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Experimental Bone Marrow Fat Necrosis

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Summary. We have studied serially by light and electronmicroscopy the development of lesions of the adipose tissue of the bone marrow of the rabbit following intravenous injections of saponin. Two types of steatonecrotic lesions were seen: in the femur giant-cell granulomas surrounding necrotic fat cells and in the sternum, small foci of fat necrosis containing calcified deposits. In both cases the lesions were of ischemic origin and secondary to the destruction of the microcirculation of the bone marrow by saponin.

These studies suggest that necrosis of the bone marrow fat cells can contribute to the pathogenesis of myelofibrosis. They also suggest that adipose tissue responds differently to ischemia depending on topography. In hematopoietic marrow necrosis of fat cells is followed by calcium deposits, whereas in fatty marrow necrosis leads to resorptive giant cell reaction.

Key words: Bone marrow – Fat necrosis – Microcirculation – Ischemia – Myelofibrosis.

Introduction

Bone marrow is an intricate structure composed of myeloid cells and a stroma. The stroma includes a number of elements: fat cells, reticular cells, nerves, connective tissue components, and a microcirculation. Adipose tissue is abundant and quantitatively the most important component of the stroma. Recently, Trubowitz and Masek (1970) have shown that the hematopoietic marrow is organized around its adipose cells and microvascular structures. It has been established that a normal microenvironment is a prerequisite for normal hema-

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topoiesis (Tavassoli, 1975) and morphologically the marrow stroma is an important feature of the microenvironment of hemopoietic tissue. There is also evidence for a contributing role of bone marrow adipose tissue in the regulation of hemopoiesis (Tavassoli et al., 1974). Though these studies indicate that pathology of stromal tissues may influence hematopoiesis, the histopathology of the stroma and of adipose cells in particular, has generally been neglected.

Recently Cazenave and Willcox (1972) and Rywlin and Ortega (1972) have described the presence of lipid granulomas in human bone marrow. Masshoff and Schettler (1975) studied fat necrosis in post mortem material from idiopathic necrosis in the marrow of the distal femur. Experimentally, Rutishauser et al. (1965) and Brookes (1971) have produced similar lesions by inducing ischemia of the marrow. Liponecrosis and giant cell lipophagic granulomas of the hematopoietic bone marrow can be induced in rabbits by intravenous injection of saponin. We have used this method to investigate successive morphological stages in production of fat cell lesions in hematopoietic marrow and yellow marrow.

Materials and Methods

Seventy-nine rabbits of the strain "Agenté anglais" of both sexes were used for the experiments. The rabbits, 6 to 7 months old, weighing 1.4 to 2.7 kg, were housed individually, fed a standard commercial rabbit chow (Sanders) and given water ad libitum. Saponin (White pure Saponin, Merck, Darmstadt) freshly dissolved in physiologic saline (9 g/l NaCl) was injected into the lateral ear vein at a dose of 1.2 mg/kg every four days for a maximum period of three weeks, as previously described by Argano et al. (1969). Twenty control rabbits received intravenous saline, every four days for three weeks, instead of saponin. Animals were killed by injection of excess sodium pentobarbital and autopsied between 1 h and 62 days after the first injection of saponin.

Femoral bone marrow was fixed in situ by intraarterial perfusion of fixative through a polyethylene catheter inserted in the abdominal aorta distal to the renal arteries, as described by Forsman et al. (1967). Fixation was preceded by washing with a solution containing 100 µ/ml (Heparin Choay, France) and 0.4 mg/ml procaine, at 4° C for 1 min, at a pressure of 145 cm of water. The fixative, 3% glutaraldehyde (Merck) in phosphate buffer pH 7.4, and 2% polyvinylpyrrolidone (P.V.P. Rhône Poulenc, France) as described by Bohman and Maunsbach (1970) was perfused at 4° C, at a pressure of 145 cm of water, and a flow rate of 90 ml/mn for 5 min. Its viscosity, measured in a Brookfield's microviscometer, was similar to that of rabbit plasma at 4° C.

