

STUDY OF CIRCULATING MEGAKARYOCYTES IN
EXPERIMENTAL TUMOURS

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ABSTRACT

The megakaryocytes are normal constituents of the bone marrow. These cells, or fragments of them, may be found in the peripheral blood of human beings and animals under physiological conditions. It has been reported that circulating megakaryocytes are more frequently seen in males than in females. In human beings, they are normally observed during the foetal stage, and less and less frequently in childhood and adult life.

Megakaryocytosis, or the increased incidence of these cells in various tissues, has been observed in diseases such as myelosclerosis, myeloid leukaemia and inflammatory conditions, as well as after accidental or sudden death. Cirrhosis causes a reduction of megakaryocytes, even in the bone marrow.

The incidence of circulating megakaryocytes is notably high in patients with cancer. Investigators in the field of cancer research have in the past frequently mistaken them for cancer cells. Then a more detailed study established a distinct morphological difference between the two cell types, and it was found that many so-called cancer cells were in fact circulating megakaryocytes. This, almost accidental, discovery drew attention to the biological importance of these cells in neoplastic disease, even if there are no bone metastases. It is not yet known why megakaryocytes are mobilized, and migrate from the bone marrow (myeloid tissue) to the blood, in malignant neoplasia.

The present investigation was designed to determine whether megakaryocytæmia is in fact related to malignant disease and, if so,

what factors cause and control it.

Various causative factors have been suggested. They include stress, changes in the blood platelet level, anoxaemia, softening of the bone marrow, and a biological reaction to neoplastic tissue. It was therefore decided to study the incidence of circulating megakaryocytes in relation to the blood platelet level, stress, adrenalectomy, splenectomy and other experimental conditions.

Male Wistar rats were used in all experiments. For transplant tumours, Walker 256 carcinoma was injected subcutaneously in the right flank. Blood for the megakaryocyte counts was taken from the heart. The simple smear technique was considered inadequate for the purpose of this investigation, and a cytological technique was used instead.

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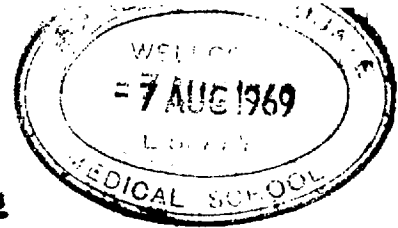
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HISTORICAL**REVIEW**



Survey of Previous Concepts

"There can hardly be any other type of cell with so many different organelles and cell inclusions, which normally only the study of many cell types would bring together. In this respect the megakaryocyte is unique." This statement was made by Rhodin in 1963. Little further attention was paid to these cells until recently. Their physiology and possible role in pathological conditions have scarcely been investigated.

Robin (1849) was the first to describe these cells. He found them in the bone marrow (Fig. 1) which, he thought, lined the sinusoids of the medullary canal and derived from that same anatomical site. Bizzozero and Newman (1905) differentiated the megakaryocytes into those with multiple nuclei and those with a single multilobed nucleus. Later some of the multinuclear cells were thought to be osteoclasts.

Aschoff (1893) found some megakaryocytes in the pulmonary bed and concluded that these cells had migrated from the bone marrow. Several other workers (Wright, 1910; Bunting, 1911; Oelhafen, 1914; Casaris-Damel, 1914; Di Guglielmo, 1917, 1920; McCullen, 1920; Pianese, 1920) also demonstrated megakaryocytes in the pulmonary bed and supported Aschoff's view that they migrate from the bone marrow in the form of emboli. This was further substantiated by other groups of investigators (Favre, 1934; Hickling, 1937; Rosenthal and Erf, 1943).

The concept that megakaryocytes migrate from the bone marrow to the lungs in the form of emboli is now more or less generally accepted. It led Bunting (1912) to look for these cells in the peripheral blood,

through which they are supposed to travel. He first detected megakaryocytes in the peripheral blood of a patient with Hodgkin's disease. Later Oelhafen (1914), Di Guglielmo (1917, 1920), Kaznelson (1917) and Pianese (1917) all confirmed Bunting's findings. Except for the work of Wright (1910), English and American authors contributed practically nothing on the subject until the early 1920s. Minot (1922) of the National Institute of Medicine, Pennsylvania, United States of America, reported finding megakaryocytes in the peripheral blood of patients with myelocytic leukaemia, polycythaemia vera and Hodgkin's disease. He suggested that the presence of circulating megakaryocytes indicated an alteration in the regulatory mechanism of the bone marrow and revealed a severe strain on the bone marrow.

Minot's study on this aspect was very thorough and intensive, especially if we consider the techniques at his disposal. He observed megakaryocytes in the peripheral blood of 35 out of 45 patients with chronic myelocytic leukaemia, and also found small megakaryocytes in the peripheral blood of patients with polycythaemia vera, Hodgkin's disease, one with fatal pneumonia and one with sepsis. He postulated the probability of migration of these cells, or tumour or tumour-like cells, to different environments. Little effort has since been made to explore the causation of this migration. Passage of the cells through the pulmonary capillaries was accepted almost without question (Minot, 1922; Minot and Buckman, 1923).

The presence of these cells in ectopic sites, that is in sites

other than the bone marrow, has been observed by various authors (Hench, 1879; Baumgartner, 1899; Askanazy, 1904; Shemori, 1904; Letulle, 1905; Assman, 1907; Lahendrof, 1907; Levy, 1919; Ricci, 1922; Koerner, 1926; Jores, 1927; Dibinskya, 1928; Hedgarn, 1928; Pincorton, 1929; Anagnostu, 1933; Jacobson, 1933; Downey, 1936; Kauffman, 1938; Bamforth, 1939; Jordan, 1941; Boiden, 1942; Weinbeck, 1942; Churg, 1944; Schwarz, 1948, 1953, 1955; Marinone, 1949; Taylor, 1950).

Schwarz (1958) is of the opinion that the presence of these cells in the spleen, liver, kidneys and lungs is independent of bone marrow disorders such as leukaemia or subleukaemic myelosis, as against Minot's suggestion (1922). The extramedullary origin and development of megakaryocytes has since been postulated and supported by other investigators.

Brill and Helpert (1948) observed megakaryocytes in the so-called ectopic site and remarked that a small number of these cells were circulating in the blood stream, but they provide no data to support their comment.

Apart from those occasional reports on the discovery of megakaryocytes in the peripheral blood of patients with haematological disorders or Hodgkin's disease, which support Minot's classical observations, little has been published on the subject between 1925 and 1950. There has been no systematic study of these cells and their appearance in ectopic sites in relation to diseases other than those already reported by Minot.

Engell (1955) studied the incidence of tumour cells in the circulating blood, and this created a lot of interest. Since then

different workers have used sophisticated and effective techniques to isolate cancer cells in the blood (Cole, Sandberg et al., 1961; Pruitt, 1962; Scheinin and Koivuniemi, 1963; Sakurai, 1962; Robinson, 1963; Delarue, 1964; Selbach, 1964). The incidence of cancer cells in the peripheral blood was found to be between 2% and 26%, and between 13% and 50% in the veins draining the tumour or the area of the tumour. These results were obtained by different investigators using different techniques.

Very recently Romsdahl (1965) used a new technique and claimed to have found cancer cells in 2 out of 35 samples of peripheral blood from cancer patients, and in only 2 out of 28 samples of blood taken from the local vein draining the tumour. He noted a remarkably low incidence in this series compared to the results previously obtained by others. Many of the cells with large hyperchromatic nuclei he found to be megakaryocytes, and great care had to be taken to distinguish these cells from circulating cancer cells. In his experience other cells, e.g. myelocytes, plasma cells, macrophages, osteoclasts, endothelial cells, tissue mast cells and trophoblastic cells, are also encountered in the peripheral blood and may be mistaken for cancer cells.

Kierszenbaum and Tres (1964) also found many cells in the blood which resembled cancer cells, but in fact were not. They identified most of these cells as megakaryocytes and 42% of their blood samples from different cancer patients showed megakaryocytes in peripheral blood. Sandberg, Moore and Schuberg (1958) demonstrated "atypical cells" in the

blood of cancer patients. They found plasma cells, myeloblasts, promyelocytes, cells in mitosis, normal tissue cells and also cells of "obscure identity", which were extremely difficult to differentiate from cancer cells. This is a difficulty encountered by most investigators looking for cancer cells in the blood.

Alexander and Spriggs (1960) comment: "After practically a century of morphological haematology, it might be thought that all the types of cells that occur in the blood would already be well known. It turns out, however, that this is not the case, and newer techniques of white cell concentration are beginning to reveal a remarkable variety of non-malignant cells which are seldom or never figured in the atlases of blood." Sandberg's difficulty in identifying all the "atypical cells" is quite understandable.

After Engell, various investigators have recorded the need for distinguishing circulating megakaryocytes from circulating tumour cells to avoid misinterpretation (Sandberg, 1957; Alexander and Spriggs, 1960; Raker, 1960; Macdonald, Roberts and Southwick, 1961; Jackson, 1962; McGrew, 1962; Harbeuval, 1962; Romsdahl, 1963; Kuper, 1964; Wast, 1964 and Sallwood, 1967).

Members of the Circulating Cancer Cell Study Group, under the presidency of Nadal (1962), reported the existence of megakaryocytes in the peripheral blood along with other marrow elements. They emphasized that it is essential to differentiate these cells from tumour cells in the blood.

Raker (1960) found a very high incidence of megakaryocytes in the peripheral blood of cancer patients. In this series of cases, megakaryocytes were found in the blood of 42% of the patients.

Sir Lionel Whitby (1948), in a review, noted that reports had been published on the appearance of these cells in the peripheral blood of patients with carcinomatosis, myelomatosis, osteosclerosis, Cooley's anaemia, and lipomatosis, as well as diseases of the bone marrow proper. Minot (1925) had suggested that the hyperplastic process of polycythaemia vera and leukaemia was the controlling factor for migration of these cells to the peripheral blood.

Leukaemia of megakaryoblastic origin has been reported by Boros and Korenys (1934). Whitby found megakaryocytes in the peripheral blood of one of his patients with leukoerythroblastic anaemia, and stated that the appearance of these cells in the peripheral blood was suggestive of myelofibrosis.

Melamed (1966) observed them in the peripheral blood of cancer patients with no demonstrable change in the bone marrow or platelet count. He noticed fragments of megakaryocytes in the blood of non-cancerous and healthy subjects.

It is only since the last decade that we are becoming familiar with the idea of associating cancer with circulating megakaryocytes. This actually emerged out of the confusion of cancer cells and "atypical cells" in the peripheral blood. There had only been sporadic reports on the existence of these cells in the blood, but after 1960 some investigators

attempted a systematic study of megakaryocytes in the circulating blood (Raker, 1960; Alexander and Spriggs, 1962; Herbauval, 1962; West, 1963; Hume, 1964; Wast, 1964).

Hume (1964) and her associates did a quantitative study of circulating megakaryocytes in human subjects. Taking blood from the antecubital vein, they found that these cells were many times more common in cancer patients than in others. According to their findings, the frequency is higher in blood taken from the tumour area than in the antecubital vein. It was noted that the incidence of circulating megakaryocytes increased after any type of surgical intervention. The same investigators also recorded a diurnal variation, as with polymorphs, with a peak at about 4 p.m. The rise after surgical operations was attributed to the diurnal variations. But most of the operations were finished around 4 p.m. in the afternoon and Hume has admitted the possibility that the stress of the operation might cause migration of these cells from the bone marrow to the peripheral blood. No critical review of her comments was given.

Williams, Balco and Warren (1953, 1955, 1960) observed increased megakaryocytes in the bone marrow and also a high platelet count in the peripheral blood following surgical operations and prolonged intravenous transfusions of ACTH. The same effect was observed by Thorn and Jenkins (1953) and Selye (1950).

Melamed (1966) took samples of blood from seventy five normal subjects and found fragments of megakaryocytes in all of them. He also

found megakaryocytes in every one of 10 peripheral blood samples taken from cancer patients. But these were mostly intact cells. The number of megakaryocytes was highest in blood taken from the azygos vein, as compared to other sites. Romsdahl noted 17.3 megakaryocytes per 10 ml. of peripheral blood from patients with advanced carcinoma, while early cases showed only some fragments. According to his observations the number of these cells in the blood increases while cancer patients are being treated with chemotherapeutic agents. Thirteen out of twenty five cancer patients showed a significant increase in the number of these cells in the blood. The average was 23.6 cells per 10 ml. of blood. Similar observations were made by other investigators (Herbeuvel, 1962; Prizhivoit, 1964; Wast, 1964).

Megakaryocytes have been found in the blood drawn from different vessels (Romsdahl, 1966). The frequency and number was highest in blood taken from the azygos vein.

Jackson (1963) used histochemical methods to identify these cells. He observed the same incidence of megakaryocytes in cancer patients and normal subjects.

Scheinin and Koivuniemi (1963) found an increased number of megakaryocytes in the pulmonary circulation of cancer patients. Anaemia and inflammatory conditions show a higher pulmonary megakaryocyte count than other pathological conditions, though it is lower than that found with neoplastic disease. The presence of these cells has also been noted by other observers (Aschoff, 1893; Goroney, 1924; Britte, 1948; Seebach, 1952; Schwarz, 1948, 1953 and 1956).

found megakaryocytes in every one of 12 peripheral blood samples taken from cancer patients. But these were mostly intact cells. The number of megakaryocytes was highest in blood taken from the azygos vein, as compared to other sites. Romsdahl noted 17.3 megakaryocytes per 10 ml. of peripheral blood from patients with advanced carcinoma, while early cases showed only some fragments. According to his observations the number of these cells in the blood increases while cancer patients are being treated with chemotherapeutic agents. Thirteen out of twenty five cancer patients showed a significant increase in the number of these cells in the blood. The average was 23.6 cells per 10 ml. of blood. Similar observations were made by other investigators (Herbeuvel, 1962; Prizhivoit, 1964; Wast, 1964).

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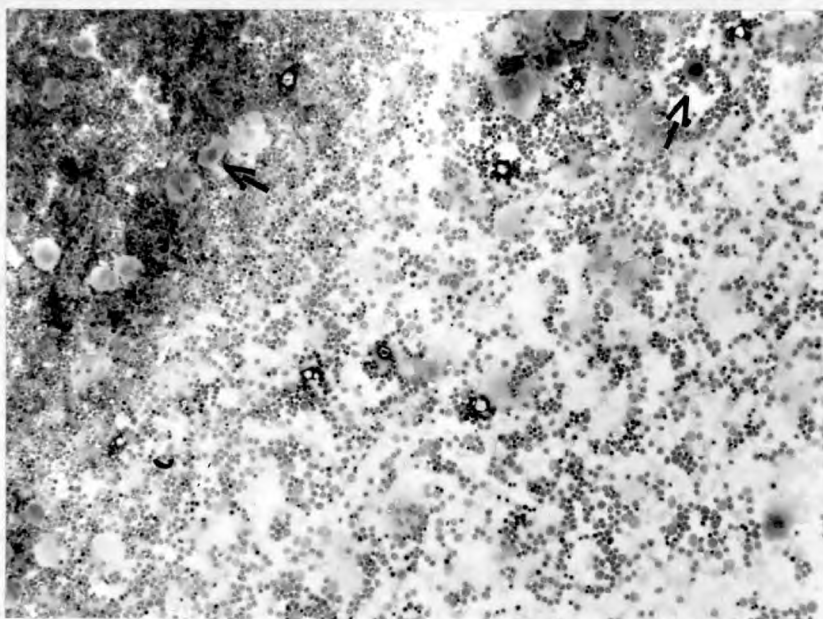


FIGURE 1.

Megakaryocytes are normal constituents of bone marrow.

It was thought that the migration of megakaryocytes was part of the process of aging (Whitby, 1948). But Cuny et al. (1961) demonstrated that the incidence of circulating megakaryocytes is in no way related to age, as there was no difference in the frequency of these cells in the peripheral blood of persons of different age groups.

There is a slight tendency for the megakaryocyte count to be higher in males than in females. Large numbers of these cells, along with other bone marrow elements, are normally seen in the peripheral blood of new born babies and foetuses (Efrati et al. 1961).

So far few experimental studies on circulating megakaryocytes have been published in the literature. Prizhivoit (1963) investigated the megakaryocytes in the bone marrow of rats with sarcoma 45, sarcoma M-1 and Walker 256 carcinosarcoma. He observed an increase in degenerate forms and a decrease in active forms. The total count (frequency) of these cells in the bone marrow had increased. Prizhivoit concluded from these observations that megakaryocytes are highly sensitive to the presence of neoplastic tissue in the body.

Traycovich (1964) examined the blood from the right heart of hamsters with melanoma, fusicellular and endothelial sarcoma. He also examined blood from the same site in 26 normal hamsters. 64% of the 62 blood samples taken from animals with tumours were found to contain large haematopoietic cells non-malignant in origin. Most of these cells were identified as megakaryocytes. 50% of the samples taken from normal hamsters also contained endothelial cells or megakaryocytes. Traycovich

mentions the presence of different cells of haematopoietic origin, but unfortunately his results are inadequate through lack of information. No mention is made of the relative frequency of different cells in animals with and without tumours.

Roszel, Prier and Koprowska (1965) examined blood from the cephalic vein of normal dogs. They used the buffy coat technique to isolate megakaryocytes and stained the smears with Wright-Giemsa stain. Every blood sample was found to contain intact cells and fragments. Haematological examination revealed that some of the dogs had a leukaemia with leukocytosis. But it is apparent from the data given that these haematological abnormalities are not related to the incidence of circulating megakaryocytes.

The authors admit that the inconsistent results obtained with the buffy coat technique are a handicap in enumerating megakaryocytes in the blood.

Roszel and his associates (1965) observed abnormal megakaryocytes in the spleen of mice bearing transmissible ascites tumours.

**Survey of Methods for the Determination of Circulating Megakaryocytes
in the Blood**

Since Minot's observation of megakaryocytes in the circulating blood, little attention has been paid to the subject until recently. Interest has been revived through the search for cancer cells in the blood and all recent information comes chiefly from investigations in this field.

Nadel and Goldblatt (1965) recently reviewed all the methods available for isolating cancer cells in the blood. To their surprise they found twenty different methods. Some of these techniques have also been used by investigators whose primary interest lay in the circulating megakaryocytes. Conventional haematological, immunological and histochemical techniques have been employed for the purpose. They can be broadly classified as follows:

- 1) Simple blood smear using conventional haematological techniques
- 2) Techniques for the isolation of cancer cells from blood with Papanicolaou's stain or conventional histological stains
- 3) Fluorescent staining and histochemical identification
- 4) Immunological technique

1) **Smear and total white cell count**

Until recently megakaryocytes in the blood were counted by the conventional haematological method. Blood is taken from the tip of the finger or ear lobule, a smear is made and stained for differential count.

A total white cell count is also done, using a counting chamber. Then the number of megakaryocytes in that total is determined. The results are inconsistent and show great variation, as the number of circulating megakaryocytes is very small. Bunting (1911) was the first to detect megakaryocytes in the peripheral blood by this method. His observation was later confirmed by Casaris-Demel (1914), Nageli (1914), Oelhafen (1914), Kaznelson (1917), Di Guglielmo (1917, 1920), Minot (1920), Pianese (1920), Minot and Buchman (1923) and Whitby (1948).

Minot (1922) did a quantitative determination of these cells in the peripheral blood with very inconsistent results. As he describes it, "at one time or other 35 out of 45 patients with myelocytic leukaemia had megakaryocytes in the peripheral blood". It is essential to examine a number of slides to detect these cells. The procedure is tedious, and time consuming, with uncertain results. The method being an indirect one, its reliability should certainly be questioned. The advantage of the technique is that it always retains a very clear morphology of the cells. It is easy and simple, but definitely unsuitable for an element for which a huge quantity of blood has to be examined.

Minot found megakaryocytes in the blood of patients with a white cell count of 50,000 per cmm or more. If the white cell count was below 50,000, these cells were found to disappear from the blood. The range of occurrence was between 1% and 8% of the total white cell count. This has been confirmed by a few authors (Hickling, 1937; Carpenter and Flory, 1941;

Favre, 1934; Rosenthal and Erf, 1943). Sir Lionel Whitby (1948) found the same proportion of megakaryocytes in the peripheral blood of a patient with leukoerythroblastic anaemia. He reported that on various occasions megakaryocytes formed 2 to 4% of the total leukocyte count for this patient.

It is remarkable that between 1925 and 1950 the literature contains nothing about circulating megakaryocytes in relation to other pathological conditions and normal health. This is no doubt due to the technical difficulties. There are reports on the association of these cells with diseases of the haematopoietic and reticuloendothelial system and with Hodgkin's disease. Recently more sophisticated techniques have been employed and the results suggest that megakaryocytes are much more frequently found in the circulating blood in both health and disease, and particularly neoplastic conditions.

2) Techniques for Isolating cancer cells from the blood and their use for the determination of megakaryocytes

The fact that Nadel (1965) found that twenty different techniques are employed for the isolation of cancer cells suggests that none of them are satisfactory. All these techniques consist chiefly in removing the vast number of normal cells, leaving the abnormal cells behind. The aim is also to preserve the morphological structure of the cells to be examined. Megakaryocytes have a well-defined morphological character, so that they are relatively easily detected in the blood and differentiated from cells of similar morphology. The general principle of all these

techniques is the removal of erythrocytes, removal of leukocytes, followed by preparation for microscopy.

Various methods have been tried for the removal of erythrocytes, e.g. water (Queensel, 1921), acetic acid (Pool and Dunlop, 1934), saponin (Engell, 1955; Whang, 1958; Kuper, 1961; Erichsson, 1962). Streptolysin O has been used for the lysis of both red and white cells (Malmgren, 1958, Romsdahl, 1961). This is an expensive, unstable and quickly destroyed enzyme. Some workers used streptolysin S, but this was shown to be detrimental to cancer cells and probably also other cells in the blood. Erythrocytes have also been separated by the process of sedimentation. This can be improved by adding bovine fibrinogen (Sandberg, 1957; Roberts, 1958, Diddle, 1959; Fletcher and Stewart, 1959; Cousineau, 1961; Watne and Lincoln, 1962; Ishikawa, 1962; Stoffberg, 1963), dextran (Alexander and Spriggs, 1960; Colombo et al., 1960; Romsdahl, 1961), or phytohaemagglutinin (De mello, 1960). These methods are not very expensive, but the possibility of losing cells is very great, so that they cannot be used for quantitative determinations.

Differential centrifugation after lysis of the white cells with streptolysin O has been tried. Cancer cells, lymphocytes and megakaryocytes have less specific gravity than erythrocytes and polymorphonuclear cells and can be separated by differential centrifugation (Roberts, 1958; Seal, 1959).

Polymorphs have been separated with a horseshoe magnet after

binding with carbonyl iron. The red cells are lysed with saponin during and after the process (Kuper, Brignall, Lucklock, 1961; Sellwood, 1964, 1967). There is sufficient evidence to suggest that this method is suitable for quantitative determination (Sellwood, 1964, 1967). It is cheap, simple and quick. For these reasons it has been chosen for this particular study and further details will be given in the following chapters.

For microscope examination, paraffin blocks of cell deposits (Engell, 1955), smears (Sandberg and Moore, 1957; Roberts, 1958) and membrane filters (Malmgren, 1958; Kuper, 1961; Sakurai, 1962; Saito, 1961; Sellwood, 1964) have been used.

Malamed (1966) and Seal (1964) used plastic sieves for isolating megakaryocytes and cancer cells respectively. They collected 10 ml. of blood from a major thoracic vessel or any peripheral vein in EDTA or a heparinized container. Immediately after collection the sample is passed through a perforated plastic membrane with 1.2×10^5 perforations of 4.5 micron diameter per cm^2 .* The sieves with the retained cells are washed in saline, followed by 50% ethyl alcohol, and then fixed in propanol. They are stained with haematoxylin and eosin, dehydrated in alcohol and cleared in xylene. The plastic sieves become transparent and are mounted on 1" x 3" glass slides in parmount.

* These are known as nucleopore filters, manufactured by the Millipore Corporation, Bedford, Massachusetts, U.S.A.

Properly collected fresh blood should pass through the sieves easily by gravity alone, taking less than 2 - 3 minutes. Seal (1964) used sieves of 1" diameter for 10 ml. of blood.

In the experience of various investigators the plastic membranes and millipore membranes with large pores (Foss, 1963) have proved reasonably precise. Membrane filters will effectively trap cancer cells and megakaryocytes and retain them for microscopy. They have proved better than other techniques (Sakurai, 1962; Taft and Arizaga-cruz, 1965; Sellwood, 1967).

Various stains have been used, depending largely on the field of research in which the investigator specialized. A pathologist normally likes to use haematoxylin and eosin, a cytologist Papanicolaou, and a haematologist Romanovsky's stain, and so all these staining techniques have been used.

3) Fluorescent staining and histochemical identification

It has been claimed that fluorescent staining with acridine is a useful method (De Mello, 1962; De Mello and Kaster, 1962). This technique produces a high proportion of false positives but is said to make megakaryocytes easily recognizable (Jackson, 1962; Stein, 1962; Pavone and Rolfini, 1963; Nagy, 1965).

John F. Jackson (1963) has described a histochemical technique using acridine orange fluorescence for the rapid identification of all suspicious cells, and subsequently periodic acid-Schiff's stain to

differentiate the megakaryocytes.

For this method, 10 ml. of blood are mixed with 5 ml. of 5% dextran in physiological saline. The mixture is left to stand for 35 to 45 minutes to allow the erythrocytes to settle. Then the supernatant fluid is withdrawn and replaced by the same volume of saline. After centrifugation, smears of the deposit are made on slides. The smears are fixed in Carnoy's fluid, rinsed with 50% ethyl alcohol and washed in distilled water. They are then placed in 0.01 acridine orange in Mailvaine's citric acid buffer at a pH of 3.8 for 3 minutes. After this they are rinsed in distilled water and mounted.

The slides are scanned for all cells showing positive acridine orange fluorescence, these cells being classified as suspicious. Then the slides are placed in 50% alcohol for 2 minutes to remove the stain, and restained for the periodic acid-Schiff reaction. The suspicious cells are examined for PAS positive granules which help to identify the megakaryocytes. According to Jackson, the detection rate is satisfactory.

Fragments of megakaryocytes also fluoresce and give a PAS positive reaction, due to the presence of glycogen. Tumour cells, mast cells and trophoblasts fluoresce in the same way as megakaryocytes. Like other histochemical methods, the technique requires great care, precision and skill. Its advantages still require evaluation.

4) The immunological technique

Herbeuval (1961) used an immunological technique for the

identification of megakaryocytes in the circulating blood. Human anti-platelet globulin adheres to all structures of the megakaryocyte series, and fibrinogen. Herbeuval used human antiplatelet serum at 1 in 640 per litre and identified the megakaryocytes under ultraviolet light. This method is certainly precise and effective, for with it few other cells or elements will give the same appearance as megakaryocytes. It is complicated but would seem to detect these cells effectively in the blood. Unfortunately the paper gives no details of the results, e.g. the incidence or total count, which would have been helpful for a comparative study.

(It is hoped that further details will be published.)

Identification of Megakaryocytes

Since Robin's (1849) original description of these cells as "megakaryocytes" there has been little dispute over their identification, either in the bone marrow or at ectopic sites. He described them as huge cells with multiple lobulations of the nucleus. He observed that these cells lined the sinusoids of bone marrow and thought they developed from the same site. Bizzozzo and Newman (1905) described two different morphological types of these cells:

1. Cells with a single multilobed nucleus
2. Cells with multiple nuclei

Many of the multinuclear cells have been identified as osteoclasts. These are only rarely seen in the blood, but may always be confused with megakaryocytes. With multinuclear megakaryocytes seen in the blood and bone marrow, the number of nuclei is usually less than 16, whereas osteoclasts contain more than 20. The osteoclast cell with the cytoplasm is also much bigger than a megakaryocyte, its diameter being about 100 micron (Rhodin, 1963).

Description of the Different Parts of Megakaryocytes (Fig. 8 & 9)

Cell membrane - The cell membrane is of medium thickness and continuous, though it appears broken at times, which is said to be due to the formation and dissemination of platelets.

The Cytoplasm - Haematoxylin - eosin and Romanovsky's stain produce a pink or purple colouration of the cytoplasm. Frequently the cytoplasm is eosinophilic and homogenous, with occasional granules or vacuoles. These granules are basophilic or azurophilic. The presence of granules in the cytoplasm has been ascribed to the fact that it is actively engaged in blood platelet formation.

The nuclear membrane - This is thin, regular and continuous around all the lobes of the nucleus. Rupture of the nuclear membrane is never seen under physiological conditions.

The nucleus - In mature megakaryocytes the nucleus is always multilobular,

with direct nucleoplasmic connection between the lobes. Frequently completely detached nuclear lobes or nuclei are seen, either singly or in numbers. The distribution of chromatin granules in the nucleus is uniform, with conspicuous condensation along the nucleoli. Nucleoli are only infrequently observed in these cells.

The nucleo-cytoplasmic ratio - The nucleo-cytoplasmic ratio is very high, very much higher than in any cell of comparable morphology and size.

Mitosis - Very rarely one may see the cells under endomitotic conditions, and in this state they are difficult to differentiate from cells of a similar type.

Megakaryoblasts (Fig. 9a)

These are huge cells, 20 to 50 micron in diameter. The nucleo-cytoplasmic ratio is high but less than ⁱⁿ mature cells. The cytoplasm is profuse, and eosinophilic or pale violet granules may be seen in it. Some of these cells may have a thin rim of basophilic cytoplasm and are frequently mistaken for cancer cells. Basophilic staining of the cytoplasm of cancer cells is not yet known.

The nucleus is comparatively larger than that of the mature cell. Lobulation of the nucleus is never encountered. The nuclei contain evenly distributed chromatin granules and nucleoli are frequently present. Valaitas and McGrew (1962) have drawn attention to the mirror image appearance of the nucleus, with the chromatin granules symmetrically arranged.

Incidence of Circulating Megakaryocytes

Megakaryocytes have been demonstrated in the blood of patients with various diseases, especially conditions involving the haematopoietic system. Until recently these cells were not thought to be a normal constituent of the blood. With the aid of modern sophisticated techniques megakaryocytes have been found more frequently in patients with various conditions and also in healthy subjects. Fragments of these cells are seen in the blood of healthy subjects, while undamaged cells are conspicuous in morbid states and particularly neoplastic disease.

Using conventional haematological methods, Minot (1922) demonstrated megakaryocytes in the blood of 77.7% of patients with myelocytic leukaemia. Several other authors reported similar findings (Hickling, 1934; Carpenter and Flory, 1941; Favre, 1934; Downey and Nordland, 1939, Rosenthal and Erf, 1943).

Raker and his associates (1960) first attempted to study the incidence of these cells in the blood of cancer patients, using Seal's cell concentration technique (1958). They found megakaryocytes in the blood of 42% of their cancer patients. But their paper gives no comparative data on the incidence of these cells in normal individuals or patients with non-malignant diseases.

Scheinin and Kolvummiemi (1963) used buffy coat smears and detected megakaryocytes in the blood of 129 (77%) of 168 patients with malignant and non-malignant diseases. 114 of the 168 patients had cancer

in different sites of the body. They took about 4 - 8 ml. of blood from the antecubital vein of these patients, and found 1.2 megakaryocytes per sample on average. The total megakaryocyte count was greater in cancer patients (0.8 cells per specimen) than in those with other diseases (0.3 cells per specimen).

Herbauval and his associates (1964) used a modification of Seal's technique and found fragments of megakaryocytes in the peripheral blood of patients with haematological disorders (43.7%). In the blood of cancer patients they found mostly intact megakaryocytes and occasional cell fragments (16.5%)

Hume et al. (1964) used the modified Seal's technique and found megakaryocytes in the blood of 50.6% of their cancer patients. Only 3.1% of patients with non-cancerous diseases showed megakaryocytes in the blood. They also observed that intact megakaryocytes were more frequent in the blood of cancer patients than in that of patients with other diseases.

Efrati et al. (1963) used the buffy coat technique and detected fragments of megakaryocytes in every one of a number of specimens taken from healthy subjects. They presented comparative findings for adults of both sexes and foetuses of both sexes. They also demonstrated the normal variation in adults of different sex. They found that 10 ml. of adult blood contained on the average 21.79 megakaryocyte fragments, compared to 602.7 fragments in the blood of foetuses. In male subjects they detected

14.6 megakaryocytes or fragments and in female subjects 28.

Wast (1963) used the Seal's technique with blood taken from the antecubital vein. He found megakaryocytes in the blood of 22 out of 82 cancer patients, and remarks that the blood of cancer patients contained mostly intact megakaryocytes, while only a few occasional fragments were found in the blood of the normal subjects.

Cuny et al. (1961) took blood from normal subjects and observed that all the samples were positive for megakaryocytes, nearly all of them showing fragments, with just occasional intact cells. In this series males had a higher count than females, 11.5 megakaryocytic elements per ml. as compared to 5.6 per ml. He could not find any difference in the number of circulating megakaryocytes in different age groups. On the other hand he admits the significantly higher occurrence of these cells in the peripheral blood of fetuses. He used the same technique as Harbeuval et al. (1962).

Malmel et al. (1966) used the buffy coat technique and found megakaryocytes or fragments of these cells in blood samples taken from both healthy subjects and cancer patients. For this investigation, 10 ml. of blood were taken from each subject. Fragments of megakaryocytes were found in the blood of every one of 75 normal subjects, the average being 144.97 per subject. Intact cells were found in all the samples taken from 12 cancer patients, with an average of 54.75 cells per specimen.

Jackson (1962) employed the histochemical technique to study

the incidence of megakaryocytes in the peripheral blood of patients with and without cancer. His material consisted of 18 cancer patients and 11 patients with other diseases. Megakaryocytes were found in blood taken from the antecubital vein of 26 of these 29 patients. The cancer patients showed 7.7 megakaryocytes or fragments per ml. of blood, patients with non-cancerous diseases 6.7 per ml. Jackson claims that with this technique he saw mostly intact megakaryocytes in the blood of non-cancerous patients. This does not agree with the findings of other observers. No specific mention is made of the frequency with which megakaryocytic fragments occurred in this series. This is important, because the fragments fluoresce and give the same positive PAS reaction as intact cells.

Traykovich (1964) studied the incidence of circulating megakaryocytes in the blood of hamsters. Blood collected from the right heart of the hamsters was placed in a phosphate buffered solution with polyvinyl pyrrolidone sodium citrate and 2% formalin. A saponin solution containing 50% ethyl alcohol and 2% formalin was added drop by drop until all the erythrocytes were lysed. The mixture was centrifuged and the deposit washed with Herbeuval and Herbeuval's solution. It was then again centrifuged, smears were made of the deposit and stained with Papanicolaou and Fontana's method.

64% of 28 animals with cancer of different tissues showed megakaryocytes or fragments of these cells in blood taken from the heart. Only 50% of the normal controls had megakaryocytes in the blood, together with other haematopoietic tissue elements.

