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STUDIES ON THE PROLIFERATIVE CAPACITY
OF MACROPHAGE PROGENITOR CELLS IN THE MOUSE

Michael G. Chen
(Ph.D. Thesis)

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categories: those claiming hematogenous origin and those claiming autochthonous or local origin. The hematogenous theories reflected the embryological concepts and hematological dogmas of the day, particularly the monophyletic (unitarian) school as championed by Maximow (1924) and the polyphyletic schools of dualism (Naegeli, 1931) and trialism (Schilling, 1929). Among the hematogenous theories, the idea of origin from a totipotential lymphocyte (unitarian school) was to prove the most influential. As first proposed by Metchnikoff in 1888, this theory was to find its strongest exponents in Maximow, Downey and Weidenreich, Pappenheim and Bloom (see Bloom, 1928). These investigators presented morphological evidence for a continuous series of transitions from lymphocyte to monocyte to macrophage, and placed the mononuclear phagocytes clearly within their unitarian scheme of blood cell formation. A modern proponent of the lymphocytic origin of the macrophage has been Rebuck and his co-workers (Rebuck and Crowley, 1955; Rebuck et al., 1964).

The dualistic scheme of blood cell formation with its separate myeloid and lymphatic lines regarded the monocyte to be of myeloid origin (Ehrlich and Lazarus, 1898; Naegeli, 1931); in view of evidence that monocytes are the precursors of the exudative macrophage, this would likewise designate the macrophage as being of myelocytic (bone marrow) origin. The dualists based their theory on the oxidase positivity of the myeloblast and the monocyte. Interest in the idea of a common progenitor for monocytes and granulocytes has been revived by Leder (1967) on the basis of his
histochemical investigations and by Virolainen and Defendi (1968) on the basis of their tissue culture and chromosome marker experiments; these studies will be discussed further at a later time.

The origin of monocytes and macrophages from elements of the reticuloendothelial system was once widely accepted and fitted in with the trialistic idea of the RES as a third independent cell-producing system (see reviews of Simpson, 1922; Sabin, Doan and Cunningham, 1925; Bloom, 1928; Jaffe, 1938). Recent studies using autoradiography, chromosome marker techniques, and electron microscopy have invalidated many of the conclusions drawn from these careful histological studies (see review of Leder, 1967). Lastly, Cunningham, Sabin and Doan (1925) favored the idea of a special stem cell for the monocyte cell line with the so-called monoblast as its earliest progenitor; they did not believe that the monocytes were related to either lymphocytes or histiocytes.

The concept that inflammatory mononuclear cells arose from cells at the local site of reaction has continued to receive consideration by some workers (Kojima et al., 1958; Kojima and Imai, 1964). Several cell types have been proposed: fibroblasts, vascular endothelium, adventitial cells, resting histiocytes, serosal mesothelium, etc. (see Maximow, 1932; Bloom, 1938; Ebert, 1965; Leder, 1967 for references). Macrophages attached to peritoneal and omental surfaces of rodents, such as occur in the "milky spots" (tâches laiteuses), have been regarded as an important source of peritoneal macrophages in normal mice (Mims, 1964) and in induced peritoneal exudates (Gappell, 1930; Felix and

The remaining sections of this chapter will be concerned with a selective review of studies on the question of hematogenous versus autochthonous origin for these exudative phagocytic mononuclear cells and on the problem of the identity of the progenitor(s) of these inflammatory cells.

1.2 Early in vitro and in vivo studies: Hematogenous versus autochthonous origin

One technique employed to find an answer to these problems was the then new method of tissue culture. The rationale was to isolate certain tissues in vitro and watch the transformations occurring therein. Observations were principally on whole blood, buffy coat cells, or lymph and essentially demonstrated that macrophages could arise from such tissues and that hematogenous origin was therefore feasible. Trowell (1965) has extensively reviewed the literature on in vitro studies, and reported the findings about equally divided between those favoring exclusive origin from lymphocytes and those favoring origin from monocytes. One observation that was difficult to explain was the appearance over 24 hr of considerable numbers of macrophages in cultures showing little mitotic activity and initially containing insufficient numbers of monocytes and few if any macrophages. Several groups of workers claimed to have observed direct lymphocyte to macrophage transformations in vitro, current reports being by Rebuck et al., (1958) and Klein (1959).
A modern correlate of this type of approach are the millipore chamber studies in which lymphocytes from various sources are "cultured in vivo" in diffusion chambers of the Algire type. Several workers have reported evidence in favor of origin of macrophages from small lymphocytes, again because the numbers of macrophages produced could not be accounted for from the few, rarely dividing monocytes seen (Holub, 1960; Petrakis, Davis and Lucia, 1961; Shelton and Rice, 1959). However, Schooley and Berman (1960) found only a few "monoscytoid" cells developing from thoracic duct lymph "cultured" in diffusion chambers and no transformation of small lymphocytes.

Direct observation in vivo in amphibians (Lewis, 1925; Clark and Clark, 1930) provided evidence for monocyte to macrophage transformation, but in amphibian larvae, tissue histiocytes (the local "resting wandering" cells of the connective tissue were also seen entering the inflamed tissue. Ebert and Florey (1939) in a classic study reported successful direct observation of the migration of carbon-labeled blood monocytes into sites of inflammation in rabbit ear chambers and their conversion to macrophages and giant cells; no small lymphocyte to macrophage transformations were observed. They presented evidence based on differential vital staining with two dyes that in mammals local tissue histocytes did not contribute to the inflammatory exudate. This confirmed earlier work of Cappell (1930), who induced a mild peritonitis in intensely vitally stained mice and guinea pigs, which had free dye present in blood and tissue fluid but little in
the peritoneal cavity; the free phagocytic cells appearing were not significantly stained, which ruled out origin from the local heavily stained tissue histocytes. He interpreted his findings as indicating hematogenous origin, and favored lymphocyte to monocyte to macrophage transition.

I.3 Pathological and Transplantation Studies: The "Skin Window" Technique and Other Studies

Continuing the work of previous investigators, the question of hematogenous versus local origin was further resolved by studies following changes in the cell composition of inflammatory infiltrates as a function of time and by studies tracing immunologically labeled or chromosome marked cells in radiation chimeras. The prototype of the former experiment was that of Kolouch (1939) who injected egg albumin subdermally in the rabbit and studied the cell infiltration in the areolar subcutaneous tissue by means of serial biopsies. He concluded that the cells in the infiltrate were of hematogenous origin and that the macrophages were derived from small lymphocytes. Pam and Spector (1962) employed similar techniques in the rat, using various macromolecular substances given as intradermal or intraperitoneal injections; again the inflammatory exudates were considered to be predominantly of blood origin. Monocytes were the chief constituents of the early peritoneal exudates and were thought to be derived from lymphocytes.
Probably the most influential of such studies in recent years have been those of Rebuck and co-workers (Rebuck and Crowley, 1955; Rebuck et al., 1964) in which they have used their "skin window" technique. The technique involves placement of glass coverslips on areas of abraded human skin. Exudate cells adhere to the "windows" and can be examined microscopically by removing the coverslips at various time intervals. By means of such serial sampling, they found large numbers of small lymphocytes between 8-12 hours and typical macrophages between 10-20 hours; they concluded that the small lymphocytes hypertrophied and became macrophages. Rebuck's technique has been extensively employed in the study of various clinical diseases (Rebuck and Crowley, 1955; Riis, 1959; Wulff, 1967).

The interpretation by morphological criteria that "skin window" macrophages develop from the small lymphocyte of the blood has received support from some investigators (Braunsteiner, Paertan and Thumb, 1958; Riis, 1959) and criticism by others (Leder, 1967; Wulff, 1963; Volkman and Gowans, 1965 a and b; Trepel and Begemann, 1966); the autoradiographic studies will be reviewed in a later section. Histochemical studies have led to divergent results: Rebuck et al. (1958) and Braunsteiner (1963) reported an increase of certain enzymes from a weak concentration in lymphocytes to a strong one in macrophages, while Leder (1967) and Wulff (1963) found histochemical similarities (notably by the naphthol AS-D chloroacetate method) only between the blood monocyte and the exudative macrophage. Braunsteiner (Schmalzl and
Braunsteiner, 1967) has recently reversed his position on this subject, so that current histochemical studies favor monocytic origin.

Final important evidence for hematogenous origin has come from studies with the radiation chimera, in which it is possible to produce an animal effectively host in its local tissues and donor in its hematopoietic tissue; cells from donor and host can be differentiated by means of specific antisera or by means of chromosome markers. Balner (1963) administered allogeneic bone marrow to lethally irradiated hosts and found that the free peritoneal macrophages produced in such chimeras were of donor (bone marrow) type. He carefully did not exclude the fixed reticuloendothelial cells of the donor bone marrow as possible cells of origin. Lubaroff and Waksman (1967, 1968) have demonstrated by a similar technique that most of the infiltrating cells in passively transferred delayed hypersensitivity reactions to PPD in allogeneic rat chimeras are of bone marrow origin. Goodman (1964) independently reported experiments involving long term radiation chimeras (3–16 months), restored with a wide variety of tissues (bone marrow, fetal liver, peripheral blood leucocytes, peritoneal fluid cells); again, the macrophages produced in the chimeras were of donor origin.

Chromosome-marker evidence in syngeneic mouse radiation chimeras has limitations in that only dividing cells can be karyotyped and morphological identification of the dividing cells is not currently possible. Virolainen (1968) has superceded the
problem of obtaining karyotypes from mature rarely dividing macrophages through use of a "macrophage growth factor" which induces mitosis in vitro, and has established bone marrow or spleen cells as the source of these dividing macrophages in peritoneal exudates, lung washings, and from organ cultures of bone marrow, spleen, lymph node, thymus and lung. Pinkett, Cowdrey and Nowell (1966) reported that in both normal lungs and in lungs exposed to an irritant, approximately two-thirds of the dividing cells which could be recovered by lung washings arose from the hematopoietic system and one-third were of pulmonary origin. Boak et al. (1968) have reported that during intense reticuloendothelial proliferation in the liver in graft versus host reaction and after stimulation with Corynebacterium parvum, dividing cells in liver macrophage preparations can be derived from either of two different precursors in populations of recirculating lymphocytes and bone marrow cells respectively. This chromosome marker study obviously involves a different macrophage population under radically different experimental conditions, though their findings are provocative. All the radiation chimera studies emphasize the remarkable proliferative and differentiative potential of some early progenitor of the mononuclear phagocytes that is contained in the hematopoietic tissues restoring the irradiated host animal.

1.4 Cell Kinetic Studies Using Tritiated Thymidine: The Problem of the Identity of the Progenitor Cell

With the advent of tritiated thymidine (H3-TdR), a specific stable cell marker for cells in DNA synthesis, a sensitive
technique became available for tracing the movements of proliferating cells and for identifying the morphological type of such labeled cells through autoradiography. This provided a possible means for resolving the question of the identity of the macrophage progenitor cell. Cronkite, Bond, Fliedner and Killmann (1960) were the first to capitalize on the different degree of labeling by \( H^3 - \text{TdR} \) of hematopoietic cells and cells indigenous to extravascular sites of inflammation; they established certain important labeling patterns which provided the basic modus operandi by subsequent investigators. By varying the time of \( H^3 - \text{TdR} \) labeling with respect to induction of sterile inflammation, they found that few cells proliferating locally took up the labeled precursor and that the majority of the cells contributing to the exudate came from blood mononuclears, but were not classical small lymphocytes (i.e. large and medium lymphocytes or similar sized cells, see Cronkite in discussion following the cited paper). Using the same rationale, Kosunen and co-workers (1963 a and b) examined the origin of cells participating in delayed hypersensitivity reactions and in experimental allergic encephalomyelitis and concluded that the infiltrating cells were of hematogenous origin and resembled medium-sized lymphocytes of lymphoid tissue or large lymphocytes of the blood. Lidén (1967) came to similar conclusions in his study of allergic contact dermatitis in the guinea pig; labelled small lymphocytes were not observed in significant numbers.