The fixed femoral bone marrow was then removed in toto from femur and divided into two parts. One was post-fixed in Holland-Cazal fixative (Cazenave, 1970) then embedded in paraffin, cut and stained by standard methods (Cazenave, 1970) with hematoxylin and eosin (H & E), Ponceau red, anilin blue or Laidlaw's reticulin silver stain.

The remainder of the fixed femoral bone marrow was processed for ultrastructural studies. Fragments of approximately one cubic millimeter were cut, and fixed in 3% glutaraldehyde in phosphate buffer at 4° C for 2 h. They were then washed and stored for 12 h at 4° C in phosphate buffered osmium tetroxide for 1 h (Sabatini et al., 1963), dehydrated in graded alcohol and finally embedded in araldite according to Luft (1961). Sections were cut with a glass knife on a Porter-Blume ultramicrotome. Fragments of sternal bone marrow which had not been prefixed in situ, were processed in the same way.

Thick sections (0.5–1 m) were stained as described previously (Oberling et al., 1972) with P.A.S. or methylene blue and examined and photographed with a conventional optic microscope or a phase contrast microscope. Thin sections were stained with uranyl acetate followed in some cases by lead citrate (Reynolds 1963) mounted on 300 mesh copper grids and examined with a Siemens Elmiskop I (electron microscope) at 60 or 80 kv.

