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THE GROWTH, MONITORING AND IMMUNOTHERAPY OF MOUSE PLASMACYTOMA

by

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SUMMARY

This work was contemplated in a centre skilled in the evaluation of paraproteins and wishing to consider immunotherapy in relation to plasmacytoma. Accordingly the four introductory chapters review tumour growth, tumour immunology, tumour immunotherapy and plasmacytoma whence evolved the advantages of studying an ascitic form of a stable plasmacytoma MP 5563 in inbred C_3H mice.

After validation of the chosen methods of study, chapters 5 & 8 described the monitoring of the actual number of tumour cells (using Cr^{51}-labelled red cells to correct the volume of the ascites) and the relationship of these to the also monitored serum paraprotein level. It was shown that following intraperitoneal transplantation, MP 5563 showed three phases of growth, a latency period (0-4 days) an exponential (5-8 days) and a terminal phase (9+ days). Reliable tumour cell counts could only be achieved between 5-8 days due to the terminal formation of a fistulous-like communication between the peritoneal cavity and the blood stream. From 5-8 days the serum paraprotein level was simply related to the number of tumour cells and this appears to be the first time that the circulating product of a tumour has been directly related to the actually counted number of tumour cells.

Following the establishment of these methods to monitor the tumour, the immune response of the rat (harvested as thoracic duct lymph) to the mouse tumour was studied (chapters 9 & 10). Washed lymphocytes were ineffective in vivo or in vitro even at ratios of 400 per tumour cell. The in vitro and in vivo effects of whole rat lymph were shown to result only in the immunized rat and to be due to the 19s fraction of cell-free lymph together with complement. Prior in vitro exposure of MP 5563 cells to this 19s fraction completely prevented their subsequent growth. In contrast in vivo administration of 19s fraction could not effect a cure, but did achieve a 3.75 log reduction (by 99.75%) in tumour cell numbers which could prolong the survival of the mouse. Possible mechanisms whereby the 0.25% survived in vivo are considered in the final chapter.

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CHAPTER I

1.1. TUMOUR GROWTH

A tumour is an abnormal mass of tissue, the net growth of which exceeds and is uncoordinated with that of the normal tissue of origin, and persists in the same excessive manner after cessation of the stimuli which evoked the change. If it infiltrates the surrounding tissue and spreads to distant organs a tumour is classified as malignant, and constitutes the disease of cancer.

In most tumours, the neoplastic tissue consists of cells of a single kind, derived from a single kind of tissue, and evidence for such a monoclonal origin is reviewed later (see p. 62). Some tumours however appear mixed or composite, in that they consist of two or more kinds of neoplastic tissues, the fibroadenoma of the breast is a familiar example.

Cancer is characterized by a cell production rate which is in excess of the rate at which cells are lost. The tumour outgrows its parent tissues slowly or rapidly forming an ever increasing superfluous mass.

There are some normal tissues the cells of which in the adult body at least rarely or never multiply, e.g. muscle fibres or nerve cells. A progressively growing tumour composed of such cells clearly possesses a proliferative power in excess of the normal rate which is zero. Other tissues

of the body, such as surface epithelia and the haemopoetic tissues, normally exhibit mitotic proliferation of their cells to replace those which are constantly lost. A tumour of such tissue exhibits mitotic proliferation in excess of this normal replacement rate. It is not yet clear whether this increased growth rate is caused by reduced time between cell divisions, by more cells dividing, or by a reduced rate of cell loss or variable combinations of these. The importance of these processes may be different in different tumours.

It is clear that tumour growth cannot be conceived as being maintained by the continuous stimulation of the tumour tissue by the same agents which evoked it. These agents must act by initially inducing in the cells invisible changes of metabolism and proliferation which are then transmitted indefinitely to the descendents of the initially changed cells; but it is not clear whether for most tumours they grow from a single cell or from a number of single cells that have undergone malignant transformation due to some common cause, directly or by stages.

1.2. TUMOUR GROWTH MODEL

The growth of a tumour could be considered in terms of parenchymal cells, bearing in mind that stromal changes are also occurring. A four compartment model of tumour growth seems to describe tumour growth adequately in the present state of knowledge. (Fig. 1.).

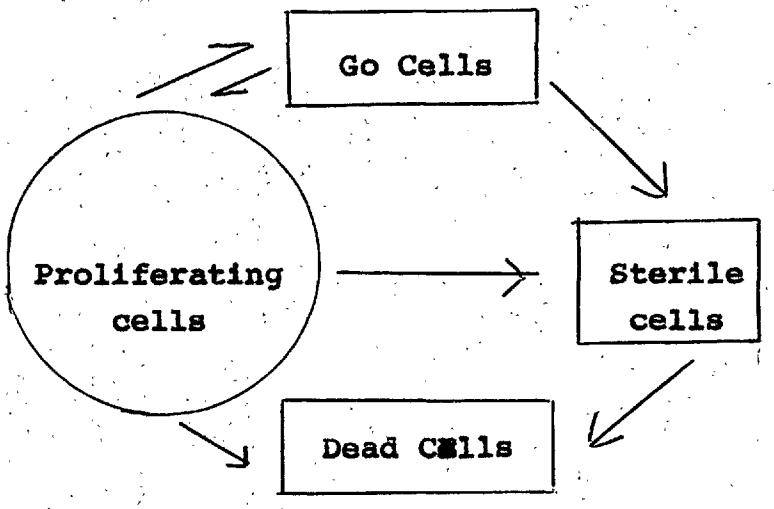


Fig. 1.

A four compartment model of a tumour cell population containing Go cells which are non-proliferating, but capable of re-entering the proliferation cycle together with proliferating cells, dead cells, and sterile cells.

1.3. METHODS OF FOLLOWING TUMOUR GROWTH

The growth of tumour is best defined in terms of serial estimates of cell number. This is only practically possible by direct counting of cells in ascitic tumours. For solid tumours one must rely either on measurements of the external dimensions using calipers, applicable to superficial tumours only (Mayneord, 1932), or on the weight of the extirpated tumours (Rosenoer and Whisson, 1964). The volume or weight then has to be related to cell numbers.

Such methods can be invalidated, as the parameters may be influenced by haemorrhage, necrosis, infiltration or stroma growth which can change the volume or weight of the tumour.

The common belief was that tumour growth followed a single exponential process, terminated when the host was no longer able to provide the nutritional requirements for growth. This was expected from the binary division of cells.occurring in closed population in which the generation time and the fraction of cells taking part in the division process was constant.

In practice however, when the logarithm of volume is plotted against time such growth curves usually bend towards the axis, and a straight line fits only a minority of solid tumours. Mayneord (1932), observing this departure from logarithmic growth in Jensen rat sarcoma, suspected this was due to central necrosis characteristic of that tumour. With a mathematical model he was able to show that cell proliferation was restricted to the outermost layer of tissues. Replotting measurements of Jensen sarcoma as a mean diameter versus time he found that they gave a straight line.

Ascites tumours are better examples of exponential growth. The logarithm of ascitic tumour cell number when plotted against time shows a linear relationship over a wide range of cell numbers though eventually the curve flattens during the last days of life of the animal(Revesz

and Klein, 1954). Klein and Revesz (1953) found an exception to this with MCIM and Ehrlich ascites tumours which conformed to poorly logarithmic growth. They were similar to solid tumours in that the cube root of cell number was linear against time; they described this as a "Cube root phase". Patt and Blackford (1954), emphasized two phases of growth of ascitic tumours, a logarithmic phase followed by a cube root phase, with neither phase only describing the entire growth. Laird (1964), described the growth of many tumours as a gompertzian finding. Following the introduction of DNA labelling with tritiated thymidine to measure the cell cycle time, new light was thrown on the problem. The slowing of tumour growth could be due to any or a combination of the following factors:

(1) Prolongation of the mitotic cycle - although there is only a little evidence for this phenomenon. Baserga (1963); Hornsey and Howard (1956); and Lala and Patt (1966), claim to have shown prolongation of the S, G_1 and G_2 phases as the tumour continues to grow.

(2) Decrease of the growth fraction - the percentages of cells taking part in mitosis where shown by Baserga (1963); Lala and Patt (1966), to decrease progressively as the tumour grows.

(3) Increase in the cell loss - this increases proportionally with time.

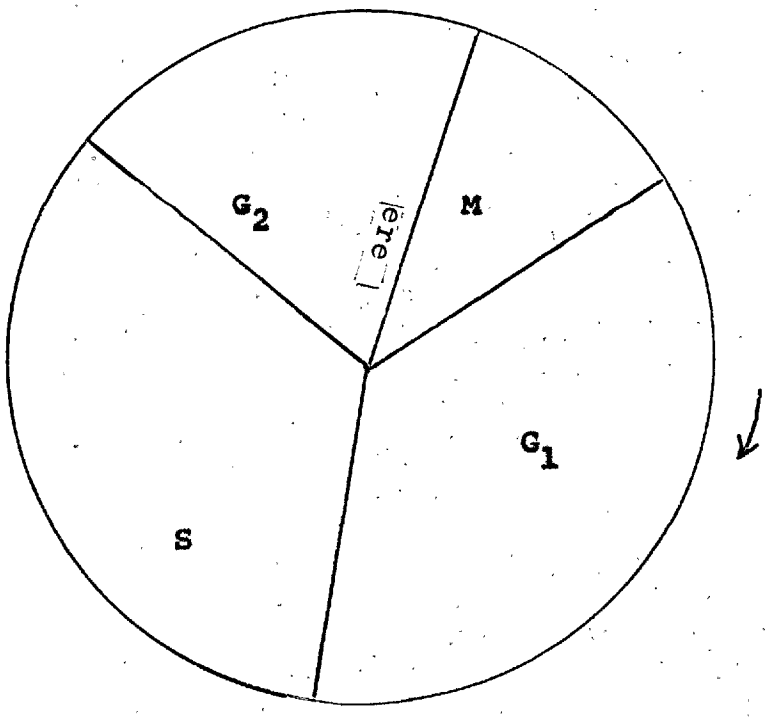


Fig. 2.

Diagram representing the four phases of the cell cycle. Arrow shows the direction in which cell progresses around the cycle.

1.4. THE CELL CYCLE

Before the advent of radioactive precursors and autoradiography, mammalian cells were described as passing through only two main phases during their cell cycle; a shorter phase, mitosis, in which the chromosomes became visible and cell division occurred and a longer interphase or resting phase. It was considered that during the mitosis phase, both replication and division of the chromosomes occurred, until Howard and Pelc (1951a and 1951b) using autoradiography with S^{35} and P^{32} labelled precursors of DNA suggested that the synthesis of DNA and of chromosome material in dividing cells occurred during a discrete portion of the long interphase of the cell cycle.

Consequently, it was suggested by Howard and Pelc(1953) that the cell cycle could be represented as a model shown in Fig.2, where the cycle is sub-divided into four phases, S, G_2, M and G_1. Conventionally S stands for DNA synthesis. G_1 and G_2 are two "gap" phases during which no DNA synthesis occur, presynthetic and postsynthetic respectively and M stands for mitosis.

1.5. CELL POPULATION KINETICS -

Cell proliferation kinetics is a new field of study which has developed as a result of the techniques which have become available for studying the rate at which cells grow within an animal. Consequently the picture of a malignant

tumour as that of a tissue growing faster than its tissue of origin has been profoundly changed in the past few years. Just like normal renewing tissues of the body, it has been emphasized that a tumour is in a state of dynamic equilibrium between cell renewal and cell loss, and it only increases in weight or volume when the rate of proliferation is higher than the rate of cell loss.

Studies of tumour cell population kinetics aim to:

1) Provide a proper kinetics of animal and human tumours.
2) Understand the regenerative capacity of normal and malignant tissues.
3) Throw new light on the nature and causes of malignancy.
4) Help in the design and assessment of treatment.

Such studies relate to many aspects of cancer therapy as well as to the underlying aetiology and nature of the neoplastic growth, and have an important bearing on the future of radiotherapy, chemotherapy and immunotherapy.

Within the classification of malignant tumours, there is a wide range of growth rates, and also a range of degrees of malignancy. In C_3H mice, the experimental plasma cell tumour MP5563 doubles its volume in 18 hours, while in man pulmonary metastases from carcinoma of the colon double their volume with median times of 25-100 days (Steel and Lamerton 1966). A wide range of histological types and degrees of differentiation is found in both human and animal tumours but

often very similar patterns can be produced. It therefore seems likely that studies on animal tumours will be relevant to human tumours, realising that human tumours generally grow much more slowly than the experimental animal tumours. This is mainly because of the growth fraction which can approach 1.00 in some experimental animal tumours but which is usually only a small fraction, 0.01 in some human tumours.

In a quantitative studies of the killing of haemopoetic and tumour stem cells by chemotherapeutic agents (Bruce 1967) has shown that the selective killing of tumour cells by many agents depends to a large extent on their proliferative state. Proliferating cell populations are much more sensitive to the toxic effects of many chemotherapeutic agents than are resting non-proliferative cells in the G_0 state. Thus one would predict that agents which appear selectively toxic for rapidly proliferating cells (e.g. AKR lymphoma) would be less effective in the treatment of slowly growing tumours because the tumour stem cells may have (i) a long generation time (ii) a significant proportion in G_0 (iii) a small growth fraction (Mendelsohn 1962). When the tumour stem cells are in rapid cell cycle they should be very sensitive to agents that are selectively toxic for proliferating cells.

1.6. THE GROWTH CURVE OF ASCITES TUMOUR AS A MODEL

A number of different tumours can be induced to grow in an ascitic form, where free tumour cells grow actively in the

peritoneal fluid and reach there a high absolute and relative concentration, with many advantages for use in investigations in various branches in experimental oncology. After inoculation there is a "latency period" of a few days which is inversely proportional to the number of transplanted tumour cells (Klein and Revesz 1953), and of unknown aetiology, during which the tumour growth could not be detected. Thereafter, the tumour growth is maximal shortly after the end of the latency period and decreases continuously. Finally followed by terminal period with an apparent fall off in tumour growth. Steel (1968) showed that cell loss is an important factor in determining the growth rate and the shape of tumour growth curve, of a number of experimental tumours. This cell loss is small when the tumour is small and progressively increases as the tumour grows in size. Several possibilities have been identified of cell loss from tumours.

1) Metastases - both living and dead cells may be lost from the tumour volume. It is obvious that living cells leave tumours, since metastases often form in sites which are distant from the primary tumours. These viable cells may be carried in the vascular system and circulating tumour cells have been located in the blood of cancer patients particularly after surgical excision of the tumour.

2) Cell death - using the results of Baserga (1963); [redacted] and separately Lala and Patt (1966), for Ehrlich ascites tumour, Steel (1968) described a slowly progressive increase in cell loss to as high as 90% before the death of the host and assumed this to be due to the death of many of the tumour cells found. Cell death occurs for variety of reasons, including inadequacy of the blood supply or vascular accidents. These dead cells may autolyse and be resorbed or they may be removed by phagocytosis.

CHAPTER 2

TUMOUR IMMUNOLOGY

After considering the immune responses, especially those capable of killing cells, evidence for antigenic differences between tumour and its parent normal tissue are reviewed, concluding with evidence for host responses to such differences.

2.1. IMMUNE RESPONSES

Normal animals show the capacity to rid themselves of infection by microorganisms, as well as rejecting many materials (designated as not-self) by the mechanisms of natural immunity, however such mechanisms are occasionally valueless against infection. Animals that recover from an initial infection are often able to resist a second invasion by the same microorganism through the immunity acquired from previous exposures to that microorganism. This depends upon the individual's active contribution and this is referred to as "Specific active acquired immunity".

Again while specific immunity usually protects the animals, it can sometimes confer unpleasant or even dangerous hypersensitivity following further exposure to the provoking antigen, the so-called allergic reaction. In certain disease states, immunological processes directly against the body's own tissues, e.g. the formation of autoantibodies.

Since the recognition of such autoantibodies, a wide range of tissue disorders have been classified as auto-immune diseases.

A most exciting field of modern immunology is evidence of the role of immunological process in controlling cancer after it became evident that the body can recognize cancer cells as foreign. Approaches to the treatment of cancer are therefore attempts to develop means by which it might be possible to evoke or enhance the immunological responses of the body against cancer, or to passively transfer immunity to cancer patients. Consideration will be given to these approaches in a later chapter.

Specific immunological reactions are mediated by humoral factors (humoral immunity) or more directly by cells (cellular immunity).

2.1.(i). HUMORAL IMMUNITY

This is mediated by certain serum proteins which run mainly as γ-globulins after electrophoretic separation and are now called immunoglobulins (Heremans, 1959). They can react directly with antigens, and in vitro this can result in precipitation of soluble antigen, or agglutination of the cells which have specific antigens on their surface. In man, the immunoglobulins have been divided into five groups known as IgG, IgA, IgM, IgD and IgE, the physico-

Chemical characteristics of which are described in chapter 3. They are made by cells of the plasma cell series, found in the bone marrow, the medulla of lymph nodes, red pulp of the spleen and the laminae propriae of the gut and respiratory system, etc. In some cases, the biological consequences of the interaction of antibodies with antigens depends upon the secondary activation of a complex system of non-specific factors present in the blood and tissue fluids known as complement.

Complement is a number of substances which interact in a definite sequence in a manner analogous to the blood clotting mechanism. When antigen and antibody react to form a complex in fresh serum, some components of complement becomes actually bound up with the complex. The reaction is initiated when the Fc part of the heavy chain becomes distorted or exposed by combination with antigen, and when more than one pair of such heavy chains should be arranged at a suitable short distance apart. Because of this IgM antibodies (with 10 heavy chains close together) are more efficient at fixing complement than are IgG antibodies.

2.1.(ii), CELLULAR IMMUNITY

This type of immunity mainly mediates delayed type hypersensitivity, the rejection of homografts (or tissues which can be recognised as foreign) and some intracellular

infections. Small lymphocytes become sensitized upon passing through the tissues, and then pass to the lymph node where they enter the paracortical area between the lymph follicles and the medulla, where they proliferate. The sensitized lymphocytes first differentiate into large basophilic cells - referred to as immunoblasts. These increase in numbers about 3 days after challenge and return to normal by day 10, (Delorme, Hodgett, Hall and Alexander (1969). On day 3 the immunologically active lymphocytes leave the local lymph node and move to other lymph nodes were they can propagate into other immunologically active lymphocytes. Meanwhile such cells can be found in the blood stream, and can be recognised by observing their reaction or exposure to the antigen.

The thymus gland plays a major role in establishing the cellular immune responses early in life. Early thymectomy can result in failure to develop cell mediated immunity. The lymphoid tissues will be depleted of lymphocytes, especially in the paracortical areas of lymph nodes "thymus dependent areas" and the white pulp of the spleen. Neonatally thymectomized animals can develop normal immunoglobulin levels and respond mainly to stimulation with soluble antigens, however there can be inhibition of certain types of antibodies such as those against sheep red cells because in such instances cell-to-cell cooperation

between cellular and antibody responses seems essential.

2.1.(iii). PRIMARY AND SECONDARY RESPONSES

When an animal becomes first in contact with an antigen, a limited response of low intensity and short duration is produced. This is termed the primary response and during it germinalcentres may develop in localized collections of lymphocytes in the lymphoid tissue called lymph follicles. At the same time plasma cells may begin to proliferate at the cortico-medullary junction and in the medullary cords. This results in the formation of sensitised cells and/or antibodies.

On a subsequent contact with antigen, a much quicker and more extensive reaction occurs, and antibody or sensitised cells are produced as a result for much longer time than the primary response. This time the antigen is localized in the germinal centres as well as in the medulla of the lymph nodes affected and a much more extensive proliferation of plasma cells occurs. The secondary response is thought to occur as a result of immunological memory residing within cells of the small lymphocyte class.

2.1.(iv). MECHANISMS OF KILLING CELLS

Of all the various mechanisms of immunity those which are of special relevance to the present thesis are those capable of killing cells.

Of the human immunoglobulins only IgM and IgG are known to have this property. When IgM antibody combines with antigens on the surface of the cell membrane it activates complement, and does so in such a way as to be very effective in causing a local lesion in the underlying cell membrane, if the antigen lies on or very close to the cell surface. IgM is so efficient in that a single molecule of IgM antibody attached to the surface of a cell, e.g. red blood cell which is incapable of repairing its membrane, can activate complement so as to lyse the cell. This is in contrast to IgG which can also cause cell lysis with complement but which appears to do so only when two antibody molecules become attached very close together on the same surface. Consequently many more IgG molecules per cell are required to produce the same amount of damage as a single IgM molecule, e.g. 7-10,000 per red blood cell requiring something like 50mg. specific IgG antibody/100ml. plasma. In practice this concentration of specific antibody is very rarely achieved, so that direct killing of cells by IgG antibodies is not seen. However certain IgG sub-classes (IgG_1, IgG_3) can become attached to cells and then such tagged cells can become opsonised by phagocytes, with subsequent destruction of the tagged cells. Complement is essential to this process, and receptors for its components and for only IgG_1, IgG_3 or IgM are demonstrable on the

phagocytes. (Huber and Fudenberg, 1968). That such complement-dependent opsonin-dependent phagocytosis can lead to the destruction of tumour cells has been shown by The (1970).

The only other known cytotoxic mechanisms are those of cellular immunity.

A characteristic feature of cell-mediated cytotoxicity is the antigen specific aggregation of lymphoid cells on target cells, brought about by antibody-like receptors on some of the lymphoid cells. Contact between lymphoid cells and target cells is essential for the cytotoxicity of the lymphoid cells. Microscopic and time lapse cinematography with target cells in monolayers show that lymphoid cells after random movements, will become attached to the targetrcells which finally seem to undergo osmotic lysis (Orita, 1969). It is possible that cytotoxic factors which nonspecifically inhibit target cell growth are released from the effector cells upon contact with target cell antigens. Such mediators may be hydrolytic enzymes, components of the complement system or other factors.

In practical terms, it is clear that events resulting from interaction of sensitized lymphocytes with specific antigen depends largely on the biological and immunological environment in which this interaction is taking place. In the animal this can result in the inflammation of delayed hypersensitivity or rejection of tissue allograft, or

the enhanced elimination of those infective agents which can reside inside macrophages or other cells.

In tissue culture, the results of interaction between target cells and sensitised lymphocytes can also be sometimes observed by the release of mediator molecules, the lymphokines. Some of these may be cytotoxic directly, and others by inducing macrophage aggregation or migration inhibition may promote death of target cells. Even others by coopting mitogenesis in other lymphocytes may add to the whole reaction.

2.1.(v). COOPERATION IN IMMUNE MECHANISMS

There is much evidence that immune reaction is regulated by cellular cooperation within the lymphoid system and that peripheral manifestations of the immune response frequently result from interaction between cell mediated and antibody mediated components. In recent studies, McLennan, Loewi, and Harding (1970), have provided evidence that humoral antibodies may induce cell mediated cytotoxic reaction in vitro. Briefly antigenic target cells when treated with specific 7s immunoglobulin will be damaged when exposed to a moderate excess of lymphoid cells from unsensitised donors. The specific immunoglobulin is effective at dilutions too weak to give rise to conventional complement mediated lysis in the absence of lymphoid cells. Furthermore there is evidence that reaction against neuroblastoma is mediated both by cellular and humoral immunity (Hellstrom, Hellstrom, Piere and

2.2. TUMOUR ANTIGENICITY

The importance of immunological factors in the growth and progression of tumours is not recently discovered. Attention has been paid to it since the beginning of this century by many distinguished scientists like Ehrlich (1909) who was the first to discover the ability of the host to reject transplanted tumours.

