# The microenvironmental organization of early B cell precursors in the femoral bone marrow of mutant SCID mice, SCID/myc transgenic mice and alternate fraction x-irradiated endocolonized mice

by

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# Microenvironmental organization of pro-B cells in murine bone marrow

## ABSTRACT

The *in situ* microenvironmental organization of early precursor B cells in mouse bone marrow has been studied using three experimental models (1) mutant mice with severe combined immunodeficiency (SCID), which develop pro-B cells but no pre-B and B cells, (2) SCID/myc transgenic mice having expanded pro-B cell populations, but no pre-B and B cells, (3) x-irradiated C3H/HeJ mice allowing early stages in the regeneration of pro-B cells in bone marrow seeded from a shielded marrow site. The *in vivo* localization of B220$^+$ cells was revealed by the binding of i.v. injected $^{125}$I-mAb 14.8 detected by light and electron microscope radioautography of femoral marrow sections. Many B220$^+$ pro-B cells were located in peripheral regions of SCID and SCID/myc bone marrow, often in clusters, associated with an electron dense extracellular matrix and with the processes of stromal reticular cells. Many B220$^+$ cells were associated with macrophages which contained numerous ingested bodies. Macrophage associations were more numerous in SCID/myc than in SCID mice, especially in the peripheral marrow regions. 3-5 day post-irradiation endocolonizing marrow contained increasing numbers of B220$^+$ cells in subosteal and peripheral regions, situated both within sinusoids and extravascularly, associated with stromal reticular cell processes and often close to nerve fibers. The results demonstrate that early B220$^+$ precursors begin to differentiate in peripheral marrow regions and develop intimate associations with reticular cells and macrophages. These findings suggest that through

these associations, the bone marrow reticular cells promote the early development of the B cell lineage, while the bone marrow macrophages play a role in the elimination of aberrant precursor B cells

# RÉSUMÉ

L'organisation microenvironementale *in situ* des precurseurs des lymphocytes B dans la moelle osseuse des souris a ete étudiee à l'aide de trois modèles expérimentaux. 1) la souris mutante SCID, qui developpe les cellules pro-B, mais pas les cellules pre-B et B, la souris transgenique SCID/myc a une population de cellules pro-B plus grand que normale, mais n'a pas de cellules pré-B et B; la souris C3H/HeJ exposée aux rayons-x et examinee durant les premiers stages de regénération des cellules pro-B en provenance d'un site de la moelle protégé La localisation des cellules pro-B a ete permise par l'attachement, *in situ*, de l'anticorps monoclonal 14.8 associé à $^{125}I$, injecté intraveineusement. Les sections de la moelle osseuse, exposées à la radioautographie, ont été ensuite examinées par microscopie optique et électronique. Plusieurs cellules B220$^+$ pro-B étaient localisées dans les régions périphériques de la moelle osseuse des souris SCID et SCID/myc, souvent en groupes, et associées avec de la matrice extracellulaire et des procès de cellules réticulaires du stroma Plusieurs cellules B220$^+$ étaient associées avec des macrophages qui contenaient plusieurs corps ingestés Les associations avec les macrophages étaient plus nombreuses chez les souris SCID/myc que chez les souris SCID, spécialement dans la région périphérique de la moelle osseuse Aux jours 3-5 après le traitement aux rayons-x et la recolonisation, la moelle contenait un nombre grandissant de cellules B220$^+$, dans les régions associées à l'os et les régions périphériques, situées dans les sinusoides et

extravasculairement, associées avec des procès de cellules réticulaires du stroma et souvent à proximité de fibres nerveuses Les résultats démontrent que les jeunes précurseurs des cellules B220$^+$ commencent leur différentiation dans les régions périphériques de la moelle osseuse et développent des associations intimes avec les cellules réticulaires et les macrophages Nous suggérons qu'à travers ces associations, les cellules reticulaires de la moëlle osseuse promouvoient le développement des cellules de lignée B tandis que les macrophages de la moelle osseuse jouent un rôle dans l'élimination des précurseurs des cellules B aberrantes.

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# 1. INTRODUCTION

The mammalian immune system consists of a multitude of celluar components responsible for eliminating potentially harmful foreign organisms and molecules from the body. One important constituent of the immune system is the B lymphocyte lineage, responsible for humoral immunity, mediated by antibody formation. The present work is concerned with the development and organization of the B lymphocyte lineage at its site of origin, the bone marrow.

## 1.1 Lymphocytes bearing surface immunoglobulin

Early radioautographic studies performed in guinea pigs showed that small lymphocytes were being rapidly and continuously produced in the bone marrow (Osmond and Everett, 1964). Most of the lymphocytes in bone marrow of both guinea pigs and rats were newly-formed cells (Osmond, 1972; Osmond and Everett, 1964; Osmond, 1967; Everett and Tyler, 1961; Rosse, 1971) derived from proliferating precursors within the marrow (Osmond and Everrett, 1964; Osmond, Miller, and Yoshida, 1973; Osmond, 1967; Everrett and Tyler, 1964; Harris and Kugler, 1964; Brahim and Osmond, 1970; Yoshida and Osmond, 1971). Migration of some of these cells was demonstrated to occur from the bone marrow to the spleen and lymph nodes via the blood circulation (Brahim and Osmond, 1970) These early findings, were followed by the demonstration of small lymphocytes bearing surface immunoglobulin receptors

in murine femoral bone marrow revealing them to be B lymphocytes (Osmond and Nassal, 1974) Collectively, the foregoing studies showed post natal mammalian bone marrow to be the primary site of B lymphocyte production. It was later demonstrated that the B lymphocytes newly generated in the bone marrow show a progressive maturation of surface immunoglobulin M (sIgM) and other B lineage associated surface molecules with time (Osmond 1975, 1980).

### 1.2 B lymphocytes express immunoglobulin μ heavy chain

A characterization of B lineage precursor cells ensued. Progenitors immediately preceding newly formed sIgM$^+$ B lymphocytes, termed pre-B cells, could be defined by the presence of free cytoplasmic μ Ig heavy chains (cμ) in the absence of surface μ chains (sμ) (Cooper, 1981). Combined mitotic arrest and immunofluorescence labeling techniques revealed the population size and kinetics of cμ$^+$sμ$^-$ pre-B cells. These cells fell into two categories, large dividing, and small-nondividing (Landreth et al. 1981; Opstelten and Osmond 1983). 75% of the pre-B cells in mouse bone marrow are small cμ$^+$sμ$^-$ post mitotic pre-B cells that mature into sIgM$^+$ cells without further division.

### 1.3 B220: a B cell lineage associated surface glycoprotein

Another marker used to categorize B lineage subpopulations was the B lineage associated surface membrane glycoprotein B220 (220 KD) (Coffman and

Weissman, 1981; Coffman 1983) detected by several monoclonal antibodies (mAb), including the mAb 14 8 developed by Kincade et al.(Kincade et al., 1981). The mAb 14.8 binds to B220 on the surface of all mature $s\mu^+$ B lymphocytes, and $c\mu^+s\mu^-$ pre-B cells. However, the total number of $B220^+$ cells in mouse bone marrow is greater than that of those bearing μ chains ($c\mu+s\mu$) (Landreth et al., 1983 ). Removal of $B220^+$ cells from in vitro cultures of bone marrow prevents the production of pre-B and B cells (Kincade and Phillips, 1985; Kincade,1981). These findings strongly suggested that B cell precursors begin to express B220 glycoprotein prior to expressing μ chains.

## 1.4 TdT: an intranuclear enzyme involved in B cell diversity

A third marker used in establishing the subsets of B cells has been terminal deoxynucleotidyl transferase (TdT). This intranuclear enzyme is involved in generating immunological diversity by inserting additional short nucleotide segments (N regions) at VJ and DJ gene segment junctions during Ig heavy chain gene rearrangements (Deciderio et al., 1984, Kunkel et al 1986). TdT is not expressed, however, during rearrangements of the Ig light chain coding gene (Tillinghost et al. 1989) Using double immunofluorescence techniques with the 14.8 mAb and anti-TdT antibodies, three distinct populations of $\mu^-$ cells were found (Park and Osmond 1987): $TdT^+B220^-$, $TdT^+B220^+$, $TdT^-B220^+$. The kinetic data of these populations along with previous evidence that B220 is expressed before μ, suggests these three

populations to be early B cell progenitors (pro-B cells) preceding the pre-B cell stage (Park and Osmond, 1987).

## 1.5 A working model for B cell development

Using these cytological markers, six B cell subpopulations have been defined. The first three precede μ chain expression: 1) early pro-B cells, $TdT^+B220^-$; 2) intermediate pro-B cells, $TdT^+B220^+$; 3) late pro-B cells, $TdT^-B220^+$. The three subsequent populations progressively express IgM components: 4) large mitotic pre-B cells, $c\mu^+s\mu^-$; 5) small post-mitotic (Go), pre-B cells, $c\mu^+s\mu^+$; 6) mature $sIgM^+$ cells. The sequential stages of these populations are represented as a working scheme of B cell development in Fig.1. The precursor product relationship between the pre-B and B cell populations has been verified (Kincade, 1987; Osmond and Opstelten, 1983). Also, following sublethal γ-irradiation, the waves of precursor B cell regeneration pass through the six phenotypic compartments in accordance with the sequence proposed in the model (Park and Osmond, 1989).

## 1.6 B lymphocyte population dynamics

With the working model of B cell development as a base, the population dynamics of B cell precursors have been studied using mitotic arrest techniques combined with double immunofluorescence labeling (Opstelten and Osmond, 1983; Park and Osmond, 1987; Park and Osmond, 1989). Data concerning

frequency, cell size and proliferation are detailed in a comprehensive review by Osmond (Osmond, 1990). Three main stages appear to be involved in precursor B cell dynamics. The first is that of a clonal expansion The clone size of B cells with any given Ig heavy chain specificity is determined by the number of mitoses occuring between the start of μ heavy chain gene rearrangement and sIgM expression. It was found that a minimum of 1 mitotic cycle occurs at each pro-B cell stage with a hightened production rate and population size at the late pro-B cell stage followed by another mitotic division at the cμ+sμ (large pre-B cell) stage. B cell clones of a specific μ chain sequence can be estimated to total 64 cells or more (Park and Osmond, 1989) However, subsequent to this substantial clonal expansion of B cells, kinetic data have indicated an important cell loss (75%) at the pre-B cell stage, the turnover of small pre-B cells being only a fraction of the predicted exit of cells from the large pre-B cell compartment (Opstelten and Osmond, 1983). In view of the lack of pre-B cells outside the bone marrow in mice, the findings suggest that an actual cell loss occurs in the marrow itself: this is the second stage of B cell dynamics This cell deletion is probably the result of non-functional rearrangements of the μ heavy chain coding gene. With a very high frequency of somatic point mutations, errors in recombonation of gene segments, and the insertion of nucleotide sequences by TdT, lead to a high incidence of genetic errors, which may lead to cell death (Tonegawa, 1983). The third stage of B cell population dynamics is the clonal expansion of B cell precursors that ceases during light

chain rearrangement, before sIgM is expressed.

## 1.7 The microenvironmental organization of murine bone marrow

A series of studies have identified $sIgM^+$ cells and $B220^+$ cells, in intact marrow, *in situ*. These studies have provided insight into B cell expansion and selection/loss by showing the localization and distribution of precursor B cells as well as their intercellular associations.

The development of the B cell lineage and other hemopoietic cell lineages, is intimately related to the marrow structure. The architecture of the bone marrow has been reviewed by Lichtman (Lichtman, 1981). The mid-diaphysis of the femur is usually chosen for the examination of bone marrow due to its lack of complex trabeculae. The surrounding cortical bone is circular in shape and the blood circulation reveals a symmetrical pattern of venous and arteriolar blood flow. All these factors facilitate the study of the intact bone marrow and of the hemopoietic lineages that develop within.

The bone marrow has a dual blood source: a) the nutrient artery, obliquely penetrating the cortical bone; b) cortical arteries, derived from muscles and other attachments leading into the bone. The nutrient artery gives rise to one or two longitudinal arteries that branch out via radial arteries extending to peripheral bone. Here, they diminish in calibre and form a capillary plexus communicating with the cortical arteries and giving rise to venous sinuses that converge towards a large central venous sinus which leads

to a nutrient vein, exiting the marrow. In contrast to its rich blood supply and venous drainage, the bone marrow is devoid of lymphatic vessels. The extravascular compartment between the anastomosing venous sinuses is the site of genesis of all hemopoietic lineages, in post-natal mice.

Another major component of bone marrow that is located within these extravascular compartments consists of stromal cells Stromal cells such as reticular cells and macrophages, are often associated with hemopoietic cells

Stromal reticular cells resemble one another morphologically, but differ on the basis of histological location, biochemical properties, and functional properties. They share the common characteristic of sending out long, extensive thin cytoplasmic processes that form an ultrastructural network or "reticulum". Adventitial re icular cells are found on the abluminal or adventitial surface of vascular sinuses. Their cytoplasmic processes form a sheath, partially enveloping the outer sinus wall (Lichtman, 1981). Peri-arterial adventitial reticular cells are characterized by having a very thin laminated cytoplasm that concentrically surrounds both nerves and arterioles (Yamazaki and Allen, 1991). Parenchymal reticular cells, not associated with any vasculature, have processes extending throughout the parenchyma, establishing contact between themselves and with hemopoietic cells "Barrier" cells have been defined as a fibroblastic, contractile stromal reticular cell (Weiss and Geduldig, 1991) They are usually found in restricted loci of trabecular bone, but can migrate away from these regions during times of stress, such as irradiation They are

multilaminar, branched and appear to envelop hemopoietic stem cells and clusters of early progenitors. They also envelop blood vessels, threading their processes through the vessel wall possibly preventing the release of immature cells.

The heterogeneity of these reticular stromal cells is also reflected by the numerous stromal cell lines that support the development of hemopoietic cell lineages in long term bone marrow cultures by secreting short range growth factors and by the expression of numerous cell-cell adhesion molecules that associate early progenitor cells to stromal cells (Simmons et al., 1992). B cell development *in vitro* has clearly been shown to be dependant on stromal cells or on stromal cell lines which secret various growth factors, notably interleukin 7 (Nishikawa et al , 1988, Nishikawa, 1990; Imhof et al, 1991).

Macrophages, another stromal cell element, also play an important role both in the development and maintenance of hemopoietic cell lineages. They are described as being actively phagocytic as well as secretory (Weiss, 1983). They are large cells, extending complex cytoplasmic processes through the parenchyma. Their nucleus are often indented and the cytoplasm contains abundant endocytic vacuoles, lysosomes, and phagolysosomes. Many mitochondria are also present. Macrophages are responsible for recognizing and disposing particulate matter such as silica, carbon and asbestos, pathogenic microrganisms coated with serum proteins and bacteria. The importance of their role as secretory cells is demonstrated by the wide range of molecules

they synthesize and secrete, i.e., enzymes, complement proteins, interferons, and many growth factors (Weiss, 1983).

The bone marrow is well supplied by nerve fibers. Early studies (Yamazaki and Allen, 1991) have revealed that: 1) the bone marrow is innervated by both myelinated and non-myelinated nerve fibers; 2) in general, nerves in the bone marrow are distributed with the blood vessels; 3) some fibers stray away from the vessels and branch into the parenchyma; 4) most of the afferent fibers are myelinated and the sympathetic efferent fibres are unmyelinated; 5) there is a lack of concrete evidence concerning direct innervation of parenchymal cells. Recent work (Yamazaki and Allen, 1990) has demonstrated the existence of a "neuro-reticular complex" in which efferent (autonomic) nerve terminals are connected via gap junctions to stromal reticular cells. They also suggest this complex to be involved in the function of the hemopoietic microenvironment, the blood-marrow barrier and transport or sorting of hemopoietic cells in the marrow. Goetzl and Sreedharan (Goetzl and Sreedharan, 1992) reviewed the communication between the neuroendocrine and immune systems; they describe bidirectional interactions between the two sytems that influence physiological activities such as tissue location of lymphocytes, antibody responses, and neural signal transmission.

## 1.8 The detection and visualization of B lymphocytes

B lymphocytes develop *in vivo* within the intricate marrow environment and recent studies have revealed associations with other cells that are influential for their proper development. The technique of radioautography has permitted the labeling and detection of cell types using specific markers conjugated with radioisotopes, while electron microscopy has been used to visualize intact marrow at high magnification with high resolution.

The earliest LM radioautographic study performed on bone marrow lymphocytes was by Osmond and Everett (Osmond and Everett, 1964) showing that small lymphocytes were rapidly produced in the bone marrow. Their approach was to use $^{3}H$ thymidine, which would be incorperated into DNA-synthesising cells and thus could be traced through successive cell divisions. More specific B cell labeling was later achieved using rabbit anti-mouse immunoglobulin conjugated to the radioisotype $^{125}I$ (Osmond and Nossal, 1974). Electron microscope radioautographic studies followed, using *in vivo* administration of $^{125}I$ anti-IgM Ab (Osmond and Batten, 1984). These studies showed, for the first time, the *in situ* localization of clearly labeled $sIgM^{+}$ B lymphocytes in intact bone marrow They found that $sIgM^{+}$ lymphocytes were located throughout the extravascular space of the marrow, either singly or in groups and that some regional concentrations were apparent. This work opened the door to the study of *in vivo* B cell localization and B cell genesis with regard to the marrow microenvironment.

## 1.9 Working model of B lymphocyte organization in the bone marrow

Studies by Jacobsen and Osmond (1990) have resulted in a working model of the microenvironmental organization of primary B cell genesis. Their work involved the detection B cells bearing the B lineage associated marker, B220 glycoprotein, by the *in vivo* administration of $^{125}$I-mAb 14.8, followed by intracardiac washout perfusion and fixation, and since B220 is expressed from the intermediate pro-B cell stage onward, a wide range of precursor B lymphocytes could thus be localized *in situ*. Three major components of the working model are as follows: 1) there is a centrally directed sequence of differentiation initiated by early B cell precursors, many of which appear to be situated in peripheral marrow adjacent to the cortical bone, 2) there are close associations between proliferating precursor B cells and stromal reticular cells; 3) macrophages are involved in the deletion of ineffective precursor B cells. These findings and proposals are the basis of the present work

## 1.10 Aims

The present project has aimed to test and to contibute further to the model of the microenvironmental organization of early progenitor B cells in the bone marrow, especially by examining selectively the early B220$^+$ stages of precursor B cell development, in situ. This aim was approached by the use of three unique murine models, (1) the SCID (severe combined immunodeficiency disease) mouse, a mutant murine model, (2) the SCID/Myc mouse, a transgenic

murine model, (3) endocolonization of irradiated bone marrow by stem cells, using the method of alternate fraction x-irradiation. All three models will have examined early B cell progenitor localization and intercellular associations, *in situ*, in the femoral bone marrow.

## 1.11 The SCID mouse

### 1.11.1 The SCID defect

A severe combined immunodeficiency mutation (SCID) was first described in the mouse by Bosma et al.(1983). Mice homozygous for this autosomal recessive mutation lack pre-B cells, mature B cells and T cells. SCID mice thus have no humoral or cell mediated immunity. However, myeloid differentiation and function are not affected by the SCID mutation and bone marrow is not impaired: it can support the development of transplanted bone marrow cells from normal mice, leading to the production of B and T lymphocytes.

The SCID mutation is associated with a defective recombinase system that leads to the improper cutting and rejoining of gene segments coding for Ig heavy chains. There are three Ig gene coding segments, V, D, J: in normal mice the D-J segments are combined first, followed by the joining of the V segment (Gellert et al , 1988) However, in the SCID mutant mouse, D and J segments are improperly joined leading to a deletion in the J gene segment. This, in turn, interferes with V-DJ joining and results in a failure to express the μ heavy

chain (Sakano and Okazak 1988; Gellert 88; Malynn et al., 1988). There is also a basic defect in a DNA repair gene, leading to an inability to repair double strand DNA breaks (Fulop and Phillips, 1990; Phillips and Spaner, 1991) This is reflected by the increased sensitivity of the SCID mouse to ionizing radiation. Since gene rearrangement seems to involve double strand breaking, the inability of SCID mice to repair double strand breaks in DNA is consistent with their inability to produce functional Ig and T cell receptors (Brown, 1991)

### 1.11.2 The use of the SCID model in research

Because of the absence of an immune response, the SCID mouse has been widely used for the study of various aspects of the immune system, lymphoid differentiation and function (McCune, 1991), graft versus host disease (Phillips and Spaner, 1991), and lymphomagenesis (Purtilo and Beisel, 1991). In other applications, SCID mice have been reconstituted with human immune or hemopoietic elements creating a SCID-human model (hu-SCID) (McCune et al.,1991) permitting the *in vivo* study of human immune function, AIDS and lymphoproliferative disorders (Torbett, 1991, J.M McCune et al , 1991, Smith et al., 1991).

The SCID mouse provides a convenient and unique model for the *in vivo* study of early B cell progenitors (pro-B cells) up to the differentiation stage at which aberrant pre B cells with defective Ig gene rearrangements are deleted.

### 1.11.3 Use of the SCID model in the present project: aims

In this present work, SCID mice have been used to examine selectively the microenvironmental localization and cellular interrelationships of pro-B cells in the SCID mouse bone marrow, and the nature of B cell deletion mechanisms. The lack of pre-B and B cells in the SCID mouse bone marrow allows for the precise identification of pro-B cells and their associations by labeling cell surface B220 *in situ*. The method used to detect pro-B cells has combined the techniques of *in vivo* radiolabeling and radioautography: $^{125}I$ conjugated mAb 14 8, binding specifically to the B cell lineage associated glycoprotein, B220 (Kincade et al., 1989) has been administered intravenously followed by intracardiac washout/fixation perfusion, and the examination of femoral bone marrow sections by light and electron microscope radioautography.

## 1.12 The SCID/myc transgenic mouse

### 1.12.1 Nature of the SCID/myc mouse

The SCID/myc mouse, only few of which have been produced, was obtained through crossbreeding the SCID mouse with the Eμ-myc mouse. The Eμ myc is a transgenic mouse in which the c-myc proto-oncogene, involved in cell proliferation (Cory, 1986), is coupled to the lymphoid specific IgH enhancer gene (Eμ) thus rendering myc expression constitutive in the B cell lineage (Langdon et al , 1986, Harris and Adams 1988). This results in an abnormal

but non-malignant expansion of precursor B cell populations, eventually leading to lymphomagenesis. The condition of the Eμ-myc mouse provides new opportunities to examine the prelymphomatous state of a differentiating B cell population under the influence of constitutive c-myc expression (Langdon et al., 1986). Recent work (Kim and Osmond, unpublished) has shown that the SCID/myc mouse, like the SCID mouse, still lacks pre-B and B cells and its B cell lineage is therefore limited to the pro-B cell population. Compared with the SCID mouse, however, the early pro-B cell population is expanded and the late pro-B cell stage is decreased. Thus the proliferation of pro-B cells appears to be enhanced by the myc transgene during the stage of μ heavy chain gene rearrangement, but the pro-B cells abort at an earlier stage than in SCID mice.

### 1.12.2 The use of the SCID/myc model in the present project: aims

The present study has examined the possible effects of constitutive myc expression on the microenvironmental localization and intercellular relationships of pro-B cells in SCID/myc bone marrow. Intermediate and late pro-B cells bearing the B220 glycoprotein have been labeled by the i v. administration of $^{125}$I mAb 14.8 followed by intracardiac perfusion/ fixation and radioautography.

## 1.13 The model of alternate fraction irradiation: endocolonization

### 1.13.1 Hemopoietic colony formation

Hemopoietic colony formation in the bone marrow has been extensively researched in the past four decades. Early studies on the radioprotective effects of bone marrow transplantation (Lorenz et al., 1951; Congdon et al. 1952) and the clonal proliferation of the transplanted cells, led to the development of colony forming assays for hemopoietic stem cells. (Till and McColluch, 1961; Pluznik and Sacks, 1966). Post-irradiation-regeneration studies (Curry et al., 1967; Wolf and Trentin, 1968), have shown the production of hemopoietic colonies in irradiated bone marrow and spleen supplied by stem cells either via donor bone marrow transplantation (exocolonization) or from an autologuous site that had been shielded during irradiation (endocolonization). An important characteristic of the regenerating colonies was that for approximately 9 days post-irradiation, marrow colonies differentiated along single hemopoietic cell lineages until 10-12 days post-irradiation, where there was a phase of mixed or multilineage colony differentiation (Congdon et al., 1952; Wolf and Trentin, 1968; Cline et al., 1977; Curry and Trentin, 1967).

### 1.13.2 Hemopoietic colony formation by alternate fraction irradiation

In vivo hemopoietic colony formation has permitted the study of early events of hemopoiesis, including the interactions of hemopoietic cells with their surrounding stromal microenvironment. In an extensive study on the

organization and regeneration of bone marrow hemopoietic cell-stromal cell interactions, Lambertsen and Weiss (1983) used the method of alternate fraction x-ray irradiation, which consists of shielding one limb of the animal while irradiating the rest of its body, followed, after a time interval to allow migration of stem cells, by irradiating the initially shielded limb alone. Their methods of examination were based purely on morphology, without the use of cytological markers. Therefore, the analysis of regenerating cells did not permit the detection of B cell colonies. A group of repopulating cells has been described as being of undifferentiated morphology, ranging in appearance from small lymphocytes to blast forms, many of which were dividing (Lambertsen and Weiss, 1983), however, the possible lymphoid identity of these cells has not been examined.

### 1.13.3 Endocolonization in the present project: aims

In the present work, the method of alternate fraction x-ray irradiation, has been used used to create a state of hemopoietic endocolonization, in order to study the earliest stages of $B220^+$ cell development from immigrant primitive precursor cells. $^{125}$I-mAb 14.8 was administered *in vivo* to identify early B lineage cells as they differentiate to and beyond the intermediate pro-B cell stage in the bone marrow. The work aims to examine the time of appearance of these cells, their localization and cellular interactions, to provide information on the migration, homing and development of mobile B lineage

precursor cells. This is relevant both in ontogeny, when B cell genesis transfers from liver and spleen to the bone marrow (Osmond and Owen, 1984; Landreth et al., 1983; Owen et al., 1977; Cooper et al., 1983) and after bone marrow transplantation initiating the lengthy period of B cell reconstitution until humoral immunity is eventually restored.

# 2. MATERIALS AND METHODS

## 2.1 Animals

Mice with the mutation SCID (severe combined immunodeficient disease) originally arose from the C.B-17 inbred strain [BALB/c.C57BL/Ka-Igh-$1^b$/ICR (N17F34)] (Bosma et al., 1983). Five week old male C B-17 SCID mice and normal C.B-17 counterparts were provided by Dr. R A. Philips (Ontario Cancer Institute, Toronto). The mice were maintained under constant specific pathogen-free (SPF) conditions being housed in sterile microisolator cages, in which sterile food and water were provided.

Male, 5 week old SCID/myc transgenic mice were obtained from Dr. Sidman (Jackson Laboratories, Bar Harbour, Maine).

Male, 5 week old Balb/c mice, were purchased from Charles Rivere Laboratories (Ontario).

## 2.2 Antibodies

Rat monoclonal antibody, mAb 14.8 (Coffman and Weissman 1981), directed against the B cell lineage glycoprotein B220, was affinity column-purified from supernatant fluid from culture hybridoma cells (ATTC) and diluted 1:150 in phosphate-buffered saline (PBS), pH 7.2. Fluorescein isothiocynate (FITC)-conjugated goat anti-rat IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was diluted 1:15 in PBS pH 7.2, and used as a secondary

antibody after cell surface binding of mAb 14.8. Rabbit anti-terminal deoxynucleotidyl transferase (anti-TdT) (Supertechs Inc., Bethesda, MD) was used at 1.20 dilution and detected with rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG F(ab')2 (1:20 dilution; Jackson Immunoresearch Laboratories Inc , Mississauga Ontario). TRITC- and FITC-conjugated affinity-purified goat antibodies to mouse Ig heavy chain constant regions (μ chains) (Southern Biotechnology Associated Inc., Birmingham, AL, Kirkegaard & Perry Laboratories, Gaithersburg, MD) were diluted 1:30 and 1:10 respectively in PBS, pH 7.2. Aggregates from all antibodies were removed prior to use by ultracentrifugation for 30 min at 120 000 x g (Beckman Instruments, Palo Alto, CA).

## 2.3 Metaphase arrest

Vincristine sulphate (Sigma Chemical Co., St. Louis, MO) was administered to the mice intraperitoneally at a dose of 0.1 ml/Kg per body weight to block cells in metaphase, by dissociating cytoplasmic microtubules (Wright and Appleton, 1980). After a 2hr 40 min interval, which represents the mid-point of the linear curve of increase in mitotic cells, the bone marrow cells were sampled.