Further characterization of the inflammatory exudate at cutaneous sites has come from the work of Spector and co-workers.
(Spector, Walters and Willoughby, 1965; Spector and Coote, 1965), who studied cutaneous responses to fibrinogen or paraffin oil, again using comparison of $^{3}H$-TdR incorporation into mononuclear leucocytes and exudate mononuclear cells, but with the addition of colloidal carbon as a marker. They concluded that almost all of the exudate mononuclear cells were derived from circulating monocytes and excluded small lymphocytes, large and medium lymphocytes, and various transitional forms as progenitors of the inflammatory macrophages. These workers have extended their studies to contact sensitivity and chronic delayed hypersensitivity reactions and have found generally similar results (see Spector, 1967); interesting evidence that small round cells contained in inflammatory granulomata are progeny of histiocytes and that these small round cells subsequently change to histiocytic or macrophage form has been reported (Spector and Lykke, 1966).

The most conclusive evidence against the concept of the small lymphocyte as the precursor of phagocytic mononuclear cells in blood or inflammatory exudates has come from Gowans' laboratory (Volkman and Gowans, 1965 a and b). Following the method of Braunsteiner, Höfer and Sailer (1961), who first combined $^{3}H$-TdR labeling with Rebuck's skin window technique, Volkman and Gowans (1965a) clearly demonstrated that exudative macrophages originate from rapidly dividing precursors continuously proliferating at sites distant from the locus of inflammation, and that these macrophages are derived from blood-borne antecedents, most probably blood monocytes. They found no correlation of percent labeled
a good deal of the confusion that had resulted from the morphological interpretations of the past.

Volkman (1966) has extended the generality of the results from the skin window studies to the origin and turnover of mononuclear cells in peritoneal exudates in rats; similar labeling patterns were seen and bone marrow established as the source of the macrophage precursors. Because of the better sampling of the cells in the exudates further characterization of the minority population of labeled small lymphocyte-like cells in the exudates was possible; these cells have the labeling features of a rapidly proliferating population unlike the majority of blood and thoracic duct lymph small lymphocytes and are bone marrow derived. The possibility that they might be immature monocytes was discussed. Van Furth and Cohn (1968) have confirmed in the mouse the findings of Volkman and Gowans (1965 a and b) in the rat. In addition they found similar labeling patterns for macrophages in unstimulated peritoneal exudates and studied the kinetics of what they call promonocytes in the bone marrow itself. Mononuclear phagocytes in bone marrow, blood, and peritoneal exudates were defined by the interesting operational criterion that such cells must stick to glass within the initial 2 hours of in vitro culture.

1.5 Summary

From the previous sections the emerging picture of the origin of phagocytic mononuclear cells in inflammatory exudates is that:
(i) The majority of these phagocytic mononuclear cells are of hematogenous origin.

(ii) These blood-borne macrophage antecedents originate in hematopoietic tissue, principally the bone marrow, and in the blood appear mostly as monocytes. Evidence for a minority population of small round cells, similar morphologically but not functionally to small lymphocytes, is accumulating.

(iii) The bone marrow-located macrophage precursors (the progenitor cells for the macrophage antecedents) are a rapidly dividing, continuously proliferating population of cells.

Recent autoradiographic data (see section 1.4) renders untenable the concept that the classical small lymphocyte of the blood and lymph is a macrophage antecedent, but also presents evidence for a small lymphocyte-like cell that may be a macrophage antecedent; this finding coupled with the increasing recognition of the functional heterogeneity of the "small lymphocyte" population affords an explanation for the controversy over the lymphocytic origin of monocyte and macrophage and emphasizes the inadequacy of pure morphological criteria for distinguishing such cells. The next chapter will review some functional criteria for differentiating morphologically similar cells important as macrophage precursors.
CHAPTER 2

RELATIONSHIP OF MACROPHAGE PRECURSORS
TO HEMATOPOIETIC STEM CELLS

2.1 Evidence from Regenerating Systems for Monocyte–Macrophage Stem Cells

This chapter will further examine the relationship of the phagocytic mononuclear cells to their precursors in hematopoietic tissue and review the data on the proliferative capacities of each of the stages in the development of the mature cell. The studies reviewed in the last chapter (sections 1.3 and 1.4) demonstrated bone marrow contains monocyte–macrophage progenitors capable of repopulating the partially or whole body irradiated animal to the extent that satisfactory response to inflammatory stimuli could be mediated. This same tissue has been shown to furnish pluripotential hematopoietic stem cells capable of repopulating both myeloid and lymphoid tissues (Ford, Micklem and Gray, 1959; Barnes et al., 1959; Micklem et al., 1966; Wu, Till, Siminovitch and McCulloch, 1967 and 1968). The obvious question is the relationship between these monocyte–macrophage progenitors and the stem cell(s) responsible for other types of hematopoiesis (erythropoiesis, granulocytopoiesis, megakaryocytopoiesis, lymphocytopoiesis). Answers to such questions have often been sought in regenerating or embryonic systems, some of the earliest studies being by Sabin and co-workers (Sabin, Doan and Cunningham, 1925; Cunningham, Sabin and Doan, 1925). As will be shown in the following
sections, it is evidence obtained by such alterations in the steady state that has provided us with some insights into these relationships.

2.2 The Stem Cell Concept: The Spleen Colony Technique

The idea of hematopoietic stem cells is an old one, and a long standing controversy over their morphological identity and ontogeny exists (Sabin, 1932; Downey, 1932; Bloom, 1938; Yoffey, 1962). Functionally defined, these cells maintain by proliferation and differentiation the integrity of the hematopoietic system in the steady state and after injury. They constitute a very small minority of all cells found in hematopoietic tissue, being greatly outnumbered by their more differentiated progeny. Clarification of some facets of the stem cell problem has recently been achieved because techniques have been developed to measure functional attributes of such cells. Pioneers in their area have been McCulloch, Till, Siminovitch and their collaborators at Toronto. They have defined four essential properties of a hematopoietic stem cell: (i) capacity for extensive proliferation, (ii) capacity for self-renewal, (iii) capacity to enter differentiation, and (iv) capacity to alter behavior in response to changing demands for hematopoiesis (Becker, McCulloch and Till, 1963). By means of their in vivo spleen colony technique, they have demonstrated these properties for what they call colony-forming units (CFU), cells closely related to if not identical to the pluripotential hematopoietic stem cell postulated in the hematological literature.
In brief, the spleen colony technique assays the capacity of certain cells in normal mouse hematopoietic tissue to proliferate upon transplantation into a suitable recipient mouse, giving rise to macroscopic nodules on the surface of the host spleen (Till and McCulloch, 1961). A linear relationship exists between the number of bone marrow or spleen cells injected and the number of nodules observed 10 to 14 days later. These nodules contain several million cells, some of which can form further nodules upon subsequent retransplantation (Simonovitch, McCulloch and Till, 1963). Histological study of these nodules revealed cells of the erythrocytic, granulocytic, or megakaryocytic series — singly or in combination of cell types, including colonies containing all three cell types (Jurášková, Tkačlecek and Drášil, 1964); some nodules contain cells not morphologically identifiable.

An important property of these spleen nodules, suggested by limiting dilution studies, is their clonal nature. The Canadian workers (Becker, McCulloch and Till, 1963; Fowler, Wu, Till, McCulloch and Siminovitch, 1967) have presented cytological evidence based on unique radiation-produced chromosome markers for the single cell origin of the spleen colony; studies using stable chromosome markers have confirmed these findings (Welshons, 1964; Chen and Schooley, 1968). Coupled with the histological observation that all three major hematopoietic cell types can occur in a single colony, clonal origin suggests that some colony-forming units are pluripotential hematopoietic stem cells.
2.3 Evidence for a Direct Ontogenic Relationship Between Colony-forming Units and Macrophage Precursors

The immediate question arises as to the possible derivation of monocytes and other macrophage antecedents from the colony-forming unit. The problems faced with direct morphological identification of monocyte precursors such as postulated promonocytes in histological sections of spleen colonies are similar to those that have plagued hematologists examining bone marrow for monocyte precursors — notably the morphological similarity between monocytes, promyelocytes and myelocytes when stained by conventional methods. Recent work by Virolainen and Defendi (1968), using entirely different criteria which circumvent these problems, presents evidence for a direct ontogenic relationship between CFU and mature macrophage. By using T6 chromosome-marked transplant tissue (bone marrow), an ingenious grid culture system for culturing individual spleen colonies, and their "macrophage growth factor" (Virolainen and Defendi, 1967), these investigators established that macrophages are produced by some progeny of the original colony-forming cell. They also established that there existed a positive correlation between colonies wholly or partly granulocytic in composition and colonies producing macrophages; pure erythrocytic colonies did not form macrophages. Besides showing a direct connection between colony-forming cells and macrophage precursors, these findings suggest a common precursor for granulocytes and monocytes and revive Naegeli's concept of a pluripotent myeloblast (1931).
Leder (1967) has presented histochemical evidence from studies of bone marrow that promyelocytes can be considered common progenitors for neutrophils and monocytes. He has assayed for the presence of two pairs of enzymes, each characteristic for monocytes or promyelocytes respectively, and found intermediate forms containing both sets of enzymes; the transitional forms were interpreted as transforming cells intermediate between promyelocytes and monocytes. This differential staining technique could have application to histochemical studies on spleen colonies with the intent of confirming the Virolainen and Defendi data.

Craddock (1967) also has argued for such a common precursor on the basis of clinical observation (the myelo-monocytic leukemias) and experimental evidence; patterns of cortisol sensitivity and resistance (Craddock et al., 1967) and recovery studies after drug - or antineutrophilic serum - induced agranulocytosis (Lawrence, Craddock and Campbell, 1967).

2.4 Dependent Stem Cell Populations: Erythropoietin-Sensitive Cells and Agar Colony-forming Cells

I should like to consider one final theoretical concept, a controversial one, before turning to a more concrete topic, that of macrophage-monocyte kinetics. It concerns the problem of control of hematopoiesis — specifically, at what level of stem cell differentiation do regulatory mechanisms act in response to changing demands for hematopoiesis? Work from several laboratories, investigating the capacity of the hematopoietic system in normal rodents (Bruce and McCulloch, 1964; Blackett, Roylance and Kay,
1964; Schooley, 1966; Schooley et al., 1967; Feldman and Bleiberg, 1967; Morse and Stohlman, 1966) and genetically anemic mice
(Fowler, Till, McCulloch and Siminovich, 1967; Bennett, Cudkowicz, Foster and Metcalf, 1968; Bennett, Pinkerton, Cudkowicz and Bannerman, 1968) to respond to stresses such as hypoxia and radiation, has led to data that suggests that control mechanisms do not act at the pluripotential stem cell level but rather at more differentiated levels. These more differentiated cell levels are characterized by a more committed pattern of differentiation (e.g. unipotentiality), different rate and capacity for proliferation, sensitivity to specific controllers such as humoral agents or antigen, and a probably close relationship to the pluripotential stem cell itself (McCulloch, 1968). McCulloch, Till and Siminovich (1965) have termed such stem cells, "dependent" stem cells, because they are dependent upon the more primitive "independent" stem cells (e.g. the CFU) for eventual replenishment.

The best example of such a dependent stem cell population are erythropoietin-sensitive cells (ESU) which were first described by Bruce and McCulloch (1964). The existence of this population of cells was postulated on the basis of evidence showing that prolonged hypoxia (assumed to produce elevated erythropoietin levels) had little if any effect on the CFU content of the bone marrow; hence, the hormone must act on some progeny of the CFU rather than the CFU itself. Schooley (1966) has restudied this phenomenon in a more controlled system and demonstrated that the growth of CFU in the spleens of the irradiated polycythemic hosts was unaffected by the presence or absence of erythropoietin.
Till, Siminovitch and McCulloch (1967) have confirmed this observation using unirradiated hypertransfused mice of the genotype $W^w/W^w$ as hosts for normal coisogenic marrow cells; granulocytepoiesis and CFU self renewal were not affected by the suppression of erythropoiesis. Differences in the rates of proliferation and recovery of ESU and CFU in the steady state polycythemic mouse before and after sub-lethal irradiation or administration of certain mitotic poisons are also evidence for two distinct cell populations (Schooley, Cantor and Havens, 1966; Hodgson, 1967; Lajtha, 1967; Schooley et al., 1968). Tentative evidence of this nature exists for dependent cell populations in megakaryocytepoiesis (Morse and Stohlman, 1966; Ebbe, 1968) and for early phases of the immune response -- e.g. antigen-sensitive cells (Kennedy et al., 1966).