With the development of various techniques it is now possible to detect megakaryocytes in the peripheral blood more frequently than before. These cells have been observed in blood samples taken from almost every part of the body, with the highest incidence and total count in blood from the azygos vein (Melamed, 1966). In many cases the identification of megakaryocytes has been based on bare nuclei or cell fragments only. This is bound to give a false impression of the actual number of cells circulating in the blood. The chances of missing solitary nuclear lobes are very high, and clumps of lymphocytes may be erroneously identified as bare nuclei of megakaryocytes. There is also the possibility of including the bare nuclei of other cells like macrophages or osteoclasts. It has been suggested that there is a diurnal variation in the number of circulating megakaryocytes, the peak being in the late afternoon. Major surgery and the continuous slow infusion of ACTH also affect the normal biology of these cells. The incidence of intact megakaryocytes in the blood is markedly greater in neoplastic conditions than in any other disease or in healthy subjects. The incidence is also higher in advanced stages of cancer than in the early ones. In disorders of the haemopoietic system the blood was found to contain both intact cells and fragments. These diseases are mainly neoplastic or anaemias of the leukoerythroblastic type which are said to develop during the terminal stage of many neoplastic conditions.

It is difficult to analyse the results of the various investigations because blood was taken from different sites and in different quantities. Very few statistical analyses have been done of the data

given by different investigators. The results have been varied and there have been discrepancies in the conclusions drawn from them. The only agreement to be found in a general review is that a greater number of intact megakaryocytes are seen in the blood of cancer patients, with increased frequency in advanced cases, and that these cells also appear in the blood of patients with various disorders of the haematopoietic system, particularly if their nature is malignant. Various authors have noted the ectopic occurrence of these cells, mainly in the lungs and spleen.

The present study was designed to determine the incidence of circulating megakaryocytes in Wistar rats with tumours, and to compare this with the incidence seen in normal rats and rats with other diseases. Other experiments have been devised to find out what causes and controls the migration of megakaryocytes, and to determine the biological effect of neoplastic tissues on these cells.

MATERIALS AND METHODS

(GENERAL)

The Animals

Male, albino, Wistar strain of Norwegian rats, weighing between 220 and 320 gms. and bred in the Animal Department of the Royal Post-graduate Medical School were used throughout.

All the animals were kept in the animal quarters for at least 2 - 3 weeks before an experiment started, to get them acclimatized to the environmental conditions. For storage, 25 - 30 rats were kept in a single big cage, but during experiments the animals were kept singly in metal cages manufactured by Bowman's (large A9 and B6). The rats were given Diet 41B, manufactured and supplied by E. Dixon and Sons of Ware, Ltd., and water ad lib.

The temperature and humidity in the animal quarters were kept within a constant range, the temperature being between 65° and 72°C, and the humidity between 45% and 50%.

The albino Norwegian rats are a strain first started in a colony at the Wistar Institute of Anatomy and Biology at Baltimore in 1921. This particular strain was selected because other workers had found them ideal for cancer research. The rats are very resistant to infection. The incidence of spontaneous tumour development varies greatly from colony to colony with this strain, but in general it is certainly less than with the Copenhagen or Albany strains (Earle, 1935).

The growth rate of transplant tumours is more regular than with many other strains.

The Tumour (Fig. 2)

Walker 256 carcinoma tissue has been used for this investigation. It was supplied by the Chester-Beatty Research Institute, transplanted and maintained in the animal colony of our laboratory.

The tumour from which this strain has developed was first found by Dr. George Walker of Baltimore in 1928, and carried on by successive subinoculation in rats. The original tumour developed in the breast of a pregnant rat, which flared up after delivery. A histological section was examined by Dr. Walker (slide 1004) and Dr. H. G. McCallam of John Hopkins University, and diagnosed as carcinoma.

These tumours are generally hard, lobulated and smoothly rounded. Subcutaneous tumours show a well defined fibrous pseudo-capsule. In the case of old or very large tumours this may be incomplete or disrupted, which indicates invasion.

On macroscopic examination a section of the tumour shows three distinct zones. The peripheral layer forming the pseudo-capsule of the tumour is relatively thin. Central to this capsule is a ring of healthy growing tumour tissue which is slightly translucent. Its thickness varies between 1 and 10 mm in different areas of the tumour. The third, central area is generally necrotic and not well defined from the second layer.

The capsule consists of collagen fibres and is completely devoid of reticulin. Numerous, slender, deeply stained connective tissue cells

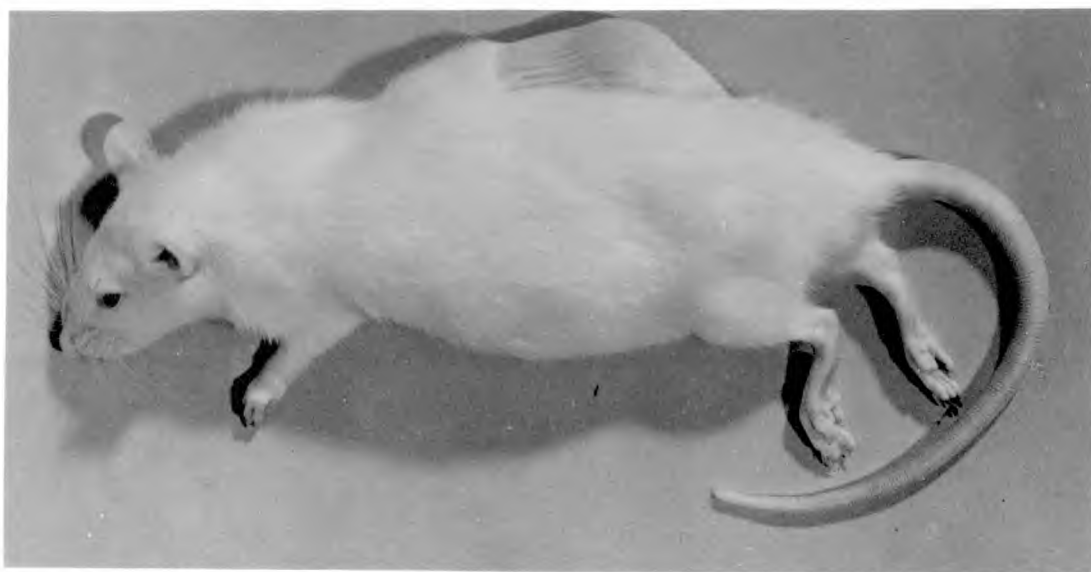


FIGURE 2.

Walker 256 tumour transplanted subcutaneously in the right flank of a Wister rat.

of fibroblastic appearance are seen on microscopic examination. Lymphocytic and monocytic infiltration of the capsule is uncommon, but a thick layer formed by a dense aggregate of small lymphocytes is seen just outside the capsule.

The second layer, the zone of healthy and active tissue, stains very deeply with H & E. It consists of larger cells which are elongated or polygonal. The nuclei are large, ovoid or spherical, with coarse chromatin granules and irregular reticulin, and always contain nucleoli. These cells are identified as epithelial cells. Another type of cell which is less numerous is associated with the stroma of the tumour. These cells are longer than the epithelial cells and have less prominent nucleoli due to the absence of chromatin. They are considered to be fibroblast-like cells. A third type of cell has elongated small nuclei with one or two nucleoli, and deeply stained cytoplasm, resembling the fibroblasts. Neither the appearance nor the number of these cells suggest the sarcomatous nature of the tumour (Earle, 1935).

Mitotic forms are abundant and are noted at a rate of 2 - 5 per oil immersion field. Abnormal mitotic forms, e.g. triploids and irregular types, are not infrequent.

In general, the cytological appearance of the intramuscular tumours is similar to that of the subcutaneous tumours. But there are certain differences, such as the absence of the capsule, a higher rate of growth, and increased thickness of the peripheral part of the growing

cell layer, giving a closer resemblance to Jensen's sarcoma (Earle, 1935). Unlike the subcutaneous tumours, the intramuscular tumours are surrounded by a layer of lymphocytes and mononuclear cells. They metastasize more frequently and behave like sarcomata.

Earle (1935) compared the structure of the tumour from Walker's laboratory with that from the Carnegie Institute of Embryology and confirmed that histologically they were similar. In our laboratory it has been established that the tumours we received about 3 years ago from the Chester Beatty Research Institute are also similar.

Subcutaneous tumours were chosen for our investigations for the various reasons discussed above, namely the infrequent metastases, constant rate of growth, and the smooth and rounded form which makes it easier to determine the tumour volume.

Anaesthesia

All operations were carried out under general anaesthesia, using ether and oxygen. Mortality and morbidity as a result of anaesthesia were practically nil.

Originally it had been attempted to anaesthetize the rats with a precisely controlled dose of 6% Nembutal in physiological saline, given by intraperitoneal injection, as it was felt that this should effectively dope the animals. But the method produced such a high mortality rate and a high incidence of pneumonia that it had to be abandoned.

Induction of anaesthesia was done by placing the animals for 1 - 2 minutes in a pyrex or perspex chamber containing ether. Then anaesthesia was maintained as described above. This technique achieved prolonged, quiet and uneventful performances.

Technique of Taking Blood Samples (Fig. 3)

For the study of circulating megakaryocytes, 1 ml. of blood was collected from the heart by the transthoracic route (Griffith and Farris, 1942). The animals were kept under light anaesthesia and placed in the supine position on a cork board, with the anaesthesia mask ready to hand in case of need. The forelegs were kept apart by the fingers of the left hand. The left index finger was used to determine the site of maximal cardiac thrill.

2 ml. Gallette disposable syringes treated with heparin citrate and fitted with size 15 needles were used. The needle was introduced into the thoracic cavity through the selected intercostal space, directed backwards and medially guided by the left index finger. It is often possible to locate the exact position of the heart by palpating carefully with the edge of the needle.

Gentle withdrawal of the plunger brought the cardiac blood into the syringe. 1 ml. of blood was always collected over a period of 1 to 1.5 minutes.

If the tip of the needle was still within the cardiac muscle, slight withdrawal followed by careful reintroduction always proved



FIGURE 3.

Technique of collecting blood from the rat's heart
by transthoracic route.

successful. It is essential (1) to avoid multiple cardiac punctures during any single session, (2) to withdraw the blood gently, and (3) to allow not less than one minute for the collection of 1 ml. of blood, to avoid the effects of trauma produced by this procedure. The collected blood was transferred to a clean universal container containing 0.1 ml. of heparin citrate. It was then gently rotated and shaken for about 2 minutes, so that the heparin was completely mixed with the blood to prevent clotting.

The Collection of Blood for Platelet Counts

The blood required for platelet counts was taken from the tail veins of the rats. The tail was shaved and cleaned with spirit. A small nick was made with a scalpel and the blood squeezed out. For successive platelet counts the site of the nick was moved from the distal to the proximal part of the tail.

The Technique of Tumour Transplantation (Fig. 4)

a) Materials

1. Tumour tissue - a portion of tumour tissue was selected from the second layer of the tumour (Fig. 4a and b), carefully avoiding necrotic material, blood clots and fibrous tissue. Healthy tumour tissue has a pink or pinkish white appearance, is translucent and of soft consistency.
2. Size 23 scalpel blade
3. Assortment of dissecting forceps

4. Two or three pairs of Petri dishes
5. A pyrex pestle and mortar
6. Endicott's filter of mesh No. 40, manufactured by Endicott's (Test Sieves) Limited, London
7. Pair of fine pointed scissors
8. 2 ml. disposable syringes
9. Size 15 needles
10. Sterile physiological saline
11. Counting chamber

b) Method

A portion of tumour tissue as described above was taken from a rat with tumour and placed in a Petri dish. The tissue was then carefully and thoroughly minced with the aid of a scalpel. The minced tissue was transferred to a mortar and very gently but thoroughly ground with the pestle for 4 - 5 minutes. It was then filtered through the Endicott's filter, and 2 - 3 ml. of sterile physiological saline were added to make a suspension (Fig. 4c,d,e). A counting chamber was used to determine the number of cells present. Approximately 5×10^6 to 6×10^6 cells were then injected subcutaneously into the right flank of each experimental animal in 4 to 6 ml. of suspension.

The transplant into other animals was done immediately after the suspension was made. Delay at this point causes failure of the tumour to grow in a very high percentage of rats. The whole procedure was

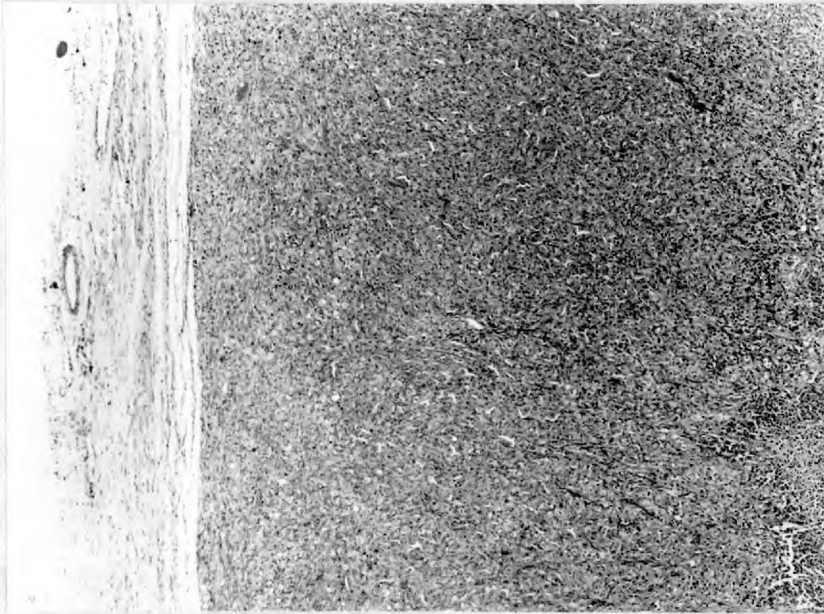


FIGURE 4a

Low power view of a section of Walker tumour X 40

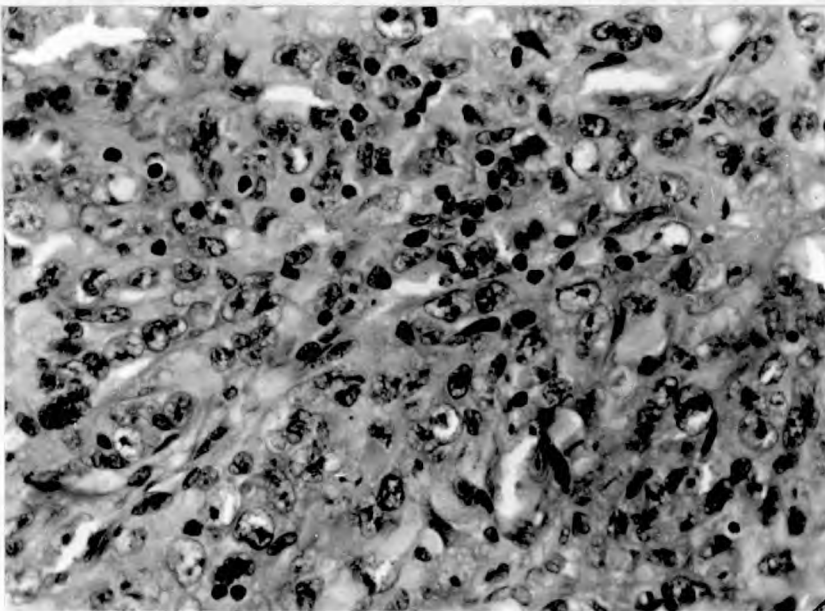


FIGURE 4b.

Same under high power X 400

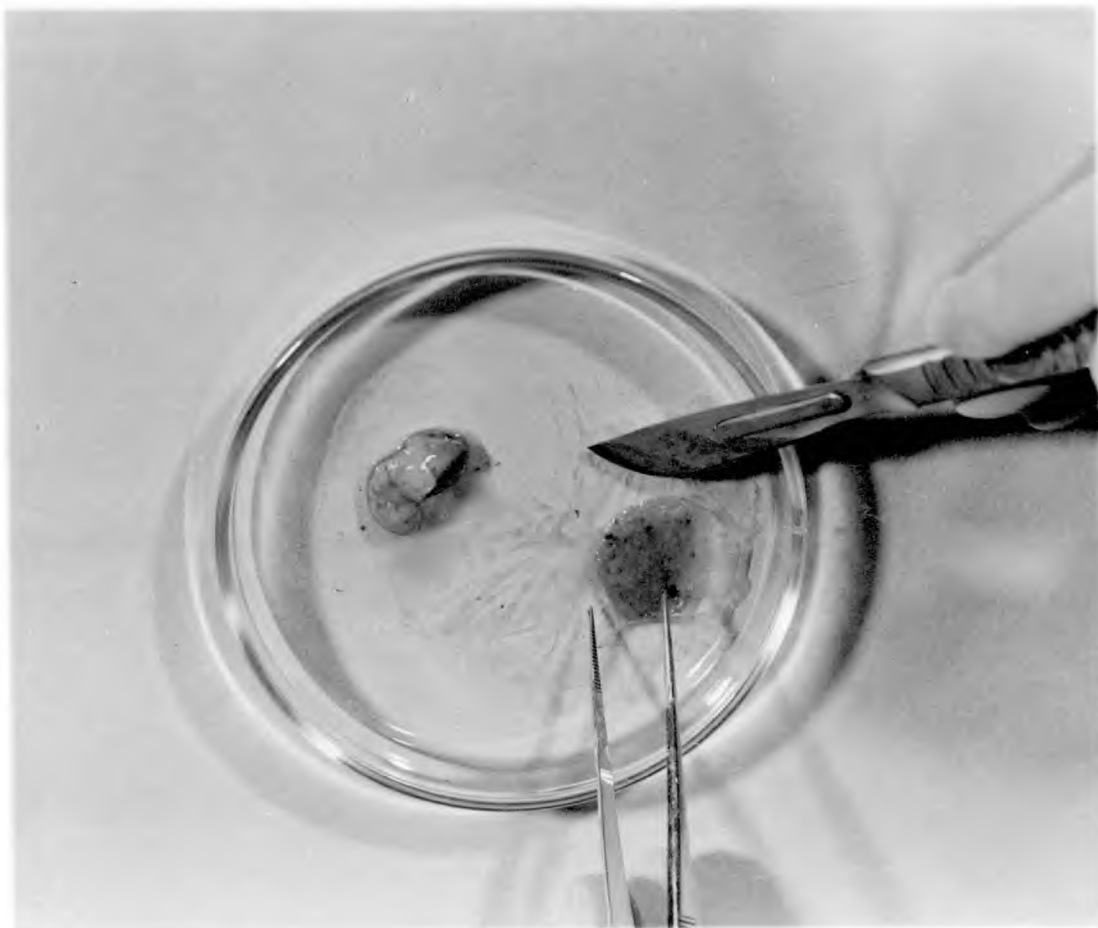


FIGURE 4c.

Technique of preparing a monocellular suspension of Walker tumour suitable for inoculation and cell count.



FIGURE 4d.

Technique of preparing a monocellular suspension of Walker tumour suitable for inoculation and cell count.

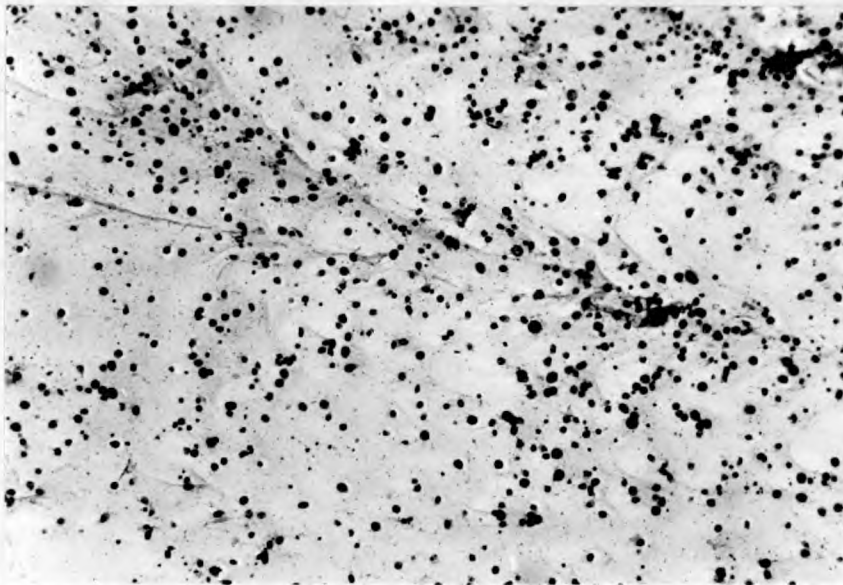


FIGURE 4e.

Smear of mono-cellular suspension of Walker tumour.

carried out under surgically sterile conditions.

The Isolation of Megakaryocytes

About 2058 blood samples from the 496 rats used in the experiment were examined. Each consisted of 1 ml. of blood mixed with 50 mg. of heparin to prevent clotting. All samples were processed within 2 - 3 hours of collecting the blood.

Approximately 100 mg. of carbonyl iron (grade SF, manufactured by Antara Chemicals, 435 Hudson Street, Hudson, New York) were added to each sample before it was incubated for 30 minutes at 37°C. During incubation the containers were placed in a roller mixer. Iron particles were phagocytosed by the polymorphonuclear leukocytes (Fig. 5).

Red cells were lysed by adding 1 ml. of 0.5% brown saponin in normal saline and shaking the container. Lysis is complete in 2 - 3 minutes, which is indicated by the sample becoming transparent.

Polymorphonuclear cells were removed with the help of a simple strong horseshoe magnet (Fig. 6). The remainder of the sample was poured into a conical centrifuge tube, centrifuged for 2 minutes at 3,000 r.p.m. and the supernatant fluid decanted carefully.

The deposit in the centrifuge tube was resuspended in 0.2 ml. of normal saline. About 2 ml. of a fixative made up of 10% formalin, 10% acetic acid and 50% absolute alcohol in distilled water was added to the suspension.

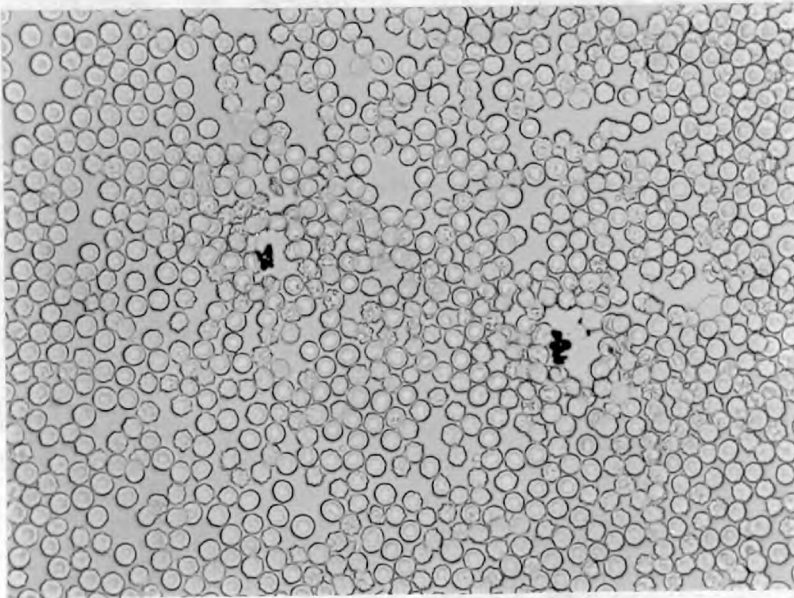


FIGURE 5.

Unstained smear of blood which shows phagocytosis of iron particles by polymorphonuclear leucocyte

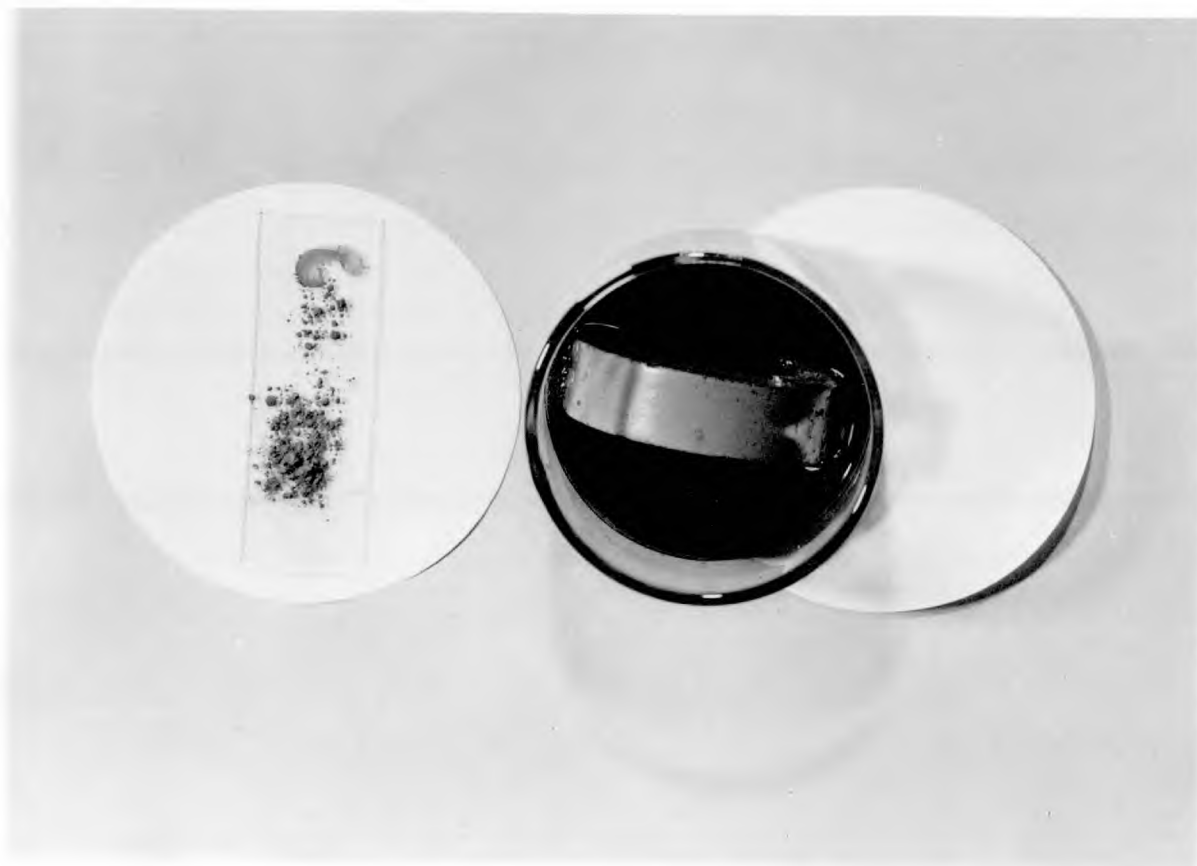


FIGURE 6.

Magnetic separation of polymorphs from the lysed blood.

Fixation was complete in about 10 minutes. At this stage the samples were sometimes left overnight for further processing on the following day. It was found that no change whatsoever occurred if a sample was left in the fixative overnight.

The magnetic process was repeated 2 or 3 times on the fixed samples. After this the suspension was filtered through a millipore filter with a pore size of 5 micron (specific no. SMWPO 190 R, manufactured by the Millipore Filter Co., Massachusetts, USA Inc.), using a suction pump.

Batches of 4 filter membranes were fitted in perspex membrane holders devised in the workshops of the Royal Postgraduate Medical School (Fig. 7). The membrane holders with the membranes were placed in formalin vapour for 30 seconds and then covered with 5% gelatin to prevent contamination between adjacent membranes.

The membranes were stained in Harris's acid haematoxylin for 5 minutes and then washed in running tap water for 5 minutes. They were differentiated in 1% HCl in 50% alcohol for 10 - 15 seconds and again washed in running tap water for 5 - 7 minutes. After placing them in Scott's tap water substitute for 5 minutes, the membranes were once more washed in tap water for 10 minutes. They were then counter-stained in 0.5% eosin and dehydrated in three changes of alcohol, first in 70% alcohol, and the last two in absolute alcohol. Finally the membranes were cleared in three changes of xylene, which made them transparent.

It was found that during the processing multiple ridges had



FIGURE 7.

Technique of filtration and setting millipore filter in the holder prior to staining.

developed in the membranes, making scanning very difficult. The difficulty was removed by placing the membranes between two filter papers and pressing with the ball of the thumb. This flattened the membranes and made scanning much easier.

The membranes were then mounted on 1" x 3" slides in Canada balsam under coverslips. Air bubbles were removed by gentle pressure. The slides were kept in a warm place, usually in an incubator at 37°C, for about 24 to 48 hours. After this they were ready for microscopic examination.

Scanning was done under low power, and the average time taken to scan one slide was 2 - 3 minutes. Intact megakaryocytes and all cells of similar morphology were looked for.

Determination of the Megakaryocyte Count

Both intact cells (Fig. 13a, b, c) and parts of megakaryocytes (Fig. 13d) have been found in the peripheral blood. These cells are either multinuclear or have a single multilobed nucleus. If such cells are denuded of their cytoplasm, it is difficult to distinguish them from other cells, for all one sees are a bare nucleus or fragments of it. A blood sample may contain a number of fragments from one and the same nucleus. If each fragment is counted separately, a false impression of the actual number of circulating megakaryocytes will be obtained. Various authors have reported that intact megakaryocytes are more frequently seen in the blood of patients with cancer. In the blood of normal

subjects, only bare nuclei of megakaryocytes were seen by some authors. With every technique employed, lymphocytes are included in the field, as they are with our technique. It has already been mentioned that a clump of lymphocytes may easily look like the bare nucleus of^a megakaryocyte (Fig. 10). The biological significance of the bare nuclei has not yet been determined. Nuclei of other nucleate haematopoietic elements, disintegrate and their nucleic acid content is used in the formation of new nuclear material.

If the megakaryocyte count in blood taken from normal subjects is higher than that found with cancer patients (Melamed, 1966), this must be attributed to fragmentation of these cells.

In the present study, only intact megakaryocytes were counted as "positive" cells. An interpretation of the stability and intactness of these cells is given in the chapter on discussion.

Determination of the Tumour Volume

The tumour volume was determined according to the technique described by Mori and his colleagues (1960). The method was one of external measurement. The measurements obtained by external palpation were consistently in agreement with the results of direct measurement. The tumour volume was calculated as follows:

$$\text{Tumour volume} = \frac{\pi}{6} \times pqr,$$

where p = the greatest external diameter

q = the smallest external diameter

r = the height of the tumour

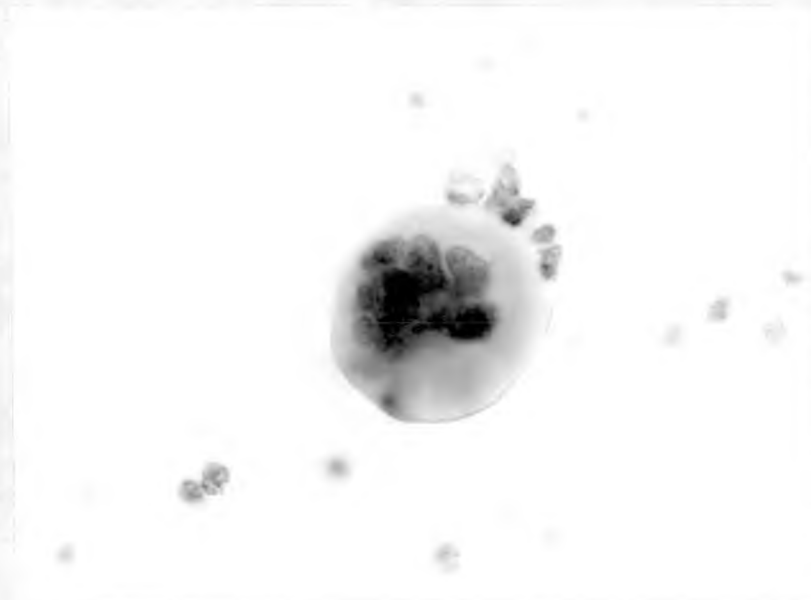


FIGURE 8.

Megakaryocyte isolated from peripheral blood



FIGURE 9.

Megakaryocyte isolated from peripheral blood.

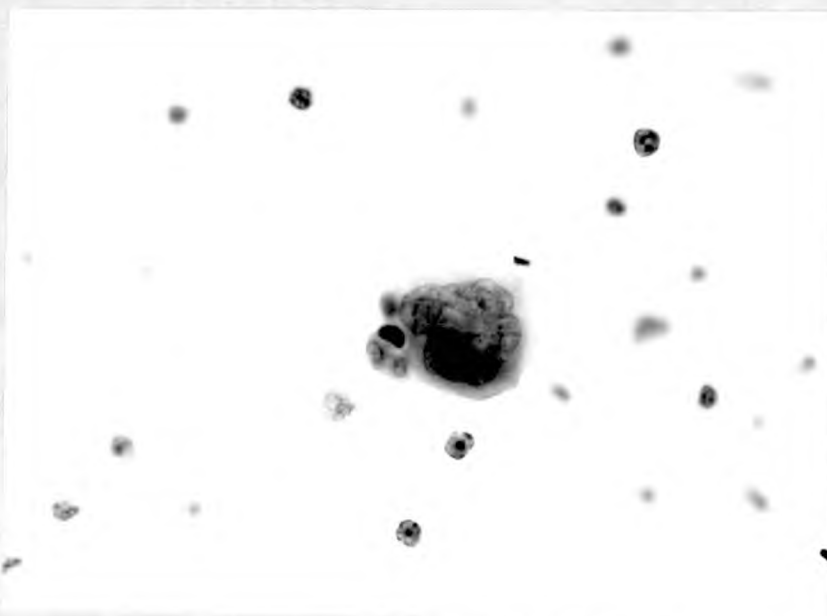


FIGURE 9a

Megakaryoblast isolated from peripheral blood.

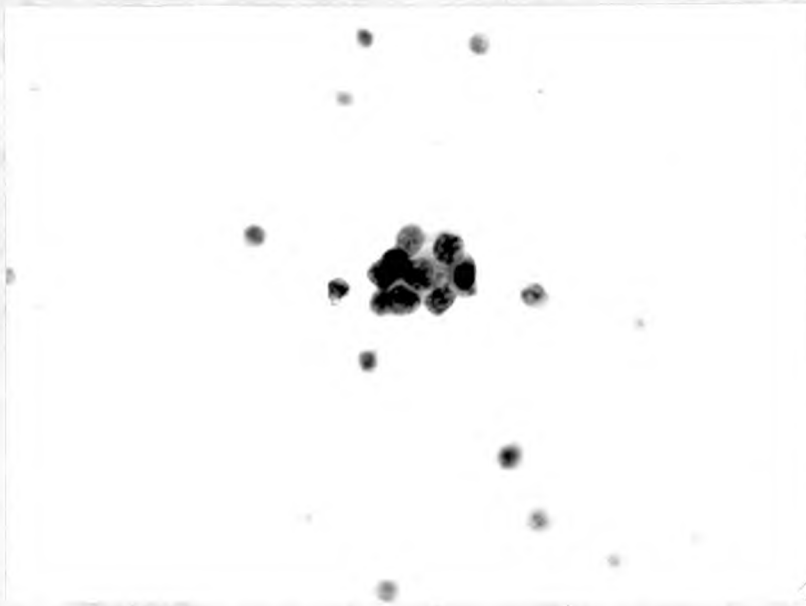


FIGURE 10

A clump of lymphocytes resembling bare nucleus
of megakaryocyte X 400

Vernier callipers were used to obtain the various dimensions. In several cases a mean of more than two measurements was taken because the tumour was lobulated.

