"MACROPHAGE PRECURSOR CELLS IN BLOOD FROM HEALTY INDIVIDUALS AND FROM CANCER PATIENTS"

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"MACROPHAGE PRECURSOR CELLS IN BLOOD FROM HEALTHY INDIVIDUALS AND FROM CANCER PATIENTS"

by

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ABSTRACT

An original method for counting macrophage precursors from peripheral blood is presented. The interest in developing this method lay in an unresolved question about macrophage behaviour in patients with cancer.

It is known that patients with advanced cancer have reduced cell-mediated immunity, as shown by poor delayed responses to intradermal injections of a wide range of microbial antigens. Furthermore it has been demonstrated that the cellular response to abrasion of the skin in cancer patients shows a significant reduction in the number of macrophages appearing, when compared with the same response in control individuals. It is likely that the reduced macrophage response and the reduced cell-mediated immune response are causally related. This reduced mononuclear response could be due to a numerical reduction of macrophage precursors in the bloodstream. The objective of this thesis was to investigate this possibility by counting the number of macrophage precursors in the bloodstream.

The method for counting macrophage precursors entails culturing a leukocyte suspension for seven days. During this period of incubation precursor cells develop into macrophages. The macrophage preparations can be fixed *in situ*, stained and the macrophages counted. No preferential loss of any type of white cell was observed during the various stages of preparation of the leukocyte suspension, thus indicating the validity of the method. Furthermore the macrophages are non-dividing cells under these conditions. Tests of reliability of the method were considered satisfactory.

The number of macrophage precursors in 24 healthy adults averaged 3-4% of the white cells and the counts were linearly related to the total number of white cells in the blood. The higher the total number of white cells, the higher the number of macrophage precursors.

In a preliminary study seven patients with cancer were compared with seven healthy control subjects; there was no significant difference in the number of macrophage precursors in the two groups. Thus, if these preliminary results are confirmed, the reduced macrophage emigration onto skin windows that has been observed by others in cancer patients can best be explained by a failure of chemotactic mechanisms. Statistical analysis of the haematologic data in the cancer group as compared with the healthy controls revealed one significant difference. In healthy people the number of macrophage precursors correlated positively with the total white cell count; on the other hand, in the cancer group there was no such correlation. This difference between correlations in healthy controls and cancer patients is statistically highly significant.

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INTRODUCTION

REVIEW OF THE RELEVANT LITERATURE

Macrophages, the topic of this thesis, are part of the system of phagocytes defined by Aschoff in 1924 which he named the Reticulo-Endothelial system (RES). The function of RES cells is well established to be that of scavengers, removing particles, tissue debris, effete cells etc... and indeed it is by such a functional test that the cells of the RES were identified. Thus the function of these cells is relatively non-specific. However macrophages are also essential for the proper functioning of the antigen-specific cells of the immune system, the lymphocytes. Here it is evident that prior treatment of some antigens by macrophages is necessary before the lymphocytes are able to respond to the antigen and mount a specific immune response.

A third involvement of marcrophages in immunity is revealed when we consider the effector function of the specific cell-mediated immune response. It is evident that non-specific cells, including macrophages, play a vital role here; in their absence an expression of cell-mediated immunity, such as the lesion of delayed hypersensitivity, cannot be elicited.

In this thesis we are interested in patients with cancer. Such patients have been shown by many investigators to have reduced cell-mediated immunity (C.M.I.). These results correlate positively in a most interesting manner with reduced macrophage emigration onto the coverslips that are employed in the "skin window" technique. Since

C.M.I. may be important in eliminating tumour cells it seemed worthwhile following these observations. Our intention was to see if the failure of macrophage emigration onto coverslips was due to a reduction in the number of macrophage precursors in the bloodstream, since it is known that macrophages adhering to such coverslips are derived from a circulating precursor cell.

Cancer: Evidence for Beneficial Role of Immune Mechanism.

(a) Spontaneous Regression: Convincingly documented cases of spontaneous regression of cancer (Smithers, 1964; Everson and Cole, 1966) and the more common instances of patients who survive for long periods in apparent biological equilibrium with their cancers suggests that there is some form of control mechanism. Naturally an immunological mechanism has been suggested. Although these regressing cancers are rare, they are nevertheless frequent enough to suggest that sometimes the growth of malignant tumours is slowed and even stopped by some type of inhibitory reaction.

(b) Tumour Antigens: Although research into possible involvement of the immune system started from the end of the last century, there was no great progress initially. The main reason was that the experiments involved transplantation of tumours into genetically different animals, where they were of course rejected like any other homograft. These studies failed to prove that the common transplantable tumours of rodents contained tumour-specific antigens (Woglom, 1929). The first recognition that pure-line mice must be used if reproducible

results were to be obtained in the study of transplantable cancers was made by Little in 1941, and it was only when tumours began to be transplanted into genetically identical (syngeneic) animals that the presence of tumour-specific antigens could be proved.

Neoplastic cells were then found to be antigenically different from the normal tissue cells of their hosts in virtually all experimental tumour systems tested; the appearance of new tumour-specific antigens was clearly demonstrated in the experiments of Foley (1953) and of Prehn and Main (1957).

Tumour-specific antigens have now been demonstrated in a number of human malignancies. For example, carcinoembryonic antigen (CEA) has been found in tumours of the human digestive system (Gold and Freedman, 1965a,b); Burkitt's Lymphoma (Burkitt, 1958) has an antigen resembling Epstein-Barr virus antigen; malignant melanoma has been shown to contain specific antigens (Lewis, 1967; 1969); neuroblastoma has its own antigen (Hellstrom et al., 1968a) and there are others which have been recently reviewed (Piessens, 1970).

(c) Specific Immune Responses Against Tumour Antigens:

(i) Circulating Anti-tumour Antibodies: Since antigens were found in tumours it was natural to enquire if there were immune responses mounted against these antigens. Serologic techniques have been employed extensively to determine tumour immunity in cancer patients. The Grahams in 1955 were the first to demonstrate anti-tumour antibodies in sera of cancer patients. Thereafter numerous investigators employing various laboratory techniques have sought and

reported the presence of antibodies in sera of patients with a variety of neoplasm. In 1965, Gold and Freedman (1965b) reported the presence of circulating antibodies to carcinomas of the digestive system. In 1968, Morton and Malmgrem (1968a) employing immunofluorescence noted a high incidence of antibodies to osteosarcomas in the sera of patients with this disease, others (Lewis, 1967; Morton et al., 1968b; Oettgen et al., 1968) using immunofluorescence techniques, found and reported the presence of circulating antibodies in serum of patients with malignant melanoma.

(ii) Cellular Immune Response in Vitro: Studies in vitro have demonstrated that animals as well as humans can mount cell-mediated immune responses against tumour cells which possess tumour specific tissue antigens.

Levy et al., in 1972 showed that patients with primary intracranial neoplasms, both well differentiated and anaplastic, intra- and extra-cerebral, possessed peripheral blood lymphocytes that were specifically cytotoxic in vitro to tissue cultured, autochthonous tumour cells. These studies also suggested that antigenic cross-reactivity existed between glioblastoma cells from different patients and moreover, between glioblastoma and melanoma cells. Other in vitro techniques, such as colony inhibition have been used extensively by the Hellstroms. In this technique tumour cells are plated onto Petri dishes so as to yield a certain number of colonies per dish. Subsequent to cell attachment, they are exposed to either sensitized (immune) or a variety of control lymphocytes and after several days the colonies

are stained and counted. Blood lymphocytes from patients with neuroblastomas have been found to inhibit the in vitro colony formation of plated neuroblastoma cells, independently of whether the lymphocytes are derived from patients with active disease or from patients who are symptom free following therapy (Hellstrom et al., 1968a, 1970a). Serum from the former group of patients, but not from the latter one, can specifically nullify the inhibitory effect of the lymphocytes, a phenomenon which is probably analogous to efferent immunological enhancement (Hellstrom and Hellstrom, 1970b).

Lymphocytes from patients with a variety of tumours other than neuroblastomas have also been found to inhibit colony formation of plated neoplastic cells of the respective types (Hellstrom et al., 1968b, 1970b,c), and they have been shown to have a direct cytotoxic effect on the tumour cells as well (Chu et al., 1967; Bubenik et al., 1970a;b). Recently, anti-tumour immune responses both humoral and cellular have been extensively reviewed by Piessens, 1970 and Southam, 1971 and will not be elaborated further in this review.

Role of Macrophages in Cell-Mediated Immunity

(a) Origin and Function of Macrophages: The mononuclear cells comprising the macrophage system are characterized by their highly developed capacity to phagocytose foreign particulate and Colloidal materials. Cells which differ in certain functional capacities or in morphological appearance from the typical tissue macrophage are usually included in this hetrogenous system. Cells such as the micro-

glia in the brain and Kupfer cells of the liver are examples of this variation. The term "macrophage" itself is now used fairly generally as a group designation for these various cells (Vernon-Roberts, 1972).

The myelogenous origin of the mononuclear phagocytes of the blood was first demonstrated in radiation chimeras given allogeneic bone marrow cells (Balner, 1963; Goodman, 1964; Virolainen, 1968). The bone marrow origin of tissue macrophages of the rat which attach to subcutaneously implanted glass coverslips was very clearly shown by Volkman and Gowans in 1965a,b. They implanted coverslips subcutaneously in rats and showed that lymphocyte depletion by either chronic drainage from the thoracic duct or 400 rads of X-irradiation failed to suppress the emigration of macrophages onto the coverslips. This showed that the circulating lymphocyte is unlikely to be the precursor of macrophages. Furthermore, X-irradiation of rats with 750 rads suppressed the emigration of the exudate macrophages. Rats were restored to normal when the tibial marrow was shielded during irradiation. They also showed that labelled monocytes were found in the blood of rats which had received injections of labelled bone marrow. These experiments conclusively showed a bone marrow origin for exudative macrophages and provided suggestive evidence that the macrophages might develop from circulating monocytes.

(b) Role of Macrophages in Delayed Hypersensitivity: Delayed Hypersensitivity is essentially a biphasic reaction with an early and transient polymorphonuclear response which is followed by an influx of mononuclear cells as shown by serial histological examination

of the lesions. Many of these mononuclear cells are macrophages, although a variable number of lymphocytes are present. It appears that the effector cells in C.M.I. reactions such as Delayed Hypersensitivity (D.H.) includes these macrophages, however, the lymphocyte is the cell which initiates the whole response. A relatively small number of specifically sensitized lymphocytes react with antigen at the site of injection and are stimulated to synthesize and release factors which elicit inflamnatory reaction and cause the accumulation of monocytic infiltrate from the blood (Kosunen et al., 1963).

Cells of the monocytic infiltrate in D.H. originate from a rapidly proliferating pool of bone marrow precursor cells. In addition, studies with H^3-Thymidine have revealed that the large majority of the cells in D.H. reactions in passively sensitized animals are host cells. Thus 80% of these cells are labelled if H^3-Thymidine is administered to the recipient animals 24 hours before passive transfer (McCluskey et al., 1963).

This nonspecific infiltrate is essential in the pathogenesis of the lesion, since irradiation of the recipient animal previous to the passive transfer of sensitized cells from another animal renders it incapable of exhibiting a D.H. reaction (Coe et al., 1966). Presumably the failure is because of destruction of the bone marrow precursor cells of the blood monocytes.

Non-Tumour Specific Changes in the Immune System in Patients with Cancer.

(a) The Antibody Response: With regard to the antibody producing

capacity of patients with cancer, Southam and colleagues (Southam and Moore, 1954; Southam and Greene, 1958) have shown apparently normal antibody formation following West Nile and other virus infections. Others (Leskowitz et al., 1957) also have reported normal antibody response to pneumococcal polysaccharide in a group of cancer patients. Levin et al. in 1970 compared the production of 7S and 19S antibody in patients with various types of carcinoma, in patients with lymphomatous neoplasms and in healthy controls, at weekly intervals following administration of 17D Yellow Fever live virus vaccine. They reported similar means and ranges of antibody titres in carcinoma patients and controls. However, they showed a slight delay in antibody production in the cancer patients. Other reports have stated results which conflict with those above. Lytton and Hughes in 1964 have reported that serum antibody responses to tetanus toxoid were significantly lower in cancer patients than in healthy controls. Similarly Lee et al. (1970) measured the antibody response to primary immunization with monomeric flagellin from Salmonella adelaide in 61 patients with cancer. Subsequently, the antibody-producing capacity of individual patients was correlated with their survival. Lee et al. reported that in 27 patients suffering from "active" cancer, antibody-producing capacity was significantly depressed ($p < 0.05$) as compared with sick but not cancerous controls; in 13 such patients who survived more than 6 months after immunization, antibody-producing capacity was moderately depressed, whereas in 14 who survived less than 6 months, the capacity was markedly depressed.

In 34 patients with cancer "cured" by surgery and/or radiotherapy, antibody-producing capacity was significantly greater than that of the "hospital" controls and the patients with "active" cancer; however, antibody formation in this group was still significantly less than that of healthy subjects. It may be concluded from these latter reports that some antibody responses in patients with cancer are depressed.

Workers in this field have used different antigens and the patients had different types of neoplasm with varying natural histories. These two discrepancies could be enough to explain the apparent disagreement in the literature.

(b) Specific Cell-Mediated Immunity: Cellular immunity is the basis of both the delayed type hypersensitivity responses (Lawrence, 1959), and the rejection of homografts (Snell, 1963). It is generally thought that cellular immunity to tumour-specific tissue antigens (T.S.T.A.) has a more significant role than humoral immunity as a host defense against neoplasia; a defect in this line of defence may be the major cause for the survival and local spread of neoplasms; antibody is probably of prime importance in preventing metastasis.

To test the cell-mediated immune system in vivo, various methods have been employed. Some of these methods involve: (a) testing the ability of a person to respond to an intradermal challenge with a battery of delayed allergens; (b) testing the ability of the person to reject a homograft or an autochthonous tumour graft or a homograft of tissue cultured cells.

(i) Delayed Hypersensitivity: Solowey et al. (1967) skin tested 150 patients with cancer, with a battery of delayed allergens which included Streptokinase-Streptodornase (SK-SD Lederle) dissolved in normal saline (10 units SK - 2.5 units SD), ppd of tuberculin (0.2 mcg) histoplasmin (NIH standard), diptheria toxoid (Mass. Biological Labs) in doses of 0.008 LF, coccidioidin (University of California, Lot 64) in dilution of 1:100, and mumps skin test antigen (Lilly Laboratories). One hundred ten patients were completely anergic to the test antigens, and of these anergic patients 94 had evidence of metastatic disease at a later operation. In contrase only 14 out of 80 healthy controls proved to be anergic by their test routine. Correlation of responses with the presence or absence of metastatic disease indicated that the anergy of patients with cancer is associated with spread of the tumour beyond local confines, and that there is only a slight difference in D.H. responses in patients with localized cancer when compared with those who are cancer free. The experiments of Logan (1956) showed that the incidence of a positive mumps skin test was low in patients with advanced carcinoma, but normal in patients with early carcinoma.

The development of delayed hypersensitivity to a previously unencountered sensitizing agent was studied by Levin et al. (1964a) in patients with advanced cancer and was compared with findings in patients with non-neoplastic diseases and with healthy control subjects. They induced sensitization by applying 0.02 ml. of 0.05 M dinitro-fluorobenzene (DNFB) in acetone and corn oil solution to the skin for 24 hours. After 10 to 15 days, patch tests with 0.01 M DNFB were applied to determine whether hypersensitivity had been established.

They found that petients with advanced cancer (other than neoplasms of the reticuloendothelial system) and without recent or concurrent anti-cancer treatment, showed a significantly lower incidence of response to DNFB than either healthy controls or patients with debilitating non-neoplastic diseases. Positive tuberculin tests were also less frequent in the cancer group, thus confirming an impairment of cell mediated immunity in patients with advanced cancer.

These experiments taken together indicate that there is a progressive depression in delayed hypersensitivity in patients with cancer which worsens as the tumour spreads.

(ii) Graft Rejection: Kelly et al. (1958), Green and Gorso (1959) and Miller et al. (1963) have described prolonged survival of skin allografts both in patients with Hodgkin's disease or other lymphomas. Snyderman et al. (1960), Gardner and Preston (1962) have conducted similar experiments in patients with non-lymphomatous malignancy with similar findings.

Another approach to testing the ability of patients to reject grafts has been to use <u>in vitro</u> cultured human cancer cells. Patients with advanced cancer exhibited delayed rejection of such transplants (Southam and Moore, 1958; Southam et al., 1957). In another study, Levin and colleagues (1964b) reported that nineteen patients with debility not due to cancer showed no delay in rejection of cultured human cancer cells, thus ruling out simple debility as a cause for the failure of rejection.

(c) Cellular Response to Mechanical Irritation of Skin: Southam and his colleagues have studied the local cellular response to skin abrasion, both by the "skin window technique" of Rebuck and by a more quantitative method. They abraded the skin with sandpaper and then placed either a coverslip or a fluid filled chamber over the lesion. The cellular exudates that appeared were then analyzed.

Dizon and Southam (1963) studied this cellular response in 30 healthy volunteers, 83 patients with advanced cancer, 18 patients with early cancer and 53 patients with diseases other than cancer. They kept the proportion of male and female patients similar in all groups except for the early cancer group in which there were females only. They observed that in healthy controls at 6 hours after abrasion, the cells on the coverslip were almost all neutrophil polymorphonuclear leuchocytes. At 24 and 48 hours the majority of cells were macrophages. In patients with advanced cancer the response at 6 hours was similar to that of the healthy controls, but at 24 and 48 hours, neutrophils were still more abundant than macrophages. Figure 1 illustrates this significant difference between the advanced cancer patients and normal controls. Mean values for the percentages of macrophages present are widely separated at all times after 6 hours, with no overlap of the one standard deviation range and little overlap even at two standard deviations in the 21 and 24 hour data.