## 2.4 Bone marrow and spleen suspensions

Mice were killed by cervical dislocation. Left and right femurs were removed and bone marrow cells were flushed out from the femoral shafts with

1 ml of cold Eagles' minimal essential medium (MEM), pH 7.2, containing 10% (vol/vol) newborn calf serum (MEM/NCS, Gibco Laboratories) which was centrifuged (10 min, 250 x g, 10°C) to remove any aggregates and debris The cells were resuspended thoroughly and were underlayed with 1 ml of NCS for 5 min, removing large particles by sedimentation. The cell suspension was centrifuged through 1 ml of NCS (10 min, 250xg, 10°C) and the remaining pellet resuspended in 1 ml MEM/NCS. After lysing red blood cells using "zapoglobin", the nucleated cells in suspension were counted with an electronic particle counter (Model Z, Coulter Electronics, Hialeah, Fl). Spleens were dissected and were gently ground through a fine mesh screen with MEM/NCS, and the harvested cells were then prepared and counted as previously described.

## 2.5 Double immunofluorescence labeling

### 2.5.1 B220 glycoprotein and μ chains

Detection of the surface membrane glycoprotein B220 was achieved by incubating 100 μl of bone marrow cells (4 x $10^7$ nucleated cells/ml) with 100 μl of mAb 14.8 (Park and Osmond, 1987). The cells were washed twice by centrifugation (10°C, 10 min, 250xg) through NCS, incubated with FITC-conjugated goat anti-rat IgG (30 min, 4°C), washed twice through NCS, and resuspended in 2.5 ml of 0.15 M NaCl, with 2.7 mM disodium EDTA (Fischer Scientific Co., Fairlawn, NJ) and bovine serum albumin (BSA; Gibco Laboratories), 5% (wt/vol). Samples of 4 x $10^5$ cells were cytocentrifuged (7000

rpm, 5 min) creating a deposit (cytospot) on glass slides previously coated by centrifugation of 100 µl PBS/BSA 3% (wt/vol) (9000 rpm, 7 min). The slides were rapidly air-dried to preserve morphology, fixed in pre-cooled (4°C) 5% (vol/vol) glacial acetic acid in absoloute ethanol for 12 min on ice, and then washed four times in PBS. Excess PBS was wiped off the slide, leaving only the area of the cytospot, each of which was incubated with 30 µl of anti-µ-TRITC for 30 min in a humidified chamber, washed four times and immersed overnight in PBS at 4°C.

### 2.5.2 Surface and cytoplasmic µ chains

Bone marrow cell suspensions were incubated with anti-µ-FITC (30 min, 4°) to label surface µ heavy chains (sµ) of immunoglobulin (Opstelten and Osmond, 1983), then washed, cytocentrifuged and fixed as described above. Labeling of cytoplasmic µ chains was carried out by incubation of each cytospot with 30 µl of anti-µ-TRITC.

### 2.5.3 B220 glycoprotein and TdT

After surface labeling with mAb 14.8 (see above), bone marrow cells were cytocentrifuged, fixed with absolute methanol for 20 min on ice and gradually rehydrated by first washing with 50% (vol/vol) $CH_3OH$/PBS (3-5 min) and then with 100% PBS (3-5 min). After washing four more times at 10 min intervals with PBS, each cytospot was exposed to 30 µl normal goat serum (NGS) for 30 min to block any nonspecific binding sites for the goat anti-rabbit antibody,

incubated overnight with rabbit anti-TdT (12hr, room temperature) and then exposed to TRITC-conjugated goat anti-rabbit IgG F(ab')2 for 30 min (Park and Osmond, 1989). Slide preperations were mounted in 90% (vol/vol) glycerol in PBS, pH 8.0, containing 0.1% (wt/vol) p-phenylenediamine to reduce fading of the fluorescein during microscopy. An epifluorescence microscope (Carl Zeiss of Canada Ltd., Don Mills, Ontario), equipped with a HBO 50 mercury lamp, phase contrast optics, and an x100 oil immersion phase contrast objective was used for analysis.

### 2.5.4 Double immunofluorescence analysis

Individual marrow and spleen cell preparations were scored for (a) single labeling with either FITC alone (B220, sμ) or TRITC alone (cμ, TdT) or (b) double labeling with FITC plus TRITC ($B220^+c\mu^+$, $s\mu^+c\mu^+$, $TdT^+B220^+$). Fluorescing cells were examined by phase-contrast to detect cells arrested in metaphase. The incidence of $TdT^+B220^-$, $TdT^+B220^-$ and $B220^+\mu^-$ pro-B cells, $c\mu^+s\mu^-$ pre-B cells, and $s\mu^+$B cells were obtained by examining at least 3000 nucleated cells. The counts included at least 100 cells of each phenotype. The proportion of pro-B cell and pre-B cell populations that were in metaphase was determined by counting 3000 nucleated cells. The absolute number of B lineage cells in the bone marrow was calculated from their incidence and the total bone marrow cellularity.

## 2.6 Radioiodination of antibodies

Aliquots of 130-140 µg of antibody were coupled to carrier-free $Na^{125}I$ (2mCi = 74MBq, specific activity 1.5 x $10^7$µCi/µg) by a modified chloramine T technique: 10µl of $Na^{125}I$ followed by 10µl of chloramine T (0.4% in $ddH_2O$) were added to the aliquot of the antibody in question. This creates a reaction mixture in which the chloramine T promotes the binding of iodine to the tyrosine residues of the protein. After a 10 min incubation period at 4°C, 10µl of Na-metabisulfate (0.96% in $ddH_2O$) was added to the mixture, reducing any unbound chloramine T, and thus arresting the binding reaction between the protein and the iodine. The excess of chloramine T was taken up by the addition of 80µl of KI (0.2 molar). The iodinated antibodies were then transferred to a Sephadex G25 column. The column seperates by particle size: beads in the column create pores that entrap any unbound $^{125}I$, allowing the passage of the larger $^{125}I$-conjugated antibody molecules. Twenty consecutive fractions of 1 min each, were collected once the flow through the column had begun. Next, 5µl samples of each fraction were measured for radioactivity (counts per minute or cpm) using a gamma counter (Gamma 4000, Beckman Instr, Fullerton, Ca.). The $^{125}I$-conjugated antibody was recovered primarily in the earlier fractions (e g 4th-5th fraction), whereas the unbound iodine molecules appeared in the subsequent fractions ( e.g. 14th-16th fractions). The bound antibody fractions were pooled, stored within lead containers, and used for *in vivo* labeling techniques.

## 2.7 *In vivo* labeling of B lineage cells in the bone marrow

Mice were anesthetized with an I.P. injection of chloral hydrate (1.6% in 0.9% sterile NaCl; 0.75ml/25g of body weight), and then were immobilized, extended on their backs, on an absorbant platform. An incision was made to reveal the subcutaneous structures of the neck and shoulder regions. Connective tissue and fascia were carefully teased away to expose the left external jugular vein. The vein was constantly kept moist with Ringer's solution to prevent desiccation and rupture of the vessel. A 28 gauge insulin syringe was loaded with 100µl of the $^{125}$I-mAb 14.8, which was then injected into the external jugular vein. A transient clearing of the vein was indicative of a successful injection of the antibody into the circulation. The antibodies were permitted to circulate for 3 min, a sufficient period of time for the antibody to penetrate the bone marrow (Osmond and Batten, 1984). An incision was then made through the skin and peritoneum, exposing the diaphragm. At the 3 min time interval, the diaphragm was incised and the ribcage was severed along each side and then lifted up to reveal the heart. The tip of a 30 gauge butterfly infusion needle was inserted into the lumen of the left ventricle, introducing a flow of cold lactated Ringer's solution (4°C) into the arterial circulation. This was delivered at a rate of 3 ml/min, for ten min, using a syringe pump (model 355, Sage instruments, White Plains, NY.). The wall of the right atrium was slit as soon as the perfusion began, allowing the escape of perfusate and consequently washing out blood and unbound $^{125}$I-mAb 14.8 from the circulation. Perfusion with fixative (2.5% or 5%

gluteraldehyde in cacodylate buffer, pH 7.4) was then carried out, at a flow rate of 3 ml/min for ten min.

## 2.8 Tissue processing and radioautography

Femurs and spleens from the perfused mice were removed and immersed in the fixative (2.5-5% gluteraldehyde in cacodylate buffer). After three hours, the spleens were washed (1x5 min) in a cacodylate washing buffer, and were post-fixed in KFeCn-reduced $OsO_4$ (1%) for 3 hours at 4°C. The femurs remained in the fixative overnight, and then were decalcified in EDTA (82.6% EDTA, 6% NaOH, pH 7.4) for 5 days and post-fixed, as above, for 4 hours. The post-fixed tissues were dehydrated in ascending concentrations of acetone (2x10min for 30%, 50%, 70%, 80%, and 95% acetone; 2x30 min for 100% acetone), followed by increasing concentrations of Epon in acetone (1:1 for 4 hours, 2:1, 3:1 overnight and pure Epon for 6 hours). They were then flat embedded in pure Epon and hardened for 60 hours at 60°C. The solid blocks were transversely cut across the mid-diaphysis of the femur, exposing the bone marrow. Using glass knives and a microtone, thick sections (1µm) were cut for light microscopic (LM) radioautography and placed on specimen slides. These were then put on a hot plate at a temperature of 80°C The tissues on the slides were initially covered with iron alum for 5 min each The stain was then removed and the slides were washed in three successive baths of distilled water. The slides were replaced on the hot plate, stained with iron hematoxylin for 5 min and washed as above. The

slides were flooded with tap water, rinsed in distilled water and allowed to dry before radioautographic processing. Thin sections (75-90nm) were cut for electron microscopic (EM) radioautography using a diamond knife and an ultratome, and were positioned on celloidin-coated slides which were then carbon coated. Stained LM and unstained EM preperations were processed for radioautography (RAG). LM slides were dipped in Kodak NTB2 emulsion and were exposed for periods of 1, 2, and 3 weeks prior to development. EM slides were dipped into Ilford L4 emulsion, exposed for 8-12 weeks, and developed using filamentous grain-developping techniques. After development, EM grids were dipped in glacial acetic acid to remove the coat of celloidin covering the tissue, at 5 sec intervals until the multicolored celloidin layer had disappeared. The grids were placed on beads of lead citrate and uranyl acetate stains for 5 min and 2 min, respectively, and were thoroughly washed after each stain in distilled water. The EM sections were examined on a Philips 301 transmission electron microscope.

## 2.9 Analysis of histological radioautographs

Entire transverse sections of diaphyseal femoral marrow from the mice were examined by light microscopy in successive square 2025$\mu m^2$ fields delineated by an ocular grid to quantitate radioautographic labeling indices throughout the bone marrow. Within each field the total number of nucleated cells (excluding megakaryocytes, endothelial cells, and adipocytes) and the number of grains over each cell were recorded. Optimal grain densities over

labeled cells with only minimal background over non-lymphoid cells were obtained after 7 days of radioautographic exposure. The number of cells labeled at various grain count thresholds was tabulated in each field and plotted on histological maps of the marrow sections. For EM radioautography, numerous tissue sections from each femur were examined at radioautographic exposure times of 8-14 weeks.

## 2.10 Alternate fraction whole body irradiation

Groups of three mice were anesthetized (1.6% chloral hydratein 0.9% sterile NaCl; 0.75ml/25g of body weight), restrained with adhesive tape on a movable platform. The right hind leg of each mouse was shielded by positioning the leg underneath an elevated lead shield (15 mm thick). The whole body, excluding the shielded leg, then received an 850 rad dose of x-ray irradiation (260 Kv, 8 mAmp, 70 rads/min). A period of 8-10 hours was allowed to elapse for the migration of hemopoietic progenitor cells from the shielded bone marrow of the right leg to the irradiated parts of the body. The previously shielded leg was then irradiated at the same dose of x-rays, the remainder of the body being protected by a 15mm thick lead shield. At days 1, 2, 3, and 5 post-irradiation, three animals were killed A control animal which had received a single whole body dose of 850 rads without any shielding was examined after 1 and 5 days. *In vivo* localization of $B220^+$ cells in the bone marrow was carried out by iv administration of $^{125}$I-mAb 14 8 followed by intracardiac washout perfusion and

fixation as described above. Left and right femurs were removed and processed histologically for LM and EM radioautographic examination.

# 3. RESULTS

## 3.1 Precursor B cell populations in femoral bone marrow of SCID mice

A preliminary double immunofluorescence labeling analysis was performed in order to establish the incidence of B cell lineage subpopulations of the SCID. Bone marrow cell suspensions from a 5 wk old SCID mouse revealed a pattern of early $B220^+$ progenitor cells similar to that seen in 11-15 wk old SCID mice (Osmond et al., 1991), with the exception that the $TdT^+$ cells were less frequent. In the 5 wk old SCID mouse, the incidences of the pro-B cell populations were: $TdT^+$ cells, 1.3% ($TdT^+B220^+$, 0.68%; $TdT^+B220^+$, 0.64%); $B220^+\mu^-$ cells, 3.5% ($B220^+TdT^-$, 2.86%). The incidence of late pro-B cells was reduced to 8% of control values, while no pre-B cells and mature B lymphocytes were detected in the bone marrow and the spleen. The results indicate that B cell development in the young SCID mouse is limited to the pro-B cell progenitor stage, i.e. early pro-B cells, intermediate pro-B cells, and late pro-B cells. In contrast to normal mice, B220 labeling therefore reveals a narrow window of early development in the B cell lineage consisting mainly of $TdT^+$ $B220^+$ pro-B cells.

## 3.2 The localization of early B cell precursors in the femoral bone marrow of SCID mice

The *in situ* binding of $^{125}$I-mAb 14.8 to B220$^+$ cells after *in vivo* administration, proved to be effective, as revealed by four representative radioautographic tissue sections from each of three different SCID mouse femurs, analyzed by light and electron microscopy.

A total of 60-86 (mean, 78) well labeled B220$^+$ cells were detected in the individual marrow sections, each of which contained about 7000 nucleated cells (excluding megakaryocytes, adipocytes and endothelial cells). A cell was considered positively labeled when it had 10 or more overlying grains. This threshold was selected as clearly distinguishing specifically labeled cells from background grains representing either unbound or nonspecifically bound material or eminating from a neighbouring cell. At this threshold, the total incidence of labeled cells per bone marrow section was 1.1% ± 0.2 (mean, ± standard deviation of four values). The incidence of B220$^+$ cells derived from immunofluorescence labeling of bone marrow cell suspensions was somewhat higher (3.5%). Therefore, the radioautographic threshold used in the study of bone marrow sections restricted observations to the most highly labeled B220$^+$ cells.

Histological mapping of labeled cells, under LM, revealed that the B220$^+$ cells were widely distributed throughout four representative bone marrow sections (fig.2). The histological maps were divided into three annular zones,

peripheral, intermediate, and central, each one containing an equal number of nucleated cells. Distributing the observed number of labeled $B220^+$ cells throughout the entire section by a random numbering procedure resulted in 34%, 33%, and 32% of the randomly generated cells being assigned to the peripheral, intermediate and central zones, respectively (not shown).

The peripheral zone of the marrow contained many of the labeled early B cell precursors, some of which were located in the subendosteal layer of the cortical bone (fig.3-5). They were seen in patchy clusters of up to three labeled cells in individual microscopic fields (2025 $\mu m^2$) (fig.3). The single outermost fields lying immediately adjacent to the endosteum around a complete section of femoral marrow contained 44% of the total number of $B220^+$ cells in the section, compared to the value of 34% expected by random distibution.

Moving towards the centre of the marrow sections, the labeled $B220^+$ cells became more dispersed (fig.2). The intermediate zone of the marrow contained 30% of the labeled cells compared to the value of 33% obtained by random distribution, considerably less than that of the periphery. The innermost fields adjacent to and surrounding the central sinus contained 26% of the labeled cells as individually scattered cells, below the value of 32%, obtained by the random distribution procedure. Individual central fields contained no more than 1 $B220^+$ cell each.

The majority of $B220^+$ cells were located within the extravascular compartment. However, in intermediate and central zones, some positive cells

were closely adjacent to endothelium of venous sinusoids, including the central venous sinus. At times they were seen in direct contact with the endothelial cells making up the sinus walls. In addition, of the 311 labeled cells that were mapped by L.M., 3 heavily labeled cells were clearly located within the lumen of small venous sinusoids (fig.6, 7).

In all the femur sections, adipocytes were prominent in peripheral zones of the marrow. About 30 of these fat cells were present per section. B220$^+$ cells were often adjacent to as many as three adipocytes (fig.4b).

Some heavily labeled cells were observed in the marginal zone and red pulp of the spleen.

### 3.3 Cellular associations and ultrastructure of B cell precursors in bone marrow of SCID mice

Thin bone marrow sections were analyzed by electron microscopy after a 45 day exposure period, to locate B220$^+$ cells precisely by radioautography. Positively labeled cells were identified by the presence of 10-50 filamentous silver grains overlying the plasma membrane and delineating the cytoplasmic boundaries (fig.8). Background grains were virtually absent at the EM level due to the thinness of the section (70nm) The B220$^+$ cells were readily observed in subosteal areas near the bone interface, where they were often in direct contact with osteoblasts (fig.9-12). These B220$^+$ cells were of medium size and generally undifferentiated morphology. The nucleus displayed an open leptochromatic

pattern with peripheral patchy chromatin condensation. The cytoplasm, small to moderate in volume, contained prominent mitochondria and rough endoplasmic reticulum (fig 9). The B220$^+$ cells were closely associated with a particularly prominent electron dense extracellular matrix in the subendosteal regions of the parenchyma. This matrix completely surrounded the B220$^+$ cells, which seemed to be embedded within it (fig 9-11). In addition, stromal reticular cell processes were often intimately associated with the B220$^+$ labeled cells in the peripheral regions of the marrow (fig.11, 12).

In intermediate zones, further removed from the surrounding bone, B220$^+$ cells, located singly among cells of other lineages, were also closely associated with stromal reticular cell processes (fig.13, 14). The labeled B cell progenitors had in some cases, a wavy indented nuclear profile and were otherwise of undifferentiated morphology.

Large macrophages with extensive processes and numerous inclusions were prominent in the bone marrow of the SCID. B220$^+$ cells were frequently associated with the cytoplasmic extensions of the macrophages which at times completely surrounded the labeled cells, suggesting that phagocytosis had been taking place (fig 15, 16, 17, 18)

In regions of the central marrow, some higly labeled B220$^+$ cells were juxtaposed to the outer wall of the sinusoids, overlying the thin layer of adventitial reticular cell processes partially covering the endothelial lining (fig.13, 14).

Electron microscopy confirmed the scarcity of highly labeled B220$^+$ cells within the lumen of sinusoids (fig.19-21). However, two such B cell progenitors were observed to be anchored to the luminal wall of the sinusoid by thin irregular cytoplasmic processes which appeared to extend through the sinusoidal wall to the extravascular space (fig.19, 20). The B220$^+$ cells located in or adjacent to the sinusoids appeared morphologically to be somewhat more mature than those situated elsewhere as revealed by their generally smaller size, more condensed nuclear pattern, electron dense cytoplasm and numerous vacuoles.

### 3.4 Localization of early B cell precursors in femoral bone marrow SCID/myc mice

Localization of early B cell progenitors in the bone marrow of SCID/ Eμ myc transgenic mice was carried out as described for the SCID mice, by the *in situ* binding of i.v. administered $^{125}$I mAb 14.8 to B220$^+$ cells The SCID/myc mice were used at 4-5 weeks of age.

Light microscopic analysis of radioautographic sections of femoral bone marrow revealed B220$^+$ cells of lymphoid morphology labeled with numerous overlying silver grains The localization of the B220$^+$ cells resembeled that seen in the SCID mouse The peripheral region of the bone marrow contained many B220$^+$ cells accumulated in patchy clusters (fig.22). However, many labeled B cell precursors were also located in intermediate regions of the marrow Labeled cells appeared to be less numerous in the area surrounding the central sinus in

central marrow regions

Labeled B220$^+$ cells were observed exclusively in the extracellular compartment· no intrasinusoidal B220$^+$ cell was seen. This finding was in marked contrast the observations made in Eμ-Myc parental strain marrow sections under LM, in which clusters of B220$^+$ labeled cells were readily seen within sinusoidal lumens (data not shown).

The intermediate regions of the marrow contained many large lymphoblastoid cells (fig 23a) Their morphology is suggestive of blast cells, i.e. they had a large ovoid or round nucleus with a prominent centrally located nucleolus. They formed distinct clusters among other hematopoietic cells, ranging from 3-6 cells within a group. The nuclear shape and high nuclear to cytoplasmic ratio of these cells appeared to be lymphoid. A small number of these cells were clearly labeled, however, at a low intensity (<10 grains) (fig.23b). The presence of these lymphoblast-like cell clusters have been observed in Eμ-myc mice marrow , but not in the SCID marrow.

### 3.5 Cellular associations and ultrastructure of B cell precursors in femoral bone marrow of SCID/myc mice

Electron microscopy once again permitted a detailed evaluation of B220$^+$ cell localization. The peripheral marrow regions revealed many B220$^+$ cells located subendosteally The labeling of these cells varied from heavy to light (15 to 4 silver grains). Even in the latter case, however, the labeling was distinct

since background grains were practically absent. The B220$^+$ cells adjacent to the subosteal layer were often found in a clusters of 3-4 cells that were lightly labeled (3-4 grains) (fig.24). The labeled cells were of medium size and undifferentiated morphology. An open leptochromatic chromatin pattern with patchy peripheral chromatin condensation was common to their nuclei, as well as a slight indentation of their nuclear profile. The cytoplasm of these cells was abundant and contained numerous organelles. Peripheral B220$^+$ cells further removed from the subosteum were of similar early B cell morphology (fig 25).

As in the SCID mouse, B220$^+$ cells were associated with stromal reticular cell processes in the peripheral zones of the marrow (fig 24, 25). However, in more intermediate and central regions of the marrow no stromal reticular cell associations were detected. The B220$^+$ cells were either adjacent to other hemopoietic cells, detached from neighbouring cells or adjacent to macrophages which were characterized by a very loose dispersed cytoplasm containing cellular debris (fig.26, 27). The majority of the B220$^+$ cells observed under EM were associated with macrophages, usually in the form of a cell-cell interface (fig 28-30). In many instances, the cell surface labeling of the pro-B cells seemed to be localized along the plasma membrane at the interface (fig 28-30) In addition to the marked relationship between the B220$^+$ cells and the macrophages, this particular cellular association occured mainly in the peripheral zones of the marrow. The morphology of the macrophage-associated B220$^+$ cells was that of normal early B cell progenitors. In Eμ-myc transgenic mice, B220$^+$ cells

phagocytosed or in contact with macrophages are usually apoptotic in appearance (Jacobsen and Osmond, 1990). There was no strong evidence of such apoptotic early B cell preursors in the SCID/myc marrow. However, one particular B220$^+$ cell in contact with a macrophage showed the features of an early stage of apoptosis (fig.28): with condensation and peripheral segration of nuclear chromatin, cytoplasmic vacuoles and concentration of organelles in one area of the cytoplasm.

### 3.6 Microenvironmental organization of femoral bone marrow in alternate fraction irradiated mice: localization, ultrastructure and cellular associations of B220$^+$ cells

The method of alternate fraction whole body X-irradiation was used to produce endocolonization of irradiated bone marrow. The mice were sacrificed at days 1, 2, 3, and 5, post-irradiation. In vivo labeling of B220+ cells in the endocolonized marrow was carried out as previously described. At each time point, both LM and EM radioautographic sections were examined.

At day 1 post-irradiation/endocolonization, the marrow was in a degenerative state There was a severe decrease in hemopoietic cell numbers (fig.31a) and the central sinus and peripheral sinusoids were markedly dilated. Erythrocytes seemed to have flooded the extravascular parenchyma, although the endothelial lining of the sinusoids was intact. Many granulocytes were scattered throughout the marrow Megakaryocytes were randomly located within the

marrow and seemed to be undergoing mitotic divisions. No labeled B220$^+$ cells were observed.

At the electron microscopic level, the LM findings were confirmed Large eosinophils were observed in close proximity to one other. Cellular debris was evidently being removed by macrophages, whose cytoplasm contained numerous large phagosomes, lysosomes, and cellular debris (fig.32)

Groups of large undifferentiated cells resembling early myeloblasts were observed (10-12 μ in diameter) (fig.32). The nuceli in some of these cells were multi-lobed and in others they were unilobar. They had isolated strands of rough endoplasmic reticulum as well as numerous mitochondria. In one such group of cells, junctional complexes appeared to interconnect the cells (fig.33).

The bone marrow of whole-body irradiated control animals resembeled that of the alternate fraction irradiated animals.

No B220$^+$ cells were detected at 1 day and cells with lymphoid morphology were completely absent.

After 2 days, the nucleated hemopoietic cells were depleted even more, so that hemopoietic cells could rarely be identified. However, stromal reticular cells were prominent, their cytoplasmic extensions being clearly seen in the depleted marrow (fig.31b). Numerous red blood cells were still present in the parenchyma.

At the EM level, cells resembling granulocytic neutrophils were found, in groups of 3-4 cells (fig.34). These neutrophils had bilobed nuclei or band-shaped

nuclei. Neutrophils were closely associated with each other as well as with stromal cells (fig 34). Megakaryocytes were now abundant, occupying large areas of parenchyma (fig.35). Macrophages were still prominent and were filled with phagosomes and residual bodies (fig.36). The parenchymal space surrounding arterioles was virtually empty except for the presence of large nerve bundles. There was still no evidence of any lymphocytes, or of B220 labeling.

After 3 days the marrow was more densely cellular and clusters of mitotic cells were present within the parenchyma (fig.37a). Under the light microscope, cells with a chromatin pattern characteristic of dividing cells appeared near the bone in subendosteal parenchyma, as well as in parenchymal cords extending away from the periphery. The dividing cells were of undifferentiated morphology.

The first evidence of $B220^+$ cells in the marrow was observed at this time interval. The cells were lightly labeled and were situated singly both intra- and extravascularly, usually near the surrounding bone (fig.37b). The number of such labeled cells was small.

Clusters of neutrophils showed possible junctional associations between themselves and were also closely associated to stromal reticular cells (fig.38). Eosinophils were present as in day 1. Large fat cells and macrophages were abundant.

After 5 days, the marrow was densely cellular in peripheral areas of the

marrow where numerous neutrophil precursors line the subendosteal layer (fig.39a) as well as arterioles further removed from the subendosteum (fig.39b).

$B220^+$ cells were relatively numerous at day 5 (fig 40, 41) Three transverse sections of mid-diaphyseal marrow were examined and analyzed field by field as described. An average of 10 labeled cells were counted per section(± s.d ). The $B220^+$ B cell precursors were located in three areas of the marrow: a) subendosteally, among granulocytes and adjacent to or near sinusoids and capillaries (fig.40a, 41a); b) within parenchymal regions isolated from those adjacent to the subendosteum (fig.40b, 41b); c) intrasinusoidaly, in the highly dilated sinusoids of intermediate zones, but not in the small peripheral sinusoids (fig.40a). The $B220^+$ cells were always observed individually rather than in clusters. In day 5 control animal that had received whole body x- irradiation, a prolonged search revealed only one $B220^+$ cell within transverse sections of femoral marrow.

By electron microscopy, two morphological categories of labeled $B220^+$ cells were found, early and late. The early $B220^+$ cells had a low nucleus-to-cytoplasm ratio and a nuclear pattern of dispersed euchromatin with a thin rim of heterochromatin in patchy clumps around the nuclear border The nucleus was sometimes slighty indented (fig 42-45). The cytoplasm contained several mitochondria and a Golgi complex. The $B220^+$ cells of more mature morphology had a large round nucleus, with increased condensation of chromatin The nuclei occupied most of the cell volume as evidenced by the extremely thin cytoplasmic

rim surrounding the nucleus. Cells of such morphology were seen within the sinusoids (fig 46), as well as the parenchyma (fig 47).

The B220$^+$ cells with early morphology were located in peripheral regions of the marrow where recolonization was occuring They were located within the extravascular parenchyma adjacent either to the endothelial wall of sinusoids (fig 42) or to other arteriolar or venous vessels (data not shown).

All B220$^+$ cells in the extravascular marrow were clearly associated with stromal reticular cell processes confirming previous findings reported in this work. The cytoplasmic processes formed thin strands both in longitudinal and cross sections (fig.42-46).

B220$^+$ cells were also located immediately adjacent or close to nerve fibers in which numerous axons were embedded in the cytoplasm of Schwann cells (fig.42, 43, 45, 47) One B220$^+$ cell of early morphology showed a particularly close moulding around a nerve bundle (fig.43). Another B220$^+$ early precursor was in contact with three separate structures having the appearance of myelinated nerve axons in cross section, but with diminished spiraling of the myelin sheath around the axon (fig.45), possibly an effect of irradiation.