In light of the possible generality of this pattern of individual control of different hematopoietic cell lines at a dependent stem cell level, it is feasible to propose a unipotential stem cell directed to monocyte-macrophage differentiation and to speculate on its relationship to the macrophage precursors of Volkman and Gowans (1965 a and b). Recent independent reports from two laboratories (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966) have shown the existence in vitro of a hematopoietic stem cell committed to granulocytic and monocyte-macrophage differentiation; these agar colony-forming cells (ACFU) satisfy some of the criteria of a dependent stem cell population. Both groups have described an in vitro method for cloning hematopoietic
cells in soft agar over a bottom feeder layer of neonatal kidney cells or embryonal cells. The colonies produced become macroscopically visible about one week after plating, contain from 50 to several thousand cells, and display cells of two morphological types: cells in various stages of granulocytic differentiation and/or large phagocytic mononuclear cells indistinguishable from macrophages or monocytes. The clonal nature of the pure macrophage colonies from mouse spleen (Pluznik and Sachs, 1966) and of pure granulocytic colonies from rat bone marrow (Paran and Sachs, 1968 in press) has been established; limiting dilution data and agreement between colonies per dish and the Poisson distribution at low concentrations of cells suggest possible clonal origin for the mixed colonies as well (Pluznik and Sachs, 1965). Macrophage and granulocytic colonies in the rat marrow system can be successfully recloned in the presence of a continued supply of inducer (Paran and Sachs, 1968); similarly, macrophage colonies can be recloned in mouse systems (Pluznik and Sachs, 1965; Metcalf and Foster, 1967), but at present not granulocytic colonies (Ichikawa, Pluznik and Sachs, 1966). This capacity to proliferate on recloning suggests self-renewal capabilities.

The characteristics of the ACFU described in the last paragraph strongly suggest a dependent stem cell population, particularly the limited differentiative and proliferative potentials, though McCulloch (1968) has pointed out that these limitations may be a reflection of the influence of environment on the expression of potential of hematopoietic stem cells rather than the existence of two classes of stem cells with different
potential. Wu, Siminovitch, Till and McCulloch (1968) have examined the relationship between ACFU and CFU by studying individual chromosome-marked spleen colonies in order to establish some ontogenic relationship between the two classes of cells and to assay each nodule for colony forming activity of each type. Their results proved that many of the clonally derived spleen nodules contained progeny capable of forming new spleen colonies and others capable of forming colonies in culture. A remarkable correlation was found in the numbers of each type of colony-forming cell in each nodule, which is compatible with a close parent–progeny relationship between CFU and ACFU respectively; the data however could neither establish nor rule out identity between the two types of stem cells. Bennett, Cudkowicz, Foster and Metcalf (1968) have reported normal numbers of ACFU's in genetically anemic mice of the W series, which bear CFU's severely deficient in numbers and in the ability to produce normal sized spleen colonies. Therefore, at the present time characterization of ACFU's as a dependent stem cell population remains conjectural; the studies of Chapter 4 will report on experiments designed to further delineate this cell population in relation to CFU's and macrophage precursors.
2.5 Studies on the Proliferative Capacities of Cells of the Monocyte-Macrophage System

Data on the rates of division and turnover times of each cell population in the development of the mature macrophage are of crucial importance to interpretation of \( H^3 \)-thymidine labeling studies (section 1.4) and establishment of possible subclasses of monocyte-macrophage stem cells (section 2.4). The macrophages and monocyte-like cells seen in non-induced (normal) and induced peritoneal exudates are generally conceded to be a rarely dividing, mature, slowly turning-over cell population. Table 2.1 summarizes data on \( H^3 \)-TdR labeling of these cells as reported in the literature; the data support this concept as applied to murine peritoneal macrophages. Van Furth and Cohn (1968) have estimated on the basis of their data that the turnover time of normal peritoneal macrophages is about 40 days. Spector and Ryan (1969) recently reported a possible 100 day life span for macrophages in carrageenin-induced granulomata as determined by \( H^3 \)-TdR autoradiography.

Similarly, the circulating blood monocyte has been shown to be a rarely dividing cell in humans (Bond, Cronkite, Fliedner and Schork, 1958; Bond, Fliedner, Cronkite, Rubini, Brecher and Schork, 1959) and in rats and mice (Volkman, 1967; van Furth and Cohn, 1968); using \textit{in vitro} \( H^3 \)-TdR labeling of leucocyte concentrates from peripheral blood, these workers found less than 1% labeling of all the blood mononuclears. A more remarkable finding, however, was the significant percentage of
<table>
<thead>
<tr>
<th>Mode of Labeling</th>
<th>Animal</th>
<th>Route</th>
<th>Dose of H(^3)-TdR</th>
<th>Length of Induction</th>
<th>Inducing Agent</th>
<th>Duration of Induction</th>
<th>% Labeled Macrophages</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>in vivo</strong></td>
<td>mouse</td>
<td>iv, ip, sc</td>
<td>? 1-2 hr none</td>
<td>1-3 Mims, 1964</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sc</td>
<td>100(\mu)C x 2 7 hr none</td>
<td>1-3 Mims, 1964</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse</td>
<td>iv</td>
<td>10(\mu)C 2 hr none</td>
<td>1-3 Mims, 1964</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse</td>
<td>iv</td>
<td>25(\mu)C 1 hr none</td>
<td>1-3 Mims, 1964</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>in vivo</strong></td>
<td>rabbit</td>
<td>ip</td>
<td>0.5(\mu)C/g 1-4 hr Klearol 4-48 hr 2-3</td>
<td>Aronson, Elberg, 1962</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse</td>
<td>iv</td>
<td>10(\mu)C 2 hr glycogen</td>
<td>1-4</td>
<td>Wiener, 1967</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rat</td>
<td>iv</td>
<td>1(\mu)C/g 1 hr glycogen</td>
<td>24 hr 0.9</td>
<td>van Furth, Cohn, 1966</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>in vitro</strong></td>
<td>mice</td>
<td>direct</td>
<td>0.1(\mu)C/ml 1 hr none</td>
<td>&lt;1</td>
<td>Forbes, Mackaness, 1963</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>direct</td>
<td>0.1(\mu)C/ml 24 hr none</td>
<td>2.2</td>
<td>van Furth, Cohn, 1968</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>in vitro</strong></td>
<td>rabbit</td>
<td>direct</td>
<td>0.5(\mu)C/ml 8 hr paraffin days none</td>
<td>Watts, Harris, 1959</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rat</td>
<td>direct</td>
<td>1(\mu)C/ml 1 hr glycogen</td>
<td>1 day 1.1</td>
<td>Volkman, 1966</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse</td>
<td>direct</td>
<td>1(\mu)C/ml 1 hr starch</td>
<td>3 days &lt;0.5</td>
<td>Virolainen, Defendi, 1967</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse</td>
<td>direct</td>
<td>0.1(\mu)C/ml 1-5 days calf serum</td>
<td>1-2 days 3.5</td>
<td>van Furth, Cohn, 1968</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
labeled monocytes that was seen within 36 hr after intravenous injection of H\textsuperscript{3}-TdR -- of the order of 25-30% or more. This was first demonstrated in humans by Fliedner, Cronkite and Bond (1961) and subsequently in animals by several groups (Volkman and Gowans, 1965a; Whitelaw, 1966; Matsuyama, Wiadrowski and Metcalf, 1966; Volkman, 1967; van Furth and Cohn, 1968). The fact that labeled monocytes appear as early as 6 hr after pulse labeling with the isotope and rapidly constitute a significant proportion of the total monocyte pool led to two conclusions: (1) there must be a brief post-mitotic maturation period at the extravascular site of production, and (2) the monocytes must be progeny of rapidly dividing precursors. Volkman (1966) has suggested that on release into the blood stream these cells are poorly differentiated because of the paucity of recognizable monocytes in the bone marrow.

Estimates of the turnover time of the monocyte in the blood were first reported in patients by Fliedner, Cronkite and Bond (1961). More extensive studies in the rat by Whitelaw (1966), using multiple labeling with H\textsuperscript{3}-TdR over a 6 day period and the criterion of peroxidase positivity for identifying monocytes from large lymphocytes, produced an estimate of about 3 days for the mean half-life of the circulating monocyte. He also concluded that the disappearance of the monocytes from the circulation occurred at random and not as a consequence of senescence. Volkman (1967), in an independent study in which H\textsuperscript{3}-TdR was given by slow continuous infusion (0.5 uC/gm/day) for 4-5 days, came to similar conclusions, but estimated the half-time of disappearance of unlabeled monocytes as 40 hr; even shorter half-times (22 hr) have been reported in
the mouse by van Furth and Cohn (1968), using stricter criteria for what constitutes a labeled cohort of cells.

An interesting conclusion drawn by Whitelaw (1966) from data on the rise in mean grain count above background during the labeling period was that the generation time of the monocyte precursor averages about 24 hr and that at least 3, possibly 4, generations exist from the primitive earliest precursor to the circulating mature monocyte. Whitelaw, Bell and Batho (1968), in a further study utilizing a single pulse of $^3\text{H}$-TdR and frequent sampling over a 3 day period, have applied a mathematical model to the resultant curves and estimated a 21 hr generation time and 12.5 hr DNA synthesis time. Van Furth and Cohn (1968) have reported in vitro and in vivo labeling data for mononuclear phagocytes of the bone marrow, designated promonocytes and defined as those bone marrow cells adhering to glass cover slips during 2 hr incubation in vitro; a detailed description of the morphological characteristics of these dividing and maturing promonocytes is to be given in a subsequent communication by these workers. If one accepts these criteria for promonocytes, their data show 32% $^3\text{H}$-TdR in vitro labeling of these cells, mostly during the initial 24 hr of culture, and 21% and 68.4% in vivo labeling 1 hr and 24 hr respectively after one intravenous injection of $^3\text{H}$-TdR. The peak of the labeled bone marrow promonocytes after in vivo labeling occurred 24 hr prior to the peak of labeled monocytes; this was interpreted as evidence of the derivation of blood monocytes from bone marrow promonocytes. Again, the significant degree of promonocyte labeling attested to their rapid turnover.
Lastly, consideration should be given to turnover rates within the compartment of hematopoietic stem cells as measured by the spleen colony technique; if one accepts the data of Virolainen and Defendi (1968), these are the most primitive progenitors of monocytes and macrophages. Becker, McCulloch, Siminovitch and Till (1965) have shown that in the steady state the fraction of CFU in adult mouse marrow or spleen in DNA synthetic cycle is very small; the vast majority of CFU are apparently in very long cell cycle (long G₁) or out of cell cycle, the G₀ state postulated by Lajtha (1963a). However, in situations where hematopoietic tissues are rapidly expanding due to environmental pressures, such as fetal liver or regenerating bone marrow or spleen transplants, a large fraction (40–65%) of the CFU are in DNA synthesis. This study supported the hypothesis of Lajtha and his collaborators that the physiologic state of stem cells with respect to the length of their generative cycle may be altered as the demand for their differentiated progeny changes (Lajtha, Oliver and Gurney, 1962; Lajtha, Gilbert, Porteous and Alexanian, 1964).

