ASSOCIATION OF ALKALINE-PHOSPHATASE-POSITIVE RETICULUM CELLS IN BONE MARROW WITH GRANULOCYTIC PRECURSORS*

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In bone marrow, an intact system of vascular sinuses and fibroblastic stroma has been shown to be the structural basis necessary for hemopoietic activity under physiologic (1, 2) and pathologic (3) conditions. The predominant cells of this hemopoietic microenvironment are believed to be reticulum cells which expand a three-dimensional network (reticulum) of cytoplasmic extensions throughout the marrow parenchyma (4). It is generally agreed, however, that the term reticulum (reticular) cell might include several cell types that have not yet been precisely characterized (5). So far, two main types of reticulum cells have been distinguished in the rodent marrow by electron microscopy: a nonphagocytic reticulum cell which is believed to be mainly fibroblastic (4, 6–8) and a phagocytic reticulum cell or macrophage, which is known to be the center of erythropoietic islets (9). Whether either cell type is a constituent of the sinus system is still a matter of controversy (4, 10).

There have been relatively few reports of studies combining electron microscopy and cytochemistry for the characterization of these two stromal cells. Initial findings indicate, however, that abundant acid phosphatase (Ac-Pase)[1] activity is localized in intraparenchymal bone marrow macrophages (11–12) and in certain endothelial cells of the vascular sinuses (13). No comparable fine structural data have been published for alkaline phosphatase (Alk-Pase), although its activity has been observed on smears of bone marrow stromal cells by several investigators (14–16).

To further define the cells in the reticular network, we obtained core biopsy samples of intact bone marrow which were fixed by immersion without disturbing the basic organization of the tissue. Some biopsy specimens were embedded in plastic, 1- to 3-micron sections were cut, and enzymatic incubation for Alk-Pase and Ac-Pase were conducted on the sections; they were subsequently examined by light microscopy. Other specimens were fixed, incubated, processed, and examined by electron microscopy. By this approach, we provide evidence that two types of stromal cells can be differentiated in the bone marrow of small rodents: (*a*) a fibroblast-type of reticulum cell which is characterized by its membrane-bound Alk-Pase associated mainly with

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[1] *Abbreviations used in this paper:* Ac-Pase, acid phosphatase; Alk-Pase, alkaline phosphatase; Al-RC, alkaline-phosphatase-positive reticulum cells; CSA, colony stimulating activity. ER, endoplasmic reticulum.

granulocytic precursors. (*b*) a macrophage type of reticulum cell which is characterized by its abundance of lysosomal Ac-Pase associated mainly with erythroid precursors.

Materials and Methods

Animals. A total of 85 mice (BALB/c and NRBI) and 56 rats (Sprague-Dawley and Wistar) of both sexes up to the age of 2 yr was studied. Out of these, 24 mice (BALB/c) and 17 rats (Sprague-Dawley) of various sexes and ages were used for electron microscopic examinations; the other animals were analyzed by light microscopic techniques.

Biopsy of Bone Marrow. The animals were killed by ether inhalation. Femoral bone marrow cores, 1 mm in diameter (mice) or 2 mm in diameter (rats), were punched out of the femoral cavity with small glass tubes and blown into the appropriate fixative within seconds. Other small pieces of marrow were squashed between two coverslips, and air-dried. To further elucidate the relationship between marrow parenchyma and bone structure, the entire femurs of 10 male and 10 female rats (Wistar) up to 18 mo old, and of 4 mice (NRBI) of both sexes were split longitudinally and were placed into the appropriate fixative en bloc.

Histochemistry of Tissues Examined by Light Microscopy. Core samples of bone marrow were fixed at 4°C for 2 h in 4% paraformaldehyde in phosphate buffer. Specimens were then dehydrated within 2 h in ethyl alcohol at 4°C, embedded in glycol methacrylate at 4°C, according to Rudell (17) (JB-4 plastic embedding medium from Polysciences, Inc., Warrington, Pa.), and sections (1- to 3-micron sections) were cut.

The split femurs of the rats were fixed at 4°C for 2 h in a mixture of equal parts of 2-hydroxyethyl methacrylate and methyl alcohol. After dehyration in 2-hydroxyethyl methacrylate, the specimens were impregnated with methyl methacrylate, and then they were postimpregnated in glycol methacrylate and embedded as above at 4°C. Undecalcified 2- to 3-micron sections were cut on a motor-driven microtome (1140/Autocut, Reichert-Jung, 6901 Nußloch, West Germany).

The method of Kaplow (14) was used, with Naphthol AS phosphate and fast blue BBN, to demonstrate the presence of Alk-Pase activity. All substrates were obtained from Sigma Chemical Co., St. Louis, Mo., unless stated otherwise. Ac-Pase activity was demonstrated by the method of Burstone (18) using Naphthol AS-BI phosphoric acid and fast garnet GBC salt. In several instances, sections were sequentially incubated, first to assay alkaline, and then acid phosphatase, with 1% methyl green as a counterstain. In addition, we simultaneously processed smears or cryostat sections of unfixed or formaldehyde-fixed tissue. Inhibition studies of Alk-Pase were carried out by adding 0.2 mg L-*p*-bromotetramisole to 10 ml of incubation medium (19). (This material was kindly provided by Dr. M. Borgers, Janssen Pharmacuetical Inc., Beerse, Belgium).

Cytochemistry of Tissues Examined by Electron Microscopy. To demonstrate Alk-Pase activity, cores of bone marrow were fixed for 20 min in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4°C. Samples were subsequently cut into 40-micron slices with the Smith-Farquhar tissue chopper (Ivan Sorvall, Inc., Norwalk, Conn.), rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) with 7% sucrose, incubated in Millonig's medium (20) with the addition of 20% glycerol for 60 min at 37°C, rinsed, and again fixed for 2 h in 1.5% glutaraldehyde. These tissue specimens were then postfixed in 1% OsO_4 in 0.05 M acetate-veronal buffer with 5% sucrose for 1 h at 4°C, and stained en bloc for 60 min at 22°C in 0.5% uranyl acetate in Michaelis buffer, pH 6.0. All tissues were dehydrated in ethanol and embedded in araldite. Grids were stained with alkaline lead citrate.

As a control for cross-reactivity with other phosphatases, the specific inhibitor for Alk-Pase, L-*p*-bromotetramisole (19), was added to the incubation mixture.

In the assays for Ac-Pase activity, the best results were obtained with a fixation time of 2 h in 1.5% glutaraldehyde at 4°C, as described above, and incubation at 37°C for 10 min (rats) or 20 min (mice) in Barka and Anderson's medium (21) at pH 5.0. To provide a control for nonspecific background staining, incubation for Ac-Pase was performed in the absence of substrate.