# 4.DISCUSSION

Early B cell progenitors, expressing B220 glycoprotein, have been localized in murine femoral bone marrow using three models in which $B220^+$ cells are restricted to the pro-B cell stage. SCID and SCID/myc mice both provide excellent systems in which to study the localization of early $B220^+$ B cell precursors in the bone marrow. The SCID defect presents a model in which no pre-B or B cells can be detected, as shown by the preliminary immunofluorescence studies in the present project and in a further published report (Osmond et al., 1991). The existing B lineage cell populations in the SCID and SCID/myc mice are pro-B cells. These consist of $TdT^+B220^-$, $TdT^+B220^+$ and $TdT^-B220^+$ cells, representing early, intermediate and late pro-B cell populations respectively. Therefore, the $B220^+$ cells detected in the SCID and SCID/myc marrow by in situ binding of $^{125}$I-mAb 14.8 represent only a narrow window of development of intermediate and late pro-B cells, many of which express TdT and are thus in the process of $V_H$ Ig gene rearrangements. Early post-irradiation endocolonization provides an opportunity to examine early B cell development during hemopoietic colony formation, when cell-to-cell interactions first begin to develop.

In the SCID and SCID/myc models, the pro-B cells undergo cell deletion at the late pro-B cell stage. Recent work (Osmond et al, 1991; Kim and Osmond, unpublished) demonstrates that differentiation and mitotic divisions

of pro-B cells occur up to the late pro-B cell stage. However, at the late pro-B cell stage there is a decrease in cell number. Since no pre-B or B cells are detected in either the bone marrow or the peripheral lymphoid tissues it is concluded that the aberrant pro-B cells are all being deleted. In the SCID/myc and endocolonization models, the pro-B cells are being stimulated. In SCID/myc mice, under the influence of c-myc expression, the intermediate and late pro-B cell populations are expanded, even though the pro-B cells still do not mature past the late pro-B cell stage (Kim and Osmond, unpublished). In endocolonizing marrow, hemopoietic colony formation may be stimulated by the post-irradiation depletion and the need to promote hemopoietic regeneration. Post-irradiation marrow regeneration experiments by Park and Osmond (1989), have shown that from 3 to 7 days after a sublethal dose (150 rads) of whole body irradiation, pro-B cells recover and exceed normal numbers. A similar overshoot was observed in work by Jacobsen, Tepper and Osmond (1990) where early B precursor cells expressing B220 were enriched at 1-3 days post irradiation (150 rads). Cell death from the irradiation probably stimulates dormant radioresistant stem cells to divide and to give rise to rapidly proliferating and differentiating precursors in order to compensate for the hemopoietic cell loss. A similar stimulation may occur in the marrow of alternate fraction x-irradiated mice. The high dose of irradiation used in this work ensures that cells repopulating the initially irradiated marrow sites will have originated from the autologous marrow that was shielded during

irradiation. Initially, the cells will be under pressure to regenerate the various marrow cell lineages, though Lambertsen and Weiss (1983) do not report an actual overshoot of hemopoietic cells in the irradiated marrow after alternate fraction x-irradiation.

The distinctive features of these three experimental systems provide models for the examination of early B cell precursors with respect to their localization and cellular associations. The systems will also reflect the effect on pro-B cells organization of the stimulation of B lineage cells in SCID/myc and endocolonized bone marrows or by their aberrancies in the SCID and SCID/myc models where improper Ig gene rearrangements lead to cell loss. The SCID and SCID/myc mice provide models to study the mechanisms responsible for cell loss at the late pro-B cell stage. Cell loss and deletion mechanisms have previously been described at later stages of B lymphopoiesis (Jacobsen and Osmond, 1991).

## 4.1 Localization of early B cell precursors in the bone marrow

Under light microscopy, histological maps of $B220^+$ cells in transverse sections of SCID mouse femoral marrow reveal the distribution of the pro-B cells. Many of the $B220^+$ pro-B cells are located in peripheral areas of the marrow. In the SCID mouse, the actual concentration of $B220^+$ cells in peripheral, intermediate and central marrow zones was compared to that of an equal number cells generated by computer at random throughout the marrow

zones. Actual B220$^+$ cells were localized in decreasing numbers from peripheral to central zones, whereas the randomly generated cell distribution was almost equal in each of the defined zones. This suggests that B220$^+$ cells are not randomly localized but are preferentially concentrated in peripheral zones. Although histological maps were not made, the same distribution was noted in SCID/myc femoral marrow. The presence of a substantial proportion of pro-B cells in the peripheral marrow of SCID and SCID/myc mice and the fact that these cells are actively proliferating suggests that the peripheral microenvironment is particularly supportive of early B cell differentiation and proliferation. The SCID/myc peripheral marrow contains large lymphoblast cells, only lightly positive for B220. These may be early pro-B cells that have just begun to express B220 glycoprotein, again implicating the peripheral marrow in being a major site of early B cell proliferation. Previous work by Jacobsen, Tepper and Osmond (1990) has shown that in post-irradiated regenerating marrow, the first wave of B220$^+$ cells are located in the peripheral marrow regions Further work by Jacobsen and Osmond (1990) revealed B220$^+$ cells to be peripherally located in the marrow of anti-IgM treated mice, in which all sIgM$^+$ cells are eliminated, leaving only pro-B and pre-B cells. In marrow of vincristine-treated mice, mitotic arrest reveals the location of many dividing B220$^+$ B cell precursors in peripheral marrow zones. In day 3 and 5 endocolonized marrow, all the B220$^+$ cells were within peripheral regions of the marrow. At the EM level, the peripheral B220$^+$ cells were of early B cell

precursor morphology. It appears that developing early B cell precursors in endocolonized marrow are preferentially located in peripheral marrow regions. The endocolonizing system further showed that B220$^+$ cells first appeared only at day three post irradiation. This may indicate that B lineage cells seed into the irradiated bone marrow from the shielded marrow only after a lag period of two or more days. Alternatively, early B220$^-$ B cell progenitors may have seeded the marrow before that time but not expressed B220 until they have undergone a further period of differentiation. The B220$^+$ cells in day 3 and 5 endocolonized marrow are undoubtedly derived from precursors in the shielded marrow since bone marrow of whole body irradiated control animals at day 5 post-irradiation has virtually no B220$^+$ cells. Furthermore, previous work (Lambertsen and Weiss, 1983) shows that endogenous hemopoietic colonies appear only after 7-8 days post-irradiation. The alternate fraction irradiation procedure ensured that migration from the shielded limb would only occur in a prescribed period of 10 hours at the onset of the experiment.

The location of B220$^+$ precursor B cells in day 3 and 5 endocolonized marrow may be related to their sites of entry into the marrow B220$^+$ cells were localized both intravascularly and within the parenchyma, often adjacent to small subendosteal capillaries. The migrating B220$^+$ cells from the shielded marrow may thus be entering the parenchyma of the irradiated marrow via the subendosteal capillaries or the walls of the peripheral sinusoids, consisting of little more than endothelium. The venous sinuses in these subendosteal regions

are highly branched and are generally the smallest in caliber (Debruyn et al., 1970). Contact between seeding B lineage precursor cells and endothelial cells may thus be promoted, initiating diapedesis in these regions (Lambertsen and Weiss, 1983). In ontogeny, hemopoietic cells that migrate from liver and spleen to bone marrow, possibly migrate through similar trajectories. Endocolonization work by Lambertsen and Weiss (1983) shows that hemopoietic microenvironments for stem cell proliferation and committment to differentiation, occur in endosteal regions, as well as periarterial regions. This work is in accord with the present findings that are indicative of a preferential microenvironment for early B cell development in the peripheral bone marrow.

Substantial numbers of $B220^+$ pro-B cells are also present in intermediate zones of the femoral bone marrow. In the SCID mouse they are less concentrated in this zone than in the periphery. Normally, as precursor B cells mature they appear to move in a centripital fashion, i.e. from periphery towards the center (Osmond et al., 1981). $B220^+$ pro-B cells located in the intermediate marrow regions are perhaps those of the late pro-B cell stage that have migrated away from the periphery in an attempt to differentiate further. However, due to the SCID defect, the pro-B cells are aborted at the late pro-B cell stage. The distribution of $B220^+$ cells in SCID/myc intermediate marrow regions differs from the SCID mouse in that many more $B220^+$ cells seem to be located in this area. This may have resulted from the proliferation of the large $B220^+$ blast cells in the SCID/myc peripheral marrow, not seen in the SCID

peripheral marrow. These blast cells may lead to the expansion of the proliferating early pro-B cell population, heightened by the constitutive c-myc expression, as has been determined by double immunofluorescence and stathmokinetic analysis in SCID/myc bone marrow (Kim and Osmond, unpublished).

The endocolonized marrow at the time intervals examined in the present work was not yet sufficiently repopulated for the formation of intermediate or central marrow regions. The central marrow of SCID and SCID/myc mice contained almost no B220$^+$ pro-B cells. Although past work has shown that there are greater numbers of early precursor B cells in peripheral marrow of normal mice, some were still located in central regions of the marrow (Jacobsen and Osmond, 1990; Jacobsen, Tepper, and Osmond, 1990). The scarcity of B220$^+$ pro-B cells in SCID and SCID/myc central marrow zones is related to deletion of late pro-B cells, the mechanisms of which will be discussed.

The patchy clusters of B220$^+$ pro-B cells in peripheral marrow of SCID and SCID/myc mice, and in intermediate marrow of SCID/myc mice possibly represent the progeny of a common progenitor cell This would concur with double immunofluorescence and stathmokinetic studies showing that B220$^+$ pro-B cells are actively proliferating. However, further experiments would be needed to test the possible clonality of the pro-B cell clusters The presence of B220$^+$ pro-B cell clusters in the intermediate marrow regions of SCID/myc mice may once again be due to the expansion of the pro-B cell population caused by

the constitutive c-myc expression (Kim and Osmond, unpublished). In endocolonized marrow, no such $B220^+$ cell clusters are seen. Those $B220^+$ cells present at day 3 and 5 of endocolonization are individual, i.e. they are singly located among other regenerating hemopoietic cells. This is perhaps an indication that the migrated $B220^+$ cells have not yet begun to proliferate. The morphology of these $B220^+$ cells is mainly that of early B cell precursors with potential to proliferate. Further endocolonization experiments are indicated to examine time points later than 5 days.

In the SCID mouse, almost all of the $B220^+$ cells are located within the extravascular space of the marrow. However, a few $B220^+$ cells are seen within sinusoidal lumens. This finding was unexpected since the SCID defect prevents the development of pre-B cell and mature B cells. There are several possible explanations for these intrasinusoidal $B220^+$ pro-B cells. First, $B220^+$ cells in the sinusoid may be pro-B cells that have been prematurely released into the circulation. Second, some defective $B220^+Ig^-$ cells may have avoided cell deletion and continued to mature enabling them to enter the sinusoids. Third, some pro-B cells may occasionally be able to properly rearrange VDJ segments, coding for Ig heavy chains, leading to normal cell development, survival, exit from the marrow and ability to function as a B cell. Electron microscopy shows these cells to be of a more differentiated appearance than extravascular $B220^+$ cells. Their cytoplasm is smaller in volume and the nuclear chromatin appears more condensed than that of early pro-B cells, suggesting that they are unlikely to

represent the premature release of pro-B cells. The B220$^+$ cells in SCID mice may rarely successfully rearrange their Ig coding genes, possibly due to a chance rearrangement, producing a "leaky" SCID (Bosma et al., 1988). However, no B cells were detected in peripheral lymphoid organs by double immunofluorescence in the present work. Thus, the second explanation seems to be the most plausible; aberrant Ig$^-$ pro-B cells having escaped deletion, continue to mature and are released into the sinusoids. Since these cells cannot express cytoplasmic or surface μ heavy chains, they are not detected as pre-B or B cells by immunofluorescence labeling. SCID pro-B cells, when transferred to in vitro cultures are capable of surviving although they are not functional (Witte et al., 1987). This is in accord with the idea that *in vivo*, the intrasinusoidal B220$^+$ cells may be rare, non-functional pre-B or B cells, escaping cell deletion and entering the peripheral circulation. The rarity of this event in SCID mice may reflect the efficiency of the normal deleting mechanisms and macrophage associations discussed below. B cells normally express sIgM before being released into the circulation (Osmond and Park, 1987; Osmond and Batten, 1984; Jacobsen and Osmond, 1990). This present work raises the question of whether B cell exit from the marrow is necessarily dependant on sIgM expression.

## 4.2 Intercellular associations of early precursor B cells in the bone marrow

### 4.2.1 Extracellular matrix

Most evident in the SCID mouse, subendosteal B220$^+$ cells of undifferentiated morphology are associated with a prominent electron dense extracellular matrix, in which they appear to be embedded. Molecules making up the extracellular matrix in bone marrow, such as fibronectin and proteoglycans, appear to be involved in the promotion of cell-cell adhesion and in restricting the range between growth factors binding the matrix and cell surfaces (Bernardi ey al., 1988; Sanderson et al., 1989). These findings suggest a specific microenvironment within which, early B cell development may be controlled via growth factors, the nature of which requires further study. The electron dense matrix was not evidently in SCID/myc and endocolonized mouse marrow. However, the matrix is not unique to the SCID mouse, since previous work shows B220$^+$ cells to be embedded in an electron dense extracellular matrix, in post-irradiation regenerating marrow (Jacobsen, Tepper and Osmond, 1990).

### 4.2.2 Osteoclasts

In all three experimental systems, B220$^+$ pro-B cells were apparently associated with osteoclasts. The B220$^+$ cells were either in contact with or in very close proximity to osteoclasts lining the endosteum. Lambertsen and Weiss (1983) show associations of undifferentiated and granulocytic colonies with

osteoclastic endosteum, and suggest that osteoclasts play a microenvironmental role in the marrow. The present findings raise the possibility that osteoclasts may promote pro-B cell development, although the specific nature of this role is open to speculation.

**4.2.3** Stromal reticular cells

The close association of peripherally located $B220^+$ precursors with stromal reticular cells in the bone marrow of the three murine models was examined in the present work The $B220^+$ cells are surrounded by and usually in direct contact with the thin cytoplasmic processes of the reticular cells. *In vitro* studies demonstrate the necessity of stromal cells to support B cell development (Kincade et al., 1989; Landreth and Dorshkind, 1988). Previous *in vivo* work in our laboratories has demonstrated that $B220^+$ B lineage are associated with stromal reticular cells in normal mice (Jacobsen et al., 1990). Since the SCID and SCID/myc marrow contains only pro-B cells, the present work now demonstrates that stromal reticular cells are associated with these early differentiation stages which include the phase of $V_H$ Ig gene rearrangement. In the SCID mouse, these stromal cell associations are also evident in intermediate and central zones of the marrow In normal mice, the contact of stromal reticular cells is probably required to support the various stages of B cell development (Jacobsen et al , 1990). The presence of stromal reticular cell processes in intermediate and central zones of the SCID marrow suggests that the stromal microenvironment has not been affected by the lack

of pre-B and B cells in these zones. In the SCID/myc mouse, the stromal reticular cell associations with B220$^+$ cells are especially prominent in peripheral regions, it being difficult to locate such associations in the more central regions of the marrow, where the B220$^+$ cells in intermediate and central regions are dissociated from other surrounding cells. Such a dissociation may be a sign of the onset of apoptosis (Fesus, L., 1991): if so, this possible onset of apoptosis would be a contributing factor to the cell loss occuring at the late pro-B cell stage. However, the B220$^+$ cells themselves did not show any morphological signs of apoptosis.

In day 5 endocolonized marrow, the B220$^+$ cells are associated with stromal reticular cell processes. In this regenerating marrow, B220$^+$ cells are developing in a microenvironment in which hemopoiesis has been depleted, but the radioresistant stromal elements still exist. Associations formed between these precursor cells and their microenvironment may thus resemble events occuring in late fetal mice, when B cell progenitors or pluripotent stem cells first migrate into the femoral bone marrow (Osmond, 1985). The associations suggest a microenvironment favouring the development of pro-B cells, in accord with previous work stressing the importance of interactions between stroma and B cell precursors As the electron micrographs demonstrate, the associations between stromal reticular cells and regenerating B220$^+$ cells are elaborate. One B220$^+$ cell with early B cell morphology had contact with a network of stromal cell processes seen in cross and tangential sections. Such

early cells do not have the continuous boundaries of contact with the stromal reticular cell processes, typical of normal, SCID and SCID/myc bone marrow, but a more interrupted pattern of contact. This may reflect the newly arrived early B cell progenitors not having yet associated fully with the microenvironment. Another B220$^+$ cell with an elongated shape suggesting that it was a motile cell, was also surrounded by a broken pattern of stromal reticular cell processes. Possibly, the immigrant B220$^+$ cells do not completely associate themselves with stromal reticular cells until they have reached an appropriate area of parenchyma presenting suitable cell adhesion molecules or cytokines, rendering the cell static. One apparently more mature B220$^+$ cell located within the parenchyma near a sinus wall, had a thin electronlucent reticular cell cytoplasmic extension completely surrounding it (fig 47) This extensive degree of association between B220$^+$ cells and stromal reticular cells resembles studies in normal mice in which stromal reticular cells often form complete contact with B220$^+$ cells in a deep goblet-shaped niche, assuring intimate cell-cell contact (Jacobsen and Osmond 1990) In the particular case of figure 47 , two explanations seem plausible: a) an early B cell precursor, having migrated to and settled in the irradiated marrow, has developed extensive, functional contact with a stromal reticular cell through time, and consequently differentiated to a pre-B or B cell; b) a more mature B220$^+$ cell migrated direct from the shielded marrow and was able rapidly to associate itself with a stromal reticular cell. To render this particular case even more

intriguing, the B220$^+$ cell along with its stromal reticular cell association, seem to be completely surrounded by another cell of uncertain identity. It appears to be neither a macrophage nor a standard stromal reticular cell, yet its extensive cytoplasm and irregular shape resemble those of stromal cells. Weiss et al. have distinguished a type of stromal cell, termed a "barrier cell" (Weiss et al., 1991) These are related to reticular cells and other fibroblasts, possibly even being fibromuscular in nature, actin and cytoplasmic filaments having been identified within their cytoplasm by immunocytochemistry. They are predominantly located in subendosteal regions of trabecular bone marrow. However, in conditions of stress, such as irradiation, they proliferate and spread from these restricted sites to other areas such as the diaphysis of the femur Some barrier cells establish intimate associations with blood vessels in the marrow insinuating slender processes of cytoplasm between endothelial and adventitial cells. They branch out from the adventitial surface of sinuses into the parenchyma, confining and enveloping differentiating hemopoietic cells. This mobilization around venous sinuses and envelopment of hemopoietic cells is proposed to be a regulatory system to prevent the premature release of cells into the circulation (Weiss et al , 1991) In the present case, the unidentified stromal cell may represent such a "barrier" cell that has enveloped the B220$^+$ cell-stromal reticular cell complex The density of its cytoplasm, its extensive cytoplasmic processes, its perisinusoidal location and the way in which it appears to surround an apparently premature B cell are all features consistent

with it being a barrier cell, possibly retaining the B220$^+$ cell within the parenchyma for its further maturation. If so, this would favour the first explanation concerning the nature and origin of the B220$^+$ cell, i.e., an early B cell precursor, having migrated into the irradiated marrow, has developed contact with a stromal reticular cell and subsequently differentiated. This would be the first in situ observation of a lymphoid cell associated with a barrier cell.

**4.2.4** Macrophages

A possible mechanism of cell loss during mouse B lymphopoiesis, as observed previously (Osmond et al., 1991), has been suggested by the present work. EM reveals many resident macrophages closely associated with B220$^+$ pro-B cells in intermediate and central marrow zones in the SCID mouse, and in peripheral marrow zones of the SCID/myc mouse The macrophages are characterized by distinctive features including large irregular dimensions, phagosomes, lysosomes, much ingested cellular debris, and, in these particular preparations, a dispersed or "loose" cytoplasm In normal bone marrow, 75% of all B cell precursors produced are lost at the pre-B cell stage and do not enter the blood circulation (Osmond and Park, 1987). In the SCID and SCID/myc models, all B cell precursors begin to differentiate and to proliferate, but they do not survive beyond the late pro-B cell stage which itself is depleted, compared to normal mice. Therefore, virtually 100% cell loss appears to occur in the SCID and SCID/myc mice but the mechanisms associated with the loss

remain unknown. The associations with macrophages now suggest that these cells are rapidly recognizing and ingesting the aberrant B cell precursors, as part of the cell loss process. In SCID marrow, one particular B220$^+$ cell was observed to be entirely within the cytoplasm of the macrophage even though only 3 min had elapsed between administering $^{125}$I-mAb 14 8 and fixing the tissue. Such a cell deletion could be occuring at the late pro-B cell stage, preventing aberrant B cell precursors from maturing and exiting the marrow. These results accord with the hypothesis that macrophages are implicated in the loss of B lineage cells in normal murine marrow (Jacobsen and Osmond, 1990). In one important respect, however, the appearances differ from those in normal marrow or in the marrow of Eμ-myc transgenic mice where most cell loss occurs at the pre-B cell stage The B220$^+$ cells associated with the macrophages in the SCID marrow are morphologically normal. Macrophages are known to phagocytose dying or dead cells (Jacobsen and Osmond, 1991). In the case of SCID mice, however, the B220$^+$ cells associated with macrophages do not show the morphological evidence of apoptosis. Their nuclei are not condensed and fragmented and they do not have a decrease in cytoplasmic volume. This in marked contrast with with Eμ-myc transgenic mice in which extreme apoptotic morphology is seen (Jacobsen, Sidman, and Osmond, unpublished). In SCID mice, the macrophages appear to detect and ingest aberrant B220$^+$ pro-B cells either without the cells undergoing apoptosis or before the morphological signs of apoptosis can become evident. The

macrophages seem to be selectively eliminating defective B cell precursors and thus playing an important role in the "quality control" of B cell genesis. In SCID/myc mouse marrow, B220$^+$ cells are associated with macrophages, even more frequently than in the SCID mouse marrow Indeed, the majority of labeled pro-B cells seen under EM are directly adjacent to macrophages This may again represent a process of recognition and phagocytosis of aberrant pro-B cells. The increased macrophage association in these regions implies a very early recognition of aberrant B220$^+$ pro-B cells The expansion of the population of TdT$^+$B220$^+$ pro-B cells, all of which are aberrant, may be a signal to increase macrophage activity. In Eμ-myc mice, B220$^+$ cells are often associated with or ingested by macrophages (Jacobsen and Osmond, 1991) and furthermore, those B220$^+$ cells are in large part apoptotic. In the SCID/myc mouse, out of all the B220$^+$ cell-macrophage associations observed, only one B220$^+$ cell had some morphological characteristics of apoptosis The rest of the pro-B cells were of normal, early B cell precursor morphology It seems that the macrophages are deleting the aberrant pro-B cells before they have a chance to manifest the morphological signs of apoptosis This implies a delicate positive or negative molecular signaling mechanism, triggering macrophages to recognize and delete aberrant cells at an early stage The present work in SCID and SCID/myc mice provide strong support for the view that macrophages are involved in the selection and deletion of non-functional B cell precursors, preventing their release into the circulation No macrophages were associated with B220+ cells

in the day 3-5 endocolonized marrow. This may suggest that in this system the early pro-B cells were differentiating normally without cell loss and that the pre-B cell stage at which cell loss can normally occur, had not yet been reached.

**4.2.5** Proximity of regenerating early precursor B cells to nerve fibers

A novel observation has been the apparent association of B220$^+$ cells with nerve fibers containing unmyelinated axons in day 5 endocolonized marrow. In almost every sighting of a B220$^+$ cell, a nerve fiber was located in the same electron microscopic field, close enough to prompt speculation that there may have been some functional communication between the two. In one instance, a B220$^+$ cell of early morphology had shaped its cytoplasm around the edge of a nerve fiber giving the impression of a "niche" or an isolated microenvironment within which signal transmissions could occur. Recent work by Goetzl and Sreedaharon (1992), *in vitro*, has demonstrated bidirectional interactions between the immune system and neuroendocrine system, influencing such diverse physiological activities as tissue localization of lymphocytes, antibody responses and neural signal transmissions. Under certain stimuli, cultured astroglial and microglial cells expressed mRNA for α-tumor necrosis factor (TNF-α), IL-1, IL-6, and ß-transforming growth factor (TGF-ß), while prolactin and VIP (vasoactive intestinal peptide) are active in regulating of lymphocyte growth and functions These findings raise the possibilities that transmitter molecules from nerve endings located near early

B cell precursors in post-irradiation endocolonized marrow, may play an important role in the recovery of the B lymphocyte population. Prolactin, a pituitary polypeptide hormone involved in maintaining B cell immune responsiveness in rodents (Friesen et al., 1991), increases the transcription of c-myc proto-oncogene and interferon regulatory factor 1 (IRF-1), both of which are involved in enhancing cellular proliferation (Yu Lee et al , 1990). Possibly these nerve fibers, whose terminals can only be conclusively identified through special staining, may be enhancing the development of the migrated B cell progenitors by secreting molecules that promote B cell proliferation Yamazaki and Allen (1991) have also demonstrated a "neuro-reticular complex" in which efferent nerve fibers are intimately associated with stromal reticular cells in the bone marrow. They suggest a nervous control of hemopoiesis through the stimulation of the stromal cells to secrete cell growth factors Such neuro-reticular cell associations, though not directly examined or identified in the present work, could serve as an additional mechanism stimulating B cell proliferation in conjunction with a direct nervous stimulation of early B cell progenitors.

## 5. Summary

The three animal models used in this project, SCID mice, SCID/myc mice and post-irradiation repopulation, have all provided information concerning the localization and intercellular associations of early B220$^+$ pro-B cell progenitors in the bone marrow. The results both substantiate previous views, and introduce new concepts that can lead to future work.

All three models have shown that many early B cell precursors expressing the B220 glycoprotein are preferentially located in peripheral marrow. Other precursor B cells can also be seen in intermediate zones of SCID and SCID/myc mouse marrow while very few are found in central marrow zones around the central sinus The peripherally located pro-B cells in the SCID and SCID/myc mice are intimately associated with an electron dense extracellular matrix and with stromal reticular cell processes both of which appear to play a role in supporting B cell development. The endocolonization system has enabled the detection of B220$^+$ cells in a situation where B cell precursors are recolonizing hemopoietically depleted bone marrow. The consistent observation of stromal reticular cell associations with B220$^+$ cells confirms the findings in the other two murine models, as well as previous work Furthermore, it reveals the development of stromal associations at an early stage of B cell development that marks the beginning of colony formation.

In the bone marrow of SCID and SCID/myc mice, prominent macrophages are associated with many B220$^+$ cells, all of which have aberrant

Ig gene rearrangements and are destined to be deleted. These findings suggest a role of macrophages in recognizing and deleting aberrant B cell precursors in SCID and SCID/myc marrow where virtually all B cell precursors are aberrant and are lost at the pro-B cell stage, as is the case in normal marrow where many B cell precursors are lost at the pre-B cell stage

The early $B220^+$ B cell precursors in post-irradiation endocolonizing bone marrow are often adjacent to nerve fibers containing unmyelinated nerve axons, previously defined as efferent autonomic fibres. This finding raises the possibility of a role for nervous factors in the post-irradiation endocolonization of early B cell precursors.

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**Figure 1.** Schematic representation of the working model of B cell development.

