrates from many strains of anaerobic coryneforms. (18) This factor has been shown to be strongly and specifically attractive to monocytes and may be responsible for the migration of monocytes into areas of C. Parvum localisation in vivo.

6. Stimulation of the Bone Marrow: The promonocyte precursors of all cells of the mononuclear phagocyte system (MPS) are found within the bone marrow. Marrow cell suspensions were obtained from the femoral shafts of experimental animals and then incubated with a source of colony stimulating factor for one week. Discrete colonies formed, each of which was taken to represent one original promonocyte. After C. Parvum there was a highly significant increase in the number of such colonies found. (19 & 20) It was also noted that strains of C. Parvum with the highest anti-tumour activity appeared to have the greatest effect in increasing the stimulation of macrophage progenitor cells. (21)

Summary

The evidence suggests that C. Parvum increases MPS cell numbers, their phagocytic capacity, their concentrations of acid hydrolase enzymes, and influences localisation of these cells.

Human Studies

Investigations have been much more limited in this field but some evidence exists to suggest that the effect of C. Parvum on the human MPS is the same as that in animals.

Patients with cancer being treated with C. Parvum showed an increased rate of clearance of ^{125}I labelled aggregated albumin from the blood. This test was shown to closely parallel the disappearance of colloidal carbon in experimental animals and by inference confirms that C. Parvum increases the phagocytic activity within the RES of cancer patients. (22)

After intravenous C. Parvum a transient monocytopaenia has been observed. This has variously been described as lasting for 24 hours, four and five days and ten days. (23, 24 & 25) It has been suggested that the monocytopaenia is due to the sequestration of blood monocytes within the RES. The subsequent monocytosis which appears at about 14 days is thought to be due to bone marrow stimulation.

Conclusion

There is clear evidence that C. Parvum can stimulate the RES in animals and humans. Whether this stimulation can be harnessed to increase immunity is not yet clear. Clarification of the role of the MPS against spontaneously arising malignancy is needed. It seems unlikely that a single cell line will prove capable of attacking malignant tissues alone. Some form of co-operative response may exist and identification of participating cells is an urgent priority.

Immunotherapy

Ehrlich's original concept of an immune surveillance mechanism has been restated more recently by Burnet. (26) According to this theory malignant transformation, rather than being a rare event, occurs frequently, but these cells are eliminated as they arise by a powerful immune mechanism. Clinically detectable cancer will only become apparent when this system is overwhelmed. As a corollary it should be possible to stimulate these putative mechanisms increasing the resistance of the host to the development and spread of cancer.

Evidence implicating the immune system in the development and spread of cancer

It seems likely that the patients immune system is capable of mounting an attack against malignant cells and that these defences are broken down by advancing disease.

Malignant cells can be found in the general circulation during the growth of primary tumours. It is probable that these cells only rarely survive to develop into secondary deposits of cancer. (27) The factors which normally prevent this development are unknown. Local factors may play a part. For example, engulfment by cells of the mononuclear phagocyte system (MPS) in the same way as they would deal with invading bacteria is possible. Macrophage and lymphocyte activity at the periphery of tumours is reported to confer an improved prognosis (28) as does sinus hyperplasia in the draining lymph nodes. (29) These histological signs of activation of the MPS seem beneficial. The opposite may be the case where activation does not, or cannot, occur.

Patients bearing small localised tumours are usually immunologically competent as measured by skin tests of cellular immunity, serum immunoglobulins and lymphocyte numbers. This is not the case in those who have advanced malignant disease, many of whom show suppression of cellular and humoral immunity. (30, 31, 32, 33, 34 & 35)

Cellular immunity can be clinically measured by the subcutaneous inoculation of a variety of skin test antigens. The substances inoculated usually include mumps virus, purified protein derivative of the tubercle bacillus, streptokinase, streptodornase, trichopyton and candida albicans. Response is measured by the development of cutaneous nodules and patients showing no reaction are termed anergic. There is a clear relationship between suppression of these responses and prognosis, patients with the most suppressed responses having the poorest outlook. This is true for various solid tumours, (30) acute leukaemia (35) and lung cancer. (34) It is also recognised that patients undergoing therapeutic immune suppression after transplant surgery have a high incidence of neoplasia, particularly lymphomas. (36)

Some oncogenic viruses induce tumours in normal neonatal animals, but will only do this in adults which have been immunosuppressed. Chemical carcinogens on the other hand, have an equal effect in inducing tumours in immune suppressed and normal animals. (27)

Reports that some malignancies undergo spontaneous regression may suggest alteration of the host/cancer relationship. This phenomenon has been observed most commonly in malignant melanoma. "Immune" sera from those fortunate patients can be transferred with benefit to those suffering from the same disease. (37)

More information is required to substantiate the view that immune surveillance is important in determining the incidence of malignant disease. Immune status may play a greater part in governing the growth pattern of tumours.

Transplanted tumours which do not normally metastasize can do so if grown in immune suppressed animals. It may be that these hosts, be they animal or human, are unable to eradicate residual tumours after surgery, chemotherapy or radiation treatment as these can all cause long lasting immunosuppression. (38, 39) Moreover these treatments may render the patients more likely to develop metastases and supports the viewpoint that restoration of immune competence would be beneficial.

The antigenicity of tumours

In 1957 Prehn and Main succeeded in demonstrating tumour specific antigens. (T.S.A.'s) (40) Since then large numbers of T.S.A.'s have been identified in carcinogen induced animal tumours. The possibility that such antigens would be present in human tumours, enabling the development of specific immunotherapy led to a resurgence of interest in this approach. The original investigations were carried out on a murine methyl cholanthrene induced sarcoma and it remains far easier to demonstrate T.S.A.'s in carcinogen induced malignancy. It has not been possible to identify T.S.A.'s in human cancer. Human tumour associated antigens (T.A.A.'s) like carcinoembryonic antigen (C.E.A.) have however been identified. (41) Although originally thought to be associated only with cancer of the colon such specificity has now been disproved. Serum C.E.A. levels are elevated in a variety of conditions.

This was the first of a group of tumour associated antigens described in malignant disease. As a group they resemble antigens found in the developing foetus. It is suggested that they are produced by malignant cells showing reversion to the less differentiated state found in the primitive cells of the developing foetus.

These are termed oncofoetal antigens and, although not absolutely specific for any given malignant condition they have proved to be useful in monitoring the progression of some cancers. The other oncofoetal antigen which is particularly important is α foetoprotein. This is elevated in many cases of testicular teratoma and all cases of hepatocellular carcinoma. However, these antigens are not at present of widespread application in the management of cancer patients.

General approaches to immunotherapy

Passive

In passive immunotherapy, the effector arm of an immune response directed against tumour specific antigens is mediated by either antibodies or lymphoid cells produced in donors, usually animals. This approach was used as long ago as 1895 by Hérecourt and Richet. (42) They raised antisera in dogs and donkeys by immunising the animals with fragments of the patients tumour. 50 patients were treated with the resulting antiserum. Diminution of tumour size, lessening of pain and improvement in general condition was claimed to result in many cases. More recently 200 patients were treated with globulins from horses immunised with human tumours. Again subjective

improvement was claimed to occur in a large number of patients with occasional objective evidence of benefit. (43)

Lymphoid cells can be prepared from an animal that has been immunised with tumour cells. These are then given to the original tumour bearing host. If the host is immunosuppressed a graft versus host reaction may ensue. This may destroy as many normal host cells as tumour cells and would therefore not be useful. However, in the immunocompetent host the graft versus host reaction does not occur since the injected lymphocytes are unable to become established. This type of procedure is of limited application. In the region of 10^9 cells require to be transferred and clinical benefit has not been demonstrated. (44)

Cell free extracts which contain RNA from immunoblasts raised against tumours in allogeneic or xenogeneic hosts exert an anti-tumour action. (45) While injection of these extracts may transfer specific immunity, it is difficult to obtain a large enough quantity to be clinically useful.

Immune macrophages from the peritoneal cavities of immunised mice can be used to control ascites, provided they are injected directly into the ascitic fluid. Monocytes may also be capable of transfer in this way and have the advantage of recirculating. However, it is difficult to obtain large enough quantities to evaluate clinical response. (46)

Lymphoid cell transfer has been used clinically in the management of malignant disease. Nadler and Moore reported cross immunisation of pairs of patients suffering from advanced malignancy (mainly melanoma). Exchange of peripheral blood lymphocytes was then carried out and a few regressions noted. (47)

Active Immunotherapy

Specific

Auto-immunisation with irradiated tumour cells leads to the transient appearance of antibodies specifically directed against the inoculated tumour. At the same time serum tumour antigen levels fall. It is argued that by altering the situation from antigen excess to antibody excess, circulating cytotoxic cells will be enabled to act against the malignant cells in the tumour since they will no longer be blocked by T.S.A.'s. (27) Whether this form of immunisation will also increase the level of circulating cytotoxic cells is unclear. To achieve increased antibody levels repeated inoculation with large numbers of cells is required.

This therapeutic approach was first reported in 1902. (48) An autologous tumour cell suspension was used as a vaccine in patients suffering from advanced malignancy. No clear cut evidence of benefit resulted. A more recently reported series of 232 patients with advanced gynaecological cancer treated with autologous irradiated tumour extract similarly failed to show any evidence of benefit. (49)

In an effort to enhance the immunogenicity of the inoculum various adjuvants have been given. Pertussis, typhoid A and B, and complete Freunds adjuvant have been used, (50) but again no clear evidence of benefit resulted.

Non Specific

Non specific immunotherapy aims to increase immunological reactivity usually by stimulation of the reticuloendothelial system. This has the effect of increasing cell mediated and humoral reactivity to a variety of unrelated antigens.

Bacille Calmette Guérin (B.C.G.) has been given regularly by dermal abrasion in the treatment of acute lymphoblastic leukaemia. Mathé's report, in 1969, that this treatment had significantly prolonged the length of remission of this disease, probably fired more optimism for immunotherapy than any other single report. (51, 52) His patients also received irradiated allogeneic leukaemic blast cells, routine anti leukaemic chemotherapy and in some cases other immunostimulants, like Poly 1, Poly C and C. Parvum. It was therefore difficult to know exactly what contributed to the improved results.

C. Parvum is an even more potent stimulant of the RES than B.C.G. Given systemically its anti-tumour action is likely to be non-specific and mediated by cells of the M.P.S.

Bacteria in the treatment of cancer

Bacteria, bacterial extracts and parasites have been quite extensively studied in the treatment of cancer. (53)

One of the first reports of benefit in cancer treatment was Coley's claim of tumour response to streptococcal endotoxin in 1906. (54) Patients suffering from advanced cancer were inoculated with the endotoxin and developed fever as a result. Tumours were noted to become smaller with improvement in the general condition of the patient in a significant number of cases. These results and others from similar trials with bacterial endotoxins have been reviewed and their use is still advocated by Nauts. (55) Although there have been persistent suggestions of benefit from this treatment, critical analysis shows an absence of control patients and improvement has often been claimed on a purely subjective basis.

The effects of repeated treatment with B.C.G., usually given by scarification, have been extensively studied in patients suffering from malignant melanoma.

This approach, particularly when treatment is given directly into the skin lesion, sometimes leads to regression of both injected and non-injected skin nodules. However, there is no evidence that this has any effect on visceral metastases, or the survival of the patient. In addition the treatment is painful and frequently causes local inflammatory reactions which may lead to dermal ulceration. This approach has been extensively evaluated in malignant melanoma, particularly by workers in the U.S.A. Reports

that repeated intradermal B.C.G. either given alone or in combination with autologous irradiated melanoma cells led to a 50% reduction in metastases and an improved remission rate, (56) prompted similar trials in this country. (57, 58) However, it rapidly became apparent that the treatment group of patients in this country, far from having improved results showed worrying evidence of early recurrence of disease and the trials were brought to an end.

Reference has already been made to Mathé's report of improved remission rates using B.C.G. in the treatment of acute lymphoblastic leukaemia. (51, 52) B.C.G. was added to the standard treatment of this condition in a large series carried out under the auspices of the Medical Research Council. (59) In their report no added benefit occurred in the B.C.G. treated group.

Both of these examples illustrate the complexity of the problem. While one group of workers claim improved results others find none, or even the suggestion of worsening of outlook.

While B.C.G. has been extensively tested, its side effects make it an unpleasant form of treatment. Cell wall extracts of B.C.G., for example, methanol extractable residue (MER) have been developed which may result in equally effective immune stimulation without causing the side effects. (60) The anaerobic diphtheriods have been shown to cause intense stimulation of the RES. C. Parvum and C. Granulosum have been investigated in the hope that this stimulation would boost anti tumour immunity.

C. Parvum as an anti tumour agent

Animal Studies

Since 1966 when first Woodruff in Edinburgh, then Halpern in Paris (61, 62) demonstrated that systemically administered C. Parvum exerted an action against experimental animal tumours its anti tumour effects have been repeatedly demonstrated. (63) However, the bulk of this work has been carried out in syngeneically transplanted murine tumours. These are either induced with chemicals or oncogenic viruses. Those arising spontaneously have been maintained by passaging usually for several generations. These animal tumour models bear little similarity to the human situation, but such experiments may elucide the mechanism by which C. Parvum exerts its anti tumour action. Improvement in understanding might reasonably be expected to clarify the naturally occurring host defence mechanisms against cancer.

Systemic Injection

Woodruff and Boak demonstrated that intravenous C. Parvum, given as a single injection 8 to 12 days after the subcutaneous implantation of a spontaneously arising mammary carcinoma, delayed the appearance and subsequent growth of the tumour. (61) Halpern then showed a similar suppression of growth of a transplanted sarcoma (Sarcoma J) if the bacterium was given within 2 days of tumour inoculation. His group also reported suppression of growth of the Ehrlichs ascites tumour when C. Parvum was given intraperitoneally at the same time as the tumour cells. (62)

The Edinburgh group then found that not only was intraperitoneal C. Parvum effective in suppressing growth when given within 3 days of inoculation of the mammary adenocarcinoma, but that the same effect was noted in two unrelated methylcholanthrene induced sarcomata. Elucidation of the possible mechanism came when exactly the same anti-tumour effect was found in T cell deprived mice. (64)

Systemically administered C. Parvum limits the growth rate of some transplanted tumours but has a more marked effect against metastases. For example the Lewis lung carcinoma is a transplantable tumour of mice, which after subcutaneous inoculation leads to a locally enlarging neoplasm and widespread lung metastases. I.V. C. Parvum given either at the time of challenge with the tumour inoculum or within 7 days caused suppression of growth of the primary tumour. If the C. Parvum was given by either the I.V. or I.P. routes 3 or 4 days prior to tumour cell challenge then a marked decrease in the subsequent development of metastases resulted. Subcutaneous inoculation of C. Parvum had no effect. (65)

Using the same animal tumour model, the mice were submitted to prior thymectomy, whole body irradiation and marrow reconstitution to render them T cell deficient. This had no influence on the effect of C. Parvum against both primary and metastatic tumours. When the animals were given silica or cortisone, both macrophage toxins, a considerable increase in metastases resulted. C. Parvum given after these toxins abrogated this effect, possibly by stimulating an increased formation of new M.P.S. cells in the bone marrow. (66) Another interesting observation made with this model was that non-specific operation (amputation of

a limb) led to an increased number of metastases and that C. Parvum given systemically abolished this effect. (66)

The closely related bacterium Corynebacterium Granulosum is effective against the development of pulmonary metastases after the intravenous inoculation of a syngeneic fibrosarcoma in mice. The bacterium in this case was administered intraperitoneally and the maximum effect against the development of metastases was only seen when inoculation was within 2 days of tumour cell challenge. (67)

Although systemic C. Parvum suppresses the development of metastases in T cell depleted animals, anti lymphocyte serum (ALS) has been shown to prevent this action of the bacterium. (68) A.L.S. acts mainly by destroying long lived T_2 lymphocytes. (69) The anti metastatic effect of C. Parvum while largely mediated by macrophages seems to require co-operation from these T_2 lymphocytes.

Using transplantable tumours which cause ascites (Meth A ascitic, S37 ascitic and Ehrlichs ascitic), intraperitoneal C. Parvum was protective provided it was given prior to tumour transplant. However, in this model prior splenectomy, thymectomy or treatment with A.L.S. had no effect. (70)

C. Parvum also limits tumour growth in complement deficient animals. (71) The animals used were congenitally deficient in C5 and were challenged with both a spontaneously arising mammary carcinoma and a methyl-cholanthrene induced sarcoma.

While repeated doses of C. Parvum stimulate an increased anti C. Parvum antibody titre this approach is no more

effective than single doses in preventing the development of pulmonary metastases in the Lewis lung cancer model. (72) Indeed, when repeated inoculations of C. Parvum were given with a further dose in the day of tumour challenge, the anti-metastatic capacity was abrogated. It is unclear whether the bacterium, when used in this way, stimulates suppressor cells, causes the appearance of circulating immune complexes blocking cell mediated immunity or simply produces a high titre of anti C. Parvum antibody which blocks its anti-metastatic effect. The observation does suggest that great caution should be taken when contemplating the clinical use of repeated C. Parvum.

Even in these animal studies systemically administered C. Parvum has not universally shown anti-tumour capabilities. (73) An investigation which closely paralleled those described on the Lewis lung cancer gave different results. Using two early generation transplantable adenocarcinomata which had arisen spontaneously and were of low immunogenicity, C. Parvum given I.P. four days prior to excision of the subcutaneously growing primary tumours had no effect on the subsequent development of lung metastases. Further efforts to induce an anti-tumour action by the inoculation of irradiated tumour cells were also ineffective. The authors concluded that there was, "no evidence that bacteria can superimpose specific antigenicity on tumour cells." Over three quarters of the tumours being used in the laboratory study of C. Parvum vaccine have been artificially induced by chemicals, radiation or oncogenic viruses. Of the other quarter, i.e. those which have arisen spontaneously, all are more than 20 years from their time of origin. For example the Lewis lung cancer arose more than 25 years ago and has been

exchanged between many laboratories and passaged in hundreds of animals. The opportunity for the acquisition of artefactual immunogenicity is very high, for example by genetic diversification or viral contamination.

When C. Parvum was given repeatedly intraperitoneally during the induction of colonic cancers using dimethyl-hydrazine, (DMH) rather than showing any protection the reverse occured. Rats given the combination developed tumours earlier than those receiving DMH alone. These rats also died earlier from more rapidly advancing tumours, they commonly developed metastases, which are unusual in this model, and showed a tendency to develop primary cancers at sites other than the gut. (74)

Local Injection

Intradermal, subcutaneous or direct injection into the tumour with C. Parvum causes negligible toxicity and is assumed to act through stimulation of cells in the regional lymph nodes. Used in this way there is no effect on the size of the liver or spleen nor is there any influence on the macrophage producing capacity of the bone marrow.

C. Parvum injected intradermally in the flank contralateral to an established transplanted methylcholanthrene induced fibrosarcoma has a small anti-tumour effect. However, when combined with intraperitoneal cyclophosphamide the suppression of tumour growth was much greater than either treatment alone. In fact in certain combinations many animals were "cured" of tumour. (75) Currie speculated that stimulated immune lymphocytes might be protected against cyclophosphamide which would then exert a proportionately greater effect on the tumour cells.

Cyclophosphamide has been shown to selectively depress the B cell areas of the spleen and lymph nodes. (76) A proportional increase in T lymphocytes after cyclophosphamide was reported with enhanced cell mediated immunity. It may be that the combination of C. Parvum and cyclophosphamide caused further enhancement.

Local injection of C. Parvum is only minimally effective if given at sites distant from tumour, but can cause marked inhibition of growth if injected nearby (i.e. where it is likely to engage the tumour draining lymph node). This is found in both mammary adenocarcinoma (77) and mastocytoma. (78) Injection directly into the tumour was most effective of all, but local injection has a significant effect provided the lymph node which drained the tumour also drained C. Parvum. This was not seen in T cell deprived mice, a finding which prompted Woodruff and his group to repeat their earlier work using local rather than systemic injection of C. Parvum. (79) They were able to confirm that the inhibiting effect of C. Parvum injected into the tumour noted in their transplantable fibrosarcoma was not present in T cell deprived mice. Animals in whom tumour growth was suppressed by this treatment were highly resistant to a second challenge with the same tumour. It was also noted that diphtheriods, which have no anti-tumour effect when given systemically are not active when inoculated directly into tumour.

When C. Parvum was injected along with an inoculum of live mouse mammary carcinoma cells tumour growth was normal in the initial stages, but at 14 days rapid and complete regression supervened. When the injection was delayed until the tumour had been established for 14 days rapid regressions of the primary and its metastases occurred.

Local injection of C. Parvum into the site of the tumour or mixed with tumour cells often causes complete regression. (79) (53) This is in distinction to its systemic use where, although suppression of tumour growth may result cures are rare. (80)

Combination of C. Parvum and tumour cells

Many attempts to stimulate specific anti-tumour immunity have been made using tumour cells whose mitotic capacity has been removed, but whose antigenic structure remains intact. Commonly a suspension of tumour cells is irradiated and re-inoculated either alone or in combination with an immune adjuvant. Used in this way C. Parvum has such an adjuvant effect (81) and boosts antibody response to a variety of antigens. Both IgG and IgM levels are augmented in animals.

The simultaneous s.c. injection of irradiated tumour cells and I.P. injection of C. Parvum to mice with small, but actively growing fibrosarcoma isotransplants caused more prolonged remission than either treatment alone. (82)

When the C. Parvum was given 7 days before the irradiated tumour cells, rather than conferring a beneficial effect, tumours grew more rapidly. (83) It appears that even in these immunogenic tumours the timing and dose levels of both tumour and C. Parvum are critical.

Mice challenged with either subcutaneous (s.c.) or I.V. methylcholanthrene induced fibrosarcoma cells derived very little benefit when given I.V. C. Parvum 2 days later. However, when the C. Parvum was combined with an optimum number of irradiated tumour cells and injected into the footpads with 6 days of the initial tumour challenge, very

marked suppression of growth and some cures resulted. This was only demonstrable in T cell intact animals and when the same tumour cells were used for inoculation and challenge. The immunity derived is highly specific. (84)

Mode of Action of Systemic C. Parvum

The anti-tumour action resulting from systemically administered C. Parvum may be largely non-specific. This action is not abrogated in T cell deprived mice (64, 85) and is also partly resistant to treatment with anti-lymphocyte serum. (70) Nor does it seem likely that this action of C. Parvum is dependent on humoral immunity as an anti-tumour effect is also seen in experimental animals selected for low antibody responses. (83, 84)

After systemic C. Parvum peritoneal exudate cells appear in both normal and thymectomised mice which inhibit tumour cell growth in vitro. (86) This activity disappears if the cells are first incubated on a glass surface and the glass adherent cells excluded from the tissue culture. It is also absent when the effector cells are lysed, (implying the action is not simply enzymic), or if they are not in intimate contact with tumour cells. The anti-tumour effect after systemic C. Parvum is paralleled by an increase in macrophage mediated phagocytosis and can occur in the presence of a high antibody titre against the organism. (87)

Other evidence suggests that the macrophage is the important effector cell. The action is still present when the peritoneal exudate cells are exposed to 4000 rads immediately before incubation with tumour cells. The surviving cells are macrophages and display the same anti-tumour action as non irradiated collections. In addition the adherent cells from such collections histologically

appear to be macrophages and such adherent cells have the same anti-tumour activity as the intact collection of cells.

Active strains of C. Parvum produce a macrophage chemotactic factor which can also activate lysozymes within these macrophages. (16) After systemic administration the bacterium localises within liver, lungs and spleen (88, 7) but the observation that up to 25% of the injected dose localised within the tumour (88) is denied by other investigators. (7) Selective localisation in liver and lungs may contribute to the anti-metastatic capacity of systemic C. Parvum.

C. Parvum has been shown to increase the number of macrophage progenitor cells in the marrow of stimulated animals. This increases the availability of M.P.S. cells. (19)

Serum lysozyme levels rose after intravenous C. Parvum in both normal and tumour bearing animals. (17) Examination of the cell population within syngeneic transplanted adenocarcinoma showed that C. Parvum increases the numbers of Fc receptor bearing cells, but cells capable of phagocytosing carbon particles were not increased. (9) It may be that non phagocytic monocytes were those bearing Fc receptors and these immature cells are the major effector cell population. (10) C. Parvum induced stimulation of bone marrow would lead to efflux of macrophage precursors. Using a transplantable mouse methylcholanthrene induced fibrosarcoma an anti-tumour effect was noted without any effect on Fc receptor positive cells within the tumour. (11) In contrast to the suggestion that the immature monocyte is the anti-tumour effector cell, it has been shown by differential sedimentation

of peritoneal exudate cells that the anti-tumour cells were found to be the heaviest cells i.e. the largest most differentiated macrophages. (89)

Mechanism of Action of local C. Parvum

Anti-tumour activity after locally administered C. Parvum must be at least partly T cell dependent. Suppression of subcutaneous growth of several transplantable murine tumours occurs in normal but not congenitally immunosuppressed (nude) mice. (90) C. Parvum may act as an adjuvant, enabling recognition by T cells of tumour associated antigens. However, at the site of C. Parvum initiated tumour destruction following its intralesional injection there is a massive mononuclear infiltrate suggesting that these cells also play a part. (91) It may be that C. Parvum evokes a T cell response against C. Parvum antigen which causes the release of substances capable of producing activated macrophages.

The ability of C. Parvum to suppress the growth of admixed tumour cells when given to athymic hosts was abrogated by concomitant administration of silica. This implies that a phagocytic cell is required either to destroy or assist in the destruction of the tumour cells. (92)

In summary

Systemically administered C. Parvum results in a moderate anti-tumour effect with considerable effect against developing metastasis in some models. The main effector cell is the macrophage and a generalised stimulation of the reticuloendothelial system results from this form of treatment, which requires a relatively high dose of the bacterium. There is augmented antibody production and the

marrow is stimulated producing increased numbers of macrophage progenitor cells.

The anti-tumour effect does not appear to require complement and is not dependent on T or B cell co-operation. However, there is evidence that the sub-population of T_2 cells is required. Anti-tumour activity is non specific in nature.

On the other hand locally administered C. Parvum acts mainly through T cells and macrophages. Its effect is to produce highly specific anti-tumour immunity, effected via the regional lymph nodes. Only low doses of C. Parvum are required. There is minimal systemic effect as a result and the marrow and reticuloendothelial system are not markedly stimulated.

C. Parvum in Humans

The use of a new agent which might be expected to cause toxic side effects in the treatment of cancer is preceeded by a phase 1 study. This means establishing the dose of the drug in a limited number of patients suffering from advanced malignancy. These studies do not set out to evaluate therapeutic benefit. Phase 1 studies using intravenous C. Parvum suggested that it could be given safely in doses of up to 10 mg/m^2. (93, 94, 95)

Side Effects

In all studies where the bacterium has been given intravenously the dose has been suspended in a diluting volume of fluid, usually normal saline. The dose given has been based on the estimated surface area of the patient, calculated with a nomogram using the height and body weight.

In most reported series the dose has been between 5 and 10 mg/m^2. Infusion usually takes between half an hour and four hours. Side effects are unusual in the first two hours. Chills, fever and occasional rigors are common between two and four hours.

These symptoms can be partly controlled with extra blankets and aspirin. However, they can neither be prevented nor completely controlled. Headaches and musculoskeletal aches and pains are also often experienced. Again aspirin helps. Nausea and vomiting is common, but can largely be controlled by the prior administration of anti-emetic drugs. The overall impression of this phase of the reaction is of an influenzal-like illness.

A more serious side effect is the marked fluctuation in blood pressure that occurs, (96, 97) although some have not found this to be a problem. (93) Both hypotension and hypertension can develop and they may follow in sequence. This side effect had been found during phase 1 studies and as a result caution was taken over patient selection for this treatment by all investigators. In most patients these blood pressure fluctuations can be managed conservatively and increasing the infusion rate of simple fluids usually controls the hypotension. However, in our own series one patient died of an acute myocardial infarction 24 hours after a period of C. Parvum induced hypotension. (96) Hedley and Currie reported a very similar mortality in their treatment of a patient suffering from malignant melanoma. (98) Although, in their case the precise cause of death was unclear it seems likely that it was similar to ours. The subsequent rigid exclusion of patients with any suspicion of cardio respiratory disease makes the systemic use of C. Parvum unsuitable for a large number of patients suffering from malignant disease.

While some workers have found that hydrocortisone will prevent these fluctuations in blood pressure (93) others have not. (96) Moreover hydrocortisone may suppress macrophage function thereby interfering with the proposed mechanism of C. Parvum induced anti-tumour activity.

Another serious side effect is that of confusion which did not appear to be directly due to hypotension. (93, 94, 96) Patients in our series have not been known to suffer from cerebral disease or metastases and confusion has only been found in two patients. However, when the drug was used in the treatment of patients suffering from known cerebral metastases severe CNS side effects occurred in two. (93) One became aphasic with a hemiparesis then recovered. The other patient became aphasic after a fit and died. This was in spite of prophylactically given hydrocortisone, which this group used in order to minimise side effects. A similar death occurred in a British study (97) where the clinical picture suggested cerebral oedema. It was suggested that as biopsies from these tumours showed acute inflammatory oedema with histiocytic infiltration, the presence of an intracranial tumour should be a contra-indication to the use of C. Parvum.

Fisher also gives an isolated report of a patient developing a proliferative glomerulonephritis and renal failure after systemically given C. Parvum. (93) Lastly there is an increased susceptibility to viral infections, particularly herpes zoster and herpes simplex.

When C. Parvum is given intradermally or subcutaneously very few side effects have been reported. Occasionally transient painful swelling of the lymph nodes draining the site of inoculation has occurred.

Haematological Alterations after C. Parvum

Systemically administered C. Parvum causes the following haematological alterations:-

Haemoglobin: Most workers do not comment on the haemoglobin level after systemic C. Parvum. However, a fall of up to 2 G with an increased reticulocyte count has been reported. (99, 94)

Monocytes: Some workers have found that blood levels of monocytes drop precipitously within hours of treatment then slowly rise to peak at greater than normal levels some 14 days after injection. (96, 99, 23) However, others did not find this. (100) No alteration in the serum level of the macrophage enzyme lysozyme follows C. Parvum therapy. (25, 99)

Lymphocytes: Lymphocytopaenia occurs within hours of the infusion. (25, 99 23)

T cell function has been analysed in several studies.

While T cell numbers were decreased in the study of Micheson and Castro (101) others found no change. (100)

The response of T cells to polyclonal activation with the plant mitogens PHA and ConA has been extensively used as an *in vitro* test for cell mediated immunity. Investigations using lymphocytes from patients who have received C. Parvum lead to confusing results.

An increased lymphoblast response was found after intravenous C. Parvum in doses from 1-5 mg. This correlated with an increased skin response to a battery of recall antigens. (23) However, the responses were suppressed more often than they were increased and the results were inconclusive. A slight increase in these responses was seen after intravenous C. Parvum in a dose of 2 mg/m^2 given to patients suffering from malignant melanoma. However, no claims of significance were made. (102)

Micheson and Castro found that these responses were significantly reduced in their patients all of whom suffered from advanced cancer. Further suppression occurred after systemic C. Parvum given at a dose of 10 mg/m^2. (101)

Similar depression was noted in patients with treated colorectal cancer receiving C. Parvum 5 mg/m^2 I.V. Here the suppressed values were statistically significant when measured 3 hours after infusion. (96)

Neither culture of lymphocytes <u>in vitro</u> with C. Parvum nor lymphocytes taken from patients with breast cancer treated with C. Parvum showed any consistent changes. These patients were receiving alternating courses of chemotherapy and C. Parvum, so that the chemotherapy might have influenced the lymphocyte responses and obscured the effect of C. Parvum. (103)

This confused picture makes it extremely difficult to draw conclusions. It is quite likely that much of the confusion is due to the large number of variables involved. For example, the patients were suffering from a variety of different malignancies and were advanced in some series while in others all known tumour had been extirpated.

Different doses of C. Parvum were used and the lymphocytes were analysed at variable times after the infusion. However, it does seem likely that any lymphocyte stimulation was modest and not of real significance.

Immunoglobulins: Serum levels of immunoglobulins are not altered by C. Parvum treatment in humans. (93, 94, 102, 100) An increase in the IgG subclass IgG_2 has been reported. (101) (104)

The overall impression is that humoral immunity is unlikely to be stimulated by this treatment. This is in contrast with the increase in IgG and IgM noted after its use in experimental animals. (105)

Skin Testing: The poorer prognosis of patients who are anergic to skin test antigens has been described. Although C. Parvum treatment occasionally converts the patients response from negative to positive, usually no alteration occurs. (100, 93, 94)

Clotting Factors: Systemically administered C. Parvum causes considerable alterations in blood clotting factors. A marked increase in fibrinolytic activity occurs within 2 hours of infusion returning to normal levels by 4 hours. A corresponding increase in the level of fibrin degradation products is noted, although there is no alteration in the total fibrinogen level. (106, 101) Whether this is of any therapeutic benefit is open to doubt. It has been argued that fibrinolysis diminishes the likelihood of circulating tumour cells successfully implanting to develop into a metastasis.

Although evidence has been presented suggesting that serial infusions lead to a state of disseminated intravascular coagulation this has not been clinically apparent in patients so treated. (101)

Biochemical change: During the 5 hours following the intravenous infusion of C. Parvum there is a rise in the serum levels of glucose, lactate and ketone bodies and a fall in serum alanine. Bilirubin, glutamyloxaloacetate transferase and urea are elevated 24 hours after the infusion while albumin and cholesterol are lowered. (107) These alterations are similar to those seen after bacterial infections of the liver with acute parenchymal cell damage.

Phagocytic Function: Since stimulation of the RES was the primary observation in experimental animals injected with C. Parvum Attié devised a measurement of this function suitable for patients. (22) Serial measurements of the level of serum radioactivity after the injection of radiolabelled microaggregated human albumin was performed. This gave a measurement of the rate of disappearance of the test substance. By comparing this rate with standard controls a measure of the uptake of the substance was obtained. These studies appeared to confirm that when C. Parvum was given to these patients, all of whom suffered from cancer, increased phagocytosis occurred.

Macrophage Function: Hedley and Currie have examined monocyte preparations obtained using Ficol-hypaque gradients according to the method of Böyum. (98) These preparations were made from blood obtained from patients suffering from disseminated malignant melanoma or those with probable micrometastatic malignant melanoma. The patients were being treated with alternating courses of the chemotherapeutic

agent imidazole carboxamide (D.T.I.C.) and intravenous C. Parvum in doses ranging from 700 µg to 7 mg. Three different ways of testing the monocytes have been used.

Monocyte Maturation: The in vitro capacity of monocytes to develop into colonies when incubated under favourable conditions.

After C. Parvum the number of colonies which developed was not increased when the tested monocytes were obtained from patients suffering from general dissemination of disease. However, the number was significantly increased in the samples taken from patients with microscopic evidence of metastatic spread into the regional lymph nodes. Moreover mitoses were sometimes seen in the culture plates, normally a most unusual phenomenon.

Nitro Blue Tetrazolium Reduction: The reduction of the dye nitro blue tetrazolium is taken to be a direct measure of the activity of the hexose monophosphate shunt and hence the metabolic rate of the cell. Monocytes from patients who had received C. Parvum showed a greatly increased reducing capacity.

Lytic Capacity: The rate of release of ^{51}Cr from antibody coated autologous red blood cells bearing this radio-isotope was measured after incubation with patient's monocytes. Monocytes from C. Parvum treated patients increased release suggesting that their lytic capacity was increased.

These in vitro tests suggested that C. Parvum is capable of "activating" macrophages. Similar results were obtained when the bacterium was inoculated by the safer intradermal route.

Interferon

It has been suggested that C. Parvum increases natural levels of interferon production and this could be a mechanism of anti-tumour action. Serum interferon levels were increased after subcutaneous C. Parvum given to healthy volunteers. (108) In this study there was also enhanced *in vitro* lymphocyte mediated cytotoxicity against K 562 leukaemia and RAJI lymphoma cells. However, the subcutaneous route is not usually noted to have particularly marked effects on cell populations and these studies were only performed on 4 volunteers.

In another recent study the proliferative rate of T lymphocytes and their interferon production was increased by the addition of C. Parvum to the culture. (109) This effect was maximal when the C. Parvum was added 7 days after the initiation of the culture. When C. Parvum is added to macrophage cultures no such interferon production occurs, nor do C. Parvum incubated T cells show further enhancement of interferon production when admixed with macrophages.

Another method of stimulating interferon production is to use the polyvalent mitogen PHA. Lymphocytes stimulated by both PHA and C. Parvum show greatly enhanced interferon production which is more than the sums of the two on their own.

Results of C. Parvum treatment in human malignancy

Clinical trials using C. Parvum against human malignant disease began in France in 1967. (95) Israel reported 414 human cancers treated with combined chemotherapy and immunotherapy.

In this early study the bacterium was given by weekly subcutaneous injection in a dose of 4 mg. 5 drug combination chemotherapy was given twice a month, the combination comprising cyclophosphamide, methotrexate, 5 fluorouracil, vinblastine and streptonigrin. This combination of treatments was used in the treatment of various disseminated solid cancers, the majority being from primaries in the lung or the breast. In the combined treatment group double the number of patients were alive at 6, 12 and 18 months. C. Parvum treated patients also had a lower incidence of leukopaenia and fewer interruptions of chemotherapy.

Israel's group also reported the results of I.V. C. Parvum in 12 patients with disseminated disease. 4 of the patients had a partial response of a very limited duration.

Using a mixture of autologous irradiated tumour cells mixed with C. Parvum, McCune reported minimal side effects. (110) The dosage employed was 30 mg C. Parvum mixed with 10^7 tumour cells. Weekly subcutaneous inoculations were used until all the vaccine was used up. Although this was a phase 1 study in 15 patients with a heterogenous group of malignancies a prolonged regression of disease was noted in 1 patient who was suffering from pulmonary metastases secondary to a resected renal carcinoma.