In order to see if simple debility was the deciding factor, Dizon and Southam correlated their results with the performance status (PS) of each person tested. PS is a rough but semi-objective indication

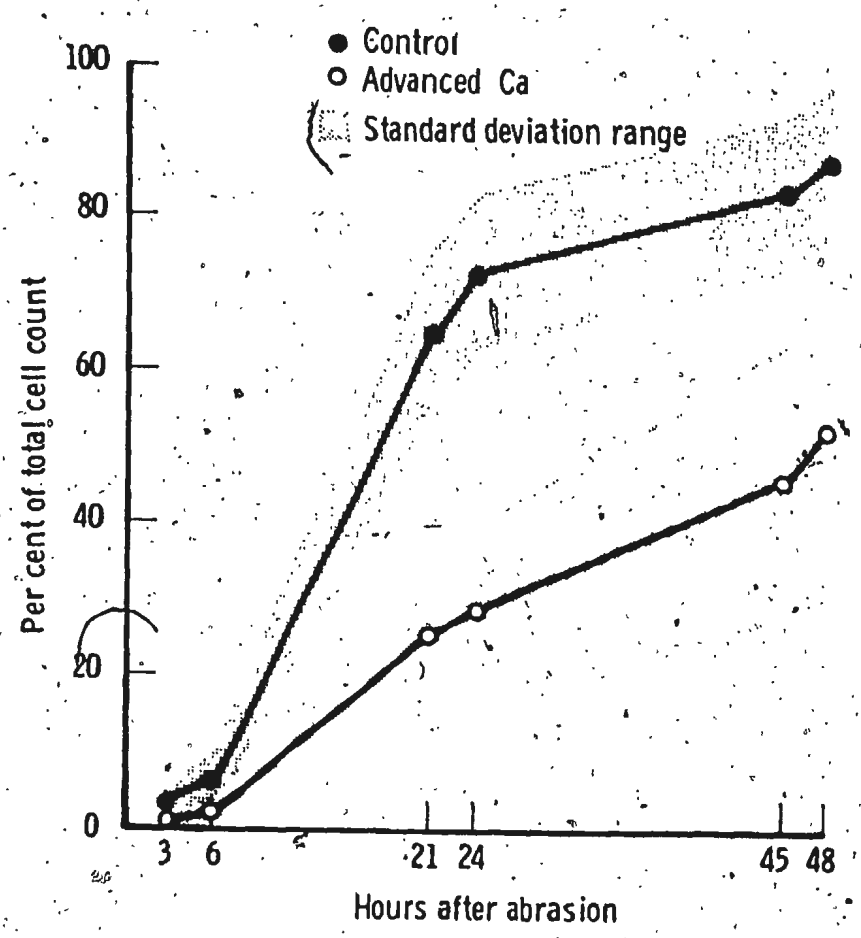

Figure 1. Rebuck Test (modified).
Macrophage responses to sandpaper test in 30 healthy controls and 83 patients with advanced cancer. The shaded areas present the one standard deviation range.

of the over-all status of a patient, reflecting the totality of pathogenic mechanisms that reduce the patient's ability to carry on normal activities. An individual with unlimited activity has a PS of 100; as need for nursing increases, the PS decreases, thus a bedridden patient would have a PS of 50, and a moribund patient a PS of 10. It was shown that with 53 non-cancer patients analyzed in a similar fashion there was no correlation between PS and macrophage response. On the other hand a good positive correlation was found in cancer patients between the various stages of their disease process and their macrophage response. Thus one cannot blame the reduced cellular response on the overall status of physical debility of the patient.

The authors summarized the results of all 4 groups of patients in the graph reproduced on the next page as Figure 2. The contrasting results between the advanced cancer patients and normal controls and the patients with diseases other than cancer can be clearly seen. It is interesting to note that results of 18 early cancer patients lay between those of the normals to those of advanced cancer; this suggests a direct relationship between the stage of cancer and the macrophage response.

Goldsmith et al. (1965) pursued this study further by testing quantitatively as well as qualitatively, the cellular responses in cancer patients. They employed the skin window technique for their qualitative experiments and a Sykes Moore chamber strapped to the forearm for counts of the total number of cells actually emigrating from the abrasion. They tested 13 healthy adults and 32 patients with advanced carcinoma. Their

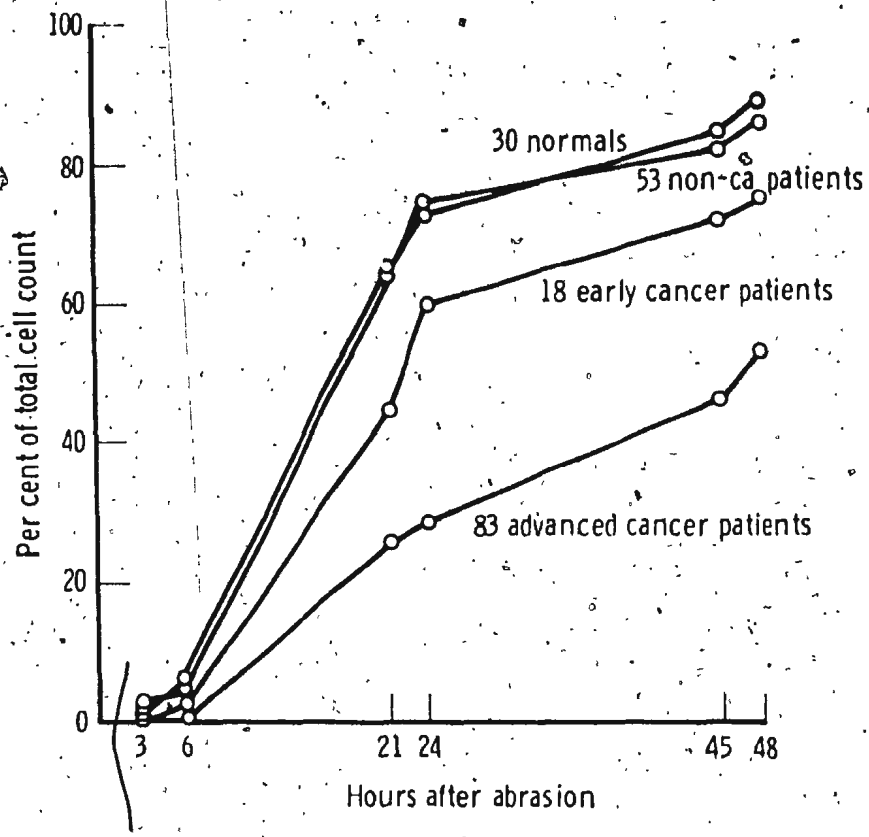

Figure 2. Rebuck Test (modified).
Mean macrophage responses to sandpaper test in each of the study groups.

results are shown in Table 1. It can be seen that there was indeed a lower mononuclear response in patients with cancer both qualitatively and quantitatively. Ninety-five percent of the cells entering the Sykes Moore chamber (presumably this is correct, the original paper is equivocal on this point) resembled macrophages: the remainder were lymphocytes. Although the total number of cells emigrating in the two groups of individuals were similar, the percentage of mono-nuclear cells that was observed was different. This study therefore confirmed the previous report by Dizon and Southam. The quantitative studies not only showed a lower percentage of macrophages but also a lower absolute number of macrophages, establishing that the percentage difference really is due to a defective macrophage response rather than an excessive polymorphonuclear reaction.

The presumed relationship between a defective macrophage response and defective cell-mediated immunity in patients with advanted cancer is discussed in the next section entitled "Objective of This Work."

QUANTITATIVE METHOD		
	Total Cells	Total Mononuclear Cells
32 Patients With Cancer	2.7 Million	0.26 Million
13 Normal	2.8 Million	0.52 Million (95 % Were Macrophages)
QUALITATIVE METHOD		
	(a) Skin Window	(b) Chamber As Above
	% Mononuclear Cells	% Mononuclear Cells
32 Patients With Cancer	42	13
13 Normal	64	27

Table 1. Modified from Goldsmith et al. (1965).
The table shows the cellular responses on cover slips and in Sykes-Moore tissue culture chambers. A reduced mononuclear cell response is evident in patients with cancer with both techniques.

2. OBJECTIVE OF THIS WORK

The preceeding review of the literature has documented that as a group, patients with cancer tend to have impaired cell-mediated immune responses. Furthermore, studies with the "skin window technique" have revealed that patients with cancer show proportionally fewer macrophages in the cellular exudate that is induced by abrasion of the skin. Since mononuclear cells are important for cell-mediated immune responses, these two findings could be linked; that is to say, the reduced cell-mediated immune responses could be due to a defect in emmigration of macrophages from the bloodstream into an area containing antigen. The work in this thesis was aimed at analysing further the reason for the poor macrophage response.

When considering the emigration of macrophages onto a "skin window", the crucial experiments are those of Dizon and Southam (1963). (These experiments have been fully described in the literature review and will only be summarized here.) They abraded the skin of patients and controls with sandpaper and then placed sterile coverslips over the abraded areas. At intervals thereafter, coverslips were removed, stained and the proportion of various cell types present was determined. They reported that patients with advanced cancer showed a proportional deficiency in macrophages on the coverslips as compared to normal healthy controls and non-cancerous patients. Modification of this technique later allowed quantitation of the cellular response (Goldsmith et al., 1965) which confirmed earlier suspicions that there was a reduced mononuclear response in patients with cancer on a quantitative as well

as on a qualitative basis. 95% of the mononuclear cells resembled macrophages and the remaining few were classed as lymphocytes. These quantitative studies showed a lower absolute number of macrophages in the exudate, thus indicating that the percentage difference in earlier experiments was due to a defective macrophage response rather than to an excessive polymorphonuclear reaction.

There are two possible explanations of this reduced mononuclear response:

(1) The macrophage precursors in the bloodstream are numerically reduced.

(2) The macrophage precursors are present in the bloodstream in adequate numbers but the emigration through capillary walls is defective.

Therefore, the object of this work has been to determine the number of macrophage precursors in the bloodstream in such patients. Such a determination could discriminate between the two possibilities mentioned above and would allow rational planning of further research into this general question. First however, we had to devise a reliable method for counting macrophage precursors in peripheral blood.

Methods for growing macrophages from peripheral blood are to be found in the literature; it was simply a question of developing one of these in a quantitative way and then applying it to a series of patients with cancer and a set of controls.

An important paper was written by Berman and Stulberg (1962) who drew attention to the macrophages which appear in peripheral blood cultures. They showed that granulocytes degenerate and disappear leaving

behind a mixture of macrophages and lymphocytes. The difficult question of the identity of the precursor cell for the macrophages was raised by the authors who felt it was most probably a lymphocyte and in a few cases a monocyte. At all events the important conclusion that they made from the point of view of our present experiments, was that the macrophages developed without the occurrence of mitosis and were themselves non-mitotic. Berman and Stulberg stated: "....in hundreds of specimens, probably representing many hundreds of thousands of cells we have not encountered mitotic figures in the macrophages." Evidently Marshall, Rigo and Melman (1966) found the same absence of mitosis in uninfected cultures. Similarly in the experiments reported in this thesis, we have never seen a macrophage in mitosis. Furthermore in an unpublished experiment, Marshall and Simmons (1973, personal communication) counted the number of macrophages per Petri dish from the second day of culture up to the eighth day and found no significant variation in number. This last experiment indicates that there is, in addition, no quantitatively important cell death in these macrophage cultures.

The above experiments and conclusions have reassured us that growing macrophages from peripheral blood and counting them after a number of days in culture is a valid procedure for determining the number of macrophage precursors originally present in the bloodstream: Evidently the cells develop from precursors in vitro without undergoing division.

EXPERIMENTAL SECTION

The aim of the experiments was to grow human blood macrophages in a suitable device and to count them, in order to make comparisons between healthy controls and patients with cancer. We had to devise a method to do this and then had to experiment with the method to see how reliable it was before we could finally use it on patients and controls. The experiments are described in the following sections:

1. BASIC METHODS AND MATERIALS.
2. EXPERIMENTS USING DIFFERENT APPARATUS FOR CULTURING MACROPHAGES.
3. EXPERIMENTS USING DIFFERENT CONCENTRATIONS OF LEUKOCYTES AND VARIOUS VOLUMS OF CELL SUSPENSION.
4. EXPERIMENTS ON THE VALIDITY AND REPEATABILITY OF THE METHOD.
5. EXPERIMENTS TO INVESTIGATE POSSIBLE ADHERANCE OF CELLS TO GLASSWARE.
6. EXPERIMENTS COMPARING AUTOLOGOUS SERUM, AUTOLOGOUS PLASMA AND FETAL CALF SERUM.
7. DETAILS OF THE FINAL METHOD EVOLVED FOR CULTURING MACROPHAGES.
8. ENUMERATION OF MACROPHAGE PRECURSORS IN THE BLOOD OF PATIENTS WITH CANCER AND IN HEALTHY CONTROL SUBJECTS.

1. BASIC METHODS AND MATERIALS.

(a) Taking Blood.

(b) Separation and Centrifugation of Plasma.

(c) Resuspending the White Blood Cells in Tissue Culture Medium.

(i) The Basic Culture Medium

(ii) Sera

(iii) Autologous Plasma

(d) Incubating the Cell Suspension.

(e) Staining and Counting.

(f) Statistical Methods.

1. BASIC METHODS AND MATERIALS.

There are 6 stages in carrying out the experiments. These stages will be described separately. Each manoevure in the laboratory was carried out using standard sterile precautions.

(a) Taking Blood: Blood was obtained from healthy males and females aged 19 - 40 years and for the final experiment from a series of cancer patients whose diseases and ages are documented later. No particular time of day was chosen for the bleeding but it was always between 9 a.m. and 5 p.m. Twenty mls. of blood was withdrawn from an antecubital vein into a 30 ml. syringe containing 2 ml. of a 4 mg. per ml. solution of preservative free heparin-sodium made up as follows: 100 ml. of saline was added to 0.4 gram of heparin-sodium U.S.P. 147 units per mg. (Connaught Medical Research Laboratories, Toronto, Canada) and the dry powder was dissolved. This solution was sterilized by passing it through a millipore filter with pore size 0.22 μ (Millipore, Bedford, Mass. U.S.A.)

After withdrawing 20 ml. of blood into a heparinized syringe, a white cell count was done on this sample in a Neubauer Haemacytometer. Into this heparinized blood, 3 ml. of Dextran '110' injection BP in sodium chloride (Dextran from Leuconostic mesenteroides NCIB 8710, 6% w/v and sodium chloride 0.9% w/v manufactured by Glaxo Laboratories Limited, Greenford, England) was added. The syringe containing blood, Heparin and Dextran was then inverted several times to mix the reagents and was dispensed in equal volumes (12.5 ml. each) into 2 Pyrex brand screw-capped glass culture tubes No. 72-9826, 15 mm. internal diameter

with rounded bottom (manufactured by Corning Glassworks, Corning, New York, U.S.A.). This mixture was allowed to sediment for 2 hours at 37°C.

(b) Separation and Centrifugation of Plasma.

The leucocyte rich supernatant plasma was removed as completely as possible with a Pasteur pipette and was put into 15 ml. screw-capped centrifuge tubes (No. 12-8082 manufactured by Corning Glassworks). A white blood cell count was done on the plasma and the total volume recorded. The leukocyte-rich plasma was then spun for seven minutes at 2000 r.p.m. at room temperature in a centrifuge (International Model HN, Needham Hts., Mass. U.S.A.), a procedure which sediments the white blood cells and leaves the majority of the platelets in suspension. After centrifugation, the supernatant was removed with a Pasteur pipette and was replaced with an appropriate amount of tissue culture medium.

(c) Resuspending the White Blood Cells in Tissue Culture Medium.

(i) The basic culture medium was Eagle's minimum essential medium (Eagle, 1959) except that it contained Earle's salt solution (Earle, 1943) as a base (obtained from BBL-BioQuest, Cockeysville, Maryland, U.S.A.). Into 100 ml. of this medium, 1 ml. of a solution containing 10 mM of L-glutamine, and 2 ml. of a solution containing 20,000 u penicillin and 20,000 mcg. streptomycin were added.

(ii) Sera: Human umbilical cord blood was collected from fresh placentas from the case room of a local hospital. The serum was pooled, inactivated by heating at 56°C. for half an hour and sterilized by filtration through millipore filter (size 0.22 μ) in swinnex-25 filters.

This was referred to as human umbilical cord serum (H.U.C.S.). Equal volumes of MEM and H.U.C.S. were mixed and were used as the standard final medium for some experiments. In other experiments, we used foetal calf serum obtained from BBL-BioQuest, again in 50:50 concentrations. These mixtures are referred to hereafter as final tissue culture medium (F.T.C.M.).

(iii) Autologous Plasma: After the white blood cells were spun down from plasma and the supernatant removed, the platelet-rich plasma supernatant was spun for 10 minutes at 3400 r.p.m. at 4°C. (International Equipment Co., model PR-6, Needham Hts., Mass. U.S.A.), a procedure which sediments the platelets from the plasma. This plasma was used for preparing our final tissue culture medium after experiment number 69.

(d) Incubating the Cell Suspension.

Appropriate volumes of F.T.C.M. were added to the cell pellet with a sterile pipette and the sedimented cells were resuspended gently. Four or five ml. of this suspension, containing the required number of white blood cells, was placed in "Integrid" Dish No. 3030, size 60 X 15 mm. style with 2 mm. grid (Falcon Plastic Division of Becton Dickinson and Company, Los Angeles, CA.) or in other tissue culture vessels as indicated by the experiments. Culture vessels were placed in large glass Petri dishes 5.5 inches in diameter containing moist gauze sponges in order to prevent evaporation of the culture medium. Cultures were placed in an atmosphere of 5% CO_2 in air at 37°C. for 7 days.

(e) Staining and Counting.

After 7 days of incubation, the culture medium and non-adherent cells were poured off. Methyl alcohol was poured in and left for 5 minutes to fix the cells. The cell layer was then stained for ten minutes in a 1 in 10 dilution of Giemsa stain in phosphate buffer pH 6.8 (SO-G-28, Fisher Scientific Company). After the stain had been poured off, the layer was rinsed in buffer for 30 seconds and air dried. Counts of macrophages were made in seven selected areas (Fig. 1); in each case an area of 0.25 sq. mm. was examined and counted using X10 objective, X12.5 eyepiece in a monocular microscope head and with the help of a net micrometer in the eyepiece. From these counts and from a knowledge of the area covered by the net micrometer, it was possible to calculate the total number of macrophages per dish.

Differential counts on the blood or on the original cell suspension were done either on stained smears and/or in wet preparations in coverslip chambers (Schrek, 1958) which were examined in an inverted phase-contrast microscope. With this last method, it was possible to differentiate between polymorphs and monocytes on the one hand, and lymphocytes on the other. With stained smears, it was possible to differentiate between polymorphs and monocytes and lymphocytes.