2.6 Characterization of Monocyte-Macrophage Progenitor Cells in the Bone Marrow

On the basis of material reviewed in this chapter and certain facts summarized in section 1.5, a plausible model of the developmental and proliferative sequences involved in monocyte-macrophage production has been designed. The model is analogous to the Type II stem cell model designed by Lajtha and co-workers.
Figure 2.1 - A proposed model of the monocyte-macrophage system (steady state conditions)

BONE MARROW

Differentiating and Dividing

BLOOD

Macrophage precursors

Non-Dividing

TISSUE

Macrophage antecedents

Mature Macrophages
to explain bone marrow stem cell kinetics after irradiation (Lajtha and Oliver, 1962; Lajtha, Oliver and Gurney, 1962). It emphasizes more what is unknown about the monocyte-macrophage system than what is fact. Figure 2 summarizes the model in a schematic fashion after the notation of Gilbert and Lajtha (1964). The boxes represent compartments of cells at identical stages of differentiation and do not represent anatomic compartments. Arrow entering indicates cells entering the population from progenitor compartments, and arrow leaving, cells leaving to more differentiated compartments. Double-headed arrow over some compartments leaving and entering indicates cells leaving on division and daughter cells entering (i.e. self-renewal divisions). Hatched areas represent the rough proportion of cells in cell cycle.

The experimental part of this dissertation will concern itself with testing the validity of the model and elucidating the proliferative capacities of macrophage progenitor cells within the bone marrow more mature than CFU and relationships of cell compartments within the bone marrow to one another. Specific questions that arise from consideration of the model are:

(i) What functional types of progenitor cells in the steady state come under the classification of "macrophage precursor cells" as defined by Volkman and Gowans (1965a)?

(ii) What is the relationship of ACFU to macrophage precursors?
(iii) What is the precise relationship of ACFU to CFU?
Are they identical or different cell populations?

(iv) Can ACFU be classified as a dependent stem cell population?

(v) Since macrophage precursors are rapidly proliferating, morphologically immature cells, what is their radiosensitivity and sensitivity to specific cell cycle poisons?

(vi) What is the tempo and timing of recovery of macrophage precursors and ACFU after radiation or drug injury?

(vii) What are the similarities and differences between monocyte-macrophage progenitor cells and precursors for the other hematopoietic cells?

The general approach to these questions will employ techniques measuring cell population kinetics. Radiation and the mitotic inhibitor, vinblastine sulfate, were used to perturb the steady state and thereby allow analysis and identification of the kinetic patterns of the cell populations under study. These techniques have been utilized with some success by Lajtha and co-workers in radiation studies (see Lajtha, 1963b) and by Bruce and collaborators in studies using specific cell cycle poisons (see Bruce, 1966). Chapters 3 and 4 describe experiments utilizing these techniques to characterize monocyte-macrophage progenitor cells and the results of those experiments.
CHAPTER 3

EFFECTS OF IONIZING RADIATION AND VINBLASTINE ON THE PROLIFERATION

OF PERITONEAL MACROPHAGE PRECURSORS IN THE MOUSE

3.1 Introduction

During the last decade much interest has centered on
the inhibitory effects of radiation and drugs on the immunological
processes, particularly in regard to the lymphoid cells of the
immune system and their response to the injection of antigen
(Taliaferro and Taliaferro, 1951; Talmage, 1955; Taliaferro,
Taliaferro and Jaroslow, 1964; Schwartz, 1965; Makinodan, 1966).
Only recently has more attention been directed to effects on
other cells, particularly the macrophage, because of increasing
recognition of the probable requirement for processing of antigen
by macrophages in antibody production (Fishman, 1961; Nossal,
Ada and Austin, 1964) and of their important but not well
understood role in cellular immunity (Elberg, 1960; Dumonde, 1967;
Turk, 1967; Mackaness and Blanden, 1967).

The mature macrophage and monocyte have been shown to
be comparatively radioresistant cells to killing (Bloom, 1948;
Brecher, Endicott, Gump and Brawner, 1948; Kornfeld and Greenman,
1966). However, several workers have demonstrated that radiation
alters the functional capabilities of the cells, such as intracellular degradation of bacteria (Donaldson, Marcus, Gyi and
Perkins, 1956; Nelson and Becker, 1959), though engulfing and
degradative capacities of peritoneal phagocytes for sheep erythrocytes appear to be resistant to supralethal levels of radiation (Perkins, Nettesheim and Morita, 1966). Of considerable immunological interest has been the recent demonstration by Gallily and Feldman (1967) and Pribnow and Silverman (1967) that preparation of antigen by macrophages for antibody production is a moderately radiosensitive process.

We have been interested in radiation and mitotic inhibitor effects on the early progenitors of the macrophages and monocyte-like cells discussed above. Volkman and Gowans (1965 a and b) demonstrated that these progenitor cells occur as rapidly dividing cells in the bone marrow, measurable as $^3$H-thymidine labeled mature macrophages on skin windows. Because the rapid proliferation and immaturity of these precursor cells suggested sensitivity to radiation and specific cell cycle poisons (e.g. vinblastine) comparable to that of other hematopoietic precursor cells, we elected to study the differential effects of these two agents on the numbers of macrophage precursors available for $^3$H-thymidine labeling and quantitatively harvestable as labeled macrophages in glycogen-induced peritoneal exudates. The results of this study show marked sensitivity as compared to the mature macrophage and a recovery pattern following injury similar to that of progenitor cells of the erythroid system. The results will be discussed in terms of the relevance of this cell population to immunological reactivity.
3.2 Materials and Methods

**Mice.** Eight to eleven week old female C₃H/0um mice weighing between 18 and 22 g were used in the experiments. They were housed in sterilized cages, 4 to 5 a group, and allowed food and chlorinated water ad libitum.

**Irradiation.** Mice were irradiated at 1.22 meters from a Co⁶⁰ source with a dose rate in air of 26.6 R/min, measured in air with a Victoreen condenser R-meter. Under these exposure conditions, the average tissue dose to the mouse was approximately 0.94 rad/R in air. After the irradiation the animals were housed in sterilized cages and allowed free access to chlorinated water and food. For the dose response study, the mice were divided into several groups of 4, each group receiving a different dose of irradiation.

**Vinblastine sulphate (VLB).** Vinblastine sulphate (Velban; Eli Lilly and Co., Indianapolis, Indiana) was obtained as a lyophilized powder and dissolved in sterile physiological saline to a concentration of 400 μg per ml (80 μg per 0.2 ml) for recovery studies or other concentrations for the dose response study. All vinblastine preparations were injected i.v. as single 0.2 ml injections.

**Tritiated-thymidine labeling.** Mice were injected in the lateral tail vein with a single dose of 1μC of tritiated thymidine (H³-TdR) per gram of body weight (specific activity, 2.0 C/mm; New England Nuclear Corp., Boston, Mass.). For the
radiation and drug dose-response studies, the labeled compound was given 24 hrs. after irradiation or vinblastine. For the recovery studies, the labeled compound was given at various times after irradiation or vinblastine.

Peritoneal exudates. Non-specific peritoneal inflammation was always induced 24 hrs. after H³-TdR injection by the intraperitoneal injection of 1 ml of a 1% oyster glycogen in water solution (Eastman Organic Chemicals, Rochester, New York). Twenty-four hours later the mice were sacrificed by ether inhalation, the skin over the peritoneum reflected, and 3 ml of sterile Tyrode's solution (Ca⁺⁺ and Mg⁺⁺ free, Difco Labs., Detroit, Mich.) containing 10 units of heparin per ml was injected into the cavity. After 3 mins of gentle abdominal massage, 2 ml of fluid were aspirated off with a needleless 1 ml tuberculin syringe into a plastic test tube immersed in an ice bath. In the early experiments peritoneal washings from each mouse were collected separately; later, the washings from 3-5 mice were pooled. Bloody exudates were discarded.

The cells were counted in a conventional hemocytometer chamber after 1:1 dilution of a 100 μl aliquot with Turk's diluting fluid. On the basis of the number of peritoneal cells harvested per ml as calculated from the hemocytometer count and data from isotope dilution studies that show negligible volume of exudate compared to the volume of injected harvesting fluid, the total number of harvestable peritoneal fluid cells per mouse was calculated.
In all experiments the washings were centrifuged lightly at 500 g for 8 mins and the cell pellet washed X 1 and resuspended in normal rat serum. Smears were prepared by painting the cell suspension on acid-cleaned slides with a 00 size sable paint brush, quick air drying in the air stream of a hair dryer, and fixation in absolute methyl alcohol. These unstained smears were later dipped into Eastman Kodak liquid emulsion NTB-2, dried thoroughly in a slow current of air, and stored at 4°C in light-tight boxes with desiccant for 2 weeks. The slides were then developed in Eastman Kodak developer D170 for 6 minutes and fixed. Subsequently the autoradiographs were stained with May-Gruenwald Giemsa (5 mins) and Leishman Giemsa (20 mins). Differential counts were performed on at least 200 cells from each preparation. The number of labeled peritoneal macrophages was calculated from the percentage of such cells present and the total cell count. A schematic summary of the experimental procedures is depicted in Figure 3.1. Plate 3.1 is a photomicrograph of a May-Gruenwald Giemsa stained autoradiograph of H^3-TdR labeled and unlabeled peritoneal fluid cells in a typical 24 hr glycogen exudate.
Figure 3. 1 - Schematic summary of experimental procedures employed in the determination of numbers of \(^{3}H\)-thymidine-labeled peritoneal macrophages in irradiated or vinblastine-treated mice.
Peritoneal fluid cells harvested

Differential count

 Autoradiographs prepared

Labeled cell count

Day of sacrifice

DBL 693-4620

Figure 3.1
Plate 3. 1 - Labeled and unlabeled peritoneal fluid cells, 24 hr glycogen exudate. $H^3$-TdR 48 hr before harvest. (M) macrophage. (L) lymphocyte. May-Gruenwald Giemsa stained autoradiograph, X 800.
3.3 Results

**Cells of the exudates.** Macrophages and monocyte-like cells comprised the vast majority of cells seen in the 24 hr exudates. For classification purposes, cells larger than 10 μm in diameter with pale, indented or partially folded nuclei were called macrophages; most of these cells were more than 15 μm in diameter. The nuclei of the cells possessed a fine chromatin pattern and the abundant pale cytoplasm contained many vacuoles. Cells classified as small lymphocytes had diameters of 5 - 7 μm with dense pachychromatic nuclei and scant bluish cytoplasm. Mononuclear cells with diameters of 7 - 10 μm, more leptochromatic nuclei and slightly more cytoplasm, were called "round cells". Common among the other cells noted were polymorphonuclear granulocytes and mast cells. Table 3.1 summarizes data on total cell number and composition of 12 separate groups of 24 hr glycogen exudates obtained from control, otherwise untreated mice.

In mice receiving irradiation or vinblastine the relative proportions of the various classifiable cell types remained about the same, but the total numbers of peritoneal fluid cells changed. Minor variations in the percentages of cells other than macrophages following irradiation or drug were not studied in greater detail since it was the primary purpose of this investigation to follow changes in the H3-TdR labeled macrophage population.
<table>
<thead>
<tr>
<th></th>
<th>Range</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells/ml X 10^6</td>
<td>4.8 - 9.6</td>
<td>7.2 ± 0.5</td>
</tr>
<tr>
<td>Macrophages and Monocytes, %</td>
<td>76 - 90</td>
<td>81 ± 1.3</td>
</tr>
<tr>
<td>Small lymphocytes, %</td>
<td>1.5 - 16</td>
<td>7.6 ± 1.3</td>
</tr>
<tr>
<td>Round Cells, %</td>
<td>4.5 - 11</td>
<td>7.9 ± 0.7</td>
</tr>
</tbody>
</table>
Sensitivity of macrophage precursors to vinblastine.

In the first series of experiments, groups of mice were given vinblastine sulphate (VLB) in saline solution i.v. in doses ranging from 0 to 100 μg. Twenty-four hours later they received 1 μC/g body weight H₃-TdR (s.a. 2 C/mm²) i.v.; twenty-four hours after the thymidine injection, peritoneal exudates were induced with i.p. 1% glycogen. After another twenty-four hours, the mice were sacrificed and peritoneal washings collected and processed as described. For each experimental run, a group of control mice was employed which did not receive VLB, but were otherwise treated identically.