Combination chemotherapy with or without C. Parvum in a dose of 5 mg/m^2 given subcutaneously was given to patients suffering from advanced breast cancer. The group receiving both treatments showed an improved survival at 11 months. These patients suffered less bone marrow suppression so that there were fewer interruptions of cytotoxic treatment. (99)

While C. Parvum has been used quite extensively in patients suffering from advanced cancer of the colon (111, 112) no definite evidence of benefit has been obtained. In many of these trials C. Parvum was given in combination with cytotoxic drugs. The resulting difficulties in interpretation are increased because of the lack of proper randomisation and the use of historic controls in some of the studies. Many of these trials have not yet been completed.

A randomised controlled study in the treatment of patients with Stage III lung cancer has been reported. (113) All patients received cytotoxic chemotherapy with adriamycin, cyclophosphamide, cis-platinum and vincristine. In addition they were randomised to no treatment, immunotherapy with MER or immunotherapy with I.V. C. Parvum. The C. Parvum treated group had a stastically significant improvement in survival rate over the other two groups. This study has been criticised on the basis of lack of comparability between the treated groups.

There is also a preliminary report suggesting benefit from combining systemic C. Parvum with chemotherapy in patients with Stage III ovarian cancer. (114) In this report the patients had as much tumour removed as possible prior to receiving chemo/immunotherapy. Nearly a third of the patients received C. Parvum alone and they all died within

a month of operation. None of the deaths were apparently directly related to C. Parvum treatment. Four out of thirty nine patients received the combined treatment and were alive and free of evidence of disease 9-12 months after operation.

There has been no unequivocal evidence of clinical benefit following the clinical use of C. Parvum.

Histochemical Staining of some Acid Hydrolases

The aim of enzyme histochemistry is to accurately demonstrate the localisation within tissues of the enzyme being studied. Alterations in concentrations and localisation of enzyme may be characteristic of disease processes and may enable an analysis to be made of functional cell populations. Both fixed tissue and circulating members of the M.P.S. contain high concentrations of acid hydrolase enzymes and various stain techniques can be applied in their investigation. Activation of these cells may increase the amounts of enzyme present, while participation in metabolic processes eg. phagocytosis, cell killing and lysis may lead to depletion. Unfortunately the plethora of enzymes present within these cells makes it difficult to select those most worthy of study. In an ideal enzyme staining reaction the substrate used is specific to the enzyme being studied. The product of the enzyme - substrate reaction is stable, non diffusible and can be clearly and accurately recognised. This ideal reaction is probably rarely achieved but various techniques render the method sufficiently accurate to permit reasonable inferences to be made about enzyme localisation.

Fixation: (115, 116) Depending on the enzyme being studied, prior fixation may improve results. For instance fixation with formal chloride for 12 hours renders β glucuronidase less diffusible and hence staining reactions after this treatment give a more precise indication of enzyme localisation. On the other hand acid phosphatase diffuses out of the tissues during fixation in formol calcium and Pearse suggests that here the best results are obtained using raw frozen sections. (117)

The results of histochemical staining of enzymes may be influenced by several factors;

1. Mitochondrial enzymes begin to diffuse within a few minutes of ischaemia. Although minor degrees of this diffusion may not be significant, if allowed to proceed the validity of enzyme localisation would be suspect. This process can be halted by rapid freezing but the formation of ice crystals can adversely affect histo-chemical reactions. Enzyme staining reactions should be performed on the freshest tissues possible, unless prior fixation is considered preferable.

2. The pH at which the staining reaction is to be carried out may also be critical. (118) For example the acid phosphatases are a group of iso enzymes having their maximal activity over separate pH ranges. At the pH of 5 which is optimal for most acid phosphatase activity the rate of coupling is too slow to prevent diffusion, so a compromise pH of 6.5 is used.

3. Coupling reagents like pararosanilin may also inhibit enzymes.

4. The substrate should be water and lipid soluble with a low molecular weight. This allows rapid diffusion into tissues permitting a high ratio of substrate enzyme. In these circumstances the reaction follows zero order kinetics and the reaction product accurately reflects the amount of enzyme present.

   The substrate should also be capable of being easily hydrolysed at a pH and temperature in the optimum range for the enzyme activity.

5. Substantivity refers to the affinity of the Primary enzyme/substrate reaction product to protein. This is important since a highly substantive complex will not diffuse away from the site of the primary reaction. If this is not the case the final reaction product will diffuse leading to poor localisation. However, if substantivity is too high there is a risk of binding to specific proteins leading to false localisations.

The techniques used in the study of enzymes follow in the appropriate sections. The methods for non specific esterase and acid phosphatase are examples of simultaneous capture coupling. Here the enzyme/substrate complex is linked to hexazotised pararosanaline as it forms giving a stable coloured precipitate. β glucuronidase has been stained by a post coupling capture method, again using hexazotised pararosanaline but adding this in a separate step subsequent to the enzyme substrate reaction.

The Macrophage

In 1868 Paul Langerhans described a dendritic cell in the human epidermis which stained with gold chloride. (119) He believed this cell to be a neural element and described it as having processes passing into the dermis. This may have been the first description of the morphologically diverse group of cells which make up the reticuloendothelial or mononuclear phagocyte system. (MPS) There are now so many roles ascribed to this group of cells that some investigators believe there must be distinct sub-populations serving separate functions. (119) The diversity of functions ascribed to the macrophage make a succinct description of this cell series difficult. They are found throughout the body either as circulating mononuclear

phagocytes (blood, peritoneal, alveolar, lymph) or fixed tissue macrophages and histiocytes (Kupffer, reticular, dendritic and mesangial cells).

Cells of the M.P.S. are capable of engulfing foreign particulate material (including bacteria) by phagocytosis. This function requires the co-operation of the complement system and B cells. Phagocytosis is a metabolically active phenomenon and is accompanied by increased oxygen consumption. There is also increased activity of the hexose monophosphate shunt and the formation of hydrogen peroxide. (120) Engulfed materials contained within vescicles are fused with lysozomes and digested. M.P.S. cells, having a high metabolic activity, have a well developed endoplasmic reticulum, Golgi apparatus and numerous mitochondria. (121) Peroxidase activity is usually present and a large variety of enzymes can be demonstrated. (122)

The Macrophage and Cancer

Macrophage mediated killing of tumour cells *in vitro* has been repeatedly demonstrated since first shown by Gorer in 1956. (123, 124) The role of the M.P.S. in spontaneously arising cancers is not known. (125)

Before examining the evidence that macrophages exert an anti-cancer action some terminology should be defined.

Normal Macrophage: These cells obtained from non activated normal animals have no anti-tumour effect *in vitro*. (126)

Immune Macrophage: Peritoneal macrophages from animals which have previously been challenged by the intraperitoneal inoculation of tumour cells are capable of lysing the same

tumour cells. This lysis is highly specific but has no implications about the mechanism of cytolysis. (126)

Armed Macrophages: When macrophages from non immune animals are incubated with immune lymphocytes they are rendered cytotoxic for specific target tumour cells. Cell lysis is not achieved. The lymphocyte factor which confers this cytotoxic capacity is called "specific macrophage arming factor" (S.M.A.F.). (127)

Activated Macrophage: Macrophages from some animals inhibit tumour cell growth non-specifically. This is seen more commonly after stimulation with bacterial products (128) chronic infection with intracellular parasites (129) or nematodes, (130) interaction with polyanions such as endotoxin or double stranded RNA (131) and stimulation by B.C.G. and C. Parvum. (129)

Anti-tumour activity can be studied in three ways:-

1. The anti-tumour effect of macrophage stimulating agents.

2. Macrophages within tumours.

3. The anti-tumour effect of macrophages injected after tumour cells.

1. The first of these modes of action has been separately considered under the heading "C. Parvum as an anti-tumour agent."

2. Macrophage within tumours

Alexander has shown in rats that the presence of large

numbers of macrophage within solid sarcomata is a favourable prognostic indicator. These tumours metastasize much less readily than those with a lower macrophage content. (132) Precisely what role these cells have within the tumour is not known. Elucidation of their action would be possible if a strain of animal deficient in M.P.S. cells was available. Such a defect might be incompatible with life and the best alternative at present is to use silica, carageenan or anti-macrophage serum. These agents are all toxic and may also influence T lymphocytes. In addition sublethal doses do not reduce tumour macrophage content.

Hopper and Pimm have shown that the mechanism of action of B.C.G. when injected into tumour deposits is dependent on macrophages. (133) Locally administered B.C.G. suppresses growth of syngeneically transplanted rat tumours. This treatment is successful even in immuno-suppressed rats and nude mice. Treatment with the macrophage toxin silica abrogates this effect of B.C.G. in both these models. These authors also found a correlation between the macrophage content of tumours and the capacity for suppression of growth by B.C.G. contact.

A method (134) of preparing tumour cell suspensions has been described by Evans. Using this technique macrophage numbers can be counted, based on the recognition of functional characteristics like adherence to glass and phagocytic capacity. Macrophage concentrations within a variety of experimental tumours vary between 4% to 56% of the total cell numbers. These cells were shown to have been derived from circulating host monocytes. Using this method Eccles and Alexander studied the macrophage contents of a variety of chemically induced rat fibrosarcomata to see if this was related to the biological behaviour of the

tumours, particularly the ability to metastasize. (135) Hooded rats were depleted of T cells by either prolonged drainage of thoracic duct lymph or thymectomy and repeated whole body irradiation before tumour implantation. A benzpyrene induced fibrosarcoma was transplanted intramuscularly in the hind limbs and excised 14 days later when the tumour macrophage content was assessed. T cell depletion considerably lowered the macrophage concentration, led to increased metastases and reduced survival.

A considerable degree of co-operation between T cells and macrophages appears to influence the growth of this particular experimental tumour. Alexander suggests that the macrophage both attempts to prevent metastatic spread and phagocytoses tumour breakdown products, thereby reducing the amount of soluble tumour antigen released into the circulation.

3. The anti-tumour effect of injected macrophages

Macrophages inoculated after a tumour cell challenge can exert an anti-tumour effect. This effect can be seen both after injection of macrophages at the tumour site or at a distance from the tumour. One criticism of this approach is that the macrophages are obtained from peritoneal exudate collections and are not necessarily tumour associated.

Macrophages injected at the tumour site

Syngeneic immune macrophages: Immune macrophages can be obtained from the peritoneal cavity of mice exposed to the L5178Y tumour. Syngeneic mice who received an intra-peritoneal dose of these cells after an intraperitoneal

inoculation of the same tumour survived twice as long as controls. (136)

Syngeneic armed and syngeneic activated - armed macrophages: Normal peritoneal cells are armed in vitro by incubation with lymphocytes. Activated - armed macrophages are prepared by in vitro incubation with immune spleen cells and target cells (in this case lymphoma cells). These cells demonstrated increased cytotoxicity in vitro. They also prolonged survival when given to syngeneic mice after they had received an intraperitoneal inoculation of the same lymphoma cell. (137)

Allogeneic immune and hyperimmune macrophages: Immune macrophages were obtained from the peritoneal cavities of mice which had previously received intraperitoneal SL2 - lymphoma cells. These cells were then used to immunise mice of a different strain against the same tumour. Immunised mice had considerably prolonged survival. Lymphocytes contaminating the peritoneal exudate cells had no anti-tumour effect on their own but did enhance the macrophage effect. (137)

Syngeneic activated macrophages: Macrophages become activated by in vitro culture with B16 melanoma cells or supernatants from culture of these cells. Intravenous injection of these activated macrophages suppressed the development of pulmonary metastases when given after I.V. challenge with the B16 melanoma. Intra-peritoneal macrophages had no such effect. This could be regarded as a local effect since the macrophages are cleared by the lung after I.V. inoculation. (138)

Macrophages injected at a distant site

Activated Macrophage: In the experiment just quoted intraperitoneal administration of activated macrophages had no effect on the development of pulmonary metastases. (138)

Macrophage in immune spleen cell suspensions: Cell suspensions of spleens taken from immune animals will suppress tumour growth if given at a different site. This effect is partly dependent on macrophage since it does not occur after treatment with silica. However, the results are equivocal. (137)

There is little firm evidence that activated macrophages have anti-tumour effects when given at a site distant from the tumour. This does not imply that in all naturally occurring cancers the circulating macrophages cannot be activated on reaching the tumour site.

The mechanism of macrophage mediated cell killing

The enhanced cytolytic capacity of macrophages by specific mechanisms like S.M.A.F. or antibody is directed against a unique target cell type. (139) Non specific activation, for example by bacterial endotoxin or C. Parvum, confers widespread cytotoxic capabilities against a variety of target cells. (140) Whatever the mechanism, cell to cell contact is probably required to cause tumour cell death. Such intimate intercellular adherence permits the release of lysozomal enzymes causing cell destruction. Arming factors, antibodies, complement and macrophage activation may alter the macrophage cell surface enabling this intimate contact to occur. (141) Apart from release of high concentrations of toxic enzymes from lysozomes, cytoplasmic

peroxidases could also cause destruction.

Another suggestion is that macrophages release arginase which has the effect of depleting the tumour cell millieu of arginine. (142) Tumour cells which are unable to stop metabolising in spite of the lack of essential amino acids, literally die of "starvation".

Macrophage function in tumour bearing hosts

Reports of studies on monocyte and macrophage function in both human and animals cancers are at first sight confused. During tumour growth many macrophage functions are enhanced including the ability to kill tumour cells in vitro, (143) in vivo resistance to tumour cell challenge, (144) and macrophage production by the bone marrow. (145) Depression of macrophage function is commonly seen in advanced cancer. (146) While it appears that during the early stages of disease macrophage function is enhanced, this is not the case in advanced cancers. Using the Lewis lung cancer as an experimental model in C57 BL mice, Otu has examined macrophage function at varying stages of disease. He assessed the rate of clearance of colloidal carbon, chemotactic locomotion of macrophages and the rate of formation of macrophage colonies from bone marrow. All these functions were depressed for the first few days after tumour transplantation. Subsequently they were enhanced until, in the presence of extensive disease, they were once more depressed. (147) Serum from mice previously grafted suppressed the in vitro formation of marrow derived macrophage colonies suggesting that a soluble factor may be responsible for macrophage inhibition in cancer.

This varying response may explain the apparently conflicting results obtained in patients with malignant disease. Early suppression by cancer cells could allow the tumour to become established. Later the host macrophages increase in numbers only to become suppressed again in the face of over-whelming malignant disease.

Interference with systemic macrophage function may also enable metastatic spread. Soluble tumour antigens, antigen-antibody complexes or small non specific factors could be responsible for paralysing the macrophage response. (148, 144)

It is simplistic to view the M.P.S. as an anti cancer mechanism in isolation. The role of complement, antibody and arming factors have been mentioned. Lymphocyte ability to recognise foreign tumour cell antigens and the subsequent release of lymphokines enhances the accumulation of M.P.S. cells. (149, 141) It may be that it is the complexity of the normal tumour defence mechanism that has prevented its elucidation.

The origin, circulation and fate of mononuclear phagocytes

The classic work of Volkman and Gowans has established the bone marrow origin of cells of the M.P.S. (150) Monocytes arise from promonocytes in the bone marrow and enter the general circulation (151, 152) eventually passing into the tissues as mature macrophages. Monocytes are able to migrate to the extravascular spaces by amoeboid movements between endothelial cells of venules. (153) Cells of the M.P.S. do not migrate through the post capillary venules of lymph nodes. This is in contrast to the major route of recirculation of lymphocytes from blood to lymph as described by Gowans. (154)

Production

M.P.S. cells within the bone marrow can be classified as promonocytes and monocytes. (151) Promonocytes develop from primitive pluripotential progenitor cells. Promonocytes have been shown by H^{-3} thymidine labelling studies to be the proliferating cells and the monocytes non dividing. This study was performed under normal steady state conditions. Similar labelling studies showed that the monocytes were the progeny of division of promonocytes and that two such daughter cells are produced for each mitosis.

Monocytes then enter the blood stream where they either remain in circulation or migrate to tissues or coelomic cavities. During a chemically induced peritonitis a threefold increase in blood monocytes with a x2.5 increase in peritoneal macrophages is noted. (155) The monocytosis is due to increased production by marrow promonocytes and the increased peritoneal macrophages all come from this source. There is no local proliferation of peritoneal macrophages.

The relationship between the peripheral blood monocyte and the tissue macrophage has been demonstrated by some celebrated names in pathology. Metchnicoff, Aschoff and Florey have all made major contributions.

The function of the circulating monocyte is diverse and unclear. It is certainly rather more than a short lived cell in transit. It forms the major defence against intracellular bacteria, fungi and parasites, plays a part in antigen processing and interacts with lymphocytes in cell mediated immunity. (119) The monocyte may also provide a defence system against some tumours. (156, 157)

Laboratory investigations into the function of circulating monocytes have been hampered by difficulties in obtaining pure samples of these cells. In normal blood the monocytes make up approximately 1% of the total white cell population. More concentrated preparations can be obtained by using Ficoll-Hypaque density gradients which allows separation of the white cells of the blood into their constituent sub-populations. Samples obtained by this method may contain cells of another sub-population and caution must be exercised in the interpretation of results obtained using these supposedly pure preparations. (158)

Tissue Macrophages

The Kupffer cell has been regarded as the classical fixed tissue macrophage. Its position in the liver sinusoids means that it is ideally situated to clear the blood of gut derived micro-organisms and endotoxins. They also remove other gut derived antigens, antigen/antibody complexes, chylomicrons and senescent red cells. (159)

Kupffer cells originate in the bone marrow. (160) There is debate about the capacity for these cells resident in the liver to divide. (160) Under normal circumstances the rate of incorporation of H^{-3} thymidine by Kupffer cells is less than 1.5% of the cell population. It is therefore unlikely that they synthesize sufficient DNA for mitosis. (159)

Studies on patients after liver transplantation have provided convincing evidence for the bone marrow origin of Kupffer cells. Three months after the transplant the hepatic Kupffer cells are of recipient origin. This is clearly shown by sex chromatin studies on liver biopsies when donor and recipient are of different sex. (159)

Stimulation by C. Parvum causes an increase in Kupffer cell number. There is some argument about the origin of the newly formed cells. Some workers claim that liver Kupffer cells retain the capacity to divide. (13) Others argue that the dividing cells are monocytes only recently recruited from the bone marrow. (161)

The fate of effete macrophages under normal circumstances is unknown. Macrophage death may occur as the result of endocytosis of toxic material. Attempts at mitosis may be self destructive as gross chromosomal abnormalities often appear at the first division. They may also form multinucleate giant cells. (153) It is not clear to what extent the tissue macrophage retains mobility.

M.P.S. cells within the lymphatic system

The cell population of lymph is altered by its passage through a lymph node. Peripheral (afferent) lymph contains a varying population of M.P.S. cells. Lymphatics leaving the liver are termed afferent prior to reaching lymph nodes. These afferent liver lymphatics can be cannulated in the sheep. (162) Large numbers of cells are harvested (up to 2×10^8/ day), approximately 20% of which are members of the M.P.S. These cells are not seen in the efferent lymph leaving the regional lymph nodes.

In lymph afferent to the popliteal lymph node in rabbits approximately 40-50% of cells belong to the M.P.S. Only 10% of these cells show phagocytic capacity, (measured by uptake of colloidal carbon) and 3% show Langerhans granules which are classically seen on ultrastructural studies of the deep layers of the epidermis.

Cells of the M.P.S. do not normally appear in thoracic duct (efferent) lymph. Indeed lymph obtained from thoracic duct fistulae has been used experimentally as a sample of pure lymphocytes. Roser has shown that this is not absolutely true. (163) During chronic lymph depletion from rats with cannulae within the thoracic duct he showed that M.P.S. cells are found at varying time intervals after cannulation. He could not explain the great variation in the numbers of these cells obtained. In some animals (25%) there were no M.P.S. cells in the lymph, whilst in others they appeared soon after commencement of thoracic duct drainage. 72 hours after cannulation 0.24% of the total cells were members of the M.P.S.. Roser also demonstrated that these cells might originate in the peritoneal cavity of the animal.

A new experimental rat model has been described as the result of work carried out in the Sir William Dunn School of Pathology in Oxford. Total extirpation of the mesenteric lymph nodes of experimental rats is performed. At least six weeks later, after allowing time for healing, cannulation of the thoracic duct is performed. The lymph obtained contains cells of the M.P.S. (164)

This model enables studies to be made on M.P.S. cells which are assumed to have been obtained directly from the gut wall. If this postulate is true a unique opportunity exists for dynamic studies on gut associated M.P.S. cells.

Materials and Methods

Animals Used

Female Wistar rats weighing 100-250G have been used in this study. This strain was selected in view of its known susceptibility to the carcinogenic effects of dimethylhydrazine dihydrochloride. Specific pathogen free rats were supplied by Olac laboratories, Bicester, Oxon, and Messrs. Banting and Kingman, Aldborough, Hull. On receipt the animals were housed in a conventional animal unit. Rats undergoing carcinogen tumour induction were kept in separate cages with wire grid floors. This allowed droppings and urine which were possibly carcinogen contaminated to pass through into a collecting tray. The rats were fed with standard pelleted rat diet.

Corynebacterium Parvum

C. Parvum was provided as a generous gift by Dr. T. J. Priestman of the Burroughs Wellcome Medical Foundation, Beckenham, Kent. The bacterium is formalin killed and made up as a suspension in 0.01% thiomersal as a preservative. Vials containing 7 mg of the dry weight of the organism are made up to 1 ml with preservative. A fresh vial was used for each experiment.

Working Solutions

The following working solutions were used:

Dulbecco A & B (D.A.B.) : Phosphate buffered saline was made by adding a one gram tablet of Dulbecco A (Oxoid, London) to 100 mls of distilled water. This was converted to the fully buffered isotonic solution DAB at

a pH of 7.3 by the addition of 0.5 ml of mineral salts (Dulbecco B) to each 100 ml.

Foetal Calf Serum (F.C.S.)

F.C.S. (Gibco bioccult, Paisley) was used as a source of opsonins and complement during phagocytosis of sensitised sheep red blood cells. It was also added to DAB in a concentration of 10% and this solution used for resuspending cell pellets after centrifugation. This method has been found to prevent clumping of cells and also minimises the loss of cells during repeated centrifugation.

Trizma Ammonium Chloride (A.C.T.)

A.C.T. was used to lyse red blood cells. White cells are less susceptible to this lysis and, provided exposure is not prolonged, no damaging effects result. A.C.T. was prepared by dissolving 3.74 G of ammonium chloride in 450 mls of distilled water then adding 0.103 G of Trizma base (trishydroxymethyl aminomethane, Sigma Chemicals, Poole) dissolved in 50 mls of distilled water. After thorough mixing the pH was adjusted to 7.2.

Phagocytosis

Phagocytosis has been measured by the ability of the lymph cells to engulf sensitised sheep red blood cells. This capacity was studied on cells obtained from thoracic duct lymph, peritoneal lavage and splenic macerates.

The method of sensitising sheep red blood cells was as follows:-

Sheep red blood cells were spun in a centrifuge at 1500 r.p.m. for 5 minutes then resuspended with DAB solution. This procedure was repeated three times and will subsequently be referred to as a "wash". 0.3 mls of the packed washed cells was added to 17.3 mls of RPMI 1640 (Gibco Europe, Glasgow) containing 25mM Hepes medium and L-Glutamine. 2 mls of 10% foetal calf serum, (F.C.S.) 0.2 mls of Penicillin and streptomycin and 0.2 mls of non essential amino acids were then added and finally 50 µl of rabbit anti-sheep haemolytic serum. (Wellcome, Beckenham, Kent) This mixture was incubated at 37°C for 30 minutes to allow the γ globulin of the haemolytic serum to coat the sheep cells. The mixture was then stored at 4°C and could be used for up to a week.

Phagocytic assay

Cell samples were washed thrice in DAB containing 10% FCS. To provide the maximum opportunity for phagocytosis, intimate cell contact must take place in the presence of opsonins. This was achieved by mixing the sensitised cells in excess with the effector cells and spinning the mixture at 1500 r.p.m. for 5 minutes. The supernatant was then discarded and replaced by 1 ml of F.C.S. The cells were resuspended and then centrifuged once more. Finally the mixtures were incubated at 37°C for 20 and 40 minutes. After this time the supernatant FCS was discarded and replaced by 1 ml of ACT. This lyses all the remaining extracytoplasmic red cells during a 5 minute incubation. After a further spin the ACT was discarded and the cells suspended in 1 ml of DAB/FCS (Cytocentrifuge slides were prepared from this solution).

Estimating the cell count of a sample

Cell suspensions were washed before being counted. On resuspending the cells after the third wash they were routinely made up to 10 mls with DAB/10% FCS. This usually gave a satisfactory working volume and made subsequent calculations simple.

Cell counts were made with a haemocytometer (Hawksley Crystalite, improved Neubaeur). 0.1 ml of the cell suspension was added to 0.9 ml of white cell counting fluid (Methylene blue in 2% acetic acid). After mixing the cells a drop of this fluid was placed adjacent to the cover slip of the haemocytometer. The solution and its contained cells became distributed under the cover slip. Using the x10 objective of a microscope the number of cells within a defined field was counted. This field comprises the four outer quadrants marked on the haemocytometer. The total number of cells within these quadrants was counted. This value divided by four and multiplied by 10^4 gave the number of cells per ml of the sample

$$\frac{n}{4} \times 10^4 = \text{number of cells/ml of sample}$$

This method was used for all cell counts and has been found to be reliable and reproducible.

Siliconising glassware

It is a characteristic of macrophages that they adhere to glass. In order to obtain samples that were not depleted of MPS cells by this phenomenon it was necessary to coat the glass with a non adherent layer. This was achieved by exposing the glassware to an atmosphere of dimethyldichlorosilane. The glassware was loaded into a large dessicator with a small beaker containing 5 mls

of dimethyldichlorosilane. After several hours a film of silicone coats the glassware. Since the fumes are toxic this procedure was carried out in a fume cupboard.

Use of the cytocentrifuge

Microscope slide preparations of cell suspensions were made using the cytocentrifuge (Cytospin, Shandon Southern). The cell suspension was placed in a well and when the cytocentrifuge chamber was rotated at high speed the suspension was forced onto a microscope slide. The cells became randomly distributed on the glass of the slide by centrifugal force, but the liquid was taken up by absorbent paper placed around the outlet from the well. The cell concentration of the suspension was adjusted to between 3 and 4 x 10^6 cells/ml. Three pipette drops of this suspension (100 µl) were introduced into the cytocentrifuge well and the cytocentrifuge was run at 6000 r.p.m. for 5 minutes. This technique was used to study cells obtained from thoracic duct lymph, splenic macerates and peritoneal exudates.

Slides prepared in this way were allowed to air dry before fixation in concentrated formalin vapour for one minute.

Mesenteric lymphadenectomy

The mesenteric lymph nodes in the rat lie in a chain at the root of the mesentery. The caecal nodes are found in a group at the base of the caecum lying separate from the remainder of the mesenteric nodes. To remove the nodes the animals were anaesthetised with ether and the abdomens shaved. A long midline abdominal incision permitted the small intestines to be delivered out of the wound. The

Lymph nodes were removed by first breaking open their peritoneal covering with fine sharp pointed forceps then dissecting them away from the mesenteric blood vessels. With care this was easily achieved and minor degrees of bleeding stopped quite quickly. Lymph nodes situated behind the duodenum were also removed. The duodenum was rotated to the left and the nodes were found running parallel with the portal vein. Great care was taken in the removal of these nodes since a traumatic laceration of the portal vein can occur.

After removal of all visible lymphoid tissue the abdomens were sutured with a double row of 4/0 black silk and the animals allowed to recover. Cannulation of the thoracic duct was not performed until at least six weeks had passed to allow time for healing. It is unclear whether disrupted lacteals can heal or whether new channels open up.

Cannulation of the thoracic duct

Cannulation of the thoracic duct was carried out under ether anaesthesia and according to the method of Bollman, Cain and Grindlay. (165) Some modifications have been adopted and the procedure will be described in full.

After the anterior abdominal wall had been shaved the rat was taped to an operating board with the hind limbs fully abducted, the upper body rotated to the right and the fore limbs taped together on the right. A left subcostal incision was made through the skin and muscles of the anterior abdominal wall. The intestines and the liver were retracted to the right and the left kidney mobilised by gentle manipulation using cotton buds. (Johnsons baby buds) These structures were then all retracted to the right and retained with a gauze swab and a mastoid retractor.

The abdominal aorta was clearly seen running between the crura of the diaphragm. Subsequent dissection was performed with a glass rod dissector which was made from pyrex glass rods. The rod was heated to melting point, then drawn out to fashion a gentle curved tip at right angles to the line of the rod. When the extreme tip is flamed it becomes bulbous with no sharp projections, facilitating atraumatic blunt dissection of tissue planes. Using this dissecting rod the fascia of the posterior abdominal wall was broken down and the aorta fully exposed. At this stage the thoracic duct could be seen as a thin, almost transparent line, running parallel, slightly behind and to the left of the aorta. Dissection just below the crura of the diaphragm allowed the rod to be passed around the duct. At this level a silk tie was placed to occlude lymphatic return and distend the duct. (Plate 1) Further dissection slightly lower down allowed a second tie to be placed loosely around the distended duct at the level of the superior suprarenal artery. With a large hollow needle the posterior abdominal wall was pierced just lateral to the vertebral bodies. This enabled placement of a length of 800/100/200/100 plastic tubing (Arnold Horwell Ltd., London), which was primed with normal saline. This tubing was previously heated and moulded into shape. Two curves were made. The first was a hair pin bend. The second was a slight dorsal angulation in the distal limb. These curves allowed the tubing to lie comfortably when the tip was within the thoracic duct and the distal limb transfixed the posterior abdominal wall. Using fine iris scissors a cut was made in the anterior wall of the distended duct. 2-3 mm of the bevelled tip of the cannula was then placed within the duct, and this was secured by tying the remaining ligature. A second retaining ligature was placed through the quadratus lumborum muscle and tied. (Plate 2) The wound was

then closed after returning the viscerae to their normal positions.

The procedure was seen to have been successful when lymph entered the tubing and fluid steadily dripped from the tip of the tubing.

In order to maintain the animal in a state of full hydration a further length of polythene tubing was introduced and then tied into the femoral vein and fluid infused. This was achieved by a similar method to cannulation of the thoracic duct though no curves were required in the tubing and finer 800/110/140/100 gauge tubing was used. In order to advance the tip of the cannula into the inferior vena cava, the limb was fully extended and retracted caudally. This makes the line of the bifurcation more obtuse and the catheter was less likely to be impeded as it was advanced.

The proximal end of this cannula was connected with a number 18 gauge Yale microlance needle and through this to tubing coming from an electric roller infusion pump. By this means a constant infusion of 4% Dextrose, 0.18% NaCl saline containing 1 unit of Heparin per ml was given. This minimised the risk of lymph clots forming in the tubing.

After placement of these cannulae the rats were confined in specially constructed restraining cages. (Plate 3) Modifications from the design of Bollman (166) have been of a minor nature and will not be described.

A purpose built unit has been designed and built in this department. (Plate 4) Each cage has its own tray for collecting urine and faeces and this was changed daily. In addition, as the cages stand on a shelf, this is easily

cleaned. As can be seen in Plate 4, the infusion unit is an integral part of the design and cannulae and tubing can be kept neatly.

The following principal problems have been faced with this experimental model:

1: Dissection of the thoracic duct as it passes through the diaphragm can cause a traumatic pneumothorax.

2: Haemorrhage. This can be troublesome during intravenous infusion of Heparin.

3: Lymph clots within the drainage cannula.

4: Twisting of the cannulae.

5: Dislodgment of the cannulae.

4 and 5 are more likely to happen if the cage is too large for the animal. Too much freedom of movement makes it almost inevitable that tail movement will cause cannula problems.

Once the animals recovered from the anaesthetic they were allowed free access to salinised drinking water and normal rat pelleted diet. The intravenous infusion rate was between 70 and 100 mls in 24 hours. Once lymph drainage was fully established output volumes of greater than 200 mls in 24 hours were commonly achieved.

Non Specific Esterase Stain

This is a diverse group of enzymes whose common link is that their substrates are esters of carboxylic acids. The

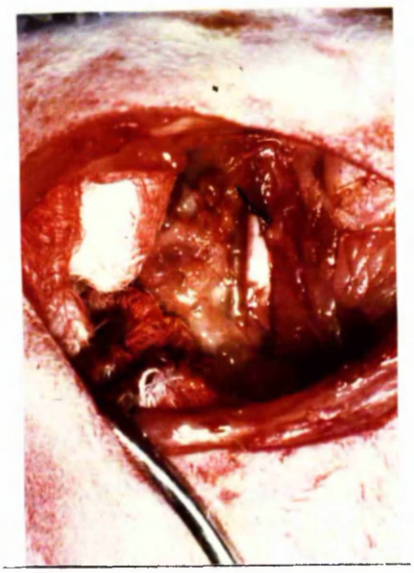


Plate 1 : Distension of the thoracic duct after placement of an occluding ligature.

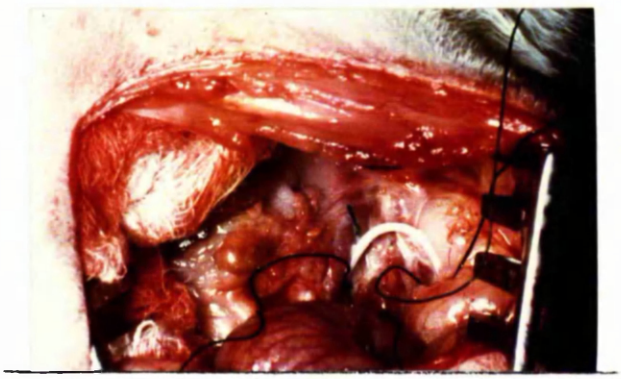


Plate 2 : Thoracic duct cannula in situ.

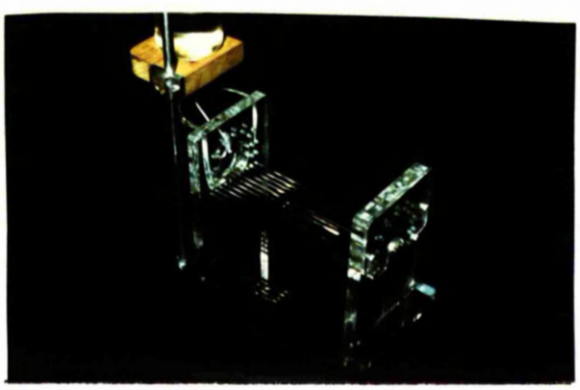


Plate 3 : The Bollman cage.

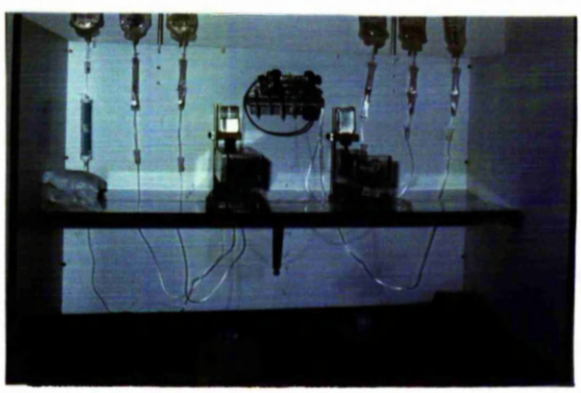


Plate 4 ; The purpose built unit allowing 6 parallel drainage procedures at any one time.

non specific esterases act on simple short chain ester substrates like methylbutyrate or naphthyl acetate, while the lipases act on higher fatty acids. This distinction is not absolute and there is overlap. (167) The naphthyl acetate method using simultaneous coupling to azo dye gives a brilliant colouring reaction but the final reaction product is soluble in both alcohol and water and hence diffusion artefact is a problem. (167) In this study the staining method used to identify non specific esterase activity employs α naphthyl butyrate as the substrate. In their report of the use of this technique, Crofton, Diesselhoff, den Dulk and Van Furth showed that it caused diffuse staining of the cytoplasm of Kupffer cells and peritoneal macrophages. (160) They also found a high correlation between cytoplasmic esterase staining and other macrophage characteristics like phagocytosis, pinocytosis and the presence of Fc receptors. MacPherson and Steer have shown that this staining method can be applied to the cells obtained in the thoracic duct lymph of mesenteric lymphadenectomised rats. (164) A positive staining reaction was seen in some lymphocytes as one or two dots in the cytoplasm. Cells in which diffuse cytoplasmic staining was present had the morphological appearance of M.P.S. cells both on light and electron microscopy. These cells were also glass adherent but showed a gradation of staining and variable phagocytic capacities. The method has found to be reliable and to give satisfactory results when used to stain cytocentrifuge preparations from thoracic duct lymph, peritoneal exudates and splenic macerates. It has also given good results when used to stain fresh frozen tissue sections fixed in formalin vapour.

The substrate solution was made by adding 250 mg of α napthylbutyrate to 48 mls of dioxan. Pararosaniline hydrochloride was made by dissolving 1G of pararosaniline in

30 mls of twice normal Hydrochloric acid. This was hexazotised by adding an equal volume (0.8 ml) of sodium nitrite solution (IG sodium nitrite to 30 mls of water). 5 mls of 0.4 N Sodium Cacodylate and 0.15 mls of Tween 20 were added and the pH of the solution adjusted to 6 with further sodium cacodylate. The final volume of hexazotised pararosaniline was made up to 15 mls with distilled water. 3 mls of methcol were added to 2 mls of ∝ napthylbutyrate substrate solution and 15 mls of hexazotised pararosaniline added. Finally this solution was made up to 35 mls and the pH again adjusted to 6.0. Slides were stained after fixation for one minute in concentrated formalin vapour. Between 30 and 40 minutes was usually required for the staining reaction. The slides were then washed with distilled water and counter stained with 1 drop of Giemsa for 30 seconds. Permanent preparations were made by dehydrating these specimens through graduated concentrations of alcohol, then through xylol and mounting with DPX.

Chapter 1

Dose level, route and timing of C. Parvum in rats

The splenomegaly induced by C. Parvum is used by the manufacturers as a quality control for activity of the bacterium. Mice have been used extensively for this purpose and 0.35 mg of C. Parvum reliably induces splenomegaly. A dose of 4.7 mg has been established as the LD_{50} for female OF_1 mice weighing 18-20 g. (169) Much less is known about the effects of C. Parvum in other rodents. A literature search failed to reveal any data of toxicity studies carried out in female Wistar rats. It was considered to be an essential preliminary step in this study to establish a dose level which would produce reticuloendothelial stimulation without unacceptable toxicity.

