LITERATURE CITED

1. V. N. Babichev and E. I. Adamskaya, Probl. Éndokrinol., No. 4, 44 (1976).
2. V. N. Babichev, L. N. Sidneva, and O. V. Abramova, Fiziol. Zh. SSSR, 68, No. 11, 1573 (1982).
3. A. Barbarino and L. De Marinis, J. Clin. Endocrinol., 51, 749 (1980).
4. M. D. Bates and P. M. Conn, J. Endocrinol., 115, 1380 (1984).
5. P. M. Conn and D. C. Rogers, J. Endocrinol., 105, 1122 (1979).
6. P. M. Conn, D. C. Rogers, and S. G. Seay, J. Endocrinol., 113, 1592 (1983).
7. P. M. Conn, A. J. W. Hsueh, and W. F. Crowley, Fed. Proc., 43, 2351 (1984).
8. G. Fink, A. Curtis, C. E. Lewis, et al., J. Endocrinol., 104, Suppl. 1, 10 (1985).
9. F. Kamel and L. C. Krey, Mol. Cell. Endocrinol., 32, 285 (1983).
10. J. Marian and P. M. Conn, Mol. Pharmacol., 16, 196 (1979).
11. S. Ozawa and O. Sand, Experientia, 34, 542 (1978).
12. S. Ozawa and S. Miyazak, Jpn. J. Physiol., 29, 411 (1979).
13. H. Rasmussen and P. Q. Barrett, Physiol. Rev., 64, 938 (1984).
14. W. A. Smith and P. M. Conn, J. Endocrinol., 114, 553 (1984).
15. P. S. Taraskevich and W. W. Douglas, Proc. Natl. Acad. Sci. USA, 74, 4064 (1977).
16. J. A. Williams, Fed. Proc., 40, 128 (1981).

CLONAL NATURE OF FIBROBLAST COLONIES FORMED BY BONE MARROW STROMAL CELLS IN CULTURE

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UDC 612.419.014.2:612.6]-085.23

KEY WORDS: bone marrow, stroma; cloning

After explantation of suspensions of hematopoietic cells into monolayer cultures the stromal colony-forming units (CFU_f) present in the bone marrow form discrete fibroblast colonies [3, 8-10].

In mixed cultures of bone marrow cells from male and female guinea pigs, dividing fibroblasts of each stromal colony have a karyotype which is only male or only female, in agreement with the hypothesis of the clonal nature of the colonies [3]. However, this has been demonstrated for cells of autobred animals and the possibility cannot be ruled out that progenies of several syngeneic, but not of allogeneic CFU_f, are combined in the colony.

The aim of this investigation was to study whether colonies of fibroblasts formed by CFU_f are in fact cell clones. Experiments were carried out to take into account three possible sources of error when interpreting the results of fibroblast karyotyping in mixed cultures, namely: cells of stromal aggregates, not dissociated into separate cells, may be found in the explanted suspensions, only syngeneic but not allogeneic fibroblasts may be combined into common colonies, and colonies may be contaminated by macrophages and hematopoietic cells.

EXPERIMENTAL METHOD

Bone marrow cells from CBA and CBAT6T6 mice were used for explantation. Contents of the medullary cavity of the femora were expelled by means of a syringe into medium DMEM with 15 mM HEPES, and single-cell suspensions were prepared either by mechanical disaggregation (passage through syringes with needles of diminishing diameter), or by trypsinization [2]. All cell suspensions were filtered through four layers of kapron and centrifuged for 10 min at 400g; the cell residues were resuspended in fresh culture medium. The efficiency

N. F. Gamaleya Research Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR P. A. Vershilova.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 103, No. 3, pp. 356-358, March, 1987. Original article submitted July 8, 1985.

 0007-4888/87/0003-0402$12.50

of stromal colony formation (ECF_f) was determined in glass flasks the bottom of which had an area of 40 cm^2, the culture medium consisting of DMEM medium with 15 mM HEPES and 20% embryonic calf serum with gaseous phase of 5% CO_2 in air [4]. To act as a feeder, $2 \cdot 10^7$ guinea pig bone marrow cells, irradiated in a dose of 60 Gy (^{60}Co) were added to the cultures. To discover whether aggregates of stromal cells were present in the explanted cell suspensions, 10^5 of these cells was introduced into T25 plastic flasks, covered with poly-L-lysine. After 1 h the flasks were fixed, stained by Giemsa's method, and examined under the microscope.

The intensity of migration of stromal fibroblasts in the cultures was estimated under conditions when the bone marrow cells at the time of explantation were able to adhere only to part of the surface of the flasks. For this purpose, drops of medium DMEM in a volume of 0.2 ml, containing 10^4-10^7 bone marrow cells, were introduced into the dry flasks. After 2 h the drops were withdrawn, their outlines traced, the cultures thoroughly washed, and covered with complete nutrient medium (α-MEM with 20% embryonic calf serum), covering the whole surface of the flask, and incubated at 37°C in an atmosphere of air with 5% CO_2. The cultures were fixed with ethanol and stained by Giemsa's method on the 12th day.

To obtain cultures for karyotypic analysis $(0.3\text{-}5) \cdot 10^5$ bone marrow cells, treated beforehand with carrageenan (400 μg/ml for 30 min at 37°C) were explanted into Leighton's tubes with coverslips. After 16 h the cultures were washed to remove nonadherent cells and the medium replaced by fresh. Culture continued in the presence of the above-mentioned feeder ($4 \cdot 10^6$ cells per sample). After 8 days the cultures were again treated with carrageenan, after which they were added for 17 h to medium containing 3.2 mM thymidine, which was then replaced by fresh medium, to which colcemid (0.5 μg/ml) was added 4 h later for 4 h. The cultures were then treated for 30 min with PBS, diluted with water (1:3), fixed with methanol and acetic acid, and stained by Giemsa's method.