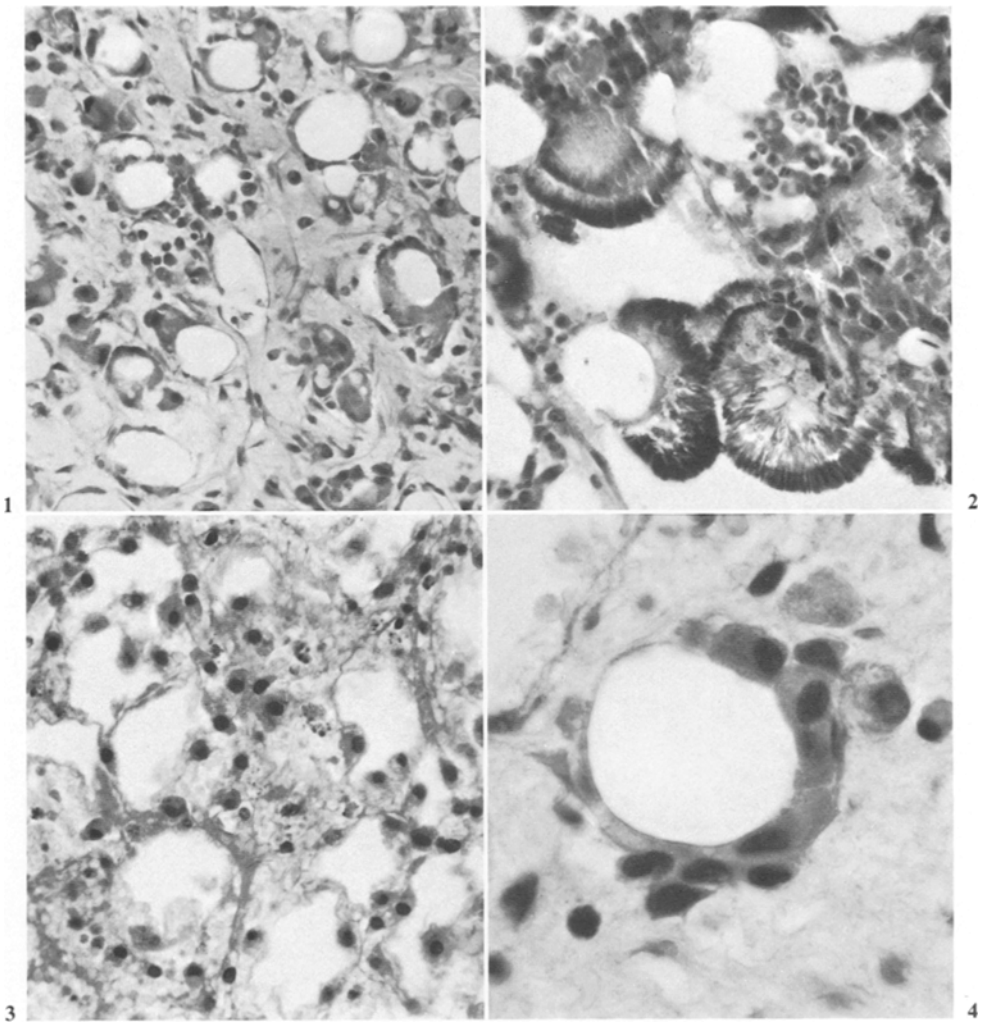

Fig. 1. Femoral bone marrow (day 26). Resorptive giant cells, at different stages of development, surrounding necrotic adipocytes in a loose fibrous network. A few mononuclear cells are seen. (H & E, ×280)

Fig. 2. Sternal bone marrow (day 17). Three pseudo-crystalline areas of calciphylaxis have developed on necrotic adipocytes. (H & E, ×480)

Fig. 3. Femoral bone marrow (day 26). Necrotic adipocytes with a folded, thick, chromophile and refringent cell wall. The interstitial space is infiltrated by mononuclear cells. The marrow is depleted of myeloid cells. (H & E, ×480)

Fig. 4. Femoral bone marrow (day 35). Monocytes are seen surrounding an adipocyte. (Thick sections, methylene blue, ×1200)

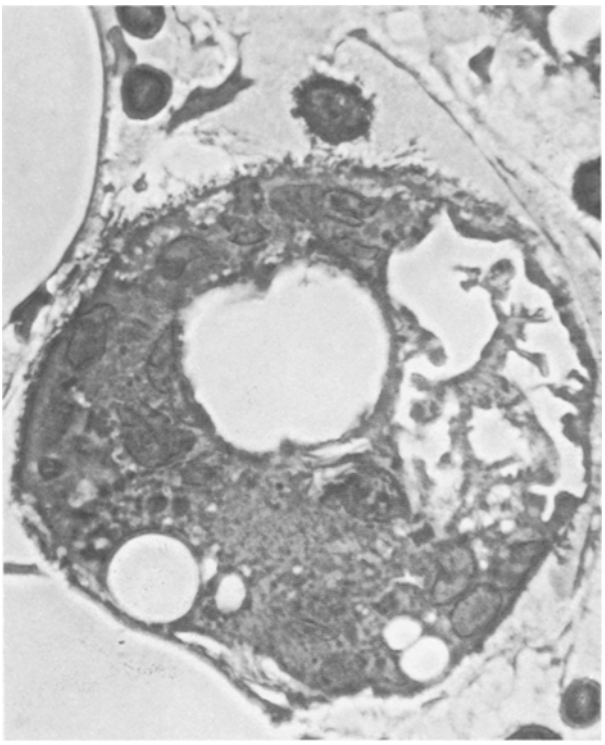

Fig. 5. Femoral bone marrow (day 59). Multinucleated giant cell penetrating an adipocyte. Fragments of the cytoplasm of the adipocytes are within the giant cell and appear optically lucent. The larger adipocyte (bottom) has been penetrated by cytoplasmic extrusions from the giant cell. The ruffled border of the giant cell is seen. (Thin section, phase contrast, methylene blue, ×1150)

Results

Light Microscopy

No significant changes in the hematopoietic bone marrow of the femur and sternum were found in 20 control rabbits. The structure of the bone marrow adipose cells in normal rabbits has been previously described (Oberling et al., 1972).

In rabbits treated with saponin the first histological changes were noted within 1 h of the first saponin injection and consisted of widening and congestion of marrow sinusoids. This was followed within a few hours by sinusoid wall rupture and appearance of interstitial hemorrhage. In the femoral bone marrow branches of the nutrient artery were frequently thrombosed. As the sinusoids ruptured, myeloid cells disappeared. We have previously reported in detail the microvascular changes which occur in bone marrow following saponin injection (Oberling et al., 1973).