Preparation of Tissue Homogenates (Fig. 12)

Tissue homogenates were prepared in an MSE (Measuring and Scientific Equipment Ltd., London, England) tissue homogenator. This has an enamel plated metal stand and a chromium plated pillar fitted with a stainless steel impeller shaft with turbine blades.

The tissue to be homogenized was weighed and placed in a 10 ml. vortex beaker. A measured volume of normal saline was added to the substance. The beaker was placed in another, larger beaker containing ice. Then the tissue in the beaker was brought under the blades which were immersed below the saline level and revolved at 6,000 r.p.m. for 2 minutes. The tissue homogenate thus prepared was drawn up into a syringe fitted with a millipore filter. This was done in the hope that only tumour homogenate without any intact cells would be injected. The filtrate was injected in the right flank, the site for tumour transplants. Before the injection a smear of the filtrate was taken, for further check-ups.

Attempts were made to carry out this process under dust free, clean and sterile conditions.

Technique of Platelet Count

Diluent used for the count:	3% trisodium citrate	3G
	1% formalin	1ml.
	distilled water	100ml.

0.02 ml. of blood taken from the tail vein was mixed with 2 ml. of the diluent in a biju bottle and well shaken for 2 to 3 minutes. Then the counting chamber was charged and platelets were counted in the 25 small central squares. The platelet count was determined as follows:

Total number of cells counted $\times 2,000$.



FIGURE 12.

Materials used for the preparation of tissue homogenates

- | | |
|---|---------------------------------|
| (1) 10 ml syringe | (4) Sterile universal container |
| (2) 13 mm diameter millipore filter of 5 μ pore size. | |
| (3) The Hypodermic Adaptor. | |

EXPERIMENTS AND RESULTS

Experiment 1

The Incidence of Circulating Megakaryocytes in Tumour-bearing Rats and Normal Controls

Rationale

A review of the literature shows that there is an association between circulating megakaryocytes and neoplastic disease. Most of the studies published have been the by-product of investigations on circulating cancer cells. No study has been made so far in a controlled animal system. There have been some animal studies, but no possible conclusions can be drawn from these, particularly as regards the incidence and significance of these cells in subjects with neoplastic tumours.

An investigation was therefore carried out to determine the incidence of circulating megakaryocytes in an experimental tumour system, and in a comparable group of normal rats which served as controls.

Materials and Methods

40 male Wistar rats were used in this experiment, their average weight being 250 gms. ($SD \pm 8.24$). They were divided into two groups of 20 each. The first group (a) served as controls and were given no treatment. The second group (b) received subcutaneous injections of a suspension of Walker tumour tissue in the right flank. The skin was shaved and painted with a 5% hibitane solution, and the animals were given very light anaesthesia, using ether and oxygen. Each animal in this group received 0.4 to 0.6 ml. of the suspension of Walker cells. That volume contained

approximately 5 - 6 million cells. The cell suspension was prepared by the method described in the previous chapter. Animals developing ulceration were excluded from this study and replaced by others with healthy looking tumours.

The date of inoculation was taken as day 1. Blood samples were taken from the rats in both groups on days 0, 7, 14 and 21, to determine the incidence of circulating megakaryocytes. 1 ml. of blood was taken from each animal on each of those days. The animals were killed after 21 days and a careful postmortem examination and histological studies were made.

Results

Only intact megakaryocytes were taken into consideration (Fig. 13a, b, c) and counted. Bare nuclei (Fig. 13d) and parts of nuclei were ignored, in accord with the considerations set out in the previous chapter. Apart from the megakaryocytes, various other cells of comparable morphology were also noted (Fig. 13a, f, g, h, i, j), but their numbers were not counted.

In the normal control group (Table 1 and Fig. 14), 5% of the 20 samples were found to be positive for megakaryocytes on day 0, nil on day 7, 10% on day 14, and again 10% on day 21. Of the 20 samples taken from tumour-bearing rats, none contained megakaryocytes on day 0, 25% on day 7, 55% on day 14, and 70% on day 21 (Table 2, Fig. 14)

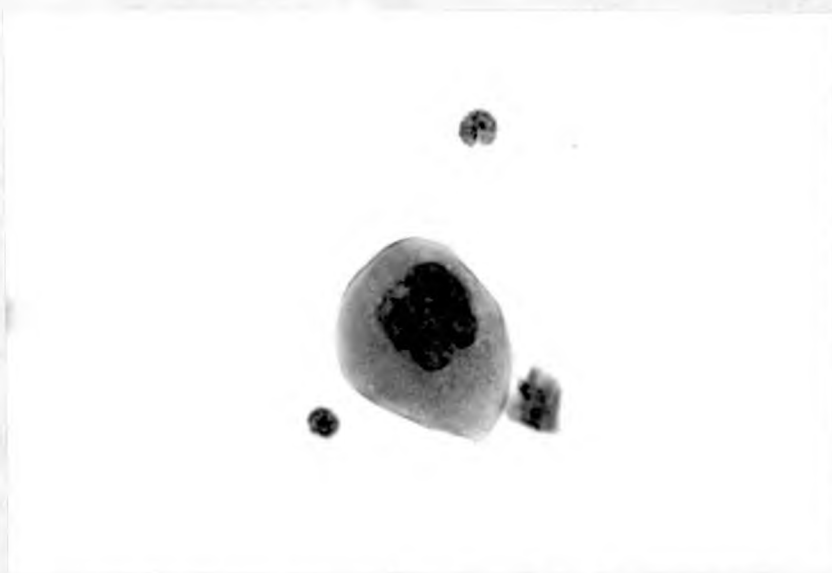


FIGURE 13a

Mature megakaryocyte isolated from blood

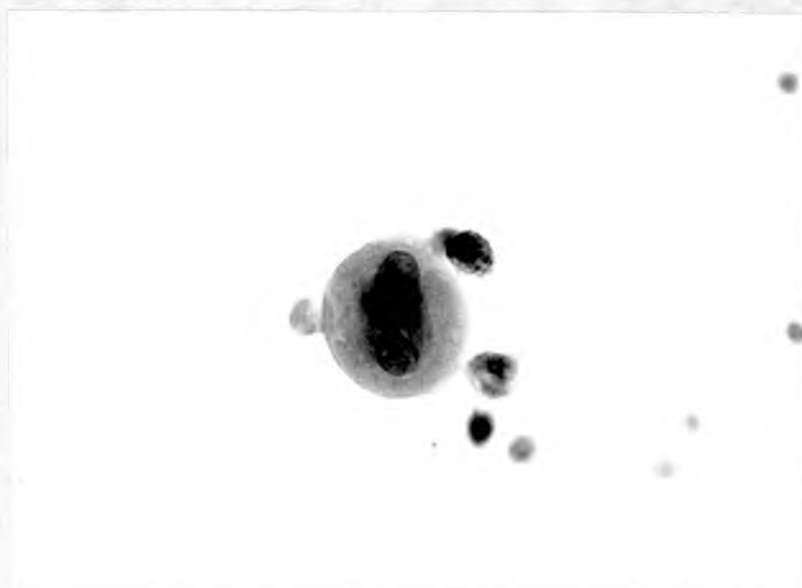


FIGURE 13b

Mature megakaryocyte isolated from blood

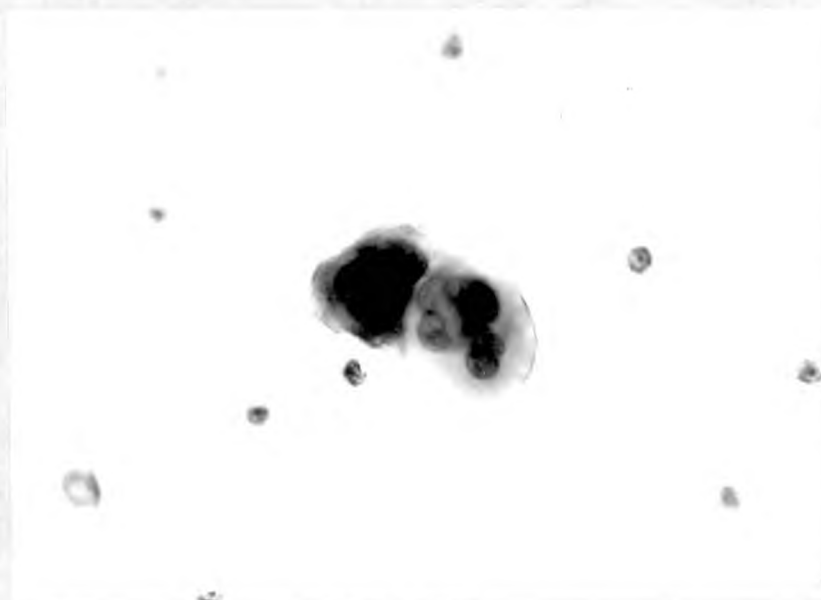


FIGURE 13c

Two megakaryocytes in blood

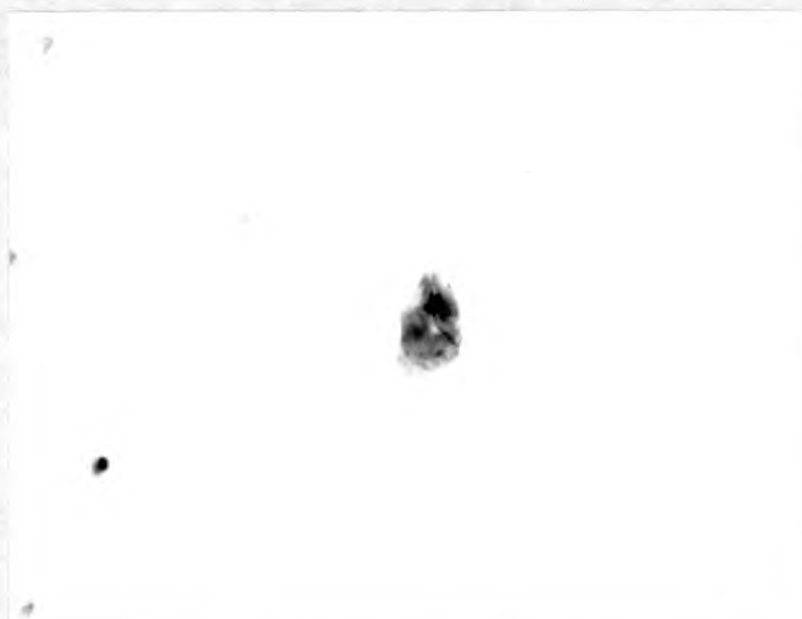


FIGURE 13d

A bare nucleus of megakaryocyte in blood

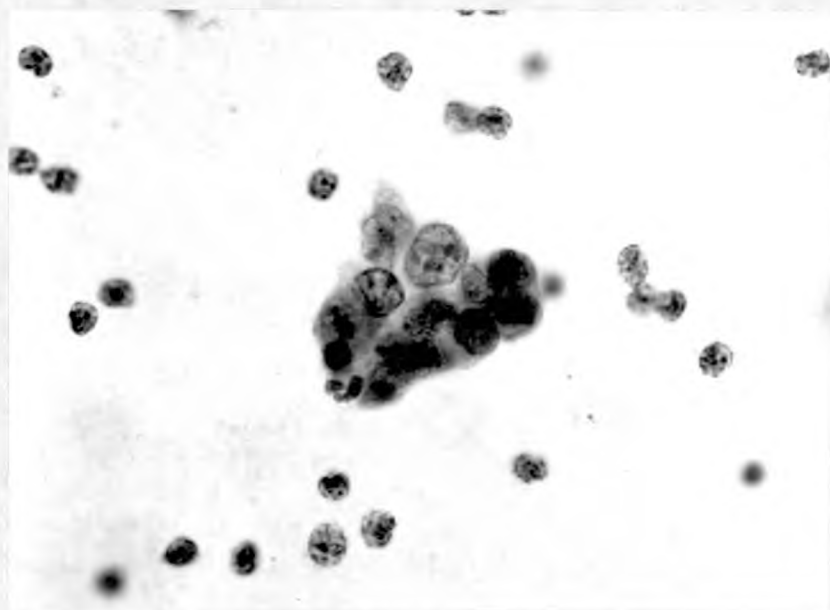


FIGURE 13e

A clump of tumour cells in blood

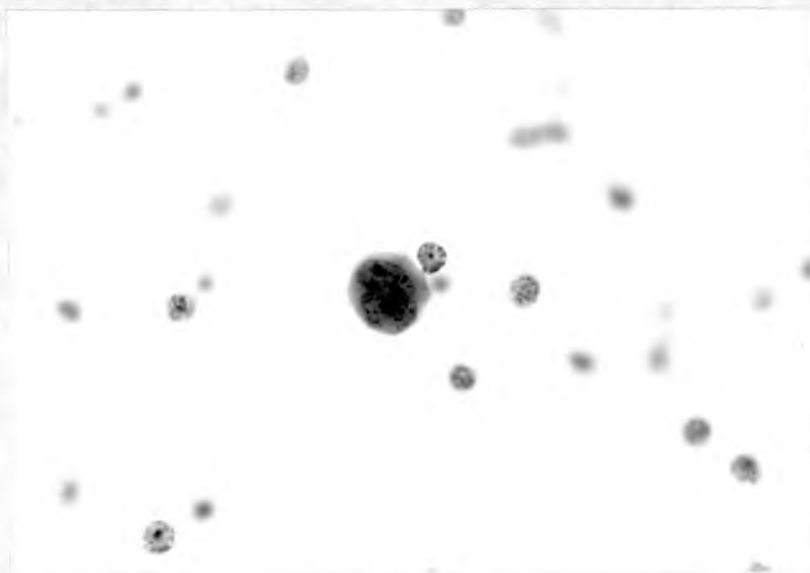


FIGURE 13f

Single tumour cell seen in blood



FIGURE 13g

One megakaryocyte and a tumour cell in blood



FIGURE 13h

Clump of bone marrow cells seen in blood



FIGURE 131.

Plasma cell in blood

Statistical Analysis

Statistical analysis showed no significant difference (at 5% level) between the results of days 0 and 7. But on day 14 ($P < 0.01$) and day 21 ($P < 0.001$) significant differences were established. If the results of the various days are taken together, the difference between the tumour and control groups was highly significant ($P < 0.001$), the incidence being higher in animals with tumours.

Discussion

Various authors have reported the presence of megakaryocytes in the peripheral blood of cancer patients, and their results have shown considerable variation. There are two obvious reasons for this discrepancy. Most investigators included the bare nuclei of megakaryocytes in their counts, and different techniques were used to isolate these cells from the blood. The danger of counting the parts of cells has already been discussed.

The results of the test show no significant difference in the incidence of megakaryocytes in the peripheral blood on the 7th day after inoculation. But a significant difference was found on the 14th day, and this became even more significant on the 21st day. So it may be assumed that as the neoplastic process advances these cells become more evident in the peripheral blood. This agrees with the findings of some investigators who state that megakaryocytes are more frequently seen in the peripheral blood of patients with advanced cancer than in early cases. The implications of this will be discussed in a later chapter.

TABLE 1. Incidence of circulating megakaryocytes in normal rats.

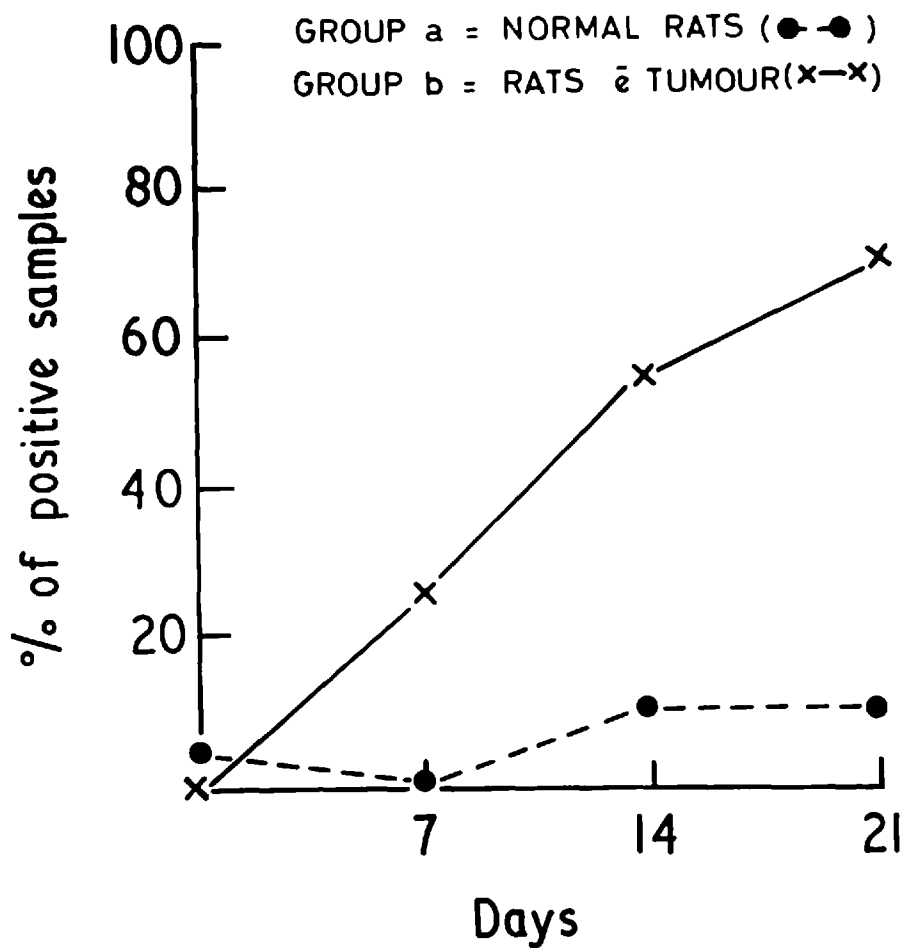
Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence
0	20	20	None	5%
7	20	20	None	0%
14	20	20	None	10%
21	20	20	None	10%

TABLE 2. Effect of transplanted tumour.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence
0	20	20	Subcutaneous Walker tumour transplant.	0%
7	20	20		25%
14	20	20		55%
21	20	20		70%

EXPERIMENT 1

Effect of transplanted tumour



ON 14 DAYS

$$\chi^2 = 7.294$$

$$P = < 0.01$$

ON 21 DAYS

$$\chi^2 = 12.604$$

$$P = < 0.01$$

FIGURE 14.

The incidence of megakaryocytes in Experiment I
(Table I and Table 2)

When the results for each group are combined and compared, the difference becomes highly significant. This indicates that the presence of a neoplastic tumour could indeed be a cause for the migration of megakaryocytes from their anatomic site to the peripheral blood.

There has been no comparative study in an experimental animal system. But the findings of this study are to some extent similar to those of Traycovich (1964), who showed that megakaryocytes were found in the peripheral blood of 64% of animals with tumours of various histological character in different anatomic sites. Unfortunately his report gives no details of the stage of the disease, and he had no normal controls.

Experiment 2

Effect of General Anaesthesia and Laparotomy

Rationale

Investigators concerned with the study of basic biological problems have been using general anaesthetics as a laboratory tool only (Wilson, 1902). It has been known for years that volatile anaesthetics may inhibit cell division. Henderson first mentioned this in 1930. The mechanism of the effect is still unknown. Recently Bruce et al. (1966) suggested that in the case of halothane an alteration of the structure and function of the cytoplasm may be the basis of inhibition of cell division. Halothane makes the peripheral cytoplasm rigid. The nature of this change is unknown. Bruce et al. observed granulocytopenia after general anaesthesia with halothane. In the bone marrow they noted an increase in the ratio of dividing to maturing marrow granulocytes. Lassen et al. (1956) reported that granulocytopenia and thrombocytopenia developed after prolonged application of nitrous oxide, and Kieler et al. (1957) described this gas as a "mitotic poison". Granulocytopenia and lymphocytopenia as the result of general anaesthesia had been observed as early as 1928 by Goldman. Recently Green (1963) reported a decrease in the peripheral leukocyte count after general anaesthesia. He also noted gross damage to all cellular elements except the megakaryocytes, which were unaffected and maintained normal morphology.

There is sufficient evidence that general anaesthesia does

cause changes in the cell count of peripheral blood and also alters the character of the bone marrow. No data are available on the effect of ether and oxygen in this respect. As these substances were used in the present experiments, it was felt that their effect on the peripheral megakaryocyte count should be determined, at least as a control study.

It has been claimed that surgical trauma causes an increase in the number of megakaryocytes in peripheral blood (Hume, 1964). This increase has also been associated with a diurnal variation in the megakaryocyte count. There is no definite evidence to suggest surgical trauma as the cause of the increase in circulating megakaryocytes. Williams et al. (1955) had suggested this possibility after demonstrating a drop in the platelet count and an increase in megakaryocytes in the bone marrow following surgical interventions.

In the course of the present investigation several operative procedures have been used. It was, therefore, essential to determine whether simple operative procedures caused any changes in the peripheral megakaryocyte count.

Materials and Methods

24 male Wistar rats with a mean weight of 267 gms. ($SD \pm 9.18$) were used in this experiment. General anaesthesia was given by the method described in the previous chapter, using ether. Anaesthesia was maintained for 10 minutes before blood was collected for examination.

Technique of Laparotomy

The anterior abdominal wall of the anaesthetized rats was shaved and painted with 5% hibitane. A midline incision was made, starting 1 cm. below the xiphoid process and 3 cm. long. The rectus sheath was split by incision and the peritoneal cavity exposed. After palpation of the intra-abdominal structures, catgut No. 1 was used to close the peritoneum and suture the rectus sheath. The skin was apposed with Mitchel's clips and the wound dressed with dry gauze. The Mitchel's clips were removed after 7 days.

Blood for the determination of circulating megakaryocytes was collected on days 0, 7, 14 and 21, as in the previous experiment.

Results

The rats in this study had been divided into two groups of 12. The first group (a) had been given general anaesthesia only and the incidence of megakaryocytes in their blood was nil on day 0, 8.3% on day 7, 8.3% on day 14, and 16.7% on day 21 (Table 3 and Fig. 15). The second group^(b) had a laparotomy done under general anaesthesia. Here the incidence of circulating megakaryocytes was 8.3% on day 7, 16.7% on day 14 and 16.7% on day 21.

Statistical analysis showed no significant difference between the results for the two groups on corresponding days. Nor was there any significant difference when the results of individual days were added up and compared. Comparison between the percentage of positives found in

TABLE 3. Effect of general anaesthesia.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in blood
0	12	12	General anaesthesia	0%
7	12	12		8.3%
14	12	12		16.7%
21	12	12		16.7%

TABLE 4. Effect of laparotomy.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in blood
0	12	12	Laparotomy	8.3%
7	12	12		8.3%
14	12	12		16.7%
21	12	12		16.7%

EXPERIMENT 2

Effect of general anaesthesia + laparotomy.

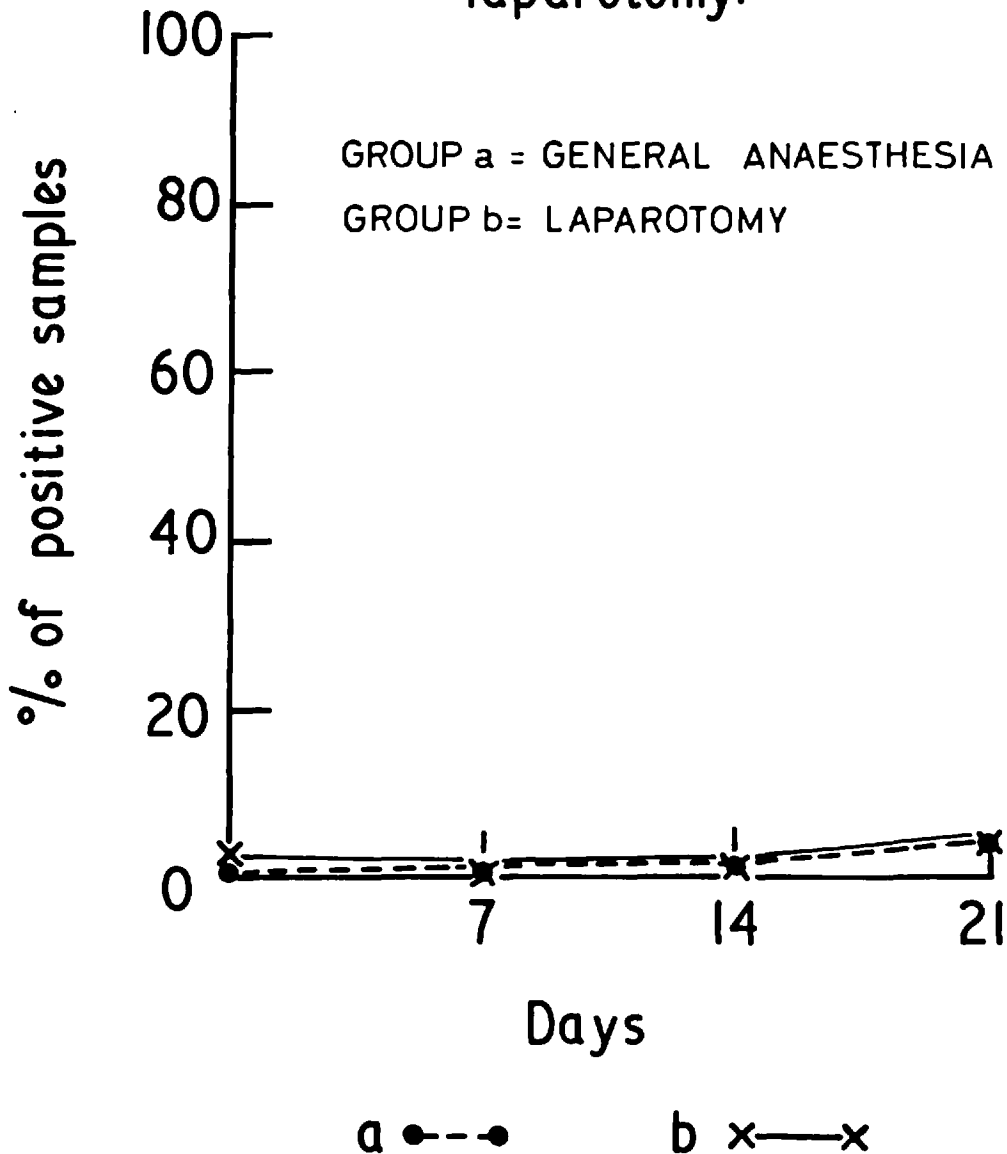


FIGURE 15.

Graphic representation of Tables 3 and 4

either of the two groups and control group (a) of Experiment 1 also failed to reveal any significant difference.

Discussion

Different investigators have shown that granulocytopenia, lymphocytopenia and thrombocytopenia occur as a result of general anaesthesia. Nothing has been reported so far on the megakaryocyte count in peripheral blood after general anaesthesia. Hume (1964) claimed to have observed an increase in circulating megakaryocytes after surgical operations in human patients, but she associated the increase with a diurnal variation in the number of these cells in the peripheral blood. Apart from a suggestion made by Belko (1959), there is no definite evidence that megakaryocytes are associated with surgical trauma.

Circulating megakaryocytes have been studied in two groups of rats, one of which had general anaesthesia only, and the other laparotomy under general anaesthesia. A comparison of the incidence of these cells in the two groups on corresponding days showed no significant difference. Both groups also failed to show significant differences when compared with a normal control group. These findings demonstrate that neither general anaesthesia nor surgical trauma affect the incidence of megakaryocytes in the peripheral blood.

Experiment 3

The Effect of Non-specific Substances and Granuloma

Rationale

There is nothing in the literature to indicate whether the subcutaneous injection of bland or irritant substances, or a subcutaneous granuloma, produces an increase in the number of circulating megakaryocytes. Subcutaneous injections of turpentine produce nasty looking ulcerating granulomata. Morphologically these give the impression of a tumour. Arachis oil is a bland oil which resembles the tumour suspension without its biological activity. Saline acts as a foreign fluid when injected subcutaneously. Thus we have three possible physical properties of a tumour or tumour cell suspension which may be studied separately.

Materials and Method

30 rats with a mean weight of 264 gms. ($SD \pm 8.73$) were divided into three groups of ten each. The first group (a) were given a subcutaneous injection of 0.2 ml. of turpentine in the right flank. Injections were given with a No. 15 needle on the 1st, 3rd, 5th and 9th days. The rats in group two (b) were given arachis oil and those in group three (c) saline, in the same amount and at the same intervals as for group one. The skin of the flank was shaved before giving an injection, and sterile procedure was maintained as far as possible.

Results

In the first group of rats (a) which had been injected with turpentine, none of the blood samples taken on days 0 and 7 were positive. On day 14, 20% of the 10 samples were positive, and on day 21 again 20% were positive (Table 5, Fig. 16).

Group two (b) were injected with arachis oil. On day 0, none of the ten blood samples taken were positive. On days 7, 14 and 21, 10% were positive (Table 6, Fig. 16).

Group three (c) were injected with saline. The incidence of positive samples was as follows: on day 0, none; on day 7, 20%; on days 14 and 21, 10% of the ten blood samples (Table 7, Fig. 16).

Statistical Analysis

Chi square analysis of the results of the three groups on corresponding days showed no significant differences. When the results of different days were combined and compared, these too showed no significant differences. Nor were significant differences observed on comparing the three experimental groups with the normal control group of rats (Expt. 1, Gr. a)

Discussion

Various toxins and drugs have been reported to cause changes in the peripheral blood cell count. But there is nothing in the literature to indicate whether the incidence of circulating megakaryocytes is affected. Three substances, turpentine, arachis oil and saline were selected for

TABLE 5. Effect of turpentine injected subcutaneously.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in blood
0	10	10	Subcutaneous injection of turpentine	0%
7	10	10		0%
14	10	10		20%
21	10	10		20%

TABLE 6. Effect of arachis oil injected subcutaneously.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in blood
0	10	10	Subcutaneous injection of arachis oil	0%
7	10	10		20%
14	10	10		30%
21	10	10		10%

TABLE 7. Effect of normal saline injected subcutaneously.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in blood
0	10	10	Subcutaneous injection of normal saline	0%
7	10	10		10%
14	10	10		10%
21	10	10		10%

reasons given above. The systemic actions of these substances appear to have produced no demonstrable effect on the incidence of megakaryocytes in the peripheral blood. So there is no reason to think that the physical properties of Walker tumour and tumour cell suspension have any influence on the incidence of megakaryocytes in the peripheral blood.

EXPERIMENT 3

Effect of non-specific substances.

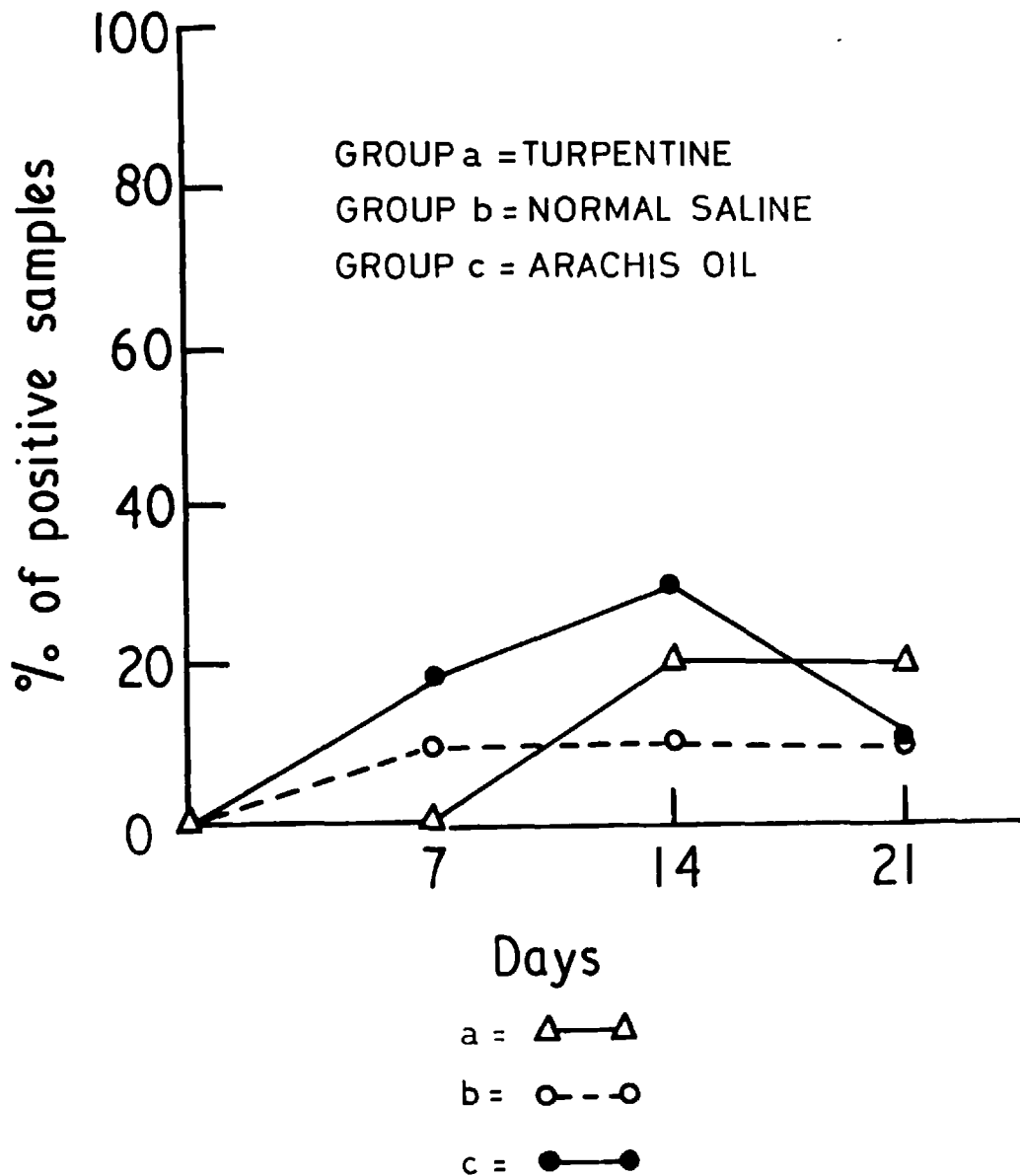


FIGURE 16.