Stained smears were made from Heparinised blood, plasma and cell suspesnion in two ways: (1) By placing a drop of cell suspension on a slide and dragging this drop behind the edge of a second glass slide held at an angle to the first and trimmed so that it was slightly narrower than the first (Dacie and Lewis, 1968); (2) By placing a drop of cell suspension

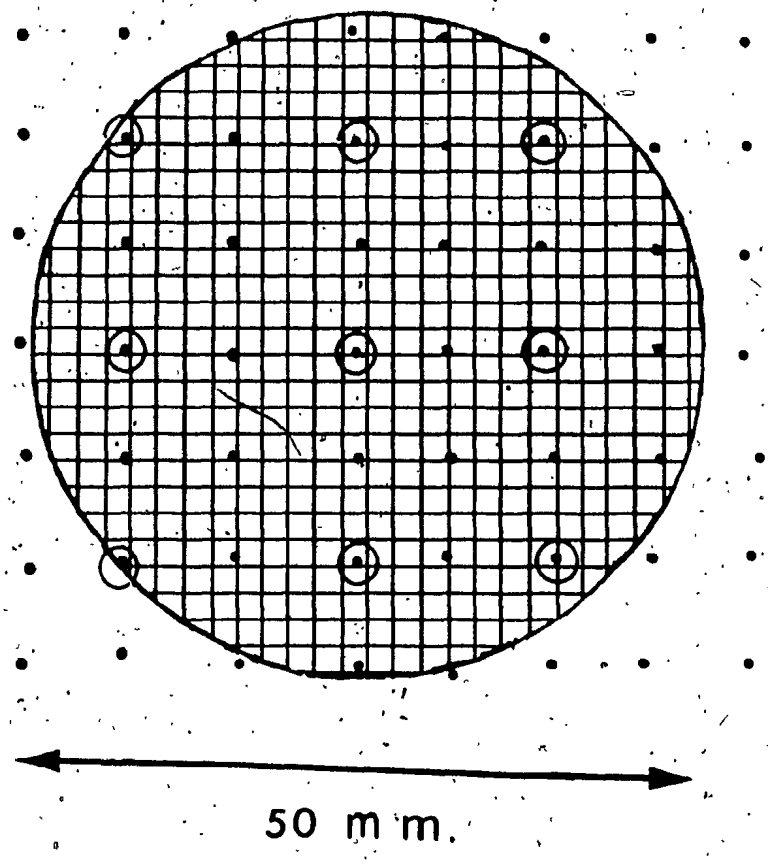

Figure 1: shows how seven randomly selected areas of the Petri dish are chosen for counting. The Petri dish is superimposed on a paper marked with dots in a 1 cm. square pattern. The areas chosen for counting are circled (2 circled areas on the edge of the plate are rejected).

on a glass slide and spreading it by hand with the point of a pin or capillary tube. Smears were air dried immediately and fixed in methyl alcohol for five minutes and stained in a 1 in 10 dilution of Giemsa stain (SO-G-28, Fisher Scientific Company) in phosphate buffer pH 6.8 for 10 minutes, then rinsed in buffer for 30 seconds, air dried and mounted with a coverslip.

(f) Statistical Methods.

(i) Analysis of Variance: For this analysis the textbook of Hope (1967) was used and the method outlined in Chapter 3 was applied.

(ii) Student's Test: For these calculations a Hewlett-Packard model 10 STAT PAC calculator was used which had been programmed to calculate the t test of means of two samples according to the method of Brownlee (1965).

(iii) X^2 Chi Square Test: For these calculations a Hewlett-Packard model 10 STAT PAC calculator was used which had been programmed to calculate the X^2 Chi Square Test according to the method of Freund (1962).

(iv) Covariance and Coefficient of Correlation: For these calculations a Hewlett-Packard model 10 STAT PAC calculator was used which had been programmed to calculate the covariance and coefficient of correlation according to the method of Hald (1960).

(v) Two-Variable Linear Regression: For drawing my graphs a Hewlett-Packard model 10 STAT PAC calculator was used which had been programmed to calculate the two-variable Linear regression.

2. EXPERIMENTS USING DIFFERENT APPARATUS FOR CULTURING MACROPHAGES.

(a) Procedure and Results.

(i) Trial of Terasaki Plates.

(ii) Trial of Drilled Perspex Plates.

(iii) Trial of Leighton Tubes.

(iv) Trial of Plastic Rings of Different Diameter.

(v) Trial of Integrid Petri Dish.

(b) Conclusion.

2. EXPERIMENTS USING DIFFERENT APPARATUS FOR CULTURING MACROPHAGES.

(a) Procedure and Results.

In nine experiments, the development of macrophages in various types of vessel was studied in order to find the best one for the proposed clinical experiments.

(i) Trial of Terasaki Plates: It was thought that a device such as a Terasaki plate (Figure 1) would be ideal for growing and counting macrophages. In experiments numbers 1, 2, 3, we tried these plates and found that macrophages did grow well in them. However, the sides of the wells are not vertical and a proportion of the macrophages adhered to the sloping sides. This made the counting entirely impossible.

(ii) Trial of Drilled Perspex Plates: A rectangular piece of perspex sheet 3 mm. thick was drilled with holes of various diameters from 1/8 inch to 3/8 inch (Figure 2). These plates were placed on top of glass slides and sealed in place with either petroleum jelly ("Vaseline") or with melted paraffin wax taking care to prevent the material from entering the holes. A small amount of culture medium was put into each hole to fill the capillary space between the drilled perspex and the glass slide; this was to prevent cells from flowing into this capillary space when a cell suspension was put in the wells.

In experiments number 4, 6, 8, we placed approximately 0.2×10^6 cells in each well. Cell survival was poor, however, as there was a tendency for the cultures to dry out and the cells that did survive were not found in discrete circular spots beneath each hole, as had been hoped. Therefore, further trials with this technique were abandoned.

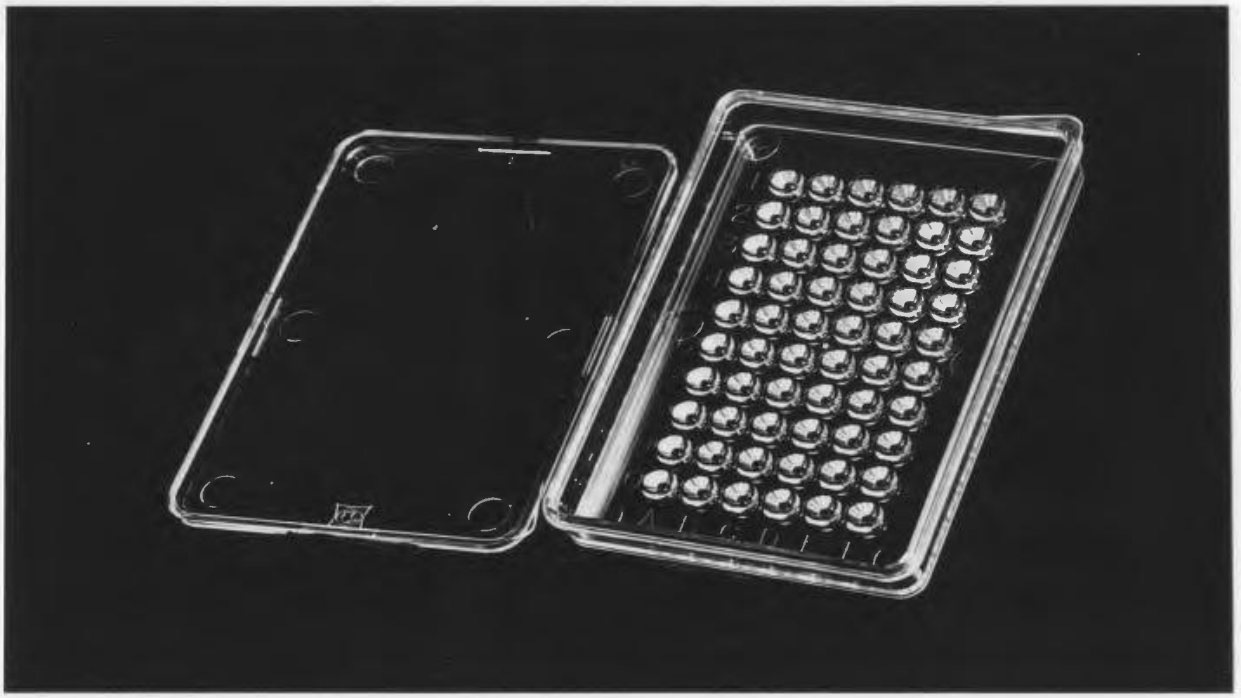

Figure 1. Photograph of a Terasaki plate: shows 60 small wells for cell culture together with a lid.

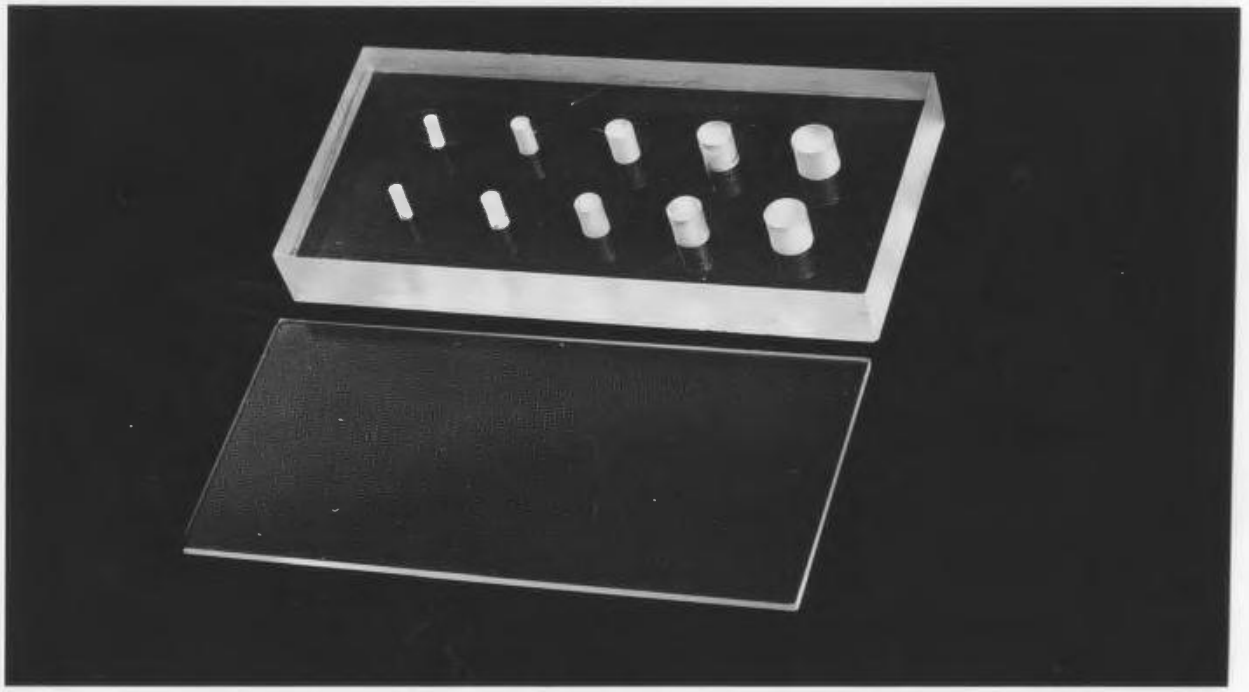

Figure 2. A drilled perspex plate of 3 mm. thickness. This plate was placed on top of a glass slide and sealed in place with petroleum jelly ("Vaseline"). Cell suspension was placed in the holes.

(iii) Trial of Leighton Tubes: In experiments 4, 5, 6, 7, 8, 9, 10, 11, 12, we placed 1 ml. volumes of cell suspension with concentrations between 4.25×10^6 - 11.250×10^6 Leucocytes per ml. in Leighton tubes (Figure 3). Macrophages grew well in all of these tubes at all the concentrations used. However, the glass floor of a Leighton tube is not completely flat and the macrophages were unevenly distributed, larger numbers were found in the peripheral gutters and fewer were to be seen in the centre of the flattened area. For this reason and because of the indifferent optical conditions that these tubes offered, it was difficult to get good counts of macrophages on stained preparations. In one experiment (No. 7), we therefore placed narrow coverslips in Leighton tubes to overcome the counting problem; no clear advantage was gained, however.

(iv) Trial of Plastic Rings of Different Diameter: Another way to get small well defined circular spots of macrophages onto glass slides that was tried was to have plastic rings of suitable size in which to hold the cell suspension. Rings were cut from plastic tubing between 1/8 inches and 1/2 inches internal diameter. These were mounted on glass microscope slides (see Figure 4). A small amount of petroleum jelly was applied at the edges of each ring as it rested on a slide, the slide was then passed over a flame; this procedure melted the jelly and allowed it to form a seal between the ring and the slide.

In one experiment (No. 8) we placed between 0.1×10^6 - 0.4×10^6 Leucocytes suspended in 0.1 ml. - 0.4 ml. tissue culture medium in a series of these rings. The problems associated with this method were:

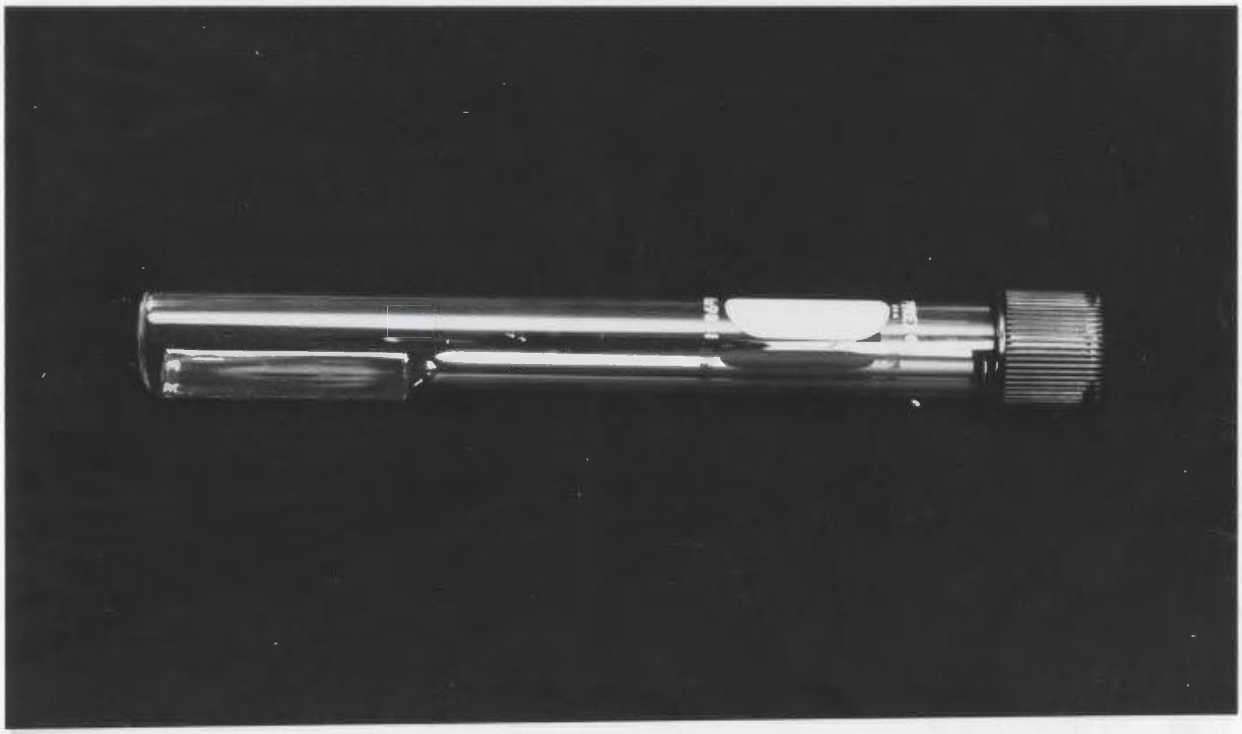

Figure 3. A Leighton tube.

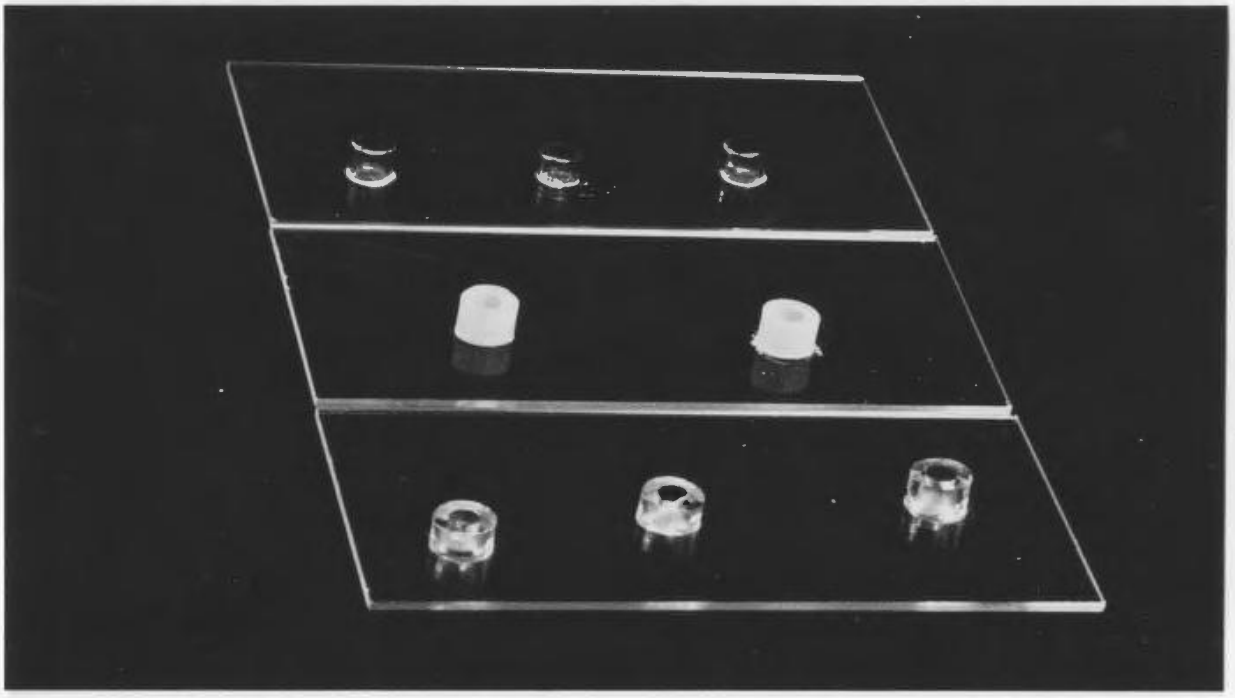

Figure 4. Plastic rings of different diameter stuck to a glass slide were used as chambers for growing macrophages.

