activated T lymphocytes, and their function is evidently linked with the supply of antigens [9].

Cells of the thickened intima have often been described in NAA as fibroblasts. However, we know that the main type of vascular cells, namely smooth muscle cells (SMC), undergo what is called modification of the phenotype under certain conditions, and morphologically they become fibroblast-like [3]. Under these circumstances they preserve some of their type-specific features of SMC, one of which, it is generally agreed, is a basement membrane, which fibroblasts do not possess [6].

Staining sections of vessels affected by NAA with antibodies to basement membrane proteins, laminin and type IV collagen, demonstrated their presence on cells of the intima (Fig. 3). In other words, although in NAA the media undergoes destruction, whereas the intima, on the contrary, undergoes hyperplasia, SMC are the principal cell type in both layers. It can be tentatively suggested that in NAA cytotoxic lymphocytes attack the SMC subpopulation in the media of the vessel.

The results thus showed that T_8-positive lymphocytes predominate in foci of inflammatory infiltration of the vessel wall in NAA, which supports the view that the disease is autoimmune in nature.

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BONE TISSUE FORMATION BY MOUSE BONE MARROW CELL SUSPENSION IN ORGAN CULTURES

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Bone marrow fragments transplanted into a diffusion chamber [4] or beneath the capsule of the kidney [5] invariably form bone tissue. A similar result can be obtained by transplantation of suspension of bone marrow cells into a diffusion chamber [6, 3]. For bone tissue to be formed as a result of transplantation of bone marrow cell suspension into an open system, a definite packing density of the cells must be ensured, and this can be done by using porous frameworks impregnated with cells [8]. The formation of typical bone tissue in organ cultures of fragments of mouse bone marrow has recently been obtained [2, 9].

The aim of this investigation was to study the possibility of bone tissue formation in organ cultures of suspensions of disaggregated bone marrow cells.

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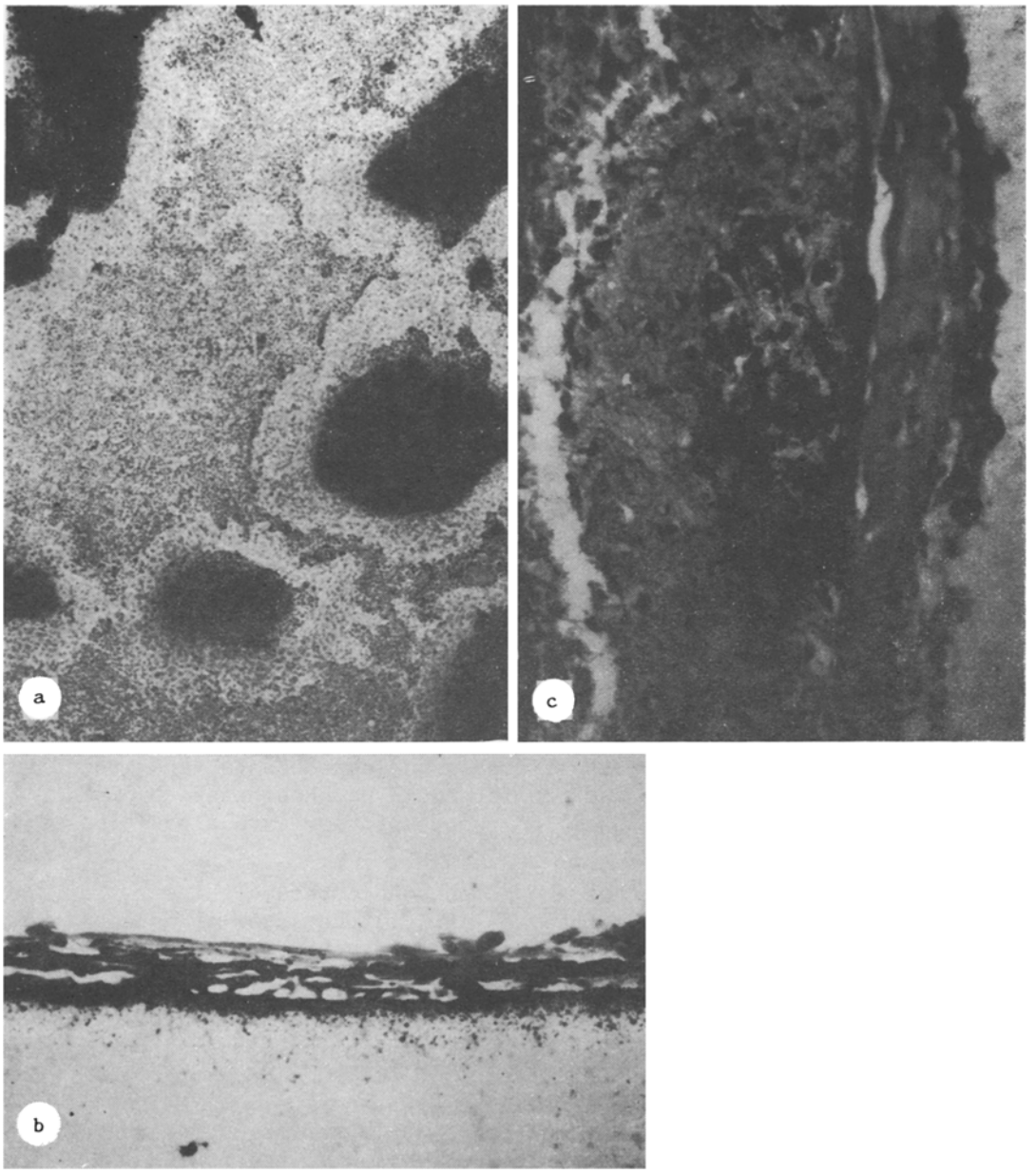

