STUDIES WITH IMPLANTED CARCINOMA IN INBRED MICE

ALAN EDWARDS, M.B., F.R.C.S.

Montreal General Hospital Surgical Clinic.

Thesis submitted in part fulfilment of M.Sc. Degree

CONTENTS

CONTENTS (2)

CONTENTS (3)

FIGURES

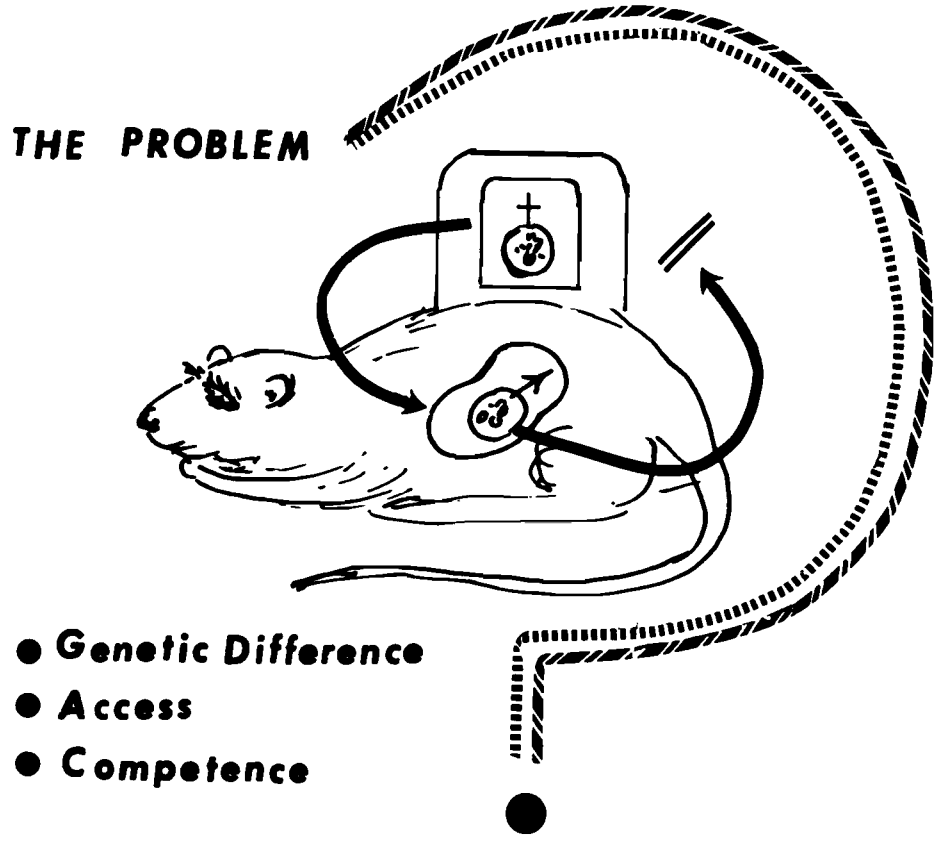

Figure 1

The tumour problem - does a tumour survive on an animal because there is insufficient genetic difference between host and tumour to provoke rejection; is there a barrier of access between tumour and R.E. system; or have the competent clone of R.E. cells been eliminated?

PART A

Introduction

Evidence from paleopathology indicates that cancer is a very ancient disease. Preserved relics of prehistoric times reveal wide geographic and zoologic distribution of malignancy. Dinosaurs of the Mesozoic period are said to have shown signs of cancer, and other evidence of its antiquity (Sambon cited by Haddow) (68) are to be found in the treasures of the pyramids, from the Etruscan tombs, from Peruvian mummies and from the cuneiform tablets of the library of Nineveh. The earliest medical record known to man (94) is the "Edwin Smith Surgical Papyrus". This anonymous relic of antiquity is generally regarded by historians as having been originally written in the Egyptian Pyramid Age in the Old Kingdom (3000 - 2500 B.C.), although speculation by Breasted and Cooper (16) suggest that this document may form part of the teachings of the oldest known physician, Imhotep, the 'patriach and sage of primitive medicine' (94) who lived in the thirteenth century B.C. In Breasted's translation (16) eight cases are reported of ulcers and tumours of the breast and treatment by cauterization with the "fire drill" recorded for the first time. The Papyrus Elbers (circa 1500 B.C.) (37) described superficial skin tumours but advised treatment only if symptomatic (i.e. ulcerated forms) by application of arsenical pastes. It may well be that in the intervening three and one half millennia, the cycle of thought has turned full circle in that perhaps the treatment of malignant disease may well, at best, be only palliative.

Nomenclature is Greek in origin for in the fifth century B.C. Hippocrates used the word 'cancer' for benign growths and carcinoma for malignant ones (112). It is, however, remarkable in view of his prolific authorship in most fields that cancer appeared to receive so little attention. However, the 'corpus Hippocraticum' referring to occult (deep seated or non-ulcerated) carcinoma makes this prophetic prognosis regarding the contra-indications of surgery: "It is better to omit treatment altogether, for, if treated, the patients soon die, whereas if let alone they may last a long time". (75). "Galen was a celebrated Greek physician who lived in the second century A.D. amongst the Romans and wrote with remarkable prolixity. The vapours of Galen became the medical heritage of antiquity which clouded the path of progress for untold generations. The incredible authority of Galen and his sect created a dynasty of medical didactics during the Dark Ages which according to some medical historians may have been responsible for the refractoriness of medical progress until after the Renaissance" (Edward F. Lewison) (94). It was not therefore until the eighteenth century that Francois le Dran defied Galen's humoral theories by declaring cancer to be a local disease that spread by the lymphatics (93). In 1773 Peyrilhe recognised the unity of various forms of cancer and began to study its nature by animal experimentation. In so doing he attempted to throw off the paralysis cast over oncological investigations by the authoritarian teaching of the great Galen that cancer was the result of an excess of black bile - melancholia. The first true appreciation of the basic nature of cancer and its differentiation

from other conditions had to await the development of the microscope. Schwam in 1839, following Schleiden's work the year before on the cellular structure of plants, recognised similar structural units in animal tissues, which paved the way for Müller to describe the cytological origin of neoplasms. In 1876 Novinsky successfully transplanted a small cell carcinoma from one dog to another to be followed thirteen years later by Hanau's transplantation of a spontaneous metastatic cancer between rats. Thus began a period of increasingly intense laboratory investigation until it has reached today's tempo and fervour. It is probably fair to say that, although awareness of the disease commands the respect due to antiquity, in less than a century of organised and systematic investigation more has been learned about this pathological entity than in all its long lineage (112). Moreover, there is every promise that even greater advances are imminent.

Inevitably a little lagging, but nevertheless within the footprints of the march of knowledge of the disease, comes the concepts and methods of its treatment without which mere knowledge becomes, in the eyes of the clinician, a worthless void. The treatment of cancer has been mediated chiefly by surgeons and therefore not unnaturally has been based on mechanistic precepts. Evidence that there was much excisional therapy for cancer in pre-Homeric Egyptian surgery is scant yet that it was extensively practiced by the early Romans is implied by the admonitions of Aulus Cornelius Celsus (24) concerning irritation of cancers by imprudent intervention. With regard to cancer of the breast he advised against removal of the pectoral muscles in mastectomy and it is interesting

that this empiric procedure should find favour again some 1800 years later. According to Aetius (2), Leonides, of the Alexandrian School, also in the first century A.D. performed skillful and courageous surgery inspite of the nihilistic attitude assumed by his contempories towards cancer. He used the cautery at first to arrest haemorrhage and then to eradicate residual disease claiming that when burning was deep prognosis was much better. Even then there was an awareness both of prognosis and its improvement by extended local excisions. Interrupted only temporarily by the Galenic paralysis, there has developed through the centuries from this time, perhaps unconsciously, the concept that the more tissue that is removed the better the procedure, and modern anaesthesia and supportive adjuncts have allowed full reign to its execution. Ambroise Pare (1510-1590) (115), although a Galenist, recognised the relationship between breast cancer and axillary metastasis although it was Francois le Dran (1685-1770) (93) who dissected glands from the axilla and appreciated the considerably worsened prognosis by their metastatic involvement. In 1867 Charles Moore (109) propounded the 'en bloc' principle of dissection and postulated the principles of radical mastectomy (except for pectoral excision) which by 1894 had been popularised as the elegant Halsted procedure (71, 72) which, with the Willy Meyer (102) addition of excision of the pectoralis minor, has remained copy book to this day. Since this surgical zenith there has been little major advance in the excisional treatment of mammary carcinoma. Over the last fifteen years or so there has been increasing dissatisfaction with the classical surgical procedures with a hardening suspicion that perhaps the biological properties of a neoplasm are more significant than its anatomical boundaries.

Yet for the surgeon who has spent many years acquiring the stringent disciplines of radical surgery, and the surgical apprentice who tends to view such procedures as indices of surgical maturity, it is difficult to set them aside. Yet there is a growing weight of evidence, moulding an enlarging body of opinion that suggests that this might possibly have to be considered. Perhaps one of the most forceful exponents of this new doctrine is Dr George Crile, Jr, of Cleveland who wrote: "In many fields our standard techniques should be re-evaluated in the light of modern insight into physiology or natural history of disease. Oftentimes such re-evaluation can lead to modified or new techniques with reduction in operative morbidity. Familiarity with a technique should not have undue influence on the choice of operation. It is the mind that should guide the hand". (29).

This challenge must be answered for the treatment of carcinoma forms a large part of Western surgical practice. Some half a million persons develop cancer annually in the U.S.A. and nearly a quarter of a million die from this disease (Ackerman) (1). While the reported cure rates are constantly improving, the death rate per 100,000 persons has not been significantly altered (105), a paradox which may possibly be explained by a better diagnosis obtaining the less malignant cases, or the fallacy of the '5 year cure'. Dr Crile (27) pointed out an analogy in the treatment of appendicitis when he showed that between 1900 (when reliable figures are first available) and 1935 (when sulphonamides came into use) the death rate from appendicitis per 100,000 people steadily increased. Since the introduction of

antibiotics it has fallen to the lowest level in history. Introduction of a valid method of controlling infection was reflected promptly in a diminution of death rate per 100,000. The pertinent point in the history of appendicitis is that in all hospitals, during the period in which the general population's mortality per 100,000 from appendicitis was steadily rising, the mortality of patients treated for appendicitis was falling. The public were educated to seek earlier treatment, surgeons learned better techniques, many borderline diagnoses of mild or innocuous appendicitis were made, and as a result the mortality of patients diagnosed as having appendicitis fell. Yet the population's death rate per 100,000 rose steadily until an effective medical treatment of peritonitis was discovered. "Today in the field of cancer we have much the same problem as we did in appendicitis before the discovery of the sulphonamides and antibiotics. This is steady improvement in the cure rate of cancer but no significant change in the age adjusted death rate per 100,000 people." (Crile) (27). As McKinnon has said "We do not alter the death rate from cancer by curing cancers that would not have been fatal even if untreated" (105).

There has been much emphasis laid on early detection of cancer and in 1946 Early Detection Centres were set up in the U.S.A. Between 1946 and 1948, 51,728 persons were examined, 406 cases were discovered, a "success rate" of 8 per 1,000 with an estimated cost of £10,000 per case. Yet one wonders if the time scale is not perhaps over emphasised. McWhirter (108) estimated that if cancer starts in a single cell it would take 20 geometric divisions to make a mass 1 mm in diameter, 30 divisions 1 cm diameter, 40

and the tumour would weigh over a pound, 50 and it would weigh 150 pounds. The growth of cancer follows an exponential curve with an initially flat but rapidly increasing gradient. A very rapidly growing tumour may double its size once per month, on the other hand a very slowly growing neoplasm, e.g. scirrhus cancer of the breast may not double its size for over a year. Correlation of these rates with cell division makes it apparent that the rapidly growing tumour must have been present for 30 months before it reached the size of 1 cm, while the schirrous growth must have been there for 30 years or more! While such calculations are salutatory, they ignore the varying fluxes of stimulation or inhibition to which such a neoplasm may well be exposed in vivo, yet they help to focus attention on the prime importance of the biological behaviour of tumour cells rather than the clinical chronology of the tumour mass.

In the traditional concept of the spread of cancer, the tumour is at first localised, later spreads to regional lymph glands and finally metastases throughout the whole body. According to this doctrine the most extensive operation performed at the earliest time would give the best chance of cure. There is, however, mounting evidence that this is not absolutely true. Galch (51) has long held the view termed "biologic predeterminism" by MacDonald (96) that the course of the disease is more dependent on the balance between the biologic properties of the neoplasm and the resistance of the host. This view is supported, for cancer of the breast by the studies of Park and Lees (114) which indicated that it is impossible to prove that the course

of cancer of the breast is influenced by any form of surgical treatment. Controversy has raged sometimes with acrimony over extended or restricted mammary amputations, yet the battle has never been for more than a marginal few per cent of survival rate, the significance of which becomes perhaps just a little blunted by the realisation that untreated breast carcinoma has an approximately 20% five year survival pattern (Greenwood (64), Forber (48), Nathanson and Welch (111)). Urban and Baker (139) and Wangensteen (142) advocate extension of the radical mastectomy to include the internal mammary chain, while others press for more restricted resections. Williams, Murley and Curwen (144) in analysing the figures for survival after surgery for cancer of the breast done at St Bartholomew's Hospital, London, in the thirties showed that the better result was obtained by Keynes using non-radical means (simple mastectomy; radium implant) rather than other surgeons employing standard radical mastectomies. A similar report was made by Meyer and Smith (101) in America a year later. Publications by Small and Dutton (130) and Byrd and Conerly (23) showed similar findings. In 1955 Deaton (35) in a survey of the World literature reported a survival rate in simple mastectomy to be 5% higher than in radical mastectomy. McWhirters classical Scottish experiment (107, 108) also appeared to show that by not dissecting the axilla dissemination was less, for simple mastectomy followed by irradiation had a ten per cent better five and ten year survival than radical mastectomy plus x-irradiation. Haagensen and Stout (66) triple biopsy technique is tending to vindicate such conclusions although arrived at from different initial criteria.

The circulation of cancer cells in the blood even in "operable cases" is a disquieting finding for the protagonists of the classical "local" concept of cancer. Engell in 1955 (38) using the Saponin technique was the first major worker in this field. Working in Stockholm, Sweden, he showed circulating cancer cells in 59% of 107 cases of operable cancers of the breast, lung, stomach and colon. Thirty five per cent of grade II cancers, seventy eight per cent of grade III and a hundred per cent of grade IV had cancer cells free in the blood stream. In the same year Fisher and Turnbull (46) found cancer cells in one third of a series of patients with operable carcinomas of the colon. Later Sandberg and Moore (125) were able to increase the percentage of positive smears in patients with more advanced disease. Such work led Denoix (36) of Paris, France, to suggest that there is probably a time in the evolution of all cancers when they are systemically disseminated. It would seem that the entry of cells into the blood stream, be it by loss of cohesion or virtue of some primitive mobility, appears implicit in the process of malignant change. "Suddenly the problem of the spread of cancer is given a reverse twist. No longer is it a question 'what makes a cancer shed its cells into the blood stream?'. Rather it is 'why do not all cancer cells that enter the blood stream survive and grow metastases?'." (Crile) (30).

There must, clearly, be some measure of host resistance and it is the courting, understanding and enhancement of this factor that may well pay greatest dividends to the cancer therapist. Forty five years ago Jones and Rous (81) experimenting with intraperitoneal injection of transplantable

tumour in susceptible mice showed that carcinosis was only achieved if the peritoneum had been previously injured or irritated. Ferguson (41) in 1940 suggested that prompt amputation of a limb bearing osteogenic sarcoma may promote rather than prevent pulmonary metastases. Unexpected clinical results of this nature led Mider (102a) in 1956 to state "A growing primary malignant neoplasm may conceivably restrict the ability of its metastases to grow." This thesis was subsequently challenged experimentally. Schatten (127) showed that, in mice, amputation of a tumour bearing, but not the healthy contralateral, limb caused a striking increase in the size and incidence of pulmonary metastasis. Martinez et al (98) obtained similar results with similar experiments and they were able to show a critical time limit before which amputation was curative and after which it was metastatogenic. Yet in other experiments a non-specific laparotomy increased the incidence of pulmonary secondaries from intravenously injected cancer cells (18 and 125). Fisher and Fisher (45) studied the growth of hepatic metastases following intra-portovenous injection of cancer cells and they were able to show twofold increase when the rats had partial hepatectomies immediately after injection, or had open liver massage two weeks later. It is tempting to suggest that these results may imply disturbed Kŭpfer cell function, but there is no evidence in support. However, all the above experiments taken together indicate a delicate dynamic equilibrium between host, primary and secondary tumour.

It is perhaps natural that the lymph gland system should be considered as sentinels of host defence, more especially because of their

known participation in infection and the grave prognosis that their gross involvement with neoplasm connotes. For many years it has been known that lymph glands are efficient filters that trap cancer cells and prevent them entering the general circulation as was elegantly shown by Zeidman and Buss (146) when they injected cells of a transplantable tumour into the popliteal lymph nodes of a chicken. As early as 1926 Murphy (110) raised the question of the role of the lymphocyte in the body's resistance to cancer. More recently Black, Oper and Speer (13, 14) have observed that the prognosis for cancers of the breast and stomach are better when the primary tumour is infiltrated with lymphocytes and the regional lymph nodes show 'sinus histiocytosis'. These reactions are similar in homograft rejection and suggest an immunological mechanism.

There is a good deal of evidence (vide infra) that tumours have different antigen mosaics. Whether this alteration in genetic composition be achieved by mutation or viral inclusion is not, from the point of view of this discussion, of moment. What is of importance is that the tumour should contain antigenic material sufficiently foreign to the host to achieve histio-incompatibility and evoke the host's immunological mechanisms. There is, however, a school of thought that believes that the cancer state is associated with a deletion of protein constituting the "identifying antigens" (Green) (62, 63), by a form of induced autoimmunity in which the carcinogen acts as a haptene. If this be so then the reticulo-endothelial system is not only unable to furnish defence to the host but has been "intimately concerned" in the establishment of the tumour.

The regional lymph nodes have long preoccupied the minds of surgeons and associated cancer therapists. In homograft rejection they play a key role. Immunity against tissue appears to be mediated by cell bound antibodies and "second set" response can only be passively transferred by cells not serum. Moreover, Billingham et al (10) showed that such immunity, in the mouse, was only carried by the immediate regional lymph glands, those from the contralateral side being without effect. This raises the whole issue as to whether regional glands constitute a defence, perhaps the only defence, against widespread dissemination of cancer cells. Does their removal convert a beach head into a major invasion?

Kaplan and Murphy (85) and Von Essen and Kaplan (140) found that local irradiation of a radio resistant metastasising tumour in mice was followed by an increase in the incidence of pulmonary metastases from 9.6% to 43.5%. That this change was a radio induced mutation was excluded by retransplantation to new hosts; that radiation did not effect the host was shown by pretransplant treatment in control series. There seems no doubt that the radiation had, in some way, affected the host tumour balance. Could it be by affecting the radio sensitive lymphocyte?

The principle of local excision or destruction of the cancer within the lymph node barrier has been employed by many cancer therapists for treatment of cancer of the lip, skin and mouth for many years. The advantage of simultaneous prophylactic resection of lymph nodes has not been proven to the satisfaction of many. In melanoma Lund (95) showed quite the converse.

The survival following simultaneous dissection of the primary melanoma and palpable lymph node metastases is practically nil, yet nearly half of the patients have been reported to survive more than 5 years when the primary is first removed and the involved lymph nodes are resected at a later operation.

Thus for the young surgeon entering the field of cancer therapy there is confusion and difficulty. He can no longer accept the classical precepts and procedures without doubt, or at least stringent critical appraisal, however attractive their technical execution might be. Moreover this is at a time when the incidence of many cancers is increasing, be it by virtue of greater life expectancy, greater urbanisation of population, pollutions of air and water, "purifications" of foods, or greater release of nuclear energy. Host resistance is seemingly important, though whether it be general or local is not yet clear. The role of the regional lymph gland is obscure but may well be crucial. What of paired organs when one develops cancer? Are both initiated with the promotion of one a little earlier than the other? It is for these reasons that the author chose to take surgical time, use money and space in a Surgical Department virgin to this work, to try to study in as simple way as possible the relationship of tumour to host.

The implantation of bright eyed, sharp toothed, bad tempered, virgin female mice with breast cancer requires no surgical techniques, yet I believe that the problems involved are of the highest surgical import.

Tumour Antigens

If resistance of the host to its neoplasm is to be mediated by immunological means in a way similar to the body's defence from other invasive processes, then it is implicit that the cancerous cells must contain antigenic material not possessed by, or exposed within, normal tissue, i.e. tumour specific antigens. There is a growing weight of evidence that this is true although, at once, there appear to be differences (whether qualitative or quantitative is as yet unknown) between the three types of experimental tumours - transplanted, induced and spontaneous. Nor is this field very well explored. "The relation of immune reactions to neoplastic growth and potential is of fundamental importance in oncology, yet our knowledge of immunology in relation to cancer is still in an elementary stage. Despite the fact that many attempts have been made to use the methodology of classical immunology and serology, the conceptual and actual tools of the discipline have more in common with transplantation biology than classical immunology." (Hirsch, 78).

Various methods have been employed to try to demonstrate tumour specific antigens. These include:

(i) Active immunisation of an animal with strain specific tumour or tumour preparation, followed at an interval by challenge by tumour inoculation.

(ii) Preparation of antibodies in a foreign or isologous host and the testing, following adsorbtion with homologous normal tissue, by carcinostatic, neutralising or cytolytic effects.

(iii) Skin sensitivity tests (e.g. Grace and Kondo) (59).

(iv) The use of anaphylactic systems in which sensitisation is achieved with tumour tissue and desensitisation with normal homologous tissue. Challenge is made with the tumour material to test for residual hypersensitivity e.g. Zilber (147, 149), Gorodilova (58), Kolmykova and Yeroshkina (89, 90), Grace and Lehocsky (60).

(v) Acquired tolerance experiments.

(vi) Classical immunological methods including complement fixation, precipitation reactions, agar gel diffusion, haemagglutination methods, labelled antibody techniques and fluorescent antibody methods.

1. Transplanted Tumours

Historically the most frequently used and almost classical method is one based on active immunisation (method (i) above). According to material used, dosage and time scale, enhancement or resistance may be achieved (Kaliss, 83). Both results, however, depend on histocompatability gene difference. However, the existence of even a slight genetic gap between tumour and host in the experiment gives results which simulate the presence of tumour specific antigens. The many pitfalls in this approach have been extensively written upon (6, 53, 74, 76, 87 and 99). Seemingly specific

antigens have been demonstrated in long transplanted tumours (40, 122) and in tumour cells propagated for long periods in tissue culture (134) and in cells with neoplastic potential derived in vitro from normal cells in tissue culture (123, 125). Although there is a great deal of suggestive evidence the known tendency for independently carried sublimes to develop isoantigenic differences make rigid proof difficult. However, much has been written on this subject (Hauschka (74), Hirsch (76), Klein (87), Revesz (122), Stern (136), Barrett (6), Witebsky (145), Southam (133), Milgrom (103), Gorer (53), Medawar (99).

2. Spontaneous Tumours

The evidence for the presence of true tumour specific antigens in spontaneous tumours of experimental animals is still tenuous. Gorer (54) found that two spontaneous leukaemias in mice that seemed to contain specific antigens. In the human, the case for host resistance, and therefore, by implication, tumour antigens rests on rather vague and circumstantial evidence as observations of spontaneous regression (39, 67, 137), the frequently observed long latency of tumour cells (52, 67, 69, 129), lymphoid infiltration near tumour sites as well as lymph node enlargement (15) and plasma cell infiltration seen in some human cancers (18). Support of these observations can be found in the work of Finney (42, 43), Bjorklund (11, 12). The isolation of lipid hapten from human tumours grown in conditioned rats which may be

tumour specific was achieved by Rapport (121). A phospho lipid hapten 'malignolipin' was demonstrated in cancer tissues by Kosaki (92). Davis and Busch (32, 33) have isolated an acid soluble nuclear protein found uniquely in neoplastic tissues. Distinct plasma components have been found both in patients and in mice with neoplastic conditions by a number of workers (Korngold (91), Makari (97), Burrows (20), Burrows and Neil (21), Miller and Bernfield (104).

3. Carcinogen Induced Tumours

The evidence for true tumour specific antigens is much stronger in the case of induced tumours and have been demonstrated by Gross (65), Foley (47), Baldwin (5), Prehn and Mann (117), Prehn (118), Revesz (122) and Old et al (113) by active immunisation techniques. Klein et al (88) also demonstrated resistance in the primary autochthonous host. Day et al (34) showed that radio labelled antihepatoma antibodies localised preferentially in the hepatoma tissue. Hoepke (79) showed that rats developing benz-pyrene tumours formed large numbers of plasma cells and lymphocytes both in their reticulo-endothelial system, and infiltrating and partially destroying the tumours. Tumour specific antigens called 'X antigens' have been demonstrated in a number of induced leukoses by Gorer (55, 56, 57) and Amos and Day (4).

The fact that it is relatively easy to demonstrate tumour specific antigens in induced tumours raises the problem as to how such a clone

of cells can flourish without being destroyed by the host's reticulo-endothelial system. Depression of this system by carcinogenic agents has been postulated by a number of workers (76, 77, 88, 117, 136). Hirsch (76) has pointed out that carcinogenic and mutogenic agents such as ionising radiation, radiomimetic chemicals, and several carcinogenic substances are known to lower immune responsiveness. Their use might thus have twofold effect both in the induction of tumour cells as well as in increased opportunities for such cell variants to establish themselves.

It would seem, therefore, that there is a fair weight of at least suggestive evidence as to the existence of tumour specific antigens. Their ability to provoke immunological response would depend on a number of factors including accessibility of the discordant antigen to the host's immunological mechanisms and their histocompatability "strength". In mice, for example, H_2 locus incompatability will cause a skin homograft to be rejected within 12 days, whereas H_2 compatability, despite any other incompatability, will allow skin homografts to survive for at least 20 days. Such factors as these may well explain apparent differences in the antigenicity between the three types of experimental tumours. Nevertheless, that a malignant condition can exist in which a tumour appears to prevail inspite of antigenic incompatability is of the greatest theoretical importance. From this consideration three questions appear pertinent (see fig. 1, page 1).

(1) Is an established and growing tumour sufficiently different

antigenically from its host to provoke immunological response?

(ii) If so, is there a barrier of access between these specific antigenic qualities and the host's reticulo-endothelial system?

(iii) Have the competent clone of cells (19) capable of attacking and destroying the neoplastic cells been eliminated from the host's R.E. system?

With this background of thought, the chance observation of Professor Haddow of spontaneous regression of animal tumours subsequent to accidental second inoculation (that I heard about orally) and the work of Ischibashi (80) in which subcutaneous injection of sarcoma inhibited the growth of Yoshida ascites tumours in mice, with his bold application of this principle to human patients, prompted this present work. It was planned to study the effects of vaccinating tumour bearing animals with tumour material. No report was found of this sort of procedure on an established tumour, although there is considerable work onvaccination prior to tumour inoculation. This is by no means a new principle in clinical practice, however. In 1902 von Leydon (quoted by Grahams) (61) administered the "juice" from tumours to three cancerous patients, two of whom were said to have improved. In 1955 Stone (138) implanted frozen blocks of cancer tissue into inoperable cancer patients to try to promote resistance without success. In 1959 the Grahams (61) used a vaccine and adjuvant in 114 patients with apparently encouraging results. In the same year Finney and Wilson from Dallas (44) administered tumour with

Freund's adjuvant to nine patients and obtained inflammatory reactions 15-25 days later at the site of their neoplasms. Blood taken at this time was readministered later to produce regression of 65% of subdermal nodules.

It was decided to see if any effect could be obtained by a planned programme of vaccination with homogenated tumour and Freund's adjuvant on an established transplantable tumour in inbred animals trying to avoid isoantigenic difficulties. By this means it washoped to present tumour antigens to the host in an assimilatable form at varying times throughout the evolution of the neoplasm and to see if the stimulated reticulo-endothelial system could modify the tumour behaviour. In this way it was hoped to try to answer the three posed pertinent questions (page 19) in this mouse system at least, by attacking the second one first.

PART B

EXPERIMENTAL PROCEDURES

SECTION I

1. Materials

(i) Host

Because it was mandatory to use highly inbred animals so that tissue could be implanted from one to the other without isoantigenic difficulties, and because only two strains of inbred rats are available and these in short supply (100), it was obligatory to use mice although their size, disposition and habits do not make them ideal. Grey female virginal DBA/2J Jax mice were chosen. These animals have histo-compatability alleles of a at H_1 and d at H_2 (131 and 132). Originally developed by Little in 1909 from mice used in colour experiments, they were obtained by Jax in 1948 at F.26. There is some incidence of spontaneous mammary tumours in old breeding females, but this has declined since 1955 (73).

They have a life span of 15-19 months and a body weight of 23.4 ± 0.6 g. at 120 days (73) and were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Habor, Maine, U.S.A. at 7-8 weeks of life. They were kept in steel cages at a temperature of 75°F with free access to water and "Purina Laboratory Micromixed Chow". They were generally kept in groups of ten, sometimes twenty. Various methods were tried at labelling

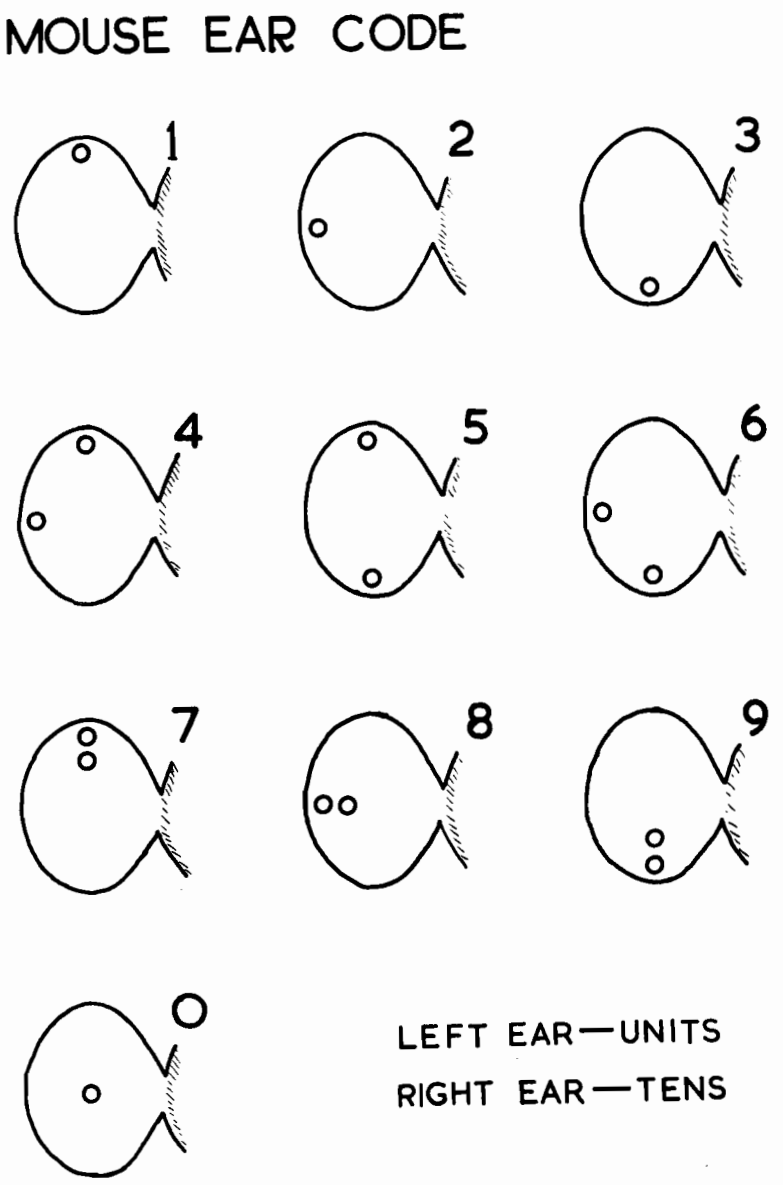

Figure 2

Ear code used for mouse identification. Circles represent holes punched in ears. Up to 100 mice can be labelled in this way but with small ears recognition requires careful unfolding of the pinna which is time consuming.

individual mice in each group. A suitable code was devised and the standard method of punching holes in the pinnae was attempted (see fig. 2, page 22). Although this was possible to do if care was exercised even with small ears, recognition of the individual mouse was sometimes tedious as it required unfolding and inspection of the ear. This manoeuvre was not always as easy as it sounds with a struggling, sharp toothed mouse and was in our hands, at least, time consuming. Hence this method was later abandoned and tail banding with marking pencils (of the 'Speedry', of Speedry Chemical Products Inc., type) employed. The bands started at the tail base and worked distally for the first five and then from the tail tip proximally for numbers 6 to 10 (see fig. 3 page 25). Different colours were initially employed for different groups and this was found quite satisfactory so that not only individual mice but also separate groups could be recognised at a glance. Occasional remarking was required when the bands began to fade. Colours were abandoned in the experimental groups, however, in favour of a uniform black, lest the individual dyes exerted different effects either by absorption through the skin or neighbourly licking. Groups I-IX were thus marked with black banding. Ten mice maintained under these conditions for eight months appeared healthy with a glossy coat, a mean weight of 27.6 g. and a mean haematocrit of 46 at the end of this period (see fig. 4 page 26).