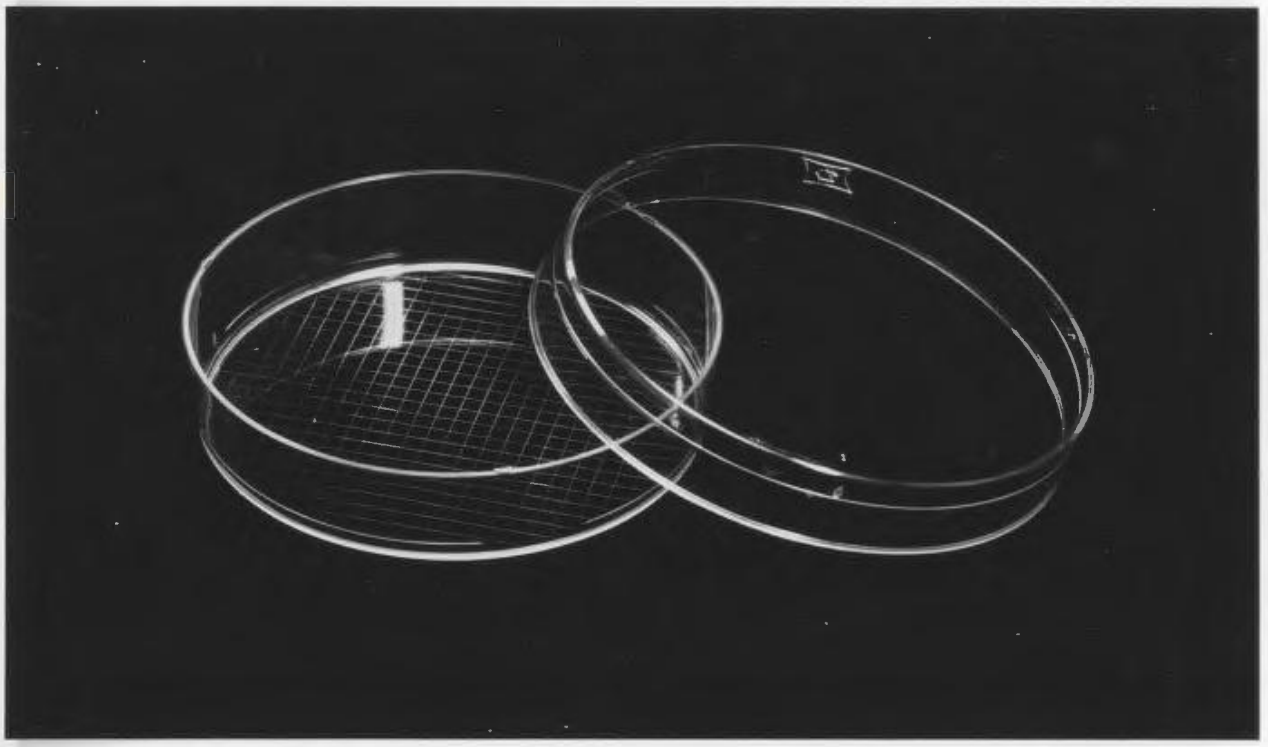

Figure 5. Integrid Petri dish with lid.

(1) a tendency for the medium to dry up; (2) small irregular patches of cells grew indifferently well. We were, therefore, discouraged from making further trials particularly since the Petri dishes (see below) by now had worked very much better.

(v) Trial of Integrid Petri Dish: In experiments 9, 10, 11, 12, we used "Integrid" plastic Petri dishes (Figure 5), and placed in them between 22.5×10^6 - 45×10^6 Leucocytes suspended in various volumes of culture medium. The result was that macrophages developed well and could be stained and counted satisfactorily. In general, the macrophages were distributed evenly and in particular, the distribution was much better than it had been in the Leighton tubes.

(b) Conclusion.

During the initial 12 experiments, various different culture vessels were tried. The best growth of macrophages was observed in Falcon Integrid No. 3030 plastic Petri dishes and in Leighton tubes. However, the only device in which macrophages could be satisfactorily counted was the Integrid Petri dish.

3. EXPERIMENTS USING DIFFERENT CONCENTRATIONS OF LEUKOCYTES AND VARIOUS VOLUMES OF CELL SUSPENSION.

(a) Procedure.

(b) Results.

(c) Conclusion.

3. EXPERIMENTS USING DIFFERENT CONCENTRATIONS OF LEUKOCYTES AND VARIOUS VOLUMES OF CELL SUSPENSION.

(a) Procedure.

In experiments (No's. 10, 11, 12, 14, 15, 16, 17, 18, 19, 21, 22, 25) the effect of varying the number of cells per culture dish was tested. Various numbers between 1.95 million and 45 million per dish were used. Here we were looking for optimal conditions where the cells would be in a healthy looking state and at the same time the population would be satisfactorily spread around the dish for counting, not overlapping nor clumped and not so sparse as to make counting inaccurate. It appeared that "healthy" macrophages were mostly well rounded with the cytoplasm spread out in a disc shape; on the other hand when culture conditions had deteriorated, the cells formed into elongated densely stained stringy shapes.

In the course of these experiments we also varied the volume of cell suspension from 2-5 mls. per dish to try and define an optimal volume.

(b) Results

The results of these experiments are summarized in Table 1 in which the experiments are arranged in order of increasing total number of white cells per Petri dish. It was clearly seen that the best looking cells were observed in the highest concentrations of white cells, but this also had its disadvantage by making the cells very difficult to count. For this reason we decided that the optimal number

TABLE 1.

EXPERIMENT NO.	NUMBER OF WHITE BLOOD CELLS/ml. IN MILLIONS IN CELL SUSPENSION	NUMBER OF ml./ DISH	TOTAL WHITE BLOOD CELLS PER DISH - IN MILLIONS	APPEARANCE OF MACROPHAGES AFTER INCUBATION	SUITABILITY FOR COUNTING
25	.650	3	1.950	Very Poor	Very Poor (too few)
25	.650	4	2.600	Very Poor	Very Poor (too few)
21	.675	4	2.700	Fair	Satis.
22	.800	4	3.200	Poor	Good
17	.870	4	3.550	Fair	Satis.
19	1.175	4	4.700	Good	Satis.
16 a	1.400	4	5.600	Poor	Very Poor (too few)
18	2.000	4	8.000	Good	Satis.
16 b	1.650	5	8.000	Fair	Poor
18	2.000	5	10.000	Very Good	Satis.
14	3.950	3	11.800	Fair	Satis.
15	3.250	4	13.000	Poor	Satis.
14	3.950	4	15.800	Good	Satis.
15	3.250	5	16.250	Poor	Satis.
11	4.250	4	17.000	Fair	Satis.
10	11.250	2	22.50	Good	Satis.
12	8.300	4	33.200	Good	Poor
10	11.250	3	33.75	Very Good	Poor
12	8.300	5	41.500	Good	Very Poor (too many)
10	11.250	4	45.000	Very Good	Very Poor (too many)

of cells per Petri dish would be 20-25 million cells, given that the number of macrophage precursors is in the normal range.

In these 12 experiments, we tried volumes of cell suspensions ranging from 2 to 5 mls. The Petri dishes which had 2 or 3 mls. of cell suspension had the tendency to dry up in the CO_2 incubator during the 7 day incubation period. We concluded from performance of these tests that 4 mls. of culture would be the most suitable for our purpose.

(c) Conclusion.

The conclusions drawn from these 12 experiments were that: (a) for best results, the volume of cell suspension should not be less than 4 ml. per Petri dish, and (b) that a satisfactory number of leucocytes was $20\text{-}25 \times 10^6$ per Petri dish. Under these conditions, the macrophages appeared in good condition and were satisfactorily distributed for counting purposes.

4. EXPERIMENTS ON THE VALIDITY AND REPEATABILITY OF THE METHOD

(a) Introduction

(b) Experiments to show the fate of cells during the preparative procedure

(i) Introduction

(ii) Experiments to prepare a balance sheet showing distribution of cells by total counts only

Procedure

Results

Conclusion

(iii) Experiments to prepare a balance sheet showing the distribution of cells by differential count

Introduction

Data Record Experiment 37

Data Record Experiment 39

Data Record Experiment 40

Discussion of Pooled Data from the three experiments

Conclusion

(c) Repeatability of the method

(i) Introduction

(ii) Procedure (Experiment 36)

(iii) Results

(iv) Procedure (Experiment 39)

(v) Results

(vi) Conclusion from both experiments

(d) Normal values from a series of healthy subjects.

4. EXPERIMENTS ON THE VALIDITY AND REPEATABILITY OF THE METHOD

(a) Introduction

The method we developed for culturing and counting macrophages involved a step where red cells and white cells were separated by sedimentation in the presence of Dextran. Our cultures used only the leukocyte-rich plasma supernatant. Thus, it was important to find out what proportion of the white cells we were dealing with and to see if there was a preferential loss of any cell type during the sedimentation procedure; this is to say, we had to test the validity of our method. In addition, it was necessary to gain information about the repeatability of the method.

To test the validity of the method, we tried to establish the fate of all the white cells in 4 separate experiments (No.'s 34, 36, 37, 39 and 40). To test the repeatability of the method, we performed duplicate experiments on two occasions (No.'s 36 and 39). In these experiments, we followed the procedure as described under Basic Methods and materials.

(b) Experiments to show the fate of cells during the preparative procedure

(i) Introduction: the objectives of this section were (1) to trace quantitatively the fate of white cells, and to prepare a balance sheet showing their distribution; (2) to trace particular types of cells by differential counts at every step of the method, to see if any particular type of cell was lost preferentially.

(ii) Experiments to prepare a balance sheet showing distribution of cells by total counts only: In this section experiment No. 34 is described fully. However we have included the results of

experiments No. 36a, b, 37 & 39a, b, and 40 in a final table in order to show average results. The details of these latter 4 experiments are given in section 4b(iii) and in 4c. It should, however, be mentioned that this analysis of cell loss was routinely used in all our experiments hereafter including the experiment on patients with cancer (see Tables 5 and 6 in section B part 8).

Procedure: (Experiment No. 34) 20 ml blood was taken from G.K., and a cell suspension containing 5.35×10^6 cells per ml was prepared as described under "Basic Methods and Materials". 4 ml of this suspension containing a total of 21.40×10^6 cells was put in each of 3 integrid petri dishes which were incubated for 7 days and then stained. White blood cell counts were done at different stages; on whole blood, on the initial plasma cell suspension, on the red blood cells plus Dextran sediment, on the discarded plasma after presumed removal of the leukocytes by centrifugation and on the final cell suspension.

Results: The results are displayed in Tables 1-4.

TABLE 1. COUNTS PER ML. (CONCENTRATION OF CELLS) AT VARIOUS STAGES OF THE EXPERIMENT

White Cell Count Per ml. Heparinized Blood (Millions)	White Cell Count Per ml. Plasma (Millions)	White Cell Count Per ml. of R.B.C. Sediment (Millions)	White Cell Count Per ml. Final Cell Suspension (Millions)	White Cell Count Per ml. of Discarded Plasma
5.675	7,650	2.060	5.350	5,000

TABLE 2. TOTAL (ABSOLUTE) COUNTS AT VARIOUS STAGES OF THE EXPERIMENT

Number of White Cells in 22 ml. of Heparinized Blood (Millions)	Number of White Cells in 13.4 ml. Plasma (Millions)	Number of White Cells in 11.6 ml. R.B.C. Sediment (Millions)	Number of White Cells in 20 ml. Final Cell Suspension (Millions)	Number of White Cells in 12.4 ml. Discarded Plasma
124.85 (22.0 ml.)	102.51 (13.4 ml.)	23.94 (11.6 ml.)	107.00 (20.0 ml.)	620,000 (12.4 ml.)

TABLE 3. BALANCE SHEET SHOWING DISTRIBUTION OF CELLS. (Figures in Millions)

Blood Collected Contains (Millions)	Plasma & R.B.C. Dextran Sediment (Millions)	Cell Suspension Plus Discarded Plasma Plus R.B.C. Sediment (Millions)
Total 124.85	Total 126.45	Total 131.56

Table 1 simply shows the cell concentration in the various suspensions examined during the course of an experiment. These counts become meaningful in Table 2 when they are converted to absolute numbers, that is to say, total count for each suspension. It can be seen in Table 2 that about 80% of the cells present in the blood sample are recovered in the plasma suspension, with some 20% sedimenting with the red cells and becoming lost. The number of cells remaining in the supernatant after centrifugation is small, amounting to just over half a million or around 0.5% of the total. Table 3 shows the result of summing the various

fractions for comparison with numbers originally present in the blood sample. Theoretically, the number of cells in each of the three columns should be the same; that they are not is presumably due to errors inherent in the counting techniques.

The result of this "balance sheet" approach in four subsequent experiments is shown in Table 4. The pooled data indicates that there has been a loss of around 10% of the cells during dextran sedimentation in an unknown manner. However, it is reassuring to note that there was no loss of cells during the latter stages in the procedure.

* TABLE 4. BALANCE SHEET SHOWING DISTRIBUTION OF CELLS (IN MILLIONS)

Blood Collected Contains	Plasma & R.B.C. Sediment	Cell Suspension Plus Discarded Plasma Plus R.B.C. Sediment
TOTAL	TOTAL	TOTAL
36a 63.75	48.89	42.57
b 63.75	52.55	44.68
37 170.50	169.70	163.60
39a 59.10	48.72	57.00
b 65.20	51.80	50.70
40 100.00	89.70	102.00
mean 99.10	90.10	90.77
% 100	91	92

* For the above calculations of mean and % the averages of a and b have been used in experiments 36 and 39.

Conclusion: Taking only total cell counts, it is shown that 20% of the leukocytes are lost in the red cell sediment, and about 0.5% are lost in the centrifugation step. In some experiments, there

appear to be 10% of leukocytes lost during the Dextran sedimentation which are not accounted for; the possibility that the loss was due to cells adhering to the glass tubes was explored in experiments described later.

(iii) Experiments to prepare a balance sheet showing the distribution of cells by differential count.

Introduction: This section includes differential counts to see if any particular type of cell was preferentially lost from the suspensions during sedimentation of the red blood cells and the subsequent experimental manipulations. In this section we have included the results of experiments No. 37, 39a, b, and 40. It should however, be mentioned that experiment No. 39 was performed in duplicate to test the repeatability of the method and some of the results will also be discussed under that heading.

The outlines of the three experiments together with the accumulated data for each will be presented separately next. The combined results from these three experiments will then be described together in the text.

Data Record Experiment 37: A cell suspension containing 6.35×10^6 wbc/ml. was prepared from subject J.C. and was put in integrid Petri dishes and incubated as under "Basic Methods and Materials".

TABLE 5. COUNTS PER ML. (CONCENTRATION OF CELLS) AT VARIOUS STAGES OF THE EXPERIMENT

White Cell Count Per ml. Heparinized Blood (Millions)	White Cell Count Per ml. Plasma (Millions)	White Cell Count Per ml. R.B.C. Sediment (Millions)	White Cell Count Per ml. Final Cell Suspension (Millions)	White Cell Count Per ml. Discarded Plasma
7.750	9.750	3.200	6.350	28,000

TABLE 6- TOTAL (ABSOLUTE) COUNTS AT VARIOUS STAGES OF THE EXPERIMENT

Number of White Cells in 22 Ml. Heparinized Blood (Millions)	Number of White Cells in 13.7 Ml. of Plasma (Millions)	Number of White Cells in R.B.C. Sediment (Millions)	Number of White Cells in Final Cell Suspension (Millions)	Number of White Cells in Discarded Plasma
170.50 (22.0 ml.)	133.57 (13.7 ml.)	36.16 (11.3 ml.)	127.00 (20.0 ml.)	383,000 (13.7 ml.)

TABLE 7 BALANCE SHEET SHOWING DISTRIBUTION OF CELLS. (FIGURES IN MILLIONS)

Blood Collected Contains	Plasma & R.B.C. Sediment	Cell Suspension Plus Discarded Plasma Plus R.B.C. Sediment
TOTAL 170.50.	TOTAL 169.70	TOTAL 163.60

TABLE 8-11 PROPORTIONS OF CELLS IN BLOOD, PLASMA, FINAL CELL SUSPENSION, R.B.C. SEDIMENT

Abbreviations: Polys. = Polymorphonuclear Leukocytes.

Lymphos. = Lymphocytes.

TABLE 8

PERIPHERAL BLOOD: Total 7.75 x 10^6 w.b.c./ml.	PERCENTAGE	TOTAL NO. OF CELLS (22.0 mls.)
100 cells were counted		(Millions)
Polys.	66%	112.53
Lymphos.	31%	52.85
Monocytes	3%	5.11

TABLE 9

PLASMA: 9.75 x 10^6 w.b.c./ml.	PERCENTAGE	TOTAL NO. OF CELLS IN PLASMA, (13.7 ml.)
99 cells were counted		(Millions)
Polys.	63%	84.15
Lymphos.	34%	45.41
Monocytes	2%	2.67

TABLE 10

RED BLOOD CELL SEDIMENT 3.20 x 10^6 w.b.c./ml.	PERCENTAGE	TOTAL NO. OF CELLS IN R.B.C. SEDIMENT (11.3 ml.)
100 cells were counted		(Millions)
Polys.	72%	26.06
Lymphos.	27%	9.77
Monocytes	1%	0.36

TABLE 11

FINAL CELL SUSPENSION 6.35×10^6 w.b.c./ml.	PERCENTAGE	TOTAL NO. OF CELLS IN CELL SUSPENSION (20 ml.)
219 cells were counted		(Millions)
Polys	68%	86.36
Lymphos.	31%	39.37
Monocytes	1%	1.27

TABLE 12 MACROPHAGE COUNTS

Counts of Macrophages expressed as number per Petri dish	Number of Macrophage Precursors per ml. Blood	Percentage of Macrophage Precursors in Blood
984,283	300,320	3.9%

TABLE 13

	% LOSS OF CELLS IN EACH SUSPENSION AS COMPARED WITH TOTAL CELLS IN THE WHOLE BLOOD SAMPLE.	
	Plasma Cell Suspension	Final Cell Suspension
Poly.	25	23
Lymph.	14	25
Mono.	48	75

NB. These results are summarized and discussed after the records of results from experiments 39 & 40 have been presented.

Data Record Experiment 39: A subject, S.S., was bled on two occasions with an interval of 10 minutes between, from different veins and using different syringes. The two samples are designated as "a" and "b". The white blood cell counts on the two samples were 5.375×10^6 wbc/ml. and 5.925×10^6 wbc/ml. respectively. Cell suspensions were prepared from each sample and were found to contain 4.75×10^6 wbc/ml. and 4.25×10^6 wbc/ml. Two integrid Petri dishes were prepared from each suspension and were cultured and stained as in "Basic Methods and Materials".

TABLE 14. COUNTS PER ML. (CONCENTRATION OF CELLS) AT VARIOUS STAGES OF THE EXPERIMENT

	White Cell Count Per ml. Heparinized Blood (Millions)	White Cell Count Per ml. Plasma (Millions)	White Cell Count Per ml. R.B.C. Sediment (Millions)	White Cell Count Per ml. Final Cell Suspension (Millions)	White Cell Count Per ml. Discarded Plasma
a*	5.370	5.000	2.350	4.750	12,500
b*	5.920	5.600	2.100	4.250	7,500

* "a" and "b" refers to the two cell suspensions prepared for this experiment.

TABLE 15. TOTAL (ABSOLUTE) COUNTS AT VARIOUS STAGES OF THE EXPERIMENT

	Number of White Cells in 11 ml. (Each) Heparinized Blood (Millions)	Number of White Cells in 7.3 ml. Plasma (Millions)	Number of White Cells in R.B.C. Sediment (Millions)	Number of White Cells in 9.2 ml. Final Cell Suspension (Millions)	Number of White Cells in Discarded Plasma
a	59.13 (11.0 ml.)	36.50 (7.3 ml.)	12.22 in (5.2 ml.)	43.94 (9.2 ml.)	850,000 (6.8 ml.)
b	65.18 (11.0 ml.)	40.88 (7.3 ml.)	10.92 in (5.2 ml.)	39.31 9.2 ml.)	510,000 (6.8 ml.)

