Michele K. Parish. THE DETERMINATION OF MACROPHAGE ORIGINS IN PARABIOTIC MICE (Under the direction of Dr. Alvin Volkman) Department of Biology, June, 1983.

ABSTRACT

An attempt was made to try to ascertain the origins of resident macrophages. The most popular view of macrophage origins is that all varieties of them are derived from circulating blood monocytes. The parabiotic system was employed to evaluate this belief. This model involved the parabiotic union of histocompatible C57bl/6J (black) and C57bl/6Jbgbg (beige) mice. The beige mouse is a mutant of the C57bl/6J that exhibits phenotypic characteristics which are expressed as a pigmentary defect and the presence of bizarre giant lysosomal granules in leukocytes. These cellular alterations characteristics were used as an in vivo marker in parabiosed mice to study the role of blood monocytes in the renewal of macrophage normally resident in tissues (resident macrophage). In the initial evaluation certain parameters of the black-beige parabiotic system were investigated. Circulatory exchange between black-beige parabionts was demonstrated, by use of infused ^{51}Cr labelled RBC, to occur 4 days after parabiosis. The optimal sampling time was found to be 18 hours after infusion with ^{51}Cr labelled RBC. Black and beige phenotypic differences observed in freshly drawn blood samples proved to be very subtle. Bone marrow and peritoneal cells from black and beige were cultured. The phenotypic characteristics of the beige mouse became greatly enhanced, whereas the

characteristics of the black mouse remained fairly normal in appearance. The principle characteristic observed by light microscopy in cells from the beige mouse was the presence of numerous large vacuoles. Cells from black mice exhibited small uniform vacuoles. These characteristics were observed in approximately 90% of each the preparations from the respective cell populations sampled. Electron micrographs of bone marrow cells from beige mice exhibited electron density variation and size differences of vacuoles. Vacuoles in bone marrow cells from black mice had relatively uniform sizes and density. When these characteristics were used in evaluating populations of cells from black and beige parabionts, it was found that after 6 weeks, 90% of the cells in a population, whether from black or beige mice, remained phenotypically unchanged. These last results offer no support for the concept of the exclusive renewal of resident macrophage populations by blood monocytes.

THE DETERMINATION OF MACROPHAGE ORIGINS IN PARABIOTIC MICE

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to

My Parents

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INTRODUCTION

The origins of mononuclear phagocytes has remained an unresolved problem since the discovery of these cells. There is reasonable certainty about the origin of blood monocytes and the macrophage (MØ) of inflammation. The rapidly dividing precursor pool in the bone marrow produces the blood monocyte which can, under the appropriate stimuli, function as a MØ of inflammation. The MØ normally resident in tissue and tissue spaces represent a large mass of cells. The mechanisms for renewal of these resident populations are still unknown. They have poorly defined precursors, although there is a large literature of strong opinions. Many of the experiments dealing with this subject are not sufficiently objective and have not yielded conclusive data. Other published results are difficult to interpret since lethal irradiation or excessive quantities of steroid hormones were used to study resident populations. Some of these details are reviewed in detail elsewhere in this thesis.

The present study employed an indelible marker of genetic origin in parabiotic mice. The marker originated by spontaneous mutation and has remained stable through many generations. The method involved was to establish a common circulation between two mice only one of which has the marker. In this way marked cells can be traced in the unmarked partner.

Other facets of this study deal with the establishment of appropriate technical parameters along with assesment of the usefulness of the marker as a tracer of cells that may leave the blood and enter tissue.

LITERATURE REVIEW

The macrophage (MØ) is a mononuclear phagocyte that has been found to be crucial in host defense directed against infection (Territo and Cline, 1976; Nelson, 1976) and possibly against cancer (Hibbs et al., 1978). There is nevertheless considerable uncertainity about the origin and fate of these cells. Mononuclear phagocytes are classified as tissue MØ, circulating blood monocytes and exudate MØ of inflammation. Tissue MØ are widely distributed and normally present in the liver, spleen, lymph nodes, bone marrow, peritoneal cavity, lung, bone tissue, nervous tissue and connective tissues (Van Furth and Cohn, 1968; Van Furth et al., 1972; Volkman, 1976).

Macrophage were once recognized simply as scavenger cells able to phagocytose dead cells, to phagocytose and kill certain micro-organisms and to store iron. It is now know that MØ have important functions as accessory and regulatory cells in the induction and expression of humoral and cellular immunity (Nelson, 1981). Macrophage also function in the uptake, catabolism, and presentation of antigen to T and B lymphocytes. Antigen presentation is a necessary step in the development of a characteristic specific antibody response. Cell-mediated responses, such as, delayed type hypersensitivity and graft rejection phenomena, appear to be regulated in an analogous manner (Feldmann, 1979; Pierce, 1980).

In addition to their ability to phagocytose and function as regulatory cells, MØ secrete important biologically active substances including a wide variety of enzymes, complement components, interferon

(Blanden et al., 1976) and other products active in inflammatory processes (Spicer et al., 1979; Pierce, 1980) such as prostaglandins (Davies and Allison, 1976; Nelson, 1981) and endogenous pyrogenes (Davies and Allison, 1976).

Macrophage have receptors on their membranes which can bind a variety of ligands including immunoglobulins and complement which in turn facilitate phagocytosis (Unanue, 1979; Nelson, 1981). These cells are also able to interact with the clotting system (Oliver and Berlin, 1976).

The ability of MØ to engulf, kill, digest and remove invading organisms represents one of the critical factors in protection of the body from the environment (Henson, 1980). In order for phagocytosis to take place there must first be phagocytic recognition. Phagocytic recognition occurs best if the organism is opsonized with immunoglobulins IgG and IgM with or without complement component C3b. A MØ is then capable to binding to the coated organism by the Fc and C surface receptors (Unanue, 1979; Van Furth and Leigh, 1980). After binding of the organism to the MØ, the cell membrane invaginates, encloses the organism to form a phagocytic vacuole. This vacuole, known as a phagosome, is internalized in the cell and becomes attached to a lysosome (Territo and Cline, 1976). The lysosome and phagosome fuse at which time lysosomes degranulate liberating the enzymes into the newly formed phago-lysosome, in which a wide variety of organisms can be digested.

It has been found that not all MØ are capable of performing the above functions at any one time. MØ exhibit differences in their

biochemistry, morphology, and function, depending upon which tissue they are located in (Walker, 1976). For example, MØ have expressed differences in bactericidal activity and responses to chemotatic stimuli.

For many years now, it has been said that the blood monocyte has a dual role capable of becoming a MØ of inflammation on the one hand and as the main source of renewal for resident populations of tissue MØ (Van Furth and Cohn, 1965, 1972) on the other. The origin of inflammatory MØ was established in 1965a by Volkman and Gowans. The findings of this discovery were developed to theorize a line of rapidly dividing precursors in the bone marrow a circulating form, the blood monocyte and to a mature form found in inflammation, the exudate MØ (Volkman, 1966). These basic observations have been amply confirmed (Van Furth and Cohn, 1968; Meuret, 1976).

These findings were extended by others into which the concept of the mononuclear phagocyte system (MPS). According to the criteria of the MPS (Van Furth et al., 1972) the blood monocyte is considered to be the sole immediate source of all populations of MØ both inflammatory and resident. There appear to be some difficulties with this concept. Much of the evidence for this system has been obtained from animals in a chimeric state in which there was lethal whole body irradiation of the recipients (Goodman, 1962; Balner, 1963). Whole body irradiation of recipients will cause extensive cell death, cell damage and reduced resistance to infection. In another set of experiments, excessive amounts of adrenal glucocorticosteroids were injected into mice exten-

sive cell death and reduced resistance to infection (Thompson and Van Furth, 1976). These factors could certainly account for monocyte attracting stimulli and give misleading results in the study of resident populations of MØ (Volkman, 1977). On the other hand there is evidence indicating that resident populations of MØ are capable of self-renewal. North (1969) demonstrated that some local Kupffer (liver) MØ replicated in situ in response to infection of Listeria monocytogenes. Volkman's (1976) study of resident peritonal MØ and Kupffer cell renewal by use of tritiated thymidine (^{3}HTdR) found evidence to indicate that the monocytes do not play a major role in resident MØ population renewal. The evidence favored the view that these populations are self-sustaining and independent of monocytes. For these reasons the concept of blood monocytes as the sole source of renewal of resident MØ populations must be reexamined.

Historical Perspectives

What are the origins of resident MØ? Since 1882, when MØ were first discovered by Metchnikoff this has been a very controversal point (Metchnikoff, 1884). Metchnikoff discovered phagocytic cells by introducing a splinter into the body of a starfish larva, devoid of blood vessels or of a nervous system, and watched the splinter become surrounded by mobile cells (Metschnikoff, 1921). After Metchnikoff's discovery, interest in the origins, functional capacities, kinetics and fate of these cells has generated an enormous body of literature.

Ebert and Florey (1939) stated very eloquently the dilemna of the MØ: "Certain investigators have looked upon the monocyte as the stem

cell of tissue MØ, other investigators have suggested almost every other type of cell of the blood and tissue as the precursor of the MØ". They further demonstrated that monocytes could migrate from the blood into an area of injury and become a MØ, by employing rabbit ear chambers in vivo.