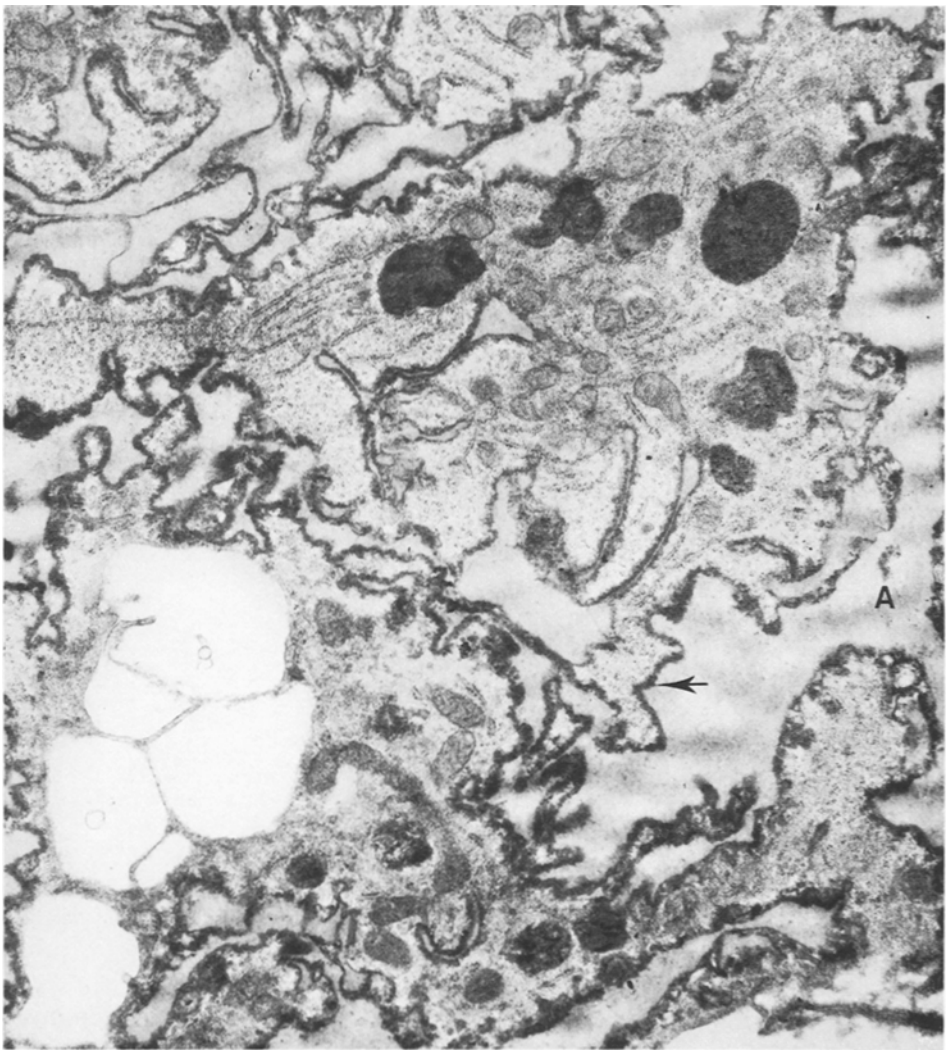

Fig. 6. Electron micrograph of the femoral bone marrow (day 39). Cytoplasmic extensions of the giant cell fragmenting an adipocyte (*A*). The contact zone between the two cells is electron dense (arrow). (×12,000)

Lesions of the bone marrow adipose tissue were visible from the 10th day of the experiment, fibrosis of the marrow becoming apparent at the same time. The extent and severity of fat cell lesions were most marked in the femoral marrow (yellow marrow), and much less in evidence in marrow from the sternum (red marrow). In femoral marrow, the lesions were composed of giant-cell granulomas with centres of necrotic adipocytes (cytosteatonecrosis). These granulomas were surrounded by scanty fibrous tissue and patchy infiltrates of lymphocytes and plasma cells (Fig. 1). The appearance of the granulomas varied markedly

