TUMOUR CELLS IN THE CIRCULATING BLOOD

by

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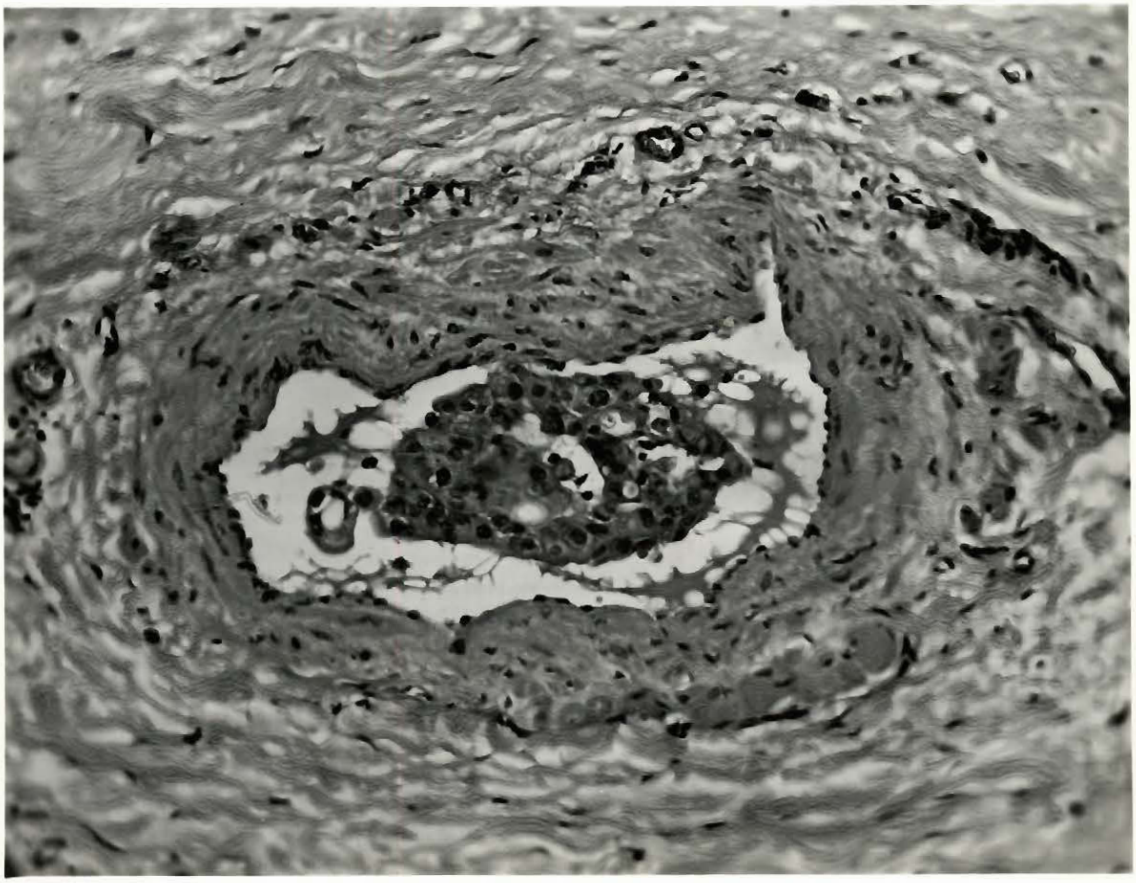

Fig. 1

Tumour cells seen within a venule of a patient operated for infiltrating and intraductal carcinoma of the breast X 200

"..... these (cancer) germs are probably carried sometimes through the lymphatic vessels to absorbent glands in the vicinity of the primary growth; but there can scarcely be a doubt that the blood is the main channel by which the seeds of this dreadful malady are conveyed from its first to its subsequent sites."

Thomas Watson,
Lectures on the Principles and
Practice of Physic.
Vol. 1, p. 207, Parker, London, 1843

Dedicated to my wife, Lise

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P R E F A C E

In my second year in the McGill University Post-Graduate Diploma Course in Surgery, being interested in the mechanism of cancer metastasis, I was assigned by Doctor Donald R. Webster, Professor of Surgery, McGill University and Surgeon-in-Chief, Royal Victoria Hospital, to do one year as a Research Fellow in a project of much interest at the present time, the identification of tumour cells in the blood stream of patients with cancer. This project had been started at McGill University in 1958 by Doctors D.R. Webster, A.C. Ritchie, S. Ritchie, J.F. Hopkirk, R.C. Long and I. Salgado and a first report of the findings had been published in the Canadian Medical Association Journal in October of 1959. The present work is a continuation of that endeavour and, at the present time, investigation in this field is still being pursued by others.

The investigations reported in this thesis were conducted between July 1959 and June 1960 and the thesis was written between July 1960 and April 1961. A grant by the National Cancer Institute of Canada made this research project possible and for this we are most grateful.

Many have contributed towards the accomplishment of this work. Doctor Webster apart from suggesting that I participate in this venture which has been very interesting and enlightening, also obtained the grant for me and kindly accepted me in the Experimental Surgery Department of McGill University. Dr. G.C. McMillan, Strathcona Professor of Pathology and Chairman of the Department at McGill, kindly permitted me to use the

facilities of the Pathological Institute to carry on my work.

Doctor A. C. Ritchie, Miranda Fraser Associate Professor of Comparative Pathology, in his capacity as director of this research project, contributed most actively towards the accomplishment of this work. By his continued interest, professional knowledge and ready co-operation, he has guided me with a firm and friendly hand and, inevitably, his suggestions cannot be clearly separated from my own work. It was he who rendered the final diagnosis of malignancy of the tumour cells. He also reviewed all the slides of carcinoma of the breast for histological gradation and supervised the preparation of this thesis. But for him, this work would have had little sense of direction.

Doctor Susan Ritchie, Lecturer in Pathology, introduced me to the subject and was especially instrumental in helping me perfect the technique that we used for the isolation of tumour cells from the blood. Her competency in cytology made her opinions concerning the interpretation of various cells invaluable and her keen interest and enthusiasm were always a source of encouragement.

Doctors J. F. Hopkirk and R. C. Long, Demonstrators in Surgery at McGill University and Assistant Surgeons, Royal Victoria Hospital contributed greatly by giving clinical orientation to this project. From their clinical experience, they would suggest the practical aspects of our results and I shall never forget our regular "Friday meetings" from which I benefited so much.

Many members of the Department of Pathology helped by conversation and co-operation. The advice of Doctor J. W. Stevenson concerning bacteriological questions and that of Doctor B. I. Weigensberg concerning

problems of a chemical nature were much appreciated. Miss Marcia Eldridge did all the staining and mounting of the slides and much of the screening was done by Miss Irene Shepshensky. All the photographic work was done by Mr. H. Coletta and Mrs. M. Paasuke very ably did the typewriting of the thesis. Mrs. Anna Zushmanis, Miss H. O'Brien and Mr. H. A. T. Nye have been especially helpful.

Finally, my association with the Royal Victoria Hospital, the Donner Building, the Department of Anatomy and the Medical Library of McGill University has been a happy one and I shall always cherish fond memories of my research year. The work done so far is, I hope, just a beginning and will be a spur to further work.

Edmond D. Monaghan

April, 1961

NOTE ON THE ARRANGEMENT OF THE THESIS

The thesis is divided into four parts:

Part I - Review of Medical Literature Pertaining to Tumour Cells in the Blood Stream

Part II - Materials and Method

Part III - Results

Part IV - Discussion and Conclusions

The Bibliography and four Appendices are at the end of the thesis

In the bibliography of this thesis, the authors are listed in alphabetical order. In the text, the references are given by the name of the author or authors and the year of publication. When the same author or authors published two or more papers in the same year, to avoid confusion, the papers are indicated in their order of publication by adding to the year small letters of the alphabet. Thus Moore, Sandberg and Watne published two papers in 1960. The first is listed as Moore, Sandberg and Watne (1960 a), and the second as Moore, Sandberg and Watne (1960 b).

PART I

REVIEW OF MEDICAL LITERATURE PERTAINING TO TUMOUR CELLS IN THE BLOOD STREAM

CHAPTER I

REVIEW OF THE DEVELOPMENT OF KNOWLEDGE OF BLOOD-BORNE METASTASIS

A. Early Concepts

It is interesting to review the evolution of thinking concerning the propagation of metastases via the blood stream. That tumour emboli are sometimes carried in the blood stream is to us a familiar and obvious conception but, surprisingly, it is only recently that this phenomenon came to light. Up until the nineteenth century, the mechanism of cancer metastasis was not understood (Long, 1928). Though pathologists taught that local and lymph node metastases were lymph-borne, they could not explain distant metastases. Gradually, however, they began to indict the blood stream. Thus in 1808, Christopher Johnson in the prize essay of the Royal College of Surgeons of England referred to those who contended that cancerous matter entered the "circulatory system" before bringing about "a taint of the whole habit". Macfarlane in 1832 could not understand how far flung metastases could be established "through the medium of the absorbents" (lymphatics). He favoured blood spread. So did Hodgkin (1836) who surmised that blood vessels were "in some way connected with the extension of the disease". Watson wrote in 1843.."... these (cancer) germs are probably carried sometimes through the lymphatic vessels to absorbent glands in the vicinity of the primary growth; but there can scarcely be a doubt that the blood is the main channel by which the seeds of this dreadful malady are conveyed from its first to its subsequent sites".

A significant contribution indeed wasthat of Joseph Claude Recamier who described the local infiltration of cancer and the invasion of veins by cancer. He was the first to use the term "metastasis". In 1829, finding secondary tumour growths in the vessels of the brain of a patient with mammary carcinoma, he called these "des métastases cancéreuses". It is interesting to remember that at that time the term "metastasis" was used to indicate transportation of any substance from one part of the body to the other. For example, Virchow in 1858 lectured on the subject of peripheral thrombi producing "metastatic deposits" in the lungs (Virchow, 1860 b). To-day the term is usually used to denote only secondary growths of tumours, though it is still occasionally used to denote the spread of non-neoplastic disease.

At any rate, as Onuigbo (1958) points out, subsequently it became accepted that "local and lymph node spread is lymph-borne, remote spread blood-borne", and this view was taught by such authorities as Sir James Paget (1853), Rokitansky (1854), Campbell de Morgan (1874), Pepper (1884) and Coats (1895).

However, the first half of the nineteenth century was a period of conjecture and speculation concerning the pathogenesis of haematogenous metastasis. The mechanism was not clearly understood. At that time, the humoral hypotheses of the nature of metastases held the field. For example, in 1829, Cruveilhier, impressed with the association of tumour invasion of blood vessels and the formation of distant secondary growths, concluded that cancer was essentially an intravascular disease, liberating a specific "cancerous juice" which was carried in the blood and could give rise to tumours in various parts. Though Müller published his classical

study of malignant tumours in 1838, and Schwann described his doctrine of cellular structure in 1839, the concept, so familiar to us to-day, that haematogenous metastases are produced by tumour cell emboli travelling in the blood stream, was not accepted.

In his tenth lecture at the Pathological Institute in Berlin on March 17th, 1858, Virchow (1860 a) taught that tumours give off "parenchymatous juices" which travel via the blood stream carrying the disease to more distant parts. It is difficult to see why Virchow was one of the advocates of the humoral method of cancer metastases when one considers that this learned man was the very founder of cellular pathology. But he exerted great influence and the humoral hypothesis was slow to disperse. Willis (1952 b) relates that, as late as 1874, there was a vigorous debate at the meeting of the Pathological Society of London, between those advocating the humoral hypothesis of metastasis and those upholding the conception of the transfer of tumour cells.

At any rate, Virchow's theories did not remain unchallenged for long. Indeed, Wilder (1956) reports that Alfred Hannover had stated even before Virchow published his book that cancer cells circulated in the blood stream and were responsible for metastasis. But the first real refutation of Virchow's theories came in 1865 when Thiersch, having observed invasion of cancerous tissue into veins and lymphatics, advocated the embolic phenomenon as the cause of secondary deposits. He recognized that cancer elements travelled in the blood and said of these "Ich sehe nicht ein, warum sie diese Fähigkeit durch einem kurzen Transport in den Strom des Lymphe oder des Blutes einbüssen sollten" (I do not see why they should lose this ability ((to reproduce)) by a

brief passage through the lymphatics, or blood stream). Waldeyer (1872) supported Thiersch and it was their writings which finally led to the rejection of Virchow's views on metastases (Wilder, 1956). From then on, most of the investigators in the late 19th century came to adept this "mechanical theory" - tumour cells voyaging in the blood stream and causing metastases wherever they stop and grow.

It was only natural, then, that attempts be made to prove this theory by actually identifying tumour cells in the blood. The earliest report is that of Ashworth who in 1869 demonstrated tumour cells in the blood of a patient after death. He described the cells as being of the same size and appearance as those found in the patient's "multiple malignant skin tumours". Ritchie (1960) surmises that these latter might have been subcutaneous metastases from a chordoma. Subsequently, medical literature began to lay the groundwork for our present concepts of metastasis. In 1889, Zahn wrote about metastases arising from tumour emboli lodging in capillaries. Stephen Paget (1889), concerned about the anomalies of tumour dissemination, likened tumour emboli to "seeds" falling in "soils" of different degrees of fertility and thereby established the famous "seed-soil" theory. Goldmann (1897) studying a well localized carcinoma of the tongue found that it had already broken into a non-thrombosed vein and therefore postulated that the finding of tumour cells in the blood stream was not restricted to advanced cases, since venous invasion can take place, as in this case, at an early stage. Schmidt in 1903 examined the lungs of 41 patients with advanced malignant disease but without visible pulmonary metstases and in 15 found tumour emboli in the pulmonary arteries. He concluded that the majority of these

cells perish by thrombotic organization but that others may pass through the pulmonary circulation and disperse throughout the body without the presence of visible metastases in the lungs.

But at the beginning of the twentieth century, though the haematogenous spread of cancer was well recognized, there had been only Ashworth's paper to report their actual identification in the circulating blood. Then came what was probably the first description of a technique for their isolation in blood samples. In 1904, Loeper and Louste, by hemolysing red blood cells with 1% acetic acid, were able to isolate tumour cells. In 1905, they replaced the acetic acid by 33% alcohol. Though they were unable to demonstrate tumour cells in the blood of patients having carcinoma, they found malignant cells in 3 cases of sarcoma. They described the cells judged to be malignant: they were larger than the polymorphonuclears and the monocytes; they stained differently to the other cells of the smear; the nucleus was larger, densely stained and rich in chromatin and finally, the nucleus appeared irregular, sometimes double. To confirm that the tumour cells found in the blood were truly tumour emboli, they compared them with a smear of the primary neoplasm. Lastly, they foresaw the value of finding tumour cells in the circulation as regards the diagnosis of the disease and the prognosis. The same year, Babés and Panea (1905) reported the finding of sarcoma cells in the blood of two patients, one with a sarcoma of the knee and one who had died of generalized metastases from a sarcoma of the ovary. Now interest was stimulated and reports began to appear more frequently in the journals. Schleip (1906) described abnormal cells which he presumed to be cancer cells in smears of the peripheral blood

of one patient dying of gastric carcinoma and of another patient who died 5 months later of generalized sarcomatosis of the bone and lymph nodes. Autopsy of the patient with gastric carcinoma revealed widespread metastases and neoplastic masses in the right atrium. Also in 1906 myeloma cells were found by Aschoff in the post-mortem blood of a patient with myelomatosis.

Not everyone was successful. Menetrier (1909) looked for cancer cells in the blood stream and reported his results to be negative. He therefore concluded that their presence must be exceptional. Parturier (1913) agreed with him, maintaining that they were identified in the blood only rarely, even in patients with widespread malignancy. "La constation n'est pas toujours facile!"

But reports continued. Reporting his findings in the blood of 4 patients with advanced carcinoma and bone metastases, Ward (1913) was impressed by the presence of large cells (20-30 micra in diameter) in smears of the peripheral blood from a patient dying of gastric carcinoma. Though he did not describe these cells except to say that they resembled the smaller giant cells seen in bone marrow films after death, he felt that they were neoplastic. Investigating post-mortem material in humans, Iwasaki (1915) verified Schmidt's (1903) finding that tumour cells were rarely free in the blood stream being, as a rule, he said, destroyed intravascularly by thrombus formation and by the direct action of the plasma. Krokiewicz (1919) countered that "Galloping" cancer could only be explained by assuming an acute dispersal of tumour cells throughout the organism. Marcus, the same year, reported "abnormal cells" in the blood taken from the finger of a patient with bronchogenic carcinoma

five days before death. These cells were extremely polymorphous in appearance and varied in size from that of a lymphocyte to that of a giant cell. As in one of Schleip's cases, autopsy revealed invasion of the right atrium. Basing himself on this last factor, Quensel (1921) extracted, at autopsy, blood from the right atrium of 50 patients having died of malignant disease in various organs. He then attempted to concentrate the cells by hemolyzing the erythrocytes with distilled water. This being done, he examined the centrifuged sediment. He discovered cancer cells in the circulating blood in 4 cases of gastric carcinoma, in one of carcinoma of the lung and one of malignant hypernephroma, all with numerous metastases. In his opinion, this method would demonstrate neoplastic cells in the blood only in advanced cases in which there were gross metastases. At the same time he supposed that the cancer cells which passed into the blood stream were destroyed there in large numbers and that this probably contributed considerably to the "cancer cachexia." Unlike previous investigators, he is probably the first to use large amounts of blood and publish a specific report on a large number of cases.

In the early twenties, doctors began to worry about the significance of these tumour cells in the blood. Knox (1922) warned medical men to avoid diagnostic or operative manipulations by underlining the importance of massage as a means of inducing metastases of tumour cells. Jolly, in his "Traité d'Hématologie" (1923), doubted if the demonstration of tumour cells in the blood had any value in clinical diagnosis. Though he admitted that they could be observed in the peripheral blood, he considered that they were found only rarely, mostly in cases of generalized carcinomatosis, only in very small quantities and that they did not always have

enough distinctive characteristics to be distinguished from leukocytes.

However, interest in the subject did not wane. Jeannée in 1925 found multiple small metastatic growths in almost all viscera following invasion of a large pulmonary vein by a bronchial carcinoma. Durante (1929) describing the tissue cells, supposedly fixed, which may be found travelling in the blood stream, included chorio-epithelioma cells. Mauclaire published an interesting article in 1929. This surgeon had been quite worried because one of his patients had accidentally received an emergency blood transfusion from her mother who had a pleural recurrence three years after operation for a carcinoma of the breast. Mauclaire feared that the patient transfused would develop a tumour secondary to cells transplanted by means of the blood transfusion. Five years afterwards, however, the patient was still alive and asymptomatic even though the mother had since died of her metastases.

One report often cited in the modern literature is that of Pool and Dunlop (1934). They found atypical cells in the peripheral blood of 17 of 40 patients having advanced cancer but could not be sure that these were tumour cells. They emphasized that it was difficult to establish the true identity of atypical cells within the blood. A year later, Oertel of McGill University, emphasized the distinction between "tumour cell transportation" by the blood and "metastasis". He pointed out that cancer cells circulating in the blood are not bound to stop and "take" and therefore should not be confused with metastasis.

From 1935 to 1954 little interest was shown in this subject. That tumour cells circulated in the blood remained unchallenged but, because their demonstration was so technically difficult and because no

clinical application had been confirmed, investigators concentrated on finding tumour cells elsewhere, particularly in other body fluids. Nevertheless, in 1942, Gruner of Montreal, studying blood smears from 787 cases of carcinoma and 89 cases of sarcoma reported finding a 9.7% frequency of occurrence of "cells like tumour cells in the blood stream". He believed that one was more likely to find tumour cells in the blood stream in the presence of sarcoma due to its well recognized intimate relation to its vascular channels, than in carcinoma which reputedly spread via the lymphatics.

The only report published between 1935 and 1954 seems to be that of Holland, who,in 1949, gave an account of a case in which mother, placenta and foetus were involved with malignant melanoma and tumour cells were found in both maternal and foetal blood vessels.

B. Contemporary Work

Major credit for re-awakening interest must be given to Engell who wrote an extensive monograph on the subject in 1955. He used saponin to hemolyze the erythrocytes of 274 blood samples from 140 patients with cancer. He discovered tumour cells or atypical tumour-like cells in the blood of 90 of these patients, that is in 63%. Endeavouring to reproduce Pool and Dunlop's results, Engell studied blood from the cubital veins of 14 patients with inoperable carcinoma and found 7 of the samples (50%) to be positive for tumour cells. Using blood drawn during surgical intervention, he discovered tumour cells in the peripheral blood in 10 of 79 patients (13%) with various malignancies. But mostly, he concentrated

on searching for tumour cells in blood drawn at operation from the vein draining the tumour site. In a group of 107 cases of cancer of the rectum and colon, Engell found malignant cells in the blood draining from the tumour in 63 cases (59%). An outstanding feature of his work is that Engell related the findings of tumour cells to the histological grading of the tumour in these patients with cancer of the rectum and colon. Tumour cells were found in 35% of Broders grade II tumours, 78% of grade III tumours and in all grade IV tumours. Also, significantly, Engell found a larger number of cells per unit volume of blood in the more anaplastic tumours. Engell also correlated his cytological findings with Dukes' classification (1932) of carcinoma of the rectum and colon. In group A, there were 41% of cases with tumour cells in the blood stream; group B included 57% cases with tumour cells and 70% of group C had tumour cells in their blood. However, Engell did not wish to draw any conclusions from this. He maintained that the above differences might be explained by the fact that the number of undifferentiated tumours increased from group A to C.

His conclusions were fourfold; firstly, the spread of cancer cells in the blood depends chiefly on the histological grade of differentiation of the tumour; secondly, cancer cells are too seldom found in the peripheral blood to be of any diagnostic value; thirdly, no prognostic importance should be derived from the presence of these cells in the blood, and lastly, that manipulation of the tumour during surgical intervention does not lead to an increase in the escape of malignant cells.

To prove his third point concerning prognosis, Engell (1959) followed-up 125 patients with cancer, most of them intestinal carcinomas, for five to nine years after operation. At operation, tumour cells had

been found in either the peripheral blood or in the venous blood from the tumour area in 61% of the cases. As he expected, 51% of the long-term survivors had had tumour cells in the blood before, during or after surgery. Engell therefore concluded that in these cases the disseminated cells must have perished intravascularly and reiterated his conclusion that the presence of tumour cells in the blood was not of prognostic significance.

Fisher and Turnbull (1955) were also interested in the vascular dissemination of colorectal carcinoma. Having isolated part of a mesenteric vein during operations for cancer of the colon or rectum, they extracted the blood trapped between 2 ligatures and in this fashion recovered tumour cells in 8 of 25 specimens (32%). They were also concerned with the inconsistencies between histological evidence of venous extension, the presence or absence of tumour cells in the blood stream, and metastases. They explained this by saying that just as "Angioinvasive neoplasms in other regions of the body, particularly the encapsulated angioinvasive carcinoma (angioinvasive adenoma) of the thyroid gland, do not consistently metastasize", so, too, the presence of intravascular tumour thrombi does not necessarily mean that tumour emboli will result, or metastases will develop.

Like Engell, Whang (1958) also used saponin to isolate tumour cells, but was less successful. He was unable to demonstrate malignant cells in the peripheral veins of 26 cases or in the femoral arteries of 11 patients. On the other hand, he did observe tumour emboli in the blood from the tumour area in 7 of 26 cases, making 11.1% positive findings out of 63 cases. He recommended that pressure and tension should be avoided during operations to prevent dissemination of cancer cells in the blood.

There were other less orthodox reports in 1958. Though Taylor and Vellios admitted that the large immature-looking cells found in the blood stream looked like and had the features of tumour cells, they felt that proof was lacking. Consequently, they set out to prove that these alleged tumour cells really were tumour cells. To do this, they gathered blood samples from 4 patients with advanced cancer, and in whose blood tumour cells had previously been demonstrated. They then re-injected subcutaneously into the donors, the buffy layers prepared from these blood samples. No secondary growths resulted. Among the reasons they gave to explain their failure to reproduce secondary tumours by this method was that too few tumour cells must have been present in the buffy layers of their preparations and secondly, the injected cells must have been destroyed by the natural resistance of the host. Peckholz and Böehm (1958) examined blood taken from the heart in a series of 74 autopsies of patients who had died of malignant tumours. Tumour cells in groups of from less than 10 to more than 50 were found in 10 of the 74 cadavers.

Coutts et al. (1959), though they used only a very small quantity of blood (2 ml.), found tumour cells in blood from the antecubital vein in 2 out of 5 patients orchiectomized for advanced prostatic carcinoma. Diddle, Sholes, Hollingsworth and Kinlaw (1959), examining the blood for tumour cells in patients with carcinoma of the cervix uteri, found none in the peripheral blood but 8 of 14 aliquots of blood from regional veins were positive. Fletcher and Stewart (1959) found tumour cells more frequently present in the regional veins (15 positive of 38 cancer cases) than in the peripheral veins (4 positive out of 24 cancer cases). Morley of Toronto (1959) identified tumour cells and cell clumps in blood samples drawn from

the superior sagittal sinus of three patients with brain tumours. One was a case of metastasis to the brain from a bronchogenic carcinoma and the other two were gliomata. It is particularly interesting that he found glioma cells in the venous blood since metastases from such tumours are extremely rare, if they occur at all.

Seal (1959), using his silicone flotation technique, examined 5 ml. samples of blood from 86 patients with advanced cancer and found tumour cells in 39.

C. The Three Main Schools

And so it is quite evident from the foregoing that there has been very keen interest in many centres concerning this subject in recent times, since Engell's monograph in 1955. But the prevalent medical opinions to-day concerning the haematogenous spread of tumour emboli have mostly been influenced by three large centres of investigation whose contributions of the medical literature have been numerous. Since 1954, they have each published a series of both clinical and experimental reports on the subject. These three important schools are: firstly, that of Warren N. Cole and Stuart S. Roberts and their associates of the University of Illinois College of Medicine, Chicago; secondly, that of George E. Moore and Avery A. Sandberg of Roswell Park Memorial Institute, Buffalo, and thirdly, the National Cancer Institute group of Bethesda, Maryland, under Doctor Richard A. Malmgren. Unfortunately in the various reports of these workers there is a good deal of repetition, and so it is not always easy to determine clearly the number of cases they have studied.

Cole (1954 a),impressed by the rate of recurrence of carcinoma of the colon and rectum,studied the mechanism of dissemination of cancer cells. Cole, Packard and Southwick (1954 b) found cancer cells in the fluid perfused through the artery and collected from the vein of a cancerous segment of colon removed at operation. Because they felt that these malignant cells had become detached intravascularly during mobilization of the tumour, they advocated ligation of both the lumen of the colon (several inches above and below the tumour) and the arteries and veins supplying the involved segment of colon BEFORE mobilization of the involved intestine. Their findings stimulated them to further attempts to demonstrate tumour cells in the circulating blood and in 1958 they published their technique for isolating cancer cells, and their preliminary results. For the purposes of their study, they divided their patients into "curable" and "incurable". In this as in all their subsequent reports, they indicate finding higher incidence of malignant cells in "incurable" cases than in "curable" cases. To them, this indicated a direct relationship between the advancing stage of the disease and the percentage of positive blood samples (Roberts, Watne, McGrath, McGrew and Cole, 1958 a). They also compared blood samples taken from the antecubital vein, the major venous trunk draining the tumour area and the vein actually draining the tumour site and found tumour cells more frequently in the veins draining the tumour site than in the peripheral blood. This seemed especially evident in malignancies of the gastrointestinal tract which only manifested cancer cells in the peripheral blood when they were "incurable". They surmised, consequently, that the liver might be a fairly effective filter of tumour cells.

(Roberts, Watne, McGrew, McGrath, Nanos and Cole, 1958 b). A possible corroboration of this last hypothesis is the observation that clumps of tumour cells are found more often in blood draining the tumour area than in the systemic blood where single tumour cells are more common. (Cole, Roberts, Watne, McDonald and McGrew, 1958 c). Until 1959, this group used the albumin-flotation method for the isolation of tumour cells, but they have since changed to a modification of Malmgren's "streptolysin-filtration method (Long, Roberts, McGrath, and McGrew, 1959). Recently, they published the sum total of their results since 1954 (Long, Jonasson, Roberts, McGrath, McGrew and Cole, 1960 b). They have examined the blood of 475 patients with malignant disease. They report that 20% of 90 curable patients and 29% of 207 incurable patients were positive by the albumin-flotation method while 25% of 59 curable patients and 39% of 119 incurable patients were positive by their simplified streptolysin method.

An interesting special report of this group was that of Grove, Watne, Jonasson and Roberts (1959) who isolated cancer cells from the circulating blood of 11 of 20 paediatric patients with malignancies. The incidence (55%) was approximately twice that (23%) of a series of 200 adults with malignant disease reported in a previous paper (Roberts and Cole, 1959 b). Another significant finding mentioned by Grove et al. is that they were able to demonstrate histological evidence of blood vessel invasion in 9 of the 11 patients having cancer cells in their blood.

Colombo, Rolfo and Maggi (1959) compared the results of their findings of tumour cells in the peripheral blood with those of Roberts, Watne, McGrew, McGrath, Nanos and Cole (1958 b) and found them to be similar. The Italian group examined 42 patients with carcinomatosis and

using a technique derived from that of Goudsmit and Van Loghem (1953), reported 10% of their operable cases to be positive as well as 30% of their inoperable cases giving a total of 16% positive.

Another important group is that of Malmgren and his associates of the National Cancer Institute in Bethesda, Maryland. Having described their "Streptolysin-filtration method" for the cytological detection of tumour cells in whole blood in 1958, they went on to study a large group of cases. Later, they published preliminary results (Pruitt, Hilberg and Kaiser, 1958; Pruitt, Hilberg and Kaiser, 1959) before beginning to draw conclusions. One of the important deductions resulting from their study of the correlation between the type of tumour and the incidence of cancer cells concerned "biologic activity" of the tumour. They found that patients with sarcomata and melanomata were more frequently positive than patients with adenocarcinoma and epidermoid carcinoma (Potter and Malmgren, 1959 a). In the same paper, they observed as did Engell (1955; 1959) and Roberts, Watne, McGrath, McGrew and Cole (1958 a) a direct relationship between the degree of extension of the patient's disease and the recovery of tumour cells. They also agreed with Engell (1955) and Long, Roberts, McGrath, McGrew and Cole (1960 a) that the percentage of blood specimens containing tumour cells was greater when blood was taken from veins draining the tumour than from the peripheral blood (Malmgren and Potter, 1959 b). Their latest statistics were derived from 981 blood specimens taken from 376 patients (Potter, Longenbough, Chu, Dillon, Romsdahl and Malmgren, 1960). These patients were divided, as those of the Illinois group, in two groups - "resectable lesions" and "non-resectable lesions". They were able to demonstrate malignant cells in the peripheral blood

of 79 (28%) of 285 patients. Of 117 patients with resectable lesions, 19 or 16% were found to be positive and of 168 patients with non-resectable lesions 60 were positive or 36% indicating a marked increase in advanced patients. They examined blood from the veins draining the tumour areas in 91 patients and found malignant cells in 39 or 43%. These 91 patients yielded the following statistical break-down: 30 or 40% were positive among 75 with resectable tumours and 9 or 56% of 16 were positive in 16 with non-resectable lesions. Further, in this same most recent paper, the Bethesda group found the following overall incidence of positive results by tumour type: melanomata gave the highest incidence of tumour cells in the blood (of 40 patients, 19 were positive for tumour cells,that is 47%) followed by breast adenocarcinoma (14 patients out of 36 were positive or 39%); choriocarcinoma (10 patients of 29 were positive or 34%); sarcoma (5 positive of 17 or 29%); epidermoid carcinoma of the cervix (9 positive patients out of 40 or 23%); genito-urinary adenocarcinoma (5 positive out of 22 or 23%); carcinoma of the head and neck (9 positive out of 55 patients or 16%), and lastly, adenocarcinoma of the gastro-intestinal tract (4 positive out of 26 patients or 15%).