Much work after Erlich came to a halt when Woglom (1929) reviewed most of the work done in that period and considered it to be valueless. The experiments had been conducted in outbred animals and the tumours were rejected like any other homograft. No real progress was made until Gross (1943) produced immunity against transplantable tumours in an inbred strain of mice. From 1948-1956 the Russian School of Zil'ber did much to show that another animal (e.g. guinea pig) could readily recognise immunogenic differences between a tumour and its parent normal tissue(e.g. of man) (Vygodchikov, 1959). Later on it was found (Foley, 1953; and confirmed by (Prehn and Main, 1957; and by (Revesz, 1960), that methyl cholanthrene induced sarcoma, but not spontaneous mammary carcinoma could induce a state of resistance in isologous mice. Since this renewal of enthusiasm, much work has been done.

It is very interesting to note here that chemically induced tumours have individual antigens, and cross immunization

is rare or absent even between tumours of similar morphology and induced in the same host genotype (Klein and Klein, 1962), or even between several tumours formed in the same host by one carcinogen. In contrast, virus induced tumours, carry a virus determined surface antigen which is common for all the tumours induced by the same virus even if they are different in morphology, and cross immunization is feasible. (Klein and Klein, 1964).

2.3. EVIDENCE FOR IMMUNE RESISTANCE BY THE HOST

Evidence that transplantation antigens exist on the surface of tumour cells and that the host is capable of recognising these antigens came from two sources:

(A) evidence from experimental animals

(B) evidence from man

A study with experimental animals on the means of unmasking the antigenecity of tumours and potentiating the host reaction against its tumour might help in devising for man immunotherapy against his primary tumours.

2.3.(A). EVIDENCE IN ANIMALS. This is derived from studies of immunised or immunosuppressed animals.

(i) IMMUNISATION

Recent advances in cancer immunology pay tribute to the establishment of inbred strains of animals, making possible tissue grafting from one animal to another. Antigenicity of tumour tissues in isogeneic hosts (unlike normal tissue) was demonstrated following immunisation by the following methods (Alexander and Fairly, 1967). (a) surgical removal of the tumour after it had been transplanted and reached a certain size. (b) injecting live tumour cells which had been rendered incapable of mitosis by previous treatment with X-ray or certain chemicals. (c) challenging the animal with a small dose of tumour cells below the threshold number which normally produced tumours.

This immunity was then demonstrated when the immunised animals failed to develop the tumour upon challenge with a dose of tumour cells that normally produced tumours in the unimmunised animal. Larger overwhelming doses did produce tumours in these immunised animals reflecting the weak antigenecity of the tumour in the isogeneic host and emphasising high-dose tolerance.

(ii) IMMUNOSUPPRESSION FOLLOWED BY CANCER

Both human and experimental animals develop tumours with increased frequency when deprived of their immune response, e.g. being treated with immunosuppressive agents. Most striking is the observation made by Gougas, Chesterman, Hirsch, Rees, Harvey and Gilchrist (1969), when they attempted to propagate human leprosy in CBA mice made immunologically deficient by adult thymectomy and repeated ALS injections. All the mice were injected in the laboratory with polyoma virus. All the thymectomised mice receiving ALS (the group with the most severe immune deficiency) developed polyoma tumours whereas not one of those only thymectomised or the controls developed the tumours. This demonstrated the effect of immunity in limiting tumour development in animals. Gershon and Carter (1970), found with hamsters transplanted with lymphoma, that those treated with ALS were shown to have tumours larger than those treated with normal serum. Metastases developed in most of the ALS treated ones but not the controls. Immunosuppression has a very significant effect in inducing virus induced cancer but not chemically induced ones.

2.3.(B). EVIDENCE IN MAN

For obvious reasons, experiments to establish the antigenicity of tumours in human beings cannot be carried out in the same way as in animals. Furthermore, except

in identical twins or a few families, inbred strain of human beings are not available. For these reasons, much of the evidence in man is indirectly based on observations of patients. These fall into three categories: (i) evidence from patients with immune depression. (ii) evidence from host reactions against established tumours and, (iii) other evidence.

(i) EVIDENCE FROM PATIENTS WITH IMMUNE DEPRESSION

(Burnet, 1967) evaluated the intact immunological mechanism in self defence against cancer. It is clearly established that there is a high incidence of lymphoma in patients with agammaglobulinaemia (Miller, 1968) and in patients with autoimmune diseases such as rheumatoid diseases (Lea, 1964).

If immunological responses are important in controlling cancer, patients receiving organ homografts and under long continued and efficient immunosuppression might be exposed to a higher risk of cancer either inadvertently received with the donor graft or arising from their own tissues.

In six patients who received kidney homografts, a donor type neoplasia has developed in or outside the homograft. This suggests that the donor graft contained tumour cells which have produced cancer in the recipient whose immunity was suppressed. One recipient of a renal homograft

from a cancer patient developed an extensive tumour of the donor type in the lung. This underwent complete regression after the patient stopped taking the immunosuppressive drugs, (Fairley, 1970).

Immunosuppressive treatment might encourage tumour to emerge from neoplastic cells present in the recipient before the homograft operation. In favour of this phenomenon is the report of Starzl, Groth, Brettschneider, Moon, Fulginiti, Cotton and Porter (1968). Their patient had a liver totally infiltrated with tumour tissue. This was removed surgically and a liver homograft was transplanted. On immunosuppressives the patient rapidly developed widespread secondary tumour and died. It is therefore questionable whether immunosuppression combined with transplantation has any place in the treatment of cancer patients.

2.4.(B)(ii). EVIDENCE OF HOST REACTION AGAINST AN ESTABLISHED TUMOUR.

Both cellular and humoral reactions against autologous tumours have been established and are considered under (a) delayed type hypersensitivity, (b) histology (c) active immunization and (d) humoral reactions.

(a) DELAYED TYPE HYPERSENSITIVITY

The presence of delayed type hypersensitivity reaction in patients towards extracts of their own malignant tumours was shown by Hughes and Lytton (1964). Similarly Stewart

(1969) showed that the reaction to a cell-free extract from their own tumours occurred in 26% of patients and that this reaction correlated with the degree of infiltration of the tumour by lymphocytes. Earlier Stewart (1968) had established that a good prognosis exists in such patients. This suggests a cell-mediated immunity directed against the growth and spread of cancer.

(b) HISTOLOGY

Pathologists have observed histological evidence of reaction between cancer cell and the host. Lymphocytes and plasma cells were sometimes seen to surround and infiltrate mostly secondary and even primary tumours. This was shown by Hamlin (1968) to be correlated to the prognosis of carcinoma of the breast; the greater the lymphocyte and plasma cell infiltration the better the prognosis. Similarly in Hodgkins disease, poor prognosis had been associated with lymphocytic depletion (Lukes, 1964), and a better prognosis was associated with lymphocytic and/or histiocytic infiltration.

(c) ACTIVE IMMUNIZATION

On rare occasions, cancer patients who have been incidentally severely infected with bacterial or viral agents have obtained partial or total remission of their malignant tumours. This effect has been explained as due to a non-specific stimulation of the immune response.

It was Old, Benacerraf, Clarke, Carswell and Stockert, (1961) who demonstrated the effect of BCG to retard the growth of chemically induced tumours, and Mathé et al (1969) who adapted this method to induce prolonged remissions in patients with acute lymphoblastic leukaemia.

(d) HUMORAL REACTIONS

Antibodies directed against specific tumour antigens have been found with melanomata and with Burkitt lymphomata. The findings of Lewis (1967) are most interesting in that serum taken from patients with melanoma with long history of the disease, but with no metastases, is toxic to the autochthonous melanoma cells in tissue culture. In contrast serum from patients with disseminated disease is not. The presence in the serum of antibodies directed against the tumour was established and these can prevent the tumour from metastasising. Antibodies to melanoma are produced by injecting irradiated cells to normal adults. (Ikonopisov, Lewis, Hunter-Craig,Bodenham, Philips, Cooling, Proctor, Hamilton-Fairley and Alexander(1970).

More important in this field is Burkitt's lymphoma. This type of lymphoma seems to be induced by virus known now as EB (Epstein, Barr and Achong, 1965) virus. Antibodies to the EB virus and to the cells containing the virus in tissue culture were detected by Klein, Clifford, Klein and Stjernsward (1966), the fluorescence technique, in the sera of patients with Burkitt's lymphoma and in some normal people in Africa and in other countries where the studies were carried out.These antibodies were

Shown to be the same as those found in other conditions associated with EB virus, e.g. glandular fever. It is not yet known whether the EB virus is a common cause for both Burkitt's lymphoma and glandular fever as the virus favours the growth in these two conditions. The study carried out by Biegler, Cohen, Marrow, Kyalwazi and Carbone,(1970), evaluated the immune status of patients with Burkitt's lymphoma. They found normal cellular immune response in treated or untreated patients, and a defective primary antibody immune response with low IgM levels in untreated patients but not in patients in remission.

2.4.(B)(iii). OTHER EVIDENCE

This evidence is less direct but immune reactions have been implicated in (a) metastatic regression (b) spontaneous regression and (c) regression of choriocarcinoma.

(a) METASTATIC REGRESSION

Following effective eradication of the primary lesions, surgeons and radiologists have observed the disappearance of some metastatic lesions completely or at least for a period of time. Usually they reappear and grow rapidly leading to the death of the host. These conditions might suggest the ability of the host to control the rest of the tumour after the tolerogenic influence of the mass of primary tumour has been removed. A possible mechanism is the release of competent lymphocytes which had been previously restrained to the lymph nodes regional to the primary tumour (Alexander, Bensted, Delorme, Hall and Hodgett, 1969).

(b) SPONTANEOUS REGRESSION

Is defined by Everson and Cole (1956) as "The partial or complete disappearance of a malignant tumour in the absence of all treatment, or in the presence of therapy which is considered inadequate to exert a significant influence on neoplastic disease". This phenomenon occurs very rarely, and Everson and Cole collected 176 cases of authentic spontaneous cure reported by surgeons throughout the United States. The incidence of this phenomenon is about 1 in 100,000. It occurred in patients with various tumours including neuroblastoma hypernephroma, chorio-carcinoma and malignant melanoma. Several patients who were reported to have spontaneous regressions of kidney tumours had actually been treated with androgens before the effectiveness of this method of treatment was recognised. Even though spontaneous cases are infrequent, they may be considered as important evidence for host resistance.

(c) REGRESSION OF CHORIOCARCINOMA

This is an unusual malignancy, since it represents a peculiar homograft of fetal tissue. In being so, it should be strongly antigenic and presents an ideal opportunity to study the host effect on the malignant tumour.

Hertz, Lewis, and Lipsett, (1961) reported that chemotherapy alone was able to cure almost 50% of the patients with choriocarcinoma. As in Burkitt's lymphoma

it would seem that the effect of chemotherapy has been supplemented by the host's own immune response, especially because a skin graft from the father was injected at the same time. Bagshawe (1963), has made similar observations after the treatment of carcinoma using chemotherapy and radiotherapy.

SUMMARY

From the known mechanisms of immunity, it can be seen that tumour cells could be directly killed by complement fixing antibodies of the IgM class. On the whole complement fixing antibodies of the IgG_1 and IgG_3 classes seem to be most effective by promoting phagocytosis. Sensitised lymphocytes are also capable in some unknown way of directly killing tumour cells and again certain lymphocytes may potentiate this process and indeed promote phagocytosis.

Such immune reactions require effective recognition of antigenic differences between the tumour and the normal tissue of the host. These differences can readily be recognised by another animal, but in the host they are either ineffectively recognised or overcome by some mechanism such as high dose tolerance.

From a review of the literature it can be seen that occasionally patients make an effective recognition of their tumour cells and it seems to be specially the case when a

large mass of tumour has been removed or when the patient's immune responses have been potentially or incidentally activated.

CHAPTER 3

TUMOUR IMMUNOTHERAPY

After considering the aims and results of existing cancer therapy, mechanisms for the presumed failure of normal immunological surveillance are reviewed. The currently developing forms of tumour immunotherapy are then discussed because of the guidance they afford to the present thesis.

3.1. AIM OF CANCER THERAPY

The aim of the cancer therapist is to stop the tumour growing, and if possible to eliminate it without causing excessive damage to the normal tissue. Overall only about one third of cancer patients survive more than 5 years. A study of the reasons for failure of the available methods to cure the incurable two thirds of cancer patients might give some guidance as to what might be more successful method. Broadly, the available methods of treatment are:

(i) Surgery - although surgery accounts for the management of 50% of the cancer patients, it can only achieve a reasonable success when the tumour is localized, and when its complete removal is possible. If the tumour has metastasized into another part of the body, then the removal of the primary tumour will usually not solve the problem, the metastases continue to grow and it is only a matter of time to

to the inevitable outcome.

(ii) Radiation therapy - radiation therapy is preferred in most cases where surgery fails. The problem of radiotherapy is like that of surgery in that it is best applied to localized rather than systemic cancer. After a surgical excision, radiotherapy may be useful to treat the bed or the regional nodes of the tumour in an effort to destroy metastases. The destructive effect of radiation is however not only confined to cancer tissues, normal tissues are vulnerable as well. Tumours most often are sited deeply in the body, and the radiation dose received by the skin and the other superficial organs may be more than that received by the tumour. This difficulty is overcome to a certain extent with the development of high energy machines in combination with the rotation therapy whereby the maximum dose may be given to the deeper cancer.

Anoxia of the tumours renders them less succeptible to irradiation (Deschner and Gray, 1959). This can be overcome to some extent by keeping the patient in a hyperbaric oxygen tank, during treatment, in an attempt to raise the oxygen level of the tumour to the maximum enhancement ratio.

Cell kinetics studies have revealed that some tumours are more slowly growing and thereby less vulnerable to the effects of radiation etc., than are

normal tissues.

(iii) Chemotherapy - In general few "cures" of cancer have as yet been achieved by chemotherapy with the available agents. In spite of that, they have produced enough promising results to justify the continuing research for other more efficient agents. The reason for the present failure is that these agents are cytotoxic to the normal tissues as well as to the cancer tissue. The goal of the chemotherapist is to eliminate the last surviving cancer cell without reducing to a dangerous or lethal level the other necessary cell populations in the patient.

Available irradiation and chemotherapy can reduce the number of tumour cells down to $\frac{1}{1000}$ of its original number. Many tumour cells are thereby left, for the patient often presents with 10^{9}-10^{11} tumour cells (see p.109). To eliminate them all would require a logarithmic increase in dosage, which is impossible in practice, as this would kill the patient.

The body's immune mechanisms, especially the cellular ones, have the ability to hunt-down everything that can be recognised as foreign and for practical purposes could eliminate all cells so recognised. However where a large cell mass has arisen because of some blindness to its foreign

nature, even should an immune recognition be brought about, the sheer mass of antigen could overwhelm or delete such an immune reaction - this is the phenomenon of high dose tolerance (Mitchison, 1964).

A logical trend becoming apparent today is to use conventional methods of cancer treatment (Surgery, radiotherapy, chemotherapy) to produce an initial reduction in the total mass of tumour (e.g. to 1/1000th. of its original size, and then to try and encourage an immune reaction against the tumour now at a level where high dose tolerance may not be a problem.

3.2. NORMAL IMMUNOLOGICAL SURVEILLANCE

As soon as it became evident that tumours induced by chemical carcinogens or viruses contain specific antigens capable of stimulating host rejection responses, it became likely that many antigenic and potentially neoplastic cells are continuously formed in the body and eliminated by immunological mechanisms before they give rise to a tumour. This being so, why did antigenic tumours still sometime develop?

-- It is estimated that about half billion cells in the human body must be replaced daily. This entails such an enormous number of mitotic divisions (5×10^{11}) that it is unlikely all are completed perfectly. At a conservative estimate mutations probably occur once in each 500 thousand

resulting in about one million abnormal cells daily. Although most of the abnormal cells formed in ordinary development are so severely anomalous that they cannot survive, other do survive in a replicable form. It is essential that these be recognized by the body as foreign and be destroyed, otherwise all of us would develop cancer early in life.

Burnet (1964) has postulated that the physiological function of the homograft reaction is this elimination of spontaneously arising neoplastic cells and other types of unwanted clones that represent potential threats against the organism. Immunological surveillance therefore exists without any doubt, and the existence of antigenic tumours therefore implies that in such cases the neoplastic cells have escaped the immunological surveillance mechanism in one way or another. Three known mechanisms exist (a) immunological tolerance (b) immune paralysis (c) enhancement.

3.2.(a) IMMUNOLOGICAL TOLERANCE

A weak immunogen e.g. from a tumour cell, can induce tolerance if presented below a threshold dose which would evoke an immunological reaction of the host (Mitchison, 1964). This could be the case early on in the development of tumours originating from a single cell, or a few cells, but eventually a threshold level of immunogen should be

reached unless the potentially responsive immune cells are eliminated. Tolerance could be obtained to an immunogen when the host is exposed to it in foetal or neonatal life.

Experimentally, evidence for this is provided by tumours arising in mice as a result of neoplastic transformation by oncogenic viruses. Tumours are produced when mice become infected in utero or soon after birth at a time when the capacity of immune response is still immature. With certain exceptions (e.g. Rous sarcoma virus of rodents and fowls) inoculation of the virus into adult animals does not result in the formation of tumours, for immunity is elicited against the virus, and when formed soon induces regression of any tumours that might arise (Klein and Klein, 1964).

Infection with oncogenic viruses might be transmitted from the mother during intra uterine life or via the milk after birth. Natural infection could occur shortly after birth, and the subsequent growth of any tumour induced by such virus could result from immunological tolerance to the virus and the surface antigens evoked by such virus. This is only true for certain types of virus, mainly those which infect lymphocytes. Other types of virus such as mouse polyoma virus which evoke antibody production in the mother might be transmitted to the foetus along with a sufficient amount of maternal antibody to suppress the virus. Such newborn mice can lose this protective action by the normal metabolism of the maternal antibody or by further inoculation of virus after

birth, and tumour can result. Similar arguments could be used for other oncogenic agents before or close to birth.

3.2.(b) IMMUNE PARALYSIS

Malignant cells can also escape from immunological surveillance when the host is sufferring from immune paresis; and indeed patients with inborn immune pareses do develop a higher incidence of many cancers than the normal population. General immune paresis can also be evoked by the same agents which are carcinogenic. The drugs used to induce immunosuppression or to treat cancer mainly act by deranging DNA in the cell attempting to divide. Such drugs are themselves carcinogenic, and should a replicable neoplastic mutation be induced it can get away to a very good start because of the influence of the drug on the host's immune surveillance. Severe immune paresis also naturally occur late in the course of many cancers, especially those of the reticuloendothelial system (Hobbs, 1968; Lowler, Pentycross and Reeves, 1967). The three above immune pareses, inborn, therapeutic or secondary, do not however account for the majority of cancer, and general immune paresis is certainly not common early in the course of most human cancers.

What however does seem probable is that specific immune paralysis occurs, i.e. in some way the host's response to the tumour immunogens is impaired. Experimentally,

Alexander, Bensted, Delorme, Hall and Hodgett (1969) induced 3:4 benzpyrene sarcoma in rats. The rats seemed immunologically competent and histology of the lymph nodes regional to the sarcoma showed a vigorous immune reaction. However after a short initial burst, no further competent immune cells (immunoblasts) could be detected in the efferent lymph. Following surgical extirpation of the sarcoma there was a marked release of immunoblasts. It seemed that once the tumour had reached a given size, its antigens could act in excess in the regional nodes and in some way restrain the immunoblasts to those nodes. In man, a corollary to this picture is seen in melanoma. With early localised melanoma, antibody and competent cells are detectable but later in the disease these can no longer be detected peripherally. This is presumably due to local specific immune paralysis for surgical removal can again reveal the immune reaction, provided the regional nodes are not removed at the same time.

3.2.(c) ENHANCEMENT

It was observed that animals which had been immunized with dead allogeneic tumour cells later accepted transplants of viable tumour cells of the same origin, at least for a while. Similarly when serum was taken from mice immunized against killed tumour tissue and administered to normal isogeneic mice at the same time as a transplant viable tumour, the recipients accepted the tumour as readily as did the original mice actively immunized against the dead tumour

tissue. In other words, serum antibodies can enhance the growth of the allogeneic tumour (Irvine, Eustace and Fahey, 1967). This is not true for all tumours, for some e.g. leukaemias, the presence of antibody is cytotoxic. to the tumour cells both in vitro and in vivo (Gorer and Amos, 1956).

Why some antibodies are enhancing to some types of tumours is explained on the basis of masking the antigenic sites on the tumour surface. Presumably certain antibodies can combine harmlessly to such sites and protect them from being recognised as foreign by competent immunoblasts or harmful antibodies. Direct evidence for this been found in a mouse system where pretreatment of mouse tumours with such antibodies prevented their destruction by sensitized lymphocytes in vivo and in vitro. Further, it has been found that tumour cells which were able to grow in the allogeneic host after treatment with protective antibody were able to be transplanted successfully to normal mice of the same strain without further treatment. This was due to the diminished capacity of the tumour cells to evoke cell mediated immunity against themselves. Charney (1968) in an in vitro study of virus induced rat sarcoma, has shown that the tumour cells are capable of eliciting antibodies directed against antigens within the tumour cells themselves. Presumably these antibodies were enhancing and protected the tumour against other host defence mechanisms.

In analogy Zimmerman and Feldman (1970), found that skin grafts previously dipped in an antisera prepared against the graft, survived longer than control untreated grafts when transplanted into allogenic recipient. They concluded that the enhancing antibody acted by blocking the antigenic site on the graft rather than by inducing a distant action at the regional nodes.

3.3. ACTIVE IMMUNOTHERAPY

Generally speaking, in order to obtain the best from immunological treatment, the tumour should first be reduced to the minimum size by the conventional methods of treatment including surgery, radiotherapy or chemotherapy. The remaining tumour should then be dealt with by immunotherapy in an attempt to eradicate the living roots of the cancer tissue. Active immunotherapy includes specific and non-specific stimulation of the patient's own immune responsiveness. Mathe, Amiel, Schwarzberg, Schneider, Cattan, Schlumberger, Hayat and Vassal (1969) introduced this type of therapy for patients with acute lymphoblastic leukaemia who had made a good initial response to chemotherapy and used BCG (non-specific) or pooled leukaemia cells (?specific) previously irradiated or rendered non-viable by pre-treatment with formalin. Some patients had prolonged remission from their disease, lasting over three years in one patient. The same method of treatment is now included in the

M.R.C. leukaemia trials in this country. Guyer and Crowther (1969) showed that B.Pertussis vaccine was not as effective as BCG in obtaining remissions. Later Mathe, Pouillart and Lapeyraque (1969a), using experimental animals showed that a combination of BCG and irradiated leukaemia cells gave a better response than either given separately. Sjorgen and Ankurst (1969) showed that it is possible to protect hamsters from transplanted tumours by non-specific stimulation of the immune responsiveness during the latent period. Again applying the non-specific principle, chemicals which produce contact dermatitis (delayed type hypersensitivity) have been used in the form of topical cream to control primary epidermal neoplasms. Williams and Klein (1970) found two agents, 2,3,5 triethylene imino-benzoquinone (TEIB) and dinitrochlorobenzene (DNCB), good for this purpose, because they produced more intense immune reactions in epidermal neoplasms than in normal skin. The reaction showed stages of erythema, edema, vesiculation, erosion, exudation, necrosis and final disappearance of the tumour in 95% of multiple superficial basal cell carcinoma tested for observation periods of up to 5 years. Healing occurred with negligible or no defect.