Results

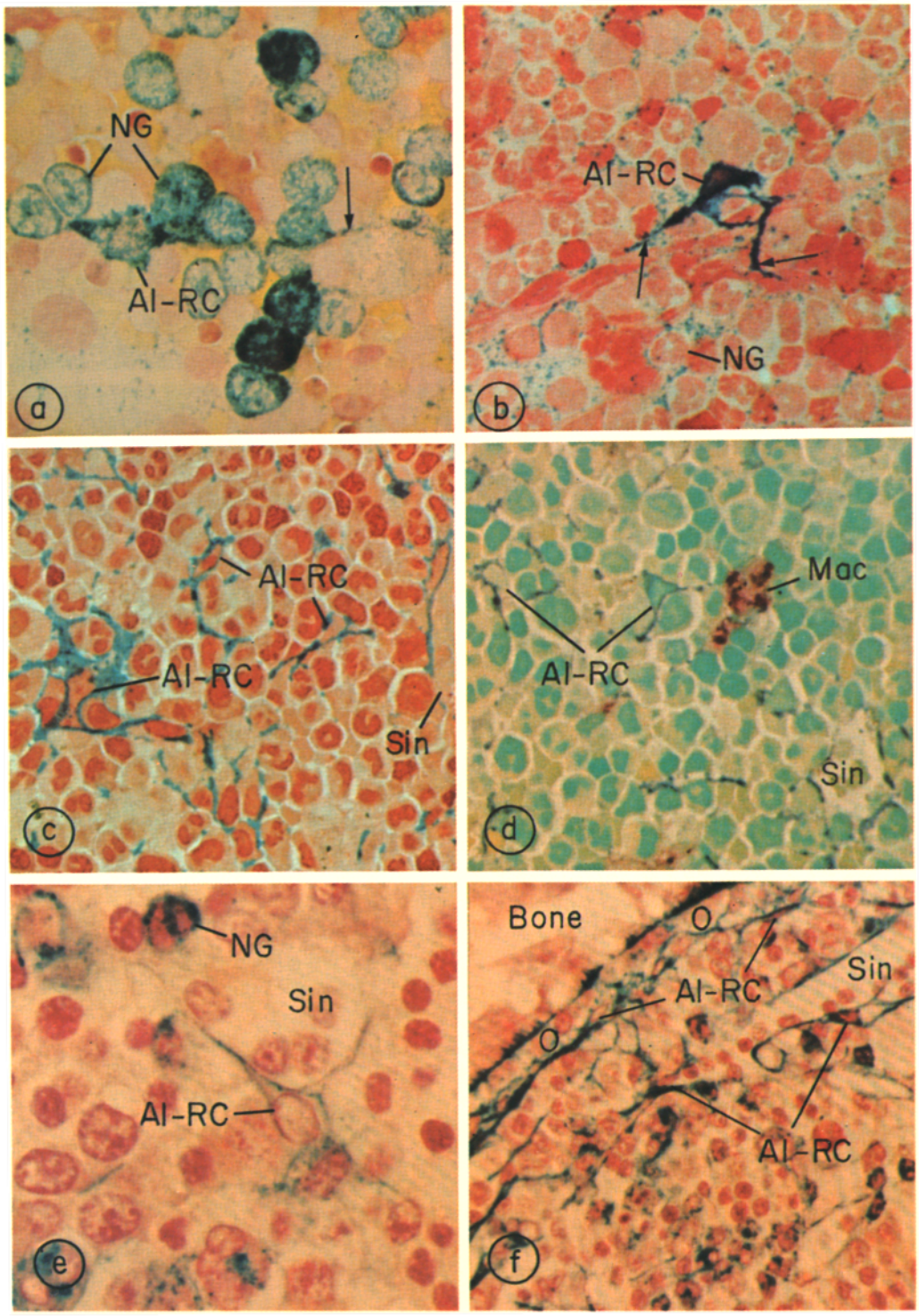
Light Microscopy. As mentioned in the Introduction, the presence of Alk-Pase in

certain stromal elements can be observed in smear preparations of bone marrow both in rats (Fig. 1 a) and mice (Fig. 1 b). However, because smears show a gross distortion of cellular architecture of the marrow, the cells that exhibited Alk-Pase activity could not be identified with certainty. Examinations of sections, however, after incubation for enzymatic activity, revealed that besides small arterioles (22), two types of stromal cells contained high concentrations of Alk-Pase: certain intraparenchymal reticulum cells (Figs. 1 c, d, e, and 2) and certain cells of the sinus wall (Figs. 1 c, d, and f). Furthermore, using a sequential double assay for both enzymes, we found that the stromal cells that stained positively for Alk-Pase were low, or lacking in Ac-Pase, and that another type of nonhemopoietic cell, probably the bone-marrow macrophage, contained large amounts of Ac-Pase but no detectable Alk-Pase (Fig. 1 d).

In the mouse, these alkaline-phosphatase-positive reticulum cells (AL-RC) were characterized by their long cytoplasmic extensions, which radiated haphazardly into the hemopoietic parenchyma. Often the bone marrow was interspersed with a fine network of the blue reactive cell processes, although the small and ovoid nuclei of these cells were mostly difficult to identify (Figs. 1 c, and d). Furthermore, parts of the sinus walls were clearly outlined by reaction product as well, sometimes over considerable distances (Figs. 1 c, d, and e). Rarely, an elongated nucleus of the corresponding cell became visible (Figs. 1 c, and d). It was not clear, however, whether these cells were a real constituent of the sinus wall itself or merely adjacent to it.

Although AL-RC were consistently found in both mice and rats, certain variations were observed with respect to strength of reactivity and overall distribution pattern in the marrow: the reactive reticular network often seemed to be generally sparser in the axial marrow region than in the marrow periphery where it usually tended to outline the sinuses. In addition, a few core biopsies of mice showed hardly any reactivity in the marrow stroma except for the small arterioles (not illustrated) which were always reactive. An intense Alk-Pase deposition, however, was usually observed at the periphery of the core biopsies, and we initially interpreted this phenomenon as a crush artifact. To clarify the nature of this activity, we made a study of the enzymatic reaction in areas close to the bony structures by embedding entire rat and mouse femurs, without prior decalcification, and then analyzing the cut sections as previously described. Our results indicated that reticulum cells that stain for Alk-Pase are consistently present in large numbers near the endosteum in both mice and rats (Fig. 1 f). In contrast, stromal cell elements heavily loaded with reaction products of Ac-Pase (macrophages), were found to be more or less evenly distributed throughout the marrow (Fig. 1 d).

Sections of rat marrow were more difficult to interpret because of the abundant Alk-Pase reaction product in the differentiating neutrophils. Occasionally, AL-RC sectioned through their nuclei were seen (Fig. 1 e). Sometimes reactive cell processes seemed to outline individual fat droplets or to directly border upon megakaryocytes (not illustrated). An abundant layer of AL-RC was always present, however, near the bony tissue (Fig. 1 f). From this point, the AL-RC extended towards the marrow parenchyma, often outlining the sinuses for considerable distances. Adjacent to the endosteum, the Alk-Pase activity of osteoblasts was evident. Note that enzyme reactivity of the AL-RC was also apparent in areas where the AL-RC were separated from the osteoblastic rim by a marginal sinus. Furthermore, abundant AL-RC were usually present in the stromal-cell layer which surrounds the large central sinus of the rat marrow (not illustrated). In the epiphysial and metaphysial region of the rat


NG
Al-RC
a
Al-RC
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b
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femur, a tight network of AL-RC which extended between the spongework of the trabecula, was consistently observed partially outlining the sinuses (not illustrated).

Electron Microscopy

ALKALINE PHOSPHATASE. (*a*) Nonvascular structures.[2] Alkaline phosphatase reaction product in nonvascular stromal cells of both species was found to be restricted to stellate nonhematopoietic cells (Figs. 2 and 3), whose major morphological characteristic consisted of long, thin cytoplasmic extensions that radiated haphazardly into the adjacent cell population. We refer to this cell type as the Al-RC. Enzyme activity was situated on the outer surface of the plasma membrane, but only portions of the membrane were reactive. There was also evidence for the presence of Alk-Pase in occasional vesicles of these cells. The cytoplasmic extensions were especially reactive in areas of intimate contact with the various types of neighboring cells (Fig. 3); these consisted mainly of granulocytic precursors. Occasionally, contact with megakaryocytes was observed, but hardly ever with erythroid precursors. Except for the far-reaching cellular extensions which frequently reached the outer wall of the sinuses (Fig. 2), the morphological aspects of these Al-RC were inconspicuous. When the nucleus was in the plane of the section, it was usually associated with a narrow rim of cytoplasm, and showed a marked condensation of heterochromatin near the nuclear envelope. Nucleoli were seldom present. Except for the one to three stacks of Golgi cisternae, generally only a scant number of organelles was found in the cytoplasm,

[2] In small bone marrow arterioles and capillaries, localized Alk-Pase activity was detected in pinocytotic vesicles, the intravascular lumina, and portions of the basement membrane, as well as between adjacent endothelial cells. These data have been published in abstract form (22), and will appear in a separate paper.