Malmgren's "streptolysin-filtration" method has been adopted for the study being carried out here at McGill University. Preliminary reports of this work have been published (Salgado, Hopkirk, Long, Ritchie, Ritchie and Webster, 1959; Ritchie and Webster, 1960) but discussion of the findings will be deferred to the body of this thesis.

The third of the <u>most influential</u> schools is that of Sandberg and Moore of the Roswell Park Memorial Institute in Buffalo, New York. They began by describing their "fibrinogen" method for the isolation

of blood-borne tumour emboli in 1957 (Sandberg and Moore, 1957). Since then, they have published many reports in various medical journals. Though at first, like Engell, they found no difference in the incidence of tumour cells in veins draining the tumour before or after surgical manipulation (Sandberg and Moore, 1957; Moore, Sandberg and Schuburg, 1957 a) they later surmised that there was unverified evidence that the frequency of tumour cells in the regional veins was increased by surgical manipulation (Moore, Sandberg, Burke, Johnston and Katz, 1958). In an early paper (Moore, Sandberg and Schuburg, 1957 b) they found almost the same percentage of tumour cells in the regional blood (55%) as in the peripheral blood (52%). Later, they affirmed that tumour cells were found more frequently in regional veins than in peripheral blood in curable lesions because the lungs, the liver and the terminal capillaries act as filters (Moore, Sandberg, Burke, Johnston and Katz, 1958). Here they specified "curable" lesions because in advanced cases of malignancy, they speculated that the liver would be inundated by cancer cells and these would "overflow" into the peripheral blood circulation.

One point which shows the importance of compiling a large number of statistics before concluding from the results is that in an early report (Moore, Sandberg, Burke, Johnston and Katz, 1958) they found the occurrence of tumour cells in both resectable lesions and non-resectable lesions to be similar (50%). However, in a later paper, of 44 patients with colorectal carcinoma, they noted a higher incidence of tumour cells (37%) in patients with non-resectable lesions than in patients with resectable lesions (16%) (Moore and Sako, 1959).

In editorials, Moore (1957; 1960) warned against "nihilistic attitudes" which surgeons might adopt secondary to the distressing reports of tumour cells found circulating in the blood even after "curative surgery". Though it has been demonstrated unequivocally that blood-borne metastases do occur and that some of the tumour cells are viable and capable of producing metastatic growths, nevertheless, he agreed with Engell (1955); Roberts and Cole (Long, Roberts, McGrath, McGrew and Cole, 1960 a) and Potter and Malmgren (1959 a) that the significance of tumour cells was not known and that not all cancer cells which embolize produce metastases. Also, since it takes many hundreds of thousands of tumour cells travelling in the blood stream to give rise to a few metastases and since there is little doubt that up to 99% of blood-borne cells released from the primary tumour are destroyed by host resistance (Moore, Sandberg and Watne, 1959), Moore maintains that no prognostic import can be attached,at present, to the presence of cancer cells in the blood nor can therapy be governed by such findings (Moore, 1960).

If the fears of surgeons were allayed by these last conclusions, they would find very little reassurance in the following figures. In cases of carcinoma of the lung tumour cells have been recovered from the pulmonary vein of 7 out of 12 patients upon whom a curable operation was done (Moore, Sandberg, Burke, Johnston and Katz, 1958). In a follow-up study of 817 patients with various types and stages of malignancy <u>after</u> palliative treatment or curative surgery, the 18-month survival was 25% for patients with tumour cells in their blood before treatment and 51% for those without demonstrable tumour cells (Watne, Sandberg and Moore, 1960). In their latest article, the Roswell Park investigators published significant

findings. Of 179 patients with advanced cancer, 50% of peripheral blood samples contained tumour cells. Also they correlated the presence of tumour cells in the blood with histological evidence of direct invasion of veins by malignant growths. It will be seen that the figures match impressively; in lung tumours there was venous invasion in 71% and tumour cells were found in the regional veins in 78%; in stomach tumours there was 57% venous invasion compared with tumour cells in the blood of 53% and in colorectal carcinomata 33% of the veins were invaded whilst tumour cells were present in the regional veins of 30% of the patients (Moore, Sandberg and Watne, 1960).

From the foregoing, it must be immediately apparent that the results of various studies cannot be compared directly. Not only are there differences in isolation techniques and diagnostic criteria, but each work has varying numbers of diverse kinds of tumours at different stages. Tumour cells have been identified in the circulating blood of from 9.7% (Gruner, 1942) to 78% (Engell, 1955) of cancer patients. They were found on an average in 31% of cancer patients. This last figure was calculated taking only the statistics of the reports of large series of cases. It would seem that the number of cancer cells in the blood of patients with lung cancer is much greater than that found in the blood of patients with colorectal carcinoma - even in terminal stages of the disease (Moore, Sandberg and Watne, 1959; Engell, 1955). On the other hand, Delarue (1960) searched for malignant cells in lung, breast and colon cancers comparing their incidence in the blood taken pre-operatively, during operative manipulation and post-operatively. Though he does not mention the total number of cases studied, he reports that tumour cells

were found in a greater percentage of patients with cancer of the colon before operation than in patients with cancer of the breast and lung. There was an increase in the findings of tumour cells in all three groups during operative manipulation but after operation, cancers of the lung led cancers of the colon by 58% to 46%.

Many contradictions and discrepancies become evident from even a cursory look at the various reports on this subject and so one must not be too quick to draw conclusions from such unevaluated observations.

CHAPTER II

CLINICAL AND EXPERIMENTAL EVIDENCE TO ESTABLISH THE PROPERTIES OF TUMOUR CELLS AS REGARDS THEIR PASSAGE TO THE BLOOD STREAM, IN THE BLOOD STREAM AND FROM THE BLOOD STREAM

A. How Do Tumour Cells Get Into the Blood?

A tumour cell has invasive properties, and the most significant of these according to Willis (1952) are its power of progressive multiplication, its motility, and its loss of adhesiveness. Let us now consider these properties in order:

Power of progressive multiplication. The continuous anarchic multiplication of tumour cells by a neoplasm results in an overpopulation of deformed cells which extend into the surrounding tissues by sheer volume or by increased intratumour tissue pressure and so infiltrate at the margins of the tumour into every nook and cranny.

Motility and decreased adhesiveness of tumour cells. Certainly a main factor responsible for tumour invasion is that cancer cells can migrate into surrounding tissues by their own ameboid motility (Coman, 1942). This aspect of invasiveness has been extensively reviewed by Coman (1953). It would seem that cancer cells, to become invasive, must become loosely attached to one another so that they can easily separate and migrate into the surrounding tissues (Coman, 1944; McCutcheon, Coman and Moore, 1948). One reason for the decreased adhesiveness of cancer cells may be that they are deficient in calcium (DeLong, Coman and Zeidman, 1950). Another is that there are structural macromolecular abnormalities on the surfaces of cancer cells (Coman and Anderson, 1955).

Power to interpenetrate normal tissues. These factors do not, however, explain the invasive growth of malignant tumours in any satisfactory way. Another factor of importance is that in tissue culture, one colony of normal tissue will not invade another, while a colony of malignant cells readily invades a colony of normal tissue (Abercombie and Heaysman, 1954).

Now keeping in mind these three invasive properties of tumour cells, it will be seen that there are three mechanisms by which they can enter the blood stream.

(i) By direct vascular invasion. A tumour may grow into a vein by destroying its wall and forming a thrombus from which tumour emboli disperse (Ackerman and del Regato, 1954). As early as 1863, Virchow had taught that the walls of veins and lymphatics could be invaded by malignant cells (Virchow, 1863 c). Two years later, Thiersch also reported cancerous invasion into veins and lymphatics (Thiersch, 1865). There have been many reports of venous invasion by carcinomata of the rectum and colon (Dukes and Bussey, 1941; Dukes, 1944; Grinnell, 1950; Sunderland, 1949; Fisher and Turnbull, 1955). Fisher and Turnbull claimed that their histopathologic criteria for judging vascular extension was the elastica of the vessel (1955). Meissner reported that blood vessel invasion was present in 57% of resected gastric carcinomas (1949). In a study of 45 specimens of carcinoma of the large intestine removed at operation, roentgenograms were taken after injecting the veins with an opaque medium. This was done to compare macroscopic and microscopic venous invasion. There was venous occlusion by roentgenogram in 51% of the cases and 36% showed microscopic infiltration (Barringer, Dockerty, Waugh and Burgen, 1954).

If the mind pictures a tumour encroaching upon the wall of a blood vessel and penetrating into its lumen, one can easily understand how tumour

cells, fragments of tumour tissue or fragments of thrombus bearing tumour cells may become detached and circulate in the blood stream. But there are discrepancies and the following should be borne in mind. The size of the vein sustaining neoplastic infiltration is important. For instance, if a tumour grows into a small vein, it might cause the venule to become immediately occluded and thus the dissemination of tumour emboli would not be favoured. A tumour proliferating into a large vein, however, might cause a thrombus, fragments of which could be detached by the rapidly passing blood. Jeannée (1925) described innumerable small metastatic growths in almost all viscera following invasion of a large pulmonary vein by a bronchial carcinoma.

It has also been found histologically that a rapidly growing anaplastic tumour has more chances of causing tumour emboli than does a slowly proliferating highly differentiated tumour because in the former case there would be rapid destruction of the vein wall, no occlusion of the lumen and minimum thrombus formation whilst in the latter case there would be slow neoplastic involvement of a vein wall, occlusion of the vessel and abundant thrombus formation (Willis, 1952). This has been confirmed by Engell (1955) who related the findings of tumour cells in the blood stream to the histological class of the tumours in patients with carcinoma of the rectum and colon. As has already been mentioned, he found grade II tumours to yield 35% positive results, grade III 78%, and grade IV 100%. Thus Engell concluded that the haematogenous spread of tumour emboli depended on the grade of differentiation of the tumour. Brown and Warren had concluded similarly in 1938.

If the tendency to venous invasion and consequent haematogenous spread depend greatly on the grade of differentiation of the tumour, the role of the <u>stage of advancement</u> of the disease is less obvious. Goldmann pointed

out in 1897 that the finding of tumour cells in the blood stream was not restricted to advanced cases since venous invasion could usually be demonstrated at an early stage. Sunderland considered a combination of factors important. "The occurrence of vein invasion by cancer of the rectum is directly related to the depth of cancer penetration of the bowel wall, the presence of lymphatic extension and the degree of microscopic anaplasia" (Sunderland, 1949). Engell (1955) studied the incidence of tumour cells in the blood occurring in 107 cases of cancer of the rectum and colon distributed into three groups accerding to Dukes' classification (Dukes, 1932). There were 41% positive in group A, 57% positive in group B, and 70% positive in group C. He felt that the increase in incidence was not due to the advancing spread of the disease but to an increase in the number of undifferentiated tumours from A to C. However, in another report published in 1959 he admitted that "venous spread is to some degree related to local extension of the tumour" (Engell, 1959).

At any rate, venous invasion is considered paramount by most authors for consequent haematogenous spread of cancer cells. Though there have been experimental studies (Baserga and Saffiotti, 1955), the following clinical data is more impressive. Moore, Sandberg and Watne (1960) correlated histological evidence of direct invasion of veins by malignant growth with the presence of tumour cells in the blood. In lung tumours, they found venous invasion in 71% and there were tumour cells in the regional veins of 78%. Fifty-seven per cent of the stomach tumours showed venous invasion and there were tumour cells in the regional vein of 53%. In tumours of the colorectal region, venous invasion was demonstrated in 33% as compared with tumour cells in 30%. Gagnon and Gelinas-Mackay (1959) studying carcinoma of the lung found that the histological type of neoplasm did not seem to influence the prognosis

but they agreed with Sunderland (1949) that the prognosis was less favourable in patients whose cancers showed microscopic blood vessel invasion.

Fisher and Turnbull (1955) noted amongst their findings that of 7 cases in which venous invasion had been histologically observed, they were unable to find blood-borne tumour cells in three. They explained such an inconsistency by the premise that the presence of neoplastic infiltration of blood vessels does not necessarily imply that embolization will result.

So far only neoplastic invasion of veins has been mentioned and nothing has been said about arteries. Arteries do sustain invasion by cancer but more rarely. Although the carotid arteries and the femoral arteries are occasionally ulcerated by tumours, the aorta is penetrated only rarely. This may be because tumour cells cannot penetrate the media (Ackerman and Regato, 1954). Large tumour cells carried away in an artery are usually quickly arrested in the first arterioles or capillary system encountered (Willis, 1952). The role of capillaries will be mentioned later.

(ii) Via the lymphatics. It has long been recognized that cancer may spread through the lymphatic system. As mentioned earlier, local and lymph node metastases were considered to be through the medium of the "absorbents" whereas distant metastases were explained by the haemotogenous route (Onuigbo, 1958). However, recently it has been shown that tumour cells may enter the blood stream by first passing through the lymphatic system. This mode of spread has been especially investigated by Zeidman (1956). There are essentially three routes by which tumour cells may enter the blood stream from the lymphatics. Firstly, having been perhaps temporarily arrested by the regional lymph nodes and lymphatics, they may pass into

the thoracic duct and thus are transported into either the innominate vein, the internal jugular vein, the subclavian vein or the vertebral vein - whichever receives the thoracic duct (Zeidman, 1955 b; Willis, 1952). Cancer cells have actually been identified in the thoracic duct lymph (Watne, Hatiboglu and Moore, 1960). Secondly, tumours may invade the thoracic duct directly giving rise to tumour emboli dispersing into the blood stream via the thoracic duct as described previously. Willis (1952) observed 18 cases of thoracic duct invasion in 500 necropsies. Lastly, lymph nodes may act as barriers arresting tumour cells which may grow and form metastases but after a time tumour cells may escape directly from the nodes into the blood by invading blood vessels near the involved node (Zeidman and Buss, 1954). Thus the passage of tumour cell emboli via the lymphatic system into the circulating blood is an important mode of cancer dissemination.

(iii) By modifications of blood pressure. One mechanism suggested to explain the "porte d'entrée" of cells into the blood stream is that of capillary distension (Durante, 1929). He proposed that by the influence of modifications of blood pressure, capillaries distend, causing not only the layers of cells in the walls of capillaries to separate but also the endothelial cells themselves to separate one from the other leaving inviting interstices for the migration of tumour cells into the blood stream. This theory has been verified and confirmed by Young and Griffiths (1950) who applied the principle to the spread of tumour emboli. They experimented with plastic models and showed that differences in hydrostatic pressure within and without vessels could explain the intravascular penetration of malignant cells. A contributing factor could be added by invoking

Bernouillie's principle of suction. The rapid passage of blood in a blood vessel could aspirate tumour cells from the neighbouring parenchyma into the vessel. At any rate, it has been suggested that sporadic findings of tumour cells in the blood stream could be explained by "des àcoups de réplétion et de dépression vasculaire ou par dès poussées congestives successives separées par des phases de décongestion". (Durante, 1929).

It has also been suggested that sudden changes in venous pressure such as those caused by coughing may dislodge cancer cells into the blood stream (Zeidman, 1957).

B. The Metastasizability of Tumour Cells

The fact that blood-borne metastases do occur demonstrates unequivocally that some tumour cells in the blood stream are capable of producing metastatic growth. But there are many conditions affecting the tumour cell's ability to form metastasis. A few of these will now be reviewed.

(i) Viability of tumour cells. To cause metastatic growth, a neoplastic cell must be viable and able to overcome the patient's natural defences. Experimental evidence testifying to the viability of tumour cells in the blood stream is slim. As previously mentioned, Thiersch in 1865 wrote "I do not see why cancer cells should lose their ability to reproduce by a brief passage through the lymphatics or blood stream". Reiss (1959) wrote "Only further experimental studies can enlighten us whether the single cancer cells observed (in the blood) are living cells, capable of multiplication under certain circumstances rather than dead cells shed by the tumour". Dr. F.W. Taylor in the discussion following Grove's report (1959) asks "Here are tumour cells which we see circulating

in the peripheral blood, and they are all good healthy cells, well preserved. Why don't we see some of the cells that are dying? Certainly, most go through the peripheral capillaries and do not form metastases, and yet we never have identified a degenerating form".

Attempts have been made to demonstrate this viability. Antologous tumour cell implantations resulted in a low incidence of "takes" even in patients with advanced disease when fragments of tumour were re-implanted in the donor (Grace and Kondo, 1958), but, as has been mentioned, no "takes" were found when blood from patients with cancer was re-injected into the donor (Taylor and Vellios, 1958). However, this could have been due to natural "host resistance" as will be seen later. Jonasson (1958) identified tumour cells in the circulating blood of C57 black line mice bearing T-150 Lewis bladder carcinomata or T-241 fibrosarcomata and in white rats with the Walker 256 tumours. She injected either the whole blood, or cancer cells isolated from the blood, of these animals into normal animals. This resulted in tumour growths in the normal animals. She then concluded that cancer cells in experimental animals are viable and capable of producing metastatic growth. Zeidman, McCutcheon and Coman (1950) and Korpassy, Kovacs and Tiboldi (1953) grew tumour cells from the blood of animals in a similar way.

Attempts have been made to grow human tumour cells in tissue culture. Using the number 199 culture medium which has as ingredients a mixture of amino acids, vitamines, purines, pyramidines, cholesterol, ATP, adenylic acid, sodium acetate, glutamine, ribose, desoxyribose and ferric nitrate (Morgan, Morton and Parker, 1950), Moore, Mount and Wendt (1959) added 10% calf or foetal calf serum but found the growth of tumour

cells isolated from the blood difficult. Of 64 blood samples from cancer patients, they isolated tumour cells in 13 but were able to grow tumour cells in only 3. They concluded that better media were required. McDonald, Chan and Cole (1960) took blood specimens from 50 patients with cancer and attempted to culture the cancer cells recovered from them. They were able to grow malignant cells in the cultures of only 4 specimens.

Thus it is clear that at least some of the tumour cells in the blood are viable.

(ii) Biological activity of the cell. It has been said that the more biologically active is a malignant cell the more likely is it to cause secondary growth (Delarue, 1960). This rather vague concept may explain the findings of various authors that the incidence of tumour cells observed in the blood is greater in patients afflicted with the more malignant types of cancers such as sarcomata and melanomata (Sandberg and Moore, 1957; Potter and Malmgren, 1959 a; Moore, 1960). Though not proven, this factor may also explain the higher incidence of tumour cells found in the blood in cases of highly anaplastic tumours (Engell, 1955; Moore, Sandberg and Schuburg, 1957 a).

(iii) The number of circulating cancer cells. If numerous tumour cells can be observed in a 5 ml. blood specimen, it is interesting to consider the millions of cancer cells that must be released daily into the cirulation. Experimental evidence (Zeidman, McCutcheon and Coman, 1950; Baserga and Saffiotti, 1955) has shown that, though tumours can rarely be transplanted by a single cell, it usually takes many, sometimes even thousands, of cells to cause even a few metastatic growths (Moore, Sandberg,and Watne and Coman, 1959; Moore, Sandberg and Schubarg, 1957 b). Using the Walker

carcinosarcoma 256 of rats, Overstreet and McDonald (1957) have shown that the more cancer cells are injected the greater the number of metastatic "takes". Fisher and Fisher (1959 b) injected tumour cells in numbers varying from 50 to 250,000 cells into the portal vein of Spragne-Dawley rats. Two weeks later, the animals were examined and it was found that only 15% of animals developed hepatic growths after injection of 50 cells whereas 82% of the animals showed metastases after injection of 250,000 cells.

It has also been claimed that a discrete single tumour cell travelling along in the blood stream is less likely to be arrested in the tissues and cause metastatic growth than are tumour cells in clumps (Watanabe, 1954). As tumour cells are found more frequently in blood samples taken from veins draining the tumour site than in the perihperal blood (Malmgren and Potter, 1959 b) and as tumour cell clumps are found more frequently in veins draining the tumour sites (Moore, Sandberg and Watne, 1960; Roberts and Cole, 1959 b), it would appear then that regional venous blood may have a greater tendency to produce metastases than does peripheral blood.

Tumour cell embolism may occur repeatedly so that metastases will be formed sporadically thereby increasing with time or tumour cells may be emitted "in showers" so that there will be an acute dispersal of tumour emboli causing the organism to be studded quickly with metastatic growths (Zeidman, 1957).

C. Arrest of Tumour Cells.

It has been said that the majority of tumour cells are of too large size to traverse the capillary blood vessels and as a result are arrested by the first arterioles or capillaries encountered (Willis, 1952). There is much

clinical evidence for this concept as haematogenous metastases are found most often in the lungs and liver, the organs in which tumour cells travelling in the blood usually meet their first capillary bed. It was formerly believed that only very small tumour emboli such as those from lymphomata or sarcomata could pass through vascular capillary networks. But these concepts need new assessment in the light of recent experimental work.

Prinzmetal, Ornitz, Simkin and Bergman (1948) introduced glass spheres of different sizes into the arterial vessels of various organs of experimental animals and recovered them from the efferent veins. They found that spheres measuring from 50 to 180 micra in diameter could pass through the liver, spheres of from 160 to 370 micra through the spleen and spheres of from 100 to 390 micra through the lungs. Tobin and Zarquiey (1950) were able to pass glass spheres of up to 500 micra in size through the vascular tree of fresh human lungs obtained at autopsy. Larger spheres lodged in the arterial vascular system. Parker, Andresen and Smith (1958) were able to pass, at physiological pressures, Lucite spheres of from 75 to 80 micra in diameter through the lungs of dogs, but Müller (1953) did not agree that such transpulmonary passage could take place at physiological pressures. From his studies, he concluded that arterio-venous shunts opened to let such spheres bypass the vascular bed only when there were intrapulmonary pressure changes subsequent to blocking or spasm of the pulmonary arterioles. The other workers considered that such shunts were always patent.

With transplantable tumours in rabbits and rats, it has been shown that some tumour cells may pass immediately through the circulation of the liver and kidney (Zeidman, Gamble and Clovis, 1956), the lungs (Zeidman and

Buss, 1952) and the spleen (Korpassy, Kovacs and Tiboldi, 1953).

Arterio-venous shunts such as postulated by the workers with spheres may well explain the passage of tumour cells and tumour cell clumps into the peripheral circulation. Tumour cells emanating from a neoplasm of the gastro-intestinal tract and recovered in the antecubital vein have obviously passed through three capillary beds, the liver, the lung and the limb, or arterio-venous anastomoses in these beds, if the tumour emboli have taken origin in the portal system or two capillary beds, the lung and the limb if the tumour emboli reached the blood through the thoracic duct.

It is conceivable that the passage through capillary networks is made easy by the plasticity of the tumour cells (Zeidman, 1959) in the capillary lumen and by the distensibility of the capillary wall (Delarue, 1960).

If tumour cells are capable of passing through three vascular filters, there is theoretically nothing to prevent them from surviving appreciable periods of time in the free circulation. Relatively enormous ascites tumour cells have been shown experimentally to recirculate for long periods of time (Ambruss and Ambruss, 1956).

But how long tumour cells can circulate is indefinite. Delarue (1960) maintains that if the tumour cells do not lodge and continue to circulate, "the trauma alone attendant upon such repeated passage through channels normally narrower than the cells themselves might be expected to produce degenerative changes if not complete destruction".

But it seems unlikely that arterio-venous shunts are the sole determinant of whether tumour cells will pass without interruption through

capillary beds, The type of tumour seems to be a contributory factor. Zeidman and Buss (1952)injected suspensions of Brown-Pearce cells or V2 carcinoma cells into the marginal ear veins of rabbits and immediately afterwards took blood samples from the aorta of these animals. These blood samples were then injected into other rabbits. They observed that 50% of the recipient animals injected withthe Brown-Pearce carcinoma developed tumour growth and only 13% injected with the V2 carcinoma did likewise. They thus concluded that the cells from both types of tumours had passed through the lungs of the donor animals but that the Brown-Pearce carcinoma cells had a greater ability for transpulmonary passage than did the V2 carcinoma cells. In another experiment, it was demonstrated that immediate transhepatic and transrenal passage occurred in both rabbits and rats with all tumours,except the Flexner-Jobling, thereby manifesting another difference due to tumour type (Zeidman, Gamble and Clovis, 1956).

The fact that tumour emboli can pass uninterrupted through capillary beds is manifestly of clinical interest. It may well explain unusual distribution of metastases. For example, secondary tumours may be absent from the lungs but present in organs farther downstream.

D. Fate of Tumour Cells.

The possible destinies of tumour cells which have entered the circulating blood are three:

(i) They may grow and form metastases. As has been mentioned above, tumour cells may encounter arterioles or capillaries where they suffer arrest and being viable may "take" and grow. This power of growth is

attributed by Willis (1948) more to clumps of cells or masses of thrombus containing cells than to isolated cells. This is the mechanism of blood-borne metastases.

(ii) Tumour cells may be destroyed. About 100 years ago Thiersch (1865) said, "Out of the numerous tumour cells that reach the blood stream, only a few are viable and able to form metastases". This opinion is still held to-day. Of the hundreds of thousands of tumour cells that may be released in the blood stream daily from a primary tumour, it has been estimated that 99.9% fail to survive and form metastases (Moore, Sandberg and Watne, 1960). Dockerty (1958) described this phenomenon well when he said many tumour cells "wither on the vine".

Histologic sections of lungs have shown that many tumour cells have been caught in the capillaries and are undergoing degeneration (Moore, Sandberg and Watne, 1959). This had been shown as early as 1903 by Schmidt who, finding tumour emboli embedded in pulmonary arteries, concluded that the majority of such cells perished. Iwasaki (1915) found that tumour cells from all malignant tumours could be destroyed in the blood vessels of both man and animals by thrombus formation and by the direct action of the plasma. Takahashi (1916) verified Schmidt's findings of tumour cell destruction in the lungs of humans by observing the destruction of tumour cells in the lungs of experimental animals. Since Quensel (1921) found tumour cells in the blood of only 6 patients out of 50 who had died of cancer, he concluded that the tumour cells in the other patients must have perished.

Many mechanisms have been incriminated to explain this destruction of intravascular malignant cells. It has been suggested that the lytic properties of the blood stream and tissues prevent the tumour cells from lodging and forming metastases (Pool and Dunlop, 1934).

But the most important factor responsible for the "in vivo" destruction of neoplastic cells is the yet unexplained resistance of the host. This resistance is better explained indirectly or in a positive way. By producing a diminution of the body's natural defences by various measures, for example, stress, it has been found that such a diminution in host resistance will increase the number of "takes" of implanted cancer cells (Grace and Kondo, 1958). Conversely therefore, the destruction of cancer cells may be due in large part to the body's natural resistance to tumour embolism. The investigations of Taylor and Vellios (1958) may be recalled here. They were unable to reproduce tumour growth by autologous tumour cell inoculations. They attributed this failure to the natural ability of the host to destroy the injected cells. Yet discussing another paper (Grove, Watne, Jonasson and Roberts, 1959) Dr. Frederick William Taylor wondered, "Why don't we see some of the cells dying? All the cells we see circulating in the peripheral blood are healthy cells and we have never identified a degenerating form". He perhaps overlooked the difficulty of identifying dead or dying cells.

Delarue (1960) suggested that the lympho-reticulo-endothelial system has an important role in the development of resistance in a patient with cancer. He reminds us of the rarity of metastatic carcinoma in the spleen. One should, however, perhaps remember the commonness of metastases in the lymph nodes. Delarue's supposition, however, supports Fould's (1932) who also suggested a possible resistant action on the part of the reticulo-endothelial system to blood-borne metastases.

It is this large destruction of tumour cells within the blood vessels that has made Moore (1960) question the prognostic import of their

isolation in the blood stream and that explains Engell's conclusion (1955) "Cancer cells were found with about the same frequency in patients who are surviving without evidence of metastases as in those who have developed metastases or recurrence".

It should also be remembered that even clumps of tumour cells which succeed in establishing a metastasis may still die leaving no persisting disease. Clinical reports of the regression of metastases are well known (Allen, 1955; Penner, 1953; Sumner, 1953) and it has been clearly shown experimentally that tumour emboli which lodge in the lung (Baserga and Saffiotti, 1955) or liver (Guha, 1960) may grow for a time, but fail to establish permanent metastases.

(iii) <u>They may lie dormant in the tissues for years.</u> This third possibility was recently stressed by Roberts and Cole (1959 b). Dormancy implies "malignant cells which, although remaining alive in the tissues for relatively long periods, show no evidence of multiplication during this time, yet retain all their former and vigourous capacity to multiply" (Hadfield, 1954). That cancer cells may lie dormant is well known. For example, Rohdenburg (1920) observed small groups of living cells from a gastric carcinoma in the liver and omentum 10 years after the operation on the primary tumour with clinical cure. A cerebral metastasis has been found in a patient operated 20 years previously for a bilateral breast carcinoma (Crouzon, 1920). Fisher and Fisher (1959 a) found that Walker 256 carcinosarcoma cells could remain in the liver of rats in a viable but dormant state until made to grow by surgical trauma. Many other examples could be quoted.

CHAPTER III

REVIEW OF THE FACTORS AFFECTING THE VASCULAR DISSEMINATION OF TUMOUR CELLS

A. Role of Surgery.

There appears to be two facets of surgery which may affect intravascular liberation of tumour cells - manipulation of tumour and operative stress.