(ii) Tumour

The transplantable adenocarcinoma Ca D2 that was spontaneous to the DBA/2J breast in 1960 was used because of its good blood supply and

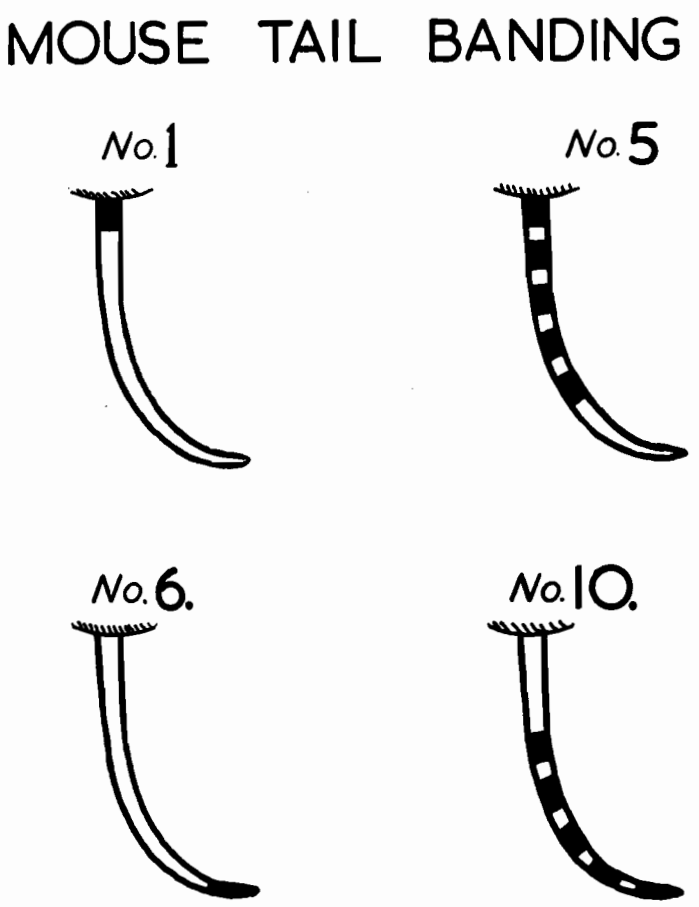

Figure 3

Tail banding used for mice identification. A simpler and easier method of marking and recognition but limited to groups of ten unless a colour code is used.

Figure 4

The DBA/2J Jax mouse at 10 months of age. Group VII.

Three of the control mice after eight months captivity under laboratory conditions.

Note apparent health and glossy coat. Compare figure 10. p. 37.

uniformity of cells. The gross appearance of the tumour is as a soft pink nodule (see fig. 5 page 28). Microscopically the tumour cells are large, uniform, arranged in alveoli and solid sheets with large vesicular nuclei exhibiting frequent mitoses (see fig. 6 page 29). The tumour is supported by a fairly fibrous stroma and a good blood supply (see fig. 7 page 30). On transplantation it becomes palpable after 4-5 days and transplantable at 14 days. It kills its host at about 3 weeks.

(iii) Freund's Adjuvant

Adjuvants are designed both to stimulate the reticulo-endothelial system and prolong the antigenic stimulus. Whether this be achieved by delayed absorption or microembolisation is still a matter of conjecture. Freund's complete adjuvant contains Aracil A a light mineral oil, Drakeol N.F. an emulsifying agent and killed tuberculosis bacilli (25, 49, 50). It was obtained from Difco Company of Detroit.

2. Proof of Histo-compatability

Full thickness skin grafts were interchanged between mice of the same age and batch and different ages and batches to demonstrate transplantation compatability. Initially single grafts were exchanged between mice more or less at random. These particular mice appeared to be very intolerant to inhaled ether developing bronchorrhoea and lethal respiratory obstruction, although chloroform was accepted. Anaesthesia was therefore by intra-peritoneal 'Nembutal' in dosage of approximately 0.02 mg. per g. body

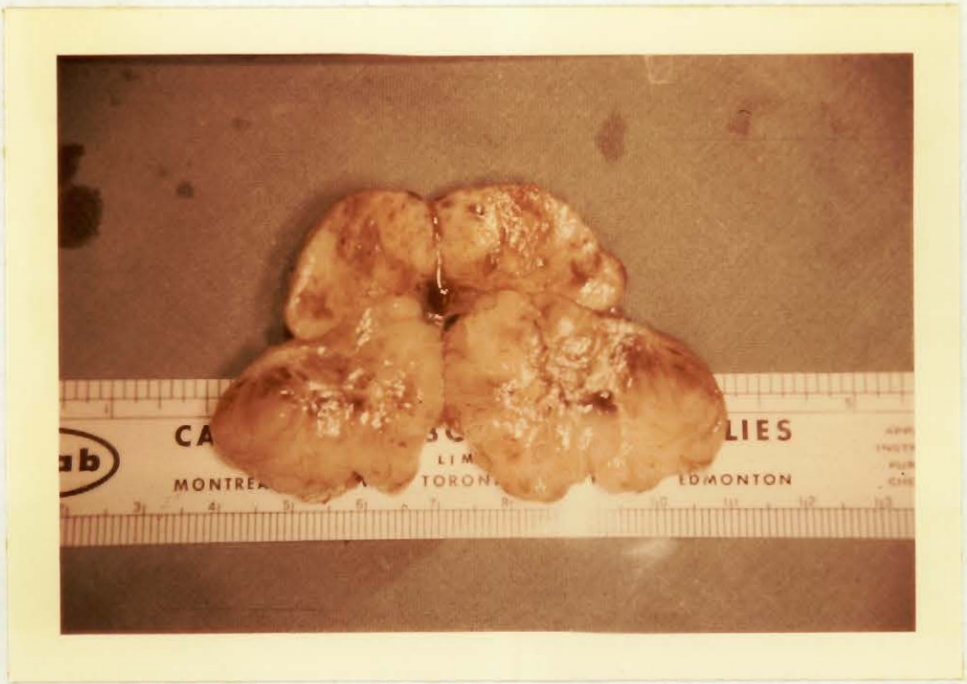

Figure 5

The CaD_2 Adenocarcinoma 4 x 4 cm. tumour bisected.

Note absence of necrosis, firm structure.

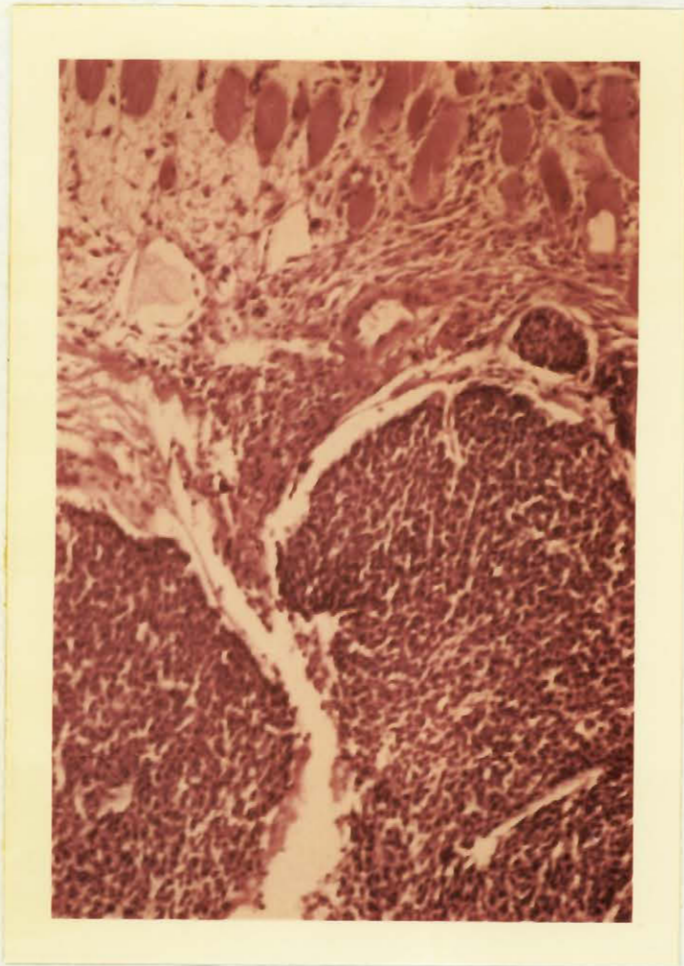

Figure 6

The CaD_2 Adenocarcinoma x160 H and E.

Note neoplasm invading striated muscle inspite of pseudocapsule.

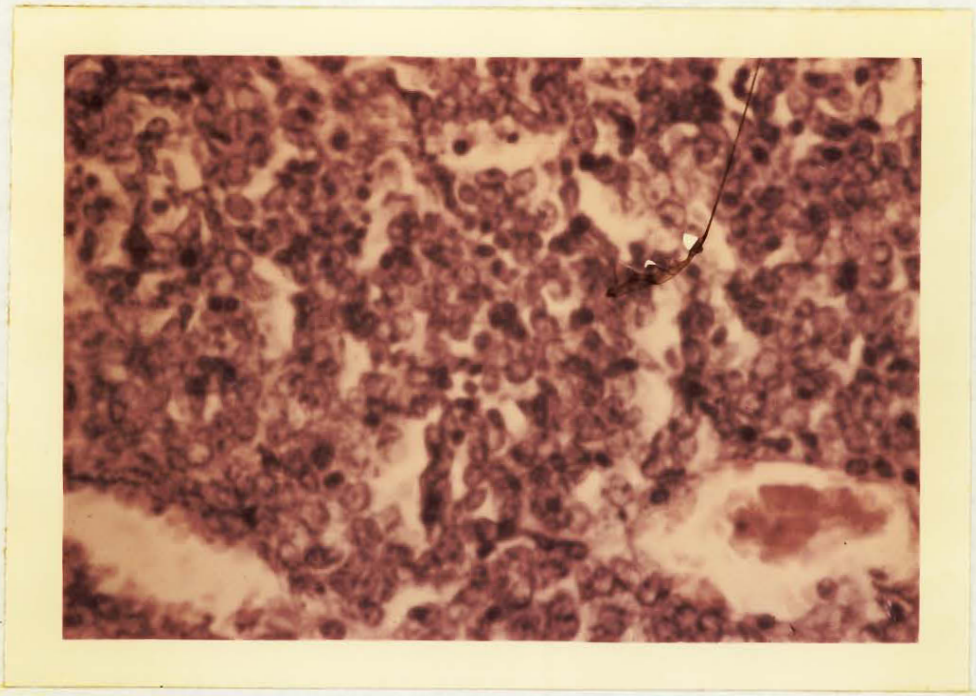

Figure 7

The CaD_2 Adenocarcinoma x 600 H and E.

Note relative uniformity of cells with good blood supply and frequent mitoses.

weight (a solution of 0.4% was used and up to 0.1 ml. given). There appeared to be considerable variation of tolerance to this drug. The fur was clipped off the dorsum of the animal's body with electric shears and a solution of 'Proiodine' painted on the exposed skin (this was, very probably, unnecessary). Clean hands and instruments were used but no attempt made at sterility. Full thickness grafts were then removed from the back of each mouse and interchanged after the panniculus carnosus had been carefully scraped off the skin. Grafts were secured in place by using black braided 000, 000 silk on an atraumatic reverse cutting needle. Stay sutures were inserted at the four points of the compass and then a continuous over and over suture run round the graft. A plaster of Paris jacket was applied over a small dressing. This I believe was probably necessary only to prevent the grafts being eaten by cage mates and this could be better circumvented by allowing convalescence in separate cages.

Subsequently, largely because mice tended to arrive in groups of four lots of fifty, a technique was developed in which skin was exchanged between four mice (one from each lot) so that each mouse had three grafts (one from each of the other three) (see fig. 8 page 32). A large cork borer was used to mark the skin so that graft and bed would be congruent, and orientation of hair adjusted by insertion of a marker suture before cutting. Grafts were exchanged not only amongst mice of the same age, but also between new and old consignments.

Figure 8

Skin transplantation technique. Graft removed from animal on right (marked out with large cork borer to achieve congruity) being applied to mouse on left. Four stay sutures have been inserted and a continuous over and over stitch (000, 000 silk) is just commenced. Two other grafts from other animals already in place on each mouse.

No skin rejections were seen although there were a few technical failures. In one instance an antibiotic spray was used and every single graft failed to take. A few grafts were lost by adherence to the plaster corset. A few mice were lost either from poor anaesthesia or respiratory embarrassment from too tight a corset. Most grafts were observed for at least two months and in all good growth of hair was obtained and the graft being visible only because the direction of the hair follicles had been altered (see fig. 9 page 34).

3. Tumour Implantation

The method of tumour implantation used at the Jackson Memorial Laboratory depends upon the insertion of "1-3 small pieces about 5 mm^3 into a number 13 trocar" (100). This was found difficult to quantitate in our hands and as a consequence a tumour suspension method was developed. The donor animal was killed by breaking its neck (although use of Nembutal, ether or chloroform had no apparent deleterious effect on tumour take) and the body immersed in 70% alcohol for five minutes. It was then removed and pinned out on a cork board covered with sterile paper. Sterile instruments were used: The skin was opened with one set, the tumour removed with a second, any necrotic or unsatisfactory portion being discarded, and then minced in a sterile petri dish with a third. The mince was then transferred to a 10 ml. syringe (with a Luer lok) and its volume measured. An equal volume of sterile physiological saline was added and the mixture squirted into a sterile rubber-

Figure 9

Skin graft eight months after exchange, only visible by altered orientation of hair (white edge on left hand mouse). This was the longest period for which a graft was observed.

capped vial through a 14 gauge needle. Positive pressure was achieved in the vial by injection of air and this enabled the tumour cell suspension to be repeatedly withdrawn and reinjected until it would easily pass through an 18 gauge needle. (I am indebted to Mr Ivor Gabor for showing me this method.) Implantation was made subcutaneously into the recipient animal using an 18 gauge needle on a tuberculin syringe. The skin was always swabbed with a skin cleaner although its efficacy appeared doubtful. 70% alcohol was initially used but had the grave disadvantage, in our hands, that the mice became very drunk - to the point of narcosis - presumably from licking each other. As a consequence aqueous Benzalkonium Chloride 1 in 1,000 was used. The needle was inserted under the skin in the right iliac fossa and run up to the right axilla where the dose of tumour cell suspension was delivered. The needle track was obliterated by pressure as the needle was withdrawn to stop escape of the cells and implantation in the skin

4. Tumour Behaviour

Study was made of the behaviour of the implanted tumour and the natural history of the cancerous process within the mouse. All possible mice (cannibalism was rife) were routinely examined post mortem although the skulls were not latterly opened. No distant (blood borne) metastases were discovered in nearly 800 examinations although spread across and dissemination within the pleural and peritoneal cavities was quite common once tumour had gained access by direct spread. Once within these serous cavities multiple

nodules would develop in pleura or mesentery and lymph glands, especially mediastinal, would become involved. Regional lymph glands were, otherwise, but rarely obviously enlarged or infiltrated. The tumour itself would grow to a good size without central necrosis (see fig. 5 page 28) and would appear to be well circumscribed and pseudoencapsulated (see fig. 6 page 29) although seemed to grow with facility through body wall of abdomen or thorax. The tumour would quite often reach one quarter to one third the weight of the animal. A haemothorax or haemoperitoneum was not uncommon once these cavities were grossly involved and seemed often to be the result of infarction or infective necrosis of a tumour plaque, although the possibility of an induced defect in clotting mechanism was not studied. It was rare, but not unknown, to see any obvious interference with vital function such as obstructive uropathy or bowel obstruction. More commonly the animal would sustain quite a large volume of tumour becoming progressively more listless with thinning and dystrophy of its coat, scaling of tail with ulceration of paws and tail and obvious signs of diarrhoea (fig. 10 page 37). A quite marked feature was the development of an angular thoracic kyphosis (see fig. 11 page 38). The superficial tumour - especially if ventral - would often undergo ulceration with necrosis and haemorrhage almost certainly precipitated or promoted by abrasion along the cage walls as the wretched animal dragged itself about. Other tumours would exhibit neatly incised punched out areas that would suggest cannibalism, especially if a sick and lively animal were confined within the same cage. Figure 12 (page 40) shows the atypical case. A large tumour

Figure 10

Mouse moribund from tumour. Note poor coat, lethargy, ulcerated hind foot and scaley tail. Perianal staining (diarrhoea) not visible in this view. Compare figure 4. p. 26.

Figure 11

Two three month old litter mates. On right normal control mouse. On left tumourous "runted" mouse. Note poor coat, thoracic kyphosis.

has invaded the thoraco-abdominal wall. The lateral thoracic vessels (or their murine equivalent) have enlarged to supply the tumour. The axillary lymph glands (the standard pair found in this mouse species is clearly seen) have become involved with neoplasm as have the upper mediastinal group. This picture is included because it so clearly shows that the tumour obtains its blood supply from the existing integumental and body wall vessels and also the unusual involvement of lymph nodes in such an anthromimetic fashion.

Despite all these variations of invasion, ulceration and intra-serous haemorrhage, the tumour host balance seemed very precise. In 90 mice, despite considerable variation in dose and site, the mean mouse survival in days remained uniform at 20.5± 0.73 S.D. (vide infra).

5. Experiments to Find a Suitable Parameter of Tumour Growth Groups I-VII

Search was made to find a convenient measure of tumour growth which could be used as an indication of any altered tumour behaviour achieved by subsequent procedures. Animals were weighed daily and their tumours measured in three diameters as done by Prince (119, 120) with vernier calipers. The geometric product of these three measurements was arbitarily called an index. Where two or more tumour masses co-existed the two indices so obtained were added together. Intra-peritoneal extension of the tumour mass (see fig. 13 page 41) could not be measured at all accurately by this method, (intra-peritoneal or intra-thoracic secondaries escaped entirely, and

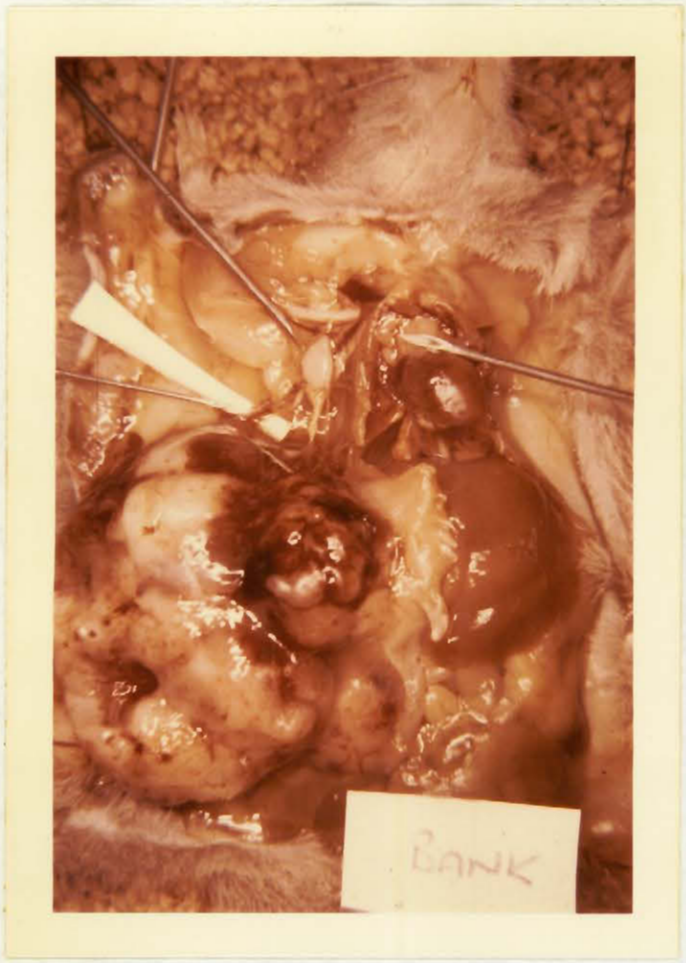

Figure 12

Post mortem examination of atypical tumour bearing mouse (tumour bank mouse). Note large tumour in right body wall supplied by lateral thoracic vessels (slither of card under these vessels). Secondary deposits of tumour in both axillary and mediastinal lymph nodes indicated by needles. Tip of large spleen just visible.

Figure 13

Tumour removed post mortem from mouse No. 5, Group V to show difficulty of "index". Halsted forceps holding abdominal wall to show intraperitoneal extension of growth as large as subcutaneous.

gross departure from the approximate cuboidal dimensions rendered the mensuration inaccurate. However, some approximate correlation was obtained between weight of tumour at death and this index (see fig. 14 page 43). In the later pilot experiments on vaccination and in the group of animals that responded best (Group I), the rate of increase of this "index" was lowered (from 1.85×10^3 to 0.68×10^3) and the period of rapid growth (as shown by index) delayed from the tenth to the sixteenth day (see fig. 15 page 44). It would therefore seem that such an "index" might have some value in the assessment of tumour growth and activity but its measurement was found to be too time consuming for single handed work.

Rather surprisingly, I thought, absolutely no relationship was found between weight of tumour at death and longevity (see fig. 16 page 45) even though the hosts were highly inbred and considered identical. However, that this tumour-host system should so reproducibly produce death at twenty days implies that some factor other than mere tumour size is operative. Rate of change of size was demonstrated to be significant from the studies with the index (fig. 15 page 44). Simple measurement of the animal's body weight gave some crude indication of tumour development (see fig. 17 page 46) although the fluctuations manifest in the control group demonstrate the inaccuracy of the method. This method might have been more uniform if the mice had been kept longer under the laboratory conditions after their air freightage to become acclimatised before being subjected to test.

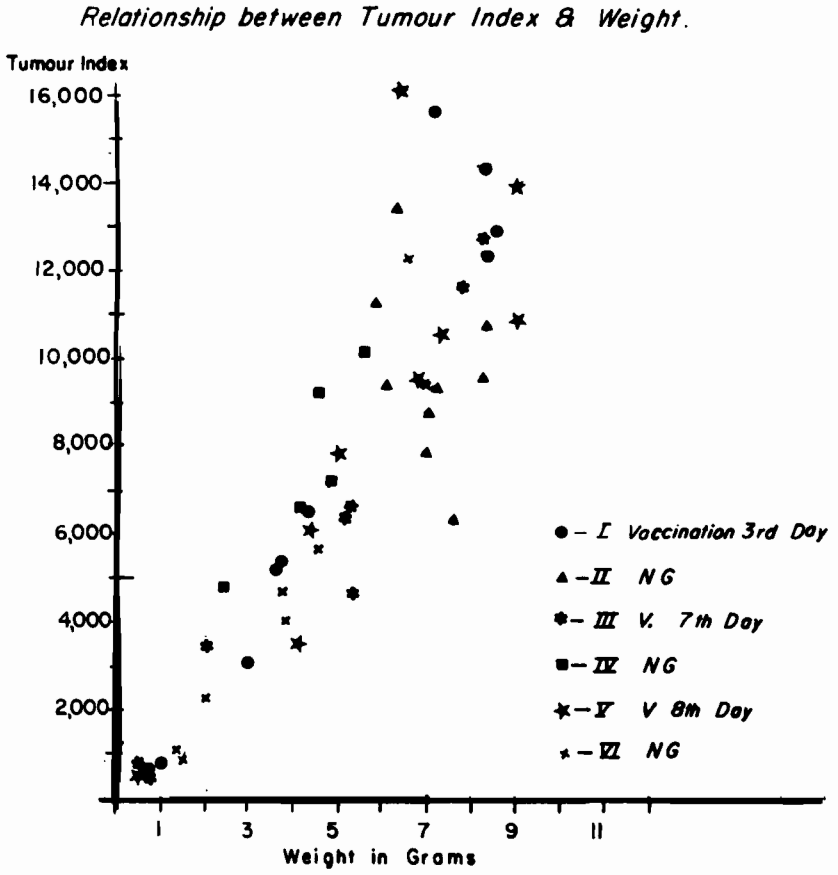

Figure 14

Relationship of "Tumour Index" and weight of tumour at post mortem - Group I-VI from Experiment 8.

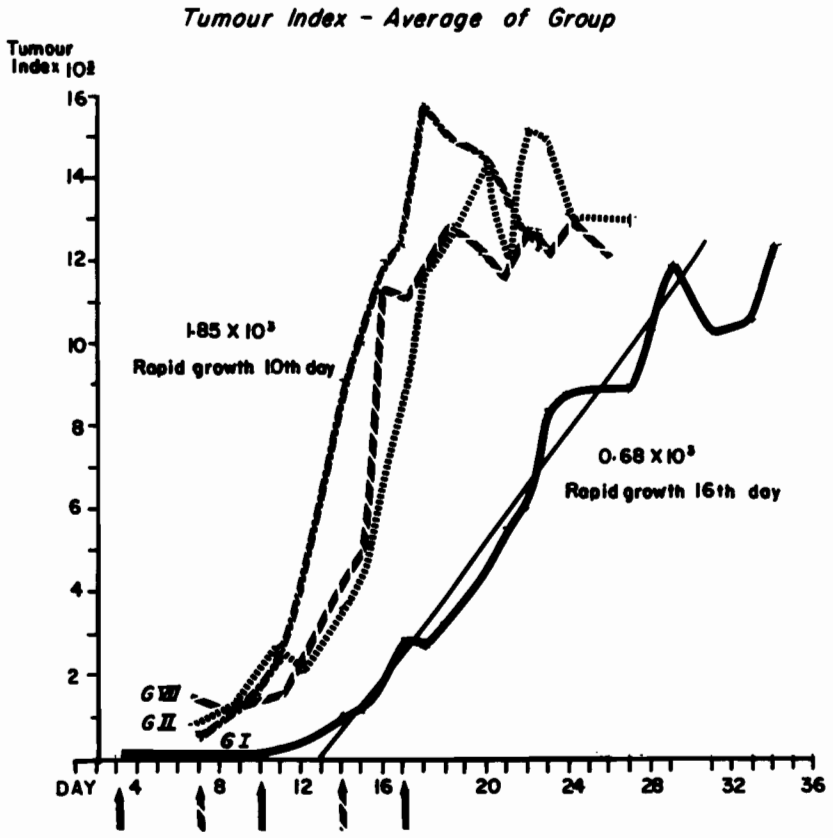

Figure 15 Rate of change of tumour index with time Groups I, II, III, IV.

Ordinate tumour index cubic mm.; abscissa - days after tumour implant; vertical arrows indicate vaccination.

Third day vaccination reduces gradient of curve from 1.85×10^3 mm^3/diem to 0.68×10^3 mm^3/diem and delays onset of rapid phase from 10th to 16th day.

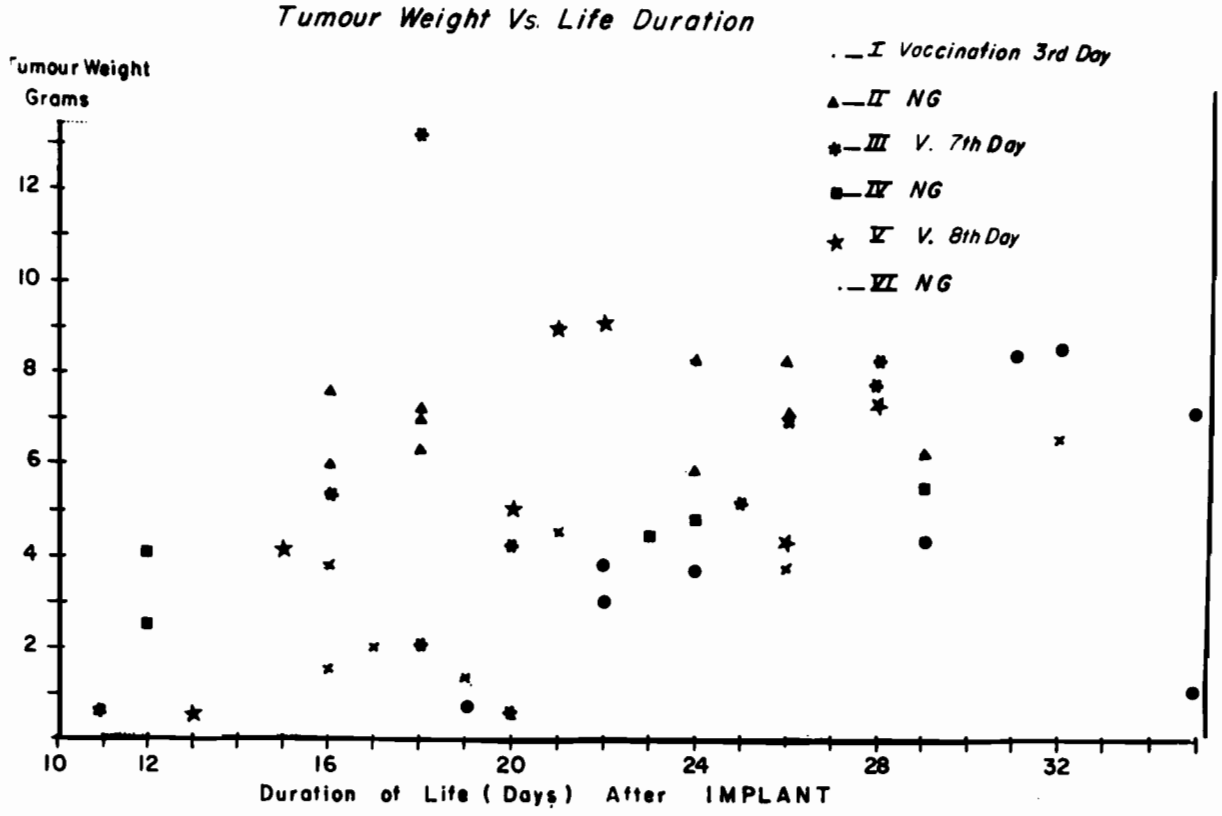

Figure 16

Tumour weight at post mortem and longevity. Groups I-VI Experiment 8.