Fig. 1. Cultures of mouse bone marrow cell suspension. a) HAWP filter: total preparation (33rd day of culture); b) HAWP filter: section (36th day of culture); c) AUFS filter: section (29th day of culture). Von Kossa's stain, hematoxylin. Magnification: a) 100, b) 240, c) 400.

EXPERIMENTAL METHODS

The femora of (CBA × C57Bl)F_1 mice weighing 18-22 g were used. Bone marrow cell suspensions were prepared by two methods: by mechanical dissociation and by trypsinization [7]. In both cases the suspensions were free from cell aggregates as a result of filtration through a four-layer Kapron filter. The cells were cultured on the boundary between two phases by the method of multiple organ cultures [1]. Millipore filters with different pore diameters were used: HAWP (0.45 μ), RAWP (1.5 μ), and AUFS (0.6-0.9 μ). Gelatin sponge (Gelfoam sponge) was applied above each filter in the form of a film 0.5-1 mm thick, with an area of 25-50 mm^2. The cell suspension was applied to the sponge by means of a glass capillary tube. The number of nucleated bone marrow cells explanted into one sponge was $(0.5\text{-}2)\cdot 10^7$. Platforms with filters on them were placed in Conway dishes, in which they were covered with culture medium of the following composition: 80% MEM medium in the α-modification, 20% fetal calf serum, with additives: L-glutamine (0.5 mg/ml), glucose (4 mg/ml), and vitamin C (0.15 mg/ml), and antibiotics: penicillin and streptomycin (60 U/ml of

each). Starting with the 6th-15th day of culture, sodium β-glycerophosphate was added to the medium in a concentration of 10 mM. Culture was carried out at 37°C in an atmosphere consisting of a mixture of air with 5-7% CO_2 and saturated with water vapor. The culture medium was completely changed twice a week. The cultures were fixed on the 2nd-39th day with 96% ethanol. Total preparations were obtained from some cultures, serial paraffin sections from the rest. Both were stained with alum-hematoxylin for alkaline phosphatase by Gomori's method and for insoluble calcium salts by Von Kossa's method.

EXPERIMENTAL RESULTS

During culture of $(1.5\text{-}2)\cdot10^7$ cells of a trypsinized bone marrow suspensin on HAWP filters a considerable number of hematopoietic cells migrated from the sponge in the first few days. By the beginning of the second week of culture most of the hematopoietic cells had degenerated, and the predominant cells on the filter were not macrophages, among which single fibroblasts and multinuclear cells appeared. The fibroblasts proliferated intensively and by the end of the second week they had become the predominant cells, and they were arranged on the filter in foci or bands, and in some places formed continuous sheets. In the intervals between the fibroblasts lay macrophages, and on the surface of the fibroblast layer, foci of myeloid cells were located in some cultures. By this time the gelatin sponge had partly absorbed, and the more intensively cell proliferation took place in the culture the faster this occurred.