1. Role of Surgical Manipulation of a Tumour.

(a) Clinical Evidence. Lawrie in 1906 was perhaps the first to warn surgeons of the danger of implantation of cancer cells into a wound, and as early as 1908 Ryall advised changing of the drapes, instruments, gloves, etc. after a biopsy was performed before proceeding with a radical resection of cancer. Practical studies since that time on the contamination of operative wounds by cancer cells provoke thought. There has been increasing evidence that up to 50% of local recurrences (Smith and Hilberg, 1957) are due in large part to the "seeding" of cancer cells on the raw surfaces of the wound and on the suture lines at time of operation. McGrew, Laws and Cole (1954) showed that surgical manipulation could cause dissemination of cancer cells since occlusive ligatures placed around the colon early in the operative procedure prevented their spread. Ackeman and Wheat (1955) cited many instances of tumour cell implantation following certain surgical procedures; needle biopsy, skin grafting for malignant tumours, rupture of capsule during attempts at enucleation of the tumour, transplantation of tumour cells from one bronchus to another during biopsy

of a bronchial adenoma using a cautery, rupture of ovarian cancer with implantation on the peritoneal surfaces, and cutting through tumour. Saphir (1936) has identified viable tumour cells on knives used in biopsy or excision. A case is reported of inoculation metastasis caused by intubation anaesthesia in surgical therapy of carcinoma of the larynx (Glaninger, 1959). But more directly there have been many contributions attesting to the inoculation of wounds at time of surgery (Meadows, 1957; Smith, Thomas and Hilberg, 1958) and a good number of investigators have concerned themselves particularly to the problem of local recurrentes following resection of tumours of the colon, sigmoid and rectum (Galigher, Dukes and Bussey, 1951; Southwick and Cole, 1955; Morgan, 1955; LeQuesne and Thomas, 1958).

Though it is well accepted to-day that surgical manipulation does cause local implantation of tumour cells, the role of operative trauma as regards intravascular dissemination of cancer cells is still the subject of much debate and conjecture. Engell in 1955 found little reason to believe that the incidence of malignant cells in the circulating blood was higher after surgery than before. However, in 1959, he admitted that operative manipulation played a minor role in intestinal carcinoma. Reiss (1959) agreed that there was no significant increase after surgical manipulation. In his series, 11% of 36 cancer patients showed tumour cells in the blood stream before operation and 15% of 14 cancer patients did so after operation. Sandberg and Moore (1957) did report that the frequency of tumour cells in the regional veins was increased by operation (e.g. gastrectomy) but otherwise their findings parallelled those of Engell - there was no apparent increase in the

frequency of tumour cells in the peripheral blood after surgery (Moore, Sandberg, Burke, Johnston and Katz, 1958).

Conversely, others have found a significant increase in circulating neoplastic cells after operative trauma. In 35 patients operated for carcinoma of the colon, cancer cells were found in the blood of the regional veins in 38% after the tumour had been manipulated but they were found in only 13% of 76 patients when the tumour had not been handled (Turnbull,1957). Ross (1958) studying carcinoma of the lung found that in 15% of the cases in which blood was drawn pre-operatively, tumour cells were present in the blood stream, whereas they were found in 70% of the cases in which blood was drawn during the operation.

Probably the most comprehensive study of this problem has been made by Cole and his associates of the University of Illinois. By taking frequent blood samples preceding, during and after various surgical procedures, they concluded to three basic types of response to operation: Firstly, a transitory appearance of tumour cells in the blood during resection of the tumour in "curable" patients in whom pre-operative and post-operative samples were negative; secondly, a disappearance of tumour cells from the blood following a curative resection of a neoplasm in cases in which tumour cells had been found in the blood of the patient pre-operatively; and thirdly, a post-operative transient decrease of tumour cells in patients whose blood samples had been positive prior to surgery and in whom the tumours were found to be unresectable at celiotomy (Roberts, Watne, McGrath, McGrew and Cole, 1958 a; Roberts, Watne, McGrew, McGrath, Nanos and Cole, 1958 b; Roberts, Watne, McGrath, McGrew and Cole, 1959 a; Roberts and Cole, 1959 b; Long, Roberts, McGrath, McGrew and Cole, 1960). What is deemed especially significant in the

above is that removal of a tumour surgically may result in disappearance of the blood-borne cancer cells, but not if metastases are present (Cole, Roberts, Watne, McDonald and McGrew, 1958 c). However, this same group have recently found an increase in blood-borne emboli during uterine cubettage of 3 of 5 patients with malignant disease of the endometrium (Roberts, Long, Jonasson, McGrath, McGrew and Cole, 1960), a finding more in keeping with Turnbull's work.

(b) Experimental Evidence. The role of massage as a means of inducing metastases of tumour cells could not be established in humans by Knox (1922) but in animals he showed that gentle massage for short periods each day for a few days set free numerous tumour particles which embolized to the lungs. Others agreed that massage of tumours favours metastasis. Tumour cells were found in the blood aspirated from the hearts of mice with transplanted sarcomata by Jonescu (1931) more readily following massage of the tumour. Ide, Harvey and Warren (1939) studied the Brown-Pearce rabbit carcinoma in rabbit ear chambers. They observed that capillaries, damaged by slight trauma, would permit entrance into the blood stream of small detached islets of tumour cells which were then carried away by the blood stream towards the bigger veins.

2. Role of Operative Stress.

(a) Clinical Evidence. In so far as operation causes alterations of host resistance, it may conceivably favour the haematogenous dissemination of cancer cells. Various factors have already been mentioned and other factors surrounding operation such as anaesthesia and metabolic alterations will be discussed in a later chapter. Some interesting experimental work can be mentioned.

(b) Experimental Evidence. Lewis and Cole (1958) found that the frequency of pulmonary metastases was increased in mice after operative stress. They injected 100,000 cancer cells in the hindfoot of mice and two to three weeks later, after having placed an elastic band around the thigh, they amputated the tumour-bearing limb. They reported the incidence of pulmonary metastases to be much greater in the operated group than in the animals similarly inoculated but not amputated. The elastic band placed proximally to the tumour in the operative group acted as a tourniquet preventing per-operative dissemination of tumour cells and led to the presumption that tumour cells were already in the blood stream before amputation. Thus the authors' purpose was not to prove that surgical manipulation caused intravascular "spillage" of cancer cells but that the stress of operation decreases the resistance of the host to the cancer cells desquamating from a tumour.

The experiments of Ketchum (1959) were in direct contradiction to those of Lewis and Cole. S-91 melanoma cells were injected into the thigh of mice and the tumour-bearing limbs of one group were amputated after three weeks. Thirty-five days after inoculation, he examined the lungs of these and of the non-amputated control group for metastases. He observed that complete removal of the primary tumour had resulted in a decreased number of metastases although the average size of the metastatic foci in the amputated group was larger than in the control group. He concluded that since the metastases that he observed in the lungs of the amputated group were larger and older than those in the non-operated group, then these must have been present pre-operatively and surgery therefore must prevent or reduce the occurrence of new metastases.

Schatten (1958) found that it is not the surgical act "per se" which results in the establishment of metastases because in a mouse with a tumour-bearing leg, amputation of the contra-lateral leg does not affect the frequency, size or number of pulmonary metastases, but he explains its role as follows: A primary tumour of sufficient size inhibits the development and growth of distant metastases. Many metastases that occur in mice become established and grow prior to the time the primary tumour becomes large. Working with S91 melanoma and DBA sarcoma 49 in mice, he observed that complete removal of the primary growth 3 weeks after implantation "unleashed" as it were these secondary foci and resulted in the rapid growth of large numbers of latent pulmonary metastases.

Buinauskas, McDonald and Cole (1958) injected 25,000 Walker 256 cells subcutaneously into 164 rats of which 85 were subjected to celiotomy just before inoculation of the cells. The remaining 79 rats were used as controls. They found 61.1% "takes" in the rats having had celiotomy compared to 31.6% in the rats not having had celiotomy. Two corollaries were thought to be significant. One, the control group of animals lived longer than the operated group and, two, there was slightly faster and larger growth of the tumour in the group having been submitted to surgery. Their experiments suggest that operative stress reduced the resistance of the animals to inoculated Walker 256 cells.

Fisher and Fisher (1959 a) found that tumour cells could remain in the liver in a viable but dormant state until made to grow by surgical trauma with its attendant metabolic alterations.

Thus the evidence would seem to indicate that both surgical trauma and alterations in host resistance due to operative stress may contribute to cancer cell dissemination.

B. Role of the Liver.

1. Experimental Evidence. Though tumour cells have been shown to pass unarrested through the liver (Zeidman, Gamble and Clovis, 1956), the prevalent opinion to-day is that the liver acts in some way as a barrier to tumour cells.

Patey (1936) injected Brown-Pearce carcinoma cell emulsions into the portal circulation of 41 rabbits and into the systemic circulation of 44 rabbits. On examination of the tissues 3 to 5 weeks after injection, he found 17% metastatic deposits in the "portal system" rabbits and 68% deposits in the "systemic circulation" rabbits. It seemed then that tumour cells reaching the blood stream via a portal vein were markedly less likely to give rise to metastatic deposits than tumour cells entering through a systemic vein. There was clinical evidence which parallelled these observations. Blood-borne metastases were reportedly much more frequent in tumours of the systemic territory such as carcinoma of the breast and naevo-carcinoma than in tumours of the portal territory such as carcinoma of the large intestine. How to explain this? Patey believed that the liver, by means of its abundant content of cells of the reticulo-endothelial system, acts to some extent as a barrier to blood-borne metastases by the portal route.

Abbott and Krementz (1957) using the Brown-Pearce and V2 carcinomas, injected fixed hematoxylin-stained tumour cells into the portal tributaries of one group of 40 rabbits and did likewise with viable tumour cells in another group of 40 rabbits. Later, they determined the fate of these cells by autopsy. In the first group, microscopic examination revealed that, while many cells, especially single cells and

those in small clumps, had passed unarrested through the liver, the majority were arrested in the liver. In the 40 rabbits sacrificed 5 weeks after injection of saline suspensions of viable cancer cells, the authors observed four interesting facts:

(a) despite large numbers of cells injected, very few resulted in growing metastases;

(b) but the metastatic growth that did occur took place mostly in the liver;

(c) tumour emboli in general followed the axial streams of the portal vein as indicated by growth in particular liver lobes;

(d) the size and extent of blood-borne metastases were dependent on inherent characteristics of the tumours, that is, those from the Brown-Pearce carcinoma grew more frequently and rapidly than those from the V2 carcinoma.

Fisher and Fisher (1959a and 1959 b) studied experimentally the factors influencing hepatic metastases. Firstly, they discovered that the greater the number of tumour cells injected into the portal vein of Sprague-Dawley rats, the more metastatic growths could be demonstrated in the liver. Secondly, they found an increased incidence of hepatic metastases over control animals when intraportal injection of varying doses of Walker 256 carcinosarcoma was immediately followed by partial hepatectomy. They suggest that, for a transient period after partial hepatectomy, tumour cells are more readily converted to metastases.

2. Clinical Evidence. Fletcher and Stewart (1959) gave special attention to pre- and post-hepatic blood. They examined blood from both sources in 9 patients having malignant tumours of the gastro-intestinal tract and found tumour cells in the portal vein blood of 4 cases and in

the hepatic vein blood in only one case. They surmised therefore that the liver was a highly but not completely efficient filter of tumour cells reaching it by the portal vein. They also believed that the liver "releases" tumour cells sporadically and that the finding of them in the hepatic vein depended on chance timing.

Roberts and Cole (1959 b) incriminated the liver to explain why they had found no cancer cells in the antecubital vein blood of 30 patients with "curable" malignancies of the digestive tract. On the other hand, since cancer cells had been isolated in the antecubital vein blood of 10 out of 36 patients with "incurable" malignant tumours of the digestive tract, they postulated that whenever tumour cells do show up in the peripheral blood of a patient with a cancer of the digestive tract, then the patient must have an "incurable" lesion.

It would seem then that tumour emboli liberated in the portal venous system are caught in the liver.

C. Role of Chemotherapy.

Chemotherapy as an adjunct for surgical treatment of cancer is currently under study (Shimkin and Moore, 1958). Investigators are especially directing their efforts towards the effect of chemotherapy on small amounts of residual tumour and on unestablished tumour cells. If cytotoxic agents can prevent or delay such free tumour cells or clumps forming tumour "colonies", their administration to patients before, during and after surgical resection would be rational. Shapiro and Fugmann (1957) have shown experimentally that with a sensitive tumour and effective chemotherapeutic compound the combined use of surgery and chemotherapy results in many more cures than the use of either singly But there are many problems.

1. Toxicity of anti-cancer agents.

Experimental studies have indicated that some compounds such as nitrogen mustard, Actinomycin D and Dakin's solution are the most effective for the local destruction of tumour cells on surgical wounds. These and relatively ineffective agents such as saline, dilute antiseptics and distilled water have been used by surgeons to irrigate surgical wounds. Clorpaclin (2% monoxychlorosene), a chemical for topical use, has been found effective experimentally but cannot be used clinically because of its toxicity. However, irrigation of wounds with some ineffective substances may actually be dangerous. Saline, water, heparin, Neomycin, Terramycin and other solutions will not damage tumour cells but instead will carry them away to crevices in the wound where they may readily grow. The first three compounds mentioned above can destroy tumour cells in concentrations that will not impair wound healing. But given systematically, higher concentrations must be used resulting in adverse effects to the organism. General toxic reactions are appreciably less when chemotherapeutic agents are applied directly to tumour cells in surgical wounds and in the body cavities than when they are given systemically (Moore, Sandberg and Watne, 1960). The successful application of the proper anti-cancer agent will depend on balancing its cytotoxic activity in malignant tissue against coincident injury to host factors. There is experimental evidence that toxic compounds may indirectly accelerate tumour growth by inhibiting normal defence mechanisms (Moore and Kondo, 1958). Delarue (1960) maintains that this diminished resistance is due to the cytotoxic effect on the lympho-reticulo-endothelial system.

2. Specificity of the Chemotherapeutic Agent and Sensitivity of the Tumour.

Not all chemotherapeutic compounds are equally effective against different kinds of tumours, individual tumours of the same type, or even all metastases of a single tumour. It will be necessary to assess the effectiveness of each compound against each kind of tumour cell. Dipaolo and Moore (1959) recommend "in vitro" tests of anti-cancer activity similar to those used for determining bacterial sensitivity to antibiotics. Administration of a non-effective chemotherapeutic agent followed by intravenous inoculation of ascites tumour cells in mice has been reported to actually increase the percentage of "takes" forming pulmonary metastases (Kondo, Moore and Bohn, 1958).

Moore, Sandberg and Watne (1960) have reported that 40,000 chemotherapeutic compounds are being tested yearly by the Cancer Chemotherapy National Service Center of the National Cancer Institute. Most are found to be toxic or ineffective. The value of this work is doubtful because there is such a narrow specificity of each agent - tumour reaction that the agents may not be tested against the right tumour (Kondo, Moore and Bohn, 1958).

3. Effectiveness Against Primary and Secondary Tumours.

Experiments have shown that unattached tumour cells in the blood, body cavities or in surgical wounds are much easier to destroy than the established tumours. It has been shown that in breast carcinoma in mice, the larger was the tumour, the less was the chemotherapeutic effect of 6-mercaptopurine. Not only was a larger tumour less responsive but it appeared to be able to protect smaller tumours in the same animal from

damage by chemotherapy (Shapiro and Fugmann, 1957).

However, the primary tumour having been removed, cytolytic agents seem to show more effectiveness. Kramer and Schatten (1958) found that TSPA (triethylenethiophosphoramide) was effective in reducing the number of S91 melanoma pulmonary metastases when administered after removal of the primary tumour. In some mice, metastases were even completely prevented. They were surprised at this effectiveness taking into consideration the reputed increased growth of distant metastases that occur after removal of a primary tumour.

Also, it has been demonstrated that compounds which are ineffective against solid tumours might completely destroy small numbers of the same tumour cells injected intravenously (Kondo, Moore and Bohn, 1958). But in general, the activity of anti-cancer agents against unestablished tumour cells parallel their effects on the same cells, growing as solid tumours. (Kondo, Moore and Bohn, 1958).

4. Clinical Trials

A review of recent pertinent experimental work in this field reveals that some investigators have been successful in their preliminary clinical trials with some anti-cancer agents. Cruz, McDonald and Cole (1956) having discovered that nitrogen mustard, administered intravenously or intraperitoneally, was effective in reducing "takes" in rats after the intraportal injection of Walker 256 carcinosarcoma cells, decided to try this agent in cancer patients at time of operation hoping to prevent both haematogenous metastases and metastases via the lymphatics. After having used HN_2 without incident on 60 patients, they recommended certain precautions and refinements to the technique (Morales, Bell, McDonald and Cole, 1957). They also advocated the intraperitoneal instillation of HN_2

as a safe procedure at the conclusion of curative operations for cancer of the stomach, colon and rectum, provided certain precautions were utilized (Economou, Mrazek, McDonald, Slaughter and Cole, 1958). Another chemotherapeutic agent which they investigated was triethylenethiophosphoramide (Thio TEPA) (McDonald, Livingston, Boyles and Cole, 1957). This chemical had previously been found more useful than amino-teropterin in reducing the spontaneous implantation and growth of free tumour from the ascitic fluid of tumour-bearing mice (Goldie, Walker, Graham and Mitchell, 1957). They found the effects of nitrogen mustard and thio TEPA to be about the same providing that each agent had been selected for its specific action against a specific tumour in a given patient (Roberts and Cole, 1959 b). In 2 patients one with a non-resectable sarcoma of the uterus and one with a non-resectable carcinoma of the stomach, no cancer cells were found in the blood stream within 72 hours following administration of nitrogen mustard in the first case and Thio TEPA in the second, but the malignant cells reappeared in the circulation 14 to 28 days later (Roberts, Watne, McGrath, McGrew and Cole, 1959 a). They advised that prophylactic and adjuvant therapy could be instituted with best advantage at time of surgery in curable patients having no tumour cells in their blood pre-operatively. (Long, Roberts, McGrath, McGrew and Cole, 1960 a).

The evolution of therapy for cancer has been compared to that of osteomyelitis (Moore, Sandberg and Watne, 1960) and so the recommended treatment at present combines perfusion of chemotherapeutic compounds into isolated segments of the body, use of radioactive agents and excisional surgery (Moore, Sandberg and Watne, 1959). Roberts and Cole (1959 b) feel that if cancer cells are demonstrated in the blood of a patient before operation, then chemotherapy should be mandatory apart from operation.

D. Role of Host Resistance

The vascular dissemination of tumour cells resembles that encountered in bacterial infections. As established, infection becomes apparent only when an overwhelming number of organisms are inoculated, when the organisms are extremely virulent or when the ability of the host to mobilize the normal defence mechanisms is lost, so too, it would appear that the development of metastatic foci depends not only upon the nature of the cells disseminated, but also upon the reaction of the host (Delarue, 1960).

Many factors contribute to the resistance shown by animals and humans to metastases. Some have been discussed already. Willis (1952) points out the natural components of resistance - "a constitutional attribute embracing all tissues", age, heredity, and good health. Delarue (1960) suggests that the lympho-reticulo-endothelial also plugs a large part in the organism's normal defences against metastases. Any factor then causing decrease in host resistance is apt to promote increased "takes" of tumour cells. Operative stress has already been mentioned (Lewis and Cole, 1958). Haemorrhage and anaesthesia have been shown experimentally by Gaylord and Simpson (1916) to produce an augmentation of metastases in mice. Chemotherapy by its cytotoxic effect on the lympho-reticulo-endothelial system has also been reported to cause increase of metastasis (Delarue, 1960). Possibly general X-irradiation (Cirio and Balestra, 1930) acts in like manner.

Malnutrition will contribute to the increased development of metastases from circulating tumour cells. Acute starvation is one form and the cachexia resulting from the depletion of nitrogen and protein

from the tissues by a large primary tumour utilizing these substances for its growth is another (Delarue, 1960).

Any stress affecting an organism's general health is likely to potentiate it to develop secondary tumour growths. Extremes of temperature have been demonstrated to promote metastases. Griffiths (1959) demonstrated an increased take of Walker 256 carcinosarcoma in rats following hypothermia and cold stress. Lucké and Schlumberger (1949) found that metastases from renal carcinoma in frogs were increased after keeping the animals at high temperatures.

Cortisone which simulates operative stress was also reported by Moore and Kondo (1958) to increase the "take" of inoculated tumour cells in mice. Pomeroy (1954) injected cortisone acetate into Swiss mice concomitant with intravenous injection of tumour cell suspensions. He observed the production of widespread metastases beyond the lung filter and surmised that this phenomenon had probably been caused by the temporary destructive effect of cortisone upon the lymphoid tissues and reticulo-endothelial cells causing changes in the resistance of these tissues.

Finally, it should be pointed out that not all investigators are in agreement with the above. In their experimental studies of post-operative tumour metastases, Schatten and Kramer (1958) found no significant effect of operative stress, anaesthesia or cortisone administration on the number of artificial pulmonary metastases of S-91 melanoma in mice, and the number of dissenters could readily be increased (Guha, 1960).

E. Role of Body Movement.

Peristaltic activity, general muscular movement (Moore, 1957), sudden changes in venous pressure as may occur in coughing (Zeidman, 1957),

respiration (Moore, Sandberg, Burke, Johnston and Katz, 1958) and body movement have all been cited as possible aetiological factors causing tumour cells to dislodge and float away in the blood stream thereby increasing the probability of secondary tumour deposits.

F. Role of Radiotherapy.

As far as could be ascertained, there has been no evidence promulgated regarding the effect of radiation against circulating tumour cells. It is generally known that the direct action of radiation is mainly on the genetic apparatus of a cell but it also affects cell metablism, growth and differentiation (Upton, 1958). It would be technically difficult to attempt to destroy radiologically tumour cells circulating in the blood as this would mean irradiation of the whole patient, using large doses and for lengthy periods of time. More practically, the use of pre-operative radiotherapy has been suggested to supplement the role of surgery in the extirption of a primary tumour. Small doses were recommended directed on tumours in an effort to kill the tumour cells so that viable cells would not enter the circulation or cause recurrences by implantation at operation. But trial tests of pre-operative radiotherapy in cases of primary mammary carcinoma gave questionable results (Delarue, 1960).

Several forms of irradiation have been used against local and systemic cancer cells. Keetel and Elkins (1956) supplemented the surgical and X-ray treatment of ovarian malignancy with the intra-abdominal instillation of radioactive gold. Moore, Sandberg and Amos (1957),experimenting with large doses of gamma and other globulins as anti-cancer agents, foresaw the use of specific carriers of radioactivity

in the future, such as isotope-tagged antibodies, but as yet no satisfactory method of combating blood-borne tumour cells by radiotherapy has been evolved.

CHAPTER IV

EXFOLIATIVE CYTOLOGY: SITES IN THE ORGANISM WHERE TUMOUR CELLS HAVE BEEN FOUND

Though this work pertains to tumour cells in the circulating blood, its scope often impinges upon the field of exfoliative cytology. Consequently, for interest and completeness this section will be devoted to the mention of but a few examples of the various other sites in the organism where tumour cells have been isolated.

In recent years, the science of exfoliative cytology has advanced rapidly to the forefront of medical technology by its advantages as a method of detection of cancer. Its scope penetrates into many areas of cyto-pathology and it is widely used to-day as a method of screening large numbers of the population in an effort to detect early cancer. Mass cytological screening of the population has been carried out mostly in the United States as a means of detecting early cervical carcinoma in women by examining endocervical smears (Nieberg, 1957).

Its effectiveness has been made possible in recent years especially by G. N. Papanicolaou of Cornell University who in 1954 described a method for the staining of cancer cells in his "Atlas of Exfoliative Cytology". Since then this and other methods have become increasingly popular to identify tumour cells in various sites in the human organism.

We will first consider the bone marrow. Reich in 1935 described the presence of cancer cells in bone marrow specimens obtained by needle aspiration of the sternum and it is believed that he was the first to do so.

Jaimet and Amy (1956) thought that the diagnosis of cancer could be made by bone marrow smears. However, Gimm and Krönke (1958) saw cytological differential diagnostic difficulties caused mainly by cells easily confused with tumour cells such as reticulum cells and megakaryoblasts. Also, they did not believe that the presence of tumour cells in the sternal punctate necessarily meant that a metastasis was present because, although their presence in the bone marrow indicated that there were some circulating in the blood stream, they recalled that most tumour emboli were destroyed. Though they encountered technical difficulties - individual malignant cells are harder to identify in the bone marrow than in the blood because of the presence in the former of a great number of immature haematopoietic cells - Sandberg, Moore, Crosswhite and Schubarg (1958) succeeded in isolating cancer cells in the bone marrow of 8% of 600 patients with cancer. Surprisingly, in 305 patients in which both peripheral blood samples and sternal marrow aspirations were examined, they found the incidence of tumour cells, though low, to be about the same in both marrow and blood.

Tumour cells have also been identified in the thoracic duct lymph. It has been reported that patients with carcinoma of the stomach, lung and breast appeared to have a high incidence of tumour cells in the lymph (Watne, Hatiboglu and Moore, 1960). Further, it has been found that tumour emboli may pass directly from the thoracic duct to nearby lymph nodes. This has been thought to explain Virchow's node in cases of abdominal cancer in man (Zeidman, 1955 a).

Neoplastic cells have been identified in cerebrospinal fluid. Studying the C.S.F. of 320 patients presenting neurological diagnostic

difficulties, McCormack, Hazard, Belovich and Gardner (1957) found malignant cells in 25.

Neoplastic cells have also been found in operative wound washings (Smith, Thomas and Hilberg, 1958); sputum (Wandael, 1944); pleural and peritoneal transudates in patients with neoplastic diseases of the serosae (Graham, 1933; Juniper and Chester, 1959); urine (Solomon, Amelar, Hyman, Chaiban and Europa, 1958); and prostatic secretions (Jönsson and Fajers, 1950).

Exfoliative cytology has been extensively used in the diagnosis of bronchogenic carcinoma. Positive bronchial washings have been recognized to be of great value in substantiating a diagnosis of lung carcinoma (Jackson, Bertoli and Ackerman, 1951; Ross, McGill, 1949). Pleural cavity washings have also revealed carcinoma cells (Spjut, Hendrix, Ramirez and Roper, 1958).

Cytological examination of breast secretions has also been found useful for diagnosis of mammary carcinoma (Saphir, 1950). Specimens obtained by aspiration of breast tumours have also been studied. Cornillot and Verhaeghe (1959) consider that diagnosis by this means is more reliable for solid than for cystic tumours, but the Russian team Shiller-Volkova and Agamova (1960) claim 96.7% accuracy in the diagnosis.

Much more difficult technically is the cytological examination of fluids from the pancreatic duct of Wirsung, duodenum, biliary tract and stomach. However, aspiration of duodenal contents has shown tumour cells in cases of carcinoma of the pancreas and biliary tract (Lemon and Byrnes, 1949). Exfoliated pancreatic cancer cells have also been found in the duct of Wirsung (Howden and Papanicolaou, 1959) and, by gastric lavage and gastric mucosa "swabbing", Witle (1959) made the cytodiagnosis of a carcinoma of the stomach.

Cole (1952) demonstrated,in washings from the colon, the presence of viable tumour cells washing away from the surface of colorectal growths by the passage of the faecal stream. Dukes and Galvin (1956) supported this finding and added that tumour cells were more likely to be shed from an ulcerating growth, and that frequently these cancer cells would implant at a lower level in the bowel wall. Cole (1952) even demonstrated tumour cells in enemata!

Finally, medical literature concerning exfoliative cytology is abundant with reports referring to diagnosis of uterine, vaginal and ovarian carcinoma by means of endocervical and vaginal smear.

CHAPTER V

INTRAVASCULAR MIGRATION OF TISSUE CELLS OTHER THAN MALIGNANT CELLS INTO THE BLOOD STREAM

For the sake of interest, it was thought pertinent at this time to mention briefly that non-neoplastic elements, supposedly fixed in the tissues, may also be found travelling in the blood stream.

Normal adipose tissue cells may be liberated into venous blood following trauma to bone (Zenker, 1862; Busch, 1866). Both normal parenchymatous liver cells (Schmorl, 1887-1888) and phagocytic Kupffer cells derived from the endothelium lining the capillaries of the liver (Durante, 1929) have been demonstrated in the circulating blood. Placental elements may travel in the blood stream. Douglas, Thomas, Carr, Cullen and Morris (1959) found trophoblastic cells in veins draining the uterus during pregnancy but could find none in the peripheral blood. Even brain tissue cells have been seen in venous channels of the lung (Krakower, 1936). Durante (1929) made a study of tissue cells "foreign" to the blood and reported observing fatty cells, placental elements, chorio-epithelioma cells, phagocytic Kupffer cells, renal cells and smooth muscle cells.

A further example is that cited by Rapoport, Raum and Horrell (1951) who saw bone marrow metastases in the lung.

P A R T II

MATERIALS AND METHODS

CHAPTER VI

ISOLATION OF TUMOUR CELLS: HISTORY AND DESCRIPTION OF VARIOUS TECHNIQUES

As may well be imagined, the recognition of tumour cells amongst the various elements of a routine blood smear is very difficult though it has been done (Finkel and Tishkoff, 1960). The early investigators using only blood smears got low percentages of positives, and only in patients with advanced disease. Consequently, for a long time most studies of the blood in cancerous patients were directed towards the discovery of better means of identification of tumour cells. At first, changes in the blood of cancerous patients were studied. In 1903, Kurpjuveit thought that the presence of a severe anaemia with an increased amount of myelocytes in the blood of a patient was significant of a primary tumour with metastases to the spinal bone marrow. Likewise Ringold (1928) suggested that the finding in the blood of bizarre monocytes with irregularly shaped nuclei was an indication of cancer. And recently, hypercoagulability has been demonstrated to be a constant finding in neoplasia of the digestive tract and of the respiratory apparatus (Fumurola and Del Buono, 1958). Morphological variations in granulocytes (Broye, 1959) and eosinophilia (Isaacson and Rapoport, 1946; Murray, 1953; and Nagel, 1954) have also been proposed as being suggestive of cancer. The significance of haematol-ogical changes (shifting of leukocytes to the left, presence of myelocytes, normoblasts and thrombopenia) as an indication of extensive metastases in the bone marrow in patients suffering from malignant disorders has been

stressed by Den Ottolander, Pelt, Hellendoorn and Gerbrandy (1958). Gruner (1942) wrote an extensive monograph on the subject.

Nevertheless, investigators were still intrigued by the challenge of finding circulating tumour cells. The first problem was to find a suitable method for their detection. Clearly, the ideal technique should fulfil the following criteria: -

(1) As large a volume of blood as practicable should be used in order to increase the chance of finding tumour cells.

(2) The technique should not destroy the tumour cells or alter their appearance.

(3) All other cellular elements of the blood should be removed or destroyed, leaving the tumour cells intact.

(4) The tumour cells should be concentrated in a small area suitable for microscopic examination.

(5) The preparation should permit the calculation of the number of tumour cells per unit volume of blood.

These ideals proved hard to achieve. Many methods were tried, and it is perhaps fitting at this time to give an account of some of the more important of these.