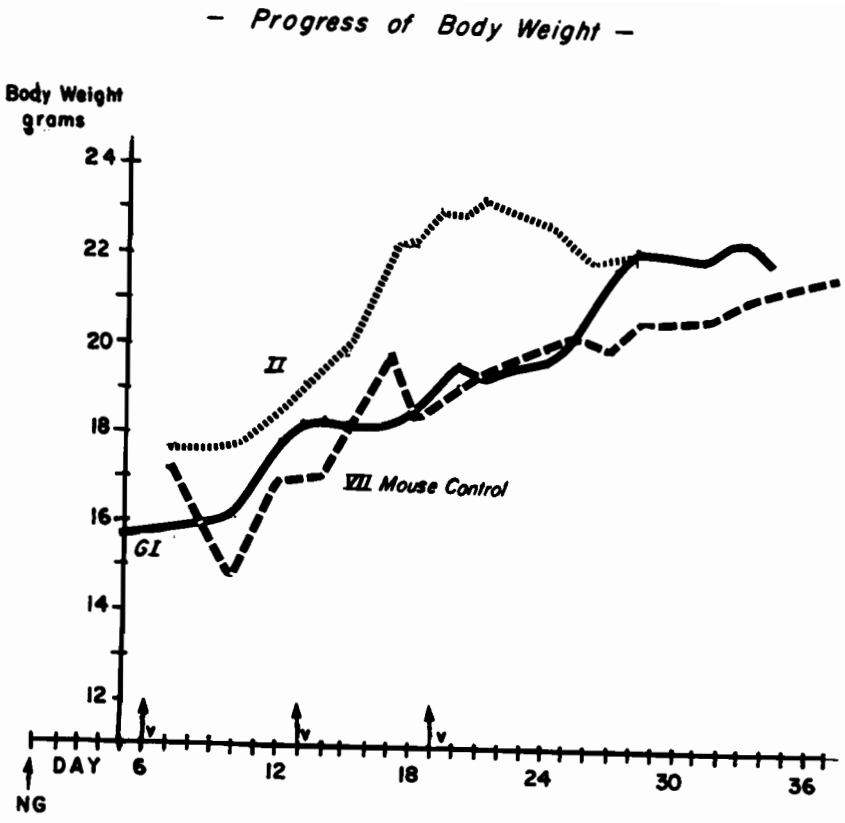

Figure 17

Progress of body weight with time. Groups I, II, VII (control).

From these studies, however, it was decided that subsequent experiments would be based solely on life duration of the tumourous animals subsequent to implantation as being the only feasible method under the prevailing conditions. In general groups of ten mice were used and the average survival time taken as the mean mouse days (M.M.D.).

6. Experiment to Find the Effect of Tumour Dosage on Life Duration

Groups IV, XI, XIII, XV

As life duration measurements were to be taken as evidence of tumour activity, it was thought necessary to investigate any variation in this parameter subsequent upon fluctuation of dosage of tumour implantation that might accrue from accident, carelessness or inaccuracy.

Animals were divided into groups of ten and each group given a different dose of tumour suspension subcutaneously varying from 0.2 ml of a 1:1 suspension to 0.1 ml. of a 1:4 suspension (8 fold difference). One group, Group VI was also included in which implantation was achieved by the trocar technique as practiced at the Jackson Memorial Laboratory (see page 33). Very little alteration in life duration was seen (M.M.D. from 19.2 - 20.5) (see fig. 18 page 48 and fig. 19 page 49).

7. Experiment to Find the Effect of Intra-cutaneous Injection of Tumour on Life Duration

Groups XVII, XVIII.

In view of Ischibashi's work (80) showing that subcutaneous

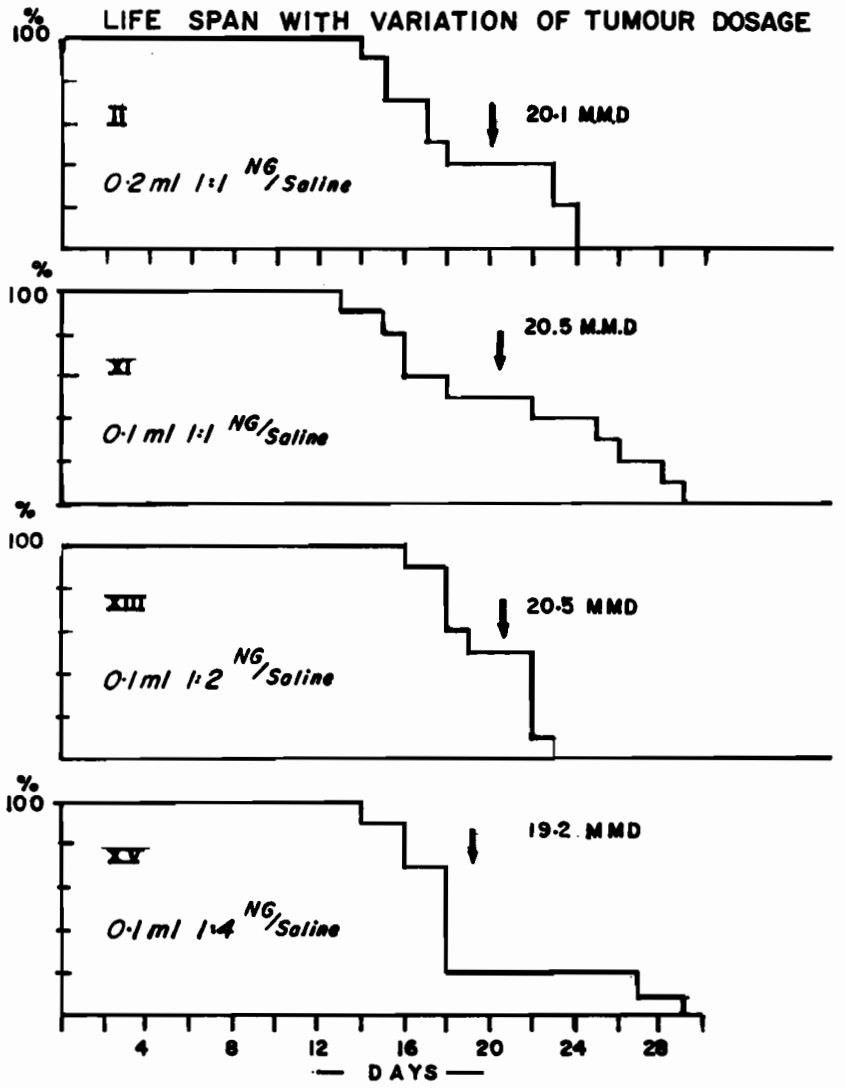

Figure 18

Life span with variation of tumour dosage. Groups II, XI, XIII, XV. 8 fold variation in dosage without significant variation of longevity.

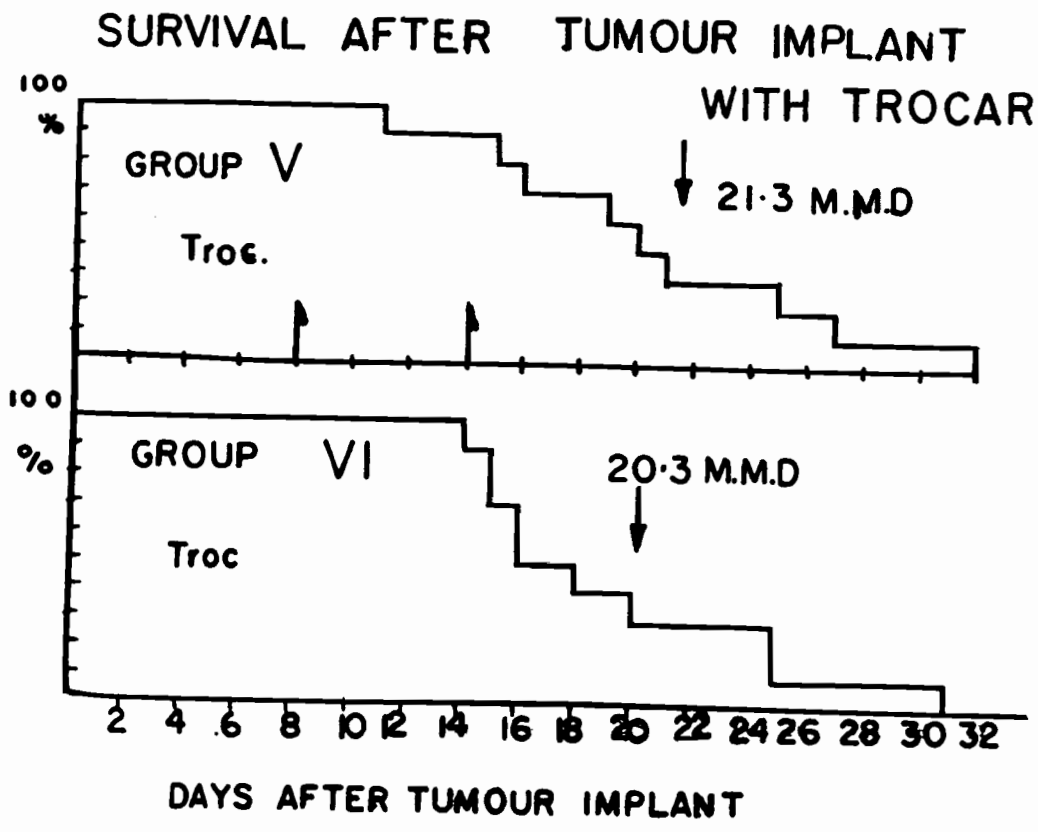

Figure 19

Survival after trocar method of implantation. No difference from saline suspension method.

No significant effect from 4th day 'vaccination'.

injections of Yoshida ascites tumour inhibited intra-peritoneal growth in rats, and his bold application of this principle by using intra-cutaneous tumour autografts in tumourous human patients, experiments were made to see what intra-cutaneous injection of tumour would achieve in the Jax mice. Two groups of ten were given intra-cutaneous injection of tumour suspension (0.05 ml. 1:1 new growth to saline suspension). The M.M.D. for the twenty mice was 21.5 days, the two groups being 21.3 and 21.7. This is only very slightly higher than for the subcutaneous group at the same dosage (20.5 days) (see fig. 20 page 51 and fig. 24 page 58).

From experiments 6 and 7 it was concluded that dosage was by no means critical and that accidental intra-dermal implantation (along the needle tract for example) was not important. Any variation in life duration of the experimental mice could not therefore be ascribed to these factors.

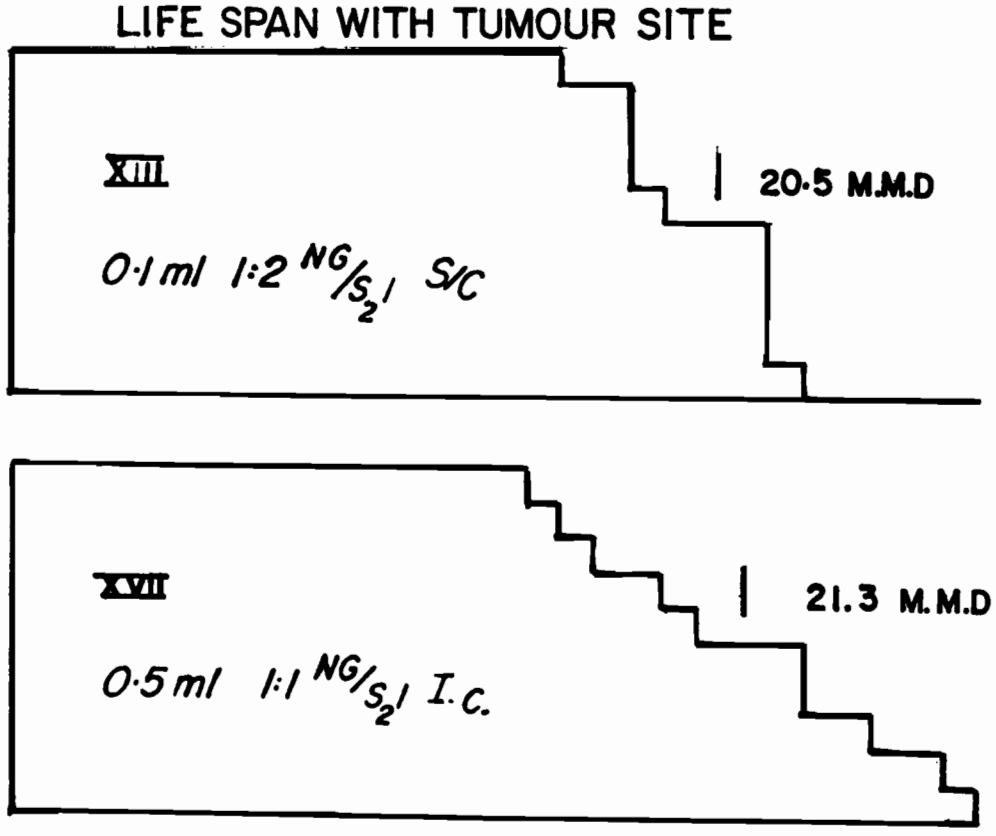

Figure 20

Comparison between subcutaneous and intracutaneous inoculation of equal doses of tumour - no significant difference.

SECTION II

8. Experiments with Aqueous Vaccine

Groups I-IX and XI-XVI

Being unaware of any report on attempts to modify an established tumour by vaccination in the experimental animal, it was thought worthwhile to attempt to vaccinate tumour bearing mice with an aqueous tumour homogenate and Freund's adjuvant. A pilot study was therefore undertaken.

"Vaccination" Procedure

Tumour was ground in a sterile Teflon homogenisor in twice its own volume of sterile water. Equal volumes of this homogenate and Freund's complete adjuvant were then homogenised together and 0.2 ml. aliquots administered subcutaneously with a sterile tuberculin syringe at weekly intervals for 3 doses (where possible) to groups of ten mice inoculated with tumour 3, 4, 7 and 8 days previously (the day of inoculation counting as the first day). In case the tumour dose/vaccination dose was critical, varying doses were implanted as in experiment 4 and the animals vaccinated on the fourth day. In the third day group (Group I controlled by Group II) a 32% increase in life duration was encountered which was statistically significant ($P < 0.001$) (see figs. 21 and 22 page 53 & 54). Rate of increase of "tumour index" was also reduced from 1.85×10^3 mm^3/diem. to 0.68×10^3 mm^3/diem. and the time of onset of the rapid growth phase delayed from the tenth to the sixteenth day (see fig. 15 page 44). Increase in body weight did not exceed

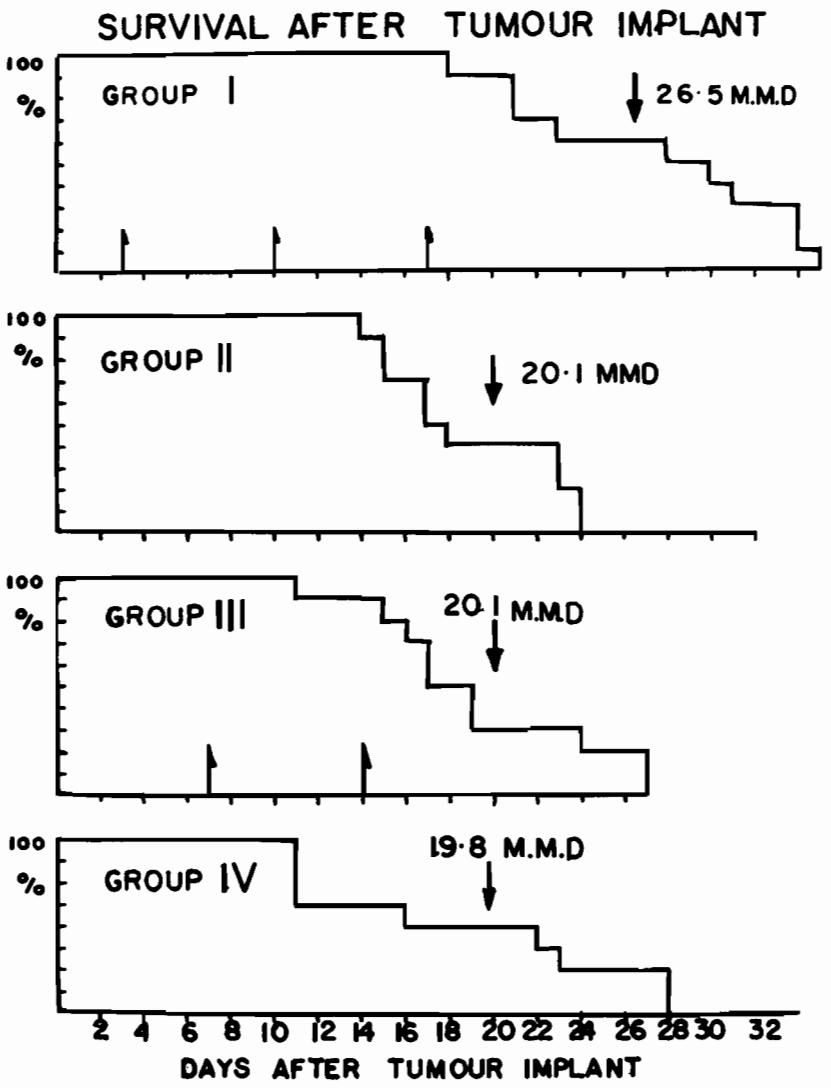

Figure 21

Pilot vaccination study. Groups I-IV.

Group I vaccinated on 3rd day post implantation Group II control.

Group III vaccinated on 7th day post implantation Group IV control.

Group IV contained only 8 animals (2 escapes). Group I has a significantly greater ($P<0.001$) life span than Group II.

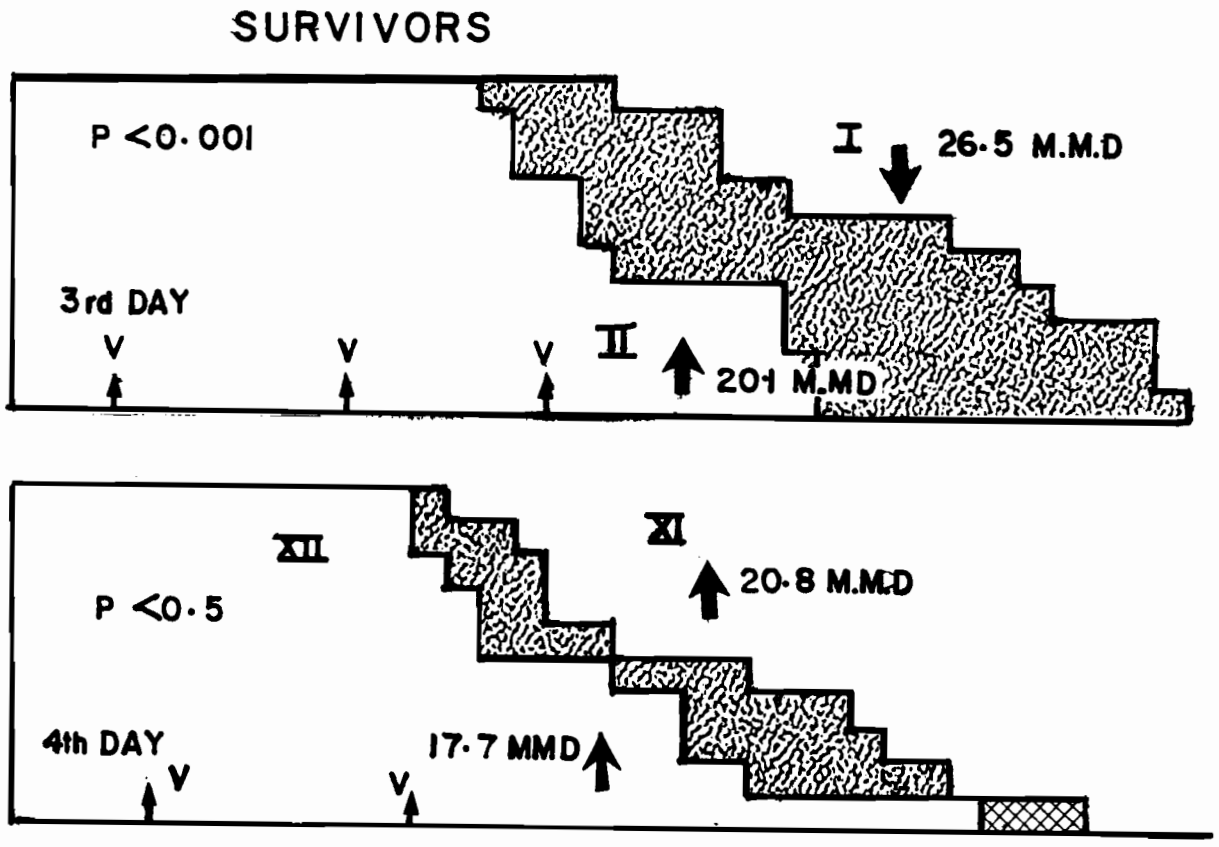

Figure 22

Pilot vaccination study. Groups I, II and XI, XII.

Group I vaccinated on 3rd post tumour implant day Group II control.

Group XII vaccinated on 4th post tumour implant day Group XI control.

Group I has a significantly greater ($P < 0.001$) life span than Group II. Group XII not significantly worse than Group XI.

the control group until the twenty-fifth day, whereas in the tumourous but unvaccinated group this occurred on the thirteenth day (see fig. 17 page 46). Vaccination at other times did not seem to have any effect (see fig. 21 page 53) and fig. 22 page 54). Fourth day vaccination appeared, especially in Groups XI and XII in which half the normal tumour implantation dose was used (0.1 ml. 1:1 suspension tumour/saline), to be disadvantageous (see fig. 23) p. 56 but this was not statistically significant. Variation of tumour dosage and subsequent fourth day vaccination appeared to be without effect within the limits of the eight fold variation studied (see table 1 page 57 fig. 23 page 56 fig. 24 page 58).

The results of this pilot experiment showed that vaccination on the third day post-tumour implantation (but not on 4, 7 or 8 day) did affect the progress of the tumour. This would seem to suggest that there may be tumour specific antigens in this particular CBA/2J - CaD_2 system, that there may well be some barrier of access and that there is an important time factor. Further experiments were therefore conducted to further explore this avenue.

9. Experiments A and AC

Groups XXXVI - LX

Two sets of 12 groups of 10 animals were selected. Groups XXXVI - XLVII falling into set A and Groups XLVIII - LIX into set AC which were to serve as controls for the effect of vaccination with Freund's adjuvant without tumour homogenate. Group LX was set up as a tumour control. The animals in these groups were all the same age (9 weeks) and approximate

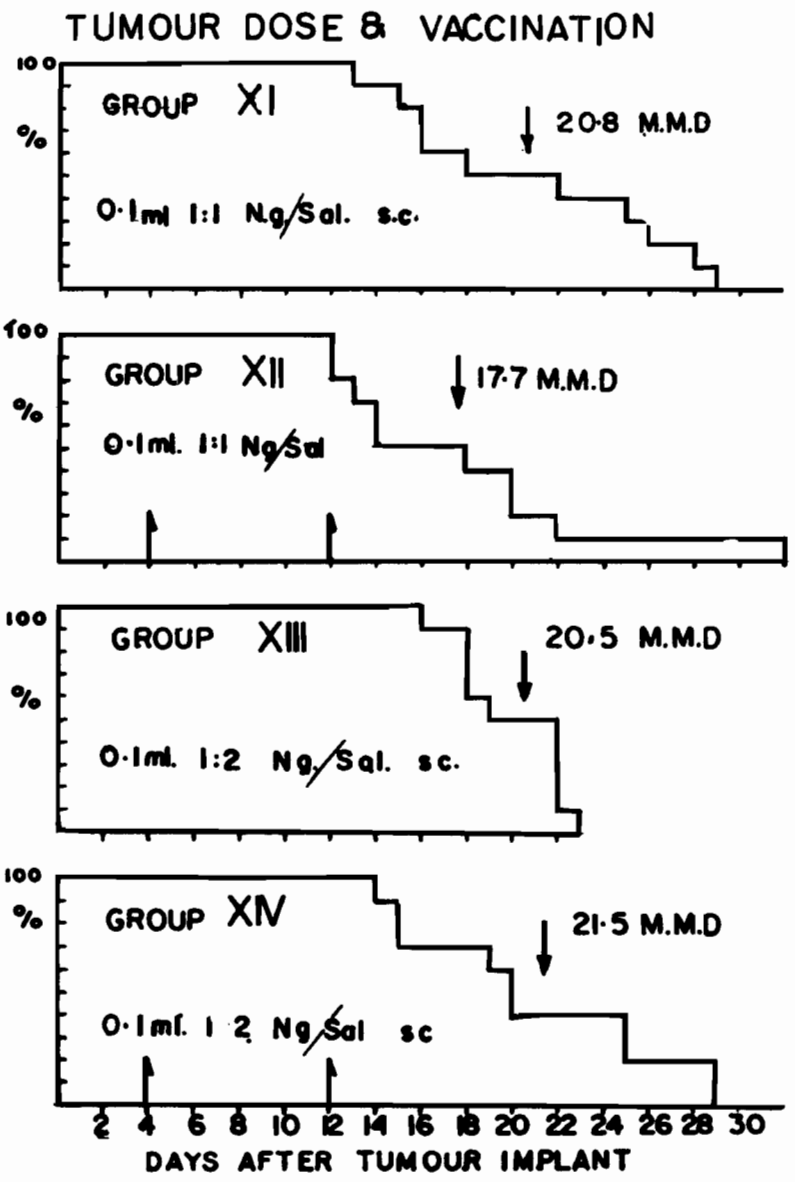

Figure 23

Variation of tumour dosage with subsequent vaccination. Groups XI, XII, XIII, XIV.

No significant difference with variation of tumour dosage and subsequent 4th day vaccination.

A. TUMOUR GROUPS		TUMOUR DOSE* (both A and B) of tumour/saline suspension v/v	B. VACCINATION GROUPS			
GROUP	M.M.D.		GROUP	DAY VACC.	M.M.D.	LIFE INCREMENT
II	20.1	0.2 ml. 1:1 S.C.	I	3	26.5	+6.4
IV	19.8	0.2 ml. 1:1 S.C.	III	7	20.1	+0.3
VI	20.3	Trocar	V	8	21.3	+1.0
XI	20.8	0.1 ml. 1:1 S.C.	XII	4	17.7	-3.1
XIII	20.5	0.1 ml. 1:2 S.C.	XIV	4	21.5	+1.0
XV	19.2	0.1 ml. 1:4 S.C.	XVI	4	17.8	-1.4
XVII	21.3	0.05 1:1 I.C.				
XVIII	21.7	0.05 1:1 I.C.				

M.M.D. = Mean Mouse Days of survival after inoculation.

TABLE 1

Note: If life span of mouse of 18 months be compared to woman of 70 years then 20 M.M.D. is approximately equivalent to 3 years 2 months.

Average survival time of untreated carcinoma of the breast is said to be 3 years 2 months. (John & Geoffrey Hadfield, British Surgical Practice, Progress vol. 1959, Butterworth, London.)

* S.C. = Subcutaneous
I.C. = Intracutaneous

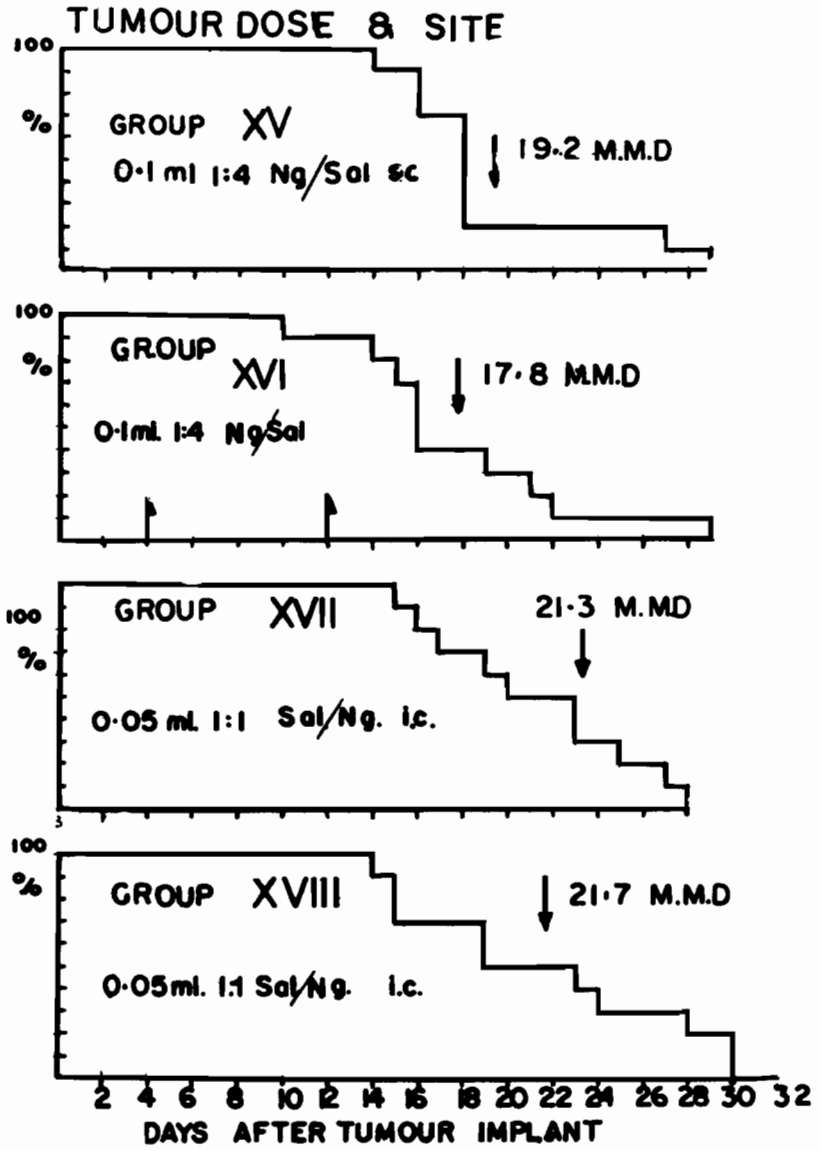

Figure 24

Variation of tumour dosage with subsequent vaccination (continued).

Groups XV, XVI.

Intracutaneous inoculation of tumour suspension. Groups XVII, XVIII.

weight. Each group of animals received a course of three vaccinations at seven day intervals (where life allowed) and one group from each set was vaccinated each day. Courses were started on each of the five days preceding the day of, and the six days succeeding, tumour inoculation which was achieved with the standard dose of 0.2 ml. suspension of 1:1 tumour in saline subcutaneously. In set A the "vaccine" was composed of one part of tumour homogenated in a sterile Teflon homogenisor with twice its own volume of sterile water mixed in equal volumetric proportions with Freund's adjuvant and administered in 0.2 ml. doses subcutaneously. In set AC the "vaccine" was composed of a homogenate of equal volumes of sterile water and Freund's adjuvant. Ten animals were set aside as controls without vaccination of any sort.