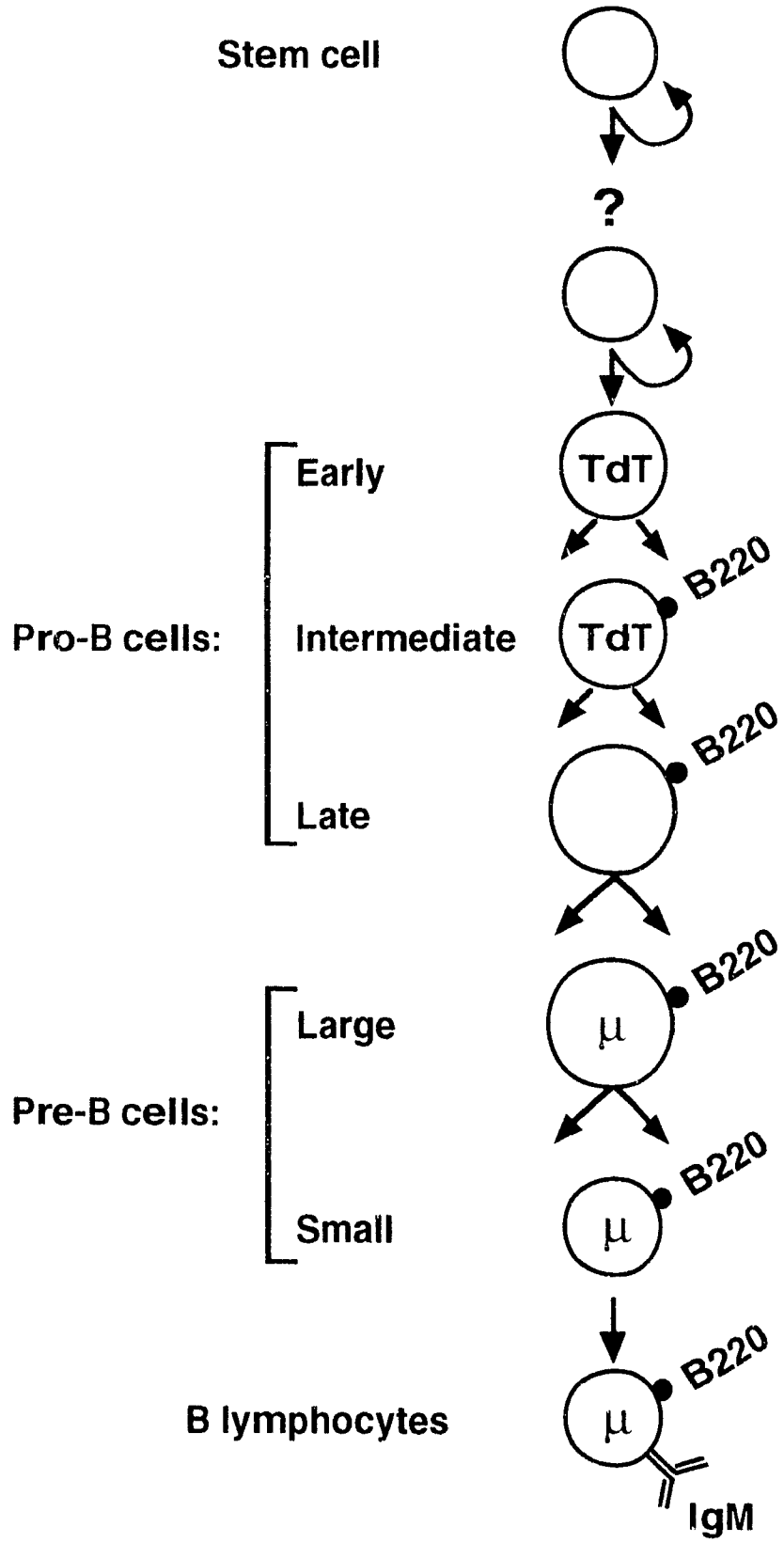

Stem cell
?
TdT
Early
B220
Pro-B cells:
TdT
Intermediate
B220
Late
B220
μ
Large
Pre-B cells:
B220
μ
Small
B220
μ
B lymphocytes
IgM

**Figure 2**. Histological maps of four representative femoral marrow sections from three SCID mice, showing the distribution of $B220^+$ early precursor B cells. Each circle represents the various numbers of $B220^+$ cells seen in individual microscopoc fields ($2025\mu m^2$).

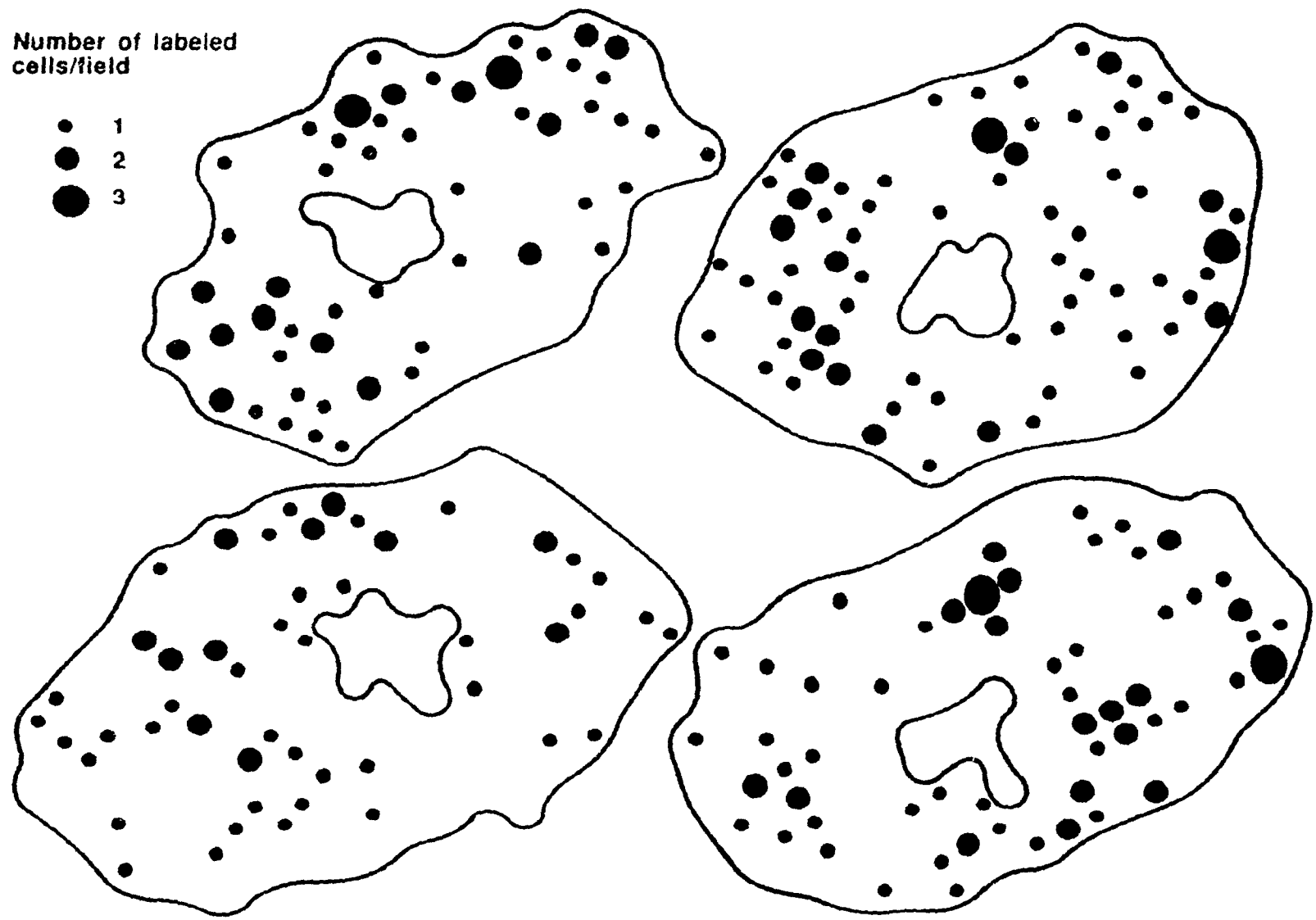

Number of labeled cells/field
1
2
3

**Figure 3.** L.M. radioautograph showing the patchy distribution of $B220^+$ early B cell precursors (**arrowheads**) in the peripheral region of the femoral bone marrow of a SCID mouse. Some of the labelled cells are near the cortical bone (**b**) and others are further away from it. A large sinusoid (**S**) is penetrating the peripheral parenchyma in which adipocytes (**A**) and megakaryocytes are also found. Two week exposure period, 40x magnification

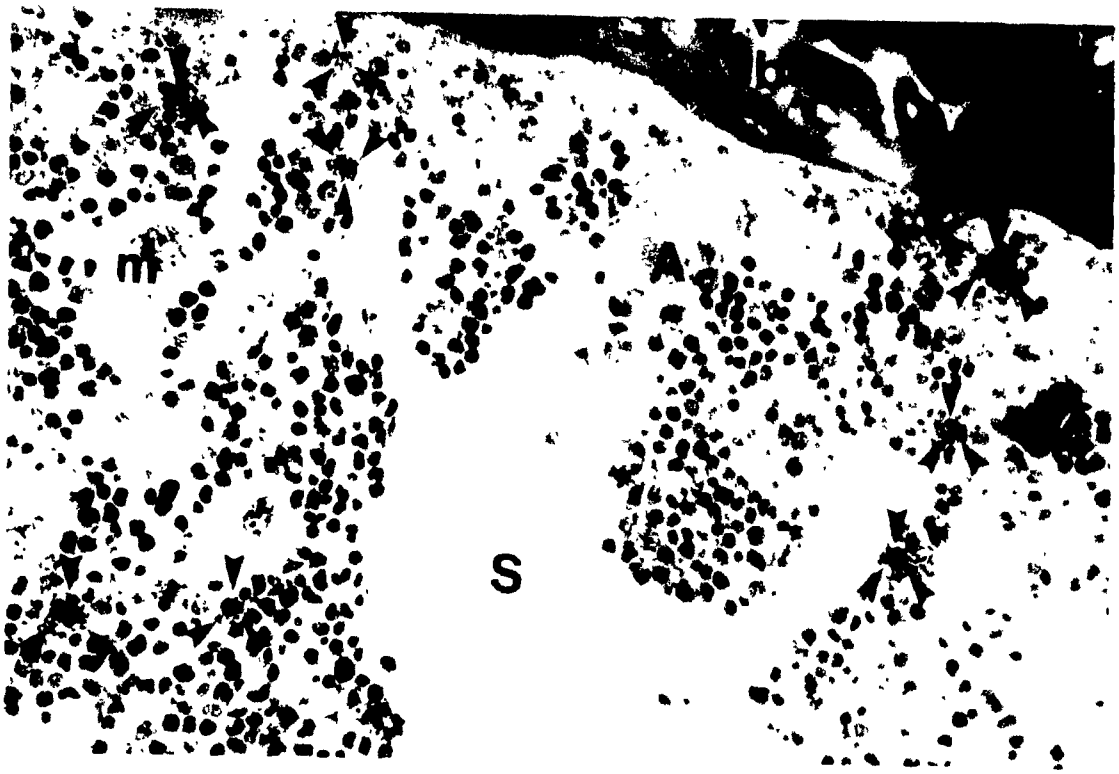
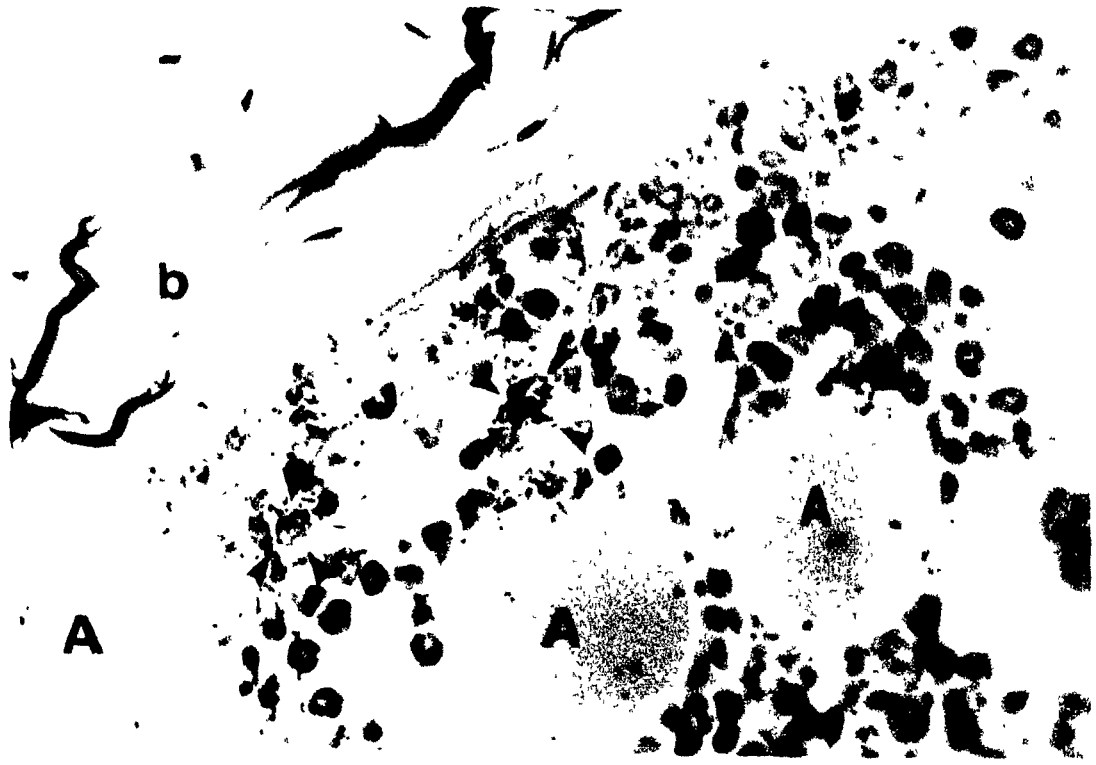

m
S

**Figure 4.** L.M. radioautographs of B220$^+$ early B cell precursors (**arrowheads**) in the peripheral region of the femoral bone marrow of a SCID mouse situated a) near the cortical bone (**b**) and neighbouring adipocytes (**A**) and b) between a small cluster of adipocytes (**A**). Two week exposure period, 80x magnification.

a)




b)

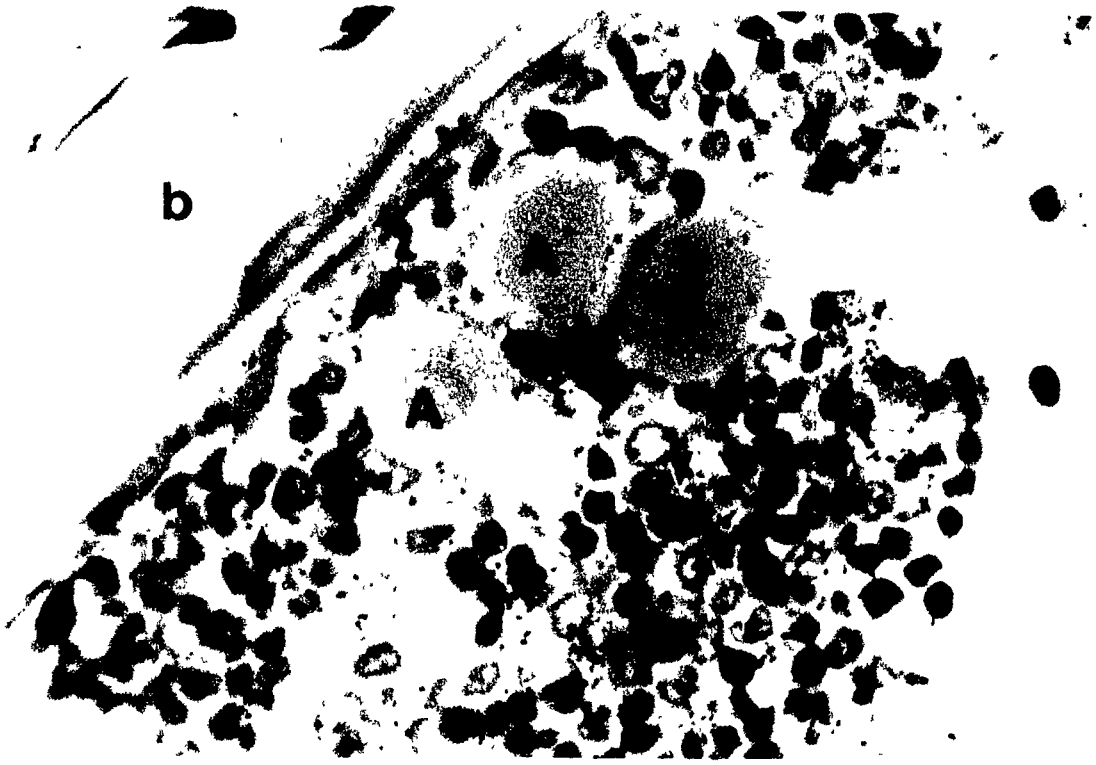
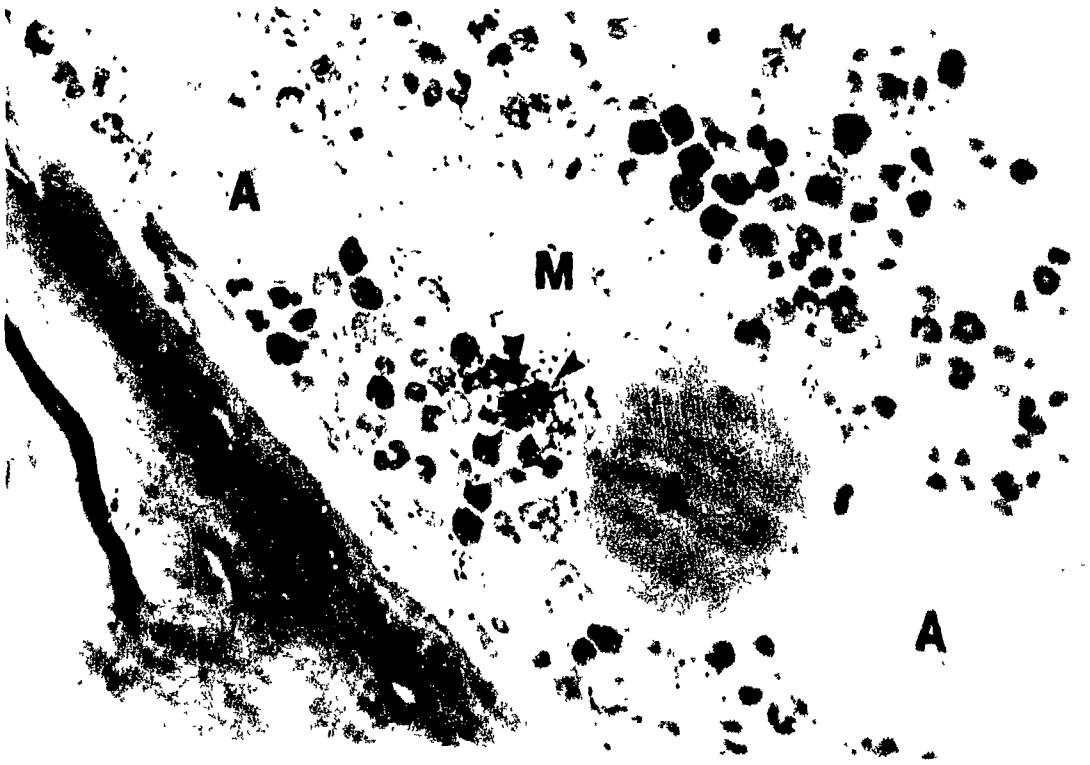


**Figure 5.** L.M. radioautographs of B220$^+$ early B cell precursors (**arrowheads**) in the peripheral region of the femoral bone marrow of a SCID mouse. Figure a) shows a positively labelled pro-B cell (**arrowheads**) located close to the cortical bone (**b**), and is also adjacent to a megakaryocyte (**M**) and an adipocyte (**A**). Figure b) shows two heavily labelled pro-B cells (**arrowheads**) adjacent to a sinusoid filled with erythrocytes, which is lying against the cortical bone (**b**). An adipocyte (**A**) is also seen in this field. Two week exposure period, a and b· 64x magnification.

a)




b)

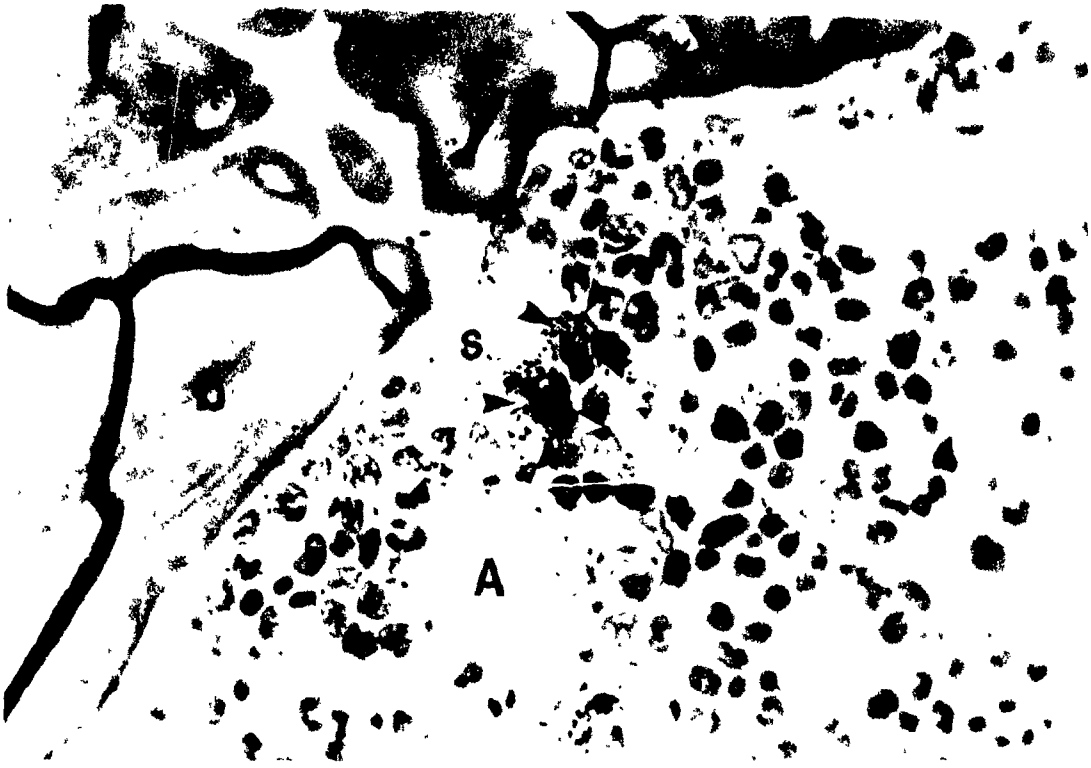


**Figure 6.** L.M. radioautograph of a heavily labelled $B220^+$ early B cell precursor (**arrowheads**) within the lumen of a sinusoid (**S**) in the femoral bone marrow of a SCID mouse. Two week exposure period, 125x magnification.

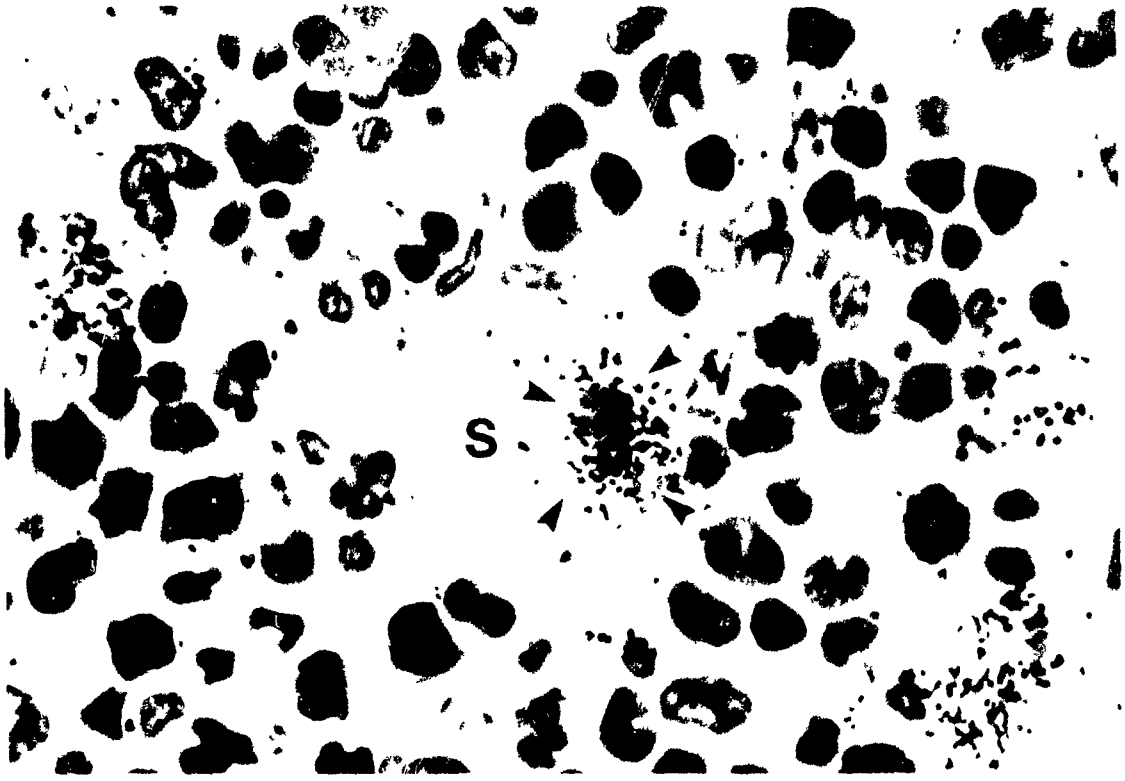
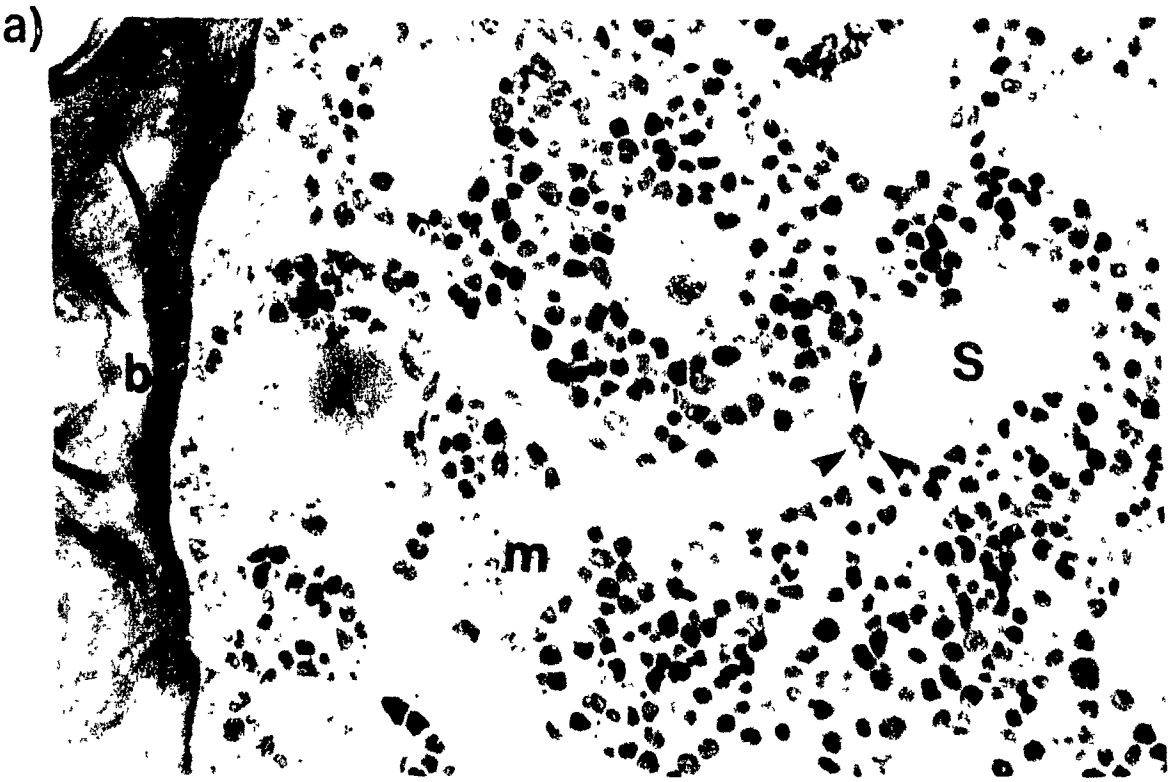

S

**Figure 7.** L.M radioautographs of heavily labelled $B220^+$ early B cell precursors (a) and b); **arrowheads**) in the peripheral region of the femoral bone marrow of a SCID mouse. In both cases, the pro-B cell is adjacent to the endothelium lining a sinusoid. This microscopic field reveals other aspects of the peripheral region of the marrow such as the cortical bone (**b**), adipocytes (**A**) and megakariocytes (**M**). Two week exposure period, a: 31.25x, and b: 50x magnification.


a)
b
S
m

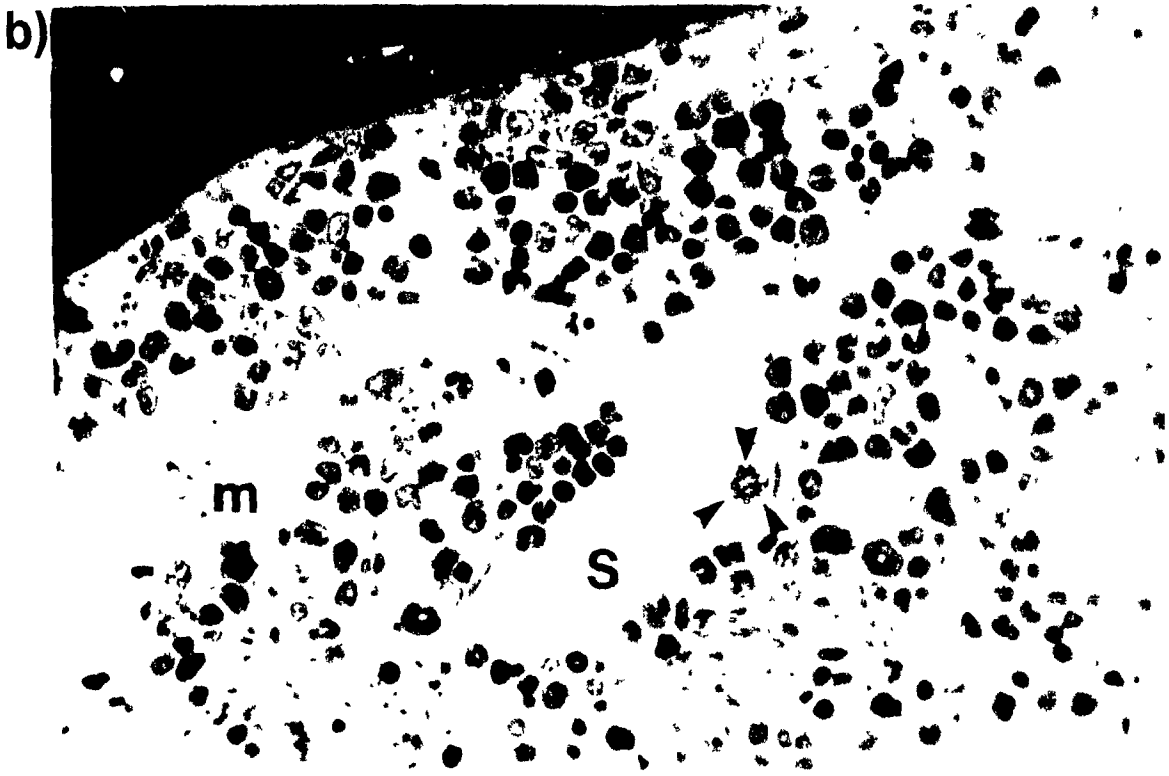

b)
m
S

**Figure 8.** Electron microscope (E M.) radioautograph of a B220$^{+}$ pro-B (**PB**) cell in the femoral bone marrow of a SCID mouse, labelled by *in vivo* administration of $^{125}$I mAb 14 8 The filamentous development of silver grains (**arrowheads**) on the photographic emulsion, due to the radiation emissions of $^{125}$I, clearly delineates the plasma membrane of the cell. Two month radioautographic exposure period, 16 250x magnification.