One of the first techniques devised to haemolyze red blood cells so as to concentrate the white blood cells and the tumour cells was that of Loeper and Louste (1904). They collected 15 drops of blood from a finger into a centrifuge tube containing 15 ml. of 1% acetic acid. After haemolysis, they centrifuged for 10 minutes and then made a smear of the "culot de cellules". Fixation and staining were carried out in the usual way. In 1905, they modified their method substituting 19 ml. of 33% alcohol

for the 1% acetic acid. Quensel in 1921 used distilled water to cause haemolysis. Extracting blood from the right atrium at autopsy, he haemolyzed the erythrocytes with distilled water and examined the centrifugal sediment. Pool and Dunlop (1934) used a variation of a method described by Mandlebaum (1917), in which the cells remaining after haemolysis were embedded in paraffin. They haemolyzed 5 ml. of oxalated venous blood with 15% acetic acid, centrifuged the specimen at high speeds for one half-hour and then fixed the cells by over-laying them with 10% formalin in alcohol. After dehydration, the specimen was embedded in paraffin and sections were made for microscopic study. The same year a warning was issued by Shear and Fogg (1934) that tumour cells themselves may sustain morphological changes by the direct influence of various fluids. They found that in vitro, swelling of tumour cells occurred in isotonic as well as in hypertonic and hypotohic solutions. In fact, distilled water did damage the tumour cells, and acetic acid often was difficult in practice, as it coagulated the proteins of the red cells (Engell, 1955).

In 1955, Engell described a means of concentrating tumour cells by haemolyzing red blood cells with a weak solution of saponin. He removed the plasma from 2 to 5 ml. of heparinized blood by adding saline, centrifuging and pipetting off the supernatant fluid. After adding more saline, the red cells were haemolyzed by the addition of 1% saponin in the same volume as was the original blood sample. Then the products of haemolysis, any excess saponin and the heparin were removed by repeated saline washings and centrifugings. The resultant cone of cells was then detached from the bottom of the test-tube by the addition of citrated plasma and 10% calcium carbonate. The disc-shaped clot was fixed in formalin, embedded in paraffin

and sections were made for staining. This method was also used by Whang (1958.

With this method the quantity of blood used was small and not all the sediment was prepared for histological study. Engell himself recognized that 15% to 20% of the cancer cells were lost. The microscopic screening of the slides took a very long time, because of the presence of the leukocytes which are not affected by the saponin. In addition, the cancer cells were distorted so that their identification was rendered more difficult.

In 1957, Sandberg and Moore published a description of their "Fibrinogen" method. This was based on a finding by Buckley, Powell and Gibson (1957) that when fibrinogen was added to whole blood, rouleaux formation of the erythrocytes took place, and there followed rapid sedimentation, leaving the leukocytes and other nucleated elements in the supernatant plasma. Sandberg and Moore mixed 5 ml.of blood with 80 mgm. of bovine fibrinogen dissolved in 2 ml. of water containing 1 mgm. of heparin. Saline was added to reduce the concentration of the red blood cells and thus accelerate sedimentation. The supernatant was then removed. The nucleated cells left in the supernatant were concentrated by centrifugation and smears made.

While this method was simple to perform, inexpensive and produced well-stained slides in which the tumour cells were not distorted, it had serious disadvantages. A relatively small amount (5 ml.) of blood was used; only part of the final sediment was used to make the slides; and there were probably some tumour cells discarded with the remainder of the sediment.

Other substances have been used to obtain rapid sedimentation of the red blood cells. One of these is Dextran (Klein, Eridani, Djerassi and Resnick, 1958). Colombo, Rolfo and Maggi (1959) concentrated the tumour cells in the blood by a method derived from that described by Goudsmit and Van Loghem (1953) for the separation of leukocytes with a Dextran solution. To 9 ml. of blood (which was kept from coagulating by the addition of 1 ml. of Plexocrom A) they added 2 ml. of a 5% solution of Dextran of molecular weight of 179,000. The test-tube was inclined at 45° and incubated at 37°C for 30 minutes. The supernatant fluid was aspirated, and centrifuged at 1000 revolutions per minute for 30 minutes. Three smears were prepared from the sediment and stained by the May-Grunwald Giemsa method.

Reiss (1959) tried to haemolyze red blood cells by means of a combination of acetic acid and hydrochloric acid but obtained slides of poor quality. He was able to obtain only incomplete haemolysis with Engell's saponin method, and so he adopted the fibrinogen method of Sandberg and Moore.

But attempts at the isolation of tumour cells in the blood have not been directed only towards the haemolysis or sedimentation of red blood cells. In 1959, Seal proposed a very interesting method. It was based on the theory of flotation which maintains that, on centrifugation, cellular elements in a liquid medium will resolve themselves into different layers depending on their specific gravity. He advocated, therefore, using silicone, a fluid available commercially in carefully graded densities, to float off cancer cells. When blood cell samples are added to the adjusted silicone medium and centrifuged, the cancer cells float to the top where they can be collected, counted and studied under the microscope.

Seal explained the mechanism as follows: if one overlays silicone (specific gravity 1.075) with blood, upon centrifugation, the cancer cells (specific gravity 1.056), lymphocytes (specific gravity 1.065), and platelets (spec. gravity 1.032) being higher than the medium come to lie on the surface of the silicone. The red blood cells (specific gravity 1.092 to 1.097) and polymorphonuclear neutrophilic leukocytes (specific gravity above 1.075) being heavier than the medium collect under the silicone. The big disadvantage of this method is, as Seal admits, that one cannot assume that all cancer cells have a specific gravity of 1.056.

Another technique for the separation of cancer cells based on the theory of flotation was that described by Roberts, Watne, McGrath, McGrew and Cole in 1958. Using 10 ml. of heparinized blood, they sedimented the red cells with bovine fibrinogen as did Sandberg and Moore (1957). The supernatant fluid containing the leukocytes and tumour cells was then decanted and layered over 5 ml. of a 35% isomotic solution of bovine serum albumin (Ferrebee and Geiman, 1946) adjusted to a specific gravity of 1.065 according to the formula of Agranoff, Vallee and Waugh, 1954. Thus the plasma of specific gravity 1.055 to 1.060 (Mitchell, 1950) rested on the albumin solution of specific gravity 1.065 which is less dense than most of the formed elements of the plasma. On centrifugation, the tumour cells, being of specific gravity less than the 1.065 of the albumin collected on top of the albumin at the plasma-albumin intersurface (Fawcett, Vallee and Soule, 1950; McGrew, 1950). The majority of the white cells, however, were of specific gravity greater than the 1.065 of the albumin (Vallee, Hughes and Gibson, 1947; Fawcett and Vallee, 1952) and so passed through the albumin to lie at the bottom of the test-tube. The tumour cells thus isolated

over the albumin layer, were aspirated, washed in saline and stained. Roberts and his associates also made smears of the heavier layer below the albumin in case some tumour cells had been carried into it.

The advantage of this method is that the nuclei and cytoplasm of the tumour cells were well preserved. Its main disadvantage is that it is not quantitative since many tumour cells may be lost in the initial sedimentation of erythrocytes. Consequently, Roberts, Cole and their colleagues have changed their method, adopting the streptolysin method described by Malmgren, Pruitt, Del Vecchio and Potter in 1958. This is the method used in the present study and is considered in detail in the next chapter.

Some authors have preferred simpler or more complicated methods of isolating cancer cells. Morley (1959) and Coutts, Silva-Inzunza, Bulnes and Rosenberg (1959) simply centrifuged their 2 to 5 ml. heparinized blood samples, pipetted off the supernatant plasma and made smears of the thin, white filmy "buffy" layer covering the red blood cells. But, on the other hand, Rosengren (1959) has developed an integrating apparatus, based on the absorption of X-rays, which is capable of determining cell mass and so counting the large tumour cells.

CHAPTER VII

THE STREPTOLYSIN TECHNIQUE FOR THE ISOLATION OF TUMOUR CELLS FROM THE WHOLE BLOOD

A. Description.

The method used for the detection of tumour cells in the blood was essentially that described in 1958 by Malmgren, Pruitt, Del Vecchio and Potter of the National Cancer Institute of Bethesda, Maryland. Experience with the method suggested improvements which were gradually incorporated into the original method. Some of these modifications are our own and others were described in later publications by the Bethesda group. The method will now be described. Further details concerning each step will be given in Appendix A. There are seven main steps.

1. Collection of the Blood.

Seventeen to twenty ml. of whole blood are taken from an antecubital vein through a size 20 needle into a non-siliconized 20 ml. syringe and mixed with 3 ml. of Heparin sodium contained in the syringe. (1,000 U.S.P. units or 10 mg. per ml.). The blood is then placed in a stoppered test-tube. Ideally, it should be processed immediately although it may be kept under refrigeration for a few hours.

2. Removal of Plasma.

The naturally occurring anti-streptolysins in the plasma must be removed by centrifuging and washing.

(a) The blood is placed in a 40 ml. pyrex centrifuge tube and centrifuged at 300 x g. (1500 revolutions on a Model PR-1 Portable International Refrigerated centrifuge) for 25 minutes to separate the cells from the plasma.

(b) The plasma is removed by means of a Pasteur pipette attached to a suction apparatus. The plasma is then replaced with 30 ml. of normal saline. The cells in saline are stirred by means of a glass rod and the resultant mixture is centrifuged again at 300 x g. for 25 minutes.

(c) The supernatant fluid is removed as previously. The cells are resuspended in 30 ml. of normal saline, and again centrifuged at 300 g. for 25 minutes. The supernatant fluid is again removed and discarded.

3. Streptolysin Digestion

The erythrocytes and most of the polymorphonuclear leukocytes are lysed with Streptolysin-O, an enzyme of the streptococcus. 500 mg. of dried Streptolysin-O reagent are dissolved in 30 ml. of 0.85% saline (Potter and Malmgren, 1959 a), then added to the packed cells remaining after the last washing. The whole is then incubated in a water bath for 10 minutes at 39°C. with gentle shaking from time to time.

4. Fixation

The digested blood is centrifuged for 15 minutes at 300 x g., and the supernatant fluid is aspirated and discarded. This leaves a small cone of packed cells (lymphocytes, monocytes and tumour cells) at the bottom of the centrifuge tube. To this are added 10 ml. of normal saline and the mixture is agitated by aspirating it through a size 18 needle into a syringe and then expelling it until the cells are uniformly suspended. Then 10 ml. of 10% formalin are added (Seal, 1959) and the mixture left for 30 minutes for preliminary fixation. The specimen may, however, be left overnight at this stage (Seal, 1959).

5. Filtration

The entire preparation is then filtered through Millipore filters

(Seal, 1956; Solomon, Amelar, Hyman, Chaiban and Europa, 1958; Del Vecchio, De Witt, Borelli, Ward, Wood and Malmgren, 1959; Juniper and Chester, 1959). The filters are mounted on Millipore filter holders, connected to filter cups which in turn are fitted into 1000 ml. Buchner flasks. Four filtration units are connected in series to a mercury manometer and to a vacuum source (Seal, 1956). Filtration is carried out under 20 mm. Hg. of negative pressure. A greater pressure should not be used as the cells become distorted on the filter and air bubbles appear. The filtration is ideally done as soon as the 30 minute period of formalin fixation is completed.

Only very small quantities of the preparation should be placed on each filter at any one time and the rate of filtration should be watched. When the passage of the fluid through the filter slows down, this means that the filter has become almost impermeable and should be changed to provent clogging. It may take anywhere from two to eight filters (depending on the density of the preparation) to filter one sample of blood but, on the average, four are required.

During filtration, tumour cells present are "caught" on the surfaces of the Millipore filters because their usual diameter is greater than 10 μ (Moore, Sandberg and Watne, 1960).

6. Staining.

The cell containing filters are then placed in numbered metal filter holders which in turn are promptly immersed in modified Carnoy's fixative for 30 minutes (De Witt, Del Vecchio, Borelli and Hilberg, 1957). to remove the retained haemoglobin and make microscopic examination easier. The filters in their filter holders are then transferred to 70% alcohol and are stained by a modification of the Papanicolaou technique (Papanicolaou, 1954).

Each filter is then mounted in Permount on a 52 x 77 mm. slide using a 48 x 60 mm. coverglass. It is important to place Permount on both the slide and the coverslip to seal the filter completely. This avoids the formation of air bubbles between the slide and the coverglass and prevents dehydration of the smear.

7. Screening

Each slide is then examined meticulously and completely for the presence of cancer cells. This was done as follows: A trained cytology technician scanned a slide by starting at the top and by moving the mechanical stage of the microscope horizontally and vertically so swept back and forth across the whole of the filter area at a power of one hundred diameters (10 x lens and 10 x objective). Any cell which aroused suspicion was examined at a higher power and marked. The circling device for marking cells described by Del Vecchio, Siegler and Hilberg in 1956 was found unsatisfactory in our hands. These slides were then secondarily reviewed by the research fellow. Again, any suspicious cell was investigated at a higher power (720 x or under oil immersion). But the final decision as to malignancy was made by a cyto-pathologist and confirmed by a pathologist.

The screening procedure took twenty to thirty minutes per slide or an average of two hours per entire specimen.

B. Advantages and Disadvantages of This Technique

After reviewing all techniques described for demonstrating tumour cells in the blood (see Chapter VI), the Streptolysin-Filtration Method of Malmgren was chosen because it has the following advantages: -

(a) A relatively large volume of blood is used in order to increase the chance of finding tumour cells.

(b) The tumour cells are concentrated on the transparent Millipore filters with minimal cell loss and morphologic damage.

(c) All the erythrocytes, fibrin and most of the polymorphonuclear leukocytes are destroyed leaving the remainder of the cellular elements of the blood evenly distributed on microscopic slides for relatively easy screening.

(d) This technique has also proved satisfactory for processing other body fluids (such as pericardial, pleural and ascitic fluids) for cytologic examination (Seal, 1956; De Witt, Del Vecchio, Borelli and Hilberg, 1957; Solomon, Amelar, Hyman, Chaiban and Europa, 1958; Del Vecchio, De Witt, Borelli,Ward, Wood and Malmgren, 1959; Juniper and Chester, 1959).

(e) It permits, if desired, quantitating the number of tumour cells per unit volume of blood (Malmgren, Pruitt, Del Vecchio and Potter, 1958a; Pruitt, Hilberg and Kaiser, 1958; Potter and Malmgren, 1959a; Malmgren and Potter, 1959b).

(f) Malmgren, Pruitt, Del Vecchio and Potter in 1958 incubated filters containing LAC tumour cells obtained from a blood specimen processed by the Streptolysin-O technique. After 21 hours incubation at 37°C in Eagle's (1955) basal tissue-culture media, they observed many of the tumour cells in various stages of mitotic activity. They took this to signify that the technique had not altered the vitality nor the viability of tumour cells.

Disadvantages

(a) The preparation of the slides is time consuming. The procedure takes approximately two hours from the time the blood is drawn till the slides are ready for staining. The staining and mounting of the slides takes approximately 90 minutes. The procedure can only be interrupted after the formalin fixation stage before filtration and after fixation in modified Carnoy's fluid before final staining by the Papanicolaou method.

(b) It is expensive. The filtration apparatus is costly ($75.00 for each filter cup with holder and there are four of them). Each blood sample requires two 25 ml. size bottles of Streptolysin-O reagent ($5.00) and an average of four filters ($2.00). In addition, there is the cost of the staining and the salary of the technician.

(c) The screening is formidable. Because of the large volume of blood used, four large slides are prepared for each specimen and up to twelve slides may have to be screened for one patient at any one time (four slides each for pre-operative, per-operative and post-operative blood specimens). Since it takes approximately 30 minutes for microscopic study for each slide, patience is imperative!

(d) One of the reasons why the screening is so difficult is that while the erythrocytes and most of the polymorphonuclear leukocytes are lyzed by the streptolysin, the lymphocytes and monocytes are not.

(e) It is erratic. At times for reasons unknown in our experience a blood specimen was lost because of various vagaries of the method such as incomplete digestion, difficult filtration or faulty staining. In this manner, blood specimens from 10 patients were lost out of the 110 done or a total of 9%.

CHAPTER VIII

IDENTIFICATION OF MALIGNANT CELLS

A. DIFFERENTIATION FROM OTHER CELLS.

The problem that one faces in any attempt to demonstrate tumour cells on blood smears is the possibility of mistaking an "atypical" cell or cells normally present in the blood stream for a cancer cell. This was emphasized by Pool and Dunlop (1934) who found atypical cells in the peripheral blood of 17 of 40 patients having advanced cancer but were uncertain that these were tumour cells. Taylor and Vellios (1958) felt that definite proof was lacking that the immature-looking cells found in the peripheral blood really were malignant cells and so they attempted to establish their identify by injecting them into humans. Having failed to reproduce tumour growths, they suggested that attempts be made to grow tumour cells in tissue culture. This was done by Moore, Mount and Wendt (1959) but these authors found the task difficult and fraught with error. Grace and Kondo (1958) investigating host resistance in cancer patients carried out autografts of tumour to hosts with advanced malignant disease. They found that these tumour cell implantations resulted in a low incidence of "takes". Jonasson (1958), however, by injecting cancer cells isolated from the blood of animals with far advanced disease, into normal animals, was successful in showing that these were viable tumour cells and capable of reproducing secondary growths.

The positive identification of tumour cells in the blood stream remains a technically difficult problem. The slides contain relatively few tumour cells scattered among a large number of blood elements and their differentiation is not always easy. Sandberg, Moore and Schubarg (1959) pointed out that the following cells may confuse the observer: immature cells of haematopoietic origin, myeloblasts, large-sized promyelocytes, macrophages, megakaryocytes, plasma cells at various stages of differentiation, cells in mitosis, and the occasional osteoclast. They also mentioned among the wide variety of atypical cells to be found in the blood, epithelial, endothelial and fibroblastic cells but since these are not usually seen in the blood and since they are more easily identifiable, they are less bothersome. Severely ill patients are apt to have an increased number of immature bone marrow cells in the blood (Sandberg and Moore, 1957). Abnormally large or immature lymphocytes and monocytes may also be confused for tumour cells (Pruitt, Hilberg and Kaiser, 1959). Wandering histiocytes may also be occasionally seen (Pruitt, Hilberg and Kaiser, 1958). Sandberg, Moore, Crosswhite and Schubarg (1958) found it easier to identify tumour cells when they were in clumps. Particularly troublesome in blood smears according to Sandberg, Woernley and Crosswhite (1959) are plasmoblasts and immature lymphocytoid cells. They are difficult to distinguish because of their dark staining cytoplasm, dark nuclei and ability to divide while in the blood stream. Also, to make matters more difficult there are unusually large numbers of plasma cells in the blood stream of cancer patients. Engell (1955) found megakaryocytes, premyelocytes and polychromatophil erythroblasts easily distinguishable from tumour cells but Raker, Tafl and Edmonds (1960), using Malmgren's technique, thought the presence of

megakaryocytes confusing. In a series of blood samples from 144 patients with cancer, they found only two patients whose blood specimens contained definitely positive tumour cells. Megakaryocytes were found in the peripheral blood of 60 patients (42%) by these authors and they report having mistaken these for tumour cells at the beginning of their study.

As supportive evidence of the accuracy of the identification, some investigators compared the tumour cells isolated from the blood with a direct cellular imprint-smear of the original tumour (Fisher and Turnbull, 1955; Sandberg and Moore, 1957; Roberts, Watne, McGrew, McGrath, Nanos and Cole, 1958b).

The identification of tumour cells in the blood must be based mainly on the morphological features of the individual cells. The diagnostic criteria of malignancy that we have employed are an application of the usual features used to recognize and detect malignant cells in other types of cytology.

B. CRITERIA OF MALIGNANCY.

These were considered to be divisible into two degrees of importance:

1. Primary Importance.

The abnormal pattern of nuclear chromatin was considered of major significance (Papanicolaou, 1954). The irregular arrangement and increase in amount of nuclear chromatin were considered to be the most important signs.

2. Secondary Importance

(a) An increase in the nucleolo-nuclear ration was looked for. This was emphasized by Engell (1955) and MacCarty and Haumeder (1934). Zadek felt

that one could prove the diagnosis of a cancer cell by direct measurement of nucleolus/nucleus ratio. A disproportionately large nucleolus was considered to signify malignancy. He estimated the usual ratio to be from 0.22 to 0.50 for tumour cells.

(b) Several irregular nucleoli may be present. MacCarty and Haumeder (1934) studied 4000 cells to compare reparative regenerative and malignant regenerative cells and found that both possess one or more nucleoli but as a rule, those of the malignant cell are much larger in proportion to the nucleus than those of the reparative cell (fibroblasts, endotheliobLasts and other immature cells).

(c) Tumour cells are usually large menacing-looking cells. They are "visual strangers" among the normal haematopoietic elements (Sandberg, Moore, Crosswhite and Schubarg, 1958). Moore, Sandberg and Watne (1960a) studied the comparative size and structure of tumour cells in the blood and found their average diameter to be 19 micra. The minimal diameter found was 10 μ and the maximal 50 μ. Though the sizes of tumour cells varied according to the type of tumour and according to whether they were found in the peripheral blood, veins draining the tumour site, bone marrow or tumour imprints, these variations were found to be minimal and the average tumour cell measurements did not vary significantly regardless of their origin.

They reported the average diameter of circulating breast carcinoma cells to be 18.3 μ.

(d) Immature appearance of cells. Tumour cells may have a sharp nuclear border and a pale foamy indistinctive cytoplasm with little or no cell border.

(e) Increased nucleo-cytoplasmic ratio. This factor was emphasized by Engell (1955), Reiss (1959) and Moore, Sandberg and Watne (1960a). Sometimes the observer sees a large nucleus with only a fringe of cytoplasm around it. Moore, Sandberg and Watne (1960) partly explained this by suggesting that large cells are fragile and susceptible to damage to their cytoplasm during procedures.

(f) Though clumps of tumour cells are more easily identifiable in blood smears, we did not rely on this factor nor find any on our preparations.partly because clumps of tumour cells are rarely found in peripheral blood samples (Moore, Sandberg and Watne, 1960b) but also because our method for the isolation of tumour cells with its centrifugings and washings would reduce the likelihood of finding clumps.

(g) For the most part tumour cells usually found in blood smears are quite undifferentiated . Certain cells show vacuoles, as in those arising from an adenocarcinoma, but in most cases their origin is difficult to recognize. However, Moore, Sandberg and Schubarg (1957a) claim that with experience, it is sometimes possible to diagnose the type of tumour, e.g. lymphosarcoma.

C. COMMENTS ON THE PREPARATIONS USED IN THE PRESENT WORK.

The great majority of cells seen in our preparations were lymphocytes and monocytes. These were well preserved and well stained. There were also a few polymorphs to be seen on the slides. These were mostly neutrophils which presented with a greenish hue from the stain, but the odd eosinophil was also to be seen. The polymorphonuclear leukocytes which are of average diameter 12.5 μ (Moore, Sandberg and Watne, 1960a)

were easily distinguishable from the other cells by their morphological and staining characteristics.

Megakaryocytes were sometimes seen but their large size, multilobulated nuclei, granular chromatin, absence of nucleoli and "foamy" cytoplasm made them relatively easy to differentiate from tumour cells.

The stratification resulting from the centrifugation and filtration was at times a nuisance on the slides because of the constant necessity to adjust and readjust the focus.

Rarely clusters of red cells persisted and most slides contained some carbon debris which at times would resemble the dense chromatin of a tumour cell nucleus.

However, most of the difficulty encountered was in differentiating tumour cells from large lymphocytes and large monocytes. The average diameter of lymphocytes is 10 μ but larger forms are common (10 to 20 μ) (Wintrobe, 1951). Also, the average diameter of monocytes is 14 μ. (Maximow and Bloom, 1952). But it may be larger - up to 20 μ. (Wintrobe, 1951). Consequently, it was found very difficult to diagnose tumour cells of minimal diameter under low power during screening. Only very careful scrutiny and secondary revision of all slides might have minimized the "escape" of a tumour cell of the same size or smaller than lymphocytes or monocytes.

When, despite examination under oil immersion on a high power microscope by several independent observers, the positive identification of a tumour cell remained doubtful, this cell would be classified as "atypical" or "suspicious" but it would be considered negative and would not be entered in the category of tumour cells.

Only cells which fulfilled the criteria of malignancy were called t u m o u r c e l l s (see Reiss, 1959).

Like Engell (1955), we sometimes saw stratified squamous epithelial cells on our blood smears and these were considered to have been aspirated in the needle while piercing the skin on drawing the blood sample.

Finally, the identification of the various cellular elements of the myelocyte, lymphocyte and monocyte series and the differentiation of tumour cells from them remains a difficult problem, and it would be a very arduous task indeed to positively assert the presence of tumour cells in the blood of patients suffering from certain blood dyscrasias.

P A R T III

R E S U L T S

CHAPTER IX

R E S U L T S

As this work is a continuation of a project started at McGill University in 1958 and previously reported by Salgado, Hopkirk, Long, Ritchie, Ritchie and Webster in 1959 and Ritchie and Webster in 1960, it is perhaps fitting to summarize the complete findings to date. These will be found in Table I.

Table I

Tumour Cells Found in the Circulating Blood.
Summary of Findings to Date.

	Total (number of) cases.	Positive	Negative
Controls (patients without cancer)	50	0	50
Cancer patients	125	66	59

This table includes as controls, the 30 patients with some non-neoplastic disease reported by Salgado, Hopkirk, Long, Ritchie, Ritchie and Webster (1959) and 20 patients with benign breast disease studied by the present worker. Tumour cells were found in the blood of 66 of the 125 cancer patients studied or in 52.8%. Tumour cells were found in none of the 50 control cases without cancer.

In 10 of the 125 cases of cancer, the blood was drawn from the vein draining the tumour site, and, as is shown in Table II, tumour cells were found in the blood in 4, or 40%.

Table II

TUMOUR CELLS FOUND IN THE BLOOD DRAINING THE TUMOUR SITE

TYPE OF TUMOUR	CASES	POS.	NEG.
CARCINOMA OF THE COLON	4	3	1
CARCINOMA OF THE RECTUM	1	0	1
CARCINOMA OF THE STOMACH	1	0	1
GENERALIZED ABDOMINAL CARCINOMATOSIS	1	0	1
FIBROSARCOMA OF THE THIGH	1	1	0
MALIGNANT MELANOMA (FOOT)	1	0	1
CARCINOMA OF THE LUNG	1	0	1
TOTAL	10	4	6

In the other 115 cases of cancer, the blood was drawn from a peripheral vein, nearly always the antecubital vein. In these, tumour cells were found in the blood in 62, or 53.9%. As can be seen in Table III, tumour cells were found in the peripheral blood in many different kinds of malignancy.

Table III

Tumour Cells Found in the Peripheral Blood

Type of Tumour	Number of Cases	Positive	Negative
Carcinoma of the breast	80	43	37
Carcinoma of the large bowel	9	5	4
Carcinoma of the rectum	1	0	1
Carcinoma of the stomach	8	3	5
Carcinoma of the lung	6	2	4
Carcinoma of the kidney	2	2	0
Retroperitoneal fibrosarcoma	1	0	1
Carcinoma of the pancreas	1	1	0
Seminoma of the testicle	1	1	0
Carcinoma of the thyroid	1	0	1
Malignant melanoma	1	1	0
Alveolar rhabdomyosarcoma	1	1	0
Carcinoma of unknown origin	2	2	0
Carcinoma of the cervix	1	1	0
Total:	115	62	53

Table III differs from the similar table in the report of Salgado, Hopkirk, Long, Ritchie, Ritchie and Webster (1959) in that 56 cases of carcinoma of the breast and 7 new cases of malignancy other than carcinoma of the breast have been added. Since the present work

is a report on 80 cases of carcinoma of the breast, 56 of which were studied by the writer and 24 of which were studied by Salgado, Hopkirk, Long, Ritchie, Ritchie and Webster (1959), there would seem to be a discrepancy if one considers that, in their results, the above authors reported 32 cases of carcinoma of the breast. That is because, since the first published findings by the McGill Group, we have changed our method of compiling results. Whereas, at first, patients were counted positive as many times as their blood samples were found to be positive for tumour cells, presently we count a patient positive only once no matter how many times malignant cells are found in different blood samples.

After preliminary studies with various types of tumour, we decided to confine ourselves to cases of carcinoma of the breast. This was done because, although several centres of Cancer Research have reported the detection of malignant cells in the peripheral blood of cancerous patients, these experiments were not reported in such a way as to be easily applied to clinical medicine because a great variety of tumours were studied without adequate follow-up and without sufficient numbers of any one type of tumour. We therefore thought it was very desirable to confine our attention to one type of tumour and ensure that the patients were followed adequately and for a long time. Since our studies were begun, Engell (1959) has reported a series of carcinoma of the large intestine with good follow-up.

Carcinoma of the breast was chosen for study for the following reasons: -

(1) It is a fairly common carcinoma, 80 to 100 new cases being seen each year in the Royal Victoria Hospital.

(2) It is subject to many possible methods of treatment, i.e. surgery, radiotherapy, chemotherapy.

(3) It can be traced from its early beginning (breast biopsy) and may be followed-up for 5 years or more

(4) It is a surface tumour, the diagnosis of which can be made with relative ease.

(5) It seemed to yield a satisfactory percentage of blood samples positive for tumour cells (Salgado, Hopkirk, Long, Ritchie, Ritchie and Webster, 1959).

For several reasons, we decided to use, in this study, peripheral blood rather than blood from the vein draining the tumour site. Firstly, because, though our series was small, the incidence of tumour cells found in venous blood draining the tumour site (40%) did not seem to be significantly different from that found in the peripheral blood (57%) (Salgado, Hopkirk, Long, Ritchie, Ritchie and Webster, 1959). Secondly, because in carcinoma of the breast, it is not clear what is the vein draining the tumour site. Thirdly, because we desired to draw samples of blood from our patients repeatedly over a period of weeks or months, and this would rarely be possible if we used blood from a vein draining the tumour. And finally, because we hoped to devise a method which would be useful clinically, and in practice it would rarely be possible to draw blood from the vein draining the tumour site.

Frequently, in assembling this series of cases of carcinoma of the breast, blood samples were taken more than once from a patient. This was done for the purpose of following up the cases and determining whether there was any pattern to the presence or persistence of tumour

cells before, during and after the time of mastectomy. It follows therefore that a difference is obtained by the compilation of the results as the proportion of positive samples in the total number of blood samples rather than as the proportion of patients in which tumour cells were found in the blood.

Blood samples were taken from 80 patients with carcinoma of the breast. Twenty-four of these were studied by Salgado et al., and 56 by the present writer. Tumour cells were found in the peripheral blood of 43, or 53.7%. As mentioned previously, 20 patients with some benign disease of the breast were studied as controls and in not one case were tumour cells found. Table IV summarizes the findings.

Table IV

Tumour Cells Found in the Peripheral Blood of Patients with Carcinoma of the Breast: Summary of Findings.

	Total (number of) Cases	Positive	Negative	% positive
Controls (patients without cancer)	20	0	20	0%
Patients with carcinoma of the breast	80	43	37	53.7%

In these 80 cases of carcinoma of the breast, 214 blood samples were taken from the peripheral blood, and tumour cells were found in 64, or 29.9%. These results are shown in Table V.

Table V

Difference in Incidence of Tumour Cells According to whether the Number of Patients or the Number of Blood Samples is Considered.

	Total number	Positive	Negative	% positive
Patients with carcinoma of the breast	80	43	37	53.7%
Blood samples	214	64	150	29.9%

In 5 blood samples, cells suspicious of malignancy were observed, but as it could not be positively affirmed that they were cance cells, they were counted as negative.

