A STUDY OF THE PHAGOCYTIC PROCESS IN VITRO IN A CULTURE OF BONE MARROW HISTIOCYTES

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(Presented by Active Member of the Akad. Med. Nauk SSSR V. L. Troitskii)
Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 53, No. 1, pp. 74 - 76, January, 1962
Original article submitted March 18, 1961

Until recently, the tissue culture method was used very rarely for studies of the interactions between microbes and macrophages. By accepted practice, the most convenient source for obtaining mammalian macrophages was considered to be exudates from the peritoneal cavity. For this purpose, animals were injected intraperitoneally with various substances (mineral oil, soluble purified casein, glycogen), causing an accumulation of exudate in the peritoneal cavity, consisting mainly of monocyte type cells [12, 15, 16, 17].

It was shown that cells of the monocyte—macrophage type can also be obtained without preliminary treatment of the animals (mice) by washing out their peritoneal cavities with a buffer solution [13, 18].

Earlier, we showed that it was possible to obtain a single-layered culture of macrophages from bone marrow cells, using the No. 199 medium with serum [10].

The purpose of this work was to investigate the possibility of using the bone marrow as a source of histiocyte-macrophages, for the study of phagocytosis in a tissue culture. Before us also stood the particular problems connected with developing methods for appraising how fully the in vitro reproduced phagocytic process is accomplished.

It was shown earlier that in a number of cases, under natural conditons, it is possible to trace the fate of phagocytized microbes, provided the latter contain a histochemically demonstrable material as a tag [5, 8, 9]. The intercellular polysaccharides that are synthesized by certain microbes may serve as this material. Because of this, we settled on Cl. butyricum, which contains a natural tag in the form of an intracellular polysaccharide, demonstrable by the Hotchkiss histochemical reaction (Schick reaction) [14] and by staining with lugol. In addition, this microbe is an obligatory anaerobe, which eliminates the danger of the microbes multiplying in the culture fluid when inoculated into the tissue culture, even in the absence of antibiotics within the fluid.

EXPERIMENTAL METHOD

The bone marrow was washed out of the diaphyses of guinea pig femurs into a culture fluid containing heparin. The homogeneous suspension of cells was diluted in the medium to the necessary concentration (400,000—620,000 cell bodies per ml) and 2 ml portions were transferred to test tubes. The cultures were grown at 36—37 deg in test tubes measuring 100x16 mm, with a one-sided depression of the wall to permit placement of an 11x32 mm cover glass. The composition of the culture medium was: 20% normal beef serum, 80% medium No. 199 with antibiotics (50 units / ml of streptomycin and the sodium salt of penicillin). The medium was replaced at intervals of 1—2 days.

We also cultivated cells that were obtained from washings of the peritoneal cavity of pigs and mice. After 2—3 days, the cell growth took on an appearance characteristic of histiocytes.

Inoculation of the macrophages was carried out with a washed suspension of a 24-hour Cl. butyricum culture, grown under anaerobic conditions at 37 deg on a potato-water medium containing calcium carbonate. Occasionally we also used Bac. anthracoides. For the inoculation, we used a suspension containing 70 million microbial bodies per ml. Prior to the inoculation, the tissue culture was washed with Earl's solution, and then we added 2 ml of the microbe suspension in medium No. 199 without antibiotics or serum. After 1 hour at 37 deg, the microbe suspension was removed, and culture medium was poured in. At 1—4 hours and 1—3 days after the inoculation, several of

the cultures were placed in fixative, including some from the inoculated and uninoculated (control) groups. Other controls consisted of cultures of macrophages that were killed by heating (at 52 deg for 10 minutes) 1 hour after the inoculation, and cultures of fibroblasts from guinea pig embryonal heart, inoculated and fixed at the same time as the macrophage cultures. Prior to staining, each cover glass, with its single-layered macrophage culture, was cut into two portions, one of which was stained with hematoxylin and counterstained according to Gram–Weigert [1], and the other–according to Hotchkiss. The cultures were fixed in Bouin's solution.

EXPERIMENTAL RESULTS

By the 4–6th day, the growth from the bone marrow cultures consisted primarily of monotypic cells, corresponding in their morphology to the histiocytes or polyblasts (functionally–macrophages) of Maximov, described by a number of authors in both in vitro and in vivo experiments [3, 4]. Judging by their morphological properties and their phagocytic activity, the cells of the histiocyte–macrophage type which we obtained from different sources (bone marrow and peritoneal cavity) were identical. This does not conflict with the position held in the literature on the nature and derivation of cells of the histiocyte [2, 4, 6].

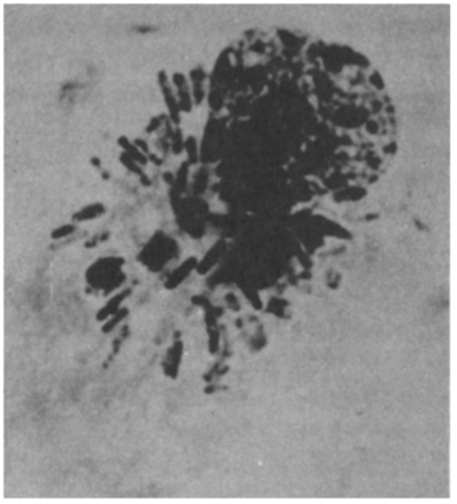

Fig. 1. Five day old culture of bone marrow macrophages, one hour after inoculation with Cl. butyricum. PAS-positive microbial cells, radially distributed in the cytoplasm of a macrophage. PAS- reaction–hematoxylin. Obj. 100x, ocul. 10x.