Materials and Methods

Intravenous route: C. Parvum was injected intravenously via the dorsal tail vein. In order to be certain that the inoculum was entering the vein it was diluted with sterile normal saline to a volume of 1 ml. Successful inoculation resulted in free emptying of the syringe. Extravasation caused resistance to injection and distension of the tail. Dilutions of C. Parvum of 0.035 mg, 0.35 mg, 0.7 mg, 1.4 mg and 2.8 mg were injected, suspended in 1 ml of normal saline. Animals were killed at weekly intervals after inoculation and their spleen and body weights recorded.

Intraperitoneal route: C. Parvum was injected intraperitoneally in identical dilutions to those given by the intravenous route. Inoculation was performed by

pinching the shaved anterior abdominal wall and introducing the needle at an angle in the midline. Entry to the abdominal cavity was accompanied by a loss of resistance and the ability to move the needle tip freely from side to side.

Intralumenal route: 0.7 mg of C. Parvum in 1 ml of normal saline was injected into the lumen of the mid small bowel or the ascending colon at laparotomy. The hypodermic needle was angulated and the inoculum did not appear to leak out of the bowel lumen after the needle was withdrawn.

Intralacteal route: Cannulation of mesenteric lacteals was performed by the supervisor of my research, Mr. H. W. Steer. This was achieved using finely drawn capillary tubes which were introduced into the lumen of the lacteal under visual control using an operating binocular microscope. A very much smaller volume was introduced by this route and in each case 0.7 mg of C. Parvum was given in 0.1 ml of thiomersal.

Results

Immediate

Intravenous: No acute side effects were noted when doses of 0.7 mg or less were used. Above this level the animals showed marked pilo-erection which passed off within 2 hours. Shivering and hyperventilation were not noted. Doses even as high as 3.5 mg caused only transient side effects and no mortality.

Intraperitoneal: No immediate side effects resulted from the intraperitoneal injection of C. Parvum. In the group of rats which had undergone prior mesenteric lymphadenectomy, treatment, particularly in the higher doses, was responsible for some long term morbidity. A number of these rats developed inflammatory adhesions and localised abscess formation. This made subsequent procedures on these animals difficult.

Intralumenal: No acute side effects resulted from this procedure.

Intralacteal: As the small volume of undiluted C. Parvum reached the draining lymph node, it was seen to distend and blanch. This node was subsequently found to be enlarged and to contain large granulomata with considerable obliteration of normal nodal architecture. That this effect was not simply due to distension of the node has been shown by control experiments when 0.1 ml of 0.01% thiomersal alone was injected into the node. These results are more fully discussed in Chapter 6.

Splenomegaly

The weight of the normal rat spleen increases with the body weight of the animal in a linear fashion (Table 1). Any observation on splenic size must therefore take into account the weight of the animal. In order to standardise animal and spleen weights the following calculation has been made

$$\text{Standard spleen weight (grams)} = \frac{100 \times \text{spleen weight (grams)}}{\text{Rat weight (grams)}}$$

C. Parvum given by both the I.V. and the I.P. routes reliably produced splenomegaly in doses of 0.7 mg and more

(Table 2). 0.35 mg also produced splenomegaly when given by either route but not in all cases. At each dose level the resulting splenomegaly was greater when the intravenous route was used. A dose of 0.035 mg did not produce any change in spleen weight.

Splenomegaly after I.V. C. Parvum in a dose of 0.7 mg is present at 1 week (Table 3), reaches a peak at two weeks and then begins to decrease although it is still present at four weeks after the injection.

The splenomegaly resulting from 0.7 mg of C. Parvum given by a variety of routes is shown in Table 4. All the results given on this table are within the first two weeks of the injection. Intralacteal C. Parvum induced splenomegaly of a similar magnitude to the I.V. route. Intralumenal C. Parvum did not cause splenomegaly. Spleen weights were measured in 12 rats which had received I.V. C. Parvum followed by thoracic duct lymph drainage for 5 days. These rats were seriously deficient in lymphocytes at the end of this procedure as measured by lymphocyte counts in thoracic duct lymph and blood. Despite this, significant splenomegaly was still present.

Discussion

A dose of 0.7 mg of C. Parvum given either I.V. or I.P. results in marked splenomegaly with little toxicity in female Wistar rats. When this bacterium has been used in clinical trials systemic doses of 5 to 15 mg have usually been given. Side effects of considerable severity result from the treatment in humans. If it is assumed that the rats weighed 250 grams and the humans weighed 70 kilograms then the rat dose of 2.8 mg/kg can be compared with the human dose of 0.14 mg/kg. It is remarkable that the rat

dose, which is 20 times the human equivalent dose causes so few side effects. Splenomegaly has not been reported following the use of C. Parvum in humans.

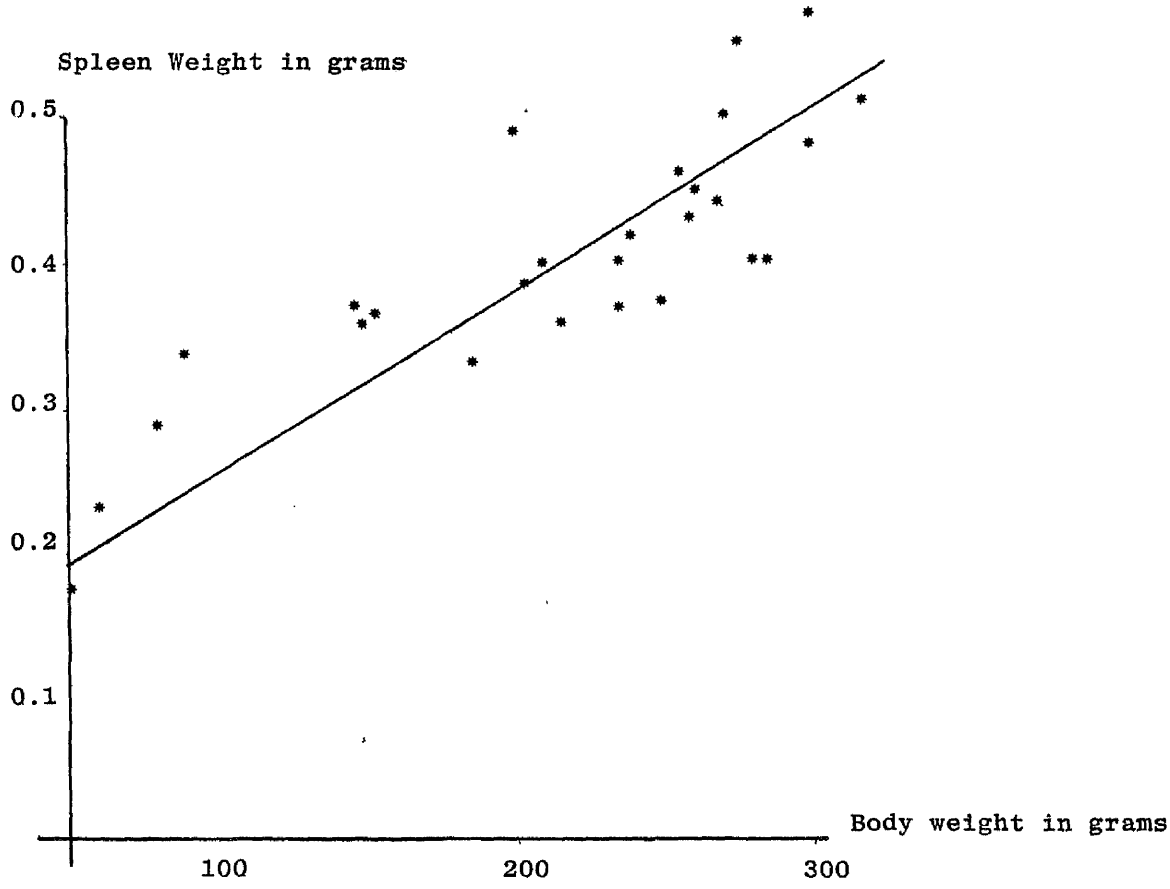
When C. Parvum is inoculated into the lumen of the large and small bowel no effect on spleen size results. This is similar to the effect of subcutaneous administration and implies that the bacterium is not absorbed by the gut mucosa. When C. Parvum is injected directly into the mesenteric lacteals however, marked splenic enlargement occurs. C. Parvum, or an active subfraction, appears capable of exerting a systemic effect when given in this way. The destruction of lymph node architecture resulting from intralacteal C. Parvum may permit its entry into the general circulation.

Splenomegaly after systemic C. Parvum remains after thoracic duct lymph depletion. This implies that the cells contributing to the increase in spleen mass are not re-circulating lymphocytes.

As a result of the work described in this section a dose of 0.7 mg of C. Parvum was selected as being an appropriate level for subsequent studies. It was decided to concentrate on the I.V. route since this gives marginally greater stimulation than the I.P. route with no risk of subsequent adhesions or abscess formation. This was of particular importance in previously lymphadenectomised rats where it was proposed to perform cannulation of the thoracic duct.

Fig. 1, 1

The Relationship of Splenic Weight to Body Weight in Female Wistar Rats



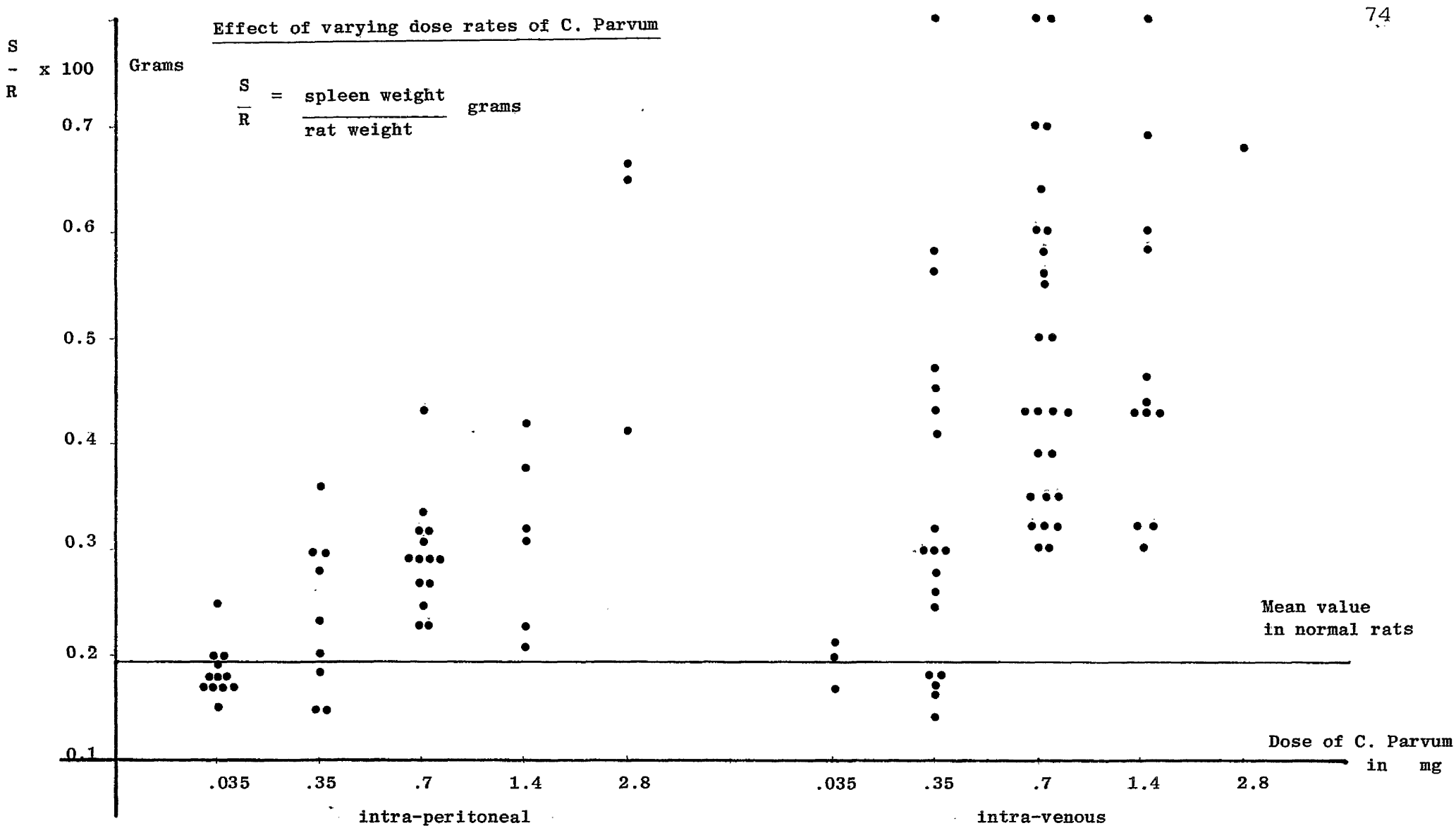

Effect of varying dose rates of C. Parvum
S/R = spleen weight / rat weight grams
S - R x 100
Grams
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Mean value in normal rats
Dose of C. Parvum in mg
.035
.35
.7
1.4
2.8
.035
.35
.7
1.4
2.8
intra-peritoneal
intra-venous

Figure 1, 2

Fig. 1,3

Time course of C. Parvum induced splenomegaly

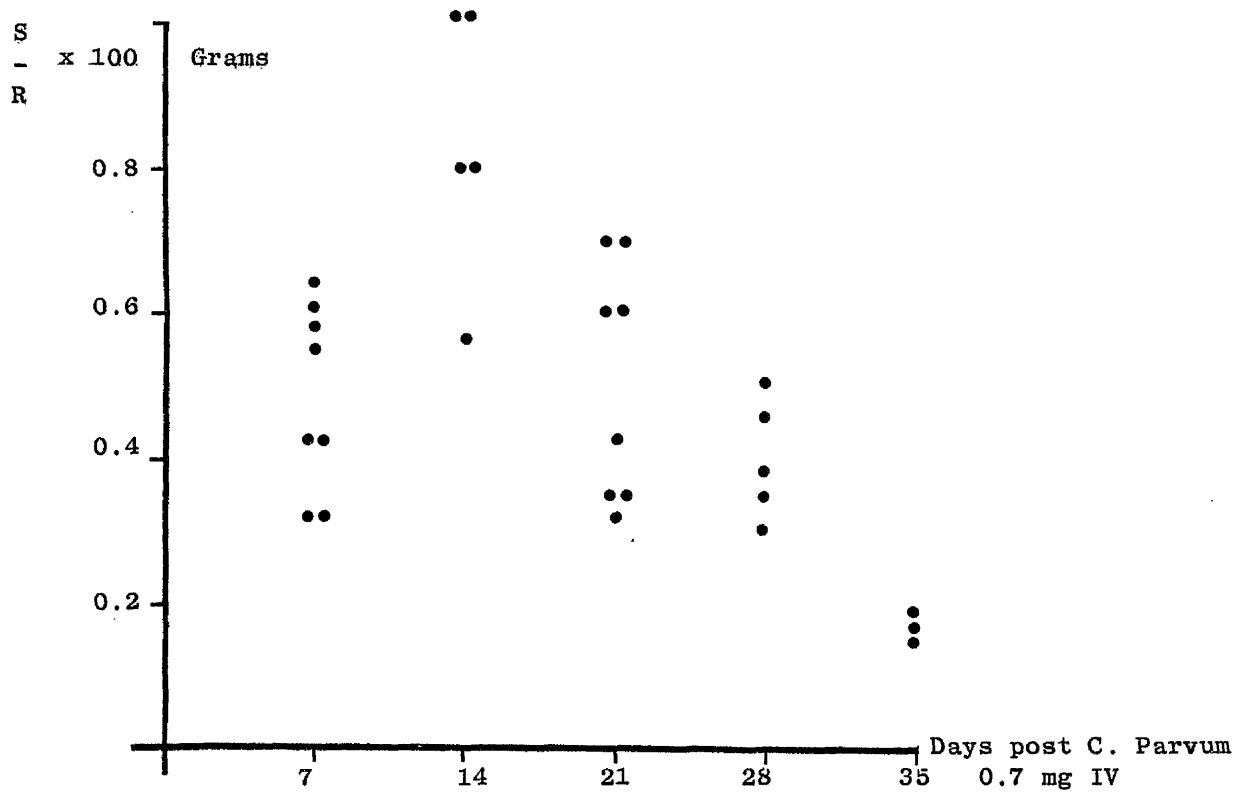
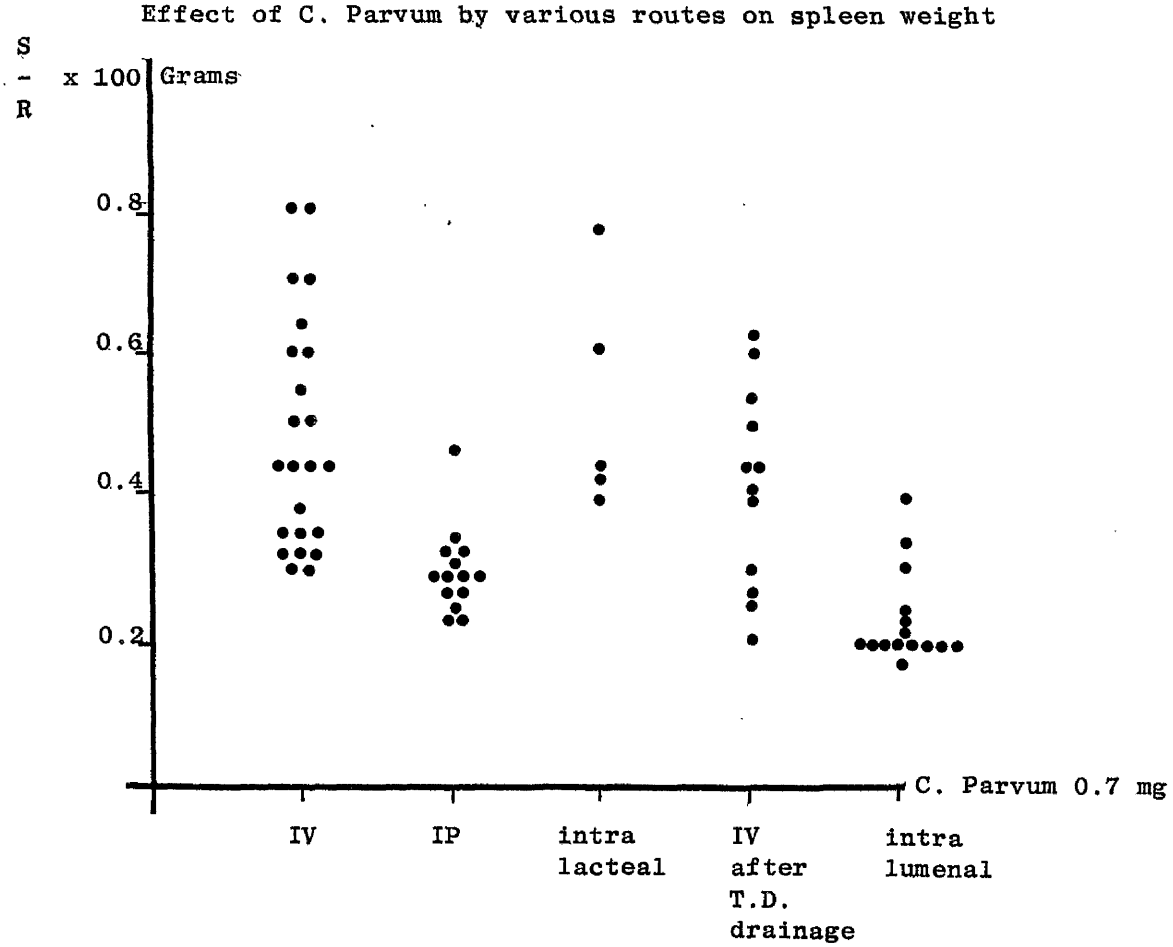


Fig. 1,4

Effect of C. Parvum by various routes on spleen weight



Chapter 2

The effect of systemic C. Parvum on the effluent lymph cells of the gastro intestinal tract

A series of experiments have been carried out which were designed to study the effects of systemically administered C. Parvum on the cell composition of the thoracic duct lymph in rats. Mesenteric lymphadenectomy was performed in these rats at least six weeks prior to these studies. The principle aim of this work was to establish whether C. Parvum has any influence on gut associated M.P.S. cells.

Normal values were obtained after collecting thoracic duct lymph from nine rats which had previously undergone mesenteric lymphadenectomy, but had received no other treatment. C. Parvum was injected in a dose of 0.7 mg in 1 ml of normal saline intravenously via the dorsal tail vein. Four groups of rats underwent thoracic duct and femoral vein cannulation at weekly intervals from one to four weeks after the injection. A further group of rats received the same dose of C. Parvum intraperitoneally and underwent cannulation one or two weeks later. Finally a group of rats underwent cannulation and once drainage of lymph was established at 48 hours, received 0.7 mg C. Parvum via the femoral vein cannula.

Five consecutive overnight collections of lymph were made after cannulation of the thoracic duct. Lymph was allowed to drain into siliconised glass flasks, standing on melting ice within polystyrene boxes. 5 mls of DAB containing 20 international units of Heparin/ml were added to the flasks prior to the collection. These precautions minimised the risk of clots forming in the collected lymph. Samples containing more than trivial blood or lymph clots

were discarded and that days results excluded from the analysis. After washing each satisfactory sample thrice an estimate of the total cell count was made using the haemocytometer. This figure taken with the total hours of lymph drainage is used to estimate the hourly output of cells from the thoracic duct fistula. A cytocentrifuge preparation was made and stained for non specific esterase activity. From the percentage of cells showing a positive staining reaction on the slide the hourly output of these cells was estimated. A portion of the cell sample was assessed for the ability to phagocytose sensitised sheep red blood cells. Cytocentrifuge preparations made after this assay permitted an estimate of the percentage and number of phagocytic cells draining hourly.

The statistical significance of the results in this and the following chapter has been carried out by comparing the variance of the regression lines. A Hewlett Packard Model 10 calculator was programmed to plot the regression line from the daily mean values. The regression line is allocated the value B and its Variance Var B. The t value for the significance of the observation is then calculated using the equation:-

$$t = \frac{B_1 - B_2}{\sqrt{Var_{B_1} + Var_{B_2}}}$$

when B_1 represents the normal value and B_2 the value from treated rats

Results

The effect of C. Parvum on the hourly cell output in thoracic duct lymph is shown in table 2.1 and figure 2.1. Over 97% of the cells in the thoracic duct lymph of

rats which have undergone mesenteric lymphadenectomy are lymphocytes. The figure shows that rats quickly became depleted of these cells during the chronic drainage of lymph. Rats which had received systemic C. Parvum had been injected between one and four weeks prior to cannulation. The third group of results shown in figure 2.1 are from rats which were injected with C. Parvum on the second day after the drainage of lymph had been established. C. Parvum given either prior to thoracic duct cannulation or during the drainage of lymph had no effect on the numbers of effluent cells.

The percentage of the total cell output having a positive staining reaction for non specific esterase is shown in Table 2.2 and figures 2.2 to 2.6. In the figures the mean percentage of these cells with the standard error of their means, have been plotted. For diagramatic convenience the standard error of the mean has been shown as its positive or negative value throughout this work. In each figure the normal results have been compared with those from rats at varying time intervals after the injection of C. Parvum. In all animals the percentage of esterase positive cells in thoracic duct lymph rose over the five day drainage period. The highest percentage found was just over 3% on days four and five of drainage in the rats which had been injected with 0.7 mg of C. Parvum two weeks previously. Comparison of the regression lines obtained by plotting the values from normal and C. Parvum treated rats showed that treatment had no influence on the percentage of esterase positive cells in thoracic duct lymph. Wilcoxons rank sum test for unpaired samples was used to compare some individual results which appeared to differ from those from normal rats, for example the values obtained on days 4 and 5 three weeks after I.V. C. Parvum (fig 2.4). The differences were not statistically significant.

The effect of systemic C. Parvum on the number of esterase positive cells in thoracic duct lymph is shown in Table 2.3

and figures 2.7 to 2.11. Although, as has been shown, the percentage of esterase positive cells rose over the five days of drainage, the absolute number slowly decreased. Comparison of the regression lines shows that C. Parvum treatment had no effect on the numbers of esterase positive cells in thoracic duct lymph in all of the groups studied except four weeks after I.V. C. Parvum. In this group there was a statistically significant increase ($P < 0.01$).

The hourly output of phagocytic cells is shown on tables 2.4 and 2.5 and figures 2.12 to 2.17. In the figures the results from the first and second weeks after C. Parvum have been combined for reasons of brevity, as have those from the third and fourth weeks after injection. C. Parvum treatment had no influence on the output of phagocytic cells in the thoracic duct lymph of mesenteric lymphadenectomised, but otherwise normal, rats. There were no marked differences noted between the number of phagocytic cells found after 20 or 40 minute incubation periods.

C. Parvum given intravenously in a dose of 0.7 mg after two days of drainage of thoracic duct lymph caused no acute alterations in the output of esterase positive or phagocytic cells. (Table 2.6) In this small group of animals drainage was slow to become established and the cell output was low for the first 48 hours. After the injection of C. Parvum the values obtained closely corresponded with those from normal animals.

Discussion

Complete surgical removal of the mesenteric lymph nodes in rats permits the collection of gut associated M.P.S. cells. Cells obtained by this method belong to two

broad classes. Firstly there is a population of cells which are the same size as medium to large lymphocytes. These adhere strongly to glass, are phagocytic and have a small amount of cytoplasm with a lobulated nucleus. The second cell population is of larger cells which are not strongly glass adherent, and are less capable of phagocytosis. They have abundant cytoplasm with "frilly" processes like pseudopodia. A capacity for metabolic activity is indicated by the numerous intracytoplasmic organelles seen with the electron microscope. While these larger cells stain diffusely and often densely for non specific esterase the smaller phagocytes either stain poorly or not at all. (164) (Plate 1, Page145) Direct cannulation of the lacteals in rats which have not undergone mesenteric lymphadenectomy permits the collection of cells similar to those found in the thoracic duct lymph after lymphadenectomy. (164) Lacteal lymph, however, has a higher percentage of M.P.S. cells than thoracic duct lymph. This may be due to dilution of thoracic duct lymph for example by the influx of hind limb lymph.

Afferent lymph carries a variable population of cells which belong to the M.P.S. This is not the case in efferent lymph which has passed through lymph nodes. Whether they are destroyed or transformed during transit through the node is unclear. Labelled M.P.S. cells in the afferent lymph become distributed throughout the thymus dependent paracortical area. They are not found in the primary follicles, medullary cords or germinal centres and have a completely different distribution from labelled lymphocytes or infused inert particles. (122)

The proportion of cells in thoracic duct lymph capable of phagocytosis varies with the method of study. (164) Values obtained using latex particles are higher than when using antibody coated red blood cells. Sensitised

sheep red blood cells have been used in this study because of the east with which they are recognised on light microscopy. Small particulate material can easily be mistaken for ingested latex but the appearance of intra cytoplasmic red cells in unlikely to lead to error. The present finding that cells which ingest sensitised sheep red blood cells usually display very little reaction with non specific esterase stains is in accordance with the report from MacPherson and Steer. This is not always the case and plate 2 (Page 145) shows a cell that is quite densely esterase stained yet contains three ingested red blood cells. Although phagocytosis was studied after two different incubation times the number of cells which showed this capacity remained the same. It is unlikely that limiting the incubation period to 20 minutes would be satisfactory for future work.

Cells staining for non specific esterase are present throughout the period of drainage, the percentage varying from 0.5 to 3%. Although the percentage of these cells rises over the five day drainage procedure the absolute count falls slowly as the total cell output falls. This was the case in all the groups studied and at no time after C. Parvum treatment was the percentage of esterase positive cells significantly altered. Nor did C. Parvum alter the number of esterase positive cells in thoracic duct lymph except in one group of animals. Whether the increase found four weeks after I.V. C. Parvum represents a true stimulation is not clear. No animals were cannulated later than this period since the other signs of reticulo-endothelial stimulation (splenomegaly and bone marrow cell cultures) are returning to normal by four weeks. (19)

The number of phagocytic cells in thoracic duct lymph is not influenced by C. Parvum treatment. This is in contrast to the observations that C. Parvum increased

clearance of colloidal carbon, (2) and radiolabelled albumin. (22) Augmented clearance of these particles may be solely due to increased uptake by fixed tissue phagocytes, for example in the liver and spleen.

The total cell output in thoracic duct lymph is not influenced by C. Parvum treatment. C. Parvum given intravenously causes an acute fall in blood lymphocyte numbers within 24 hours of injection. (23, 25, 99, Chapter 4) It is of interest that cells in the thoracic duct, which are 97% recirculating lymphocytes, are not affected by C. Parvum. The observations of Gowans and Knight (154) showed that these cells are mainly small T lymphocytes that continually recirculate from the blood to the lymph by crossing the walls of the post capillary venules of the lymph nodes. These lymphocytes re-enter the blood when the thoracic duct lymph passes into the subclavian vein. It is not clear whether the acute lymphocytopaenia in the blood after C. Parvum treatment is due to the suppression of another group of lymphocytes. In a group of five rats C. Parvum was injected after two days of lymph drainage. No immediate alterations resulted in the lymphocyte output in thoracic duct lymph. Nor did C. Parvum given previously either intravenously or intraperitoneally have any effect on the thoracic duct lymphocyte output.

Conclusions

Systemic C. Parvum has no influence on:-

1. the output of thoracic duct lymphocytes
2. the percentage of esterase positive cells in thoracic duct lymph

3. the number of esterase positive cells in thoracic duct lymph up to three weeks after treatment. The number of esterase positive cells in thoracic duct lymph four weeks after I.V. C. Parvum was increased.

TABLE 2.1

HOURLY OUTPUT OF CELLS IN THORACIC DUCT LYMPH

Normal Rats		1	2	3	4	5	Days
	n	9	9	9	7	4	
	$\bar{x}$	13.4	8.7	4.6	4.8	2.1	$\times 10^6$
	$\pm$ SEM	8.7	2.7	1.8	2	0.7	$\times 10^6$

After C. Parvum 0.7 mg IV		1	2	3	4	5	Days
	n	28	26	28	28	25	
	$\bar{x}$	17.3	14.9	8.7	5.6	1.9	$\times 10^6$
	$\pm$ SEM	11.4	8.9	4.5	3.2	0.9	$\times 10^6$

C. Parvum on day two of drainage		1	2	3	4	5	Days
	n	5	5	5	5	5	
	$\bar{x}$	10.3	7.7	6.5	6	2.7	$\times 10^6$
	$\pm$ SEM	8.7	4.3	0.9	2.7	1.5	$\times 10^6$

Figure 2.1

Mean hourly cell output in thoracic duct lymph on days 1 - 5 of drainage

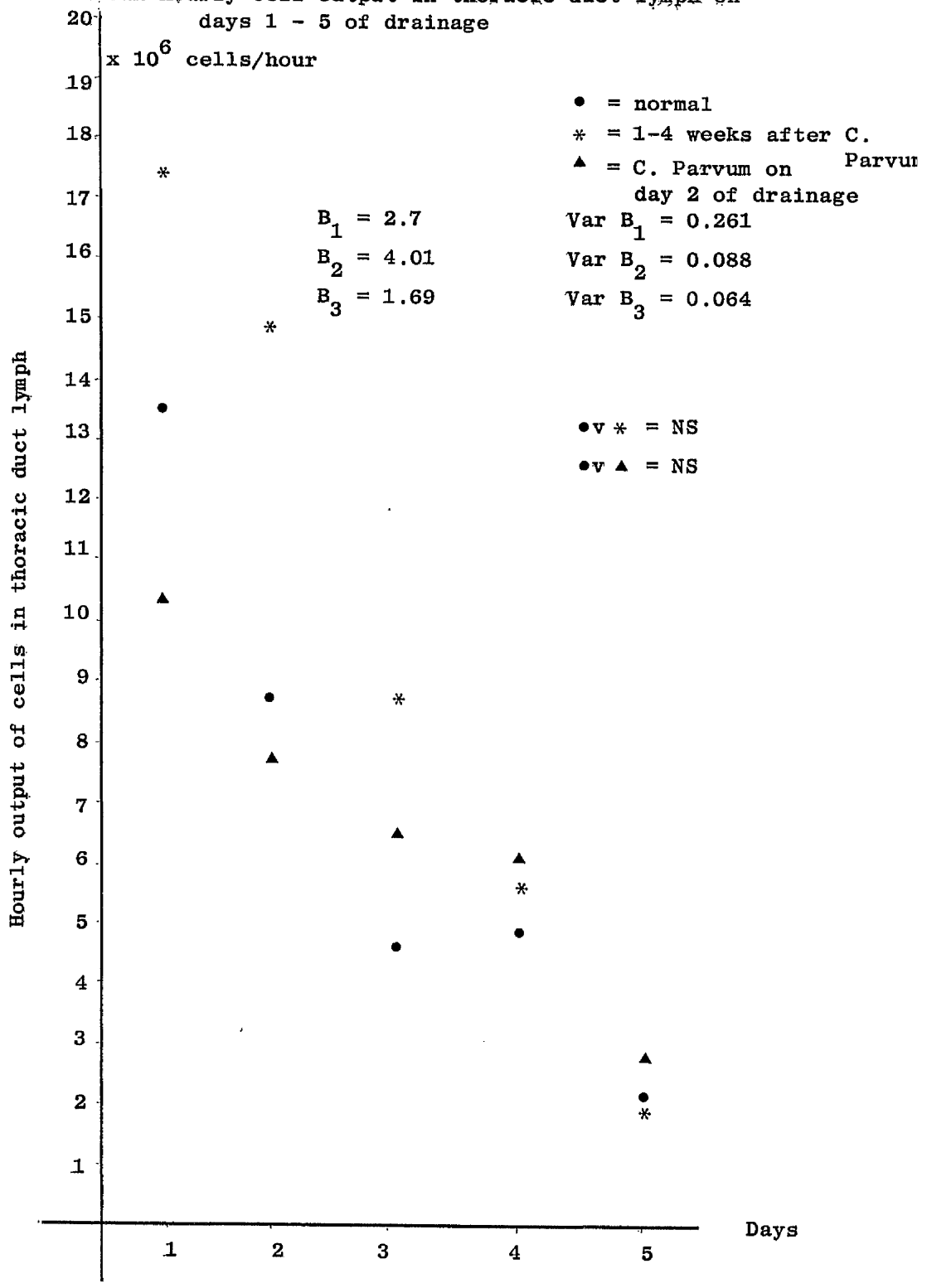


Table 2.2 Percentage esterase positive cells in thoracic duct lymph

	Day	1	2	3	4	5
No prior C. Parvum	n	9	12	12	10	4
	$\bar{x}$	1.27	1.4	1.85	1.82	2.2
	$\pm$SEM	0.23	0.14	0.29	0.23	0.45
C. Parvum 0.7 mg IV x one week prior to cannulation	n	8	7	7	7	7
	$\bar{x}$	0.9	1	1.76	1.76	2.07
	$\pm$SEM	0.09	0.15	0.17	0.17	0.17
C. Parvum 0.7 mg IV x two weeks prior to cannulation	n	7	6	8	6	4
	$\bar{x}$	1	1.7	2.01	2.01	3.03
	$\pm$SEM	0.23	0.38	0.38	0.29	0.86
C. Parvum 0.7 mg IV x three weeks prior to cannulation	n	6	8	9	8	6
	$\bar{x}$	0.96	1.27	2.3	3.13	3.18
	$\pm$SEM	0.29	0.29	0.48	0.52	0.76
C. Parvum 0.7 mg IV x four weeks prior to cannulation	n	9	9	9	9	7
	$\bar{x}$	1.02	1.6	1.5	2.4	2.97
	$\pm$SEM	0.14	0.21	0.24	0.3	0.45
C. Parvum 0.7 mg IP x two weeks prior to cannulation	n	8	7	6	6	6
	$\bar{x}$	1.2	1.5	2.1	1.97	2.1
	$\pm$SEM	0.16	0.23	0.27	0.19	0.42