On the specific side, Ikonopisov, Lewis, Hunter-Craig, Bodenham, Philips, Cooling, Proctor, Hamilton-Fairley and Alexander(1970), have used autologous stored melanoma to boost or reactivate the patient's immune reaction, and has succeeded in bringing about a reappearance

of circulating cytotoxic antibodies. Attemps are also being made to render tumours more immunogenic. Cunningham, Olson, Laffin, Horton and Sullivan (1969), did not get any benefit from treating patients (whose own immune response was normal), by immunizing them with their autologous tumour coupled to rabbit gammaglobulin.

3.4. PASSIVE IMMUNOTHERAPY

The use in patients of (a) competent cells or (b) antibodies is complicated by the ability of the patient to reject such donor cells or even some alloantibodies.

(a) THE USE OF SENSITIZED LYMPHOID CELLS

The cytotoxic effect of sensitized lymphocytes is of particular interest in tumour immunotherapy. Their effect on target cells in vitro is demonstrated when tumour cells are mixed with specifically sensitized lymphocytes and then fail to grow when injected into the syngeneic host(Klein and Sjogren, 1960); unsensitized lymphocytes have no effect, and the tumour grows normally. In vivo, the effect(on tumours) of foreign immunologically competent cells will depend on (i) the antigenic constitution of the tumour bearing host and the cell donor (a closely related donor's cells would survive longer). (ii) the recognition of differences between the tumour and its parent normal tissues(a closely related donor might barely see any). (iii) the percentage of the donor cells specifically committed against the tumour and

the number of them gaining access to the tumour. (iv) finally there is a risk that donor cells might gain the upper hand and attack the normal tissue of a patient whose own immunity was weak.

Woodroff and Symes (1962) were able to get complete regression of mammary carcinoma in A-strain mice, by subjecting them to a sublethal dose of irradiation, and infusing them with allogeneic lymphoid cells. They could not however prolong the survival of these animals as those who did not die from the tumour, died from graft versus host disease.

In vivo, the growth of a chemically induced fibrosarcoma in the rat, could be retarded by the intravenous injection of a large number of competent thoracic duct lymphocytes. These were obtained from rats of the same strain previously immunized with the same tumour. However, no complete cure was achieved (Delorme and Alexander, 1964).

After a number of experiments (Alexander, Delorme and Hall, 1966), showed that the cytotoxic action in vivo was different from that in vitro. It could be carried out indirectly by a subcellular fraction from the sensitized lymphocytes. This fraction was similar to the transfer factor of Lawrence (1969) and was most probably messenger RNA, capable of being adapted by the lymphocytes of the host.

In man, Nadler and Moore (1965) took patients with incurable malignant diseases divided them into pairs and cross immunized them with one another's tumour cells. After 10-14 days they collected the respective white blood cells cross-transfusing them between the paired patients. By this procedure they were able to benefit 7 out of 26 patients, two of whom had remissions over 2 years.

Symes, Riddel, Immelman and Terblanche (1968), used lymphocytes derived from specifically immunized pigs to treat four patients with advanced malignant diseases, with unsatisfactory results. Newton and Donaldson (1969) also failed in treating two patients with lymphocyte transfusions. Denton and Symes (1969) studied lymphocytes from different donors immunized with mouse lymphoma and were not able to detect a significant cytotoxic effect by these lymphocytes.

Spleen cells derived from immunized animals were shown to be as effective as lymphocytes if given in high doses to tumour bearing animals. Alexander, Connel and Mikulska (1966), eradiated tumour by giving a dose at 200 allogeneic spleen cells per tumour cell, when the number of tumour cells was not more then 10^4-10^5 cells. Similarly Glynn, Halpern and Fifir (1969), used spleen cells in conjunction with cytoxan to treat lymphoma in BALB/C mice.

b) THE USE OF ANTIBODIES

Specific tumour antibodies are usually elicitable against virally induced leukaemias, but less often against chemically or physically induced sarcomas or carcinomas. In vitro such antibodies can be cytotoxic to the tumour cells (Klein and Klein, 1964). In vivo, antibodies can be enhancing to the tumour growth rather than being cytotoxic, Bubenik and Turano (1968).

Gorer and Amos (1956) raised antisera against transplantable leukaemia in an allogeneic host. They found that the purified specific antibody could protect the animal against subsequent challenge with the tumour but its therapeutic value was limited. On the other hand, immune sera injected into syngeneic animals where shown to have enhancement activity (Hellstrom and Moller 1965), located in the 7s γ2-globulin component (Irvin, et al 1967). This component was shown to encourage the growth of the tumour in all allogeneic hosts when injected after transplantation of the tumour. In that the non-complement fixing immunoglobulins IgA, IgD, IgG_2, IgG_4 are 7s and have fast electrophoretic mobility (2) it may well be that enhancing bodies belong to these classes. In contrast 7s IgG_1, IgG_3 and 19s IgM have slower electrophoretic mobilities (3, 4) and are known to be capable of fixing complement and killing cells.

3.5. IMMUNOCHEMOTHERAPY

As already stated, immunotherapy has only been successfully used against small tumours, or small numbers of tumour cells. Often this could only be achieved following treatment by conventional methods. However, chemotherapy is the initial treatment of choice in many kind of neoplasia, particularly with leukaemia or sarcoma. Most of the chemotherapeutic agents have immunosuppressive activity as well. The results of the treatment therefore depend upon whether there is synergism between the drugs and the immunological responses of the host.

Minich (1969) treated sarcoma-180 in Swiss mice using 6-MP and found that the results depended upon altering the balance between immunologic enhancement and immunologic rejection of the tumour graft. Thymectomised animals had a lower incidence of complete regression of the tumours than did sham operated animals, inferring that cellular immunity was an important asset to rejection. Splenectomised animals showed a higher incidence of complete regression than sham operated animals. Hyperimmune sera decreased the percentage of complete regressions, while no effect was observed using normal serum. The presence of the spleen or a strong humoral response seemed to protect the tumour.

It is therefore important to assess whether the body's immunological reaction is cytotoxic or enhancing to the growth of the tumour. A cytotoxic reaction inhibited by chemotherapy could facilitate tumour growth. On the other hand an enhancing reaction inhibited by chemotherapy could impair tumour growth.

Recently Currie and Bagshawe (1970) used a combination of cyclophosphamide and non-specific immunotherapy (corynebacterium parvum) against a chemically induced isogneic tumour. They stated that the time interval between the administration of chemotherapy and the active immunization is quite critical, finding that 12 days was the optimum time for the induction of immunotherapy following cyclophosphamide treatment.

3.6. CONCLUSION

Except for conveniently localised primary tumours conventional treatments are unable to eliminate cancer. At best they can effect a reduction to about 1/1000th of the original size, but in terms of cell numbers, (10^{9}-10^{11}) at clinical presentation, 10^{-3} is but the top of the tumour iceberg. Thus such methods are unlikely to effect a cure unless some further process (such as an immune rejection) can eliminate the residual tumour cells. Because of high dose tolerance it would still be very important to apply immune rejection when the major mass of the tumour had been

reduced by conventional methods.

Throughout most previous attempts there has been a sad lack of quantitation. Few workers knew how much residual tumour their patients still had, few measured the competence of any white cells or antibodies they used in treatment, nor were the latter studied for size, class or ability to fix complement. Because passively transferred immunity is itself likely to be soon rejected by the recipient, it is imperative to measure its mode of action and estimate the first (and possibly the only) dose so that it may be given at an effective level.

CHAPTER 4

PLASMACYTOMA

Plasmacytoma represents autonomous neoplasias arising from those cell populations which are normally responsible for the synthesis of the circulating immunoglobulins.

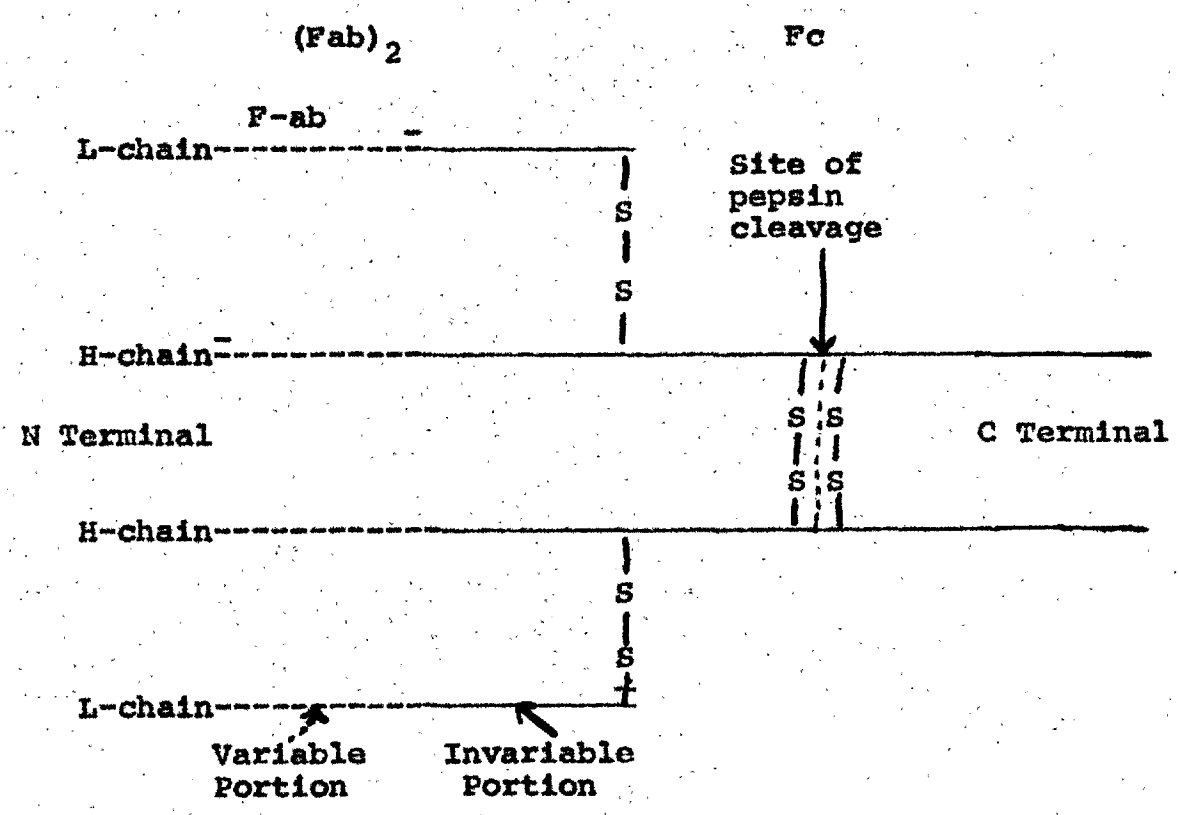
Thanks to Potter many transplantable plasma cell neoplasms have been developed in mice. Each neoplasm is associated with a different but characteristic globulin in high concentration in the serum or urine. (Potter and Fahey, 1960). These neoplasms grow progressively in normal mice. Most are transplanted as soft-tissue tumours but some give rise to the other characteristic changes associated with multiple myeloma in man. The study of these mouse tumours is relevant to the pathogenesis and natural history of plasmacytoma in man, and also provides a unique opportunity to assess the effect of different antitumour agents or other forms of treatment.

The neoplastic proliferation of the cells which produce immunoglobulins, results in the elaboration of large quantities of proteins which are related to their normal immunoglobulin counterparts by physico-chemical and immunochemical properties, but most are apparently devoid of any antibody specificity.

4.a) THE NORMAL IMMUNOGLOBULINS

The antibody activity of normal serum is associated

with the immunoglobulins. They comprise an extremely heterogeneous group of proteins, which are classified according to the antigenic differences. In man five major classes occur (G,A,M,D, and E), in order of their normal concentration in the blood. They all consist of similar basic four chain units consisting of two heavy (mo.wt.50.000-70.000) and two light (mol.wt.20.000) polypeptide chains, linked by interchain disulphide bonds (see fig.3).



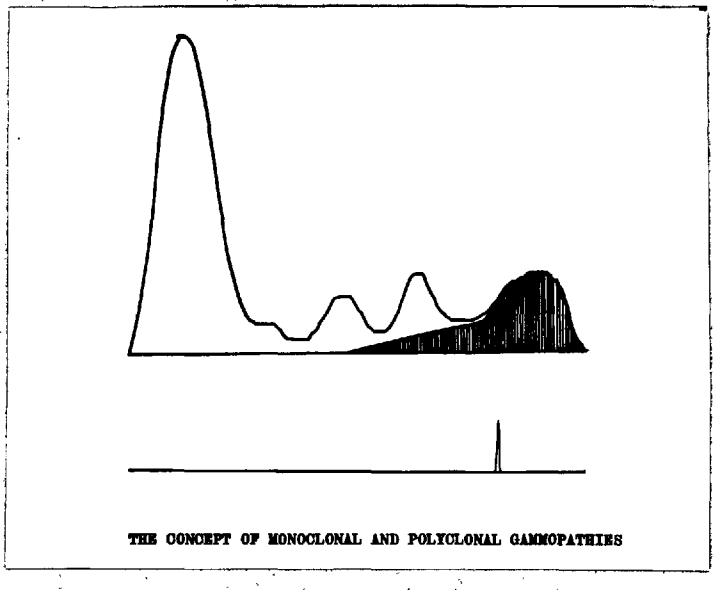
(Fig. 3.
Human 7s IgG_1 illustrating the structural basis of immunoglobulins.

This structure was first proposed by Porter (1962) for rabbit immunoglobulins and later was shown to apply to all vertebrates having recognizable antibody activity. The five major classes of immunoglobulins have distinct antigenic, physico-chemical and biological properties which are due to the differences in their heavy chain structure. (γ α μ δ and ε). . The two types of light chains (κ & λ) are present in a proportion close to 2K:1L in the different immunoglobulin classes except for D which shows a 1K:9L ratio. Each class of immunoglobulins may contain several subclasses of chains which are not allelic products since they occur in all individuals. Moreover, it has been revealed that light chains and heavy chains have C-terminals sections with sequences defined by isotypic and allotypic specificities alone. On the whole the N-terminal ends of both heavy and light chains appear to be specific for the clone of origin. This position of these chains is therefore called the variable region (see fig. 3). Only once to date (Wang, Wilson, Hopper, Fudenberg and Nisonoff, 1970), have two variable regions proved to be of identical structure. In that instance the two chains (G & M) are believed to be derived from a single parent clone. In the mouse, the three major classes G, A and M have been clearly identified, and subclasses of G(γ_1 & γ_2) are also well recognised (Fahey, Wunderlich and Mishell (1964).

4.b) THE MONOCLONAL CONCEPT

According to the clonal selection theory of Burnet, a single antigen could stimulate the proliferation of a single clone of cells to produce antibody. The protein molecules produced should all have the same structure and on electrophoretic separation they should run as a solitary narrow band. In practice this is observed in rare instances, when a single hapten has been used as antigen, enabling the isolation and purification of its antibody(Singer 1965). More usually such pure haptens evoke several overlapping antibodies and even more appear with time, in other words one antigen can evoke a spectrum of antibodies. This situation is more evident with pure protein antigens because they have more than one antigenic site in the one molecule, and even more evident following the challenge of an infecting organism containing many proteins which can therefore evoke many more antibodies. Thus natural challenges result in diffuse polyclonal increase of immunoglobulins which on electrophoretic separation with mobilities which stretch from $2\alpha_2 - \gamma$ (Figg.4.)

Figure 4.



THE CONCEPT OF MONOCLONAL AND POLYCLONAL GAMMOPATHIES

Thus when in practice a homogeneous spike of protein is found by electrophoresis, and shown to be due to immunoglobulin or part thereof, it is believed to represent the product of a monoclone of plasmacytoma and is called a paraprotein. This hypothesis owes much to the work of Waldenstrom (1968) and in 1968, Marchalonis and Nossal proved that the immunoglobulin produced from a single cell was indeed homogeneous. Therefore there can be no reasonable doubt that myeloma proteins represents the result of a single mutant plasma cell. Its descendants continue to produce the same immunoglobulin as the ancestral cell. In man, it was calculated by Hobbs (1969), that on average it takes 33 years for a single mutant cell to form a clone of cells that would cause a patient to present with clinical symptoms of myelomatosis. If the properties of a large number of unselected myeloma proteins from different individuals are averaged out, they are found to correspond very closely to the properties of the total serum immunoglobulins in any one normal individual. Furthermore, the frequency with which myelomas producing a given kind of immunoglobulin arise in a large population of people reflects the average abundance of each kind of immunoglobulin in the serum of normal people drawn from the same population (Hobbs 1968). It is as if any type of proliferating plasma cell was subject to the equal possibility of mutation.

In some 2% of myelomatosis more than one paraprotein can be clearly identified (different heavy and/or light chains) so that presumably more than one clone of cells occurs. Such clones may be related to one another by a common parenthood (Wang et al, 1970) and can be called a "tribe of clones". Others may be unrelated and can be called a "mosaic of clones".

Not all the monoclones will cause symptoms in the host, indeed it may be that the majority are benign and do not result in malignant invasive neoplasia in the natural population for the latter was only detected in 1 of 52 followed for 2½ years. (Axelson and Hallén, 1968). In hospital populations with longer follow-up more like 75% of patients with monoclonal proteins prove to have malignant neoplasia (Hobbs, 1969a).

4.c. THE CONCEPT OF BIOCHEMICAL DEDIFFERENTIATION

Askonas and Williamson 1967 using a stable mouse plasmacytoma line showed that in the process of immunoglobulin synthesis the light chains were formed on one size of polyribosome and the heavy chains on a larger polyribosome. Free light chains could be released within the endoplasmic reticulum of the plasma cell. In normal antibody-forming cells and well differentiated plasmacytoma cells radiolabelled synthesis was so well balanced that less than 1% of the radioactivity escaped from the cell as free light chains, and whole immunoglobulin only was released.

A prominent feature of plasmacytomata and allied diseases with malignant dedifferentiation is imbalance in immunoglobulin synthesis, detectable in some 80% of patients. As a result of such biochemical dedifferentiation, instead of (1) whole monoclonal immunoglobulin fragments occur such as: (2) excess light chains (3) light chains only (4) heavy chains only (5) half-molecules (6) incomplete molecules (7) 7s IgM and finally (8) no paraprotein at all.

(2) Excess monoclonal light chains are usually excreted through the kidney as monomers (mol.wt. 22,000) or dimers (mol.wt. 44,000) and have been known as Bence Jones proteins since 1848. In a survey of 402 patients in whom Bence Jones proteinuria was detected, malignant neoplasia of plasma cell lines was confirmed on biopsy or at post-mortem in 400 within 3 years (Hobbs, 1969a). In contrast no Bence Jones proteinuria was found in patients' with benign monoclonal immunoglobulins. This led to the concept that biochemical dedifferentiation (imbalanced immunoglobulin synthesis) was paralleled by malignant dedifferentiation (invasive neoplasia).

(3) Light chains only are found in some 20% of myelomatosis (Osserman and Takatsuki 1963; Williams, Brunning and Wollheim 1966; Hobbs 1966), and in such patients fluorescent

studies of the malignant plasma cells only reveal light chains. There has been a complete loss of heavy chain synthesis. The tumours of these patients present earlier grow faster, have more complications and carry a worse prognosis than with (1) (Hobbs, 1969a).

(4) Heavy chains only. Franklin (1964) first recorded patients in whom no light chain synthesis could be detected. Their neoplastic plasma cells only produced a dimer part of the heavy chain (mol.wt. 80-120,000). Both C and N terminal sequences of the heavy chain were detected indicating that it was the product of heavy chain gene with deletion of the mid portion. In the first 8 patients this heavy chain was of the γ-class and associated with malignant soft-tissue plasmacytoma (Ellman and Bloch, 1968). Later Seligmann, Danon, Hurez, Mihaesco and Preud'homme (1968) discovered plasmacytomata producing only heavy chain of the α-class. These tumours mainly invade the wall of the small gut of young adult patients of Arabian ancestry. (Tanigaki, Yagi, Moore and Pressman, 1966), noted only μ-chain synthesis in a fluorescent study of some lymphoma lines, and μ-chain plasmacytomata are now recorded by others (Ballard, Hamilton, Marcus and Illes, 1970).

(5) Half-molecules of IgA were first recorded with mouse plasmacytoma (Potter and Kuff, 1964) and the full half molecule of IgG (one γ-chain plus one K-chain) has been

found in association with malignant plasmacytoma in man (Hobbs and Jacobs, 1969).

(6) Other incomplete molecules of IgG with variable amounts of deletion are also recorded (Snigurowicz, Murawski, Kraj, Jozwiak, and Zupanska, 1968) as are half light chains (mol. wt. 10,000) (Solomon, Killander, Grey and Kunkel, 1966).

(7) Normal IgM is a 19s pentamer of five 7s units, but this final step in immunoglobulin synthesis can also be deranged, and malignant lymphomata are encountered producing only monoclonal 7s IgM (Solomon and Kunkel, 1967), or a variable amount of 7s together with 19s IgM (Bush, Swedlund and Gleich, 1969).

(8) All the above derangements (2-7) of immunoglobulin synthesis can result in a lower overall output of paraprotein from a malignant plasma cell compared to a normal plasma cell, so that it is not suprising that eventually dedifferentiation occurs to the extent where no paraprotein at all is released. In about 1-2% of clinically and microscopically typical myelomatosis no free paraprotein can be detected. In most of these cases fluorescent studies do not reveal any synthesis of recognisable heavy or light chains but in some cases positive fluorescence (retained immunoglobulin) is seen.

All the above forms of biochemical dedifferentiation of immunoglobulin synthesis have to date only been recorded

together with malignant dedifferentiation. The presence of immunoglobulin fragments can also be associated with reduced overall paraprotein production, so that estimates of the amount of tumour based on paraprotein levels could be misleading.

4.d. RELEVANCE OF ANTIBODY-LIKE ACTIVITY IN MONOCLONAL IMMUNOGLOBULINS

The existence of antibody-like activity in a number of Waldenstrom macroglobulinaemia (Kritzman, Kunkel, McCarthy, and Mellors (1961) provided some evidence for the possibility that the myeloma proteins themselves represent individual antibodies, the products of a clone of myeloma cells that originated from a normal antibody response in an individual plasma cell. Studies of the physiochemical structure of most myeloma proteins and Waldenstrom macroglobulins indicate these are closely related to the normal immunoglobulins, suggesting that most are normal synthetic products resulting from a massive production of one globulin selected at random from the family of immunoglobulins. Several paraproteins have been described which resemble authentic antibodies by many criteria. The subtle variations in specificity occur only in the amino-terminal ends of heavy and light chains the variable regions. The Waldenstrom macroglobulin binds human γG proteins, but it readily discriminates between human γG and all γG's except those from the most closely related

primates. The binding is exclusively in the variable regions, with one combining site to each molecule of Fab. The human myeloma protein γG-Bry binds certain nitrophenyl derivatives with considerable specificity, again confined only to the Fab region with one combining site per molecule of Fab. The BALB/C mouse A myeloma protein 315 binds nitrophenyl derivatives with a high binding constant and great specificity, again still confined to the Fab fragment. These observations were important because it was known that most animals are capable of making antidinitrophenyl antibody of exceedingly high affinity when immunized. Osserman and Takatsuki (1965) pointed out that the induction of plasmacytoma in mice (by chronic peritoneal irritation) would be analogous to cases of multiple myeloma in man, possibly arising subsequent to recurrent inflammatory disease. The subsequent development of a plasmacytic dyscrasia could be relevant to the host's previous immunologic history. In this respect the report of Seligmann, Danon, Basch and Bernard, (1968) is highly relevant. They described a patient who had had three recurrent attacks of rheumatic fever, and then developed an IgG myeloma. The myeloma protein exhibited high antistreptolysin O activity, an antibody activity specifically related to rheumatic fever. If such cases could be repeatedly demonstrated, chronic inflammatory disease might be implicated as an important factor in initiating plasmacytoma. There is good evidence that

paraproteinaemia is commoner in areas with a high incidence of infection (Michaux and Heremans, 1969). The reason why not so many specific cases are reported maybe the very long latent period for the disease to become clinically manifest. It may need 33 years for a single myeloma cell to give rise to a clone which is detected clinically (Hobbs, 1969) a time which is sufficient to mask any correlation with the previous history at the time when the transformation started.