Graphic representation of Tables 5, 6 and 7

Experiment 4The Effect of ACTHRationale

The effects of adrenocorticotrophic hormone on blood and on the haematopoietic system are well documented. Antopal (1950) noted lymphopenia in the peripheral blood and lymphoid hyperplasia after injection of massive doses of ACTH. Eosinopenia and lymphopenia after injections of cortisone and adrenocorticotrophic hormone were reported by several investigators (Hill, 1948; Quittner, 1951; Yoffee, 1956). Repeated injections of ACTH cause an increase in the cellularity of bone marrow (Yoffee, 1967). Only the myeloid and erythroid elements were found to be increased, with lymphocytes remaining unaffected (Yoffee, 1952). Adrenocorticotrophic hormone directly or indirectly stimulates the formation of cells in the bone marrow (Hudson, 1952). Eosinopenia and thrombocytopenia were observed together with increased megakaryocyte activity in the bone marrow as a result of surgery and also as a result of single or repeated doses of adrenocorticotrophic hormone (Williams, 1961). Unfortunately nothing has been reported on the effect of ACTH on the incidence of circulating megakaryocytes. It has been demonstrated that surgical trauma increases ACTH activity in the blood (Sayer, 1949).

Investigations on the incidence of megakaryocytes in the peripheral blood after injections of adrenocorticotrophic hormone therefore seemed

indicated. It was also thought necessary to study the combined effect of this hormone and surgical trauma.

A controlled experiment was planned to compare the effect of ACTH with that of a substance of high molecular weight, so that the real effect of ACTH could be elucidated.

Materials and Methods

40 rats with an average weight of 257 gms. ($SD \pm 7.57$) were divided into four groups of ten each. The first group (a) were given subcutaneous injections of 10% W/V Rheomacrodex which contains Dextran BMI 10gms., NaCl .9 gm. and water for injection to make up 100 ml. Each animal received 0.2 ml. in the right flank on the 1st, 4th, 7th, 10th, 13th, 16th and 19th days, so that the local concentration of the drug was maintained.

The second group of rats (b) were injected with 5 units of ACTH in the right flank on the 1st, 4th, 7th, 10th, 13th, 16th and 19th days. The repeated injections served to maintain a high local and systemic level.

The rats in group three (c) had a laparotomy done on the 1st day and were given the same Rheomacrodex injections as group (a).

Group four (d) also had a laparotomy done on the 1st day and received the same ACTH injections as group (b). The technique of laparotomy was exactly the same as described for the earlier experiment.

Blood was taken from the heart on day 0, 7, 14 and 21, and examined for megakaryocytes.

Results

In the first group of rats (a), who had been given subcutaneous injections of Rheomacrodex, 0% of the 10 blood samples were positive on days 0, 7, 14 and 21 (Table 8, Fig. 17). In group two (b) who had received subcutaneous injections of ACTH, 0% of the 10 blood samples were positive on days 0 and 7, 10% on days 14 and 21 (Table 9, Fig. 17).

In group three (c), laparotomy and Rheomacrodex, the results were 0% on day 0, 30% on day 7, 20% on day 14 and 20% on day 21 (Table 10, Fig. 17). In the fourth group (d), laparotomy and ACTH, 0% of the ten blood samples were positive on day 0 and 20% on day 7, and days 14 and 21 had 30% and 10% positive respectively (Table 11, Fig. 17).

Statistical Analysis

The combined results of the different days were statistically analysed. No significant differences were found between any two groups. Comparison of the results of each group with the normal control group also revealed no significant differences.

Discussion

Other workers have observed that after ACTH therapy or surgical operations the thrombocyte count in the blood goes down and the megakaryocyte count in the bone marrow goes up. The results of the above experiment indicate that no significant change occurs in the incidence

TABLE 8. Effect of Rheomacrodex.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence
0	10	10	Subcutaneous Rheomacrodex	0%
7	10	10		0%
14	10	10		20%
21	10	10		20%

TABLE 9. Effect of Adrenocorticotrophic hormone.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence
0	10	10	Subcutaneous injection of ACTH	0%
7	10	10		10%
14	10	10		10%
21	10	10		0%

TABLE 10. Effect of Laparotomy and Rheonaerodex.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in blood
0	10	10	Laparotomy and Rheonaerodex	0%
7	10	10		30%
14	10	10		20%
21	10	10		20%

TABLE 11. Effect of Laparotomy and Adrenocorticotrophic hormone.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in blood
0	10	10	Laparotomy and ACTH	0%
7	10	10		20%
14	10	10		30%
21	10	10		10%

of circulating megakaryocytes after the administration of ACTH, either alone or in combination with surgical operation. It is therefore obvious that ACTH on its own does not effect the megakaryocyte level in the blood. Combining our results with those of others it may be assumed that the incidence of circulating megakaryocytes is not related to thrombocytopenic conditions of the blood or to an increased megakaryocyte count in the bone marrow. It has been suggested that the megakaryocyte count is controlled by a feed back mechanism of the blood platelet level (Prizivoit, 1964). Detailed data on the relation between the blood platelet level and the incidence of circulating megakaryocytes are given in a later chapter.

EXPERIMENT 4

Effect of surgery, ACTH and macro-molecular substances.

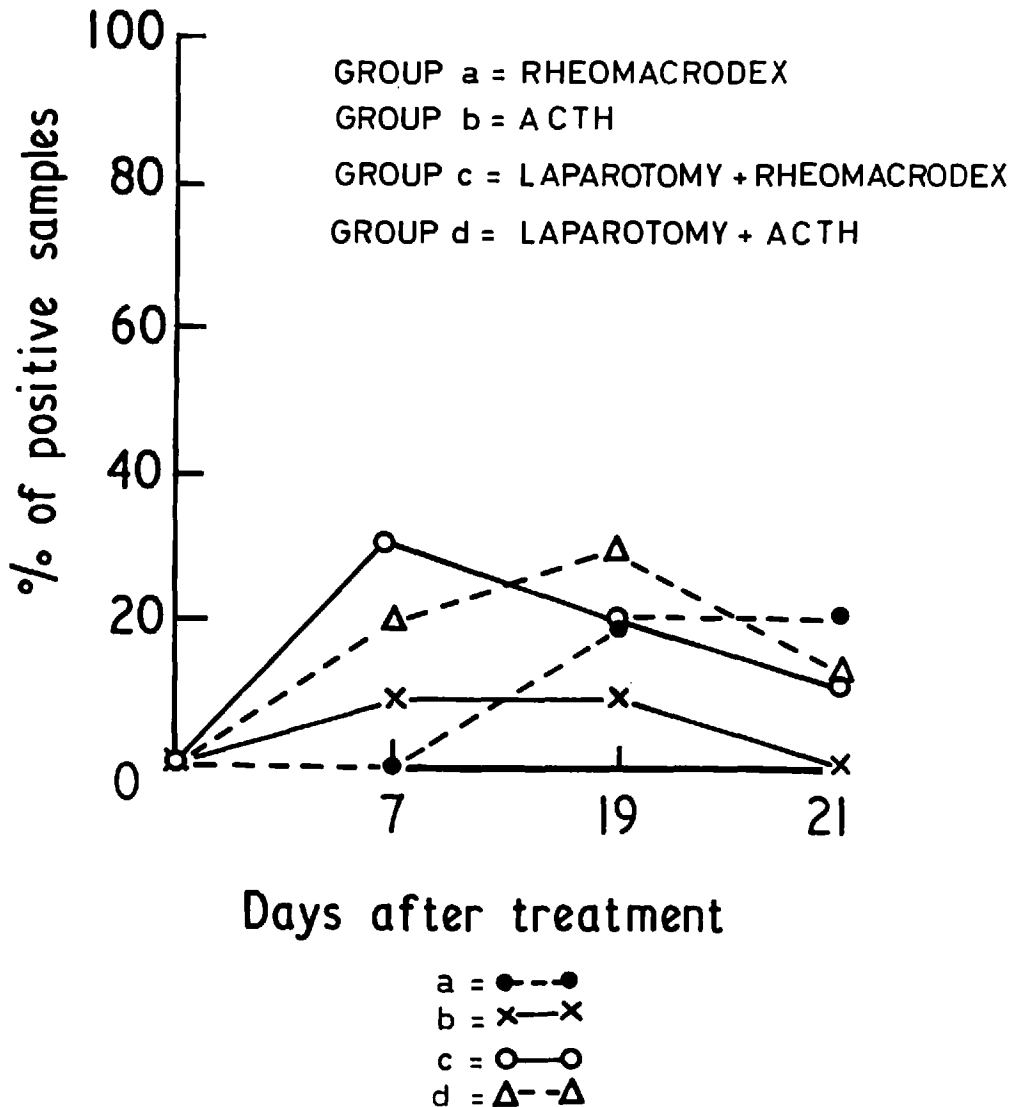


FIGURE 17.

Graphic representation of Tables 8 , 9 , 10 and 11.

Experiment 5

The Effect of Excising the Tumour

Rationale

Several investigators have reported that in patients with cancer the incidence of circulating megakaryocytes, particularly intact ones, is increased. This has already been discussed in the historical review given in the first chapter. Experimental evidence from animal studies so far has been inadequate. In the present study, the first group of experiments have already shown that the incidence of circulating megakaryocytes is significantly increased in animals with tumours. Various other control experiments have revealed that the deviations observed in the control groups were of no significance.

As it appeared that the significant increase in the incidence of circulating megakaryocytes was related to the presence of the tumour, it was decided to observe the changes that occur after excision of the tumour.

Materials and Methods

44 male rats were divided into three groups. Group one (5a) consisted of 12 normal rats, and a simple skin excision was made in the flank of each. Group two consisted of 20 rats (5b) with inoculation tumours which were later removed completely. Group three (5c) consisted of 12 rats only. These were given tumour inoculations and later part of

the tumour was excised. The mean weight of these rats was 298.30 gms. (SD $\pm$ 9.58).

The technique of tumour inoculation was the same as in the previous experiments. It had then been found that about 14 days after the tumour transplantation circulating megakaryocytes would be found in approximately 50% of the samples. It was decided, therefore, to grow the tumours for 14 days and then excise them either completely or incompletely.

Technique of Skin Excision

The rats were anaesthetized and the skin was painted with 5% hibitane. A spindle-shaped piece of skin, 3 cm. by 2 cm., was removed together with the subcutaneous tissue. The wound margins were apposed in two layers, as in the other two groups. Michel's clips and a light sterile dressing were applied and removed on the 7th day.

In group one (5a) which had only had the skin excision, blood was drawn on days 0, 7, 15, 21 and 28. In group two (5b) with total excision of the tumour, blood was taken on days 0, 7, 14, 21, 28 and 50, and in group three (5c) with partial excision, on days 0, 7, 14, 21 and 28. In the group with partial excision all the rats died shortly after the 28th day, so that further follow-up became impossible.

Technique of Complete Excision of the Tumour

The animals were given general anaesthesia. The skin over the tumour and in the surrounding area was shaved with electric clippers.

Great care was taken to avoid handling the tumour more than necessary and to avoid traumatizing the local skin.

The operation was performed under completely sterile conditions. The tumour was excised together with a 1 cm. margin of healthy skin. The whole thickness of subcutaneous tissue was removed with the tumour, leaving a clean fascial base. Bleeding points were clamped and ligated. The wound margins were apposed and closed in two layers, first the subcutaneous layer and then the skin. Good apposition was achieved without difficulty, because the skin of rats is very loose. Michel's clips were used to hold the skin together, and the wound was covered with clean gauze and a small piece of elastoplast. This dressing and the Michel's clips were removed on the 7th day.

Partial Excision of the Tumour

The technique used for partial removal of the tumour was essentially the same as that of total excision. The tumour was dissected out, keeping its blood supply intact. The so-called capsule of the tumour was incised beforehand. Then approximately two thirds of the tumour were removed and the capsule was sutured. Closure of the wound and dressings were as for total excision.

Results

Group one (5a) consisted of normal rats who had had a skin excision only. On day 0 none of the 12 blood samples were positive for

megakaryocytes. On day 7, 8.3% and on day 14, 16.7% of the 12 samples were positive; on day 21, 25% of 12 samples and on day 28, 16.7% of 12 samples were positive (Table 12, Fig. 18).

Group two (5b) had had the total tumour removed. On day 0, the tumours were 15 days old and the average volume was 4.818 cc ($SD \pm 1.102$). 60% of the 20 blood samples taken on day 0 were positive for megakaryocytes. On day 7, i.e. 7 days after complete excision of the tumour, 70% of the 20 samples taken were positive. On day 14, 45% of 20 samples were positive, on day 21, 26.3% of 19 samples, and on day 28, 33.3% of 19 samples. The rats were kept alive until the 50th day, when the last sample was drawn. On day 50, 27.8% of 18 samples were positive. (Table 13, Fig. 18).

A careful postmortem examination of every rat was done and suspected tissues were examined for possible metastases. In fact, two rats died before the 50th day and both had lung metastases (Fig. 19). One of them had also a local recurrence at the site of excision.

Group three (5c) consisted of 12 rats with partial excision of the tumour. On day 0, the tumours were 15 days old, with an average volume of 4.846 ($SD \pm .921$). On day 0, 58.3% of 12 blood samples were positive for megakaryocytes. On day 7, i.e. 7 days after partial excision of the tumour, 50% of the 12 samples were positive; on day 14, 58.3% of 12 samples; on day 21, 66.7% of 12 samples, and on day 28, 87.5% of 8 samples. By the 28th day, 4 of the 12 rats had died and all the rats were dead by the 35th day (Table 14, Fig. 18).

Careful postmortem examinations and histological studies were done on these rats. Two of the 12 also had lung metastases. The rest died because of the huge local tumour.

Statistical Analysis

In group two (5b) with complete excision of the tumour, comparison of the results of individual days after excision with those of day 0 showed no significant differences on days 7, 14 and 28. But a significant difference at 10% level was found on days 21 and 50. On day 21, $\chi^2 = 3.230$ and on day 50, $\chi^2 = 2.706$.

Similar comparisons in group three, with partial excision, and group one, normal rats with skin excision, showed no significant differences between the results obtained before and after the operation.

When the results of the group with complete tumour excision were compared with those of the group with skin excision, significant differences were seen on days 0 ($P < 0.01$) and 7 ($P < 0.01$). The other days were not significant.

Comparison of the results for the group with partial excision of the tumour and the group with skin excision showed a significant difference on day 0 ($P < 0.01$), no significant difference on days 7, 14 and 21, and again a significant difference on day 28 ($P < 0.01$).

When the results of complete excision were compared with those of partial excision, no significant differences were seen on days 0, 7

TABLE 12. Effect of excision of skin.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in blood
0	12	12	None	0%
7	12	12	Excision of skin	8.3%
14	12	12		16.7%
21	12	12		25%
28	12	12		16.7%

TABLE 13. Effect of complete excision of tumour.

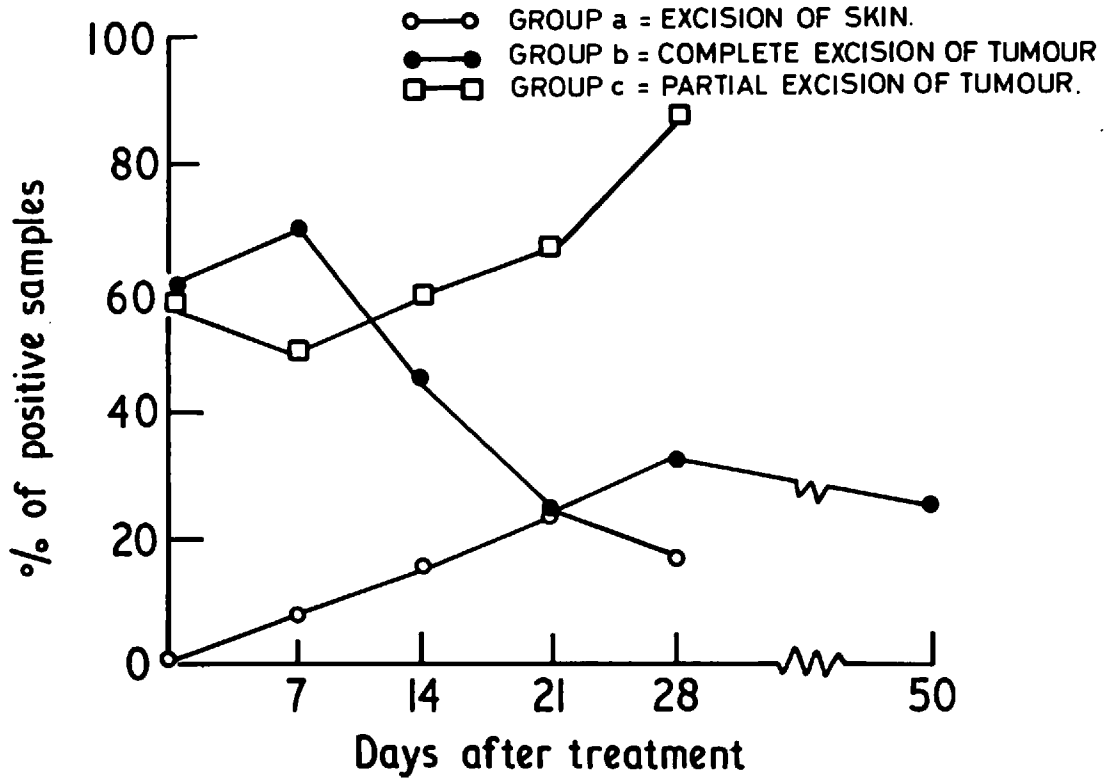
Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in blood
0	20	20	None	60%
7	20	20	Complete excision of tumour	70%
14	20	20		45%
21	19	19		26.3%
28	19	19		33.3%
50	18	18		27.8%

TABLE 14. Effect of partial excision of tumour.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in blood
0	12	12	None - tumour transplanted 19 days ago	58.3%
7	12	12	Partial excision of tumour	50%
14	12	12		58.3%
21	12	12		66.7%
28	8	8		87.5%

EXPERIMENT 5

Effect of excision of tumour



GROUP b V GROUP a (EXPT 1)

'0' DAY $\chi^2 = 11.396$ $P < 0.001$

21 DAY = NO SIGNIFICANT DIFFERENCE

GROUP c V GROUP a (EXPT 1)

'7' DAY $\chi^2 = 9.244$ $P < 0.01$

FIGURE 18.

Graphic representation of Tables 12, 13 and 14.

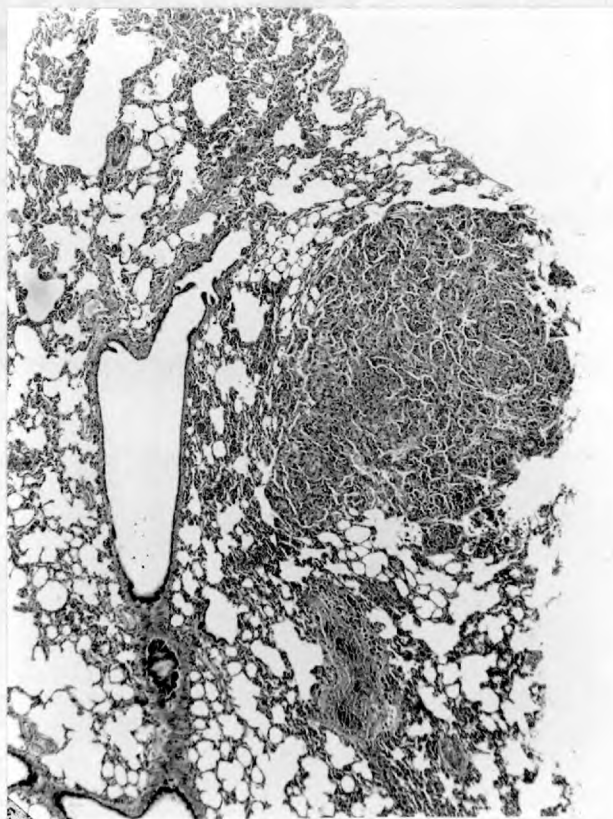


FIGURE 19.

Secondary deposit of Walker tumour from subcutaneous transplant in rat's lung.

and 14. But the results of days 21 ($P < 0.05$) and 23 ($P < 0.05$) were significantly different, the complete excision group being lower.

Comparison of the results of complete excision with those of the normal control group (a) of Experiment 1 gave significant differences on day 0 ($P < 0.05$) and day 7 ($P < 0.05$), but none on the later days.

Discussion

Several authors have reported an increase in circulating megakaryocytes in association with advanced carcinoma. Recently Malmgren et al. (1966) have described the effect of chemotherapy and irradiation on the incidence of circulating megakaryocytes in cancer patients. Nothing has been reported so far on the incidence of circulating megakaryocytes after surgical excision of the tumour.

In this experiment, the animals were divided in three groups. Those in group (a) had a skin excision only. In group (b) an inoculation tumour was completely removed, and in group (c) the tumour was partly removed.

In the complete excision group, repeated megakaryocyte counts at regular intervals showed a steady decrease in the incidence of these cells. An initial rise 7 days after excision of the tumour may be attributed to other factors apart from any remaining tumour activity, among them ^{the} added effect of surgical trauma and changes due to general anaesthesia. The tumour may have been producing an effect which regulated

megakaryocyte circulation, and this might have continued for some time after the tumour was removed. Or it may be that the changes occurring in the anatomical sites of megakaryocyte production take some time to return to the normal once the influence of the tumour and its products is removed.

After excision of the tumour the incidence of circulating megakaryocytes decreases slowly but steadily. In animals where the whole tumour had been removed the decrease became significant 21 days after excision of the tumour. The same result was obtained 50 days after the operation. On both these days the difference was significant at the level of 10 per cent.

In the case of the animals which had had partial excision of the tumour, the incidence of circulating megakaryocytes showed no significant changes on any day after the operation when compared to that before operation. The tumour continued to grow at the usual rate and all the animals had died by the 35th day after partial excision. In fact, on the 7th day after the operation the incidence of circulating megakaryocytes dropped from 58% to 50%. This was no significant reduction, but there is some indication that the tumour volume is to some degree related to the incidence of circulating megakaryocytes. This relation may not be a straightforward one.

On postmortem examination, lung metastases were found (Fig. 19) in two animals of the group which had had complete excision, and one of these two also had a local recurrence of the tumour. In the group with

partial excision, again two had lung metastases, and in both these cases the survival time was shorter than for the rest of the group. There was no evidence of bone metastases, though it has been suggested that any condition which involves the bone marrow and causes softening (Minot, 1922, 1923) may cause megakaryocytes to enter the circulating blood.

It is apparent that it takes longer for the incidence of circulating megakaryocytes to go down after excision of the tumour than it took for it to increase during tumour growth. The volume of the tumour appears to show a direct relation to the incidence of megakaryocytes, but it would seem that other factors are also involved, which are initiated by the presence of the tumour.

Experiment 6

The Effect of Tumour and Other Tissue Homogenates

Rationale

In 1948 Nakahara and Fakuoka isolated a substance from tumour tissue which caused a decrease in liver catalase activity when injected into normal animals. This finding was soon confirmed by others (Green-Field and Miester, 1951; Kwamorita, Kosuge and Kikawa, 1951; Okushima, 1952). This active principle of tumour tissue was named toxohormone. It was thought to be common to all tumour tissue (Nixon, 1966). However, Green-Field (1951) found that toxohormone can also be isolated to a limited extent from normal tissue. This lowers the liver catalase activity to a limited degree (Kempschmidt et al., 1959 and 1960). Kemp Schmidt and his colleagues recorded various effects due to toxohormone. They suggested that it may cause involution of the thymus, lower the plasma iron level,^{producing} anaemia, reduce the kidney catalase activity and increase the weight of the liver, spleen and adrenals. In their opinion the depression of the plasma iron level was a more sensitive index than the reduction of liver catalase activity.

Bacteria and bacterial extracts produce effects very similar to those caused by toxohormone, such as a reduction in liver catalase activity and in plasma iron (Kempschmidt, Schults, McKinzie, 1962). In the case of tumours contaminated with bacteria it is possible that those

two factors, the toxohormone and the bacterial factor, might be additive. The striking changes in liver catalase activity and plasma iron observed by Kemp Schmidt et al. (1963a, 1963b) with bacteria-contaminated tumours might therefore be attributed to an additive effect.

Matsuoka et al. (1964) have shown recently that a toxohormone prepared from a fibrosarcoma free from bacterial contamination produced a marked depression of liver catalase activity in vivo. Nixon (1966) found that toxohormone from uncontaminated tumour tissue showed significantly higher activity than toxohormone from contaminated tumours. This might signify that bacterial contamination antagonizes the activity of toxohormone from tumour tissue. Nixon used a Walker 256 carcinosarcoma, where repeated cultures from tumour tissue had shown no bacterial growth. The toxohormone prepared from this bacteria-free tumour tissue showed consistently similar results.

Extracts of bacteria-free normal kidney and splenic tissue were also found to depress the serum iron level in mice (Nixon, 1966). Nixon established that normal kidney and splenic tissue has a protein content comparable to that of many tumour extracts, whereas liver and muscle extracts yield a much lower protein concentration. In his opinion the activity resides in a heterogenous group of the protein-like moiety.

Apart from this toxohormone activity, the current medical literature gives considerable support to the suggestion that some malignant tumours may actively secrete a hormone-like substance with biological

activity similar to that of some known endocrine gland. Oat-cell carcinoma has on many occasions been reported to produce Cushing's syndrome with nothing to indicate involvement of the suprarenal gland (Estridge et al., 1965; Golub, 1964).

No report is available on the effect of these tumours on the bone marrow, particularly in relation to megakaryocytes at that site or in circulation. Very recently Catayee and Bicheieray (1966) demonstrated an increase in the number of megakaryocytes in the splenic pulp and inside the vascular lumen after injecting animals with tumour extract. This increase resembles that found in animals with tumours.

This study, therefore, has been designed to determine the effect produced by tumour and other tissue homogenates on the megakaryocyte count.

Materials and Methods

65 male rats with an average weight of 296.40 gms. ($SD \pm 11.79$) were used for this experiment. Group one (6a) consisted of 20 rats. These were given injections of 0.2 ml. of Walker 256 carcinoma tissue homogenate in the right flank on the 1st, 3rd, 5th, 7th, 9th, 11th, 13th, 15th, 17th and 19th day.

Group two (6b) consisted of 15 rats. These received subcutaneous injections of 0.2 ml. of muscle homogenate at the same site and in the same frequency as group one. The muscle tissue for the homogenate was obtained from a normal isogenic rat of the same weight as the mean weight

of the group.

15 rats formed group three (6c). They were given injections at the same site and in the same frequency of 0.2 ml. of a liver homogenate. The liver tissue was obtained from an isogenic rat of approximately the same weight as the rats in this group.

Group four (6d) also consisted of 15 rats, who were given 0.2 ml. of spleen homogenate in the right flank. The frequency of the injections was the same as for group one. The splenic tissue for the homogenate was obtained from an isogenic rat of approximately the same weight as the average for this group.

The technique of tissue homogenate preparation has been described in a previous chapter. Before injecting the homogenate, a smear was made and wet stained with 0.5% methyl blue to make sure that it contained no intact cells. The whole procedure was carried out under sterile conditions. Blood was collected from the rats on day 0, 7, 14 and 21.

Results

In group one (6a), injected with Walker tumour homogenate, none of the 20 blood samples taken on day 0 were positive for megakaryocytes. On day 7, 20% were positive, 40% on day 14, and 50% on day 21. (Table 15, Fig. 20).

In group two (6b), injected with muscle homogenate, none of the 15 samples were positive on day 0, 26.7% on day 7 and again on day 14, and 13.3% on day 21 (Table 16, Fig. 20).

In group three (6c), injected with liver homogenate, again none were positive on day 0. On day 7, 26.7% of the 15 samples were positive, on days 14 and 21 likewise 26.7% (Table 17, Fig. 20).

In group four (6d), injected with splenic tissue homogenate, none of the 15 samples were positive on day 0, 13.3% on day 7, 20% on day 14 and 26.7% on day 21 (Table 18, Fig. 20).

Statistical Analysis

Statistically, no significant difference could be established when any two groups were compared.

Comparison of the first group, injected with tumour homogenate, with the normal control group (a) of Experiment 1 showed no significant differences on days 0, 7 and 14. But the results of day 21 appear to be significantly different ($P < 0.02$), with the higher incidence in the tumour homogenate group.

Comparison of the second, third and fourth groups with the control group (1a) revealed no significant differences.

Again, when the results of different days in the tumour homogenate group were compared with those of group (b) of Experiment 1, that is the group of rats with Walker tumour, no significant difference was found.

When the results of different days were combined and compared between two groups, a significant difference was observed between the group of rats injected with Walker tissue homogenate (6a) and the normal control group (1a) ($P < 0.001$), and also between the group given liver

TABLE 15. Effect of Walker tumour homogenate.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in blood
0	20	20	Subcutaneous injection of Walker tumour homogenate	0%
7	20	20		20%
14	20	20		40%
21	20	20		50%

TABLE 16. Effect of muscle tumour homogenate.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in blood
0	15	15	Subcutaneous injection of muscle tissue homogenate	0%
7	15	15		26.7%
14	15	15		26.7%
21	15	15		13.3%

TABLE 17. Effect of liver homogenate.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in Blood
0	15	15	Subcutaneous injection of liver homogenate	0%
7	15	15		26.7%
14	15	15		26.7%
21	15	15		26.7%

TABLE 18. Effect of splenic tissue homogenate.

Day	No. of Rats	No of Samples	Nature of Treatment	Incidence of MGK in Blood
0	15	15	Subcutaneous injection of splenic tissue homogenate	0%
7	15	15		13.3%
14	15	15		20%
21	15	15		26.7%

Experiment 6

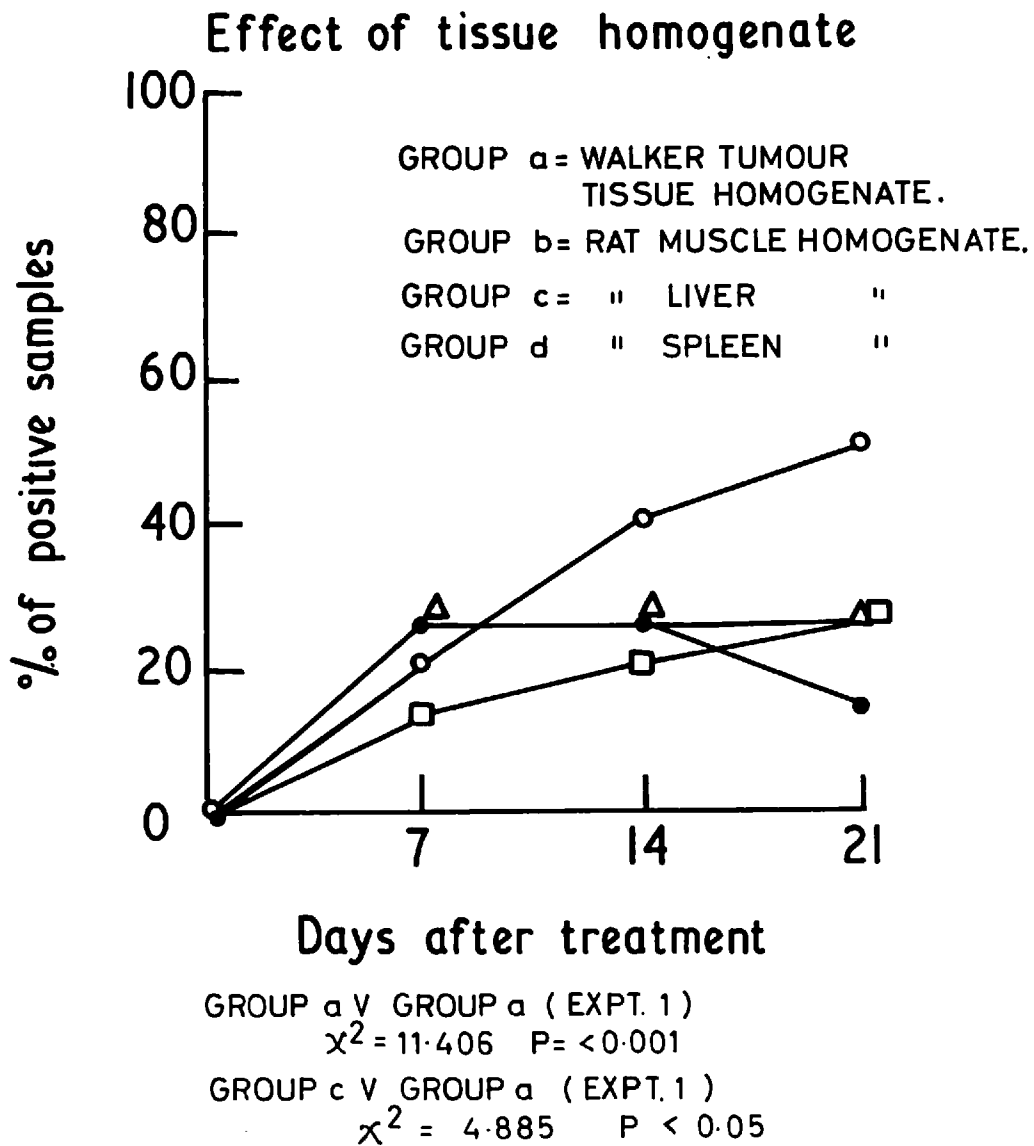


FIGURE 20.

Graphic representation of Tables 15, 16, 17 and 18.

homogenate injections and the normal control group (1a) ($P < .05$).

Discussion

The recent observations by Catayee and Biechleray (1966) on the effect of tumour extracts on megakaryocytes have been of considerable interest. They observed that both tumour and tumour extract increase the number of megakaryocytes in the splenic pulp and the vascular lumen of the spleen to a considerable extent, whereas adrenocorticotrophic hormone failed to produce such an effect. This study has established that subcutaneous injections of a tumour homogenate cause a significant rise in the incidence of circulating megakaryocytes. It is most likely that megakaryocytes have migrated from that site to the blood and other tissues.

The observations made in this experiment therefore fit in very well with those of Catayee.

Nixon (1966) observed that injections of tumour extract, normal kidney and splenic extract have a similar effect on the serum iron level. It appears that these tissues contain a common factor. Could this be a product of the mitotic cells? The number of mitotic cells is much higher in tumour tissue than in any normal tissue. Nixon also demonstrated that tumour tissue extract depresses serum iron protein to a greater extent than kidney and splenic extracts do. In the present study, no significant difference was observed in the incidence of circulating megakaryocytes when groups injected with muscle and splenic homogenate respectively were compared with the normal control group (1a). But comparison of a

group injected with liver homogenate with the normal control group (1a) did show a significant difference ($P < 0.05$). All the experimental groups showed a slight increase in circulating megakaryocytes, but this was not statistically significant. It would be interesting to follow up the group of animals injected with normal tissue homogenate for a much longer period. The significance was much lower with liver homogenate than for the group with tumour homogenate. Perhaps the active principle in question is contained in the rat liver rather than the spleen.

The results of this study justify the statement that the Walker tumour contains some substance which induces megakaryocytes to migrate from the bone marrow or reduces the fragility of these cells. It is possible that the tumour shares the same active principle with the liver of the rat, which produces a similar effect.

Experiment 7The Suprarenal Glands and Cortisone and Their Influence on theMegakaryocyte CountRationale

The previous experiments have established that the malignant tumour directly influences the incidence of circulating megakaryocytes in the rat. This effect may be produced directly by the tumour, or indirectly by some other agents which are influenced by the tumour. One factor which may well be involved in this phenomenon is the stress produced by the tumour (Romsdahl et al., 1964). It is known that two very important endocrine glands are responsible for the reaction of the body to any external or internal stress. These are the pituitary gland and the suprarenal glands. Minot (1922, 1923) suggested that the migration of megakaryocytes from the bone marrow to the blood suggests a considerable strain on the bone marrow. If this is true, it will be worth investigating whether this effect is mediated through the pituitary and suprarenal glands.