TABLE 16. BALANCE SHEET SHOWING DISTRIBUTION OF CELLS. (FIGURES IN MILLIONS)

	Blood Collected Contains	Plasma & R.B.C. Sediment	Cell Suspension Plus Discarded Plasma Plus R.B.C. Sediment
	Total	Total	Total
a	59.10	48.72	57.00
b	65.20	51.80	50.70

TABLES 17-26. PROPORTIONS OF CELLS IN BLOOD, PLASMA, FINAL CELL SUSPENSION, R.B.C. SEDIMENT.

TABLE 17

PERIPHERAL BLOOD (a): TOTAL 5.375 x 10^6 wbc/ml	PERCENTAGE	TOTAL NUMBER OF CELLS IN BLOOD (11.0 ml.)
148 cells were counted		(Millions)
Polys.	59%	34.88
Lymphos.	39%	23.06
Monocytes.	2%	1.18

TABLE 18

PERIPHERAL BLOOD (b): TOTAL 5.925 X 10^6 wbc/ml	PERCENTAGE	TOTAL NUMBER OF CELLS IN BLOOD (11.0 ml.)
212 cells were counted		(Millions)
Polys.	58%	37.80
Lymphos.	38%	24.76
Monocytes.	4%	2.61

TABLE 19

PLASMA (a) TOTAL 5.0 x 10^6 wbc/ml	PERCENTAGE	TOTAL NUMBER OF CELLS IN PLASMA (7.3 ml.)
95 cells were counted		(Millions)
Polys.	60%	21.90
Lymphos.	39%	14.24
Monocytes.	1%	0.36

TABLE 20

PLASMA (b) TOTAL 5.6×10^6 wbc/ml	PERCENTAGE	TOTAL NUMBER OF CELLS IN PLASMA (7.3 ml.)
204 cells were counted		(Millions)
Polys.	59%	24.12
Lymphos.	37%	15.13
Monocytes.	4%	1.64

TABLE 21

RED BLOOD CELL SEDIMENT (a)	PERCENTAGE	TOTAL NUMBER OF CELLS IN R.B.C. SEDIMENT
Smear was not countable	--	--

TABLE 22

RED BLOOD CELL SEDIMENT (b) 2.10×10^6 wbc/ml.	PERCENTAGE	TOTAL NUMBER OF CELLS IN R.B.C. SEDIMENT (5.2 ml.)
203 cells were counted		(Millions)
Polys	61%	6.66
Lymphos:	34%	3.71
Monocytes.	5%	0.55

TABLE 23

CELL SUSPENSION (a) 4.75×10^6 wbc/ml.	PERCENTAGE	TOTAL NUMBER OF CELLS IN FINAL CELL SUSPENSION (9.2 ml.)
186 cells were counted		(Millions)
Polys.	46%	20.21
Lymphos.	53%	23.28
Monocytes.	1%	0.44

TABLE 24

CELL SUSPENSION (b) 4.20×10^6 wbc/ml.	PERCENTAGE	TOTAL NUMBER OF CELLS IN FINAL CELL SUSPENSION. (9.2 ml.)
134 cells were counted		(Millions)
Polys.	60%	23.58
Lymphos.	38%	14.94
Monocytes.	2%	0.79

TABLE 25. THIS SHOWS COUNTS OF MACROPHAGE IN THE 4 PETRI DISHES PREPARED FOR THIS EXPERIMENT - TWO FROM EACH OF THE TWO BLOOD SAMPLES a and b.

COUNTS OF MACROPHAGES EXPRESSED AS NUMBER OF CELLS PER PETRI DISH		NUMBER OF MACROPHAGE PRECURSORS PER ML. BLOOD	PERCENTAGE OF MACROPHAGE PRECURSORS IN BLOOD
a	1,162,866	328,966	6.1%
a_1	1,155,008	326,746	6.1%
b	997,864	347,785	5.9%
b_1	990,007	345,047	5.8%

TABLE 26

% LOSS OF CELLS IN EACH SUSPENSION AS COMPARED WITH TOTAL CELLS IN THE WHOLE BLOOD SAMPLE.

	Plasma Cell Suspension	Final Cell Suspension
A. Poly.	37	42
Lympho.	38	0
Mono.	69	63
B. Poly.	36	38
Lympho.	39	40
Mono.	37	70

NB. The results in all the above tables are summarized and discussed after presentation of the data from experiment 40.

Data Record Experiment 40: 20 ml. blood containing 4.55×10^6 wbc/ml. was taken from J.H. and a Cell Suspension containing 4.225×10^6 wbc/ml. was prepared. 3 integrid Petri dishes were prepared from this suspension, one containing 5 ml. and the other two 4 ml. each. In addition to the normal differential on smears, in the case of the plasma-cell suspension and the final cell suspension, differential counts were made by phax contrast microscopy on wet preparations in coverslip chambers as described in under "Basic Methods and Materials".

TABLE 27. COUNTS PER ML. (CONCENTRATION OF CELLS) AT VARIOUS STAGES OF THE EXPERIMENT

White Cell Count Per Ml. Heparinized Blood (Millions)	White Cell Count Per Ml. Plasma (Millions)	White Cell Count Per Ml. R.B.C. Sediment (Millions)	White Cell Count Per Ml. Final Cell Suspension (Millions)	White Cell Count Per Ml. Discarded Plasma
4.550	5.100	1.600	4.225	30,000

TABLE 28. TOTAL (ABSOLUTE) COUNTS AT VARIOUS STAGES OF THE EXPERIMENT

Number of White Cells in 22 Ml. Heparinized Blood (Millions)	Number of White Cells in Plasma (Millions)	Number of White Cells in R.B.C. Sediment (Millions)	Number of White Cells in 20.0 Ml. of Cell Suspension (Millions)	Number of White Cells in Discarded Plasma
100.00 (22.0 ml.)	72.42 (14.2 ml.)	17.28 (10.8 ml.)	84.50 (20.0 ml.)	366,000 (12.2 ml.)

TABLE 29. BALANCE SHEET SHOWING DISTRIBUTION OF CELLS (FIGURES IN MILLIONS)

Blood Collected Contains	Plasma & R.B.C. Sediment	Cell Suspension Plus Discarded Plasma Plus R.B.C. Sediment
Total 100.00	Total 89.70	Total 102.15

TABLES 30-37. PROPORTIONS OF CELLS IN BLOOD, PLASMA, FINAL CELL SUSPENSION, R.B.C. SEDIMENT

Abbreviations: Polys. = Polymorphonuclear Leukocytes.

Lymphos. = Lymphocytes

TABLE 30

PERIPHERAL BLOOD TOTAL 4.45×10^6 wbc/ml.	PERCENTAGE	TOTAL NO. OF CELLS IN BLOOD (22.0 ml.)
175 cells were counted		(Millions)
Polys.	49%	49.00
*Lymphos.	49%	49.00
Monocytes	2%	2.00

* Large number of Large Lymphocytes were observed.

TABLE 31

PLASMA TOTAL 5.100×10^6 wbc/ml.	PERCENTAGE	TOTAL NO. OF CELLS IN PLASMA (14.2 ml.)
244 cells were counted		(Millions)
Polys.	56%	40.56
Lymphos.	43%	31.14
Monocytes	1%	0.72

TABLE 32. PHASE CONTRAST DIFFERENTIAL COUNT

PLASMA TOTAL 5.100 x 10^6 wbc/ml.	PERCENTAGE	TOTAL NO. OF CELLS IN PLASMA (14.2 ml.)
200 cells were counted		(Millions)
Polys & Monocytes	52%	37.66
Lymphos.	48%	34.76
*Monocytes	--	---

* Monocytes could not be counted or distinguished separately with this method.

TABLE 33.

RED BLOOD CELL SEDIMENT TOTAL 1.600 x 10^6 wbc/ml.	PERCENTAGE	TOTAL NO. OF CELLS IN R.B.C. SEDIMENT (10.8 ml.)
107 cells were counted		(Millions)
Polys.	59%	10.20
Lymphos.	41%	7.08
Monocytes	0%	0.00

TABLE 34.

FINAL CELL SUSPENSION 4.225 x 10^6 wbc/ml.	PERCENTAGE	TOTAL NO. OF CELLS IN FINAL CELL SUSPENSION (20.0 ml.)
188 cells were counted		(Millions)
Polys.	53%	44.78
Lymphos.	45%	38.03
Monocytes	2%	1.69

TABLE 35. PHASE CONTRAST DIFFERENTIAL COUNT

CELL SUSPENSION 4.225×10^6 wbc/ml.	PERCENTAGE	TOTAL NO. OF CELLS IN FINAL CELL SUSPENSION (20.0 ml.)
305 cells were counted		(Millions)
Polys./Monocytes	62%	52.39
Lymphos.	38%	32.11

TABLE 36. MACROPHAGE COUNTS

	COUNTS OF MACROPHAGES EXPRESSED AS NUMBER OF CELLS PER PETRI DISH.	NUMBER OF MACROPHAGE PRECURSORS PER ML. OF BLOOD	PERCENTAGE OF MACROPHAGE PRECURSORS IN BLOOD
Petri dish 1	829,720	(Average of 3 Dishes) 234,809	5.2%
Petri dish 2	911,435		
Petri dish 3	880,006		

TABLE 37.

	% LOSS OF CELLS IN EACH SUSPENSION AS COMPARED WITH TOTAL CELLS IN THE WHOLE BLOOD SAMPLE. (CALCULATED FROM COUNTS ON SMEARS)	
	Plasma Cell Suspension	Final Cell Suspension
Poly.	17	9
Lymph.	36	22
Mono.	64	15

TABLE 38. DISTRIBUTION OF CELL TYPES IN THE VARIOUS CELL SUSPENSIONS AS PERCENTAGES

M - Monocyte

L - Lymphocyte

P - Polymorphonuclear Leukocyte

	BLOOD			PLASMA			RED CELL SEDIMENTS			CELL SUSPENSION			RESULTS OF X^2 (CHI SQUARE) TESTS
EXP. NO.	M	L	P	M	L	P	M	L	P	M	L	P	
37	3	31	66	2	34	63	1	27	72	1	31	68	*x^2(df=6) = 3.740 p > .05
39a	2	39	59	1	39	60	-	--	--	1	53	46	x^2(df=6) = 11.118 p > .05
39b	4	38	58	4	37	59	5	34	61	2	38	60	x^2(df=6) = 4.206 p > .05
40	2	49	49	1	43	56	0	41	59	2	45	53	x^2(df=6) = 5.726 p > .05
MEAN %	3	39	58	2	38	60	2	34	64	1	42	57	

* NB. x^2 (Chi Square) test was performed on raw counts and not on the percentage values

Discussion of Pooled Data from the Three Experiments: In Table 36 are presented the pooled data from the four experiments, in which the percentage distribution of cell types in the four suspensions is given. A x^2 (Chi Square) test was performed on the results from each experiment separately to see if there was any significant difference between the counts. It should be pointed out here that the x^2 tests were performed on our primary data and not on the derived percentage values. The Chi Square values are given in the last column of Table 38 and it can be seen that there is no significant difference between the various counts. Thus we can conclude that there has not been a preferential loss of any particular cell type during the preparation of the final cell suspension.

Conclusion: No statistically significant loss of any particular cell type was observed during the preparation of the final cell suspension, thus indicating the validity of the method.

(c) Repeatability of the method

(i) Introduction: To test the repeatability of the method, we performed duplicate experiments on two occasions (Experiment No. 36 and 39). Analysis of variance was performed between duplicate integrid dishes in a single experiment and between dishes from experiments set up in parallel.

(ii) Procedure: (Experiment No. 36) 20 ml. blood was taken from H. B. into a heparinized syringe and was immediately separated into 2 equal portions. Cell suspensions containing 3.210×10^6 wbc per ml. and 3.375×10^6 wbc per ml. respectively were prepared from the two

blood samples. Each suspension was dispensed into two integrid Petri dishes making a total of 4 dishes for the whole experiment. Two dishes from one sample were called a and a_1, and from the other sample, b and b_1. After 7 days of incubation, the Petri dishes were stained and counted as in "Basic Methods and Materials" and analysis of variance was performed in a four group 2 x 2 (two by two) factorial design (for reference, see Basic Methods and Materials).

(iii) Results: The figures obtained are displayed in Tables 39-42 below.

TABLE 39. COUNTS PER ml. (CONCENTRATION OF CELLS) AT VARIOUS STAGES OF THE EXPERIMENT.

	White Cell Count Per Ml. Heparinized Blood (Millions)	White Cell Count Per Ml. Plasma (Millions)	White Cell Count Per Ml. of R.B.C. Sediment (Millions)	White Cell Count Per Ml. Final Cell Suspension (Millions)	White Cell Count Per Ml. Discarded Plasma
a*	6.375	5.825	1.975	3.210	175,000
b*	6.375	6.200	2.125	3.375	167,500

* "a" and "b" refer to the two cell suspensions prepared for this experiment.

TABLE 40. TOTAL CELL (ABSOLUTE) COUNTS AT VARIOUS STAGES OF THE EXPERIMENT.

	Number of White Cells in Heparinized Blood (Millions)	Number of White Cells in Plasma (Millions)	Number of White Cells in R.B.C. Sediment (Millions)	Number of White Cells in Final Cell Suspension (Millions)	Number of White Cells in Discarded Plasma
a.	63.75 (11.0 ml.)	39.61 in. (6.8 ml.)	9.28 in. (4.7 ml.)	32.10	1.19 (6.8 ml.)
b.	63.75 (11.0 ml.)	42.78 in. (6.9 ml.)	9.77 in. (4.6 ml.)	33.75	1.16 (6.9 ml.)

TABLE 41. MACROPHAGE COUNTS

COUNTS OF MACROPHAGES EXPRESSED AS NUMBER OF CELLS PER PETRI DISH.		NUMBER OF MACROPHAGE PRECURSORS PER ML. OF BLOOD.	PERCENTAGE OF MACROPHAGE PRECURSORS IN BLOOD.
a	463,576	230,157	(Average of two)
a_1	416,431	206,754	3.4%
b	495,003	233,710	
b_1	424,288	200,302	3.4%

TABLE 42. ANALYSIS OF VARIANCE

Source of Variance	Degree of Freedom	Sums of Squares	Mean Squares	F.	Probability
Between the parallel experiments	1	43.75	43.75	0.357	$p > 0.05$
Integrid Petri dishes (a) vs. (b)	1	393.75	393.75	3.211	$p > 0.05$
Interaction: a or b x experimental group	1	15.75	15.75	0.128	$p > 0.05$
Within the samples	24	2943.00	122.62		
Total	27	3396.25			

Table 39 simply shows the cell concentrations in the various suspensions examined during the course of the experiment. From the volumes of the various suspensions it was possible to calculate the total number of cells in each suspension; these are displayed in Table 40. The implications of these counts together with a balance sheet have been presented in the previous section. Table 41 shows the results of macrophage counts in each of the 4 Petri dishes. When one compares these results it is evident that there is a close similarity between these parallel experiments. Table 42 shows details of the analysis of variance which clearly indicate that there is no significant difference either between duplicated dishes or between the separately processed halves of the original blood sample (parallel experiments).

(iv) Procedure: (Experiment No. 39) A subject, S. S., was bled on two occasions with an interval of 10-15 minutes between the bleedings. The blood came from different veins and involved the use of different syringes. Samples were designated "a" and "b". The white blood counts on the two samples were 5.375 and 5.925 x 10^6 wbc/ml respectively. Cell suspensions were prepared from each sample and were found to contain 4.75 x 10^6 wbc/ml and 4.25 x 10^6 wbc/ml. Two integrid Petri dishes were prepared from each suspension and were stained and counted as in Basic Methods and Materials.

(v) Results: The counts are shown in tables 43-45 below.

TABLE 43. COUNTS PER ML. (CONCENTRATION OF CELLS) AT VARIOUS STAGES OF THE EXPERIMENT.

White Cell Count Per ml. Heparinized Blood (Millions)	White Cell Count Per ml. Plasma (Millions)	White Cell Count Per ml. R.B.C. Sediment (Millions)	White Cell Count Per ml. Final Cell Suspension (Millions)	White Cell Count Per ml. Discarded Plasma
a. 5.375	5.000	2.350	4.750	12500
b. 5.925	5.600	2.100	4.200	7500

TABLE 44. TOTAL (ABSOLUTE) COUNTS AT VARIOUS STAGES OF THE EXPERIMENT

	Number of White Cells in 11.0 ml. (each) (Millions)	Number of White Cells in Plasma (Millions)	Number of White Cells in R.B.C. Sediments (Millions)	Number of White Cells in Final Cell Suspension (Millions)	Number of White Cells in Discarded Plasma
a.	59.12	36.5 (7.3 ml.)	12.22 in (5.2 ml.)	43.94 (9.25 ml.)	850,000 (6.8 ml.)
b.	65.18	40.88 (7.3 ml.)	10.92 (8.2 ml.)	39.31 (9.25 ml.)	510,000 (6.8 ml.)

TABLE 45. MACROPHAGE COUNTS

	Counts of Macrophages Expressed as Number of Cells Per Petri Dish	Number of Macrophage Precursors Per Ml. of Blood	Percentage of Macrophage Precursors in Blood
a	290,716	328,966	6.1%
a_1	288,752	326,746	6.1%
b	249,466	347,785	5.9%
b_1	247,502	345,047	5.8%

TABLE 46. ANALYSIS OF VARIANCE

Source of Variance	Degree of Freedom	Sums of Squares	Mean Squares	F.	Probability
Between the parallel experiments	1	3087	3087	9.414	$p < 0.05$
Integrid Petri dish (a) vs. (b)	1	7	7	0.00213	$p > 0.05$
Interaction: a or b experimental group	1	0	0	0	$p > 0.05$
With the samples	24	7868	327.9		
Total	27	109.62			

Tables 43 and 44 show the cell concentrations and the total number of cells present in the various suspensions. It should be pointed out here that there was a difference in the total white cell counts in the two samples of blood.

Table 45 shows the counts of macrophages obtained in each Petri dish and the calculated percentages and absolute number of macrophage precursors present in the blood.

When analysis of variance was performed (Table 46), no difference was seen between Petri dishes obtained from the same cell suspension; however, a significant difference was observed between the results of the parallel experiments performed on entirely separate blood samples with a probability value of $p < 0.05$.

(vi) Conclusion from both experiments: These experiments are reassuring in that the processing of a blood sample does not introduce significant variation even if the sample is split into halves which are processed separately. There is some variation in macrophage count, however, if separate bleedings are performed on the same subject, which in this experiment amounted to a difference of six percent. This difference may be due to variability of the original white cell count in the blood which in turn could be technical or possibly due to a biologic variation in the subject.

(d) Normal values from a series of healthysubjects

In the experiments performed up to this point, 24 were technically satisfactory and the counts of macrophage precursors from these are shown in table 47. Calculations from this data indicate that the average macrophage precursors count was 225,000 per ml. blood with a standard deviation of 100,000. When these counts are expressed as a percentage of the total white cell count it appears that the average macrophage precursors count was 3.7% of the white count.