4.e. THE EXPERIMENTAL MODELS

Several mouse plasma cell tumours which are used as experimental models were found spontaneously (Nielsen and Gormsen, 1951; Potter and Fahey, 1960).

Other plasma cell tumours, many of which are transplantable have been induced. Merwin and Algire, (1959), implanted BALB/CAn mice intraperitoneally with millipore diffusion chambers containing C_3H mammary-tumour tissue and subcutaneously with diethyl stilbosterol pellets. Many developed multiple plasma cell tumour nodules in the viscera and on the peritoneal surface, with bloody ascites, and many of these tumours were established by transplant.

Potter and Robertson (1960), found that BALB/C mice have a predeliction to develop an apparently unlimited array of biologically distinctive plasma cell tumours when given an intraperitoneal injection of mineral oil or mineral oil

adjuvants. The initial response in such mice to these stimuli was the formation of widespread lipogranulomata. After a period of 6-24 months these evolved into multiple discrete plasmacytomata and sometimes as many as five different neoplastic plasma cell lines could be isolated from a single mouse. Each neoplasm produced antigenically different proteins. Many of the lines could be continued through several intraperitoneal or subcutaneous transplantations in the same strain. The large number of these tumours now available has made possible the study of this disease as a model system. Hopefully, the information thus derived can be directed towards healing clinical myelomatosis, particularly since the mouse plasma cell tumours resemble human myelomatosis.

From the viewpoint of tumour immunology it has been found by Paraskevas, Lee and Israels (1970), that BALB/C mouse plasmacytoma cells have shown antigenic deletion, i.e. the loss of or block of certain antigenic receptor sites on the cell surface as compared to normal mouse cells. These authors in fact suggest this loss of a potential feedback mechanism predisposes to the uncontrolled immunoglobulin synthesis and sustained proliferation of the plasmacytoma line.

4.f. PROTEIN PRODUCTION OF MOUSE PLASMACYTOMA

The occurrence of abnormal homogeneous proteins in

mice bearing plasma cell neoplasms received a great deal of attention, and many questions have been presented, some of which are still unanswered. Whether this myeloma protein was formed by the tumour cells themselves or by other tissues in response to the tumour was answered by Nathans, Fahey and Potter, (1958), who proved the protein was synthesized by the tumour cells. Osserman, Rifkind, Takatsuki and Lawlor, (1964), found that the increase in serum level of the abnormal protein was directly related to the increase in tumour size. However, above a 2-3gm. size of the tumour the relationship failed, and this was found to be due to central necrosis of the tumour mass. Furthermore a significant decrease in the amount of protein production by a given mass of tumour was observed in the course of 75 transplantations over a six year period. This diminution in fractional differentiation was associated with cytological changes and an increase in growth rate of the tumour.

SUMMARY OF INTRODUCTORY CHAPTERS

From a study of the literature on tumour growth it appeared that an ascitic form of a tumour provided a very convenient model for experiments as tumour cell counts could readily be made. It also became obvious that the stability of a transplantable tumour required checking at frequent intervals.

Mouse plasmacytoma afforded another parameter which might be used to monitor tumour growth, this being the paraprotein produced. Expertise in such measurements was readily available in our laboratory. It was however important to use a well-differentiated tumour synthesising its protein as completely and as well balanced as in the normal plasma cell. Production of immunoglobulin fragments would in fact confuse measurements.

In the field of tumour immunology it appeared spontaneous tumours were more reliable and afforded a better model of natural cancer than did chemically or virus induced tumours. Accordingly the mouse plasmacytome 5563 was primarily chosen for this work, although the chemically induced lines in BALB/C mice were to be tried as the helpful Chester Beatty Institute had much experience with such lines.

From the work on tumour immunotherapy it seemed that in man some success had followed the introduction of immunotherapy at a time when the mass of the tumour had been reduced in size by conventional treatment. In myelomatosis the paraprotein affords an index of such reduction and could indicate the best time to attempt immunotherapy, as well as a parameter whereby it could be monitored. Since the cellular immunity of plasmacytoma hosts is well maintained, the passive introduction of competent lymphocytes seems to offer little risk of graft-versus-host reaction. This does mean that only one dose from a given donor could be used, and would need to be effective within the first week. The mouse model offered a chance to see if a single effective dose could be used successfully. From the literature it became imperative to try and measure both the humoral and cellular components of any immune response and also to assess other factors such as class and size of antibody and ability to fix complement, or potential enhancement, or damaging cross-reactions against the host's normal tissues.

CHAPTER 5

MATERIALS AND METHODS

5.a. MONITORING TUMOUR GROWTH USING CELL COUNTS

Tumour: The tumour MP-5563, a plasmacytoma in ascitic form was used in all the experiments and was kindly supplied by Dr. B.A. Askonas, of the National Institute for Medical Research, Mill Hill. This tumour originally arose spontaneously in the ileocecal lymph node of an ovariectomised 22 months old female C_3H/HC mouse. It was first noticed by Dr. Ira Pilgrim at the National Cancer Institute, Bethesda, Maryland, and was passaged by Dr. M. Potter by subcutaneous and intraperitoneal implantation in the same strain of mice, but failed to grow in other strains. It was described as slow growing, well differentiated plasmacytoma, bearing similar histological picture to human plasma cell neoplasms. It produces a 7s IgG_{2a}-globulin which is demonstrable in the serum of the tumour bearing animals in high concentrations after the tumour attains certain size. It does not produce Bence Jones protein.

Mice: A colony of inbred C_3H mice was established. The breeding pairs were originally supplied by the Medical Research Council's Laboratory Animal Centre of Carshalton. The animals used throughout the whole experiments were males or females 8-12 weeks old derived from the colony.

They were kept on B_{41} diet, and 4-6 to a cage depending on the experiment.

Tumour Transplantation: The tumour was collected from tumour-bearing animals which had been transplanted 7-8 days earlier. These were sacrificed by cervical dislocation, and ascitic fluid was aspirated from the peritoneal cavity using a sterile 2 ml. syringe. This was transfered into a sterile plastic Universal container containing 5 ml. of normal saline. Samples were taken from this aliquot for counting, and the aliquot was then adjusted with normal saline to contain the required number of tumour cells per ml. For most purposes it was adjusted to contain 5×10^6 cells per ml. The required number of cells were then injected intraperitoneally into the required number of mice.

Cell Counting: 0.02 ml. of the aliquot to be counted was transferred into a Universal containing 10 ml. of the counting fluid, mixed, and counted in a Coulter counter model A set at ACS2, threshold 42. The count obtained represented the actual number of cells in 0.5 ml. of the aliquot/counting fluid mixture. 10 ml. of this mixture contained 0.02 ml. of the original aliquot. Thus, the count obtained represented:-

$$\frac{20}{1000} \times \frac{0.5}{10} = \frac{1}{1000}$$ per ml. of the original aliquot

In order to obtain the actual count of the original aliquot, the count on the Coulter had to be multiplied by 1000.

Counting Fluid: The following formula was used to prepare an isotonic solution of suitable pH for use in Coulter counting of the tumour cells. This formula was supplied by Dr. T. Connors of the Chester Beatty Research Institute:

Disodium EDTA	1.58 gm.
Trisodium EDTA	1.82 gm.
Tetrasodium EDTA	0.32 gm.
Sodium Chloride	6.5 gm.
Formaldehyde Solution	1 ml.
Distilled water up to	1000 ml.

The solution was filtered twice through sintered glass.

Normal peritoneal cells counted at the Coulter setting for tumour cells

With the Coulter counter set at ACS2 threshold 42, contaminating red cells are not counted. This was easily verified by counts made before and after hypotonic shock treatment (see p. 83). However larger cells from the peritoneal lining could contribute to the overall count in three ways (1) those "washed off" by the mere presence of excess fluid in the peritoneal cavity (ii) those

migrating into the peritoneal cavity as an inflammatory response to the tumour (iii) those gaining the peritoneal cavity due to loss of integrity of blood vessels. The last could be excluded by largely working in the phase of tumour growth before bloody ascites developed. In order to check (i) and (ii) the peritoneal cavity of normal mice was washed with physiological saline at two volumes, 1 ml. and3 ml. covering the extremes of ascitic volume encountered in the working phase of tumour growth. These washings were Coulter counted and then examined microscopically in a counting chamber.

Using the same microscopic method tumour cell containing ascites was also examined to see if any excess of normal peritoneal exudate cells could be detected.

Loss of tumour cells from the ascites: It is possible that tumour cells could become attached to or even invade the peritoneal lining. Histological examination of sections of the peritoneal wall taken from mice sacrificed early and late in the phases of tumour growth was therefore undertaken. (see fig. 5,7&8) I am grateful to Dr. David Evans of our Morbid Anatomy department for his help.

5.b. DETERMINATION OF THE TOTAL VOLUME OF ASCITIC FLUID USING ISOTOPE DILUTION

By aspiration alone it was not expected that full

recovery of all ascitic fluid could be achieved. By labelling autologous red cells with Cr^{51} and injecting them into the ascites, isotope dilution offered a means of estimating the total ascitic volume. This assumes that such red cells mix evenly and that neither they nor their Cr^{51} label leave the peritoneal cavity during the time of the determination. As a check on this peripheral blood was sampled and counted for Cr^{51}.

Red cell labelling: Following the method of Mollison and Veal (1955), about 1 ml. of blood obtained from the orbital plexus of a C_3H mouse, was transferred immediately into a universal container in which 2 ml. of dilute heparin solution (2 mgm/100 ml. of normal saline) was already placed. The universal was centrifuged and the supernatant was discarded. About 5 µc of Cr^{51} solution was added to the packed red cells remaining in the universal and the mixture was left at room temperature for 30 minutes. The cells were then washed twice in normal saline, and re-suspended in 5 ml. of normal saline. The labelling was freshly done for every experiment.

Cr^{51} isotope: The isotope was supplied by the Radiochemical Centre at Amersham in the form of hexavalent sodium chromate solution of high specific activity.

Method: With the use of 1 ml. insulin syringe and a fine needle (Gauge 25), an exactly known volume of isologous labelled red cell suspension (0.5-1 ml.) was injected intraperitoneally. After 10 minutes, a sample was drawn from the ascites for counting. An exactly known volume of this ascitic fluid was counted for radioactivity in a well-scintillation crystal. For the standard an exactly known volume from the stock red cell suspension from which the injection was given was counted under identical conditions. After subtracting the background activity from the counts of the sample and standard, the total volume of the ascites was determined from the following formula:-

$$C_1 \times V_1 = C_2 \times V_2$$

where C_1 = count of the standard per ml.

V_1 = ml. of the labelled red cell suspension injected

C_2 = counts of the ascitic fluid per ml.

V_2 = ml. of total ascites

Blood samples from the mice were obtained from the orbital plexus immediately after sampling the ascites. The mice were anaesthesized first with Penthrane to make the manipulation easy. The tip of a Pasteur pipette was then passed behind the eye ball and the plexus was punctured and the blood was sucked out using a teat fitted at the end of the Pasteur pipette. The blood was then checked for radioactivity.

Recoverable ascites: In a group of 5 mice sampled as above, the remaining ascites was then aspirated as completely as possible. The mice were then sacrificed, the abdomen swiftly opened and any further drops of fluid aspirated as completely as possible. A careful measurement was made of the total volume, a measured aliquot was counted enabling an estimate of the total radioactivity recovered. This was then expressed as a percentage of known radioactivity injected. In this particular experiment the actual syringe used to give the dose of Cr^{51} red cells was counted before and after the dose was administered. The difference in counts was the exact amount injected.

5.c. MONITORING TUMOUR GROWTH USING PARAPROTEIN MEASUREMENTS

Blood collected as above from the retroorbital plexus was transferred into small plastic centrifuge tubes, allowed to clot for 10-15 minutes and was then centrifuged in the Spinco microfuge. The serum obtained was transferred into another small plastic tube, labelled and either analysed fresh or kept at $-20^{\circ}C$ and only thawed once before analysis.

Serum Electrophoresis: This was carried out on cellulose acetate strips, using barbitone buffer pH 8.6, 0.05M, for 65 minutes at 25v/cm. The serum was applied as a fine line on the edge of a microscope slide, which was then touched onto the cellulose acetate strip. Straight bands resulted with clear resolution of the paraprotein from the normal β_1 (Fig.12).

The strips were then stained for 5 minutes with Naphthalene Black 10B, having the following formula:-

Naphthalene Black 10B	0.25 gm.
Trichloroacetic acid	3 gm.
Methyl alcohol	100 ml.

This stain is known to bind reasonably uniformly to the proteins of serum so that reliable estimates of albumin or globulin can be made (Hobbs, 1965).

After staining, the strips were then washed twice with a mixture of 9 parts of methanol and 1 part of glacial acetic acid allowing five minutes for each washing, and following twice with pure methanol. This washing procedure will result in clearing the background of the strip from any unwanted stain.

Total Protein estimation:- This was done on the basis of specific gravity because a biuret method was shown to underestimate IgG-globulin by some 6% against an albumin standard.

Using the method of Lowry and Hunter (1945), a small drop of the serum was delivered from a Pasteur pipette into a specific gravity column which had been previously calibrated. The protein content of the serum was obtained from comparison against a series of standards of known specific gravity.

Estimation of the Paraprotein:- The paraprotein band was estimated as a percentage of the total dye uptake measured by transmission scanning using the Zeiss Extinction recorder mark II. The paraprotein percentage of the total protein was then calculated in gm/100 ml. and by the above method this could be reproduced to within 0.05 gm/100 ml. Since the paraprotein was superimposed on the residual normal immunoglobulins, the overall accuracy was probably only within 0.1 gm/100 ml.

5.d. IMMUNOLOGICAL PROCEDURES

Rats: Male albino rats 5-6 months of age were supplied by Scientific Products Ltd., Canterbury-Ash. These were found to have a very little subcutaneous fat which made the access into the thoracic duct more feasible. They were kept on B_{41} diet and six to a cage.

Immunization of rats:- Plasmacytoma cells were collected fresh from tumour bearing mice in the usual way, and red cells, if any were lysed using the method of Janowsky, Rosenau and Moon (1964). The ascitic fluid was centrifuged,

and the supernatant was discarded. To the packed cells 10 ml. of 0.35% sodium chloride solution was added and the mixture was shaken gently. After 60 seconds, 1.2 ml. of 5% sodium chloride solution was added to restore the isotonicity of the mixture. The mixture was then centrifuged, the supernatant discarded and the tumour cells were resuspended in normal saline. The suspension was counted.

About 30×10^6 tumour cells were then given to each rat by three injections. One subcutaneous injection into each of the hind legs and one intraperitoneal injection. Similar booster doses were given one week later.

Collection of whole thoracic duct lymph:- Lymph was collected by cannulation of the thoracic duct of the rats using the Gowans technique(see figs. 6-8). Immunized rats were cannulated three days after the booster immunization dose. A cannula made of two parts (fig.9) was found most convenient for the purpose:-

1) The head was made from a piece of nylon intravenous cannula O.D. 1.02 mm. (Portex Ltd.). This was bent to a hook-like shape, over a very gentle flame, and its tip was sharpened by cutting it obliquely. This facilitated penetration of the thoracic duct



Fig.6. Gowan's technique.
This picture shows the thoracic duct of the rat ligated and swollen.

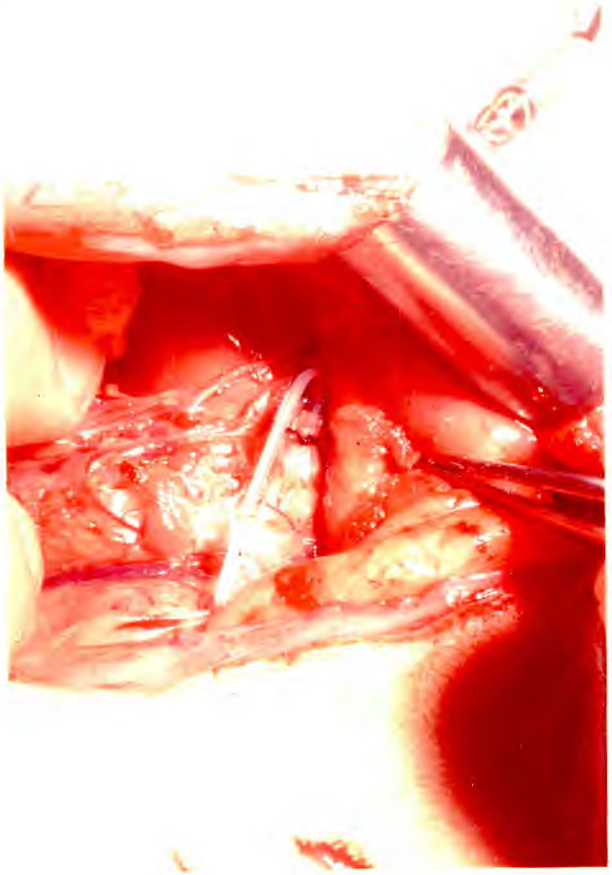


Fig.7. Gowan's technique.
This picture shows the cannula after being placed in the thoracic duct and ligated in position.



Fig.8. Cowan's technique.
After the operation, the rat is kept in a restraining cage, fed 5% glucose saline from a feeding bottle, and the draining lymph is collected in the flask.

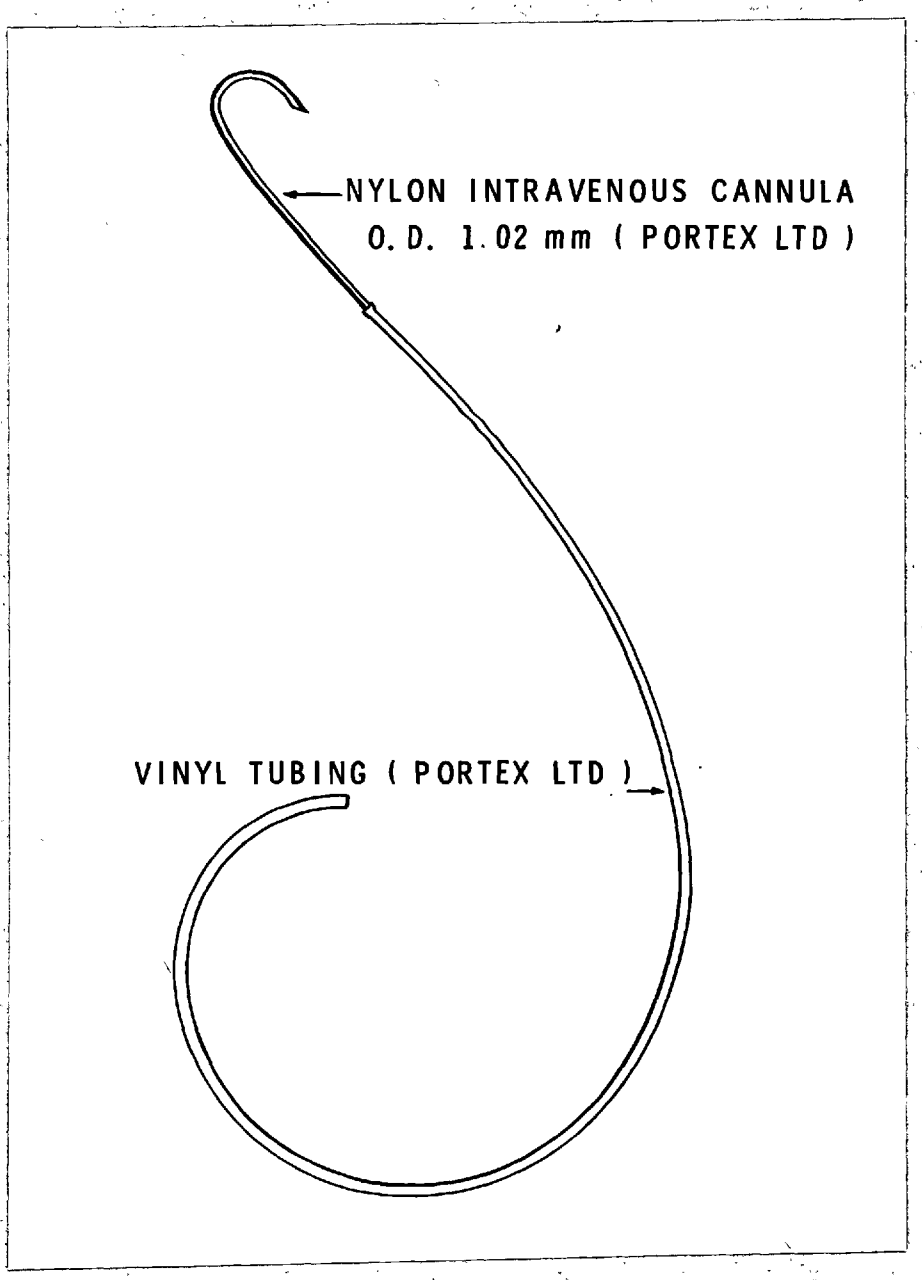


Fig.9.
A suitable cannula for thoracic duct cannulation of the rat, made from two pieces.

directly without any previous puncture which might cause the duct to collapse.

2) The head was attached to transluscent vinyl tubing (Portex Ltd. size 1), which was very soft and absorbed any shock to the external part of the cannula. This prevented the head from being dislodged from the duct. The cannula was siliconized and sterilized by irradiation.

The animals were given intramuscularly 0.1 mgm. vit K prior to cannulation. They were fed 5% glucose in normal saline or water with the usual B_{41} normal diet.

The lymph was collected in 100 ml. siliconized sterile glass flasks into which 1 ml. of heparin (200 mgm/100 ml.) was first placed. The lymph that came out in the first few hours after cannulation was very viscous and tended to clot if not agitated every few minutes. The cannula was prone to be blocked in the first few hours after cannulation. It could be reopened by light aspiration of the lymph from the cannula by a syringe and a suitable needle. If the cannula could not be opened by this method, replacement was necessary. The rat was reanaesthesized, and the old cannula was cut at about 1" from where it emerged from the skin and a new cannula was connected to the remaining piece by a suitable junction fitting both ends tightly. The lymph was collected every 12 hours.

Preparation of cells from lymph:- The lymph was centrifuged at a low speed for five minutes. The supernatant was discarded or used as cell-free lymph below. The cells were then washed in medium TC 199 and resuspended in the same medium to contain the required number of lymphocytes in not more than 1 ml. of medium. The viability of the cells was determined by the dye exclusion technique using 1% trypan blue solution.

Chromatographic fractionation of the cell-free lymph:-

Amounts of 10 ml. of cell-free thoracic duct lymph from an immunized rat were applied to a column of Sephadex G200 which had been previously calibrated with normal rat serum. The three main peaks were collected and concentrated by ultra filtration back to the original volume of the lymph. The fractions were then dialysed against phosphate buffer saline (pH 7.4) for 48 hours at 4°C and finally sterilised by ultra filtration. They were then distributed in 2 ml. amounts in sterile universal and kept frozen at -20°C. The required number of bottles were thawed once immediately before use.