FIG. 1. Light micrographs of rat and mouse bone marrow preparations reacted for Alk-Pase (blue reaction product) (a–c, and f) or both Alk-Pase and Ac-Pase (reddish reaction product) (d). (a) Squash preparation of rat bone marrow reacted for Alk-Pase. Individual stromal-cell elements (Al-RC) with long blue cytoplasmic extensions (arrow) are heavily reactive for Alk-Pase, as are the granulocytic precursors (NG), which often are associated with Al-RC. The tissue was fixed in paraformaldehyde-acetone, incubated with naphthol-AS phosphate and fast blue BBN (pH 9.2), and counterstained with neutral red. × 650. (b) Squash preparation of mouse bone marrow reacted for Alk-Pase. Reaction product is heavily concentrated on individual stromal cells (Al-RC) with long cell processes (arrows). In contrast to the rat, the granulocytic precursors (NG) are not reactive in this species. A light overall distribution of reaction product (granular blue) appears between the hemopoietic cells. Preparation as in (a). × 500. (c) Bone marrow section (1 micron) of a mouse reacted for Alk-Pase. Several reticulum cells (Al-RC) of the axial marrow region show enzyme activity in the hemopoietic parenchyma and along the sinus (Sin) walls. The tissue was fixed in buffered formalin, embedded in glycol methacrylate, and incubated as in (a). (d) Bone marrow section (1 micron) of a mouse consecutively reacted for Alk-Pase and Ac-Pase. The reddish-brown reaction product of Ac-Pase marking the fixed macrophages (Mac) has a different distribution pattern than that of the blue deposits which mark the Alk-Pase-positive reticulum cells (Al-RC) and their cytoplasmic processes in the hemopoietic parenchyma and at the sinus (Sin) walls. Tissue treated as in (c), and then incubated in naphthol AS-BI phosphoric acid and fast garnet GBC (pH 5.0), counterstained with methyl green. × 500. (e) Section (2 microns) of rat bone marrow reacted for Alk-Pase. Star-like reticulum cell (Al-RC) with reaction product on its cell membranes, located in the axial marrow parenchyma, and bordering upon a sinus (Sin). Neutrophilic granulocytes, (NG). The tissue was fixed in methanol-hydroxyethyl methacrylate and embedded in glycol/methyl methacrylate without previous decalcification. Incubation as in (a). × 1,050. (f) Section (2 microns) of rat bone marrow reacted for Alk-Pase. Alk-Pase-positive reticulum cells (Al-RC) are abundantly present in areas near bony structures (Bone), and partially outlining sinuses (Sin). They form a stromal cell layer that is adjacent to osteoblasts (O) which are also reactive at the bony rim. Preparation as in (e). × 420.

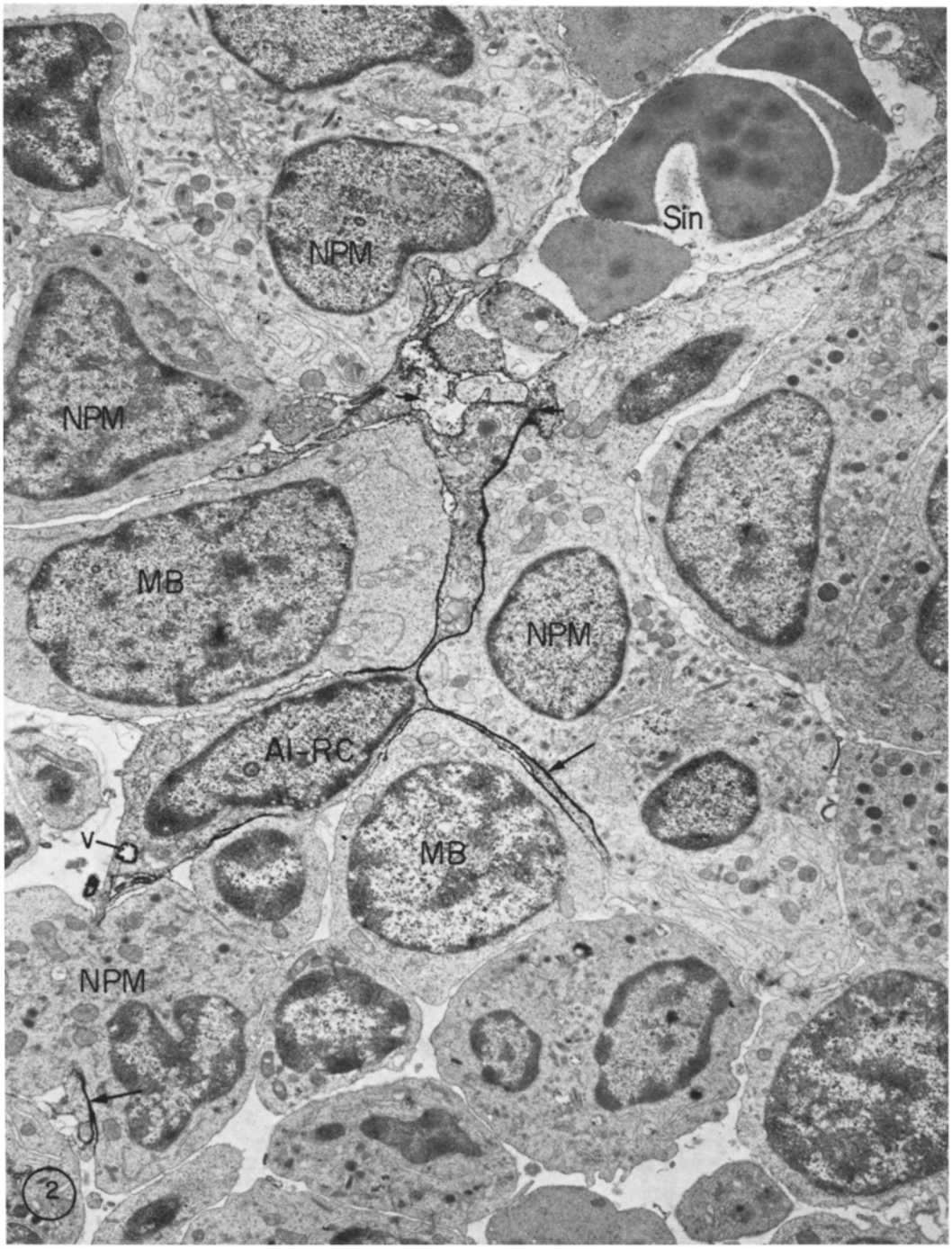

FIG. 2. Low-magnification electron micrograph of rat bone marrow reacted for Alk-Pase. Reaction product (large arrows) is visible as a black precipitate on portions of the plasma membrane of a fibroblast-like reticulum cell (Al-RC), and is particularly prominent on the elongated cytoplasmic extensions. The center of the cell is located intraparenchymally but parts of the cell processes reach the outer wall of a sinus (Sin), where some intra- and extra-cytoplasmic (small arrows) leakage of enzyme has occurred. Typically, reaction product is also present in individual cytoplasmic vesicles (v). Cells of the neutrophilic series almost completely surround the reticulum cell. Some are obvious promyelocytes (NPM) and myelocytes, whereas others are blast-like cells, or presumably myeloblasts (MB). The tissue was fixed for 10 min in 1% glutaraldehyde, incubated for 60 min in Millonig's medium, and subsequently postfixed for 2 h in 1.5% glutaraldehyde and for 1 h in OsO_4, then embedded in araldite. × 8,000.