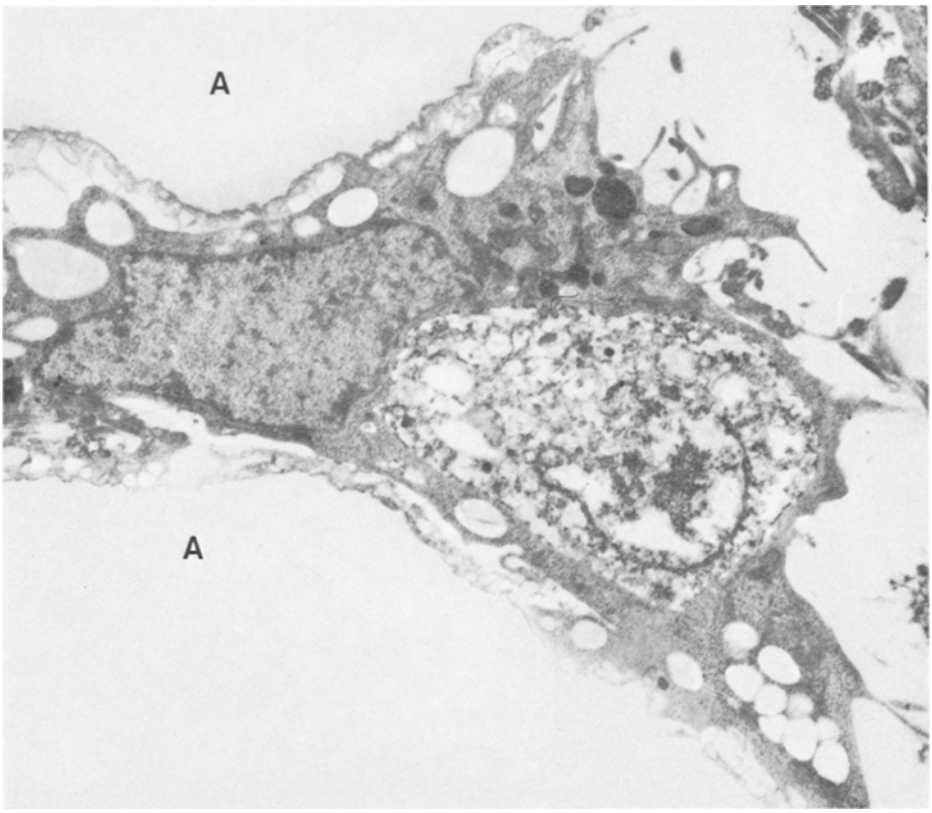

Fig. 7. Electron micrograph of the femoral bone marrow (day 12). Phagocytosing macrophage squeezed between two necrotic adipocytes (*A*). (× 8000)

from one animal to the other and within the same bone marrow. They were randomly dispersed and their stage of development was variable. They persisted for some time before disappearing spontaneously, and were rarely found in marrow examined at the 62nd day of the experiment. At this period the bone marrow microcirculation was restored.

Sternal marrow was much less depleted of its myeloid cells than femoral marrow and there were no areas of cytosteatonecrosis surrounded by lipophagic giant cells. Areas of fat necrosis containing calcified deposits were seen (Fig. 2), lesions which were not found in femoral bone marrow. The von Kossa's staining coloured these deposits in black.

The development and evolution of giant cell lipophagic granulomas appeared to involve a number of stages, though these could be defined only tentatively, due to the coexistence of lesions of different age in the same marrow sample. The adipocytes of a territory decreased in size and their periphery became irregular and folded. The folded cell margin appeared thicker, more chromophilic and refringent (Fig. 3) and collapsed progressively, forming protrusions inside