CHAPTER 6

VALIDATION OF METHODS

A. RESULTS

1. Recovery of ascitic fluid:- From a 1 ml. insulin syringe 99 to 99.5% of a given dose of Cr^{51} labelled red cells could be delivered into the peritoneal cavity. Knowing the dose actually given it was a simple calculation from the radioactivity and tumour cell numbers in a subsequent sample of ascitic fluid to estimate the total number of tumour cells within the total ascitic fluid. In 10 animals complete aspiration of the ascitic fluid followed by opening the abdomen for the last few drops, only recovered from 92 to 94% of the given dose of radioactivity. It can therefore be seen that simple aspiration would be likely to underestimate the total number of tumour cells. The use of the Cr^{51} red cells allowed more complete estimation of the ascitic volume.

2. Validity of Cr^{51} red cells over 10 minutes:- Up to the end of 8 days after the tumour inoculation, Cr^{51} labelled red cells could not be detected (<0.1%) in the blood taken from the orbital plexus 10 minutes after their intraperitoneal administration. However, by the end of the ninth day, Cr^{51} red cells were regularly found in the systemic circulation within 10 minutes of inoculation.

The Cr^{51} method therefore is valid up to 8 days but not after 9 days.

3. Validity of tumour cell counts:-

(a) Washing the peritoneal cavity of normal C_3H mice released only a total of 0.6-1.0 million cells counted at the Coulter setting used whether a volume of 1 ml. or 3 ml. of saline was used.

(b) Microscopic examination, in a cell counting chamber, of ascitic fluid containing MP5563 cells did not reveal any excess of recognisable epithelial cells or macrophages compared to the washout of the peritoneal cavity of a normal mouse. Such cells only accounted for up to 1 million of the total cell count.

4. Tumour cells adherent to or invading the peritoneal wall:-

Macroscopic and histological examination of the peritoneal wall revealed no adherent masses or frank invasion by tumour cells, up to 8 days after inoculation. Thereafter invasion was regularly found (see fig. 5) 5a; 5b).

1 - 4 therefore indicate that tumour cell counts and Cr^{51} red cell corrected ascitic volume measurements yield reliable estimation of the total of tumour cells up to 8 days after transplantation, but not thereafter, nor with low cell counts approaching the level of 1 million.

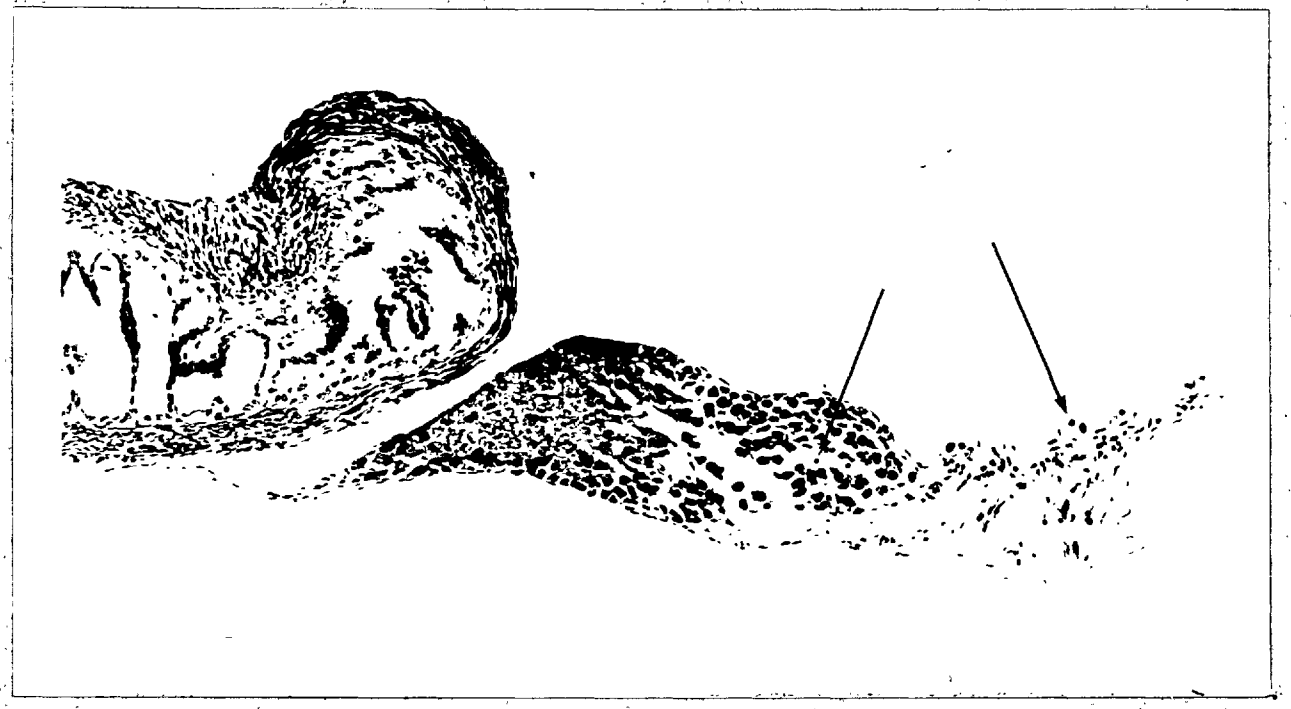


Fig. 5.
Photomicrograph showing invasion of the peritoneal lining by the plasmacytoma cells (⟶) 10 days after transplantation.



Fig. 5a.
Macroscopic examination of the peritoneal wall, during the exponential growth phase. It shows no adherent masses of frank invasion by tumour cells.



Fig. 5b.
Macroscopic examination of the peritoneal wall, following the exponential growth phase, showing frank invasion of the tumour cells.

B. DISCUSSION

The utmost care was taken when collecting the ascitic fluid from the peritoneal cavity. Firstly, aspirating as much as possible by syringe to relieve the tension of the abdomen(loss of fluid could result if the abdomen was opened while tense) and then collecting the last few drops with a Pasteur pipette after opening the abdominal wall. Inspite of these precautions the total volume of fluid collected and measured by this method fell short by about 7% of the results obtained when measurements were done by the isotope dilution technique. What could have accounted for this difference was fluid left sticking to the walls of the abdominal cavity and the viscera, because of the high viscosity of the ascites due to its high protein content. With the use of insulin syringe, the delivery of a precise volume of labelled red cells was insured. The relatively high amount of activity ($\simeq$1μC) gave a high count rate and a low standard error of counting. The whole procedure took only few minutes during which time the elution of the chromium from the red cells was negligible, as was loss of red cells from the peritoneal cavity. The sample of ascites was taken while the abdomen was still closed and loss of ascites was excluded.

Cell counting was done subsequent to calibration of the Coulter counter. At the setting used, red cells were not counted, but macrophages and some epithelial cells from the peritoneal cavity were counted together with the plasmacytoma cells. It was therefore necessary to establish the cell counts from peritoneal washouts of control mice, and this number was subtracted from the total cell count to yield the plasmacytoma cell count.

CHAPTER 7

THE GROWTH CHARACTERISTICS OF MP 5563

Klein and Revesz (1953) stressed the advantages of using ascitic forms for studying tumour growth. After inoculation there was a "latency period" of a few days and of unknown aetiology during which tumour growth could not be recorded. Thereafter they described a "cube rate phase" in which they found the cube roots of the cell numbers were linear against time and similar to findings with solid tumours. Finally followed a "terminal period" with an apparent fall-off in tumour growth. Steel (1968) using the results of Baserga (1963) and Lala and Patt (1966) for Ehrlich ascites tumour described a slowly progressive increase in cell loss to as high as 90% before death and assumed this was due to the death of many of the tumour cells formed. None of the above workers used an internal standard to control the assessment of the ascites and its cells content. The present study used intraperitoneally injected isologous Cr^{51}-labelled red cells to monitor the recovery of ascitic fluid and the integrity of the peritoneal cavity. With this safeguard a simple exponential growth was observed between 4-8 days after inoculation until a fistulous communication occurred between the peritoneal cavity and the blood-stream which invalidated all sub-

sequent cell counts.

MAIN EXPERIMENT

Harvested ascitic fluid containing tumour cells was adjusted with saline to contain 5 million cells/ml. Into each of 40 C_3H mice, 8-12 weeks old, 0.2 ml. of the fluid (1 million cells) was injected intraperitoneally. The mice were kept on a B41 diet, and six to a cage. At the same time, each day following the inoculation, four mice were given the Cr^{51} red cells, sacrificed 10 minutes later by cervical dislocation and the above tests were performed.

The whole experiment was repeated in the same colony six months later, and again six months after that, in order to assess the stability of the experimental model.

EXPERIMENTAL STUDY OF THE LATENCY PERIOD

A separate group of 20 mice were given whole body irradiation (550 r, 250 kv X-rays, known to suppress their immune responses) and subsequently transplanted and studied as above.

RESULTS

After inoculation of $1x10^6$ tumour cells, three phases of growth were noted similar to those described by Klein and Revesz (1953) and these are seen in fig.10 and table I.

Latency period:- For four days it was not possible to record a significant difference in total cell number, allowing for 1 million inoculated tumour cells and the average of 0.6 - 1.0 million cells which could be washed out of uninoculated mice using the same procedure.

Exponential period:- Between 4-8 days the cell numbers increased in simple exponential fashion with a doubling time of 18 hours. During this phase the peritoneal cavity became distended as a tight ball (fig.11), no Cr^{51} labelled red cells were detected gaining the circulation within 10 minutes and the mice were alive and in good shape. The ascitic fluid had lower albumin and higher paraprotein levels than the serum (fig. 12). Histological examination revealed no invasion of the peritoneal lining (fig. 5).

Terminal phase:- Usually after 8 days and always after 9 days a visible change occurred (fig. 11). The mice became listless, were now triangular in shape due to subcutaneous oedema and the tension was lost not only from the abdomen but also from the eyeball (noticed when bleeding the mice). The ascitic fluid was not often blood stained and Cr^{51} labelled red cells were rapidly detected in the retroorbital blood,

Table 1.

Total tumour cell counts (corrected by Cr^{51} red cell estimation of ascitic volume) following transplantation of 1 million MP 5563 cells.

Day	Millions of tumour cells	Mean	S.E.of Mean
1			
2			
3	1.2, 1.1,	1.15	
4	2.3, 1.2, 1.8, 1.9, 2.7, 3.4, 1.3, 1.7, 2.2, 4.0, 2.0, 2.5,	2.25	0.2
5	2.7, 5.4, 10.6, 6.8, 5.6, 5.8, 6.4, 5.6, 6.8, 6.9, 5.5, 6.2,	6.2	0.6
6	9.4, 19, 10,0, 10.5, 14, 21, 15, 18, 12, 14, 16, 18,	14.7	1.3
7	30, 19, 36, 39, 56, 59, 60, 110, 46, 52, 37, 48,	49	7.5
8	60, 74, 100, 160, 140, 72, 86, 98, 92, 88, 105, 110,	99	8.4
9	100, 106, 111, 131, 124, 151, 159, 95, 154, 108, 123, 137,	125	6.4
10	78, 68, 105, 100,	88	26.5

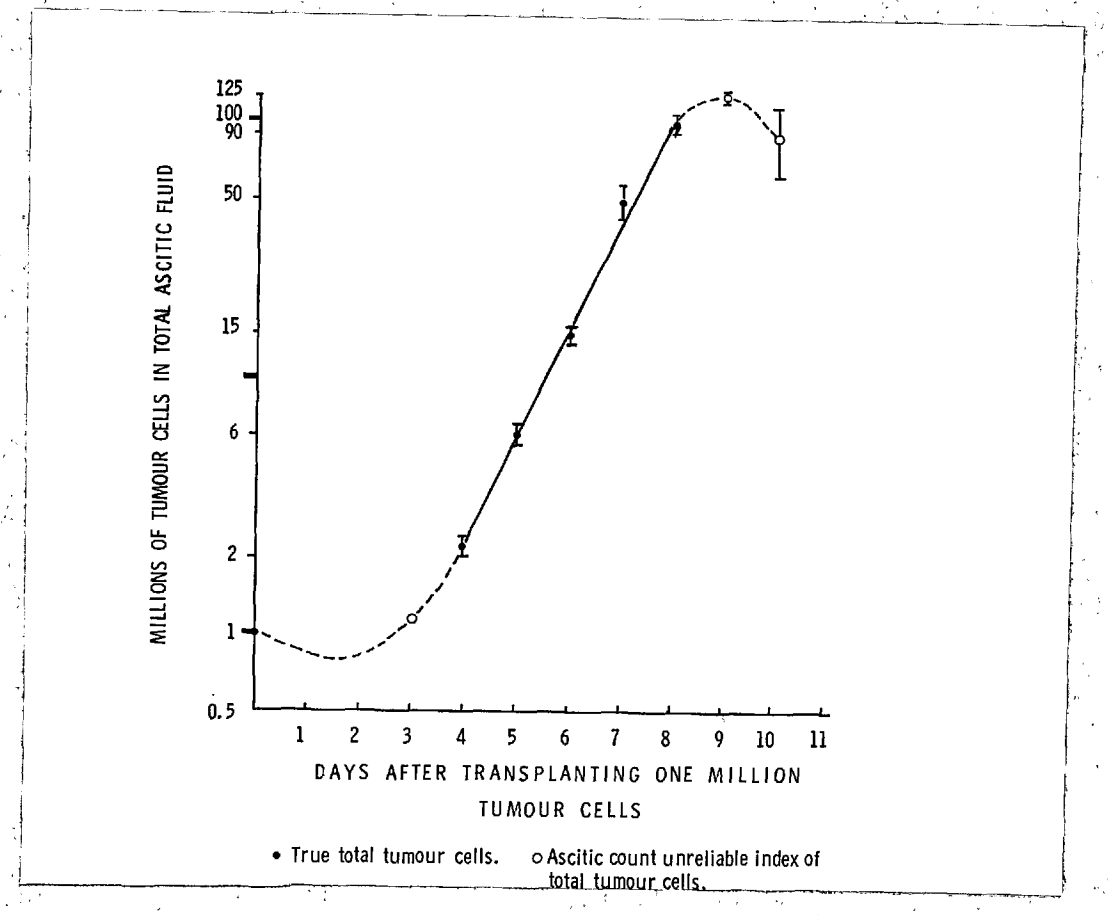


Fig. 10.

Tumour cell numbers over the days following transplantation of 1 million cells. If the exponential phase (4-8 days) is back-extrapolated it cuts the ordinate at 5000 cells, i.e. if only 5% of the transplant survived, growth would have been exponential up till day 8, whereafter fistulous communication invalidates further counts.

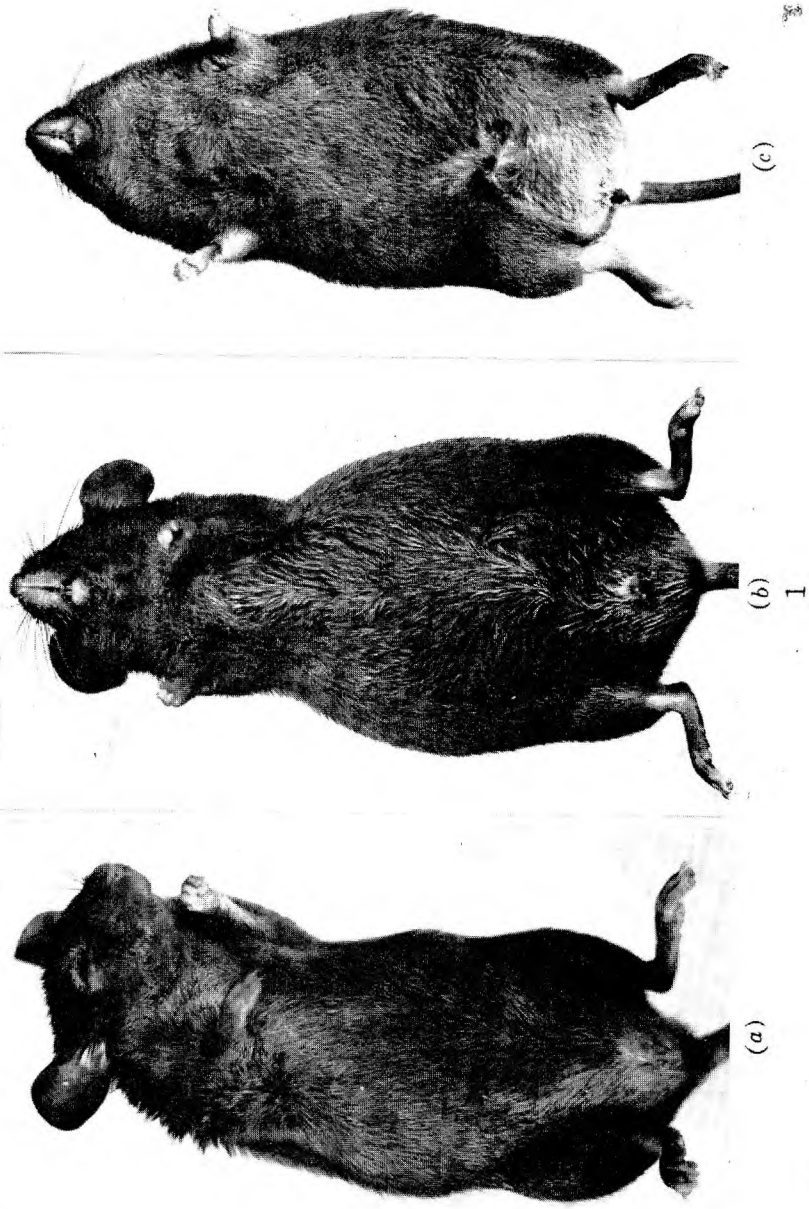


Fig. 11.

Inoculated mice during the three growth phases. (a) Latency period, day 3. (b) Exponential phase, day 8. (c) Terminal phase, day 9. Observe the tight ball of distension confined to the abdominal cavity during the exponential phase. This has gone in the terminal phase, the shape is more triangular with oedema extending up over the chest to the face.

indeed calculated as radioactivity /1gm. red cells, there was no difference between ascitic and systemic red cells at ten minutes. There was also no difference between the ascitic and serum levels of albumin or paraprotein (see fig. 12). Histology regularly showed invasion of the peritoneal wall and tumour cells were seen in clusters adherent to the lining (fig. 5). The mice usually died on the tenth day.

Stability of this experimental model:- There was no significant difference in latency period or growth rates at two six-months intervals, representing 25 and 50 passages of our initial sample of the tumour. This itself must have had some 600 passages since it was first found as a spontaneous tumour in 1956. Accordingly all our data have been pooled in fig. 10 . During the exponential phase the doubling time was 18 hours, and in all the individual experiments this only varied from 18-20 hours. However, it was observed that whereas 1 million cells were initially needed for a certain take, and an inoculum of 800,000 cells was usually a failure, by the end of the year a take could be achieved with 150,000 cells. At 18 months, inoculation had once been successful with only 20,000 cells. Despite this apparent increase in "virulence" of the tumour, the growth rate and protein production remained stable.

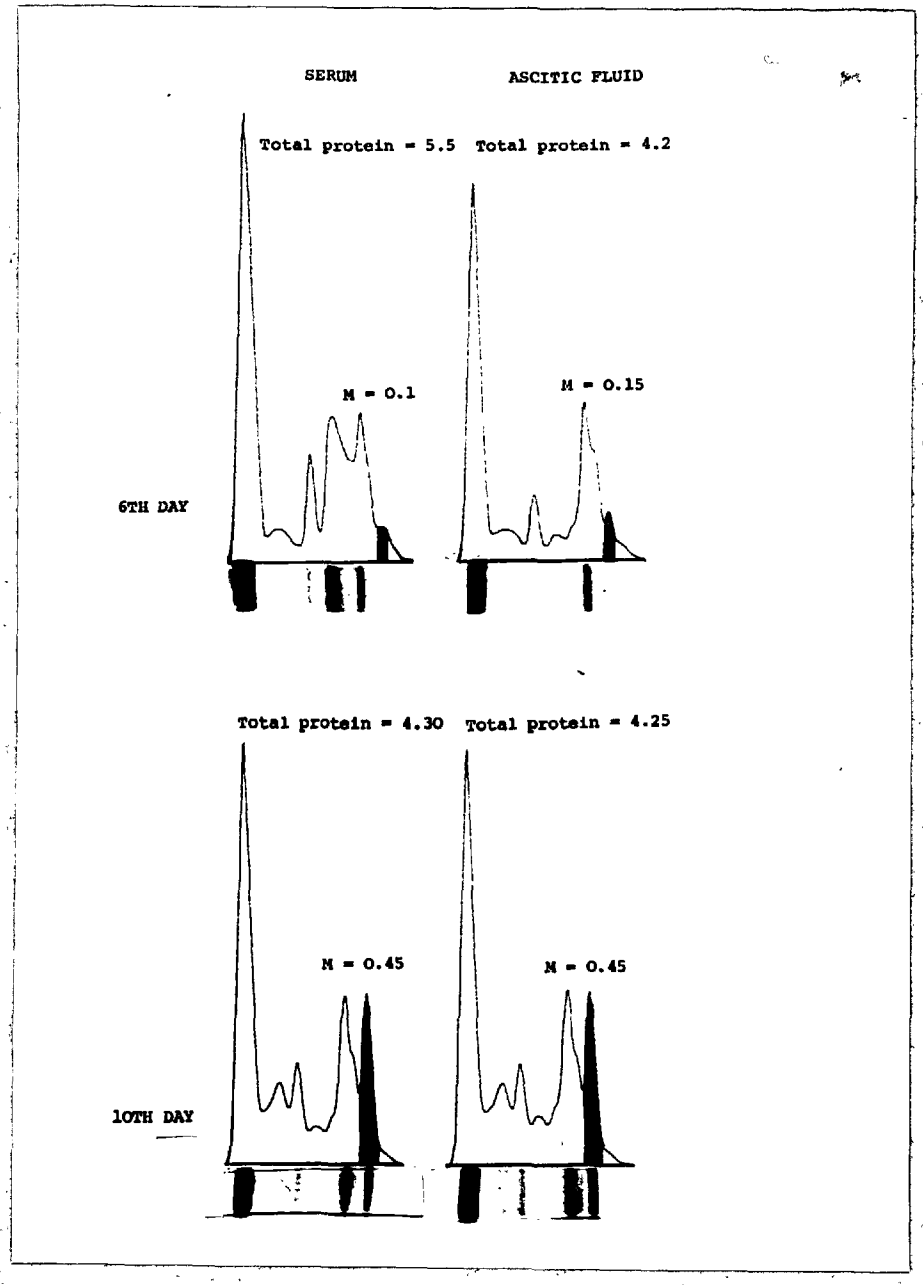


Fig. 12.

Electrophoresis of serum and ascitic fluid during exponential (above) and terminal (below) phases. Above, the difference in levels of total protein, large mol.wt. a_2-globulins and paraprotein indicates the relative integrity of the peritoneal membranes. Below equilibration of all levels confirms fistulous communication between the peritoneal cavity and the circulation.

(a) Total protein = 5.5g./100ml. M protein = 0.1g./100ml.
(b) Total protein = 4.2g./100ml. M protein = 0.15g./100ml.
(c) Total protein = 4.30g./100ml. M protein = 0.45g./100ml.
(d) Total protein = 4.25g./100ml. M protein = 0.45g./100ml.