PB

**Figure 9.** E.M. radioautograph of a B220$^+$ pro-B (**PB**) located in the peripheral region of the femoral bone marrow of a SCID mouse. The positive cell is directly adjacent to an osteoblast (**Ob**) that separates it from the cortical bone (**B**) An electron dense extracellular matrix surrounds the cell (**arrowheads**) The open leptochromatic pattern of the nucleus, the abundance of cytoplasm and the numerous organelles in the cytoplasm are typical of early B cell morphology. Six week exposure period, 16 250x magnification.

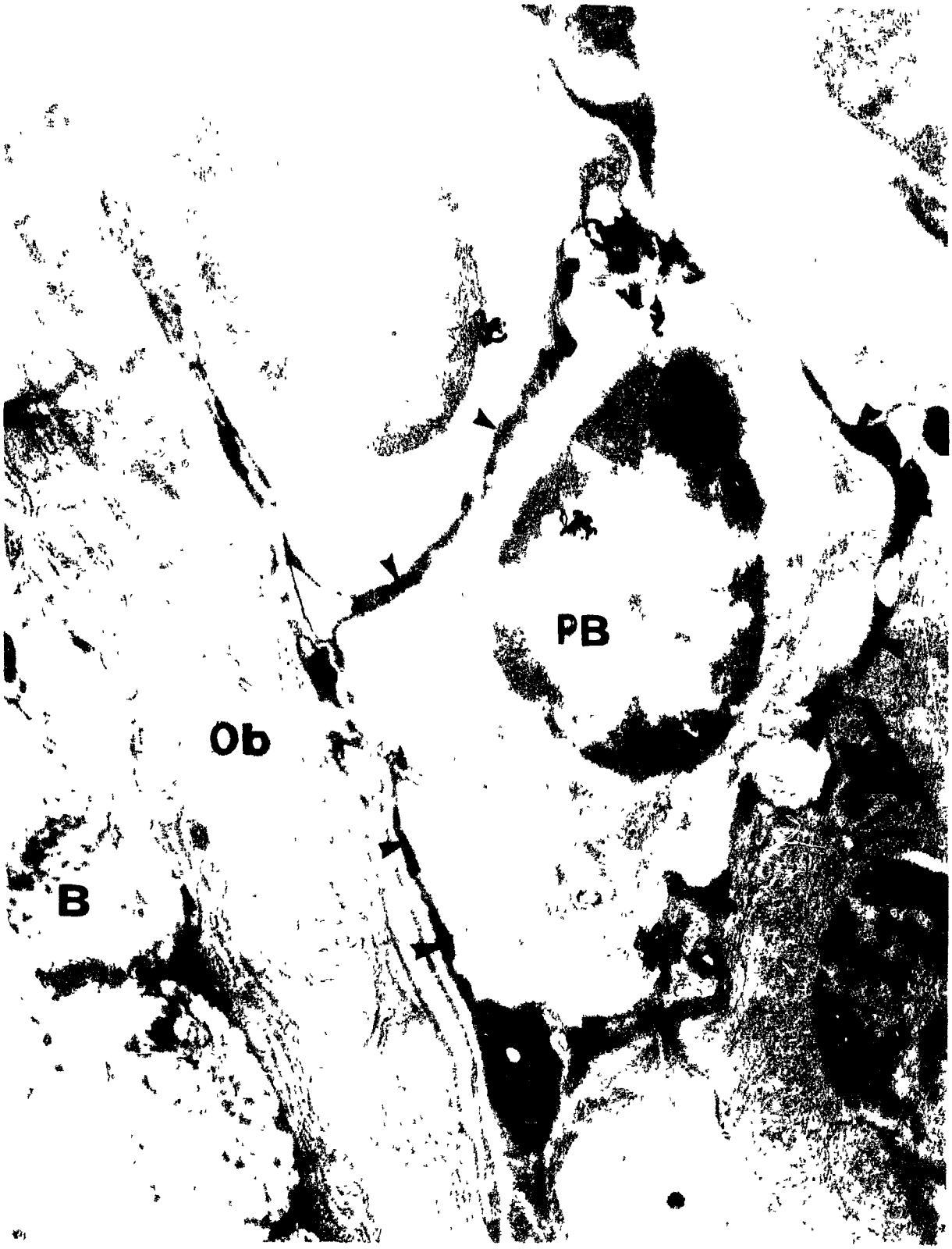

PB
Ob
B

**Figure 10.** E.M. radioautograph of a tangential section of a peripherally located B220$^+$ cell of early precursor morphology (**PB**) in the femoral bone marrow of a SCID mouse. The cell is adjacent to the layer of osteoblasts (**Ob**) underlying the bone (**B**) and is surrounded by an electron dense extracellular matrix (**arrowheads**). Six week exposure period, 10 000x magnification.

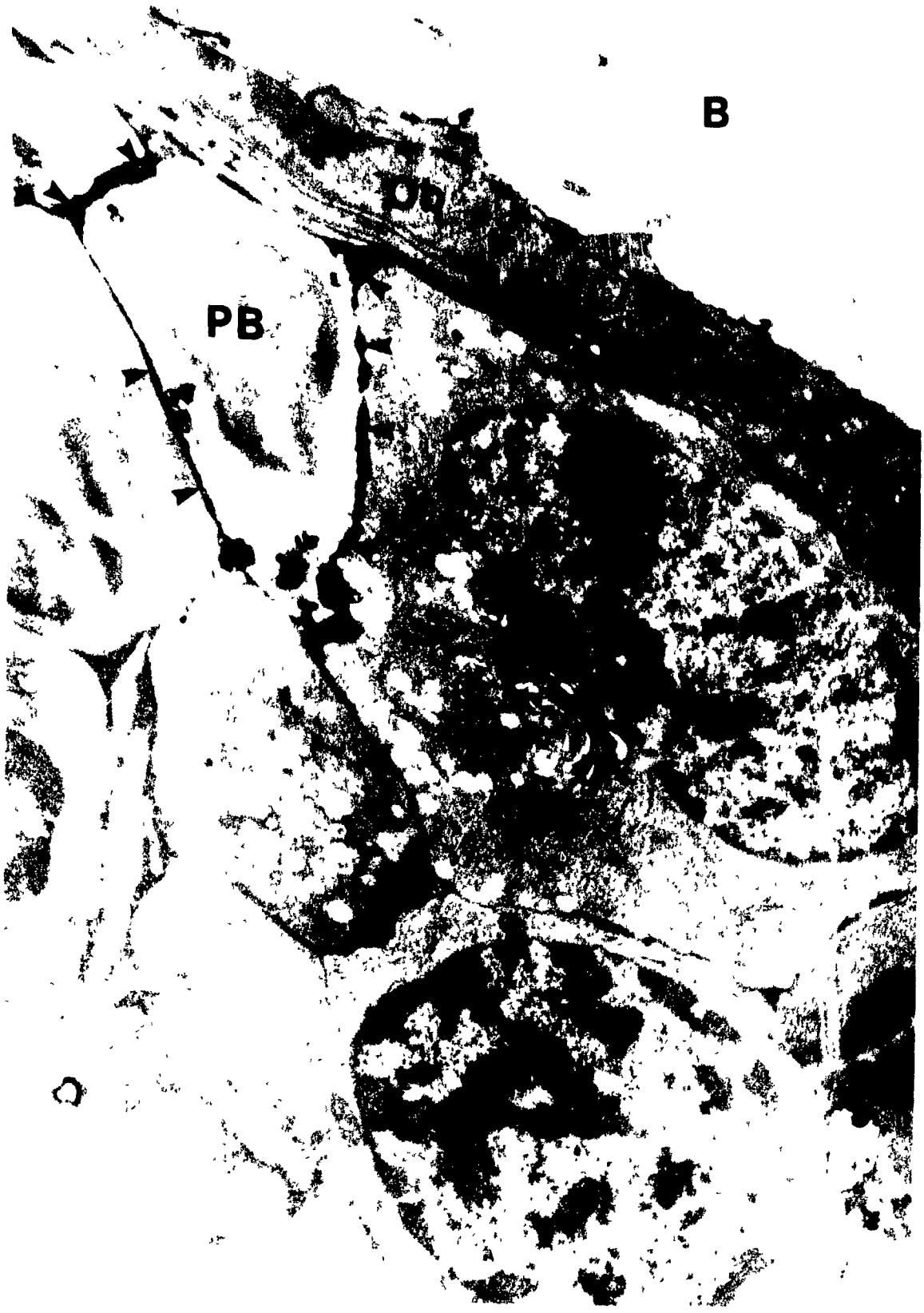
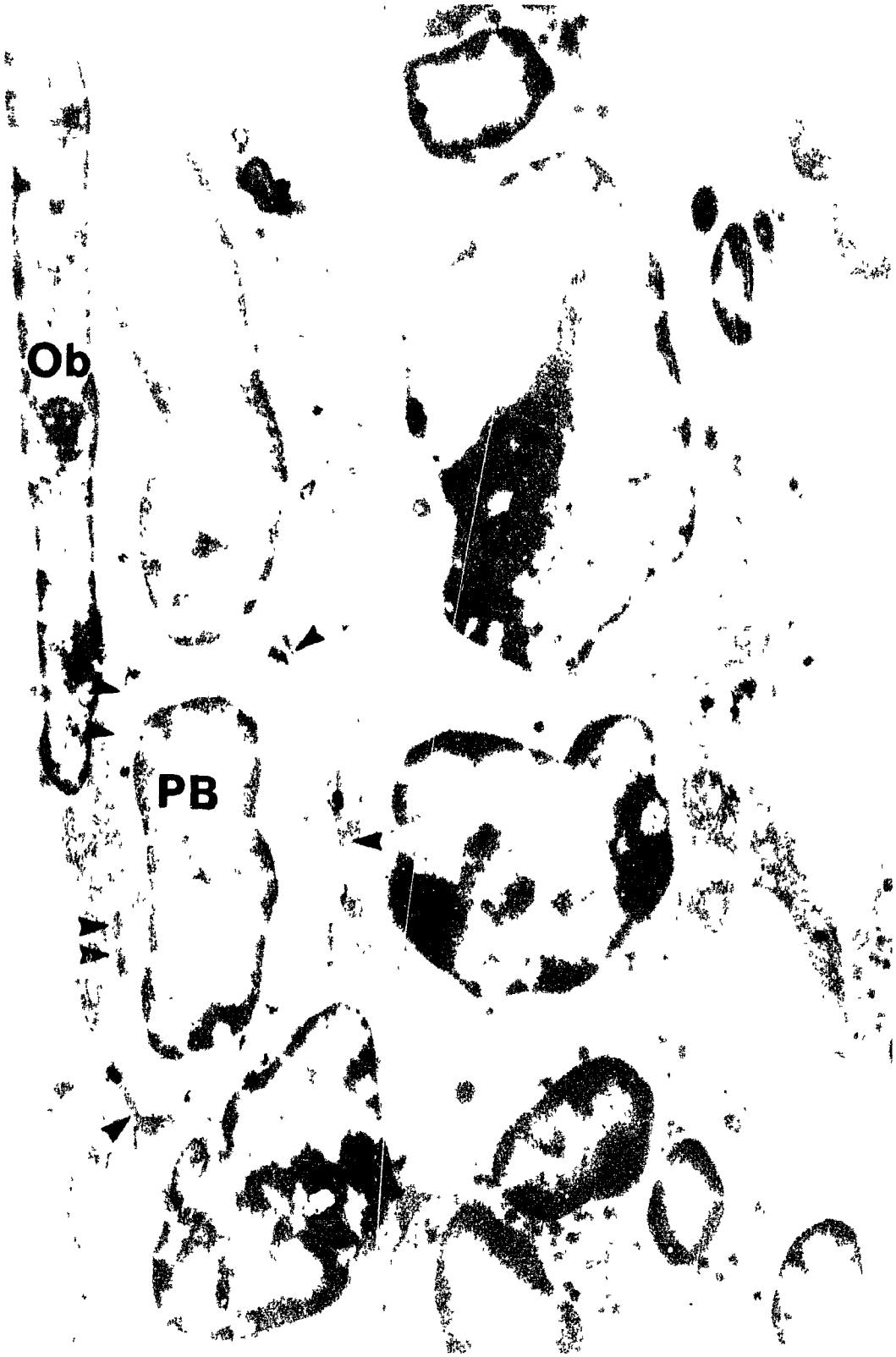

B
PB

**Figure 11.** E.M. radioautograph of a B220$^+$ early B cell precursor (**PB**) located near the cortical bone of the femoral bone marrow of a SCID mouse. This heavily labelled pro-B cell is surrounded by distinct thin cytoplasmic processes (**arrowheads**) that could be easily traced, leading away from the B220$^+$ cell. An electron dense extracellular matrix is visible between the pro-B cell and the cytoplasmic processes of the stromal reticular cell as well as between the pro-B cell and a neighbouring lymphoid cell (**L**) (**arrows**). Six week exposure period, 11 250x magnification.


B
L
PB

**Figure 12.** E.M. radioautograph of a $B220^+$ early B cell precursor (**PB**) located in the peripheral femoral bone marrow of a SCID mouse. The cell is adjacent to an osteoblast underlying the cortical bone (**B**). The pro-B cell is surrounded by various strands of cytoplasm (**arrowheads**) originating from stromal reticular cells. Three month exposure period, 8 500x magnification.




B

**Figure 13.** E.M. radioautograph of a B220$^+$ early B cell precursor (**PB**) situated in the intermediate region of the femoral bone marrow of a SCID mouse. This pro-B cell is partially encompassed by distinct cytoplasmic processes characteristic of stromal reticular cells (**arrowheads**). This positive cell is adjacent to an adventitial reticular cell (**AR**) that is lining the adluminal aspect of an endothelial cell (**E**). The latter is separating the parenchyma from a sinusoid (**S**). Six week exposure period, 12 000x magnification.

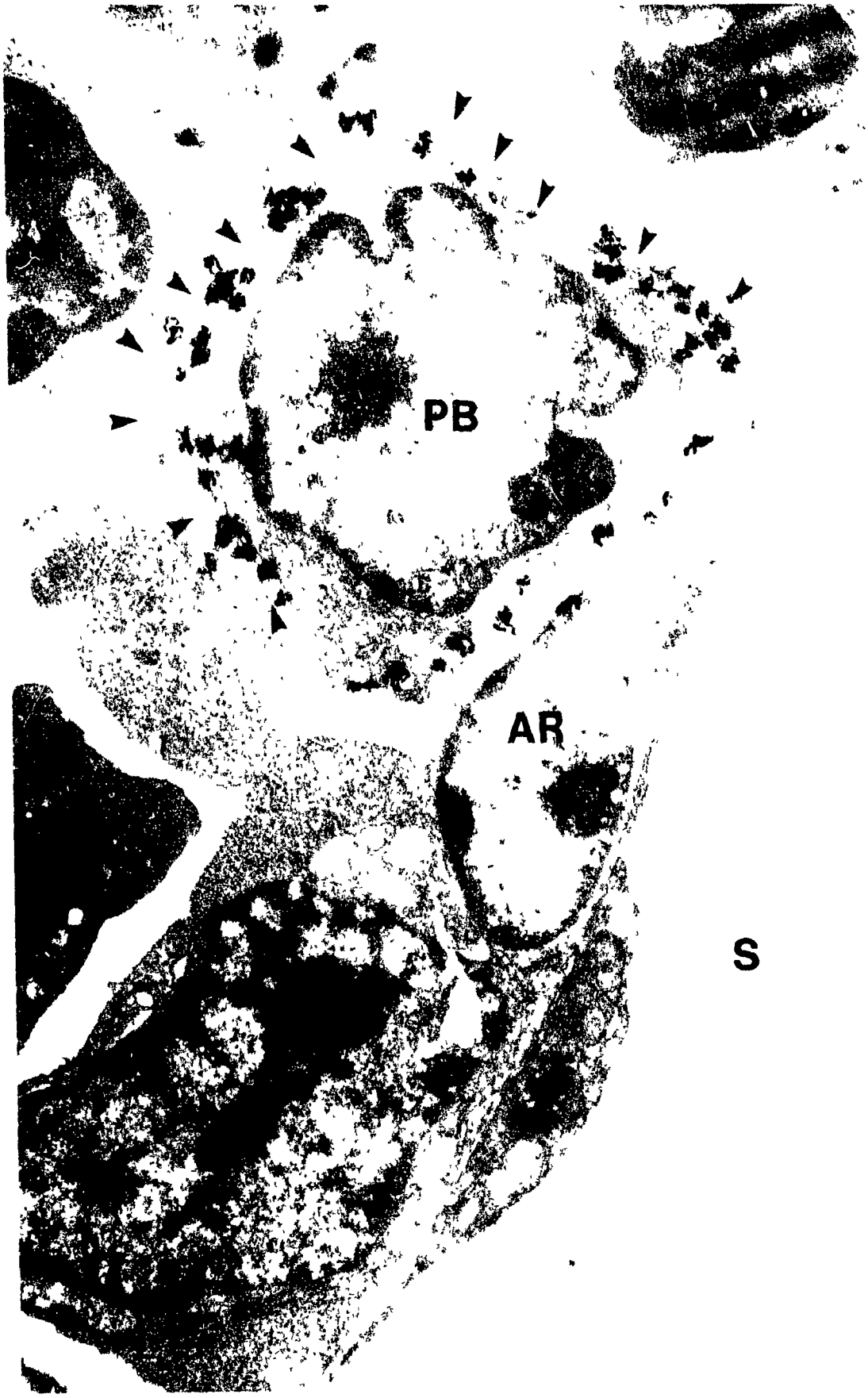

PB
AR
S

**Figure 14.** E.M. radioautograph of a B220$^+$ early B cell precursor (**PB**) in the intermediate region of the femoral bone marrow of a SCID mouse. The B200$^+$ cell is adjacent to an endothelial cell (**E**) separating the pro-B cell from a sinusoid (**S**) filled with red blood cells. The B cell precursor is in partial contact with stromal reticular cell processes (**arrowheads**). Six week exposure period, 12 000x magnification.


PB
E
S

**Figure 15.** E.M. radioautograph of a $B220^+$ early B cell precursor (**PB**) located in the intermediate region of the femoral bone marrow of a SCID mouse. The labelled cell is in direct contact with the cytoplasm of a macrophage (**M**). The macrophage has a very dispersed cytoplasm which contains many mitochondria, lysosomes, and cellular debris. Three month exposure period, 13 600x magnification.


PB
M

**Figure 16.** E.M. radioautograph of a B200$^+$ early B cell precursor (**PB**) in the intermediate region of the femoral bone marrow of a SCID mouse. A macrophage (**M**) containing numerous phagosomes and cellular debris is completely surrounding the intact B220$^+$ cell. Three month exposure period, 8 500x magnification.

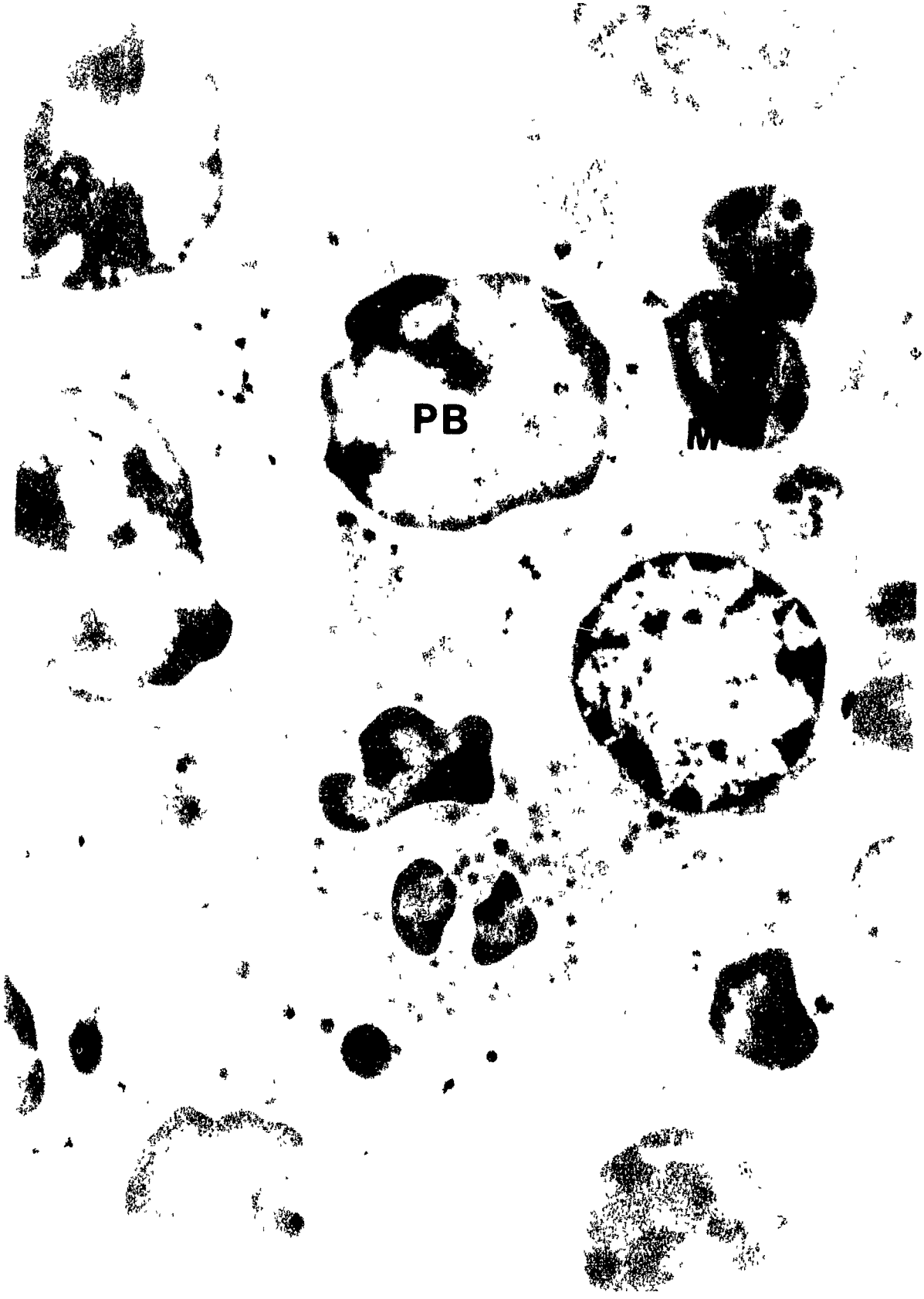

PB
M

**Figure 17.** E.M. radioautograph of a B220$^+$ early B cell precursor (**PB**) located in the intermediate region of the femoral bone marrow of a SCID mouse. The B220$^+$ cell is surrounded by a loose cytoplasmic matrix that extends throughout the length of the micrograph (**arrows**). This characteristic has been attributed to the cytoplasm of macrophages (**M**) in such histological preparations. Dispersed mitochondria, lysosomes, and cellular debris can easily be identified within the cytoplasm of the macrophage. Three month exposure period, 4 700x magnification


PB
M

**Figure 18.** E.M. radioautograph of a $B220^+$ early B cell precursor (**PB**) associated with a macrophage (**M**) in the intermediate region of the femoral bone marrow of a SCID mouse. The cytoplasm of the macrophage seems to be surrounding most of the pro-B cell (**arrows**). Large condensed nuclei of dead cells are present in the cytoplasm of the macrophage Three month exposure period, 8 500x magnification.

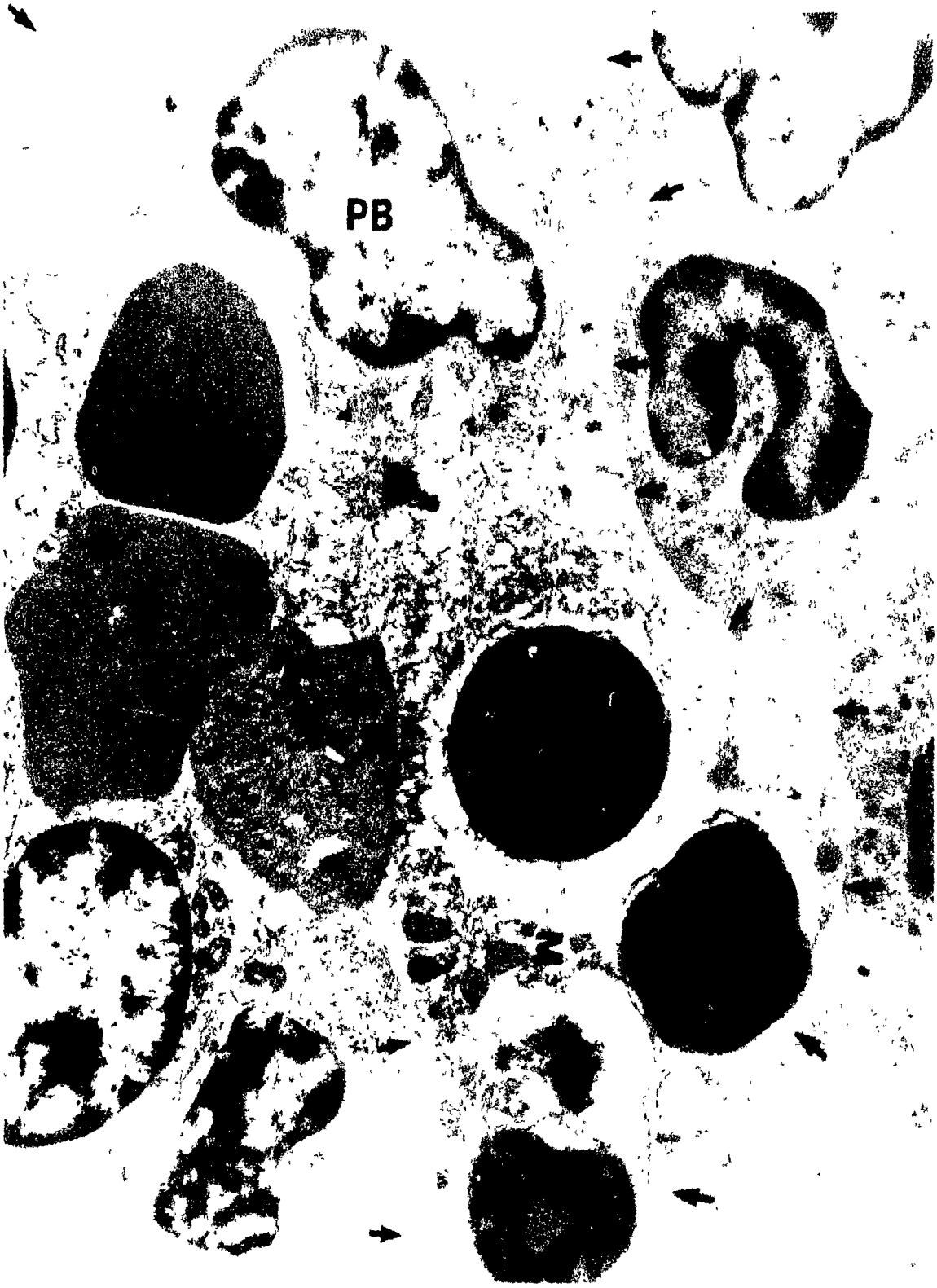

PB

**Figure 19.** E.M. radioautograph of a heavily labelled B220$^+$ early B cell precursor (**PB**) in a sinusoid (**S**) of the peripheral region of the femoral bone marrow of a SCID mouse. The labelled cells appears slightly more mature as revealed by its morphological features, the high nuclear to cytoplasmic ratio as well as the overall condensed nature. The cell seems to be trailing thin cytoplasmic processes that traverse the endothelium from the parenchyma (**arrowheads**). Six weeks exposure period, 9 000x magnification.