Figure 2.2

Percentage of esterase + ye cells in thoracic duct lymph

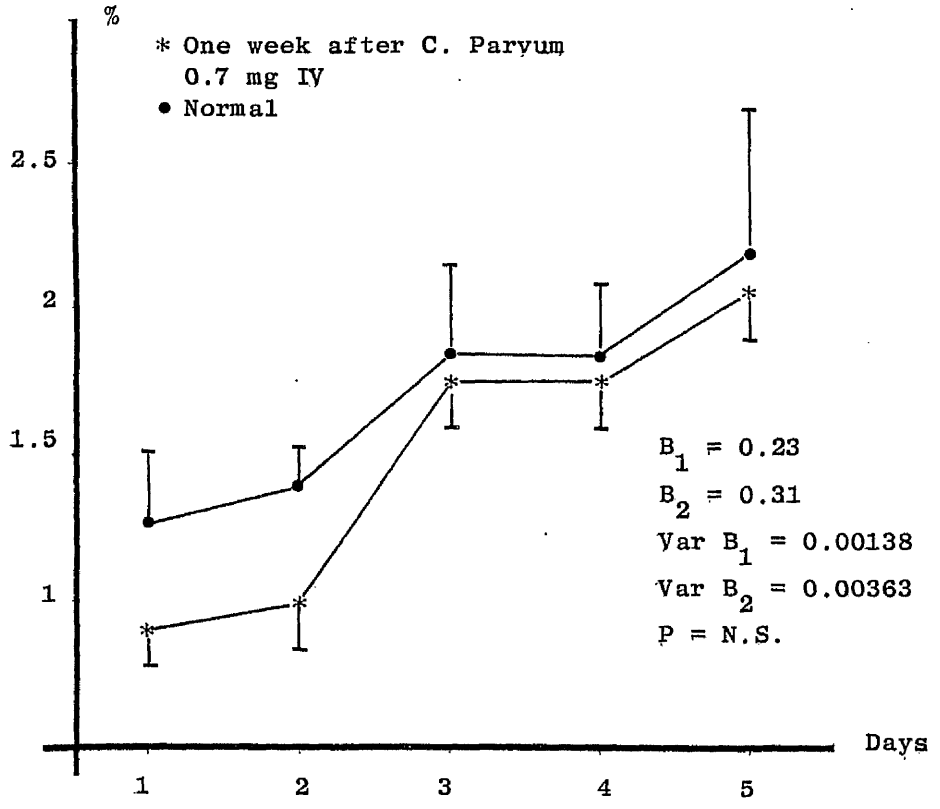


Figure 2.3

Percentage of esterase + ve cells in thoracic duct lymph

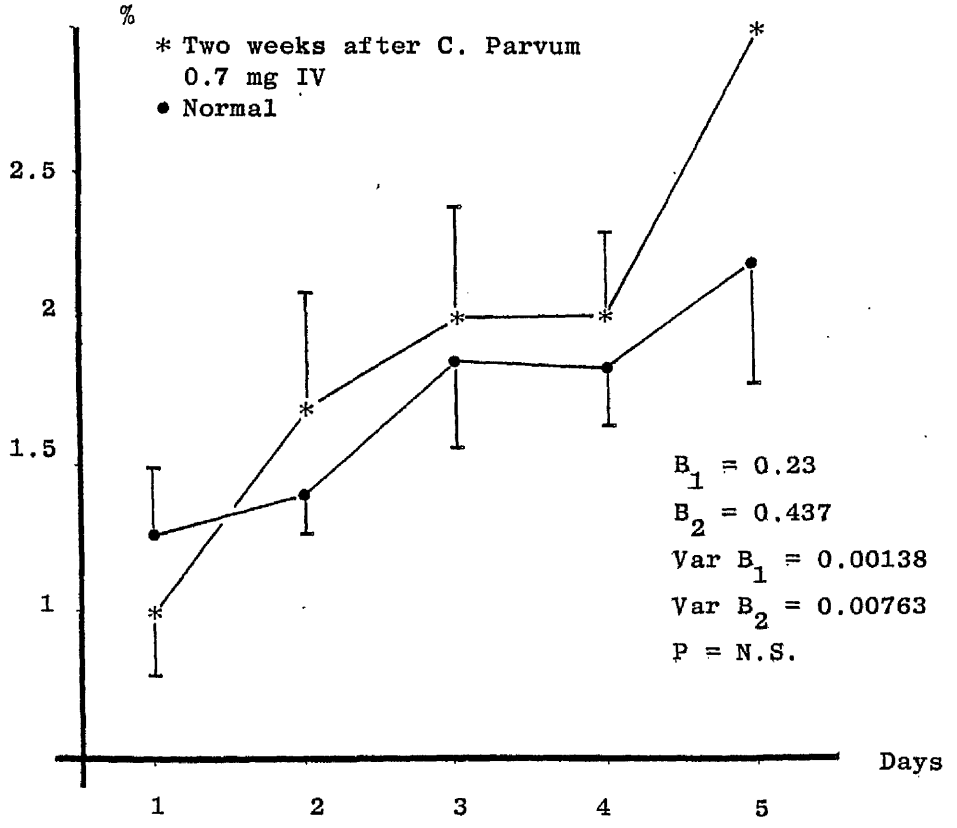


Figure 2.4

Percentage of esterase + ve cells in thoracic duct lymph

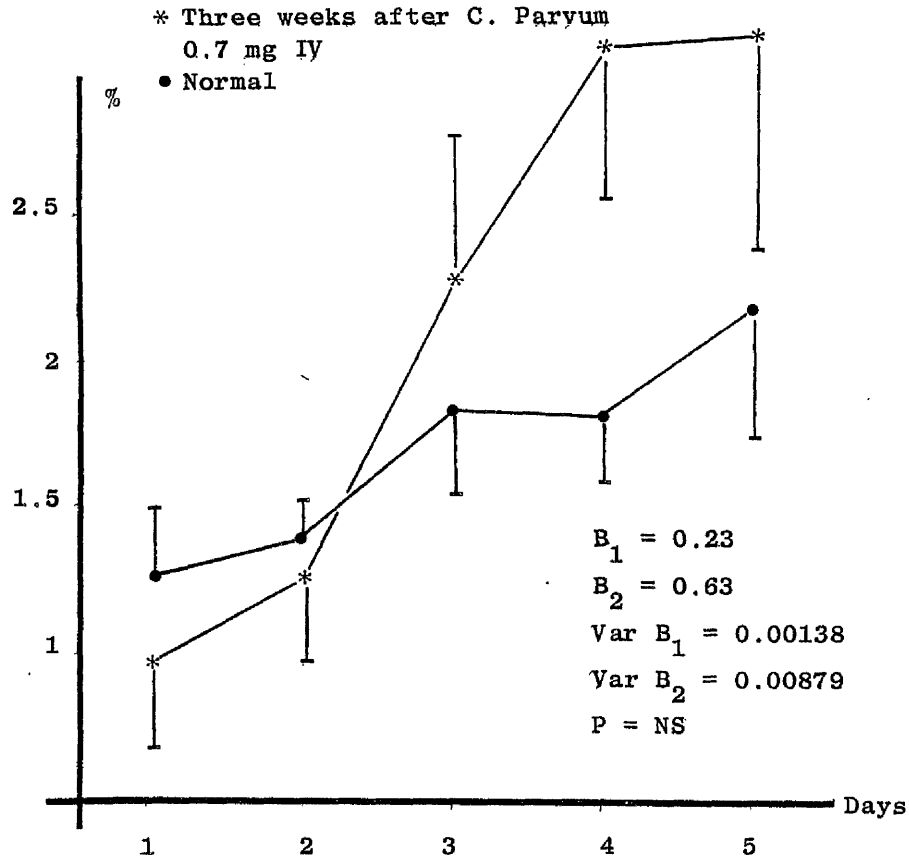


Figure 2.5

Percentage of esterase + ve cells in thoracic duct lymph

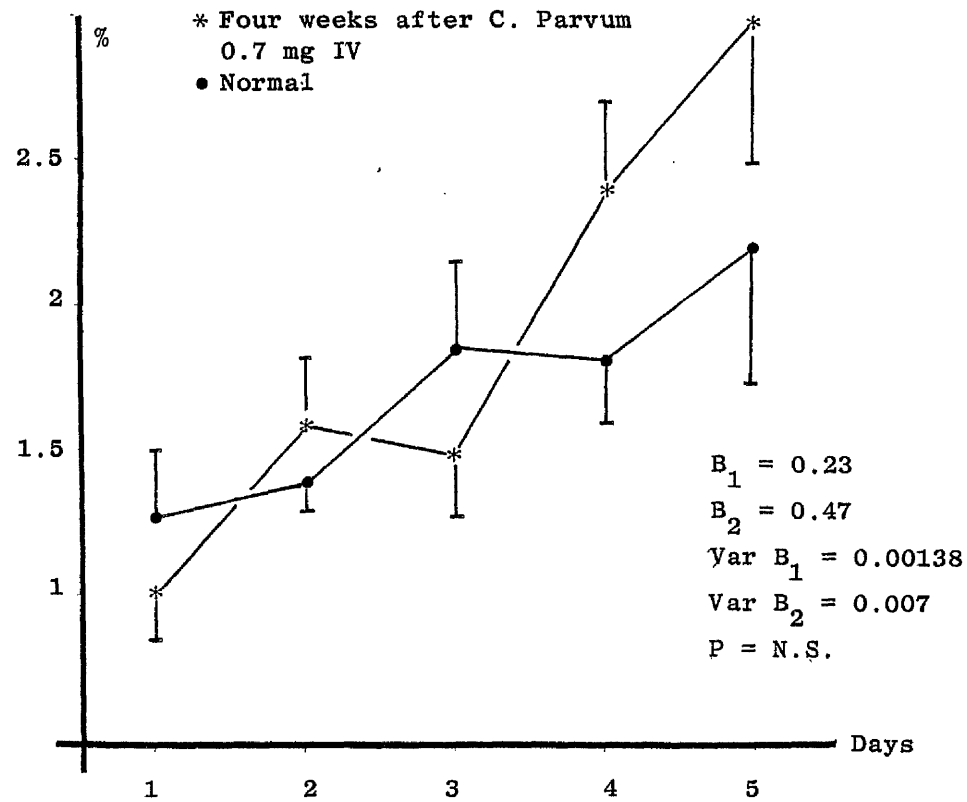


Figure 2.6

Percentage of esterase + ve cells in thoracic duct lymph

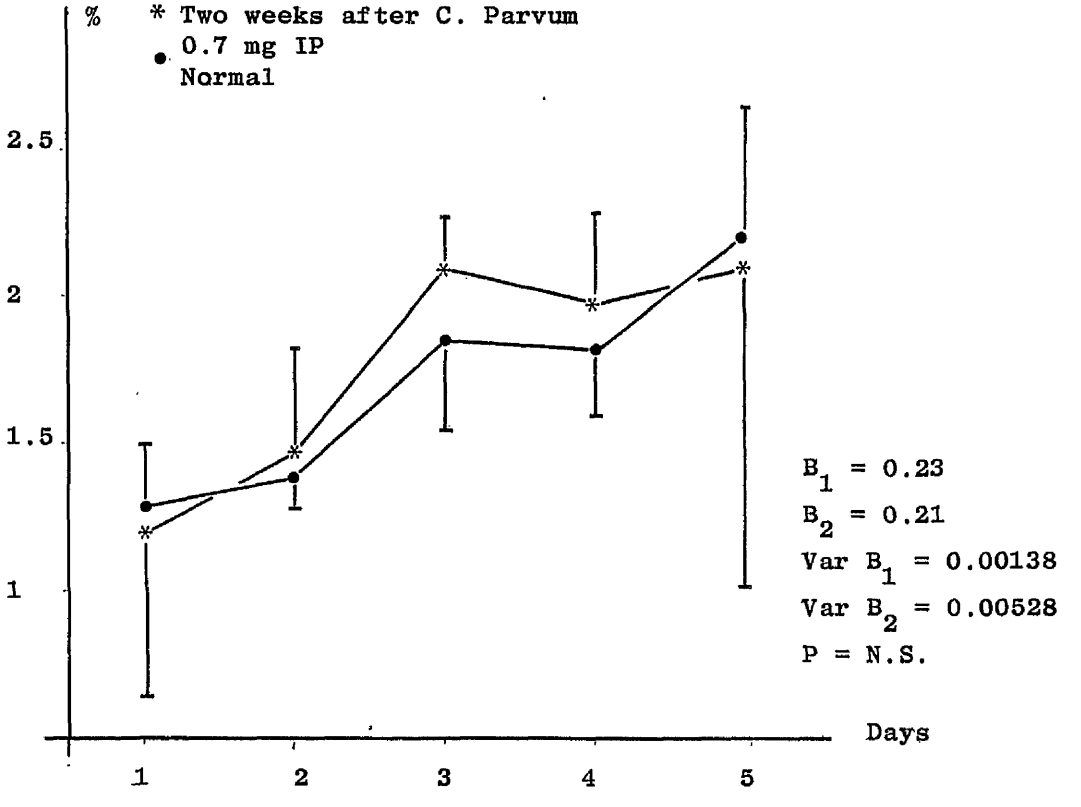


TABLE 2.3

Number of esterase positive cells in thoracic duct lymph
Hourly cell output x 10^6

	Day	1	2	3	4	5
No prior C. Parvum	n	9	11	11	9	9
	$\bar{x}$	15.9	15.2	8.7	8.4	4.5
	$\pm$SEM	3.2	2.3	1.1	1.3	0.9
C. Parvum 0.7 mg IV x one week prior to cannulation	n	8	7	7	8	7
	$\bar{x}$	13.8	17.9	13.5	10.8	3.1
	$\pm$SEM	3.2	5.5	8.4	3.5	0.6
C. Parvum 0.7 mg IV x two weeks prior to cannulation	n	7	7	7	6	4
	$\bar{x}$	9.2	25.5	16.5	11.3	5.3
	$\pm$SEM	2.03	6.9	4.6	3.2	1.8
C. Parvum 0.7 mg IV x three weeks prior to cannulation	n	7	7	9	8	5
	$\bar{x}$	15.3	12.6	15.5	11	3.8
	$\pm$SEM	4.6	3.5	3.6	2.2	0.7
C. Parvum 0.7 mg IV x four weeks prior to cannulation	n	9	8	9	9	6
	$\bar{x}$	19.2	28.6	13.8	15.1	10.1
	$\pm$SEM	4.1	7.0	3.6	3.3	5.8
C. Parvum 0.7 mg IP x two weeks prior to cannulation	n	10	9	9	9	8
	$\bar{x}$	15.9	13.2	7.5	5.5	5.8
	$\pm$SEM	2.2	2.9	1.6	1.1	1.1

Figure 2.8

Number of esterase + ve cells in thoracic duct lymph

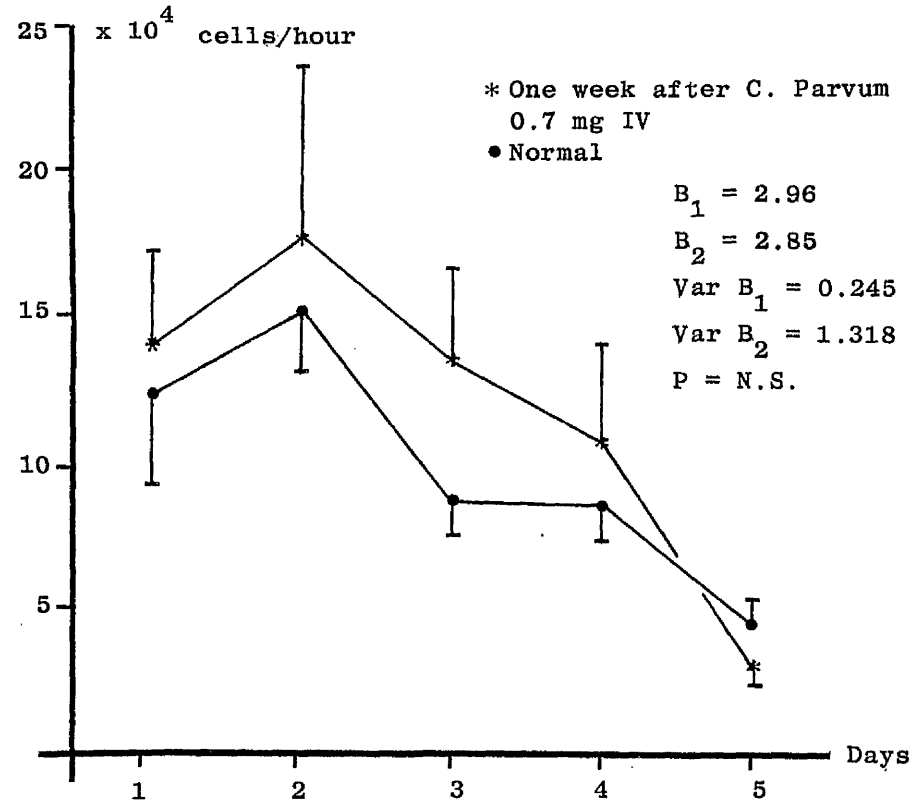


Figure 2.9

Number of esterase + ve cells in thoracic duct lymph

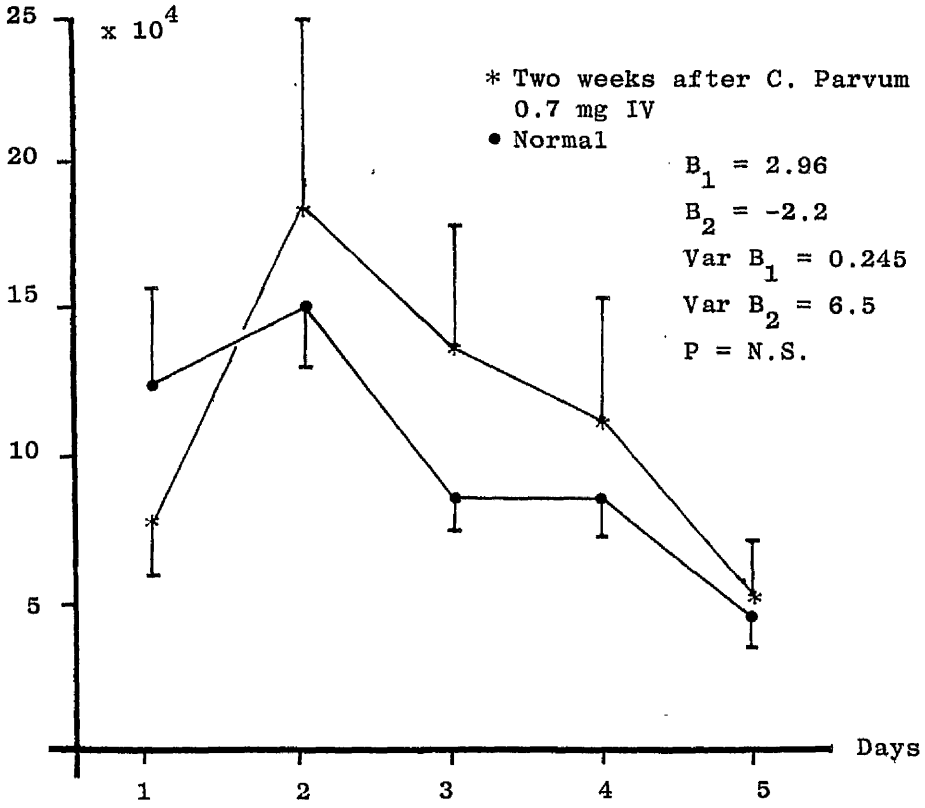


Figure 2.10

Number of esterase + ve cells in thoracic duct lymph

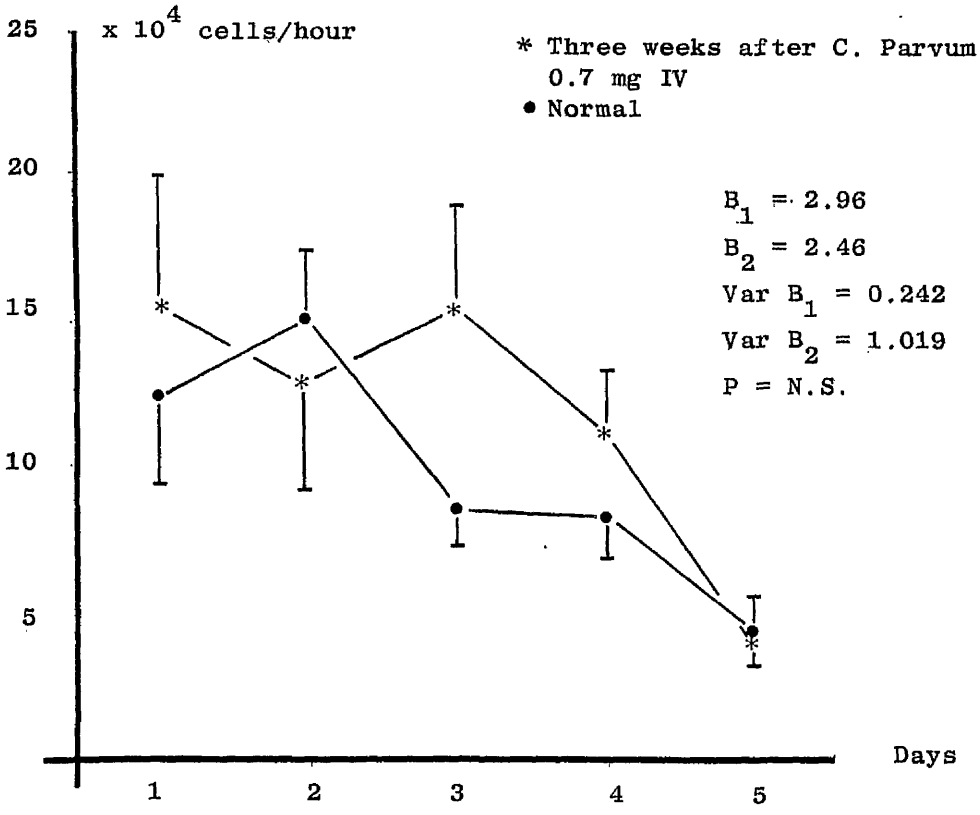


Figure 2.11

Number of esterase +ve cells in thoracic duct lymph

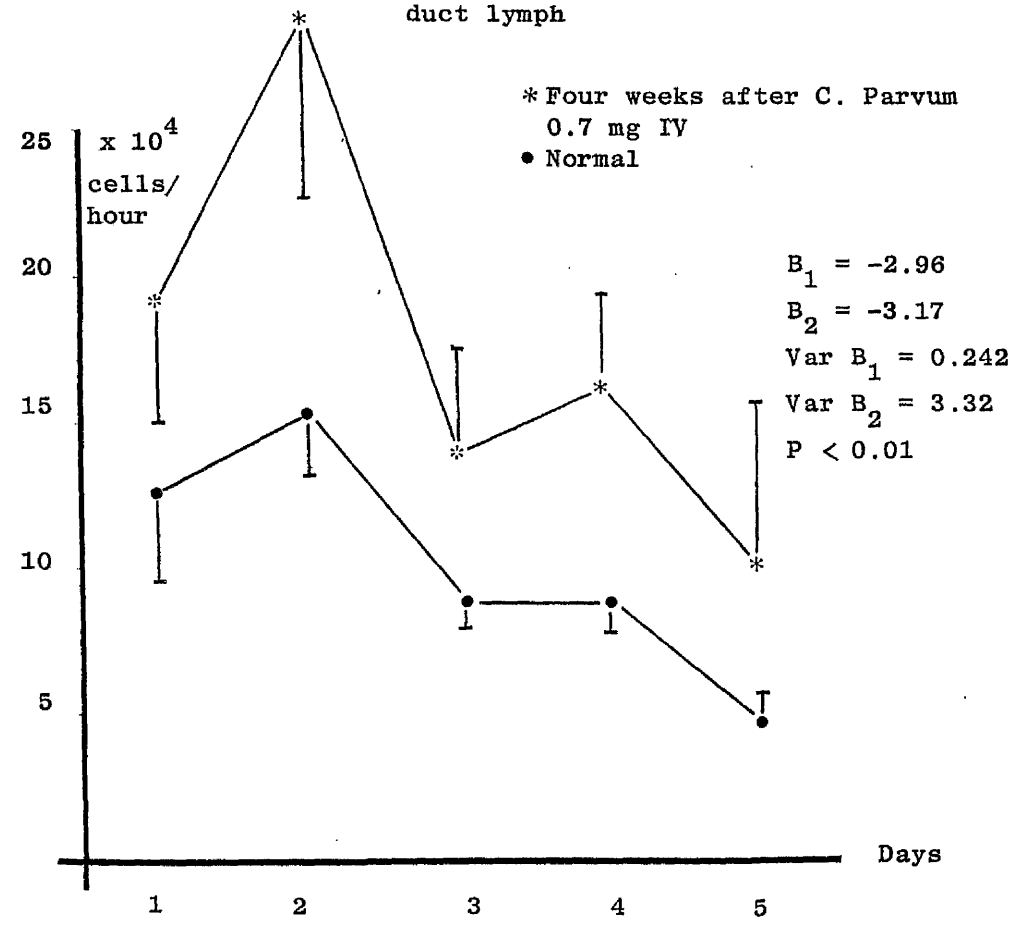


TABLE 2.4

Numbers of phagocytic cells in thoracic duct lymph
Hourly cell output x 10^6

	Day	1	2	3	4	5
No prior C. Parvum						
20 minute incubation	n	9	9	9	5	3
	$\bar{x}$	4.5	3.0	1.2	1.5	0.4
	$\pm$SEM	0.93	0.8	0.3	0.86	0.05
40 minute incubation	n	9	9	9	5	3
	$\bar{x}$	3.5	3.6	1.8	1.8	0.4
	$\pm$SEM	0.7	0.6	0.6	0.6	0.02
C. Parvum 0.7 mg IV one and two weeks previously						
20 minute incubation	n	10	8	11	11	9
	$\bar{x}$	4.1	3.3	2.1	1.53	0.9
	$\pm$SEM	1.3	1.2	0.59	0.5	0.37
40 minute incubation	n	10	9	11	11	9
	$\bar{x}$	3.79	2.8	2.87	1.6	0.88
	$\pm$SEM	1.1	1.23	1.1	0.54	0.42

TABLE 2.5

Day		1	2	3	4	5
C. Parvum 0.7 mg IV three and four weeks previously						
20 minute incubation	n	16	16	17	17	11
	$\bar{x}$	1.88	2.72	2.26	1.62	0.4
	$\pm$SEM	0.6	0.89	0.5	0.39	0.11
40 minute incubation	n	15	16	18	17	10
	$\bar{x}$	4.8	2.69	2.23	1.59	0.53
	$\pm$SEM	1.1	0.75	0.5	0.37	0.14
C. Parvum 0.7 mg IP two weeks previously						
20 minute incubation	n	10	9	10	7	4
	$\bar{x}$	3.9	1.8	1.4	0.6	0.6
	$\pm$SEM	1.4	0.6	0.4	0.2	0.35
40 minute incubation	n	9	9	10	8	4
	$\bar{x}$	3.7	2.8	1.9	0.9	0.8
	$\pm$SEM	0.8	1.3	0.5	0.3	0.25

No. of phagocytic cells on a 20 minute incubation

1 and 2 weeks after C. Parvum 0.7 mg I.V.

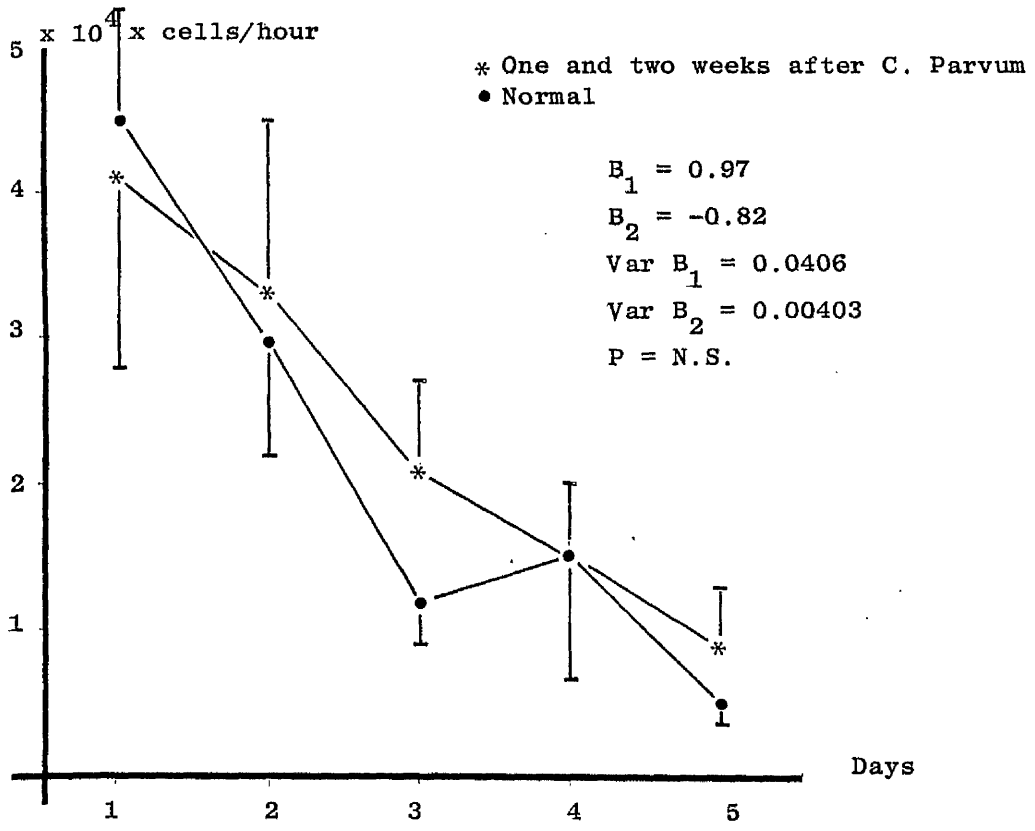


Figure 2.13

No. of phagocytic cells on a 40 minute incubation

1 and 2 weeks after C. Parvum 0.7 mg I.V.

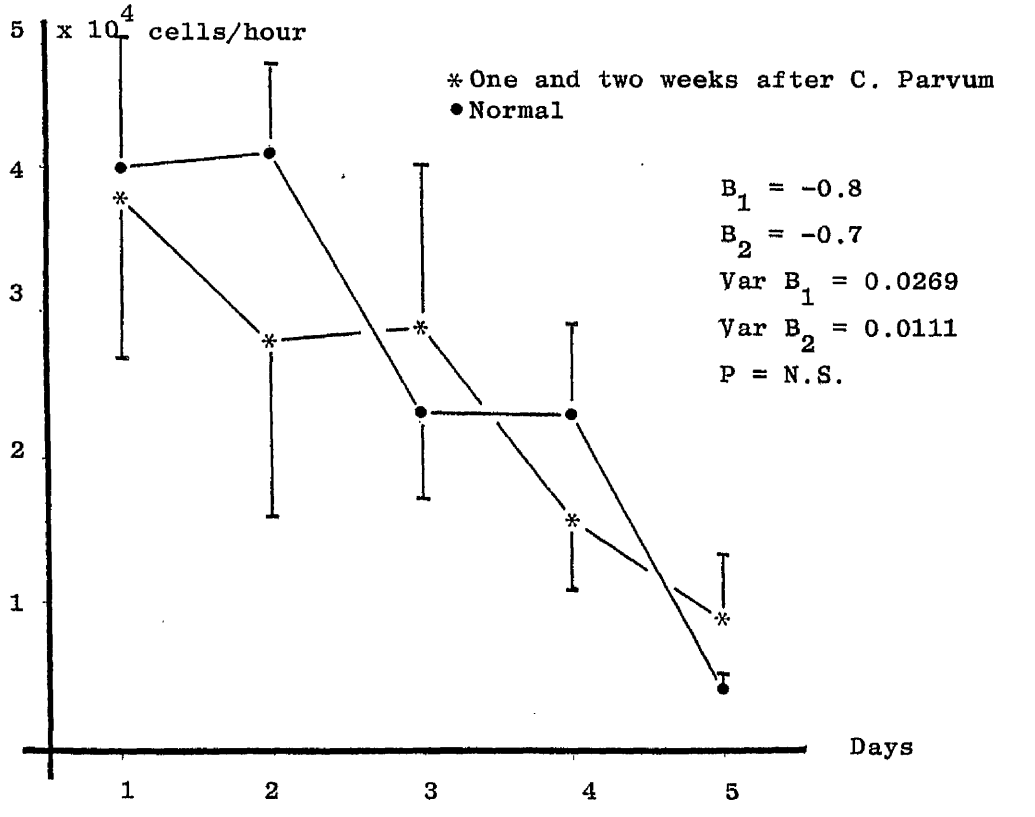


Figure 2.14

No. of phagocytic cells on a 20 minute incubation

3 and 4 weeks after C. Parvum 0.7 mg I.V.

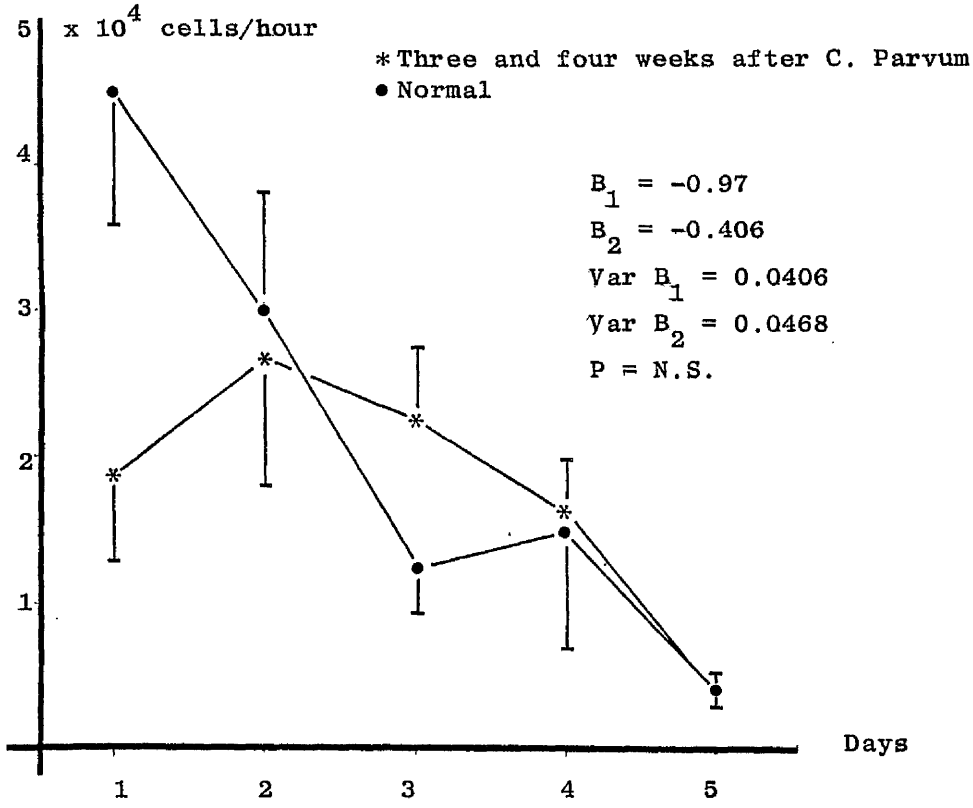


Figure 2.15

No. of phagocytic cells on a 40 minute incubation

3 and 4 weeks after C. Parvum 0.7 mg I.V.

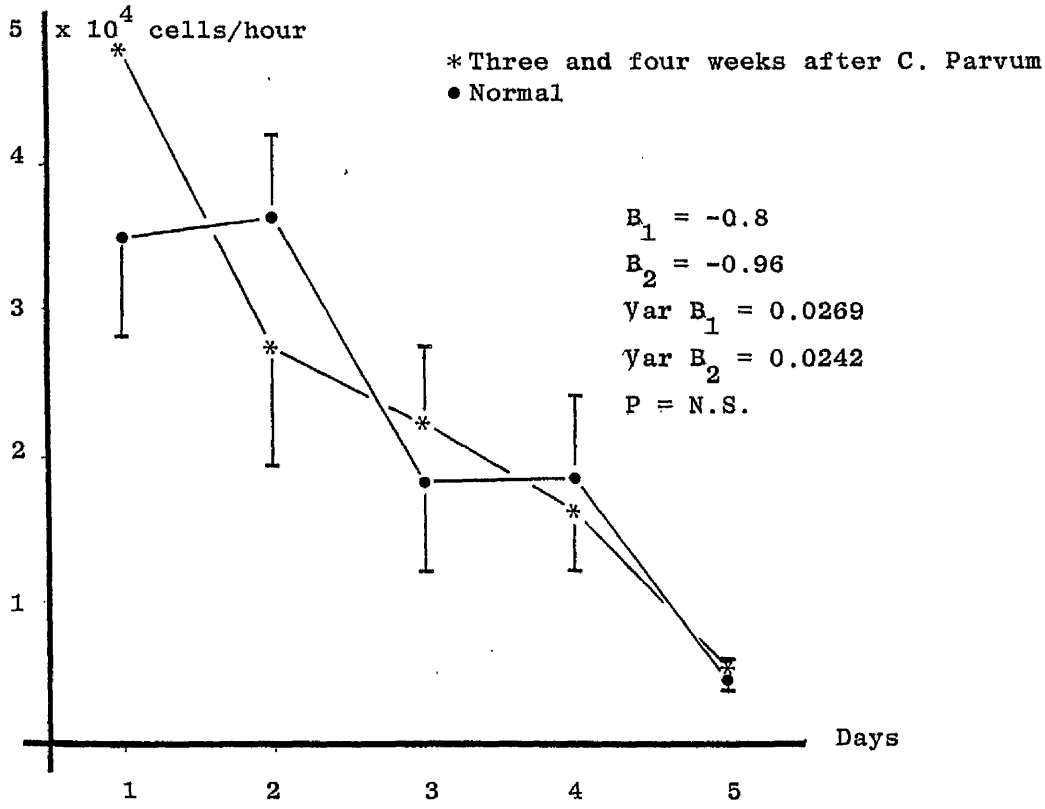


Figure 2.16

No. of phagocytic cells on a 20 minute incubation after C. Parvum 0.7 mg I.P.

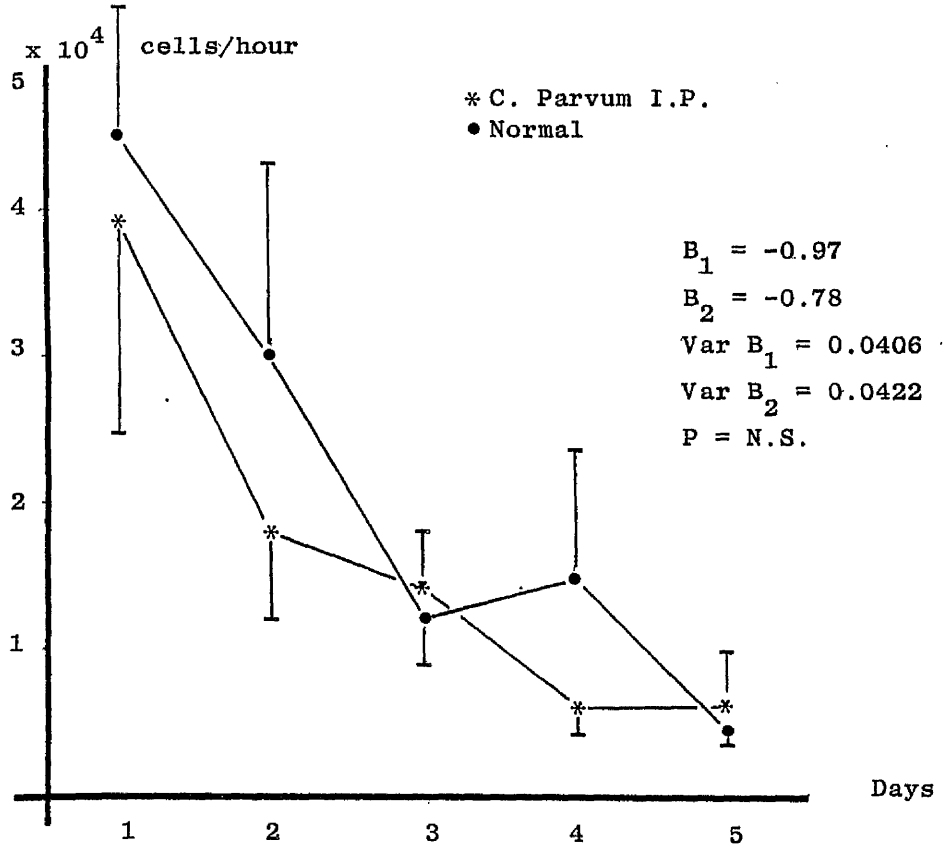


Figure 2.17

No. of phagocytic cells on a 40 minute incubation after C. Parvum 0.7 mg I.P.

