Colonization of Lipid-Laden Cells from Aortic Intima of Cholesterol-Fed Rabbits (36016)

H. YAMAMOTO, S. C. SPRARAGEN, AND H. HAMEL
(Introduced by M. L. Littman)
Nuclear Medical Service, Veterans Administration Hospital, Brooklyn, New York 11209

The cultivation of lipid-laden cells from atherosclerotic lesions of cholesterol-fed rabbits has been associated with great difficulties (1). The lipid-laden cells exhibited little or no proliferative activity and were rapidly overgrown by the faster-growing fibroblastic cells. The recent observations of Tume *et al.* (2) agreed with the foregoing in that foam cells were unable to form colonial growth in culture.

This paper reports on the first successful colonization of lipid-laden cells from single cell suspensions prepared from aortic intima of cholesterol-fed rabbits using collagenase and describes the culture conditions necessary for colonial growth of these cells.

Materials and Methods. The entire aorta was removed from adult New Zealand white female rabbits prefed 150 g of Special Purina Rabbit Chow pelleted with 500 mg of cholesterol (Gen. Biochem.) daily for 15 to 20 weeks. The animals were killed by air embolism. The cell suspensions from the aorta were prepared by stripping the intimal layer and the segments were cut into pieces 1–2 mm^2. The pieces were placed in dissociating medium of 10% fetal bovine serum in calcium and magnesium-free medium F12 saline and 5 mg/ml of collagenase (Nutritional Biochem. Corp.) incubated at 37° for 1–1.5 hr with occasional agitation. The resulting suspension was centrifuged at 400 rpm for 30 sec to remove tissue fragments. The remaining suspension was centrifuged at 1200 rpm for 3 min. The supernatant was discarded and the cells were resuspended in clonal medium. The cells were again centrifuged at 1200 rpm for 3 min. The supernatant was discarded and the cells were resuspended in clonal medium and counted in a hemacytometer. The final cell suspension contained $15–18 \times 10^6$ cells/ml.

The clonal medium consisted of a combination of 40% medium F12 (3) (Grand Isl. Biol. Co.) and 40% Eagle's minimum essential medium (4) (Grand Isl. Biol. Co.) supplemented with 10% rabbit serum (Flow Lab.) and 10% fetal bovine serum (Flow Lab.). Penicillin-G, 100 units/ml; streptomycin sulfate, 100 μg/ml; and amphotericin B, 2.5 μg/ml were used in all solutions including the clonal medium.

Cell suspensions were diluted in clonal medium to the desired cell concentrations and were inoculated into 60 mm plastic petri dishes (Falcon Plastics) containing 5 ml of prewarmed equilibrated clonal medium. The cultures were incubated at 37° in an atmosphere of 5% carbon dioxide in air with 80% humidity. After 14 days incubation, the clonal medium was removed and the colonies were fixed with 10% neutral formalin and stained with Giemsa and Sudan black B for direct visualization. Isolation colonies were picked by means of the steel cylinder (5) and cover-slip (6) techniques.

Results. The collagenase digestion technique yielded a suspension of $15–18 \times 10^6$ single cells. In early experiments, 1.0 mg/ml of collagenase was preceded by 0.2% trypsin. Subsequently a mixture of 0.1% trypsin and 2.5 mg/ml of collagenase was used. Experiments to determine the dissociation contribution of trypsin indicated that better yields were not obtainable by the addition of trypsin. Hence collagenase alone was used for primary dispersion of cells.

The initial objective was to determine the constancy of plating efficiencies so that planting levels of sufficient dilution could be established that would yield isolated colonies. If successful, this would relieve the slow-growing endothelioid cells from the competition of the faster-growing fibroblastic cells.

TABLE I. Efficiency of Plating of Freshly Isolated Rabbit Atherosclerotic Lesions.[a]

Expt.	Inoculum (total no. of cells/plate)	No. of plates	Efficiency of plating (colonies/inoculated cell)
325	10.3×10^3	9	5.5×10^{-4}
347	2.1×10^4	9	7.0×10^{-4}
3257	3.4×10^4	8	7.6×10^{-4}
3471	4.2×10^4	7	8.1×10^{-4}
34	6.7×10^4	8	6.1×10^{-4}
225	9.0×10^5	7	7.3×10^{-4}
2257	16.9×10^5	7	4.7×10^{-4}

[a] Single cell suspensions were prepared with collagenase and cell concentration determined by hemacytometer count. The cells were plated, incubated at 37° in 5% carbon dioxide in air and 80% humidity for 14 days, then stained, and the colonies were counted.