Results are graphed in figures 25-31 inclusive (see page 60-66). The control group (LX) had a M.M.D. of 21 which agreed well with our earlier studies (M.M.D. 20.5) so that the system appeared to be working normally. Once again the only significant difference is on the third day ("second day post" group XLIII (A8)) with a mean mouse survival of 26.7 days, an increase of 5.7 days over control (27% increment) (figs. 26 and 31 page 61 & 66). This is not quite as significant as in Group I and II. $P < 0.01$ ($T18 = 3.4$). Where Freund's adjuvant was used alone, better survival was obtained in the earlier three groups but this was not significant. From this experiment it was concluded that the striking third day effect is a reality for it was repeated so closely. This implied that the provision to the host of non-viable tumour

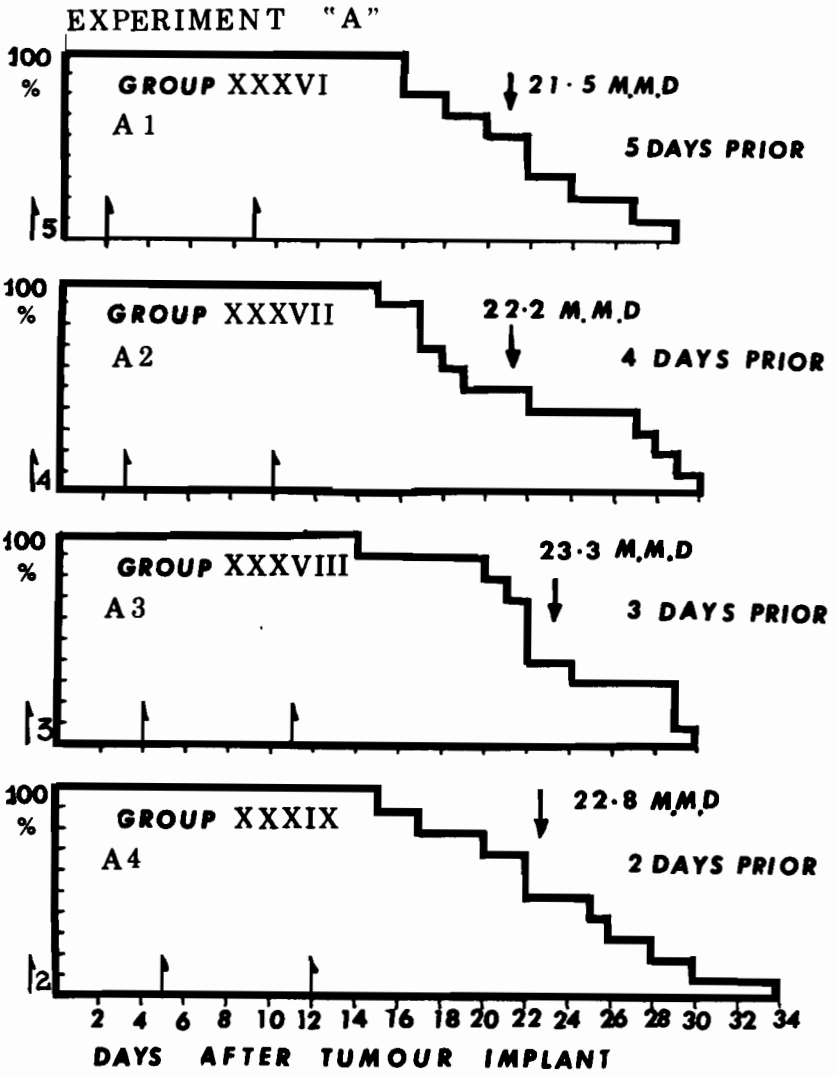

Figure 25

Experiment "A" Effective of "Active Immunisation" on tumour inoculation.

Vaccinations commenced at various times and were at 7 day intervals. Vertical arrows indicate vaccination. Groups XXXVI-XXXIX A 1-4. (5th - 2nd day preceeding inoculation).

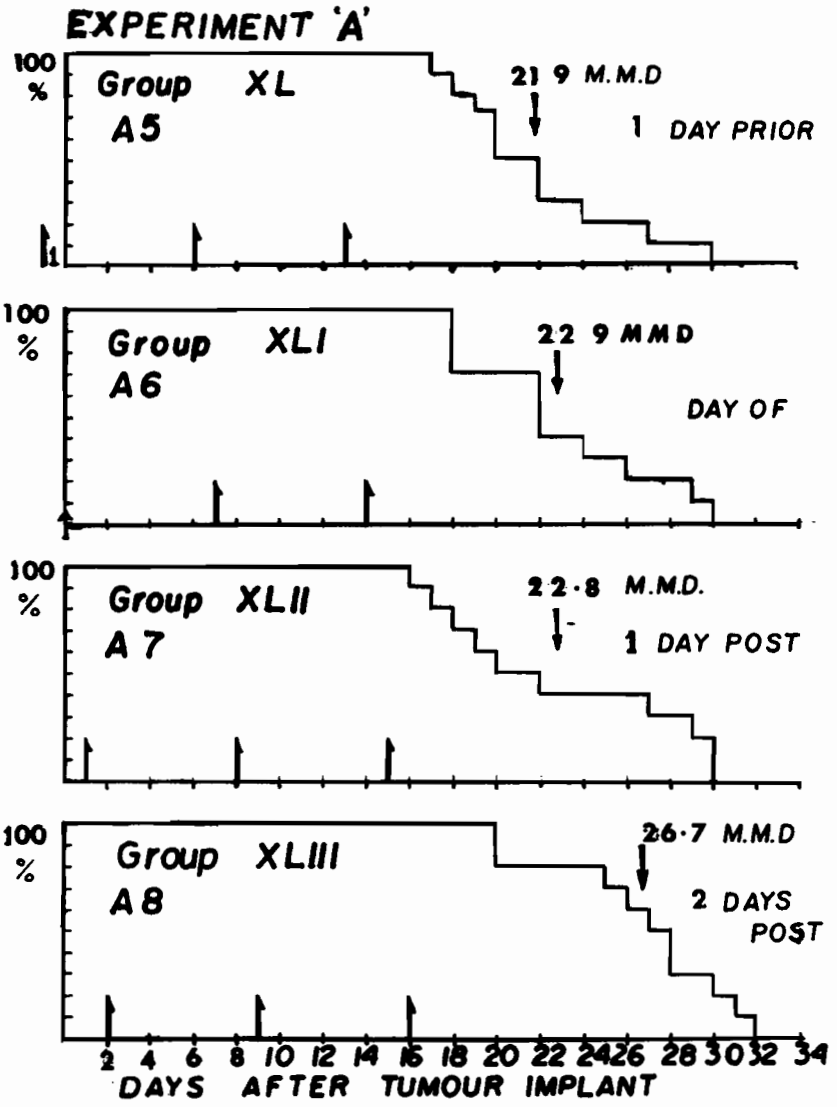

Figure 26

Experiment "A" continued.

Groups XL-XLIII A 5-8 (1 day preceeding to 2nd day following inoculation).

Group XLIII (A8) shows significant prolongation of life.

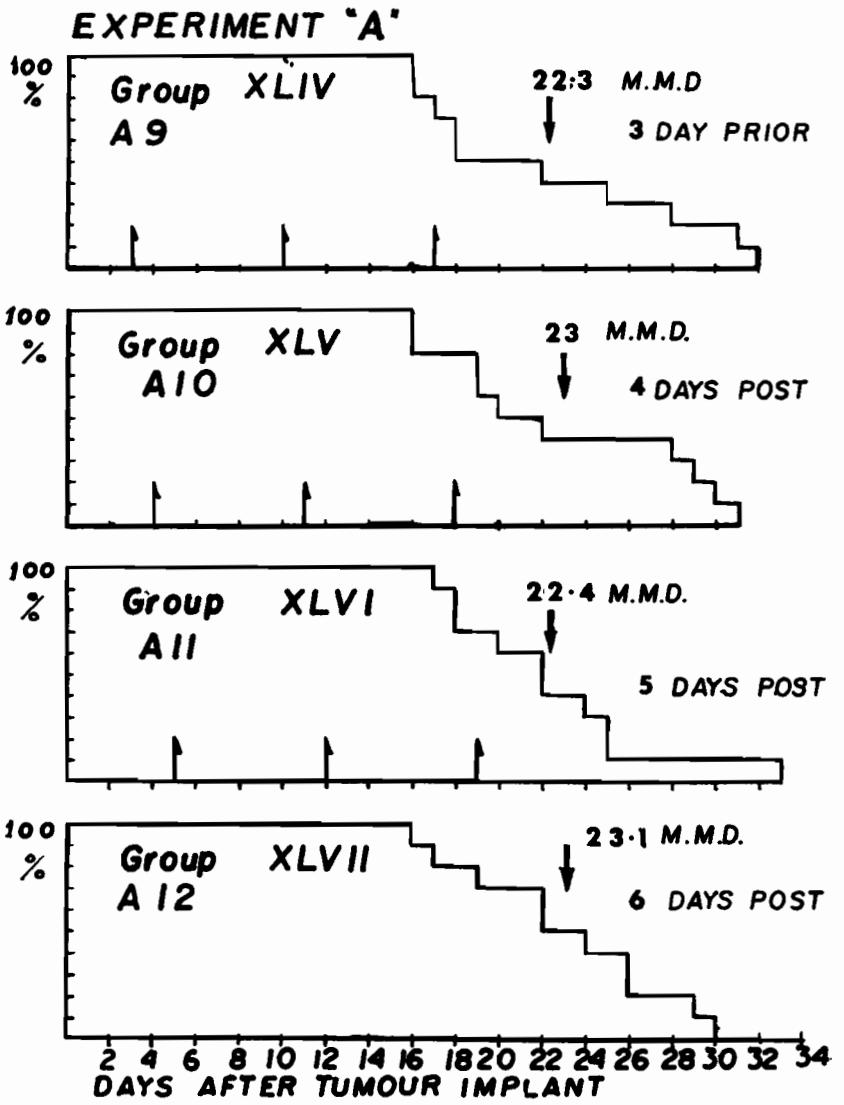

Figure 27

Experiment "A" continued.

Groups XLIV-XLVII A 9-12. (3rd - 6th day post inoculation).

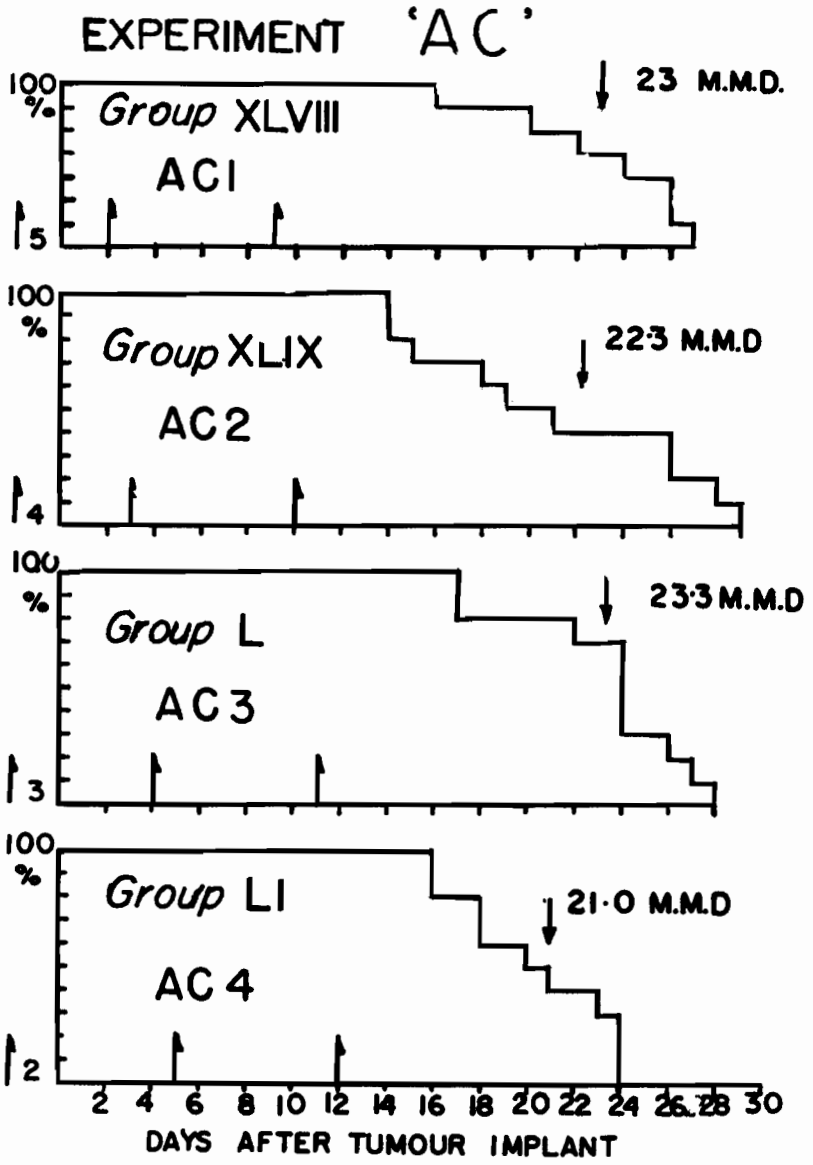

Figure 28

Experiment "AC" As a control to Experiment "A". Effect of vaccination with Freund's adjuvant and water in tumour inoculation.

Groups XLVIII-LI AC 1-4. Control to Groups XXXVI-XXXIX (fig. 25).

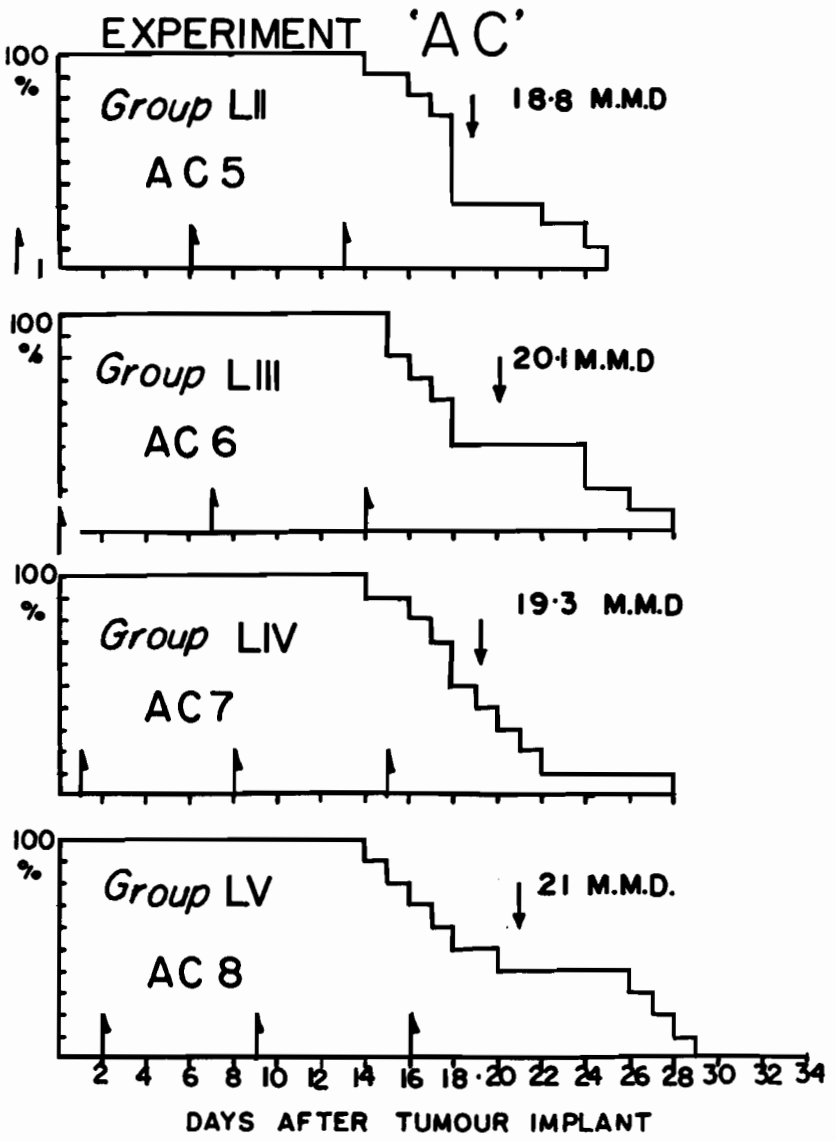

Figure 29

Experiment "AC" continued.

Groups LII-LV AC 5-8. Control to Groups XL-XLIII (fig. 26).

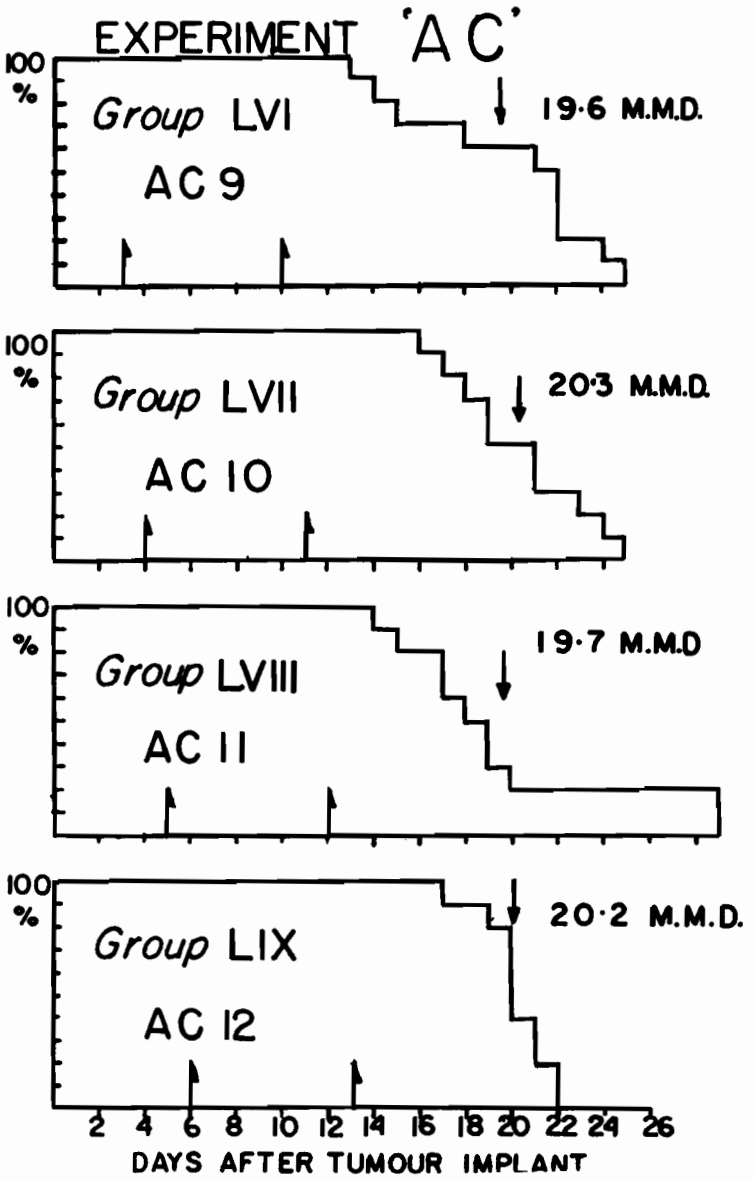

Figure 30

Experiment "AC" continued.

Groups LVI-LIX AC 9-12. Control to Groups XLIV-XLVII (fig. 27).

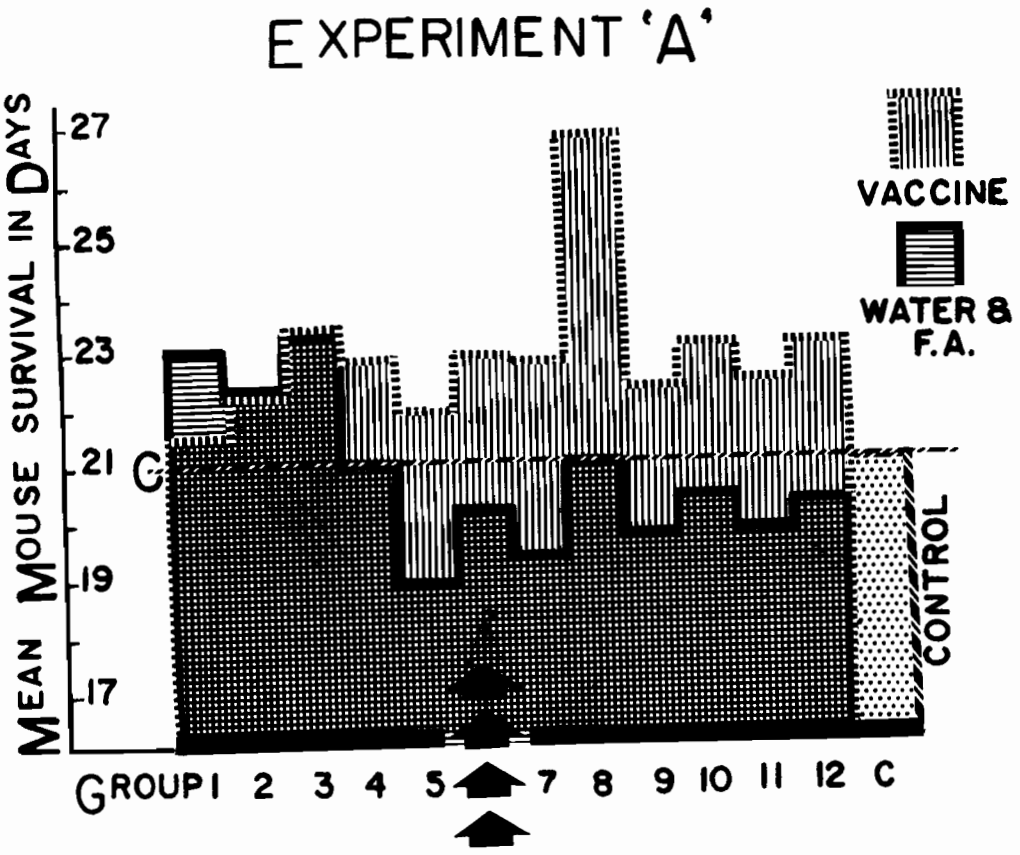

Figure 31

Experiment "A" Summary of Groups.

A 8 Group XLIII 3rd day after tumour inoculation (where day of inoculation is day 1) shows significant prolongation of life (cf figures 21 and 22).

Vertical arrows represent tumour inoculation.

material in some way stimulated resistance and that this was not merely the result of non-specific reticulo-endothelial system stimulation as by Freund's adjuvant alone. It also implied that the susceptibility, availability or effectiveness of that system must vary with time, and attempts were made to study this.

10. Experiments to Study the Effect of Passive Immunisation with Heterologous Immune Gamma Globulin.

Groups LXI-LXVII P1-7 Control Group LXIX

Because of the efficacy of the R.E. system might well be inhibited by the developing tumour (the anergic state has been well documented in some malignant conditions (136, 136a, 153, 154, 155)) and because of the work of Dr Gordon Murray in Toronto in which he has treated women with cancer of the breast with "immune" horse serum, attempts were made to study the effect of heterologous immune gamma globulin.

Six large rabbits were treated with four injections of a mixture of homogenised tumour in Freund's adjuvant at greater than weekly intervals. 0.2 ml. were given into each forepad and 0.5 ml. intra-muscularly into the thigh. The rabbits were then sacrificed and bled under sterile conditions. The gamma globulin was then obtained by cold ethanol precipitation in the following manner. The serum was diluted with three volumes of distilled water per unit volume and the pH adjusted to 7.7 (using 0.5 M acetic acid or 0.05 M disodium hydrogen phosphate and an electric pH meter). 50% ethyl

alcohol was then added to give a final concentration of 20% and the mixture allowed to stand in the refrigerator for 20 minutes and then centrifuged in the cold for 30 minutes at 3,000 r.p.m. The precipitate so obtained was dissolved in 30 ml. of 0.0425% sodium chloride to give a protein concentration of approximately 1%. The pH was then readjusted to 5.0-5.2 using 0.05M acetic acid and the alcohol concentration made up to 10% using 50% ethanol. The mixture was allowed to stand in the refrigerator for 20 minutes and then centrifuged for 30 minutes at 3,000 r.p.m. The supernatant obtained was measured and its pH adjusted to 7.3 with 0.05 M disodium hydrogen phosphate. 1 ml. of 50% ethanol was added to every ml. of phosphate and 0.6 ml. for every ml. of supernatant. The mixture was cooled in the refrigerator for 15 minutes and then centrifuged for 20 minutes at 3,000 r.p.m. The precipitate so obtained (the gamma globulin) was suspended in 20 ml. normal saline and frozen and stored at -20°C.

The six rabbits were done in three pairs. They were anaesthetised with Nembutal and the abdomen opened and the aorta cannulated under sterile conditions. From the first pair 45 ml. of serum was obtained yielding 141.6 mg. of gamma globulin (0.708 gm. % in 20 ml. saline). The second pair gave 55 ml. of serum and 120.6 mg. of gamma globulin (0.603 gm. % in 20 ml.) and the third yielded 58 ml. of serum and 119.6 mg. of gamma globulin (0.598 gm. % in 20 ml.).

Five groups of ten animals were given four daily subcutaneous doses of 0.2 ml. (varying from 1.2-1.4 mg.) of immune rabbit gamma globulin commencing on the 7, 8, 9, 10 and 11 days after standard tumour inoculation. A sixth group (P6 Group LXVI) received three daily injections starting on the 12th day and a seventh group (P7 Group LXVII) received one injection because of shortage of globulin (fig. 33 page 71).

Prolongation of M.M.D. to 25.6 was seen on the 9th day post-implant (P3 Group LXIII) group but this was not statistically significant. Unfortunately, something went wrong with the control group in this experiment for the M.M.D. appeared at 23.7 in contrast to the close conformity of 20.5 ± 0.73 found in nine other control groups. Moreover, as can be seen from the graph (fig. 33 page 71) the animals commenced to die early and proceeded uniformly for the first five mice. It was the last five that caused the discrepancy with a five day interval between the deaths of the fifth and sixth mouse. It may or may not be significant that five mice implantations (0.2 ml. each) represent one tuberculin syringe (1.0 ml.) and it is therefore conceivable that, although sterile, a syringe may have contained some tumour static agent, or perchance because the control group was, perhaps wrongly, inoculated at the end of a trying inoculation schedule that tumour vitality may have declined while on the bench. However, whatever the explanation of this anomaly may be the results obtained do not vary significantly from either the experimental control group or

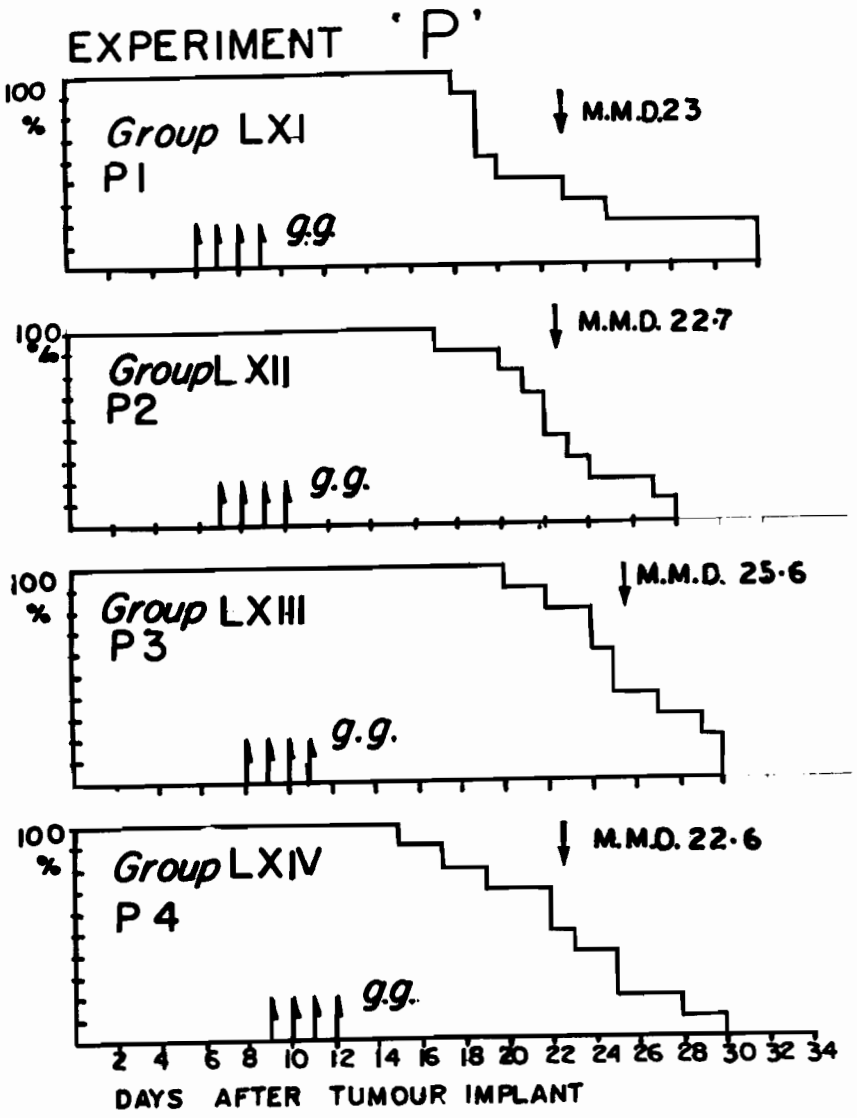

Figure 32

Experiment "P" Effect of passive immunisation with immune heterologous gamma globulin. Groups LXI-LXIV (Pl=4). Vertical arrows marked g.g. indicate administration of 0.2 ml. (1.2-1.4 mg.) of gamma globulin subcutaneously.

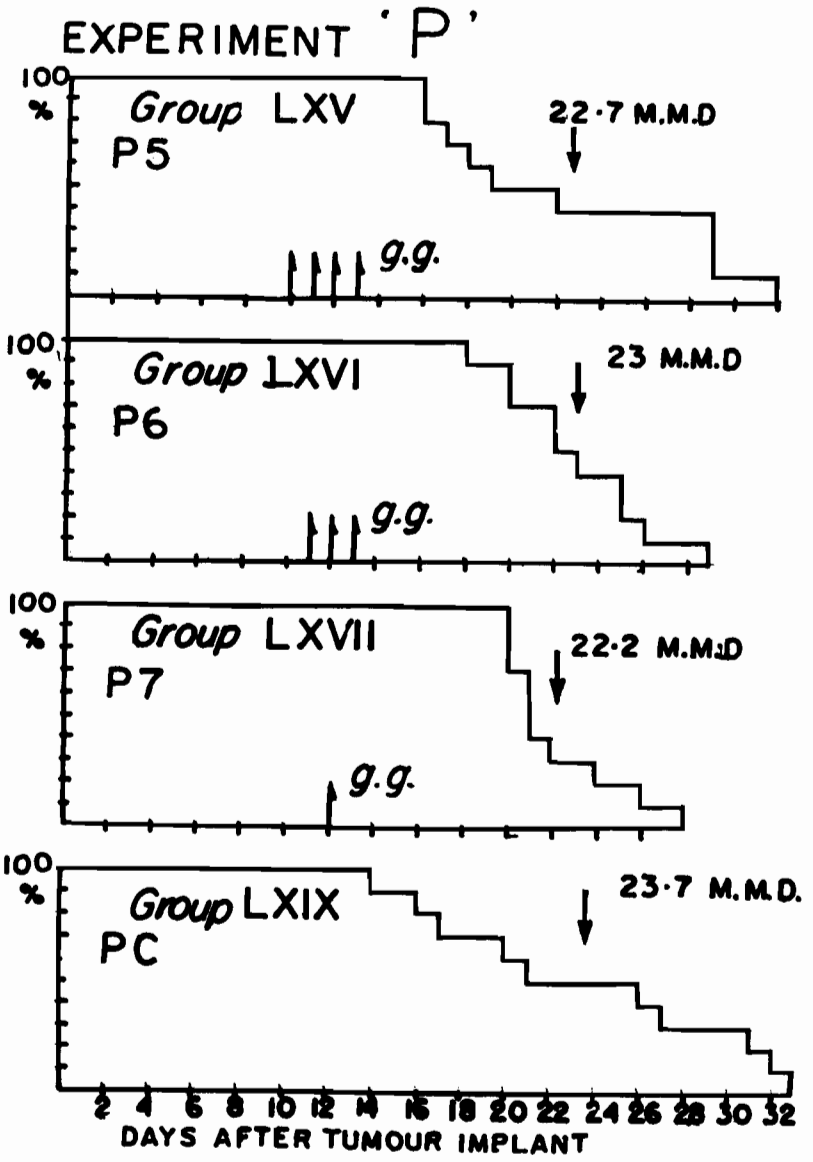

Figure 33

Experiment "P" continued.