PB
S

**Figure 20.** E.M. radioautograph of a heavily labelled B220$^+$ early B cell precursor (**PB**) in a sinusoid (**S**) of the femoral bone marrow of a SCID mouse. The cell seems to be anchored to the parenchyma by strands of cytoplasm that are traversing the endothelium, however, the origin of the strands is unknown (**arrowheads**). Six week exposure period, 7 200x magnification.

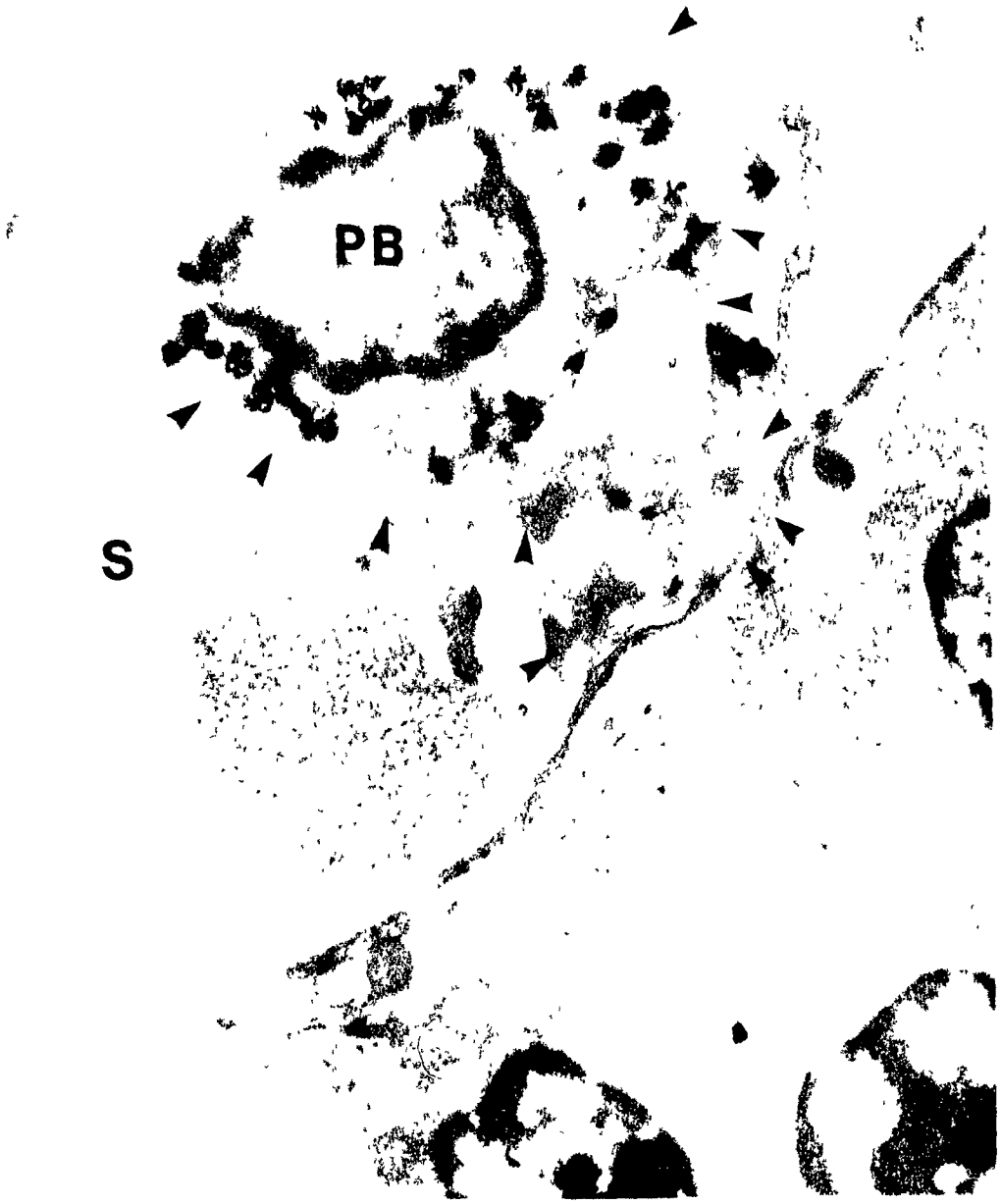
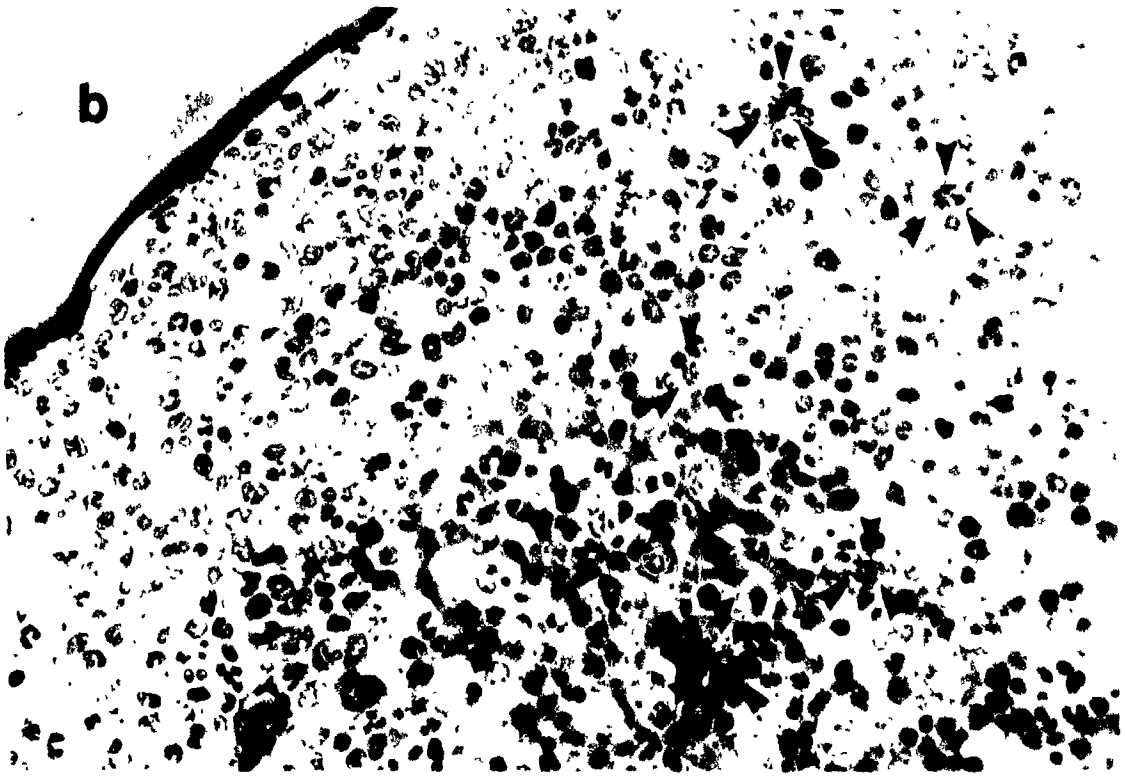

PB
S

**Figure 21.** E.M. radioautograph of a B220$^+$ early B cell precursor (**PB**) free in the lumen of a sinusoid (**S**) lined by endothelium in the femoral bone marrow of a SCID mouse. Six week exposure period, 9 600x magnification.


S
PB

**Figure 22.** L.M. radioautographs (a and b) showing the patchy distribution of $B220^+$ early B cell precursors (**arrowheads**) in the femoral bone marrow of a SCID/myc mouse. The cortical bone (**b**) reveals the peripheral nature of these marrow regions. Three week exposure period, 40x magnification

a)




b)

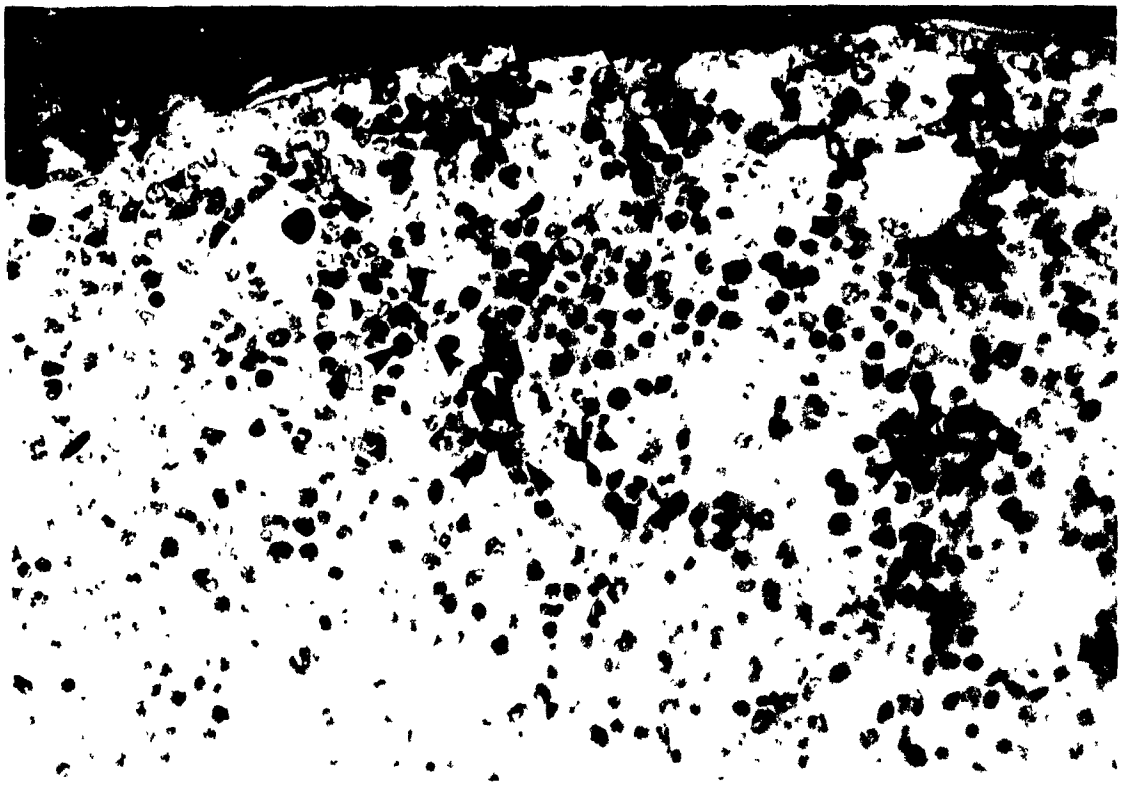
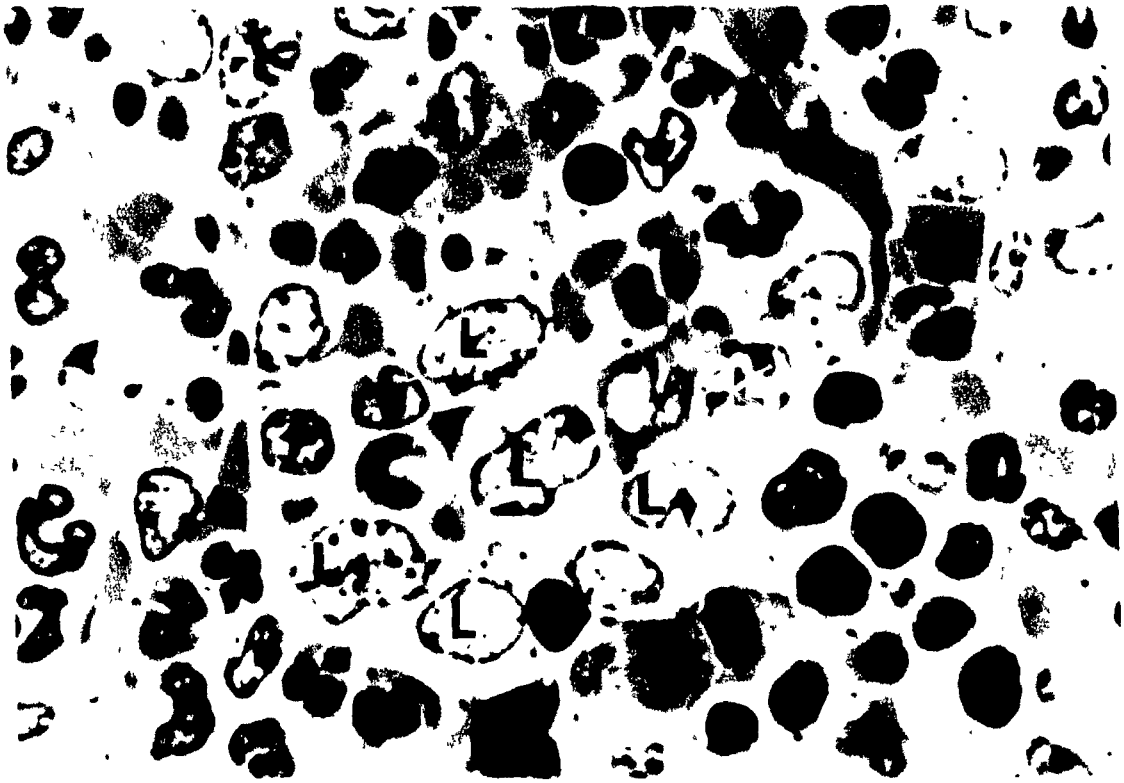


**Figure 23.** L.M. radioautographs of SCID/myc femoral bone marrow. Figure a) shows the large lymphoblast-like cells found in the peripheral region of the marrow. Figure b) shows two of the lymphoblast cells that are lightly labelled for the B220 glycoprotein (**arrowheads**). Three week exposure period, a and b: 125x magnification.

a)



b)

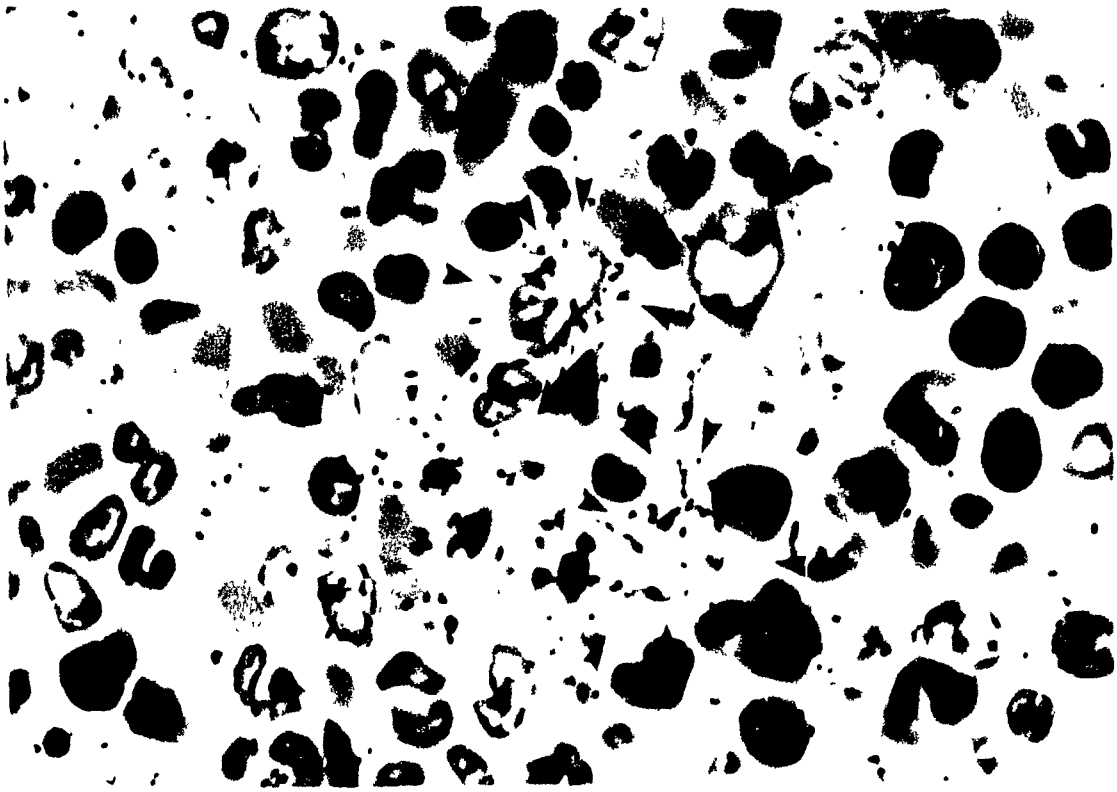


**Figure 24**. E.M. radioautograph of a B220$^+$ early B cell precursor (**PB**) adjacent to osteoblasts (**OB**) underlying the cortical bone in the femoral bone marrow of a SCID/myc mouse. The pro-B cell is not, however, directly adjacent to the subosteal layer but is in contact with a cytoplasmic process which runs parallel to the osteoblast layer. A similar process is lining the cell on its opposite side (**arrowheads**). The pro-B cell's neighbouring cells are quite probably pro-B cells, but are very lightly labelled (*). Three month exposure period, 8 500x magnification

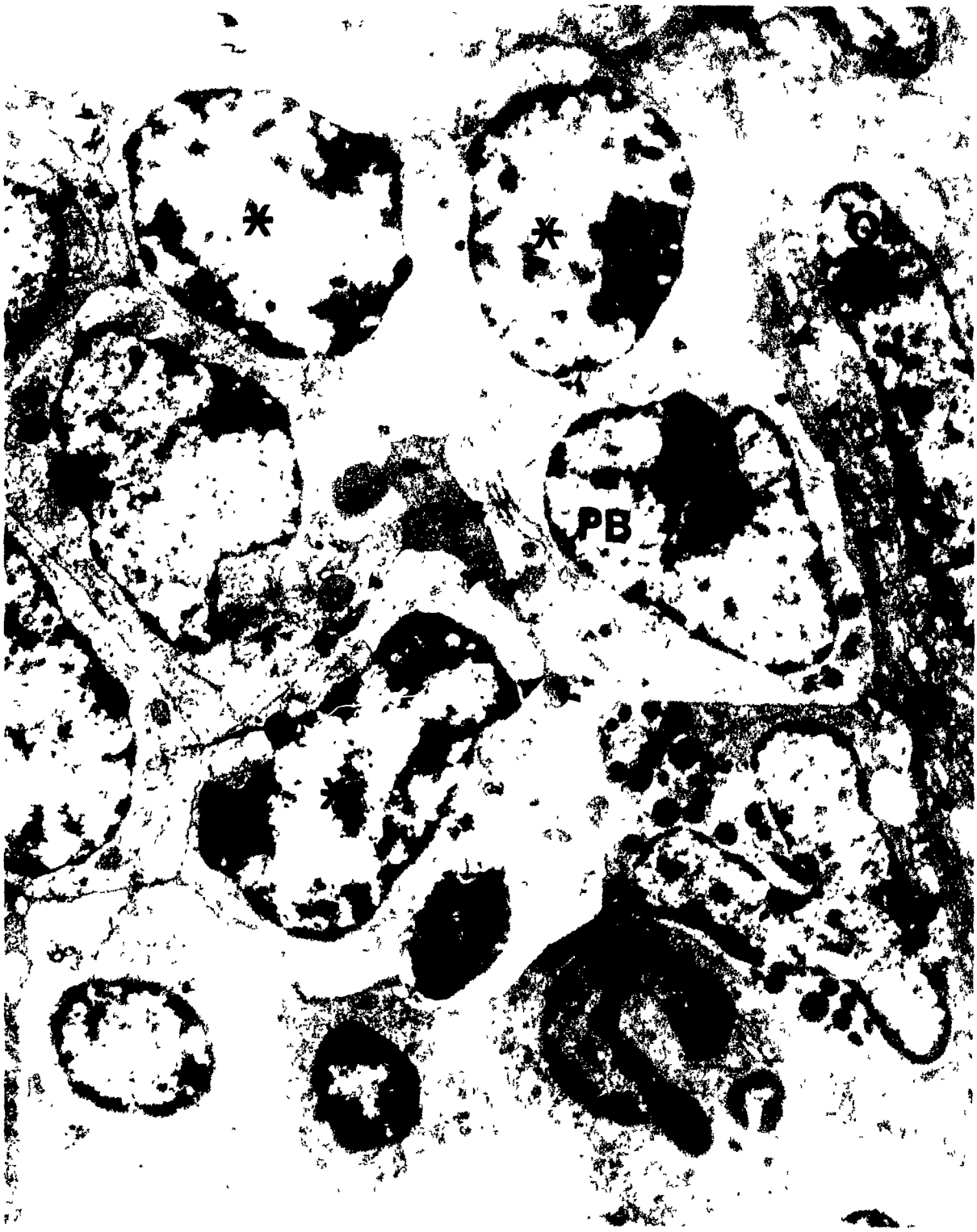

PB

**Figure 25.** E.M. radioautograph of a lightly labelled B220$^+$ early B cell precursor (**PB**)in the peripheral region of the femoral bone marrow of a SCID/myc mouse. The B220$^+$ cell, which is about 2 cell lengths away from the subosteum is lined by a distinct strand of cytoplasm which would stem from stromal reticular cells (**arrowheads**). Three month exposure period, 10 200x magnification.


PB

**Figure 26.** E.M. radioautograph of a B220$^+$ early B cell precursor (**PB**) that is partially dissociated from its neighbouring cells. The labelled cell is in the intermediate region of the femoral bone marrow of a SCID/myc mouse. Three month exposure period, 13 800x magnification.

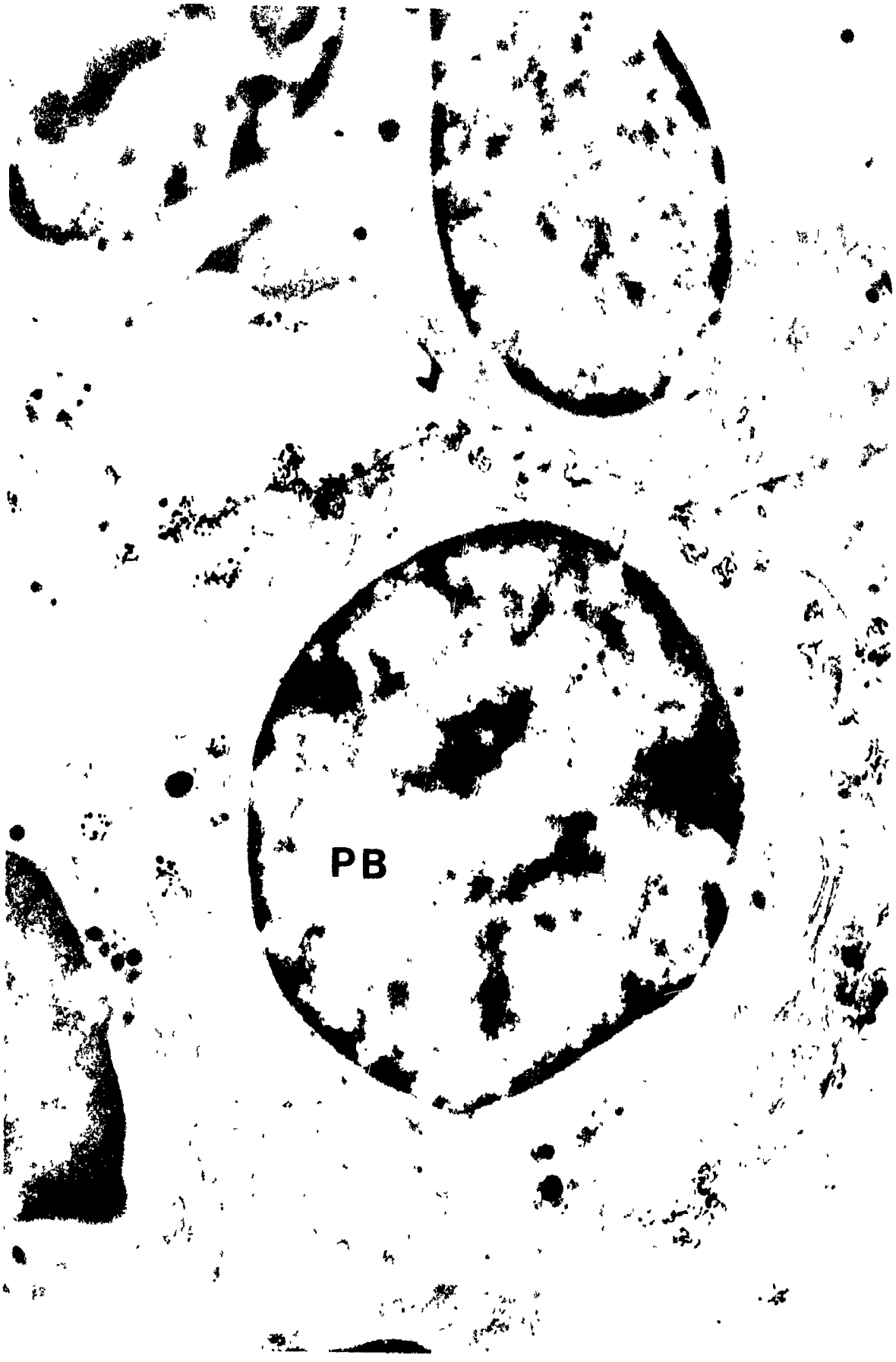

PB

**Figure 27.** E.M. radioautograph of a B220$^+$ early B cell precursor (**PB**) in the intermediated region of the femoral bone marrow of a SCID/myc mouse. The cell seems to be in the process of dissociating from its neighbouring hemopoietic cells. Three month exposure period, 18 500x magnification


PB

**Figure 28.** E.M. radioautograph of a B220$^+$ early B cell precursor (**PB**) in the peripheral region of the femoral bone marrow of a SCID/myc mouse. The cell is simultaneously in contact with a macrophage (**M**) on one side, and with a stromal reticular cell process on the other side (**arrowheads**). The pro-B cell is itself slightly apoptotic as seen by its condensed nuclear chromatin and aggregated organelles Three month exposure period, 8 500x magnification.