It was interesting and reassuring to note that the second series of healthy individuals that were tested (details given in table 7 of section [B]8) gave results which were very close to the above, namely 216,000 macrophage precursors per ml. of blood with an S.D. of 103,000 and 3.5% of the total white cells were macrophage precursors.

TABLE 47. RESULTS OF MACROPHAGE COUNTING IN 24 HEALTHY SUBJECTS

EXPERIMENT NO.	WHITE CELL COUNT PER ML. BLOOD (MILLIONS)	NUMBER OF MACROPHAGES PRECURSORS PER ML. BLOOD (THOUSANDS)	MACROPHAGE PRECURSORS IN BLOOD AS A PERCENTAGE OF THE TOTAL WHITE BLOOD CELLS
11	5.12	109	1.9
12	12.40	472	3.8
14	6.55	270	4.1
15	5.70	108	1.9
16	4.45	167	3.8
17	6.15	272	4.4
18	7.50	369	4.9
19	5.80	213	3.7
21	6.80	328	4.8
22	5.85	219	3.7
26	5.45	261	4.8
27	5.20	53	1.0
29	5.52	238	4.3
30	4.65	128	2.7
31	6.15	334	5.4
32	5.90	130	2.2
33	5.20	180	3.5
34	5.67	198	3.5
35	5.87	181	3.1
36	6.37	217	3.4
37	7.75	300	3.8
38	4.32	91	2.1
39	5.64	337	5.9
40	4.55	235	5.2
MEAN	6.023	225	3.7
ST. DEV.	1.61	100	1.2

5. EXPERIMENTS TO INVESTIGATE POSSIBLE ADHERANCE OF CELLS TO GLASSWARE

(a) Introduction

(b) Experiments

(i) Direct observations.

(ii) Preparation of cell suspensions at 4°C.

Experiment No. 41

Experiment No. 42

Combined results of experiments 41 and 42

Conclusion

(iii) Experiments with E.D.T.A.

Procedure

Results and Conclusion

(c) General conclusions

5. EXPERIMENTS TO INVESTIGATE POSSIBLE ADHERANCE OF CELLS TO GLASSWARE

(a) Introduction

Because monocytes and polymorphs are cells with the ability to stick to glassware, it was important to find out if we were losing cells from this cause during the preparation of the final cell suspension. Having demonstrated that some cells remained on the glassware, some experiments were performed in the cold and some with E.D.T.A. in an attempt to remedy this situation.

(b) Experiments

(i) Direct observations: The glassware from several experiments was rinsed in saline after use and was treated with Methanol to fix any adherent cells and then stained with Giemsa. There was in all cases a variable sized population of cells sticking to some parts of the glassware. In one experiment some of the tubes were cooled to 4°C. after the blood had been added; this showed an apparent reduction in the number of cells adhering to the tubes. The number of cells adhering was not easily quantitated, so other experiments were undertaken in which cells were processed in the cold.

(ii) Preparation of cell suspensions at 4°C. Two experiments were performed in which half the blood sample was processed at 4°C. and half at room temperature. The outline of each experiment together with the accumulation data for each will be presented next. The combined results will then be described in the text together.

Experiment No. 41: 20 ml. blood was taken from K.H. into a heparinized syringe, dextran was added and immediately the sample was dispensed equally into 2 test tubes. One tube was incubated for 2 hours at 37°C. and followed thereafter as in "Basic Methods and Materials".

The second tube was incubated in an ice bath for 2 hours, and was processed thereafter at 4°C. Cell suspensions containing 3.575×10^6 w.b.c. per ml. from the cold procedure and 5.000×10^6 w.b.c. per ml. from the 37°C. incubation method were prepared.

TABLE 1. PROPORTION OF CELLS IN BLOOD

Peripheral Blood Total 5.55×10^6 w.b.c./ml.	Percentage
203 cells were counted	
Polys.	60.0%
Lymphos.	36.0%
Monocytes	1.5%
Unclassified mononuclear cells	2.5%

TABLE 2. COUNTS PER ML. (CONCENTRATIONS OF CELLS) AT VARIOUS STAGES OF THE EXPERIMENT

	White Cell Count Per ml. Heparinized Blood (Millions)	White Cell Count Per ml. Plasma (Millions)	White Cell Count Per ml. R.B.C. Sediment (Millions)	White Cell Count Per ml. Final Cell Suspension (Millions)	White Cell Count Per ml. Discarded Plasma
Cold	5.550	6.750	2.850	3.575	100,000
37°C.	5.550	6.500	3.400	5.000	100,000

TABLE 3. TOTAL (ABSOLUTE) COUNTS AT VARIOUS STAGES OF THIS EXPERIMENT

	Number of White Cells in 11 ml. Heparinized Blood (Millions)	Number of White Cells in Plasma (Millions)	Number of White Cells in R.B.C. Sediment (Millions)	Number of White Cells in Final Cell Suspension (Millions)	Number of White Cells in Discarded Plasma
Cold	61.05 (11.0 ml.)	37.13 (5.5 ml.)	19.95	35.75 (9.0 ml.)	550,000
37°C.	61.05 (11.0 ml.)	44.85 (6.9 ml.)	19.04	50.00 (9.0 ml.)	600,000

TABLE 4. MACROPHAGE COUNTS

	Counts of Macrophages Expressed as Number of Cells Per Petri Dish	Number of Macrophage Precursors Per ml. of Blood (Average of 2 dishes)	Percentage of Macrophage Precursors in Blood
Cold a	675,719	280,550	5.0%
Cold b	770,006		
37°C. a	809,292	234,390	4.0%
37°C b	880,006		

These results will be discussed in combination with those of Experiment No. 42 below.

Experiment No. 42: 20 ml. blood was taken from M.C. into a heparinized syringe. The dextran treated blood was separated into 2 equal portions and cell suspensions containing 3.05×10^6 w.b.c. per ml. from the cold method and 4.15×10^6 w.b.c. per ml. from the 37°C. incubation were prepared respectively as in the previous experiment.

TABLE 5. PROPORTIONS OF CELLS IN BLOOD

Abbreviations: Polys = Polymorphonuclear Leukocytes

Lymphos. = Lymphocytes

Peripheral Blood Total 4.325×10^6 wbc/ml.	Percentage
253 cells were counted	
Polys.	57.0%
Lymphos.	35.8%
Monocytes	6.5%
Unclassified mononuclear cells	0.7%

TABLE 6. COUNTS PER ML. (CONCENTRATION OF CELLS) AT VARIOUS STAGES OF THE EXPERIMENT

	White Cell Count Per ml. of Heparinized Blood (Millions)	White Cell Count Per ml. Plasma (Millions)	White Cell Count Per ml. R.B.C. Sediment (Millions)	White Cell Count Per ml. Final Cell Suspension (Millions)	White Cell Count Per ml. Discarded Plasma
Cold	4.325	4.700	2.325	3.050	60,000
37°C.	4.325	5.275	2.475	4.150	127,500

TABLE 7. TOTAL (ABSOLUTE) COUNTS AT VARIOUS STAGES OF THE EXPERIMENT

	Number of White Cells in 11.0 ml. Heparinized Blood (Millions)	Number of White Cells in Plasma (Millions)	Number of White Cells in R.B.C. Sediment (Millions)	Number of White Cells in 8.0 ml. Final Cell Suspension (Millions)	Number of White Cells in Discarded Plasma
Cold	47.58 (11.0 ml.)	24.44 (5.2 ml.)	16.97	24.4 (8.0 ml.)	282,000
37°C.	47.58 (11.0 ml.)	38.80 (7.35 ml.)	12.75	33.2 (8.0 ml.)	895,500

TABLE 8. MACROPHAGE COUNTS

	Counts of Macrophages Expressed as Number of Cells Per Petri Dish	Number of Macrophage Percursors Per ml. Blood (Average of 2 dishes)	Percentage of Macrophage Percursors in Blood
Cold (a)	652,148	264,616	6.1%
Cold (b)	840,720		
37°C. (a)	919,292	227,231	5.2%
37°C. (b)	825,006		

TABLE 9. MACROPHAGE COUNTS AND BALANCE SHEETS SHOWING DISTRIBUTION OF CELLS (FIGURES IN MILLIONS).

	BLOOD COLLECTED CONTAINS	(1) PLASMA (2) R.B.C. SEDIMENT (3) TOTAL	(1) CELL SUSPENSION (2) DISCARDED PLASMA (3) R.B.C. SEDIMENT (4) TOTAL	PERCENTAGE OF MACROPHAGE PRECURSORS IN BLOOD
EXP. 41 a. In Cold	61.05	(1) 37.12 (2) 19.95 (3) 57.07	(1) 35.75 (2) .55 (3) 19.95 (4) 56.25	5.0%
b. 37°C.	61.05	(1) 44.85 (2) 19.04 (3) 63.89	(1) 50.00 (2) .69 (3) 19.04 (4) 69.73	4.2%
EXP. 42 a. In Cold	47.57	(1) 24.44 (2) 16.97 (3) 41.41	(1) 24.40 (2) .28 (3) 16.97 (4) 41.65	6.1%
b. 37° C.	47.57	(1) 38.80 (2) 12.75 (3) 51.55	(1) 33.20 (2) .89 (3) 12.75 (4) 46.84	5.2%

Combined Results of Experiments 41 and 42: It can be seen in Tables 3 and 7 that the volumes of plasma obtained after red blood cell sedimentation in the cold were considerably less than those obtained by sedimentation at 37°C. It was possible to obtain between 1.5–2 ml. more plasma in the latter case.

Tables 1 and 5 show the proportion of different white cells in the blood. Tables 2, 3 and 6, 7 show the concentrations of white cells and the total white cell counts in the various cell suspensions. It can be seen that fewer cells were obtained with the cold procedure as compared with the warm. This is a second difference between the two methods.

Tables 4 and 8 show macrophage counts from each experiment separately which are then tabulated together in Table 9. It is evident that there was a 15 per cent increase in the calculated number of macrophage precursors when the cell suspension was prepared in the cold.

Two further differences of a non-quantitative nature which led to the abandonment of the cold procedure are (1) that a floccular clumping of cells and platelets occurred during the sedimentation procedure when it was performed at 4°C. (2) that the cell monolayer in the Petri dishes was patchy and uneven evidently resulting from the earlier clumping of cells. Thus the macrophage count was not as reliable as we would have liked.

Conclusion: Sedimenting and processing cells at 4°C. gave (1) less plasma cell suspension. (2) Smaller total yield of white cells presumably due to the smaller volume of plasma obtained (the cell concentration was the same). (3) When the number of macrophage precursors per ml. of blood was calculated, a higher figure was obtained from the cold procedure. (4) Cell clumping in the plasma and consequent patchy

distribution of macrophages in the dishes led us to abandon the cold procedure.

(iii) Experiments with E.D.T.A.

Procedure: Eleven experiments (Nos. 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53) were performed substituting Ethylenediamine-tetra Acetic Acid (E.D.T.A.) for heparin. This was to test whether or not the addition of E.D.T.A., with its known effect of preventing white blood cells from sticking to glassware, would increase the number of macrophages grown from a sample of blood; that is to say E.D.T.A. could reduce possible losses of precursor cells during the preparation of the cell suspension.

E.D.T.A. was made up as follows: 100 ml. of distilled H_2O was added to 2.7 gm. of E.D.T.A. (manufactured by British Drug Houses) to dissolve the dry powder. This solution was sterilized by passing through a millipore filter. 10 ml. blood samples were obtained in syringes containing various doses of E.D.T.A. ranging from 0.4 ml. to 1.5 ml. of the 2.7% w/v stock solution. Cell suspensions were otherwise prepared, incubated for seven days, and stained as in "Basic Methods and Materials".

Results and conclusions: Examination of the glassware in two experiments did indeed show that E.D.T.A. had prevented adherence of cells. However, in one experiment after another there were no macrophages or only very scanty macrophages, small in size and unhealthy looking, to be found in the Petri dishes. This was initially thought to be due to a toxic effect of E.D.T.A. However in Experiment 49 part of the blood sample was taken into a heparinized syringe and this top—

produced virtually no macrophages. Thus we had to consider other causes for the failure of macrophage development. It seemed likely that the batch of human cord serum we were using could be at fault. We tried Fetal calf serum for the next several experiments, again with poor results. Utimately the F.C.S. was shown to be toxic when it was compared with fresh autologous serum. (See next chapter for work on the serum problem).

Thus there are virtually no results from this series of experiments and the thesis Supervisor recommended that the E.D.T.A. line of inquiry be halted.

(c) General conclusions

When the used glassware was rinsed and stained, cells were found adhering. Thus there was evidence of a loss of cells, even though the validity experiments (Section B.4) showed no statistically significant prefetential loss of any one cell type. Nevertheless it seemed worthwhile trying to correct this loss.

Attempts to prevent cells adhering to glassware by a) preparing the cell suspension in the cold and b) substituting E.D.T.A. for heparin were considered to be unsatisfactory. Sedimentation in the cold caused clumping of white cells whilst the E.D.T.A. experiments were spoiled by serum toxicity.

6. EXPERIMENTS COMPARING AUTOLOGOUS SERUM, AUTOLOGOUS PLASMA AND FETAL CALF SERUM

(a) Introduction

(b) Methods

(c) Experiments

(i) Fetal calf serum

(ii) Comparison of F.C.S. with autologous serum

(iii) Comparison of autologous serum with autologous plasma

Procedure:

Results:

(d) Conclusions

6. EXPERIMENTS COMPARING AUTOLOGOUS SERUM, AUTOLOGOUS PLASMA AND FETAL CALF SERUM

(a) Introduction

It was remarked in Section B.5, that for reasons not determined, the human umbilical cord serum which had been used in several experiments appeared to be toxic to the cultured cells.

This section summarizes trials made with plasma and various sera made in order to find the best material for the cell cultures.

(b) Methods

For these experiments the techniques described earlier under the heading "Basic Methods and Materials" were applied, with the exception that the preparation of the final tissue culture medium involved addition of the various sera or plasma preparations under test. In some experiments defibrination was performed by rotating glass marbles in the freshly collected blood.

(c) Experiments

(i) Fetal calf serum (F.C.S.): In seven experiments (No. 61, 62, 63, 65, 66, 67, 78) F.C.S. was tried, both fresh and inactivated. The results obtained from these experiments were uniformly poor. There were virtually no cells surviving in culture after a week.

(ii) Comparison of F.C.S. with autologous serum: In experiment No. 65, the blood was immediately defibrinated. This sample was then processed, and 3 final cell suspensions were prepared one with 50% F.C.S. and the other with autologous serum. The macrophages obtained by these two methods were examined and compared. It was clearly seen that the cell suspension cultured in the presence of F.C.S. showed no macrophages. On the other hand, when cells were grown in the presence of autologous serum, macrophages were seen in abundance and they appeared

to be in good condition. This comparison definitely linked toxicity with the F.C.S.

Similar results were obtained in a second experiment of this sort (Experiment No. 67) with the single difference that the F.C.S. had been heat inactivated. Macrophages appeared in the cultures with autologous serum but not in those with inactivated F.C.S.

(iii) Comparison of autologous serum with autologous plasma:

In experiments Nos. 65 and 67 described above it was shown that autologous serum was suitable for growing macrophages. It seemed important to determine if autologous serum was superior to autologous plasma for this purpose. In this experiment white blood cells from a single donor were cultured separately in autologous serum and autologous plasma.

Procedure: (Experiment No. 71) 30 ml. blood was taken from D.S. into a heparinized syringe. Subsequently a further 10 ml. blood was taken this time into a dry syringe and allowed to clot for the preparation of autologous serum. 4.5 ml. of dextran was added to the heparinized blood and the mixture incubated for two hours at 37°C. Cell suspensions containing (a) 6.900×10^6 wbc/ml. in serum and (b) 6.375×10^6 wbc/ml. in plasma were prepared. 4 ml. aliquot of these suspensions were placed in each of two integrid Petri dishes (4 dishes in all) and were cultured and stained as usual.

Results: The results are displayed in the tables 1-5 below which show that both autologous serum and plasma were suitable for growing healthy macrophages. The results of the macrophage counts in table 5 indicate that the macrophage yield was about 3 times greater when white blood cells were grown in the presence of autologous plasma instead of autologous serum.

TABLE 1. PROPORTIONS OF CELLS IN BLOOD

Peripheral Blood Total 8.4 X 10^6 w.b.c./ml.	Percentage
460 cells were counted	
Polys.	77.0%
Lymphos.	21.5%
Monocytes.	1.5%

TABLE 2. COUNTS PER ML. (CONCENTRATION OF CELLS) AT VARIOUS STAGES OF THE EXPERIMENT

White Cell Count Per ml. Heparinized Blood (Millions)	White Cell Count Per ml. Plasma (Millions)	White Cell Count Per ml. R.B.C. Sediment (Millions)	White Cell Count Per ml. Final Cell Suspension (Millions)	White Cell Count Per ml. Discarded Plasma
8.400	11.225	5.250	(a) 6.90 (serum) (b) 6.375 (plasma)	272,500

TABLE 3. TOTAL (ABSOLUTE) COUNTS AT VARIOUS STAGES OF THE EXPERIMENT

Number of White Cells in 33 ml. Heparinized Blood (Millions)	Number of White Cells in 21.8 ml. Plasma (Millions)	Number of White Cells in 15.7 ml. R.B.C. Sediment (Millions)	Number of White Cells in 2(9) ml. Final Suspension (Millions)	Number of White Cells left in 2.1 ml. discarding Plasma
277.20 (33.0 ml.)	244.70 (21.8 ml.)	82.42 (15.7 ml.)	(a) 62.10 (b) 57.38 (9.0 ml. each)	5.70 (2.1 ml.)

TABLE 4. BALANCE SHEET SHOWING DISTRIBUTION OF CELLS (FIGURES IN MILLIONS)

Blood Collected	Plasma & R.B.C. Sediment	Cell Suspension Plus Discarded Plasma Plus R.B.C. Sediment
Total: 277.20	Total: 327.10	Total: 207.65

TABLE 5. MACROPHAGE COUNTS

	Counts of Macrophage Expressed as Number of Cells Per Petri Dish		Number of Macrophage Precursors Per ml. Blood	Percentage of Macrophage Precursors in Blood
Petri dish 1	408,574	Serum	124,345	1.48
Petri dish 2	440,003		133,913	1.59
Petri dish 3	1,375,010	Plasma	452,945	5.39
Petri dish 4	1,398,581		460,706	5.48

(d) Conclusions

From the above experiments in which comparisons were made between F.C.S., autologous plasma and autologous serum it was observed that F.C.S. was toxic whilst autologous plasma proved to be a better material for culturing macrophages than autologous serum.

7. DETAILS OF THE FINAL METHOD EVOLVED FOR CULTURING MACROPHAGES

7. DETAILS OF THE FINAL METHOD EVOLVED FOR CULTURING MACROPHAGES

Following the experiments described earlier in this thesis, it was decided that the best method for growing macrophages from peripheral blood in order to count them is as follows:

20 ml. of blood are withdrawn from an antecubital vein into a 30 ml. syringe containing 2 ml. of a 4 mg per ml. solution of preservative free heparin-sodium made up as described under "Basic Methods and Materials". A white cell count is done on this sample in a Neubauer Haemocytometer. Into the heparinized blood is added 3 ml. of Dextran and the red cells are allowed to sediment at 37°C. for 2 hours as described under "Basic Methods and Materials".