Table I shows that the plating efficiency remained constant over the tested inocula range of 10.3×10^3 to 16.9×10^5 cells. This constancy holds at a relatively low inoculum size. Figure 1 is a photomacrograph of a culture of 9×10^5 single cells following a growth period of 14 days. Sixty-six discrete colonies were counted. Colony size varied from barely visible to 7 mm in diam. Colonies were divisible into 4 morphological types based on whether fibroblastic or endothelioid: Type 1, fibroblastic compact, spindle-shaped fibroblastic cells forming compact colonies (Fig. 2A). Type 2, fibroblastic diffuse, similar to type 1 cells but forming diffuse colonies (Fig. 2B). Type 3, endothelioid compact, flattened, endothelioid, polygonal cells forming colonies similar to type 1 (Fig. 2C). Type 4, endothelioid diffuse, similar to type 3 cells but forming colonies similar to type 2 (Fig. 2D). Type 4 cells contained large lipid granules which stained with Sudan black B (Fig. 3). Figure 4 is an electron micrograph of foam cells in similarly fed rabbit aortic intima revealing nucleus and lipid vacuoles.

Optimal colonial growth required the presence of an equal mixture of 10% rabbit serum and 10% fetal bovine serum. At a concentration of 20% fetal bovine serum only small colonies were obtainable. When 20% rabbit serum was used without fetal bovine serum no colonies were formed. A number of sera used for clonal growth, including horse, human, calf, and swine were tested for their ability to replace rabbit and fetal bovine serum. None of the tested sera was as effective as rabbit serum mixed in equal portions with fetal bovine serum.

Discussion. The previously reported attempt to obtain colonial growth of lipid-laden cells from atherosclerotic lesions of cholesterol-fed rabbits utilized defined medium originally designed for survival of mass populations of cells (2). Medium F12 used in these experiments was originally formulated as a completely defined medium for clonal growth (3). The nutrient concentrations are generally lower than for those media designed for growth of mass population of cells. The F12 medium has been used successfully in clonal growth of other primary tissues (7, 8).

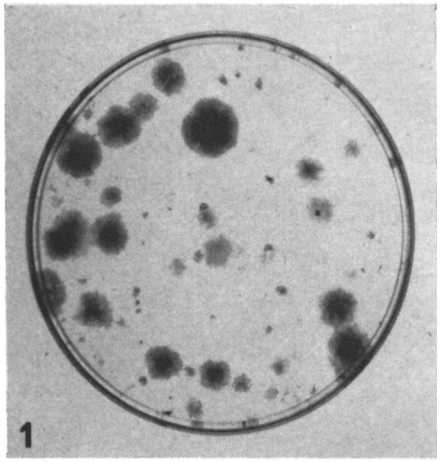

FIG. 1. Plate initiated with 9×10^5 single cells from atherosclerotic lesions of rabbits prefed cholesterol for 15 to 20 weeks. Formalin fixed and Giemsa stained after 14 days; ×1.

Although medium F12 with 10% rabbit serum and 10% fetal bovine serum was able to yield colonial growth, the addition of Eagle's minimum essential medium and identical serum supplements caused healthier colony formation. A number of other widely employed media for clonal growth, including Waymouth MB 752/1, medium 199, Puck N16, and Eagle's basal medium were tested. None of these were able to replace medium F12.