In 1962, Goodman conducted studies in radiation chimeras. Mice were lethally irradiated and then subsequently injected with bone marrow or blood from normal and foreign mouse strains. It was found that donor cells from the blood of normal mice could repopulate lymphoid tissues of irradiated bone marrow chimeras.

Before 1965, it was still thought that the monocyte was derived from small lymphocytes (Rebuck and Crowley, 1955); but, in 1965a, Volkman and Gowans proved otherwise. They used "skin windows" (glass coverslips applied to the skin after irritation) in rats and found conclusive evidence to dismiss the small lymphocytes as precursors of MØ. Their experiments with parabiotic rats employing both skin windows and ^{3}HTdR labelling showed unequivocally that exudate MØ were derived from the blood. Volkman and Gowans (1965b) tried to determine the possible origins of MØ by removal of spleen, thymus, lymphocyte depletion, whole body irradiation and irradiation with bone marrow shielding. The results indicated that the thymus and lymphoid tissues are not sources of MØ precursors and the spleen was not considered to be a major source of MØ. Whole body irradiation with bone marrow shielding gave evidence of the bone marrow as a source of exudate MØ. The total evidence indicates that blood monocytes function as inflammatory MØ and originate from a rapidly dividing radiosensitive precursor pool in the bone marrow.

Pinkett et al (1965) used a system involving radiation chimeras employing the T6 marker in the model CBA/T6T6 ⟶ CBA where T6 is a stable, visually identifiable, chromosome translocation. Chromosomal analysis of lung washes gave evidence that alveolar MØ originated from more than one source. They found in lungs subjected to an irritant that approximately one-third were of pulmonary origin, derived presumably from the alveolar lining or mesenchymal cells, and the other two-thirds had origins in the hemapoietic system, probably blood mononuclear phagocytes.

Van Furth and Cohn (1968) also tried to ascertain the origin of mononuclear phagocytes in mice. They found that whole body irradiation caused a decrease in the numbers of ^{3}HTdR labelled monocytes and peritoneal cells. Partial shielding of bone marrow showed a moderate loss of monocytes. They concluded that monocytes came from the bone marrow and eluded to the possibility of resident peritoneal MØ also arising from bone marrow progenitor cells. In another set of experiments inflammation of the peritoneal cavity was induced. Approximately 1% of peritoneal cells were still labelled with ^{3}HTdR at 5 weeks and an occasional peritoneal cell labelled at 8 weeks. Their conclusion was that this was strong evidence to link the monocyte as a precursor to resident "fixed" cell populations. It was not strongly considered that these labelled cells could themselves have incorporated ^{3}HTdR easily accounting for 1% of labelled cells.

North (1969) conducted a study to look at the resident MØ population of the liver. He found that upon infection of an animal with

Listeria monocytogenes; previously labelled with colloidal carbon, that resident liver cells underwent mitotic divisions. This evidence was indicative of the capacity of resident MØ to proliferate.

Thompson and Van Furth (1969) conducted studies on the origins of mononuclear phagocytes by employing the glucocorticosteroid, hydrocortisone. Their results showed that in normal mice given an injection of hydrocortisone there was a large reduction of the circulating blood monocyte pool with only a moderate decrease in the numbers of resident MØ. The moderate reduction of MØ in resident populations was attributed to the absence of influx of blood monocytes along with the normal turnover of resident MØ. They suggested that this was positive evidence of monocytes renewing the peritoneal MØ populations, since the disappearance of blood monocytes and decrease in resident MØ occurred during the same time intervals.

Daems et al., (1973) observed the peroxidatic activity of resident peritoneal MØ and exudate MØ of guinea pig after ingestion of latex particles, and found differences between the two cells. Resident MØ containing vacuoles with latex particles showed no peroxidatic activity; but, lysosomal material was found in these vacuoles. The exudate monocytes showed peroxidatic activity in the vacuoles. Evidence of this type suggest that monocytes play no role in the renewal of the fixed populations of cells in tissue.

Bodel et al., (1977) conducted similar experiments on blood monocytes and resident peritoneal MØ, except the peroxidose reactivity within the rough endoplasmic reticulum of rabbit cells was studied. Their results indicated that after culturing blood monocytes, the

majority exhibited the same amount of peroxidase activity as seen in resident peritoneal MØ . They state there is a possibility of different enzymes being observed in various cells. They concluded nevertheless, that this supports the theory that resident MØ are derived from blood monocytes.

Van Furth (1970) found an increase in sequential labelling of blood monocytes and resident peritoneal MØ after 4 injections of ^{3}HTdR and concluded that resident peritoneal MØ came from monocytes. Takahashi (1973) replicated Van Furth's experiments finding that resident peritoneal MØ had double the mean grain counts as compared with those of blood monocytes, therefore indicating that blood monocytes could not give rise to resident peritoneal MØ.

Many methods have been proposed to study mononuclear phagocytes such as ^{3}HTdR labelling, x-irradiation of bone marrow, and bacteriological studies. When studying monocyte distribution there are two very important requisites: first an indelible marker which can survive mitosis and secondly a model which involves minimal pertubation of normal physiology (Sawyer and Volkman, 1981).

Other methods have been employed in the study of mononuclear phagocytes. Strontium 89 (^{89}Sr) has been used to study the size of selected pools of resident MØ after depletion of blood monocytes. Monocyte depletion was obtained by injections of ^{89}Sr, a bone seaking isotope which will replace calcium in hydroxyoaptite synthesis when new bone is formed. The ^{89}Sr was found to depress the proliferation of cells for up to 7 months. Sawyer and Volkman (1981) showed that this occurred without changes in the resident MØ populations. This observa-

tion suggests that such populations are, in large part, self-sustaining and not directly dependent upon monocytes for renewal (Volkman, 1976).

Parabiosis of litter mate rats and mice have been employed in the study of resident MØ pools (Volkman and Gowans, 1965). Parabiosis provides a means of exchanging blood cells and increases the size of a donor contribution without the difficulties of removing or concentrating nucleated cells. The model involves no irradiation, steroids or other potentially harmful substances (Sawyer and Volkman, 1981). In the past ^{3}HTdR has been injected into a donor animal to label monocyte precursors. The actively proliferating precursor cell will incorporate thymidine, a precursor of DNA, during DNA synthesis (Volkman, 1976). There are a few disadvantages in using ^{3}HTdR labelling. Salvage or reutilization of labelled thymidine can occur following death of a labelled cell and enzymatic degradation of DNA. Another potential disadvantage is that tritium emits a β particle with an energy of 0.0186 MeV and a mean path length of only 1 μm. The absorption of β energy is therefore largely in the nucleus with the potential for chromosomal damage. Even with these problems, ^{3}HTdR has been effectively used in the study of cell population kinetics. To study populations with slow rates of turnover such as resident peritoneal MØ , ^{3}HTdR is not as desirable, because of the low level of ^{3}HTdR incorporation by resident cells and the length of time required to label a significant portion of the population (Sawyer and Volkman, 1976).

A possible alternative to ^{3}HTdR labelling would be the parabiotic union of the black mouse (C57bl/6J) to the beige mouse (C57bl/6J bg-bg). The black and beige mice are histocompatible (accepting skin grafts from

each other) with small genetic differences. The beige mouse is a murine representative of the Chediak-Higashi syndrome occurring in humans (Strausbauch et al., 1982) and is characterized phenotypically as partial albinism and bizarre giant lysosomal granules in leukocytes namely granulocytes, monocytes and lymphocytes (Mahoney et al, 1982). The bizarre shaped lysosomes are believed to be caused by abnormal fusion of odd shaped lysosomes with phagocytic vacuoles (Higashi, 1979; Mahoney et al., 1982).

Since black and beige mice are histocompatible partners with the beige trait on the black mouse background, parabiotic union of these mice could possibly provide a suitable model for the study of mononuclear phagocytes. The black-beige parabiotic union has many advantages, such as: 1) parabiosis will give a naturally occurring exchange of cells between the two animals via a common circulation; 2) the model does not alter blood volume as would intravenous injections; 3) the beige mouse contains bizarre lysosomes which could possibly serve as in internal genetic marker to distinguish between cells of black and beige mice in a mixed pool; 4) if blood monocytes are the sole source of resident MØ, then there should be equal opportunity for the exchange and migration of cells between the two animals eventually resulting in a theoretical 50% mixture of marked and unmarked MØ .

Most studies used in the past to visualize Chediak-Higashi type cells have employed either fluorescent dyes or electron micrographs to visualize the lysosomal granules.

In the preparation of the black/beige parabiotic model it is first

necessary to establish the earliest time after parabiosis of black and beige mice that a common circulatory system is established. Establishment of a common circulation can be determined and quanitated by injecting ^{51}Cr labelled RBC into a donor mouse to later determine an equilibrium of red cells between donor and recipient animals. The next step is to establish the method by which the beige marker can be easily visualized.