Table 3.2 summarizes data on actual total number of peritoneal fluid cells (PFC) ± S.E.M., number of labeled macrophages determined by percentage labeled macrophages on the autoradiographs and total cell count, and percentage of control number of labeled macrophages. Figure 3.2 shows these results in the form of a survival curve, in which the logarithm of the percentage of control number of labeled macrophages is plotted against vinblastine dosage. The supranormal labeling seen after 10 μg VLB suggested reversible mitotic inhibition; a similar apparent enhancement of H₃-TdR labeling has been seen after colcemid, 1 μg/gm i.v., a known reversible mitotic inhibitor, using the same sequence of labeling and production of inflammation (Chen, unpublished data).

Sensitivity of macrophage precursors to radiation. In this experiment groups of mice were given whole-body γ-radiation in doses ranging from 0 to 700 R. Following the irradiation the
**TABLE 3.2**

**EFFECT OF VARIOUS DOSES OF VINBLASTINE ON LABELING OF PERITONEAL MACROPHAGE PRECURSORS WITH H³-THYMIDINE**

<table>
<thead>
<tr>
<th>Dose VLB i.v. (micrograms/mouse)</th>
<th>Total No. of Peritoneal Fluid cells \times 10⁶/mouse</th>
<th>No. H³-TdR Labeled Macrophages \times 10³/mouse</th>
<th>Percent of Control Labeled Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>0(Control)</td>
<td>7.8 ± 0.4*</td>
<td>180 ± 20</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>9.3 ± 1.0</td>
<td>270 ± 40</td>
<td>150 ± 20</td>
</tr>
<tr>
<td>40</td>
<td>5.6 ± 0.2</td>
<td>110 ± 4</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>60</td>
<td>4.9 ± 0.8</td>
<td>20 ± 6</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>80</td>
<td>3.3 ± 0.5</td>
<td>7 ± 3</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>100</td>
<td>3.3 ± 0.1</td>
<td>8 ± 3</td>
<td>4 ± 2</td>
</tr>
</tbody>
</table>

*Standard error of the mean*
Figure 3: 2 - H\textsuperscript{3}-thymidine-labeled peritoneal macrophages as a function of dose of vinblastine, relative to values obtained for control mice not exposed to the drug. Standard errors of the mean for 3 - 5 separate determinations at each dose level.
$^{3}$H-thymidine labeled peritoneal macrophages, % of control

Vinblastine per mouse, $\mu$g

Figure 3
same temporal sequence of labeling, induction of inflammation, and harvesting of peritoneal exudates was employed. Table 3.3 summarizes the data on the number of PFC harvested, number of labeled macrophages, and percentage of control number of labeled macrophages. Figure 3.3 depicts these results in the form of a survival curve, in which the logarithm of the percentage of control number of labeled macrophages is plotted against radiation dosage. The initial portion of the curve shows a small shoulder and the linear exponential portion has a D$_{37}$ value of approximately 130 R or 122 rads.

Recovery of macrophage precursors following 80 µg vinblastine. For the studies on the recovery of $^3$H-TdR labeled macrophages following 80 µg vinblastine i.v., the tritium labeled nucleoside was given at various intervals after the drug; induction of inflammation and harvesting of the exudates were as before. Table 3.4 reports the results from two such experiments, and these data are depicted by the points with standard errors of the mean on Figure 3.4; the other points on the graph are from 3 other experiments. Within 24 hr, the relative number of labeled peritoneal macrophages reaches a minimum, followed by a fairly exponential rise to supranormal values by day 5; an approximate doubling time for the regenerating macrophage precursor population measured during this exponential recovery phase is 16 hr.

Following recovery at 5 days, the data suggest a mild overshoot and oscillatory behaviour as seen in the recovery of erythropoietin sensitivity following 200 R whole body irradiation (Schooley, 1968).
**TABLE 3.3**

**EFFECT OF VARIOUS DOSES OF GAMMA-RADIATION ON LABELING OF PERITONEAL MACROPHAGE PRECURSORS WITH H$^3$-THYMIDINE.**

<table>
<thead>
<tr>
<th>Dose (R)</th>
<th>Total No. of Peritoneal Fluid Cells x10$^6$/mouse (pooled)</th>
<th>No. H$^3$-TdR Labeled Macrophages x10$^2$/mouse (pooled)</th>
<th>Percent of Control Labeled Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>8.8</td>
<td>170</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>8.5</td>
<td>200</td>
<td>110</td>
</tr>
<tr>
<td>100</td>
<td>7.7</td>
<td>220</td>
<td>130</td>
</tr>
<tr>
<td>150</td>
<td>6.8</td>
<td>170</td>
<td>100</td>
</tr>
<tr>
<td>200</td>
<td>5.4</td>
<td>140</td>
<td>81</td>
</tr>
<tr>
<td>250</td>
<td>4.9</td>
<td>130</td>
<td>76</td>
</tr>
<tr>
<td>300</td>
<td>3.7</td>
<td>95</td>
<td>57</td>
</tr>
<tr>
<td>400</td>
<td>2.4</td>
<td>41</td>
<td>24</td>
</tr>
<tr>
<td>500</td>
<td>2.0</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>600</td>
<td>1.4</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>700</td>
<td>1.6</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 3. $\mathrm{H}^3$-thymidine-labeled peritoneal macrophages as a function of radiation dose, relative to values obtained for control mice not exposed to irradiation. Point at each dose level represents value from pooled exudates from 4 mice.
$^{3}$H-thymidine labeled peritoneal macrophages, % of control

$\gamma$-radiation dose, $R$

Figure 3.3

$D_{37} = \text{approx } 130 \text{ R}$
### Table 3.4

**Recovery of H³-Thymidine-Labeled Peritoneal Macrophage Precursors Following 80 μg Vinblastine Per Mouse**

<table>
<thead>
<tr>
<th>Day of H³-Tdr Labeling After VLB</th>
<th>Total No. of Peritoneal Fluid Cells x10⁶/mouse</th>
<th>No. of H³-Tdr Labeled Macrophages x10³/mouse</th>
<th>Percent of Control Labeled Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>7.3 ± 0.4</td>
<td>96 ± 6</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>3.3 ± 0.6</td>
<td>11 ± 2</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>2</td>
<td>2.9 ± 0.4</td>
<td>25 ± 6</td>
<td>26 ± 7</td>
</tr>
<tr>
<td>3</td>
<td>4.7 ± 0.5</td>
<td>45 ± 10</td>
<td>47 ± 10</td>
</tr>
<tr>
<td>4</td>
<td>5.6 ± 0.6</td>
<td>52 ± 6</td>
<td>54 ± 7</td>
</tr>
<tr>
<td>0 (Control)</td>
<td>5.4 ± 0.6</td>
<td>59 ± 6</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>8.8 ± 1.0</td>
<td>120 ± 30</td>
<td>200 ± 60</td>
</tr>
<tr>
<td>7</td>
<td>5.2 ± 0.5</td>
<td>49 ± 2</td>
<td>84 ± 10</td>
</tr>
<tr>
<td>9</td>
<td>7.4 ± 1.1</td>
<td>100 ± 10</td>
<td>180 ± 30</td>
</tr>
</tbody>
</table>

*Standard error of the mean
Figure 3.4 – $^3$H-thymidine-labeled peritoneal macrophages as a function of time of $^3$H-thymidine labeling after 80 µg vinblastine i.v. per mouse. The results are expressed as percent of control values from corresponding groups of mice not treated with the drug. Standard errors of the mean are shown for the experiment in which peritoneal fluid cells from each mouse of the group were collected and processed separately.
Figure 3

Day of $^3$H-thymidine injection after 80 μg vinblastine i.v.
Recovery of macrophage precursors following irradiation.

As for the studies on recovery following vinblastine, whole body gamma-irradiation (200 R or 800 R) was given at various times before $^3$H-TdR labeling, induction of inflammation and harvesting of the exudate cells. Table 3.5 summarizes the data from the irradiation studies and Figure 3.5 depicts the temporal recovery pattern. Following 200 R, more than 5 times as many precursor cells were labeled on day 1 than after 80 mg vinblastine and recovery was complete by day 2; recovery is probably faster because the initial injury to the precursor compartment was much less with 200 R. Irradiation with 800 R, however, caused considerable suppression of labeling, and recovery to normal values did not occur during the period of study.
<table>
<thead>
<tr>
<th>Day of H³-TdR Labeling After 200 R</th>
<th>Total No. of Peritoneal Fluid Cells x10⁶/mouse (pooled)</th>
<th>No. of H³-TdR Labeling Macrophages x10³/mouse (pooled)</th>
<th>Percent of Control Labeled Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>5.9</td>
<td>140</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>4.1</td>
<td>74</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>5.8</td>
<td>220</td>
<td>160</td>
</tr>
<tr>
<td>3</td>
<td>4.7</td>
<td>110</td>
<td>79</td>
</tr>
<tr>
<td>0 (Control)</td>
<td>4.8</td>
<td>89</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>5.3</td>
<td>160</td>
<td>180</td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
<td>120</td>
<td>140</td>
</tr>
<tr>
<td>7</td>
<td>4.4</td>
<td>100</td>
<td>110</td>
</tr>
<tr>
<td>9</td>
<td>4.6</td>
<td>120</td>
<td>140</td>
</tr>
</tbody>
</table>
Figure 3.5 - $H^3$-thymidine-labeled peritoneal macrophages as a function of time of $H^3$-thymidine labeling after irradiation (200 R or 800 R). The results are expressed as percent of control values from corresponding groups of unirradiated mice.
Day of $^3$H-thymidine injection after 200 R or 800 R $\gamma$-radiation
3.4 Discussion

The experimental results presented in this paper are consistent with the view that the precursors of phagocytic mononuclear cells in inflammatory peritoneal exudates are radiosensitive and mitotic inhibitor-sensitive cells, similar in many ways to other hematopoietic progenitor cells. The degree of radiosensitivity exhibited by the tritiated-thymidine-labeled precursor cells of this study ($D_{37} = 122$ rads) is comparable to that reported for cultured normal and neoplastic mammalian cells (Puck and Marcus, 1956; Hewitt and Wilson, 1959; Elkind and Sutton, 1960), hematopoietic "stem" cells (McCulloch and Till, 1962; Gurney and Wackman, 1961; Hodgson, 1962), and cells mediating various parameters of the immune response (Makinodan, Kastenbaum and Peterson, 1962; Celada and Carter, 1962; Smith and Vos, 1963; Kennedy, Till, Siminovitch and McCulloch, 1965). Radiation sensitivity of this order of magnitude suggests that it is the capacity for continued proliferation that is affected by the radiation, and that this causes the reduction in numbers of labeled mature peritoneal macrophages. The original papers that established the existence of this rapidly proliferating bone marrow cell population did not report a proportional decrease in $H^3$-TdR labeling of skin window macrophages in the rats receiving 400 rads, but did report essentially no labeling after 750 rads (Volkman and Gowans, 1965 a and b). Volkman and Collins (1968) have recently found a significant lowering of the $H^3$-TdR labeling index for mononuclear exudate cells at the foot pad test sites of $S._{gallinarum}$-vaccinated mice 24 hr after 400 rads $X$-irradiation. The fairly
quantitative method used for harvesting the cells of the inflammatory exudates in our experiments has permitted a more sensitive estimate of macrophage precursor cell radiosensitivity.

Implicit in the assumption that labeling patterns seen in the peritoneal exudative macrophage are a reflection of $H^3$-TdR uptake by dividing progenitor cells in the bone marrow is that there be comparable labeling in the intermediary circulating pool of macrophage antecedents, the blood monocytes. Blood monocytes have been demonstrated to have a fairly rapid turnover in the circulation, but represent a post-mitotic, non-proliferating population (Whitelaw, 1966; Volkman, 1967; van Furth and Cohn, 1968). Volkman and Collins (1968) have demonstrated a marked reduction following 440 rads in the absolute number of monocytes in the peripheral blood of $S. gallinarum$ sensitized mice, attributed to the reduced input from the proliferating bone marrow precursor compartment and continued random loss from the circulation. In normal, unstimulated mice van Furth and Cohn (1968) have reported marked reduction of $H^3$-TdR labeled monocytes (10% of the normal value at 84 hr) following 435 rads total-body X-irradiation, which further supports the thesis that radiation primarily affects the monocyte-macrophage precursors in the bone marrow.