Tables VI, VII, VII A, and VIII are presented to give the reader a detailed list of the 80 patients with carcinoma of the breast studied. It will be noted that both the stage of advancement of the disease and the histological gradation of the tumour are given. The classification used for the degree of clinical progression of the carcinoma of the breast is that of Portmann (1950). It was preferred because it is based on both clinical criteria and the histo-pathological evaluation of the metastatic involvement of axillary lymph nodes. In it the stages are defined as follows: -

Stage I - The tumour is limited to the mammary gland. It is movable, the skin is not involved and there are no metastases.

Stage II - Same as Stage I, but the axillary lymph nodes show secondary metastases as established by examination of the surgical specimen.

Stage III - There may be a diffuse infiltration or oedema of the breast, fixation of the chest wall to the tumour, extensive ulceration, inflammation, secondary nodules in the skin, or voluminous, numerous or fixed axillary lymph node metastases.

Stage IV - Regardless of local extent, there are supraclavicular or distant metastases.

The histological gradation of the surgical specimens used was according to the degree of anaplasia (Broders, 1926). For purposes of consistency all the slides were reviewed by the same person, Doctor A. C. Ritchie.

A histological diagnosis of the type of tumour and a column for "follow-up" have also been included in these tables. When only the word "carcinoma" appears, this should be taken to mean a polymorphous tumour but of predominantly scirrhus nature.

Each patient was given a number (A-5; B-17; etc.) which appears on the patient's record, slides, etc. The letter "A" was given to patients studied during the first year of this project and "B" to patients studied during the second year. When a patient has two denominating letters, it is because blood samples were drawn twice with long intervals in between.

Perhaps some of the abbreviations used in the tables should be clarified: -

pos. = positive = tumour cells found in peripheral blood

neg. = negative = tumour cells <u>not</u> found in peripheral blood

susp. = suspicious cells found in peripheral blood.

Blood samples were drawn from 57 patients with carcinoma of the breast at time of mastectomy and a record of this is listed in Table VI.

Table VI

Record of Patients with Carcinoma of the Breast from Whom Blood was drawn at Time of Mastectomy

No.	Case No.	Extent of tumour (Portmann classification)	Histological diagnosis	No. of times blood was drawn	Occurrence of tumour cells before (b), during (d), & after (a) operation			Follow-up
					b	d	a	
1	A-4	I	Adenocarcinoma Grade III	2			(+)	neg. 17 days later
2	A-5	I	Scirrhous carcinoma Grade II (intraductal with invasion)	3			(+)	pos. 5 days post-op. neg. 10 days post-op.
3	A-6	I	Scirrhous carcinoma Grade II	1			(+)	
4	A-8	III	Scirrhous carcinoma Grade II	2	(-)		(+)	
5	A-9	II	Carcinoma Grade III	1			(+)	
6	A-10	I	Carcinoma Grade III	1			(+)	
7	A-11 B-50	III	Adenocarcinoma Grade II	2			(+)	pos. 9 months later, 30 mins. before patient died of carcinomatosis

Table VI - Cont'd

8	A-12 B-87	IV	~~C~~arcinoma Grade II	2			(+)	neg. 1 year later
9	A-13 B-86	I	Carcinoma Grade II	3	(-)		(-)	neg. 1 year later
10	A-14	I	Bilateral simultaneous breast intraductal carcinoma Grade III	2	(-)		(-)	
11	A-15 B-88	II	Scirrhous carcinoma Grade II	3	(-)		(+)	pos. 1 year later
12	A-16	I	Intraductal scirrhous carcinoma Grade II	2	(-)		(+)	
13	A-18	I	Scirrhous and intraductal carcinoma Grade III	1		(-)		
14	A-19	III	Carcinoma Grade II	1			(-)	
15	A-20 B-93	I	Carcinoma Grade III	2			(-)	neg. 13 months later
16	A-21	II	Scirrhous carcinoma Grade III	1			(-)	Died 8 months later
17	B-14	II	Carcinoma Grade IV	1			(+)	

Table VI - Cont'd

18	B-21	I	Carcinoma Grade III	2		(-)	(-)	
19	B-22	I	Carcinoma, left breast Grade III	1	(-)			Patient also had had right radical mastectomy for carcinoma solidum 8 years previously
20	B-25	I	Scirrhous carcinoma Grade II	2		(-)	(-)	
21	B-26	I	Carcinoma Grade II	2	(-)		(-)	
22	B-27	II	Carcinoma Grade II	2	(+)		(+)	
23	B-28	I	Intraductal carcinoma with invasion Grade II	1			(-)	
24	B-34	I	Scirrhous carcinoma Grade II	1			Susp.	
25	B-38 B-54	II	Carcinoma Grade III	3	Susp.		(+)	Susp. 5 weeks later
26	B-39	II	Carcinoma Grade III	1			(+)	
27	B-40	I	Intraductal carcinoma with invasion Grade III	1			(+)	

Table VI - Cont'd

28	B-41	I	Carcinoma, right breast, Grade III	1			(+)	Patient had had left radical mastectomy for carcinoma 16 years previously
29	B-42	II	Scirrhous and intraductal carcinoma Grade II	2		(+)	(-)	
30	B-44	II	Adenocarcinoma Grade II	2	(-)		(-)	
31	B-45	II	Adenocarcinoma, left breast Grade II	2	(-)	(-)		Patient had had left radical mastectomy 2 years earlier for carcinoma
32	B-46	I	Scirrhous carcinoma Grade III	2	(-)		(-)	
33	B-49 B-89	II	Scirrhous carcinoma Grade II	3		(-)	(+)	Neg. 3 months later
34	B-52	I	Partly scirrhous, partly medullary carcinoma Grade II	14	(+)	(-)	(+)	See Case No. 1, Table XII. Pos. on 16th day post-operatively
35	B-53	II	Carcinoma Grade II	3	(-)	(-)	(-)	
36	B-58	I	Carcinoma Grade III	12		(-)	(-)	Pos. on 4th and 10th days post-operatively See Case No. 2, Table XII

Table VI - Cont'd

37	B-61	I	Carcinoma Grade II	7				Neg. from 4th to 10th days post-operatively See Case No. 3, Table XII
38	B-62	I	Intraductal carcinoma with no invasion Grade IV	2	(-)		(+)	Patient had been receiving hormones for 10 years
39	B-64	III	Carcinoma Grade II	9	(+)	(+)	(-)	Pos.on 2nd, 5th, 9th & 12th days post-operatively See Case No. 4, Table XII
40	B-67	I	Carcinoma Grade II	9	(-)	(-)	(-)	Pos. on 10th day post-operatively See Case No. 5, Table XII
41	B-68	II	Carcinoma Grade II	6		(-)	(-)	Pos. on 2nd day post-operatively See Case No. 6, Table XII
42	B-69	I	Carcinoma Grade III	9	(-)	(-)	(+)	Pos.on 9th day post-operatively See Case No. 7, Table XII
43	B-70	I	Scirrhous and intraductal carcinoma Grade II	4	(-)	(-)	(-)	Neg. 3 days later Had Had 4 breast biopsies previously for bilateral mammary fibro-cystic dysplasia

Table VI - Cont'd

44	B-71 B-77	IV	Scirrhous carcinoma with invasion. Changes of pregnancy Grade II	4	(+)		(-)	Neg. 3 days later Pos. 20 days later
45	B-72	III	Adenocarcinoma Grade III	8	(-)	(+)	(+)	Pos. on 5th & 10th days post-operatively See Case No. 9, Table XII
46	B-73	I	Adenocarcinoma Grade III	5	(-)	(-)	(-)	Pos. on 2nd day post-operatively Susp. on 4th day post-operatively
47	B-74	II	Carcinoma Grade III	7	(-)	(-)	(-)	Pos. on 2nd,4th and 7th days post-operatively See Case No. 8, Table XII
48	B-75	II	Large scirrhous carcinoma Grade III	2	(-)		(-)	
49	B-78	II	Scirrhous carcinoma Grade II	2	(+)		(+)	
50	B-79	I	Markedly atypical intraductal carcinoma with invasion Grade II	2			(+)	Neg. on 9th day post-operatively

Table VI - Cont'd

51	B-81	I	Scirrhous and intraductal carcinoma Grade II	3	(-)	(-)	(-)	
52	B-82	II	Encephaloid carcinoma Grade III	2		(-)	(-)	
53	B-83	II	Carcinoma Grade III	3	(-)	(-)	(-)	
54	B-90	III	Carcinoma Grade III	10	(-)	(-)	(-)	Neg. from 1st till 8th days post-operatively See Case No. 10, Table XII
55	B-91	I	Scirrhous Carcinoma Grade III	1			(-)	
56	B-92	II	Scirrhous carcinoma Grade II	2	(+)	(-)		
57	B-95	I	Intraductal and infiltrating carcinoma Grade II	2	(-)		(-)	

Blood samples were drawn from 23 patients with carcinoma of the breast from one month to several years after mastectomy. A list of 16 of these patients is given in Table VII.

Table VII

Record of Patients with Carcinoma of the Breast from whom Blood was Drawn from One Month to Several Years after Mastectomy

No.	Case No	Extent of Tumour (Portmann classification)	Histological diagnosis	Interval between mastectomy and drawing of blood sample	Occurrence of Tumour Cells	Comments
1	A-1	IV	Scirrhous carcinoma of both breasts Grade III	(1) 4 yrs.& 2 mos. (2) 4 yrs.& 4 mos.	Susp. (-)	Died 9 months later of carcinomatosis. Also treated by radiotherapy and androgens
2	A-2 B-94	IV	Carcinoma Grade II	(1) 7 yrs.& 3 mos. (2) 8 yrs.& 9 mos.	(+) (+)	Treated by radiotherapy, thyroid, corticosteroid and oestrogenic hormones
3	A-3	IV	Carcinoma solidum simplex Grade III	5 yrs.& 4 mos	(+)	Treated by radiotherapy. Died 6 months later of carcinomatosis
4	A-7 B-85	III	Invasive carcinoma Grade II	(1) 5 months (2) 1 yr. & 8 mos.	(+) (-)	Treated by radiotherapy
5	A-22	IV	Scirrhous carcinoma Grade III	(1) 2 yrs. & 2 mos. (2) 2 yrs. & 3 mos.	(-) (-)	Had bilateral oophorectomy, hypophysectomy, radiation and hormonotherapy. Died 3 weeks later of carcinomatosis

Table VII - Cont'd

6	B-2	IV	Carcinoma Grade II	7 months	(-)	Treated by radiotherapy
7	B-3	IV	Carcinoma Grade II	14 years	Susp.	Had a right radical mastectomy for carcinoma in 1945. Had carcinoma of left breast since 1956. Treated by radiotherapy, cobalt therapy and chemotherapy. Died Nov.1, 1959
8	B-4	I	Medullary carcinoma Grade II	6 weeks	(+)	Also treated by radiotherapy
9	B-6	IV	Carcinoma Grade III	6 months	(-)	Had also had bilateral oophorectomy and adrenalectomy and had been treated by radiotherapy
10	B-7	I	Infiltrating duct carcinoma Grade II	3 yrs. & 4 mos.	(+)	Also treated by radiotherapy
11	B-13	II	Infiltrating duct carcinoma exhibiting invasion of vascular channels Grade II	2 months	(+)	
12	B-16	I	Sclerosing & medullary carcinoma simplex Grade III	4 yrs. & 6 mos.	(+)	No clinically evident recurrences at time blood was drawn

Table VII - Cont'd

13	B-17	I	Infiltrating duct carcinoma Grade IV	1 year & 10 mos	(-)	No clinically evident recurrences at time blood was drawn. Patient had had bilateral mastectomies
14	B-29	III	Scirrhous carcinoma Grade II Lobular carcinoma	8 years	b=(-) a=(+)	These blood samples were drawn before (b) and after (a) the patient sustained a bilateral oophorectomy
15	B-33	IV	Carcinoma Grade III	4 years	(-)	
16	B-84	IV	Adenocarcinoma	5 months	(-)	Died 2 weeks later

As there were 7 patients who had blood samples drawn at both time of mastectomy and from one month to several years after mastectomy and as these patients had been previously entered in Table VI, to avoid confusion, they have been entered separately in Table VII-A.

Table VII-A

Record of Patients with Carcinoma of the Breast from Whom Blood was Drawn from One Month to Several Years After Mastectomy.

(N,B. Since these patients had previously had blood drawn at time of Mastectomy, they are also included in Table VI).

No.	Case No	Extent of Tumour (Portmann classification)	Histological diagnosis	Interval between mastectomy and drawing of blood sample	Occurrence of Tumour Cells	Comments
1	A-11 B-50	IV	Adenocarcinoma Grade II	9 months	(+)	See Table VI, No. 7.
2	A-12 B-87	IV	Carcinoma Grade II	1 year	(-)	See Table VI, No. 8
3	A-13 B-86	I	Carcinoma Grade II	1 year	(-)	See Table VI, No. 9
4	A-15 B-88	II	Scirrhous carcinoma Grade II	1 year	(+)	See Table VI, No. 11
5	A-20 B-93	I	Carcinoma Grade III	13 months	(-)	See Table VI, No. 15
6	B-38 B-54	II	Carcinoma Grade II	5 weeks	Susp.	See Table VI, No. 25
7	B-49 B-84	II	Scirrhous carcinoma Grade II	3 months	(-)	See Table VI, No. 33

Table VIII gives a record of 7 patients with advanced carcinoma of the breast who have never submitted to mastectomy.

Table VIII

Record of Patients with Advanced Carcinoma of the Breast Who Have Never Submitted to Mastectomy

No.	Case No.	Extent of Tumour (Portmann classi-fication)	No. of times blood was drawn	Tumour Cells	Follow-up	Comments
1	A-17 B-35	Stage IV	2	(+)	Pos. 7 months later	Treated by androgenic and oestrogenic hor-mones and radio-therapy Biopsy showed Grade III carcinoma
2	A-23	Stage IV	1	(-)	Patient died 5 weeks later of carcinomatosis Autopsy reveal-ed Grade II carcinoma of breast with histological venous in-vasion	Hypophysectomy was performed 6 months before blood sample was drawn
3	A-24	Stage IV	1	(-)	Died 11 months later No autopsy	Biopsy showed Grade IV carcinoma. Treated by radiotherapy
4	B-8	Stage IV	1	(+)		Biopsy showed infil-trating fibrosing carcinoma. Grade III Treated by radio-therapy
5	B-12	Stage IV	1	(-)		Biopsy showed infil-trating duct carcino-ma. Grade II. Treated by radiotherapy

Table VIII - Cont'd

6	B-15	Stage IV	1	(+)		Biopsy showed carcinoma Grade III. Treated by radiotherapy
7	B-51	Stage IV	1	Susp.		Treated by radiotherapy. Biopsy showed carcinoma Grade II

As mentioned in Chapter III, several writers have reported that operation, or even manipulation of a tumour, increases the number of tumour cells in the blood. We therefore devided to verify this in our cases of carcinoma of the breast by drawing blood samples pre-operatively, per-operatively and post-operatively at time of mastectomy. The pre-operative blood sample was usually drawn after the patient was anaesthetized, the per-operative sample was usually drawn during manipulation of the tumour and the post-operative sample in the recovery room an hour or so after the end of the operation. Table IX shows our findings.

Table IX

Tumour Cells Found in the Peripheral Blood of Patients with Carcinoma of the Breast When Blood was Drawn at the Time of Mastectomy

	Positive	Negative	% Positive
Blood drawn pre-operatively	6	24	20.0%
Blood drawn per-operatively	3	19	13.6%
Blood drawn post-operatively	29	24	54.7%

Tumour cells were found in 20.0% of the 30 samples drawn pre-operatively, 13.6% of the 22 samples drawn per-operatively and 54.7% of the 53 post-mastectomy samples. Unfortunately, there is a certain lack of consistency in this table because pre-operative, per-operative and post-operative samples were not all drawn in every case. Therefore a further table (Table X) was drawn up showing the results in the 27 patients in whom both pre-operative and post-operative samples were drawn.

Table X

Tumour Cells Found in the Peripheral Blood of Patients with Carcinoma of the Breast at the Time of Mastectomy When Blood When Blood was Drawn b o t h Pre-operatively and Post-operatively

	Number of Cases	Positive	Negative	% Positive
Pre-operatively	27	5	22	18.5%
Post-operatively	27	14	13	51.8%

The results shown in Table X are similar to those shown in Table IX. Tumour cells were identified in 18.5% of the 27 blood samples drawn pre-operatively and in 51.8% of the 27 blood samples drawn post-operatively.

We became interested in the hazards of incisional biopsy versus excisional biopsy. Though defended by Haagensen (1954), incisional biopsy has been attacked by some authors, notably Ryall (1908), Saphir (1936), and Ackerman and Wheat (1955) because it is thought more apt to cause dissemination of tumour cells.

Table XI

Incidence of Tumour Cells Found in the Peripheral Blood of Patients with Carcinoma of the Breast at time of Mastectomy After Incisional and Excisional Biopsy

	Positive	Negative	% Positive
Incisional biopsy	3	3	50.0%
Excisional biopsy	5	10	33.3%

The operative reports were reviewed and when it was noted whether a patient had had an excisional or incisional biopsy, this patient's record was studied to see if tumour cells had been found in a sample drawn after the biopsy but before the major operation. In only 21 cases were blood samples drawn immediately after the breast biopsy. The per-operative blood samples have not been counted because a positive result could be interpreted as being due to manipulation of the tumour during the main operation. Three of 6 blood samples taken immediately after an incisional biopsy showed tumour cells in the peripheral blood and 5 of 15 blood specimens taken immediately after an excisional biopsy were positive for tumour cells. The numbers are, of course, too small to allow any conclusion.

We have also attempted to determine how long after mastectomy tumour cells remain detectable in the blood. We took 10 cases of carcinoma of the breast and tried to get from each a sample of blood daily for 10 days post-operatively. Table XII shows the findings. The gaps are due to difficulties in obtaining samples.

Table XII

TUMOUR CELLS FOUND IN THE PERIPHERAL BLOOD OF PATIENTS WITH CARCINOMA OF THE BREAST WHEN SAMPLES WERE DRAWN PREOPERATIVELY, PEROPERATIVELY, AND REPEATEDLY FOR THE FIRST 10 DAYS OR MORE POST-OPERATIVELY

Case No.	Preoperatively	Peroperatively	Day Postoperatively																			
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	+	-	+	-	-	-	-	-	-	-	-	-			-				+				
2		-	-	-	-	-	+	-	-	-	-	-	+										
3	o	o	o	o	o	o	-	-	-	-	-	-	-										
4	+	+	-	-	+			+		-		+			+								
5	-	-	-	-		-		-		-	-		+										
6		-	-		+		-		-			-											
7	-	-	+	-	-		-	-	-			+											
8	-	-	-		+		+			+		-											
9	-	+	+	-		-		+		-			+										
10	-	-	-	-	-		-	-	-	-	-												

As can be seen some specimens were found to be positive for tumour cells on the 9th, 10th, 12th and even the 16th day after operation. Of the 91 blood samples shown in Table XII, 22 were positive for tumour cells or 24.1%. But 8 of the 10 (80%) patients studied showed tumour cells in their circulating blood at one time or other. Of the 75 blood samples (shown in this table) drawn post-operatively, tumour cells were found in 18 or 24.0%. Again in the 16 blood samples drawn pre- and per-operatively, tumour cells were found in 4 or 25.0%. In the 7 pre-operative blood samples, 2 were found to contain tumour cells or 28.5%. Finally in the 9 per-operative blood

samples, 2 were found to contain tumour cells or 22.2%. The constancy of the percentage of positive blood samples is striking especially when one considers that, as shown in Table V, tumour cells were found in 29.9% of the over-all total of 214 blood samples.

This provoked us to collect the 103 blood samples which were drawn before and after mastectomy in the 27 patients mentioned in Table X, and the results were compiled for comparison with those given in that table.

Table XIII gives the incidence of tumour cells found in all the blood samples taken pre-operatively and post-operatively in the 27 patients mentioned in Table X. Whereas Table X gave the number of patients with carcinoma of the breast who were found to be positive for tumour cells, Table XIII gives the number of blood samples found to be positive for tumour cells.

Table XIII

Incidence of Tumour Cells Found in the Peripheral Blood of 27 Patients with Carcinoma of the Breast When Blood was Drawn both Before Mastectomy and One or More Times Until One Month After Mastectomy. The Number of "Positive Blood Samples" is Considered and not the Number of "Positive Patients".

	Number of Patients	Number of Blood Samples	Positive	Negative	%Positive
Blood samples taken before mastectomy	27	27	5	22	18.5%
Blood samples taken after mastectomy	27	76	24	52	31.5%

Of the 27 blood samples drawn before mastectomy, 18.5% were positive whereas tumour cells were found in 31.5% of the 76 specimens drawn after mastectomy. It should be noted that this percentage, 31.5%, is similar to that (29.9%) mentioned in Table V when all 214 blood samples were being considered.

We thought also that we might gain some indication of the prognostic significance of tumour cells in the blood by comparing the frequency with which we found them in a group of cases of carcinoma with relatively poor prognosis with their frequency in a group of cases of carcinoma with a relatively good prognosis. As is well known, the prognosis is worse in cases of carcinoma of the breast in which there is axillary involvement at the time of operation.than in cases in which the axilla is not involved. Therefore, if the presence of tumour cells in a sample of blood were to be of value in prognosis, tumour cells should be found more frequently in cases of carcinoma of the breast with axillary metastases than in those without. First of all, this possibility was studied in patients at time of mastectomy. Table XIV is a composite table showing the incidence of tumour cells found in the peripheral blood of patients with carcinoma of the breast <u>with and without metastases</u> when blood was drawn before, during and after mastectomy.

Table XIV

Tumour Cells Found in the Peripheral Blood of Patients with Carcinoma of the Breast With and Without Metastases When Blood was Drawn at the Time of Mastectomy

	Number of Cases	Blood drawn pre-operatively			Blood drawn per-operatively			Blood drawn post-operatively		
		Pos.	Neg.	% Pos.	Pos.	Neg.	% Pos.	Pos.	Neg.	% Pos.
Without axillary metastases	30	1	13	7.1%	0	10	0.0%	14	14	50.0%
With axillary metastases	27	5	11	31.2%	3	9	25.0%	15	10	60.0%
Total:	57	6	24	20.0%	3	19	13.6%	29	24	54.7%

In all three circumstances were tumour cells found more frequently in patients with axillary metastases than in those without. The differences are, however, probably too small to be significant.

In patients from whom blood was drawn from one month to several years after mastectomy, tumour cells were found in 50.0% of the 10 patients without evident metastases whereas they were found in only 38.4% of the 13 patients with widespread metastases. This finding is shown in Table XV.

Table XV

Tumour Cells Found in the Peripheral Blood of Patients with Carcinoma of the Breast With and Without Metastases When Blood was Drawn from One Month to Several Years after Mastectomy

	No. of Cases	Positive	Negative	% Positive
Without evident metastases	10	5	5	50.0%
With widespread metastases	13	5	8	38.4%

Seven patients with advanced carcinoma of the breast who had never submitted to mastectomy were also studied. If the presence of tumour cells in the blood were to be of prognostic importance, we would expect a very high percentage of positive blood samples in these advanced cases. The results will be found in Table XVI.

Table XVI

Tumour Cells Found in the Peripheral Blood of Patients with Advanced Carcinoma of the Breast Who Have Never Submitted to Mastectomy

Number of Cases	Positive	Negative	% Positive
7	3	4	42.8%

Despite the fact that all 7 of these patients had distant metastases (see Table VIII), tumour cells were found in only three giving a 42.8 percentage.

As we were interested in establishing whether the frequency of the finding of tumour cells was directly proportional to the degree of

advancement of the disease, we collected all of our 80 cases of carcinoma of the breast and divided them into 4 stages according to Portmann's (1950) clinical classification. The occurrence of tumour cells in patients of each stage was then compared. The results are given in Table XVII.

Table XVII

Tumour Cells Found in the Peripheral Blood of Patients with Carcinoma of the Breast According to the Clinical Advancement of the Disease Using Portmann's Classification

Degree of Advancement	No. of Cases	Positive	Negative	% Positive
Stage I	34	17	17	50.0%
Stage II	20	13	7	65.0%
Stage III	9	7	2	77.7%
Stage IV	17	6	11	35.2%
Total:	80	43	37	53.7%

Tumour cells were found in the blood samples of 50.0% of the 34 cases of Stage I, 65.0% of the 20 cases of Stage II, 77.7% of the 9 cases of Stage III and 35.2% of the 17 cases of Stage IV. Once again, the differences are probably not significant.

Though the degree of progression of a carcinoma of the breast does not necessarily follow the duration of the disease, we thought it interesting to relate the incidence of tumour cells found in the peripheral blood to the length of time the patients presented clinical symptoms. Though these results are fraught with error because of the uncertainty in many patients concerning the time of onset of their

illness and due to the different interpretation of symptoms by different patients, Table XVIII was drawn up.

Table XVIII

Tumour Cells Found in the Peripheral Blood of Patients with Carcinoma of the Breast According to the Duration of Clinical Symptoms (presence of lump in breast, etc.)

Interval between onset of Symptoms and withdrawal of the Blood Sample	Number of Cases	Positive	Negative	% Positive
Less than 1 month	24	13	11	54.1%
One month to three months	12	8	4	66.6%
Three to 6 months	6	4	2	66.6%
Six months to 1 year	4	2	2	50.0%
More than 1 year	10	6	4	60.0%

Twenty-four patients were not included in this table either because their history was one of long duration with intervening mastectomy or radiotherapy or because they had previously had bilateral mammary cystic dysplasia and the onset of their malignancy was unsure.

As can be seen, tumour cells were found in 54.1% of patients with symptoms of less than 1 month's duration; and in 66.6% of patients presenting symptoms from one month to 6 months' duration. Fifty per cent of the patients whose symptoms ranged from 6 months to 1 year presented tumour cells in the blood and of those with complaints of more than 1 year, 60.0% were positive for tumour cells. Again, there is little difference between these figures.

We also attempted to confirm or disprove the contention of Brown and Warren (1938) that venous invasion and consequent haematogenous spread of tumour cells depend greatly on the grade of differentiation of the tumour. Consequently, the slides of 79 of our breast carcinoma cases were reviewed, graded histologically according to Broders' (1926) classification and the relationship between the incidence of tumour cell findings and the degree of differentiation of the tumour was studied. The results are shown in Table XIX.

Table XIX

Tumour Cells Found in the Peripheral Blood of Patients with Carcinoma of the Breast According to the Broders' Histological Gradation of Tumours

	No. of Cases	Positive	Negative	% Positive
Grade I	0	0	0	0
Grade II	44	25	19	56.8%
Grade III	31	16	15	51.6%
Grade IV	4	2	2	50.0%
Total:	79	43	36	

The tumours were predominantly of grades II or III differentiation there being no grade I tumour and only 4 grade IV tumours. Tumour cells were found in 56.8% of patients with grade II tumours and in 51.6% of patients with grade III tumours. It was not possible to

obtain the slides of one patient (Case No. B-84), hence the total 79 instead of 80.

Finally, like Potter, Longenbaugh, Chu, Dillon, Romsdahl and Malmgren (1960), and Sandberg and Moore (1957), it was desired to establish the relationship of tumour type and the incidence of cancer cells in the blood. It was thought possible that the more biologically active the tumour, the larger would be the percentage of positive blood specimens. Consequently, the occurrence of tumour cells was related to the various types of carcinoma of the breast. The usual general classification was used. There were 63 cases of carcinoma solidum simplex, 7 cases of adenocarcinoma and 10 cases of papillary duct carcinoma. Of the 10 cases of intraductal carcinoma, 8 were, as expected of the infiltrating and invasive type buttwo were markedly atypical. Case No. A-14 had had bilateral breast biopsies and each had revealed intraductal carcinoma. The patient subsequently submitted to bilateral radical mastectomies. Case No. B-62, on histological section, had shown intraductal carcinoma with no invasion. (Tumour cells were not found in Case No. A-14 but they were found post-operatively in Case No. B-62).

Table XX shows the relationship between the occurrence of tumour cells in the blood and the type of tumour.

Table XX

Occurrence of Tumour Cells According to the Various Types of Carcinoma of the Breast

Type of Carcinoma	No. of Cases	Positive	Negative	% Positive
Carcinoma simplex: (1) Scirrhous	60	32	28	53.3%
(2) Medullary or Encephaloid	3	2	1	66.6%
Adenocarcinoma	7	4	3	57.1%
Papillary carcinoma of duct	10	5	5	50.0%
Total:	80	43	37	53.7%

Summary of Results

(1) Tumour cells were found in the blood of 66 of the 125 cancer patients studied or in 52.8%. Malignant cells were found in none of the 50 control patients without cancer.

(2) In 10 of the 125 cancer patients, the venous blood draining the tumour site was studied for tumour cells and these were found in 4 cases.

(3) In the other 115 cases, the peripheral blood was studied for tumour cells and these were found in 53.9% of the patients. Many different kinds of malignancy were studied but attention was focussed on 80 patients with carcinoma of the breast.

(4) Tumour cells were identified in the peripheral blood of 53.7% of the 80 patients with carcinoma of the breast. Of 214 blood samples drawn

at various times from these 80 patients, 64 or 29.9% were found to be positive for cancer cells.

(5) Though our series is small, the incidence of tumour cells found in post-mastectomy blood samples seemed to be higher than in those drawn before operation.

(6) In 10 cases, blood samples were drawn pre-operatively, per-operatively and repeatedly for the first 10 days or more post-operatively and this experiment revealed the presence of tumour cells at one time or other in 8 of the 10 patients.

(7) In attempting to establish the prognostic significance of the occurrence of cancer cells in the blood stream, blood samples were taken from patients with carcinoma of the breast at various stages of their disease at time of operation, from one month to several years after mastectomy and even when the cancer was widespread. The differences found are probably not significant.

(8) The incidence of tumour cells according to histological gradation and type of tumour was also studied and again, though our figures are small, very little difference was observed.

P A R T IV

DISCUSSION, CONCLUSIONS AND SUMMARY

CHAPTER X

D I S C U S S I O N

A. Carcinoma vs. Sarcoma

The first finding which is striking on consideration of the results given in Chapter IX is the frequent isolation of carcinoma cells from the blood stream. It has long been held that the distant spread of a sarcoma takes place by the blood vessels and its spread by the lymph stream is reputedly uncommon, occurring only in 5 to 10% of the cases. In the case of a carcinoma, however, most pathologists teach that though spread by the blood stream is common, lymph node involvement is generally the rule, the tumour spreading primarily by the lymph spaces and lymphatic vessels. Though it is admitted that the finding of carcinoma cells in the blood stream does not necessarily mean that the tumour will establish haematogenous metastases, the high incidence of tumour cells found in the blood stream of our cases of carcinoma of the breast is certainly of sufficient import as to arouse some caution. Perhaps our present day concepts concerning the spread of carcinoma should be reviewed, especially when one considers that the modern approach to any surgical procedure for carcinoma is the wide excision of all local and regional lymph nodes and pathways. It is rather frightening to consider that 18.5 to 20.0% of our cases of carcinoma of the breast already had tumour cells in their circulating blood prior to mastectomy (See Tables IX and X). So it would seem insufficient in cases of carcinoma to consider only lymph spread and not apportion enough importance to blood spread.