MATERIALS AND METHODS

Parabiosis

The parabiosis technique follows the procedure described by Sawyer and Volkman (1981). Mice C57bl/6Jbgbg with control litter mates of the same sex, 6-8 wks of age, were purchased from Jackson Laboratories, Bar Harbor, Maine. The animals were shaved on juxtaposed sides taking care to remove the medial whiskers since they are more tranquil post-operatively if these tactile sensors have been removed. Under ether anesthesia, the animals were positioned back to back and a lateral incision was made in the skin of each animal at a point extending from the knee to the base of the ear, and the skin reflected. A 4-0 absorable gut suture was used to connect fascia of each animal at the shoulder, over the lower rib cage (with care not to perforate the peritoneal cavity), and the hips. These sutures prevent excess stress on skin margins during healing. Opposing edges of skin from each partner are brought together, everted with fine toothed forceps, and joined with closely spaced autoclips. Every other autoclip was removed one week after surgery and the remainder were removed in two weeks. Mice were given water containing tetracycline HCl (1.33 mg/ml) to minimize post-operative infection. Occasionally one partner was found dead, the survivor was sacrificed immediately.

Isotopic Labelling

Chromium 51 (as Sodium Chromate, New England Nuclear, Boston, Mass.; specific activity of 454.78 mc/mg at a concentration of 1 mc/ml) was used to label RBC. These isotopically labelled cells were used as

tracers in the evaluation of circulatory equilibrium between donor and recipient parabiont.

Six to eight etherized mice were bled from the oribital plexus by use of a sterile heparinized pasteur pipet or by removal of the eye. Employing these methods, blood was collected into a sterile conical 50 ml centrifuge tube. Cells were suspended in Hank's Balanced Salt solution (HBSS)(M.A. Bioproducts, Walkersville, Maryland). Cells were diluted to a concentration of 5 x 10^8 RBC/ml and approximately 230,000 counts per minute (cpm) of ^{51}Cr was added. The ^{51}Cr containing red cell suspension was then incubated for 1 hour at 37° in a shaking water bath. Following incubation, ^{51}Cr labelled RBC were washed 3x with HBSS by centrifugation at 600 g for 10 minutes each. After the third wash, cells were resuspended to contain approximately 15,000 to 26,000 cpm per 0.2 ml of 5 x 10^8 RBC. Parabiosed donor mice were then injected via a tail vein with 0.4 ml ^{51}Cr/RBC suspension. A parabiosed donor mouse is one half of the parabiotic pair chosen to receive the injection. Care was taken to label donor and recipient mice; a recipient mouse being the matching partner of the donor. After allotted periods of time blood samples were collected from both partners by making an incision in the lateral tail vein and collecting 40 µl of blood. Blood samples were suspended in HBSS for a total volume of 0.2 ml in 12 x 75 mm plastic centrifuge tubes. Tubes containing blood samples are then counted for 20 minutes each in a CG4000 Gamma Counter (Interchniques) set for ^{51}Cr gross counts program, with the background at zero.

A broad time span of 7, 14 and 28 days after parabionts was first

assessed to determine approximately when the circulatory exchange became maximal in parabiotic mice. More closely spaced studies were then conducted within a selected time interval after parabiosis. After determination of optimal exchange in days a bracketing time span of 30 minutes, 1 hour, 5 hours, and 18 hours were tested for a best sampling interval after injection with isotopically labelled cells.

Study of the Distribution of Radioactivity in Blood Samples

Microhematocrit collection from normal mice. Black mice were anesthetized with ether and an incision was made in a lateral tail vein. Blood samples were collected in 7mm microhematocrit tubes/plain (Clay Adams, Parsippany, N.Y.) and sealed with "seal-ease" (Clay Adams, Parsippany, N.Y.). Tubes were then spun in an Autocrit centrifuge for intervals of 5 minutes. The packed RBC level was measured as a percentage of total volume set for 100%.

Microhematocrit separation of whole blood collected from black and beige mice at 1, 5 and 18 hours after injection with syngeneic ^{51}Cr labelled RBC. Isotopic labelling of RBC was carried out as before. Microhematocrit tubes were prepared in triplicate from each animal blood sample. The samples are then spun for 5 minutes in an Autocrit centrifuge. After hematocrit readings were made, the tubes were cut at the RBC-plasma junction and the two pieces placed in separate 12 x 75 mm tubes for reading in the CG 4000 Gamma counter.

Microfuge separation of whole blood collected from black and beige mice at 1, 5, and 18 hours after injection with syngeneic ^{51}Cr labelled RBC. Isotopic labelling was carried out as before. One to two ml of

blood was collected in test tubes containing 1000 u/ml of heparin. A 0.5 ml sample of blood was placed in a microfuge tube and centrifuged for 1-3 minutes. Plasma and RBC of microfuged samples were drawn off separately and placed in separated 12 x 75 mm centrifuge tubes and counted as before.

Preparation of Cell Culture Media

RPMI-1640 powdered tissue culture medium with L-glutamine (Gibco, Grand Island, N.Y.) was prepared with 2.0 g $NaHCO_3$ and 10 mM of commercially available Hepes made to a volume of 1000 ml with dH_2O. The medium was filter sterilized by passage through a 0.20 micrometer filter (Nalgene Co., Rochester, N.Y.). After sterilization, 1 ml each of GRS Garamycin (10 mg/ml) reagent solution (Schering Corporation, Kenilworth, N.Y.) and Fungizone (Amphotericin B-250 µg/ml)(Gibco, Grand Island, N.Y.) was added per 1000 ml of medium.

Medium 199 powdered tissue culture medium with Earle's salts and L-Glutamine (Gibco, Grand Island, N.Y.) was prepared with 2.2 g $NaHCO_3$, by the procedure described for RPMI-1640 medium. RPMI-1640 (2x) was prepared as above but diluted to only 500 ml.

Calcium (Ca^{++}) and Magnesium (Mg^{++}) free buffered saline was prepared according to Garvey et al., (1977) and sterilized by filtration before 2% of mycoplasma tested and virus screened Fetal Bovine Serum (FBS) (Gibco, Grand Island Laboratories, Grand Island, NY) was added. Fetal Bovine Serium was heat inactivated for 30 minutes at 56°C in a water bath before use.

One percent agarose gel (Sea plaque, Marine Colloids Div., Rockland, ME) was prepared with dH_2O and sterilized by autoclaving.

Lavage and wash media contained 10 u/ml of preservative free heparin. Media used for culture in addition contains FBS at a concentration of 15%. ml of medium.

L-cell culture filtrate was obtained from stock solutions prepared by our laboratory. A flask was seeded with 3 x 10^5 cells per 5 ml of culture media. The culture media contains 9:1 suspension of Dulbecco's (Gibco, Grand Island, N.Y.) with FBS (active). Cell cultures were then incubated at 37°C at high humidity in an atmosphere of 10% CO_2 for 7 days. The media was then collected and centrifuged for 10 minutes at 160g. After centrifugation the supernatant was filtered through a 0.20 micrometer filter.

In Vitro Culture of Peritoneal Cells (pc) and Bone Marrow (bm) in Liquid Suspension and Agarose Gel.

All culture procedures were performed in a laminar flow hood.

Culture in liquid media

Peritoneal Cells

Mice were sacrificed by cervical dislocation. The abdomen was swabbed with 70% ethanol and the skin carefully reflected to reveal the peritoneal cavity. The peritoneal cavity was lavaged with 5ml of Ca^{++} and Mg^{++} free phosphate buffered saline containing 2% FBS and 10 u/ml of preservative free heparin. The abdomen was kneaded 60 times gently and the media drawn off. The media containing the pc were kept on ice at all times, and aseptic techniques were practiced. Cells were washed

2-3x by centrifugation for 10 minute intervals at 350 g. After washing cells were resuspended in Medium 199. The cell number was determined and dilutions were made so that each inoculum contained 1 x 10^6 cells. Cells were plated into 35 mm wells with 2.5 ml of Medium 199 containing 15% FBS. Cell cultures are incubated at 37°C at high humidity in an atmosphere of 10% CO_2 for 3 days. The cells were washed every other day by very gentle aspiration with a sterile pasteur pipet, and the medium was replaced.

Bone marrow

Femurs were removed from mice with aseptic techniques under a laminar flow hood. This includes wetting the animal with 70% ethanol and using sterile instruments with flamingo of instruments between uses. Immediately after removal, femurs were placed in a sterile petri dish and kept on ice until use. Excess tissue on the femur was removed by rubbing the bone gently with sterile gauze sponges until the bone is completely free of tissue. The femur was washed in a sterile petri dish containing RPMI-1640 medium. The ends of the femurs were clipped off and the bone marrow flushed from the bone with 5ml of RPMI-1640 medium containing 10 u/ml preservative free heparin into a sterile petri dish. The cell suspension was then passed through a sterile nylon mesh (100 μ) filter and collected in a 15 ml conical centrifuge tube. Cells were washed 2x by centrifugation for 10 minutes at 350 g. After washing, cells were resuspended, cell number determined and an appropriate dilution made. Plating and incubation conditions were identical to those for PC except BM were cultured for 7 days.

Culture in gel media

Agarose gels of PC and BM were collected in the same manner as for culture in liquid medium. The final culture media contains a 3:2:2:3 suspension of RPMI-1640 (2x) L-cell filtrate:FBS:1% agarose, respectively. Peritoneal Cells were cultured for 14 days while BM are cultured for 7 days. These are incubated at 37°C at high humidity in an atmosphere of 10% CO_2

Preparation of bone marrow cells cultured in gel medium for electron microscopy.