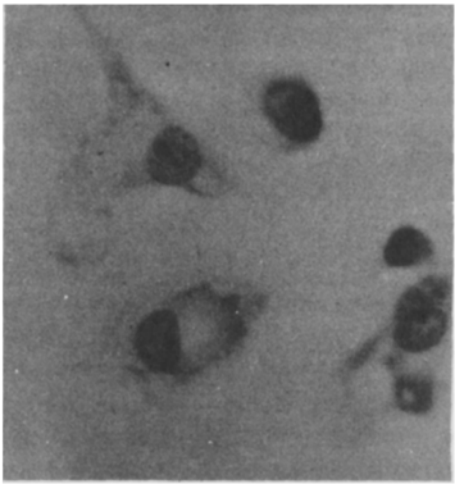

Fig. 2. Five day old culture of bone marrow macrophages, 45 hours after inoculation with Cl. butyricum. Terminal phase of phagocytosis – complete digestion of the microbial cells. Absence of staining in the paranuclear zone. Hemaotxylin–Gram-Weigert. Obj. 45x, ocul. 10x.

However, in using bone marrow cultures, several times (10–15) fewer animals and less time is required than in working with cultures of peritoneal cells.

Within ½–1 hour after inoculation, almost all the macrophages contain a varying number of phagocytized microbes–from 1–2 to 20–30 and more. The consumption is seen in both the mononucleated and the bi–trinucleated and gigantic multinucleated cells. Curiously, in a number of cases the microbes show a characteristic, orderly arrangement within the phagocytes. This pertains only to Cl. butyricum, which are completely digested by the macrophages, and not to Bac. anthracoides, which, although actively phagocytized, are not digested. Fig. 1 shows that the microbes are, in a definite manner, orientated about a certain paranuclear center. In these "centers" themselves, one most frequently encounters agglomerates of phagocytized microbes and gram-positive granules, which also take the Hotchkiss stain. In certain cases, one clearly sees the distribution of the microbes and the products of their digestion within alimentary vacuoles of the phagocytes. It should be noted that with the Hotchkiss procedure, the paranuclear zone is the area of most intense staining. However, in contrast to the periphery of the cell, this zone does not stain with hematoxylin (Fig. 2).

In the control, uninoculated cultures, it was also possible to encounter intracellular granules, but these did not take the stain for polysaccharides. These granules are the result of digestion of leukocytes and other cellular

fragments that are entrapped by the macrophages. Capture and digestion of single leukocytes is a picture which may be encountered under natural conditions and in inoculated cultures. Using different methods of staining, one can establish the nature of the aforementioned intracellular inclusions.

At 1–3 days after inoculation of the histiocytes with Cl. butyricum, complete digestion of the phagocytized microbes occurs (see Fig. 2).

There is no doubt that in this case active digestion of the microbes by the macrophages takes place. In the control cultures of macrophages that were killed by heating, the Cl. butyricum survives (Fig. 3). A large number of undigested microbes are observed in the fibroblast cultures from the guinea pig embryonal heart.

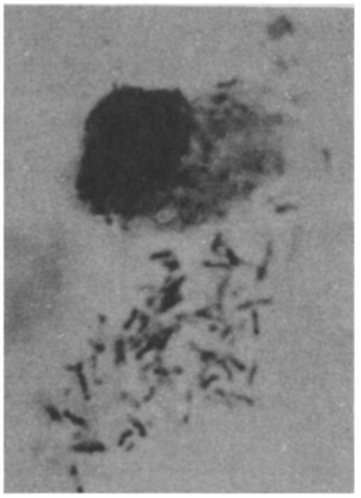

Fig. 3. Five day old culture of bone marrow macrophages, killed by heating 1 hour after inoculation with Cl. butyricum. Disintegrating macrophage with surviving microbial cells, 45 hours after inoculation. Hematoxylin–Gram-Weigert. Obj. 100x, ocul. 10x.

The tissue culture method does not yet enjoy widespread application in microbiology and immunology. Only this fact can explain why such a convenient source of macrophages as the bone marrow is not utilized for the study of macrophage phagocytosis.

In the present state of our knowledge, we still do not know the answer to one of the basic questions of natural immunity, specifically: which factors are responsible for the rapid death of some microorganisms within the phagocytes, and which permit others to survive and multiply in the intracellular milieu [11].

The material presented in this work shows outlook for using cultures of bone marrow histiocytes (macrophages) for analyzing the phagocytic process. Apart from the conveniences in method which are created by the use of anaerobic clostridia, containing histochemically demonstrable polysaccharides, for the test microbe, the expeditious nature of using saprophytic microbes in similar investigations should be especially emphasized.

One must agree with B. P. Tokin that, in the study of natural defense properties of an organism, "one must never limit experimentation only to parasites, only to pathogenic forms. This follows from more general concepts of immunity" [7].

SUMMARY

A study was made of the phagocytic process effected by the macrophages obtained in vitro from the bone marrow or by washing of the peritoneal cavity of guinea pigs. As shown, the bone marrow histiocytes are a convenient model for studying the macrophageal phagocytosis in vitro Cl. butyricum, a saprophytic microorganism, is used as an object for phagocytosis. A natural label in the form of histochemically detectable intracellular polysaccharide contained in this organism makes it possible to trace the fate of the phagocytized microbial material and to assess the degree of the phagocytic process's completion. Apart from the aforementioned positive aspects the use of such test-microbes has also the advantage of their being obligatory anaerobes; therefore, they do not multiply in the tissue culture and do not require for their depression the use of antibiotics, which is not always desirable.

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