Recently Smith (1967) produced benign and malignant tumours in rats by injecting DMBA (9-10-Dimethyl-1, 2-Benzanthracene). Smith claimed that he found significantly heavier pituitary glands in the group of rats with benign tumours than in those with malignant tumours. At the same time the ovaries were significantly smaller in the rats with benign tumours. The adrenals were also weighed, but no significant variation

was observed.

The fact that the pituitary and suprarenal glands respond to stress is well established (Selye, Johnston, 1946). Sayer and Sayer (1949) classified the reactions to stress in four categories. It is probably the third of Sayer's categories which is encountered in malignant neoplasia, with hypertrophy of the suprarenal glands, and very low levels of sudanophilic substance, cholesterol and ascorbic acid in these glands.

The effect of cortisone and adrenocorticotrophic hormone on the blood and bone marrow has been studied by various investigators. In mice, massive doses of cortisone produced lymphoid hyperplasia and lymphopenia in the peripheral blood (Antopol, 1950). A decrease in eosinophils and lymphocytes was also noted by Hill (1948), Quittner (1951) and Yoffee (1967). Quittner studied the effect of cortisone on cellular proliferation in the bone marrow and was unable to demonstrate any inhibitory action. Yoffee (1935) demonstrated increased cellularity in the bone marrow after repeated injections of ACTH. He found that the myeloid and erythroid elements in the bone marrow increased, but the lymphoid elements remained unaffected. ACTH may directly or indirectly stimulate the formation of new cells in the bone marrow (Hudson, 1952).

Eosinopenia and thrombocytopenia together with increased megakaryocyte activity in bone marrow were observed by William, Balke and Warren (1955, 1967) after surgical operations, single or repeated doses of ACTH. Unfortunately no data are available on the effect of ACTH and cortisone on the incidence of circulating megakaryocytes.

In view of the fact that adrenocorticotrophic hormone and cortisone control some cell elements in the blood and bone marrow under certain physiological conditions, it was decided to study the effects of adrenalectomy and injections of cortisone on the incidence of circulating megakaryocytes under normal and pathological conditions.

Materials and Methods

92 rats with a mean weight of 292 gms. ($SD \pm 11.71$) were used in this experiment. They were divided into five groups.

Group one (7a) consisted of 12 rats. These had a bilateral adrenalectomy and the first blood sample was taken about 14 days after the operation, the day of the first collection counting as day 0. After that blood was collected on days 7, 14 and 21.

In group two (7b) a bilateral adrenalectomy was done on 20 rats. 15 days after the operation they received a tumour transplant, and the day of the transplant counted as day 1. Blood was collected on days 0, 7, 14 and 21.

The third group (7c) also consisted of 20 animals. These had a bilateral adrenalectomy and 15 days later a tumour transplant. In addition they were given 1 microgram of cortisone intraperitoneally on alternate days from the day after the tumour transplant until the 21st day. (The cortisyl was supplied by Roussel, London, England). The day of the tumour transplant was taken as day 1. Blood was collected from the heart on days 0, 7, 14 and 21, and examined for megakaryocytes.

Technique of Adrenalectomy

The rats were anaesthetized on the same general principle as followed throughout this study. They were placed in the prone position and the skin of the back was shaved and painted with 5% hibitane. A single midline incision was made along the spines of the vertebral column from the 10th thoracic to the 3rd lumbar vertebra. This corresponds to the level of the kidney. The skin was retracted on the right side to expose the right paravertebral muscles and the back of the abdominal parietes on that side. A longitudinal incision was made in the fascial covering of the muscle, parallel to and about 1 cm. distant from the spine. A further incision was made along the lateral border of the dorsal muscles and the kidney exposed. Full attention was paid to haemostasis. The adrenal gland was identified at or near the upper pole of the kidney. In most cases it is found to be separate from the kidney itself. The gland was freed from the surrounding fatty tissue, its blood vessels were clamped, ligated and divided, and the gland complete with its capsule was excised.

The second adrenal gland on the other side was removed in the same manner. During exploration a search was made for accessory adrenal tissue, but this was never found to be present. The incision in the muscle was closed with catgut No. 1, using an atraumatic needle. The subcutaneous layer was sutured in the same way. The skin was apposed and held together with Michel's clips, the wound dressed with clean gauze

and strapped with leucoplast. The dressing and clips were removed after the 7th day.

All adrenalectomized rats were given saline to drink. In most cases they were given normal saline until they died or were killed at the end of the experiment,

Results

In the first group (7a) of 12 rats with bilateral adrenalectomy, none of the 12 blood samples taken on day 0 were positive for megakaryocytes. On days 7, 14 and 21, 8.3% of the 12 samples were positive (Table 19, Fig. 21)

Group two (7b) consisted of adrenalectomized rats who were later given a tumour transplant. In this group, 5% of the 20 samples were positive on day 0, 20% on day 7, 35% on day 14, and 60% on day 21 (Table 20, Fig. 21).

The 20 rats in group three (7c) had been adrenalectomized and later given a Walker tumour transplant. In addition they received injections of cortisone. Out of 20 samples, 5% were positive on day 0, 35% on day 7, 50% on day 14, and 65% on day 21 (Table 21, Fig. 21).

Group four (7d) consisted of 20 rats who had had a bilateral adrenalectomy and later were given injections of Walker tumour homogenate. In this group, none of the 20 samples were positive on day 0. On day 7, 20% of the 20 samples were positive, on day 14, 30% and on day 21, 55% (Table 22, Fig. 21).

TABLE 19. Effect of bilateral adrenalectomy.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in Blood
0	12	12	Bilateral adrenalectomy	0%
7	12	12		8.3%
14	12	12		8.3%
21	12	12		8.3%

TABLE 20. Effect of adrenalectomy and Walker tumour transplanted subcutaneously.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in Blood
0	20	20	Adrenalectomy+ subcutaneous tumour transplant	5%
7	20	20		20%
14	20	20		35%
21	20	20		60%

TABLE 21. Effect of adrenalectomy, subcutaneous tumour and cortisone.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in Blood
0	20	20	Adrenalectomy + subcutaneous tumour + cortisone	5%
7	20	20		35%
14	20	20		50%
21	20	20		65%

TABLE 22. Effect of adrenalectomy and Walker tumour homogenate.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in Blood
0	20	20	Adrenalectomy + Walker tumour homogenate injection	0%
7	20	20		20%
14	20	20		30%
21	20	20		55%

TABLE 23. Effect of adrenalectomy, Walker tumour homogenate and cortisone.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in Blood
0	20	20	Adrenalectomy + tumour homogenate + cortisone	5%
7	20	20		35%
14	20	20		55%
21	20	20		70%

EXPERIMENT 7

Influence of adrenal glands

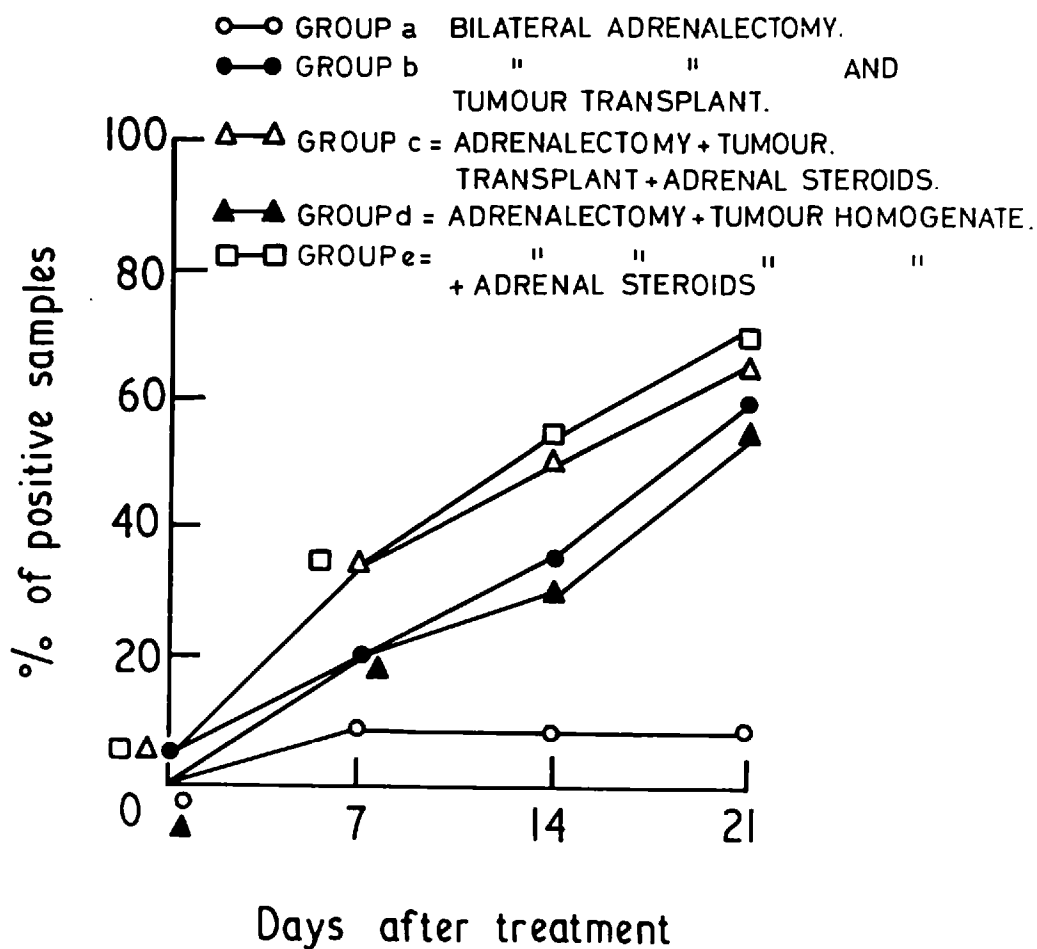


FIGURE 21.

Graphic representation of Tables 19, 20, 21, 22 and 23.

In group five (7e), 20 rats had had a bilateral adrenalectomy. Later they were given subcutaneous injections of Walker tumour tissue homogenate and intraperitoneal injections of cortisone. On day 0, 5% of the samples were positive, on day 7, 35% , on day 14, 55% and on day 21, 70% (Table 23, Fig. 21).

Statistical Analysis

For comparison between the rats given bilateral adrenalectomy only and the normal controls i.e. group (a) of Experiment 1, the results of the different days were combined. All other comparisons were for each day separately. No significant difference was found between the rats with bilateral adrenalectomy and the control group (a) of Experiment 1.

Comparison between the rats with tumour in group 1b and the other groups in this experiment showed no significant differences. Similarly, comparisons between groups two (7b), three (7c), four (7d) and five (7e) revealed no significant differences.

Discussion

The adrenal glands respond to trauma and other forms of stress. This has been established conclusively by other investigators. Apart from this response to stress, they will also affect the bone marrow and blood cells, under certain conditions. Changes in the peripheral blood count have been demonstrated after injections of cortisone. The adrenal glands therefore play a most important role in "stress response" and also in the

blood cell count. It must be assumed that tumours produce stress effects in the system just as other diseases do. Perhaps this is even more so in cases of advanced carcinoma. It has already been reported that in cases with advanced tumours the incidence of megakaryocytes is high. The earlier experiments in this study have demonstrated how the incidence of these cells increases with the progress of the tumour. With this association between tumours and stress and changes in the peripheral blood, the question arose whether these effects were produced by way of the adrenal glands.

This last series of experiments has demonstrated that tumours produce the same effect on the incidence of circulating megakaryocytes in the peripheral blood in rats with or without adrenals. The increase in the incidence of megakaryocytes in the blood of rats with tumours is therefore independent of the influence of the adrenal glands.

Tumour homogenate produces the same effect on the megakaryocytes in the blood, as the tumour does. When tumour homogenate was injected in rats with or without adrenal glands, again no significant change was observed.

To confirm the hypothesis that the adrenals have no connection with the increased incidence of circulating megakaryocytes occurring with tumours, adrenalectomized rats with inoculation tumours or injected with tumour homogenate were given supplementary cortisone injections. Again no significant differences were observed.

One point of interest is that in the group of adrenalectomized

rats given tumour homogenate only, 55% of the 20 samples were positive on day 21. But in the group given the same treatment and in addition cortisone injections, 70% of the 20 samples were positive on day 21. This difference may not be significant, but it does warrant scrutiny. The incidence is obviously higher in the group given cortisone. Though this is a matter of opinion, the products of interaction between the tumour homogenate and the cortisone may to some extent be responsible for helping the cells to migrate from their anatomical site. This needs further study.

Experiment 8The Effect of Splenectomy on Circulating MegakaryocytesRationale

A review of the literature revealed that the incidence of circulating megakaryocytes is not related to the proportion of these cells in the bone marrow. An increased number of megakaryocytes in the bone marrow does not necessarily mean a higher incidence of these cells in the peripheral blood. It is therefore reasonable to look for some other source from which megakaryocytes may disseminate into the blood.

Under normal circumstances (Fig. 22) the spleen does not function as a haematopoietic organ. It does act as a haematopoietic centre during intrauterine life and the early postnatal period, and under certain conditions where the bone marrow fails to produce blood cells in adequate numbers. Apart from the spleen, the liver, lymph nodes and even the lungs have been observed to have haematopoietic centres under some abnormal conditions.

Multinucleate giant cells identical with megakaryocytes do not normally occur in the spleen, but small amounts are found during embryonic life (Downey, 1938). In some mammals, i.e. rats, mice, bats, chipmunks, bears and hedgehogs, megakaryocytes are frequently seen in the spleen. Jolly and Rosello (1909) traced the megakaryocytes of the embryonal spleen to the lymphoid basophil stem cells. The same observation

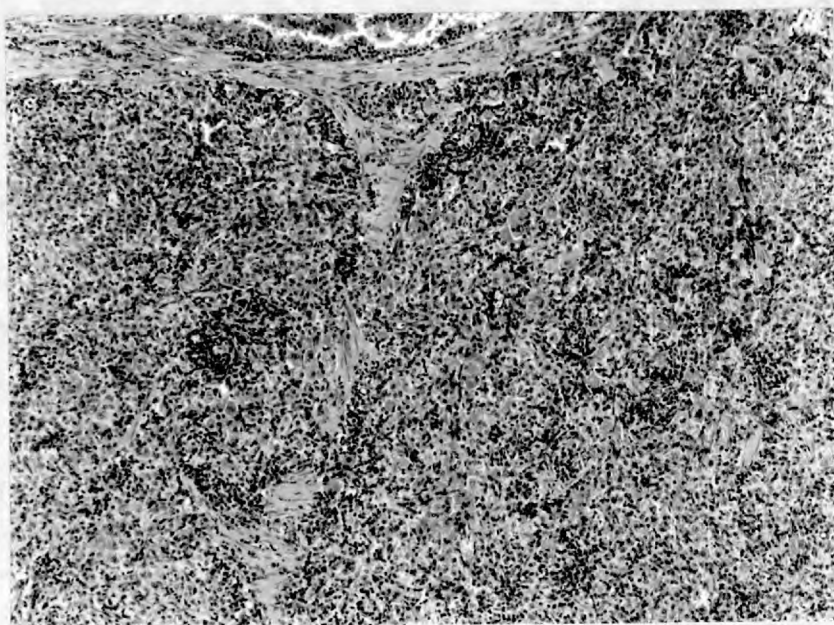


FIGURE 22.

Section of normal spleen.

was made by Klaaschen (1922) and by Rothermel (1930). Numerous megakaryocytes were also observed in the spleen of rabbits as a result of sapotoxin poisoning (Custer, 1933).

Under pathological conditions many megakaryocytes are observed in the spleen, especially with myeloid metaplasia. Extramedullary myeloid leukaemia, anaemia, infectious conditions like scarlet fever, diphtheria, erysipelas, pneumonia, meningitis, pulmonary tuberculosis, and various blood conditions like polycythaemia vera, thrombocytopenic purpura and Banti's disease.

Myeloid metaplasia of the spleen was produced experimentally by repeated bleeding (Jaffe, 1921). The repeated use of various haemotoxins like phenylhydrazine, pyrocin and pyrogallol also produces myeloid metaplasia of the spleen (Morris, 1907, Sternber, 1909), and injections of various bacterial vaccines have been shown to^{do} the same (Dickmann, 1922; Lang, 1930).

Virchow's (1863) earlier observation on the mobilization of megakaryocytes as emboli has been challenged by several investigators. Experimental observations have led to the suggestion that in cats megakaryocytes originate in the spleen through fusion of lymphocytes (Downey, 1939). Some investigators have observed differentiation of megakaryocytes from local haemocytoblasts or stem cells in the spleen (Downey, Palmer and Powell, 1930). The suggestion that megakaryocytes migrate from the bone marrow to the spleen and other extramedullary sites has been refuted

(Schwarz, 1954, 1956). If migration takes place at all, there is no evidence to support the statement that the extramedullary megakaryocytes proliferate to maintain the observed population of these cells.

Schwarz (1953) said that the megakaryocytes seen in the vascular system are inactive. He suggests a remote but possible connection between megakaryocytes and gas exchange.

Megakaryocytes are seen in the spleen under some stress conditions. Recently Catayee and Coll (1966) found that there was a significant increase of megakaryocytes in the spleen after tumour transplants and also after injections of tumour tissue extract. The real implications of this observation are not yet clear. An experimental study was required to establish the relationship between circulating megakaryocytes and the spleen.

Haematological Changes following Splenectomy

Changes in the blood have been observed after splenectomy in normal animals and human subjects. The mechanism involved remains obscure. Both anaemia and polycythaemia have been noted. Morphological changes and changes in the physical behaviour of red blood cells were observed after splenectomy in normal subjects (Krumbhaar, 1932; Singer, Miller, Dameshek, 1941).

Leukocytosis is more protracted after splenectomy than after other operations. This has been observed in both man and animals (Lauda, 1933). In albino Wistar rats the total white cell count increased by up

to 100% within 7 days after splenectomy, remaining significantly high for 70 to 90 days and then returning to normal (Palmer, 1951). The actual role of the spleen in haematopoiesis and its influence on the bone marrow and blood are still a matter of debate (Palmer, Kemp, 1951).

Materials and Methods

40 male rats with a mean weight of 301.95 gms. ($SD \pm 10.45$) were used in this experiment. They were divided into two groups of 20 each. The rats in group one (8a) underwent splenectomy, the day of splenectomy counting as day 1. Blood was taken on days 0, 7, 14 and 21.

The rats in group two (8b) also underwent splenectomy and in addition received a tumour transplant in the right flank on the same day. The quantity of tumour cells injected was the same as in the previous experiments. The day of splenectomy and tumour transplant was taken as day 1. Blood was drawn on days 0, 7, 14 and 21.

Technique of Splenectomy

The rats were anaesthetized and placed on the operating table in the supine position. The anterior abdominal wall was shaved clean and painted with a 5% hibitane solution. A left paramedian incision was made, extending 2.5 cm. below the left costal margin. The muscles of the anterior abdominal wall were split and retracted on both sides. An incision was made in the peritoneum and ^{the} spleen delivered through this. The splenic pedicle was ligated and the organ excised. Where more than one blood vessel was found attached to the splenic hilum, each vessel was ligated

and divided separately.

After excision of the spleen the peritoneum was closed with catgut, using atraumatic needles. The skin wound was closed and apposed with Michel's clips, covered with clean gauze and strapped with leucoplast. The dressings and Michel's clips were removed on the 7th day after splenectomy.

Results

In the first group (8a) none of the 20 blood samples taken on day 0 were positive for megakaryocytes. On day 7, 15% of the 20 samples were positive, on day 14, again 15%, and on day 21 only 5%. This was the group in which splenectomy only had been done (Table 24, Fig. 23).

The second group (8b) had had a splenectomy and on the same day a Walker tumour transplant. On day 0, none of the 20 samples were positive. On day 7, 35% were positive, on day 14, 60% and on day 21, 45% (Table 25, Fig. 23).

Statistical Analysis

Comparison of the combine results for each group showed a significant difference ($P < 0.001$). No significant difference was found between the splenectomy and the normal control group (a) in Experiment 1, nor between the group given splenectomy and a tumour transplant and the tumour group (b) in Experiment 1. Comparison on individual days for these two groups also revealed no significant difference.

TABLE 24. Effect of splenectomy.

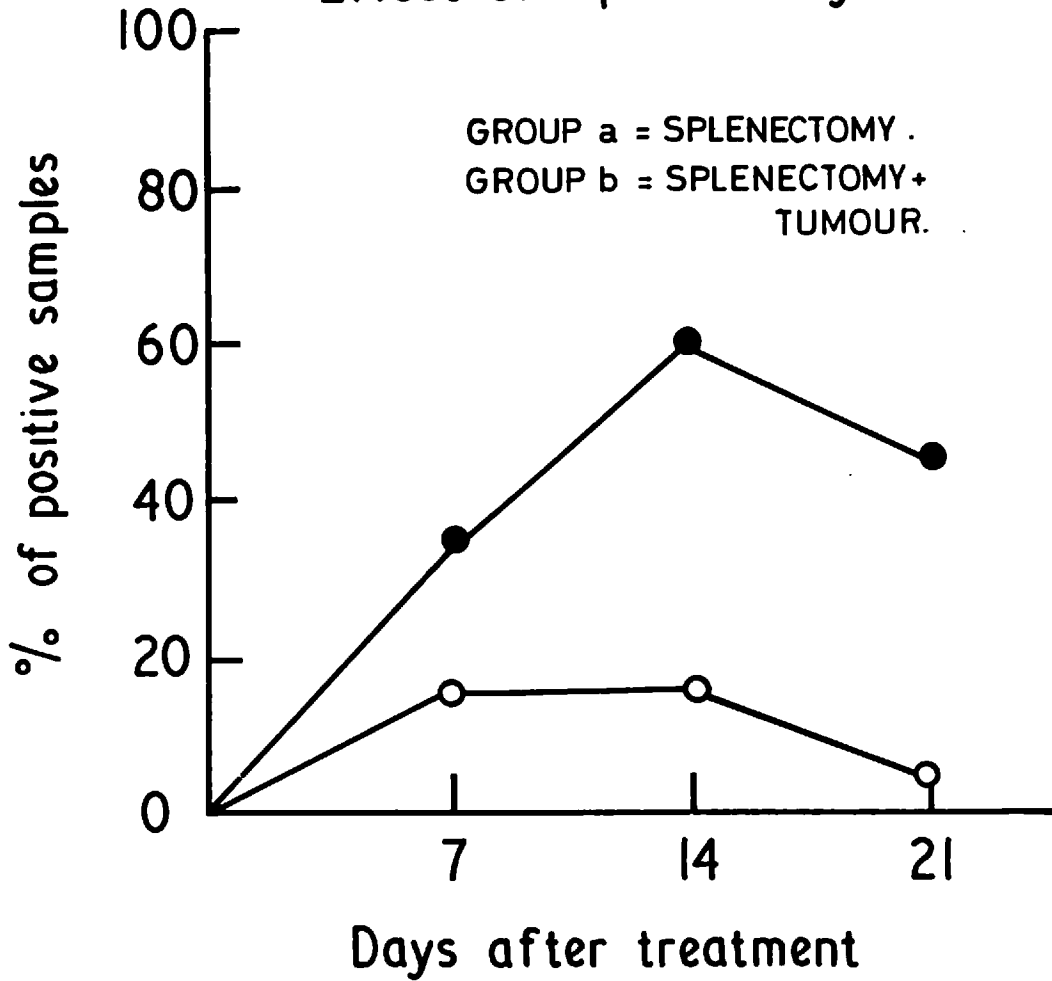
Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in Blood
0	20	20	Splenectomy	0%
7	20	20		15%
14	20	20		15%
21	20	20		5%

TABLE 25. Effect of splenectomy and Walker tumour.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in Blood
0	20	20	Splenectomy + subcutaneous tumour	0%
7	20	20		35%
14	20	20		60%
21	20	20		45%

EXPERIMENT 8

Effect of splenectomy.



GROUP a V GROUP b
 $\chi^2 = 14.628$
 $P = < 0.001$

FIGURE 23.

Graph showing the effect of splenectomy on the incidence of circulating megakaryocytes (Tables 24 and 25)

Discussion

The group of rats with splenectomy and the normal control group (1a) showed a similar incidence of circulating megakaryocytes. This indicates that the spleen does not influence the incidence under normal conditions.

In this last group of experiments, the incidence of circulating megakaryocytes showed a significant difference between the group of rats who had had splenectomy only and the group given splenectomy and a tumour transplant ($P < 0.001$). This shows that after removal of the spleen a tumour still affects the megakaryocyte count in the same way as it did before splenectomy.

One important observation is that in the group of rats with tumour and splenectomy only 45% of the 20 samples were positive on the 21st day. Tumour group 1b had shown 70% of positive samples on day 21. In the group of rats which had had a bilateral adrenalectomy and a tumour transplant (7b), 60% of the 20 samples had been positive on day 21. In these three groups the growth rate of the tumours had been similar. In the first, the mean tumour volume was 19.88 cc ($SD \pm 2.74$), in the second, 21.36 cc ($SD \pm 5.11$), and in the third, 17.48 cc ($SD \pm 4.98$), always on the 21st day after the tumour transplant. The tumour volumes of these three groups show no significant difference.

In rodents, the spleen acts as a pump, discharging its contents into the circulation at regular intervals (Snell, 1941). Various factors

may modify this activity. Recently Catayee and his colleague (1967) have noticed increased numbers of megakaryocytes in the splenic pulp and also in the lumen of the blood vessels of rats with tumours, and also in those of rats given tumour extract at regular intervals. Splenomegaly was observed in these rats. Postmortem examination of the rats with tumours used in this study also revealed splenomegaly.

Extramedullary myeloid metaplasia has been observed in infectious diseases, diseases of the blood and neoplastic conditions (Hickling, 1937; Carpenter and Flory, 1941). This is independent of any influence or condition of the bone marrow (Schwarz, 1954). The spleen is the most common site for extramedullary myeloid metaplasia (Schwarz, 1954). Both mature and immature megakaryocytes have been seen in these foci. Apart from developing in these extramedullary sites, megakaryocytes may differentiate from the haemocytoblasts or stem cells present in the splenic pulp (Downey, Palmer and Powell, 1930).

21 days after the tumour transplant the size of the tumours in the present experiment was such that the disease could be regarded as advanced. On postmortem examination, 17 out of the 20 rats given a tumour transplant only showed splenomegaly. It may be postulated that a portion of the circulating megakaryocytes seen in advanced neoplastic disease are controlled by the spleen. This may be due either to myeloid metaplasia, or to increased differentiation of these cells from the splenic stem cells or haemocytoblasts in the spleen (Fig. 24, 25). It

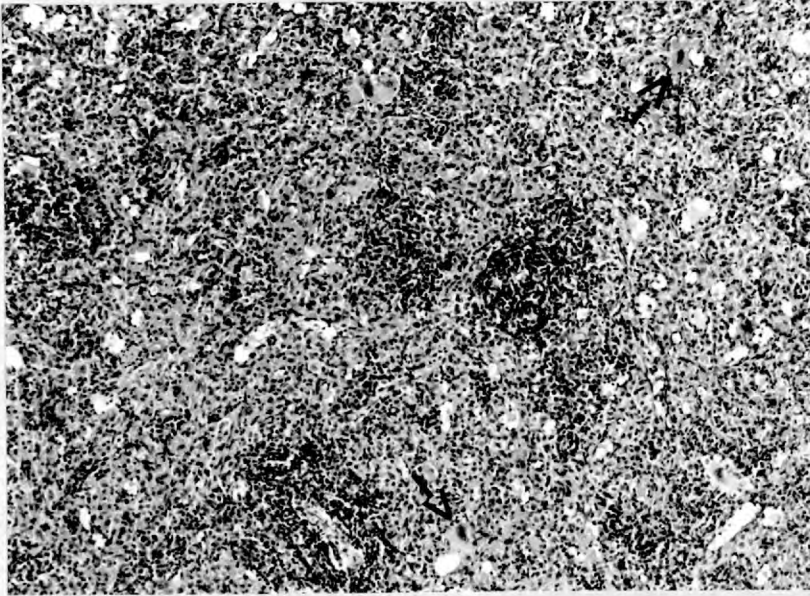


FIGURE 24.

Megakaryocyte in spleen X 40

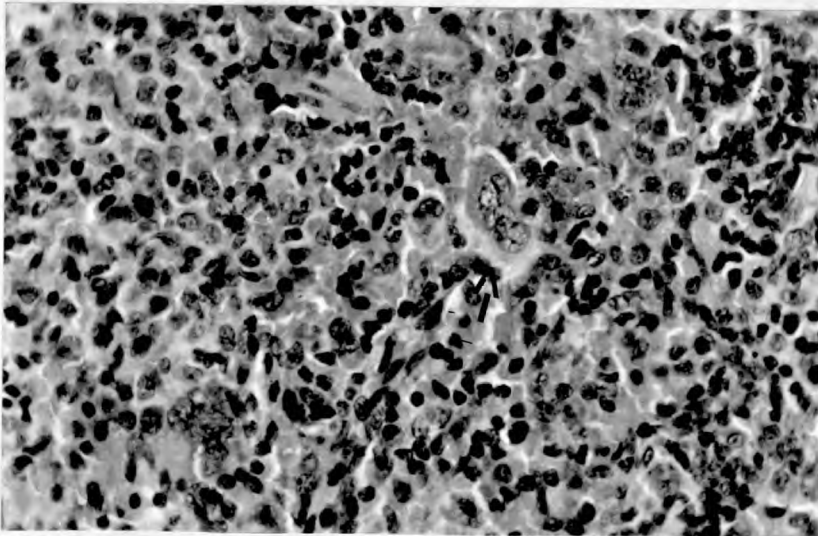


FIGURE 25.

Megakaryocyte in spleen (high magnification) X 400

is possible that the lower incidence of circulating megakaryocytes in rats with advanced tumours who underwent splenectomy was due to this.

It has been suggested that the increased platelet count after splenectomy may be attributed to the increased megakaryocyte count, and vice versa. In the present experiment no significant rise in circulating megakaryocytes was seen which might account for the increased blood platelet count observed by other authors (Steiner, 1931; Krumbhaar, 1932; Lauda, 1933).

Experiment 9Relations between Blood Platelet Count, Megakaryocytes and TumourRationale

Blood platelets were first described by Donne in 1842, who thought they originated from the fatty particles of chyle. In 1882 Bizzozero studied the physiology of the platelets, their adhesiveness and their role in thrombus formation and blood coagulation. It was Wright (1911) who conclusively demonstrated that the megakaryocytes are the precursors of blood platelets.

Recently Thiery and Bessis (1956) used microcinematography of megakaryocyte cultures and demonstrated convincingly that platelets are formed from the filiform pseudopodia of these cells. Before that, Howell and Donahue (1937) had also noted that the platelets originated from megakaryocytes. They described the platelets as "solid secretions" from a unicellular gland.

It is generally accepted that the megakaryocytes of the bone marrow are the precursors of platelets, but Howell and Donahue (1937) and Kauffman et al. (1965) have suggested that the megakaryocytes of the pulmonary bed also produce platelets. Fidler et al. (1941) opposed this idea. They considered the megakaryocytes of the pulmonary bed to be effete cells. The relation between platelets and megakaryocytes from extramedullary tissue has not yet been fully investigated.

Prigivoit (1964) found that during tumour growth in man, and in animals with experimental tumours, the thrombocytogram was lower (2%) and the number of megakaryocytes in the bone marrow increased. Other investigators reported a general increase of the platelets level in the blood of cancer patients (Odell, 1956; Theiry and Bessis, 1956). They also noticed degenerative changes in the megakaryocytes of the bone marrow. In experimental animals with tumour, increased numbers of megakaryocytes were found in various tissues, especially the spleen (Catayee et al., 1966).

The relationship between bleeding and the blood platelet level has been thoroughly investigated. Craddock (1955) noted that in dogs the blood platelet level rose for a brief period after haemorrhage. He suggested that this phenomenon might be due to the formation of platelets from megakaryocytes in extramedullary sites. Matter et al. (1960) noticed a decrease in the platelet count for 24 to 48 hours after bleeding. Craddock et al. (1955), Matter et al. (1960) and Odell (1962) found that after bleeding the total number of cells of the megakaryocyte series increased. It takes 2 to 3 days for megakaryocytes to mature (White, 1953; Rosenthal, 1951), and during this period thrombocytopenia was observed after bleeding.

Odell (1951) noticed a brief period of thrombocytopenia after bleeding, and after that the platelet count rose to between 140 and 150% of the initial count, gradually returning to normal within 9 to 10 days. He also found that the number of megakaryocytes in the bone marrow increased after bleeding. Cronkite (1958) suggested that the presence of large

numbers of platelets in the blood arrested the mechanism which initiates the rapid formation of platelets and maturation of megakaryocytes.

Thrombocytopenia occurs after major surgery and is immediately followed by thrombocytosis. The early thrombocytopenia may be reproduced in normal subjects by giving ACTH. (Warren et al., 1953). William (1952) observed that ACTH injections produced a similar effect on the bone marrow megakaryocytes as major surgery. He noticed that the number of megakaryocytes in the bone marrow increased during the phase of thrombocytopenia and decreased during the phase of thrombocytosis. Prizivoit (1964) suggested that a low platelet count during tumour growth might act as a feed-back mechanism to stimulate the megakaryocytes to migrate to the circulation for quick delivery of the platelets.

Changes in the blood platelet level of subjects with tumours have been reported. Thrombocytopenia has been noticed by many investigators (Prizivoit, 1964). This change might have some influence on the mechanism leading to the higher incidence of megakaryocytes in the blood of cancer subjects. In the present experiment, an attempt has been made to determine whether a relation exists between the platelet level and the incidence of circulating megakaryocytes in animals with transplant tumours and also in normal animals.

Materials and Methods

Bleeding is followed by a brief period of thrombocytopenia and after 5 to 7 days by thrombocytosis. That has been the experience so far

with the rats used in our laboratories, and also agrees with the findings of Odell (1951) and Matter (1960). The present experiment was designed to study the relation between circulating megakaryocytes and the blood platelet level.

Rats with a mean weight of 298.00 gms. ($SD \pm 14.76$) were depleted of 2.5 ml. of blood, roughly 1/12th of the total blood volume in rats of this type. The blood volume was then restored by injecting 2.5 ml. of sterile saline intraperitoneally. The first day on which blood was withdrawn counted as day 0 in this experiment. A megakaryocyte count was done on 1 ml. of blood, and a blood platelet count was done on the same day. In groups 1 (9a) and 2 (9b), these determinations were carried out on days 1, 7 and 21, in group 3 (9c), on days 1, 7 and 14.

It was expected that the thrombocyte count would be low on the first day after bleeding, high on the 7th, and return to normal on the 14th and 21st day. This had been the pattern in previous experience.