Individual colonies were removed one at a time by either a pasteur pipette or a 40 l pipette and transferred to a vial for a total of 20-25 colonies per vial. The colonies were fixed for 10 minutes with one part 3% glutaraldehyde and 2 parts 1% osmium in 0.1 M NaCaCo at a pH of 7.4. The colonies were fixed with fresh fixative for an additional 30 minutes (on ice). The colonies were transferred into another tube and rinsed with cold saline. Enbloc stain in 0.4% uranyl acetate was added and the mixture kept in the dark for 30 minutes. Dehydration, infiltration and embedding in epon plastic was performed according to standard technique (Luft, 1961). Thick section (0.5 microns) of each colony of cells were stained in toluidine blue. Thin sections (0.06-0.07 microns) were supported on copper grids and stained with uranyl acetate followed by lead nitrate. Electron micrographs were taken in Phillips 201 electron microscope operating at 60kV.

Staining

Acridine Orange (AO) fluorescent staining. Cultures were removed

from the incubator, medium decanted and the excess washed off with 0.9% normal saline. Culture wells were flooded with a layer of methanol for 3 to 5 minutes to the fix cells. The methanol was decanted, and the plates allowed to completely air dry. Failure to do so will subsequently cause quenching of the AO fluorescence. Two ml of 0.05% AO (Sigma Chemical Co., St. Louis, MO.) were added per well. Plates were incubated at 37°C for 25 minutes in total darkness. After incubation the AO solution was decanted and the plates were washed with normal saline followed by dH_2O. Cells cultured on glass were allowed to air dry (in darkness) and coverslips were then mounted with permount. If cells were cultured on plastic, the coverslip was mounted with 1% FBS in saline solution and rimmed with clear nail polish. Cells were observed under a Leitz Dialux microscope with a mercury lamp adjusted for epi-illumination, a Bg-12 filter and, a fluoresceindichroic mirror for excitation along with a #53 barrier filter. Color pictures were taken using Kodak 400 ADA film.

May-Gruenwald Gimesa staining (MGG) Staining was done according to standard laboratory procedures. In brief cells were stained according to the following protocal.

1:1	0.025% May-Gruenwald:dH_2O	8 minutes
1:1:19	Gimesa:methanol:Sorenson's phosphate buffer pH 5.8	20 minutes
	Sorenson's phosphate buffer	5 dips
	dH_2O	5 dips

After staining plates were allowed to air dry.

RESULTS

Isotopic Labelling for the Evaluation of Parabiotic Circulation

Chromiun 51 labelled red cells were employed to determine the establishment of a common circulatory system and the optimal sampling period, which is dependent upon the establishment of an equilibrium of isotopically labelled RBC between donor and recipient mice. This was measured as the amount of radioactivity in counts per minute (cpm) of blood samples collected from both the donor and the recipient mouse. Equilibrium is dependent upon when a common circulation is established and the rate of blood flow between donor and recipient mouse. The flow rate of blood from donor to recipient mouse will determine the time needed for circulation of isotopically labelled cells. In the first experiment, a broad time span of 8, 14 and 28 days after parabiosis was tested at 18 hours after injection of labelled cells. These data suggested (Table 1) that a common circulating system had been established by at least 8 days but counts were to low to be conclusive. Mice were then tested 4 days after parabiosis at time intervals of 30 minutes, 1 hour, 5 hours and 18 hours after injection. Table 2 shows that a common circulation had probably become established at only 4 days after parabiosis. Sampling was optimal at 5 hours and remained so until at least 18 hours after injection. However, counts remained low. An additional experiment was set up to test mice parabiosed for 2, 4 and 7 days, with more highly labelled RBC. These parabionts were sampled at 1, 5 and 18 hours after injection. The mean (X) cpm in blood collected from both donor and recipient mice is seen in Table 3. In summary, Table 3 shows

TABLE 1

DISTRIBUTION OF RADIOACTIVITY IN THE BLOOD OF PARABIOTIC MICE SAMPLED 18 HOURS AFTER INFUSION OF ^{51}Cr LABELLED RBC

	Days after Parabiosis		
	8	14	28
D[b,d]	1003[a,c]	427	659
R	936	483	704
D	548	282	420
R	680	303	413
D		501	
R		725	
D		520	
R		500	

[a] cpm per 40 μl whole blood
[b] D and R represent donor and recipient, respectively
[c] Background 315 cpm
[d] Dose injected (0.4 ml; i.v.) contained 26,602 cpm/$5x10^8$ RBC

TABLE 2

DISTRIBUTION OF RADIOACTIVITY IN THE BLOOD OF MICE PARABIOSED FOR 4 DAYS SAMPLED AT VARYING HOURS AFTER INFUSION OF ^{51}Cr LABELLED RBC

	Sampling Intervals			
	30 minutes	1 hour	5 hours	18 hours
D[b,d]	555[a,c]	453	527	468
R	342	338	537	432

[a] cpm per 40 μl whole blood
[b] D and R represent donor and recipient mice, respectively.
[c] Background 293 cpm
[d] Does injected (0.4 ml; i.v.) contained 14,779 cpm/$5x10^8$ RBC.

TABLE 3

EXCHANGE OF HIGH ACTIVITY ^{51}Cr LABELLED RBC IN PARABIOSED MICE

		Days after parabiosis		
		2	4	7
Hour[b]				
1	D[d,e]	5644±2637[a,c]	5696±686	2998±213
	R	384± 42	969±498	2293±483
5	D	1937	2573±111	2199±626
	R	1101	2121±248	1962±626
18	D		1929±109	1674±475
	R		1963±146	1866±273

[a] cpm per 40 μl whole blood
[b] time of sampling after injection of RBC.
[c] Background 350 cpm
[d] Dose injected (0.4 ml; i.v.) contained 106,086 cpm/$5x10^8$ RBC.
[e] D and R represent donor and recipeint mice, respectively.

that a common circulation had been established in all three groups, even as early as two days. The equilibrium was best shown in the 18 hour samples. Although this interval could not be evaluated in the two day group, the counts in the 5 hour sample clearly showed a significant exchange. The distribution of radioactivity in blood samples was evaluated to ascertain if cpm represented labelled cells or label lost to plasma.

Distribution of radioactivity in blood samples

In order to study the distribution of radioactivity in blood samples the plasma and RBC fractions were separated. Microhematocrit and microfuge samples were used as 2 possible means of separating and measuring the amount of radioactivity in plasma and RBC fractions. Microhematocrits were chosen as a separation method for blood, since only small samples are required, collection of sample is easy, and a sharp demarcation between plasma and packed RBC can be seen through the glass capillary tube. The microfuge was chosen as a possible alternative for separation of blood. The centrifugation provides a greater gravity force and more effective separation of cells from plasma will occur; and, the larger volume of blood used will enhance the sensitivity of the test.

Microhematocrit values of normal mice

Before microhematocrit could be used for a means of blood separation, a base line for packed RBC and centrifugation time needed to be determined. Table 4 shows mean microhematocrit values of 36 for 5

TABLE 4

MICROHEMATOCRIT VALUES IN NORMAL C57Bl/6J MICE

		Centrifugation intervals in minutes		
		5	10	15
Mouse	1	36	36	36
	2	36	37	37
	3	37	37	37
	4	34	34	34
	5	36	35	35
	6	38	38	38
	X SD	36±1.3	36±1.5	36±1.4

[a] Packed red cell expressed as a percent of total red blood cell volume.
[b] Mean ± standard deviation of packed RBC.

minutes centrifugation time. Increased centrifugation times showed no differences from the shorter spins.

Microhematocrit separation of whole blood collected from black and beige mice at 1, 5 and 18 hours after injection with syngeneic ^{51}Cr labelled RBC.

Table 5 shows that Beige mouse microhematocrit values increased by 2 units from 1 to 18 hours after injection, while black mouse microhematocrit values showed a decrease of 4 units for the same time intervals. The amount of radioactivity in plasma and RBC was compared for both the beige and black mouse samples. The plasma cpm were just below background values while the RBC cpm were just above background. This data clearly shows that the method is not very sensitive, as exhibited by the low background cpm.

Microfuge spearation of whole blood collected from black and beige mice at 1, 5 and 18 hours after injection with syngeneic ^{51}Cr labelled RBC.

Table 6 shows that the amount of radioactivity found in the plasma and RBC fractions is significantly different. There was a 10 fold difference in the amount of radioactivity found in each fraction. This was expressed by background cpm levels in plasma and 10x higher cpm in RBC; therefore, indicating no significant loss of radioactivity into surrounding plasma. This validates the use of whole blood for measurements of circulatory exchange since nearly all radioactivity is in the red cells and not in the plasma.

TABLE 5

MICROHEMATOCRIT VALUES OF INDIVIDUAL BLACK AND BEIGE MICE INFUSED WITH ^{51}Cr LABELLED RBC

	Sampling Interval[a] in hours					
	1		5		18	
Mouse	A[b]	B[c]	A	B	A	B
1	34	34	34	28	33	33
	34	23	34	28	33	33
	33	24	34	31	33	32
2	34	12	33	27	30	30
	33	12	33	27	31	31
	33	13	32	--	31	30
3	31	25	26	23	34	19
	33	23	28	23	34	19
	31	24	26	22	35	21
4	33	28	24	28	31	30
	34	29	--	28	30	32
	34	29	--	29	31	31
5	36	28	--	--	30	28
	37	27	--	--	31	29
	36	27	--	--	29	30
6	--	--	--	--	22	--
	--	--	--	--	22	--
	--	--	--	--	23	—
X SD[d]	34±1.7	26±2.5	30±3.9	27±2.8	30±3.9	28±4.8

[a] Sampling time intervals after injection of isotopically labelled cells.
[b] Black mouse packed red cell percentage.
[c] Beige mouse packed red cell percentage.
[d] mean ± standard deviations of black and beige mice.