Groups LXV-LXIX P5-7 and PC.

Note (1) P6 only had 3 doses. P7 only 1.

(2) Group LXIX as control group with abnormal result and 'biphasic' response between first five and last five mice.

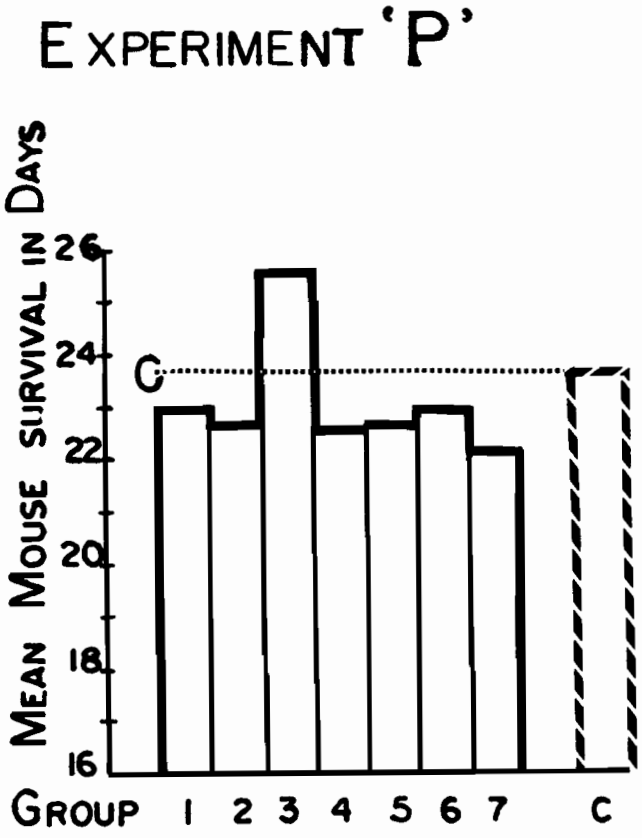

Figure 34 Experiment "P" Summary of Groups.

Administration of immune heterologous gamma globulin on 6th-12th days post tumour implant in dosage given was without significant effect.

the pattern of the control groups more usually obtained. It thus may be fairly concluded that the exhibition of gamma globulin in this experiment was without significant effect on life duration. However, a fuller exploration of both time and dose scale would have to be made before this conclusion could be generally applied. It is of course well known that homograft rejection is only achieved by cell bound antibodies, that even in autoimmune disease humoral antibody cannot disrupt organised tissue (unless indirectly, viz as antigen-antibody precipitates in the kidney glomerulus) with the exception of endothelium and single cell suspensions. Hence it would be surprising if tumour rejection could be achieved by heterologous humoral antibody unless such a gamma globulin in some way advantageously affected the tumour versus host struggle and thus indirectly promoted tumour rejection. To test such a thesis would, in my belief, require the exhibition of the antisera at an earlier stage in the development of the tumour for it is hoped to show later that the reticulo-endothelial system, or at least the regional lymph nodes, begin to decline anatomically by the 12th day and are therefore presumably functionally overpowered before this. However, at the time of this experiment this was not known and because of the time and expense involved in rabbit immunisation procedures and perhaps a false belief that this approach was fruitless, no further attempts were made at passive immunisation with different dose or time schedules.

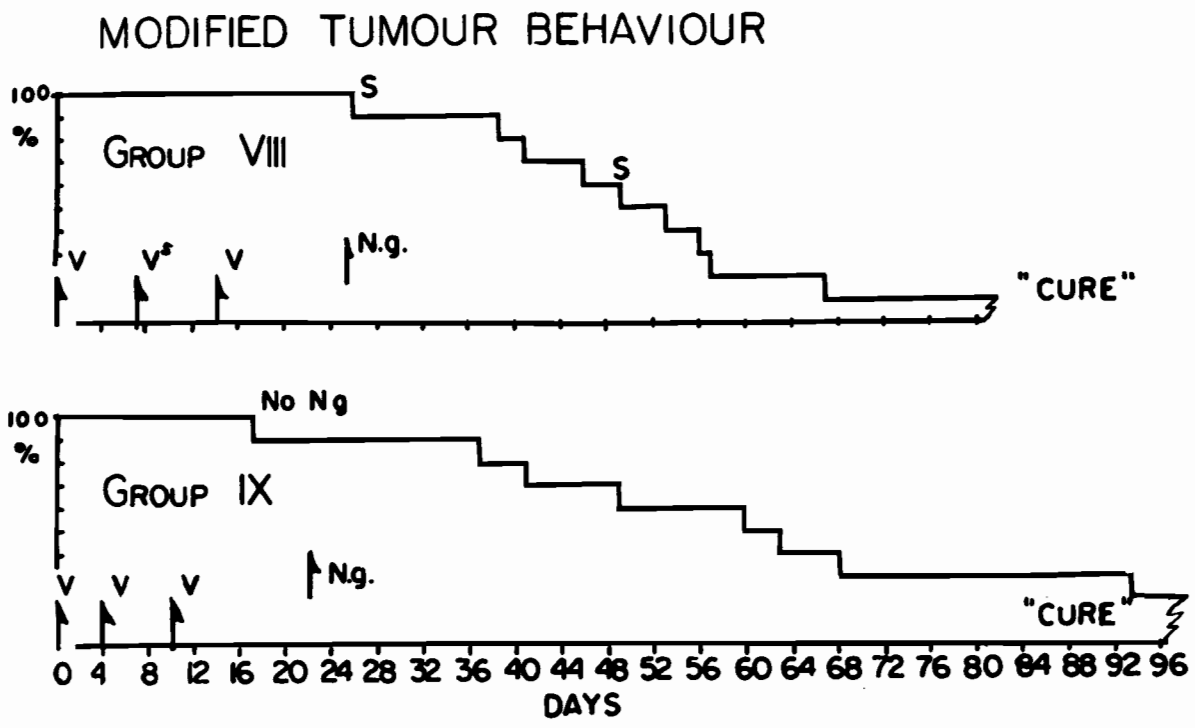

Figure 35 The "Accidental" Tumours Groups VIII and IX

Vertical arrows marked 'V' indicate vaccination with aqueous homogenate of tumour and Freund's adjuvant.

V^S indicates homogenate was with saline instead of water.

S indicates sacrifice of animal for blood.

N.g. indicates new growth palpable on all animals.

Note: scale half size of other figures.

11. The Accidental Tumours

Groups VIII and IX

In groups VIII and XI an unexpected effect was obtained. These two groups were set up as vaccine controls to Groups I and III in the pilot study in that they received three weekly doses of "vaccine". It is known that in Group VIII an error was made in that saline was used instead of water as a diluent fluid in the second inoculation. Nothing of a similar nature was observed in Group IX however. The rationale of using water instead of saline was based on the perhaps naive idea that osmotic forces would assist the homogenisor in breaking open the tumour cells. However, that viable cells were injected into these two groups of animals was shown by the development of tumours in all of the mice within 25 days of the first vaccination. Unfortunately two of the animals were sacrificed for blood (~~one~~ in a search for complement fixing antibodies) before their importance was appreciated. Of the remaining 18 mice, one was killed by her friends and 3 had their neoplasms regress and recovered completely. The remainder had a M.M.D. of 43 days from the last vaccination although which of the three injections was responsible for the tumour inoculation is unknown (see fig. 35 page 74). This result is puzzling but exciting for these three animals were the only 'cures' obtained in over 800 cancerous mice studied. The tumours grew to a fair size on all these animals. Mouse 8 Group VIII was vaccinated on 25.10.61;

Figure 36

The two survivors of Group IX who developed tumours after vaccination, but overcame their neoplasms. Photograph taken seven months after their first vaccination. Note scar on dorsum of right hand animal at site of previous tumour.

1.XI.62 and 8.XI.62. The tumour was first palpable on 19.XI.62 (after 11 days from last injection). It became ulcerated on 3.XII.62 (after 26 days) and at this time the tumour mass extended the full width of the animal's trunk (a grade ii tumour as we were then trying to grade tumour size without actual measurement). By 28.XII.62 the ulcer had healed and no growth was palpable (51 days). Mice in Group IX were vaccinated on 29.X.62, 5.XI.62 and 12.XI.62. Mouse 4 Group IX first developed a palpable lump on 23.XI.62 (11 days from last inoculation), became grade ii (as wide as the trunk without bulging beyond it) by 3.XII.62 (after 22 days) but thereafter steadily declined in size without ulceration until it was impalpable on 7.1.63 (59 days). Mouse 6 Group IX developed a palpable tumour on 15.XI.62 (3 days after last injection) which became ulcerated on 20.XI.63 (18 days), healed by 13.XII.62 and from then on steadily decreased in size until it was impalpable on 31.XII.62 (50 days). There was no histological proof obtained that these masses were tumours, except they appeared morphologically similar to all the other tumours and the size to which they grew was too large for an abscess. Where ulceration occurred the tissue exposed had just the look and feel of tumour. After healing had occurred the mice appeared to be in good health (see fig. 36 page 76).

Seven months after their first vaccination these three survivors were rechallenged by tumour inoculation. One suffered trauma and died while being photographed (a book fell on her) on the 19th post-inoculation day at which time she looked very sick; the other two died on the 26th day. (It is interesting to note that in 6 other groups of ten mice that were challenged after "vaccination"

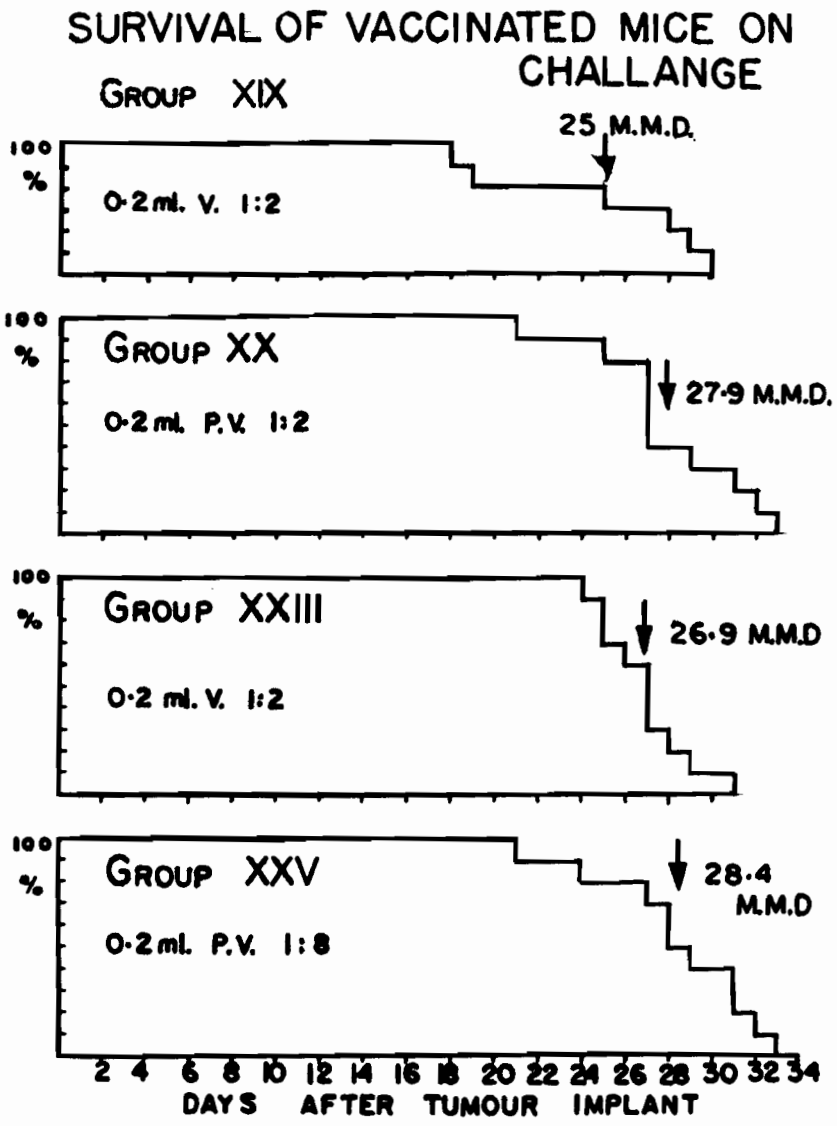

Figure 37

Survival of immunised animals.

Groups XIX, XXIII vaccinated x3 with 0.1 ml. of 1:2 suspension NG and water with 0.1 ml. Freund's adjuvant. Challenged 3 months after last vaccination.

Group XX vaccinated x3 with 0.1 ml. 1:2 suspension NG and water "pasteurised" at 46^{o}C for 30 mins. and 0.1 ml. Freund's adjuvant.

Group XXV vaccinated x3 with 0.1 ml. 1:8 suspension of NG and water "pasteurised" at 46^{o}C for 30 mins. and 0.1 ml. Freund's adjuvant. Challenged 10 weeks after last vaccination.

the average M.M.D. was 26.9). Thus what ever degree of immunity developed in these animals was not permanent. Whether the tumour cells mutated to a less malignant form is a matter of conjecture, but it is difficult to see if this be so, why the tumour should at first develop and then regress. Re-implantation into another animal might have shown this but would have been difficult to do because regression was not anticipated until it had occurred, and even if residual tumour could have been obtained, any reduced vitality, as measured by behaviour in another susceptible animal, might have been either the cause or the result of rejection.

It may have been that the combination of a few viable cells with a relatively large amount of antigenic material enabled the host to develop adequate resistance before the biological fervour of the neoplasm became sufficiently established to obtund the homograft rejection mechanism. At later rechallenge, perhaps, sufficient neoplastic cells or tumourous products were implanted to overcome this resistance either by "passive" methods of immuno-paresis or as a product of some specific vital neoplastic activity.

12. Experiments to Study 'Active Immunisation' Prior to Tumour Cell Inoculation and Subsequent Challenge.

Groups XIX, XX, XXIII-XXVI, XXXIV

This sort of experiment is one of the classical methods of trying to show tumour specific antigens. Groups XIX, XXIII and XXXIV were vaccinated (using the standard 0.2 ml. of the homogenate of 1 part tumour, 2 parts

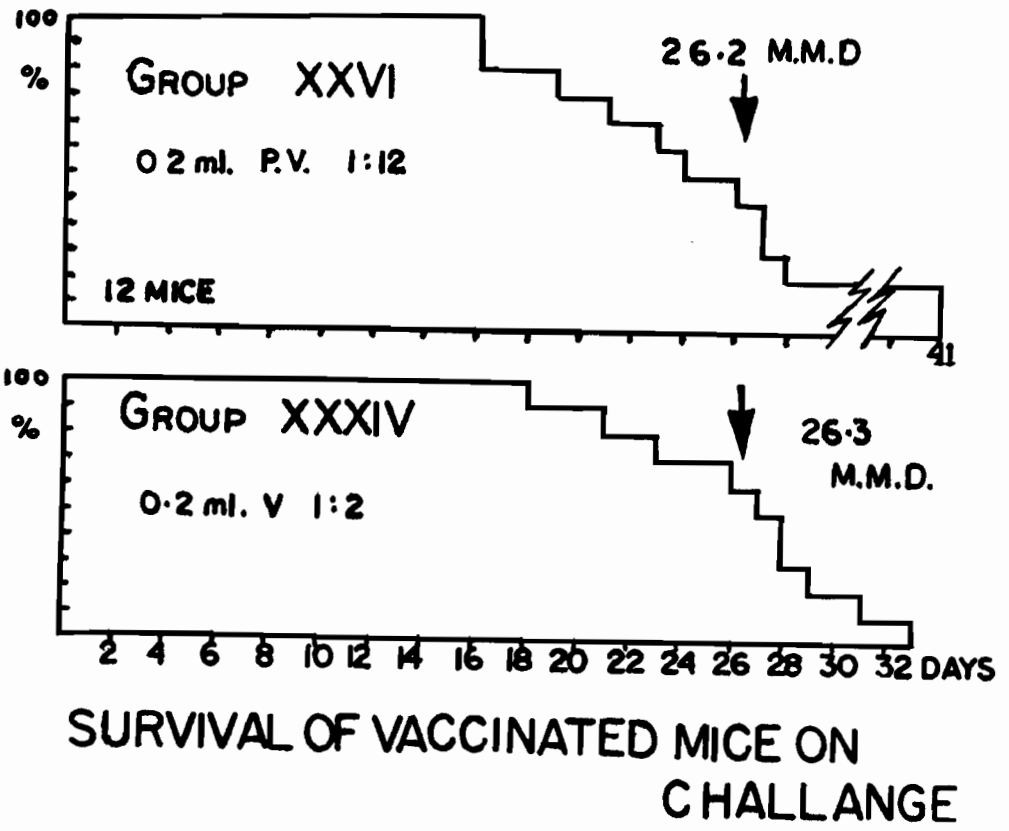

Figure 38

Survival of immunised animals.

Group XXVI vaccinated x3 with 0.1 ml. 1:12 suspension NG and water "pasteurised" at 46°C for 30 mins. and 0.1 ml. Freund's adjuvant. Challenged 10 weeks after last vaccination.

Group XXXIV vaccinated x3 with 0.1 ml. 1:2 suspension NG and water with 0.1 ml. Freund's adjuvant. Challenged 24 days after last vaccination.

water, 3 parts Freund's adjuvant) as vaccine control animals without apparent ill effect. They appeared well and healthy for the four months for which they were observed. Between four and five months after the last of their vaccinations, these mice were challenged by standard tumour inoculation. The implants took normally and Mean Mouse Day survivals of 26, 26.9 and 26.3 were obtained (see fig. 37 page 78 fig. 38 page 80). In Group XXXIV frozen tumour homogenate was used instead of fresh but apparently without effect.

Because of the experience of Groups IX and VIII in which vaccine proved to be live, and in view of Crile's work on heat sensitivity of cancer cells (31) our attempt was made to produce a "pasteurised vaccine" by heating the tumour homogenate to 46°C for 30 minutes with the idea that this might kill the cancer without destroying the histocompatability antigenicity. Graded amounts of tumour tissue were used. The vaccine consisted of 0.2 ml. of a mixture of equal parts Freund's adjuvant and a homogenate of 1 part tumour and 2 parts water in Group XX, 1 part tumour and 4 parts water in Group XXIV, 1 part tumour and 8 parts water in Group XXV and 1 part tumour and 12 parts water in Group XXVI.

Unfortunately, the results of Group XXIV have been lost but the others were: Group XX - M.M.D. 27.9; Group XXV - M.M.D. 28.4; Group XXVI - M.M.D. 26.2. This would seem to suggest that the 'pasteurised vaccine' was at least as effective, if not slightly more so, than the ordinary

straight tumour homogenate.

There is one observation that appears pertinent from these experiments and that is the very similar results obtained with "effectively immunised animals". In Groups I and XLIII in which third day vaccination was given to tumour inoculated animals, the M.M.D. was 26.5 and 26.7 respectively. In the two 'cures' obtained from accidental tumours in Groups VIII and IX (ignoring the mouse that died from trauma) the rechallenge allowed a survival of 26 days each. Challenge of previously vaccinated animals produced M.M.D. survivals of 25 (Group XLX), 26.9 (Group XXIII) and 26.3 (Group XXXIV). The "pasteurised vaccine" groups fall a little outside this range but are of the same order. It would almost appear as if an immunised reticulo-endothelial system is capable of deploying a certain amount of defence, but that this can be overcome after a relatively short, but remarkably uniform, delay. Hirsch (76, 78) reports an experiment which was 'concerned with testing the effect of pre-treatment of inbred mice with a strain specific tumour against challenge with this same tumour in an isologous system, i.e. a system in which the tumour and the host were as nearly isogenic as could be obtained with inbred mice'. Inbred 'C' strain mice were subjected to repeated implants of strain specific transplanted mammary tumours of very recent spontaneous origin into ears and tails followed by surgical removal of these organs (a procedure designed to minimise the chance of pulmonary metastases). These mice were then challenged with a suspension of cells from

the same tumour. Comparison of experimental and control mice did not show statistically significant differences in the total number of tumours or in the time at which they appeared. However, the experimental animals had a significantly longer survival time than the controls, i.e. mean survival time for experimental group 124 ± 8.7 days and for the controls 91 ± 7.3 $P = < 0.01$. It will be noted that this represents a prolongation of survival of life by about 30% which is just the figure obtained in these experiments. That similar results should be obtained in different laboratories at different times by different workers using different tumours in different host animals, may well be coincidence. Alternatively, it may suggest a mechanism that we do not understand. Berg (8) writing on cancer cells in 1959 wrote "cells that are antigenically different but have a growth potential so great that they can 'keep ahead' of the hosts' immunological defence mechanisms." If this be true then a primed immunological system may only be able to slow the vigour of that potential without destroying it. Bickis (156) has shown that heterologous antibodies could inhibit the uptake and utilisation of C14 labelled amino acids by ascites tumour cells. Alternatively one stage of the tumour efflorescence may be affected and delay ensues until this is overcome. The fact that neither Hirsch, nor I, observed any delay in the appearance of tumours in 'immunised' mice and more convincingly the shape of the tumour index curves in the pilot study (Groups I, II, III and IV see fig. 15 page 44) in this work would be against this and in favour of a continually acting

inhibitory factor.

It might be, of course, that the ultimate failure of this resistance is a reflection of some essential co-factor destroyed by the neoplasm and the enhancement of some of the ancillaries of the immunological armamentarium, for example complement in which the mouse is notoriously low, might reinforce this effect and promote a retardation into a total inhibition of tumour growth. Experimental work on these lines might well be worthwhile.

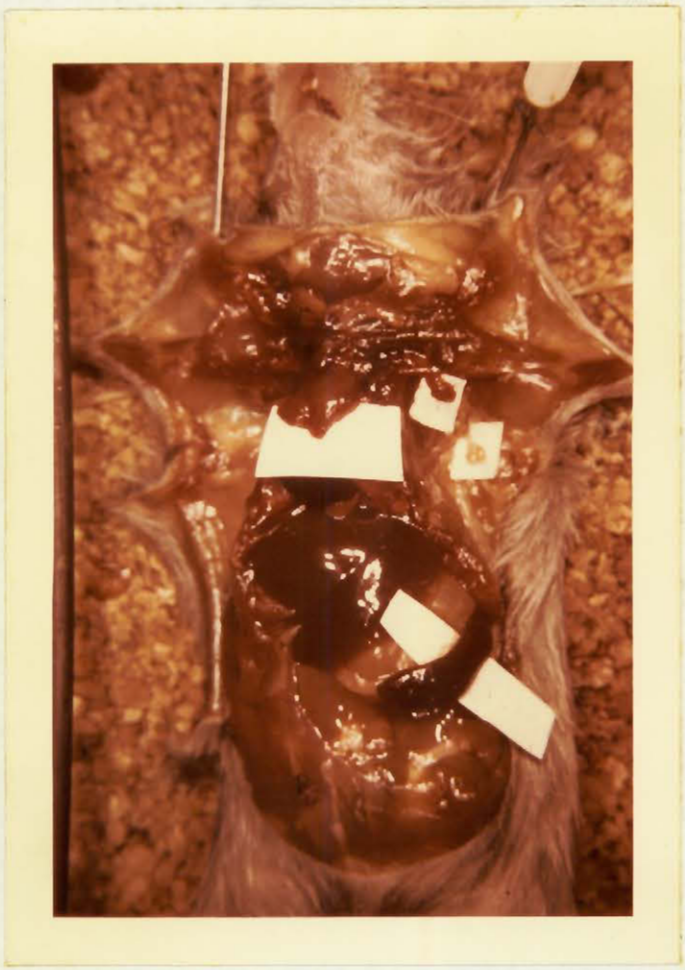

Figure 39

Post mortem dissection of normal CBA.2J mouse showing axillary lymph glands, spleen and thymus. (cf fig. 40)

Figure 40

Post mortem dissection of cancerous mouse showing axillary lymph glands, spleen and thymus. (cf. figure 39)

Note splenomegaly, thymic and apparent lymphatic atrophy.

SECTION III

13. The 'Runt' Postulate

During the routine post-mortem examinations it was observed that all animals dying from neoplasm exhibited stunting of growth, roughness and thinness of coat with scaliness and ulceration of the tail and paws, terminal diarrhoea, intense anaemia and apparent atrophy of lymphatic glands and splenomegaly (see figs. 10, 11, 39, 40, 41 p. 37, 38, 85, 86, 88). This syndrome was suggestive of Billingham's 'runting effect' (homologous disease) in which a heterologous graft of immunological competent cells reacts against the host and destroys it. If it could be shown that a tumour was capable of producing some humoral substance against the host's reticulo-endothelial system, then this would explain the puzzling time factor obtained in these experiments, and also the well documented anergic state of patients with lymphomas and other neoplastic conditions (153, 154, 136, 136a, 155). That the runting effect can occur in an animal by virtue of loss of a competent immunological system is shown by Miller's work in which he obtained the effect in some of his neonatally thymectomised mice without the necessity of a transplant of heterologous tissue. Attention was therefore directed towards the reticulo-endothelial system in the tumour-mouse system. It was decided to collect and weigh the thymi, spleens and lymph glands of tumourous animals to see if indeed the impression of change of size obtained during the post-mortem

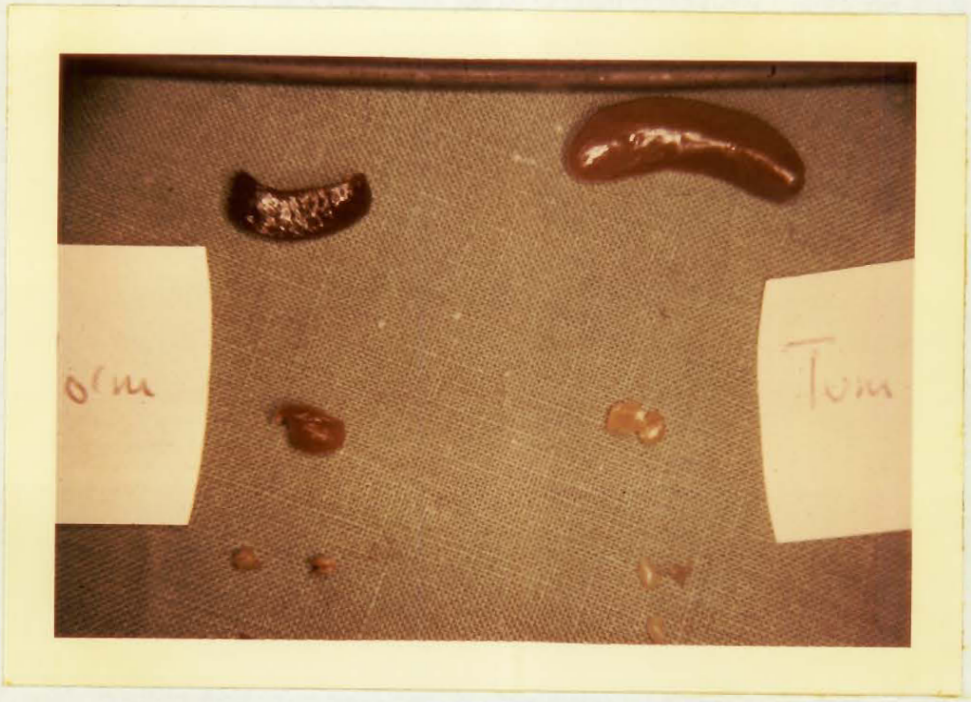

Figure 41

Spleen, thymus and lymph glands of normal (on ~~right~~ left labelled "orm") and cancerous mouse (on ~~left~~ right labelled "tum"). Lower pair of normal lymph glands unfortunately cut off print. Only 3 cancerous glands are present so that this animal is useless for weight study (experiment 15).

Note splenomegaly and thymic atrophy.

examinations was well founded. At once collection of lymph glands presented difficulty and after considerable trial dissection in various regions, it was decided to arbitrally select the axillary lymph glands in the hope that their behaviour would be indicative of the lymph glands throughout the body. Even if this were not true, however, it would provide evidence of the gross behaviour of the regional glands in a tumour bearing animal.

14. Experiment to Find a Method of Locating Lymph Glands

As this study was to depend on weight, it was of the utmost importance that all the lymph glands in a region could be found and collected. It was therefore decided to use some form of vital staining.

a. Intravital Staining

Subcutaneous injection of indian ink into the front paws of a mouse under general anaesthesia and subsequent exercise of these animals stained the axillary lymph glands well after two hours (see fig. 42 page 90). The difficulty of this manoeuvre in a moribund animal was found to be considerable for not only was anaesthesia hazardous, but the degree of physical activity necessary to get the carbon particles into the axilla was hard to obtain. Pre-tumour inoculation lymph gland staining was therefore considered and to a group of ten mice (Group XXX) was injected with ink. They did not appear to be incommoded by this procedure and appeared healthy. However, it was found that there was considerable enlargement of axillary lymph glands and splenomegaly presumably as a consequent of the endotoxic content of the indian ink.

Figure 42

Intravital staining of axillary lymph glands with indian ink. White arrow on right shows subcutaneous ink, central arrows show the two axillary lymph glands, black with ink, and left arrow shows unstained mediastinal lymph gland.