DISCUSSION

During the exponential phase (4-8 days after inoculation) the evidence supports the integrity of the peritoneal cavity, and shows that ordinary attempts to recover all the tumour cells are likely to fall short by some 7%. Isotope dilution of Cr^{51} labelled isologous red blood cells overcame this problem in the present study and also clearly showed the development of a fistulous communication between the peritoneal cavity and the blood-stream during the terminal phase (9-10 days). This was also supported by the other evidence of change in shape, low blood pressure in the orbital plexus, loss of tension of the ascites and equilibration of cell and protein contents. Cell counts of ascitic fluid beyond the 8th day therefore no longer represented the total number of tumour cells.

The growth curve of this tumour is similar in many aspects to what other workers have shown. The latency period described by Klein and Revesz has been thought to be due to immunological resistance by the host. If this had been true, the tumour in the irradiated group should have grown without this phase and the number of cells would be 8-16 times that of the control group after a few days (doubling time 18 hours). This was not the case. Sparck (1969) has also shown that prior sublethal irradiation of the host far from enhancing subsequent tumour growth in

general impairs it. Similarly cortisone which can suppress the immunological reactivity of the host was also found not to affect the tumour growth. An alternative explanation for this period could be that only some 5% of the inoculated cells survived as viable, replicable cells and then simply grew exponentially. The inoculum usually does come from a spent host.

Simple exponential growth (4-8 days) can be explained by the binary division of cells and will occur in any closed population in which the generation time and the fraction of cells partaking in the divison process is constant (Frindel, Malaise, Alpen and Tubiana, 1967). It seems that the cell loss during this short growth period is very small, and that most cells undergo mitosis so that the doubling time is similar to the time of the complete mitotic cycle. The present observed time of 18 hours is identical to that found in other mouse tumours (Baserga (1963),; Frindel, et al (1967).

In the terminal phase (9-10 days) an asymptote is reached. There appears to be a complete equilibrium between the blood and ascites. This will lead to a major loss of the tumour cells into the blood stream. A relatively physiological limit was placed on the follow-up by the death of the host.

Other causes for the asymptote have been shown. Baserga, studying the Ehrlich ascites tumour claimed a constant mitotic

cycle of 18 hours, but found a progressive decline in the growth fraction. Lala and Patt studying the same tumour claimed that in addition there was a lengthening of the mitotic cycle to 22 hours, however their claim for an initial mitotic cycle of 8 hours is unconfirmed. Steel also showed the contribution of cell death with a cell loss fraction claimed to approach 95%. It is therefore possible that the use of terminal ascites for further inoculations may be followed by a great deal of cell death, and that this best accounts for the latency period.

SUMMARY

The growth of ascitic mouse plasmacytoma 5563 has been monitored by Cr^{51}-RBC dilutions within the peritoneal cavity together with differential cell counts and protein measurements.

Following the intraperitoneal transplantation of one million tumour cells, there is a latency period of 4 days. This is followed by logarithmic growth from 4-8 days with a doubling time of 18 hours, possibly explained as only a 5% survival of the inoculum, always similar in different animals. Eventually after 8 days and after about 100 million cells an asymptote is reached which is due to a fistula-like communication between blood stream and peritoneal cavity. This terminal phase (9-10 days) is rapidly followed by death of the mouse.

Reliable tumour cell counts can only be achieved between 4-8 days.

CHAPTER 8

THE SERUM PARAPROTEIN LEVEL RELATED TO THE NUMBER OF MP 5563 CELLS

Nathans, et al (1958) showed that the total daily production of paraprotein by a solid plasma cell tumour in mice was proportional to the weight of the tumour. Later, Osserman, et al (1964) showed that simple measurement of the serum level of the paraprotein was directly proportional t to the weight of tumour, up to about 2 g. Thereafter necrosis of the tumour probably accounted for a failure of the relationship.

The present study was made using an ascitic form of plasmacytoma in mice to see if the serum level could be directly related to the actual number of tumour cells; i.e. whether the serum level could be used to monitor the tumour growth.

RESULTS

The paraprotein was readily detected by serum electrophoresis as a narrow band just behind transferrin (β_1-globulin), see fig.12. This was measured ($\pm$ 0.05 g/100 ml.) as described in Chapter 5.

The paraprotein was first seen in the serum when the ascitic fluid contained 3 million plasma cells. The level

of the protein in the serum increased as the tumour grew, showing a simple linear relationship (fig. 13). The paraprotein level still showed this relationship even though the total serum protein levels often fell. In control mice the total protein values ranged from 5.3-6.5 g/100 ml. In inoculated mice with 5-120 million cells the total protein levels ranged from 3.8-6.2 g/100 ml. Above 120 million cells, total proteins fell to 2.5-4.5 g/100 ml.

It is worth recording here that two ascitic plasma cell tumours in BALB/C mice were initially studied. It was found for both these tumours (PC5, PC6) that the paraprotein production fell off with subsequent transplants. There also occurred a shortening of the time (from 16 to 9 days) in which the transplant killed a mouse, with an increase in the amount of haemorrhage into the ascitic fluid. The reduction of paraprotein synthesising capacity was paralleled by an increase in the aggressive behaviour of the tumour; biochemical dedifferentiation paralleled neoplastic dedifferentiation.

Furthermore it was possible to grow the C_3H tumour MP 5563 in the BALB/C strain after challenge with a high dose (over 5 million cells) but the tumour failed to produce protein in the strange host.

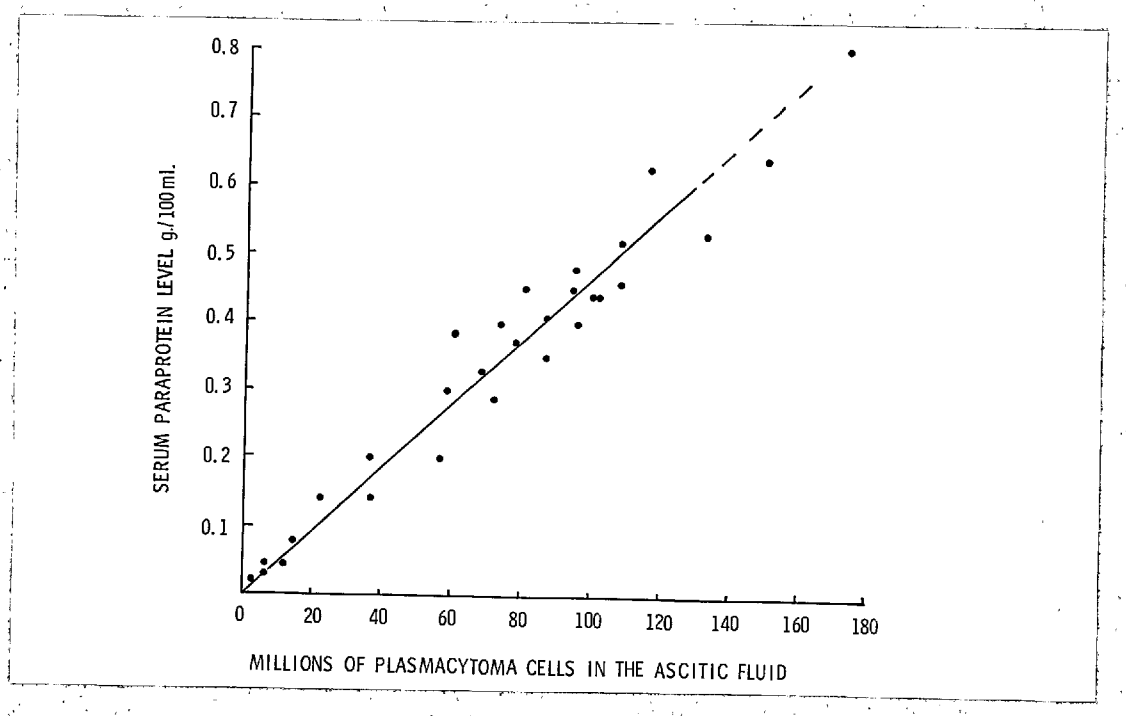


Fig. 13.

The serum paraprotein level is directly proportional to the number of tumour cells between 4 and 8 days after transplantation. Cell counts are unreliable before and after this period.

DISCUSSION

the measurement of tumour growth is essential in the assessment of anti-tumour agents and the serum level of paraprotein has proved useful for this purpose (Rosenoer and Whisson, 1964).

The stable ascitic form of the present tumour and the present isotope dilution method enabled an accurate count of the total number of tumour cells. The extent of growth of this tumour up to 120 million cells is reflected in the serum by the level of the paraprotein it is producing. Few mice survived after 120 million tumour cells were reached, but it could be predicted that above these numbers the serum paraprotein level would no longer simply relate to the number of tumour cells because of the late formation of a fistulous-like communication between the peritoneal cavity and the blood stream. This could also account for the late fall in the level of the serum total protein which was observed in some mice. Earlier falls in serum total protein could be expected simply by transudation into the ascitic fluid but this did not significantly effect the serum level of the paraprotein itself.

In a 70 kg. man it was estimated that the earliest detection of paraprotein in the serum would occur with some 43 g. of myeloma or 9,000 million cells (Hobbs, 1967). In 22-25 g. C_3H mice it was observed that the earliest detection

of paraprotein occurred at 3 million cells (see fig. 13). Since the mouse is about 1/3000th of the weight of the man this experimental observation supports the above estimate.

SUMMARY

An ascitic form of plasmacytoma (MP 5563) in C_3H mice has proved stable on transplantation. A simple linear relationship was demonstrated between the serum level of the paraprotein produced and the total number of plasmacytoma cells within the ascitic fluid.

CHAPTER 9

THE EFFECT OF PRIOR IN VITRO INCUBATION WITH IMMUNE RAT LYMPH ON SUBSEQUENT TRANSPLANTATION OF MP 5563

A method using prior in vitro exposure of the tumour MP 5563 to immune components offered full access of antibodies and/or competent immune cells to the tumour cells. Their effect on the tumour could be monitored in two ways, subsequent transplantation in C_3H mice and, where only antibodies were used, by trypan blue exclusion study of the exposed tumour cells.

METHODS AND RESULTS

Fresh harvested tumour cells were always used. They were gently centrifuged, the supernatant was discarded and the cells were resuspended in saline to contain 5 million cells per ml. By this stage of this study it was known that transplantation of 100,000 (10^5) of these tumour cells would always take, and kill a C_3H mouse within 14 days.

Incubations were done in sterile siliconized glass universals at room temperature. The lymph used was fresh and always from the first 4-6 hours post-cannulation output, collected at the presumed optimum time following immunization (Delorme, et al, 1969).

Experiment 1: The effect of whole lymph fluid:

In this experiment 10^6 tumour cells in 0.2 ml. saline were incubated for 10 hours with 0.8 ml. whole lymph which contained $40x10^6$ lymphocytes per ml. freshly collected from an immunized rat. Similarly tumour cells were incubated with whole lymph from an unimmunized rat. At the same time the same number of tumour cells were incubated with 0.8 ml. normal saline in a similar bottle as control. Then 0.1 ml. of the mixture from each bottle, containing 10^5 tumour cells, was transplanted into each of groups of 5 mice.

Table 2

Results of experiment 1, in vitro effect of immune rat thoracic duct lymph against MP 5563

INCUBATION WITH	Saline control	Thoracic duct lymph from	
		Normal rat	Immunized rat
SURVIVAL	All dead within 14 days	All dead within 14 days	All alive at 6 months

This showed that thoracic duct lymph from an immunized rat was effective in vitro against MP 5563.

Experiment 2: The effect of washed lymphocytes or cell-free lymph.

In this experiment 10^6 tumour cells in 0.2 ml. saline were tramsferred into each of 4 bottles (A,B,C,D) to which the following additions were made:

A) 0.8 ml. saline

b) 0.8 ml. of whole lymph fluid containing $250x10^6$ lymphocytes from an immunized rat

c) 0.8 ml. of lymphocyte-free lymph from an immunized rat

d) $250x10^6$ lymphocytes from an immunized rat

they were washed and resuspended in 0.8 ml. normal saline. After 10 hours incubation, 0.1 ml. from each bottle (10^5 tumour cells) was transplanted into each of groups of 5 mice.

Table 3

Results of experiment 2, the in vitro effect of immune lymph components against MP 5563

INCUBATION WITH	Saline control	Immune rat lymph		
		Whole lymph	Cell free lymph	Washed voable lymphocytes 250 per tumour cell
	A	B	C	D
SURVIVAL	All dead within 14 days	All alive at 5 months	All alive at 5 months	All dead within 15 days

This showed that the effectiveness of immune lymph resided in the cell-free supernatant.

Experiment 3: Determination of the effective fraction after separation through Sephadex G200.

12 ml. of cell-free lymph from an immunized rat was applied to a Sephadex G200 column. The three main peaks were collected and concentrated by vacuum ultrafiltration down to the original volume of the lymph. The fractions were then dialysed against phosphate buffered saline for 48 hours at $4^{\circ}C$ and finally sterilized by millipore filtration. Into 5 bottles (E,F,G,H,I) 10^6 tumour cells in 0.2 ml. saline were transferred and the following additions were made:

E) 0.8 ml. normal saline

F) 0.8 ml. fraction 19s

G1) 0.8 ml. fraction 7s

H) 0.8 ml. fraction 4s

I) 0.8 ml. whole lymph

Subsequently bottle G2 was set up using 0.8 ml. of fraction 7s which had been recycled through Sephadex G200 to free it from the originally contaminating tail of the 19s fraction.

Each bottle was then incubated for 10 hours at room temperature, and thereafter 0.1 ml. (10^6 tumour cells) was inoculated, using groups of 5 mice for each bottle.

Table 4

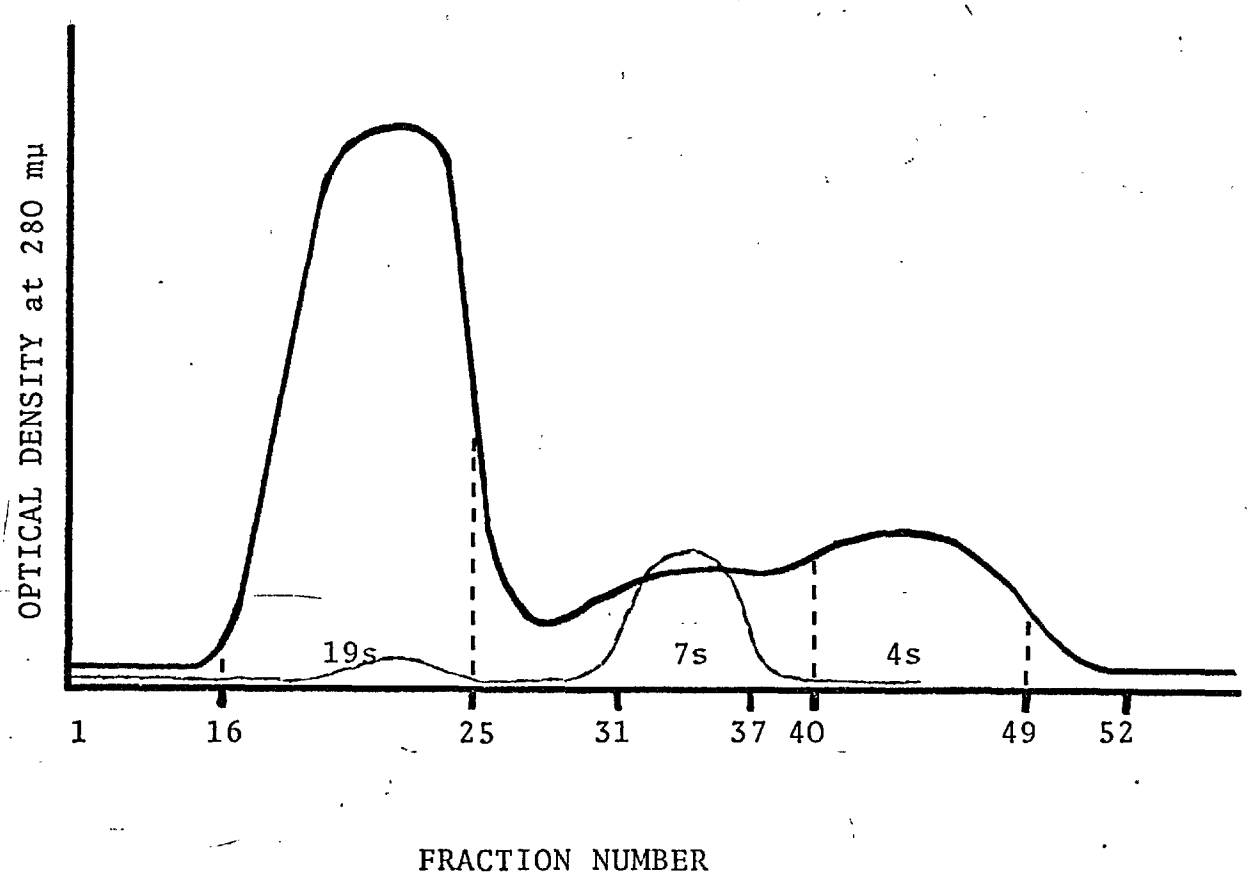
Results of experiment 3, the fraction of cell-free lymph effective in vitro against MD 5563

	Sephadex G200 fraction of immune rat lymph					
INCUBATED WITH	Saline control E	19s F	First 7s (+ trail 19s) G1	Second 7s (recycled no 19s) G2	4s H	Whole lymph I
SURVIVAL	All dead within 14 days	All alive at 4 months	3/5 mice developed the tumour and died at 22,23 & 35 days. 2/5 alive at 4 months	All dead within 14 days	All dead within 14 days	All alive at 4 months

This showed that the effectiveness of cell-free lymph depended on its 19s fraction. The recycling of the first 7s fraction which appeared partially effective, removed contaminating 19s and showed that pure 7s alone was ineffective. Furthermore there was no evidence that the pure 7s fraction enhanced the tumour growth, in that these ascites developed at the usual rate, and the mice died at the same time as the saline controls.

Experiment 4: Need for complement in the action of 19s fraction from immune lymph.

Into 3 bottles (J,K,L) 10^6 tumour cells in 0.2 saline were transferred and the following additions were made:



(Fig. 13a)

Chromatograph of the first run (——) of rat lymph through Sephadex G-200. The 3 peaks correspond to 19s, 7s and 4s proteins. Subsequently fractions 31-37 were rerun,(———) because of presumed tailing of the 19s into them, and only the second fractions 32-35 were used as the pure 7s for bottle G2.

J) 0.8 ml. normal saline

K) 0.8 ml. 19s fraction

L) 0.8 ml. 19s fraction which had been previously heated at 56°C for 30 minutes. (Some complement is contained in the front peak from Sephadex G200).

Each bottle was left at room temperature for 10 hours and thereafter 0.1 ml. (10^5 tumour cells) was inoculated, using groups of 5 mice for each bottle.

Table 5

Results of experiment 4, the need for complement

		19s fraction of immune lymph	
INCUBATED WITH	Saline control J	Unheated K	Previously heated at 56°C for 30 min. L
SURVIVAL	All dead within 14 days	All alive at 5 months	All dead within 14 days

This showed that inactivation of complement prevented the action of the 19s fraction.

Experiment 5: Target cells of the 19s fraction.

A suspension of normal C_3H mouse cells was obtained in two ways: (M) after adding dextran to fresh heparnised blood collected from C_3H mice it was possible to harvest a white cell rich supernatant, count the cells and adjust to 10^6 cells per 0.2 ml. saline (O) peritoneal washouts with sterile saline had already (Chapter 7) been shown to contain epithelial cells and macrophages and these could also be adjusted to 10^6 cells per 0.2 ml. saline. Accordingly bottles (M,O,P,Q,R,S) were set up containing 0.2 ml. of cell suspension, and the following additions made:

M) 0.2 ml. normal C_3H white cells + 0.8 ml. normal saline

O) 0.2 ml. normal C_3H white cells + 0.8 ml. 19s fraction

P) 0.2 ml. normal C_3H peritoneal cells + 0.8 ml. normal saline

Q) 0.2 ml. normal C_3H peritoneal cells + 0.8 ml. 19s fraction

R) 0.2 ml. MP 5563 cells + 0.8 ml. normal saline

S) 0.2 ml. MP 5563 cells + 0.8 ml. 19s fraction

After 10 hours at room temperature, trypan blue was added to each bottle and then a count of remaining viable cells was made.

Table 6

Results of experiment 5, susceptibility of normal C_3H cells and MP 5563 cells to immune rat 19s fraction

	Normal C_3H white blood cells	Normal C_3H peritoneal cells	MP 5563 cells
+ Normal saline	M	P	R
% Viable	98%	94%	64%
Viable cells in 0.2 ml.	9.8×10^5	9.4×10^5	6.4×10^5
+ 19s fraction	O	Q	S
Viable cells in 0.2 ml.	1.4×10^5	1.5×10^5	$< 1 \times 10^3$
% Kill due to 19s fraction	ca.86%	ca. 84%	ca.100%

This shows that the immune rat 19s fraction is directed not only against the tumour MP 5563 cells but also against the normal cells of C_3H mice. It does appear however to be more effective against the former.

Experiment 6: The effect of a larger inoculation of MP 5563 following in vitro treatment with rat 19s fraction.

Experiment 3 (F) was repeated, but using $5x10^6$ tumour cells in a bottle and then inoculating 1 million tumour cells per mouse. With this tenfold increase in tumour cell dose, still no mice developed the tumour.

Experiment 7: The effect of dilution of the 19s fraction.

In order to see if the concentration of 19s fraction was critical in experiment 6, experiment 3(F+I) was repeated using $1x10^6$ tumour cells in a bottle, but adding 0.8 ml. of 20% strength 19s fraction or whole lymph. With this five-fold reduction in immune lymph, still no mice developed the tumour..

CONCLUSIONS

Immunisation of rats with mainly live but irreproducible MP 5563 cells has resulted in a clear difference between thoracic duct lymph from immune rats compared to unimmunised rats. Prior exposure in vitro to this immune lymph could completely prevent the usual take of MP 5563 cells. This effect was shown to reside in the cell-free supernatant, was confined to its 19s fraction and required complement for its action. The lymph was very potent - 0.16 ml. preventing the take of 1 million tumour cells. The 19s fraction was not entirely tumour-specific also being lethal to normal C_3H mouse cells. Whether or not the differential effect of experiment 5 (100% lethal to MP 5563 cells, 85% lethal to normal cells) might be exploited by varying the dose in vivo is studied in the next chapter.

CHAPTER 10

THE IN VIVO EFFECT OF IMMUNE RAT LYMPH ON MP 5563 IN C_3H MICE

Some of these experiments were in fact begun before those in chapter 9. However for the sake of clarity the in vivo experiments are collected under one title.

METHODS AND RESULTS

A large batch of C_3H mice were taken and with some being used as controls, the remainder were each inoculated with 2.5×10^5 tumour cells. (It was later shown that 1×10^5 cells would take). Thereafter, at different stages (0-5days) of development of the tumour, different forms of immunotherapy were attempted.

It was therefore not possible to use the same batch of immunized rat lymph (for days 0 - 5 after transplantation) 46 rats immunized and boosted 2-3 days previously were cannulated. The thoracic duct lymph collected was used for treatment of mice at day 0, 1 and 2. After tumour transplantation the booster dose of immunization was given to another group of 3 rats at day 0. These were cannulated at day 2, and thoracic duct lymph was collected for 3 days and used as treatment for days 3, 4 and 5 after tumour transplantation. Aliquots of the whole lymph used in all these in vivo experiments were saved and subsequently using experiment 5 of chapter 9, each shown to be

effective in vitro at the doses used.