The superiority of mixed sera for primary

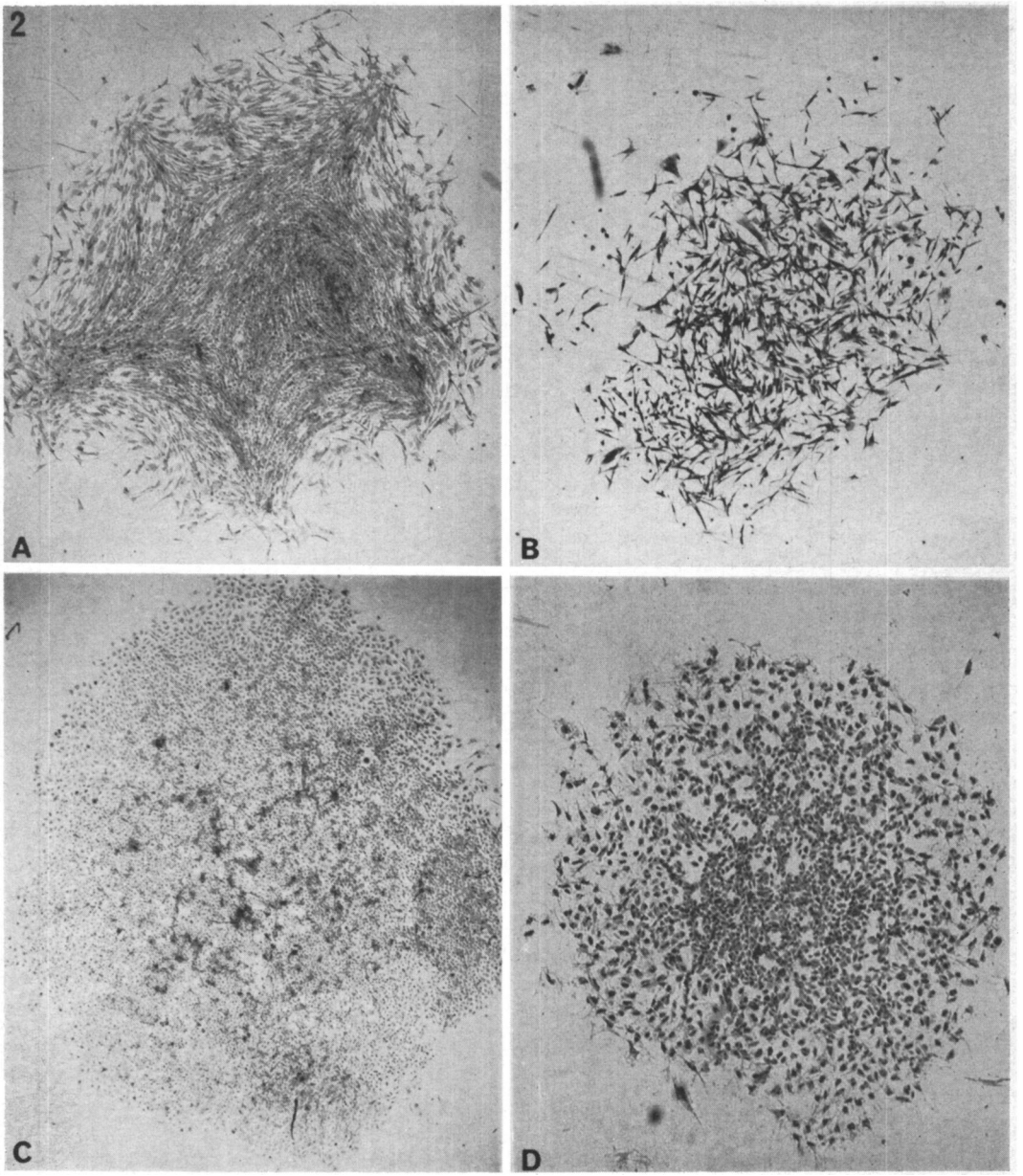

FIG. 2A–D. Colonies developed from single cell suspensions as in Fig. 1: formalin fixed and Giemsa stained after 14 days; ×17. (A); fibroblastic cells, compact (Type 1). (B) fibroblastic cells, diffuse (Type 2); (C) endothelioid cells, compact (Type 3); (D) endothelioid cells, diffuse (Type 4).