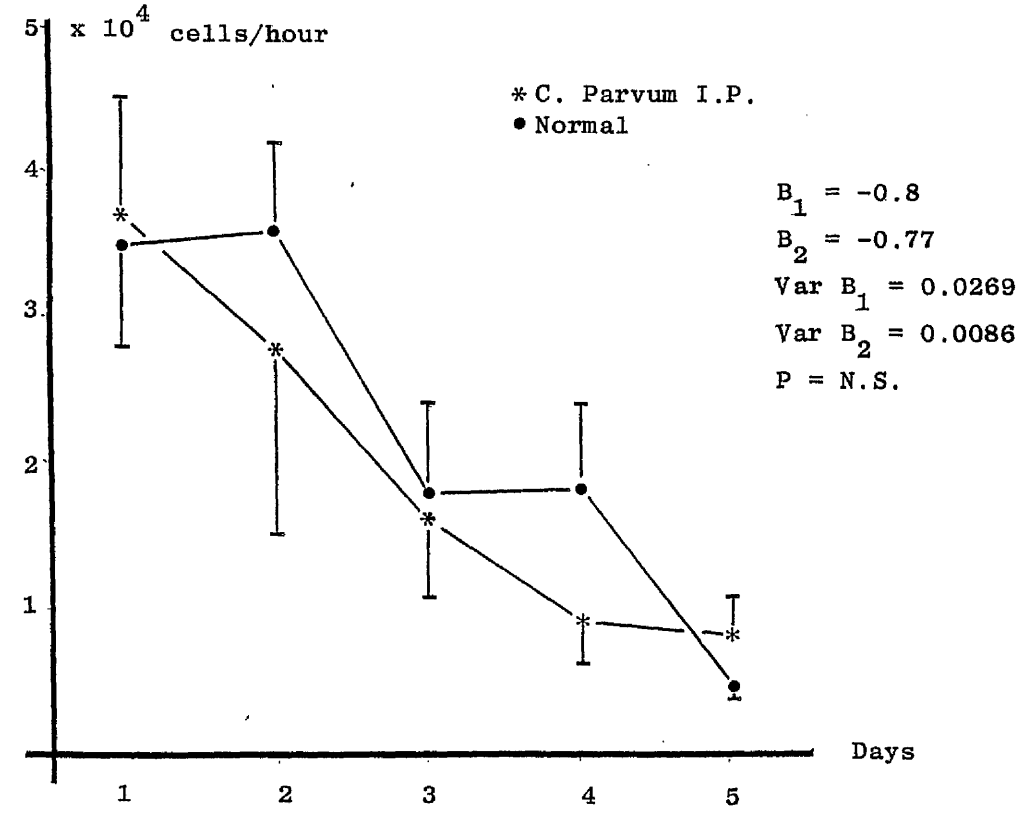


TABLE 2.6

The effect of 0.7 mg C. Parvum given I.V. after two days drainage of thoracic duct lymph

	Day	1	2	3	4	5
Percentage of esterase positive cells	n	5	5	5	5	5
	$\bar{x}$	0.6	1.1	1.2	1.4	2.3
	$\pm$SEM	0.13	0.22	0.4	0.3	0.58
Number of esterase positive cells hourly output x 10^6	n	5	5	5	5	5
	$\bar{x}$	6.5	7.7	8.2	9.3	5.3
	$\pm$SEM	2.9	1.8	3.26	4.2	1.8
Number of phagocytic cells after 20 minute incubation Hourly output x 10^6	n	5	5	5	5	5
	$\bar{x}$	3.4	2.7	2.5	2.2	0.9
	$\pm$SEM	1.1	2.8	1.2	1.4	0.5
Number of phagocytic cells after 40 minute incubation Hourly output x 10^6	n	5	5	5	5	5
	$\bar{x}$	3.4	2.7	1.7	4.3	1.0
	$\pm$SEM	1.1	1.1	0.4	3.0	0.9

Chapter 3

The effect of systemic C. Parvum on cells in the thoracic duct lymph of rats bearing colonic cancers.

Thoracic duct cannulation has been carried out in nine mesenteric lymphadenectomised rats bearing small dimethylhydrazine (D.M.H.) induced colonic cancers. The mesenteric lymph nodes were removed before beginning tumour induction. The numbers of esterase positive and phagocytic cells draining hourly were calculated using the method described in Chapter 2. A further group of six tumour bearing mesenteric lymphadenectomised rats were injected with 0.7 mg of C. Parvum intravenously between 7 and 14 days before undergoing cannulation of the thoracic duct.

D.M.H. (Sigma Chemicals, Poole) was injected subcutaneously once weekly in a dose of 30 mg/kg body weight for fifteen weeks. Crystalline D.M.H. was refrigerated until use. A suspension of the carcinogen was prepared by adding 0.35 g of D.M.H. to 100 mls 0.9 N saline and 1.5 g ethylenediamine tetracetic acid. The pH of the solution was adjusted to 6.5 with 0.1 M sodium hydroxide. Since D.M.H. is a potent carcinogen this procedure was carried out wearing gloves and a face mask and the chemicals were decanted and mixed inside a fume cupboard. This minimised the risks of absorption of D.M.H. through the skin or mucous membranes. Rats were injected weekly with 2 mls of freshly prepared solution. As most of the rats being used weighed between 200 and 250G the dose of 7 mg was equivalent to 30 mg/kg body weight. The total accumulated dose was 450 mg/kg. During tumour induction the rats were housed in cages with metal grid floors. Faeces and urine passed through the grid to a collecting tray. Technicians cleaning the collecting tray were advised to wear gloves. The cages were kept in a separate area of the animal house and marked with carcinogen

precaution notices. After finishing the series of D.M.H. injections the animals were closely monitored for signs of tumour development, for example, weight loss and bloody diarrhoea. As the tumour bearing animals were to undergo cannulation of the thoracic duct it was important that they be in as good a physical condition as possible. Random laparotomies were performed from 30 weeks after the beginning of tumour induction. When small tumours were found, other rats from the same group underwent thoracic duct cannulation. The presence of large bowel tumours was confirmed in each of these rats at the end of the experiment when, after killing the rat, the whole of the large bowel was removed and examined.

Results

The percentage and number of esterase positive cells in the effluent lymph of tumour bearing rats did not differ significantly from normal rat values. (Table 3.1, figs. 3.1 & 3.3) Nor did the systemic administration of C. Parvum in a dose of 0.7 mg between seven and fourteen days before cannulation have any effect on these results. (Table 3.2, figs. 3.2 & 3.4)

There was a significant depression in the numbers of phagocytic cells in the thoracic duct lymph of all of the tumour bearing rats whether or not C. Parvum had been given. (Table 3.1 & 3.2, figs 3.5 - 3.8) There was no difference in the degree of suppression between the two groups of tumour bearing rats. C. Parvum did not influence the output of esterase positive or phagocytic cells in thoracic duct lymph. The phagocytic cells found in the thoracic duct lymph of those rats bearing tumours were similar in appearance to those from normal lymphadenectomised rats. Other phagocytic cells like polymorphonuclear leukocytes were not seen. As has been stated, it was aimed to find

developing tumours early before the rats become unfit to undergo the cannulation experiment. In these animals the tumours were confined to the large bowel. (Plates 3, 4 Page 145) Prior extirpation of the mesenteric lymph nodes did not appear to facilitate early metastatic spread of cancer. Malignant cells were not identified in the thoracic duct lymph. In a separate series of D.M.H. tumour inductions three male DA rats and one female Wistar rat developed squamous carcinomas of the middle ear.

Discussion

C. Parvum has been reported to increase the phagocytic uptake of colloidal carbon (2), latex particles (87) and sensitised red blood cells (168) (12). Thomson and his co-workers reported that Fc receptor bearing cells within tumours are increased by C. Parvum treatment, (9) but found that there was no clear effect on cells capable of phagocytosing carbon. It is uncertain if M.P.S. cells capable of exerting an anti tumour action are in fact phagocytic. Phagocytosis is a characteristic of mature macrophages, which would not be increased in numbers of functional capacity in the first two weeks after C. Parvum treatment. (168) Studies on peritoneal exudate cells suggest that anti tumour activity is exerted by the largest cells present, while immune responsiveness is dependent on medium sized and small cells. (89)

The observation that the number of phagocytes in efferent gut lymph is suppressed in rats suffering from colonic cancer is of considerable interest. It seems unlikely that this is directly due to toxic depression caused by the carcinogen since the last dose of D.M.H. was at least 10 weeks prior to the cannulation experiment. Although the half life of D.M.H. in the circulation is unknown, it seems unlikely that a chemical suppression would be selective for an individual type of cell. Nor do the low levels of phagocytes appear to facilitate metastatic spread since this tumour rarely metastasises. (74) C. Parvum

given between one and two weeks prior to the cannulation procedure had no influence on the suppressed levels of phagocytes. It is not clear from these experiments whether C. Parvum given longer before cannulation would have affected the phagocytic cells. However, in Chapter 2 it was shown that even four weeks after C. Parvum there were no alterations in phagocytic cells. It does not seem likely that C. Parvum exerts an effect on the numbers of these cells present in thoracic duct lymph.

Mice and rats bearing transplantable tumours show increased rates of clearance of carbon particles from the blood. (169) This is not universally found and using the Lewis lung carcinoma model Otu reported depression of carbon clearance shortly after tumour transplant. (147) He also noted suppression of macrophage locomotion and bone marrow macrophage colony formation.

Increased phagocytosis has been reported in studies on peritoneal exudate cells (PEC) of mice bearing transplanted tumours. In mice bearing recently transplanted methylcholanthrene induced fibrosarcomas in their footpads PEC showed increased phagocytosis of ^{51}Cr labelled sheep red blood cells coated with IgG. (170) In another report PEC from mice receiving an inoculum of sarcoma - 180 showed increased uptake of staphylococcus aureus. (171) These workers have also identified a serum factor which blocked this enhancement of phagocytosis. Other phagocytosis inhibiting factors have been identified. Serum from cancer patients and tumour bearing animals can inhibit the lipid ingesting capacity of macrophages. (172) Nematode activated macrophages show enhanced <u>in vitro</u> phagocytosis of Walker carcinosarcoma cells. (173) This enhancement is abrogated by adding serum from the same rats to the incubation. The blocking factor in this case belongs to the

IgG 2 subclass of immunoglobulins. This group of immunoglobulins has also been found to prevent the attachment and phagocytosis by macrophages of leukaemia cells both in vitro and in vivo (174). It may be that tumours have different capacities to stimulate the development of blocking factors. Whether any such factors were present in the serum or lymph in the rats in this series is unknown.

CH_3 - N H. H Cl

CH_3 - N H. H Cl

1, 2 Dimethyl-hydrazine Dihydrochloride (D.M.H.)

D.M.H. is one of several related hydrazine derivatives which induce intestinal tumours in laboratory animals. 1, 1 Dimethylhydrazine and methylhydrazine are both naturally occurring substances. The former is found in cigarette smoke and the latter in edible fungi. Both are used in space rocket propellants. (175) D.M.H. itself has not been identified as a naturally occurring substance.

When D.M.H. is injected subcutaneously into a wide variety of rodents in a dose of between 20 mg/kg and 40 mg/kg weekly a high proportion develop tumours of the large and small bowel. (176) This dose level causes transient hepatotoxicity but no acute deaths. Most workers have given a total dose of less than 400 mg/kg body weight injected weekly in 10 to 15 equal doses. (176, 177, 178, 179)

Small areas of dysplasia begin appearing in the colon after the 12th week of tumour induction. (177) From the 19th week onwards frank carcinomas begin to appear either as plaques or sessile polyps. All grades of mucosal change can be recognised from dysplasia to carcinoma in situ and finally invasive carcinoma. At a later stage similar adenocarcinomata may appear in the small bowel. Alteration in goblet cell mucin can be demonstrated by special stains as early as 7 weeks after tumour induction has commenced. High iron diamine-alcian blue stains enable a distinction to be made between sialomucins and sulphomucins. (180) As epithelial dysplasia increases sialomucins begin to predominate and this is accompanied by a decrease in sulphated material. Similar changes have been demonstrated in the human large gut not only in apparently normal mucosa adjacent to colorectal cancers, but also at distant sites. These changes may precede the appearance of the more classical histological features of malignancy.

Mechanism of Carcinogenesis - Precisely how D.M.H. induces adenocarcinoma of the bowel is not known. Alteration of cellular differentiation could be mediated by interaction with nucleic acids or protein. (177) Changes are described in the nucleic acid metabolism and proliferative capacity of the colonic epithelium in D.M.H. treated mice, (181) which are similar to those found in human colonic mucosa adjacent to carcinoma and neoplastic polyps.

Some evidence suggests that D.M.H. is metabolised to a more active carcinogen in the liver. (178) Although only small amounts of radiolabelled D.M.H. appears in the bile the effects of chronic intoxication with the drug have not been studied. Histological signs of acute hepatic toxicity result from use of D.M.H.

Dietary factors - The low incidence of colonic cancer in native Africans has been attributed to their high fibre diet. While epidemiologists continue their debate, a controlled clinical trial would obviously be impossible in humans. Cruse has studied the effects of the alteration of dietary fibre intake in rats undergoing tumour induction with weekly D.M.H. (182)

Wistar rats were divided into three groups. All were inoculated with subcutaneous D.M.H. 40 mg/kg weekly for 13 weeks. The groups were fed on diets containing 20% w/w fibre (high fibre), 4.8% w/w fibre (normal laboratory diet and 0% w/w fibre respectively. No significant alterations in the rats of tumour induction or survival resulted.

In contrast to this work, rats undergoing similar tumour induction were protected by an all liquid elemental diet. (178) In this study rats were divided into two groups, one on a normal laboratory diet and the other on the commercial low residue diet Vivonex (Eaton laboratories). Each received D.M.H. 20 mg/kg weekly by subcutaneous injection. Rats fed on normal diets developed extensive tumours by week 24 of this regimen. In addition to tumours of the large bowel, the small bowel and liver were frequently involved. Several rats in this group did not survive the full term of the experiment. In the Vivonex fed group all rats survived the full term and developed predominantly large bowel tumours.

In the same paper D.M.H. was shown to cause acute histological changes in the liver and the Vivonex treated group appeared to be protected as these changes were less marked. In the present study all rats were allowed free access to a standard pelleted rat food and water and received 30 mg/kg D.M.H. subcutaneously by weekly injection

for 15 weeks. It is of interest to compare the differing time intervals between beginning a course of injections of D.M.H. and the appearance of colonic tumours. Castleden and Shilkin (178) and Cruse et al (179) used the same dosage of D.M.H., 20 mg/kg week, although the former workers gave 24 injections and the latter 20. The control group of animals in both studies received a normal pelleted rat diet and water. In the former study none of the rats survived for the full 24 weeks of the experiment and at post mortem they all had extensive tumours of the large and small bowel. In the report from Cruse, using the same strain of rats (Wistar) the control group of rats all survived until 36 weeks, although they all had tumours. In the present study using a higher weekly dose for a shorter period, Wistar rats developed small large bowel tumours between 30 and 35 weeks after the beginning of the course of injections. There seems to be a considerable variation in the time interval required before large bowel tumours develop. This may be due to variations in the method used to prepare the carcinogen. The method adopted in the work described in this chapter was the same as that of Cruse et al.

The Immunogenicity of D.M.H. induced colonic cancer - Colorectal cancers induced in rodents by D.M.H. possess tumour associated antigens demonstrable by *in vitro* assays. (183) Tissue specific antigens induce cellular immunity and humoral antibodies in rats developing primary bowel carcinomas, in recipients of isografts of such tumours and in animals immunised with irradiated tumour cells. Sera from tumour bearing rats block lymphocyte toxicity against the tumour cells. (183)

Rats undergoing D.M.H. tumour induction are partly protected by prior immunisation with D.M.H. induced tumours. (184)

A similar, but lesser protective effect is seen by immunisation with N-methyl-N-Nitroguanidine induced colorectal tumours. Female rats allowed to have a number of litters during carcinogenesis were also partially protected. These results suggest not only the presence of a tumour associated antigen in these chemically induced tumours, but that these antigens are partially shared, because weak cross immunisation occurs. There is also a possible shared antigen with embryonal cells although other protective effects of pregnancy cannot be discounted.

C. Parvum and D.M.H. :- C. Parvum given four times in a dose of 3.5 mg intraperitoneally to rats after week 13 of D.M.H. tumour induction resulted in a marked acceleration in the rate of tumour development. (74) Rats which received C. Parvum not only developed early and extensive tumours, but also developed metastases and other malignant tumours, particularly of the middle ear. Concern was voiced as to the effect of C. Parvum in human colonic cancer on the basis of these results. However, the dose of C. Parvum used in this animal study was greatly in excess of the human equivalent dose and also far greater than the dose required to cause reticuloendothelial stimulation in rats (as manifest by splenomegaly). These workers extrapolated from tumour inhibition studies in mice where 350 µg had been shown to result in maximal tumour inhibition. (84) Since the mice had weighed 15-20 G a tenfold dose of C. Parvum was selected for the rat studies (weight 150-200 G). This logic applied to the use of C. Parvum in humans would call for a dose of over 500 mg which is greatly in excess of the usual dose of about 10 mg. It is particularly worthy of

note that these workers had originally intended to administer C. Parvum once every three weeks, but found that the animals were failing to gain weight after 4 doses and treatment was stopped. It seems possible that this group of rats fared badly as a direct result of the toxic action of high doses of C. Parvum. The dose of an immune modulator required to enhance the immune response is critical. Dose responses are non-linear in nature and excessive doses can lead to immunosuppression. (185)

Conclusions

1. The non specific esterase positive cell output in the thoracic duct lymph is the same in tumour bearing and normal mesenteric lymphadenectomised rats.

2. The number of phagocytic cells in the thoracic duct lymph of tumour bearing rats is suppressed.

3. C. Parvum treatment has no influence on the output of non specific esterase positive cells or phagocytes in the thoracic duct lymph of tumour bearing rats.

4. Wistar rats had been shown to have an increased susceptibility to D.M.H. tumour induction when pre-treated with C. Parvum. (179) The significance of this observation is questioned in view of the very high dose levels of C. Parvum used in the experiment.

TABLE 3.1

M.P.S. cells in the thoracic duct lymph of rats with colonic tumours

	Day	1	2	3	4	5
% est + ve	n	9	9	9	9	9
	$\bar{x}$	1.3	2.2	2.7	2.16	2.87
	$\pm$SEM	0.15	0.42	0.28	0.26	0.41
Number of est + ve $\times 10^4$ cells/hour	n	9	9	9	9	9
	$\bar{x}$	18.47	13.98	19.37	9.8	9.78
	$\pm$SEM	2.68	2.45	2.91	1.92	2.3
Number of phagocytic cells on a 20 minute incubation $\times 10^4$ cells/hour	n	9	9	9	9	4
	$\bar{x}$	0.43	0.12	1.75	0.84	1.68
	$\pm$SEM	0.42	0.12	0.41	0.35	0.21
Number of phagocytic cells on a 40 minute incubation $\times 10^4$ cells/hour	n	9	9	9	9	4
	$\bar{x}$	0.96	1.13	1.09	0.89	1.55
	$\pm$SEM	0.63	0.55	0.42	0.38	0.32

TABLE 3.2

The effect of C. Parvum on M.P.S. cells in thoracic duct lymph of rats with colonic tumours
C. Parvum injected I.V. 7 - 14 days prior to the experiment

	Day	1	2	3	4	5
% est + ve	n	5	6	6	6	6
	$\bar{x}$	1.2	1.78	2.88	2.48	3.31
	$\pm$SEM	0.25	0.68	0.56	0.42	0.57
Number of est + ve $\times 10^4$ cells/hour	n	5	6	6	6	6
	$\bar{x}$	13.2	12.58	11.8	6.82	3.82
	$\pm$SEM	2.64	5.18	1.68	1.20	0.9
Number of phagocytic cells on a 20 minute incubation $\times 10^4$ cells/hour	n	5	6	6	6	6
	$\bar{x}$	1.2	0.9	0.5	0.3	0.27
	$\pm$SEM	1.2	0.58	0.31	0.19	0.09
Number of phagocytic cells on a 40 minute incubation $\times 10^4$ cells/hour	n	5	6	6	6	6
	$\bar{x}$	2.34	0.88	0.38	0.17	0.25
	$\pm$SEM	0.97	0.57	0.26	0.17	0.09

Figure 3.1

Percentage of esterase + ve cells in the thoracic duct lymph of tumour bearing rats

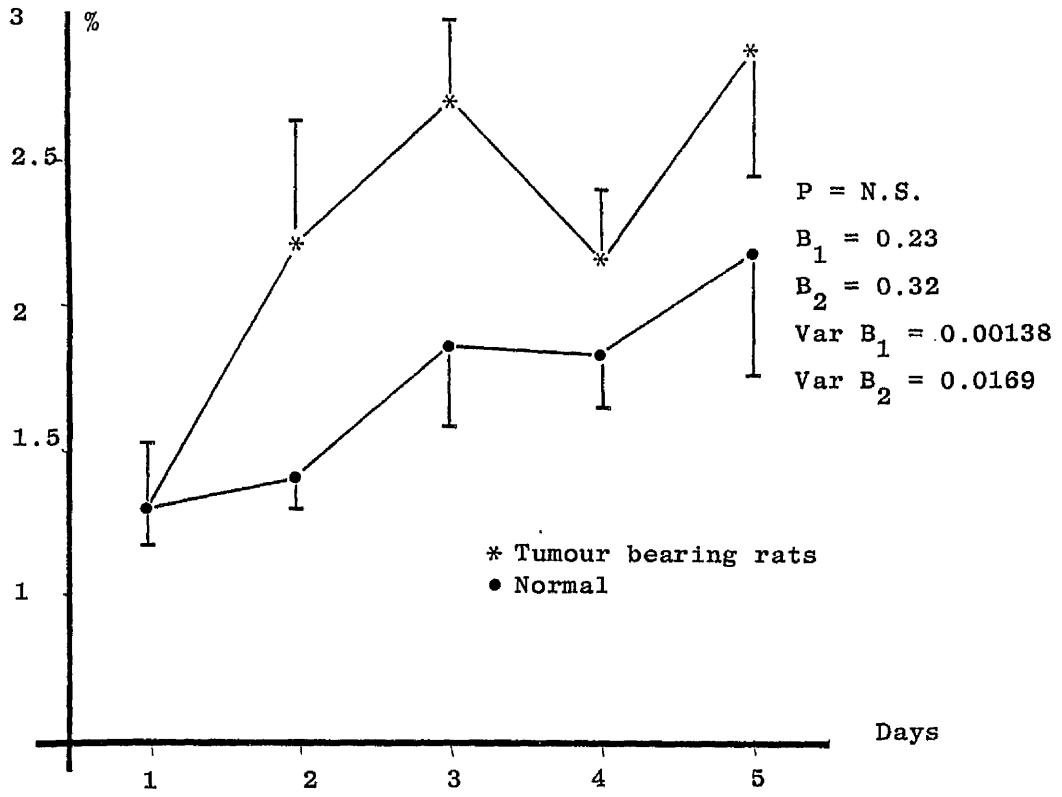
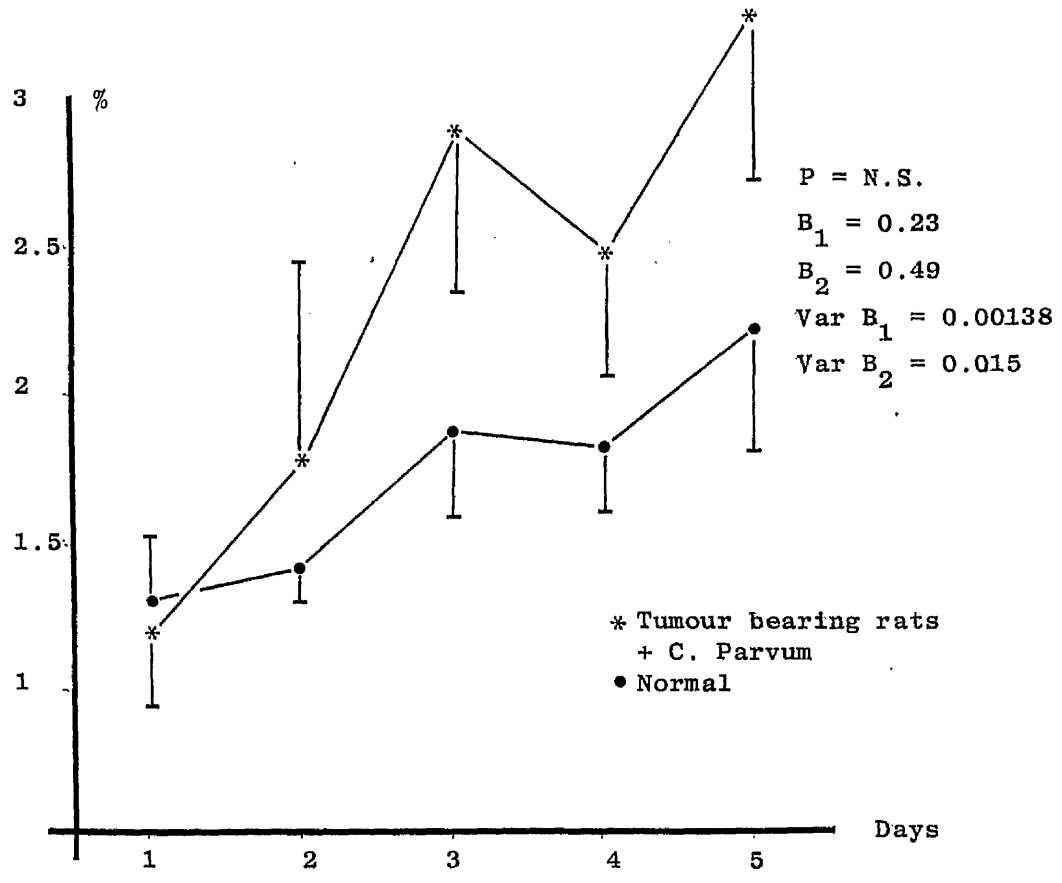


Figure 3.2



Percentage of esterase + ve cells in thoracic duct lymph of tumour bearing rats given C. Parvum 0.7 mg I.V.

Figure 3.3

No. of esterase + ye cells in the thoracic duct lymph of tumour bearing rats

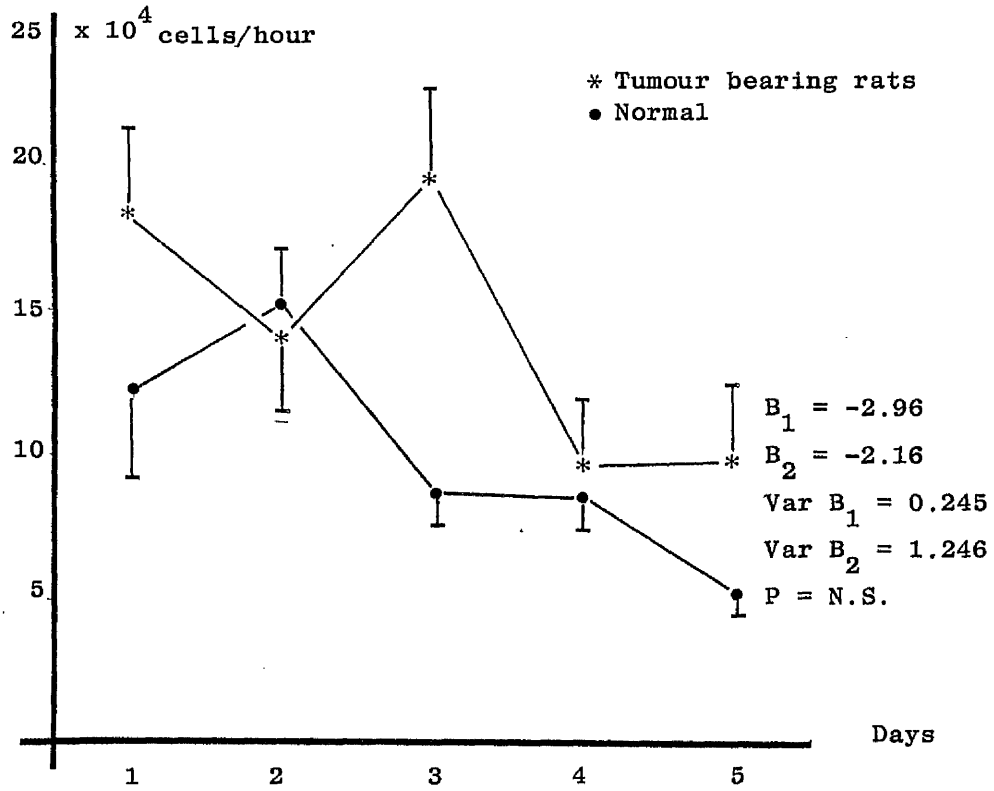


Figure 3.4

No. of esterase + ve cells in the thoracic duct lymph of tumour bearing rats given C. Parvum 0.7 mg I.V.

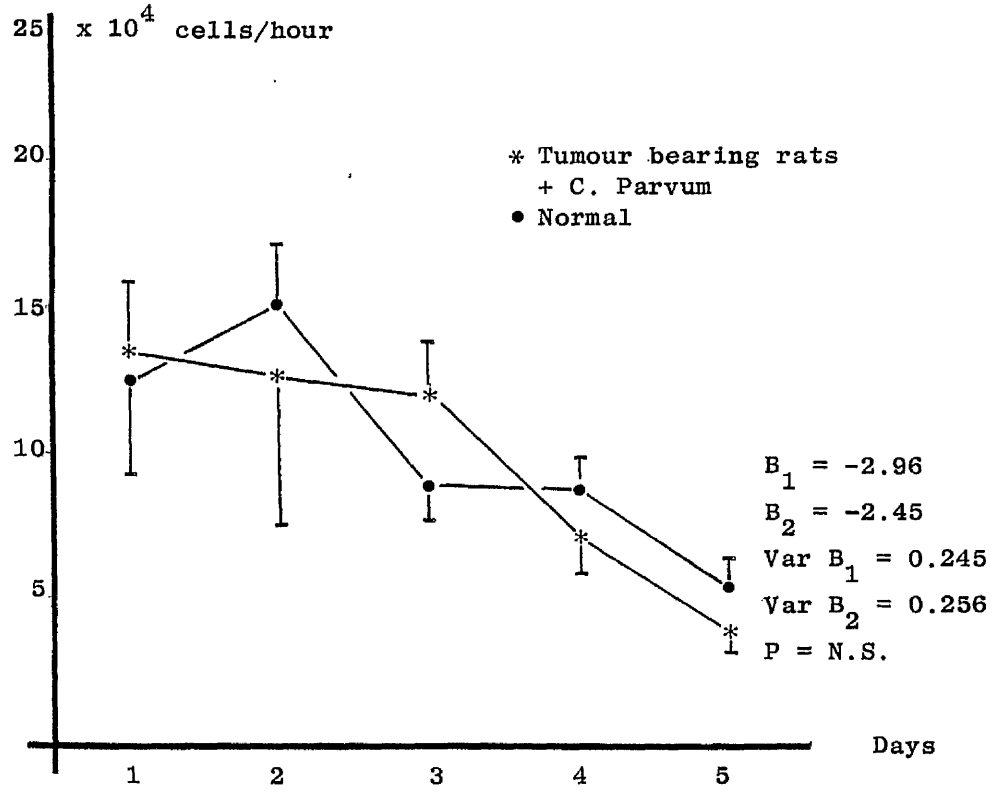


Figure 3.5

No. of phagocytic cells on a 20 minute incubation in tumour bearing rats

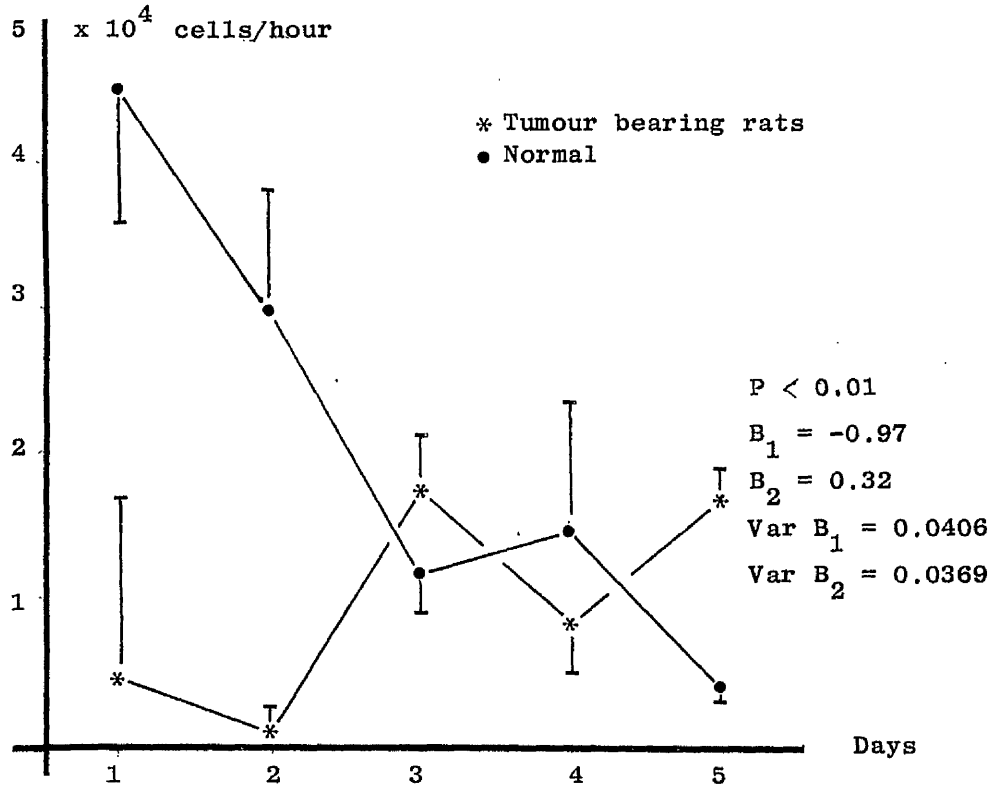


Figure 3.6

No. of phagocytic cells on a 40 minute incubation in tumour bearing rats

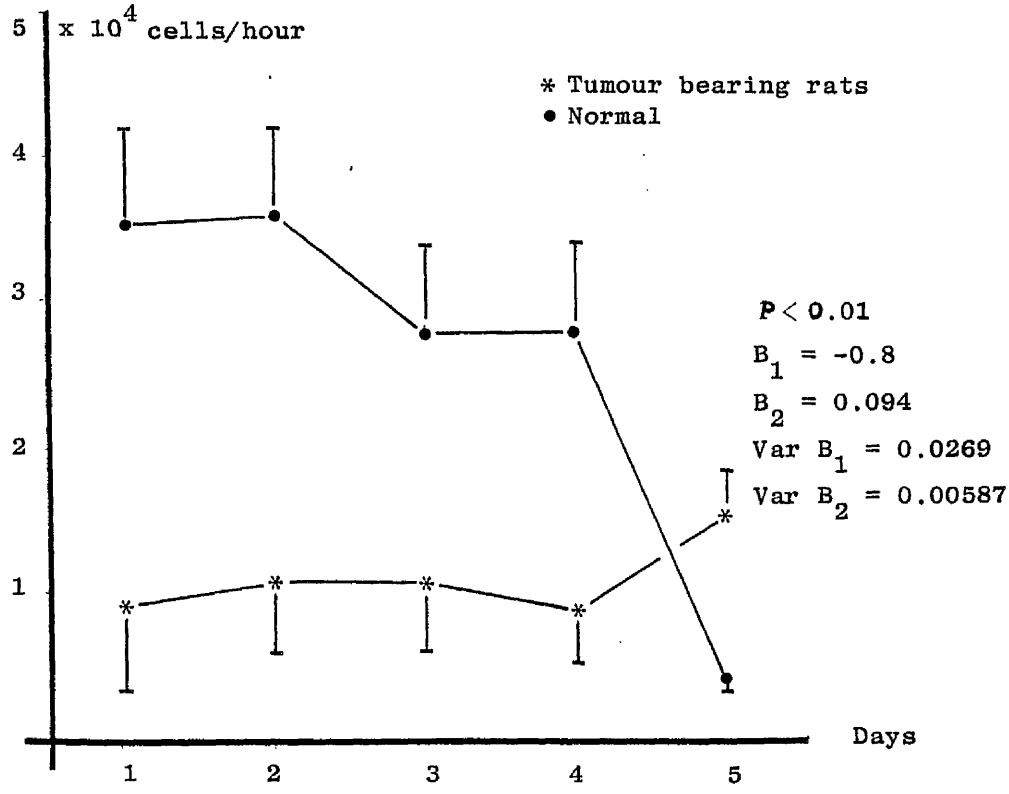


Figure 3.7

No. of phagocytic cells on a 20 minute incubation in tumour bearing rats given C. Parvum 0.7 mg I.V.