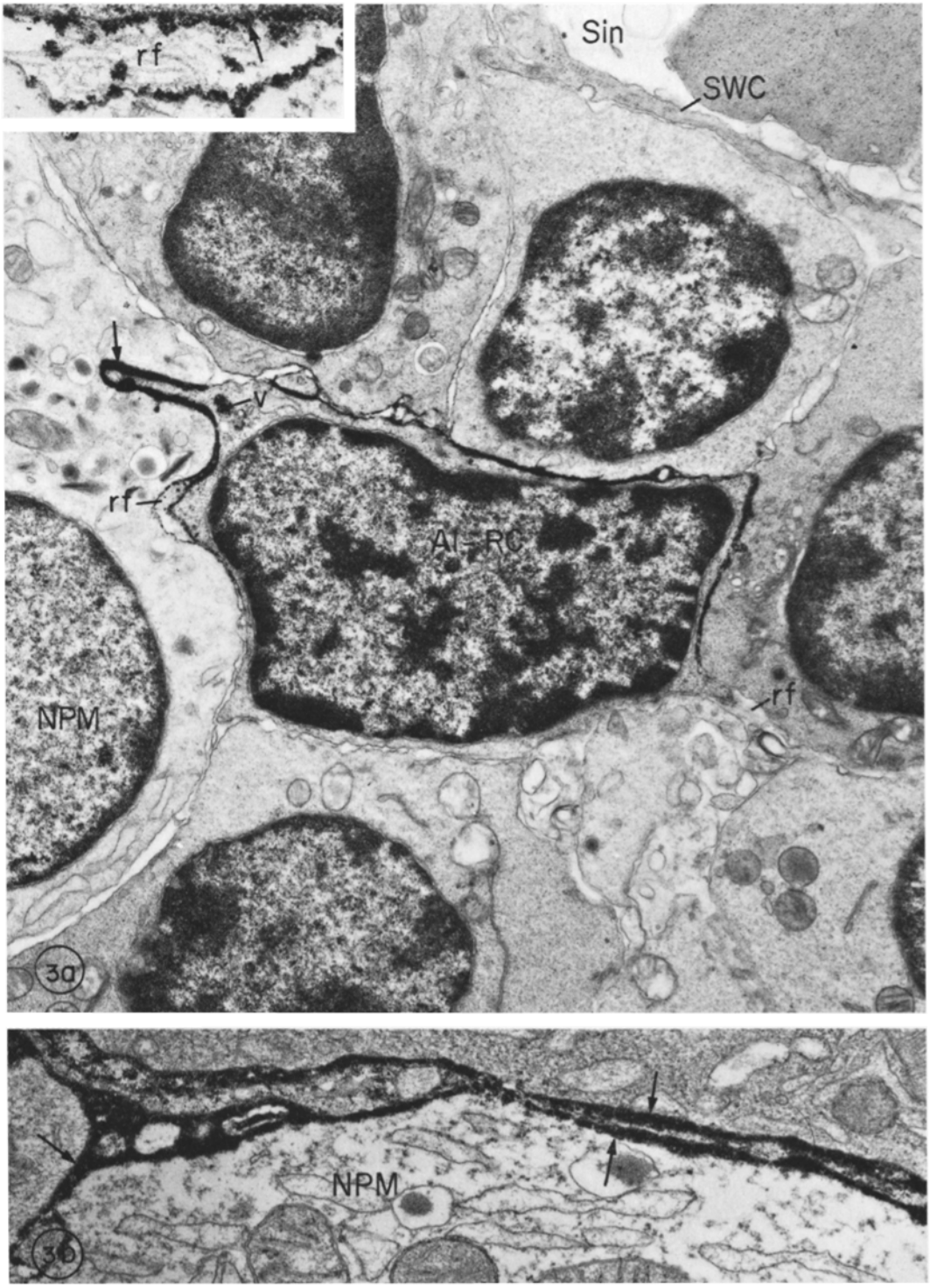

FIG. 3. (a) Higher magnification electron micrograph of rat bone marrow reacted for Alk-Pase. An intraparenchymal reticulum cell (Al-RC) contains reaction product on parts of the plasma membrane (arrow) and in an intracytoplasmic vesicle (v). It is encircled by cells of the granulopoietic line, mainly myeloblasts and promyelocytes (NPM), and closely apposes several of them. In addition, its intimate relationship with intercellular reticulin fibers (rf and inset) is obvious. No reaction product is visible in the sinus wall cell (SWC). Sinus (Sin). Tissue preparation as in Fig. 2. × 1,000; inset, × 35,000. (b) The Alk-Pase-positive cytoplasmic extensions of the reticulum cells are in intimate contact with neighbouring cells (three seen here), one of which is a neutrophilic promyelocyte (NPM). In this example it is impossible to conclude whether or not the cell membranes of the myeloid cells are also reactive. × 22,000.

i.e., a scarcely developed endoplasmic reticulum (ER), small, slightly ovoid mitochondria, and some indistinct vesicles. We rarely observed a single small vesicle filled with reaction product (Figs. 2 and 3), but several times, a few nonreactive multivesicular bodies were visible (Fig. 4 b). Occasionally, microfilaments were present, especially inside the stretched-out cytoplasmic extensions, and they were more conspicuous in the rat than in the mouse. We did not see any microtubules, probably because these cells were fixed at 4°C. A notable feature was the close proximity of Al-RC to extracellular fibrillar material (Fig. 3 a inset, reticulum fibers). The fine structure of these fibers was not well preserved after incubation in alkaline medium, but was more intact in the specimens analyzed for acid phosphatase activity (Fig. 5 b).

Only in rare cases could we observe an Al-RC that had been sectioned through its nucleus. In contrast, reactive processes which either extended into the hemopoietic parenchyma (Fig. 3 b) or which were adjacent to the inner lining cell of the sinuses (Fig. 4 a) or close to arterioles (not illustrated) were readily observed. Furthermore, in both species, a greater number of reactive cell extensions was found in the peripheral areas of the biopsy cores than in the femoral axis, as observed before by light microscopy. It should be stressed, however, that multiple fine-cellular processes were always present between hematopoietic cells, with no evidence of plasma-membrane enzymatic activity. (See the acid phosphatase Results section).

(*b*) Sinuses. In both species, Alk-Pase-positive cellular extensions were often found adjacent to the sinus wall (Fig. 4 a), where they sometimes accompanied the inner lining cells for a considerable distance. Occasionally, the location of these cells deviated from this position, and they bordered directly upon the sinus lumen, as shown in two fortuitously obtained serial sections (Figs. 4 a and b). Note that there was a heavy deposit of lead phosphate, as usual, between the inner and outer sinus-wall cells in the first section, (Fig. 4 a) whereas in the other section of the same cell, there was no visible enzymatic activity on the segment of the plasma membrane of the Al-RC that was in contact with the sinus lumen. (Fig. 4 b).

In the first micrograph (Fig. 4 a), the nucleus is irregular in shape whereas the cytoplasm has few distinctive organelles. However, at a different sectioning level, (Fig. 4 b), the same cell reveals a more regular-shaped nucleus as well as several organelles, including well-defined multivesicular bodies, a sidersome, and ER.

ACID PHOSPHATASE. (*a*) Nonvascular structures. In mice and rats, reaction product for Ac-Pase was abundant in stromal cells functioning as macrophages in the bone marrow. The activity was so great, in fact, that only after brief incubation could enzyme activity be specifically localized within large lysosomes. The Golgi cisternae, too, usually exhibited strong enzymatic activity (not illustrated).

In the hematopoietic parenchyma, two types of highly reactive macrophages warranted particular attention: (1) a vast number of phagocytic reticulum cells typical of erythropoietic islets (Fig. 5 a) with far-reaching irregular processes; (2) a very rare, rounded type of macrophage with no signs of cellular extensions (Fig. 6 a). Once, in the center of an erythropoietic island, a reticulum cell dense with Ac-Pase reaction product was observed undergoing mitosis (Fig. 6 b).