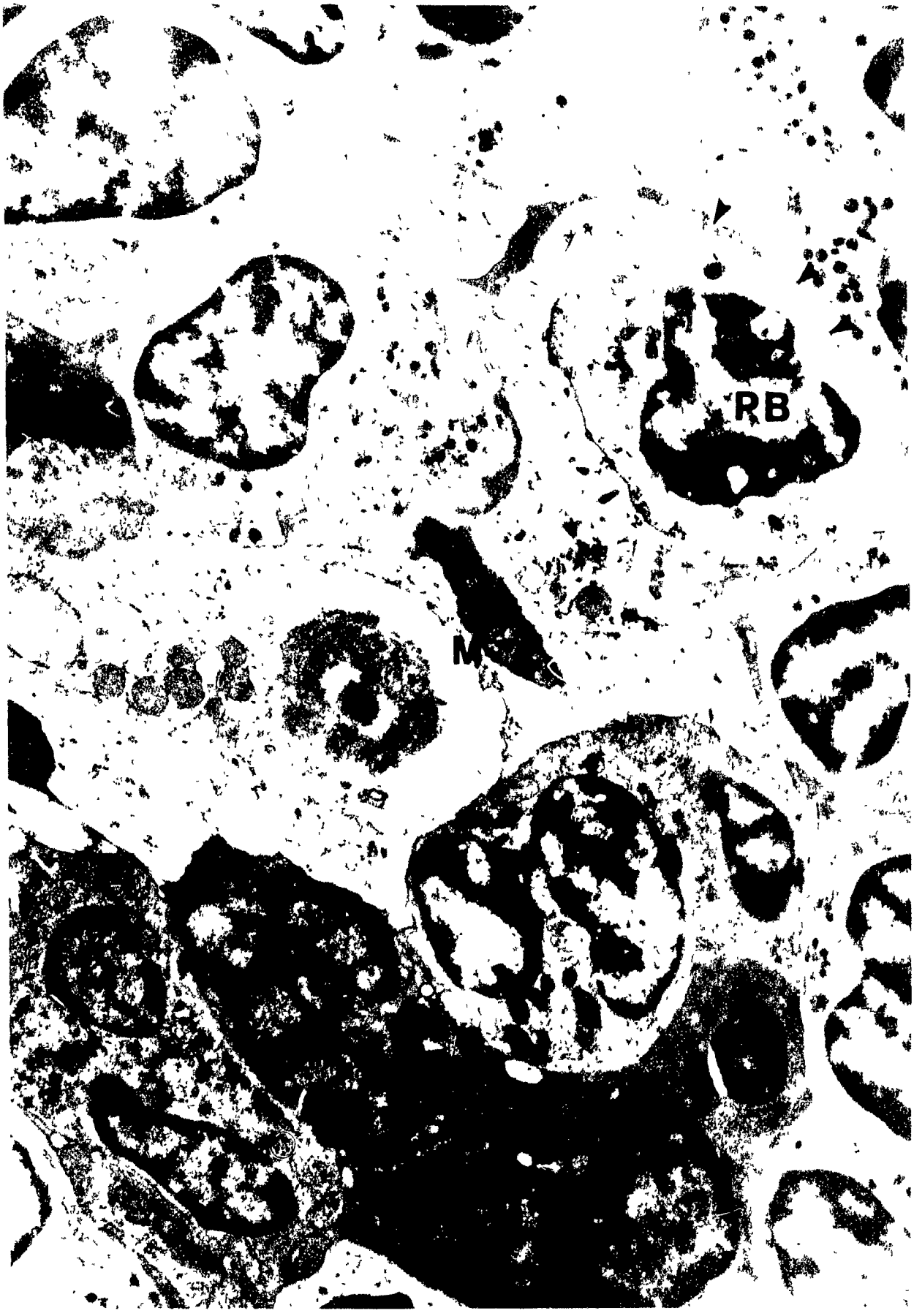

RB
M

**Figure 29.** E.M. radioautograph of a B220$^+$ early B cell precursor (**PB**) in contact with a macrophage (**M**) in the peripheral region of the femoral bone marrow of a SCID/myc mouse. The silver grains, representing the B220 glycoproteins, are present on the pro-B cell at the interface with the macrophage. Three month exposure period, 8 500x magnification.

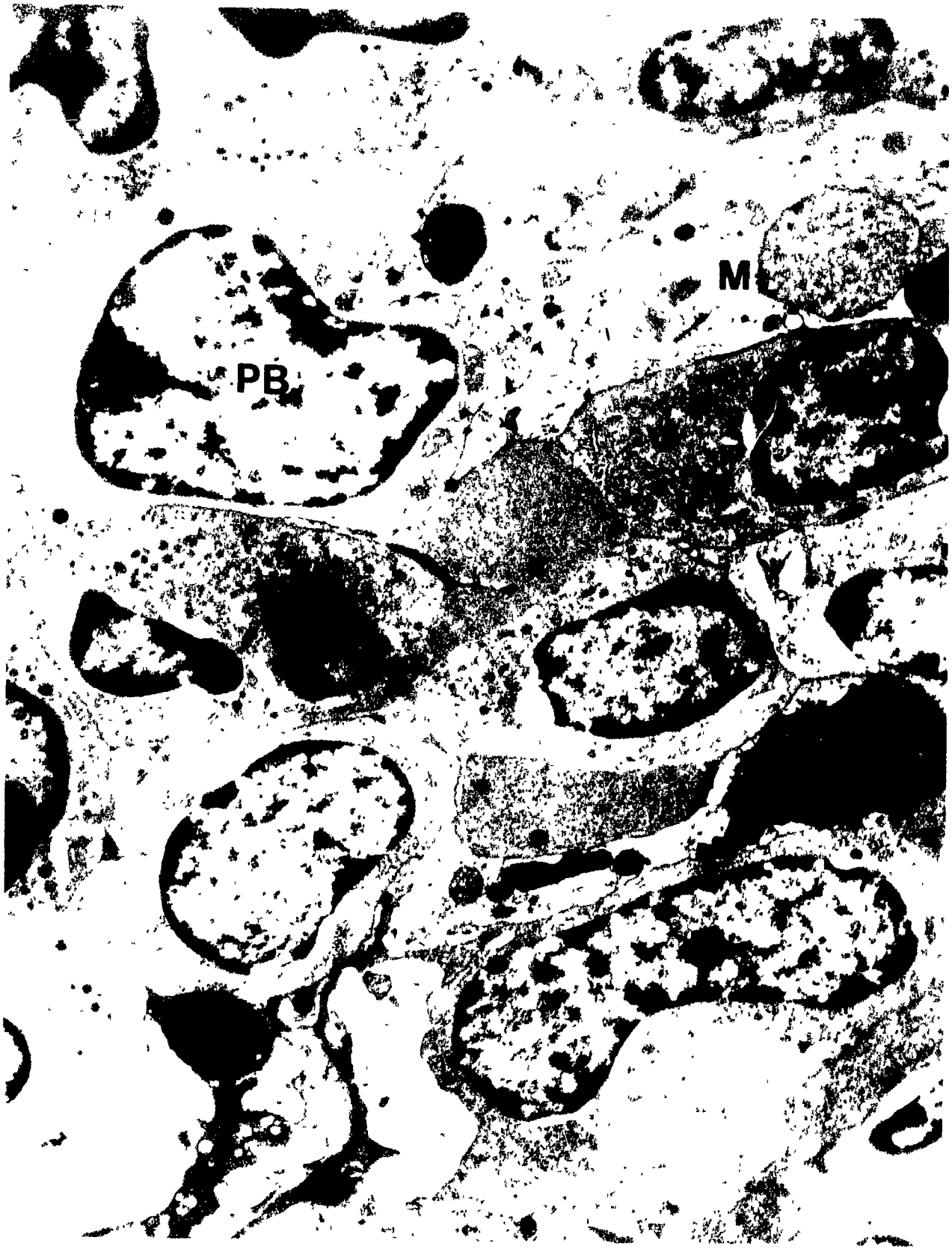

M
PB

**Figure 30.** E.M. radioautograph of a mitotic B220$^+$ early B cell precursor (**PB**) in the peripheral region of the femoral bone marrow of a SCID/myc mouse. The pro-B cell is interfacing with a macrophage (**M**) that extends away from the labelled cell (**arrows**). The silver grains are at the interface between the labelled cell and the macrophage. Three month exposure period, 8 500x magnification.

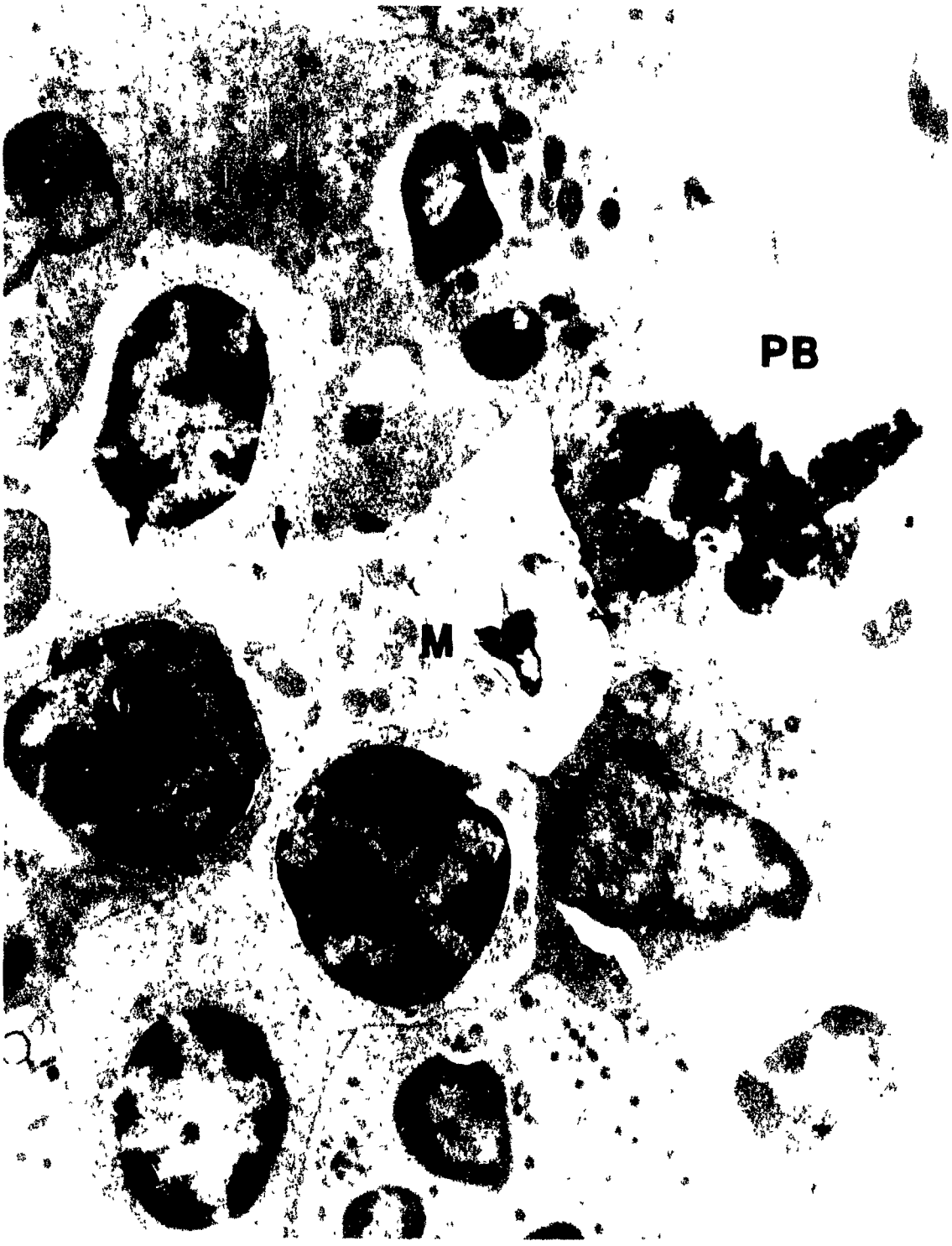
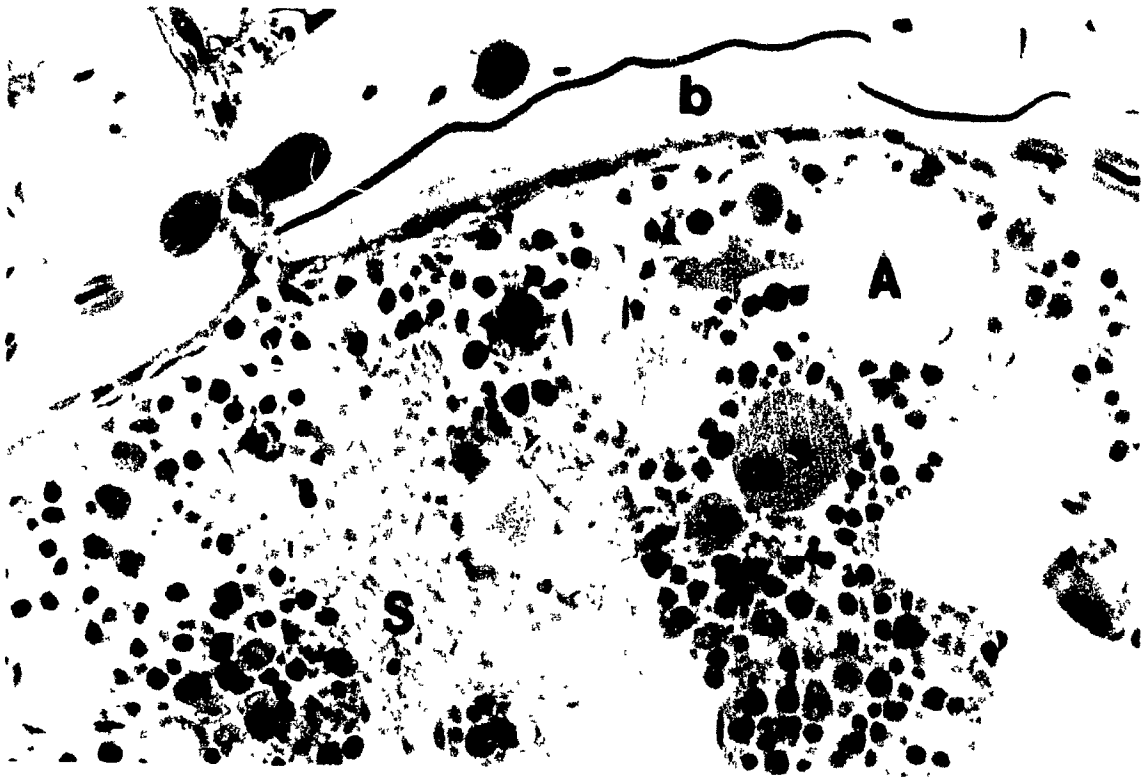

PB
M

**Figure 31.** L.M. radioautographs of day 1 (fig.a) and day 2 (fig.b) post irradiation/endocolonization of murine femoral bone marrow. Figure a) shows the decreased number of hemopoietic cells, however, some are still present in the parenchyma. Figure b) shows a parenchyma severely depleted of hemopoietic cells as compared to day 1. In both figures, adipocytes (**A**) and megakariocytes (**M**) are present, and the sinusoids (**S**) are greatly expanded due to irradiation. The cortical bone (**b**) is seen in both radioautographs Three week exposure period, a and b: 31 25x magnification

a)




b)

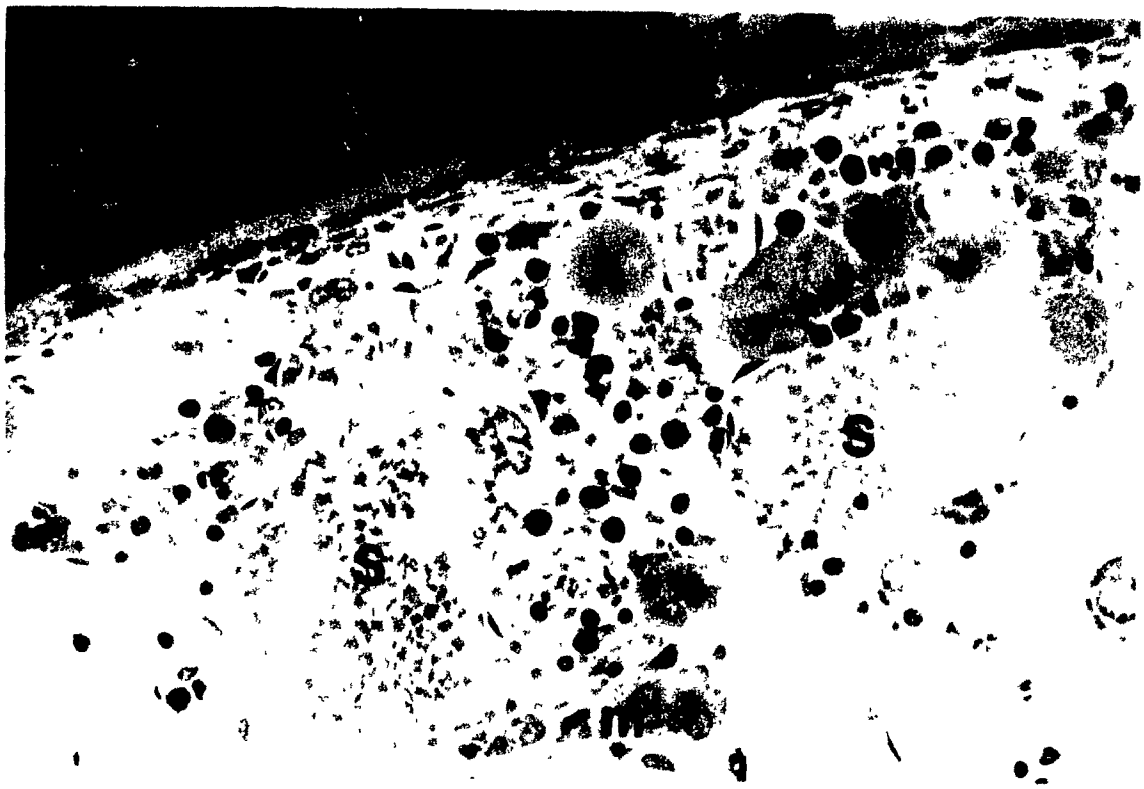


**Figure 32.** E.M radioautograph of day 1 post alternate fraction irradiation/endocolonization of murine femoral bone marrow. A cell depleted parenchyma reveals 2 large cells that seem to be of a young myelocytic (**YM**) nature. One of the cells is bordered by a macrophage (**M**) containing numerous phagocytic bodies Three month exposure period, 4 700x magnification.


M
M

**Figure 33.** E.M. radioautograph of day 1 post irradiation/endocolonization of murine femoral bone marrow. A colony of neutrophilic progenitor cells (**N**) are in intimate contact with each other as well as with a central cell that seems stromal (**St**) in nature, however difficult to define. The neutrophilic cells may be connected by junctional complexes (**arrowheads**). Three month exposure period, 7 500x magnification.


St
N

**Figure 34:** E.M. radioautograph of day 2 post irradiation/endocolonization of murine femoral bone marrow. A small neutrophilic colony (**N**) containing a band neutrophil (**NB**) Three month exposure period, 10 200x magnification.

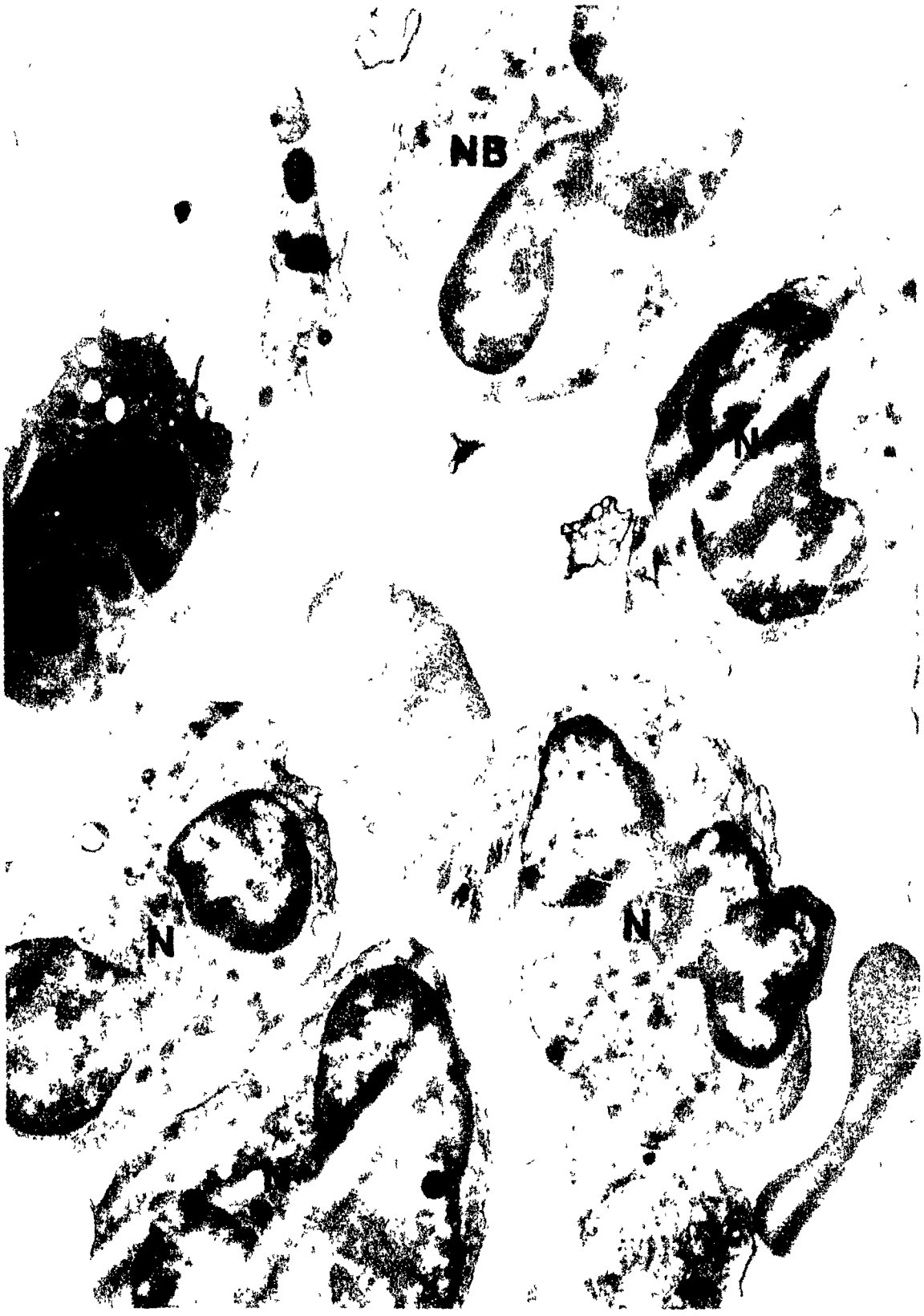

NB
N
N

**Figure 35.** E.M. radioautograph of day 2 post irradiation/endocolonization of murine femoral bone marrow showing a large megakaryocyte adjacent to the endothelium (**E**) of a sinusoid (**S**). Three month exposure period, 4 700x magnification.

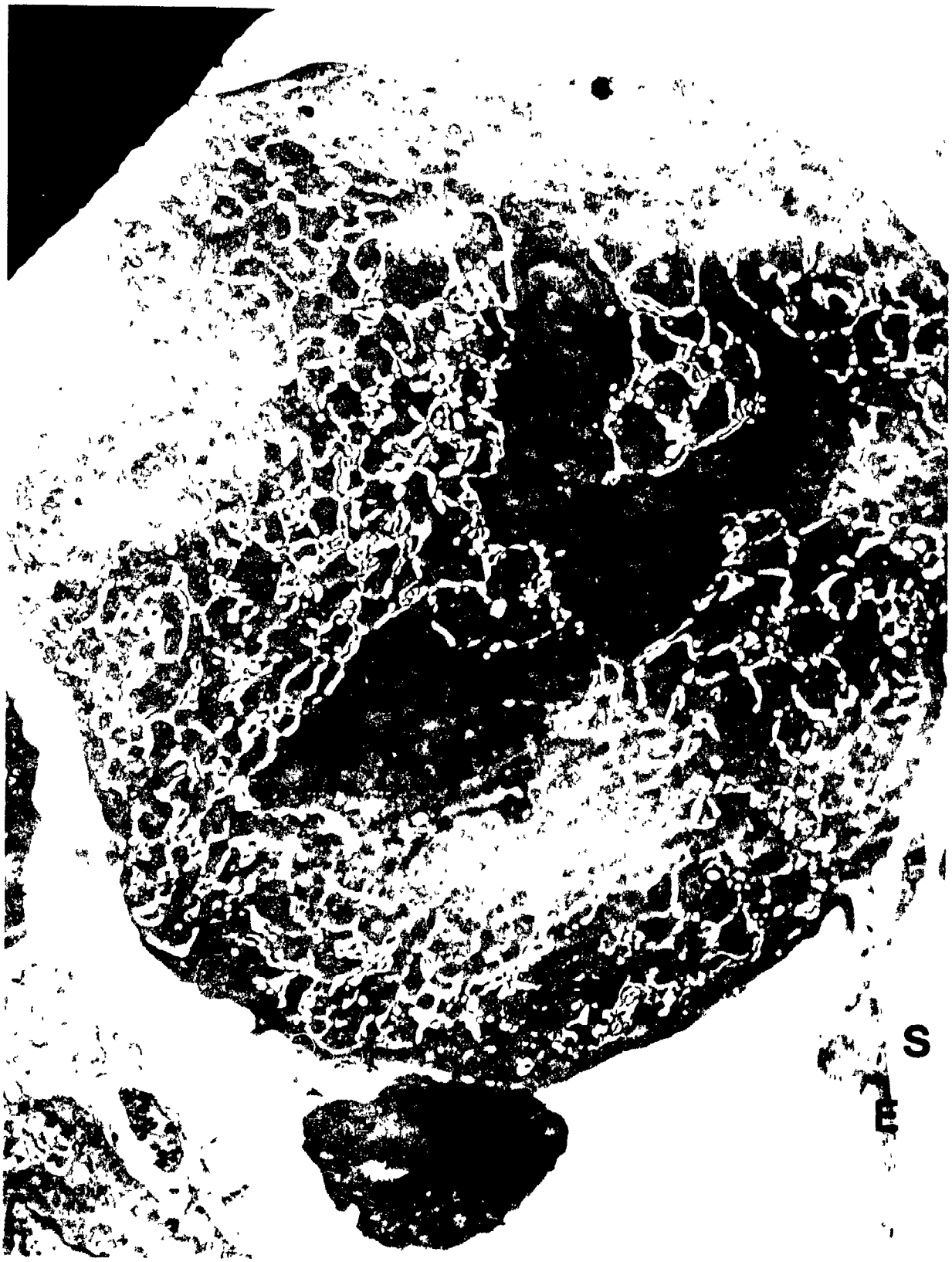
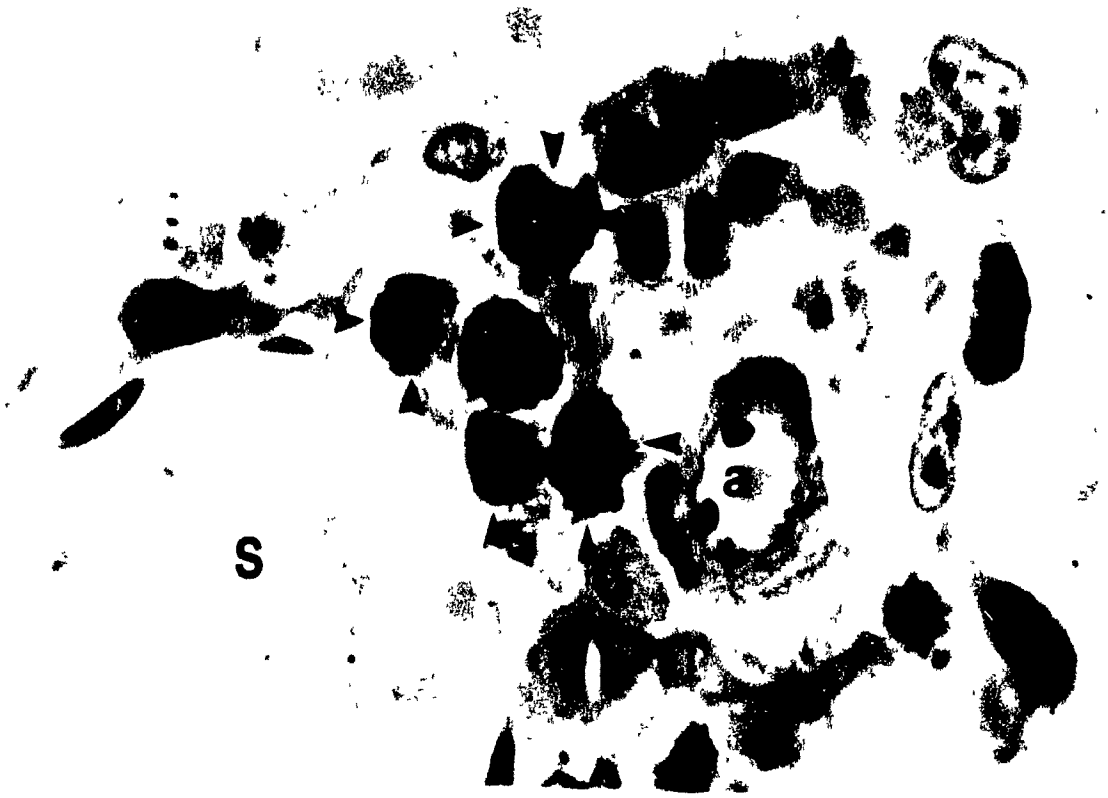

S
E

**Figure 36.** E.M. radioautograph of day 2 post irradiation/endocolonization of murine femoral bone marrow showing 2 large macrophages (**M**) scavenging for debris as revealed by the numerous phagosomes and lysosomes. Three month exposure period, 6 250x magnification.