Controls

Throughout the following experiments the following controls were used:-

a) Tumour cells in saline without any other addition were inoculated to check the viability and take of the tumour being used. In every case the tumour took.

b) Whole thoracic duct lymph or washed lymphocytes from unimmunized litter mates of the immunized rats were used to screen for any natural immune reaction. At no time was such a natural reaction detected. Furthermore the toxic effect of lymph noted by Alexander (1968) was not seen in any of these experiments.

c) Mice not inoculated with tumour were given the lymph (or its products) from immunized rats to check for any obvious side-reactions against the normal tissues of the mouse. At single doses up to 2.0 ml. of whole lymph, or 10^8 lymphocytes no side-reactions were observed in the mice. Alexander (1968) did stress it was protracted dosage that had had bad results. No evidence of rat graft versus mouse host reaction was seen up to 3 months following

the treatment.

d) Where rat lymphocytes were washed prior to injection, an aliquot was checked for viability by examining for trypan blue exclusion. In all such cases over 95% of the cells appeared viable.

To avoid needless repetition only controls (a) are shown in the tables which follow.

All the animals transplanted with the tumour and given the treatments in vivo developed the tumour and died as a result of its growth. The following tables below show the duration of survival (in days since transplantation) of each individual animal in the different experiments.

Treatment (1). The effect of washed immune rat lymphocytes given as a single intraperitoneal dose.

Table 7

A. Using 25×10^6 rat lymphocytes

	Controls (a)	Day after transplantation that treatment was given					
		0	1	2	3	4	5
SURVIVAL	10	11	10	9	10	10	10
in	10	10	10	10	11	11	10
days	10	12	10	10	11	13	11
	12	15	12	12	11	14	11
	14	16	12	12	11	14	12

Table 8

B. Using 50 x 10^6 rat lymphocytes

	Controls (a)	Day after transplantation that treatment was given					
		0	1	2	3	4	5
	9	11	10	11	10	10	10
SURVIVAL	10	11	11	12	11	10	11
in	10	13	12	14	11	11	11
days	12	13	12	15	12	12	12
	13	13	13	16	13	13	12

Table 9

C. Using 100 x 10^6 rat lymphocytes

	Controls (a)	Day after transplantation that treatment was given					
		0	1	2	3	5	5
	10	12	12	12	9	10	11
SURVIVAL	12	13	12	13	10	10	11
in	12	13	13	15	11	10	11
days	13	14	14	15	12	12	12
	13	14	23	15	13	12	13

It can be seen intraperitoneal washed rat lymphocytes up to doses of 100x10^6 (i.e. 400 per tumour cell) have no

significant effect on survival.

Treatment (ii). The effect of washed immune rat lymphocytes given as a single intravenous dose.

Because intraperitoneal lymphocytes might not pass on any potential transfer substance, rat lymphocytes were also tried intravenously. This would give the best possible chance of any such transfer to host cells in the bone marrow, spleen or lymph nodes.

Table 10

Using 50 x 10^6 rat lymphocytes

	Controls (a)	Day after transplantation 0	2
SURVIVAL in days	9	11	10
	11	12	11
	11	12	11
	14	12	11
	12	13	13

At this stage of the work there had been complete failure of in vivo therapy using washed viable lymphocytes (from immunized rats at a suitable time) in large doses

from within half an hour after tumour transplantation. Washed lymphocytes had been used in the classical manner of Alexander's group, in case of any direct toxic effect of whole lymph. However it seemed possible some cofactor (s) might be essential for the rat lymphocytes to express any activity. The work then proceeded as in chapter 9, ensuring intimate contact of lymph components and tumour cells by an in vitro phase. When it had been established that any activity resided in the 19s fraction, the following in vivo experiments were done. Just in case some other undiscovered combination of cellular and humoral components was needed, the effect of whole lymph was compared to that of an equivalent amount of 19s fraction.

Treatment (iii). The effect of immune 19s fraction.

Four groups (control, T,U,V) of 5 mice were inoculated with 10^5 tumour cells. (T) was given 0.5 ml. of 19s fraction intraperitoneally 30 minutes later. (U) received 0.5 ml. doses of 19s fraction at 30 minutes 1 day, 2 days and 3 days after inoculation. (V) was given 0.5 ml. of 19s fraction at 6 days, 7 days and 8 days after inoculation.

Table 11

Results of 0.5 ml. rat 19s fraction given at intervals after inoculation of MP 5563

	Controls (a)	at 30 mins. T	at 30 mins. and on days 1,2,3. U	on days 6,7,8. V
SURVIVAL in days	13	20	20	15
	13	23	21	16
	14	24	25	16
	15	25	25	16
	15	26	26	17

There is little effect after that of the dose given at 30 minutes.

Treatment (iv). The effect of whole immune rat lymph.

Treatment (iii) was repeated using 0.5 ml. whole lymph instead of the 19s fraction. Each dose contained some $10\text{-}30 \times 10^6$ lymphocytes , the first doses being the highest content.

Table 12

Results of 0.5 ml. whole immune rat lymph given at various intervals after inoculation of MP 5563

	Control (a)	at 30 mins. Tw	at 30 mins.& on days 1, 2,3 Uw	on days 6,7,8 Vw
SURVIVAL in days	13	21	19	14
	14	23	22	16
	14	23	23	16
	14	25	24	16
	15	26	25	16

These results are not significantly different from those in table (iii) so that the effect can all be attributed to the 19s fraction.

CONCLUSIONS

Throughout the treatment, the controls revealed that (a) 1 or 2.5×10^5 tumour MP 5563 cells were regularly lethal to C_3H mice within 14 days (b) rat lymph from unimmunized litter mates was without effect (c) in the doses given the immune components showed no obvious effect on whole mice (although 19s fraction could kill normal C_3H mouse cells in vitro - (see chapter 9) (d) the rat lymphocytes

appeared viable and (e) aliquots of the immune lymph that had been used in vivo were effective when subsequently tested in vitro.

From these controls and the results in tables 7 - 12 it can be concluded that the effect of immune rat lymph is confined solely to its 19s cell-free fraction, presumably in concert with complement. With the guaranteed contact of prior in vitro incubation this 19s fraction can completely eliminate the successful take of MP 5563 (0.16 ml. neutralising 1 million cells). In vivo a dose rate 31 times higher (0.5 ml./10^5 cells) distributed intraperitoneally can only prolong survival and not effect a cure. It is a chastening concept to tumour therapy that the 99.976% kill calculated from fig.14 is not enough to cure, the surviving 0.024% growing on to kill the mouse. Even this effect seems largely confined to the first dose given at 30 minutes after tumour transplantation, subsequent doses prolonging survival no better.

It may be that this early dose of the rat 19s fraction is largely used up by the normal tissues of the mouse before it can reach all the tumour cells. Accordingly larger doses have not been given for fear of side-effects. With later doses, either well established tumour may have acquired a protective coating of some kind (e.g. enhancing antibodies) or, with a series of doses, mouse antibodies may have arisen to neutralise the rat 19s fraction.

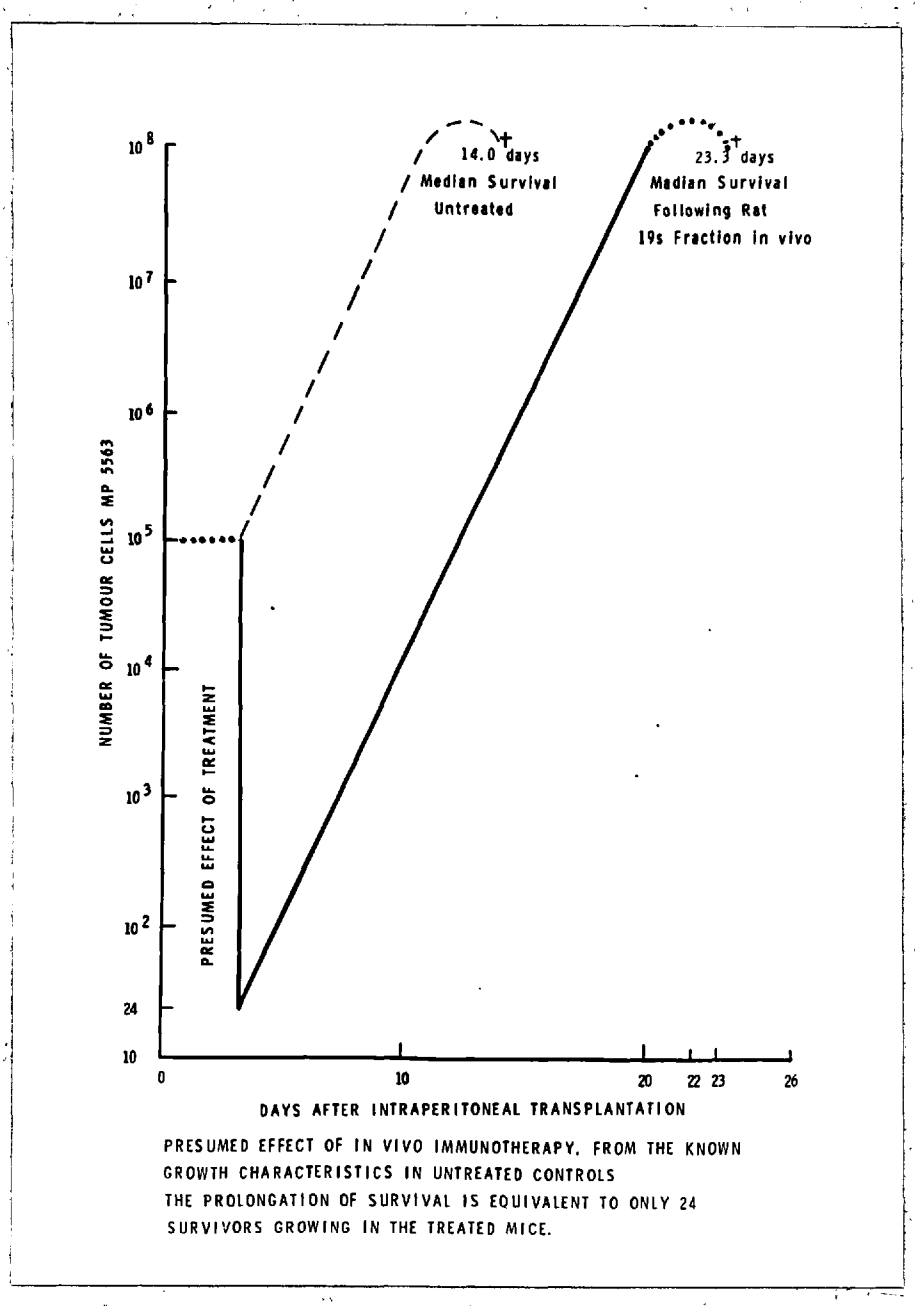

14.0 days
Median Survival
Untreated
23.3 days
Median Survival
Following Rat
19s Fraction in vivo
NUMBER OF TUMOUR CELLS MP 5563
PRESUMED EFFECT OF TREATMENT
DAYS AFTER INTRAPERITONEAL TRANSPLANTATION
PRESUMED EFFECT OF IN VIVO IMMUNOTHERAPY. FROM THE KNOWN
GROWTH CHARACTERISTICS IN UNTREATED CONTROLS
THE PROLONGATION OF SURVIVAL IS EQUIVALENT TO ONLY 24
SURVIVORS GROWING IN THE TREATED MICE.

Fig. 14.

CHAPTER 11

GENERAL DISCUSSION

Before discussing the results of this work in detail, it is relevant to review the information obtainable from such studies and its possible uses. A fundamental study of the mode of growth and monitoring of plasmacytoma, and the possible application of immunotherapy to this kind of tumour could have a bearing on human myelomatosis and its further study in a centre where other work on this disease is carried out. It is also hoped to emphasize those areas where further knowledge is required.

The rate of growth of a tumour depends at the cellular level on the cell cycle time, the growth fraction, the degree of differentiation and the rate of cell loss. The fastest growing tumour would be one having a short cell cycle time, a growth fraction of unity and no cell loss. Such a situation is rarely found, except in the early stages of growth of ascites tumours (Lala and Patt, 1966; Frindel et al, 1967), and this might account for the differences between our experimental model and human myeloma, which grows much more slowly. This difference may be caused by a longer cell cycle time, a smaller growth fraction, some loss of cells from tumour volume or all these. The growth rate of a tumour is important clinically since it

may determine the time at which symptoms appear, and also the life span of the patient. It may also determine what kind of protracted course of treatment can be used.

The paraprotein produced by a plasmacytoma can often be measured in the serum and has offered a reliable yardstick of the progress of the tumours. Since monitoring the growth of tumours has long been an obstacle to assessing the effectiveness of antitumour agents, the present validation of such measurements of the paraprotein level in the serum could provide the cancer therapist with a useful tool to study the effectiveness of the treatment of myelomatosis.

The available methods of treatment of human myelomatosis, as with other types of cancer, do not produce a cure. They include the use of cortisone and melphalan or other chemotherapeutic agents with or without radiotherapy. With the diagnosis made at the average clinical presentation, the expected longevity of the patients with older treatments was 18 months. (Hobbs, 1969). With the intensive chemotherapy used in the M.R.C. myeloma trial this at best can be extended by only 2-5 years. The search for a more effective method of treatment is obviously needed.

It is wellknown that while patients with myelomatosis often have defective humoral antibody formation, their cellular immune mechanisms appear to be intact. If it were

possible to raise a colony of foreign lymphocytes capable of specifically rejecting the myeloma cells, it is likely that within two weeks or so of their administration such donor lymphocytes would be rejected by the host. It would therefore be essential to know in quantitative terms how many donor lymphocytes would be needed to reject a given estimate of tumour. Any alternative use of antibodies should be similarly studied.

Monitoring plasmacytoma

The serum paraprotein level was shown to be directly related to the actual number of plasmacytoma cells. This is probably the first time that the product of a tumour has been directly related to the actually counted number of tumour cells in a living animal. The stable ascitic form of this tumour and the use of the isotope dilution method enabled an accurate count of the total number of tumour cells. The extent of growth of this tumour up to 120 million cells was reflected in the serum by the level of the paraprotein it was producing. Few mice survived after 120 million tumour cells were reached, but it could be predicted that above these numbers the serum paraprotein level would no longer simply relate to the number of tumour cells because of the late formation of a fistulous like communication between the peritoneal cavity and the blood

stream. This could also account for the late fall in the level of the serum total protein which was observed in some mice. Earlier falls in serum total protein could be expected simply by transudation into ascitic fluid, but this did not significantly effect the serum level of the paraprotein itself.

In a 70 kg. man it was estimated that the earliest detection of paraprotein in the serum would occur with some 43 gm. of myeloma or 9000 million cells (Hobbs, 1967). In 22-25 gm. C_3H mice it was observed that the earliest detection of paraprotein occurred at 3 million cells. Since the mouse is about 1/3000 of the weight of the man, this experimental observation supported the above estimates (Hobbs, 1969).

It therefore seemed that the paraprotein level could be used to monitor plasmacytome level. However, the experience with the present studies of PC5 and PC6 in BALB/C mice (p. 107) did show that this relationship was only valid provided the tumour did not show further biochemical dedifferentiation. Indeed three human patients have since been encountered who showed the same loss of paraprotein production (biochemical dedifferentiation) as their tumours in the same manner changed to more rapidly growing and more frankly invasive tumours, resembling reticulosarcoma (malignant dedifferentiation). Such a

change has however only been seen in a minority (3/500) of patients with myelomatosis, and in them may have been induced by prolonged chemotherapy.

The immunological approaches to plasmacytome therapy

Immunization was done by injecting the tumour cells subcutaneously and intraperitoneally, below the diaphram so that the lymph from the lower end of the thoracic duct should offer a good harvest.

The dose ($30x10^6$) of tumour cells used for immunization was chosen so as not to result in immune tolerance. In one experiment, the rats had been given $1000x10^6$ tumour cells in the same manner, and when the rats were opened for cannulation, tumour foci were seen infiltrating many organs of the body like kidneys, liver and intestine. A dose of that order had been high enough to render the rats tolerant to the mouse tumour.

Immunoblasts were shown to increase significantly by day 3 after immunization (Delorme et al, 1969), and by day 8-10 the percentage of immunoblasts returns to pre-immunization levels. Cannulations were therefore done in the period where the immunoblasts were expected at the highest levels, and only for three days. This period was enough to exhaust the rat of lymphocytes, the output being greatly reduced thereafter.

During cannulation, a nylon cannula was first used. This was unsatisfactory because of its rigidity for small movements of the animal dislodged it from the thoracic duct. It was therefore decided to use the type of cannula shown on p. which had two advantages:-

(a) the penetration of the duct by the hard and sharp head.

(b) the soft part which acted as a shock absorbent.

Vit. K was given to prevent hypoprothrombinaemia which develops in rats with chronic thoracic duct fistulae and which can cause blood to appear in the lymph (Mann, Mann and Bollman, 1949), particularly when kept on glucose saline alone. The latter is used to promote the flow of the lymph. The lymphocyte output is nearly doubled when the rat is kept on salt solution compared to when kept on water. The volume of the lymph is increased 5 - 10 times, with a lowering of its protein content, (Shrewsbury and Reinhardt, 1952), so that clotting of the lymph in the cannula rarely occurred. Anticoagulants were not needed, this being fortunate for doses adequate to prevent coagulation of the lymph were also prone to cause post-operative bleeding.

In the experiments performed, the number of tumour cells transplanted were kept to a minimum level in order to increase the rat lymphocyte/tumour cell ratio to its

highest level. Even if as suggested only 5% of the transplanted tumour cells grew, the other 95% would still have used up immunoblasts since they still had immunological determinants on their surface. Thus, on day 0, the administration of 25, 50 and 100 million lymphocytes corresponded to 100,200 and 400 lymphocytes per tumour cell. Assuming 8% of these lymphocytes were immunoblasts (collected at day 3 after the booster dose) then this would correspond to 8, 16 and 32 immunoblasts per tumour cell respectively. In that dose range they should destroy the tumour, if they are going to be effective, for in some experimental models as little as only one immunoblast was shown to be needed to destroy each tumour cell (Perlmann and Holm, 1969). In spite of the present experimental design offering the best chance of success, no effect was observed.

If the mouse's own immune mechanisms had reacted against the tumour, and this reaction had been of a delayed hypersensitivity type, then synergism could have been shown between the administered lymphocytes and the mouse's own lymphocytes at any period of the 0-5 days during which the treatments were tried. None was observed, and further evidence that there was no such reaction can be deduced from the experiments showing mice given irradiation of 550r showed the same latent period and doubling time as the non-irradiated mice.

Established tumours had been proved not to respond to immunotherapy (Alexander et al, 1967), so that treatment of the tumour more than 5 days after transplantation was not attempted. Since Alexander et al, believe that the effect of lymphocytes need not be a direct effect, but can be due to an indirect effect initiated by the donor lymphocytes and completed by the host, intravenous injections were tried. These also proved to be ineffective.

What accounts for this failure is unknown. Different workers have found different kinds of immunological reactions to the tumours in the models they used. Lymphocytes, or their RNA, were proved by Alexander's group to be effective against chemically-induced (3.4 benzpyrene) tumours in rats. In other systems (Brunner, Mauel, Cerrotini and Chapuis, 1968), lymphocytes were prevented from acting on target cells by the presence of enhancing antibody. This is unlikely to be the case in our system, since the lymphocytes were still not effective even when previously mixed and injected with tumour cells which had been kept in tissue culture before enhancing antibody could develop and find its way onto the tumour cells. In still other systems particularly in Leukaemia, Burkitt lymphoma and melanoma in man, and in virally-induced leukaemia or lymphomas in experimental

animals, antibodies directed against the tumour specific transplantation antigens have been proved to be cytotoxic.

The ineffectiveness of the washed rat lymphocytes alone to affect the tumour led us to search for an opsonizing factor (like that described by McLennan, Loewi and Howard, 1969), which might act in conjunction with the lymphocytes. Due to the complexity of in vivo reactions and the possible involvement of unknown factors, studies were first made under in vitro conditions. A critical analysis of the cytotoxicity which was found offered a possibility of applying it as an in vivo treatment.

The cytotoxic effect found was shown to be mediated only by the 19s fraction which presumably contained IgM antibodies. This reaction was inactivated by heating to 56^{o}C for 30 minutes and presumably involved the fixation of complement. This would be expected for IgM antibodies.

The partial cytotoxic effect of the first 7s fraction was shown by recycling to be due to the presence of some 19s fraction (tailing from the front peak). It could have been that some cytotoxic effect had switched over from a primary 19s IgM response to a secondary 7s IgG one, as the rats were given more than one dose of immunization. However, the effect was abolished when the 7s fraction was recycled through the column and freed from

any contamination from the 19s fraction.

The possible application of the 19s fraction in therapy of the plasmacytoma was considered, and there is an interesting anomaly between the in vitro and in vivo results. The prior in vitro exposure of MP 5563 to 19s fraction completely prevented subsequent tumour growth. Use of 19s fraction in vivo, even only 30 minutes after intraperitoneal inoculation of MP 5563, could not achieve this. The calculations in fig. 14, p. 130 , suggest that in vivo dosage probably prevented 99.976% of the tumour cells from growing, however the remaining 0.024% did eventually kill the mouse. It is encouraging that immunotherapy appears to have here effected a 3.75 log reduction in tumour cell numbers as against the 3 log reductions achieved with chemotherapy or radiotherapy, but nevertheless this was still not enough. In some way 0.024% of the tumour cells have escaped the action of the 19s fraction in vivo. With the guaranteed contact of prior in vitro exposure they did not.

One possible mechanism was an in vivo reduction in the effectiveness of the 19s fraction, because as was shown in expt. 5, p. 118 , this fraction also acted against normal cells of C_3H mice. The normal tissues of the mouse could therefore have used up much of the fraction, even though the dosage rate was some 30 times greater than in vitro.

No obvious ill effect on the mice was noted, but one group of mice given larger doses appeared to die from something resembling runt disease. A full study of increasing the dosage of 19s fraction was therefore not undertaken. Rather attempts are continuing to prepare a tumour specific treatment, by absorbing the 19s fraction with normal C_3H mouse cells. An attempt is also to be made to encourage in addition a cellular reaction by chemically modifying the MP 5563 antigen, for in theory competent immunoblasts could hunt out the in vivo tumour more effectively than a 19s antibody.

Another possible explanation for the in vitro - in vivo anomaly is that in vivo some tumour cells became inaccessible to the 19s antibody. They might have reached some pocket which did not subsequently allow access of 19s molecules, but eventually the mice died with free tumour cells in the ascites. What seems more probable is that vulnerable sites on the tumour cell surfaces acquired a protective coating of some kind. Should enhancing antibodies eventually protect the late tumour, it is conceivable that a few molecules could have been available ab initio, and could have protected the 0.024% of cells apparently surviving the first dose of 19s fraction. This hypothesis is to some extent supported by the observation (p. 126), that the first dose of 19s fraction is the one that is most

effective, subsequent doses prolonging survival no further. The relative ineffectiveness of treatment first given after 5 days also suggests the tumour cells have become protected in some way. Of course as dosage of rat 19s fraction is continued, it could be anticipated that mouse antibodies would arise and in fact neutralise the rat proteins, so that treatment was not continued for more than 4 days. Furthermore serum sickness could have complicated larger or extended doses.