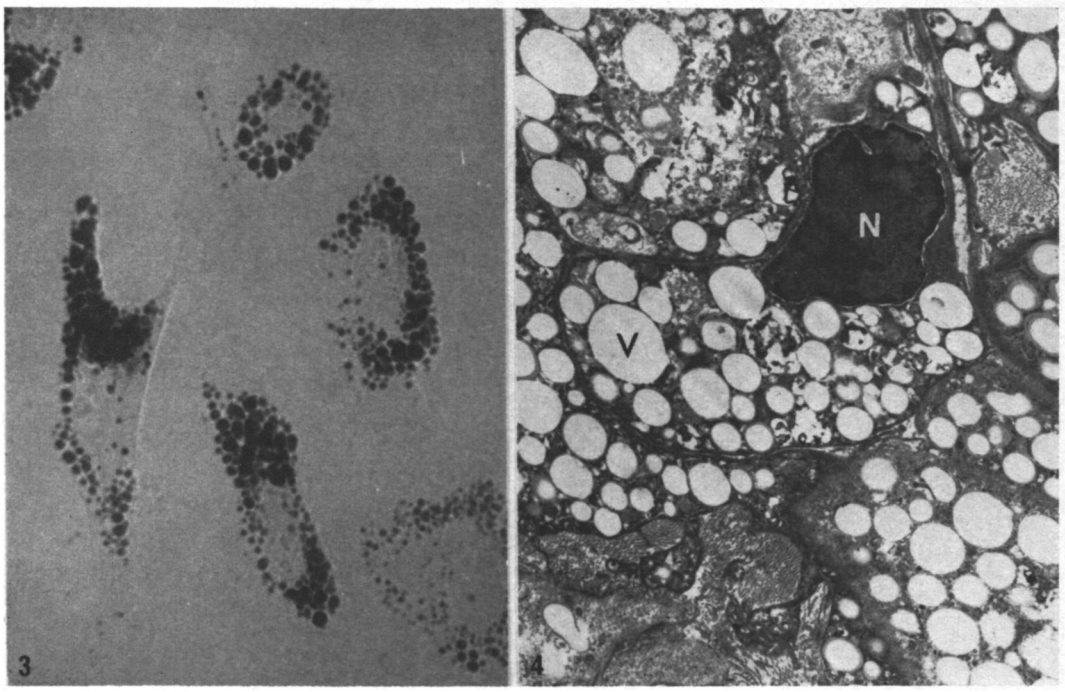

FIG. 3. Endothelioid cells from diffuse colony (Type 4). Formalin fixed and stained with Sudan black B after 14 days; ×506.

FIG. 4. Electron micrograph of foam cells in atherosclerotic lesions of rabbits prefed cholesterol for 15 weeks. Nucleus (N), lipid vacuoles (V); ×2389. (Tissue was fixed in 5% glutaraldehyde, postfixed in 2% osmium tetroxide, embedded in Epon, sectioned and double stained with uranyl acetate and lead citrate.)

colonial growth emphasizes the critical need for chemically defined media. Such media will permit precise analysis of the conditions necessary for growth of the lipid-laden cells.

These experiments differed in specific details from that of Tume *et al.* (2). The media employed were different. Tissue disaggregation was achieved with collagenase rather than with collagenase and elastase, and perhaps the difference in results was basically due to the handling of the initial cell suspension. Their methods required numerous long preparative manipulations which could contribute to degradation of cellular structures. The procedures used in our experiments were designed to avoid harsh and prolonged mechanical treatment. Manipulations outside the incubator were carried out with minimum handling in clonal medium and the inoculations were made in equilibrated prewarmed medium so that normal metabolism would resume quickly.

The demonstrated ability to obtain colonies of lipid-laden cells from atherosclerotic lesions of cholesterol-fed rabbits makes it possible to develop populations of foam cells which are known to be derived from specific cell types.

Summary. Successful colonization of lipid-laden cells were obtained from diluted single cell suspensions prepared from atherosclerotic lesions of cholesterol-fed rabbits using collagenase digestion followed by incubation in medium F12 and Eagle's minimum essential medium supplemented with equal proportions of rabbit and fetal bovine serum. Colonies of endothelioid cells containing Sudan black B staining particles appeared similar to foam cells in the rabbit aortic intima by electron microscopy.

The growth in culture of colonies of lipid-laden cells opens new possibilities for controlled cell studies on the origin of foam cells in atherosclerotic lesions.

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