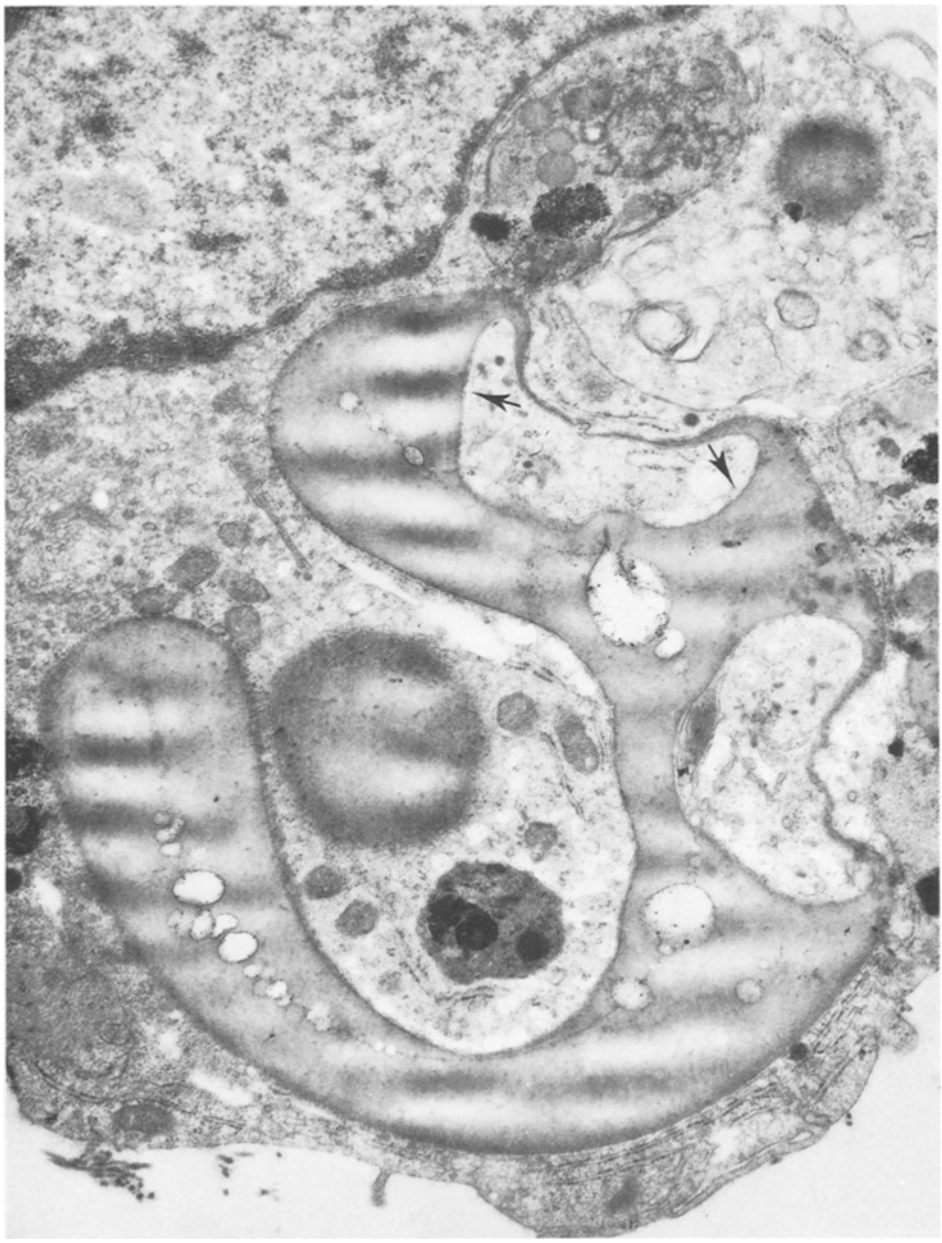

Fig. 8. Electron micrograph of the femoral bone marrow (day 59). Relationships between a giant cell and an adipocyte. The fat, containing lucent areas, is limited by a single unit membrane (arrow). ($\times$10,000)