There still remain unanswered questions. If indeed late tumour becomes protected with enhancing antibodies, then it follows that the ascites harvested for continued experiments should contain protected cells. Yet the in vitro tests show no protection! It could be that enhancement is important only in the exponential phase, and that as the tumour gains the upper hand even enhancing antibody formation is suppressed by high dose tolerance. Thereby the late harvest would no longer be protected in vitro. Yet, in vivo treatment at this late stage seems ineffective. What kind of protection can appear to be so evanescent when compared in vivo and in vitro?

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THE GROWTH CHARACTERISTICS OF AN ASCITIC PLASMACYTOMA (MP 5563) TERMINATING BY FISTULOUS COMMUNICATION WITH THE BLOOD-STREAM

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SUMMARY.—The growth of ascitic mouse plasmacytoma 5563 has been monitored by ^{51}Cr-RBC dilutions within the peritoneal cavity together with differential cell counts and protein measurements.

Following the intraperitoneal transplantation of one million tumour cells, there is a latency period of 4 days, possibly explained as only a 5% survival of the inoculum. This is followed by logarithmic growth from 4 to 8 days with a doubling time of 18 hours, always similar in different animals. Eventually after 8 days and after about 100 million cells an asymptote is reached which is due to a fistula-like communication between blood-stream and peritoneal cavity. This terminal phase (9–10 days) is rapidly followed by death of the mouse.

Reliable tumour cell counts can only be achieved between 4 and 8 days.

THE growth characteristics of a transplantable protein-producing plasma cell tumour (MP 5563) in ascitic form were studied before assessing the paraprotein level in the serum as an index of tumour growth.

Klein and Revesz (1953) stressed the advantages of using ascitic forms for studying tumour growth. After inoculation there was a " latency period " of a few days and of unknown aetiology during which tumour growth could not be recorded. Thereafter they described a " cube rate phase " in which they found the cube roots of the cell numbers were linear against time and similar to findings with solid tumours. Finally followed a " terminal period " with an apparent fall-off in tumour growth. Steel (1968) using the results of Baserga (1963) and Lala and Patt (1966) for Ehrlich ascites tumour described a slowly progressive increase in cell loss to as high as 90% before death and assumed this was due to the death of many of the tumour cells formed. None of the above workers used an internal standard to control the assessment of the ascites and its cell content. The present study used intraperitoneally injected isologous ^{51}Cr-labelled red cells to monitor the recovery of ascitic fluid and the integrity of the peritoneal cavity. With this safeguard a simple exponential growth was observed between 4–8 days after inoculation until a fistulous communication occurred between the peritoneal cavity and the blood-stream which invalidated all subsequent cell counts.

MATERIAL AND METHODS

The tumour MP 5563, a plasmacytoma in ascitic form, from C3H mice was kindly supplied by Dr. B. A. Askonas of the National Institute for Medical Research, Mill Hill. A single colony of C3H mice bred from pairs supplied by

the Medical Research Council's Laboratory Animal Centre of Carshalton was used throughout the experiments reported here. Other colonies were tried and gave similar but not exactly comparable results.

Isologous red cells were freshly labelled with ^{51}Cr by the method of Mollison and Veal (1955). These were injected intraperitoneally and left for 10 minutes before recovering ascitic fluid. The syringe used was counted before and after the injection so that an exactly known dose was delivered. At the time of the peritoneal wash-out, blood was also taken from the orbital plexus using a pasteur pipette, and this was checked for radioactivity to see if any of the intraperitoneally injected red cells could be detected.

Recovery of ascitic contents from mice killed by cervical dislocation was made by using a syringe containing 1 ml. of counting fluid (Disodium EDTA 1·58 g., trisodium EDTA 1·82 g., tetrasodium EDTA 0·32 g., sodium chloride 6·5 g., 40% formaldehyde 1 ml., distilled water to 1 litre). The fluid was injected intraperitoneally and then the syringe was gently pulled to and fro three times in one minute before finally aspirating an aliquot for counting both its radioactivity and then its cell content. Cell counting was done using a Coulter counter set at ACS2, threshold 42. Control cell counts were made from uninoculated mice, to assess the number of normal peritoneal cells that could be aspirated by the same procedures.

In a few animals the final aspiration was made as completely as possible, opening the abdomen to collect the last few drops. The radioactivity recovered was then used to check whether this long used method could satisfactorily estimate the total ascitic cell content.

Samples of the peritoneal wall and gut were prepared and examined histologically for invasion by the tumour cells, thanks to the kindness of Dr. D. J. Evans.

Protein studies were made of the serum and the ascitic fluids using methods described elsewhere (Fakhri and Hobbs, 1970).

Main Experiment

Harvested ascitic fluid containing tumour cells was adjusted with saline to contain 5 million cells/ml. Into each of 40 C3H mice, 8–12 weeks old, 0·2 ml. of the fluid (1 million cells) was injected intraperitoneally. The mice were kept on a B41 diet, and six to a cage. At the same time, each day following the inoculation, four mice were given the ^{51}Cr red cells, killed 10 minutes later by cervical dislocation and the above tests performed.

The whole experiment was repeated in the same colony 6 months later, and again 6 months after that, in order to assess the stability of the experimental model.

Experimental Study of the Latency Period

A separate group of 20 mice were given whole body irradiation (550 R, 250 kV X-rays, known to suppress their immune responses) and subsequently transplanted and studied as above.

RESULTS

Recovery of ascitic fluid

From a 1 ml. insulin syringe 99 to 99·5% of a given dose of ^{51}Cr-labelled red cells could be delivered into the peritoneal cavity. Knowing the dose actually

given it was a simple calculation from the radioactivity and tumour cell numbers in a subsequent sample of ascitic fluid to estimate the total number of tumour cells within the total ascitic fluid. In a few animals complete aspiration of the ascitic fluid followed by opening the abdomen for the last few drops, only recovered from 92 to 94% of the given dose of radioactivity. Up to the end of 8 days after the tumour inoculation, ^{51}Cr-labelled red cells could not be detected (<0·1%) in the blood from the orbital plexus, but were regularly found by the end of the ninth day.

Three phases of growth were noted similar to those described by Klein and Revesz (1953) and these are seen in Fig. 1.

Latency period

For 4 days it was not possible to record a significant difference in total cell numbers, allowing for 1 million inoculated tumour cells and the average of 0·6

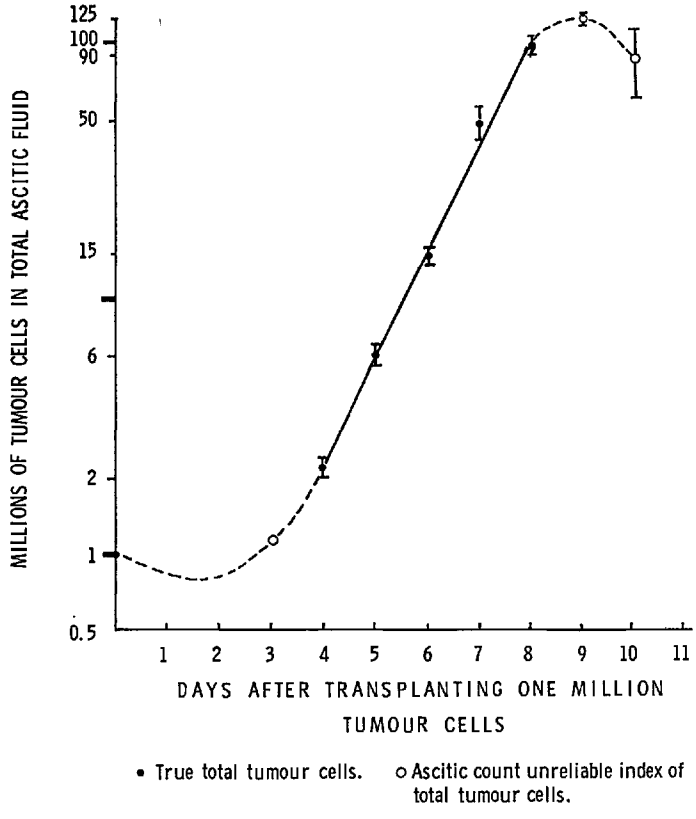


FIG. 2.—Tumour cell numbers over the days following transplantation of 1 million cells. If the exponential phase (4–8 days) is back-extrapolated it cuts the ordinate at 5000 cells, i.e. if only 5% of the transplant survived, growth would have been exponential up till day 8, whereafter fistulous communication invalidates further counts.

to 1·0 million cells which could be washed out of uninoculated mice using the same procedure.

There was no significant difference between the growth curves of the tumour in irradiated and normal mice. If the exponential phase growth line (Fig. 2) is back-extrapolated, it cuts the inoculation time at 5000 cells.

Exponential phase

Between 4 and 8 days the cell numbers increased in simple exponential fashion (Fig. 2). During this phase the peritoneal cavity became distended as a tight ball (Fig. 1), no ^{51}Cr-labelled red cells were detected gaining the circulation within 10 minutes, and the mice were alive and in good shape. The ascitic fluid had lower albumin and higher paraprotein levels than the serum (Fig. 3). Histological examination revealed no invasion of the peritoneal lining.

Terminal phase

Usually after 8 days and always after 9 days a visible change occurred (Fig. 1). The mice became listless, were now triangular in shape due to subcutaneous oedema and the tension was lost not only from the abdomen but also from the eyeball (noticed when bleeding the mice). The ascitic fluid was now often blood-stained and ^{51}Cr-labelled red cells were rapidly detectable in the retro-orbital blood; indeed, calculated as radioactivity/g. red cells there was no difference between the ascitic and systemic red cells at 10 minutes. There was also no difference between the ascitic and serum levels of albumin or paraprotein (Fig. 3). Histology regularly showed invasion of the peritoneal wall and tumour cells were seen in clusters adherent to the lining. The mice usually died on the tenth day.

Stability of this experimental model

There was no significant difference in latency period or growth rates at two 6-month intervals, representing 25 and 50 passages of our initial sample of the tumour. This itself must have had some 600 passages since it was first found as a spontaneous tumour in 1956. Accordingly all our data have been pooled in Fig. 2. During the exponential phase the doubling time was 18 hours, and in all the individual experiments this only varied from 18 to 20 hours. However, it was observed that whereas 1 million cells were initially needed for a certain

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EXPLANATION OF PLATES

FIG. 1.—Inoculated mice during the three growth phases. (a) Latency period, day 3. (b) Exponential phase, day 8. (c) Terminal phase, day 9. Observe the tight ball of distension confined to the abdominal cavity during the exponential phase. This has gone in the terminal phase, the shape is more triangular with oedema extending up over the chest to the face.

FIG. 3.—Electrophoresis of serum and ascitic fluid during exponential (above) and terminal (below) phases. Above, the difference in levels of total protein, large mol. wt. α_2-globulins and paraprotein indicates the relative integrity of the peritoneal membranes. Below equilibration of all levels confirms fistulous communication between the peritoneal cavity and the circulation.

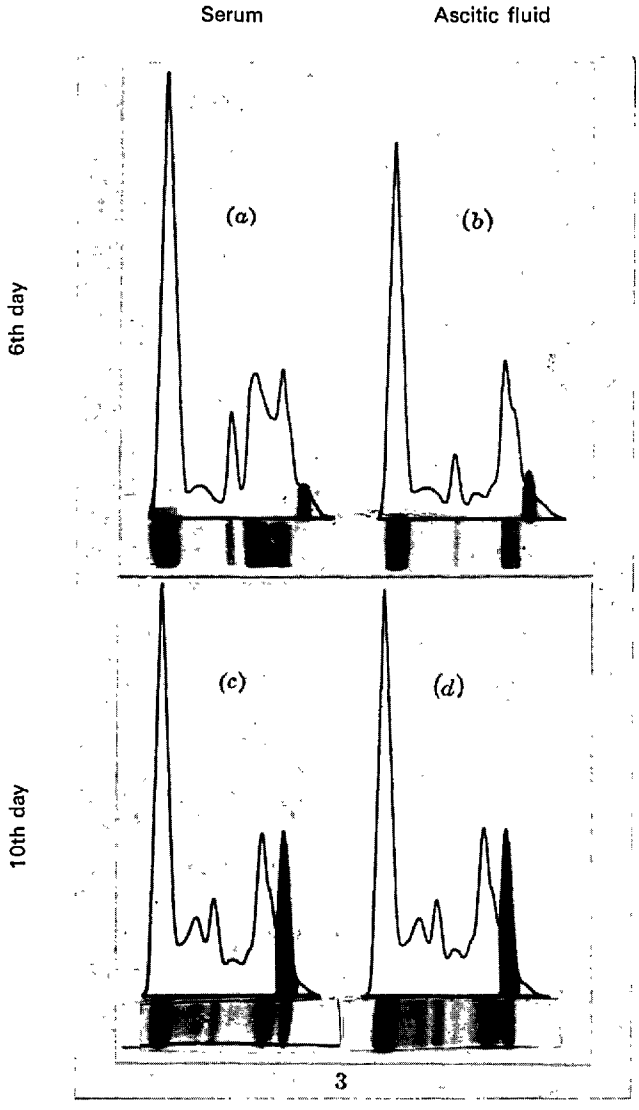
(a) Total protein = 5·5 g./100 ml. M protein = 0·1 g./100 ml.
(b) Total protein = 4·2 g./100 ml. M protein = 0·15 g./100 ml.
(c) Total protein = 4·30 g./100 ml. M protein = 0·45 g./100 ml.
(d) Total protein = 4·25 g./100 ml. M protein = 0·45 g./100 ml.



(*a*) (*b*) (*c*)

1

Fakhri.


Serum
Ascitic fluid
6th day
10th day
(a)
(b)
(c)
(d)
3

take, and an inoculum of 800,000 cells was usually a failure, by the end of the year a take could be achieved with 150,000 cells. At 18 months, inoculation has once been successful with only 20,000 cells. Despite this apparent increase in " virulence " of the tumour, the growth rate and protein production remained stable (Fakhri and Hobbs, 1970).

DISCUSSION

During the exponential phase (4–8 days after inoculation) the evidence supports the integrity of the peritoneal cavity, and shows that ordinary attempts to recover all the tumour cells are likely to fall short by some 7%. Isotope dilution of ^{51}Cr-labelled isologous red blood cells overcame this problem in the present study and also clearly showed the development of a fistulous communication between the peritoneal cavity and the blood-stream during the terminal phase (9–10 days). This was also supported by the other evidence of change in shape, low blood pressure in the orbital plexus, loss of tension of the ascites and equilibration of cell and protein contents. Cell counts of ascitic fluid beyond the 8th day therefore no longer represented the total number of tumour cells.

The growth curve of this tumour is similar in many aspects to what other workers have shown. The latency period described by Klein and Revesz (1953) has been thought to be due to immunological resistance by the host. If this had been true, the tumour in the irradiated group should have grown without this phase and the number of cells would be 8–16 times that of the control group after a few days (doubling time 18 hours). This was not the case. Sparck (1969) has also shown that prior sublethal irradiation of the host far from enhancing subsequent tumour growth in general impairs it. An alternative explanation for this period could be that only some 5% of the inoculated cells survived as viable, replicable cells and then simply grew exponentially. The inoculum usually does come from a spent host.

Simple exponential growth (4–8 days) can be explained by the binary division of cells and will occur in any closed population in which the generation time and the fraction of cells partaking in the division process is constant (Frindel *et al.*, 1967). It seems that the cell loss during this short growth period is very small, and that most cells undergo mitosis so that the doubling time is similar to the time of the complete mitotic cycle. The present observed time of 18 hours is identical to that found in other mouse tumours (Baserga, 1963; Frindel, Malaise, Alpen and Tubiana, 1967).

In the terminal phase (9–10 days) an asymptote is reached. There appears to be a complete equilibrium between the blood and ascites. This will lead to a major loss of the tumour cells into the blood-stream. A relatively physiological limit was placed on the follow-up by the death of the host.

Other causes for the asymptote have been shown. Baserga studying the Ehrlich ascites tumour claimed a constant mitotic cycle of 18 hours, but found a progressive decline in the growth fraction. Lala and Patt (1966) studying the same tumour claimed that in addition there was a lengthening of the mitotic cycle to 22 hours; however, their claim for an initial mitotic cycle of 8 hours is unconfirmed. Steel (1968) also showed the contribution of cell death with a cell loss fraction claimed to approach 95%. It is therefore possible that the use of terminal ascites for further inoculations may be followed by a great deal of cell death, and that this best accounts for the latency period.

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THE SERUM PARAPROTEIN LEVEL RELATED TO THE NUMBER OF PLASMACYTOMA-5563 CELLS IN C3H MICE

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SUMMARY.—An ascitic form of plasmacytoma (MP-5563) in C3H mice has proved stable on transplantation. A simple linear relationship was demonstrated between the serum level of the paraprotein produced and the total number of plasmacytoma cells within the ascitic fluid.

NATHANS, FAHEY AND POTTER (1958) showed that the total daily production of paraprotein by a solid plasma cell tumour in mice was proportional to the weight of the tumour. Later, Osserman, Rifkind, Takatsuki and Lawlor (1964) showed that simple measurement of the serum level of the paraprotein was directly proportional to the weight of tumour, up to about 2 g. Thereafter necrosis of the tumour probably accounted for a failure of the relationship.

The present study was made using an ascitic form of plasmacytoma in mice to see if the serum level could be directly related to the actual number of tumour cells, *i.e.* whether the serum level could be used to monitor the tumour growth.

MATERIAL AND METHODS

The tumour MP-5563, a plasmacytoma in ascitic form from C3H mice was kindly supplied by Dr. B. A. Askonas of the National Institute for Medical Research, Mill Hill. This tumour has been shown to produce a γG_{2a} paraprotein with an electrophoretic mobility in the post-β_1 region (Fakhri, 1970, Fig. 3).

The transplantation was carried out by adjusting the ascites with saline to contain 5 million tumour cells: 0·2 ml. of this fluid was injected intraperitoneally into 8–12 weeks old C3H mice. The mice were kept on B_{41} diet, six to a cage.

Measurements were made starting 4 days after transplantation, when the ascites was not yet obvious, and then daily. A blood sample was first collected with a pasteur pipette by puncture of the orbital plexus; this manipulation was easily done if the animal was anaesthetized. The animal was then killed by cervical dislocation, the ascites was collected, measured and the cell content enumerated as previously described (Fakhri, 1970).

Total protein was estimated using the specific gravity gradient tube of Lowry and Hunter (1945), as the biuret method was shown to underestimate the γG paraprotein by some 3% against an albumin standard and also required more serum for the estimation. Serum was electrophoresed at 25V/cm. for 65 min. on cellulose acetate strips in barbitone buffer pH 8·6, 0·05 M. The serum was applied as a fine line on the edge of a microscope slide, which was then touched onto the cellulose acetate strip. Straight bands resulted with clear resolution of the paraprotein from the normal β_1 (Fakhri, 1970, Fig. 3).

After staining with naphthalene black 10B (Hobbs, 1965) the paraprotein band was estimated as a percentage of the total dye uptake measured by transmission scanning using the Zeiss Extinction Recorder Mark II. The paraprotein percentage of the total protein was then calculated in g./100 ml. and by the above methods this could be reproduced to within 0·05 g./100 ml. Since the paraprotein was superimposed on the residual normal immunoglobulins the overall accuracy is probably only within 0·1 g./100 ml.

RESULTS

The growth characteristics and cell kinetics of this tumour are recorded elsewhere (Fakhri, 1970).

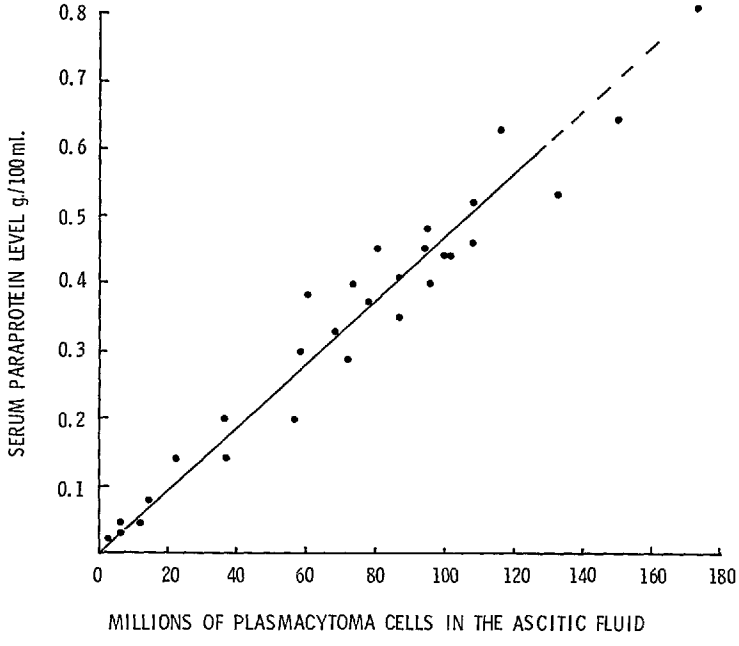


FIG. 1.—The serum paraprotein level is directly proportional to the number of tumour cells between 4 and 8 days after transplantation. Cell counts are unreliable before and after this period.

The paraprotein was first seen in the serum when the ascitic fluid contained 3 million plasma cells. The level of the protein in the serum increased as the tumour grew, showing a simple linear relationship (Fig. 1). The paraprotein level still showed this relationship even though the total serum protein levels often fell. In control mice the total protein values ranged from 5·3–6·5 g./100 ml. In inoculated mice with 5–120 million cells the total protein levels ranged from 3·8–6·2 g./100 ml. Above 120 million cells, total proteins fell to 2·5-4·5 g./100 ml.

It is worth recording here that two ascitic plasma cell tumours in BALB/c mice were initially studied. It was found for both these tumours (PC5, PC6) that the paraprotein production fell off with subsequent transplants. There also occurred a shortening of the time (from 16 to 9 days) in which the transplant

killed a mouse, with an increase in the amount of haemorrhage into the ascitic fluid. The reduction of paraprotein synthesising capacity was paralleled by an increase in the aggressive behaviour of the tumour; biochemical dedifferentiation paralleled neoplastic dedifferentiation.

Furthermore it was possible to grow the C3H tumour MP-5563 in the BALB/c strain after challenge with a high dose (over 5 million cells) but the tumour failed to produce protein in the strange host.

DISCUSSION

The measurement of tumour growth is essential in the assessment of anti-tumour agents and the serum level of paraprotein has proved useful for this purpose (Rosenoer and Whisson, 1964).

The stable ascitic form of the present tumour and the isotope dilution method of Fakhri (1970) enables an accurate count of the total number of tumour cells. The extent of growth of this tumour up to 120 million cells is reflected in the serum by the level of the paraprotein it is producing. Few mice survived after 120 million tumour cells were reached, but from previous work (Fakhri, 1970) it could be predicted that above these numbers the serum paraprotein level would no longer simply relate to the number of tumour cells because of the late formation of a fistulous-like communication between the peritoneal cavity and the blood stream. This could also account for the late fall in the level of the serum total protein which was observed in some mice. Earlier falls in serum total protein could be expected simply by transudation into the ascitic fluid but this did not significantly effect the serum level of the paraprotein itself.

In a 70 kg. man it was estimated that the earliest detection of paraprotein in the serum would occur with some 43 g. of myeloma or 9000 million cells (Hobbs, 1967). In 22–25 g. C3H mice it was observed that the earliest detection of paraprotein occurred at 3 million cells (Fig. 1). Since the mouse is about 1/3000th of the weight of the man this experimental observation supports the above estimate (Hobbs, 1969).

This investigation has been supported by a grant from the British Empire Cancer Campaign for Research. We are also grateful to Dr. T. Connors and Dr. M. Whisson for their help and cooperation.

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