TABLE 6

RADIOACTIVITY IN CELLS AND PLASMA OF MICE INJECTED WITH ^{51}Cr LABELLED RBC

		Microfuge separation				Microhematocrit separation			
		A		B		A		B	
		Plasma	RBC	Plasma	RBC	Plasma	RBC	Plasma	RBC
Hours[b]									
1	X	390[a]	2384	410	1872	364	520	353	523
	SD	6.4	161	12	585	53	56	10	49
5	X	370	1687	382	1840	355	487	451	483
	SD	15	557	16	507	75	183	133	117
18	X	373	1701	378	2062	353	503	368	495
	SD	21	352	22	492	14	77	62	100

Background 217 cpm
A represents black mice
B represents beige mice
Black mice received 8593 cpm/$5x10^8$ RBC/0.4 ml., while beige mice received 8425 cpm/$5x10^8$ RBC/0.4 ml. per injection of syngeneic RBC.
[a] cpm mean (X) standard deviation (SD).
[b] time after injection of ^{51}Cr labelled RBC.

The Beige Mouse Marker

Earlier studies in this laboratory failed to show any detectable phenotypic differences on light microscopy of freshly isolated peritoneal MØ. It was speculated that perhaps culturing of peritoneal and bone marrow cells from black and beige mice respectively would bring out distinguishable differences, using light microscopy. Many different culture media were tested before one was found that was condusive to peritoneal cell growth of both beige and black cells. Medium 199 was found satisfactory for the culture peritoneal cells. The bone marrow cells were easily cultured in RPMI-1640. It was observed that the cells in culture developed vacuoles presumably due to endocytosis. These vacuoles and some cell granules appeared morphologically different in cells from black and beige mice.

Light microscopy visualization of morphological characteristics of cultured cells from black and beige mice

The cell colonies (Figure 1) in culture were visualized either by light microscopy or fluorscent microscopy after staining of the cells with MGG or AO, respectively.

Acridine Orange fluorescent staining

Acridine Orange stains the cell nucleus green and the lysosomes and phagolysosomes a bright orange. The following morphological characteristics were seen and used subsequently to identify other cultures of PC and BM cells from black and beige mice. Bone marrow (Figure 2) and PC (Figure 4) from black mice were seen to contain numerous vacuoles and

lysosomal bodies which were uniform in shape and appearance. Bone marrow (Figure 3) and PC (Figure 5) from beige mice contained numerous large vacuoles and lysosomes. The lysosomes were frequently irregular in size, shape and appearance. The number of large vacuoles gave cells of the beige mouse the appearance of being frothy, and they remained unstained.

May-Gruenwald Gimesa Staining

May-Gruenwald Gimesa staining does not allow for the identification of lysosomal bodies as does AO. Vacuoles were visible and observed as nonstaining inclusions. May-Gruenwald Gimesa proved to be a useful alternative to staining with AO.

Morphological characteristics of cultured bone marrow cells from black and beige mice visualized by EM.

Cells cultured in agarose gels will form colony forming unit-clones (CFU-C). These individual colonies can be removed and processed for electron microscopy (EM). This study was conducted to see if the characteristic differences observed in with liquid media would also be visualized in cultures with agarose gel.

Vacuoles in bone marrow cells from black mice appeared relatively uniform in size. Some size range differences were observed. Most vacuoles could be classified as either small or large with few intermediates. Vacuole shapes were nearly circular and uniform throughout the cell. Density of the material within the vacuoles ranged from being frothy to containing dense offcenter areas (Figure 6).

Figure 1. Bone marrow colony cells from black mice cultured in liquid medium and stained with acridine orange. The nucleus is stained green, lysosomal granules are orange and vacuoles are nonstaining. X 700.

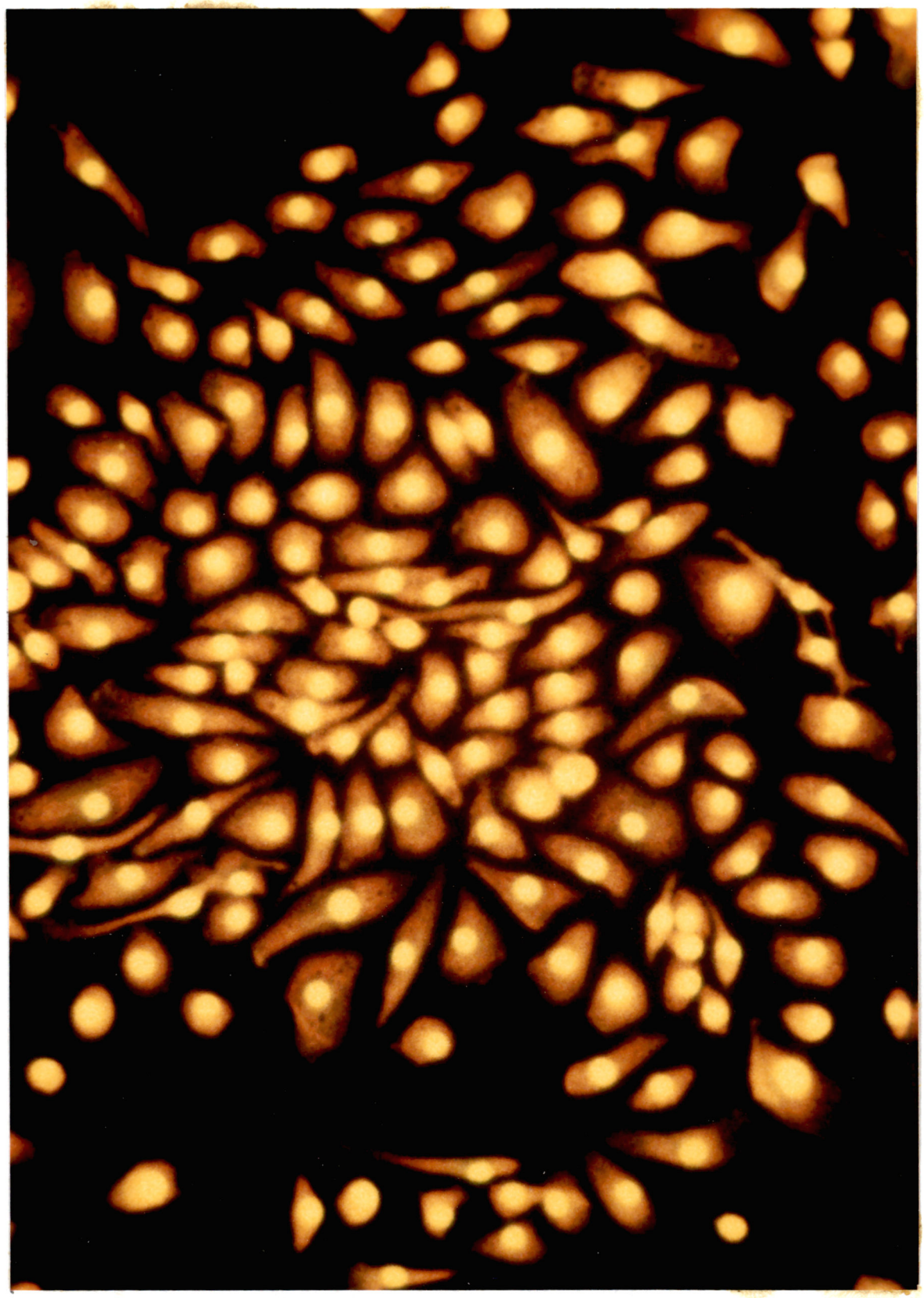
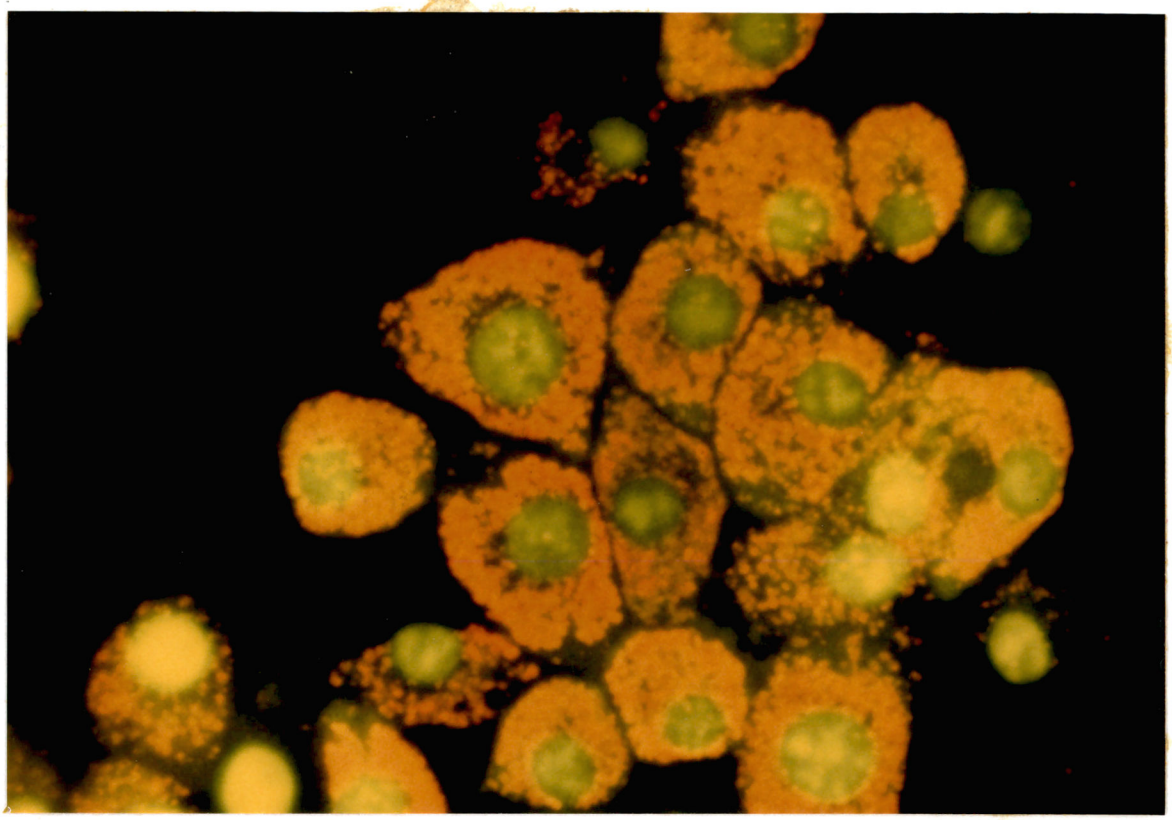