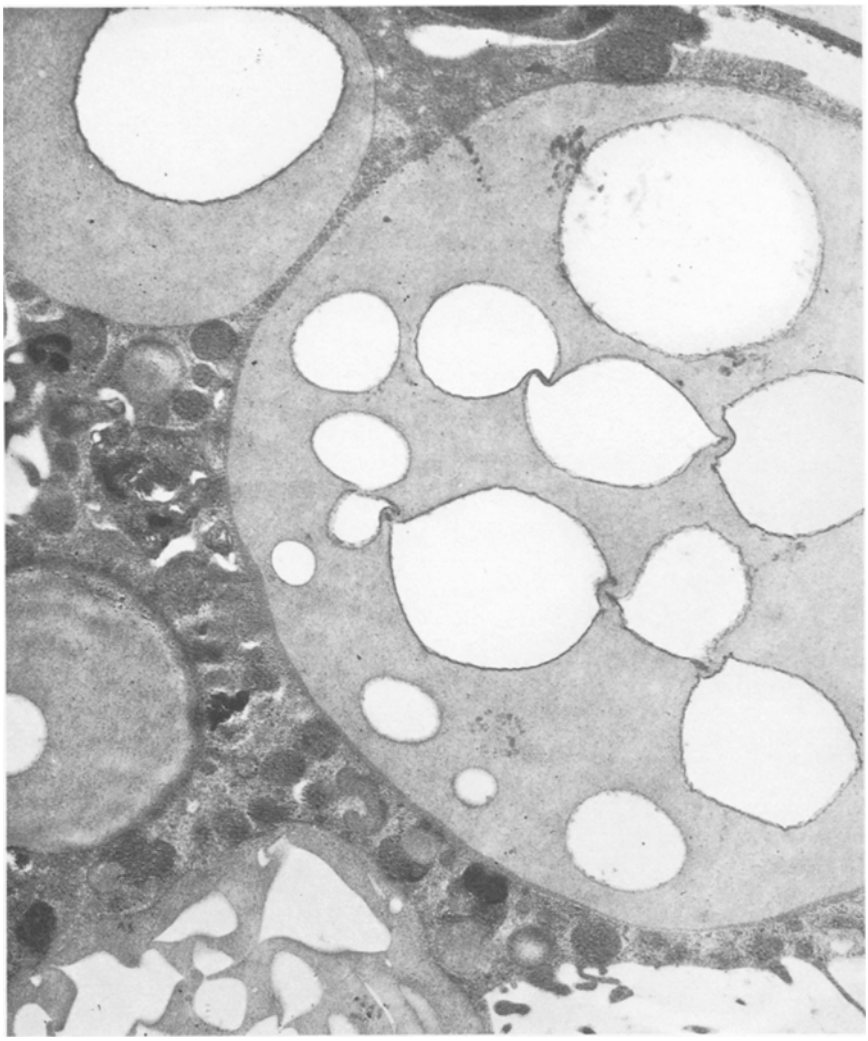

Fig. 9. Electron micrograph of the femoral bone marrow (day 59). Fat "globules" formed from fragmented adipocytes located in the cytoplasm of a giant cell, surrounded by numerous dense bodies. (× 22,000)

the adipocyte. At the same time, altered adipocytes were infiltrated by mononuclear cells (Fig. 4). These then fused, forming a multinucleated plasmod which penetrated the adipocyte progressively by cytoplasmic extension (Fig. 5). The multinucleated giant cell so formed was of the resorptive type. Once phagocytosis of the adipocyte was complete, the giant cell occupied the space which had formerly contained the adipocyte (Fig. 5).

Electron Microscopy Study

We have previously reported in detail the ultrastructural morphology of the adipose tissue of the normal hematopoietic femoral bone marrow of the rabbit (Oberling et al., 1972). The general ulstrastructural morphology of the giant resorptive multinucleated cells was similar to the appearances previously described by others (Mariano et al., 1974) (Sutton et al., 1966). Two adjacent mononuclear cells, forming part of the giant cells, were frequently seen to be separated by thin or foliated digitations which had fused, or which limited a space between the cells (Fig. 6). Early lesions of adipose tissue were characterized by a loss of the usual cytoplasmic structures of the adipocyte (glycogen, mitochondria). The fragment of cytoplasm of the fat cell adjacent to the lipid inclusion, was modified by thickening and folding inside its limiting plasma membrane. The membrane was not ruptured at the level of the inclusion. Macrophages were seen infiltrating the spaces between fat cells and phagocytosing necrotic material (Fig. 7). At a later stage of the lesion, while the multinucleated plasmods were being formed, their digitations were seen to have deeply penetrated the fat vesicle, met, fused, and divided the lipid inclusion into smaller masses of varying size (Fig. 6, 8).