**Figure 37.** L.M. radioautographs of day 3 post irradiation/endocolonization of murine femoral bone marrow Figure a) shows a cluster of mitotic hemopoietic cells (**arrowheads**) They are situated in a parenchymal islet, surrounded by sinusoids (**S**) in the peripheral marrow. The cluster is adjacent to an arteriole (**a**). Figure b) shows two B220$^+$ cells (**arrowheads**) within a parenchymal islet, surrounded by sinusoids (**S**) and near the cortical bone (**b**). Three week exposure period, a: 64x, and b: 125x magnification.

a)




b)

**Figure 38.** E.M. radioautograph of day 3 post irradiation/endocolonization of murine femoral bone marrow. A probable neutrophilic progenitor is shown associated with another undifferentiated hemopoietic cell (**UH**), an extensive stromal reticular cell process (**SR**)(**arrowheads**) and a macrophage (**M**) The neutrophil and its neighbouring hemopoietic cell seem to be joined by junctional complexes (**arrows**). Three month exposure period, 11 250x magnification

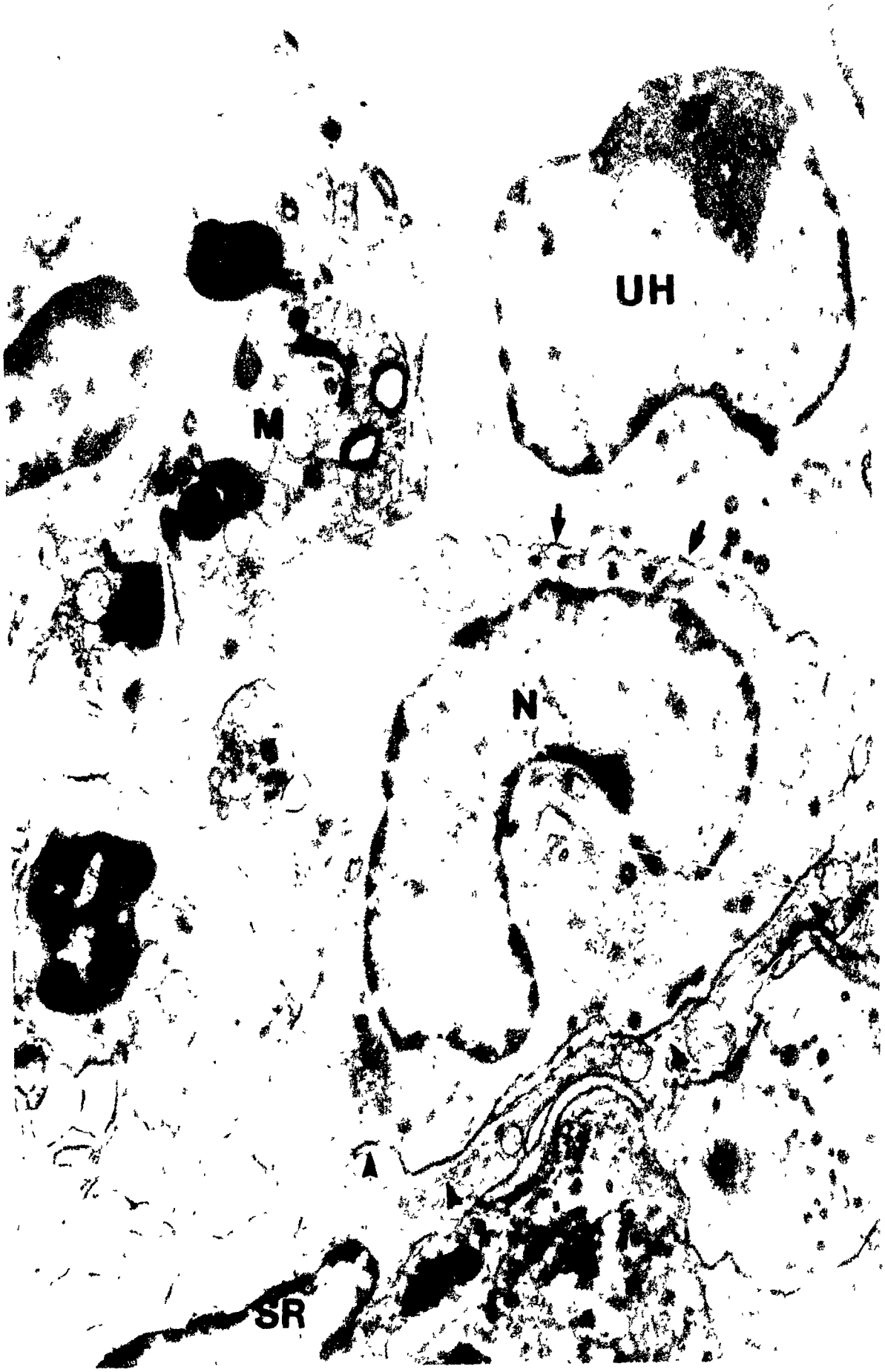
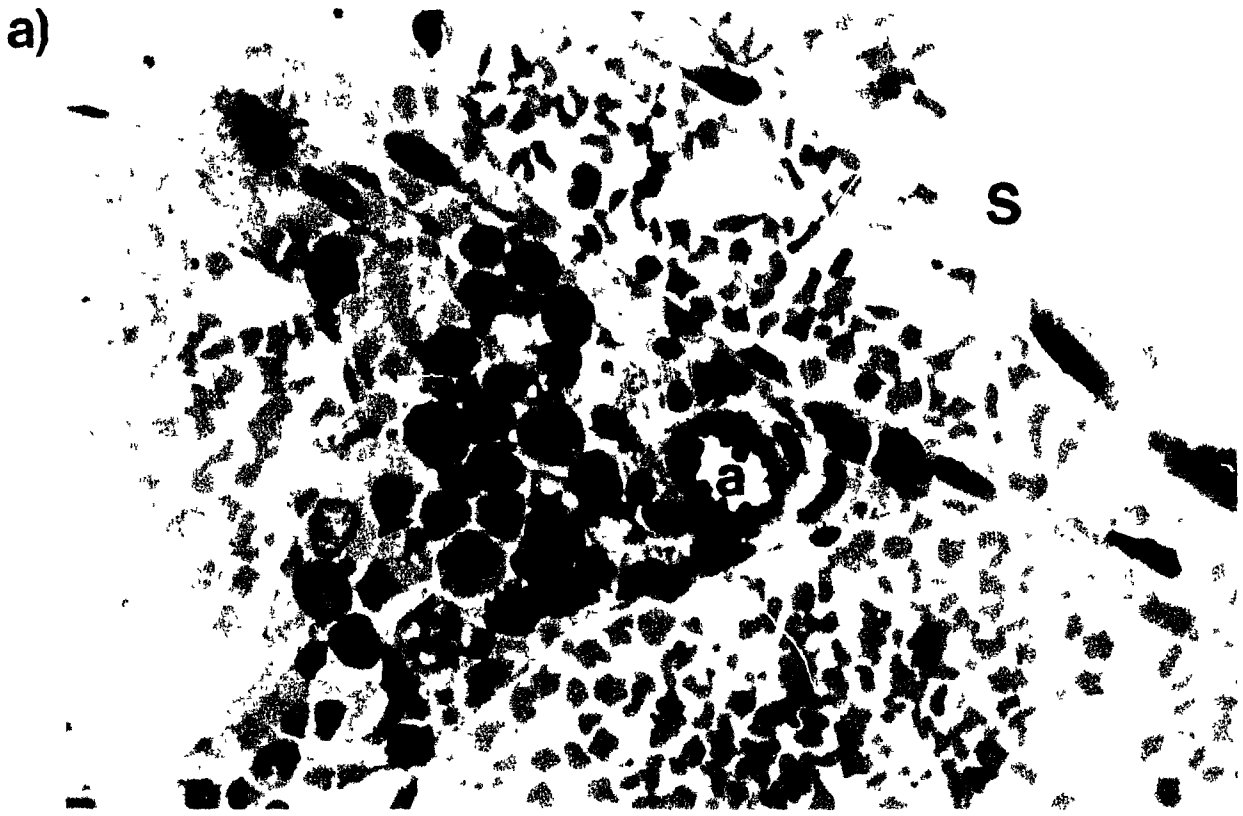

UH
M
N
SR

**Figure 39.** L.M. radioautographs of day 5 post irradiation/endocolonization of murine femoral bone marrow Figure a) shows a colony of hemopoietic cells, within which, 4 mitotic cells can be seen (**arrowheads**). The cells are close to an arteriole (**a**) and are part of a parenchymal islet in the peripheral region of the bone marrow. Figure b) shows hemopoietic colony formation along the cortical bone (**b**). The marrow sinusoids (**S**) are still distended. Three week exposure period, a: 80x, and b 50x magnification


a)
S
a

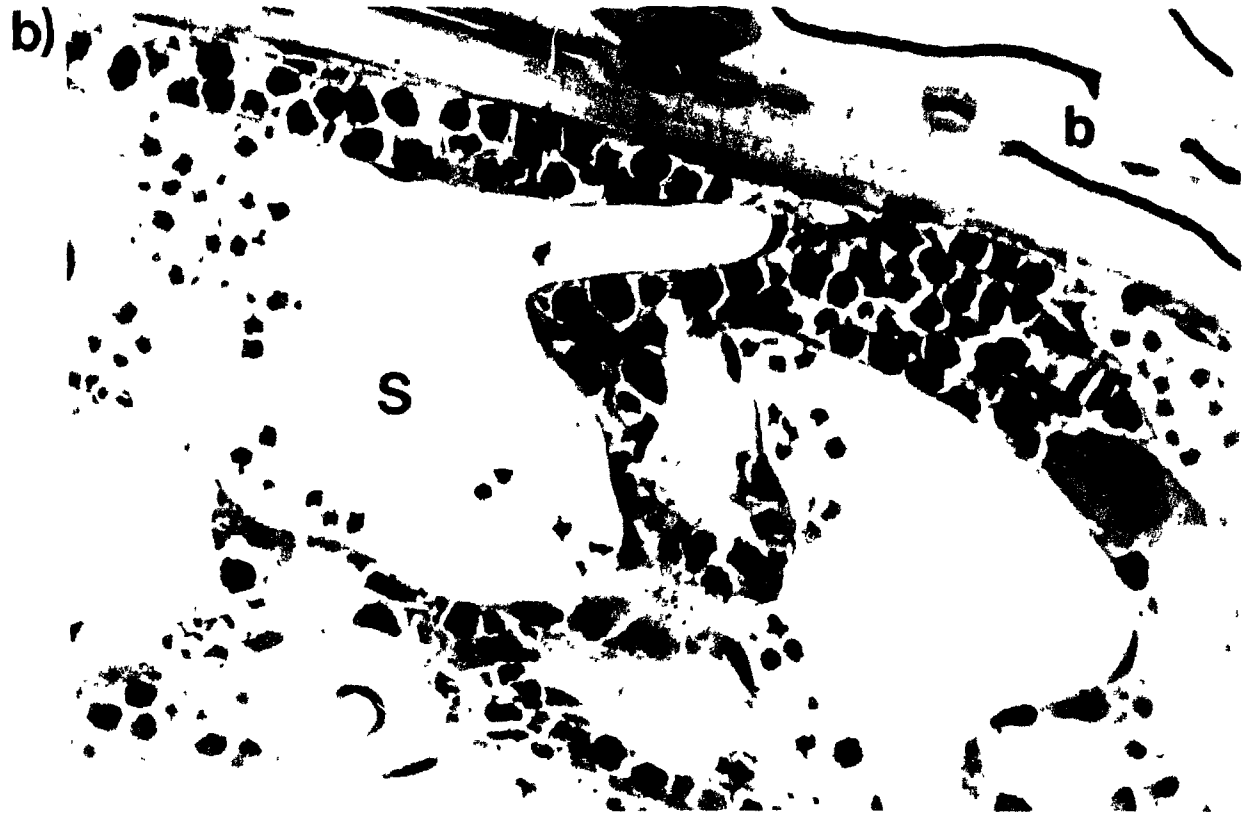
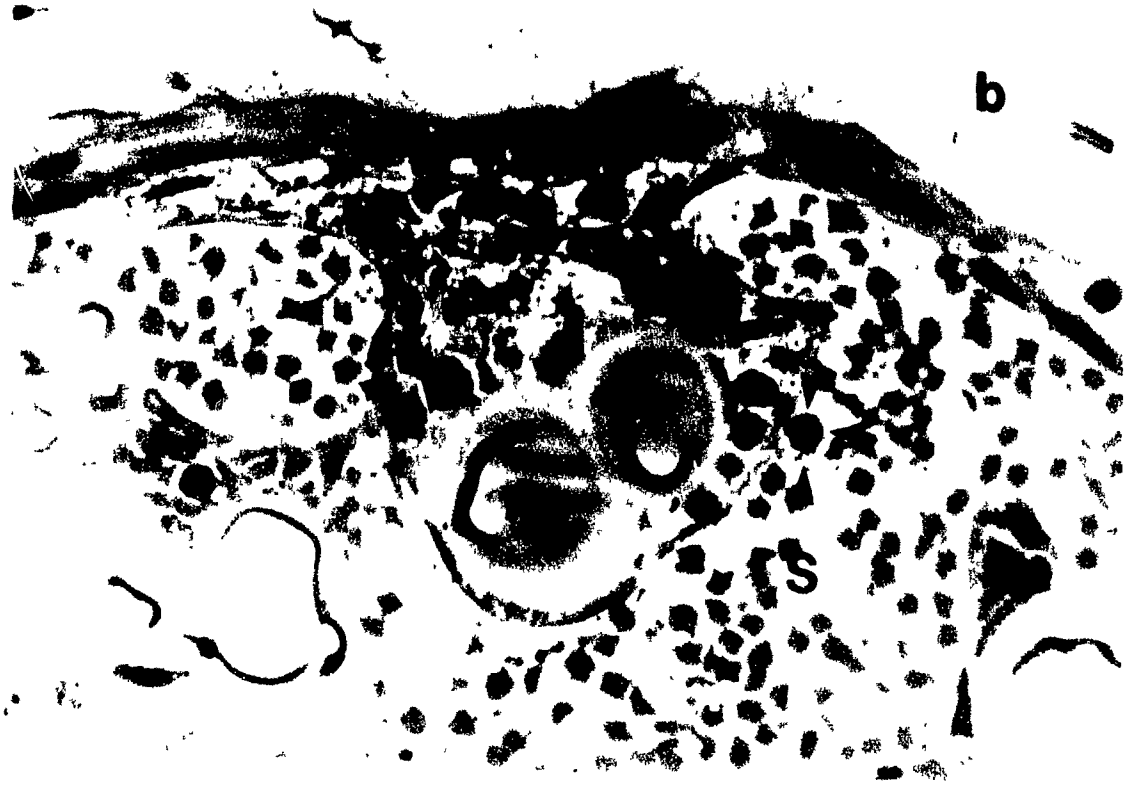

b)
b
S

**Figure 40.** L.M. radioautographs of day 5 post irradiation/endocolonization of murine femoral bone marrow. Figure a) shows 3 B220$^+$ cells. The first cell is seen within a sinusoid (**S**), adjacent to the endothelium. The second cells is within the parenchyma, adjacent to a sinusoid and the third cells is within the parenchyma, adjacent to the osteoblast layer underlying the cortical bone (**b**). Figure b) shows a B220$^+$ cell adjacent to the endothelial lining of a sinusoid (**S**) and also adjacent to an adipocyte (**A**). Adipocytes (**A**), seen in both figures, were abundant. Three week exposure period, a. 50x, and b. 64x magnification.

a)




b)

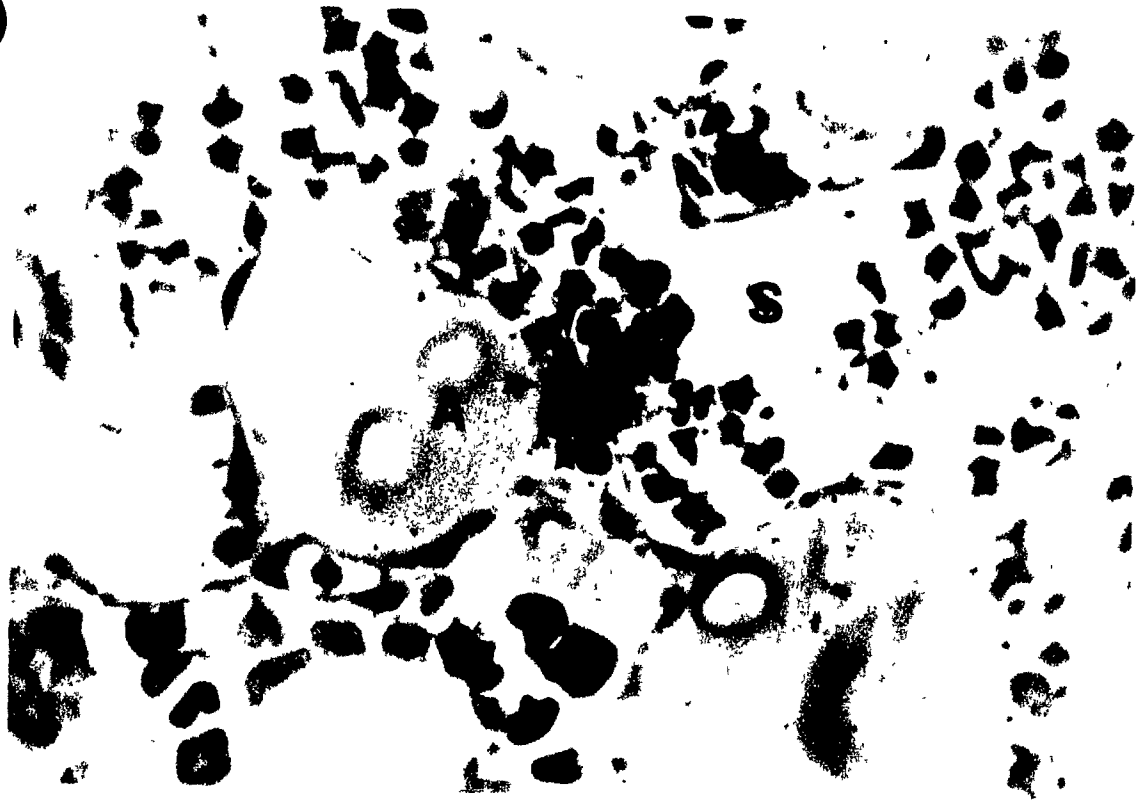
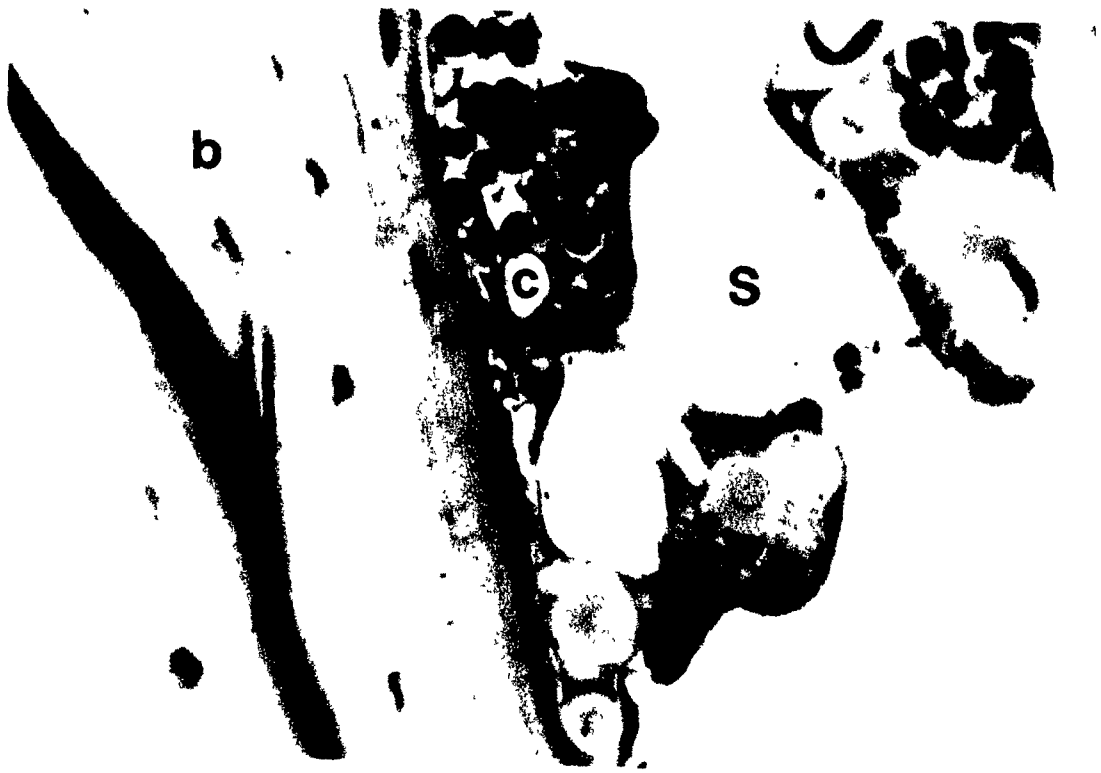


**Figure 41.** L.M. radioautographs of day 5 post irradiation/endocolonization of murine femoral bone marrow. Figure a) shows a B220$^+$ cell (**arrowheads**) adjacent to the cortical bone (**b**) as well as a small capillary (**c**). Figure b) shows 2 B220$^+$ cells (**arrowheads**), one alonside the cortical bone (**b**) and the other in a parenchymal islet, next to an arteriole (**a**) Three week exposure period, a and b: 50x magnification

a)




b)

**Figure 42**. E.M. radioautograph of a B220$^+$ early B cell precursor (**PB**) at day 5 post irradiation/endocolonization in murine bone marrow. The labelled cell is located in the peripheral region of the femoral bone marrow. The cell is of typical pro-B cell morphology; it is in contact with many thin strands of stromal reticular cell processes (**arrows**). At one end, the pro-B cell is in contact with an endothelial cell (**E**) lining a sinusoid (**S**) and the other end is in close proximity with a nerve fiber containing unmyelinated axons (**N**) Three month exposure period, 22 800x magnification.


N
PB
E
S

**Figure 43.** E.M. radioautograph of a lightly labelled B220$^+$ early B cell precursor (**PB**) at day 5 post irradiation/endocolonization in murine femoral bone marrow The positive cell has characteristics of early B cell precursor morphology and is situated in the peripheral region of the marrow, where hemopoietic regeneration is mostly occuring. The pro-B cell is in contact with thin processes of stromal reticular cells (**arrowheads**) and seems to be shaping its cytoplasm around one end of a nerve fiber (**N**) containing unmyelinated axons. Three month exposure period, 10 000x magnification


PB

**Figure 44.** E.M. radioautograph of a B220$^+$ early B cell precursor (PB) at day 5 post irradiation/endocolonization in murine femoral bone marrow. The cell has an elongated shape suggesting motility. The cell is in contact with many strands of stromal reticular cell processes (**arrowheads**). The B cell precursor is situated within a thin strip of parenchyma, adjacent to a sinusoid (**S**) on either side. Three month exposure period, 11 500x magnification.


PB
S

**Figure 45.** E.M. radioautograph of a B220$^+$ early B cell precursor at day 5 post irradiation/endocolonization in murine bone marrow. The B cell precursor is in contact with a macrophage (**M**), stromal reticular cell processes (**SRP**, **arrowheads**) and three circular structures that may be cross sections of nerve fibers (**arrows**). Three month exposure period, 22 800x magnification.


SRP
PB

**Figure 46.** E.M. radioautograph of a B220$^+$ mature B cell (**B**) at day 5 post irradiation/endocolonization in murine bone marrow. The B cell is within a capillary (**C**) lined by an endothelial cell (**E**) which is adjacent to the cortical bone. Three month exposure period, 13 500x magnification.

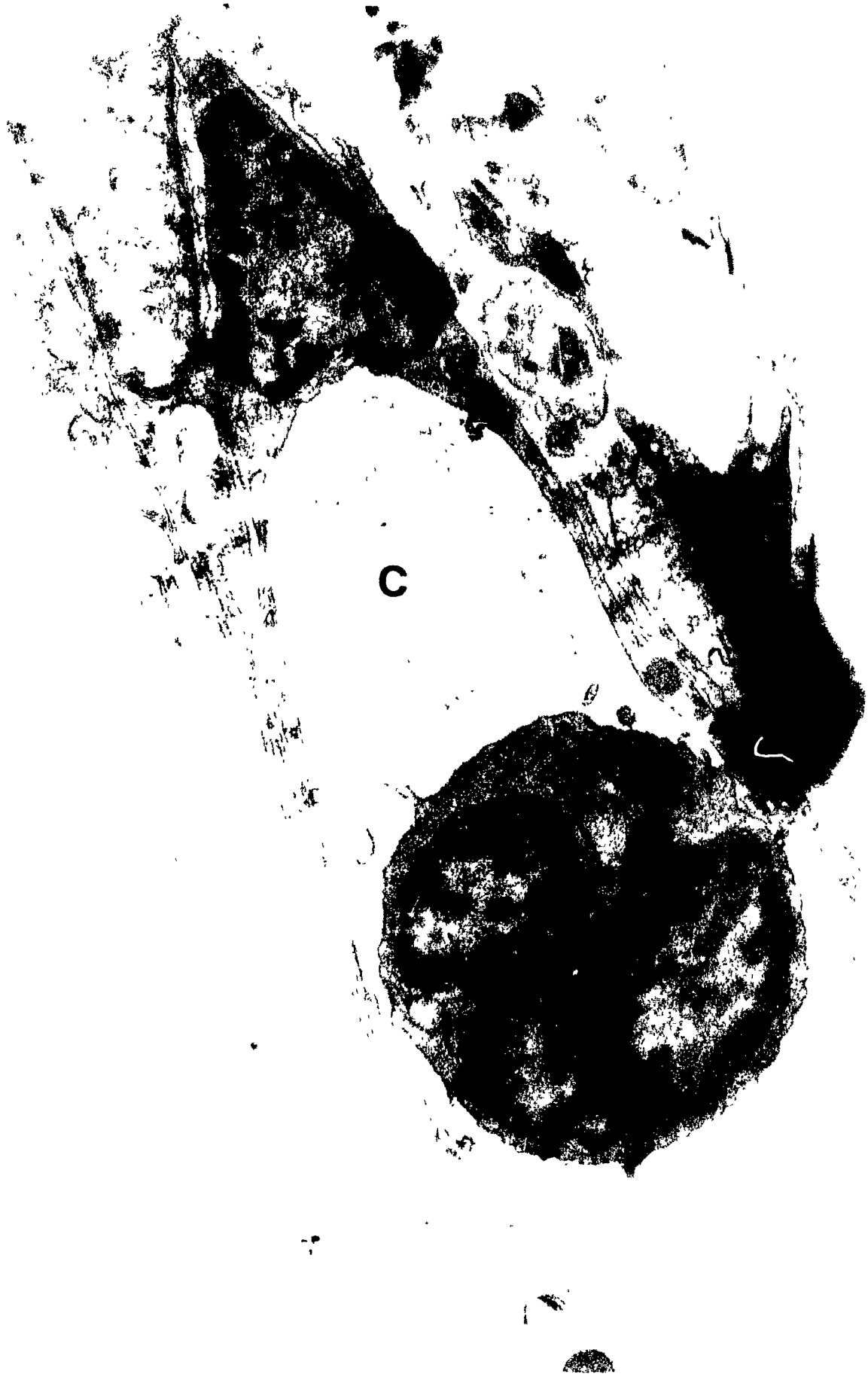

C

**Figure 47.** E.M. radioautograph of a B220$^+$ mature B cell (**B**) at day 5 post irradiation/endocolonization in murine bone marrow. The B cell is surrounded by a thin layer of cytoplasm typical of stromal reticular processes (**arrowheads**) The B cell-stromal cell process complex is in turn enveloped by the cytoplasm of an adjacent cell: this cell is possibly a barrier cell (**Ba**) The B cell is in close proximity to a sinusoid (**S**) as well as a nerve bundle (**N**). Three month exposure period, 8 500x magnification


S
Ba
N