This feature persisted for some time after the ink had been injected. No figures are offered because the numbers of animals are too small to justify that. Search was then made for other materials like ground carbon and lamp black, but no satisfactory suspension was achieved and injection was unsatisfactory. Because of its supposedly more "physiological" nature 'Imferon' an iron dextran complex was used because of its known propensity to stain the skin when used clinically. Small quantities of 'Imferon' were injected into the forepaw pads under anaesthesia. At any time greater than two hours thereafter, the axillae could be dissected and the lymph glands located by use of the Prussian Blue reaction using an equal mixture of 2% Hydrochloric acid and 2% Potassium Ferrocyanide to flood the axilla. Figures 43 (page 92) and 44 (page 93) shows how this method aids identification. Imferon appeared to have no effect on the size of the lymph glands and spleen and histological studies of even quite blue glands showed such a paucity of iron granules as to make it fairly certain that no degree of histiocyte blockage was achieved by this method.

b. Anatomy of the Axillary Lymph Glands in the CBA.2J Mouse

All these procedures had rendered the mouse axilla so familiar that in fact in subsequent work no vital staining procedure was ultimately found necessary. However, its initial use ensured that no glands were missed. There are apparently only two lymph glands present in the axilla - (1) a large central gland closely associated with the lateral thoracic vessels and often found

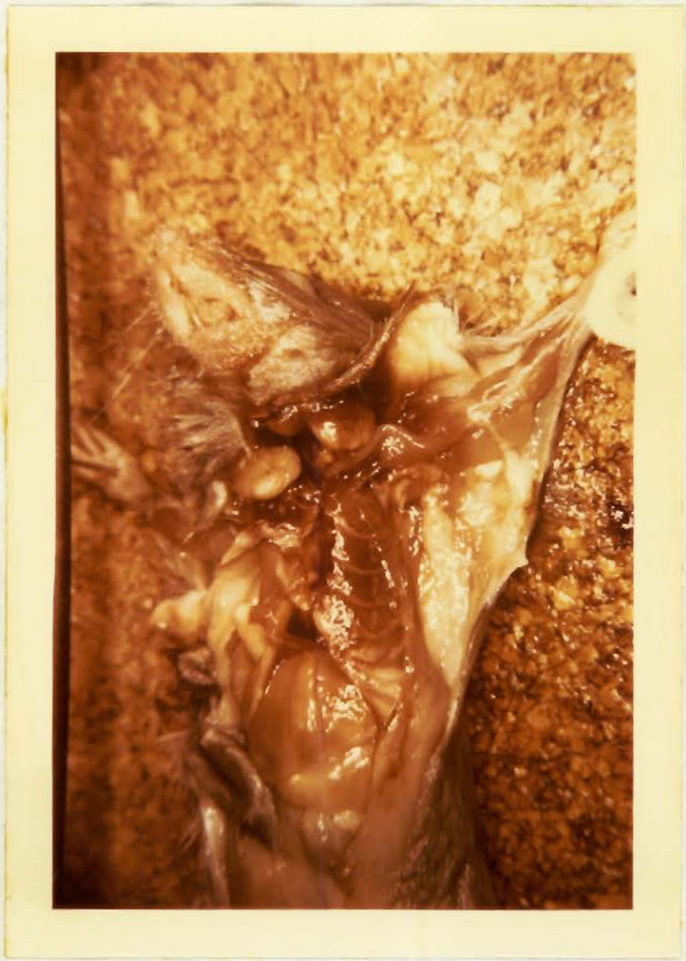

Figure 43

Intravital staining of lymph glands with Imferon. Axilla dissection for glands (compare figure 44).

Figure 44

Same axilla as figure 43 after flooding with Prussian Blue reagent. Prior injection with Imferon causes glands to stain blue.

in the angle formed between the axillary and lateral thoracic veins, (2) a smaller gland just lateral to the tendon of the murine equivalent of the latissimus dorsi as it inserts into the humerus. This gland is deeply embedded in fat in the normal animal, but fortunately much of this adiposity had disappeared in tumourous mice, and the gland was therefore more obvious. This anatomy was found to be most uniform and by using a dissecting microscope the glands could be identified and removed with reasonable facility. The spleen was removed under direct vision but the dissecting microscope was employed for the removal of the thymus since it was sometimes difficult to differentiate between mediastinal fat or lymph glands and the thymic lobes and it was important that the whole gland should be removed as cleanly as possible.

15. Experiment to Compare the Weights of Thymus, Spleen and Lymph Glands in Normal and Tumour Bearing Mice.

Thymus, spleen and lymph glands were removed froma group of normal CBA.2J mice and a group that had died from implanted tumour. Figure 41 (page 88) shows the results pictorially and demonstrates the difficulty in that if all four lymph glands are not located in the axillae, then that mouse is useless for the experiment. The numbers of the various organs are different because mice were used whose organs were being utilised in some biochemical studies. This was necessary because of expense for each animal cost nearly $1.25. Thus if the thymus was wanted for biochemistry it could not be used for this purpose since its vitality might be compromised either by meticulous

dissection or the weighing procedure. Weighing of such small amounts of tissue on a gravimetric electrical balance was rendered inaccurate by dessication which occurred during the procedure. This was overcome by standing screens of wet blotting paper in small beakers inside the weighing cabinet. By this method the air became saturated and the weight of a small piece of tissue remained constant. That this was so was periodically checked by observing the weight of a small quantity of tissue left on the weighing scale for 5 minutes. A change of less than 1 mgm. was accepted. Similar precautions were adopted during the period of dissection. The excised organs were placed in a closed petri dish containing a circle of filter paper moistened with normal saline and kept in a "wet box" (a cold, water-containing, steriliser). When the organs were weighed they were placed momentarily on a dry filter paper to take off any excess saline and weighed in small aluminium foil 'dishes'.

Results

(a) Normal Viscera

90 normal spleens were weighed and the mean was found to be 79.2 mg. $\pm$ 15.2 S.D. 30 normal thymi were weighed and the mean found to be 45.8 mg. $\pm$ 12.9 S.D. Because of the age effect on the thymus, 10 thymi of mice six months old were weighed with a mean 35.0 $\pm$ 8.5 mg. 80 sets (four glands to a set) of lymph glands were weighed and the mean found to be 11.5 $\pm$ 4.2 mg.

(b) Viscera from Tumourous Animals

115 cancerous spleens were weighed with a mean weight 198.2 ± 78 mg.

70 thymi were weighed with a mean 6.29 ± 5.3 mg.

74 sets of lymph nodes were weighed with a mean 11.0 ± 6.6 mg.

81 tumours were weighed with a mean 4.98 ± 4.5 g.

Thus, from comparison between (a) and (b) above it can be seen that there was a real degree of splenomegaly ($P < 0.001$ $t_{204} = 14.13$) and thymic atrophy ($P < 0.001$ $t_{98} = 21.48$) but that the lymph nodes were no different. Figure 45 p97 shows pictorially the variation of spleen size with tumour implantation and also the greater splenomegaly that developed in previously "immunised" animals challenged with tumour. The tumour weights were obviously, from the standard deviation, most variable. This is surprising in view of the uniform nature of the animals and the standardisation of the tumour dose.

16. Experiment to Study Effect of Known Immunological Stimulation on Murine Organ Weights.

An attempt was made to see the effect of other antigenically active procedures on the weight of lymph glands, spleen and thymus.

(i) Skin Homograft

Full thickness skin grafts were exchanged between the agouti CBA.2J and white CF.1 mice using the method described in Part B, Section I, Experiment 2. Rejection occurred within 12 days demonstrating H_2 locus

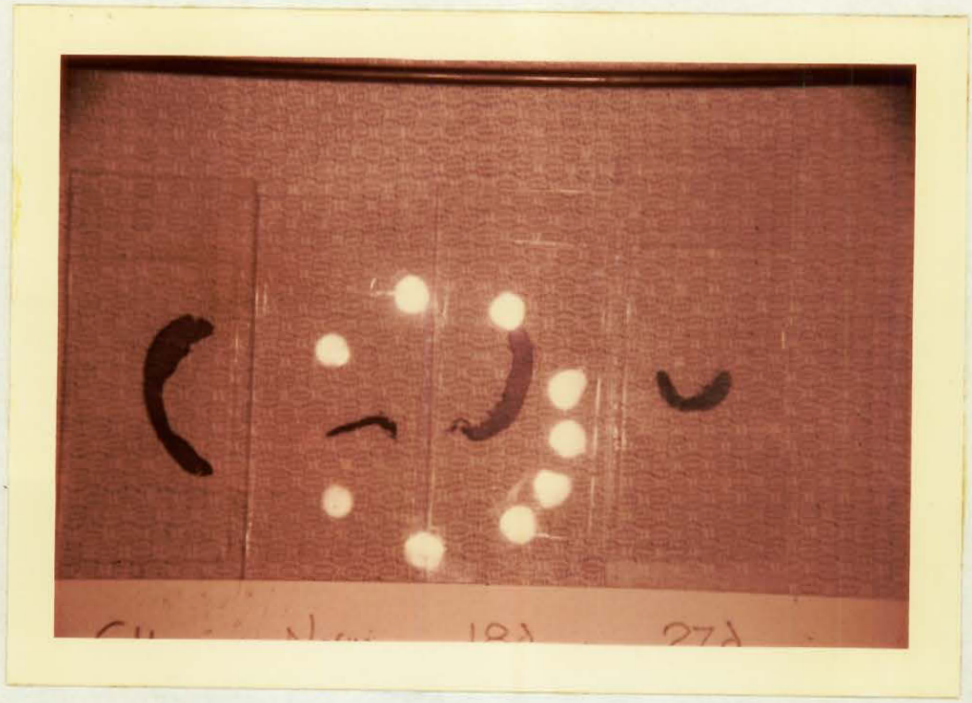

Figure 45

Mounted histological preparations of spleens illustrating variation in size. From left to right:

1. Immunised mouse challenged with tumour (at death).
2. Normal control mouse.
3. Mouse 18 days after tumour implant.
4. Mouse 27 days after tumour implant.

White spots are reflection of flash gun.

incompatability. Unfortunately, only three grey mice came to mensuration at 3o days post graft because of an unfortunate accident. White mice were not considered to be of value because of the lack of a control series of their normal organ weights.

Results

Mouse No.	1	2	3	Average
Lymph glands	.034 g	.016 g	.026 g	0.025 g
Thymus	.036 g	.037 g	.035 g	0.035 g
Spleen	.313 g	.172 g	.246 g	0.244 g

Extraneous factors such as subclinical infection (grafting was not a sterile procedure) might well affect these results but it looks, if any conclusion can be drawn from these three animals, as if splenomegaly and lymph gland enlargement occurred. I think this should, and I hope will be, amplified with isotransplant and autotransplant controls, with proper attention to time factors.

(ii) Injection of Freund's Adjuvant and Water

Five mice were injected with Freund's adjuvant and water (0.2 ml. of a homogenate of equal quantities by volume), thirteen and three days before sacrifice. One mouse developed a purulent peritoneal exudate and was disgarded.

Results (in grams)

					Average
Lymph glands	.018	.022	.020	.015	.017
Thymus	.043	.045	.053	.042	.046
Spleen	.090	.107	.096	.073	.092

Slight increase in both spleen and lymph glands was achieved but this is not significant. No conclusion can be drawn from this because the amount of antigenic material (killed TB bacilli) administered was very small, and quantitative as well as qualitative factors were important (see histological studies).

(iii) Injection of Heterologous Antithymic Serum

Two rabbits were immunised with a suspension of CBA.2J mouse thymic cells in Krebs Ringer Solution. Three injections were given at weekly intervals and a fourth one month later. 0.5 ml. was given into each forepaw and 0.5 ml. intramuscularly in the thigh. The suspension of cells was made from between 50-100 mg. of thymic tissue per ml. and had between 60-200 cells per high power field when smeared. The rabbits were bled from ear veins and the serum obtained. Mice were given three daily doses of 0.5 ml. of serum subcutaneously in the hope of producing some runting effect. This was not observed nor were any deaths obtained

Three mice, eight months old, were treated with the following result (in grams):

				Average
Lymph glands	.029	.028	.036	.031
Thymus	.012	.010	.018	.013
Spleen	.244	.198	.272	.238

Three similar eight month mice (untreated) that were cage and litter mates of the above animals:

				Average
Lymph glands	.009	.020	.013	.014
Thymus	.030	.020	.043	.031
Spleen	.078	.095	.107	.093

Three mice aged ten weeks were also treated and yielded these results:

				Average
Lymph glands	.013	.010	.009	.011
Thymus	.020	.023	.016	.020
Spleen	.157	.132	.155	.148

From these few animals it would seem that the older mice exhibited more marked lymphatic and splenic hypertrophy and thymic atrophy; the younger mice did not in fact show lymphatic gland hypertrophy at all. Whether this is due to a more vigorous response to, or a slower destruction of, the antiserum is not obvious. In any case, the results bear some

similarity to the response of these mice to tumour inoculation and may suggest slender support to the concept of an antilymphocyte action, on an immunological basis, of tumour cells, viz runting.

Experiment 15 was concerned with the post mortem studies of animals who had died from tumour. Curiosity was then aroused as to what happened to these organs throughout the evolution of the tumourous process when, it is our postulate, the struggle for supremacy between host and tumour must be fought. Consequently the following experiment was set up.

17. Experiment to Measure the Weights of Thymus, Lymph Glands and Spleen During Tumour Growth.

Groups of three mice were sacrificed at intervals throughout the evolution of a tumour and their spleen, lymph glands and thymus dissected and weighed. This was done on the 4, 5, 6, 7, 9, 12, 14, 18, 21, 22, 24, 25, 27 and 28 day after tumour inoculation. These days were arbitrary for it was originally intended to do daily sacrifice, but this was found impossible because of other commitments of time.

Results

It will be seen from figure 46 (page 102) that there was a progressive fall in the weight of the thymus, although there was a point of inflection between the 5th and 12th days. The spleen steadily increased in

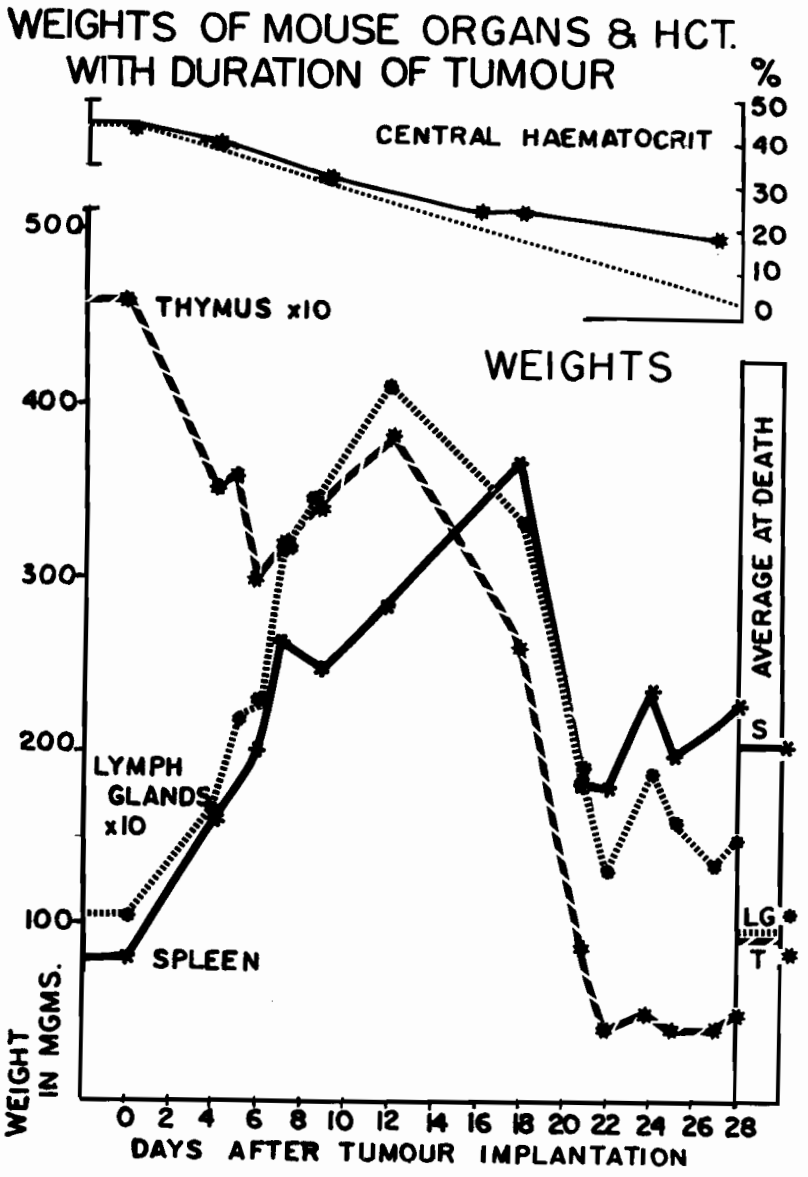

Figure 46

Upper graph: Central haematocrits (cardiac blood) of mice with duration of tumour. Dotted line represents physiological death cells.

Lower graph: Weights of thymus, spleen and axillary lymph glands with duration of tumour. Box on right represents average weight of organs in group of animals at death, ordinate on left represents values for group of normal mice.

size until the 18th day when it decreased sharply. The lymph glands rapidly enlarged until the 12th day whereafter they too atrophied away. This might suggest that the initial response of the reticulo-endothelial system is one of hypertrophy in an effort to combat the tumour invasion, but that after the 12th day the system is overcome by some agent, or agents, and the battle is lost. The initial hypertrophy might be similar to that seen in homograft rejection, but this mechanism is thwarted by an involution of its chief mediators - the regional lymph glands. Whether the agents involved could be antibodies and the condition a true runting, or some form of toxohormone (161) is a matter of conjecture. If the former, then it would ascribe immunological competence and capacity to the supposedly primitive adenocarcinoma cells which must have repercussions on our concepts of both immunity and neoplasia.

18. Experiment to Measure Mouse Haematocrits.

One of the features of runting is sometimes a haemolytic anaemia and with this in mind, and because the cancerous mice looked so anaemic, the haematocrits of these animals were measured. This was done by means of the standard microhaematocrit machine. The tip of the mouse tail was amputated cleanly with a sharp razor blade, and the drop of blood that forms sucked up the capillary tube by capillary action. The tube was sealed with heat or plasticine and spun for 15 minutes at 5,000 r.p.m. The tail was

cauterised with a hot needle. This estimation was repeated on the control group of mice (Group VII) when they were 8 weeks, 3, 5, 8 and 10 months old. The haematocrit was seen to gradually subside being 52.1 ± 1.5, 49.6 ± 1.2, 46.7 ± 1.1, 44.3 ± 1.6 and 43.6 ± 2.1 respectively. Whether this was the result of ageing, nutrition, blood loss (from the repeated estimations) or diminishing stress from acclimatisation to the procedure, and therefore less vasomotor response, is unknown. Mice under chloroform anaesthesia at 12 weeks of age had a haematocrit of 46.6 ± 1.9 (3% lower than comparable conscious animals). Group XXIV (vaccine control group) when aged 14 weeks and two weeks after their third vaccination had a mean haematocrit of 46.5 ± 1.6 which was taken to exclude the possibility that such vaccination with unabsorbed tumour tissues might induce autoimmune haemolytic anaemia.

Central haematocrits (cardiac blood) of 11 week mice under ether anaesthesia was found to be 43 ± 2.7, some 6% less than their tail values. Central haematocrits were estimated on cancerous mice on the 4, 9, 16, 18 and 27 days after the implantation of a tumour (meanof 3 animals) (see fig. 46 page 102). A steady decline in values was obtained to levels below 20%. Normal mouse red cells have a life of 30 days, and as this fall did not exceed the gradient of a line drawn to represent the physiological death of cells (dotted line, fig. 46) there was no evidence of any haemolytic component of this anaemia (nor was it excluded).

SECTION IV

Histological Studies

Histological studies of the various organs of the mice examined post mortem were made in an effort to see if any pattern of activity could be recognised. I am grateful to Mrs Farrell of the University Surgical Clinic of Montreal General Hospital for her skill in cutting and staining these sections. Some of the material from the cancerous mice was particularly difficult to handle because, not only of its very small size, but also because of its rather friable character. Sections were stained with haemotoxylin and eosin and also with Pappenheim stain with detects pyroninophilic cells and stains desoxyribonucleic acid (D.N.A.) ~~pink~~ green and ribonucleic acid (R.N.A.) ~~green~~ pink. Unfortunately, the colour prints shown here are not quite true for there has been considerable loss of red hues both at the slide to transparency and the transparency to print stages.

19. Thymus Gland

The thymus gland showed least change histologically, but appeared merely to decrease in size. It was impossible, for technical reasons, to examine the very atrophied glands, and this may be the reason that I was unimpressed with the thymus for only those glands in which reaction was not marked could we adequately examine. The thymocytes decreased in numbers quite markedly, but the distinction between cortex and medulla remained clear. Some pyroninophilia was observed in the medullary reticular

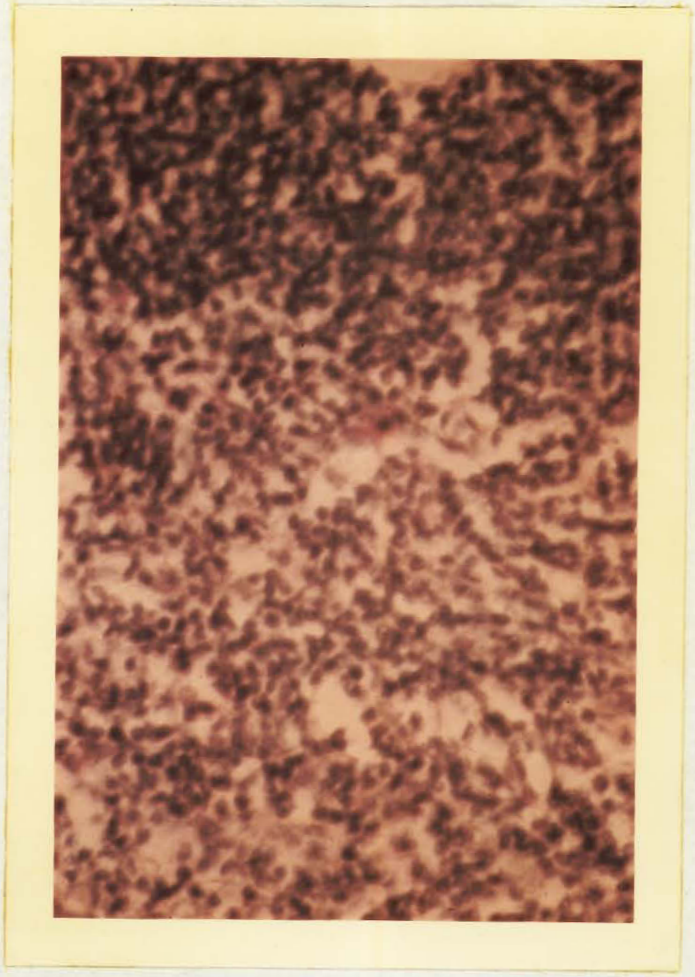

Figure 47

Thymus of mouse that had received an incompatible skin graft - CFI donor, CBA.2J recipient - 30 days after grafting.

Note pyroninophilia. Pappenheim stain x600 (Experiment H_1).

cells, but to a much less degree than lymph nodes or spleen. The corpuscles of Hassall did not appear very conspicuous at any time. The thymocytes did, to a much lesser degree, show some of the changes exhibited by the lymphocytes with a loss of deep nuclear staining and an opening of the nuclear pattern. That the thymus is involved itself in immunological reaction may be seen in figure 47 (page 106) in which a small extent of D.N.A. activity can be seen in the reticular cells in response to a mismatched skin graft. It is not, however, impressive.

20. Lymph Glands

At death the axillary lymph gland showed a loss of demarcation between cortex and medulla. No lymphoid follicles could be seen. The predominate cell was larger than in the normal gland with paler staining, open nucleus and prominent nucleoli. Elongated reticular cells could be seen but no true fibrosis was obvious. Normal looking lymphocytes were very uncommon (see fig. 48 page 108). Earlier in the malignant process there was the appearance of sinus histiocytosis with distension of the sinusoids and prominence of the reticular cells which showed a great increase in D.N.A. synthesis staining bright red with Pappenheim technique. Giant cells appeared with vivid pyroninophilia (see fig. 49 page 109). These big cells showed large multilobed nuclei with open coarse nuclear reticulum and prominent pale nucleoli. (see fig. 50 Page 110). All these features suggest that these cells might well be Fragrens type of immunologically active cells and represent

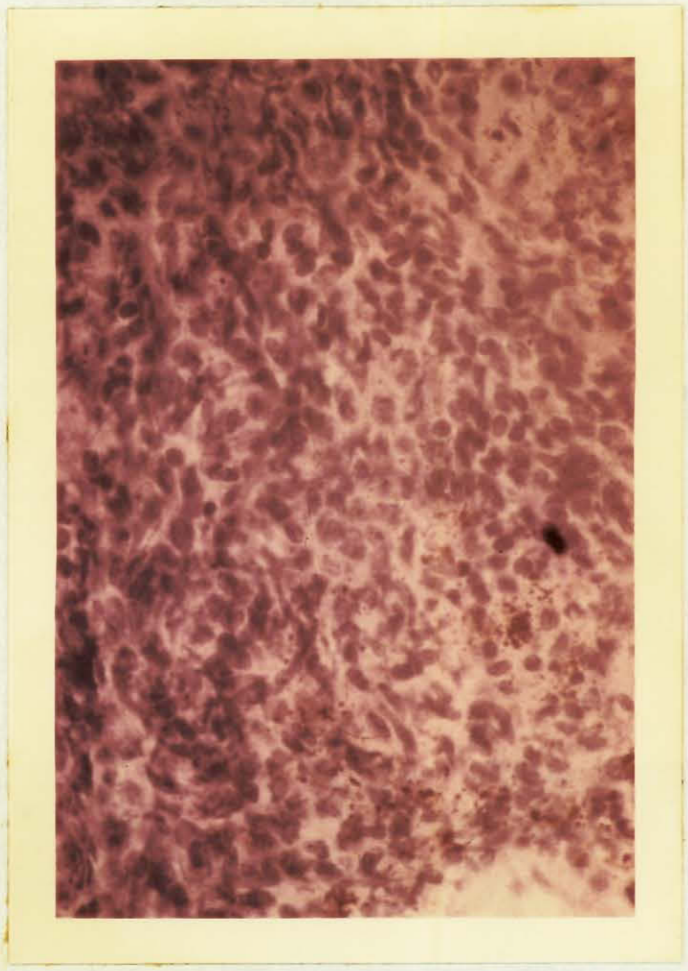

Figure 48

Lymph gland from mouse that died from tumour (Group XXXII)

H and E x600.

Note (1) Absence of typical lymphocytes.

(2) Carbon particles from previous intravital staining.

Compare with figure 49.

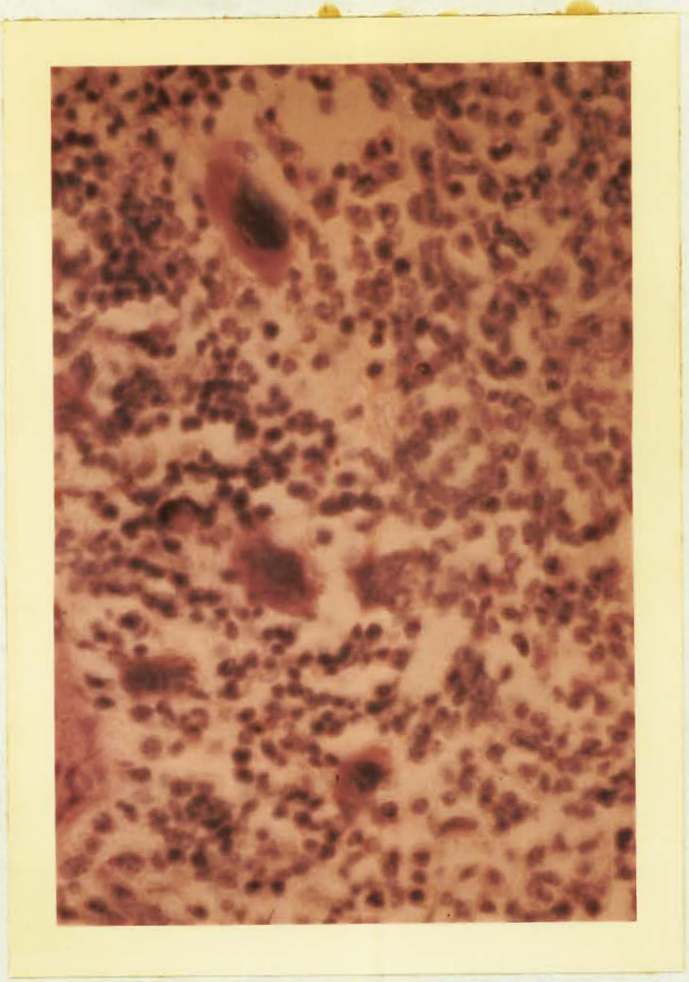

Figure 49

Lymph gland from mouse that had been implanted with tumour 12 days before sacrifice.

Note giant cells, plasma type cells and pyroninophilia. Pappenheim x600 (Exp. H9).

Compare figure 48 to see difference between lymph glands at death and during the neoplastic process.

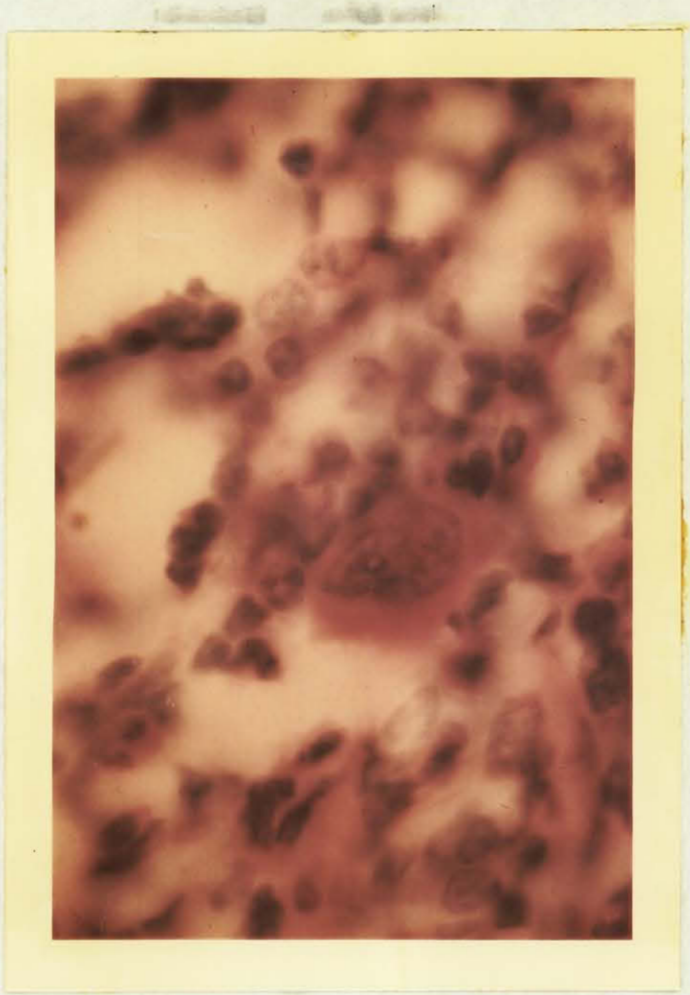

Figure 50

Lymph gland from mouse that had tumour implanted 18 days prior to sacrifice. Pappenheim x1600 (Exp. H10). Showing giant cells with prominent pale nucleoli and pyroninophilia cytoplasm.

histological evidence of the homograft type of response. If so, then it implies tumour specific antigens.

21. Spleen

The spleen lost the Malpighian prominence and appeared uniformly cellular (compare fig. 51 page 113 and fig. 52 page 114).

Sinusoids appeared distended and there was apparently generalised tissue oedema. Pyroninophilic reticular and giant cells were common (see fig. 53 page 115) especially during the middle third of the natural history of the disease. (8-15 days). The dominant cell appeared similar to that described for the lymph nodes. Giant cells of this nature in the mouse spleen (where the majority of the erythropoesis occurs) must be interpreted with extreme caution because of their resemblance to megakaryocytes. However, the enormous increase in these giant cells with tumour implantation and the lack of evidence of any haemolytic anaemia (and therefore possibly immune thrombocytopenia) makes their dismissal as megakaryocytes difficult especially as their appearance may be achieved with other immunological stimulants (vide infra). Figure 54 (page 116) and figure 55 (page 117) show some of these giant cells in rather atypical form with pale intranuclear halos that appeared under the microscope to be pale green. The electron microscopic studies of L.J. Journey and D.B. Amos (82) of the struggle between histiocytes and ascites tumour cells showed that loss of nuclear detail was the first sign of cellular damage. Could these cells be manifesting the long range toxic

Figure 51

Spleen from normal mouse. H and E x60 (Exp. H28).