Compared with the stellate reticulum cells which reacted for Alk-Pase, the majority of these macrophages had a considerably lower nuclear:cytoplasmic ratio and a much larger content of organelles, i.e., mitochondria, vesicles, and especially lysosomes of various sizes; also, nucleoli were more frequently seen. These cells were often found to

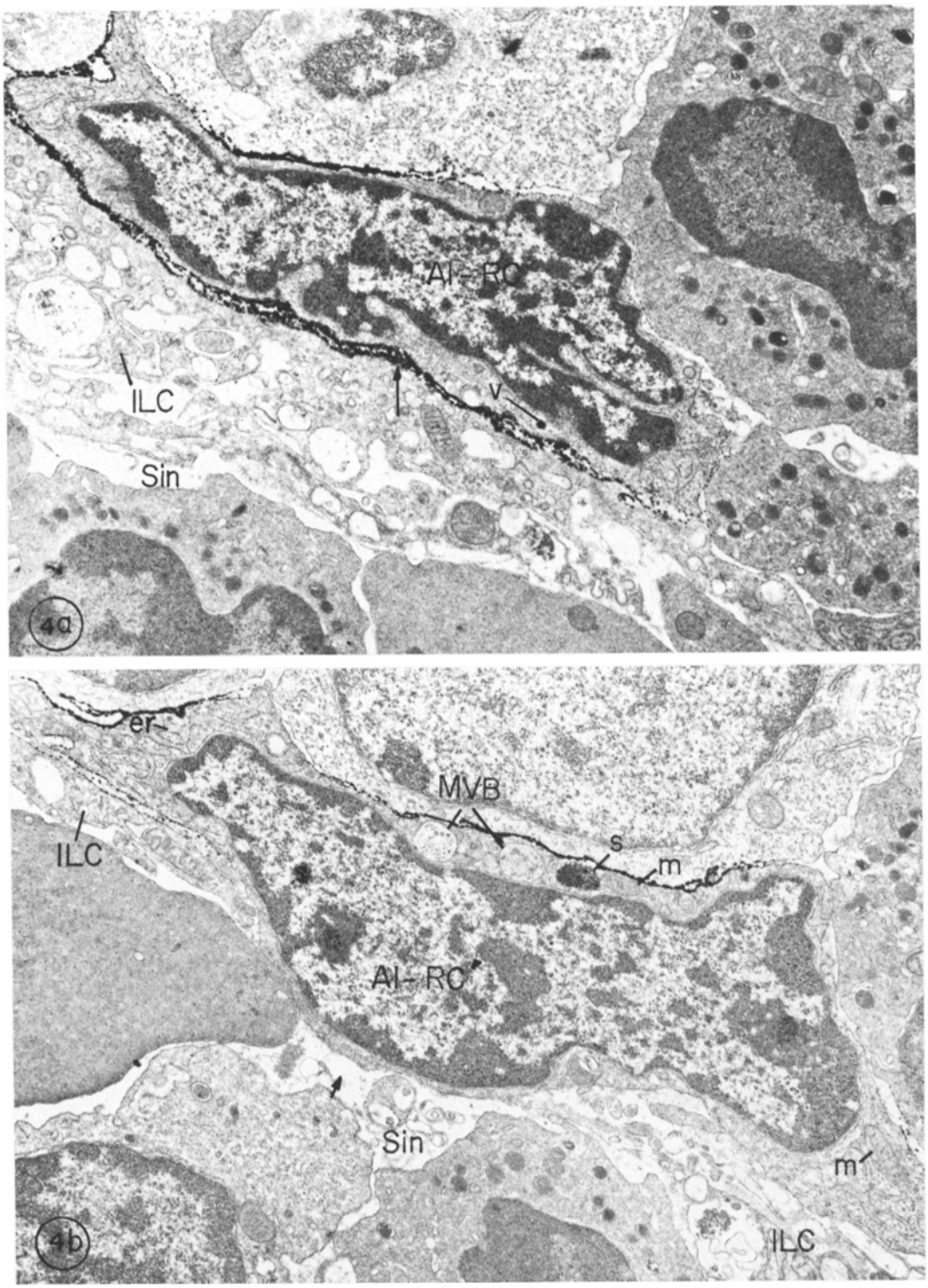

FIG. 4. Mouse bone-marrow reticulum cells reacted for Alk-Pase. The two electron micrographs illustrate serial sections prepared from the same block of tissue. (a) An Alk-Pase-positive reticulum cell (Al-RC) lies adjacent to the sinus (Sin) but is separated from it by the inner lining cells (ILC). × 15,000. (b) In a serial section, the same reticulum cell (Al-RC) as in (a) is seen to be part of the lining of the sinus wall itself. In this micrograph, the reaction product of the Al-RC is not present where direct contact between the cell and the sinus lumen is apparent (short arrow). The cytoplasm of the reticulum cell, inconspicuous in (a) except for its one reactive vesicle (v), displays multivesicular bodies (mvb), a siderosome (s), a few mitochondria (m), and some endoplasmic reticulum (er). Tissue fixed for 30 min in 1.5% glutaraldehyde, otherwise treated as in Fig. 2. × 15,000.