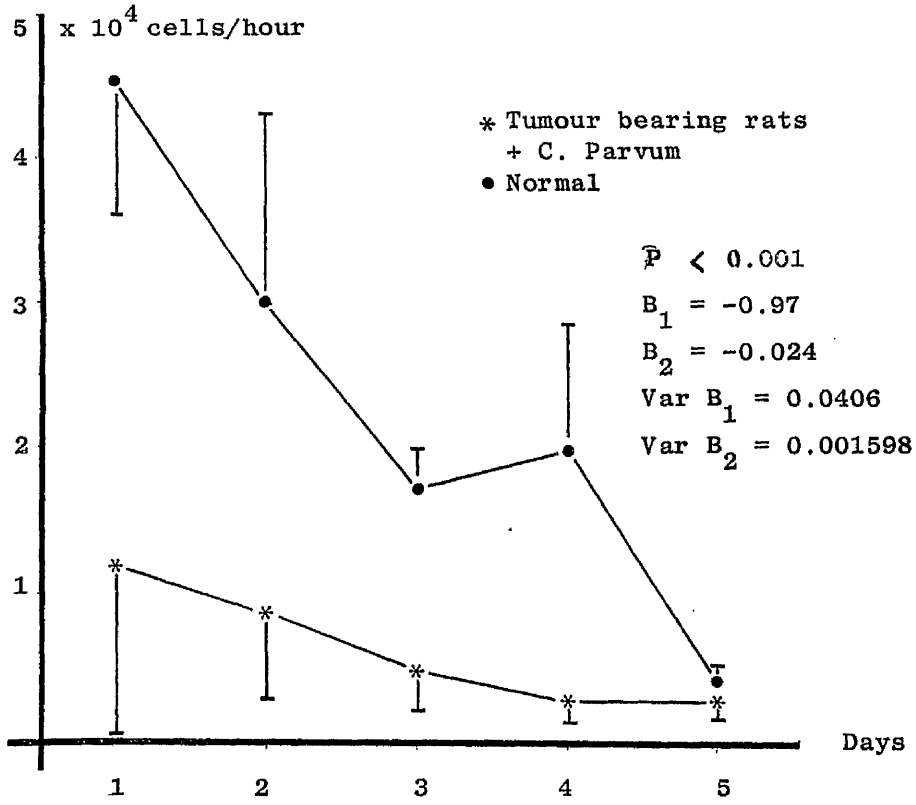
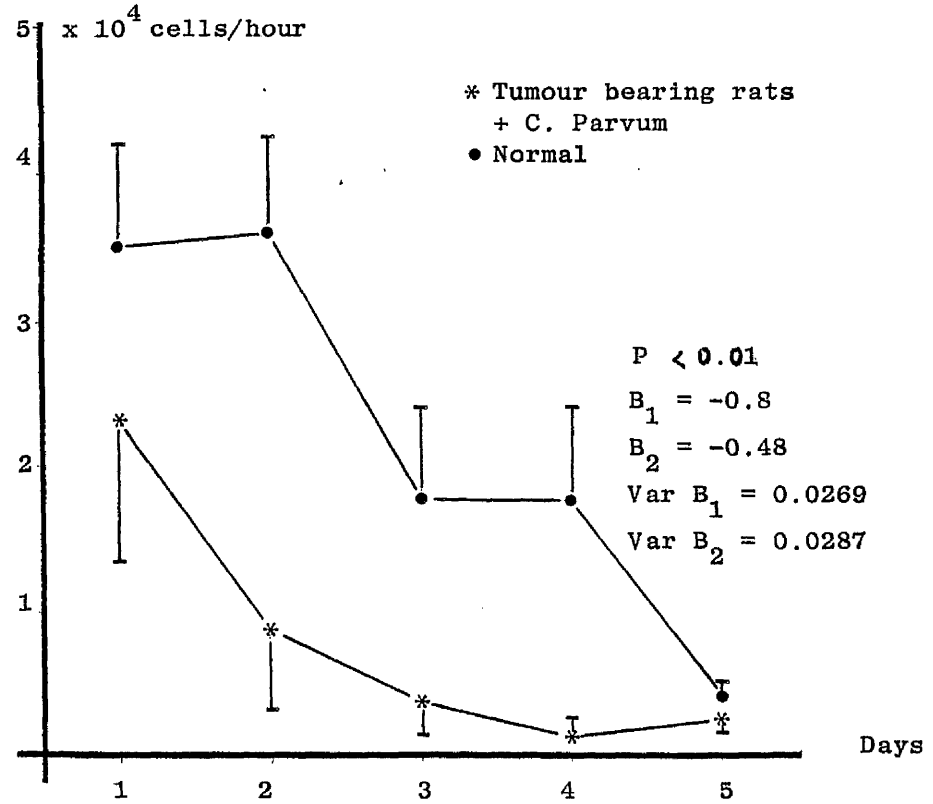


Figure 3.8

No. of phagocytic cells on a 40 minute incubation in tumour bearing rats given C. Parvum 0.7 mg I.V.



Chapter 4

The haematological effects of systemically administered C. Parvum

The effect of systemically administered C. Parvum on the various cell types of the blood varies widely both from species to species and between strains of the same species. Professor Halpern, commenting on this phenomenon at the International Conference on the effects of C. Parvum in experimental and clinical oncology, emphasized that the Mérieux strain of the bacterium appears to be more toxic and to have greater biological effects than the Wellcome strain. (186) Since the alterations in the blood of virgin female Wistar rats after a single intravenous dose of 0.7 mg of C. Parvum have not been reported it seemed important to establish these values. This was of particular interest when, as a result of the work described in Chapter 2, it became apparent that this dose of C. Parvum has no influence on the output of lymphocytes or M.P.S. cells in thoracic duct lymph.

Blood was collected after cutting off the tail tip of ether anaesthetised female Wistar rats weighing 150-200G. 0.5 mls of blood was collected in plastic bottles containing a small quantity of potassium ethylenediamine-tetracetic acid (Brunswick) to prevent clotting. After the initial blood collection, 0.7 mg of C. Parvum in 0.1 ml of thiomersal was suspended in 0.9 mls of saline (0.9% w/v) and injected into the dorsal tail vein of twenty experimental rats. Serial blood samples were taken, one hour, one day, one week and two weeks after the inoculation. At the end of the series of blood collections the rats were killed, then their spleens removed and weighed. C. Parvum induced stimulation of the reticuloendothelial system was confirmed by the presence of splenomegaly. After formalin fixation, paraffin sections

of these spleens stained with haematoxylyn and eosin were used in the studies described in Chapter 6.

A series of four control rats underwent the same procedure for collection of bloods but instead of C. Parvum, 1 ml of 0.9% w/v saline was injected into the dorsal tail vein.

The blood samples were processed in an automatic analyser (Coulter S plus). Differential white cell counts were performed on Leishman stained blood smears which were allocated a number, but gave no indication as to the source of the sample. This number corresponded with the number on the automatic print out from the Coulter counter enabling subsequent interpretation of the results. The statistical significance of the results was carried out using students t test.

Results

The total white cell count was not influenced by treatment with C. Parvum. (Table 4.1, figure 4.1) There was a considerable difference between the baseline, pre treatment value in the two groups. As the control group comprised only four rats this difference can be attributed to chance. However, the numerical values obtained for polymorphonuclear leucocytes and lymphocytes in the control group are influenced by the low total white cell count. It is probably of more value to study the trends in the figures rather than to compare the numerical values. In the statistical evaluation of the results the serial values were compared with the baseline values in each group.

C. Parvum treatment caused an immediate and highly significant increase in the count of polymorphonuclear leucocytes ($P < 0.001$) within an hour of treatment. (Table 4.1, figure 4.2) This level had returned to normal within 24 hours of treatment. In the control group no significant alterations occurred in the polymorph count.

Treatment with C. Parvum caused a highly significant suppression of lymphocyte numbers within an hour of injection ($P < 0.001$) and the following day ($P < 0.001$) (Table 4.1, figure 4.3) Subsequently lymphocyte numbers rose and by one week were just significantly higher than baseline values ($P < 0.05$). In the control group lymphocyte numbers were suppressed within an hour of the anaesthetic ($P < 0.01$) but thereafter were normal.

Both groups of rats showed similar marked falls in haemoglobin. (Table 4.1, figure 4.4) The C. Parvum treated group did not show a greater decrease than the control group. By the second week these levels had almost returned to normal.

No statistically significant alteration occurred in the number of monocytes in either group of rats. (Table 4.1)

Platelet counts were only performed in the treatment group. (Table 4.1, figure 4.5) A fall occurred within a day of treatment. Elevated levels were seen after one week followed by another fall at two weeks.

Discussion

In these studies the effects of repeated ether anaesthesia and loss of blood must be borne in mind. The control group of animals was small and was used to provide a basis for

the comparison of trends. The normal values which were obtained from the pre treatment results on the 20 treatment group rats are closely similar to published figures for specific pathogen free albino rats. (187)

Normal haematology values for virgin female Wistar rats weighing 150-200G are not available. The pre treatment figures were therefore taken to be the standard values for these rats.

Lymphocytopaenia after C. Parvum treatment has been reported in animals (188) and humans (25). The control group of rats in this study also sustained a fall in lymphocyte numbers immediately after ether anaesthesia. However, the magnitude and the duration of the fall was much less than in the treatment group. C. Parvum treatment as given in these experiments thus causes a significant decrease in the numbers of circulating lymphocytes. It is possible that these become redistributed to tissues, for example lymph nodes and spleen. (Ch. 6)

As was discussed in Chapter 2 the number of lymphocytes in the thoracic duct is not influenced by treatment with C. Parvum. Since lymphocytes in the thoracic duct and the blood are continuously recirculating it is difficult to understand why the low blood levels are not reflected in thoracic duct lymph. Confirmation that circulating blood lymphocytes are depleted by the loss of thoracic duct lymph was obtained by sampling venous blood after two days of drainage. A mean value of 1.8×10^9 lymphocytes/litre was obtained from three rats confirming that loss of thoracic duct lymphocytes results in lymphocytopaenia.

C. Parvum injection not surprisingly results in a rapid immediate rise in polymorph numbers. This is the anticipated response to bacterial challenge. Few reports of the

granulocyte response to C. Parvum are available. In human studies, Gill reported no alterations in these cells when studied at similar time intervals after intravenous C. Parvum in a dose of 5 mg/m^2. (96) This observation is somewhat surprising in view of the side effects suffered by these patients which had all the features of the release of pyrogens. Milas reported that the granulocyte count was not influenced by treatment with a variety of doses of intraperitoneal Corynebacterium Granulosum in mice. (188) However, in this series of experiments the polymorph count was not studied until three days after treatment. The immediate alterations noted in the present study would not have been seen by these workers. It must also be remembered that the rats received a higher dose weight for weight.

Anaemia has been reported after C. Parvum treatment in mice (168). It has been speculated that C. Parvum may stimulate the development of anti red blood cell antibodies which destroy autologous red cells in conjunction with activated phagocytes. The anaemia found in the present study can be attributed to the sequential loss of blood and the haemodiluting effect of the 1 ml inoculum, as the fall in each group was the same.

Monocyte levels in peripheral blood are low and usually they compromise less than 2% of white cells. This makes it difficult to be certain about a decrease in the number of these cells although an increase might be apparent. Monocytopaenia is said to occur after C. Parvum treatment, (106, 25), but no statistically significant alterations occurred in this study. There is more universal agreement that C. Parvum increases bone marrow macrophage colony formation. (19, 188, 189)

The triphasic response of platelets to C. Parvum is in agreement with the findings of Castro and his co-workers. (190) In their experiments high doses of C. Parvum (0.46 mg)

were given intraperitoneally to mice. These animals were acutely distressed for two hours after the inoculation. Subsequent histological changes in the liver, lungs and spleens of these animals were found which had the appearance of disseminated intravascular coagulation.

Systemic C. Parvum in humans causes a marked fall in the serum level of fibrinogen with an increase in fibrin degradation products. (190, 191) Thrombocytopaenia is also sometimes found. There has been some speculation that the anti metastatic effect of C. Parvum noted particularly in the Lewis lung carcinoma model, may be due to altered coagulation rather than immune stimulation. (190)

Conclusions

As a result of this study it has been shown that when 0.7 mg of C. Parvum is given intravenously to virgin female Wistar rats the following alterations occur in the blood.

There is an immediate lymphocytopaenia with a slight rebound increase by one week.

There is an immediate increase in the polymorph count, probably as a response to bacterial challenge. This rise is not sustained and there are no long term alterations.

The anaemia which resulted could not be attributed to C. Parvum treatment.

No firm conclusions can be drawn about the effect of C. Parvum on monocytes.

The platelet response was similar to another study where a much higher dose of Corynebacterium Parvum was used.

Figure 4.1

White cell count after intravenous C. Parvum 0.7 mg IV

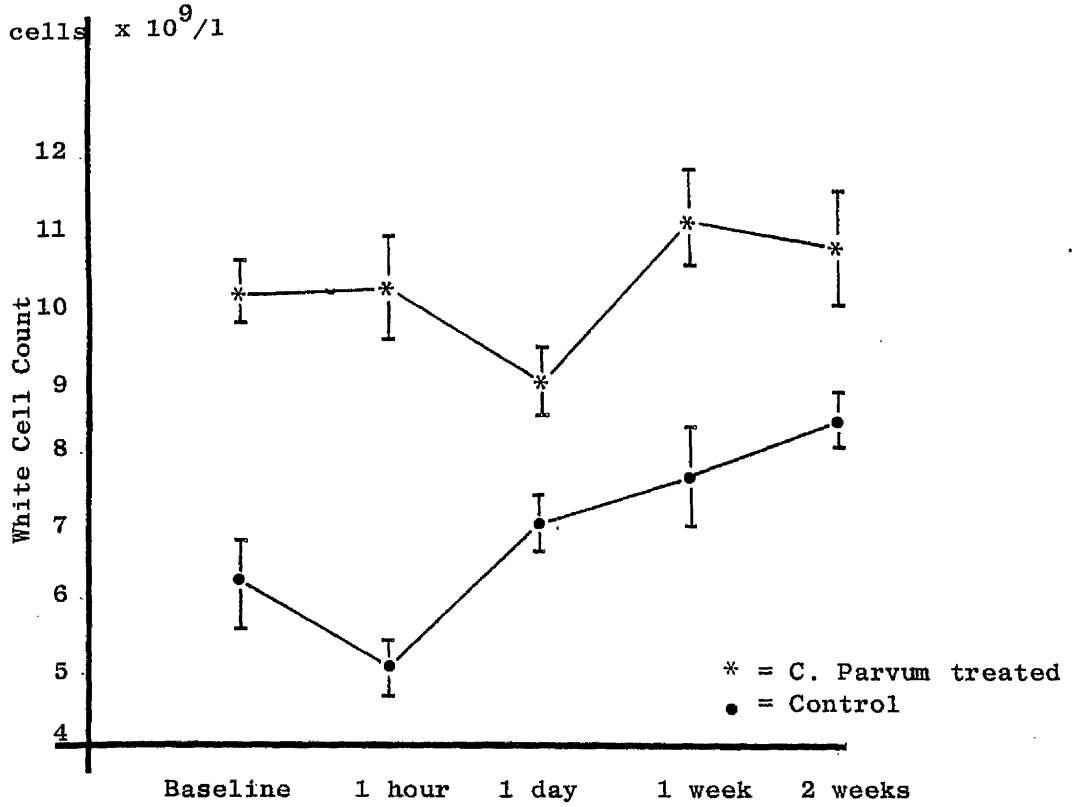


Figure 4.2

Polymorph count after intravenous C. Parvum 0.7 mg IV

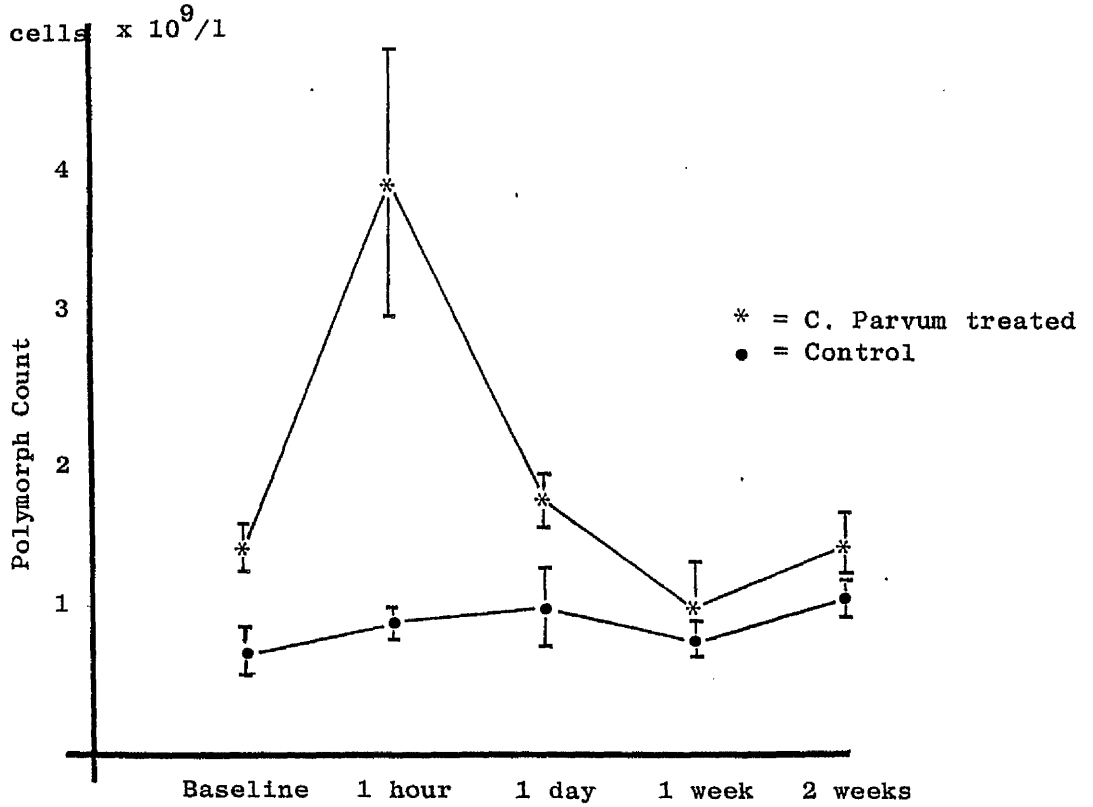


Figure 4.3

Lymphocyte count after intravenous C. Parvum 0.7 mg IV

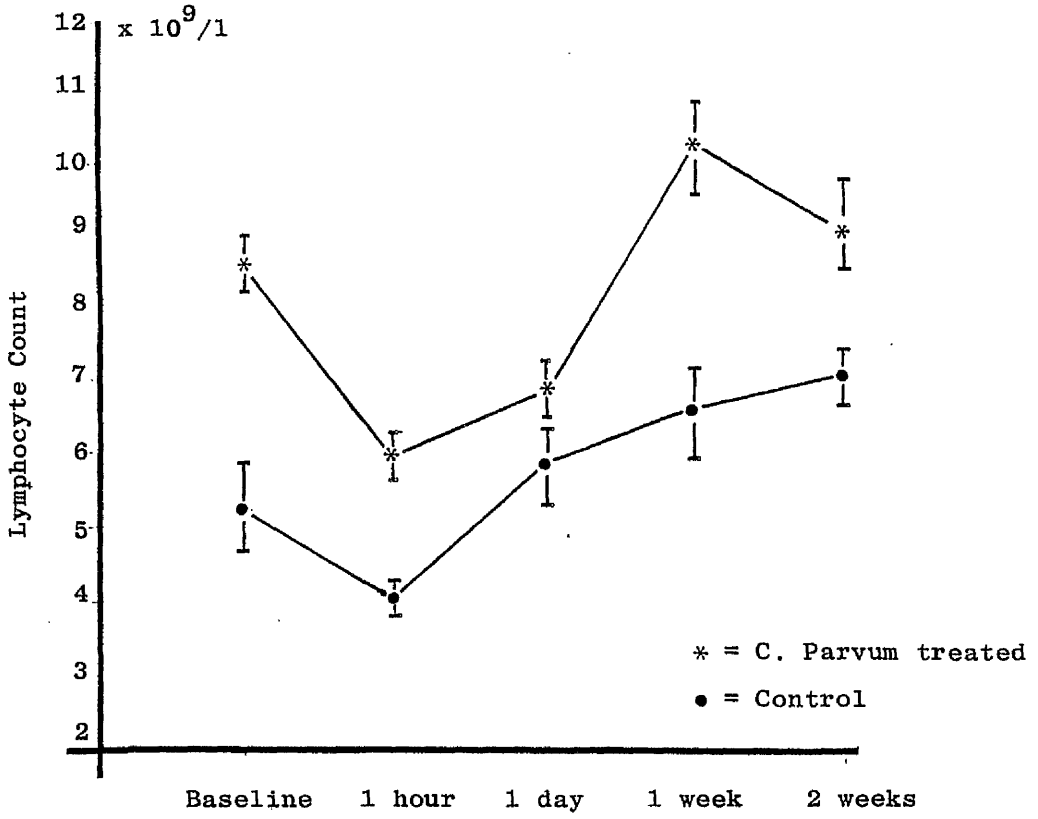


Figure 4.4

Haemoglobin changes after I.V. C. Parvum 0.7 mg I.V.

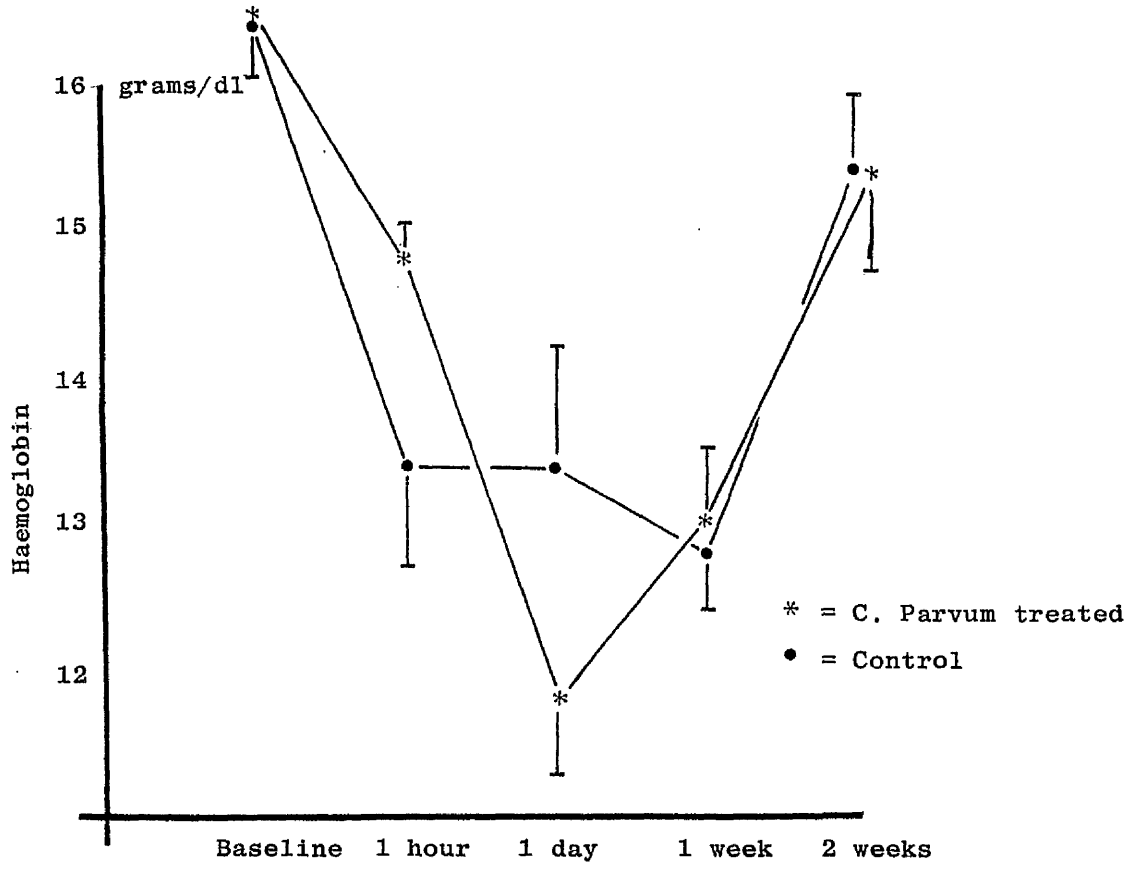
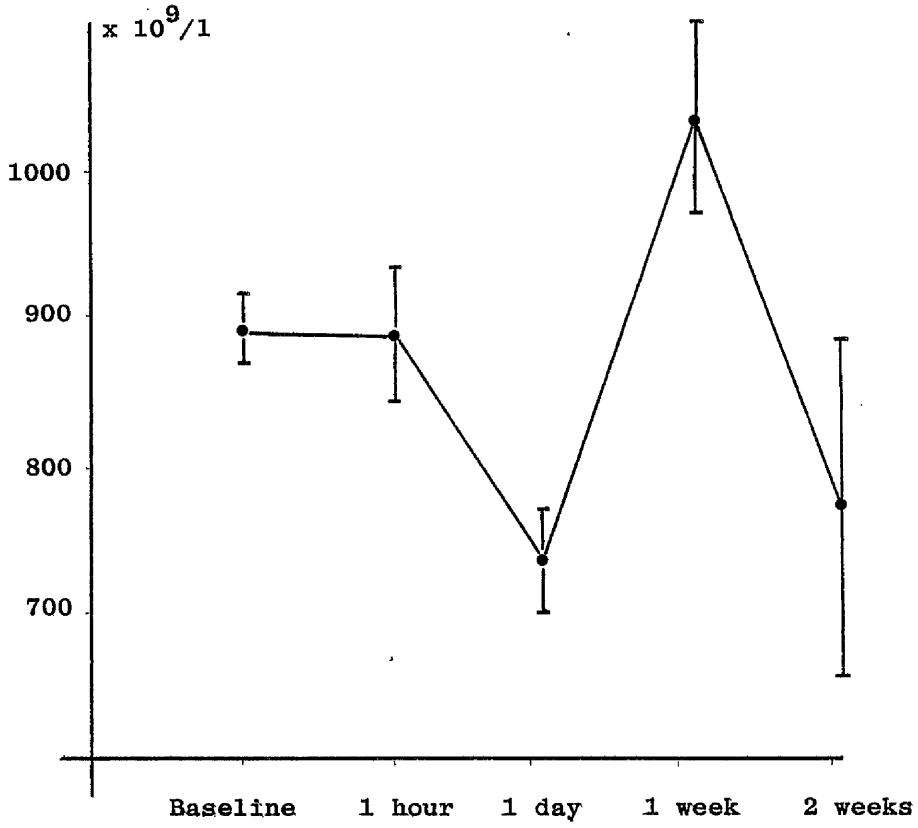


Figure 4.5

Platelet count after intravenous C. Parvum 0.7 mg IV



HAEMATOLOGICAL CHANGES IN SERIALLY BLED RATS

20 rats given C. Parvum 0.7 mg IV

	White Cell count		Red Cell count		Haemoglobin		Polymorphs		Lymphocytes		Monocytes	
	$\bar{x}$	SEM$^{+}_{-}$	$\bar{x}$	SEM$^{+}_{-}$	$\bar{x}$	SEM$^{+}_{-}$	$\bar{x}$	SEM$^{+}_{-}$	$\bar{x}$	SEM$^{+}_{-}$	$\bar{x}$	SEM$^{+}_{-}$
BASELINE	10.2	0.4	7.9	0.11	16.2	0.2	1.4	0.16	8.6	0.37	0.13	0.04
ONE HOUR POST	10.2	0.7	7.4	0.28	14.7	0.34	3.9	0.88	6.0	0.34	0.12	0.05
ONE DAY POST	9.0	0.49	7.4	0.27	14.7	0.38	1.7	0.16	6.9	0.4	0.2	0.07
ONE WEEK POST	11.1	0.65	7.1	0.15	14.4	0.24	0.73	0.1	10.2	0.66	0.12	0.04
TWO WEEKS POST	10.7	0.83	7.9	0.14	15.7	0.23	1.4	0.2	9.1	0.7	0.18	0.06

4 serially bled normal rats

BASELINE	6.25	0.6	8.88	0.05	16.25	0.12	0.74	0.15	5.3	0.58	0.16	0.06
ONE HOUR POST	5.03	0.39	8.24	0.08	15.4	0.07	0.94	0.04	4.09	0.2	0.11	0.04
ONE DAY POST	7.03	0.41	7.23	0.15	13.9	0.25	1.05	0.25	5.8	0.55	0.12	0.05
ONE WEEK POST	7.6	0.75	7.5	0.2	14.5	0.25	0.99	0.3	6.6	0.6	0.16	0.06
TWO WEEKS POST	8.45	0.34	7.87	0.13	15.7	0.29	1.08	0.16	7.18	0.38	0.18	0.12

Chapter 5

The influence of intravenous C. Parvum on cells in peritoneal exudates and splenic macerates.

C. Parvum treatment stimulates the anti tumour activity of peritoneal exudate cells (86, 192, 193, 194) and increases the uptake of particulate materials by the spleen. (2) In this section the effect of intravenously administered C. Parvum on M.P.S. cells in the peritoneal cavity has been studied. Activation of these cells could suppress the development of malignant cells in the peritoneal cavity, carrying particular implications for the use of I.V. C. Parvum in human gastro intestinal cancer.

The increased uptake of colloidal carbon after C. Parvum is attributed to an augmented fixed tissue macrophage population in the sinusoids of the liver and spleen. A series of experiments have been carried out to study whether the non fixed cell population of the spleen might also show increased phagocytosis after C. Parvum.

Peritoneal exudate cells (PEC) were harvested from ether anaesthetised female Wistar rats weighing 100-250G. 10 mls of 0.9% w/v sodium chloride solution containing 5 international units of heparin/ml was injected into the peritoneal cavity via the midline. After gently massaging the abdomen for five minutes the injected fluid was retrieved along with P.E.C. The abdomen was incised in the midline and the fluid aspirated with a siliconised glass pipette. No attempt was made to retrieve the entire inoculum and 5 mls was usually considered to be an adequate volume. Blood stained samples were discarded. After washing P.E.C. thrice a count was performed. Cytocentrifuge slides were prepared both from the washed cell sample and

from P.E.C. which had been used as the effector cells in the phagocytic assay with sensitised sheep red blood cells. Subsequently these slides were stained for non specific esterase reactivity and the percentage of cells staining for esterase and showing phagocytosis counted. From the original cell count the total number of esterase positive and phagocytic cells present per ml of the lavage fluid was calculated.

This study was performed in normal rats and in rats which had received 0.7 mg of C. Parvum I.V. one and two weeks previously.

In the same group of rats after peritoneal lavage had been performed the spleens and caecal lymph nodes were removed and weighed. The caecal lymph nodes lie separate from the rest of the mesenteric lymph chain and can easily be removed. 200 mg of each spleen was accurately weighed and subsequently macerated. This was carried out by forcing minced splenic tissue through a fine wire mesh into plastic petri dishes. A cell suspension was prepared by carrying this out repeatedly and rinsing the cells through the mesh with a fine spray of DAB/5%FCS from a plastic syringe. At the end of this procedure a small amount of tough tissue remains. It was assumed that this represents the fibrous stroma of the spleen. The spleen cell suspension was washed thrice and subsequently treated in the same way as the peritoneal lavage fluid.

Statistical comparisons were carried out with student's t test.

Results

The weights of the spleens and lymph nodes were adjusted to the weight of a 100 G rat. As was described in Chapter 1 this eliminates the influence of variations in the size of the animal. Stimulation of the reticuloendothelial system of these rats was confirmed by the increase in splenic weight after C. Parvum treatment. This became statistically significant by two weeks ($P < 0.01$) and showed an upward trend in the first week (Table 5.1, fig. 5.1) C. Parvum treatment did not influence the weight of the caecal lymph nodes.

Splenic macerates : (Table 5.1, fig. 5.2) Maceration of the spleen yielded a cell suspension containing low concentrations of both esterase positive cells and phagocytes. Mononuclear phagocytes showed a slight decrease a week after treatment but were significantly increased by two weeks. ($P < 0.001$) Esterase positive cells showed a similar initial downward trend followed by an increase after two weeks which did not attain statistical significance. Most of the cells, other than red corpuscles, seen in these preparations were large and small lymphocytes.

Peritoneal lavage : (Table 5.3, figs. 5.3 and 5.4)

A high percentage of P.E.C. had a positive staining reaction for non specific esterase. In untreated rats nearly 40% of P.E.C. were positive. Numerous large mast cells were seen in these samples, but not included in the cell counts. Large and small lymphocytes and polymorphonuclear leukocytes made up the remainder of cells. Phagocytic polymorphs were not included in the counting of phagocytes. They were clearly distinguished from M.P.S. cells by their segmented nuclei and bright pink cytoplasm. C. Parvum treatment resulted in a significant fall in the percentage of esterase positive cells within a week ($P < 0.001$) with a significant increase in this value after two weeks ($P < 0.01$).

Two weeks after C. Parvum treatment over 50% of P.E.C. were esterase positive. Phagocytic cells showed a similar trend of an early fall followed by a subsequent increase, although the figures are not statistically significant. Nearly a quarter of the P.E.C. obtained from untreated rats were phagocytic for sensitised sheep red blood cells confirming that this is an appropriate method of studying phagocytosis.

Discussion

In this series of experiments the splenic weight increase confirmed that reticuloendothelial stimulation had occurred. This dose of C. Parvum given by the intravenous route did not affect the weight of the caecal lymph nodes. Other workers have reported lymph node hypertrophy after systemic C. Parvum. In male CBA mice injected with 1.4 mg of C. Parvum I.P. the mean weight of the pooled axillary and brachial lymph nodes was increased, particularly after one week. (3) C. Granulosum had a similar effect on the weight increase of the inguinal nodes in mice. (188) The dosage of C. Parvum employed by these workers was much greater than that used in the present studies. Other workers, again using mice, have found the response of lymph nodes to C. Parvum to be highly dependent on the dose employed and the route of the injection. (168) No reference has been found on the effects of intravenous C. Parvum on lymph nodes draining the gut. As will be described in Chapter 6, these nodes do show histological changes consistent with a response to C. Parvum.

I.P. C. Parvum results in a 7 to 10 fold increase in the cell yield after peritoneal lavage. (192) 85% of these appear to be M.P.S. cells based on morphological criteria and non specific esterase staining. C. Parvum stimulated PEC show an enhanced tumour cytotoxicity both in vitro (192, 86 194) and in vivo (193). The cells exerting

this effect adhere to glass and intimate cell to cell contact is required. (72) Since the cytotoxicity is present in T cell deficient mice (64) and is impaired by agents selectively toxic to macrophages like silica (195) carrageenan (196) and gold salts, (197) it is probably mediated by M.P.S. cells. C. Parvum stimulated PEC when inoculated with methylcholanthrene induced fibrosarcoma cells caused significant delay in subsequent tumour development. (193) This action was inhibited by adding inert plastic microspheres to the inoculum which may have interfered with PEC - tumour cell contact. The cytotoxicity of spleen cell suspensions was not enhanced by C. Parvum treatment in the same group of experiments. These suspensions were prepared in the same manner as in the present study. If the tumour cytotoxic effect was entirely mediated by M.P.S. cells as these workers suggest their results would be consistent with the present observations. Although C. Parvum treatment does influence the numbers of M.P.S. cells present in spleen cell suspensions their actual number is small. It is probable that insufficient quantities would be obtained to show in vitro tumour cytotoxicity.

The increased phagocytosis seen after C. Parvum treatment has usually been measured by the rate of disappearance of intravenously injected substances, like colloidal carbon. Whole body irradiation given prior to, but not after C. Parvum treatment prevents this increase in phagocytosis. (198) This has led Woodruff to conclude that the cells responsible for the increase in phagocytosis after C. Parvum treatment are mature fixed tissue macrophages. These mature macrophages are relatively insensitive to radiotherapy unlike newly formed M.P.S. cells. Provided the C. Parvum treatment is given four days or more prior to irradiation the newly formed M.P.S. cells from the bone marrow are radio resistant.

The phagocytic activity of PEC is probably not measured by the carbon clearance test. No reference has been found on the direct measurement of the phagocytic capacity of these cells after I.V. C. Parvum.

I.V. C. Parvum causes an initial fall in the esterase positive and phagocytic cell populations in both peritoneal lavage samples and splenic macerates. Subsequently these cells are present in increased numbers. Although not all of the values achieve statistical significance it is noteworthy that the trend is the same in each population studied. The initial suppression followed by a rebound increase is very similar to the observations made by Gill on the esterase positive cell population in human peripheral blood after intravenous C. Parvum. (25)

Intravenous C. Parvum causes a marked increase in the percentage of esterase positive cells in peritoneal exudates. However, the value of just over 50% does not approach the 85% level seen after I.P. C. Parvum (193) I.P. C. Parvum may act as a chronic inflammatory stimulus rather than an immune stimulant. Intrapleural C. Parvum has been successfully employed in the treatment of human malignant pleural effusions. (T.J. Priestman : personal communication). Used in this way the treatment is probably no more specific than the time honoured procedure of instilling talcum powder to cause an inflammatory reaction leading to subsequent fusion by fibrosis of the parietal and visceral pleura.

As a result of the observations made in this study it is concluded that:-

1. C. Parvum causes a significant increase in the percentage of esterase positive cells in peritoneal exudates after an initial fall.

2. Non fixed tissue phagocytes in the spleen are increased as a result of C. Parvum treatment, but also show an initial fall. It is unlikely that these non fixed phagocytes are present in sufficient numbers to make a significant contribution to the increased splenic phagocytosis previously reported.