Figure 2. Bone marrow cells from black mice cultured in liquid medium and stained with Acridine Orange. The nucleus is stained green, lysosomal granules are orange and vacuoles are nonstaining. X 2269.

Figure 3. Bone marrow cells from beige mice cultured in liquid medium and stained with Acridine Orange. The nucleus is stained green, lysosomal granules are orange and vacuoles are nonstaining. X 2269.



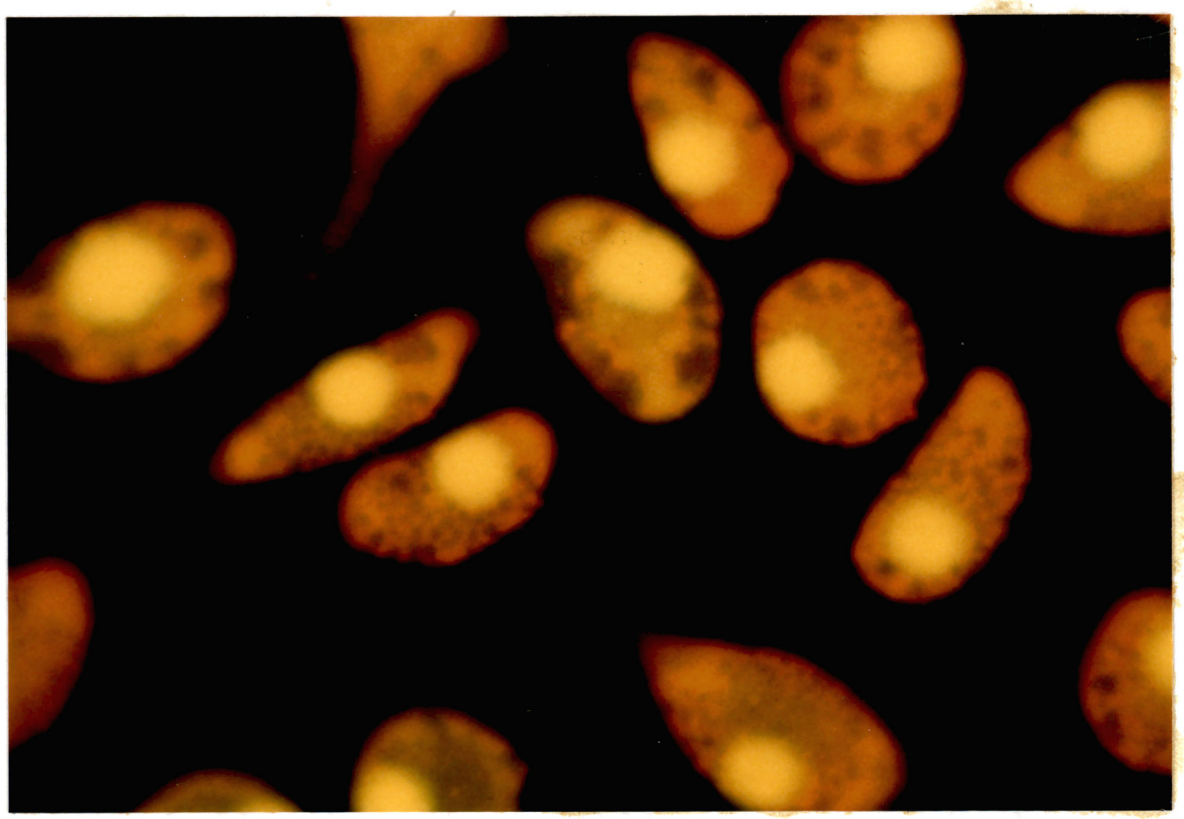


Figure 4. Peritoneal cells from black mice cultured in liquid medium and stained with Acridine Orange. The nucleus is stained green, lysosomal granules are orange and vacuoles are nonstaining. X2269.

Figure 5. Peritoneal cells from beige mice cultured in liquid medium and stained with Acridine Orange. The nucleus is stained green, lysosomal granules are orange and vacuoles are nonstaining. X2269.