To show malpighian corpuscles (colour a little distorted in printing).

Figure 52

Spleen from mouse dying from neoplasm. H and E x60 (Group XXIV).

To show loss of malpighian corpuscles with increase in cellularly and basophilia.

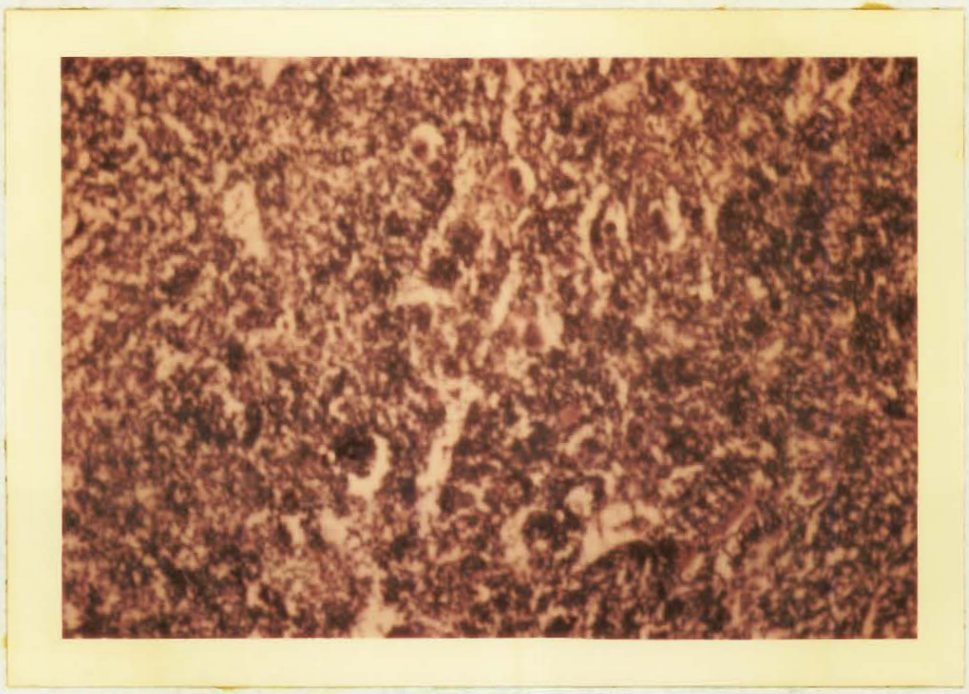

Figure 53

Spleen from mouse that had tumour implanted 27 days prior to death. (Exp. H17).

Note frequent giant cells. Pappenheim x160.

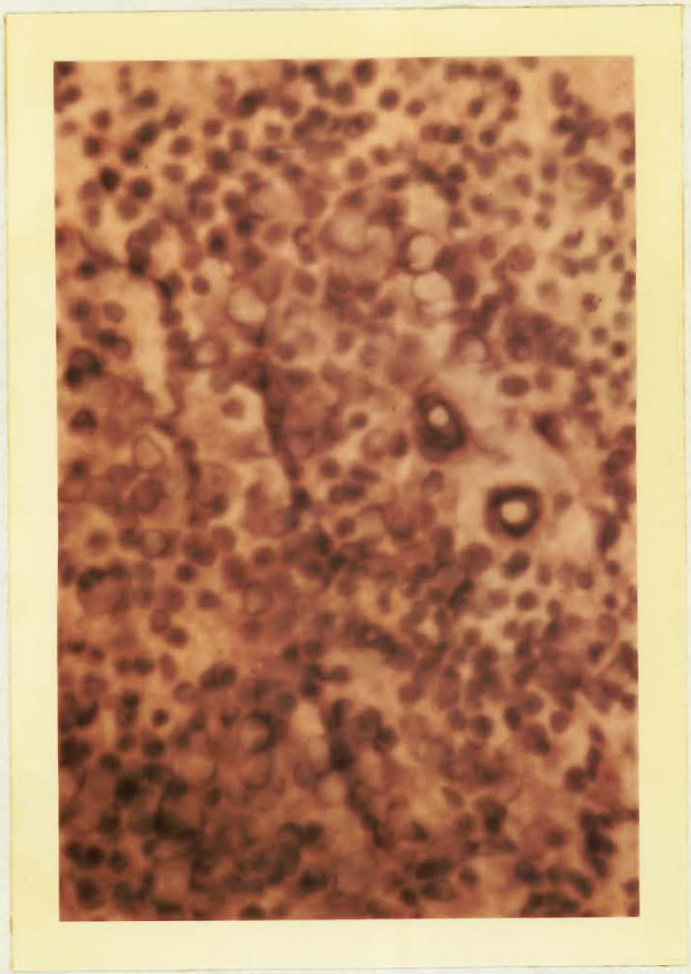

Figure 54

Spleen from mouse that had been implanted with tumour 14 days prior to sacrifice.

Note giant cells with intranuclear halos. Exp. H23 Pappenheim x600.

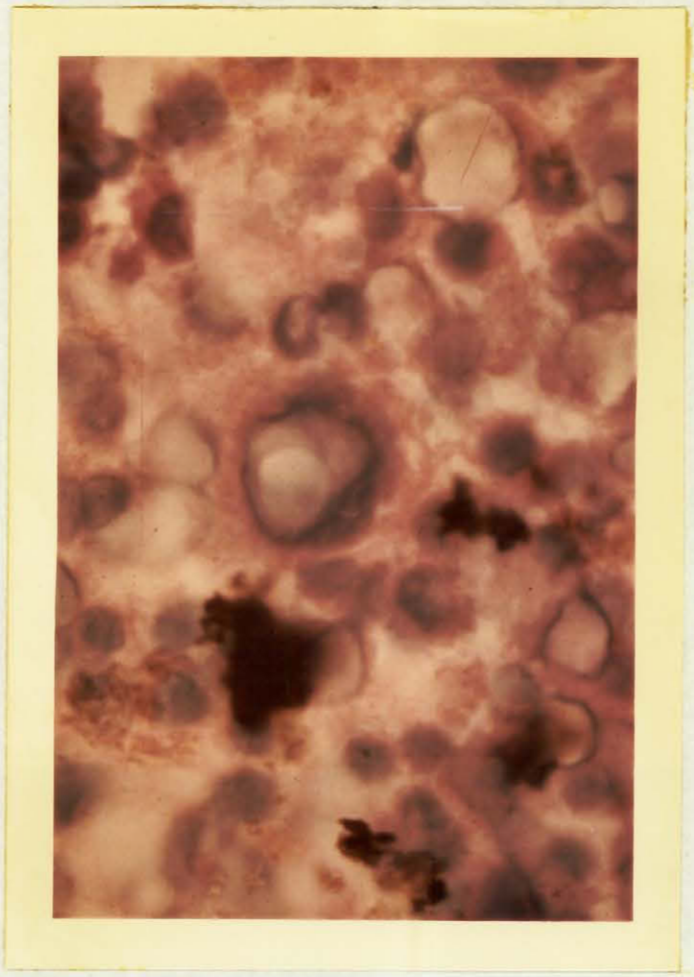

Figure 55

Spleen from mouse that had been implanted with tumour 14 days prior to sacrifice.

Note 'haloed' giant cells. Pappenheim stain x1600 (oil immersion) Exp. H23

effects of the tumour? Or is this feature merely artefactual?

22. Mice with Incompatable Skin Grafts.

The thymus, spleen and lymph nodes in mice who had had mismatched skin grafts (see Experiment 16 (i)) previously were studied histologically. The regional lymph nodes showed enlargement with widely dilated sinusoids and marked pyroninophilia of the reticular cells and occasional giant cells (see fig. 56 page 119). The germinal centres were, of course, present and normal except that they were a little overshadowed by the medullary changes. Even the thymus showed some pyroninophilic change (see fig. 47 page 106). The spleen showed changes rather similar to the lymph nodes. Giant cells were present and obvious (see fig. 57 page 120) to a far greater degree than the occasional megakaryocyte seen in the normal spleen, although it was not possible with the preparations studied to be certain of the nature of these cells (see fig. 58 page 121).

23. Mice Vaccinated with Freund's Adjuvant and Water

Pyroninophilic cells, some of them large, enlarged pink staining reticular cells, with dilated sinusoids and obvious increase in interstitial fluid was also observed in the spleen and lymph nodes of the mice which received Freund's adjuvant (see Experiment 16 (ii), fig. 59 page 122 and fig. 60 page 123).

24. Mice Inoculated with Antithymic Antiserum

In those animals that received antithymic heterologous anti-

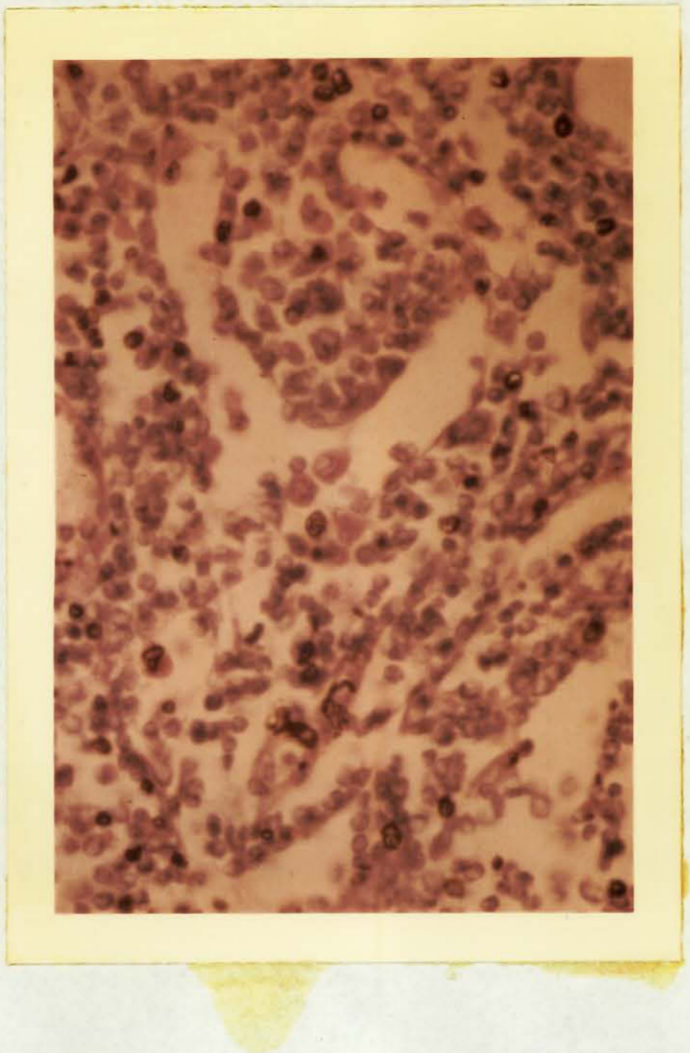

Figure 56

Regional (axillary) lymph gland from a CBA.2J mouse that had received a skin homograft from a CFI donor 30 days prior to sacrifice (Exp. H1). Note distension of sinusoids and pyroninophilia of reticular cells with some larger cells present. Pappenheim x600

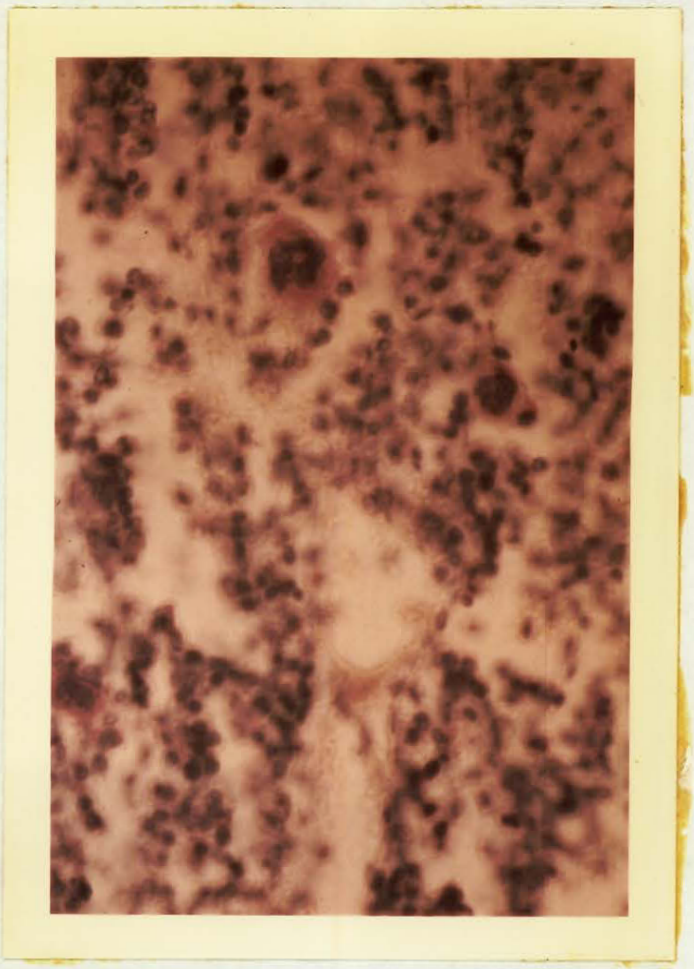

Figure 57

Spleen from CBA.2J mouse that had received a skin homograft from a CFI mouse 30 days prior to sacrifice (Exp. H1).

Note distended sinusoids and pyroninophilic giant cells. Pappenheim x600.

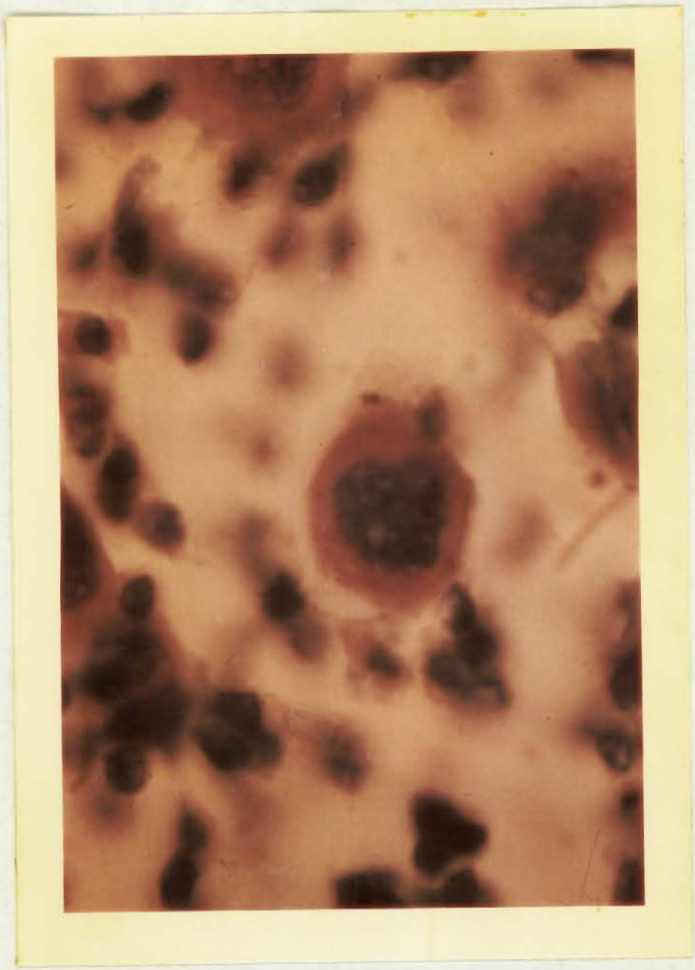

Figure 58

Spleen from CBA.2J mouse that had received skin homograft (CFI donor) 30 days prior to sacrifice. Exp. H1.

Note giant cells and pyroninophilic reticular cells. Pappenheim x1600 (oil immersion)

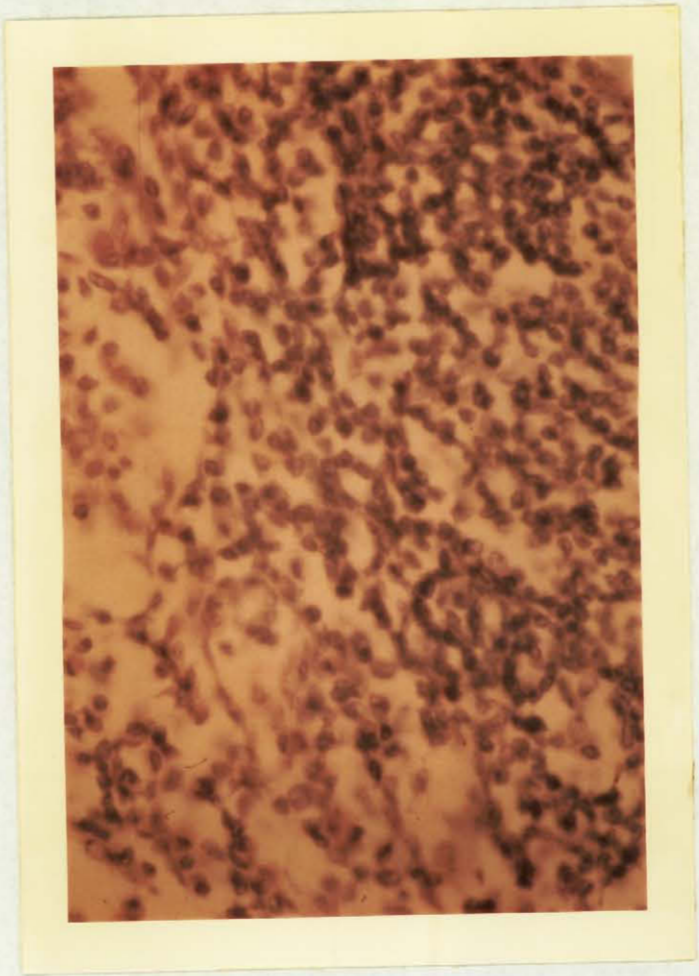

Figure 59

Lymphatic gland from mouse that had received 2 injections of Freund's adjuvant 13 and 3 days prior to sacrifice (exp. H31).

Note pyroninophilia of reticular cells. Pappenheim x600

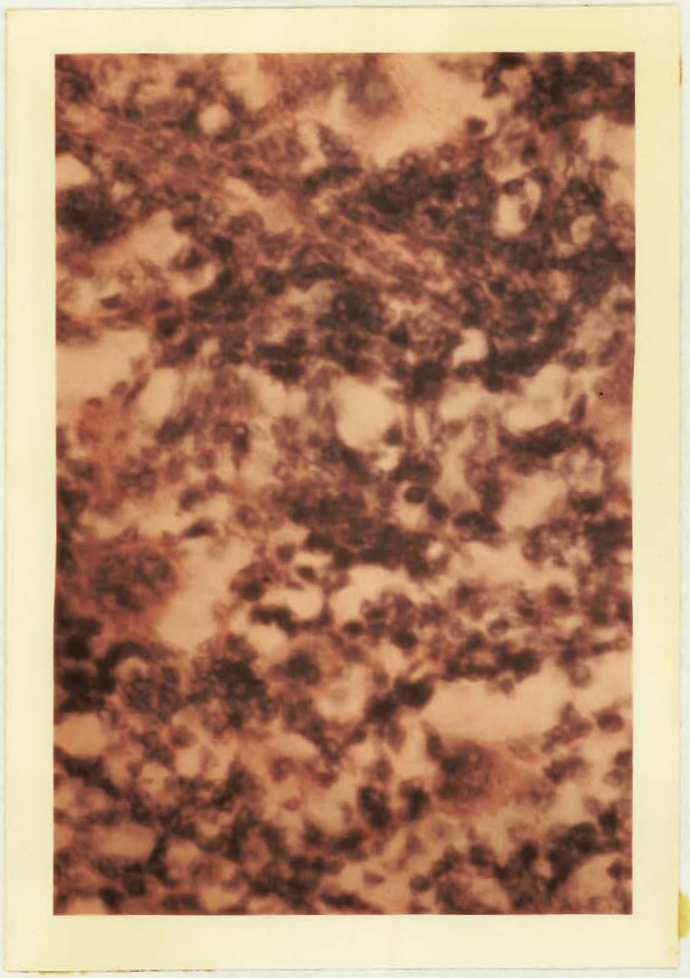

Figure 60

Spleen from mouse that had received 2 injections of Freund's adjuvant 13 and 3 days prior to sacrifice (exp. H31).

Note giant cells with pyroninophilic cytoplasm. Pappenheim x600

serum, a picture was seen somewhat similar to that in tumour bearing animals especially in the early stages of the disease. Normal lymphocytes were of course to be seen. The spleen lost the corpuscular configuration at low power and had sinusoids and appearance of cellular activity with many pyroninophilic giant cells (see fig. 61 page125). The lymph glands had a similar tendency but were not so impressive as in the other studies.

25. Histology of Other Organs

(a) Kidney

Intensive study was made of the kidney with haemotoxylin and eosin, Pappenheim and special basement membrane stains in the hope of finding evidence of intra-vascular immunological conflict (like the lupus erythematosis syndrome, for example). This proved fruitless and no specific abnormality could be detected in the kidneys of cancer bearing animals.

(b) Gut

Initial study of the gut showed a considerable loss of lymphocytic infiltration and an apparent thinning of the mucosa (see fig. 62 page126) (fig. 63 page127). This study is not complete, however, as there has not been careful matching of gut levels that would be necessary before firm statements could be made.

Conclusions from Histological Studies

The similarity of the histological pictures obtained in tumour bearing animals, incompatable skin grafts, injection of Freund'sadjuvant and

Figure 61

Spleen from mouse that had received heterologous antithymic immune serum (three doses of 0.5 ml. daily). Exp. H27.

Note frequent pyroninophilic giant cells. Pappenheim x60

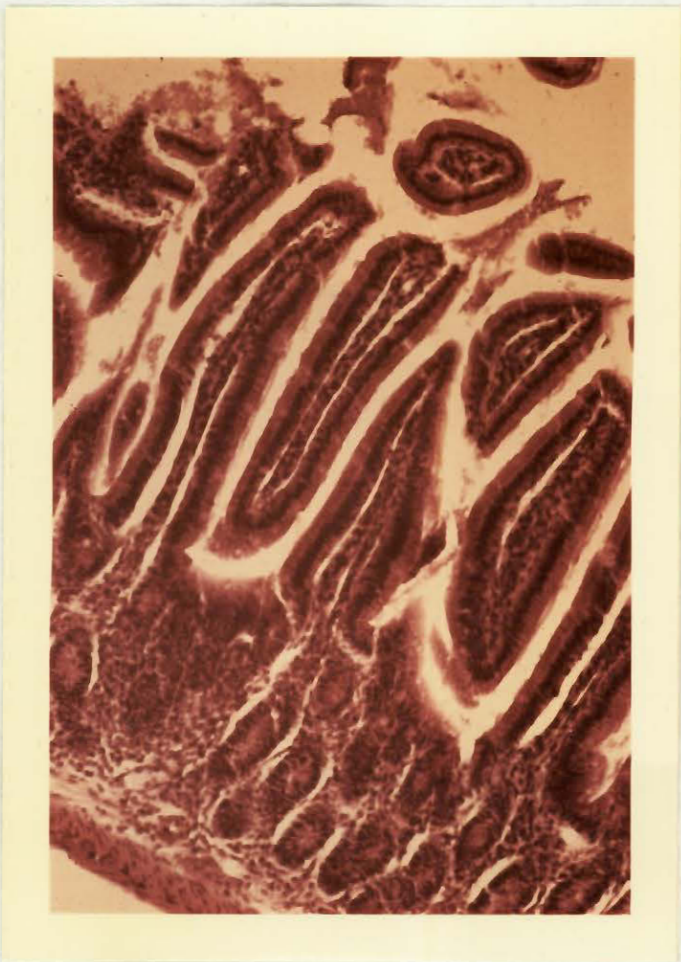

Figure 62

Small intestine from normal CBA.2J mouse. H and E x600

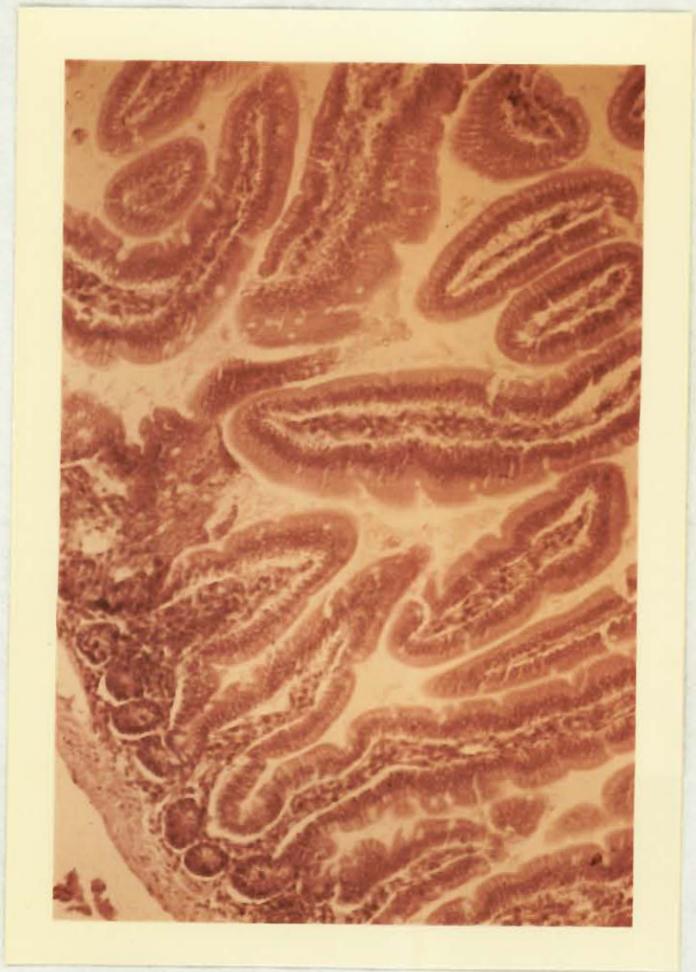

Figure 63

Small intestine from CBA.2J mouse dying from tumour.

Note thinning of mucosa and loss of lymphocytes. H and E x600

injection of antithymic heterologous serum seems to suggest that perhaps there is a common mechanism of immunological response operative. The fact that there was, in those mice with tumours, an obvious time factor is, I believe, of importance for the histology of the spleen and especially the lymph glands at death was considerably different from that in the earlier stages of the disease. The pyroninophilia of the reticular cells and the appearance of giant cells was best seen between the 6th and 14th day, and was almost totally extinct at death. This would imply an initial stimulation of D.N.A. activity - possibly an immunological response, followed by a suppression in the later stages of the disease process. This finding is compatable with the results of the gravimetric studies of lymph glands and spleen in Experiment 15.

PART C

General Conclusion

The tumour-host relationship has been studied in a system as nearly isologous as inbred mice can be and continually monitored by skin graft exchange. It was found that under the prevailing conditions, a closely reproducible survival pattern could be achieved and that this was unaffected by either considerable variation in tumour cell dosage or intracutaneous inoculation. Yet despite this, there was no relationship between size of tumour at death or longevity. It was found that in the absence of much assistance that survival time was probably the best experimental parameter to study. Experiments with "vaccination" of animals before and after tumour inoculation suggest that the anamnestic response can prolong survival time a remarkably constant amount and this was supported by challenge of the only three 'cures' obtained. However, there was a critical time factor in animals who already have established tumours and only third day vaccination significantly prolonged life. Immune heterologous gamma globulin in the dosage and time schedule used did not appear to affect the tumour. Weighing of the spleen, thymus and lymph glands of animals with tumours showed a progressive thymic atrophy, and an initial hypertrophy of spleen and regional lymph glands followed by a subsequent involution. Histological studies supported an increased R.N.A. activity in spleen and lymph

glands during the mid third of the tumour growth and this was thought to probably parallel the increase in organ size.

Woodruff, studying spleen size in spontaneous and transplanted tumours in 'A' strain mice, showed splenomegaly and histological changes of pyroninophilia and plasma cell aggregation in mice with successful but not rejected tumours (157). He interpreted this as evidence of immunological activity and proposed that splenic size was a measure of the degree of this response. The numbers of mice used was, however, fairly small and he did not study changes with time. (Estimations were made on the 14th day post-implant in a system that killed 35-50 days.) This was most interesting for me, for I did not learn of his work till I had completed my own and I was glad to find confirmatory results. In a later report (158), Woodruff showed that after many passages, the tumour was no longer able to elicit splenomegaly and he attributed this to loss of tumour specific antigens. "If this hypothesis is correct it would seem to provide an important clue to the understanding of the behaviour of malignant tumours, not only in experimental animals, but also in man. During the phase of antigenic difference a tumour may conceivably be held in check, or even destroyed, by immunological mechanisms, whereas after deletion of all tumour specific antigens the natural immunological defences have no point d'appui and any restraining effect they may have had is lost." (Professor M.F.A. Woodruff) (158).

The work here presented, however, would seem to indicate changes within the host's reticulo-endothelial system, rather than the tumour, that countenances the persistence of histo incompatability by destroying the rejection mechanism itself. To study a tumour-host system at one stage only in its efflorescence is to arrest the moving picture by studying a single frame and by so doing miss the all important factor of change. The laborious and painstaking post mortem dissections and measurements made in this study I do not now believe to be as valuable as the later time scaled studies even though the numbers involved are so different.

The conclusions that can be drawn from these experiments are necessarily guarded for the results are suggestive rather than conclusive. However, it looks as if in this system immunological mechanisms are involved and therefore tumour specific antigens exist, but that the host rejection mechanism is in someway overcome by humoral means. Whether this is on a runting basis is conjectural, but certainly the moribund cancerous mouse has all the classical macroscopic features of this condition. The lymphocyte, the generally accepted mediator of homograft rejection, virtually disappears, at least in its usual guise, from lymph glands, small intestine, thymus and spleen, and it would be tempting to suggest that this is a direct effect of the neoplastic cells. To recast these conclusions in terms of the three pertinent postulated question (fig. 1 page 1) posed in the introduction (page 19) it would seem that in our system:

(i) There was antigenic difference between host and tumour sufficient to provoke immunological response.

(ii) There was some barrier of access between host reticulo-endothelial system and the tumour cells, or at least, some delay until

(iii) There appears to be an elimination of the immunologically competent cell and in this way tumour rejection is circumvented.

The surgical importance of such views, were they to prove applicable to man, is enormous. The "innocuous" nature of circulating cancer cells in the blood would be explained for metastases would only result if the host's immunological mechanism were overcome. This would imply that once metastases had occurred, that attempts to enhance host resistance are futile, because the mediators of this defence are already overpowered, but that prior to this critical point therapy should be directed towards removing the bulk of the tumour mass without prejudicing host resistance. The question of replacement of competent cells then becomes pertinent and it is interesting that Woodruff has already done work on this with mice (159) without prolongation of life and he has used allogenic spleen cell implants in 8 patients with advanced cancer, with results that appear encouraging but are complicated by the use of cytotoxic drugs or prednisone to delay homograft rejection (160). It is along these lines, rather than bold extended surgery, that I believe cancer therapy of the future will find most success.