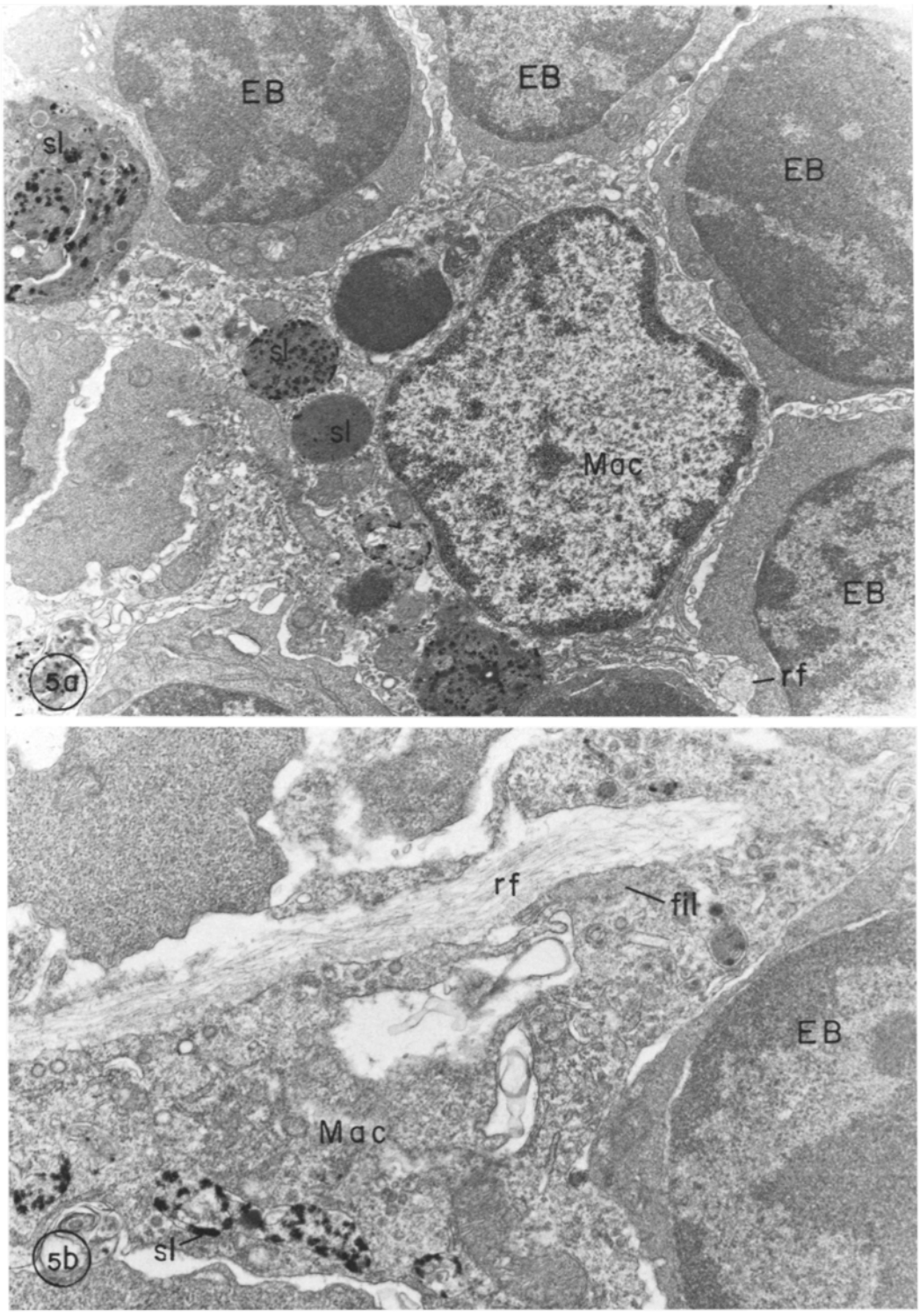

FIG. 5. (a) Electron micrograph of macrophages from mouse bone marrow reacted for Ac-Pase. An intraparenchymal macrophage (Mac) surrounded by erythroblasts (EB) forms a typical erythropoietic island. Huge secondary lysosomes (sl) are filled with reaction product, while others contain very little. The close relationship with intercellular reticulum fibers (rf) is obvious. × 18,000. (b) High magnification electron micrograph of mouse bone marrow macrophage reacted for Ac-Pase. The picture reveals the intimate contact of an intraparenchymal macrophage, forming an erythropoietic island, with intercellular reticulin (rf). Note also the intracellular foci of filaments (fil). Small reactive secondary lysosomes (sl) are also present. Tissue fixed for 2 h in 1.5% glutaraldehyde, incubated for 20 min in Barka and Anderson's medium, and embedded in araldite. × 22,000.

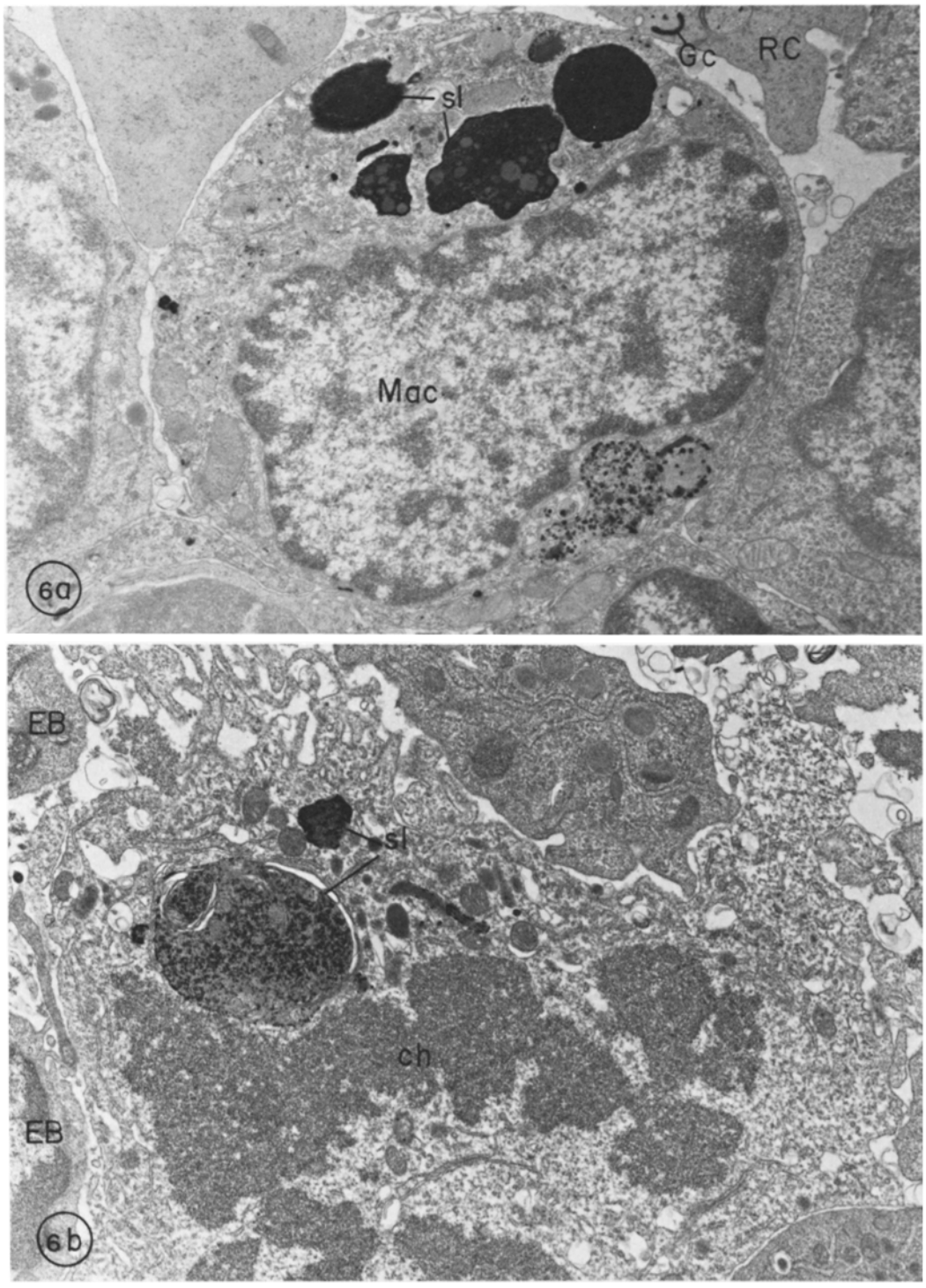

FIG. 6. (a) Electron micrograph of a macrophage from rat bone marrow reacted for Ac-Pase depicting a rare type of a rounded macrophage (Mac), without obvious cell extensions. The secondary lysosomes (sl) of the Mac and a few lysosomes and remnants of a Golgi cisternae (Gc) of a neighbouring reticulocyte (RC) also show signs of enzyme reaction. Tissue preparation as in Fig. 5. × 15,000. (b) Electron micrograph of a macrophage in mitosis in mouse bone marrow reacted for Ac-Pase. This macrophage, clearly marked by its heavily reactive secondary lysosomes (sl), is undergoing cell division, as evidenced by aggregated chromatin (ch) and the absence of a nuclear membrane. This was a rare observation. EB = erythroblast. Tissue preparation as in Fig. 5. × 9,000.

extend their cytoplasmic processes for long distances before making contact with the sinus-wall cells. Significantly, the vast majority of the abundant stromal cell extensions between the hematopoietic cell compartments displayed huge, reactive lysosomes. An intimate association of these phagocytic reticulum cells with extracellular fibrillar material (reticulum fibers), similar to that seen in the case of the Al-RC, was frequently noted (Fig. 5 b). In a few instances, the extracellular reticulum fibers seemed to be extensively surrounded by the cytoplasmic extensions of phagocytic reticulum cells which formed an erythropoietic islet (Fig. 5 b).