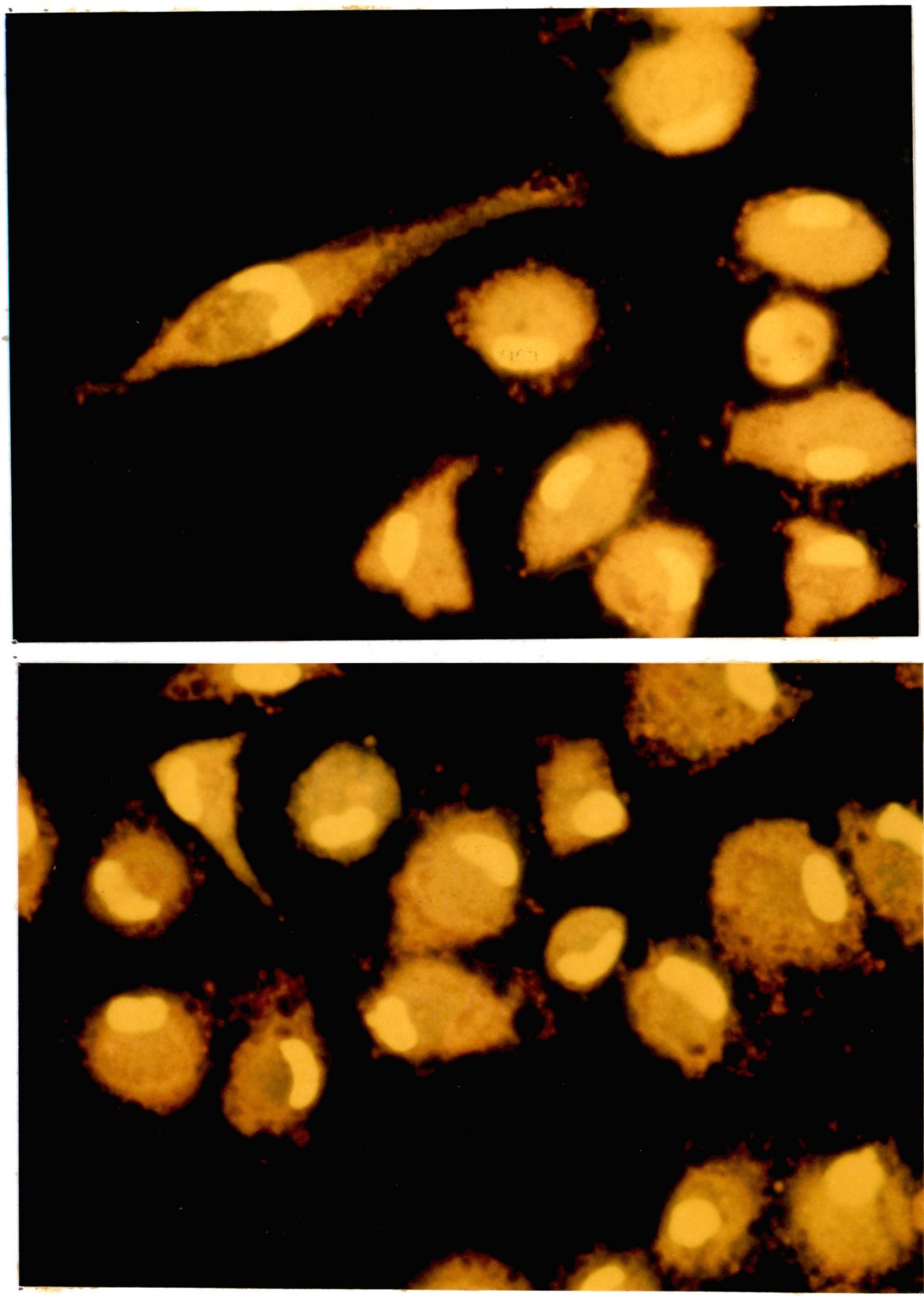
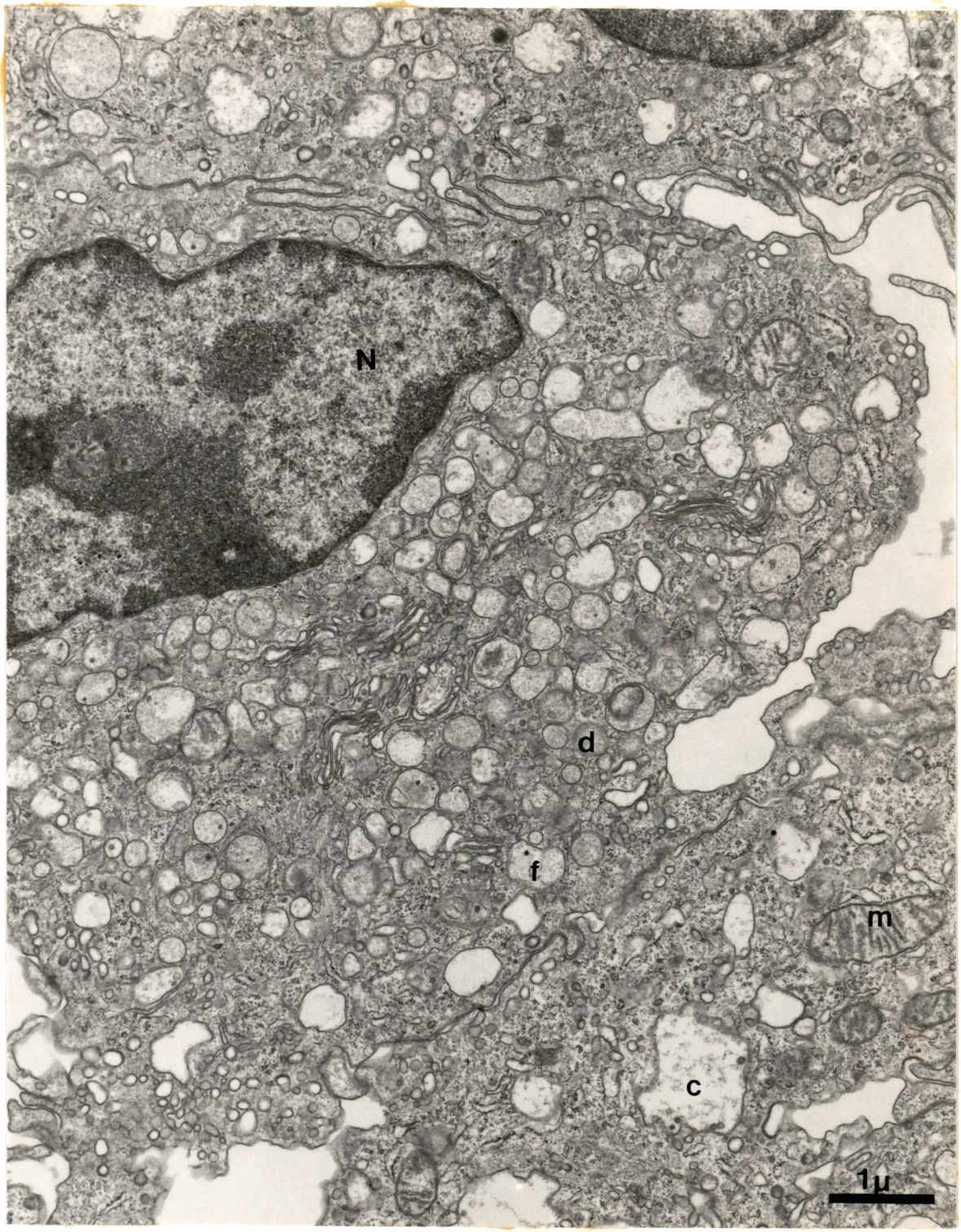


Figure 6. Electron micrograph of bone marrow cells from black mice cultured in agarose gel medium. The surrounding cytoplasma contains the nucleus (N), mitochondria (M) and vacuoles identified as clear (c), frothy (f) and dense (d), X 17,024.


N
d
f
m
c
1µ

Bone marow cells from beige mice exhibit a wide range of different vacuole sizes. The vacuoles ranged in diameter from 0.006 to 2.6 microns at 17,024X magnification, while bm cell vacuoles from black mice ranged in diameter from 0.006 to 0.9 microns. Vacuoles from beige mice varied in content from appearing clear, to frothy, to dense. Some vaucoles also had density differences within, as exhibited by a clear area surrounded by dense material (Figure 7).

When comparing bm cells from black and beige mice by EM, the major characteristic employed for identification of the two cell types was the electron density variations of vacuole content. Cells from beige mice exhibited a cytoplasm crowded with vacuoles varying in size, shape and electron density. The cells from black mice appeared more uniform in comparison.

Evaluation of bm and pc populations in black and beige mice parabiosed for 2, 4 and 6 weeks

Bone marrow and PC were collected from both the black and beige mouse of a parabiotic pair. These cells were then cultured separately. After culturing the cell populations were graded in appearance as either typical (T) or atypical (AT). Typical populations remained either phenotypically black (bl) or beige (bg) after culturing. Atypical populations were those which did not remain phenotypically black or beige but appeared to be a mixture in excess of the normal 90:10 ratio. Control groups of black-black and beige-beige parabionts were also classified by this method.

Figure 7. Electron micrograph of bone marrow cells from beige mice cultured in agarose gel medium. The surrounding cytoplasma contains the nucleus (N), mitochondria (M) and vacuoles identified as clear (c), frothy (f) and dense (d). X 17,024.