The sensitivity of the macrophage precursors to the mitotic poison, vinblastine sulfate, was greater than that reported for normal spleen and marrow colony-forming units (Valerio, Bruce and Meeker, 1966a) and antigen-sensitive precursors of hemolysin-producing cells (Syklocha, Siminovitch, Till and McCulloch, 1966),
but less than that for erythropoietin-sensitive cells (Hodgson, 1967) and lymphoma colony-forming units (Valeriote, Bruce and Meeker, 1966a). The degree of agreement with the various types of non-neoplastic progenitor cells is remarkable considering the different ways the drug was administered and the disparate methods of assay. Applying the model of Valeriote, Bruce and Meeker (1966b) for the in vivo action of vinblastine to the data of the vinblastine dose-response study and bearing in mind the several assumptions for that model — all of which are not satisfied by the experimental system, one can obtain an estimate of approximately 8.5 hr for the generation time of the macrophage precursors; a threshold dose of 15 µg was used and a dose of 80 µg was assumed to cause 100% killing.

Irreversible loss of capacity for proliferation as reflected in colony-forming ability has been proposed as the mechanism of action of the drug (Bruchovsky, Owen, Becker and Till, 1965; Valeriote and Bruce, 1965); whether this is accomplished by disruption of the mitotic apparatus (Cutts, 1961) or by blocking DNA synthesis (Van Lancker, Flangas and Allen, 1966; Luyckx and Van Lancker, 1966) is a moot point in the present experiment. The data suggest that the action of vinblastine on this capacity for proliferation is the principal cause for the decrease in labeling observed.

In the temporal recovery studies, vinblastine was employed in the hope that its selective action on cells passing through mitosis would produce a different response than radiation,
which can be assumed to have direct effects on resting $G_0$ cells as well. It would be particularly desirable to selectively inhibit progenitor cells committed to monocyte-macrophage differentiation rather than to affect the more primitive pluripotential hematopoietic stem cells as well. Becker, McCulloch, Siminovitch and Till (1965) have demonstrated that most spleen colony-forming cells (a measure of pluripotential hematopoietic stem cells) in the steady state are in a state of no cell cycle (Lajtha's $G_0$ state, 1963) or at least a cell cycle of long duration, and hence would not be affected by the mitotic poison. The selectivity of effect of vinblastine was only partially realized because the 80 $\mu$g dose used for the recovery studies probably maintained effective levels of the drug for at least 10 hr (estimated by using a biological half-life for the drug of 3.5 hr; Valeriote and Bruce, 1965). For this duration of exposure, a significant killing of the small fraction of cycling spleen colony-forming cells (CFU) did occur (see Chapter 4). The recovery pattern of $H^3$-TdR labeled macrophages following 80 $\mu$g vinblastine was characterized by rapid recovery to control numbers of labeled cells by day 4 and an overshoot beyond normal values for a few days thereafter. The rapid recovery resembles the temporal pattern of recovery of erythropoietin responsiveness in polycythemic mice after the same dose of vinblastine as reported by Hodgson (1967) and Schooley (1968), and is faster than the recovery of erythropoietin responsiveness seen after 150–200 R (Gurney, Lajtha and Oliver, 1962; Schooley, Cantor and Havens, 1966). Because there is less damage to the CFU compartment with
80 µg vinblastine than with 200 R x-radiation (see Chapter 4),
the rapid recovery may be due to input of newly differentiated
macrophage precursor cells from the resting pool of CFU. Recovery
of macrophage precursors by self renewal within that population
of cells affords another mechanism of recovery.

The temporal recovery pattern after 200 R showed prompt
recovery by day 2; that dose of radiation caused less injury to
the H³-TdR labeling macrophage precursor compartment than did
80 µg vinblastine, but far greater damage to their more primitive
progenitors, the CFU (see Chapter 4). Again, recovery could be
explained by input from a progenitor compartment or by self
renewal. If the former explanation is favored, one must suppose
that differentiation of CFU to macrophage precursors must have
priority over CFU repopulation since CFU levels remain low despite
rapid proliferation of the surviving CFU (Valeriote and Bruce,
1967). The 800 R data show that given enough damage to all
progenitor compartments (the macrophage precursors were suppressed
to a level below that seen after 80 µg vinblastine), sustained
recovery during the first two weeks after irradiation cannot occur.

Efforts to relate the results of this study to changes
in the immune system following radiation or chemotherapeutic agents
would be purely speculative. The sensitivity to loss of prolif-
erative capacity of a small but important progenitor population
of cells considered to play a role in the immune response has been
demonstrated and the rapid recovery in numbers of these cells
following injury has been delineated. Studies on the functional
or physiological states of the injured or recovering population were not carried out. Nevertheless, there are hints from the results of other workers that the sheer numbers of newly recruited functional phagocytic mononuclear cells may be an important variable in the degree and timing of immunological reactivity. Muramatsu, Morita and Sohmura (1965) presented data that suggested a certain number of functional phagocytic cells must accumulate at the site of antigen administration before formation of antibody in detectable amounts can proceed. Volkman and Collins (1968) have shown that after 400 rads x-radiation return of reactivity of the delayed hypersensitivity type coincides with the onset of cellular regeneration as reflected in the return to normal values of the percentage of labeled mononuclear exudate cells. The temporal parameters of recovery of reactivity in both immunologically specific systems are very similar to the recovery in numbers of new phagocytic mononuclear cells seen in the non-specific system examined in this study, which suggests that the newly formed macrophages are an important component of those cells mediating immune responses. Hill (1969) has shown that the presence of mononuclear cells induced by non-specific stimuli can accelerate, intensify and prolong a delayed-type hypersensitivity reaction. He suggests that the rate-limiting step in a delayed-type reaction is the process of accumulation of mononuclear cells to an effective level, and that this process is independent of the presence of specific antigen.
Lastly, we would like to emphasize the similarity in sensitivities and recovery patterns of these monocyte-macrophage progenitors to early cells of the other hematopoietic cell lines, especially the erythroid line. Based on such similarities, it should be possible to predict behavior in one system from knowledge gained about similar phenomena in the other system and acquire hints as to ways to approach unanswered questions.
CHAPTER 4

RECOVERY OF PROLIFERATIVE CAPACITY OF AGAR COLONY-FORMING CELLS AND SPLEEN COLONY-FORMING CELLS FOLLOWING IONIZING RADIATION OR VINBLASTINE

4.1 Introduction

Pluznik and Sachs (1965) and Bradley and Metcalf (1966) have independently reported methods for the in vitro culture of mouse spleen or bone marrow cells. Both methods involve growth in solid agar medium supported by a feeder layer of mouse embryonic cells or neonatal kidney cells or by the presence of certain growth factors in the medium itself. Colonies produced by this technique consist of populations of granulocytic cells in varying stages of differentiation and/or large phagocytic cells indistinguishable from macrophages.

It would be of considerable interest to explore the relationship between these agar colony-forming units (ACFU) and the spleen colony-forming units (CFU) of Till and McCulloch (1961). Wu and co-workers (1968) have suggested there exists a close relationship between these stem cell-like populations on the basis of chromosome marker work. We have elected to study this relationship by means of radiation or drug-induced perturbation of the normal steady state system with the particular goal of establishing or ruling out identity between these two measures of hematopoietic stem cells. Evidence will be presented that favors two distinct stem cell populations and these findings will be
discussed in terms of independent and dependent stem cell criteria (McCulloch, Till and Siminovitch, 1965).
4.2 Materials and Methods

Mice. Eight to eleven-week-old female C\textsubscript{3}H/Cum mice, obtained from Cumberland Farms, were used as bone marrow donors in all experiments. Neonatal kidneys were obtained from 8-10 day-old male and female C\textsubscript{3}H/Cum mice bred from Cumberland stock in this laboratory. Three to five month-old female C\textsubscript{3}H/Cum mice were used as recipients in the spleen colony assays.

Irradiation. In the radiation studies donor C\textsubscript{3}H/Cum mice were irradiated at various times before sacrifice and collection of bone marrow. Irradiations were at 1.22 meters from a Co\textsuperscript{60} source with a dose rate in air of 26.6 R/min, measured in air with a Victoreen condenser R-meter under these exposure conditions, the average tissue dose to the mouse was approximately 0.94 rad/R in air. After irradiation, the animals were housed, five to a cage, in sterilized cages and allowed food and chlorinated water \textit{ad libitum}. For the spleen colony assay, recipient mice received 1100 R under the above conditions. Terramycin (Chas. Pfizer and Co., Inc., New York, N.Y.), 0.5 to 1.0\%, was added to the chlorinated drinking water. The endogenous spleen colony level was approximately 1 colony per spleen.

Vinblastine sulphate (VLB). Vinblastine sulphate (Velban; Eli Lilly and Co., Indianapolis, Indiana) was obtained as a lyophilized powder and dissolved in sterile physiological saline to a concentration of 400 \textmu g per ml (80 \textmu g per 0.2 ml). All vinblastine preparations were injected i.v. as single 0.2 ml injections at various times before sacrifice and collection of bone marrow.
Bone marrow culture technique.

The method for cloning bone marrow cells in soft agar was essentially that of Bradley and Metcalf (1966). The reagents used in the culture technique had the following composition:

- **E2020 medium** (Grand Island Biological Co., Oakland, Calif.):
  - Mixture of minimum essential medium with L-glutamine and without sodium bicarbonate (Earle's salts) 10 X, 100 ml; sodium bicarbonate 7.5%, 30 ml; sodium pyruvate 100 X, 10 ml; non-essential amino acids 100 X, 10 ml; penicillin/streptomycin (5000 U of each/ml), 2 ml; 3 X glass distilled water, 148 ml; fetal calf serum, 100 ml; total volume 400 ml.

- **Trypticase soy broth (TSB)**: 3 g trypticase soy broth powder (Baltimore Biological Lab., Baltimore, Md.) in 100 ml 3 X glass distilled water, autoclaved 15 minutes at 121°C (15 lbs. pressure) and stored at 4°C for no more than 2 weeks.

- **Bacto agar** (Difco Labs., Detroit, Mich.): 1.0 g agar in 100 ml distilled water, boiled for 5 minutes, thoroughly dissolved, and held at 41°C in a water bath. Agar for bone marrow cell overlay was prepared in the same manner except that 0.6 g agar in 100 ml distilled water was used. **Bone marrow-collecting fluid**: E2020 medium, 40 ml; trypticase soy broth, 10 ml; distilled water, 50 ml.

Feeder layers were prepared from the kidneys of 8-10 day old C3H/Cum mice one day before adding the bone marrow cell overlay. The kidneys were removed aseptically, rinsed in Eagle's minimum essential medium, Spinnex (Grand Island Biological Co., Grand Island, N.Y.) with 50-70 U/ml penicillin/streptomycin, and minced with fine iridectomy scissors into a solution containing...
0.25% trypsin in solution A (Grand Island Biological Co., Oakland, Calif.), 50 U/ml penicillin-streptomycin, and 0.04% DNAse solution (Worthington Biochemical Corp., Freehold, New Jersey, w/v in phosphate buffered saline), 0.3 ml added per 10 cc trypsin solution (after the method of Madden and Burk, 1961). The mixture was stirred gently at room temperature for not more than 1 hour and occasionally pipetted up and down to break up larger tissue clumps. The mixture was then centrifuged at about 500 X g for 7 minutes and the cell pellet washed twice with bone-marrow collecting fluid. The kidney cells were then counted by hemocytometer after suitable dilution in Turk's diluting fluid; clumps of 3 or more cells comprised about 10% of an otherwise single cell suspension. The kidney cells were spun down by centrifugation (500 X g for not more than 4 minutes), resuspended at appropriate cell concentration in E2020 medium with trypticase soy broth (4 parts E2020: 1 part TSB) previously warmed to 37°C. The suspension was finally mixed with an equal volume of 1% Bacto agar to a final cell concentration of 1-2 X 10^6 kidney cells per ml 0.5% agar suspension. Two ml of this suspension was plated in each 35 X 100 mm plastic Petri dish (Falcon Plastics, Los Angeles, Calif.) and allowed to sit undisturbed at room temperature for 15 minutes; incubation was at 37°C in a humidified incubator with constant flow of 5% CO₂ in air.