The rosette formation test with sheep's red blood cells, sensitized with IgG, was carried out in cultures grown on coverslips [7].

EXPERIMENTAL RESULTS

When 10^5 filtered bone marrow cells were introduced into flasks covered with poly-L-lysine, all the cells were adherent after 60 min. Among them there were aggregates of 8-12 cells in the flasks. The aggregates were islets consisting of three or four proerythroblasts, and they did not contain stromal cells. Thus the concentration of stromal aggregates among the filtered bone marrow cells was under 10^{-5}.

ECF_f for cells of the mechanically deaggregated bone marrow averaged $7 \cdot 10^{-5}$, compared with $11 \cdot 10^{-4}$ for cells of trypsinized bone marrow. Hence it follows that if some of the colonies are formed as a result of stromal aggregates entering the cultures, there must have been fewer than 14% of these colonies in the case of explanation of cells from the mechanically deaggregated bone marrow and fewer than 1% in the case of trypsinized bone marrow.

In mouse bone marrow cultures aged 10-12 days fibroblast colonies contained from 10 to 30% of macrophages, depending on the original explantation density. Macrophages located outside the colonies and on their territory formed rosettes with sheep's red blood cells sensitized with IgG, whereas fibroblasts did not form rosettes.

Contamination of the carrageenan-treated cultures with macrophages, used for chromosome analysis was on an extremely small scale — not more than one rosette-forming cell was found in 7% of the colonies. Hematopoietic cells also were virtually absent from the cultures. It could therefore be firmly concluded that all cells of the colonies being typed were fibroblasts which, being syngeneic, differed in their chromosome sets.

Karyotypes of dividing cells were identified in 18 colonies in mixed (1:1) cultures of CBA and CBAT6T6 cells. In each colony from five to eight metaphase plates were typed: 12 colonies contained only CBA plates, six colonies only CBAT6T6. Under these circumstances, two tetraploid metaphase plates of the same karyotypes were found in one of the colonies with cells of the CBA karyotype. Consequently, within each colony all the metaphase plates tested belonged to the same karyotype. Consequently, most fibroblast colonies (at least 99% in cultures of trypsinized, and 86% in cultures of mechanically deaggregated bone marrow) in the colony formation test were cell clones.

The possibility that stromal fibroblasts can be cloned during explantation of bone marrow cells into monolayer cultures is evidently based on the fact that no significant mig-

ration of CFU_f and their progenies takes place in such cultures. This was demonstrated by experiments with adhesion of cells to part of the surface of the culture flasks. When drops of cell suspensions were placed on the surface of the dry flasks they spread over an area of about 3.1 cm^2. Subsequent culture for 12 days in medium covering the whole surface of the flask led to the formation of a confluent layer of fibroblasts within the outlines of the drops, which initially contained 10^6 cells or more, whereas inside drops containing $5 \cdot 10^4$ cells, discrete foci of fibroblasts were formed. Outside the limits of the drops macrophages were distributed, but no colonies of fibroblasts nor single fibroblasts were found outside the boundaries of the drops.

Bone marrow CFU_f are thus clonogenic cells and the colonies of fibroblasts formed by them in monolayer cultures are cell clones. It was shown previously that CFU_f possess high proliferative potential [1] and that cells of one colony form all types of bone marrow stromal mechanocytes — reticular, fat, and bone cells, thus enabling the formation of bone marrow organs to take place, in which the hematopoietic microenvironment is established [6]. It can therefore be concluded from the clonal nature of the colonies of bone marrow fibroblasts that stromal stem cells belong to the CFU_f category [1].

LITERATURE CITED

1. I. V. Keilis-Borok, N. V. Latsinik, S. Yu. Epikhina, and A. Ya. Fridenshtein, Tsitologiya, No. 11, 1402 (1971).
2. N. V. Latsinik, S. Yu. Sidorovich, and A. Ya. Fridenshtein, Byull. Éksp. Biol. Med., No. 9, 356 (1981).
3. A. Ya. Fridenshtein, R. K. Chailakhyan, and K. S. Lalykina, Cell Tissue Kinet., 3, 393 (1970).
4. A. Ya. Fridenshtein, Yu. F. Deriglazova, N. N. Kulagina, et al., Exp. Hematol., 2, 3 (1974).
5. A. Ya. Fridensthein, Int. Rev. Cytol., 47, 327 (1976).
6. R. K. Chailakhyan, Yu. V. Gerasimov, and A. Ya. Fridenshtein, Byull. Éksp. Biol. Med., No. 2, 705 (1978).
7. T. I. Goude, C. Schotte, and R. van Furth, J. Exp. Med., 142, 1180 (1975).
8. D. Metcalf, J. Cell. Physiol., 80, 409 (1972).
9. A. H. Piersma, R. E. Ploemacher, and K. G. M. Brockbank, Br. J. Haematol., 54, 285 (1983).
10. F. D. Wilson, L. O'Grady, C. J. McNeill, and S. L. Munn, Exp. Hematol., 2, 343 (1971).
11. C. X. Xu, J. H. Hendry, N. G. Testa, and T. D. Allen, J. Cell Sci., 61, 453 (1983).