The leukocyte-rich supernatant plasma is then removed as completely as possible with a Pasteur pipette and is put into 15 ml. screw-capped centrifuge tubes (No. 12-8082 manufactured by Corning Glassworks). A white blood cell count is made on the plasma and the plasma volume recorded.

The leukocyte-rich plasma is then centrifuged (as described under Basic Methods and Materials) to sediment the white blood cells and leave the majority of the platelets in suspension. After centrifugation, the supernatant is removed with a Pasteur pipette and is replaced by an appropriate amount of final tissue culture medium to give a concentration of cells between 4.0×10^6 and 7.0×10^6 per ml. The purpose of this step is to assure that each Petri dish contains not less than 15×10^6 white cells and preferably between $20\text{-}25 \times 10^6$ white cells.

The final tissue culture medium is 50:50 Eagles minimal essential medium and autologous plasma with antibiotics added (See "Basic Methods and Materials").

After the white cells have been resuspended in the tissue culture medium and another white cell count performed, 4 ml. of this suspension is put into each of 2 or 3 "Integrid" Petri dishes. The cultures are then placed in large glass Petri dishes 5.5 inches in diameter containing moist gauze sponges to prevent evaporation of the culture medium. Cultures are incubated in an atmosphere of 5% CO_2 in air at 37°C. for 7 days.

After 7 days of incubation, the macrophage layers are rinsed, fixed, stained and counted as described under "Basic Methods and Materials". From these counts, it is possible to calculate the total number of macrophages per dish and with the aid of the total white cell counts, the number of macrophages grown from each ml. of blood.

Other details of the method, including the statistical methods used, are described earlier in the thesis under "Basic Methods and Materials". Two photographs of a macrophage monolayer are presented in Figure 1.

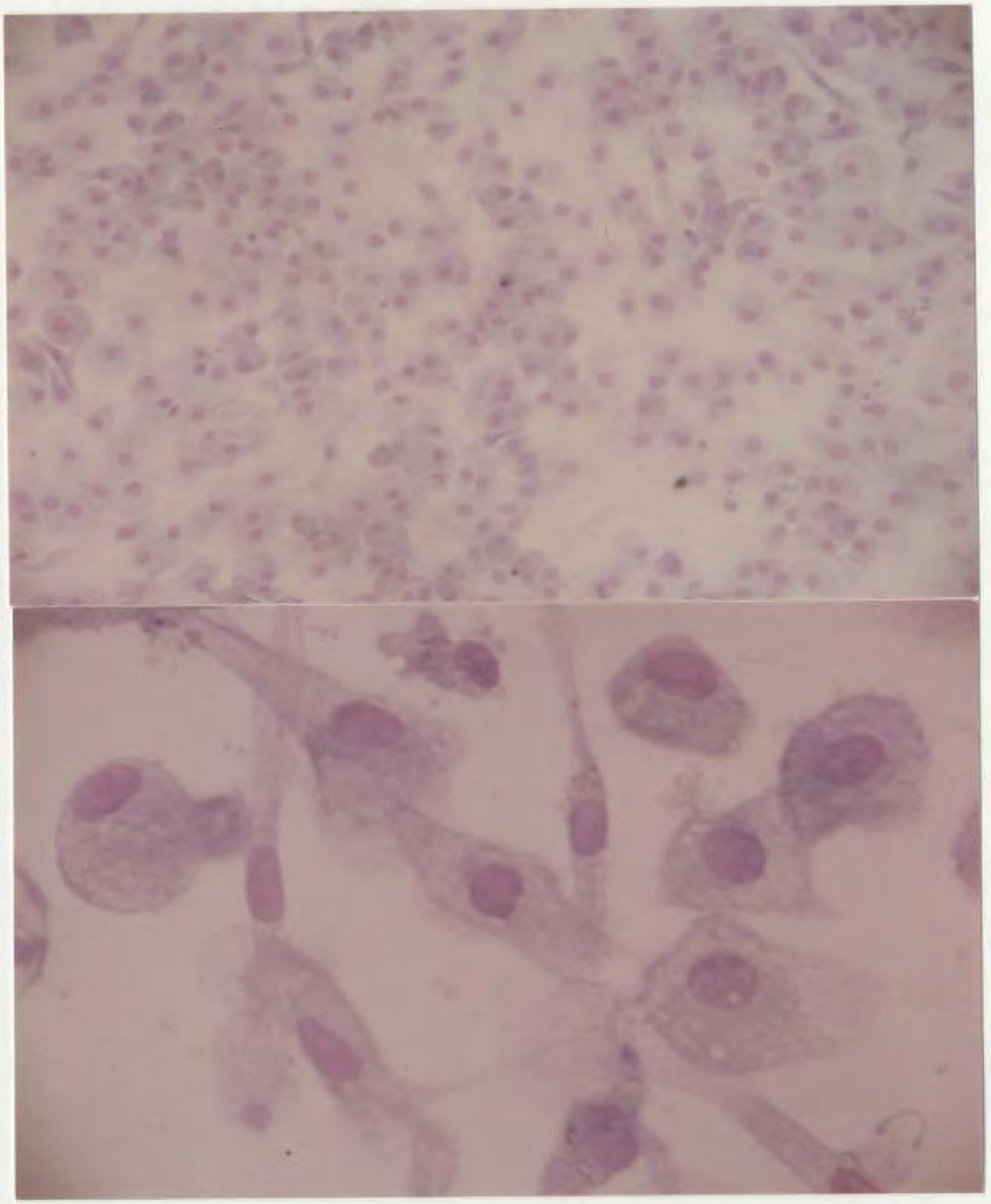

Figure 1. Photographs of a macrophage monolayer. Upper picture magnified 145 times, lower magnified 550 times.

8. ENUMERATION OF MACROPHAGE PRECURSORS IN THE BLOOD OF PATIENTS WITH CANCER AND IN HEALTHY CONTROL SUBJECTS

(a) Introduction

(b) Methods

(c) Results

(i) Patients and controls

(ii) Preparation of cell suspensions

(iii) Macrophage counting

(iv) Statistical analyses on all the haematologic data

(d) Discussion

8. ENUMERATION OF MACROPHAGE PRECURSORS IN THE BLOOD OF PATIENTS WITH CANCER AND IN HEALTHY CONTROL SUBJECTS

(a) Introduction

In an earlier section of this thesis (Section 2) the objective and rationale of these experiments was explained in detail. The problem came down to one of investigating patients with advanced cancer, who are known to have a reduced macrophage response to skin abrasion and a reduction in their cell-mediated immune responses, to see if they have reduced numbers of macrophage precursors in their blood. Previous sections of the thesis describe the development and evaluation of a method for counting macrophage precursors. In the final weeks of experimental work it was possible to apply the test to 7 patients with cancer and to a series of healthy controls.

(b) Method

The technique used for these experiments has been described in a separate chapter entitled "Detail of the Final Method Evolved for Culturing Macrophages". For statistical methods see page 29.

(c) Results

(i) Patients and controls: In table 1 the relevant details of the patients are presented. It can be seen that the types of cancer are variable, that 2 patients has no metastases evident and four patients had definite metastases. The seventh patient had lymphosarcoma. The sex and ages of the healthy controls are given as part of Table 8. Tables 2 and 3 present the relevant haematological data from both groups of individuals.

TABLE 1. DETAILS FROM PATIENTS RECORD

Exp. No.	Cancer Patient No.	Sex	Age	M.C.P. No. or Date of Birth	Chart No.	Admis. No.	Diagnosis
72	1 A.S.	M	57	9, Nov. 1914	14-11-09	3453	Lymphosarcoma
76	2 H.T.	M	67	1904	04-11-31		Bronchopneumonia. Suspected Carcinoma of lung.
79	3 M.S.	F	80	20, Jan.1891	91-01-20	30	Carcinoma of breast, removed 1966. Lung Metastasis now.
80	4 E.M.	F	62	07, Aug.1909	09-08-07	3555	Carcinoma of stomach. Metastasis in liver.*
91	5 M.W.	M	78	848-930-610-011			Carcinoma of stomach. Metastasis in Lymph nodes.
92	6 H.B.	M	77	21, Aug.1894		3624	Carcinoma of prostate. No Metastasis.
93	7 H.K.	M	84	468-881-570-013	88-06-03	3675	Adenocarcinoma of Sigmoid Colon. No Metastasis at operation.+

* Macrophage count one week postoperative.

\+ Macrophage count five days postoperative.

TABLE 2. PROPORTION OF CELLS IN HEPARINIZED PERIPHERAL BLOOD (ABSOLUTE NUMBERS)

Healthy Controls

EXP. NO.	WHITE BLOOD CELLS ($/mm^3$)	GRANULOCYTES ($/mm^3$)	LYMPHOCYTES ($/mm^3$)	MONOCYTES ($/mm^3$)	MAC. PRECURSORS ($/mm^3$)
70	4650	2325	2232	93	246
73	5700	3574	2041	86	88
77	4660	2868	1646	149	188
78	13350	9545	3505	300	455
81	6625	4174	2054	397	227
83	4475	2707	1544	224	228
86	4350	2967	1144	239	165
MEAN	6259	4023	2024	213	228
STD. DEV.	3232	2509	0751	113	113

TABLE 3. PROPORTION OF CELLS IN HEPARINIZED PERIPHERAL BLOOD (ABSOLUTE NUMBERS)

Cancer Patients

EXP. NO.	WHITE BLOOD CELLS (mm^3)	GRANULOCYTES (mm^3)	LYMPHOCYTES (mm^3)	MONOCYTES (mm^3)	MAC. PRECURSORS (mm^3)
72	10175	7930	2137	102	132
76	4200	2730	1344	126	323
79	4325	3610	0519	195	168
80	10675	9100	1200	374	191
91	9100	7550	1183	364	43
92	7625	5260	1982	381	197
93	5600	4700	0620	280	91
MEAN	7386	5840	1284	260	163
STD. DEV	2717	2387	0614	120	90

(ii) Preparation of cell suspensions: Table 4 simply shows the cell concentrations in the various suspensions examined during the course of the experiments. These counts become more meaningful in Tables 5 and 6 when they are converted to absolute numbers, that is to say total counts for each suspension. It can be seen in Tables 5 and 6 that about 65% (69% in healthy controls and 63% in cancer patients) of the cells originally present in the blood samples were recovered in the plasma suspensions, with some 26% sedimenting with the red cells. There were at this stage about 10% cells unaccounted for. When the cells in the plasma were further processed to produce the final cell suspension it is evident that further cells were lost, some in the discarded plasma supernatant and some unaccounted for. The final overall yields of cells were 55% and 49% for normal and cancer patients respectively. The cells not accounted for amount to 16% of the total for healthy subjects and 25% for patients with cancer.

(iii) Macrophage counting: In Tables 7, 8 and 9 the results of macrophage counting are presented. Table 7 presents the number of macrophage precursors in ten healthy subjects. Only seven of these (Table 8) were used as controls for comparison with the seven cancer patients; the results from experiments 85, 87, 88 were arbitrarily removed from the control series since it was necessary to remove two males and one female from the healthy group to maintain the same sex ratio as in the experimental group.

It can be seen that both the average number of macrophages per ml. of blood as well as the average percentage of the white cells which were macrophage precursors are lower in the cancer group. However

TABLE 4. COUNTS PER ML. (CONCENTRATION OF CELLS) AT VARIOUS STAGES OF THE EXPERIMENT

Healthy Controls

EXP. NO.	WHITE CELL COUNT PER ML. HEPARINIZED BLOOD (MILLIONS)	WHITE CELL COUNT PER ML. PLASMA (MILLIONS)	WHITE CELL COUNT PER ML. R.B.C. SEDIMENT (MILLIONS)	WHITE CELL COUNT PER ML. IN FINAL CELL SUSPENSION (MILLIONS)	WHITE CELL COUNT PER ML. DISCARDED PLASMA SUPERNATENT (THOUSANDS)
70	4.65	6.57	2.40	4.35	172
73	5.70	5.87	4.12	5.47	25
77	4.66	4.77	3.67	6.47	117
78	13.35	9.67	5.70	6.25	62
81	6.62	6.42	4.42	6.77	165
83	4.48	5.40	2.85	5.70	77
86	4.35	7.47	2.85	4.47	122
MEAN	6.26	6.60	3.72	5.64	106
STD. DEV.	3.23	1.61	1.14	0.95	54

Cancer Patients

72	10.17	7.45	8.25	6.35	50
76	4.20	3.60	1.65	3.80	13
79	4.32	2.55	3.37	4.95	10
80	10.67	10.77	5.10	7.02	172
91	9.10	9.32	4.42	4.80	120
92	7.62	5.70	5.55	5.82	140
93	5.60	4.95	3.45	6.00	130
MEAN	7.39	6.34	4.54	5.54	91
STD. DEV.	2.72	3.00	2.08	1.09	65

TABLE 5. TOTAL (ABSOLUTE) COUNTS AT VARIOUS STAGES OF THE EXPERIMENTS

Healthy Controls

EXP. NO.	NUMBER OF WHITE CELLS IN 22 ML. HEPARINIZED BLOOD (MILLIONS)	NUMBER OF WHITE CELLS IN PLASMA (MILLIONS)	NUMBER OF WHITE CELLS IN R.B.C. SEDIMENT (MILLIONS)	NUMBER OF WHITE CELLS IN FINAL CELL SUSPENSION (MILLIONS)	NUMBER OF WHITE CELLS LEFT IN DISCARDED SUPERNATANT PLASMA AFTER CENTRIFUGATION (MILLIONS)
70	102.30	93.36 (14.2ml)	25.92 (10.8ml)	52.20 (12ml)	2.41 (14.0ml)
73	125.40	87.54 (14.9ml)	41.66 (10.1ml)	76.65 (14ml)	0.36 (14.5ml)
77	102.57	70.67 (14.8ml)	37.48 (11.2ml)	64.75 (14ml)	1.74 (14.8ml)
78	293.70	142.22 (14.7ml)	58.71 (10.3ml)	125.00 (20ml)	0.92 (14.7ml)
81	145.75	96.37 (15.0ml	44.25 (10.0ml)	94.85 (14ml)	2.48 (15.0ml)
83	98.45	74.25 (13.75ml)	32.06 (11.25ml)	57.00 (10ml)	1.05 (13.5ml)
86	95.70	104.65 (14.0ml)	31.35 (11.0ml)	62.65 (14ml)	1.71 (14.0ml)
MEAN	137.70	95.58	38.78	76.16	1.52
STD. DEV.	71.11	23.80	10.82	25.78	0.79

FIGURES EXPRESSED AS PERCENTAGE OF CELLS REMOVED FROM PATIENTS					LOST BUT UNACCOUNTED FOR
100%	69%	28%	55%	1.1%	16%

TABLE 6. TOTAL (ABSOLUTE) COUNTS AT VARIOUS STAGES OF THE EXPERIMENT

Cancer Patients

EXP. NO.	NUMBER OF WHITE CELLS IN 22 ML. HEPARINIZED BLOOD (MILLIONS)	NUMBER OF WHITE CELLS IN PLASMA (MILLIONS)	NUMBER OF WHITE CELLS IN R.B.C. SEDIMENT (MILLIONS)	NUMBER OF WHITE CELLS IN FINAL CELL SUSPENSION (MILLIONS)	NUMBER OF WHITE CELLS LEFT IN DISCARDED SUPERNATANT PLASMA AFTER CENTRIFUGATION (MILLIONS)
72	142.45 (14.0±1)	66.30 (8.9±1)	56.10 (6.8±1)	63.50 (10±1)	0.42 (8.5±1)
76	92.40	55.08 (15.3±1)	16.00 (9.7±1)	34.20 (9±1)	0.19 (15.0±1)
79	95.15	36.97 (14.5±1)	35.44 (10.5±1)	39.60 (8±1)	0.14 (14.0±1)
80	234.85	156.24 (14.5±1)	53.55 (10.5±1)	140.50 (20±1)	2.41 (14.0±1)
91	200.20	173.44 (18.6±1)	28.32 (6.4±1)	96.00 (20±1)	2.23 (18.6±1)
92	167.75	83.22 (14.6±1)	57.72 (10.4±1)	58.25 (10±1)	2.04 (14.6±1)
93	123.20	93.06 (18.8±1)	21.39 (6.2±1)	84.00 (14±1)	0.13 (18.8±1)
MEAN	150.86	94.90	38.36	73.72	1.08
STD. DEV.	53.43	51.36	17.40	36.81	1.08

FIGURES EXPRESSED AS PERCENTAGE OF CELLS REMOVED FROM PATIENTS					LOST BUT UNACCOUNTED FOR
100%	63%	25%	49%	1%	25%

TABLE 7. RESULTS OF MACROPHAGE COUNTING IN 10 HEALTHY SUBJECTS*

EXPERIMENT NUMBER (CONTROLS)	AGE & SEX	WHITE BLOOD CELL COUNT PER ML. BLOOD (MILLIONS)	NUMBER OF MACROPHAGE PRECURSORS/ML. BLOOD (THOUSANDS)	MACROPHAGE PRECURSORS IN BLOOD AS %AGE OF THE TOTAL WBC'S
70 C.W.	44 M	4.650	246	5.3%
73 E.S.	58 M	5.700	88	1.5%
77 H.C.	64 F	4.660	188	4.0%
78 G.C.	40 F	13.350	455	3.4%
81 A.H.	52 M	6.625	227	3.4%
83 R.M.	48 M	4.475	228	5.1%
85 M.P.	50 F	7.175	190	2.6%
86 C.G.	51 M	4.350	165	3.8%
87 J.H.	56 M	8.025	278	3.5%
88 K.R.	48 M	3.875	101	2.6%
MEAN	51.1	6.289	216	3.5%
STD. DEV.	6.9	2.835	103	1.1

* The number of macrophage precursors were counted in 10 healthy controls. Only seven of these were later compared with our seven cancer patients. Experiment Numbers 85, 87, 88 were arbitrarily deleted since it was necessary to remove 2 males and 1 female to maintain the same sex ratio as in the experimental group.