**Preparation of bone marrow suspensions.** Bone marrow was collected from both femurs using the method of Schooley (1966), and marrow from the 4 mice (8 femurs) of each group pooled. Marrow was obtained by flushing bone marrow-collecting medium backward and forward through the femoral marrow cavity by means of a 23-gauge
needle inserted in the proximal end of the femur. The resulting cell suspension was then passed about 10 times through a 25-gauge needle and finally filtered through a nylon gauze sack. The pooled marrow suspension was made up to a volume equivalent to 1 ml bone marrow suspension per each femur. Cells were counted in a hemocytometer chamber after suitable dilution in Turk's diluting fluid. All cell suspensions were kept at ice water temperature throughout these procedures.

For assay of the spleen colony-forming unit (CFU) content of each sample (Till and McCulloch, 1961), an aliquot was taken and diluted to a suitable concentration with minimal essential medium, Eagle (Grand Island Biological Co., Grand Island, New York); 0.5 ml of this dilution was then injected into the lateral tail veins of recipient mice which had received 1100 R whole body irradiation less than 3 hours earlier. Groups of 15–20 recipient mice were used for each dilution, and a group of irradiated but uninjected control mice was included in each experimental run in order to determine endogenous CFU levels. Seven to ten days later the mice were sacrificed, and their spleens removed and fixed in Buon's solution. Counts of macroscopically visible colonies on the surface of the spleen were performed under 10 X magnification.

For assay of agar colony-forming unit (ACFU) content, an aliquot of the original bone marrow suspension was taken (usually 100–250 µl) and diluted with 20–50 volumes of a 4:1 solution of E2020 medium and trypticase soy broth; two dilutions were usually prepared. The cell suspensions were held at 37°C,
and then mixed with an equal volume of 0.8% agar held at 40°C. One ml aliquots of this mixture were pipetted over the kidney feeder layers prepared the day before, and left at room temperature for 15 min to allow gelling. Incubation was at 37°C in a humidified incubator with a constant flow of 5% CO₂ in air. Colony counts were performed routinely after 10 days of incubation under 10 X magnification. A schematic summary of the experimental procedures is depicted in Figure 4.1. Plate 4.1 contains a photograph of agar colonies as they appear in the Petri dish and photomicrographs of individual agar colonies of the two types.
Figure 4.1 – Schematic summary of experimental procedures employed in the parallel determination of spleen colony-forming units/femur and agar colony-forming units/femur after 200 R whole body Co gamma rays or 80 µg vinblastine i.v.
SPLEEN COLONY-FORMING UNIT ASSAY

DONOR MOUSE

Day 0

$^{60}$Co (200 R) OR
80 $\mu$g vinblastine i.v.

DONOR MOUSE

Day of sacrifice

Bone marrow suspension

AGAR COLONY-FORMING UNIT ASSAY

Neonatal kidney cell feeder layer

10 days incubation
@ 37°C and 5% CO$_2$
in moist air

Agar colonies counted

LETHALLY IRRADIATED RECIPIENT MOUSE

$^{60}$Co (1100 R)

8-10 days

Spleen colonies counted

Figure 4.1

DBL 693-4619
Plate 4.1 - (a) Colonies in agar from adult bone marrow on a supporting layer containing medium conditioned with neonatal kidney cells. X1.6 (b) Granulocyte colony and (c) macrophage colony. Both X50; (a), (b), and (c) unstained.
4.3 Results

Recovery patterns of ACFU and CFU following 80 μg vinblastine. The data on the recovery of proliferative capacities of ACFU and CFU as a function of time after intravenous injection of 80 μg VLB, simultaneously measured in femoral bone marrow, are summarized in Table 4.1 and Figure 4.2. Twenty-four hours after the drug, both ACFU and CFU were at a minimum. The ACFU were about 20% of control after this dose of mitotic inhibitor, preliminary dose–response studies have shown an exponential decrease in ACFU survival with a D_{37} value of 50 μg. Over the next 48 hr, recovery to supranormal levels was exponential with a doubling time of about 18 hr, and survival remained at normal levels (except for days 7 and 8) for the remainder of the experiment. The pattern of recovery of ACFU is similar to that for recovery of erythropoietin responsiveness following 80 μg VLB reported by Schooley (1968) and for recovery of numbers of H^{3}-thymidine labeled peritoneal macrophages reported in Chapter 3 (see Figure 3.4).

Recovery of CFU followed a similar time course and a doubling time of 24 hr during the exponential phase of recovery was found. Smith, Wilson and Fred (1968) have reported qualitatively similar data with less initial depression at 24 hr and recovery to normal levels by 60 hr; the greater survival at 24 hr and slightly later recovery to normal levels may be due to the different mode of injection of the drug (intraperitoneal) and different strain of mouse. Valeriote, Bruce and Meeker (1966) reported approximately 20% survival at 24 hr compared to the 40%
TABLE 4.1

A COMPARISON OF BONE MARROW SPLEEN COLONY-FORMING UNITS (CFU) AND AGAR COLONY-FORMING UNITS (ACFU) AS A FUNCTION OF TIME AFTER 80 µG VINBLASTINE I.V. PER MOUSE

<table>
<thead>
<tr>
<th>Day Post-VLB Injection</th>
<th>Nucleated Cells Per Femur x 10^6</th>
<th>CFU Per Femur</th>
<th>CFU Per 10^5 Nucleated Cells</th>
<th>ACFU Per Femur</th>
<th>Percent of Control No. of ACFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.5</td>
<td>4830 ± 570</td>
<td>35.8</td>
<td>14,200 ± 420</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>3.8</td>
<td>1940 ± 210</td>
<td>51.0</td>
<td>4,150 ± 65</td>
<td>29.0 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>1.9</td>
<td>4070 ± 330</td>
<td>214</td>
<td>7,660 ± 840</td>
<td>54.0 ± 6</td>
</tr>
<tr>
<td>3</td>
<td>3.7</td>
<td>7130 ± 950</td>
<td>194</td>
<td>18,200 ± 1900</td>
<td>130 ± 10</td>
</tr>
<tr>
<td>4</td>
<td>8.7</td>
<td>6050 ± 1100</td>
<td>69.5</td>
<td>12,100 ± 870</td>
<td>85.0 ± 7</td>
</tr>
<tr>
<td>5</td>
<td>12.5</td>
<td>4060 ± 260</td>
<td>32.5</td>
<td>16,600 ± 1600</td>
<td>120 ± 10</td>
</tr>
<tr>
<td>6</td>
<td>10.5</td>
<td>4860 ± 740</td>
<td>46.3</td>
<td>11,000 ± 910</td>
<td>77.0 ± 7</td>
</tr>
<tr>
<td>7</td>
<td>15.4</td>
<td>-</td>
<td>-</td>
<td>9,070 ± 720</td>
<td>64.0 ± 5</td>
</tr>
</tbody>
</table>

*Standard error of the mean
Figure 4. 2 - A comparison as a function of time of agar colony-forming units and spleen colony-forming units in the bone marrow of mice treated with 80 µg vinblastine i.v. on day 0. Agar colony-forming units are expressed as percent of control values from corresponding groups of untreated mice. Standard errors of the mean of 2 to 6 separate experiments are shown for agar colony-forming units, and for spleen colony-forming units, standard error of the mean for an average of 12 replicate determinations.
Figure 4.2

DBL 693-4617
found in this experiment. The doubling time of 24 hr for the CFU recovering from the 80 µg VLB is identical to the doubling time of transplanted marrow CFU in the spleens of lethally irradiated normal and polycythemic mice (McCulloch and Till, 1964; Schooley, 1966).

Recovery patterns of ACFU and CFU following 200 R whole body \( ^\gamma \)-irradiation. The data on the recovery of proliferative capacities of ACFU and CFU as a function of time after irradiation, simultaneously assayed in femoral bone marrow, are summarized in Table 4.2 and Figure 4.3. As in the previous experiment, minimum values of ACFU and CFU were attained within 24 hr after injury. The striking finding, however, was the rapid (if abortive) recovery of ACFU to near control values during the first week after irradiation, while the CFU remained suppressed at values 10% of control. The pattern of recovery of ACFU is similar but more rapid than that reported for recovery of erythropoietin responsiveness after 150–200 R (Gurney, Lajtha and Oliver, 1962; Schooley, Cantor and Havens, 1966). The pattern of recovery of CFU confirms similar findings by several groups (McCulloch and Till, 1961; Schooley, Cantor and Havens, 1966; Smith, Wilson and Fred, 1968).

A minimum value of 14% of control was found for ACFU 24 hr after 200 R; dose response studies in this laboratory, measuring ACFU 24 hr after various doses of whole-body \( ^\gamma \)-irradiation, have shown an exponential decrease in survival of ACFU with a \( D_{37} \) value of approximately 95 rads. Robinson,
TABLE 4

A COMPARISON OF BONE MARROW SPLEEN COLONY-FORMING UNITS (CFU) AND AGAR COLONY-FORMING UNITS (ACFU) AS A FUNCTION OF TIME AFTER 200 R WHOLE BODY GAMMA IRRADIATION

<table>
<thead>
<tr>
<th>Day Post-200 R</th>
<th>Nucleated Cells Per Femur x10^6</th>
<th>CFU Per Femur</th>
<th>CFU Per 10^5 Nucleated Cells</th>
<th>ACFU Per Femur</th>
<th>Percent of Control No. of ACFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.6</td>
<td>4700 ± 850*</td>
<td>34.6</td>
<td>8,930 ± 930</td>
<td>100</td>
</tr>
<tr>
<td>1/4</td>
<td>9.4</td>
<td>367 ± 36</td>
<td>3.9</td>
<td>1,100 ± 89</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>3</td>
<td>9.1</td>
<td>533 ± 51</td>
<td>5.9</td>
<td>9,530 ± 460</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>5</td>
<td>9.6</td>
<td>433 ± 46</td>
<td>4.5</td>
<td>6,560 ± 420</td>
<td>73 ± 9</td>
</tr>
<tr>
<td>8</td>
<td>14.7</td>
<td>450 ± 26</td>
<td>3.1</td>
<td>2,780 ± 330</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>12</td>
<td>13.8</td>
<td>980 ± 120</td>
<td>7.1</td>
<td>1,790 ± 150</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>15</td>
<td>10.2</td>
<td>1630 ± 180</td>
<td>15.9</td>
<td>7,710 ± 190</td>
<td>86 ± 9</td>
</tr>
<tr>
<td>21</td>
<td>12.0</td>
<td>3670 ± 450</td>
<td>30.6</td>
<td>12,300 ± 380</td>
<td>140 ± 10</td>
</tr>
</tbody>
</table>

*Standard error of the mean
Figure 4. 3 - A comparison as a function of time of agar colony-forming units and spleen colony-forming units in the bone marrow of mice irradiated with 200 R whole body Co gamma rays on day 0. Agar colony-forming units are expressed as percent of control values from corresponding groups of unirradiated mice. For agar colony-forming units, standard errors of the mean of 2 experiments are shown, and for spleen colony-forming units, standard error of the mean for an average of 8 replicate determinations.
Figure 4.3
Bradley and Metcalf (1967) have reported a \(D_{37}\) value of 85 rads for ACFU measured 48 hr after various doses of x-rays to C57Bl mice. In this experiment a doubling time of about 12 hours was observed for ACFU during the exponential recovery phase.
4.4 Discussion

The experimental results presented in this paper are consistent with the view that agar colony-forming cells (ACFU) are a population of cells distinct from spleen colony-forming cells (CFU). This was clearly demonstrated by the recovery patterns of each type of stem cell after 200 R; the rate and timing of recovery of the ACFU were faster and earlier than that of CFU. There was also resemblance to the pattern of recovery after 200 R of erythropoietin-sensitive cells (ESU) in relation to CFU as demonstrated by Schooley, Cantor and Havens (1966), which suggests that ACFU may exist in a similar relationship to CFU.