30 male rats were used and divided into three groups of 10 each.

In group one (9a), 2.5 ml. of blood were taken from the heart on day 0. Blood was also taken from the tail vein for a platelet count. Then 2.5 ml. of sterile saline were injected intraperitoneally.

Group two (9b) consisted of rats with subcutaneous tumours approximately 7 days old on day 0. The average tumour volume on that day was 0.7 cc ($SD \pm 2.12$). 2.5 ml. of blood were taken from the heart on day 0, and a blood sample from the tail vein for a platelet count. 2.5 ml. of

sterile saline were given intraperitoneally to restore the blood volume.

The third group (9c) also had a subcutaneous tumour of 7-day size on day 0. The mean tumour volume was 0.58 cc ($SD \pm 0.16$). On day 0, 2.5 ml. of blood were taken from the heart, and blood for a platelet count was taken from the tail vein. 2.5 ml. of sterile saline were injected intraperitoneally to restore the blood volume.

Results

The first group (9a) consisted of normal rats. The megakaryocyte incidence was 0% out of 10 on day 0, 10% out of 10 on days 1, 7 and 21 (Table 26, Fig. 26). The mean platelet count was 752,200 on day 0, 534,000 on day 1 and 1,490,000 on day 7 and 914,800 on day 21 (Table 29, Fig. 21).

The second group (9b) consisted of rats with tumour. On day 0, 40% of the 10 samples were positive for megakaryocytes, on day 1, 50% were positive, 70% on day 7, and 87.5% on day 21 (Table 27, Fig. 20). The mean platelet count was 647,800 on day 0; 521,800 on day 1; 944,200 on day 7 and 642,200 on day 21 (Table 30, Fig. 27).

The third group (9c) also consisted of rats with tumour. 40% of the 10 samples were positive for megakaryocytes on day 0, and 50% on day 1. No blood was taken on day 7. On day 14, 90% were positive (Table 28, Fig. 26). The mean platelet count on day 0 was 770,000, on day 1, 576,800, on day 7 it was 856,400 and on day 14, 646,400 (Table 31, Fig. 27).

TABLE 26. Effect of bleeding in normal rats.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of HGM in Blood
0	10	10	Bleeding	0%
1	10	10		10%
7	10	10		10%
21	10	10		10%

TABLE 27. Effect of bleeding in rats with tumour

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of HGM in Blood
0	10	10	Bleeding	40%
1	10	10		50%
7	10	10		70%
21	8	8		87.5%

TABLE 28. Effect of bleeding in rats with tumour.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of MGK in Blood
0	10	10	Bleeding	40%
1	10	10		50%
14	10	10		90%

TABLE 29. Platelet count of normal rats after bleeding.

Day	No. of Rats	No. of Samples	Nature of Treatment	Mean platelet count
0	10	3	Bleeding	752,200
1	10	3		534,000
7	10	3		1,490,000
21	10	3		941,800

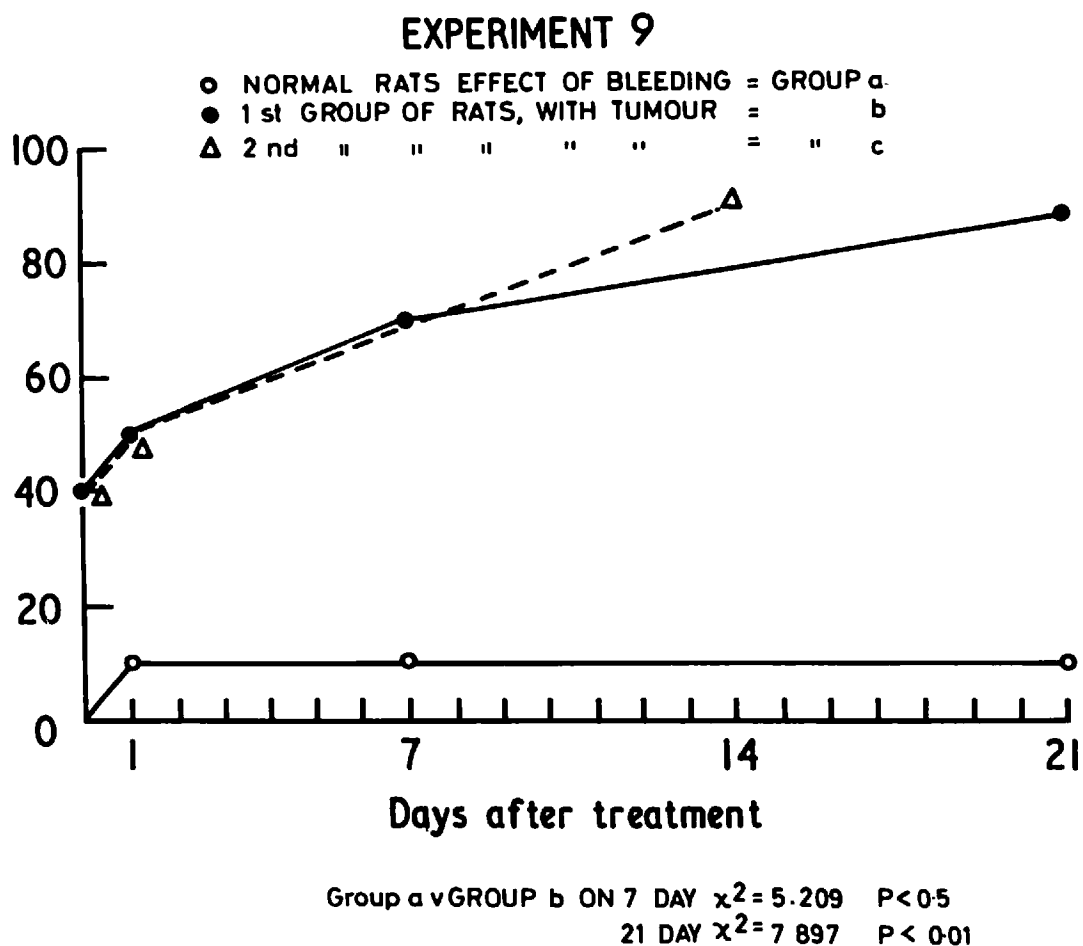


FIGURE 26.

Incidence of megakaryocytes in the blood of rats with and without tumour after bleeding

TABLE 30. Platelet count of rats with tumour after bleeding.

Day	No. of Rats	No. of Samples	Treatment	Mean platelet count
0	10	10	Bleeding	647,800
1	10	10		521,800
7	10	10		944,200
21	8	8		642,200

TABLE 31. Platelet count of rats with tumour after bleeding.

Day	No. of Rats	No. of Samples	Treatment	Mean platelet count
0	10	10	Bleeding	770,000
1	10	10		576,800
7	10	10		856,400
14	10	10		646,400

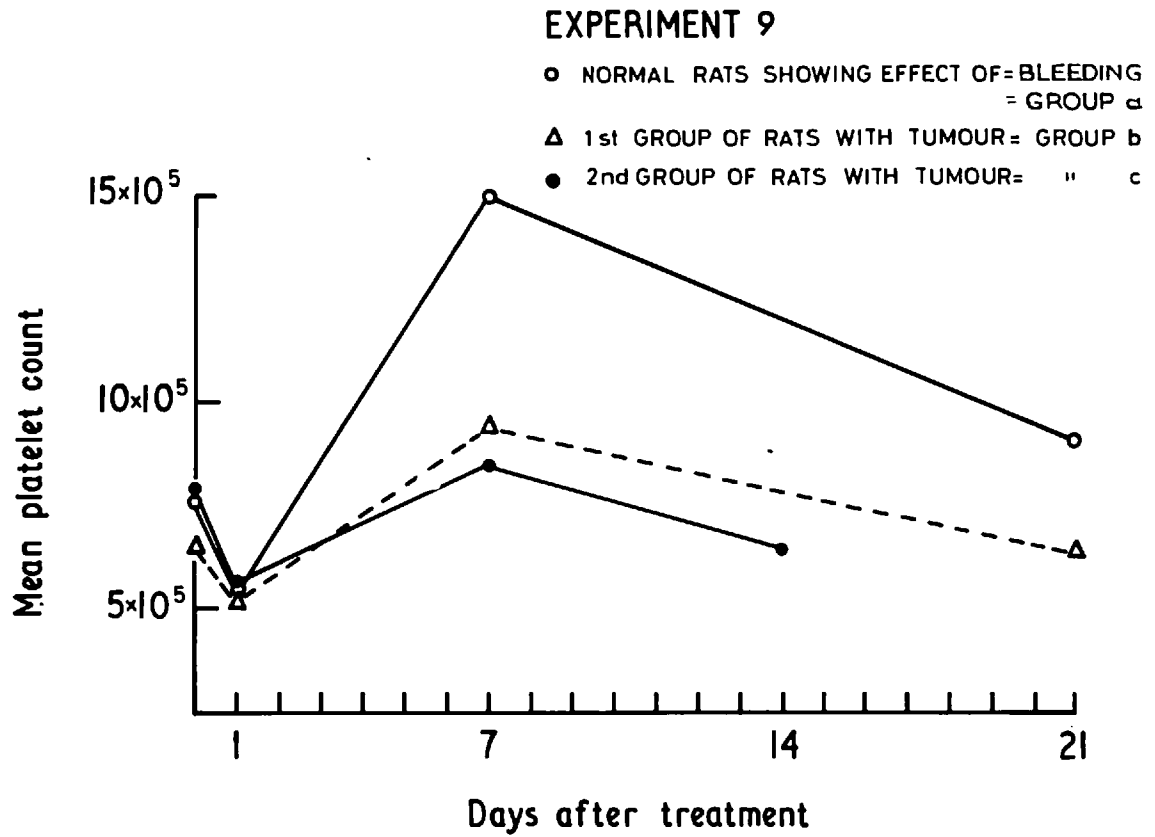


FIGURE 27.

Platelet count after bleeding of rats with and without tumour.

Statistical Analysis

Comparison of the incidence of circulating megakaryocytes between groups (9a) and (9b) showed no significant differences on days 0 and 1. But a significant rise became apparent on days 7 ($P < 0.05$) and 21 ($P < 0.01$). Comparison between groups two and three showed no significant differences. This seems comparable with the results of the tumour group (1b)(Experiment 1) only.

Discussion

In group (9a), the thrombocyte level was low on the day after 2.5 ml. of blood were taken and replaced with normal saline. The incidence of megakaryocytes was 10% on that day. On the 7th day after bleeding the platelet count was very high (200%). On the 21st day, the incidence of circulating megakaryocytes was 10% and the platelet count was within the normal range.

In the second group (9b), consisting of rats with tumour, the thrombocyte count was lower than the initial count and lower than the normal range on the day after bleeding, but the incidence of circulating megakaryocytes was 50%. On day 7, the thrombocyte count was higher than the initial value (30%) and the incidence of circulating megakaryocytes had risen to 70%. On day 21, the incidence of megakaryocytes was still higher, though the platelet count was falling. The same pattern was found in group three. There the results on day 14 were the same as on day 21 in

group two. Group three was in fact a repetition of group two, in order to confirm the results.

This experiment has demonstrated clearly that the incidence of circulating megakaryocytes in rats with transplant tumours and in normal rats is not related to the blood platelet level. The response of the platelet level to bleeding was similar to that observed by other investigators. The rising megakaryocyte count noted during the period of tumour growth is not affected by any rise or fall in the blood platelet count. This agrees with the observations made by Malméd et al. (1966) on cancer patients.

Experiment 10Relations between Immune Reactions and MegakaryocytesRationale

"Tissue immunology directly affects the outgrowth of tumours in which a distinct abnormal antigen can be detected. The immunological phenomena of tolerance or enhancement may be necessary prerequisites for the outgrowth of such a tumour. However, evidence that displaced or aged tissue is removed by a mechanism sharing some characteristics with the mechanism of classical immunology suggests that a related effect may occur with tumours in which no clear 'tumour antigen' can be detected." This immunological concept of the tumour was first suggested by Professor Green in 1954. After years of work a response mechanism in some ways similar to that of normal immune reaction has been postulated.

Rejection of a skin graft occurs when there is a minor degree of genetic disparity between the donor and the host. Infiltration of the graft by small lymphocytes is prominent in this process, and is believed to play a major role in the destruction of the graft. With a second graft, rejection is accelerated, there is a similar lymphocyte response, and the immune mechanism may be transferred to isogenic animals by lymphocytes instead of serum (Gowans and McGregor, 1965).

Macrophages play no prominent role in the reactivity to skin homografts, but they do to some extent in tumours (Groner, 1956; Old, 1962).

The mechanism of function is not yet clear, and it is not known whether the macrophages play a role in sensitization or whether they engulf the opsonized tumour cells (Granger and Weisen, 1966).

Eosinophilia has been observed in asthma, nasal polyps and in various intestinal conditions. These cells also play a part in hypersensitive conditions (Gell and Coombe, 1963).

There is no evidence that megakaryocytes are immunologically active cells. According to Green, the immune mechanism in tumours has some factors in common with the normal immune reaction, but there is nothing to indicate that the megakaryocytes are directly or indirectly involved with the immunological processes of the tumour.

It was decided to determine experimentally whether the incidence of circulating megakaryocytes changes after skin auto-, homo- and heterografts.

Materials and Methods

36 rats with a mean weight of 261.00 gm. ($SD \pm 5.48$) were used in this experiment. They were divided into three groups of 12 each.

The first group (10a) had a skin autograft. The day before the operation was taken as day 0. Blood for a megakaryocyte count was taken on days 0, 7, 14 and 21.

The second group (10b) had skin homografts on day 1. Blood was taken on days 0, 7, 14 and 21.

The rats in group three (10c) were given a skin heterograft. Blood was drawn the day before the operation and on the 7th, 14th and 21st days after it.

Technique of Skin Autotransplant

The rats were anaesthetized, both flanks were shaved and painted with a 5% hibitane solution. A circular piece of skin 2 cm. in diameter was excised from each flank and replaced by the piece taken from the other flank. The skin margins were sutured with black silk No. 1. The wound was dressed with gauze and covered with a bandage, to give protection for the time being. The progress of the skin grafts was checked daily.

Technique of Skin Homotransplant

Isogenic rats were taken two at a time. They were anaesthetized and placed side by side, on their left sides. The right flank of each rat was shaved and painted with a 5% hibitane solution. A skin disk of 2 cm. diameter, devoid of subcutaneous tissue, was taken from the flank of each rat and transplanted to the flank of the other. The grafts were sutured with black silk. The wound was covered with a clean piece of cloth and daily checks were made to follow the progress of the skin graft.

Technique of Skin Heterotransplant

This was exactly the same as for the skin homotransplant, except that skin taken from hamsters was transplanted into the flanks of the experimental animals. The hamsters had been bred in the Animal Department

of the Royal Postgraduate Medical School. Their average weight was 120 gms. and they were 8 to 10 weeks old. The rats given transplants of hamster skin were examined daily to check on the progress of the transplant.

Results

The rats in the first group (10a) had had autotransplants. All healed properly, without rejection or infection. The incidence of megakaryocytes was 0% out of 12 samples on day 0 and 8.3% on days 7, 14 and 21 (Table 32, Fig. 28).

The second group (10b) had been given skin homografts from isogenic animals. These were only successful in two rats, and the remaining 10 grafts were rejected in seven days on the average. There was no evidence of infection and the wounds healed gradually after rejection. In this group, the incidence of circulating megakaryocytes was 0% on day 0, 8.3% on day 7, 25% on day 14, and 8.3% on day 21. (Table 33, Fig. 28)

The rats in group three had received skin heterotransplants. All the rats rejected the hamster skin and the average period of rejection was 4 days. The incidence of megakaryocytes was 0% on day 0, 25% on day 7, 16.7% on day 14 and 16.7% on day 21 (Table 34, Fig. 28).

Statistical Analysis

When the results of the different days were combined for each group and compared with those of the normal control group 1a, no significant differences were found. Nor did comparison between any two groups in this experiment show any significant differences.

TABLE 32. Effect of skin autograft

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of HNK in Blood
0	12	12	None	0%
7	12	12	Skin autograft	0.3%
14	12	12		0.3%
21	12	12		0.3%

TABLE 33. Effect of skin homograft.

Day	No. of Rats.	No. of Samples	Nature of Treatment	Incidence of HNK in Blood
0	12	12	None	0%
7	12	12	Skin homograft	0.3%
14	12	12		25%
21	12	12		0.3%

TABLE 34. Effect of skin heterograft.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of H G K in Blood
0	12	12	Nil	0%
7	12	12	Skin heterograft	25%
14	12	12	-	16.7%
21	12	12	-	16.7%

EXPERIMENT 10

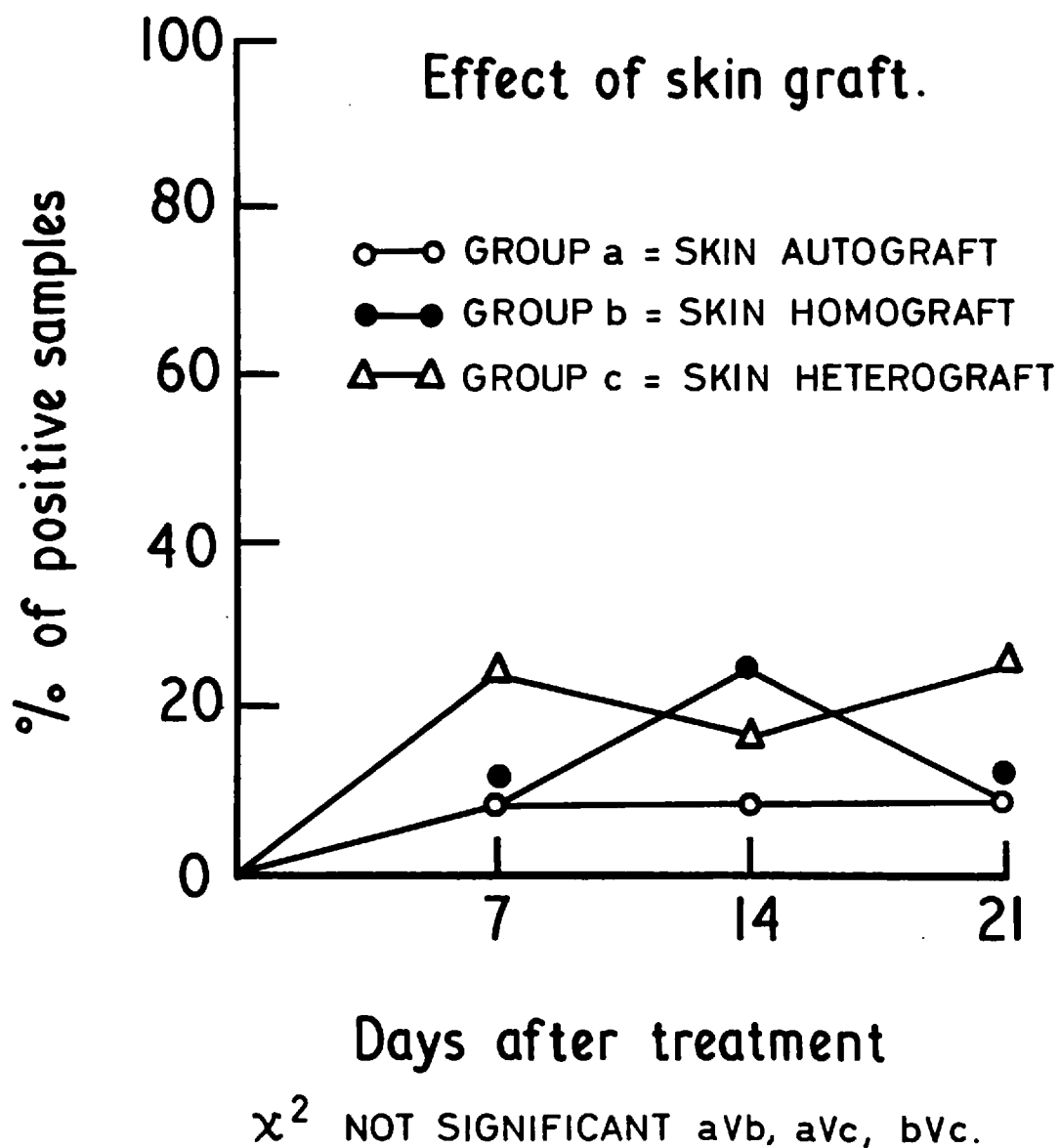


FIGURE 28.

The incidence of megakaryocytes in blood following skin grafts (Tables 32, 33 and 34)

Discussion

Small lymphocytes and macrophages are known to be immunologically active cells. Eosinophilia has been observed with hypersensitive conditions. There is no evidence that the megakaryocytes are immunologically active. In immune reactions, lymphocytes and macrophages are seen in the tissue and the blood count of these cells may be increased.

The present experiment has shown that there is no relation between the incidence of circulating megakaryocytes and the phenomena associated with the rejection of skin grafts. Biopsies from the rejected areas showed no megakaryocyte infiltration.

The spleen itself is related to the immune mechanism through its small lymphocytes and macrophages. Cathey (1966) observed splenomegaly, and also found that the number of megakaryocytes in splenic tissue is very much increased if the splenomegaly occurs as the result of a tumour. This increase still requires further investigation. If the megakaryocytes are in any way related to the immune mechanism, this certainly does not show itself in an increased incidence of these cells in the circulation.

Experiment 11The Effects of Irradiation and Cancer ChemotherapyRationale

Hieneka (1905) first demonstrated the effect of irradiation on the blood and haematopoietic system, and the radiosensitivity of the haematopoietic system. Later his observations were confirmed by other investigators (Langendorff, 1939; Brecher et al., 1948). The effect of radiation on individual components varies greatly. The differences observed are presumably due to the variation of species, and particularly to the different techniques of bone marrow evaluation (Dunlap, 1942; Dunstad, 1943). The marked effect of irradiation on erythroid, myeloid and lymphoid elements has been extensively studied (Langendorff, 1939; Bauer et al, 1940; Dunstad, 1943; Bloom, 1947). Less attention has been given to the megakaryocytes, reticulin cells and plasma cells, which are relatively less sensitive (Rosenthal et al., 1951).

Sprague-Dawley rats were given whole body irradiation with 700 r. After one day the number of megakaryocytes in the bone marrow showed no change. During the next five days there was a gradual decrease, and from the 7th to the 15th day there was complete absence of megakaryocytes. From the 15th day onward these cells started to reappear, and by the 21st day about 50% reappeared. The erythroid and myeloid elements require very much longer to regenerate (Rosenthal, 1951).

With regard to chemotherapy, Krumbhaar (1919) and Hermann (1919) reported the effect of mustard gas on the haematopoietic system. During the Second World War, the chemical warfare services of Canada, Great Britain and the U.S.A. carried out extensive studies on the biological effects of mustard gas and the newly developed nitrogen mustard. Gilman and Phillips (1946) reported on the effect of these substances as antineoplastic agents. Since then nitrogen mustard has been used in the treatment of cancer. Basically, the mustards cause a transient hypoplasia of the lymphoid system and bone marrow, accompanied by inhibition of mitosis of the blast cells in the bone marrow. This produces leukopenia of the circulating blood, with marked lymphopenia, followed by neutropenia (Kindred, 1946). When Wistar rats were treated with nitrogen mustard, Kindred observed no changes in the megakaryocytes of the bone marrow. He pointed out that the megakaryocytes suffer the least change of all the cells of the haematopoietic system.

Cyclophosphamide, which is used also as an antineoplastic agent, produces a short-term depression of myelopoiesis, followed by excessive myeloid cell production. Cyclophosphamide causes more damage to the dividing compartment than the other elements. Lajtha (1961) showed that both anti-metabolites and alkylating agents produce marked lymphopenia, granulopenia and eosinopenia. Unfortunately there is no record on the effect of these agents on the megakaryocytes.

Elson (1958) observed thrombocytopenia 8 to 10 days after

treatment with Myleran (chlorambucil). He also found that after treatment with chlorambucil and busulphan there was a reduction of megakaryocytes in the bone marrow and thrombocytopenia.

Methotrexate, a folic acid antagonist, produces leukopenia (Berlin, 1963). In the bone marrow, its exhibition led to qualitative changes in red blood cells within a few days, producing megaloblasts and intermediate cells of maturation (Dacie, 1949). There is a marked absolute and relative decrease in early granulocytes and a slight increase in late megakaryocytes in the bone marrow. During treatment a progressive depletion of megakaryocytes was noticed (Speck, 1967). The marrow lymphocytes showed no depletion during the course of treatment (Speck, 1967).

Kollar (1958) studied the response of the blood-forming system to cancer chemotherapy and observed that the megakaryocytes are extremely sensitive to such agents. He found that pyknotic degeneration occurred within 24 hours.

Romsdahl (1966) observed that patients with cancer showed an increase in circulating megakaryocytes. He found 17.3 megakaryocytes per 10 ml. of blood in these patients, and after treatment with chemotherapeutic agents this rose to 23.6 per 10 ml. The agents used were A-8103 and uracil mustard.

Cancer is treated by surgery, irradiation or chemotherapy, or with any combination of these. Blood has been examined for circulating megakaryocytes on several occasions, either during or immediately after

treatment (Wast, 1963; Hume, 1964; Romsdahl, 1967, a.o.). As irradiation and chemotherapy are known to affect haematopoiesis, it was decided to study their effects in an animal system. The incidence of circulating megakaryocytes after both irradiation and chemotherapy was determined in normal rats and in rats with tumours.

Materials and Methods

60 male Wistar rats with an average weight of 275.53 gms. ($SD \pm 6.85$) were used in this experiment. They were divided into four groups of 15 each.

The first group (11a) were given irradiation of the whole body, the dose being 250 r per minute for 2 minutes on two consecutive days. The total dose for each rat was 1,000 r.

Group two (11b) received 1.5 mg./kg. of body weight of methotrexate (4-Amino-N¹⁰-methyl pteroyl glutamic acid sodium supplied by Lederle Laboratories Division) intraperitoneally on days 10, 11, 12 and 13. In both these groups day 1 was the day after the first blood sample was taken. Blood was collected on days 0, 7, 14, 21 and 28.

The rats in group three (11c) were given a tumour transplant. The first sample of blood was taken on the day before and this counted as day 0. The tumour was irradiated with 500 r daily for two days, on the 12th and 13th day.

Group four (11d) also received tumour transplants on day 1.

Intraperitoneal injections of 1.5 mg./kg. of body weight of methotrexate were given on days 10, 11, 12 and 13. In these last two groups, blood was collected on days 0, 7, 14, 21 and 28.

Technique of Irradiation

The set was a Marconi industrial model, with electrically operated shutter, running at 250 KV and 15 mA, to give a dose rate of 250 r per minute. A 0.5 mm. copper and 10 mm. aluminium shield was used for both tumour and whole body irradiation.

The rats were immobilised by giving 0.1% Nembutal intraperitoneally. They were then placed in a Thomlinson's pressure chamber (Thomlinson, 1960). The pressure was kept normal and air was allowed to pass instead of O₂, to maintain the animals breathing normally. A dose of 500 r was given and repeated on the following day. A total of 1,000 r was given to all rats, both for whole body and for tumour irradiation. For whole body irradiation three rats at a time were placed in a perspex chamber.

the Preparation of/Chemotherapeutic Agent, Methotrexate, for Injection

Methotrexate, a popular antimitotic agent, was selected for this experiment. An adequate supply of this drug was available for experimental purposes. 4-amino-N¹⁰-methyl pteroyl glutamic acid, or methotrexate, is a folic acid antagonist commercially known as methotrexate. It is manufactured by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.

The drug is provided in powdered form. 2 ml. of sterile distilled water are added to a 5 mg. vial to reconstitute it for parenteral use.

The rats were weighed before each injection and 1.5 mg./kg. of body weight was injected intraperitoneally.

Results

Group one (11a) consisted of normal rats given whole body ^{blood} irradiation. None of the 15/samples were positive for megakaryocytes on days 0 and 7. On day 14, 13.3% were positive, and on day 21, 20% (Table 35, Fig. 29).

The second group (11b) had been treated with methotrexate. On day 0, none of the 15 samples were positive. 6.7% were positive on day 7, 33.3% on day 14 and 26.7% on day 21 (Table 36, Fig. 29).

The rats in group three (11c) had been given tumour transplants and irradiation. No sample was positive on day 0. On day 7, 26.7% of the samples were positive, on day 14, 40% and on day 21, 53.3% and day 28, 73.3% (Table 37, Fig. 29).

Group four (11d) consisted of rats given a tumour transplant and treated with methotrexate. 0% of the 15 samples were positive on day 0, 26.7% on day 7, 66.7% on day 14, 86.7% on day 21 and 93.3% on day 28 (Table 38, Fig. 29).

Statistical Analysis

The incidence of circulating megakaryocytes showed no significant

TABLE 35. Effect of whole body irradiation.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of M G K in Blood
0	15	15	None	0%
7	15	15	Whole body irradiation	0%
14	15	15	-	13.3%
21	15	15	-	20%

TABLE 36. Effect of Methotrexate injection.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of M G K in Blood
0	15	15	None	0%
7	15	15	Intraperitoneal methotrexate injection	6.7%
14	15	15	-	33.3%
21	15	15	-	26.7%

TABLE 37. Effect of irradiation of tumours

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of M G K in Blood
0	15	15	None	0%
7	15	15	Irradiation of tumour	26.7%
14	15	15	-	40%
21	15	15	-	53.3%
28	15	15	-	73.3%

TABLE 38. Effect of injection of methotrexate in rats with tumour.

Day	No. of Rats	No. of Samples	Nature of Treatment	Incidence of H G K in Blood
0	15	15	None	0%
7	15	15	Rats with tumour treated with methotrexate	26.7%
14	15	15	-	66.7%
21	15	15	-	86.7%
28	15	15	-	93.3%

EXPERIMENT 11

Effect of irradiation and chemotherapy

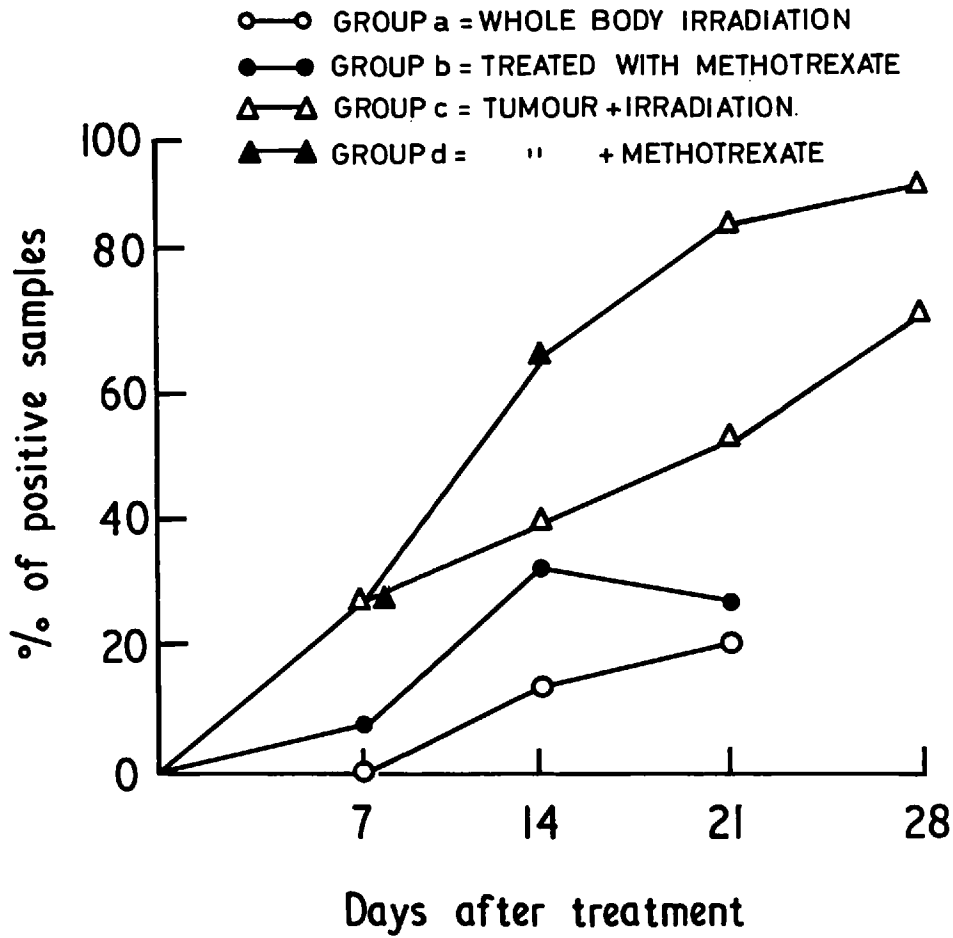


FIGURE 29.

Effect of irradiation and chemotherapy on the incidence of circulating megakaryocytes in rats with and without tumour.

differences when the groups treated with irradiation or injections of methotrexate were compared with the normal control group 1a. Comparison between the results of the group with tumour and irradiation or the group with tumour and methotrexate and the results of the tumour group 1b also revealed no significant differences. Similarly, no significant difference was seen between the group with tumour and methotrexate and the group with tumour and irradiation. The difference between the group treated with methotrexate and the group with tumour plus methotrexate was found to be significant, with a higher incidence in the latter ($P < 0.01$). A significant difference was also established between the group given whole body irradiation and the group where the tumour only was irradiated. The group with irradiated tumours showed a higher incidence ($P < 0.01$). The results of day 28 showed no significant differences between the two groups with treated tumours.

Discussion

In this experiment, the incidence of circulating megakaryocytes in rats with tumours treated with irradiation showed no significant difference from that in rats with tumours given no treatment. Similarly no significant difference was seen when normal rats given whole body irradiation were compared with normal rats given no treatment. It may be concluded therefore that the incidence of circulating megakaryocytes is not influenced to any significant degree by irradiation of the whole body or of the tumour and its effect on the haematopoietic system. This would

seem to confirm Rosenthal's (1951) observation that the megakaryocytes are less sensitive to radiation than other bone marrow cells.

In the groups of rats with tumours treated with methotrexate, the incidence showed no significant difference from that in rats with tumours given no treatment. It therefore appears that in rats with tumours the incidence of circulating megakaryocytes is not significantly influenced by either irradiation or methotrexate. On the 21st day, the incidence in the irradiated tumour group and that in the tumour group treated with methotrexate differ by 34.4%, the latter group having the higher incidence. On the 28th day, the incidence was 20% higher in the methotrexate than in the irradiated group. At the same time the irradiated tumour group gave results 26.4% lower than the tumour group given no treatment.

Irradiation changes the incidence to some extent, though the results are not statistically different.

On the 28th day, the total megakaryocyte count in the group of rats given chemotherapy was 480 cells per 100 ml. of blood. In the group treated with irradiation it was only 220 cells per 100 ml. of blood. It would appear therefore that tumours treated with irradiation have a different effect on the incidence of circulating megakaryocytes from that of tumours treated with chemotherapeutic agents. In the bone marrow, the response of megakaryocytes to irradiation is a slow one (Rosenthal, 1951). During treatment with methotrexate, progressive depletion of these cells has been observed (Speck, 1967). Kollar (1958) noted that megakaryocytes are

extremely sensitive to cancer chemotherapy. In the blood of normal rats treated with irradiation or methotrexate, the incidence of megakaryocytes showed no significant difference.