(*b*) Vascular structures. No sign of Ac-Pase activity was observed in arteries, arterioles, or capillaries after brief periods of incubation. In contrast, large secondary lysosomes (Fig. 7) and, occasionally, the Golgi cisternae and vesicles, as well as the sinus-wall cells, were intensely reactive for Ac-Pase. Even at sites that were distant from the nucleus, enormous reactive lysosomes were conspicuous. There were obvious histochemical similarities between these sinus-wall cells and the intraparenchymal macrophages, i.e., both types of cells are Alk-Pase-negative and contain abundant Ac-Pase, respectively. In these cell types, close contact with the aforementioned intercellular reticulum fibers was apparent (Fig. 7). When there was more than one cell layer in the lining of the sinus, Ac-Pase activity seemed to be restricted primarily to the cells lining the lumen.

(*c*) Enzymatic controls. 1. Alkaline phosphatase. L-*p*-bromotetramisole completely inhibited the deposition of reaction product as judged by examination of specimens at both the light and electron microscopic levels. To exclude the possibility that the fixation or embedding procedures were inhibiting significant quantities of enzyme, unfixed cryostat sections were incubated together with the usual smears and plastic sections of bone marrow of the same animal. Examination of this material disclosed no detectable differences in the amount or distribution pattern of Alk-Pase. Also, no significant differences in either the distribution pattern or the intensity of Alk-Pase activity were observed when we compared data obtained from specimens analyzed by electron microscopy and the results from light microscopic studies of plastic sections.

2. Acid phosphatase. As a control for the determination of Ac-Pase background activity, we omitted substrate during the incubation and there was no demonstrable formation of reaction product.

Discussion

In this study, we have identified the morphology and distribution of a distinct group of nonphagocytic reticulum cells which have high concentrations of Alk-Pase on their plasma membranes (Figs. 2–4). This cell type was easily distinguishable from another stellate stromal cell, the macrophage (phagocytic reticulum cell), which was negative for Alk-Pase but contained numerous large secondary lysosomes identifiable by their abundant content of Ac-Pase (Fig. 5 a).

It is significant that the Al-RC were consistently found in close association with granulocytic precursors (Figs. 2 and 3), whereas the acid phosphatase-positive macrophages were associated mainly with developing erythroid precursor cells (Fig. 5 a), as reported by others (23, and 24).

The fine-structural features of the Al-RC reported here resemble those of a similar cell type which was first described by Ito (6) and Huhn (7) in the rat, and was later

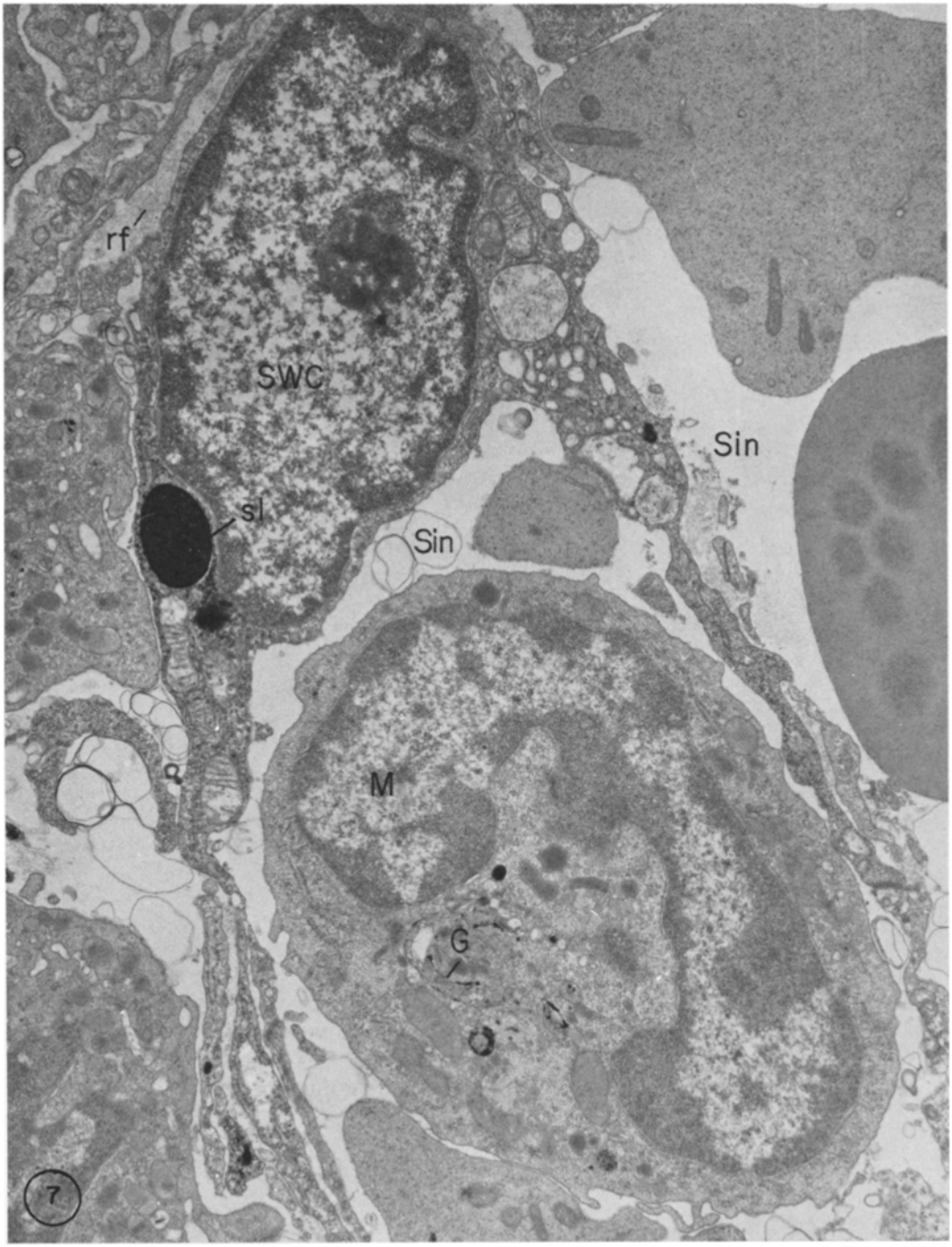

FIG. 7. Electron micrograph of a sinus-wall cell from rat bone marrow reacted for Ac-Pase. The depicted sinus wall cell (SWC) shows a large secondary lysosome (sl) with a heavy enzyme reaction. Also reactive are parts of the Golgi complex (G) and some vesicles of a monocyte (M) within the sinus. Reticulin fibers (rf) can usually be seen close to the sinus-wall cell as well. Sin = Sinus. Tissue preparation as in Fig. 5. × 16,000.

extensively characterized by Weiss (4) who called it a fibroblast-like reticular cell. It is likely to be identical with the nonphagocytic reticulum cell with intracytoplasmic fibrils as described in the human (25).

The considerable content of cytoplasmic filaments detected by all cited authors led them to the conclusion that these filaments were an important distinguishing feature of such cells. In the specimens examined by us, the number of filaments in the Al-RC showed wide variations, and although the amount was never very striking, these filaments were more obvious in the rat than in the mouse. This discrepancy from previous findings may be partly a result of morphologic changes induced by the fixation at 4°C and subsequent incubation procedure required by our method. It must, therefore, remain open whether or not the Al-RC is fully identical with the reticular cell of the aformentioned authors. Tanaka (25), for example, when dealing with reticulum cells of the nonphagocytic type, differentiated between cells with intracytoplasmic fibrils and cells with an immature appearance of both nucleus and cytoplasm. On the basis of electron microscopical observations, Huhn (7) similarly found a close resemblance of reticular stromal elements, i.e., nonphagocytic reticulum cells, fibroblasts, angioblasts, and osteoblasts. Thus, the variations in morphology of the Al-RC may reflect different functional states of the fibroblast-like reticulum cells or its participation in one of the other stromal functions in the marrow ranging from supporting hemopoiesis to bone formation (see below).