TABLE 8. RESULTS OF MACROPHAGE COUNTING IN 7 CONTROL SUBJECTS

EXPERIMENT NUMBER (CONTROLS)	AGE & SEX	WHITE BLOOD CELL COUNT PER ML. BLOOD (MILLIONS)	NUMBER OF MACROPHAGE PRECURSORS/ML. BLOOD (THOUSANDS)	MACROPHAGE PRECURSORS IN BLOOD AS A %AGE OF THE TOTAL WBC'S
70 C.W.	44 M	4.650	246	5.3%
73 E.S.	58 M	5.700	88	1.5%
77 H.C.	64 F	4.660	188	4.0%
78 G.C.	40 F	13.350	455	3.4%
81 A.R.	52 M	6.625	227	3.4%
83 R.M.	48 M	4.475	228	5.1%
86 C.G.	51 M	4.350	165	3.8%
MEAN	51.0	6.259	228	3.8%
STD. DEV.	8.1	3.232	113	1.3%

TABLE 9. RESULTS OF MACROPHAGE COUNTING IN 7 CANCER PATIENTS

EXPERIMENT NUMBER (CANCER PATIENTS)	AGE & SEX	WHITE BLOOD CELL COUNT PER ML. BLOOD (MILLIONS)	NUMBER OF MACROPHAGE PRECURSORS/ML. BLOOD (THOUSANDS)	MACROPHAGE PRECURSORS IN BLOOD AS %AGE OF THE TOTAL WBC'S
72	57 M	10.175	132	1.3%
76	67 M	4.200	323	7.7%
79	80 F	4.325	168	3.8%
80	62 F	10.675	191	1.8%
91	78 M	9.100	43	0.5%
92	77 M	7.625	197	2.6%
93	84 M	5.600	91	1.5%
MEAN	72.1	7.386	164	2.7
STD. DEV.	10.1	2.717	90	2.4

when a 't' test was performed on these figures it is evident that they are not significantly different.

(iv) Statistical analyses on all the haematologic data: In Table 10 the product-moment correlation coefficient between various white cell types is presented. These correlation tests were performed in the three groups which are (1) the initial group of healthy subjects described earlier in this thesis, (2) the cancer patients and (3) the healthy controls subjects.

It is evident from Table 10 that a significant positive correlation ($\phi =< 0.05$) exists between the total polymorph count and the total white blood count in all three groups of subjects. Thus, the higher the total white cell count, the higher was the polymorph count. In addition no significant difference was observed when an analysis of covarience was performed between the results from patients and the healthy individuals. This means that the slopes of the two lines in Fig. 1 are not significantly different. Similar results were seen when total Lymphocyte values and total white blood cells were compared in the two groups (Fig. 2).

When however, the total macrophage values and the total white blood cells were correlated a positive correlation was found in healthy subjects (both groups of these) but in the cancer group this positive correlation was not observed. The analysis of covariance showed a significant difference between these correlation in the two groups of people. This is illustrated in Figure 3.

The remaining correlations are probably of less importance since in lymphocyte count and polymorph count are each linearly related to total white count and if macrophage count in health is also linearly related to total white cell count then one could expect a linear

TABLE 10. PRODUCT-MOMENT CORRELATION COEFFICIENT BETWEEN:

	Initial Exp.	Control (7 exp.)	Cancer (7 exp.)	
Total Macrophages value per ml. blood and total white blood cell count per ml. blood.	0.746 $p < 0.05$* (24 exp.)	0.838 $p < 0.05$	-0.404 n.s.+	Analysis of covariance showed that the cancer group differ highly significantly from the control group.
Total Lymphocytes value per ml. blood and total white blood cell count per ml. blood.	-0.149 (10 exp.)	0.913 $p < 0.05$	0.543	No significant difference was found between the cancer and control groups.
Total Polymorphs value per ml. blood and total white blood cell count per ml. blood.	0.968 $p < 0.05$ (9 exp.)	0.995 $p < 0.05$	0.979 $p < 0.05$	No significant difference was found between the cancer and control groups.
Total monocytes value per ml. blood and total white blood cell count per ml. blood.	0.010 (10 exp.)	0.447	0.375	
Total macrophage value per ml. blood and total polymorph value per ml. blood.	0.233	0.819 $p < 0.05$	-0.488	

TABLE 10. PRODUCT-MOMENT CORRELATION COEFFICIENT BETWEEN: (CONTINUED)

	Initial Exp.	Control (7 exp.)	Cancer (7 exp.)	
Total macrophage value per ml. blood and total lymphocyte value per ml. blood.	0.681 $p < 0.05$ (9 exp.)	0.799 $p < 0.05$	0.183	
Total macrophage value per ml. blood and total monocyte count per ml. blood.	-0.196 (9 exp.)	0.470	-0.366	

* $p = < 0.05$ mean the probability of this correlation appearing by chance in less than 5%.

Thus there is a significant correlation between the two counts.

\+ not significant.

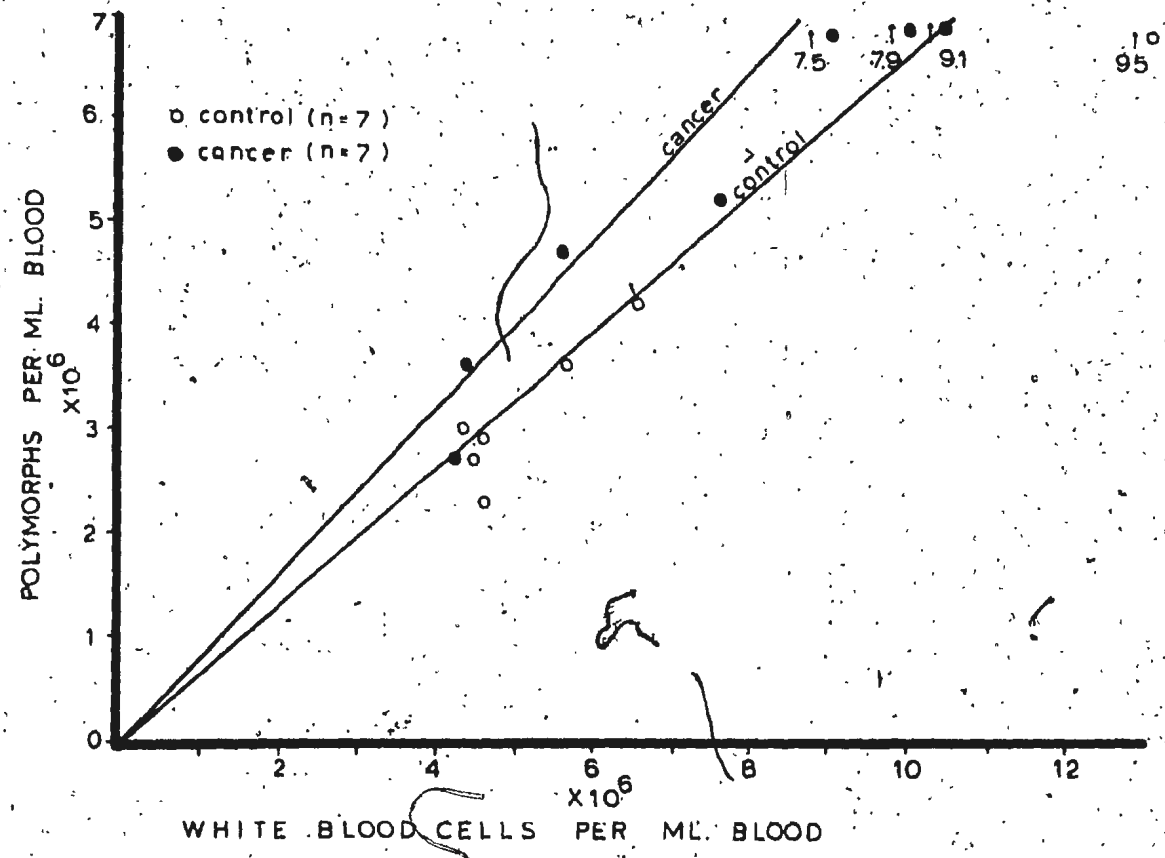

Figure 1. The equations for the above linear regressions are y = 0.66.X for the controls and y = 0.80.X for the cancer patients group. The difference between the two slopes is not significant.

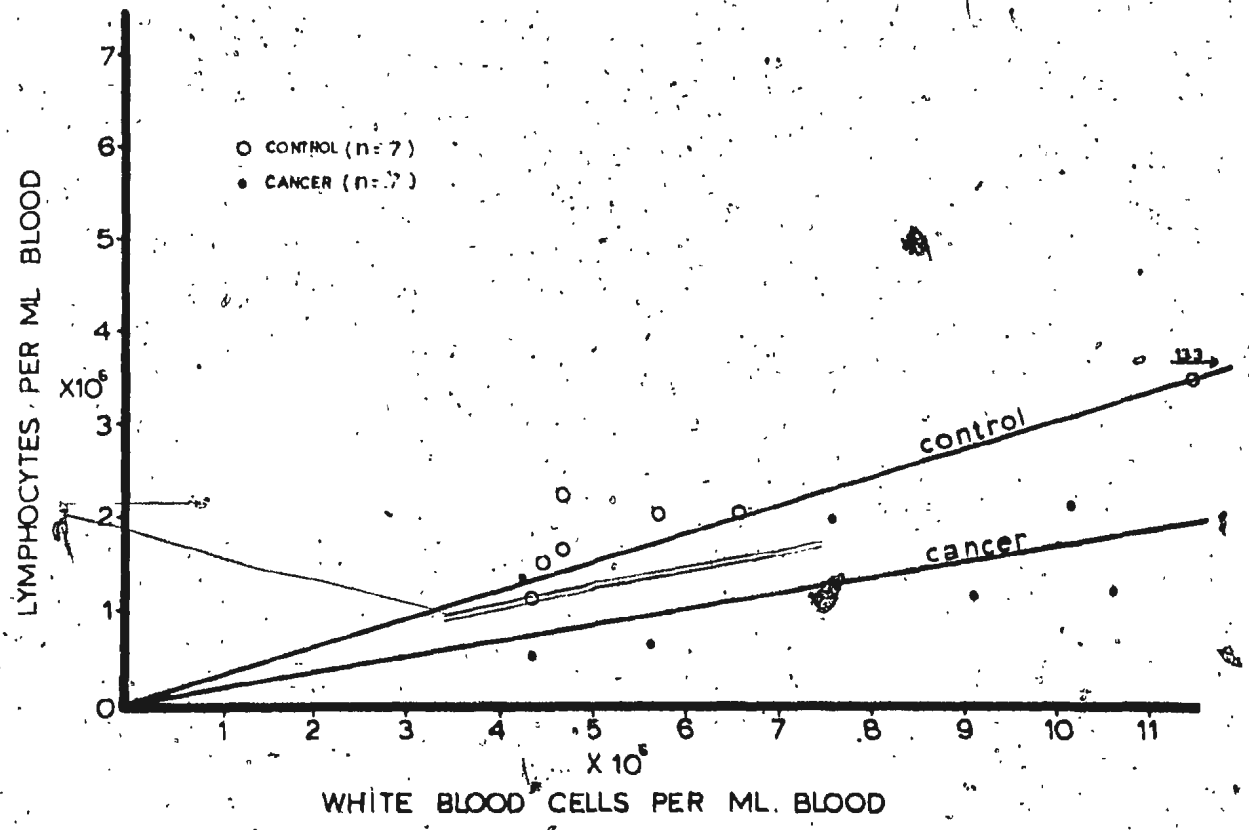

Figure 2. The equations for the above linear regressions are y = 0.303.X for the controls and y = 0.168.X for the cancer patients group. The difference between the two slopes is not significant.

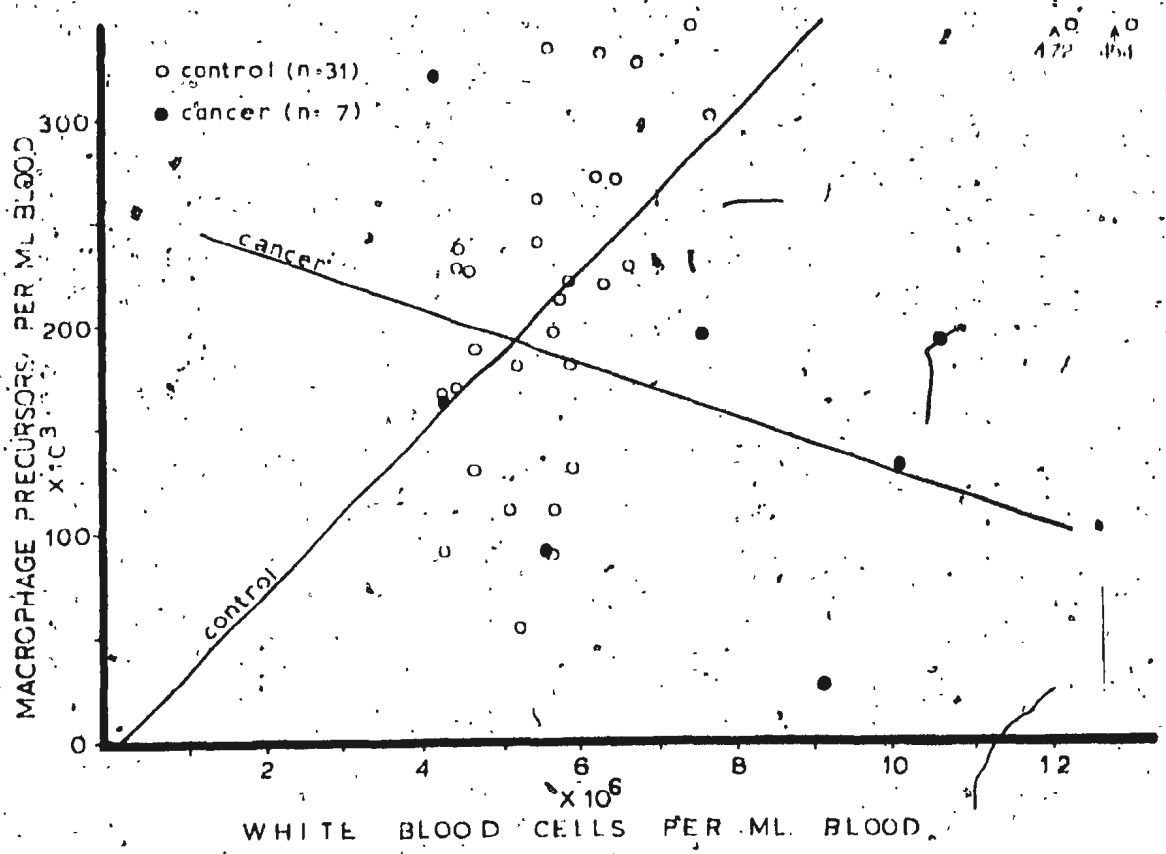

Figure 3. The relationship between macrophage precursors and total white cell count in the blood in the two groups. The equations for the above linear regressions are y = 37.567X - 2.22 for the controls and y = 13.348X + 262.20 for the cancer patients group. The difference between the two slopes is highly significant.

relationship between lymphocyte count and polymorph count on the one hand and macrophage count on the other. To some extent this expectation has been realized. It is interesting that in no case is the number of monocytes correlated with the number of macrophage precursors.

(d) Discussion

These experiments describe measurements of the number of macrophage precursors in a small series of patients for comparison with similar measurements in healthy individuals. There was no statistically significant difference between the number of macrophage precursors in the two groups. Thus we must conclude that the failure of macrophage emigration onto skin windows, that Southam and his colleagues described, is unlikely to have been due to a shortage of precursor cells in the blood. This suggests that the defect in cancer patients is due either to (1) defective emigration of these precursors across the capillary walls or (2) failure of macrophages to develop *in vivo* from the precursor cells. Since the cultures were set up with autologous plasma, the latter explanation seems unlikely. If therefore the defect is poor emigration across capillaries, there are various possible explanations; for example (1) the strength of chemotactic stimuli might be reduced or (2) the responsiveness of macrophage precursors to chemotactic stimuli might be less than normal or (3) there may possibly be an obstruction of some sort in the capillary wall preventing cells from emigrating. Polymorphonuclear leukocytes emigrate satisfactorily so the third possibility is less attractive than the first two. Experiments with chemotactic systems *in vitro* are available to test these possibilities.

The correlations between the various cell counts are of interest particularly because it seems at first sight that a departure from normal was observed in the cancer patients. Whereas the count of macrophage precursors correlated positively with the total white cell count in healthy individuals, in cancer patients on the other hand there was no such correlation or maybe even a negative correlation between the counts. These relationships between the two cell counts in the two groups are significantly different. However a note of caution should be introducted before too many interesting interpretations of this finding are made. The simplest explanation is that the cancer patients may have undergone stresses, traumas or infection which had not affected the healthy controls. For example, 2 of the patients had undergone surgery within the week. This preliminary study must obviously be repeated with full precautions to exclude surgery or drug treatment of the cancer patients and to include as controls, patients who are in hospital for non-cancerous conditions.

The nature of the macrophage precursor has not been precisely determined. In the literature review at the beginning of this thesis reasons were put forward for concluding that it is a cell that has been recently formed by cell division in the bone marrow and that it may resemble a monocyte. Experiments with this system <u>in vitro</u> are limited, but some relevant experiments were performed by Rabinowitz and Schrek (1962), who showed that macrophage precursors a) could be removed on a glass bead column and b) were not killed by doses of x-irradiation which killed lymphocytes. Furthermore, with repeated phase-contrast microscope observations of leukocyte cultures in slide

chambers, they said they were able to see monocytes develop into macrophages but never saw a lymphocyte transform in this manner. These experiments are qualitative rather than quantitative. The culture technique reported in this thesis offers the possibility of a quantitative analysis with the production of a balance sheet at the end showing the number of monocytes plated out, the number of macrophages which develop, and so on. In unpublished experiments Marshall, Simmons and Warrington, (1973 personal communication) have begun this analysis and have shown that macrophages do not grow from the polymorph fraction obtained by Ficoll-Isopaque separation of leukocytes, but grow in abundance from the mononuclear fraction.

Finally one must consider the possible importance in tumour immunity of macrophages. There is evidence that macrophages can destroy tumour cells and also evidence that R.E.S. function in man is actually increased in patients with cancer. Granger and Weiser (1964) have shown that the intitumor activity of macrophages from immunized animals is immunologically specific. Killing of tumor cells by macrophages is effected in two stages by an immunologically specific interaction which is followed by a non-specific lethal reaction. Similarly Holtermann et al (1972) have shown that cells obtained by peritoneal lavage from rats which previously had been stimulated by intraperitoneal injections of PPD were found to destroy syngeneic polyoma tumor cells in vitro. Hibbs (et al 1972) have reported similar results by employing toxoplasma instead of PPD. Thus macrophages maybe important in destroying tumor cells in vivo. The increase in R.E.S. function in cancer patients was documented by Magarey, (1972) who described a method of measuring this activity by injecting radioactively labelled aggregated human albumin

and measuring the rate of its removal from the plasma. 160 patients with cancer were studied and for comparison 126 patients with non-malignant chronic disorders of similar organs were selected. Magarey reported that, in general, patients with metastases had a more active phagocytic capacity than those with primaries only and that when serial studies with time were performed on selected patients, their phagocytic capacity tended to increase as the tumour spread.

Our preliminary results suggest that there may be a defect in macrophage emigration from the bloodstream in patients with cancer. The finding of an increased functional capacity of the R.E.S. in cancer patients is not easy to reconcile with this conclusion. However, it could be that the macrophage precursors present in the blood have a predilection for settling in the small vessels and sinuses of the liver, spleen and lymph nodes rather than for emigrating into inflamed tissues.

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