The reduction after irradiation may be attributed to effective control of the biological activity of the tumour. However, the fact that both the incidence and the total count increased after methotrexate does not prove that this agent is not effective against the tumour. This aspect requires more detailed study.

Study of Tumour Volume in Experiment 1

Relations between the Tumour Volume and the Incidence of Megakaryocytes

Rationale

Megakaryocytes are more common in the blood of cancer patients and their incidence increases as the disease advances. This has been observed by several authors. One of the experimental series in this study has demonstrated that tumour tissue homogenate injected into normal rats is equally effective in raising the incidence of circulating megakaryocytes. It is logical to presume at this stage that the tumour produces some substance which causes this increase, and that a large tumour would be more active in this respect than a smaller one. It was therefore decided to study the relationship between the tumour volume and the incidence of circulating megakaryocytes.

Materials and Methods

The results obtained with the animals in group 1b were analysed. The group consisted of 20 rats with a mean weight of 250 gms. ($SD \pm 8.24$). All of them had a subcutaneous tumour in the right flank. The tumours had been produced by injecting 5×10^6 to 6×10^6 cells in 0.4 to 0.6 ml. of sterile normal saline subcutaneously. 1 ml. of blood had been drawn from the heart on the day before this inoculation was given, this day counting as day 0. Further samples had been taken on days 7, 14 and 21. The tumour volume was also determined on days 7, 14 and 21, using the

technique described by Mori et al. (1964).

Results

In the first instance the relation of the tumour volume to the progress of the disease was studied. Table 39 shows that on day 0 the tumour volume was 0. 7 days after inoculation the mean tumour volume was 0.620 ml. ($SD \pm 1.357$), and on the 21st day 29.838 ml. ($SD \pm 10.766$). The tumour volume therefore increased significantly with the progress of the disease, as can be seen from Fig. 24.

Table 39 shows that on day 0 the incidence of megakaryocytes in the blood was nil, and obviously the average number of cells per ml. of blood was also nil. On the 7th day, the incidence was 25%, with an average of 0.50 cells per ml. On the 14th day, the incidence was 55%, with an average of 1.60 cells per ml. On the 21st day, the incidence was 70%, with an average of 4.00 cells per ml. These results have been plotted on two graphs. On the first (Fig. 30) the number of cells was plotted against the volume of each individual tumour, including the negative counts. In Fig. 31, only the positive counts were plotted against the tumour volume. A positive relation was established between the tumour volume and the number of cells, when the negative observations were included as '0' cells. This is even more clearly apparent in Fig. 32, where a comparison of regression of total numbers of megakaryocytes in each sample of blood taken from the rats in Experiment 1 has been drawn.

TABLE 39. Increase in tumour volume and number and incidence of megakaryocytes in blood.

Day	No. of Rats	Treatment given	Mean volume of tumour	Incidence of MGK in blood	No. of MGK per ml. of blood
0	20	Subcutaneous Walker tumour	0	0%	0.00
7	20	-	0.620 S.D. ± 0.273	25%	0.50
14	20	-	5.685 ± 1.367	55%	1.60
21	20	-	29.839 ± 10.766	70%	4.00

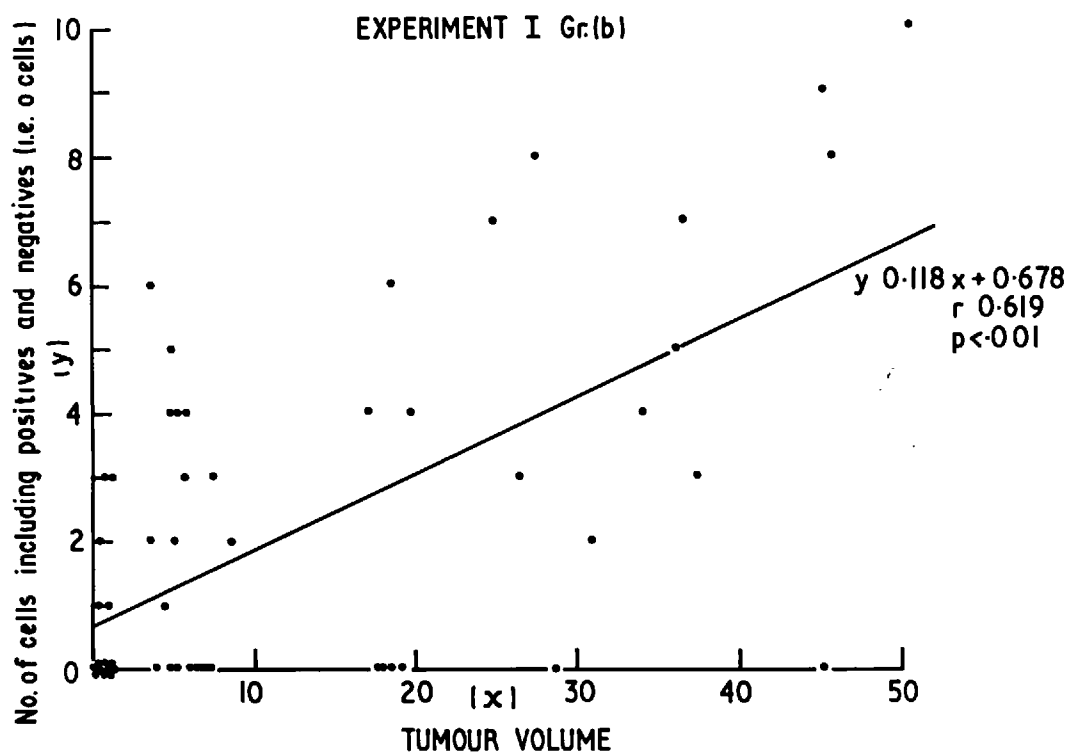


FIGURE 30.

Scatter of tumour volume both positive and negative of megakaryocytes in blood.

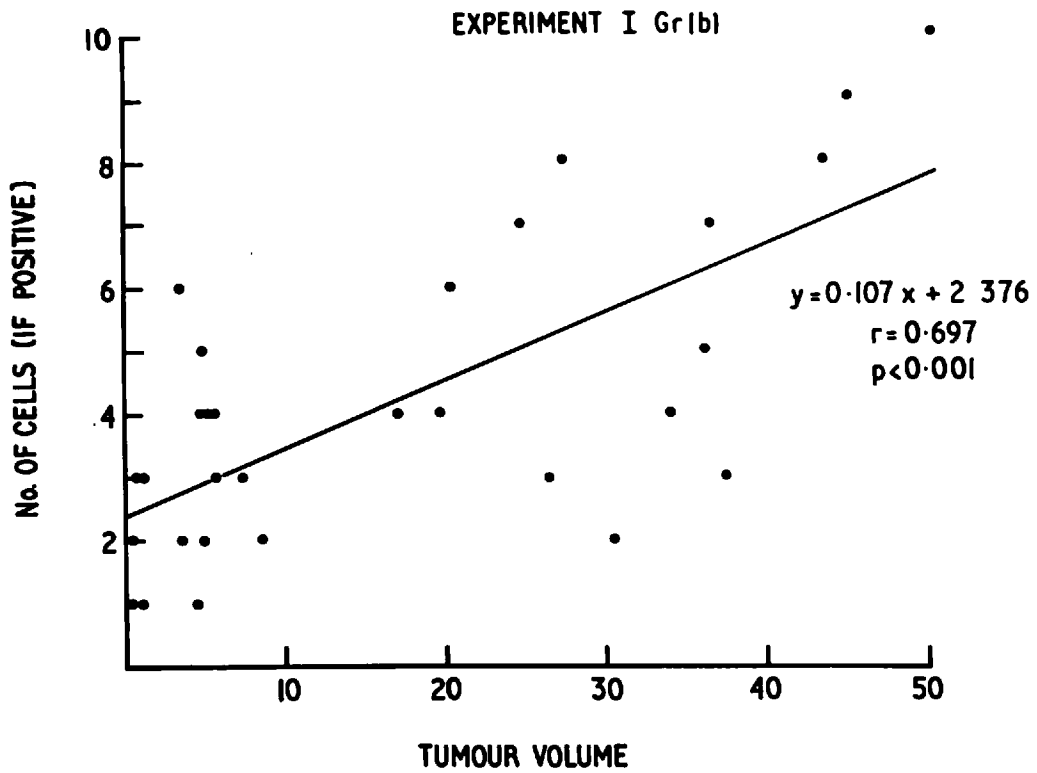


FIGURE 31.

Scatter of tumour volume of only positive
of megakaryocytes in blood.

EXPERIMENT 1 Gr(b)

Comparison of the regressions of no. of cells on tumour volume
a) when just positives are included and b) when positives and
negatives are included.

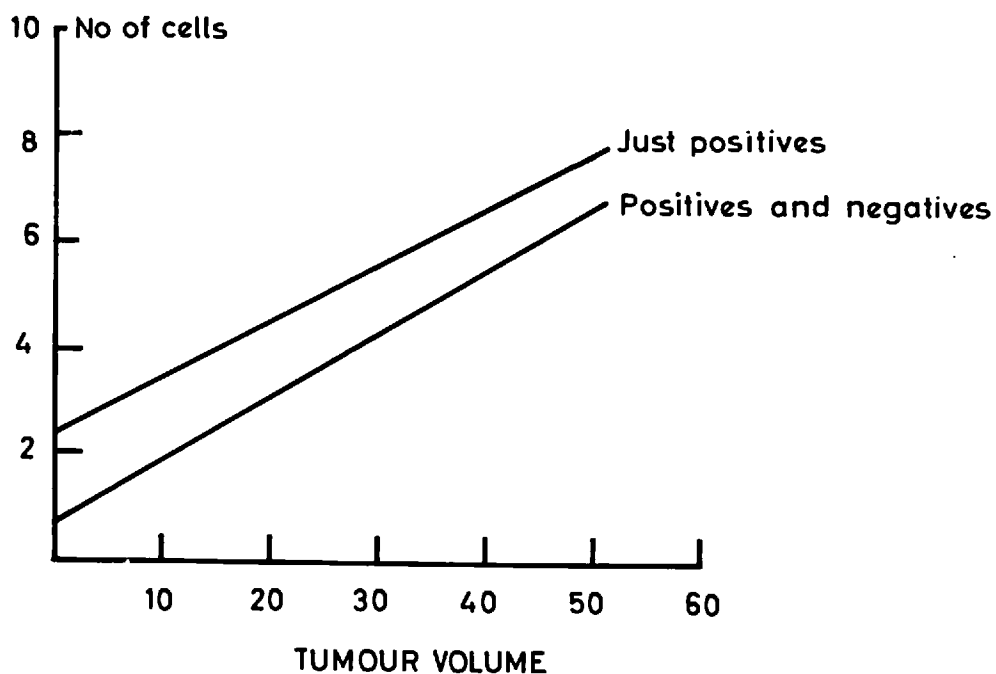


FIGURE 32.

Comparison of regression of total number of megakaryocytes in
each samples of blood taken from rats of Experiment I.

Discussion

The tumour volume was found to show a positive correlation to the number of circulating megakaryocytes. This is in agreement with the findings of several other investigators, that the incidence of circulating megakaryocytes is higher in patients with advanced carcinoma. This study has shown that there is no obvious difference in the tumour volume of rats with positive and with negative counts. Once an animal became positive it consistently remained positive. Reversal to a negative count is very rarely observed in tumour-bearing animals. Some of the rats were late in becoming positive and some never did become positive. In relation to the megakaryocyte response in the presence of a malignant tumour these animals may be divided as follows:

- 1) those responding early
- 2) those responding late
- 3) those which never respond

An explanation for the variable incidence of circulating megakaryocytes is given in the following chapter.

DISCUSSION

ISOLATION AND IDENTIFICATION

Megakaryocytes have been detected in the peripheral blood by various techniques. As early as 1922 Minot detected these cells by making simple smears and using Giemsa's staining technique. For morphological identification, the conventional haematological techniques proved adequate. It is possible to confuse various cells of haematopoietic origin, tissue cells and cancer cells with megakaryocytes, but the morphology of the latter is very characteristic and identification is simple. The megakaryoblasts may give rise to some difficulty, but this can be circumvented by identifying the basophilic and very scanty cytoplasm of these cells. With most techniques, the morphological identity of the megakaryocytes is well maintained. It is also often possible to identify megakaryocytic nuclei in different preparations. The number of these cells in the blood is much lower than that of other nucleate elements. It is necessary at times to examine several slides before a single megakaryocyte is found. This makes the technique of detecting megakaryocytes in smears very tiring and tedious.

Several authors have mentioned that megakaryocytes are not a normal constituent of the blood. This comment would be no surprise when it comes from those who tried to find these cells by the smear technique. With the development of the newer technique of leuko-concentration it is now possible to detect megakaryocytes more frequently in the blood of normal individuals. In the present series of experiments, intact cells were found occasionally in the blood of normal rats, which would have

been impossible with the smear technique. That technique was avoided because the chances of missing these cells are very high. Apart from maintaining clearer morphology of megakaryocytes, the smear technique has not proved superior to any other. The problem of these cells even with high megakaryocyte counts is first one of identification and then of enumeration. So far inconsistent results have been the main source of frustration in any attempt to determine the quantity of these cells in the peripheral blood.

With the leuko-concentration technique, detection and enumeration become easier, because it is possible to examine a large volume of blood. The morphology of these cells is quite well preserved with the technique. The smears are made from unfixed cells. The process itself may be detrimental to the cells. Fragility is more marked in megakaryocytes (Hume, 1964), because of the eventual formation of platelets by cytoplasmic disintegration. The trauma of processing may cause damage to the cells, and bare nuclei are usually difficult to identify. In fact, most of the publications in relation to circulating megakaryocytes include various nuclear segments in the count as fragments of megakaryocyte nuclei, as they resembled the bare nuclei of megakaryocytes. It must be realised that a clump of lymphocytes, nuclear fragments from osteoclasts and nuclear fragments or bare nuclei of other immature haematopoietic cells may well resemble the bare nuclei of megakaryocytes or fragments of these. Several observers reported the presence of bare nuclei of megakaryocytes

only in the peripheral blood of normal individuals. In the case of cancer patients, they found intact cells to be very common. It is reasonable not to accept the figures of those who included what they thought to be nuclear fragments of megakaryocytes in the total count and incidence. In the present study, fragments of these cells have justifiably been excluded from the count. The technique employed has been that of leuko-concentration. The cells required for examination are trapped on the surface of millipore filter membranes, having been fixed before filtration. The possibility of damaging these cells is practically nil, unlike with the buffy coat technique. Furthermore the total cell concentrate is trapped in the millipore membrane, so that this is a quantitative method, which has been lacking so far. The morphology of the megakaryocytes is well maintained when stained with haematoxylin and eosin. This technique enabled us to study both the incidence and the total count of megakaryocytes in the peripheral blood.

The plastic sieve technique of Seal (1964) and Romedahl (1954) is equally satisfactory. The histochemical technique described by Jackson always tends to give a higher count, simply because megakaryocytic fragments give the same reaction as intact cells and are counted as such. The immunological technique described by Daheille et al. (1962) is also satisfactory in detecting these cells and their parts. The incidence could be more precisely established. As far as the total count is concerned, this technique is of no advantage, because parts or fragments of mega-

karyocytes fluoresce in the same way as intact cells. But detection is very precise and the technique is superior to many that have so far been used.

Cytogenetics and Cytokinetics

Megakaryocytes as a bone marrow element were first discovered by Robin in 1849. His idea was that these cells act as lining cells of the marrow sinusoids and develop from there. His findings were soon confirmed by several other authors. The bone marrow has been the undisputed site of development and maturation of these cells. Aschoff (1893) described the embolization of these cells. According to him, substances with positive chemotaxis for leukocytes also stimulated amoeboid movement of the megakaryocytes from the bone marrow, propelling them through the thin walls of small vessels and capillaries into the blood stream. Most of these migrants are arrested in the lungs and mechanically stripped of their cytoplasm in the narrow and tortuous capillaries (Achenbach, 1949). Foa expressed this view much earlier (1899). Only a few pass through the pulmonary capillaries and lodge in other organs. This idea was also expressed by Lubarsch (1893). A controversy about the origin of these cells started in 1937 when Howell and Donohue observed giant cells in the pulmonary tissue and found more platelets in the pulmonary veins than in the arteries. They concluded that megakaryocytes were generated in the lungs. This view was quickly challenged by other investigators (Jordan, 1940; Fidler and Waters, 1941). Later studies showed that megakaryocytes are almost always present in the lungs of both humans and animals. Infection and other conditions involving increased/megakaryocyte formation have also been associated with an increase in the number of pulmonary megakaryocytes (Sharnoff, Kin, 1958; Orbison,

Laipply, 1945). Riechard, Kauffman et al. (1965) carried out experimental studies and concluded that these cells do not originate in the lungs, but are carried to the lungs in the venous blood. They further noted that surgery is a potent stimulant to megakaryocyte production. Postoperatively the number of these cells in the lungs had increased significantly.

Lengemann (1901) stated that softening of the bone marrow through hyperplasia, hyperaemia and haemorrhage is responsible for the mobilization of megakaryocytes. This "myelokinesis", as he designated it, may be experimentally valid, but cannot be applied to clinical experience (Schwarz, 1955), for most of the conditions accompanied by megakaryocyte embolization show only slight changes in the bone marrow, or none at all (Schwarz, 1956). The blood of normal individuals also contains megakaryocytes. Foa (1899) posed the question whether the pathological instance should not be regarded as the mere exaggeration of a normal occurrence. Schwarz (1954) suggested that this event, the mobilization of megakaryocytes, should be considered as of physiological significance rather than an aimless epiphenomenon. He also suggested that in the search for megakaryocytes, blood from the right auricle, obtainable during routine cardiac catheterization, might also throw some light upon the subject.

Apart from the pulmonary origin of megakaryocytes, other extra-medullary sites have also been suggested (Bushnell and Hall, 1906; Hagedorn, 1926; Downey et al., 1930; Anagnostu, 1933; Jordan, 1941; Japa, 1943; Kopac, 1943; Churg et al., 1944; Taylor Simpson, 1950; Schwarz, 1954).

In the bone marrow, either cells of the reticulum (haemohistoblasts) or the myeloblasts (haemocytoblasts) or both are at present regarded as the ancestors of megakaryocytes (Schwarz, 1955). In ectopic sites, either fixed cells or myeloblasts have been claimed as the parent cells. In the first case, the extramedullary cells would be exclusively autochthonous, in the second, the myeloblasts may either have arrived by way of the blood stream or have formed locally through metaplasia, but the transformation itself would always proceed in the ectopic site. Schwarz has accepted a variable origin and formative process for a cell morphologically and functionally as defined and specified as the megakaryocyte. A twofold origin from the reticulum and the myeloblast is not quite incompatible with the statement because of their close position in the genetic line (Schwarz, 1956). Cesa-Bianchi (1921), Di Guglielmo (1928) suggested that these cells might originate through direct fusion of mononuclear precursors. This is unacceptable, because it is the tendency of the intrasinusoidal myeloblasts to agglutination which gives this deceptive impression (Schwarz, 1954). Kopac (1943) admits the fixed reticulum as the only source of ectopic megakaryocytes, but the cases reported by Schwarz (1954) suggest a myeloblastic origin. A local transformation of myeloblasts from reticulin cells is also possible.

The transformation of the myeloblast into a cell as specified as the megakaryocyte is an instance of differentiation in its strictest sense. It is effected by the response of the pluripotent precursor cell

to a differentiating factor. In chronic myelosis, the myeloblast has preserved its competence, whether the case is regarded as reactive or neoplastic hyperplasia, because all types of granulocytes and megakaryocytes are part of the leukaemic population. Zajicek (1957) carried out histochemical studies of the localization of acetylcholinesterase and concluded that the erythropoietic and megakaryocytic systems derive from a cell of common origin. This has been confirmed by autoradiographic studies (Tempka et al, 1965). It therefore appears that the precursors of megakaryocytes are still the subject of dispute.

Megakaryocytosis occurs either in the presence of an unusually powerful local or diffuse differentiator, or through an increased propensity on the part of the cells to respond to the megakaryocyte differentiator. Perhaps the same factor may be responsible for the increase of circulating megakaryocytes in cancer cases. The increase is stimulated by the presence of a tumour, not essentially directly by any tumour product. When tumour extract was used in the experiments, ^{it} probably had the same effect on the megakaryocyte differentiator, and produced a marked increase in the incidence of megakaryocytes in the blood and spleen. The detection of the megakaryocyte stimulating factor in the blood by Stienberg and Cheng (1965) supports this idea.

As regards a local tissue factor which enhances megakaryocytosis, the only information on this seems to come from Klempner (1938). He found an accumulation of megakaryocytes in a leukaemic spleen in the

neighbourhood of an anaemic infarct, and also in two instances of thrombosis of the portal and splenic veins in non-leukaemic persons. A similar idea was also put forward by Rothermel, giving anaemia as a possible triggering factor (1930). But Schwarz (1934) reported a whole series of cases where infarcts had developed in the spleen, but no relation between the infarcts and the location of megakaryocytes was demonstrable. Catayee (1966) detected megakaryocytes in the spleen after injection of tumour extract, but their distribution appeared completely unrelated with the ischaemia or infarction. In the first of his cases, Schwarz (1934) noticed numerous megakaryocytes in the hyperaemic lymph nodes and this clearly rules out that hypothesis.

Response

Hypertrophy and hyperplasia, excluding neoplastic growth, are linked with functional stress. It is necessary to know cellular function in order to understand cellular reactions and their causes. Conversely in the absence of such knowledge, the circumstances of a reaction may give a hint of the role of a cell in the body.

The response of other cell components of the blood and bone marrow has been studied, and some definite biological activity of these cells could be detected. Polycythaemia in response to hypoxia, reticulocytosis to high altitude and hypoxia (Yoffee, 1966). Anaemia with abnormalities in erythrocytes has been well documented, and so have neutrophil leukocytosis in infections; neutrophil leukopenia with chronic protozoal diseases, some specific bacterial infections such as typhoid fever and brucellosis, and drug reactions; lymphocytosis with chronic infections; lymphopenia after whole body irradiation and the exhibition of ACTH. In addition, monocytosis has been associated with protozoal infections like malaria, bilharziasis and leishmaniasis; eosinophilia with atopic hypersensitivity and helminthic infestation; plasma cells or plasmacytosis with rubella, measles and chicken pox (Waher and Israel, 1966). All these cells respond to these specific conditions with some specific function, like phagocytosis, antitoxic or immune response. None of these functions are known to be performed by megakaryocytes. In the present study there has been no evidence to suggest that the megakaryocytes

are capable of phagocytosis in the way that neutrophils or macrophages are. In the series of experiments with different skin grafts, the incidence of circulating megakaryocytes showed no increase, whether auto-, homo- or heterografts were involved. Nor did biopsy from the site of rejection show any evidence of megakaryocyte infiltration, while lymphocytes are known to infiltrate under these circumstances. A review of the available literature provided no indication of megakaryocytes being associated with hypersensitive reactions. Nor has any antitoxic reaction of these cells been reported. The results of the present study have shown that megakaryocytes increase in the blood after injections of tumour tissue extract, so that they may be involved in neutralizing the effect of the extract. But it is also probable that the tumour tissue extract and/or the serum factor acts on normally circulating megakaryocytes and alters their fragility, thus producing the increased incidence. Halothane reduces the fragility of megakaryocytes by hardening their cell membranes (Bruce et al., 1966). It is not yet known whether this substance increases the number of megakaryocytes in the blood. In this study ether has been used for general anaesthesia, but no increase in the incidence of circulating megakaryocytes was observed after giving general anaesthesia to rats.

So far the only known function of the megakaryocytes is platelet formation. Platelet production and its participation in the clotting mechanism is a well established fact. The normal organism has many precautions to prevent clotting, so that it only occurs under abnormal

conditions like inflammation, embolism and bleeding. Few data are available on the relation between the platelet count and the incidence of circulating megakaryocytes. Megakaryocytosis was not found to be associated with a high platelet count (Schwarz, 1954), and subnormal and even decidedly low counts have been reported in such cases. The megakaryocyte level in the spleen frequently surpasses that of the bone marrow (Cataee et al., 1966). Downey and Nordland (1933) reported on a patient with a spleen highly infiltrated with megakaryocytes and no rise in the thrombocyte count, in fact, the count was low. The bone marrow was hypocellular. Apparently the extramedullary megakaryocytes do not assist in maintaining the blood platelet level (Schwarz, 1956). Epstein et al. (1934) suggested that the bone marrow megakaryocytes are in fact the real precursors of the blood platelets. This has been supported by Hutt (1953). A very interesting observation was made by Firkat and Llampos (1922) who found a complete aplasia of the bone marrow, possibly due to exposure to benzol fumes; there was an extensive myeloid metaplasia of the spleen, liver and lymph nodes. Although megakaryocytes were the most numerous elements in the myeloid tissue, platelets were practically absent from the blood.

Frey (1928) regarded spheroid megakaryocytes as inferior where platelet formation was concerned. In the present study most of the circulating megakaryocytes were spheroid. Any distinction between spheroid and "polymorphous" megakaryocytes was impossible. This is probably due to the fact that the semisolid cell assumes a physical equilibrium when suspended in blood plasma.

The inactivation of platelet formation does not involve the vegetative process, for mitosis was fairly frequently observed in adult intrasinusoidal megakaryocytes (Frey, 1928; Schwarz, 1955). The fact that remnants of megakaryocytes surrounded by accumulated platelets occur in the blood does not oppose the concept that these cells are a deviation from platelet formation. These structures are mobilized shreds of megakaryocytes, that have performed their function at the original site in the tissue. This agrees with the findings in the present experiment that the megakaryocyte level in the peripheral blood is independent of the platelet level and in no way associated with platelet production, and that the cells are to some extent derived from extramedullary sites. Moreover, Gautier (1963) considers that platelet formation in megakaryocytes is a cyclic phenomenon.

Paulas (1967) observed that 100% viable megakaryocytes could be obtained from the bone marrow^{of}/rats after collagenase digestion. It is possible that these cells become fragile and form platelets whenever necessary, but are otherwise quite stable. Prizivoit's (1964) observation of thrombocytopenia in rats with experimental tumours has no bearing on the circulating megakaryocytes. One wonders if this thrombocytopenia is due to the reduced fragility of megakaryocytes (Hume et al., 1964), but so far there has been insufficient evidence in support of this thesis.

The fibrinolytic mechanism is activated in cancer patients (Dillard and Chanutin, 1949; Tagnon et al., 1953; Sirridge et al., 1958;

Nilehn, 1966). This has been regarded as a defensive mechanism on the part of the body, to prevent the tumour from spreading by inhibiting thrombus formation and subsequent seeding in distant sites. Thrombocytopenia in malignancy may be explained that way, as the result of reduced formation from megakaryocytes. These would be made less productive by increasing their stability. This is probably controlled by some humoral factor, as Stienberg and Cheng (1965) have recently demonstrated the presence of some albumin bond-stimulating factor in megakaryocytopoiesis. This stimulating factor is mainly involved in the maturation of megakaryocytes and not in the migration of the cells from the bone marrow or platelet formation. It is possible that the maturation factor was unaffected in patients with neoplasms, but that an alteration takes place in the factors controlling platelet formation. Prigivolt's observation that the blood platelet level was reduced in megakaryocytosis of the bone marrow may therefore be valid. In some other thrombocytopenic conditions, e.g. thrombocytopenic purpura, abnormal basophilic, degranulated megakaryocytes and a reduction in numbers were noted in the bone marrow (Rosenthal and Erf, 1943).

In the case of thrombocytopenia following bleeding and surgical operations, an increased rate of maturation and proliferation of these cells have been observed. But the observation of old forms in circulation could not be confirmed in this experimental study.

When animals with tumour have a minimal thrombocytopenia, and

megakaryocytosis in the bone marrow, compensation for the reduced platelet count does not seem apparent (Prizivoit, 1964). It is possible that the tumour itself causes proliferation of the bone marrow megakaryocytes. Extramedullary megakaryocytosis is not dependent on the bone marrow megakaryocytes, and that is possibly true in their circulating counterparts. So far the experimental evidence has been that the incidence of circulating megakaryocytes is not influenced by the blood platelet level and the bone marrow megakaryocytes. We are now confronted with two problems: 1) what is the stimulus for these cells to increase, and 2) what is the source of these cells. Extramedullary megakaryocytes are found in a number of conditions. Experimental myeloid metaplasia may be produced by several methods (Sternburgh, 1909; Jaffee, 1921; Dickmann, 1922; Lang, 1930, Morris, 1951). Megakaryocytosis may be seen in the extramedullary sites. Apart from being part of the myeloid elements in extramedullary tissue, these cells have on several occasions been seen in extramedullary sites without being part of the myeloid elements (Schwarz, 1954; Catayse, 1966). Virchow (1863) observed that these cells do proliferate in other sites from emboli of bone marrow. This has been seriously challenged and there is sufficient proof to establish its fallacy (Schwarz, 1954, 1956). Local formation from mononuclear precursors has already been suggested, but that has also been questioned, because there is no direct evidence.

The response of megakaryocytes to the phenomenon of gas exchange

has been studied. That is how a link between the megakaryocytes and the lung, the only surface designed for gas exchange between the organism and the atmosphere, was conceived. There is very little on the subject in the literature. Tigerstedt (1913) assumed that platelets have a high metabolic activity. Such a high metabolic rate presumes an adequate oxygen supply and prompt removal of carbon dioxide, so that the lung might be regarded as an excellent site for such demands. An accumulation of platelets at this site, either through increased production or a slower rate of decay, would be understandable. Clinical observations supported by experimental findings indicate a relation between impaired venous circulation and local megakaryocytosis (Schwarz, 1954). Rosin and Rachmilewitz (1948) made the interesting observation that when human bone marrow cultures are kept under reduced oxygen pressure all the cells are severely damaged, with the exception of the megakaryocytes which alone preserve their normal appearance.

There is no reason to believe that the same fact is also responsible for megakaryocytæmia in cancer. In that case blood or serum should be responsible for this condition. One does not find increased megakaryocytes in hypoxæmic conditions where polycythæmia is seen. No direct study has so far been done. But it is possible that the blood oxygen level may remain subnormal through increased metabolic activity of malignant tumours, though local anoxæmia is more likely. We very rarely observed increased numbers of megakaryocytes in tumour sections. Hume (1964)

suggested that it is possible to get a high incidence of circulating megakaryocytes in blood draining the tumour. But she later concluded that these cells came from the bone marrow rather than the tumour itself. Pruitt and his colleague (1962) also noticed a higher incidence of megakaryocytes in the blood draining the tumour area. If higher metabolic activity were the cause for attracting megakaryocytes to the tumour area, the reverse would be the case. Fewer megakaryocytes are found in the blood of the pulmonary vein than that of the pulmonary artery. But again this effect could be due to the mechanical sieving effect of the pulmonary capillaries. Relations between the gas exchange and this effect and tumours are still difficult to explain. A thorough study should be made under various conditions of blood and tissue gas exchange to confirm or refute this postulate.

Megakaryocytæmia as a terminal sign of malignant disease has been associated with leukoerythroblastic anaemia (Minot, 1922; Whithy, 1949). But in a considerable proportion of cancer cases no anaemia at all is seen (Shen, Homburger, 1951). Extensive bone marrow involvement through tumours may produce a striking hæmatological effect. This is usually leukoerythroblastic anaemia and may be associated with splenomegaly and hæmatopoietic activity in the spleen. Pillar (1965) and his associates found that only 37 out of 601 patients (approx. 5%) had cancer cells in the sternal bone marrow. Anaemia has been seen in patients with cancer of the left side of the colon (Lahay, 1939), cancer of the rectum

and anal canal (Raven, 1958) and cancer of the stomach (Raven, 1958). Patients with lung cancer may also present with anaemia (Britton, 1963). But the majority of superficial cancers are not associated with anaemia. Anaemia may be seen in cancer of the breast and cancer of the prostate because of bone involvement. With disseminated malignant diseases haemolytic anaemia may be seen with increased iron turnover and increased plasma volume (Lockner, 1960).

In the present experiment all the tumours were superficial, and careful postmortem examination gave no evidence of bone involvement. In the rats of Experiment 1, group b, the incidence of circulating megakaryocytes was high on the 7th day, when the tumour volume was 0.620 cc ($SD \pm 0.273$). The increase became statistically significant on the 14th day, when the mean tumour volume was 5.685 cc ($SD \pm 1.357$). Though no assessment of anaemia was done, anaemia was unlikely at that stage, when the tumours were so small. Blood smears never showed any abnormality in red blood cells; reticulocytes and megaloblasts were just occasionally seen. Though one finds increased numbers of megakaryocytes in the blood of patients with advanced carcinoma, and also leukoerythroblastic anaemia, anaemia cannot be associated with this increase in early cases, or even after injecting tumour homogenate. But there are still gaps in these premises, to prove the validity of my observation.

The response of these cells as a result of splenomegaly has been studied by Schwarz (1954). But there has been no report suggesting

that the spleen may be one of the sources of circulating megakaryocytes. Splenomegaly has been seen as a result of sepsis, bleeding and other chronic conditions (Britton, 1961). Extramedullary haematopoietic centres were noted, and megakaryocytes have been seen in association with these centres. It is possible that under these conditions the spleen, and particularly that of rodents, being a mechanically functioning organ (Snell, 1941), discharging its contents into the blood at intervals, could be one source of megakaryocytes in the blood. But Catayee injected tumour extracts and found increased megakaryocytes in the splenic pulp even though there was no extramedullary haematopoietic centre in the spleen. In the present study, increased megakaryocytes were observed in the blood after tumour homogenate injections. Interestingly enough, there was also an increase after liver tissue homogenate injections. However, this shows that anaemia and megakaryocytaemia when present in neoplastic disease are controlled by a different stimulus.

Paulus (1967) described the megakaryocytes as polyploid cells. Polyploidy is common in plants, and normally associated with differentiation and inhibition of reproductive mitosis (Palay, Das, Skoog, 1957; Torrey, 1958). In megakaryocytes polyploidy may have many additional consequences. Some of these are morphological, e.g. the large size of both the nucleus and the cytoplasm may be explained by polyploidy. More important, however, may be the metabolic and genetic disturbance associated with a high chromosome number. As Frankhauser (1952) pointed out, the increase in

cell size could influence the relationships between cytoplasm and nucleus, and cytoplasm and environment. In tetraploid yellow corn the amount of carotenoid pigment per gene is increased. In triploid humans abnormal development has also been observed, showing the abnormal cumulative effect of an extra gene. Perhaps this difference in morphology and consequently in cell physiology renders these cells more prone to migration from the bone marrow to the circulation, because of their role in the body's reaction to the pathogenesis of neoplasia.