Although the plasma membrane-bound Alk-Pase serves as a useful marker to differentiate these stromal-cell elements, its biological function is as yet unknown. Alk-Pase is a rather nonspecific enzyme with many molecular forms and probably many functions (26, 27). In intestine and kidney, Alk-Pase has been related to the sodium pump and associated with the rapid passage of ions across cell membranes (27). When correlating the morphological findings of this study with the different functions which have been hypothesized for Alk-Pase throughout the body (27, 28), the following biological roles of the Al-RC may be postulated: (*a*) The membrane-bound reaction product of Alk-Pase may be related to the production of intercellular fibrillar material which in this study was consistently observed in the vicinity of the Al-RC. It is well known that Alk-Pase can be induced in cultured fibroblasts (26), and that this enzyme may be indicative of collagen synthesis (18, 26–29). The possibility that reticulum cells are the source of the intercellular reticulum fibers in the bone marrow has long been postulated (30), and was re-emphasized by Weiss (4). The presence of Alk-Pase on the plasma membrane would then merely reflect a certain functional state of the fibroblast-like nonphagocytic reticulum cell observed by others (4, 6–8). It may also be closely related to the fiber-associated reticulum cell which is thought to be reactive for Alk-Pase when actively producing extracellular fibrous material in the human spleen, as proposed by Heusermann and Stutte (28).

(*b*) The intimate association of Al-RC with granulocytic precursors (Figs. 2, and 3), and their concentration at sites typical of myelopoiesis such as bone surface (31–33), and blood vessels (32, 34) are very suggestive of a functional relationship with granulopoiesis. The well-known regulatory influence of stromal cells on hemopoiesis (3) was proposed to be mediated by cell contact processes and/or humoral factors (35) such as colony-stimulating activity (CSA). This glycoprotein induces granulopoietic and/or monocytic colonies in vitro (36), and was shown to be produced in greater amounts from stromal cells adjacent to bone (35). Thus, the proper microen-

vironment for the differentiation of granulocytes probably requires a local source of CSA as well as contact with an appropriate type of stromal cell. These stromal cells adjacent to bone could provide the local source of CSA, although it is now evident that CSA is produced by several types of cells, including macrophages (35). The intimate association of AL-RC with early precursors of granulocytes makes it a strong candidate for the stromal cell responsible for their differentiation. Furthermore, it is also possible, although at present completely speculative, that the function of a membrane-associated Alk-Pase could be to enzymatically modify glycoproteins[3] in its immediate vicinity. The substrates could be integral glycoproteins of the plasma membrane of adjacent cells, or a substance in the immediate microenvironment, such as CSA. Recently, two distinct stromal cells were identified in granulopoietic and erythropoietic spleen colonies by LaPushin and Trentin (38) who assumed that these cells may regulate the hemopoietic inductive microenvironment of such colonies. Whether or not these cells correspond cytochemically to the two stromal cell types described in this paper remains to be investigated.

(*c*) Finally, as AL-RC were seen to be especially concentrated in subendosteal regions (Fig. 1 f), their possible function as osteoprogenitor cells (39) cannot be excluded. This latter cell type was reported to have the appearance of undifferentiated mesenchymal cell elements and to be located within a certain distance of the bone surface. Preosteoblasts have also been shown to be reactive for Alk-Pase (40). Perhaps pertinent to this point is the work of Amsel et al. (41), who disrupted the center of femoral bone marrow with a small needle and subsequently observed the formation of new cancellous bone within the injured cavity, far away from the endosteum (and obvious osteoblasts). In this regard, Friedenstein et al. (42) have isolated from bone marrow a self-perpetuating population of determined osteogenic precursors. In monolayer cultures, these cells form clones of fibroblast-like cells. Upon transplantation subcutaneously, they initiate a certain cascade of events which includes the formation of bone, resorption, the formation of stroma, and overt hematopoiesis. Furthermore, the stroma is of donor origin whereas the cells of the host repopulate hematopoiesis. Further study will be necessary to clarify the possible interrelationships of these mesenchymal cells. Finally, it should be mentioned that it is unlikely that Al-RC are in any way related to another stellate-shaped cell, the dendritic cell, recently identified by Steinman et al. (43), because mature dendritic cells have not been found in bone marrow but are present in spleen and lymph nodes.

Although the new findings of this paper focus on Al-RC, a few comments should also be made about the phagocytic reticulum cells, i.e., macrophages, which are characterized by their abundance of Ac-Pase within large secondary lysosomes. Fixed marrow macrophages are known to be closely associated with developing erythroblasts, as reviewed by Ben-Ishay and Yoffey (24). Their function as the nursing cell of erythropoiesis or in iron metabolism remains to be further clarified, whereas their role in the removal of expelled erythroblastic nuclei by phagocytosis has clearly been demonstrated by Bessis (23). In addition, we have noted a close relationship between marrow macrophages and extracellular reticulin (Fig. 5 b), as reported by Watanabe (44) and Marton (12). In view of present-day knowledge regarding macrophage

[3] In this regard, it has ben demonstrated that treatment with highly purified Alk-Pase converts high-uptake forms of β-glucuronidase to a less acidic, low-uptake form; that is, the enzymatic modification affects the rate of pinocytosis of the altered enzyme by fibroblasts (37).

function, it seems more likely that these cells may remove reticulin fibers rather than produce them.

The current consensus is that bone marrow macrophages are part of the mononuclear phagocyte system (45, 46). However, only very few data dealing with the possible origin and fate of fixed macrophages of the bone marrow (47) have been published thus far. One interesting finding of our study is that macrophages forming erythropoietic islets are capable of mitosis (Fig. 6 b). However, it should be emphasized that this was a rare finding.

As for the sinuses, the findings of this study show that they are composed mainly of endothelial cells which contain a large amount of lysosomal Ac-Pase (Fig. 7), and a fibroblast type of adventitial cells characterized by their membrane-bound Alk-Pase (Fig. 4). The cytoplasmic extensions of the Al-RC usually surround the endothelial cell but occasionally also border directly upon the sinus lumen. This is of interest because the sinus wall has previously been described as a continuous squamous endothelium, the cells being held together by maculae occludentes (4, 48).[4] The close association between these sinus-wall cells and extracellular fibrillar material was also remarkable (Fig. 7). One function of the sinus-wall cells has been shown to be the removal of particulate material from the circulation (13). The function of the fibroblast-like adventitial cell, on the other hand, may be the production of extracellular reticulin to provide structural support for the sinus.

Summary

In the bone marrow, an elaborate stroma forms the structural basis of the hemopoietic microenvironment. In this study, two different types of stromal cells were identified with certainty on tissue sections of intact bone marrow of rats and mice using light and electron microscopic histochemistry:

(*a*) a fibroblast-type of reticulum cell which is characterized by having alkaline phosphatase associated with its plasma membrane. We refer to this cell as the alkaline-phosphatase-positive reticulum cell (Al-RC). It is closely associated with granulocytic precursors, particularly myeloblasts and neutrophilic promyelocytes. These reticulum cells may be found throughout the marrow but are concentrated near the endosteum.

(*b*) a macrophage-type of reticulum cell which is characterized by its abundance of lysosomal acid phosphatase and is mainly associated with erythroid precursors (as observed by others). In contrast to the above-mentioned cell type, this latter cell was found to be distributed uniformly throughout the marrow.

We speculate that the Al-RC are mesenchymal stromal cells necessary for granulocytic differentiation in bone marrow.

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[4] By freeze fracture, the tight junction strands can frequently be demonstrated to be incomplete. (Westen, H. and D. S. Friend. 1979. Loosely structured tight junctions and focally aggregated fenestrations in the sinuses of rat bone marrow. A freeze-fracture study. Manuscript in preparation.)

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