Also, if the results shown in Table III were to be extended so as to obtain a larger series, perhaps more types of tumours would be included in the category of those which spread commonly by the blood stream. Fifty per cent of our cases of carcinoma of the large bowel were found to have tumour cells in the blood, and both cases of carcinoma of the kidney had likewise. This certainly agrees with the claims of Ackerman and del Regato (1954) and others that carcinomata of the colon and kidney metastasize mostly by the blood stream. But it must be pointed out that the following tumours, as shown in Table III, also revealed in various incidences tumour cells in the blood; carcinoma of the stomach, carcinoma of the lung, carcinoma of the pancreas, and carcinoma of the cervix uteri. It is suggested then that perhaps greater attention should be given to the haematogenous spread of these types of carcinoma.

B. Interpretation of Our Negative Control Cases.

As shown in Tables I and IV, we have never found what we thought was a tumour cell in the blood of a patient without cancer. In our 50 control patients, not a single tumour cell was found. Some might find it surprising that we found no false positives. It must be remembered that the slides were each reviewed several times and by several observers and only cells which met the criteria of malignancy were diagnosed as tumour cells. Any atypical, doubtful or suspicious cell found was classified as negative. That is why we elected not to have a "suspicious cell" category in our results. More important, this probably means that we have missed some cells that were tumour cells in the blood of patients with cancer.

C. Are Tumour Cells Found More Frequently in the Blood of Patients with Advanced Cancer?

Though Roberts, Watne, McGrath, McGrew and Cole (1958 a), finding a higher incidence of tumour cells in the blood of patients with "incurable" disease than in those with "curable" cancer, saw a direct relationship between the advancing stage of the disease and the percentage of positive blood samples, our findings show no such correlation.

As shown in Tables XV, XVI, XVII and XVIII, we found tumour cells in no greater frequency in patients with Stage IV carcinoma than in those with Stage I. As a matter of fact it is when considering the stage of advancement of the patient's cancer that we met the greatest number of discrepancies. For example, case No. B-79 (No. 50, Table VI) had a very small carcinoma of the breast, 10 mm. at its greatest diameter and was excised completely at biopsy, there being no residual tumour in the mastectomy specimen. Yet tumour cells were found in the peripheral blood after operation. However, case No. B-3 (No. 7, Table VII) had extensive Stage IV metastases and the patient expired of carcinomatosis only one month after the blood sample had been taken, yet tumour cells were not found in this patient.

Others have also studied this matter. Engell (1955) in cases of carcinoma of the large bowel found tumour cells in 41% of group A (Dukes' classification,1932) carcinoma of the colon and rectum, in 57% of group B and in 70% of group C. He refrained from drawing any conclusions, because he maintained that the differences might be explained by the fact that the number of undifferentiated tumours increased from group A to C. Potter, Longenbough, Chu, Dillon, Romsdahl and Malmgren (1960) demonstrated

malignant cells in the peripheral blood in 16% of 117 patients with resectable carcinomata and in 36% of 168 patients with non-resectable lesions indicating a marked increase in advanced patients. Moore and Sako (1959) studying 44 patients with colorectal carcinoma, noted a higher incidence of tumour cells (37%) in patients with non-resectable lesions than in patients with resectable lesions (16%).

Experimentally, in 36 hamsters bearing malignant melanoma transplants, circulating tumour cells could be detected in the blood stream only after gross metastases had appeared. By that time, the tumour had been growing for 39 days (Wilson, 1959).

Finding no correlation between the tumour's extension and the presence of tumour cells in the blood, we, like Engell (1955), Moore, Sandberg, Burke, Johnston and Katz (1958), and Moore (1960), can only conclude that no prognostic significance can be attached to the presence of tumour cells in the blood.

Also, it must be remembered that if tumour cells are more frequently encountered in advanced lesions, this could be the result, rather than the cause of the widespread dissemination. We were particularly impressed by the slides of one patient, case No. B-50 (see Tables VI and VII-A). A blood sample was drawn from this patient 30 minutes before she died of carcinomatosis secondary to a carcinoma of the breast. The slides were literally full of carcinoma cells.

D. Significance of Degree of Differentiation of the Tumour.

Brown and Warren in 1938 proclaimed that the tendency to venous invasion and consequent haematogenous spread depended greatly on the grade of differentiation of the tumour. Engell in 1955 concluded that the spread of cancer cells in the blood depended chiefly on the histological grade of differentiation of the tumour. He found 34% of specimens positive in grade II, 76% positive in grade III and 100% positive in grade IV lesions. Our findings do not support these conclusions. Our series of patients with carcinoma of the breast included 44 with grade II tumours, 31 with grade III tumours and 4 with grade IV tumours. Though our figures are too small to be conclusive, the incidence of tumour cells was in the vicinity of 50% in each category (see Table XIX).

E. Relationship of Tumour Type and Biological Activity of a Tumour.

Sandberg and Moore (1957) supposed that the more biologically active the tumour (e.g. malignant melanoma) the more likely were tumour cells to be found in the blood. This view was endorsed by Delarue (1960). Again our series is too small to confirm or disprove these opinions but we did study the relationship of tumour type in carcinoma of the breast to the incidence of blood-borne tumour cells. As expected, there was a slightly higher incidence (66.6%) of tumour cells in the blood of patients afflicted with a medullary or encephaloid type of mammary carcinoma than in those with scirrhous carcinoma (53.3%). Surprisingly, 57.1% of the patients with adenocarcinoma of the breast revealed tumour cells in their blood. This tumour, of supposedly slow growth and lower malignancy,

perhaps should have shown a lesser incidence of malignant cells. Fifty per cent of our 10 cases of intraductal carcinoma were found to have malignant cells in the blood. As expected, 8 of these were of the infiltrating and invasive type but two appeared to be confined to the duct. (see Page 112).

It appeals to one's sense of logic to suppose that the more anaplastic the tumour, the longer it has been growing, the greater its degree of advancement, the more virulent its biological activity, the more likely would we be to find its cells circulating in the blood and the more sombre would its prognosis be. However, our findings support none of these suppositions. There are conceivably a multitude of reasons why this is so. Perhaps our series is too small, perhaps we have not drawn enough blood samples, perhaps the quantity of blood drawn has been too small, perhaps we have failed to find tumour cells in some of the samples or failed to recognize the cells for what they were. This last factor is especially noteworthy. Often, only one tumour cell is found in a blood sample and rarely do we find many. It could easily be, therefore, that they are present in such small numbers that we failed to catch any in some samples. But it seems to this writer that if these errors could be corrected, the proportional increase in the incidence of tumour cells would be relatively the same in each category and, though the figures would show a greater frequency of tumour cells, there would still be very little difference in the results regardless of whether the stage of advancement, the degree of anaplasia, the duration of symptoms or the type of tumour is considered.

F. Diagnosis of Cancer from the Identification of Cancer Cells in the Blood.

What about diagnosis? Could the diagnosis of cancer be made as a result of the identification of neoplastic cells in the blood? It has been suggested that this could be possible. Because of our conservative attitude in diagnosing a cell as malignant and because of our very strict application of the criteria of malignancy, it is felt that the negative finding of neoplastic cells in the blood is inconclusive but the positive identification of these certainly would give strong indication of a malignancy.

A good illustration of this can be found in one report which relates the case of a patient suffering from an undiagnosed lung ailment. Malignancy was suspected but could not be proven even after the standard array of laboratory tests. The patient could not tolerate bronchoscopy. But a routine blood smear was found to contain a single clump of bizarre spindle-shaped cells cytologically compatible in appearance to oat-cell carcinoma cells. Later, a pathology report of a vertebral metastasis after decompressive laminectomy verified this by announcing "Undifferentitated carcinoma consistent with metastatic oat-cell carcinoma of the lung" (Finkel and Tishkoff, 1960).

G. Significance of Tumour Cells Found in the Blood at Time of Operation.

Tables IX, X, XII, XIII and XIV all show the results of blood samples taken before and after mastectomy. In all cases, a higher incidence of tumour cells was found in patients after mastectomy than before the

surgical procedure. Twenty per cent of the patients in whom blood was drawn before mastectomy had neoplastic cells in the blood whereas 54.7% of the patients had positive blood samples drawn after mastectomy (see Table IX). This shows a significant increase in tumour cells in the blood during mastectomy. Again, in 27 patients from whom blood was drawn both before and after mastectomy, 18.5% had positive blood samples pre-operatively and 51.8% post-operatively (See Table X).

The presence or absence of axillary metastases was observed to have some significance when considered at time of operation. Of 30 patients without axillary metastases, 7.1% had tumour cells in the blood before operation and 50.0% were positive after mastectomy. In the 27 patients with axillary metastases, 31.2% had positive blood samples before operation and 60.0% had tumour cells in the blood after operation. However, because blood samples were not taken consistently before, during and after mastectomy in all these patients it is felt that no conclusions can be drawn from Table XIV.

Consequently, we cannot suggest that a prognostic significance can be given to the finding that a higher incidence of tumour cells was found both pre-operatively and post-operatively in patients with axillary metastases.

But our findings of an increase in the incidence of tumour cells after surgical manipulation might be significant. These results parallel those of Roberts and Cole (1959b),Turnbull (1957) and Ross (1958). Engell, who in 1955 found little reason to believe that the incidence of malignant cells in the circulating blood was higher after surgery than before, later (1959) admitted that "operative manipulation

played a minor role in intestinal carcinoma". Likewise Sandberg and Moore (1957) who maintained that there was no apparent increase in the frequency of tumour cells in the peripheral blood after surgery, reported that the frequency of tumour cells in the regional veins was increased by operation (e.g. gastrectomy).

An interesting thought comes to mind on considering the fact that in some patients with carcinoma of the breast, tumour cells were found immediately before operation. One wonders if pre-operative palpation of the breast tumour had anything to do with dislodging tumour cells so that they became detectable in the blood. Tables IX and X show that 18.5 to 20% of patients with carcinoma of the breast had tumour cells in their peripheral blood before operation. One wonders then, had there been no palpation or physical examination whatsoever, if the percentage might have been much lower since tumour cells would not have been dislodged by pre-operative palpation of the tumour. Consequently, the results of post-operative blood samples would loom larger and be of more significance. However, if surgical manipulation were to be an important factor in the dissemination of tumour cells, we find it somewhat surprising that blood samples taken during mastectomy did not more frequently reveal tumour cells. As Table IX clearly illustrates, only 13.6% of patients were shown to have circulating cancer cells during mastectomy. We cannot explain this. Perhaps there is a delay between the dislodging of tumour cells from the primary tumour and their appearance in the peripheral blood. Perhaps because of this delay, tumour cells are found mainly an hour or two after final excision of the tumour. Perhaps this delay is caused by the

fact that tumour cells must pass through the liver and lungs before reaching the antecubital vein and the liver has been shown to be a fairly effective filter of tumour cells (Roberts, Watne, McGrew, McGrath, Nanos and Cole, 1958 b). It has also been shown by Roberts and Cole (1959 b) who took frequent blood samples from both the vein draining the tumour site and from the peripheral blood before, during and after various surgical procedures that there were three types of response to surgical measures (See Page 41) but in all three cases, they found a high incidence of tumour cells during operative manipulation of the tumour. This could be because they were studying blood samples from veins in the region of the tumour, a study which we did not do in our series.

It would seem then that both surgical manipulation and operative stress would have some role in the dissemination of tumour cells in the blood stream. Though there seems to be an increase in tumour cells after operative trauma, again we can attach no prognostic import to this fact. For example, we found tumour cells in the samples of blood drawn post-operatively in 60.0% of the cases of carcinoma of the breast with axillary metastases treated by radical mastectomy (See Table XIV) and even the most gloomy prognosis would not suggest that 60.0% of these cases will develop distant metastases.

Yet we cannot help but heed the warning of Moore (1957) who cautioned surgeons not to adopt "nihilistic attitudes" in view of the many reports of the post-operative findings of tumour cells in the blood. He advised that surgeons should not abandon curative and palliative operations because of these unevaluated observations.

It has been only logical then, that certain authorities recommend clinical applications of these findings and so they suggested that tumours be gently examined, that they be not needlessly palpated, that afferent and efferent blood vessels be ligated before any surgical manipulation and lastly, they deemed per-operative chemotherapy a useful adjunct to the treatment of malignancy (Cole, Packard and Southwick, 1954 b; Cole, Roberts and McDonald, 1959 c).

H. Tumour Cells Found in the Peripheral Blood of Patients with Carcinoma of the Breast when Samples Were Drawn Pre-operatively, Per-operatively and Repeatedly for the First 10 Days or More Post-operatively (Table XII)

How long tumour cells can continue to circulate is indefinite (See Page 34). In attempting to determine how long after mastectomy tumour cells remained detectable in the blood, several points of interest arose from our study of the 10 cases of carcinoma of the breast from which blood samples were taken daily for 10 days or more post-operatively. Firstly, in no case were the tumour cells found in the blood consistently, though in 2 they were consistently absent, if we presume so to interpret our failure to find them. There seems to be two reasons why tumour cells were found only intermittently. It could be that they were present in the blood only intermittently, but it is perhaps more probable that we failed to find them in some of the samples. Secondly, some contradictions seemingly arise. Cases Nos. 1, 2 and 7, though patients with only Stage I were each positive several times whereas in case No. 10 who had Stage III degree of tumour spread, not one positive blood sample was found. Case No. 4 followed more closely the pattern expected in that this was a patient with a Stage III

carcinoma of the breast and 6 out of 9 blood samples were positive for tumour cells. Finally, it seemed quite clear that the tumour cells remained in the blood for at least 10 days in several of the 10 cases. It could be that the persistence of tumour cells in the blood would prove to be of more importance than their presence in the immediate post-operative period.

But one possibility seems to emerge out of even a cursory perusal of Table XII and that is that the intermittent finding of positive blood samples could be explained by the supposition that though tumour cells may be always present in the circulating blood of a patient with cancer, they might be identified only sporadically. Several lines of reasoning allow one to arrive at this conclusion. Firstly, if blood samples had been drawn (as in the case of the 27 patients listed in Table X) from the 10 patients with carcinoma of the breast listed in Table XII, only before and immediately after operation, we would have found only 4 patients to be positive for tumour cells. As it was, by drawing many samples from each of the 10 patients, more blood samples were found to be positive and the number of positive patients rose to 8, double the number that would have been found had blood samples been drawn only pre-operatively and post-operatively. Had blood samples been drawn from these 10 patients only on post-operative day number 1, not a single positive blood sample would have been found and these patients would have been classified as not having tumour cells in their blood!

It would seem then that the more frequently are blood samples taken from patients with carcinoma, the higher will be the incidence of tumour cells found in the circulating blood. As shown in Table V,

214 blood samples were taken from 80 patients with breast cancer and 64 blood samples or 29.9% were found to contain tumour cells. These 64 positive blood samples were found in 43 of the breast carcinoma patients making 53.7% of the 80 patients positive for tumour cells. It seems surprising that such a low percentage of positive blood samples (29.9%) yields such a high percentage of positive patients. What would it have been if 314 blood samples had been taken from these 80 patients? Or 500? Or 1000 blood samples? One can imagine a double-line graph with one line ascending parallel to a subjacent curve. The superior curve would be the number of "positive patients" increasing in proportion to the inferior curve representing increasing numbers of "positive blood samples".

Another line of reasoning that may be considered is that to anyone engaged in attempting to demonstrate tumour cells in the circulating blood, the most difficult fact to understand is why patients with very small and early cancers can sometimes be shown to have malignant cells in the blood whereas sometimes, patients with extensive tumours and widespread metastases cannot. One wonders then if this fact cannot also be explained by the possibility that any patient with a malignant tumour must at some time or other have tumour cells in the circulating blood and that only frequent samplings of the blood would reveal this. If tumour cells are not found in patients with advanced disease, the explanation may lie in the possibility that (a) an isolated blood sample just happened not to contain any, (b) they might not have been recognized, or (c) they might have been missed throughout the screening procedure.

This brings us to the conclusion that in the great majority of cases of carcinoma of the breast, tumour cells are present in the blood at some time, at least temporarily. But it should be emphasized that the presence or absence of tumour cells in a sample of blood in cases of carcinoma of the breast is of little or no prognostic significance. In this we are in accord with Engell (1959) who came to a similar conclusion from his follow-up study of cases of carcinoma of the colon and rectum.

We also doubt whether the determination of the presence of tumour cells in the blood is a good guide to therapy. If there is little or no correlation between prognosis and the presence or absence of tumour cells in the blood, or if they are present in the blood at some time in most cases of carcinoma of the breast, and if they are not found in all cases which do develop blood-borne metastases, there seems to be no reason to select for special treatment those cases in which the cells are found.

Finally, the presence of tumour cells in the blood does not mean that haematogenous metastases will inevitably follow.

CHAPTER XI

C O N C L U S I O N S

1. The finding of carcinoma cells in the circulating blood in such high frequency suggests that carcinoma may spread more commonly by the blood stream than previously supposed.

2. Tumour cells were found in no greater frequency in patients with advanced carcinoma of the breast than in those with early lesions. Nor were they demonstrated more often in patients with clinical symptoms of long duration than in patients with comparatively short duration of symptoms.

3. Because this series is small, only tentative conclusions can be drawn but it does seem likely that the presence or absence of tumour cells, as shown by this technique, is not related to prognosis, and so is not a good guide to treatment.

4. There seems to be no relationship between the degree of histological differentiation of a tumour and the incidence of blood-borne tumour cells.

5. Because we have not compiled a sufficiently large number of cases, a conclusion cannot be reached concerning the relationship of tumour type to the incidence of cancer cells in the blood.

6. To date, the negative finding of neoplastic cells in the peripheral blood is inconclusive, but the positive identification of these certainly would give a strong indication of the presence of malignancy.

7. A higher incidence of tumour cells was found in patients after mastectomy than before the surgical procedure. It would seem, then, that physical palpation, surgical manipulation and operative trauma have a significant role in the dissemination of malignant cells into the venous blood directly or by way of the lymphatics.

8. In the great majority of cases of carcinoma of the breast, tumour cells are present in the blood at some time, at least temporarily. We have reason to believe that they would be found even more frequently if we took repeated blood samples.

9. The presence of tumour cells in the blood does not mean that haematogenous metastases will inevitably follow.

10. The results reported in this work are too small to permit other than tentative conclusions. It must be realized that the study must be continued for many years in order to obtain a large enough number of cases to be significant and that these must be carefully followed.

CHAPTER XII

S U M M A R Y

The present study was designed in the hope that if the presence of malignant cells in the peripheral blood of patients having cancer could be found with any consistency, this could lead to (a) new methods of making the diagnosis of malicancy; (b) a convenient method of making follow-up studies on cancerous patients; (c) the opportunity to be able to predict the probability of metastases; (d) the evaluation of cancericidal agents, and (e) their therapeutic use in selected cases. In Part I, the medical literature pertaining to tumour cells in the blood stream is reviewed. In Part II, a description of the Malmgren Streptolysin-Filtration technique for the isolation of tumour cells from the blood is given. The results are given in Part III of the thesis. Two hundred and fourteen peripheral blood samples were drawn from 80 patients with carcinoma of the breast and tumour cells were demonstrated in 53.7% of the patients. In Part IV, the significance of these findings is discussed.

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A P P E N D I X A

NOTES CONCERNING THE STREPTOLYSIN-FILTRATION METHOD

NOTES CONCERNING THE STREPTOLYSIN-FILTRATION METHOD

Supplementary details concerning our experience with the isolation of tumour cells from the blood using the method described in the text (cf. Chapter VII) may be of interest and thus will be considered here:

(A) GENERAL:

(1) The blood should be stirred frequently with a glass rod during the technique. This permits a better cellular suspension and may prevent clumping of the tumour cells.

(2) Because our apparatus could only centrifuge 8 tubes at once, the maximum number of blood specimens that we could process at any one time was eight.

(3) Care must be taken during the procedure to avoid "contaminating" the blood specimen with tumour cells from another. Thus a single glass stirring rod should not be used for more than one specimen.

(4) Between bloods, the stirring rods, test-tubes, rubber stoppers, centrifuge tubes and filter caps are all washed with distilled water. Before placing a blood specimen in a pyrex centrifuge tube for processing, the latter is rinsed with a 0.85% saline solution. Periodically the centrifuge tubes and test-tubes are washed with concentrated sulphuric acid. All these are measures taken to prevent tumour cell transfer from one specimen to the next.

(5) One of the advantages of this method is that the blood is kept in the same centrifuge tube up until the time of filtration, lessening chances of losing tumour cells.

(B) COMMENTS CONCERNING EACH STEP OF THE TECHNIQUE.

(1) Collection of the Blood

(a) In this series, blood was taken from either antecubital vein.

(b) It is advisable to process the blood as soon as possible after venopuncture because it undergoes changes which make it difficult to filter and to screen.

(2) Removal of Plasma

It is better to use a refrigerated centrifuge (0 to 5°) because the blood sediment forms a better "block" and it is easier to pipette off the supernatant fluid without removing the buffy layer containing the cancer cells. Particular care must be taken if a mechanical suction pipette is used.

(3) Streptolysin Digestion

(a) Streptolysin-O reagent comes as a dry powder and is processed and standardized by the manufacturers for the titration of streptolysin-O antibody occurring in the serums of persons with group A streptococcal infections. It is meant by the manufacturers to be reconstituted in distilled water and used in the presence of Streptolysin-O Buffer Concentrate, an isotonic phosphate buffer solution at pH 6.6, which is employed for making serum dilutions and for preparing red blood cell suspensions.

However, Malmgren, Pruitt, Del Vecchio and Potter (1958 a) took advantage of the haemolytic and leukocidal properties of streptolysin-O as described by Todd in 1942 and in this Method it is used to lyse the erythrocytes and polymorphonuclear leukocytes.

(b) We have used both the Bacto-Streptolysin O Reagent of Difco Laboratories (Detroit, Michigan, U.S.A.) and the Streptolysin-O Reagent of

Hyland Laboratories (Los Angeles, California) and find no difference.

(c) It is important to note that both laboratories prepare their streptolysin by unit activity and not by weight and there is not a constant relationship between activity and weight. Therefore the activity of 500 mgms of streptolysin varies from sample to sample. Now we found as did Malmgren, Pruitt, Del Vecchio and Potter (1958 a) that there was a moderately wide range of both weight and concentration of the Streptolysin-O reagent which was effective in producing haemolysis and leukolysis. To Malmgren and his associates (1958 a) it therefore seemed simpler to express the quantity of Streptolysin-O to be used in terms of milligrams rather than the biologic nomenclature of test dose. He recommended that 500 mgms. of Streptolysin-O be used to lyse the erythrocytes and polymorphonuclear leukocytes of a 20 ml. blood sample. The amount of streptolysin required for digestion may also be calculated by using 30 mg. per ml. in the original specimen (Potter and Malmgren, 1959 a) or 50 mg. per ml. of packed cells after the third washing by centrifuge. The 500 mgms. of Streptolysin-O usually required are approximately contained in two 25 ml. size bottle or in one-half a 100 ml. size bottle of dried Streptolysin-O reagent.

(d) Re: Phosphate Buffer. Instead of reconstituting the reagent with distilled water, we reconstitute with 30 ml. of normal saline. In our experience, the use of the isotonic phosphate buffer is unnecessary. Its presence is theoretically required to make a pH of 6.4 to 6.6 because that is the optimum pH for the action of the haemolysin on the red cells and the leucocidin on the leukocytes (Todd, 1942).

When Malmgren, Pruitt, Del Vecchio and Potter recommended the use of an isotonic phosphate solution in their first paper on this subject (1958 a), presumably it was to maintain the pH of the blood specimen at 6.4 while undergoing "digestion" during the incubation period. In later reports, (Pruitt, Hilberg and Kaiser, 1958; Potter and Malmgren, 1959 a) they abandoned the use of this buffer and replaced it with saline. The same was done in our laboratory. Normal saline seems to produce as satisfactory a "milieu" for haemolysis as does the isotonic phosphate buffer solution and its pH is not as critical, its preparation is easier and it is more easily used.

The pH of the 0.85% solution of sodium chloride used in our laboratory was 6.8. This seemingly produced a medium of sufficient acidity for satisfactory haemolysis. The differences of 0.4 pH that it has with Malmgren's (1958 a) isotonic phosphate solution of pH 6.4 and of 0.2 pH that it has with the Streptolysin-O isotonic phosphate buffer of pH 6.6 as prepared by Hyland Laboratories are probably negligible.

However, it must be noted that the pH of an isotonic normal saline solution is dependent on its CO_2 content and the above findings may only hold true for the specific preparation that we used in our laboratory (1000 ml. bottles of 0.85% normal saline by Baxter Laboratories). It is, nevertheless, interesting to note that others are now using normal saline solutions instead of phosphate buffers (Pruitt, Hilberg and Kaiser, 1958; Potter and Malmgren, 1959 a).

(e) Streptolysin-O is very "<u>oxygen labile</u>", that is, it loses all haemolytic power when oxidized. Consequently, to maintain its full activity, it should always be used in the presence of reducing agents like $Na_2S_2O_4$ (Todd, 1942).

However, if one is careful to dissolve the dried streptolysin-O reagent (which is stable in powder form) only immediately before using and if one is careful not to agitate the solution too vigorously or make bubbles while pouring the solution, then there seems to be little or no danger of inactivating the streptolysin by oxidation.

Also, the manufacturers may have included in their commercial preparations of dried streptolysin-O reagent some reducing agents like cysteine. But this cannot be verified due to individual company trade secrets.

(f) Re: Water Bath. To avoid the growth of fungi in the water bath and to avoid deposits caused by creating a "Battery Cell", the use of distilled water is recommended and the application of a gram or two of benzoic acid in the water every four or five days will keep the water nicely clear.

(4) Fixation.

(a) The 10% formalin solution is prepared by adding 1 ml. of 100% neutral formalin to 9 ml. of distilled water.

(b) The 10 ml. of saline are added to the packed cells and the mixture is aspirated into and expelled from a syringe through a size 18 needle so as to obtain a uniform cellular suspension. The saline should be added first to the packed cells and the cells uniformly suspended by agitation before adding the 10% formalin. This sequence is necessary because the packed cells are in a block and if the formalin is added before the saline, the formalin will fix the cells in clumps. Consequently, it is necessary to suspend the cells in saline first.

(c) The formalin must be filtered before using. There was no need by our method to filter the distilled water nor the normal saline.

(d) The sample of blood may be allowed to fix overnight in the 10% buffered formalin. This is one stage where the method can be delayed.

(e) Because the tumour cells are fixed and hardened as a result of formalin fixation, one needs to be less strict concerning pressure regulation upon filtration as there is less chance of distorting the cells.

(f) Cells fixed in formalin, when stained, have a rather generalized hazy appearance but do preserve good differential staining. However, for those who prefer sharp, crisp nuclear detail, ethyl alcohol fixation is preferred (Seal, 1959).

(5) Filtration.

(a) Millipore Filters. Filtration of the preparation after fixation takes place through Millipore filters (Millipore filter No. SM, Millipore Filter Corporation, Bedford, Mass.). This filter is a membrane of cellulose ester which is 150 micra thick. The tumour cells are collected on this membrane and are stained directly on it. There are several types of filter membranes available whose average pore diameters range from 10 millimicra to 5 micra. The membranes used for this study were the white plain SM type of constant pore size 5 /u, were 47 mm. in diameter and each presented a total filtering area of 9.6 sq.cm.

These filter membranes are mounted on white porous discs to support them throughout filtration. It has been said that the glossy surface of the millipore filter should be placed superiorly but, in our experience, it does not matter which side faces upwards.

(b) To Avoid Debris on the Filter Membranes. To avoid debris on the filter membranes, the following points are noteworthy: -

(i) The filters and filter pads must be placed on the apparatus only at the last minute to prevent the settling of dust on their surfaces.

(ii) The formalin solution should be freshly prepared using filtered pure neutral formalin and distilled water.

(iii) The centrifuge tubes and glass rods must be well cleansed and rinsed in saline before use. The filter apparatus must be well rinsed. It is suggested that the centrifuge tubes be washed in acid after they have been used in order to prevent any transfer of cancer cells to the next blood sample processed.

(c) Clumping. As mentioned elsewhere in this work, the blood should be stirred frequently with a glass rod during the technique to avoid the formation of clumps.

(d) An equal volume of water may be placed in each filtration flask to collect the filtrate.

(e) It is preferable not to attempt to "force" a suspension of blood cells through the filter membranes either by using too few filters or by using unreasonable negative pressures. If a cellular suspension is forcefully filtered, this results in the formation of air bubbles, the multi-layering of the cells, the distortion of the tumour cells and clogging or crowding of the filter membranes.

(f) As much as possible, the filter membranes should not be allowed to dry after filtration before placing them in Carnoy's fixative.

(g) It was found throughout our experiments that post-operative samples of blood were more difficult to filter because of their increased

denseness and cellularity. This would agree with the findings of Pepper and Lindsay (1960) who observed a marked increase in leukocytes during and following surgical procedures.

(h) It should be noted here that some investigators completely bypass this step in the procedure. As mentioned earlier in this work, Roberts, Cole and their colleagues have changed their method of isolating tumour cells from the blood (the albumin flotation method described by Roberts, Watne, McGrath, McGrew and Cole, 1958 a) adopting the streptolysin method described by Malmgren, Pruitt, Del Vecchio and Potter (1958 a). However, they modified it by substituting a centrifuge process for the filtration method of collecting cells for examination. Instead of fixing the cells in formalin after the post-incubation centrifuge and then filtering them, they simply add 10 ml. of isotonic sodium chloride to the packed cells, recentrifuge, decant the supernatant fluid and the remaining sediment is placed on 12 ordinary glass slides and fixed immediately in ether-alcohol solution (Long, Roberts, McGrath and McGrew, 1959). They consider their version to be comparatively simplerthan Malmgren's. In their last paper (Long, Jonasson, Roberts, McGrath, McGrew and Cole, 1960 b), they claim to have processed blood samples from 178 cancer patients by the new method compared to 297 patients using the discontinued albumin flotation method.

(6) Staining.

(a) Numbered Metal Filter Holders. Following filtration the cell containing filters are mounted in numbered metal filter holders which in turn are placed in glass carriers for immersion into the Carnoy's fixative and Papanicolaou stain. Polyethylene millipore filter holders manufactured

by the Duke Bontz & Co., (8409 2nd Avenue, Silver Spring, Maryland) have also been described by Del Vecchio, De Witt, Borelli, Ward, Wood and Malmgren (1959), but we have found the metal ones to be most satisfactory.