In the final stages the cytoplasm of the giant cell was found to contain lipid inclusions, isolated or grouped inside a phagosome (Fig. 8, 9). Some inclusions were found outside the giant cell, in the intercellular space and were probably taken up by other macrophages.

Discussion

The intravenous administration of saponin induces two types of lesions of the bone marrow adipose tissue. One consists of small necrotic foci containing calcified deposits, the other of large necrotic lesions which induce a giant cell lipophagic reaction. These two types of lesion do not occur together, the former being found in red marrow (sternum) while the latter occurs in yellow marrow (femur).

The lesions are the result of ischemia and occur secondarily to the destruction of the microcirculation of the femoral marrow by saponin (Oberling et al., 1973). They are not due to a direct toxic action of saponin on the adipose tissue, though saponin is known to possess cytotolytic action on the endothelium of bone barrow sinusoids (Osogoe, et al., 1965). Rutishauser (1960, 1965) and Brookes (1971), who induced bone marrow ischemia by a different method, also noticed the presence of steatonecrosis with giant cell lipophagia in the bone marrow. During the present investigation, we have not found this type of lesion in adipose tissue from other organs such as mesentery or perirenal fat, and the delay in appearence of lesions of the adipose tissue is further evidence that the lesion is not due to a direct action of saponin on fat cells.

These experiments clearly demonstrate that the lesions of steatonecrosis are secondary to an injury to the microcirculation of the bone marrow. This leads

to an influx of mononuclear phagocytes into the necrotic areas, followed by phagocytosis of the lipid contents of the fat cells and formation of phagosomes. The phagocytic process is certainly associated with the release of lysosomal enzymes in the interstitial space which contribute to the inflammatory reaction. We have previously described in detail (Cazenave, 1970; Oberling et al., 1973) how saponin-induced damage of the microcirculation of the rabbit bone marrow was followed after 8 to 13 days by the development of fibrosis of the marrow. The formation of giant cell lipophagic granulomas which occurs around the 10th day may contribute to the pathogenesis of the myelofibrotic process.

It is hard to explain the difference between adipocytic necrosis with calcium deposit, as seen in sternal bone marrow and the adipocytic necrosis with giant cell macrophagia observed in femoral bone marrow. Both types of fat cell necrosis may be observed at the same time in the same animal. It is simply a matter of topography. Bone marrow adipocytes may vary in biochemical properties according to their topographical distribution and a clear histochemical difference between adipocytes in sternal bone marrow and in the centromedullary part of the femur in rabbits has recently been demonstrated by Tavassoli (1976) using the "performic acid Schiff" reaction. In an active haematopoietic site such as the sternum, adipocytes are red coloured whereas they are not coloured in the centromedullary part of the femur. Such an histochemical difference suggests a biochemical difference and may implay different behaviour of adipocytes in an ischaemic situation.

That two types of adipocytes with different histo-chemical reactions exist in different areas of the bone marrow may also account for the different outcome of ischemic adipo-necrosis in the sternum and in the femur. In the former calcification occurs in areas of adipocytic necrosis. It has been shown by Berczi (1970) that some fatty acids show "calciphylatctic" activity. Unsaturated fatty acids are "calciphylactic". Adipocytes in haematopoietic bone marrow are performic acid schiff positive, evidence for the presence of unsaturated fatty acids. Perhaps an indirect effect occurs following saponin-induced haemolysis, in support of this Evans et al. (1955) have shown that haemolysis induced in rabbits by acethyl-phenyl-hydrazin provokes the mobilization of unsaturated fatty acids from the marrow.

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