Bennett, Cudkowicz, Foster and Metcalf (1968) have shown in genetically anemic mice of the W series ACFU content of bone marrow was normal in numbers and in vitro colony-forming ability, while CFU were severely deficient in numbers and in the ability of producing normal-sized spleen colonies. They suggest that ACFU are an intermediate stage of differentiation between CFU and myeloblasts. McCulloch (1968) has also proposed this concept with the reservation that the differences in proliferative and differentiative potential expressed in the two assay systems may be a reflection of the influence of environment on the expression of potential rather than the existence of two different classes of stem cells with different potentials. We feel the results of this investigation provide more support for the concept of two functionally distinct stem cell populations.
Wu, Siminovitch, Till and McCulloch (1968) examined the relationship between ACFU and CFU in a study of the ability of individual chromosome-marked spleen colonies to produce progeny capable of clonal proliferation in vivo (CFU) and in vitro (ACFU). Their findings demonstrated that many spleen colonies possessed such capabilities and that a remarkable correlation existed between the numbers of each type of colony-forming cell in each spleen colony. Both findings are compatible with the idea that a close parent-progeny relationship exists between CFU and ACFU respectively. To our knowledge, no one has shown that ACFU can produce spleen colonies upon transplantation of agar colonies into a suitable recipient animal (i.e. form CFU).

In 1965, McCulloch, Till and Siminovitch proposed the idea of independent and dependent stem cells in order to explain data from studies on the action of erythropoietin on primitive hematopoietic cells (Bruce and McCulloch, 1964); their work and that of Schooley (1966) showed that erythropoietin did not act on CFU, but rather on a different cell population, called erythropoietin-sensitive cells (ESU). These ESU were classified as dependent stem cells, characterized by a more committed pattern of differentiation, different rate and capacity for proliferation, sensitivity to specific inductive agents, and a close ontogenic relationship to cells of the independent stem cell class (e.g. CFU). In light of studies reviewed here and accumulating evidence for hormonal agents in granulocytopoiesis and monocytopoiesis, it is appropriate that ACFU should also be included in this class of dependent stem cells.
We have been interested in ACFU as progenitors of monocytes and inflammatory macrophages. As assayed in the present experiments (colonies counted on day 10 of incubation), the colonies in agar are composed almost entirely of phagocytic mononuclear cells indistinguishable from inflammatory macrophages; this has been reported by Ichikawa, Pluznik and Sachs (1966) and Metcalf, Bradley and Robinson (1967). Volkman and Gowans (1965 a and b) have described a rapidly proliferating bone marrow cell population, called macrophage precursors, which are identifiable after H$^3$-thymidine labeling in skin window or peritoneal exudates as labeled macrophages. Comparison of the data on recovery as a function of time after 80 μg vinblastine of these macrophage precursors (see Chapter 3) with that for the ACFU of this study supports the idea that ACFU constitute a subpopulation within the class of macrophage precursors.

Macrophage precursors in the steady state must include progeny of ACFU differentiating along monocytic lines and new daughter ACFU resulting from self renewal divisions within the ACFU compartments — if such divisions occur. After radiation or vinblastine injury, the rapidly dividing CFU compartment survivors must also contribute to this H$^3$-thymidine-labeled pool of cells. For this reason and because of several other problems involved with the use of H$^3$-thymidine-labeling as a measure of cell proliferation (e.g. changing endogenous thymidine pool sizes or altered transport of the labeled nucleoside after injury), ACFU provide a more accurate measure of specifically committed monocyte-macrophage progenitor cells. Finally, the macroscopic
agar colony represents a measure of reproductive integrity after injury, not only an assay for reproductive capacity, as has been emphasized by Puck and Marcus (1956) in their studies on irradiated mammalian cells in clonal culture.

The application of functional criteria to characterization of primitive hematopoietic cells, as pioneered by McCulloch, Till, Siminovitch and their co-workers in *in vivo* systems, has relevance to *in vitro* studies as well. With recognition of other dependent stem cell populations and agents regulating their behavior and with development of new *in vivo* and *in vitro* techniques, a more complete picture should emerge of an important but poorly understood area of hematopoiesis.
CHAPTER 5

MONOCYTE–MACROPHAGE PROGENITOR CELLS

5.1 Summary of Experimental Findings:

From the theoretical considerations of Chapter 2, a model of the monocyte–macrophage system was proposed, and experiments were initiated to test that model. Particular objectives of these studies were verification and further characterization of the developmental sequences of cell populations identifiable as monocyte–macrophage progenitors and information about the kinetics of these populations in the steady state and after injury. Functionally, three precursor populations were defineable:

(i) Macrophage precursors – rapidly proliferating cells of bone marrow origin, which label with $^{3}H$-Tdr and are autoradiographically measurable as mature macrophages in inflammatory exudates.

(ii) ACFU committed to macrophage differentiation – cells in bone marrow capable of forming in agar after 7 – 10 days macroscopic colonies of phagocytic mononuclear cells indistinguishable from macrophages. We have demonstrated in vitro phagocytosis of colloidal gold by cells of this type of agar colony (Schooley, Roser and Chen, unpublished observation). These cells are a subclass of the general class of agar colony–
forming cells (ACFU), another subclass of which can differentiate along granulocytic lines and produce granulocyte colonies viable for about 4 - 5 days after initial plating. Clonal origin for mixed agar colonies (macrophage and granulocytic) has not been conclusively demonstrated.

(iii) CFU - cells in bone marrow (and spleen) capable upon transplantation of forming macroscopic nodules on the surface of the spleens of suitable host animals. At the present time this in vivo technique provides the best measure of the pluripotential hematopoietic stem cells postulated in the hematological literature.

The findings of the experimental studies are compatible with the interpretation that

(a) In the steady state, macrophage precursors are largely made up of differentiated progeny of ACFU and daughter cells produced by self-renewal divisions within the ACFU compartment. CFU do not contribute a proportionately significant number of cells to that pool. After radiation or drug-induced injury, surviving CFU contribute significantly to this \( R^3 \) -TdR labeling cell population.

(b) ACFU committed to macrophage differentiation probably represent the most primitive of the
differentiated progenitors of monocytes and macrophages and provide the best measure of these cells. ACFU are a population of cells functionally distinct from CFU and fulfill many of the criteria of a dependent stem cell population as defined by McCulloch, Till and Siminovitch (1965).

(c) Macrophage precursors are radiosensitive and mitotic inhibitor-sensitive cells, similar in many ways to other hematopoietic progenitor cells. Radiation and vinblastine sulfate induce loss of the reproductive capacity and integrity of these cells. The tempo and timing of recovery of macrophage precursors and ACFU after these agents is very similar to patterns of recovery of erythropoietin-sensitive cells, another postulated dependent stem cell population.

5.2 Relationship and Analogies to Other Hematopoietic Cell Lines

Figure 5.1 diagrammatically depicts the monocyte-macrophage system in relation to the other hematopoietic and lymphoid cell lines; it is an obvious oversimplification of what in reality must be a very complex system and several items in the diagram are as yet hypothetical. The notation is that of Figure 2.1.
Figure 5.1 - A diagrammatic representation of the monocyte-macrophage system in relation to other hematopoietic and lymphoid cell lines.
It shows the pluripotential hematopoietic stem cell (CFU) giving rise to several dependent stem cell populations: ACFU, ESU (erythropoietin-sensitive cells), TSU (thrombopoietin-sensitive cells), and PC₁ (progenitor cell-1 of the antibody-producing cell scheme of Makinodan and Albright, 1963). These dependent stem cells are then acted on by humoral agents (GP, granulocytopoietin; EP, erythropoietin; TP, thrombopoietin) or antigen, which trigger off the subsequent proliferative and maturation steps in the production of functional mature end cells (monocytes, macrophages, granulocytes, erythrocytes, platelets, plasma cells). Willoughby, Coote and Spector (1967) have reported a monocytogenic hormone (MH) elaborated by appropriately stimulated lymphoid tissue, which conceivable could act on those ACFU committed to monocyte-macrophage differentiation. Self-renewal within dependent stem cell compartments has only been clearly demonstrated for ESU (Schooley, 1968); evidence for self-renewal in ACFU is still equivocal (Paran and Sachs, 1969).

5.3 Regulatory Mechanisms

An exceedingly important area only briefly touched on in this investigation is that of homeostatic mechanisms which operate to meet changing demands for blood cell production. Because of the existence of a pluripotential stem cell, which is the immediate progenitor for the various dependent stem cell populations, there is evidence that demands on the dependent stem cell pool for either increase or decrease in activity can influence the activity of other dependent progenitor populations.
Hellman and Grate (1967, 1968) have demonstrated that erythropoietic stress (acute blood loss anemia, administration of erythropoietin, or irradiation) can reduce production of granulocytic progeny. Bradley, Robinson and Metcalf (1967) have reported that blood loss anemia can reduce numbers of marrow ACFU, while polycythemia can cause a significant increase in numbers of ACFU. These data can be interpreted as evidence for competing proliferative demands on the common stem cell pool or as conservative efforts on the part of hematopoietic regulatory systems to maintain the common stem cell pool at a certain size.

McCulloch and co-workers (1965) have proposed that regulatory mechanisms operate at the level of the dependent stem cell — their "units of control"; much of their theory is based on the idea and evidence for erythropoietin-sensitive cells (ESU). There are data, however, from the work of McCulloch and Till (1961) and Schooley (1966) that show that independent stem cells (CFU) can regulate the length of their generative cycle (or proportion of cells in cell cycle) in response to environmental stresses demanding rapid proliferation and differentiation. In consideration of macrophage precursors (particularly the ACFU), there is a suggestion from our studies that regulation of proliferatory rate and compartment size of these dependent stem cell-like populations may be mediated by a similar alteration of generative cycle as operating at the CFU level. If the generative cycle for macrophage precursors is close to 21 hr as calculated by Whitelaw, Bell and Batho (1968), then the doubling times of macrophage precursors and ACFU in recovery phase post-irradiation or
vinblastine are significantly shorter; it should be remembered that these doubling times represent upper limits for generation times of differentiating cell populations.

Finally, there is mounting evidence at the in vitro level for several factors, specific and non-specific, which influence the proliferation and expression of differentiative potential of ACFU. The relevance of these factors to in vivo regulation is at best very speculative. Besides, factors in conditioned medium elaborated by a variety of normal and neoplastic tissues (e.g. Pluznik and Sachs, 1966; Paran, Ichikawa and Sachs, 1968), substances occurring in normal and leukemic sera have been reported which can induce and maintain growth of ACFU (Robinson, Metcalf and Bradley, 1967). These factors suggest similarity to the "macrophage growth factor" of Virolainen and Defendi (1968), but confirmatory studies are still forthcoming. In addition to these inducer substances, Ichikawa and co-workers have reported inhibitor substances which can preferentially inhibit development of one or the other type of agar colony (Ichikawa, Pluznik and Sachs, 1966; Ichikawa, Pluznik and Sachs, 1967; Paran, Ichikawa and Sachs, 1968 in press). They have shown that macrophages produce a substance that inhibits the activity of the inducer required for the development of macrophage and granulocyte colonies, and that macrophage colonies are more effectively inhibited than granulocyte colonies; recently they have reported a granulocyte colony inhibitor produced by granulocytes. Their results suggest that a control mechanism which governs the growth and development of a particular
differentiated cell type includes a feedback inhibition of the activity of the inducer by an inhibitor produced by what are presumably the mature differentiated cells of that same type.

The domain of regulatory mechanisms in hematopoiesis or in other cell renewal systems presents some of the most intriguing and challenging problems in contemporary biology. With recognition of more functional compartments within the category of "stem cells" and suggestions for sites of action for control mechanisms at that level, there is promise that answers to some of these problems will be obtainable.
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