Puck and Marcus (1956) showed that polyploid cells are more susceptible to virus accumulation. The same has been shown for megakaryocytes (de Haven, Friend, 1960; Dalton, Law, Moloney, Manaker, 1961; Jerushalmy, Kaminski, Kohn, de Vries, 1963). De Haven and his colleagues carried out electron microscopic studies in animals with virus leukaemia and found numerous characteristic particles inside the channels of megakaryocytes which were different from intracellular organelles. If these particles are accepted as a morphological representation of oncogenous agents, then we have an example of non-malignant cells functioning as a site of replication for oncogenous viruses. Megakaryocytes may have served as a site of virus replication.

Paulus described both platelets and megakaryocytes as endocytic elements. Platelets have been described as sponges (Adelson, Rheingold, Crosby, 1961). The endocytosis of platelets has been clearly established through electron microscope pictures of phagocytosis (David-ferreirg, 1964)

as well as of pinocytosis (Bessis, 1955). In vitro, endocytic intervention may induce the liberation of ADP and the loss of platelet granules. In vivo it may lead to a more prolonged thrombocytopenia (Tail, Elvidge, 1926). Thrombocytopenia in malignant disease may be ascribed to this phenomenon (Prisivoit, 1964). The megakaryocytes may be acting like a mop and also as an endocytic element for the products of neoplastic tissue. In the absence of malignant disease their presence in the blood is not essential. That is why not all thrombocytopenic conditions are associated with megakaryocytæmia. A process of microphagocytosis of virus particles (de Haven et al., 1960) is suggested, rather than a site of virus replication, but the phenomenon requires confirmation by other investigators. In fact, characteristic inclusion bodies are seen in cells where virus replication takes place. But no report so far published refers to any characteristic inclusion bodies in megakaryocytes.

Minot (1922)(1923) suggested that the appearance of megakaryocytes in the peripheral blood was an indication of the bone marrow being under extreme stress. Recently Malméd (1967) found megakaryocytes and their fragments in the blood of normal subjects with no changes in the bone marrow.

Surgery and ACTH injections have been shown to increase the number of megakaryocytes in the bone marrow. Hume and her colleague (1964) and Pruitt (1964) also noticed a higher incidence of circulating megakaryocytes after surgery, but there is insufficient evidence to link this phenomenon with the effects of surgery.

"Stress" has been said to cause the megakaryocytes to migrate from the bone marrow to the blood, but there is no evidence that this stress factor is related to the stress response of the adrenal and pituitary axis. In the present study, no significant alteration in the incidence of megakaryocytes was observed after removing the suprarenal glands from both normal and tumour-bearing rats. Nor did any differences become apparent when such rats were given cortisone. Some changes were observed in adrenalectomized rats injected with tumour homogenate. The increase was low, but statistically significant. This clearly proves that tumour products are either directly or indirectly involved in causing the migration of megakaryocytes. And the megakaryocytes are cells which for some biological reason respond to neoplastic growth.

One should be aware of the fact that a definite response of megakaryocytes is associated with neoplastic conditions. If the incidence of circulating megakaryocytes is increased in the absence of any clinical signs, the possibility of an occult neoplastic condition should be considered.

In the study of circulating cancer cells, care must be taken to distinguish these cells from circulating megakaryocytes. Earlier investigators did find up to 52% of cancer cells in the peripheral blood, but these figures decreased when more became known of various other haematological responses to tumours.

CONCLUSIONS

The incidence of circulating megakaryocytes is greater in Wistar rats bearing Walker tumours than in normal rats. It increases in direct relation to the volume of the tumour. Surgical trauma and general anaesthesia do not affect the incidence of megakaryocytes in the blood of Wistar rats. Subcutaneous injections of the bland substances arachis oil and normal saline also have no effect. Subcutaneous injections of turpentine produce granulomatous ulcers, but do not affect the incidence of circulating megakaryocytes. The ulcers produced by injections of turpentine were left open and it was possible for them to become infected. It was found that these open granulomatous ulcers did not affect the incidence of circulating megakaryocytes.

Laparotomy, ACTH injections following laparotomy and ACTH injections without laparotomy had no effect on the incidence of circulating megakaryocytes. It is of interest that other investigators have demonstrated that in the bone marrow the number of megakaryocytes increases after injections of ACTH and after surgery.

The present study has shown that the suprarenal glands have no direct effect on the incidence of circulating megakaryocytes. It is assumed that ACTH injections cause increased secretion of cortisone from the suprarenals. Normal rats infected with ACTH showed no increase in the incidence of circulating megakaryocytes. However, when adrenalectomized rats were injected with Walker tumour homogenate and cortisone, the incidence of circulating megakaryocytes was higher than in a group of adrenalectomized rats who had received Walker tumour

homogenate only. This suggests that a factor other than tumour, tumour homogenate and cortisone secreted in the rat's organism may be responsible but that injected cortisone causes an increase. If the injected cortisone is not the cause, then the activity of this other factor appears to be increased by a tumour or by tumour tissue homogenate, and heightened further by injections of cortisone.

Apart from tumour homogenate, liver homogenate from normal rats is also capable of increasing the incidence of circulating megakaryocytes. This may be due to a product of cell division, a phenomenon common to both tumour and normal tissue. Other investigators have already demonstrated a serum protein factor which increases the number of megakaryocytes in the bone marrow without altering the blood platelet count. It is not yet known whether the same factor is responsible for the increased incidence of circulating megakaryocytes. If the effect is produced by a tumour factor, it may be assumed that the liver of rats also contains this factor, although in a lesser concentration. Other workers have demonstrated that the protein content of tumour tissue is similar to that of the kidney and spleen in mice. In this study it has been observed that liver as well as tumour homogenate from rats produces an increase in the incidence of circulating megakaryocytes, whereas spleen homogenate does not. This shows that tumour and liver homogenate have some similar effect.

In both normal rats and rats with tumours, the incidence of circulating megakaryocytes is in no way related to any changes in the blood platelet level.

When normal rats were treated with irradiation and cancer chemotherapeutic agents, the incidence of circulating megakaryocytes was not affected and was comparable to that of normal rats given no treatment. Rats with tumours given irradiation and cancer chemotherapy showed the increase in circulating megakaryocytes which is normally seen in rats with tumours given no treatment. This demonstrates that megakaryocytes are sensitive to tumour tissue or its products, but not to the effect of irradiation or cancer chemotherapy.

The nature of the immune response to tumours is not yet understood, but it has been suggested by other workers that there is some similarity between the immune response to tumours and that to skin grafts. In this study the immune reaction produced by skin grafts did not affect the incidence of circulating megakaryocytes.

Circulating megakaryocytes derive primarily from the bone marrow. In the present study it has been shown that in advanced stages of Walker tumour in Wistar rats the spleen also contributes to the increased incidence of these cells in the blood.

So far we know that the function of megakaryocytes is to form blood platelets. In this study it has been observed that the incidence of circulating megakaryocytes increases in rats with transplant tumours. This increase may be due to the effect of the tumour or its products, or possibly megakaryocytes are active against the tumour products and this causes their increase.

These findings would suggest that in humans the possibility of an occult neoplasm should be considered if the megakaryocyte count in the peripheral blood continues to be positive in the absence of any clinical signs.

Summary

1. The incidence of circulating megakaryocytes is significantly higher in Wistar rats with Walker 256 tumours than in normal rats.
2. The incidence of circulating megakaryocytes is directly proportional to the volume of the tumour.
3. General anaesthesia and surgical trauma have no effect on the incidence of circulating megakaryocytes.
4. Arachis oil and normal saline, injected subcutaneously, have no effect on the incidence of circulating megakaryocytes.
5. The irritant substance turpentine, injected subcutaneously, produces granulomatous ulcers, but this apparently has no effect on the incidence of circulating megakaryocytes.
6. Subcutaneous injections of adrenocorticotrophic hormone and Rheomacrodex (Dextran RMI 10 G, NaCl 0.9 G, water for injection 100 ml.) have no effect on the incidence of circulating megakaryocytes.
7. When Rheomacrodex or ACTH are injected after laparotomy, the incidence of circulating megakaryocytes is not significantly changed.
8. Complete excision of the tumour reduces the incidence of megakaryocytes in the blood, which had originally increased after the tumour transplant.
9. Partial excision of the tumour is followed by a temporary drop in the incidence of circulating megakaryocytes, but later it continues to increase as the volume of the tumour increases.

10. Subcutaneous injections of tumour tissue homogenate also increase the incidence of circulating megakaryocytes.
11. Injections of a homogenate of liver tissue from normal rats increase the incidence of circulating megakaryocytes, but the level of significance is much lower in this case than with tumour tissue.
12. Homogenates of muscle and splenic tissue of rats injected subcutaneously seem to have no effect on the incidence of circulating megakaryocytes.
13. In normal rats, bilateral adrenalectomy appears to have no effect on the incidence of megakaryocytes in the blood.
14. When rats who have undergone bilateral adrenalectomy are given tumour transplants, the incidence of circulating megakaryocytes increases in a similar way as it does in rats with tumours who have not undergone this operation.
15. The incidence of circulating megakaryocytes is slightly higher in the group of adrenalectomized rats with tumours who were given injections of corticosterone. But this incidence is still in the same range as that of rats with tumour only.
16. When adrenalectomized rats are given injections of Walker tumour tissue homogenate, the incidence of circulating megakaryocytes is significantly higher.
17. When adrenalectomized rats are given injections of Walker tumour tissue homogenate and of cortisone, the incidence shows an increase which is slightly higher than that seen in adrenalectomized rats given tumour tissue homogenate only.

18. In normal rats, splenectomy has no effect on the incidence of circulating megakaryocytes.
19. In rats with tumours, splenectomy apparently does not affect the incidence during the earlier stages of tumour growth. During the later stage of tumour growth, the group shows a lower incidence.
20. Immune reactions produced by skin homografts and heterografts have no effect on the incidence of circulating megakaryocytes.
21. In normal rats, irradiation and cancer chemotherapy does not affect the incidence of circulating megakaryocytes.
22. When rats with transplanted tumours are treated with irradiation and cancer chemotherapy, the increase in the incidence of circulating megakaryocytes is comparable to that observed in the group of untreated rats.
23. Haemorrhages do not affect the incidence of circulating megakaryocytes in the blood.
24. The withdrawal of blood causes an initial drop in the blood platelet count, which is followed by a rise to up to 180 to 200 percent of the initial count by the 3rd to 5th day, and return to the normal by the 9th to 12th day.
25. The blood platelet level shows no relation to the incidence of circulating megakaryocytes.
26. In rats with transplant tumours, the blood platelet level remains on the low side of the normal range, or slightly below the normal.

27. A serum factor has been postulated, which is responsible for the increased incidence of circulating megakaryocytes. This factor may be activated or stimulated by the products or secretions of the tumour, and it may be a protein.
28. This activator is not unique for malignant cells, but may be common to all dividing cells. It is probably present in the liver cells of Wistar rats and in the spleen and kidney of mice.
29. When studying malignant cells in the blood, care must be taken to differentiate these from circulating megakaryocytes.
30. The presence of intact megakaryocytes in the blood of human patients might indicate an occult neoplastic condition.

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APPENDIX

**Data from which figures and tables in the
chapter of "Experiments and Results" were
drawn.**

	Group a				Group b			
	No Treatment				Transplanted Walker Tumour			
No. of rats	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days
1	-	-	+ (1)	-	-	+ (2)	+ (1)	+ (3)
2	-	-	-	-	-	-	+ (3)	+ (4)
3	-	-	-	+ (2)	-	-	-	+ (2)
4	-	-	-	-	-	-	+ (2)	-
5	-	-	-	-	-	-	-	-
6	-	-	-	-	-	+ (1)	+ (4)	+ (7)
7	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	+ (6)
9	-	-	-	-	-	-	-	-
10	+ (1)	-	-	-	-	+ (3)	+ (5)	+ (8)
11	-	-	-	-	-	+ (3)	+ (4)	+ (8)
12	-	-	-	-	-	-	-	+ (3)
13	-	-	-	-	-	-	+ (4)	+ (5)
14	-	-	-	+ (1)	-	-	-	-
15	-	-	-	-	-	-	+ (2)	+ (7)
16	-	-	-	-	-	+ (1)	+ (3)	+ (9)
17	-	-	-	-	-	-	-	-
18	-	-	+ (1)	-	-	-	+ (6)	+ (4)
19	-	-	-	-	-	-	-	+ (4)
20	-	-	-	-	-	-	+ (2)	+ (10)

(Number in parenthesis represents real cell count)

EXPERIMENT 2

APPENDIX 2

	Group a				Group b			
	General Anaesthesia				General Anaesthesia & Laparotomy			
No. of rats	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days
1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5	-	-	-	-	+	+	-	-
					(2)	(2)		
6	-	-	-	-	-	-	-	-
7	-	+	-	-	-	-	+	+
		(2)					(1)	(2)
8	-	-	+	+	-	-	-	-
			(1)	(2)				
9	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	+	-
							(2)	
12	-	-	-	+	-	-	-	+
				(1)				(1)

(Number in parenthesis represents real cell count)

EXPERIMENT 3

APPENDIX 3

	Group a				Group b				Group c			
	Subcut Turpentine				Subcut Normal Saline				Subcut Arachis Oil			
No. of rats	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days
1	-	-	-	-	-	-	-	-	-	+	-	-
2	-	-	+	-	-	-	-	-	-	(2)	+	-
3	-	-	(3)	-	-	-	-	-	-	-	(1)	-
4	-	-	+	+	-	-	-	-	-	-	-	-
5	-	-	(2)	(1)	-	-	-	-	-	-	-	-
6	-	-	+	+	-	-	-	+	-	-	-	-
7	-	-	(3)	-	-	-	-	(2)	-	-	-	-
8	-	-	-	-	-	-	+	-	-	+	+	+
9	-	-	-	-	-	-	(1)	-	-	(1)	(2)	(2)
10	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	+	-
	-	-	-	-	-	-	-	-	-	-	(1)	-

(Number in parenthesis represents real cell count)

EXPERIMENT 4

APPENDIX 4

	Group a				Group b				Group c				Group d			
	Subcut Rheomacrodex				Subcut ACTH				Laparotomy & Subcut Rheomacrodex				Laparotomy & Subcut ACTH			
No. of rats	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days
1	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-
2	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-
4	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
6	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
10	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+

(Number in parenthesis represents real cell count)

EXPERIMENT 5

APPENDIX 5

	Group a						Group b						Group c				
	Excision of Skin						Complete Excision of Tumour						Partial Excision of Skin				
No. of rats	0 days	7 days	14 days	21 days	28 days	50 days	0 days	7 days	14 days	21 days	28 days	50 days	0 days	7 days	14 days	21 days	28 days
1	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	+
2	-	-	-	-	-	+	+	+	+	dead	+	-	-	-	+	+	+
3	-	-	-	-	-	dead	-	-	-	-	-	-	-	+	+	+	dead
4	-	+	+	+	+	-	+	+	+	+	dead	-	-	-	-	+	+
5	-	-	-	-	-	-	+	+	+	-	+	-	+	+	+	-	+
6	-	-	+	-	-	-	-	+	-	-	-	-	+	+	+	-	-
7	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
8	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	dead
9	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+
10	-	-	-	-	-	-	+	-	-	-	-	+	+	-	+	+	dead

(Number in parenthesis represents real cell count)

Continued/....

	Group a						Group b						Group c				
	Excision of Skin						Complete Excision of Tumour						Partial Excision of Skin				
No. of rats	0 days	7 days	14 days	21 days	28 days	50 days	0 days	7 days	14 days	21 days	28 days	50 days	0 days	7 days	14 days	21 days	28 days
11	-	-	-	-	-	-	+	+	-	-	+	+	+	+	+	-	+
12	-	-	-	+	-	-	(1)	(1)	-	-	-	-	(1)	(1)	(1)	+	(3)
13				(1)			-	-	-	-	-	-	(2)	(6)	-	(5)	dead
14							+	+	+	+	+	+					
15							(7)	(3)	(3)	(3)	(2)	(1)					
16							+	+	-	-	-	-					
17							(3)	(1)									
18							+	+	+	-	-	-					
19							(4)	(2)	(1)								
20							-	-	-	-	-	-					
							+	+	+	+	+	+					
							(2)	(3)	(1)	(2)	(3)	(2)					
							+	+	-	-	-	-					
							(5)	(2)									

(Number in parenthesis represents real cell count)

EXPERIMENT 6

APPENDIX 6

	Group a				Group b				Group c				Group d			
	Subcut Injection of Walker Tumour Homogenate				Subcut Injection of Muscle Homogenate				Subcut Injection of Liver Homogenate				Subcut Injection of Spleen Homogenate			
No. of rats	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days
1	-	-	+	+	-	-	-	+	-	-	-	+	-	-	-	-
			(2)	(5)				(1)				(1)				
2	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-
						(1)				(1)	(3)					
3	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+
										(3)				(2)	(1)	(3)
4	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-
		(3)	(2)	(3)			(1)									
5	-	-	-	-	-	+	+	+	-	-	+	+	-	-	-	-
						(2)	(2)	(3)			(1)	(2)				
6	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-
						(2)					(1)	(1)				
7	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-
				(1)						(2)						
8	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+
			(5)	(9)										(1)		(2)
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	+	-	+	-	-	+	-	-	-	-	+	-	-	+	+
		(1)		(4)			(3)					(1)			(3)	(4)

(Number in parenthesis represents real cell count)

Continued/....

EXPERIMENT 6

(Continued/....)

APPENDIX 6

	Group a				Group b				Group c				Group d			
	Subcut Injection of Walker Tumour Homogenate				Subcut Injection of Muscle Homogenate				Subcut Injection of Liver Homogenate				Subcut Injection of Spleen Homogenate			
No. of rats	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days
11	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-
		(2)	(4)	(1)			(3)									
12	-	-	+	+	-	-	-	-	-	+	+	-	-	-	-	-
			(1)	(7)						(1)	(3)					
13	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
						(2)										(1)
14	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		(4)														
15	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-
			(3)	(10)											(3)	
16	-	-	-	-												
17	-	-	-	+												
				(2)												
18	-	-	+	+												
			(5)	(5)												
19	-	-	+	-												
			(1)													
20	-	-	-	-												

(Number in parenthesis represents real cell count)

EXPERIMENT 7

APPENDIX 7

	Group a				Group b				Group c				Group d				Group e			
	Bilateral Adrenalectomy				Bilateral Adrenalectomy and Transplanted Tumour				Bilateral Adrenalectomy, Transplanted Tumour and Injected with Corticosterone				Bilateral Adrenalectomy and Walker Tumour Homogenate				Bilateral Adrenalectomy, Tumour Homogenate Subcut and Subcut Corticosterone			
No. of rats	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days
1	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+
			(2)	(1)			(3)	(3)			(1)	(3)			(1)	(1)			(2)	(4)
2	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	+
											(5)	(4)						(3)	(2)	(3)
3	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	-	-	-	+
								(2)		(2)	(2)	(5)		(1)	(3)	(3)				(5)
4	-	-	-	-	-	-	+	+	-	+	+	+	-	-	-	+	-	-	-	-
							(1)	(1)		(1)	(3)	(1)				(2)				
5	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
						(2)	(4)	(7)												
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
																		(1)	(3)	(1)
7	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	-	-	-	-
										(3)	(7)	(9)		(2)	(2)	(4)				
8	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-
								(6)				(7)								
9	-	-	-	-	+	+	+	+	-	-	-	+	-	-	-	-	+	+	+	+
					(2)	(1)	(5)	(2)				(11)					(2)	(4)	(5)	(9)
10	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+
		(1)														(2)			(2)	(4)

(Number in parenthesis represents real cell count)

Continued/....

	Group a				Group b				Group c				Group d				Group e			
	Bilateral Adrenalectomy				Bilateral Adrenalectomy and Transplanted Tumour				Bilateral Adrenalectomy, Transplanted Tumour and Injected with Corticosterone				Bilateral Adrenalectomy and Walker Tumour Homogenate				Bilateral Adrenalectomy, Tumour Homogenate Subcut and Subcut Corticosterone			
No. of rats	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days
11	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	+
								(5)							(4)	(1)				(1)
12	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
										(1)						(2)				(1)
13					-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
							(3)	(4)	(2)								(2)	(7)	(5)	
14					-	-	-	+	-	-	-	-	-	+	+	+	-	+	+	+
								(5)						(2)	(3)	(4)		(2)	(6)	(4)
15					-	-	-	-	-	+	+	+	-	-	-	+	-	+	+	+
										(4)	(2)	(4)				(1)		(5)	(9)	(7)
16					-	+	+	+	-	+	+	+	-	-	-	-	-	+	+	+
						(2)	(2)	(1)		(3)	(4)	(3)						(4)	(2)	(8)
17					-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-
								(3)		(2)	(1)	(10)								
18					-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-
								(5)						(3)	(5)	(7)				
19					-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+
											(5)	(2)							(1)	(11)
20					-	+	+	+	-	-	+	+	-	-	-	+	-	-	+	+
						(1)	(3)	(9)			(2)	(7)				(3)			(1)	(3)

(Number in parenthesis represents real cell count)

No. of rats	Group a Splenectomy				Group b Splenectomy & Transplanted Tumour			
	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days
1	-	+	-	-	-	+	+	+
		(1)				(2)	(2)	(2)
2	-	-	-	-	-	+	-	-
						(5)		
3	-	-	-	-	-	-	+	-
							(1)	
4	-	-	+	+	-	-	+	+
			(2)	(1)			(3)	(3)
5	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-
7	-	-	-	-	-	+	+	+
						(1)	(5)	(9)
8	-	-	-	-	-	-	+	-
							(1)	
9	-	-	-	-	-	-	+	+
							(3)	(6)
10	-	-	-	-	-	-	+	+
							(7)	(7)
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	+	+	+
						(1)	(4)	(6)
13	-	+	-	-	-	-	-	-
		(1)						
14	-	+	+	-	-	-	-	-
		(2)	(1)					
15	-	-	+	-	-	-	-	-
			(2)					
16	-	-	-	-	-	+	+	+
						(3)	(11)	(4)
17	-	-	-	-	-	+	+	+
						(7)	(4)	(13)
18	-	-	-	-	-	+	+	+
						(2)	(3)	(3)
19	-	-	-	-	-	-	+	-
							(1)	
20	-	-	-	-	-	-	-	-

(Number in parenthesis represents real cell count)

EXPERIMENT 9

APPENDIX 9

No. of rats	Group a Normal Bleeding				Group b Tumour with Bleeding (a)				Group c Tumour with Bleeding (b)		
	0 days	1 days	7 days	21 days	0 days	1 days	7 days	21 days	0 days	1 days	14 days
1	-	-	-	-	+	-	+	+	-	+	+
2	-	-	-	-	(1)	+	(2)	(3)	+	(1)	(7)
3	-	-	-	-	(6)	(2)	(1)	(7)	(3)	-	(2)
4	-	+	+	-	-	+	-	+	+	-	+
5	-	(1)	(2)	-	-	(2)	-	(11)	(2)	-	(10)
6	-	-	-	-	-	-	+	+	-	-	-
7	-	-	-	-	-	-	(7)	(6)	-	+	+
8	-	-	-	-	-	-	-	-	-	(2)	(6)
9	-	-	-	-	-	-	+	-	-	-	+
10	-	-	-	+	+	+	+	+	+	+	+
				(1)	(2)	(3)	(4)	(8)	(5)	(6)	(10)
					+	+	+	-	+	+	+
					(4)	(4)	(5)	-	(4)	(2)	(9)
					-	+	+	+	-	+	+
						(3)	(3)	(1)		(1)	(1)

(Number in parenthesis represents real cell count)

EXPERIMENT 10

APPENDIX 10

No. of rats	Group a Skin Autograft				Group b Skin Homograft				Group c Skin Heterograft			
	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days
1	-	-	-	-	-	-	+	-	-	-	+	-
2	-	+	+	+	-	+	-	-	-	-	-	-
3	-	(2)	(1)	(3)	-	(3)	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	+
5	-	-	-	-	-	-	-	-	-	-	-	(1)
6	-	-	-	-	-	-	+	-	-	-	-	-
7	-	-	-	-	-	-	(3)	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	+	-	+
9	-	-	-	-	-	-	-	-	-	(1)	-	(3)
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	+	-	+	+	+
							(1)			(3)	(4)	(1)

(Number in paranthesis represents real cell count)

EXPERIMENT 11

APPENDIX 11

No. of rats	Group a Whole Body Irradiation				Group b Intraperitoneal Injection of Methotrexate				Group c Irradiation of Tumour					Group d Treatment by Methotrexate in Rats with Tumour				
	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	28 days	0 days	7 days	14 days	21 days	28 days
1	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	+	+
							(2)	(2)					(2)				(2)	(5)
2	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+
										(3)	(2)	(2)	(4)		(2)	(2)	(5)	(3)
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
																(1)	(2)	(4)
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+	+
										(1)	(1)	(3)	(1)			(4)	(7)	(3)
6	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-	+	+	+
							(1)	(1)				(1)	(2)			(1)	(1)	(6)
7	-	-	+	+	-	-	+	+	-	-	+	+	+	-	-	+	+	+
			(3)	(3)			(3)	(4)			(4)	(4)	(5)			(5)	(9)	(11)
8	-	-	+	+	-	-	-	-	-	+	-	+	+	-	-	-	+	+
			(3)	(5)						(2)		(1)	(7)				(5)	(9)
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
																(7)	(4)	(3)
10	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+
													(2)				(6)	(5)

(Number in parenthesis represents real cell count)

Continued/....

No. of rats	Group a Whole Body Irradiation				Group b Intraperitoneal Injection of Methotrexate				Group c Irradiation of Tumour					Group d Treatment by Methotrexate in Rats with Tumour				
	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	0 days	7 days	14 days	21 days	28 days	0 days	7 days	14 days	21 days	28 days
11	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+
											(1)	(1)	(3)		(3)	(3)	(3)	(7)
12	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+
													(2)		(1)	(5)	(8)	(4)
13	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+
							(1)									(1)	(9)	(2)
14	-	-	-	+	-	+	+	-	-	-	+	+	+	-	+	-	+	+
				(1)		(2)	(2)				(1)	(5)	(3)		(4)		(5)	(7)
15	-	-	-	-	-			+	-	+	+	+	+	-	-	+	-	+
								(4)		(2)	(3)	(4)	(2)			(3)		(3)

(Number in parenthesis represents real cell count)

EXPERIMENT 2

APPENDIX 12

PLATELET COUNT

No. of rats	Group a in Thousands				Group b in Thousands				Group c in Thousands			
	Day 0	Day 1	Day 7	Day 21	Day 0	Day 1	Day 7	Day 21	Day 0	Day 1	Day 7	Day 14
1	780	800	1,842	1,000	700	680	800	672	980	670	1,011	586
2	920	480	1,622	990	458	548	724	620	810	558	999	771
3	610	510	1,390	878	590	610	798	550	1,000	780	978	997
4	690	400	1,364	1,100	650	480	956	700	780	520	832	660
5	554	390	1,550	900	498	530	624	530	570	386	485	522
6	780	480	1,420	724	586	638	960	692	688	704	621	610
7	1,110	734	1,210	866	688	480	1,200	Dead	820	553	1,240	600
8	888	620	1,722	990	1,020	326	1,400	924	950	689	1,100	755
9	610	600	1,400	780	700	468	980	Dead	610	488	790	561
10	580	326	1,380	920	588	458	1,000	450	492	390	508	402
Mean	752,200	534,000	1,490,000	914,800	647,800	521,800	944,200	642,250	770,000	567,800	856,400	646,400

EXPERIMENT 1

APPENDIX 12

Group b

Measurement of Tumour

No. of rats	Day 7	Day 14	Day 21
1	1.8 x 1.0 x 0.5	3.0 x 2.4 x 1.2	5.6 x 4.0 x 3.2
2	1.2 x 1.0 x 0.5	3.8 x 2.5 x 1.5	4.9 x 3.8 x 3.5
3	1.4 x 1.0 x 0.75	2.9 x 2.2 x 1.5	5.0 x 3.9 x 3.0
4	1.6 x 1.2 x 1.0	3.2 x 2.5 x 1.2	5.3 x 3.7 x 2.8
5	1.7 x 1.0 x 1.0	3.8 x 2.7 x 1.2	6.2 x 3.0 x 1.9
6	1.4 x 0.7 x 0.9	3.8 x 2.1 x 1.3	6.5 x 4.0 x 2.7
7	1.8 x 1.1 x 0.9	2.7 x 2.6 x 1.9	4.5 x 3.8 x 2.0
8	1.5 x 1.3 x 1.0	2.6 x 2.0 x 1.5	4.8 x 3.2 x 2.3
9	1.2 x 0.9 x 0.7	3.7 x 2.5 x 1.5	4.2 x 3.0 x 2.9
10	1.8 x 1.2 x 0.8	3.0 x 2.1 x 1.5	6.8 x 4.1 x 3.0
11	1.5 x 1.3 x 1.0	3.0 x 2.1 x 1.5	5.0 x 3.5 x 3.0
12	1.2 x 0.9 x 0.7	2.7 x 1.9 x 1.9	5.1 x 3.2 x 3.1
13	1.0 x 1.0 x 0.9	2.5 x 2.0 x 2.0	5.7 x 3.8 x 3.2
14	1.4 x 1.0 x 0.7	3.2 x 1.9 x 1.9	4.8 x 3.0 x 2.5
15	1.5 x 1.0 x 0.6	2.9 x 2.4 x 1.0	5.8 x 4.1 x 2.0
16	1.6 x 1.2 x 1.0	3.1 x 2.5 x 1.4	6.0 x 4.8 x 3.0
17	1.3 x 1.2 x 0.5	3.5 x 2.1 x 1.9	6.7 x 4.3 x 3.0
18	1.2 x 0.9 x 0.5	2.4 x 1.9 x 1.5	4.2 x 3.2 x 2.8
19	1.0 x 1.8 x 0.5	2.9 x 2.5 x 1.7	3.9 x 3.1 x 2.7
20	1.3 x 0.7 x 0.5	3.1 x 2.8 x 1.9	6.7 x 4.8 x 3.0

EXPERIMENT 11

APPENDIX 14

Group c

Measurement of Tumour

No. of rats	Day 7	Day 14	Day 21	Day 28
1	1.1 x 0.9 x 0.7	2.2 x 1.2 x 1.0	3.9 x 2.5 x 2.0	4.5 x 3.5 x 2.5
2	1.2 x 0.5 x 0.8	2.4 x 1.3 x 1.2	4.0 x 3.0 x 2.1	5.4 x 3.9 x 3.1
3	1.5 x 0.7 x 0.4	2.6 x 1.5 x 1.2	3.7 x 2.9 x 2.5	6.2 x 3.4 x 3.5
4	1.3 x 1.0 x 0.4	3.0 x 2.1 x 1.0	4.6 x 3.0 x 2.5	7.1 x 4.0 x 3.4
5	1.5 x 1.0 x 0.6	3.0 x 1.8 x 0.9	4.5 x 3.2 x 2.7	5.0 x 4.1 x 3.2
6	1.4 x 1.0 x 0.7	2.8 x 1.4 x 1.2	4.6 x 3.4 x 3.0	5.4 x 3.9 x 3.5
7	1.2 x 1.1 x 0.6	2.5 x 1.8 x 1.0	5.0 x 3.2 x 3.1	5.6 x 4.2 x 3.9
8	1.7 x 1.1 x 0.5	3.1 x 1.9 x 1.4	4.2 x 3.8 x 3.0	6.2 x 4.5 x 4.0
9	1.1 x 1.1 x 0.8	3.2 x 2.2 x 1.6	5.0 x 3.2 x 2.9	7.2 x 4.2 x 3.5
10	1.3 x 0.9 x 0.7	3.4 x 1.6 x 1.2	3.9 x 2.6 x 2.0	6.5 x 3.9 x 3.8

Continued/....

Group c

Measurement of Tumour

No. of rats	Day 7	Day 14	Day 21	Day 28
11	1.4 x 1.0 x 0.7	2.4 x 1.6 x 1.0	3.7 x 2.7 x 2.0	5.5 x 4.7 x 3.1
12	1.7 x 1.2 x 0.9	2.3 x 2.0 x 1.5	4.1 x 3.2 x 2.1	7.0 x 5.1 x 3.1
13	1.0 x 0.9 x 0.6	3.0 x 1.9 x 1.0	3.6 x 3.1 x 2.2	5.8 x 3.9 x 3.1
14	1.2 x 0.7 x 0.5	2.5 x 1.6 x 1.2	4.3 x 3.5 x 2.7	7.0 x 5.1 x 3.8
15	1.7 x 1.2 x 0.9	3.6 x 2.1 x 1.5	4.7 x 2.9 x 2.5	6.1 x 4.0 x 3.6

EXPERIMENT 11

APPENDIX 15

Group d

Measurement of Tumour

No. of rats	Day 7	Day 14	Day 21	Day 28
1	1.0 x 0.9 x 0.6	2.3 x 1.9 x 1.0	4.9 x 3.9 x 3.2	6.1 x 3.11 x 3.0
2	1.9 x 1.0 x 0.5	2.7 x 2.0 x 1.1	5.1 x 4.0 x 2.9	5.9 x 4.5 x 3.2
3	1.5 x 1.2 x 0.7	3.1 x 1.9 x 1.2	5.2 x 4.1 x 2.5	6.5 x 4.5 x 3.0
4	2.0 x 0.75 x 0.6	2.8 x 2.4 x 1.1	4.2 x 3.1 x 2.6	5.1 x 3.5 x 3.2
5	1.8 x 1.0 x 0.9	3.4 x 2.7 x 1.5	5.4 x 4.1 x 2.4	6.9 x 5.0 x 3.1
6	1.5 x 1.0 x 0.7	2.5 x 2.2 x 1.4	6.5 x 4.2 x 3.1	6.9 x 5.4 x 4.0
7	1.9 x 1.2 x 1.0	2.7 x 2.4 x 1.75	6.2 x 4.4 x 3.0	6.4 x 5.8 x 3.2
8	2.0 x 1.1 x 0.9	2.9 x 2.5 x 1.9	4.2 x 3.6 x 2.7	6.2 x 4.2 x 3.5
9	2.1 x 0.9 x 0.9	3.5 x 2.4 x 1.4	4.8 x 3.7 x 2.9	6.1 x 4.2 x 3.5
10	2.1 x 1.4 x 0.6	3.6 x 2.6 x 1.7	5.2 x 4.4 x 3.0	5.7 x 5.0 x 3.8

Continued/....

Group d

Measurement of Tumour

No. of days	Day 7	Day 14	Day 21	Day 28
11	1.8 x 1.1 x 0.9	3.2 x 2.8 x 1.6	4.1 x 4.0 x 2.9	6.3 x 4.7 x 3.1
12	1.7 x 1.2 x 1.0	3.3 x 2.7 x 1.9	4.6 x 4.1 x 3.1	5.2 x 4.2 x 3.2
13	1.2 x 1.1 x 0.5	2.7 x 2.5 x 1.2	5.1 x 4.0 x 2.9	5.9 x 4.9 x 3.7
14	1.9 x 1.4 x 0.9	3.0 x 2.1 x 1.7	6.2 x 5.0 x 3.1	6.5 x 5.8 x 3.6
15	1.8 x 1.5 x 1.0	3.7 x 2.8 x 1.9	4.0 x 4.0 x 3.1	6.0 x 4.2 x 3.5