3. Gut associated lymphoid tissue does not hypertrophy after the dose of C. Parvum employed in this study.

TABLE 5.1

The effect of C. Parvum on spleen and caecal lymph node weight

		Rat weight grams	$\frac{\text{Spleen weight}}{\text{Rat weight grams}} \times 100$	$\frac{\text{Node weight}}{\text{Rat weight milligrams}} \times 100$
Normal Rats n = 11	$\bar{x} \pm$ SEM	104.8 ± 6.5	0.22 ± 0.01	1.68 ± 0.74
C. Parvum 0.7 mg IV x one week n = 12	$\bar{x} \pm$ SEM	213 ± 66	0.3 ± 0.02	1.74 ± 0.9
C. Parvum 0.7 mg IV x two weeks n = 6	$\bar{x} \pm$ SEM	269 ± 10.7	0.42 ± 0.04*	1.59 ± 0.7

* $P < 0.01$

TABLE 5.2

The effect of C. Parvum on splenic macerate cells

		Cell count of macerate suspension	% esterase positive cells	Number of esterase positive cells	% phagocytic cells	Number of phagocytic cells
		x 10^5 cells/ml		x 10^5 cells /ml		x 10^5 cells/ml
Normal rats n = 11	$\bar{x} \pm$SEM	120 ± 1.87	1.2±0.12	1.5±0.3	1.5 ± 0.18	1.9 ± 0.42
C. Parvum 0.7 mg IV x one week n = 12	$\bar{x} \pm$SEM	112 ± 10.1	0.9±0.12	1.0±0.12	1.0 ± 0.2*	1.2 ± 0.26
C. Parvum 0.7 mg IV x two weeks n = 6	$\bar{x} \pm$SEM	138.6±13.1	1.1±0.33	1.8±0.65	2.6 ± 0.2***	3.8 ± 0.69**

* P < 0.05
** P < 0.02
*** P < 0.01

FIGURE 5.1

The effect of 0.7 mg C. Parvum IV on the weight of the spleen and caecal lymph nodes

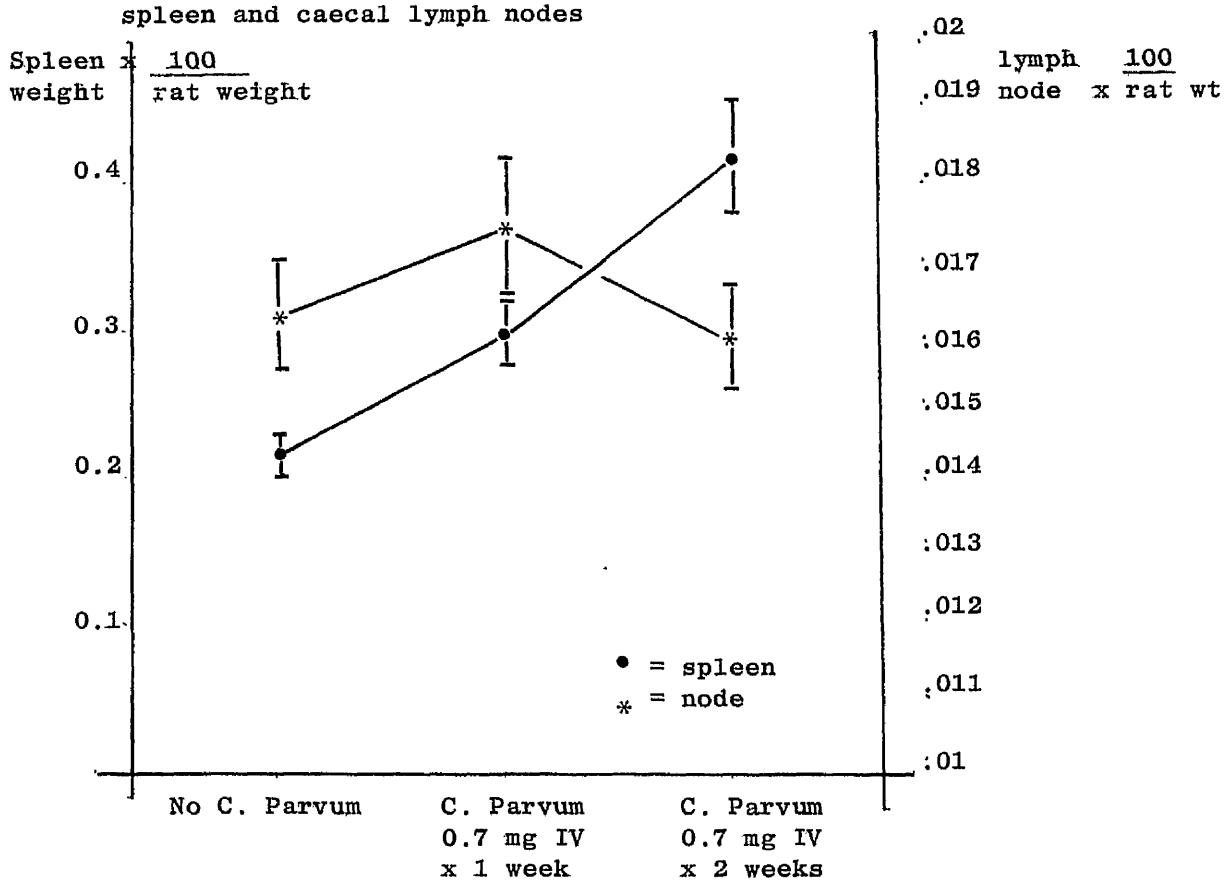


FIGURE 5.2

The effect of 0.7 mg C. Parvum on phagocytes in spleen cell suspensions

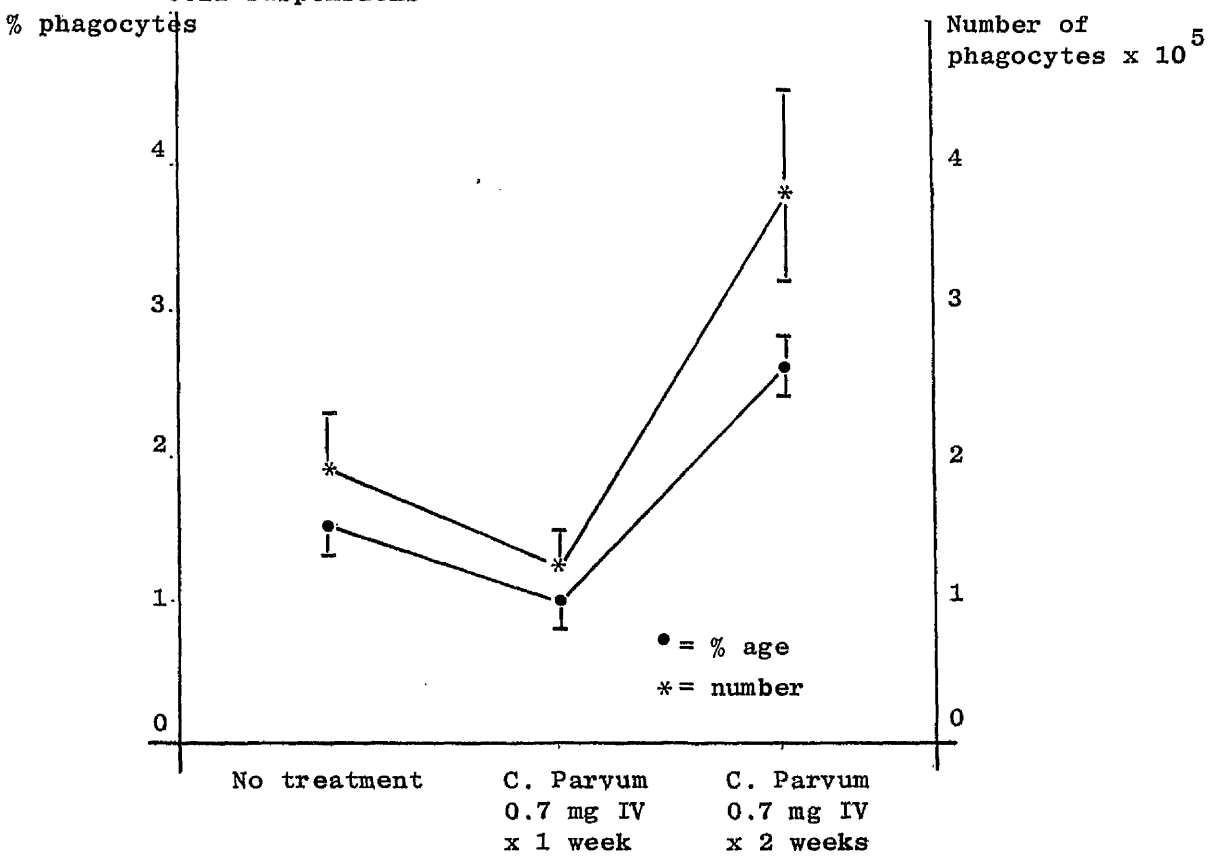


TABLE 5.3

The effect of C. Parvum on peritoneal exudate cells

		Cell count of lavage fluid x 10^5 cells/ml	% esterase positive	Number esterase positive x 10^5 cells/ml	% phagocytes	Number of phagocytes x 10^5 cells/ml
Normal rats						
n = 8	$\bar{x} \pm$ SEM	7.3 ± 1.08	38.8 ± 2.3	2.8 ± 0.45	23.8 ± 2.53	1.5 ± 0.06
C. Parvum 0.7 mg IV x one week						
n = 12	$\bar{x} \pm$ SEM	7.1 ± 0.55	21.7 ± 2.6***	1.4 ± 0.29*	18.1 ± 2.7	1.3 ± 0.4
C. Parvum 0.7 mg IV x two weeks						
n = 6	$\bar{x} \pm$ SEM	7.8 ± 0.49	50.4 ± 4.2**	3.9 ± 0.4	23.6 ± 4.3	1.9 ± 0.4

* $P < 0.02$
** $P < 0.01$
*** $P < 0.001$

Figure 5.3

The effect of C. Parvum 0.7 mg IV on esterase positive PEC

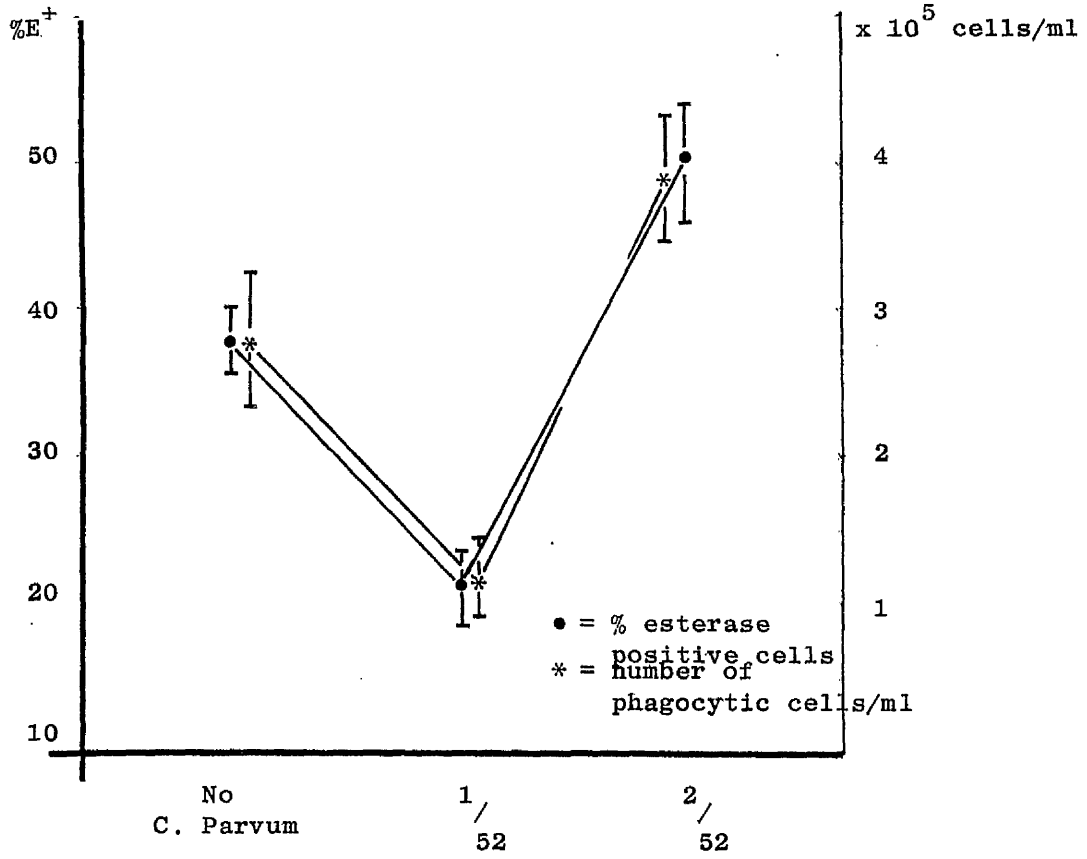
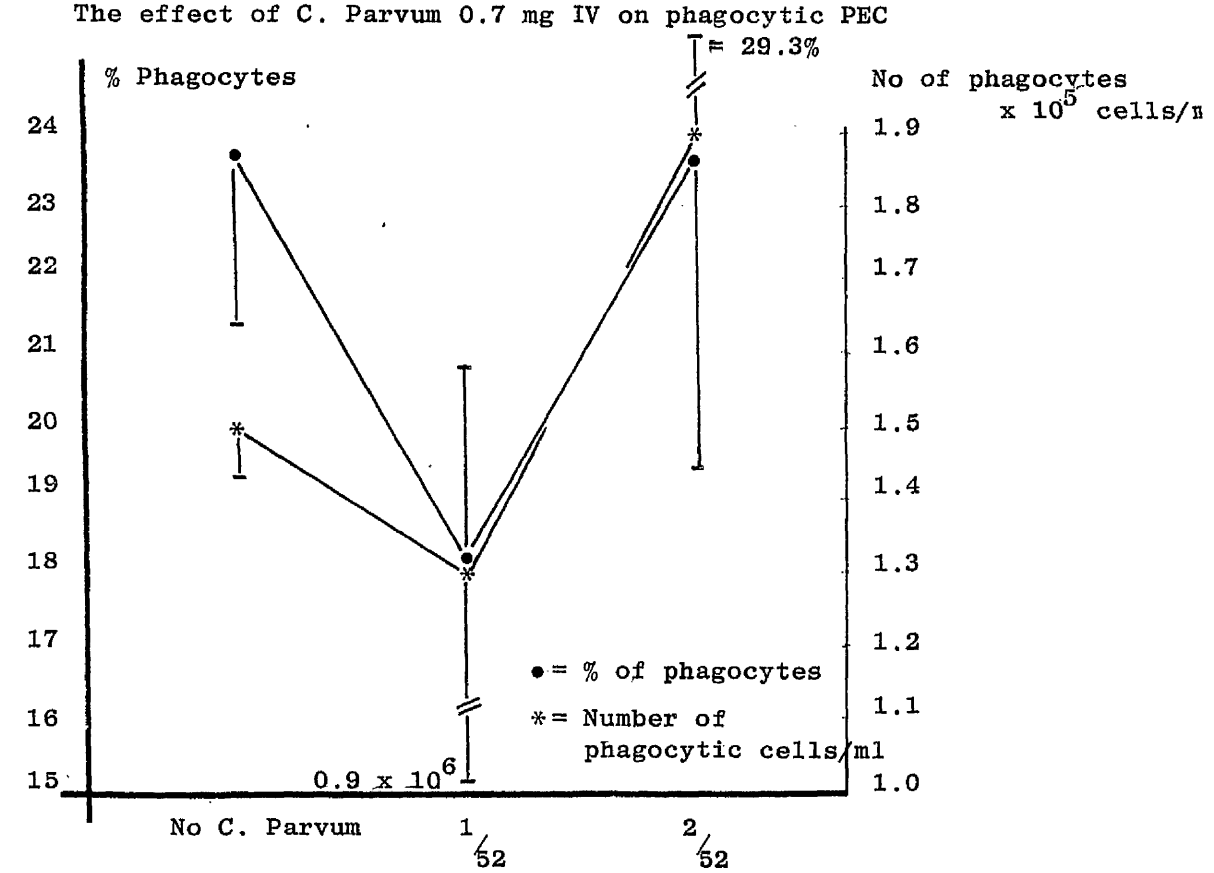


Figure 5.4

The effect of C. Parvum 0.7 mg IV on phagocytic PEC



Chapter 6

Histological alterations after C. Parvum treatment

Tissue sections have been studied to compare the histological changes caused by the dose and route of injection of C. Parvum employed in this study with those noted by other workers. This has been principally assessed by noting structural alterations in haematoxylin and eosin stained sections. Acid hydrolase stains have also been used to see if there were any alterations in the numbers and the distribution of positive staining cells.

Treated rats either received 0.7 mg of C. Parvum in 0.1 ml of 0.01% thiomersal preservative made up to 1 ml with 0.9% w/v saline for intravenous injection, or 0.7 mg of C. Parvum in 0.1 ml of 0.01% thiomersal injected directly into a lacteal. Inoculation was performed during the two weeks preceeding the study. Sections were taken from the spleen, liver, caecal lymph node and the gut. These specimens were immediately flash frozen in liquid nitrogen and cryostat sections (Slee Medical Equipment, London) of between 4 and 6 microns thickness cut by an experienced technician. The slides were stained using the following methods:

Haematoxylin and Eosin (H & E) : The slide was immersed in Carnoy's fixative for two minutes, rinsed in distilled water and placed in Bullard's haematoxylin solution for a further two minutes. After another wash in distilled water the slide was immersed in a 1% solution of acid alcohol for 30 seconds, then washed again until it appeared blue in colour. After 10 seconds in eosin solution the slide was dehydrated in graduated concentrations of up to 100% alcohol prior to mounting with DPX.

Acid Phosphatase: This was carried out by a simultaneous capture-coupling reaction and was done using a reagent pack (Sigma chemicals, Poole), designed for human leukocyte acid phosphatase. 2 mls of the substrate solution naphthol AS - BI phosphoric acid was added, with fast garnet crystals, to 46 mls of distilled water and 2 mls of acetate solution. After filtering the mixture the staining reaction proceeded in a dark oven at 37°C for twenty minutes. The section was counterstained with acid haematoxylin for 5 minutes to stain the nuclei. After washing in distilled water the slides were air dried. A water soluble mountant was used before applying a cover slip.

β Glucuronidase: A stock substrate solution was made up by dissolving 28 mg of naphthol - AS - BI Glucuronic acid in 1.2 ml of 0.05 M Sodium hydrogen carbonate. The volume was adjusted to 100 ml with 0.2 M acetate buffer. 10 mls of the substrate solution were added to 0.6 ml of hexazotised pararosaniline prepared as for the non specific esterase stain. The pH of the resulting staining solution was adjusted to 5.2 with 0.1 M NaOH and the final volume was made up to 20 mls with distilled water. The staining solution was filtered and applied to the slides which were fixed in formalin vapour for 30 seconds. Staining took about 30 minutes and was carried out at 37°C in a dark oven. Finally the sections were washed, counterstained with Giemsa and then mounted with DPX.

Non specific esterase: The method used was the same as has been described for the staining of cytocentrifuge preparations (Chapter 2). Before staining, the tissue sections were fixed in formalin vapour for 1 minute.

The area measurements of the splenic white pulp were calculated with the aid of a computer linked microscope (MOP AM03). After placing the slide under the microscope the image was projected onto a metal grid surface. Using an electronic pen, linked to the computer, a line was drawn round the perimeter of the area to be measured. A value for this area was then computed and a typed print out obtained. The statistical significance of the observations was calculated from the standard error of the difference of their respective means.

Results

Splenomegaly was present in all rats which had received C. Parvum, whether by the I.V. or the lacteal route. This was taken to confirm that reticuloendothelial stimulation had taken place.

As the lacteal inoculum of C. Parvum reached the caecal lymph node it immediately distended and blanched. When the node was removed between 7 and 14 days later it was found to be considerably enlarged and surrounded by inflammatory adhesions. C. Parvum is provided as a suspension of the killed organism in thiomersal 0.01% as a preservative. As a control experiment, 0.1 ml of 0.01% thiomersal was injected into the lacteal. No acute alterations occurred in the node, which appeared macroscopically normal when removed between 7 and 14 days later. Splenomegaly did not occur in the control rats. Direct injection of C. Parvum into the caecal lymph node caused a considerable increase in the weight of the node. (Table 6.1) Intravenous C. Parvum caused no alterations in the node weight but histological changes were seen. Marked expansion of the sinus was noted both one and two

weeks after treatment. (Plates 6.1) There were also increased numbers of developing follicle centres in the cortex. (Plate 6.2)

After direct intralacteal C. Parvum the node suffered considerable disruption. Large areas showed complete architectural destruction with necrosis. (Plate 6.3) The residual lymphoid tissue around the central zone of necrosis contained granulomas and giant cells. None of these features were present in the control group of rats.

The spleen showed considerable expansion of both the red and white pulps. (Plates 6.4 and 6.5) A comparison of the mean areas of the white pulp in normal and treated rats has permitted a statistical comparison to be made. (Table 6.2) Both I.V. and I.P. C. Parvum caused a highly significant increase ($P < 0.001$) in the area of the white pulp compared to normal. In addition the I.V. C. Parvum induced increase was statistically greater than the I.P. increase. ($P < 0.025$)

These measurements were only made on the white pulp but the red pulp appeared to show a similar size increase.

C. Parvum treatment resulted in considerable expansion of the lymphoid follicles. Large perivascular cuffs of pale staining macrophages were seen. Numerous developing granulomata were present. (Plate 6.6) By three weeks after the injection there were numerous vacuolated cells throughout the red pulp having the appearance of macrophages and the overall picture was that of a brisk reactive process.

Cells showing a positive reaction for acid hydrolase stains were virtually restricted to the splenic red pulp,

(Plates 6.7, 6.8 and 6.9). Acid phosphatase stains showed some activity in the mantle zone of the white pulp. The density of staining and the number of positive staining cells did appear to be increased by C. Parvum treatment, but no quantitation of this response has been attempted. The increase appeared to be in proportion to the increased size of the red pulp.

When these staining methods were used on cryostat sections of the liver extensive positive staining was obtained. In the gut a few positive cells were found at the bases of the crypts of Lieberkuhn. Scattered positive staining was found in the lymph nodes. In none of these tissues was it apparent that C. Parvum treatment had influenced the extent of the staining reaction.

Discussion

In Professor Halpern's original report of the increased phagocytosis resulting from C. Parvum treatment the changes in histology were also described. (2) This and subsequent studies describing histological alterations were performed in mice. (3, 168, 188) In Halpern's study the mice were injected repeatedly with C. Parvum at a dose of 255 µg. An increase was reported in cells which ingested carbon in both the liver and the spleen. Numerous granulomata were found in both of these organs. Although the liver and spleens continued to increase in weight throughout the eight injections the increased phagocytic capacity began to fall after the fourth injection.

The increased weight of the caecal nodes noted after intralacteal C. Parvum may have been largely due to

surrounding inflammatory tissue. When these nodes were excised they were densely adherent to surrounding structures making accurate dissection difficult. As can be seen from Plate 6.3 there was no evidence that this form of treatment had any stimulatory effect on the draining node. In fact after treatment it was difficult to identify normal functioning lymphoid tissue.

Increased activity in the germinal centres of the lymph nodes has also been reported by Milas. (188) In his study Corynebacterium Granulosum was given in high doses. This bacterium is closely related to C. Parvum and its systemic use results in similar stimulation of the reticuloendothelial system. Marked depletion of the cell content in the splenic white pulp resulted from this treatment which became most marked after two weeks. However, the dose employed was as high as 0.5 mg and the most extreme depletion resulted from repeated injections. The lower doses of C. Parvum employed by McBride and his co-workers resulted in histological changes in the spleen which were very similar to the present study. (168) Using 70 μg - 240 μg I.P. in mice they found expansion of both the red and white pulps of the spleen. The acid phosphatase activity of glass adherent spleen cells showed a threefold increase over that of controls.

It seems probable that low doses of C. Parvum stimulate lymphocytes while higher doses are toxic.

The dose of C. Parvum employed in this study does appear to have caused major alterations in not only the size but the cell population of lymphoid organs. No alterations were seen in the sections taken from the large bowel and liver. No granulomata were found in the liver

but the dose used in this study was considerably lower than Halpern's. Direct inoculation of C. Parvum into a lymph node has not been previously reported. The dramatic destruction of the lymph node which results may indicate that some acute hypersensitivity reaction occurs. Animals and humans (87) show background anti C. Parvum antibody titres which suggests that prior exposure to the organism is common. It seems probable that the splenomegaly which resulted from this treatment was the result of the injected C. Parvum gaining access to the general circulation rather than local processing in the lymph node.

It had been proposed to examine the dimethylhydrazine induced colonic cancers using these staining techniques. However, some tumour cells take up non specific esterase stains and the assessment of tumour macrophage populations on the basis of this stain reaction could be misleading. (199)

Whether acid hydrolase and non specific esterase stains can act as markers for M.P.S. cells is open to question. Other cells, for example polymorphonuclear leukocytes can show positive staining reactions. Although these can be distinguished on morphological grounds it is likely that much improved specificity of staining will result from the use of monoclonal antibodies.

Conclusions

1. Systemic C. Parvum results in considerable expansion of both the red and white pulps of the spleen. There is evidence to suggest that M.P.S. cells are increased in number.

2. Systemic C. Parvum causes sinus hyperplasia and increased activity in the germinal centres of lymph nodes.

3. Intralacteal C. Parvum is directly toxic to the draining lymph node and subsequently exerts a systemic action.

4. It was not possible to quantitate C. Parvum induced alterations in tissue sections using acid hydrolase stains.

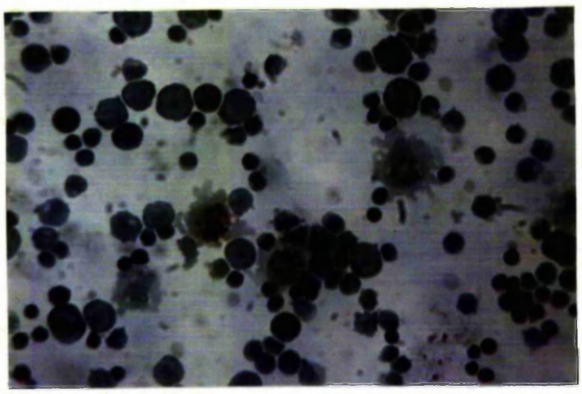


Plate 1 : Cytocentrifuge preparation of thoracic duct lymph x 40 Giemsa. Large and small lymphocytes, MPS cells showing processes and cytoplasmic staining for N.S.E..

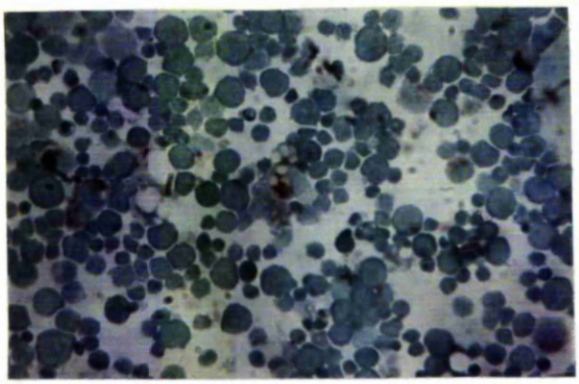


Plate 2 : Cytocentrifuge preparation of thoracic duct lymph after phagocytosis of sheep red blood cells. Note; one phagocytic cell shows cytoplasmic staining for N.S.E. x 40 Giemsa.

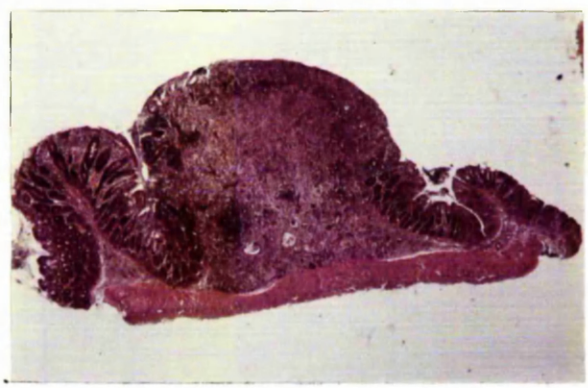


Plate 3 ; Carcinoma of rat colon x 10 H & E. The typical appearance of a DMH induced colonic cancer.

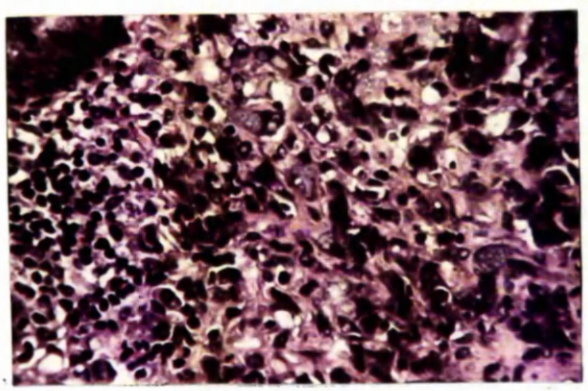


Plate 4 : Carcinoma of rat colon x 40 H & E. Pleomorphic cells hyper-chromatic nuclei, signet ring cells and abnormal mucin deposition.

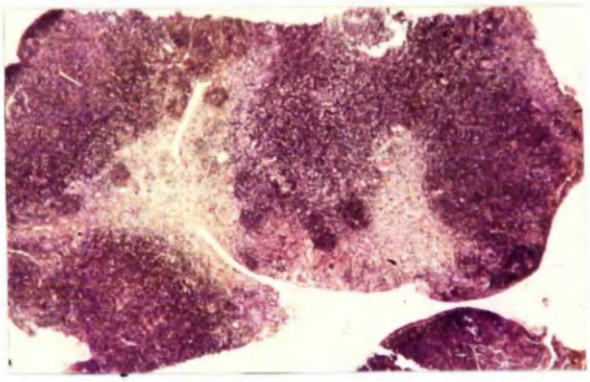


Plate 6.1 : Caecal lymph node x 12 H & E. Sinus hyperplasia after I.V. C. Parvum.

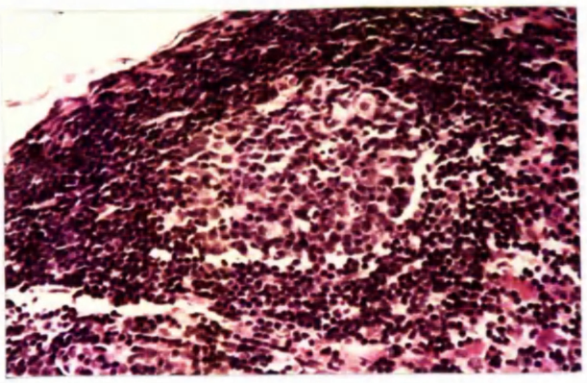


Plate 6.2 : Caecal lymph node x 40 H & E. Developing follicle centre after I.V. C. Parvum.

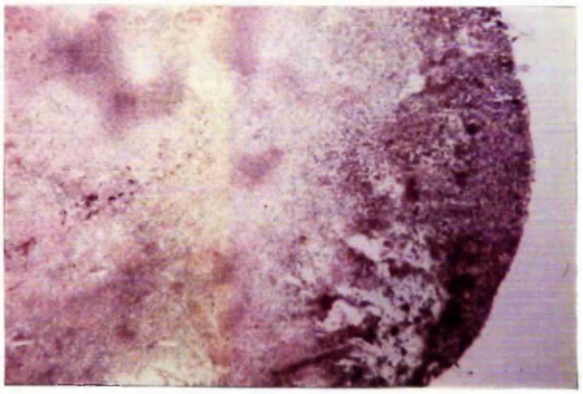


Plate 6.3 : Caecal lymph node x 15 H. & E.. Extensive necrosis after intralacteal C. Parvum.

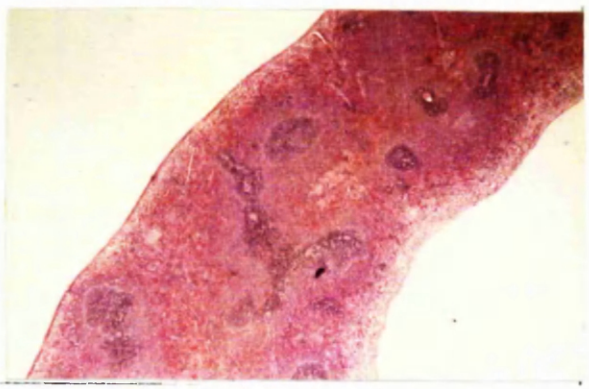


Plate 6.4 : Normal spleen x 10 H. & E..

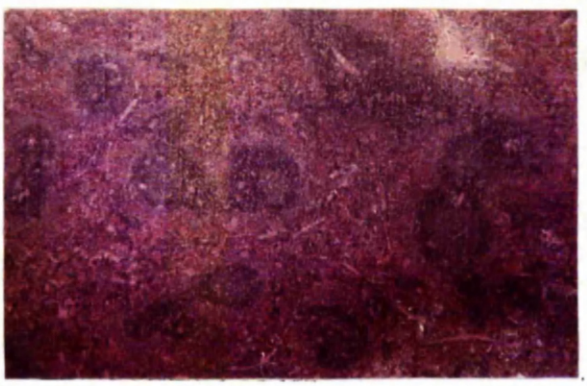


Plate 6.5 : Spleen two weeks after I.V. C. Parvum x 10 H. & E..

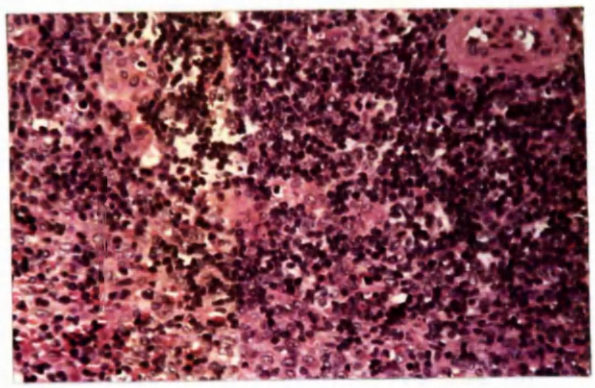


Plate 6.6 : Spleen H & E x 40. Granulomata developing in white pulp of spleen after I.V. C. Parvum.

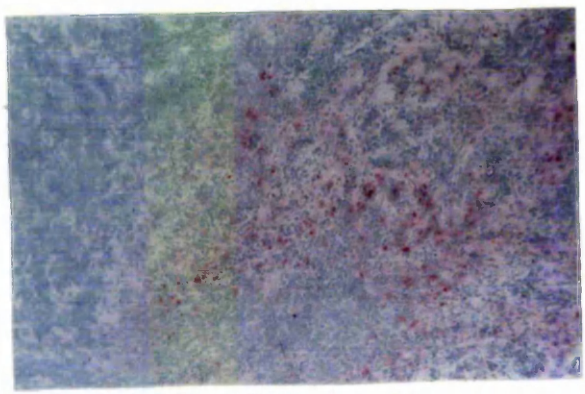


Plate 6.7 : Spleen x 40 B glucuronidase. Positive staining in red pulp.

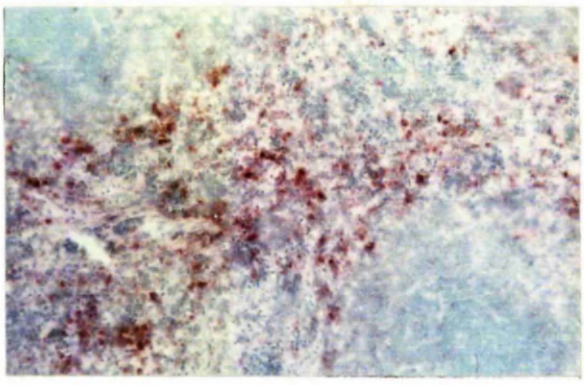


Plate 6.8 : Spleen x 40 non specific esterase. Positive staining in red pulp.

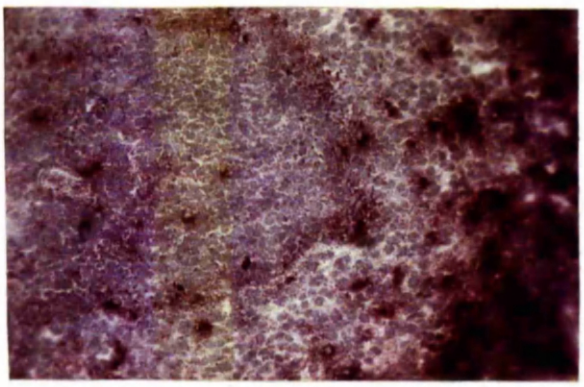


Plate 6.9 : Spleen x 40 acid phosphatase. Positive staining in red pulp and mantle zone of white pulp.

TABLE 6.1

The effect of C. Parvum on the mean weight of the caecal lymph nodes.

	Number of nodes weighed	Mean weight in mg	±SEM mg
Normal	8	1.68	0.74
C. Parvum 0.7 mg IV x one week	4	1.74	0.9
C. Parvum 0.7 mg IV two weeks before	4	1.59	0.7
Intralacteal Thiomersal 0.01% 0.1 ml 1-2 weeks before	3	1.97	0.55
Intralacteal C. Parvum 0.7 mg in 0.1 ml of 0.01% Thiomersal 1-2 weeks before	3	8.7	2.3

Table 6.2

The effect of C. Parvum on the mean area of splenic white pulp

	Number of areas of white pulp counted	Mean area	Standard deviation
1. Normal	57	860	542
2. Normal	31	763	521
3. C. Parvum 0.35 mg IV two weeks previously	33	1585	764
4. C. Parvum 0.7 mg IV two weeks previously	26	2872	1325
5. C. Parvum 0.7 mg IV two weeks previously	63	2399	1468
6. C. Parvum 0.7 mg IP two weeks previously	59	1915	1237

4. v 1.
4. v 2. $P < 0.001$
5. v 1.
5. v 2.
3. v 1.
3. v 2.

4. v 6. $P < 0.0027$
5. v 6.