By the end of the 3rd week of culture certain areas of the fibroblastic layer had become significantly thicker and some of the cells in them had changed from spindle-shaped into polygonal, thereby acquiring the morphology of osteoblasts. By the 21st-23rd day these cells had begun to lay down ground substance, which by the 26th day had become partially mineralized, with the accumulation of insoluble calcium salts. In total preparations stained by Von Kossa's method deposits of calcium salts at these times appeared as a scattering of tiny granules of metallic silver. Fibroblasts, osteoblasts, and ground substance in zones of thickening gave a positive reaction for alkaline phosphatase by Gomori's test. Among fibroblasts surrounding these zones, only some of them gave a positive stain for alkaline phosphatase.

By the 28th-30th day of culture the ground substance became thicker and disk-sphaped, with the formation of cavities containing osteocytes in it. The layer of osteoblasts with ground substance grew out above the spindle-shaped fibroblasts. Deposition of calcium salts increased: in the uppermost areas of ground substance they appeared in the form of tiny, delicate granules, whereas in deeper areas lay regions of larger, coarser granules. Zones of osteogenesis were partly surrounded by numerous giant multinuclear cells. In the latter stages (33rd-36th days of culture) the zones of osteogenesis were even thicker and acquired the shape of bony trabeculae and scales (Fig. 1a).

In transverse sections these structures had a characteristic appearance. Immediately next to the filter there was a layer of cubical osteoblasts, the processes of which penetrated deeply into the pores of the filter. Above this layer and parallel to the surface of the filter there was a layer of ground substance, whose thickness in the central zones reached 10-20 μ, the numerous cavities of which contained osteocytes. Above this again was a single layer of osteoblasts which, in turn, was covered by a layer of spindle-shaped fibroblasts.

The ground substance of the newly formed bone was intensively mineralized (Fig. 1b) and calcium salts also impregnated the upper part of the filter to a considerable depth (to half its thickness). In the zones of osteogenesis the ground substance, all the layers of cells, and the cellular processes insinuating into the pores of the filter, exhibited high alkaline phosphatase activity by Gomori's test (although the processes were stained to a much smaller depth than the deposits of insoluble calcium salts).

The development of bone tissue in the system described above thus obeys the same rules and proceeds through the same sequence of stages as in cultures of mouse bone marrow fragments on HAWP filters [2, 9]. It will be noted, however, that during culture of suspensions, the proportion of "positive" cultures in which bone tissue formed was definitely less, and amounted to not more than half of all the explants. Intensive proliferation of fibroblasts was observed in the remaining cultures but there was no thickening or deposition of ground substance. With a decrease in the number of explanted cells [to $(0.5\text{-}1)\cdot10^7$ cells of trypsinized suspension per sponge], or when suspensions obtained by the mechanical method were used ($2\cdot10^7$ cells per sponge), the proportion of cultures with osteogenesis was reduced even more.

Another difference between the system described and cultures of bone marrow fragments is that deposition of ground substance and its mineralization took place later: on the 20th-23rd and 26th-28th days compared with the 16th-19th days for cultures of fragments.

The third difference relates to the thickness of the bone lamina formed: in a considerable proportion of cultures of cell suspensions the ground substance of the newly formed bone was significantly thinner than in cultures of bone marrow fragments, but deposition of insoluble calcium salts in the filter was much more intensive.

When RAWP filters were used as the cultural substrate, cell growth took place on both surfaces of the filter. Most cells were macrophages, contamination by fibroblasts was very slight, and the zone of growth remained single-layered, without any thickenings or deposition of ground substance.

In cultures on AUFS filters the cells not only covered the filter on both sides, but also penetrated throughout its thickness. Toward the end of the 3rd week of culture a multilayered bony structure formed on the top of the filter. Immediately next to the filter there was a thick (up to 30 μ) lamina of ground substance of bony tissue with osteocytes and an osteoblastic layer. Insoluble calcium salts were not found in this ground substance however, in the thickness of the filter there was yet another region of ground substance, less compact, but well mineralized. This ground substance was of considerable thickness (up to 80 μ), it included osteocytes, and was surrounded by osteoblasts (Fig. 1c).

The culture system thus developed can be used to study osteogenic potential of bone marrow cell suspensions and it shows that, just as under transplantation conditions in vivo, the realization of this potential necessitates preservation of the original structure of the medullary tissue. An advantage of this system over cultures of bone marrow fragments is that it permits a quantitative approach to the analysis of osteogenesis in vitro, by the use of explantation of different numbers of bone marrow cells. If the prospects of obtaining analogous results with human bone marrow are taken into account, there is evidently a real possibility that bone marrow obtained by puncture can be used to study osteogenesis.

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