(b) Modified Carnoy's Fixative. Carnoy in 1887 described his fixative as comprising 60 ml. of 100% alcohol, 30 ml. of chloroform and 10 ml. of glacial acetic acid. According to Lillie (1952) this fixative gives excellent nuclear fixation. The Nissl granules are well preserved and many cytoplasmic structures are adequately fixed. However, Carnoy's fluid occasions a considerable shrinkage of the cells and dissolves acid soluble cell granules and pigments (Lillie, 1952).

De Witt, Del Vecchio, Borelli and Hilberg (1957) modified Carnoy's solution and adapted it for use in the isolation of tumour cells. They mixed 70 ml. of 95% ethyl alcohol with 25 ml. of chloroform and 5 ml. of glacial acetic acid. They suggested that it be stored in a tightly stoppered brown bottle at room temperature. It was considered by these investigators that their modification of Carnoy's fluid gave better cytological detail.

(c) Papanicolaou Stain. In 1954, Papanicolaou of Cornell University described a technique for the staining of cancer cells especially those of vaginal, endocervical and endometrial smears or aspirations. His three objectives were to obtain (i) definition of nuclear details, (ii) transparency of the cells, and (iii) differentiation of the cells.

The following is a description of the Modified Papanicolaou technique as used for staining the filters in their filter holders throughout the present work:

(1) After fixation in Modified Carnoy's fluid, the filters are transferred to a series of alcohol rinses for rehydration. They are dipped 10 times in 70% alcohol, 10 times in 50% alcohol and 10 times in distilled water.

(2) The cells are then stained in undiluted Harris haemotoxylin for one minute. This is the basic stain for the nuclei.

(3) The excess dye is then washed off in distilled water.

(4) The cell containing filters are then dipped in 0.25% HCl (0.5% aqueous HCl + 70% alcohol) for decolorization. This removes some of the dye thereby differentiating the nuclei.

(5) The filters are then washed in tap water the pH of which is on the alkaline side.

(6) The filters are then dipped for a few seconds in saturated Lithium carbonate which is a faster alkalising agent than tap water.

(7) The filters are then run in 10 dip rinses through a series of alcohols which gradually increase in concentration as follows: 50%, 70%, 80%, and 95%.

(8) The cytoplasm is then stained by a 30-second immersion in O.G. 6 solution. This is composed of 0.015 grams of phosphotungstic acid dissolved in 100 ml. of 0.5% Orange G solution in 95% alcohol.

(9) The excess stain is then removed in two rinsings of 10 dips each in 95% alcohol.

(10) The cytoplasmic stain is then supplemented by a 45-second immersion in E. A. 50 solution (prepared and marketed by the Ortho Pharmaceutical Corporation, Raritan, N.J.).

Its composition is: -

Light Green SF yellowish - 0.1% solution in 95% alcohol ..... 45 cc.

Bismark Brown - 0.5% solution in 95% alcohol ..... 10 cc.

Eosin yellowish (water and alcohol soluble) - 0.5% solution in 95% alcohol ..... 45 cc.

Phosphotungstic acid ..... 0.2 gm.

Lithium carbonate, saturated aqueous solution ..... 1 drop

(11) Dehydration is then accomplished by two rinsings of 10 dips each in 95% alcohols, and two washings of 10 dips each in absolute alcohols (100%).

(12) The filters are then passed through a solution made up of one part absolute alcohol and one part toluol or xylol.

(13) After two cleansings of ten minutes each in toluol or xylol to remove the alcohols, the Millipore filters are ready for mounting.

Note: This method of staining differs from the original as described by Papanicolaou (1954) in that concentrated Harris Haemotoxylin is used and there are shorter immersions of the filters in O. G. 6 and E. A. 50.

(d) To Mount the Filters. The filter is allowed to dry slightly upon removal from the xylol or toluol. It is mounted as described in Chapter VII. However, it should be added that a minimal amount of Permount should be used and the sides of the Millipore filter should be trimmed so that it fits snugly between the slide and the coverslip. This precaution avoids the formation of air bubbles between the slide and the coverglass and prevents dehydration of the smear.

A P P E N D I X B

EXPERIMENTS CONDUCTED TO TEST THE SENSITIVITY TO TUMOUR CELLS TO VARIOUS PHASES OF THE TECHNIQUE

EXPERIMENTS CONDUCTED TO TEST THE SENSITIVITY OF TUMOUR CELLS TO VARIOUS PHASES OF THE TECHNIQUE

The mouse melanoma S91 was rubbed on a stainless steel screen of 60 or 80 wires to the inch and the cells were collected in a beaker by washing them from the screen with normal saline. The cellular concentration was then estimated using a haemocytometer. Samples of the cellular suspension were then tested as follows: -

1. Controls

Seventeen ml. of the cells in saline were added to 3 ml. of heparin and the whole was processed in the normal manner by the technique described in Chapter VII. Another 5 ml. was added to 12 ml. of human blood mixed with 3 ml. of heparin and again processed in the usual manner. Both these samples were used as controls and in both cases, complete recovery of almost all the cells was accomplished.

2. Durability of Cancer Cells.

Various concentrations of the suspension were kept under refrigeration in stoppered test-tubes and samples were taken at regular intervals, filtered, stained and examined in order to investigate the durability of the cancer cells.

Cells were found to be intact even after one month's refrigeration.

3. Preservation in Heparin.

Five ml. samples of the cellular suspension were added to four aliquots of 15 ml. of whole human blood each mixed with 3 ml. of heparin sodium (10 mg. per ml.). The mixtures were kept in stoppered test-tubes

under refrigeration for periods of from one week to one month before processing.

Unfortunately after more than five to eight days, a thick rust-coloured jelly-like substance was observed to form rendering filtration almost impossible. The slides showed a thick reddish substance and were unscreenable. This was considered to be due to the fixed products of haemolysis (on placing the filters in Carnoy's fluid, much of the brown chocolate-like fluid would be removed from the filters and would turn Carnoy's fluid dark brown). There was a moderate amount of debris on the slides and in not one case were the cancer cells recovered.

4. Preservation in Formalin

Four mixtures were prepared each consisting of 5 ml. of mouse melanoma cells added to 15 ml. of whole human blood mixed with 3 ml. of heparin. These were processed by the streptolysin method until the formalin stage. They were then kept under refrigeration in stoppered test-tubes for increasing intervals of time before filtration and staining to find the length of cellular preservation in 10% formalin.

After one week, the slides prepared from specimen number one were quite satisfactory and the tumour cells, lymphocytes and monocytes were well preserved. After two weeks refrigeration, the slides of the specimen were quite screenable although the cells were not so well preserved and presented a paler appearance. After three weeks, there was a considerable decrease in cellular detail and definition. There was a considerable amount of debris on the slides and screening was rendered cumbersome by the vagueness and "foggy" appearance of the cellular elements. The cells were pale and never seemed to be in focus. However, one could

still recognize the odd melanoma cell. After one month, it was difficult even to get past the filtration stage. The filtration was long and tedious and required eleven filter membranes. The slides were poor in quality and were unscreenable.

5. Effect of Streptolysin on Cancer Cells.

Four 5 ml. samples of the malignant cellular suspension were subjected to the streptolysin digestion procedure each sample undergoing incubation in the presence of various doses of the Streptolysin-O enzyme. This wad done to test the possible effects that high concentrations of streptolysin would have on the cancer cells.

On screening the slides after they had been stained by the Papanicolaou technique, the tumour cells seemed to be unharmed. Even the sample that had been subjected to 2000 mg. of Streptolysin-O seemed intact. There would seem, then, to be considerable variations possible of streptolysin dosage without harm to the tumour cells. This was also observed by Malmgren, Pruitt, Del Vecchio and Potter (1958a).

6. The Use of Staphylococcic Toxin in the Technique for the Isolation of Tumour Cells from the Blood.

Because haemolysis by Streptolysin-O was found to be incomplete (it only lyses the erythrocytes, part of the polymorphonuclear leukocytes and only a few of the monocytes and lymphocytes), it was desired to find a better haemolytic agent. The first to be considered was the toxin of the Staphylococcus. Staphylococci are well known to produce a variety of toxins and a few of these were studied. Alpha-Haemolysin is described as being active against rabbit erythrocytes but not againt human red cells. Beta-lysin acts on the red cells of sheep, oxen and humans, and perhaps

might also have a leucocidal effect. Gamma-lysin causes a rapid lysis of red corpuscles in a variety of animals. There is direct microscopic evidence of the destructive action of leucocidin on human leukocytes. Whether leucocidin, Beta-lysin and Alpha-lysin are all one same faction or three separate toxins is a subject of much debate and confusion. (Topley and Wilson, 1946). Enterotoxin and staphylocoagulase are further toxins of this micrococcus.

Because of its haemolytic capabilities it was therefore decided to attempt to use staphylococcal toxin both in conjunction with Streptolysin-O and separately, to isolate cancer cells from the blood.

A strain of high toxigenic capacity was chosen and the preparations consisted of 5 ml. of staphylococcic toxin in the dry form preserved in glass ampules. Ten samples of 15 ml. of human whole blood mixed with 3 ml. of heparin and 5 ml. of the mouse melanoma cellular suspension were prepared for processing with the staphylococcic toxin. One, two, three, four and five ml. samples of staphylococcic toxin were each dissolved in 30 ml. of saline and each of these five dilutions of staphylococcic toxin was incubated at $39^{o}C$ for 10 minutes with a sample of blood and tumour cells as previously mentioned.

The dilutions of one, two and three ml. of staphylococcic toxin in 30 ml. of normal saline gave very unsatisfactory results. There was very poor digestion of the red cells rendering filtration impossible. A smear done before filtration showed many half lyzed erythrocytes bound together by a yellow, transparent substance which could have been coagulated plasma caused by the toxin staphylocoagulase. There were many polymorphonuclear leukocytes, monocytes and lymphocytes in clumps.

The more concentrated solutions of four and five ml. of staphylococcic toxin in 30 ml. of normal saline required an extra saline washing by centrifugation before filtration. The 4 ml. of staphylococcic toxin specimen yielded a very cellular slide with very few erythrocytes and the polymorphonuclear leukocytes, lymphocytes and monocytes were of good definition and detail and stained well. The cancer cells could not be outlined amongst the multicellular mass.

The slides of the specimen digested by 5 ml. of staphylococcic toxin were very beautiful. There were no erythrocytes, only rare polymorphs and the lymphocytes and monocytes were of good definition and well stained. The cancer cells were identifiable, well preserved and well stained. These slides were indistinguishable from slides of samples of blood lyzed by streptolysin.

It is possible that the reason why the weak solutions of one, two and three ml. of staphylococcic toxin were ineffective in lyzing the erythrocytes is that the incubation period was too short. Topley and Wilson (1946) mention that Beta-haemolysin is active against red cells only if the tubes have stood at room temperature overnight. However, since the 5 ml. strengths were satisfactory, no other experiments were done with weaker dilutions.

Finally, 5 ml. of staphylococcic toxin and 500 mg. of streptolysin were dissolved together in 30 ml. of normal saline for use in the lysis of blood samples but no significant advantage was found.

In conclusion, staphylococcic toxin appears no better than streptolysin. Both are haemolytic and polymorphonuclear leukocytic but their leucocidin leave the monocytes and lymphocytes unharmed.

A P P E N D I X C

P H O T O G R A P H S

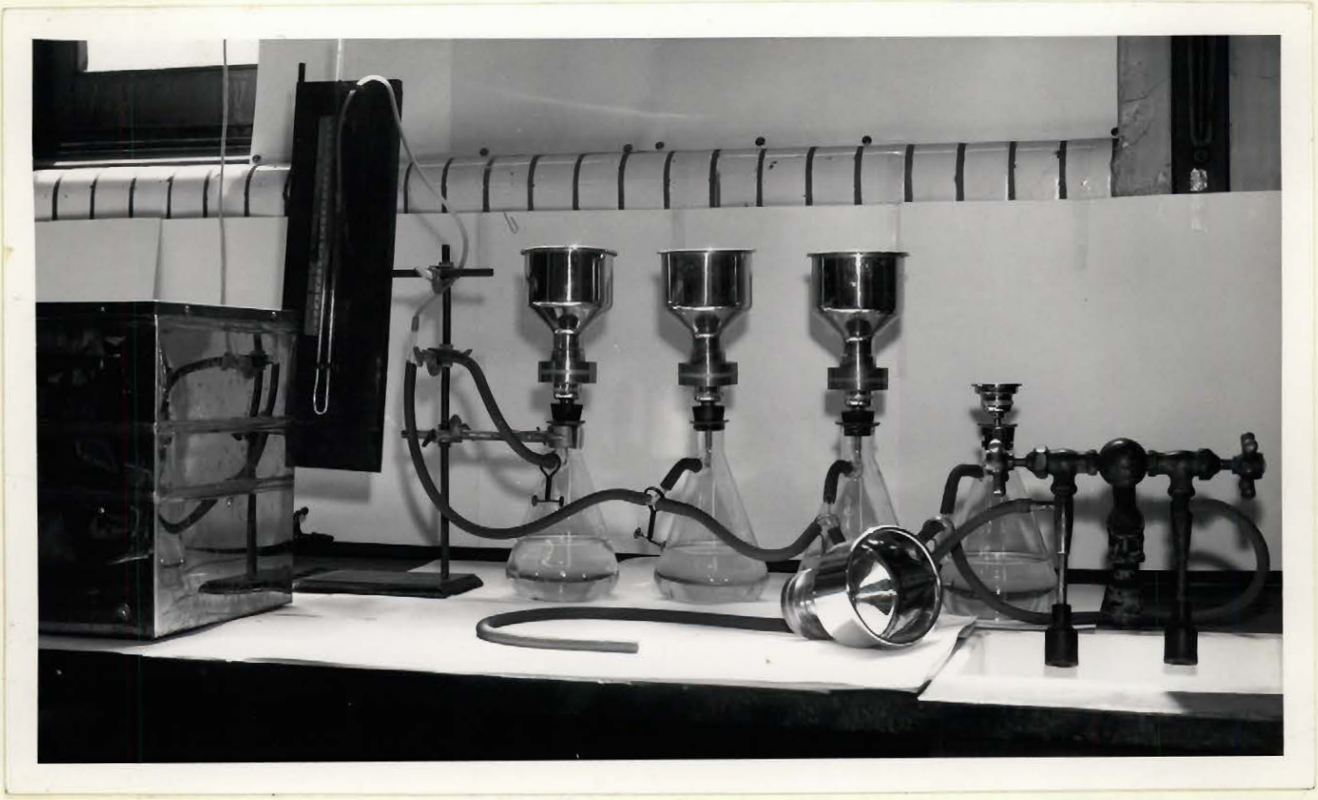

Fig. 2

FILTER APPARATUS

From left to right: The water bath; the mercury manometer connected to a series of four 1000-cc. filter flasks each mounted with a filter cup and filter holder. The filter cup on the right has been removed to show the filter holder. The water taps on the right provide the vacuum source.

Note the clamps on the tubing of each filter flask. These are used when it is desired to discontinue negative suction in an individual flask. Note also the water in each flask.

The tubing shown coiled on the table in front of the filter apparatus is connected to a Pasteur pipette (not shown) and is used to aspirate the supernatent fluid.

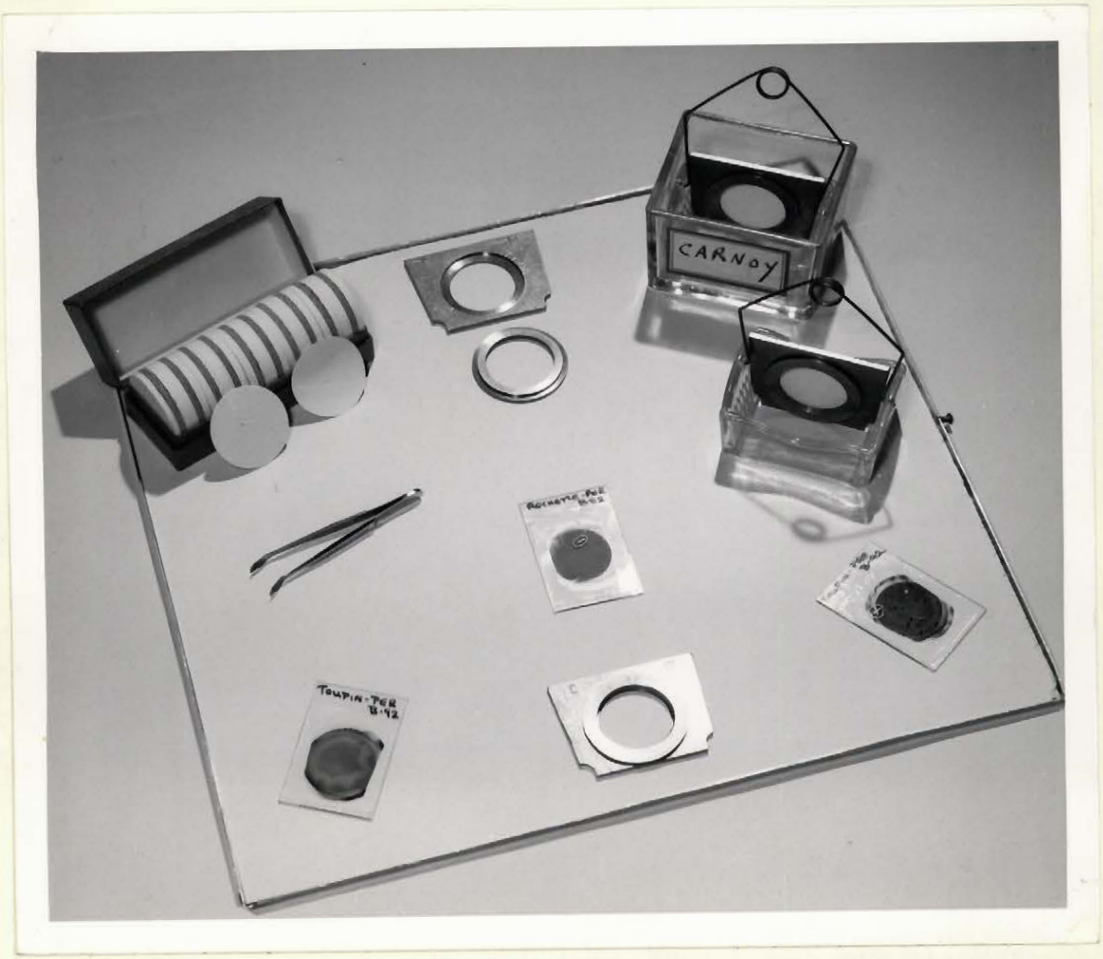

Fig. 3

Some of the materials used in the manipulation of the filter membranes are shown

- A box of 100 SM Millipore Filters and filter pads
- The numbered metal filter holders
- The tray for carrying the filter holders through the various fixation and staining dishes
- The dish used to immerse the filter holders in modified Carnoy's fluid.
- Three completed slides
- A pair of tissue forceps for the handling of the filter membranes.

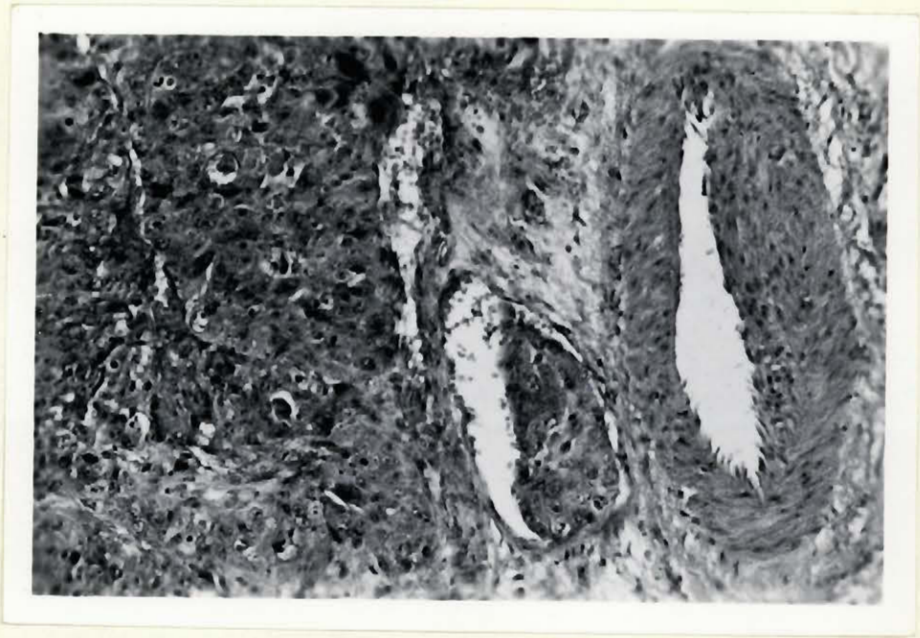

Fig. 4

Carcinoma of the oral mucosa invading venule X 200

Fig. 5

Low grade fibro-sarcoma shown invading an artery X 100

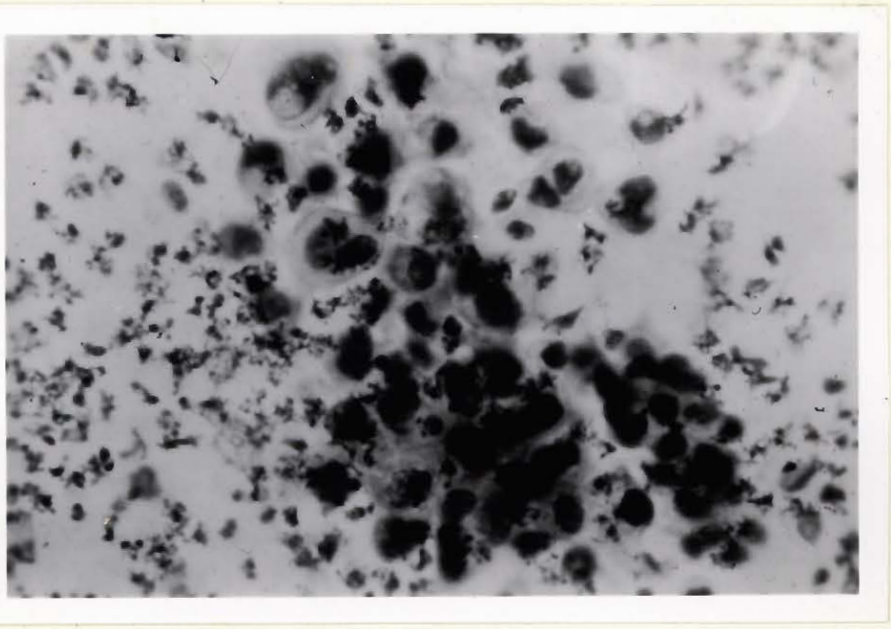

Fig 6.

Tumour cells seen in the pleural fluid of a patient with mesothelioma of the lung X 400

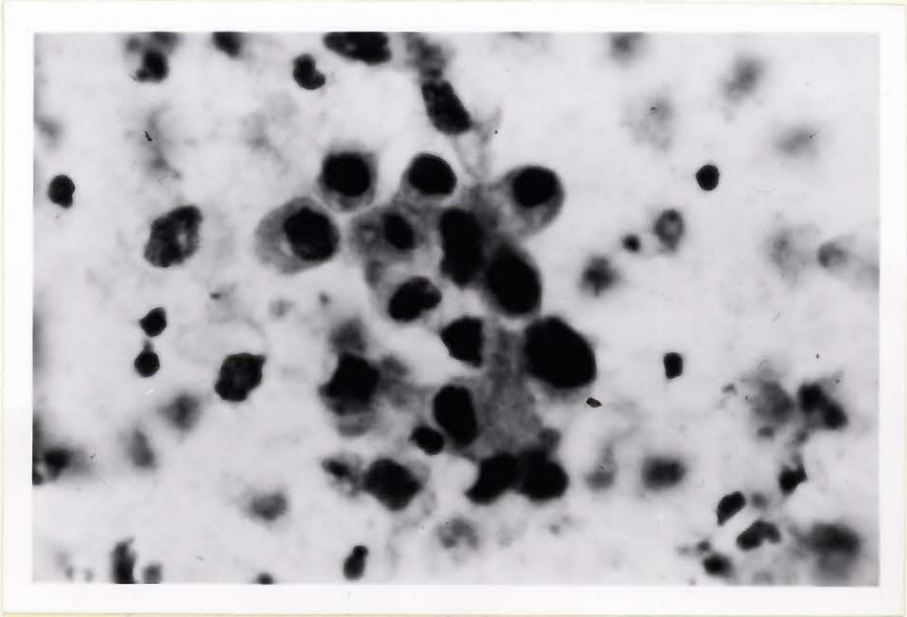

Fig. 7

Tumour cells in the pleural fluid of a patient with mesothelioma of the lung X 500

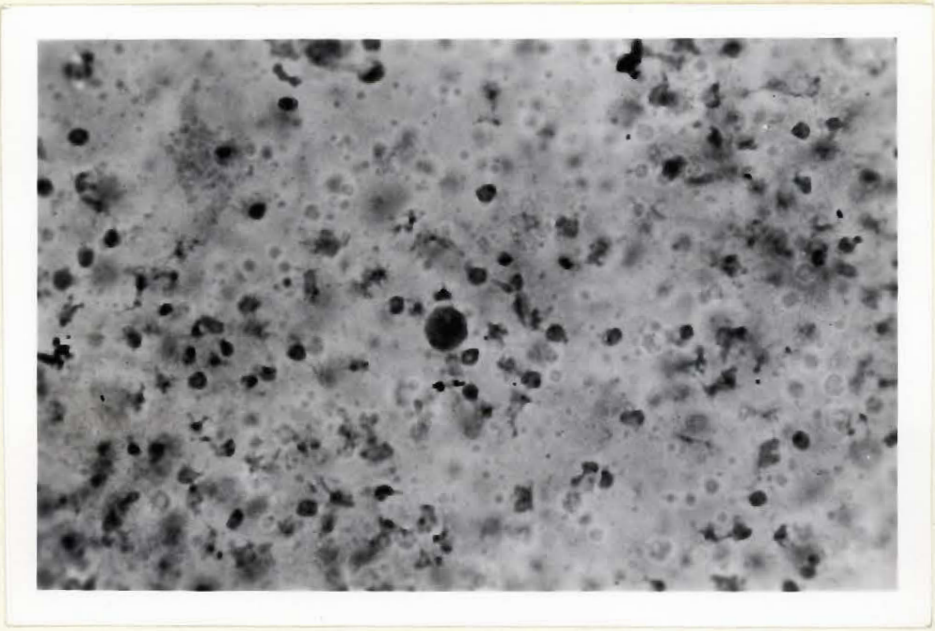

Fig. 8

Tumour cell found in the venous blood draining the tumour site in a patient with adenocarcinoma of the left colon X 200

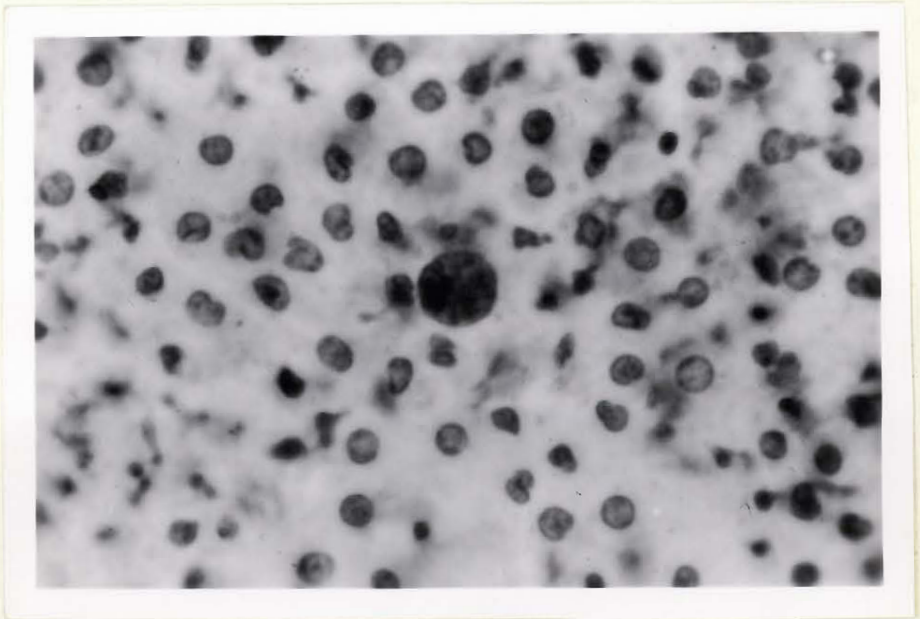

Fig. 9

Tumour cell found in a vein draining the tumour site in a patient with adenocarcinoma of the sigmoid colon X 400

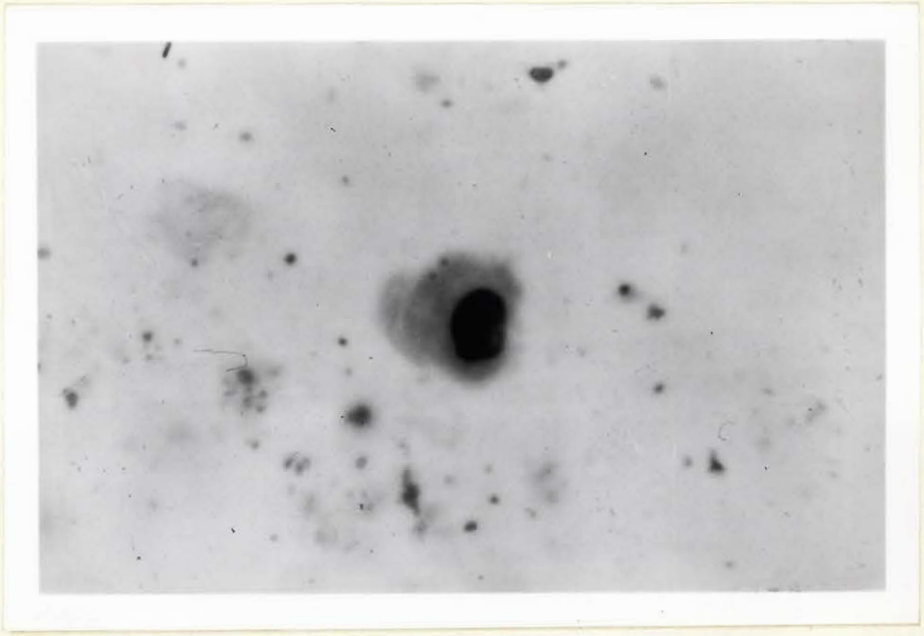

Fig. 10

Tumour cell in the peripheral blood of a patient with carcinoma of the kidney with metastases to the lungs X 500