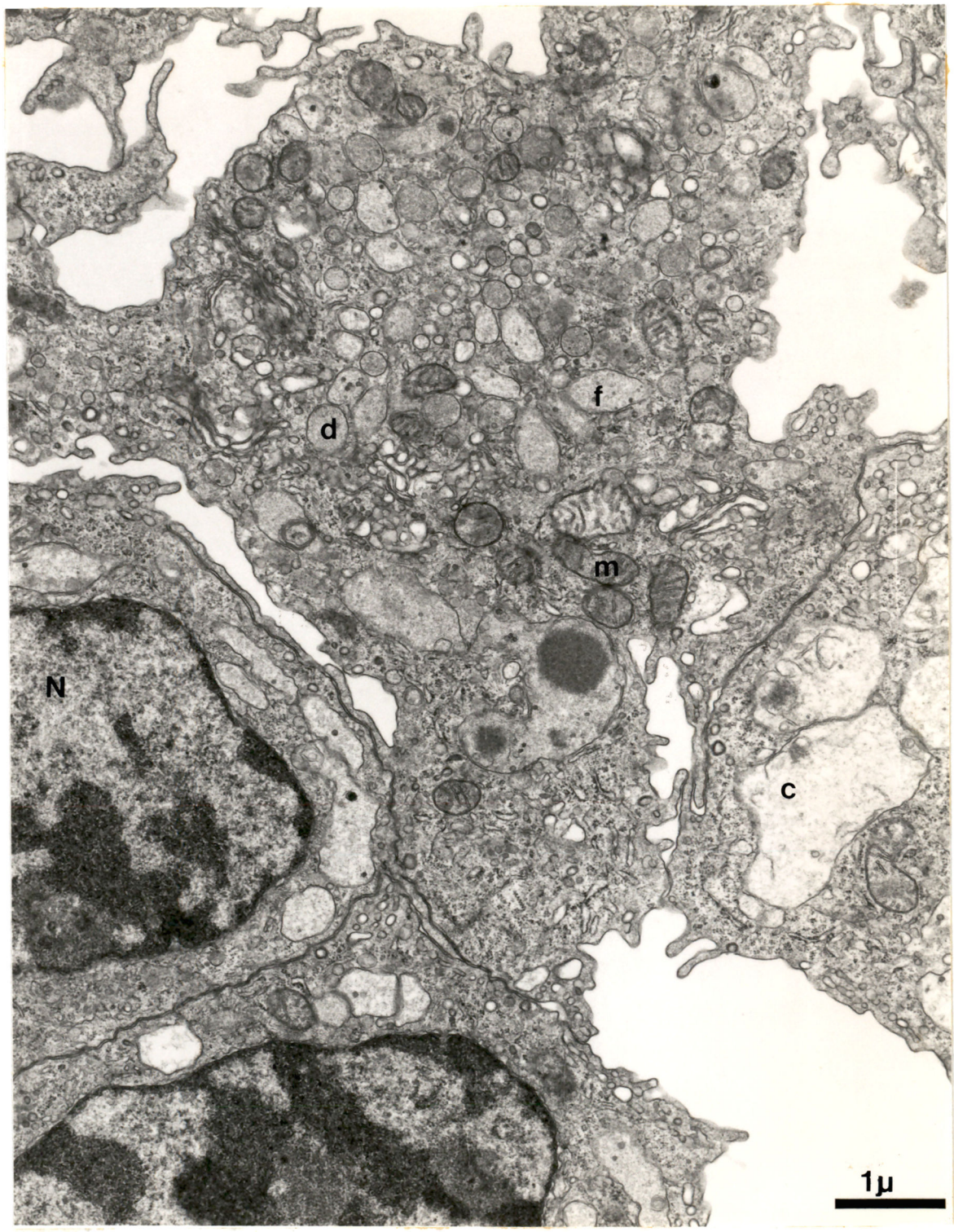

d
f
m
N
c
1µ

Table 7 shows that 2 weeks after parabiosis, PC of two of the 4 bl-bg pairs remained phenotypically bl and bg. In the bm only 1 bl-bg pair was phenotypically normal. Normal (T-T) was defined when cells from bl and bg mice in culture remained phenotypically bl and bg, respectively. The 4 week group of pc exhibited 1 out of 3 as normal, while the bm exhibited 3 our of 4 as normal. Cultured PC of mice parabiosed for 6 weeks exhibited 2 out of 3 as normal while cultured bm from 6 wk parabionts were 100% normal. Control group bl-bl gave normal results for both pc and bm cultures. The bg-bg group was totally atypical for both pc and bm cells cultured.

TABLE 7

CELL POPULATION EVALUATION OF PARABIOSED MICE

		Weeks parabiosed				
		2	4	6	>14	
	Pair[d]	bl - bg[a]	bl - bg	bl - bg	bl - bl[b]	bg - bg[c]
PC[g]	1	AT[e] - AT	T - T	T - T	T - T	AT - AT
	2	T[f] - T	AT - T	AT - AT	T - T	AT - AT
	3	T - AT	AT - T	T - T	T - T	AT - AT
	4	T - T	-- --		T - T	AT - AT
BM[h]	1	-- --	AT - T	T - T	T - T	AT - AT
	2	AT - T	T - T	T - T	T - T	AT - AT
	3	AT - T	T - T	T - T	T - T	AT - AT
	4	T - T	T - T	-- --	-- --	-- --

a. Black-Beige Parabionts
b. Black-Black Parabionts
c. Beige-Beige Parabionts
d. Parabiotic pairs 1 thru 4 cultured for either peritoneal cells (pc) and bone marrow (bm) cells.
e. Atypical: For explanation see results section.
f. Typical: For explanation see results section.
g. Peritoneal cells.
h. Bone marrow cells.

DISCUSSION

The foundation of this project was the parabiotic system and in view of this, it was felt that the appropriate conditions of the parabiotic model must be established before evaluating the effectiveness of the beige marker. Determination of the time at which a common circulation was established and the means by which this could be domonstrated was an important consideration for evaluation of the system. Volkman and Gowans (1965a) showed that the vessels anastomose at 4 weeks after parabiosis in rats. McBride (1965) demonstrated that this occurs substantially earlier after parabiosis in mice. Syngeneic ^{51}Cr labelled RBC were employed as a tracer for these purposes. Initially in the present study there were technical problems encountered with the use of ^{51}Cr labelling. A major problem was the low radioactivity in the collected blood samples. This was remedied by increasing the initial activity of the ^{51}Cr dose to the cells to be incubated. Approximately 8000 cpm of ^{51}Cr per injection dose were required to give higher values in blood samples collected from the mice. It was also found that a larger volume of blood collected from the ^{51}Cr/RBC labelled mice increased the sensitivity of the test. By day 4 after parabiosis of black and beige mice an equilibrium was established which was best demonstrated by waiting 18 hours after injection before sampling. The use of whole blood for the study had to be validated, since there was the possibility of leakage of isotope from the RBC into the surrounding plasma. The results from blood separation showed that nearly all the cpm were in the RBC fraction with only insignificant levels in the plasma fraction.

Additional information was obtained when comparing methods for separation of whole blood. Microhematocrit values were determined for both black and beige mice. There were decreases or increases seen in mean sample values of bl and bg mice, respectively, at 1, 5 and 18 hours after injection with ^{51}Cr labelled RBC. This can be attributed to an individual mouse having a higher or lower value than the other mice sampled. The bl mouse microhematocrits were found to be several units higher than bg mouse microhematocrit values.

These experiments clearly demonstrated the time of establishment of a common circulatory system and an optimal sampling interval. They also validated the use of whole blood and showed that larger collected blood samples increase the sensitivity of measuring cpm. These results set the foundation for kinetic studies of cell exchange in parabionts, specifically in the evaluation of the renewal of resident MØ populations. Sampling if needed could begin as early as 4 days after parabiosis.

It was necessary to develop the beige marker, in a quanitative, easily visualized manner before monocyte traffic studies could be conducted. Since the beige defect is expressed by bizarre lysosomes, believed to be due to abnormal phago-lysosome fusion (Akabane, 1979) it was felt that this would be the appropriate morphological characteristic to explote. The black mouse is normal and therefore should not express any abnormalities.

Previous studies used for identification of cells from beige mice have employed the EM (Akabane, 1979; White and Clawson, 1979), (Strausbauch et al., 1982). Electron microscope studies are tedious,

expensive, and a poor technique for processing large samples. It was desired to visualize cells by light microscopy. Kielan and Cohn (1980) showed phago-lysosome fusion in resident peritoneal MØ , cultured with either three molar sucrose or 15% FBS, expressed an increase in the relative size of lysosomes due to phago-lysosome fusion.

Peritoneal cells cultured with Medium 199 grew vigorously. Culturing of cells from bl and bg mice with high percentages of FBS brought out identifiable differences between the two cell types. The characteristic means for the identification of individual cells from bl and bg mice became based on the size of vacuoles observed instead of on an increase of lysosome size. Once could speculate that cells from beige mice, due to the defect in phago-lysosome fusion, were not able to process the phagocytosed material as fast as normal cells. Closer observation of the cells in culture showed that approximately 90% of the cells remained typical for the partner they represent whether bl or bg. There were occasional cells from black mice that contained large vacuoles. This could be an indication that these cells had just engulfed large amounts of FBS but had not yet digested the material. In occasional cultures, there were cells from beige mice observed with relatively few vacuoles. These cells could possibly have been in a resting state, not phagocytizing. These results show potential difficulties in precise quantification of cell populations of black-beige parabionts.

The bm colonies from bl and bg mice represent a pure MØ population. Electron micrographs of cells from bg mice exhibited vast differences in

the electron density of the cellular inclusions, while those from the bl mouse appeared relatively uniform. Strausbach et al., (1982) in studies of resident peritoneal MØ found odd dumbbell shaped lysosomes along with double membrane ring forms. These were not observed in resident peritoneal cells in black mice. White and Clawson's EM studies (1979) had also documented double membrane ring forms in circulating blood monocytes in CH patients. It appears that culturing with agarose gels affects the expression of the double membrane rings. It would be interesting to see what characteristics would be expressed by resident peritoneal MØ cultured in agarose gels. If dumbbell shaped lysosomes in resident peritoneal MØ were still seen after culturing, it could be evidence to show that these cells may be lineally distant from blood monocytes.

The model employing the beige marker in its preliminary state, does not yet satisfy the requisites of a model to test the role of monocyte influx in the renewal of resident populations. One reason for this was that in many instances there appeared to be cell types of black mice in cultures of cells from bg mice and vise a versa. Therefore, one could only assess the population semiquantitatively on the basis of dominant phenotype. In these experiments, the proportion of macrophage from bl and bg mice were examined at intervals of 2, 4 and 6 weeks following parabiosis. If the blood monocyte is functioning as a source of renewal in resident peritoneal MØ , then progressively increasing mixtures of cell populations up to 50% of each, should be seen in each partner. Trasher (1966) demonstrated that the turnover time, for complete replacement of a total population of peritoneal MØ is approximately 40

days. Thompson and Van Furth (1970) estimate that the half time for the turnover of PC is 28 days. Although additional development and refinement of the model is needed, there was useful information gained from the studies. No evidence was found to indicate mixing in resident macrophage populations which could account for renewal exclusively by blood monocytes. At 6 weeks after parabiosis, one would therefore predict that a high proportion of the cells would be atypical in phenotype. The most that was ever observed was a 10% mixture in any population. These data suggest strongly that monocytes do not renew macrophages directly as believed, but, suggest a more complex mechanism that is consistent with the views Sawyer and Volkman (1982). By employing the ^{89}Sr model they also found little evidence for the influx of blood monocytes functioning in renewal. Evidence was found to indicate that local proliferation was a mechanism of resident population renewal.

In summary, the results of the study have demonstrated that a common circulatory system is established in mice 4 days after parabiosis and is best demonstrated by sampling blood 18 hours after the unilateral infusion of isotopically labelled RBC. The use of whole blood for these tracer studies was found to be valid. In addition, it was found that PC and BM cells from bl and bg mice exhibit differences observable by both light and electron microscopy. One of the major questions addressed in this project has been answered if only in a preliminary way. In brief, the data suggest that contrary to widely held belief, renewal of the resident peritoneal MØ population does not depend exclusively on the influx of monocytes from the blood.

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