"It seems strange indeed that, despite the tremendous strides of our healing art, cancer mortality has not yet reflected to any major degree the scientific solace of the wonder drugs or the bountiful benefits of an ever improving surgical skill. Nevertheless, living in an age of modern medical miracles we may rightly hope that this malignancy, like many sinister scourges, may ultimately be conquered..... The ancient Roman Seneca once said 'our forefathers have done much, but they have not finished everything' ".

Edward F. Lewison March 1953

Acknowledgments

I would like to express my very great appreciation to all those people whose help and support made this work possible.

Dr H. Rocke Robertson who extended to me the very great privilege of inviting me across the ocean to work in his department, and who started and supported me in the work.

Dr Fraser Gurd for allowing me the freedom of his department and affording me encouragement and guidance.

Dr Ian Henderson, my immediate supervisor, whose help and instruction was invaluable.

Mr Bill Mersereau for his ready advice on many matters, including teaching me how to take photomicrographs.

Mrs M. Farrell for her histological skill in preparing sections from material that was by no means easy to handle.

Mr Ivan Gabor who taught me how to handle animals, showed me his tumour implantation technique and helped me with some of the routine.

Miss Wendy Smith for her patient and skilful secretarial work.

BIBLIOGRAPHY

1. ACKERMAN, L.V. and REGATO, J.A. de.
Cancer, Diagnosis, Treatment and Prognosis. 3rd edition, 1962. Kimpton, London.

2. AETIUS quoting Leonides.
Graeci contractae ex Veteribus Medicinae Tetrabiblos - Basileae, impensis Hier. Froben and N.C. Episcopii, 1542, Sermo IV, cap XLV, p. 884.

3. AMOS, D.B.
Genetic Studies of Tumour Immunity: An Analysis of C3H Sublines and their Tumors.
Ann. N.Y. Acad. Sci., 71, 1009-21, 1958.

4. AMOS, D.B. and DAY, E.D.
Passive Immunity Against Four Mouse Leukoses by Means of Isoimmune Sera.
Ann. N.Y. Acad. Sci., 64, 851-58, 1957.

5. BALDWIN, R.W.
Immunity to Methyl-cholanthrene - induced tumours in Inbred Rats following Atrophy and Regression of the Implanted Tumours.
Brit. J. Cancer, 9, 652-7, 1955.

6. BARRETT, M.K.
The Nature of Tumour Immunity in M.G. Sevag, R.D. Reid, and O.E. Reynolds (editors).
Origins of Resistance to Toxic Agents p. 308-33, Academic Press, New York, 1955.

8. BERG, J.W.
Inflammation and Prognosis in Breast Cancer. A Search for Host Resistance.
Cancer, 12, 714-20, 1959.

9. BERKSON, J., HARRINGTON, S.E., CLAGETT, O.T., KIRLIN, J.W., DOCKERLY, M.B. and McDONALD, J.R.
Mortality and Survival in Surgically Treated Cancer of the Breast. A Statistical Summary of Some Experience of the Mayo Clinic.
Proc. Mayo Clin., 32, 645-70, 1957.

10. BILLINGHAM, R.E., BRENT, L. and MEDAWAR, P.B.
Quantitative Studies on Tissue Transplantation Immunity - Origin Strength and Duration of Actively and Adoptively Acquired Immunity.
Proc. roy. Soc. Med., Series B, Biological Sciences, 143, 58-80, 1954.

11. BJORKLUND, B. and BJORKLUND, V.
Antigenicity of Pooled Human Malignant and Normal Tissues by Cyto Immunological Techniques. Presence of an Insoluble, Heat Labile Tumour Antigen.
Intern. Arch. Allergy Appl. Immunol., 10, 153-84, 1957.

12. BJORKLUND, B., LUNDBLAD, G. and BJORKLUND, V.
Antigenicity of Pooled Human Malignant and Normal Tissues by Cyto Immunological Techniques II. Nature of Tumor Antigen.
Intern. Arch. Allergy Appl. Immunol., 12, 241-61, 1958.

13. BLACK, M.M., OPER, S.R. and SPEER, F.D.
Microscopic Structure of Gastric Carcinoma and their Regional Lymph Nodes with Relationship to Survival.
Surg. Gynec. Obstet., 98, 725-34, 1954.

14. BLACK, M.M., OPER, S.R. and SPEER, F.D.
Survivals in Breast Cancer Cases in Relationship to Structure of Primary Tumour and Regional Lymph Nodes.
Surg. Gynec. Obstet., 100, 543-51, 1955.

15, BLACK, M.M. and SPEER, FD.
Immunology of Cancer.
Surg. Gynec. Obstet. Intern. Abstr. Surg., 109, 105-16, 1959.

16. BREASTED, J.H.
The Edwin Smith Surgical Papyrus, Chicago 1930,
University of Chicago Press, vol. 1, case 39, p. 363 and case 45, p. 463.

17. BRINKLEY, D. and HAYBITTLE, J.L.
Results of Treatment of Carcinoma of the Breast.
Lancet, 1, 86-8, 1959.

18. BUINAUSKA, P., McDONALD, G.O. and COLE, W.
Role of Operative Stress in the Resistance of the Experimental Animal to Inoculated Cancer Cells.
Ann. Surg., 148, 642-48, 1958.

19. BURNET, F.M.
The Clonal Selection Theory of Acquired Immunity.
Cambridge University Press, 1959.

20. BURROWS, D.
Schultz-Dale test for Detection of Specific Antigen in Sera of Patients with Carcinoma.
Brit. med. J., 15, 367-70, 1958.

21. BURROWS, D. and NEILL, D.W.
Investigation of the Immunologically Active Constituent of Serum of Patients with Carcinoma.
Brit. med. J., 15, 370-1, 1958.

23. BYRD, B.F. and CONERLY, D.B.
Role of Simple Mastectomy in the Treatment of Carcinoma of the Breast.
Ann. Surg., 141, 477-81, 1955.

24. CELSUS, A.C.
Translated by Greive, J.
Book V, chap. 28, pp27 and 303.

25. COHN,
Method in Clinical Research, 5, 271, 1952.

26. COOPER, W.A.
The History of Radical Mastectomy.
Ann. med. Hist., 3, 36-54, 1941.

27. CRILE, G. (Jr)
Problems in the Treatment of Cancer.
Maryland State Medical Journal, vol. 7, No. 9, 1958.

28. CRILE, G. (Jr)
Cancer of the Breast: Evaluation of Current Methods of Treatment.
Postgraduate Medicine, 26, 64-72, 1959.

29. CRILE, G. (Jr)
The Danger of Becoming Satisfied with Standardised Techniques.
Surg. Gynec. Obstet., 3, 99-100, 1960.

30. CRILE, G. (Jr)
The Cancer Problem.
The Graduate Journal, 4, n0. 1, 142-70, 1961.

31. CRILE, G. (Jr)
Heat as an Adjuvant to the Treatment of Cancer. Experimental Studies.
Cleveland Clinic Quarterly, vol. 28, no. 2, 75-89, 1961.

32. DAVIS, J.R. and BUSCH, H.
Isolation of Radioactive Acid-soluble Nuclear Proteins Uniquely found in Neoplastic Tissues.
Proc. Ann. Assoc. Cancer Research, 3, 104, 1960.

33. DAVIS, J.R. and BUSCH, H.
Chromatographic Analysis of Cationic Nuclear Proteins of a Number of Neoplastic Tissues.
Cancer Research, 20, 1208-13, 1960.

34. DAY, E.D., PLANINSEK, J.A. and PRESSMAN, D.
Specific Localisation in vivo of Antihepatoma Antibodies in Autochthonous Rat Hepatomas.
J. Nat. Cancer Inst., 27, 1107-14, 1961.

35. DEATON, W.R.
Simple Mastectomy of Breast - reported results.
Surgery, 37, 720-5, 1955.

36. DENOIX, P.F.
Monographic de l'Institut National d'Hygiene, No. 5, De la Diversite de Certains Cancers. Published by Ministere de la Sante, publique Paris, 1954.

37. EBERS PAPYRUS.
Translated by Ebbell, B.
Copenhagen 1937, Levin and Munksgaard.

38. ENGELL, H.C.
Cancer cells in the Circulating Blood: A clinical study on the occurrence of cancer cells in the peripheral blood and in the venous blood draining the tumour area at operation.
Acta chir. Scandinav., 1955, supp. 201, p. 70.

39. EVERSON, T.C. and COLE, W.H.
Spontaneous Regression of Cancer.
Ann. Surg., 144, 366-83, 1956.

40. FELDMAN, M. and SACHS, L.
The Antibody Response to Successful Tumour Homografts.
J. Nat. Cancer Inst., 18, 529-53, 1957.

41. FERGUSON, A.B.
Treatment of Osteogenic Sarcoma.
J. Bone Jt. Surg., 22, 92-6, 1940.

42. FINNEY, J.W., BYERS, E.H. and WILSON, R.H.
Studies in Tumour Autoimmunity.
Cancer Research, 20, 351-6, 1960.

43. FINNEY, J.W., CLARK, D.A., RUSSELL, H.W. and WYSS, O.
Tissue Autosensitivity in Animals: Antitumoural Autodefences in Man and their Possible Therapeutic Effect.
Fed. Proc., 17, 219, 1958.

44. FINNEY, J.W. and WILSON, R.H.
J. Lab. & Clin. med., 54, 814, 1959.

45. FISHER, B. and FISHER, E.R.
Experimental Studies of Factors Influencing Hepatic Metastases.
III Effects of Surgical Trauma with Special Reference to Liver Injury.
Ann. Surg., 150, 731-44, 1959.

46. FISHER, E.R. and TURNBULL, R.B.
The Cytologic Demonstration and Significance of Tumour CElls in the Mesenteric Venous Blood in Patients with Colo-rectal Carcinoma.
Surg. Gynec. Obstet., 100, 102-8, 1955.

47. FOLEY, E.J.
Antigenic Properties of Methylcholanthrene-induced Tumours in Mice of the Strain of Origin.
Cancer Research, 13, 835-7, 1953.

48. FORBER, J.E.
Ministry of Health Reports of Public Health and Medical Subjects No. 66, London, H.M. Stationery Office, 1931.

49. FREUND, J., LIPTON, M.M. and THOMPSON, G.E.
Aspermatogenesis in the Guinea Pig Induced by Testicular Tissue and Adjuvants.
J. exp. med., 97, 711-26, 1953.

49. FREUND, J., THOMPSON, G.E. and LIPTON, M.M.
Aspermatogenesis, Anaphylaxis and Cutaneous Sensitisation Induced in the Guinea Pig by Homologous Testicular Extract.
J. Exp. Med., 101, 591-603, 1955.

50. FREUND, J.
Mode of Action of Immunoligic Adjuvants.
Adv. Tuberc. Res., 7, 130-148, 1956.

51. GALCH, W.D.
Changing Outlook on Cancer and its Treatment.
J. Indiana State Med. Ass., 47, 973-6, 1954.

52. GORDON-TAYLOR, G.
On Cancer of the Breast.
Ann. Roy. Coll. Surg. Engl., 2, 60-8, 1948.

53. GORER, P.A.
The Antigenic Basis of Tumour Transplantation.
J. Pathol. Bacteriol., 47, 231-52, 1938.

54. GORER, P.A.
The Immunogenetics of Cancer.
Jaarb. Kankeronderzoek en Kankerbestrijding Ned 90-97, 1959.
Vereeniging het Nederlandsch Kankerinstituut, Amsterdam.

55. GORER, P.A.
The Value of Ascites Tumours in the Problems of Tumour Immunity.
Ann. N.Y. Acad. Sci., 63, 882-92, 1956.

56. GORER, P.A.
Some Recent Work on Tumour Immunity.
Advances in Cancer Research, 4, 149-86, 1956.

57. GORER, P.A.
Some Reactions of H2 Antibodies in vitro and in vivo.
Ann. N.Y. Acad. Sci, 73, 707-21, 1958.

58. GORODILOVA, V.V.
Specific Antigen of Breast Cancer.
Problems Oncol., 5 (ii), 59-68, 1959.

59. GRACE, J.T. and KONDO, T.
Investigations of Host Resistance in Cancer Patients.
Ann. Surg., 148, 633-41, 1958.

60. GRACE, J.T . and LEHOCZKY, A.
Tumor-host Relationship Factors in Man.
Surgery, 46, 238-46, 1959.

61. GRAHAM, J.B. and GRAHAM, R.M.
The Effect of Vaccine on Cancer Patients.
Surg. Gynec. Obstet., 109, 131-8, 1959.

62. GREEN, H.N.
The Immunological Theory of Cancer. Some Implantations in Human Pathology.
J. chron. Dis., 123-36, 1958.

63. GREEN, H.N.
Immunological Basis of Carcinogenesis.
Brit. med. Bull., 14, 101, 1958.

64. GREENWOOD, M.
Ministry of Health Report of Public Health and Medical Statistics, No. 33.
H.M. Stationery Office, London, 1926.

65. GROSS, L.
Intradermal Immunisation of C3H Mice against a Sarcoma that Originated in an Animal of the Same Line.
Cancer Research, 3, 326-34, 1943.

66. HAAGENSEN, C.D. and STOUT, A.P.
Carcinoma of Breast III. Results of Treatment 1935-42.
Ann. Surg., 134, 151-72, 1951.

67. HACKMANN, C.
Experimentelle Studien ueber Heilungsvorgaenge bei boesartigen Geschwuelsten.
Z. Krebsforsch, 57, 164-90, 1950.

68. HADDOW, A.
Historical Notes on Cancer From the M.S.S. of Louis Westerna Sambon.
Proc. roy. Soc. Med., 29, 1015-29, 1936.

69. HADFIELD, G.
The Dormant Cancer Cell.
Brit. med. J., 2, 607-10, 1954.

70. HADFIELD, J. and HADFIELD, G.
The Natural History of Breast Cancer.
British Surgical Practice, Progress vol. 1959, p. 221, Butterworths, London.

71. HALSTEDT, W.S.
The Treatment of Wounds with Especial Reference to the Valve of the Blood Clot in the Management of Dead Spaces.
Johns Hopkins Hosp. Rep., 2, 255-314, 1890-1 (reprinted).

72. HALSTEDT, W.S.
The Result of Operations for the Cure of Cancer of the Brease Performed at the Johns Hopkins Hospital from June 1889 to January 1894.
Johns Hopkins Hosp. Rep., 4, 297-350, 1894-5.

73. HANDBOOK on Genetically Standardised Jax Mice.
Rosco B. Jackson Memorial Laboratory, Bar Habor, Maine, 1962.

74. HAUSCHKA, T.S.
Immunologic Aspects of Cancer: A Review.
Cancer Research, 12, 615-33, 1952.

75. HIPPOCRATES Works (4 volumes) edited by Jones, W.H.S. and Withington, E.T.
Loeb Classical Library, New York, 1923-31. Putnam's Sons.

76. HIRSCH, H.M.
Tumour Isoimmunity.
Experientia, 14, 269-71, 1958.

77. HIRSCH, H.M.
Tumour Immunity and Tissue Transplantation.
J.Lancet, 79, 340-47, 1959.

78. HIRSCH, H.M.
Some Aspects of the Problem of Immunity Against Transplanted and Spontaneous Tumours.
Bacteriological Reviews, vol. 26, no. 3, p. 336, 1962.

79. HOEPKE, H.
Wehrt sich der Korper gegen Geschwulste?
Strahlentherapie, 93, 196-212, 1954.

80. ISCHIBASHI, Y., HATTORI, G., FUJII, K. OKADA, M. SEKIGUCHI, K. ASHIKAWA and K. MOTOYA.
Studies on the Tumor Auto-immunity.
Japanese Journal of Experimental Medicine, 31, no. 4, 319-331, 1961.

81. JONES, F.S. and ROUS, P.J.
On the Cause of Localisation of Secondary Tumours at Points of Injury.
J. exp. Med., Lancaster 20, 404-13, 1914.

82. JOURNEY, L.J.
An Electron Microscope Study of Histiocyte Response to Ascites Tumour Homogenate.
Cancer Research, 22, no. 8, 998, 1962.

83. KALISS, N.
Immunological Enhancement of Tumour Homografts in Mice: A Review.
Cancer Research, 18, 992, 1958.

84. KALISS, N. and BRYANT, B.F.
Factors Determining Homograft Destruction and Immunological Enhancement in Mice Receiving Successive Tumour Inocula.
Journal of National Cancer Institute, 20, 691-704, 1958.

85. KAPLAN, H.S., MURPHY, E.D.
The Effect of Local Roentgen Irradiation on the Biological Behaviour of a Transplantable Mouse Carcinoma.
I. Increased Frequency of Pulmonary Metastasis.
Journal of National Cancer Institute, 9, 407-13, 1949.

86. KAPLAN, H.A. and SMITHERS, D.W.
Autoimmunity in Man and Homologous Disease in Mice in Relation to Malignant Lymphomas.
Lancet 2, 1-4, 1959.

87. KLEIN, G.
The Usefulness and Limitations of Tumour Transplantation in Cancer Research: A Review.
Cancer Research, 19, 343-58, 1959.

88. KLEIN, G., SJORGREN, H.O., KLEIN, E. and HELLSTROM, K.E.
Demonstration of Resistance Against Methylcholanthrene Induced Sarcomas in the Primary Autochthonous Host.
Cancer Research, 20, 1561-72, 1960.

89. KOLMYKOVA, V.N. and YEROSHKINA, A.M.
The Similarity and Differences in the Antigenic Composition of Tumour, Embryonal and Adult Tissues in Man.
Problems Oncol., 5 (2) 1-6, 1959.

90. KOLMYKOVA, V.N. and YEROSHKINA, A.M.
The Similarity and Differences in Antigenic Composition of Human Sarcoma and Leukaemic Tissues.
Problems Oncol., 5 (12) 1-6, 1959.

91. KORNGOLD, L.
The Distribution and Immunochemical Properties of Human Tissue and Tumor Antigens.
Ann. N.Y. Acad. Sci, 69, 681-97, 1957.

92. KOSAKI, T.
A New Phospholipid, Malignolipin in Human Malignant Tumors.
Science, 127, 1176-7, 1958.

93. LE DRAN, H.F.,
Memoire avec un Precis de Plusieurs Observation sur le Cancer.
Mem. de l'Acad. roy. de Chir., Paris 3, 1-56, 1757.

94. LEWISON, E.F.
The Surgical Treatment of Breast Cancer.
Surgery, 34, no. 5, 905, 1953.

95. LUND, R.H., IHNEU, M.
Malignant Melanoma: Clinical and Pathologic Analysis of 93 Cases. Is Prophylactic Lymph Node Dissection Indicated?
Surgery 38, 652-9, 1955.

96. MACDONALD, I.
Biological Predetermination in Human Cancer.
Surg. Gynec. Obstet., 92, 443-52, 1951.

97. MAKARI, J.G.
Detection of Antigens in Sera of Patients with Neoplastic Disease by Schultz-Dale Test.
Brit. med. J., 2, 358-61, 1958.

98. MARTINEZ, C., MIROFF, G. and BITTNAR, J.J.
Effect of Size and/or Rate of Growth of Transplantable Mouse Adenocarcinoma on Lung Metastases Production.
Cancer Research, 16, 313-5, 1956.

99. MEDAWAR, P.B.
The Immunology of Transplantation.
Harvey Lecture 1956-7, Academic Press Inc. New York, p. 144-76.

100. MEIER, H.
Personal Communication.
Rosco B. Jackson Memorial Laboratory, Bar Harbor, Maine.

101. MEYER, A.C. and SMITH, S.S.
Some Concepts in Treatment of Breast Cancer.
A.M.A. Arch. Surg., 69, 707-10, 1954.

102. MEYER, W.
An Improved Method of the Radical Operation for Carcinoma of the Breast.
Med. Rec., 46, 746, 1894.

102a. MIDER, G.B.
Some Developments in Cancer Research.
J. chr. Dis., 4, 296-320, 1956.

103. MILGROM, F.
A Short Review of Immunological Investigations on Cancer.
Cancer Research, 21, 862-8, 1961.

104. MILLER, E.E. and BERNFELD, P.
Abnormal Plasma Components in C3H Mice Bearing Spontaneous Tumours.
Cancer Research, 20, 1149-53, 1960.

105. McKINNON, N.E.
Limitations in Diagnosis, Treatment of Breast and Other Cancers: A Review.
Canad. M.A.J., 73, 614-25, 1955.

106. McWHIRTER, R.
Discussion - Treatment of Cancer of Breast.
Proc. roy. Soc. Med., 41, 122-9, 1948.

107. McWHIRTER, R.
Simple Mastectomy and Radiotherapy in the Treatment of Breast Cancer.
Brit. J. radiol., 28, 128-39, 1955.

108. McWHIRTER, R.
Some Factors Influencing Prognosis in Breast Cancer.
J. Faculty radiol., 8, 220-34, 1957.

109. MOORE, C.H.
On the Influence of Inadequate Operations on the Theory of Cancer.
Roy. Med. & Chir. Soc., London, 1, 244-80, 1867.

110. MURPHY, J.B.
The Lymphocyte in Resistance to Tissue Grafting Malignant Disease and Tuberculosis Infection. An Experimental Study. Monographs of the Rockefeller Institute for Medical Research, 21, 1926.

111. NATHANSON, I.T. and WELCH, C.E.
Life Expectancy and Incidence of Malignant Disease. 1 Carcinoma of the Breast.
Amer. J. Cancer, 28, 40-53, 1936.

112. OLCH, P.D. and LEIKINA, M.C.
Some Land Marks in the History of Cancer Research.
Exhibit. American College Surgeons, 1963.

113. OLD, L.J., BENACERRAF, B., CLARKE, D.A., CARSWELL, E.A. and STOCKERT, E.
The Role of the Reticuloendothelial System in the Host Reaction to Neoplasia.
Cancer Research, 21, 1281-1301, 1961.

114. PARK, W.W. and LEES, J.C.
Absolute Curability of Cancer of the Breast.
Surg. Gynec. Obstet., 93, 129-52, 1951.

115. PARE, A.
Les Oeuvres de M. Ambroise Pare.
Paris, 1575, G. Buon.

116. POWER, Sir d'Arcy.
The History of the Amputation of the Breast to 1904.
Liverpool Med. chir. J., 42, 29, 1934.

117. PREHN, R.T. and MAIN, J.M.
Immunity to Methylcholanthrene-induced Sarcomas.
J. Nat. Cancer Inst., 18, 764-78, 1957.

118. PREHN, R.T.
Tumor-specific Immunity to Transplanted Dibenz (a, L)anthrocene-induced Sarcomas.
Cancer Research, 20, 1614-7, 1960.

119. PRINCE, J.E., FARDON, J.C., NUTIMI, L.G. and SPERTI, G.S.
Induced Resistance to an Indigenous Transplantable Mouse Tumour.
Cancer Research, 17, 312-6, 1957.

120. PRINCE, J.E., MORGAN, C.M., FARDON, J.C., NUTINI, L.G. and DUARTE, A.G.
Induced Susceptibility and Resistance to an Indigenous Transplantable Mouse Tumour with Autolysed, Alcohol Killed Mouse Tumour Tissue.
Cancer Research, 18, 760-3, 1958.

121. RAPPORT, M.M., GRAF, L., and SKIPSKI, V.P., ALOUZO, N.F.
Immunochemical Studies of Organ and Tumour Lipids VI.
Isolation and Properties of Cytolipin H.
Cancer, 12, 438-45, 1959.

122. REVESZ, L.
Detection of Antigenic Differences in Isologous Host-tumour Systems by Pretreatment with Heavily Irradiated Tumour Cells.
Cancer Research, 20, 443-51, 1960.

123. SALK, J.E. and WARD, E.N.
Some Characteristics of a Continuously Propogating Cell Derived from Monkey Heart Tissue.
Science, 126, 1338-9, 1957.

124. SANDBERG, A.A., MOORE, G.E. and SCHUBERG, J.R.
Clinical and Experimental Observations of the Occurrence and Rate of Tumour Cells in the Blood Stream.
Ann. Surg., 146, 580-7, 1957.

125. SANDBERG, A.A. and MOORE, G.E.
Examination of Blood for Tumour Cells.
J. Nat. Cancer Inst., 19, 1-5, 1957.

126. SANFORD, K.K, MERWIN, R.M., HOBBS, G.L., FIORAMONTI, M.C. and EARLE, W.R.
Studies on the Difference in Sarcoma-producing Capacity of Two Lines of Mouse Cells Derived in vitro from One Cell.
J. Nat. Cancer Inst., 20, 121-45, 1958.

127. SCHATTEN, W.E.
An Experimental Study of Post-operative Tumour Metastases.
I. Growth of Pulmonary Metastases following Total Removal of Primary Leg Tumour.
Cancer, 11, 455-9, 1958.

128. SHIMKIN, M.B.
Duration of Life in Untreated Cancer.
Cancer, 4, 1-7, 1951.

129. SHIMKIN, M.B.
Natural History of Neoplastic Disease.
In.F. Homburger (ed), The Physiopathology of Cancer, p. 855-71, 2nd ed. Hoeber-Harper, New York. 1959.

130. SMALL, R.G. and DUTTON, A.M.
Survival of Patients with Carcinoma of Breast.
J. Amer. med. Ass., 157, 216-9, 1955.

131. SNELL, G.D.
Histocompatability genes in the Mouse. I. Demonstration of Weak Histocompatability Differences by Immunisation and Controlled Tumour Dosage.
J. Nat. Cancer. Inst., 20, 787-24, 1958.

132. SNELL, G.D.
Histocompatability genes in the Mouse. II. Production and Analysis of Isogenic Resistant Lines.
J. Nat. Cancer Inst., 21, 843-77, 1958.

133. SOUTHAM, C.M.
Relationship of Immunology to Cancer: A Review.
Cancer Research, 20, 271-95, 1960.

134. SOUTHAM, C.M. and MOORE, A.E.
Induced Immunity to Cancer Cell Homografts in Man.
Ann. N.Y. Acad. Sci, 73, 634-51, 1958.

135. SOUTHAM, C.M.
Are There Host Defences Against Cancer?
Med. Clin. N. Amer., 45, 733-52, 1961.

136. STERN, K.
Immunologic Approaches to the Cancer Problem.
Chicago Med. Sch. Quart., 14, 68-77, 1953.

136a. STERN, K. and DAVIDSOHN, I.
Heterohaemoantibodies in Inbred Mice with Transplanted Tumours and Leukaemias.
J. Nat. Cancer Inst., 24, 1319-39, 1960.

137. STEWART, F.W.
Experiences in Spontaneous Regression of Neoplastic Disease in Man.
Texas Repts. Bisl. Med., 10, 239-53, 1952.

138. STONE, H.B. and SCHNAYFER, L.
Attempts to Induce Resistance to Cancer.
Ann. Surg., 142, 329-333, 1955.

139. URBAN, J.A. and BAKER, H.W.
Radical Mastectomy in Continuity with enbloc Resection of Internal Mammary Lymph Node Chain: New Procedure for Primary Operable Cancer of the Breast.
Cancer, 5, 992-1008, 1952.

140. VON ESSEN, C.F. and KAPLAN, H.S.
Further Studies on Metastasis of a Transplantable Mouse Mammary Carcinoma after Roentgen Irradiation.
J. Nat. Cancer Inst., 12, 883-92, 1952.

141. VYGODDUKOX, G.V. (Editor) (trans. Crawford).
Pathogenesis and Immunology of Tumours.
Sergamon Press, London.

142. WANGENSTEEN, O.H.
Discussion of H.G. Bell: Cancer of the Breast.
Ann. Surg., 130, 310-7, 1949.

143. WEILER, E.
Antigenic Difference Between Normal and Cancerous (Stilboestrol Induced) Hampster Kidney. Complement Fixation with Cytoplasmic Particles.
Brit. J. Cancer, 10, 553-9, 1956.

143. WEILER, E.
Antigenic Differences Between Normal Hamster Kidney and Stilboestrol Induced Kidney Carcinoma. Histological Demonstration by Means of Fluorescing Antibodies.
Brit. J. Cancer, 10, 560-3, 1956.

144. WILLIAMS, I.G., MURLEY, R.S. and CURWEN, M.P.
Carcinoma of Female Breast: Conservative and Radical Surgery.
Brit. med. J., 2, 787-96, 1953.

145. WITEBSKY, E., ROSE, N.R. and SHULMAN, S.
Studies of Normal and Malignant Tissue Antigens.
Cancer Research, 16, 831-41, 1956.

146. ZEIDMAN, I. and BUSS, J.M.
Experimental Studies on Spread of Cancer in Lymphatic System.
I. Effectiveness of Lymph Nodes as a Barrier to Passage of Embolic Tumour Cells.
Cancer Research, 14, 403-5, 1954.

147. ZILBER, L.A.
Specific Components of Malignant Tumours.
Uspekhi Sovremennoi Biol., 30, 188-221, 1950. Translated at N.I.H., Bethesda, Maryland.

148. ZILBER, L.A.
Some data about the Immunisation against Mammary Cancer of Mice.
Vop Onkol, 1, 14, 1955.

149. ZILBER, L.A.
Specific Tumour Antigens.
Advances in Cancer Research, 5, 291-329, 1958.

150. ZILBER, L.A.
Studies on Tumour Antigens.
Paper read at the 7th International Cancer Congress, London, 1958.

151. ZILBER, L.A.
Some Aspects of Anti-tumour Immunity.
Neoplasma, 6, 337, 1959.

152. ZILBER, L.A.
Study of Tumour Antigens.
Acta Un. int. Cancr., 15, 933, 1959.

153. KELLY, W.D., LAMB, D.L., VARCO, R.L. and GOOD, R.A.
An Investigation of Hodgkin's Disease with Respect to the Problems of Homotransplantation.
Ann. N.Y. Acad. Sci., 87, 187-202, 1960.

154. MILLER, D.G., LIZARDO, J.G. and SHYDERMAN, R.K.
Homologous and Heterologous Skin Transplantation in Patients with Lymphomatous Disease.
J. Nat. Cancer Inst., 26, 569-83, 1961.

155. MURPHY, W.H. and SYVERTON, J.T.
Relative Immunoligic Capacity of Leukaemic and Low Leukaemic Strains of Mice to Resist Infection.
Cancer Research, 21, 921-8, 1961.

156. BICKIS, I.S., QUASTEL, J.H., VAS, S.T.
Effect of Ehrlich Ascites Antisera on the Biochemical Activities of Ehrlich Ascites Carcinoma Cells in vitro.
Cancer Research, 19, no. 6, 602-7, 1959.

157. WOODRUFF, M.F.A. and SYMES, M.O.
The Significance of Splenomegaly in Tumour Bearing Mice.
Brit. J. Cancer, 16, 120-30, 1962.

158. WOODRUFF, M.F.A. and SYMES, M.O.
Evidence of Loss of Tumour Specific Antigen on Repeatedly Transplanting Tumour in the Strain of Origin.
Brit. J. Cancer, 16, 484-8, 1962.

159. WOODRUFF, M.F.A. and SYMES, M.O.
The Use of Immunologically Competent C[illegible]lls in the Treatment of Cancer.
Brit. J. Cancer, 16, 707-15, 1962.

160. WOODRUFF, M.F.A. and NOLAN, M.B.
Preliminary Observations on Treatment of Advanced Cancer by Injection of Allogenic Spleen Cells.
Lancet, ii, 426-9, 1963.

161. BUSCH, H.
An Introduction to Biochemistry of the Cancer Cell.
Academic Press, New York, 1962.