Chapter 7

A clinical trial of specific active immunotherapy using C. Parvum with autologous irradiated tumour cells in surgically treated Stage 1 and 2 lung cancer

While surgical excision remains the cornerstone in the management of potentially curable cases of lung cancer, it is depressing to note that this most favourable group of patients has a five year survival rate of only 25-30%. (200) These patients, suffering from what is considered to be 'early cancer', i.e. localised to the lung or, at most involving the draining lymph nodes, commonly harbour more extensive disease than is revealed by surgery. Indeed a survey of post mortems on these patients dying of post operative complications within a month of operation revealed that 35% harboured residual disease (local plus metastatic (201)). Some form of additional treatment therefore seems justified. Chemotherapeutic agents which appear to possess activity against advanced lung cancer can cause considerable long and short term toxicity. In addition these drugs are of low patient acceptability when used in this post surgical adjuvant role. They may also be responsible for increased morbidity, especially as many of these patients are in poor general health. Nor is there any clear evidence to suggest that this approach improves survival. (202)

Immunotherapy

The occurence of post operative empyema may improve the prognosis after surgery in lung cancer. (203) When this action was mimicked using post operative intrapleural B.C.G. a similar improvement in prognosis was observed, though only in patients with Stage 1 disease. (204) The

appearance of sinus hyperplasia in the draining lymph nodes (29) and active macrophages around the primary tumour (28) apparently indicate a favourable prognosis, and suggests that the patients immune system has recognised a tumour specific antigen and is capable of reacting against it. On the other hand depression of cell mediated immunity in advanced malignant disease is both common and of poor prognostic significance. (30, 205)

A clinical trial was planned using autologous irradiated lung cancer cells which were re-injected into the dermis of the thigh, along with a very small dose of C. Parvum during convalescence. This route has been shown to induce strong specific anti tumour immunity in experimental animals resulting in prolonged survival and immunity to rechallenge by the same tumour. (206)

Patients

95 patients with histologically confirmed cancer of the lung were included in the study. Randomisation was carried out per-operatively by the surgeon responsible for the procedure, when he was of the opinion that all macroscopic tumour had been resected and anticipated a relatively smooth post operative course. Randomisation tables were used and slips placed in sealed envelopes which were opened in sequence. Allocation was to a no treatment arm when routine follow up alone was carried out, or to treatment with autologous tumour cells. Patients were staged subsequently, after pathological analysis of the resected specimens. Stage 1 patients were those who had tumour localised within the lung and Stage 2 had involvement of the draining nodes but no distant dissemination.

If a patient was randomised to treatment then a sample of fresh tumour was taken immediately from theatre and a cellular suspension prepared under sterile conditions. No enzymes such as collagenase were used, and the procedure consisted of fine maceration followed by several washes in Eagles Medium. The final suspension was tested for cell viability by exclusion of trypan blue, a count performed and then stored, after controlled rate freezing, at -140 degrees centigrade. Treatment was given during convalescence, between 14 and 28 days after surgery. On the day of treatment a sample containing 20×10^6 tumour cells was irradiated with 10,000 rads and 30 µg C. Parvum added with medium to a final volume of 1.5 ml. The rationale for combining these tumour cell numbers with a small dose of C. Parvum is based on animal studies showing that this relationship is critical and that higher doses of C. Parvum can enhance tumour development. (84, 207)

The trial was approved by the ethics committee of the Oxford Area Health Authority and informed consent was obtained from all patients randomised to immunotherapy. The statistical significance of the results has been analysed by the log rank test. Life tables have been prepared as described by Peto and Pike and by using a computer programme designed by these authors. (208)

Results

A total of 95 patients have been included in the trial, 49 in the control group and 46 in the treatment group. In each group the average age of the patients was similar and patients were predominantly male. 9 out of a total of 14 female patients are in the control group. If all the patients randomised to therapy are considered then the

treatment group has shown an apparently poorer survival but this is not significant (Table 7.1, figure 7.1). However, in this group, 12 patients did not receive treatment. 6 patients were either too unwell or died post operatively, 2 did not attend the follow up clinic on the day of treatment, 2 were suspected of harbouring residual disease on review and in 2 a satisfactory cell suspension could not be prepared. In the control group 3 patients died within 3 months of operation and the remainder were well at the first review. If patients not surviving 3 months after operation are excluded from the analysis then the groups are even more similar. (Table 7.2)

When the outcome is assessed according to the stage of spread of the tumour at operation, the anticipated poor results are seen after surgery in patients with involvement of the regional lymph nodes (Table 7.3, figure 7.2). Treatment does not appear to have affected the outcome in either Stage 1 or Stage 2 disease. It can be seen that 13/46 of patients in the treatment group had Stage 2 disease compared with 11/49 in the control group.

8 of the 13 treatment patients with Stage 2 tumours are dead at a median time interval of 9.1 months after operation. This compares with 6 of the 11 control patients with this stage of disease who had a median survival of only 6.2 months.

Table 7.4 shows that there was an equal distribution of histological types in the two groups. Although it had been intended to exclude patients with small cell tumours from this trial, 3 such tumours were diagnosed on the histology of the resected specimens after randomisation. These have

been retained and the two patients with this histology in the treatment group were given immunotherapy. The table shows that treatment has not improved survival in any histological type. It is of interest to note that all 7 patients with a diagnosis of adenocarcinoma who survived by 3 months are still alive at median time intervals from surgery of 23 months in the treatment group and 15.5 months in the control group.

Four out of five patients with anaplastic cancer in the treatment group survived longer than 3 months from surgery. All of these patients are still alive at a median time of 10 months. The small numbers involved in this group make conclusions difficult.

When survival is assessed according to the nature of the operation (Table 7.5) performed there is no significant difference in the results. This analysis has been made on patients surviving more than 3 months after the operation and 17/40 (42.5%) treatment patients had a pneumonectomy compared with only 10/46 (22%) controls. It is apparent that the patients randomised to immunotherapy were suffering from rather more extensive disease. This may explain the greater number of post operative deaths in the treatment group of patients.

Discussion

Attempts to increase anti tumour immunity in human malignancies must be at least partly based on empiricism. The results of laboratory experiments may be helpful but are not necessarily of direct clinical relevance. While tumour specific antigens can be identified in artificially induced malignancies in experimental animals (40) this is not yet true in human cancer. Although there is indirect

evidence both for the presence of tumour specific antigens and host immune reactivity against these putative antigens, the mechanism of the host response to cancer remains unclear.

It has been claimed that immunotherapy with allogeneic tumour cells and B.C.G. given intradermally after surgery for Stage 2 malignant melanoma resulted in a 50% reduction in metastases as compared with an histological control group (56). However, a very similar trial in this country using concurrent control patients showed an alarming trend towards early recurrence in the treatment group and the trial was brought to an early halt (58).

Numerous trials of immunotherapy have been reported in both early and late cases of lung cancer. In many of these trials immune stimulation was combined with either chemo or radiotherapy, thus making the result somewhat difficult to interpret (209).

B.C.G. along with irradiated allogeneic tumour cells has been given intradermally to patients suffering from all stages of lung cancer. The results have been compared with groups who received B.C.G. alone or no additional treatment. There was no benefit from treatment in advanced disease, but patients with Stage 1 and 2 disease have not yet been evaluated (210). The interpretation of the results of this treatment was complicated in Stage 3 patients as radiotherapy or chemotherapy were added as considered clinically indicated.

Claims of benefit after surgery for Stage 3 tumours have been made after using autologous tumour cell vaccine treated with vibrio cholera neuramidase and concanavalin A then injected intradermally with Freunds complete adjuvant. However, as the treatment group of patients

required less extensive surgery and had a higher proportion of patients with adenocarcinoma the interpretation of the claim of benefit is open to doubt (211).

A controlled randomised trial of intrapleural B.C.G. in surgically resected lung cancer carried out in this country failed to confirm the experience of McKneally (212). The authors point out various minor differences in the treatment given but it seems unlikely that these were sufficient to explain the differences in results between the two trials.

By administering a cellular suspension of autologous tumour cells which were irradiated to prevent local implantation or dissemination, and combined with a small dose of C. Parvum, it was hoped to augment host resistance to any residual lung tumour. By injecting this vaccine in the thigh where it would drain to lymph nodes unlikely to have had previous exposure to the tumour antigen, and by using C. Parvum for its adjuvant effect (105) it was hoped that these immunocompetent nodes might produce specific anti tumour antibodies. Careful analysis of the results fails to show that any such beneficial effect has occurred. However, it does not appear that there have been any adverse effects from this type of therapy and side effects were minimal.

In reviewing the results of this trial it was clear that the interpretation was made more difficult because of the large number of patients (almost 25%) who although randomised to treatment did not receive it. This could probably have been avoided if tumour samples had been prepared from all patients considered by the surgeon to be suitable for inclusion in the trial. Randomisation could

then have been deferred until the patient had made a satisfactory recovery from the operation. Prior to discharge those patients who had drawn treatment could be given a full explanation of the proposed immunotherapy with the option of declining at this stage. It is likely that more treatment patients would actually have received the vaccine had this approach been adopted.

The overall results of immunotherapy in lung cancer are not encouraging. We do not feel that any long term benefit is likely to result from the approach we have adopted in this trial. It seems unlikely that this form of treatment will become relevant until lung tumour specific antigens can be identified, assuming they exist. The recent advances using hybridoma derived monoclonal antibodies suggest that this may not be such a remote long term aim (213).

Table 7.1

Overall Death Rate

	Treatment	Control
Number	46	49
Deaths	20 (43.5%)	17 (35%)
Mean time to death	1.25 mths	13.1 mths

Figures in parenthesis indicate percentage of total in the group

Table 7.2

Death rate excluding patients dying within 3 months of surgery

	Treatment	Control
Number	40	46
Deaths	14 (35%)	14 (30%)
Mean time to death	17.3 mths	15.6 mths

Figures in parenthesis indicate percentage of total in the group

Table 7.3

Overall survival according to tumour stage at operation

		Treatment	Control
Stage 1	alive	21	27
	dead	12 (14.8)	11 (16.8)
Stage 2	alive	5	5
	dead	8 (9.1)	6 (6.2)

Mean time interval in months in parenthesis

Table 7.4

Histological type and death rate of patients surviving 3 months

	Treatment	Control
Squamous	33 (14)	34 (9)
Adenocarcinoma	3 (0)	4 (0)
Anaplastic	4 (0)	8 (5)

Table 7.5

Survival according to operation of patients living 3 months post-op

	lobectomy		pneumonectomy	
	No	Time in months	No	Time in months
Treatment				
alive	17		9	
dead	6	20.3	8	13.5
Control				
alive	27		5	
dead	9	11.4	5	12.2

Figure 7.1

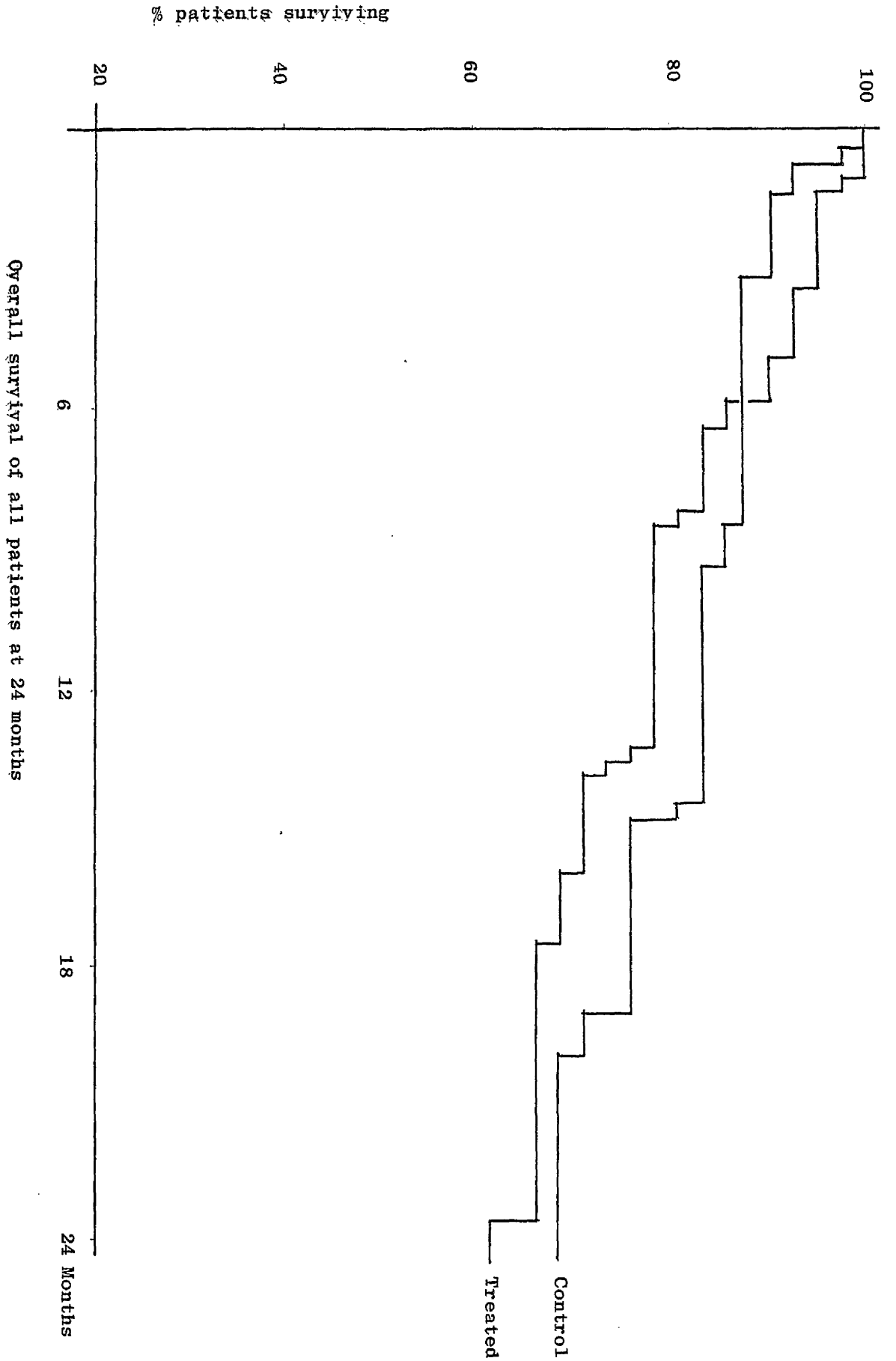
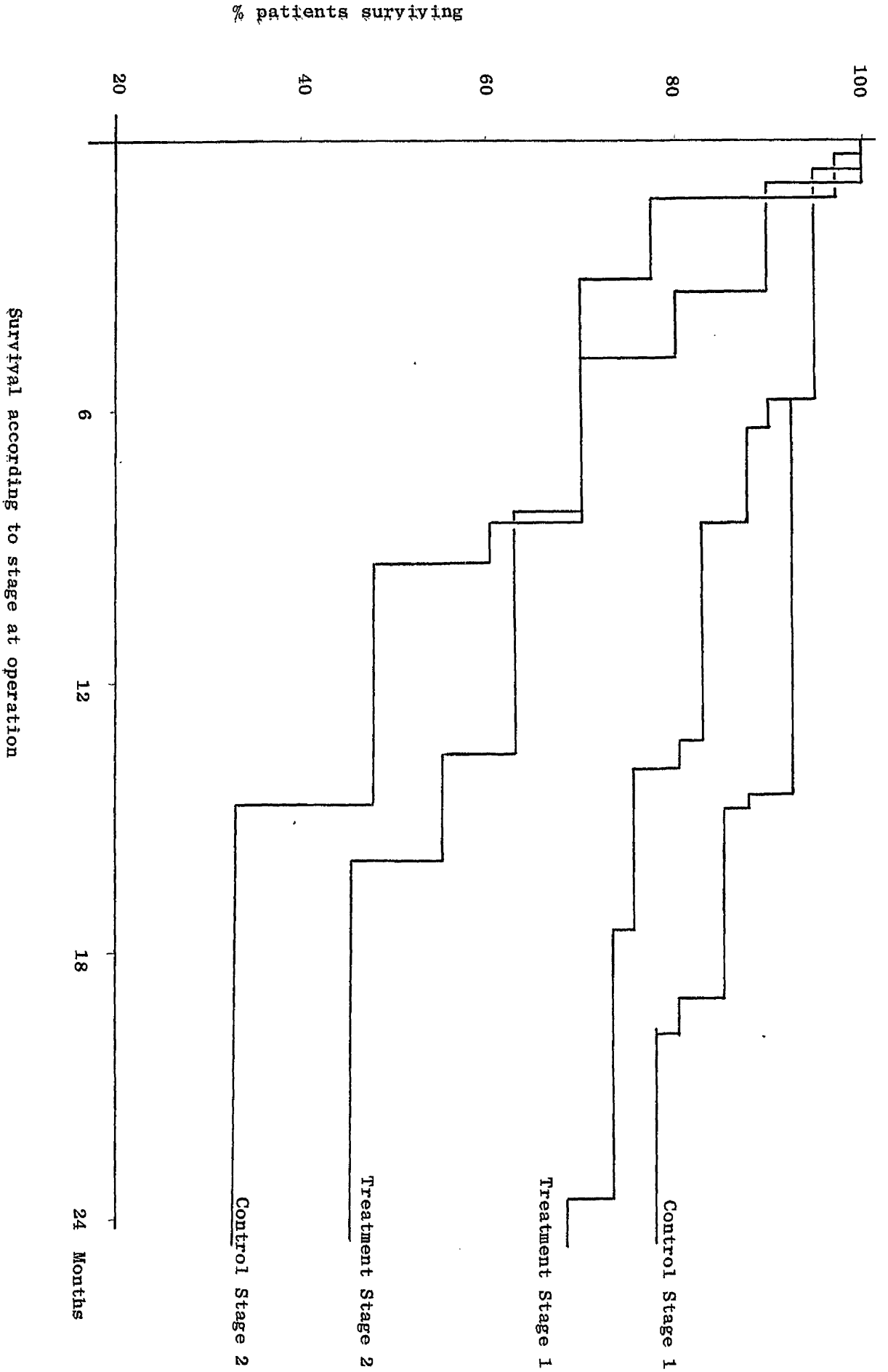

% patients surviving
20
40
60
80
100
6
12
18
24 Months
Overall survival of all patients at 24 months
Treated
Control

Figure 7.2



Survival according to stage at operation

Chapter 8

A trial of non specific immunotherapy using systemic C. Parvum in treated patients with Dukes B and C colorectal cancer

In 1977 cancer of the colon and rectum caused 16,462 deaths in England and Wales (214). Advances in surgical technique have made no significant difference to the prognosis of the patient presenting with this disease. An analysis of the results of surgery in the Oxford region (215) gave figures similar to those from Birmingham (216) and Bristol (217). After excision of Dukes B cancers patients had an age adjusted 5 year survival rate of 58%. In Dukes stage C this dropped to 27.1%. While specialised centres of referral can report figures considerably better than these (218) it seems likely that our own results are more representative of those of the country as a whole. These results are particularly depressing when it is considered how often the surgeon is confident that he has removed all traces of malignant tissue.

It is against this background of disappointment that consideration has been given to additional treatment after surgical excision of these cancers. (111, 112)

Colorectal Cancer and the Immune System

The recognition of a circulating antigen in the serum of patients suffering from colorectal cancer suggested that a genuinely tumour specific antigen had been found in a human malignant disease (41). Although this specificity has now been disproven, the association is nevertheless of importance. Elevated serum levels of "carcinoembryonic antigen" are found in most cases of colorectal cancer and,

perhaps of more practical importance, revert to normal after tumour excision, becoming elevated again when secondary cancer develops.

Although tumour specific antigens have not yet been demonstrated in human cancer, considerable supportive evidence suggests that they are present.

Patients lymphocytes are cytotoxic for cultured tumour cells. (219, 220) This is true when the tumour cells were autochthonous (the patients own tumour), or allogeneic (tumour from another human). These lymphocytes are not cytotoxic when cultured with normal cells or tumours from organs other than large bowel and pancreas.

The presence of a strong capacity for lymphocyte mediated tumour cell destruction might be expected to confer an improved prognosis. That this is not the case may be explained by further in vitro experiments. Autologous serum admixed to the lymphocyte/tumour cell culture prevents the lymphocyte mediated tumour cell destruction. (221) The serum components responsible are termed "blocking factors". Circulating tumour antigens and antigen - antibody complexes have been implicated.

Soluble extracts of tumour cell membranes have been prepared from human colorectal tumours. These preparations have been used as skin test antigens. A delayed type of hypersensitivity response results when these extracts are used on patients suffering from colorectal cancer, with no response in normal patients. No such response occurs when similar preparations are made with normal colons. (222) This again suggests that recognition and response to tumour antigens has occurred.

Colon cancer cell preparations suppress the migratory capacity of mononuclear cells from tumour bearing patients. (223) Monocytes from normal patients are not affected. Preventing the influx of mononuclear cells might further depress cell mediated immunity.

Immune reactivity, as measured by skin testing of patients, lymphocyte numbers and the response of lymphocytes to mitogen stimulation, is suppressed even in early colorectal cancer. (224)

These in vitro studies support the viewpoint that tumour specific antigens are present in colorectal cancer and that they evoke a cell mediated immune response which can be blocked. Although the clinical importance of these observations is unclear, it seems reasonable to assume that the restoration of immune responsiveness should be beneficial.

The effect of C. Parvum in animal and human tumours has been fully discussed. (Introduction) In view of the poor prognosis for patients who present with Dukes B and C colorectal cancers this group was selected for a trial of regular systemic infusions of C. Parvum in the two years after surgery. In this time period many relapses occur and immune stimulation should be of maximal benefit when used in the patient bearing minimal residual disease. (111) It was also of interest to see if C. Parvum treatment would influence the development of distant metastases. Animal experiments suggested that systemic use of C. Parvum would have a greater effect in suppressing metastases than local recurrence of tumour. (63)

Patients

Patients were considered for inclusion in the trial during convalescence after surgery for pathologically confirmed Dukes B and C carcinoma of the colon or rectum. Dukes stage A patients were excluded in view of their excellent prognosis and Dukes stage D patients because of the improbability of immune stimulation being effective against an extensive bulk of tumour tissue. Treatment was with a series of intravenous infusions of C. Parvum in a dose of 5 mg/m^2 given in a diluting volume of 100 mls of normal saline over a period of 30 minutes. The first treatment was within a month of operation and in each patient it was planned to give ten infusions in the two years after surgery. Treatment usually involved an overnight stay in hospital, but some patients were able to be allowed home within 8 hours of beginning the infusion. At the beginning of the trial the reported side effects of intravenous C. Parvum included hypertension and hypotension and in view of this, patients who were known to suffer from cardiovascular disease, hypertension or pulmonary disease were not considered for treatment. Patients with a history of allergy and those over the age of 75 years were also excluded. The side effects of this treatment were similar to those previously reported (93, 94, 95) and have already been discussed in the introduction. The statistical analysis of the survival curves has been carried out by drawing life tables and applying the log rank test. (208)

Results

A total of 44 patients were randomised into the treatment group and 48 into the control group. (Table 8.1) The average age of the control patients was rather less than

that of the treatment group, being 58.9 years and 63.4 years respectively. The sex distribution between the groups was unequal. 38.6% (17/44) of the treatment group were males compared with 66.6% (32/48) in the control group.

When the Dukes stage of the tumour is considered there have been almost the same number of Dukes stage B tumours, but more Dukes C lesions in the control group of patients. (Table 8.2)

Recurrence of cancer has occurred to date in 25% of the treatment patients (11/44), but nearly 30% (13/44) of this group are dead. (Table 8.3, figure 8.1) Three of these have been from causes other than colorectal cancer. One patient suffered from a myocardial infarction after a period of C. Parvum induced hypotension early in the study. (96) One other patient died from an unexpected myocardial infarction during an apparently uneventful convalescence and before receiving C. Parvum treatment. The third patient died from the effects of a second malignancy in the uterus, histologically confirmed as unrelated to her colonic cancer. An almost identical percentage of the control group of patients have developed signs of recurrent cancer. Although the recurrence rate is 27% (13/48) the percentage who are dead is rather lower than in the treatment group, being 25% (12/48). Only one death in the control group was not due to recurrent cancer. This was in a patient who died from acromegalic cardiomyopathy. The mean time interval till recurrence is virtually identical, being 17.3 months in the treatment group compared with 17 months in the controls. Nine patients developed recurrent cancer during the course of C. Parvum infusions. When the life table analysis method was applied to the survival figures according to the Dukes

stage there was no difference between the two groups (Figure 8.2).

Table 8.4 shows the principal sites where tumour recurrence first became evident. It does not appear that treatment has had any influence on the pattern of recurrence.

Although it was planned to give ten infusions of C. Parvum, it became apparent that in many patients it would not be reasonable to pursue this. Tolerance to the side effects of C. Parvum was anticipated to increase after the first few infusions. However, this only occurred in the minority of patients and in only 7 out of the 44 patients has the full course been given. However, over half of the group have received five or more infusions. Eight patients randomised to treatment did not receive any. These have been included in the analysis of the figures as treatment patients. One of these was the lady who died of a myocardial infarction during convalescence. The other seven patients declined treatment having previously given informed consent prior to randomisation. As a full discussion of the side effects and nature of the proposed treatment took place prior to randomisation it is understandable why these seven patients had second thoughts about undergoing the infusions once they were discharged from hospital. No subsequent attempt was made to persuade these patients that they should have treatment.

Discussion

The poor outlook for Dukes stage B and C colorectal cancer has prompted many other clinical trials of adjuvant treatment after surgery. (93, 94) In several of these, chemotherapeutic drugs, commonly 5- fluorouracil, (5-FU) have been used.

A significant improvement in the 5 year disease free survival has been reported in both Dukes B and C cancers by using 5-FU in two intensive five day courses. (225) In this report, 81.6% of Dukes B and 57.5% of Dukes C patients were alive with no evidence of disease five years after surgery. These results appear to have been confirmed by two randomised controlled trials of post operative 5-FU treatment. (226)

In many trials chemotherapy, usually with 5-FU, was combined with attempted stimulation of the immune system. The most commonly adopted means of immune stimulation has been repeated doses of B.C.G. usually administered by scarification. As this treatment was often very unpleasant, painful and sometimes caused local ulceration, fractions of the B.C.G. cell wall have also been used since these were thought to be less likely to cause side effects. The results from most of these trials are inconclusive or not yet published. Where claims of benefit have been made, for example by the M.D. Anderson group, the randomisation procedure has been open to question. (226) This group of workers have treated patients after surgery for Dukes C cancers in two ways. Some patients received repeated scarification with B.C.G. alone while others had the same treatment combined with oral 5-FU. B.C.G. treatment involved scarification weekly for three months then on alternate weeks. 5-FU treatment involved 150 mg/m^2 of the drug taken orally four times a day for five days, once every four weeks for two years. Each group had a prolonged disease free interval and a survival curve that matched that of an historical control group of patients who suffered from Dukes stage B cancer. Since both treatment groups had similar disease free intervals and survival times it seemed reasonable to attribute this to the B.C.G. scarification rather than the

5-FU. However, these figures have been criticised mainly because of the use of historical control groups. The apparent improvement in the treated Dukes C patients could well have been due to some other alteration, for example a change in the type of patient presenting for surgery. This seems particularly relevant when the survival curves of the control group are carefully considered. The control group of Dukes C patients appears to have fared particularly badly hence the improvement in prognosis for both treatment groups may be more apparent than real.

Israel's report of the beneficial effect of systemic C. Parvum in advanced human malignancy engendered considerable enthusiasm for this approach. (95) However, proof of clinical benefit is now difficult to find. The ability of a treatment to influence the growth and spread of tumours in laboratory animals may support the investigation of its use against human malignancy. But laboratory results are not necessarily of clinical relevance. Even in laboratory animals systemic C. Parvum has a relatively weak action against large tumour masses and is more effective in suppressing the development of metastases. C. Parvum as used in our trial has not influenced the pattern of recurrence of disease. In clinical practice it is often impossible to be sure of the extent of recurrence of cancer and for the purpose of this trial the most obvious sites of recurrence have been assumed also to be the main sites. The majority of recurrences in both groups have been within the abdominal cavity. In a few cases recurrence appeared to be genuinely localised, for example at the site of the anastomosis or involving structures near the site of the original tumour excision. Two patients in the control group had second resections for locally recurrent cancer and both are still alive at

time intervals of 30 and 23 months from their second operations. This supports the view that "second look" surgery in colorectal cancer is justified.

This trial was carried out over a time course of 54 months. All patients randomised into the trial have been followed up until the closing date. Both the treatment and the control groups of patients show improved survival figures when compared with the retrospective study on patients presenting with colorectal cancer in Oxford reported by Gill et al (215). This is probably explained by considering the highly selected nature of the relatively fit patients who were included in the trial. The observation also serves to emphasise the dangers in the use of historical control groups.

In the early part of the trial one patient suffered a myocardial infarction 24 hours after his second pulse of C. Parvum treatment. The first infusion had been tolerated well. During the second infusion the patient became hypotensive but appeared to recover from this uneventfully. Some 12 hours after the infusion the patient developed crushing chest pain and subsequently died with irrefutable electrocardiographic evidence of acute myocardial infarction. Rather than bring the trial to a halt, a more rigid exclusion policy was adopted. Any patient with the least suspicion of cardio pulmonary disease was thereafter not considered for randomisation. With this policy no further tragedies have occurred. However, this has resulted in very low input into the trial. A high percentage of otherwise suitable patients have been excluded because of concern about the side effects of therapy. Another effect of this policy has been to result in a very highly selected group

of patients being studied. The recently reported side effects of human lymphoblastoid interferon (227) seem very similar to those of C. Parvum. This may seriously limit the number of patients in whom this interesting form of treatment can be evaluated.

At the beginning of this trial it seemed reasonable to aim for a course of infusions. Unfortunately the tolerance which was reported to build up after the first few infusions only developed in the minority of our patients. Indeed some patients suffered from increasingly severe side effects. Seven patients had the full course of ten infusions and only one of these has developed recurrent cancer. Nine patients developed signs of recurrence during the two years of treatment. Most of the other patients who did not complete the course of treatment were withdrawn by the investigating team, but two found the side effects intolerable and requested that treatment be stopped.

One objection to the use of immunostimulants is that of lack of specificity for target cells. If the proposed anti-cancer drug were to increase a population capable of blocking naturally occurring tumour defences, for example suppressor T cells, then enhanced tumour growth might result. None of the early human studies on advanced human cancers supported this viewpoint, but there was a clear need for caution in monitoring the progress of the patients in our trial. Some patients in both groups developed recurrent tumours within 12 months of surgery, but there was no difference in the mean time taken to tumour recurrence in either group.

Input to this trial has now ceased. There has been no evidence that C. Parvum has influenced the development

of tumours used in this way in this group of patients. It is still premature to say what the long term effects will be and the results should be reassessed in the future.

TABLE 8.1

	Treatment	Control
Total	44	48
Male	17	32
Female	27	16
Average Age	63.4	58.9

TABLE 8.2

Dukes Stage	Treatment	Control
B	35	31
C	9	17

TABLE 8.3

	Treatment	Control
Deaths	13	12
Recurrences	11	13
Dead due to recurrence	10	11
Dead from other causes	3	1

TABLE 8.4

Principal sites of recurrence	Treatment	Control
Local	2	3
Intra-abdominal	8	9
Extra abdominal	1	1

Figure 8.1 Life table analysis of deaths in the two groups

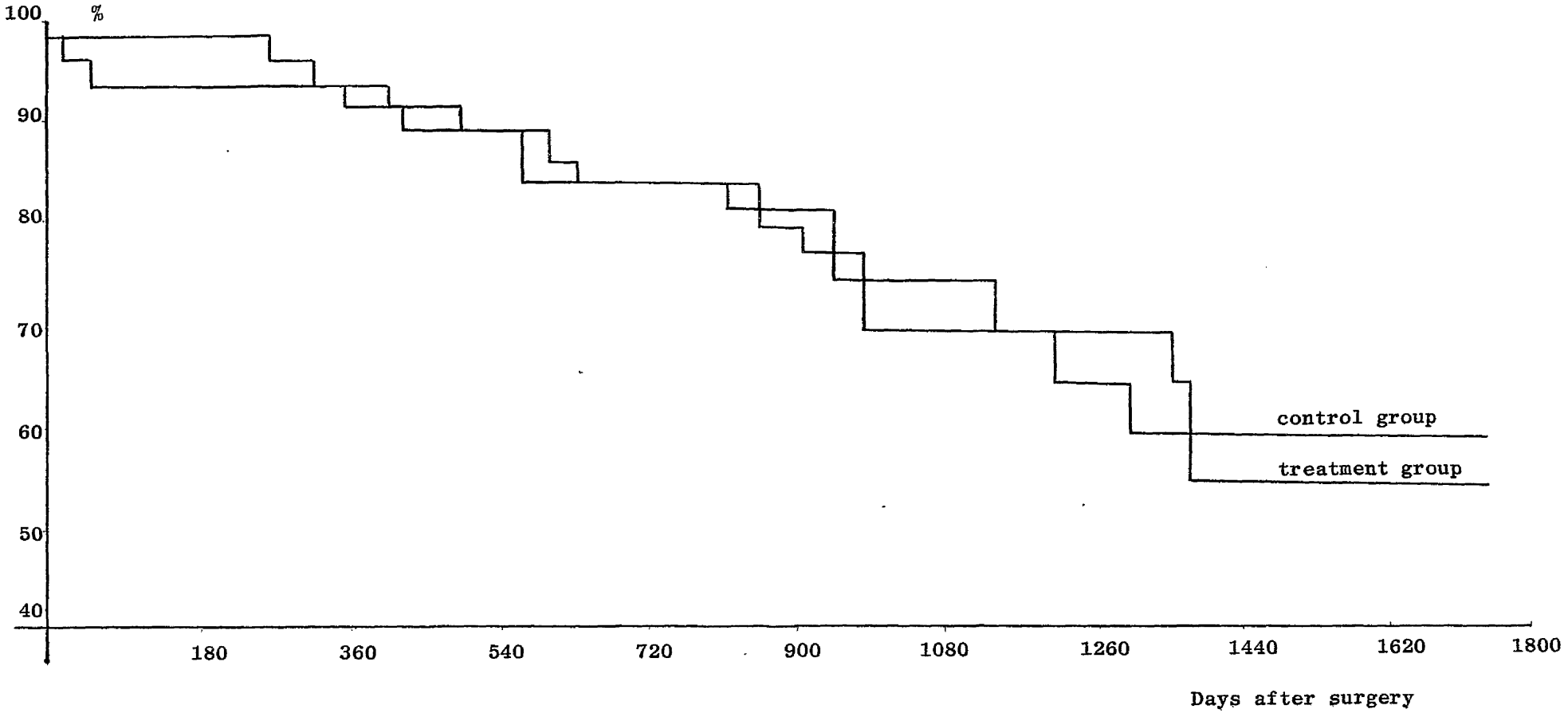
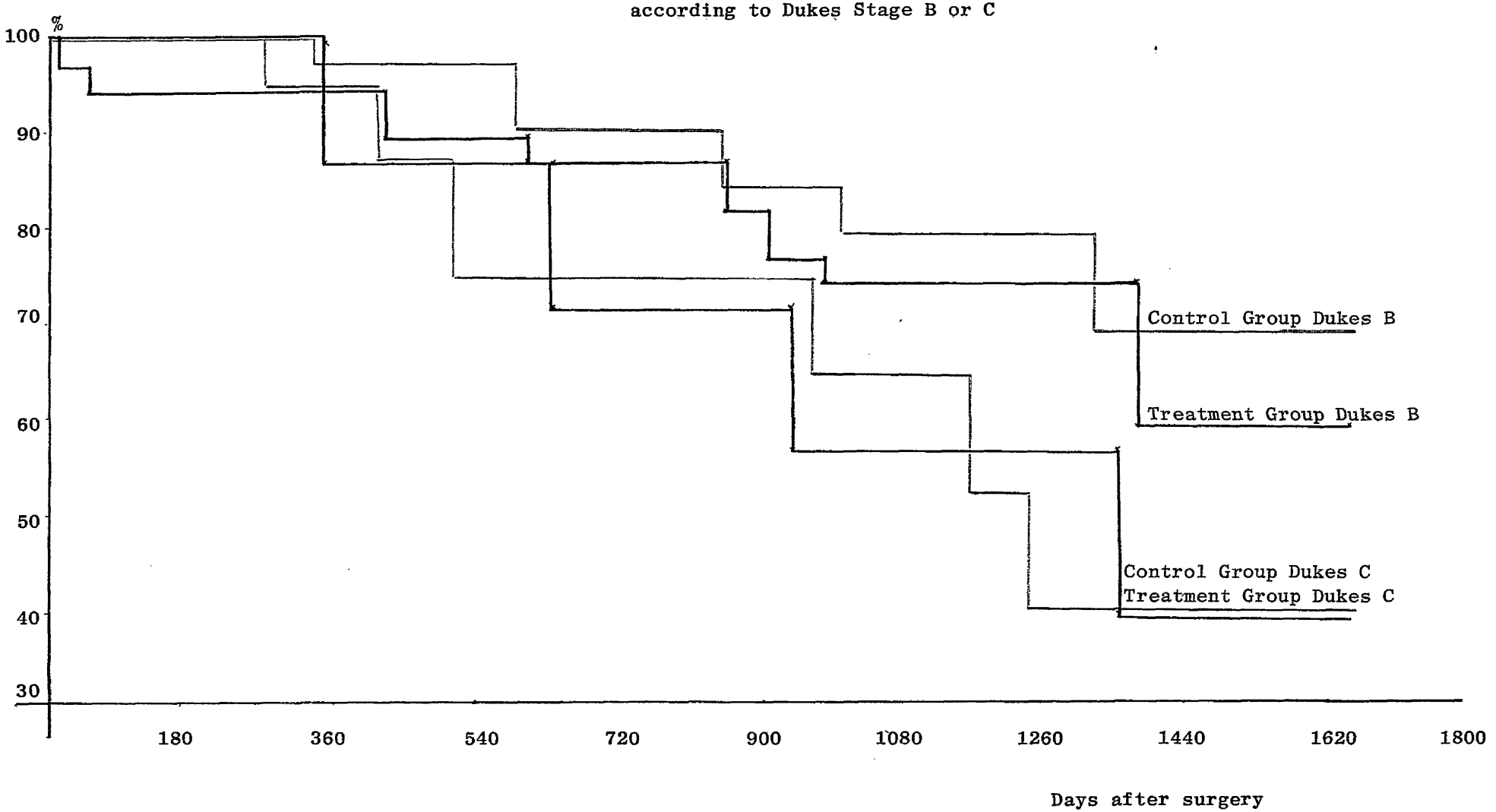


Figure 8.2 Life table analysis of deaths in the two groups according to Dukes Stage B or C



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