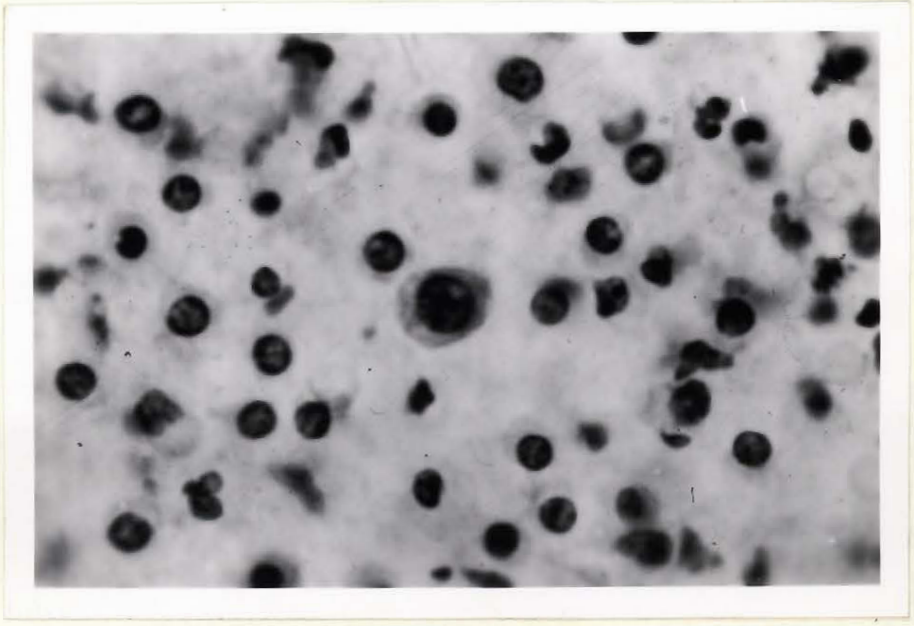

Fig. 11

Tumour cell in the peripheral blood of a patient with carcinoma of the stomach X 500

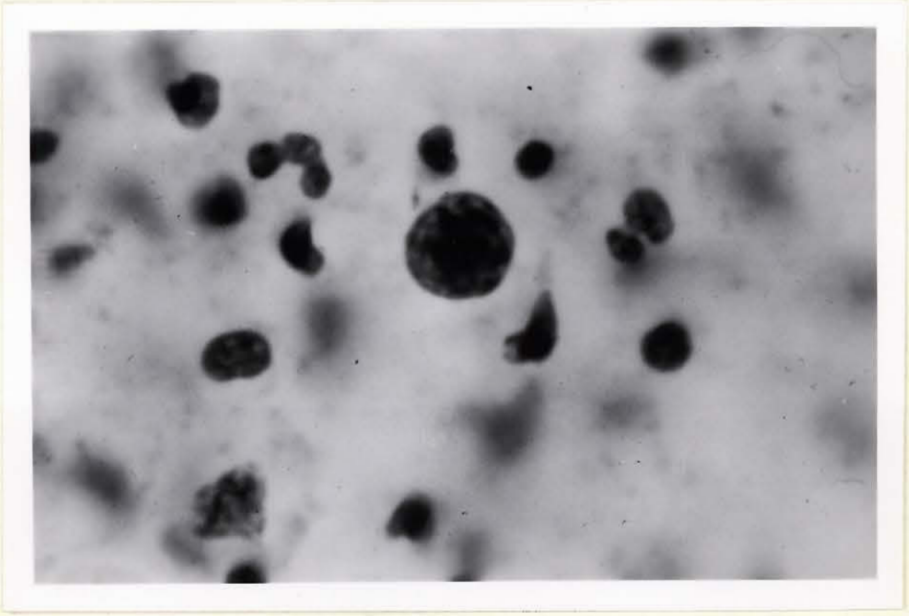

Fig. 12

Tumour cell in the peripheral blood of a patient who sustained a radical mastectomy for stage I carcinoma of the breast 2 years previously and who, at the time of taking the blood sample, had local skin recurrences
Case number A-7 X 400

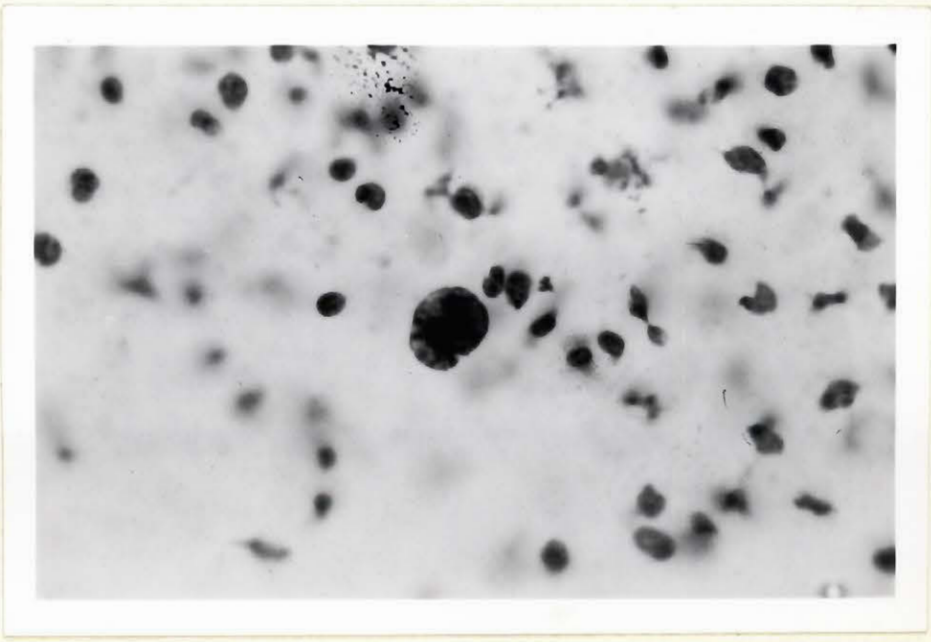

Fig. 13

Another tumour cell found in the peripheral blood of the same patient (See Fig. 12) X 500

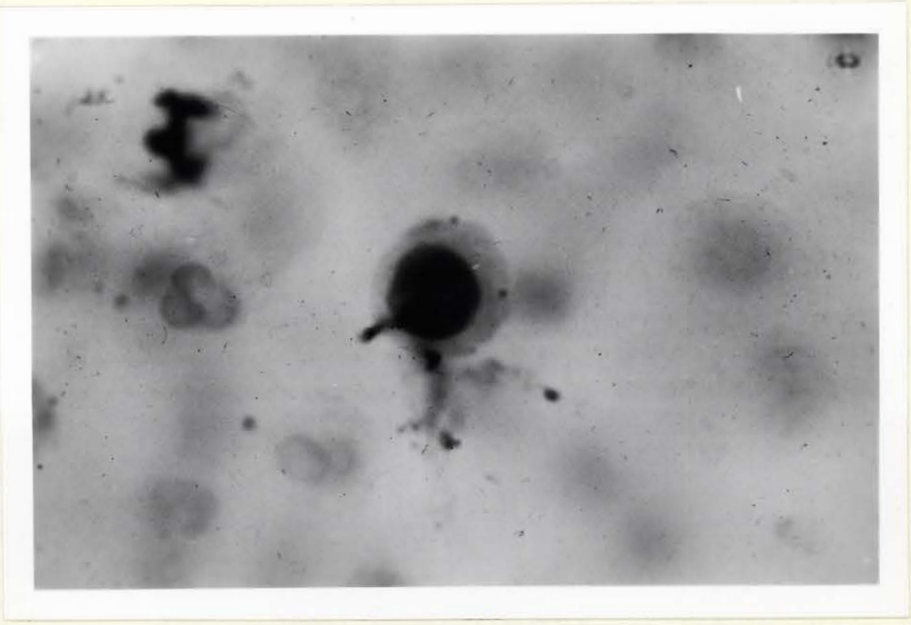

Fig. 14

Tumour cell found during the immediate post-operative period in the peripheral blood of a patient with scirrhous carcinoma of the breast involving 7 of 22 axillary lymph nodes. Case number B-49 X 1000

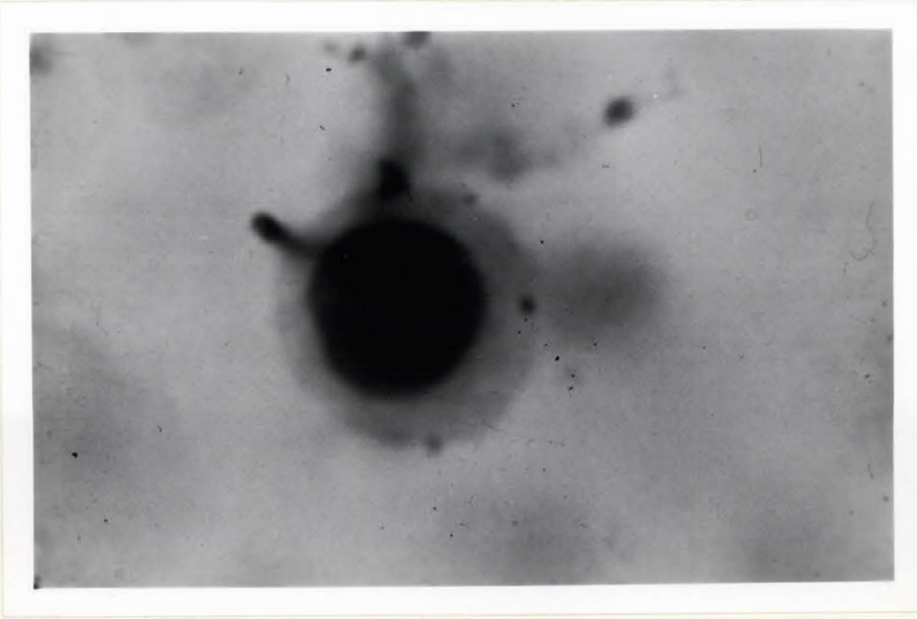

Fig. 15

Same tumour cell as in Fig. 14 but at a higher power X 2000

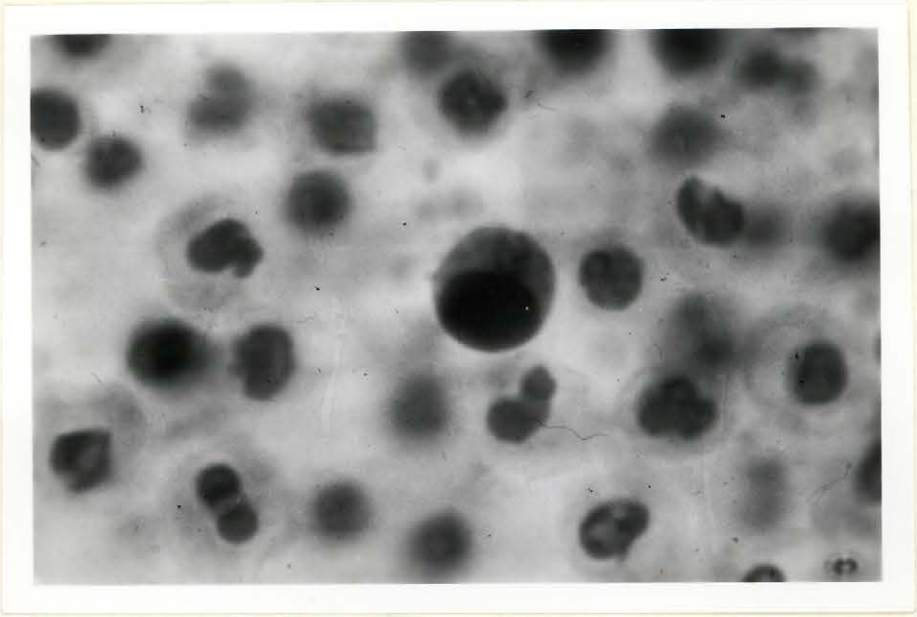

Fig. 16

Tumour cell found in the peripheral blood of a patient who had sustained a radical mastectomy one year previously for an infiltrating duct carcinoma exhibiting invasion of vascular channels and secondary carcinoma in the axillary lymph nodes. Case number B-13 X 1000

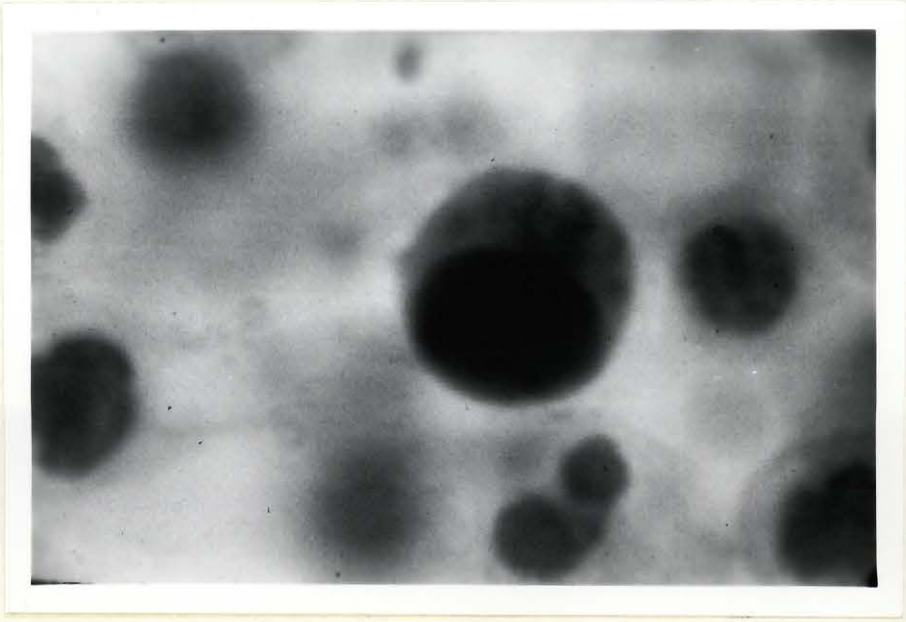

Fig. 17

Same tumour cell as in Fig. 16 but at a higher power X 2000

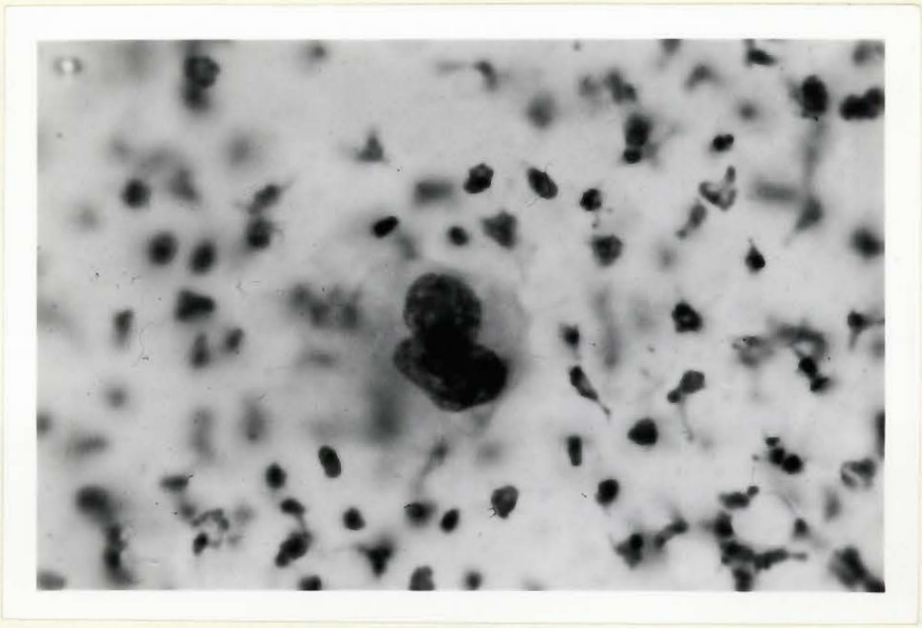

Fig. 18

Two tumour cells seen in a sample of peripheral blood drawn during the immediate post-operative period from a patient with carcinoma of the breast involving axillary lymph nodes. Case number A-9 X 500

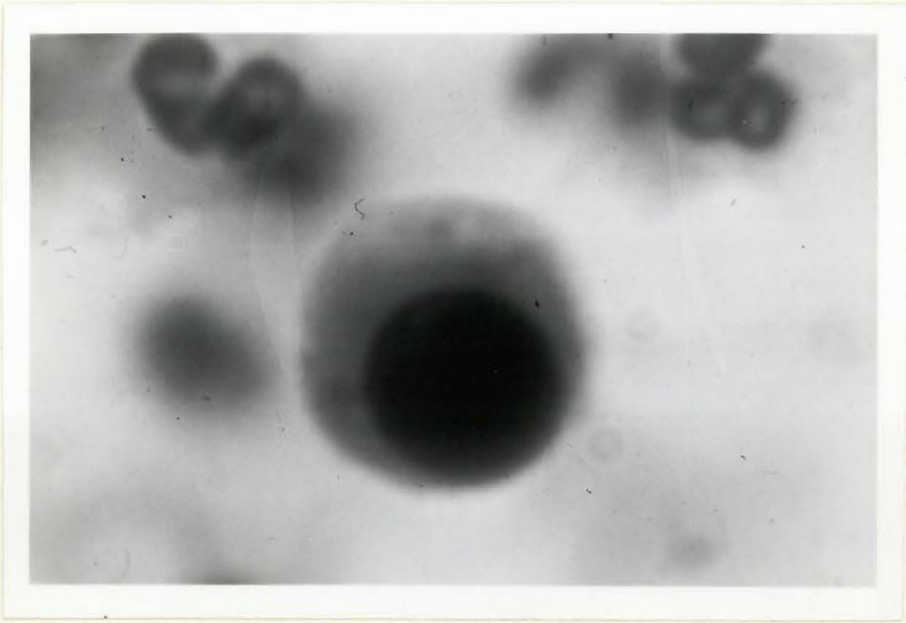

Fig. 19

Tumour cell found in the peripheral blood of a patient with advanced infiltrating fibrosing carcinoma of the left breast. Case number B-8 X 2000

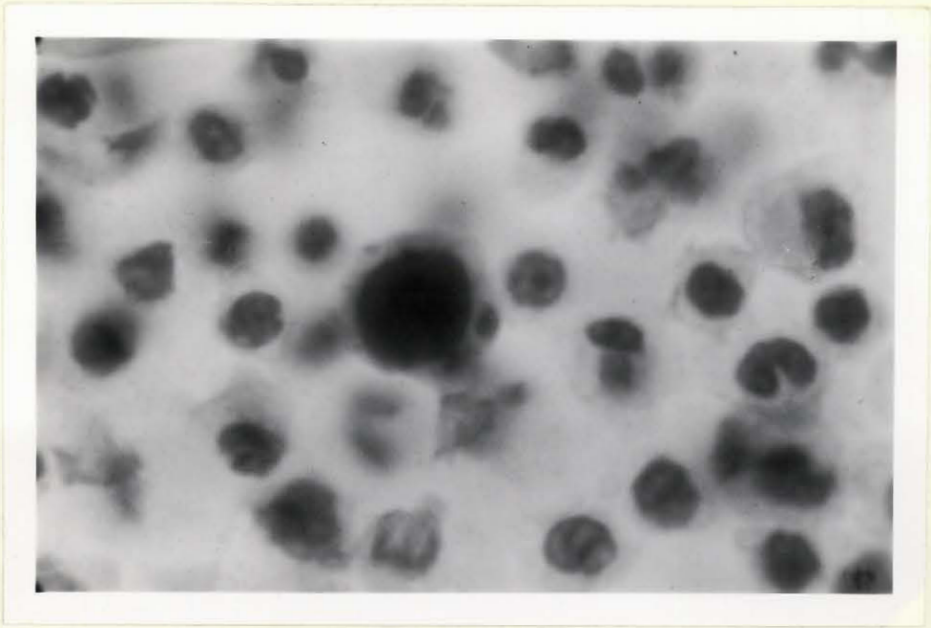

Fig. 20

Tumour cell in the peripheral blood of a patient who underwent a radical mastectomy 11 days previously for a carcinoma of the breast with secondary carcinoma in the axillary lymph nodes. Case number B-14 X 1000

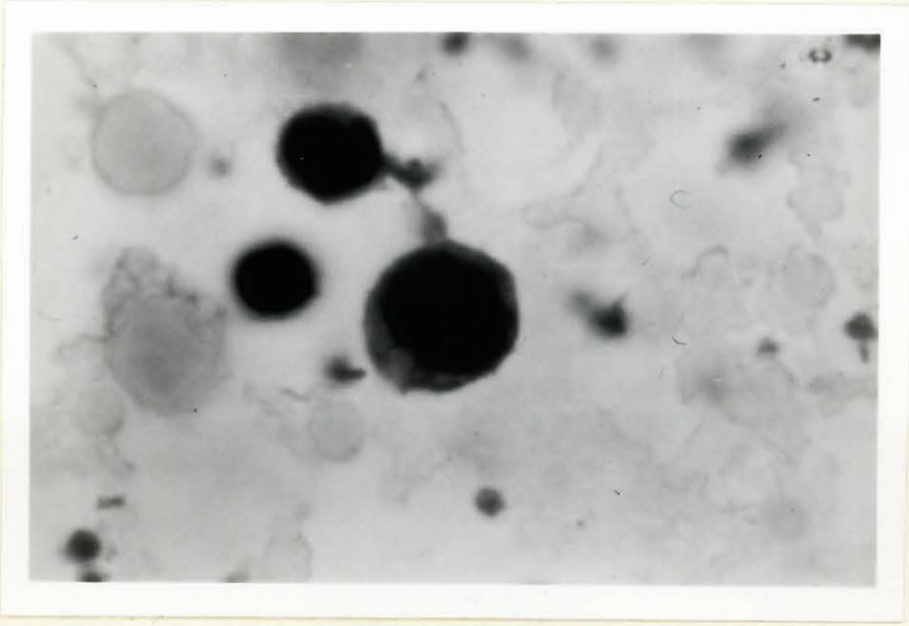

Fig. 21

Tumour cell found during the post-operative period in the peripheral blood of a patient who had a simple mastectomy for intraductal carcinoma of the left breast. The axillary lymph nodes were not involved but the exfoliative cytology smears made from the nipple discharge had shown adenocarcinoma. Case number B-62 X 1000

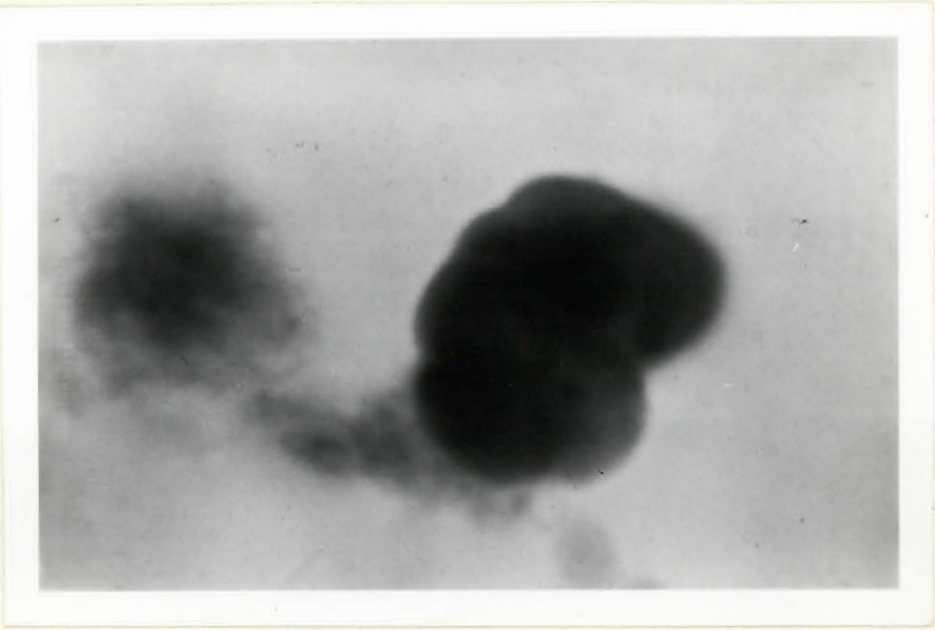

Fig. 22

Two tumour cells are shown. These were found in a peripheral blood sample taken from a patient who, 3 days previously, had sustained a right radical mastectomy for stage I carcinoma of the breast.
Case number B-41 X 2500

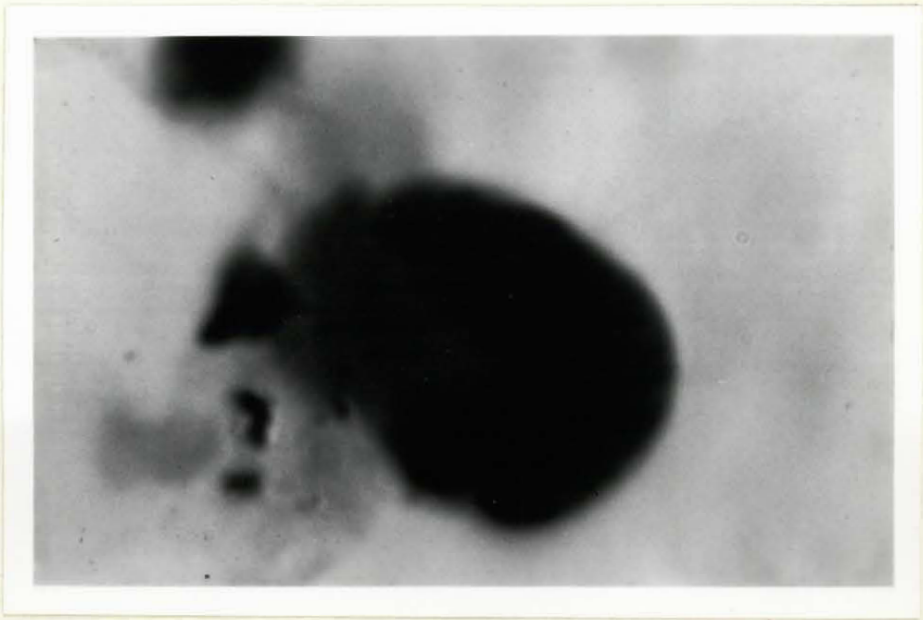

Fig. 23

Tumour cell shown under high power. This tumour cell was found in the peripheral blood of a patient who had had a radical mastectomy 3 years previously for a stage I infiltrating duct carcinoma of the right breast. When this blood sample was drawn, the patient felt well and there were no signs of recurrence. Case number B-7 X 2500

A P P E N D I X D

TABLES SUMMARIZING THE REVIEW OF THE LITERATURE

Table XXI

Summary of the Findings by Various Investigators of Tumour Cells in the Circulating Blood of Patients with Cancer. The Differences in the Incidence of Tumour Cells in Peripheral Blood and Regional Blood are shown

Authors	Date	No. of Cases Total	Peripheral Blood			Regional Blood		
			No. of Cases	Positive	% Positive	No. of Cases	Positive	% Positive
Ashworth	1869	1		1	100%			
Schmidt	1903	41				41	15	36%
Loeper & Louste	1904	?	?	3	?			
Babes & Panea	1905	2		2	100%			
Schleip	1906	2		2	100%			
Aschoff	1906	1		1	100%			
Ward	1913	4	4	1	25%			
Marcus	1919	1		1	100%			
Quensel	1921	50				50	6	12%
Pool & Dunlop	1934	40	40	17	42%			
Gruner	1942	876	876	85	9.7%			
Holland	1949	2		2	100%			
Engell	1955	140	93	17	18.2%	125	75	60%
Fisher & Turnbull	1955	25				25	8	32%
Whang	1958	63	37	0	0%	26	7	27%
Taylor & Vellios	1958	?		4				
Peckholz & Böehm	1958	74				74	10	13.5%
Colombo, Rolfo, & Maggi	1959	42	42	7	16.6%			
Seal	1959	86	86	39	45%			

Table XXII - Cont'd

Authors	Date	No. of Cases Total	Peripheral Blood			Regional Blood		
			No. of Cases	Positive	% Positive	No.of Cases	Positive	% Positive
Coutts et al.	1959	5	5	2	40%			
Morley	1959	3				3	3	100%
Diddle	1959					14	8	57%
Fletcher & Stewart	1959	62	24	4	16.6%	38	15	39%
Reiss	1959	50	50	6	12%	6	3	50%
Finkel & Tishkoff	1960	1		1	100%			
Potter et al.	1960	376	285	79	28%	91	39	43%
Long et al.	1960a	328	297	78	26%	69	22	32%
Moore et al.	1957b	288	179	93	52%	109	60	55%
Salgado et al.	1959	70	60	34	57%	10	4	40%

Table XXII

Summary of the Findings by Various Investigators of Tumour Cells in the Circulating Blood of Patients with Cancer. The Differences in the Incidence of Tumour Cells in the Blood of Patients with "Incurable" and "Curable" Lesions are Shown

Authors	Date	Total			Curable			Incurable		
		No. of Cases	Positive	% Positive	No. of Cases	Positive	% Positive	No. of Cases	Positive	% Positive
Ashworth	1869	1	1	100%				1	1	100%
Schmidt	1903	41	15	36%				41	15	36%
Loeper & Louste	1904								3	
Babes & Panea	1905	2	2	100%				2	2	100%
Schleip	1906	2	2	100%				2	2	100%
Aschoff	1906	1	1	100%				1	1	100%
Ward	1913	4	1	25%				4	1	25%
Marcus	1919	1	1	100%				1	1	100%
Quensel	1921	50	6	12%				50	6	12%
Pool & Dunlop	1934	40	17	42%				40	17	42%
Gruner	1942	876	85	9.7%						
Holland	1949	2	2	100%				2	2	100%
Engell	1955	140	90	63%	126	83	65%	14	7	50%

Table XXII - Cont'd

Authors	Date	Total			Curable			Incurable		
		No.of Cases	Positive	% Positive	No.of Cases	Positive	% Positive	No.of Cases	Positive	% Positive
Fisher & Turnbull	1955	25	8	32%						
Whang	1958	63	7	11.1%						
Taylor & Vellios	1958		4						4	
Peckholz & Boehm	1958	74	10	13.5%				74	10	13.5%
Colombo, Rolfo, & Maggi	1959	42	7	16.6%	20	3	15%	22	4	18.1%
Seal	1959	86	39	45%				86	39	45%
Coutts et al.	1959	5	2	40%				5	2	40%
Morley	1959	3	3	100%				3	3	100%
Diddle	1959	14	8	57%						
Fletcher & Stewart	1959	62	19	30%						
Reiss	1959	50	6	12%	50	6	12%			
Finkel & Tishkoff	1960	1	1	100%				1	1	100%
Potter et al.	1960	376	118	31.4%	192	49	25%	184	69	37%
Long et al.	1960b	475	142	29%	149	33	22%	326	109	33%
Moore & Sako	1959	44					16%			37%
Salgado et al.	1959	70	38	54%				8	2	25%

Table XXIII

Summary of Findings by Various Investigators of Tumour Cells in the Circulating Blood of Patients with Carcinoma of the Breast

Author	Date	Number of Cases	Positive	% Positive
Gruner	1942	207	12	6%
Engell	1955	6	3	50%
Colombo, Rolfo, & Maggi	1959	5	1	20%
Seal	1959	18	6	33%
Fletcher & Stewart	1959	25	6	24%
Reiss	1959	21	3	14%
Potter et al.	1960	36	14	39%
Long et al.	1960a	62	15	24%
Salgado et al.	1959	32	17	53%