

In Vivo Cultivation of Leukocytes in Diffusion Chambers: Requirement of Ascorbic Acid for Differentiation of Mononuclear Leukocytes to Fibroblasts

By NICHOLAS L. PETRAKIS

RECENT EXPERIMENTS dealing with the *in vivo* cultivation of leukocytes in subcutaneous diffusion chambers have demonstrated that certain mononuclear leukocytes (lymphocytes and/or monocytes) from the blood of human adults, when so cultivated, have a "mesenchymal" capacity and are capable of undergoing differentiation to macrophages and to collagen-producing fibroblasts.¹ The cells possessing this capacity for differentiation are probably identical with those mononuclear leukocytes which undergo mitosis in tissue cultures of blood² and which become rapidly labeled with tritiated thymidine, *in vivo*.³

The present studies were undertaken to evaluate the role of ascorbic acid upon this system, since it is known that buffy coat leukocytes are rich in ascorbic acid, and that this vitamin is essential for normal phagocytosis, wound healing and collagen formation.^{4,5} Mononuclear leukocytes from both normal and scurvy guinea pigs were cultivated subcutaneously in diffusion chambers in vitamin-C deficient guinea pigs. Under these conditions, the chambers functioned as tissue culture vessels in host animals deficient of ascorbic acid, permitting an evaluation of the significance of leukocyte ascorbic acid in the differentiation of mononuclear leukocytes to fibroblasts.

MATERIALS AND METHODS

Young adult guinea pigs weighing 250 to 350 Gm. were made ascorbic acid-deficient by dietary restriction for at least three weeks. The diets consisted of rabbit pellets without supplementary greens, and water *ad lib*. With this diet, the plasma ascorbic acid levels fell from control values of 0.6 to 1.0 mg. per cent to levels of 0.2 to 0.3 mg. per cent in three weeks. Most of the animals succumbed to scurvy between the fourth and fifth weeks after beginning the diet. Blood was drawn by cardiac puncture from normal controls and ascorbic acid-deficient guinea pigs when the plasma ascorbic levels of the latter had fallen below 0.3 mg. per cent. Buffy coat leukocytes were obtained by gentle centrifugation of the heparinized blood, and aliquots of plasma containing approximately 50,000 leukocytes were sealed in the diffusion chambers. Differential leukocyte counts revealed no abnormal nor immature leukocytes in the blood of the ascorbic acid-deficient donor animals at three weeks with plasma ascorbic acid levels of 0.2 to 0.3 mg. per cent.

The diffusion chambers employed, similar to those described previously,¹ consisted of leucite washers covered by Millipore membranes (Type HA, 18 mm. diameter, 150 μ thick, with a pore size of $0.45 \mu \pm 0.05 \mu$). These were sealed to the washers with acrylic B-7 diluted 2:1 with 1,2 dichloroethane.

Diffusion chambers containing leukocytes from ascorbic acid-deficient animals and leukocytes from control animals were implanted aseptically in the subcutaneous tissue

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Submitted Apr. 12, 1961; accepted for publication June 14, 1961.

along the back of host guinea pigs deficient in ascorbic acid. The chambers were removed at two-day intervals over a period of 10 days following implantation. Fourteen experiments were conducted, which included four to six animals in each study. Four to eight diffusion chambers were implanted in each guinea pig. The following experiments were made: (1) Ascorbic acid-deficient leukocytes in ascorbic acid-deficient hosts; (2) normal leukocytes in ascorbic acid-deficient hosts; (3) ascorbic acid-deficient leukocytes in normal hosts; (4) normal leukocytes in normal hosts. Beginning ten days after implantation of the chambers, four scorbutic host guinea pigs bearing normal and ascorbic acid-deficient leukocytes were treated with 100 mg. ascorbic acid, subcutaneously, daily for five days, and were returned to normal diets. The membranes were stained with hematoxylin and eosin, and the pattern of differentiation was observed.

RESULTS

It was found that mononuclear leukocytes from guinea pigs deficient in ascorbic acid did not differentiate to fibroblasts in the manner previously described;¹ but within 5 to 7 days many colonies of hyperplastic, often strikingly abnormal cell forms were found to be growing over the surfaces of the membranes. These cells were characterized by marked nuclear enlargement, fine chromatin, and active mitoses (fig. 1). By 10 days, numerous, large, often bizarre, multilobular giant cells were noted which frequently contained multipolar and abnormal mitotic figures (fig. 2). The nuclei of these cells often measured up to 30 to 60 microns in diameter. Nucleolar enlargement was not prominent. Fibroblasts having less disturbed morphology were also found growing in the chambers, which cells tended to be larger and more immature

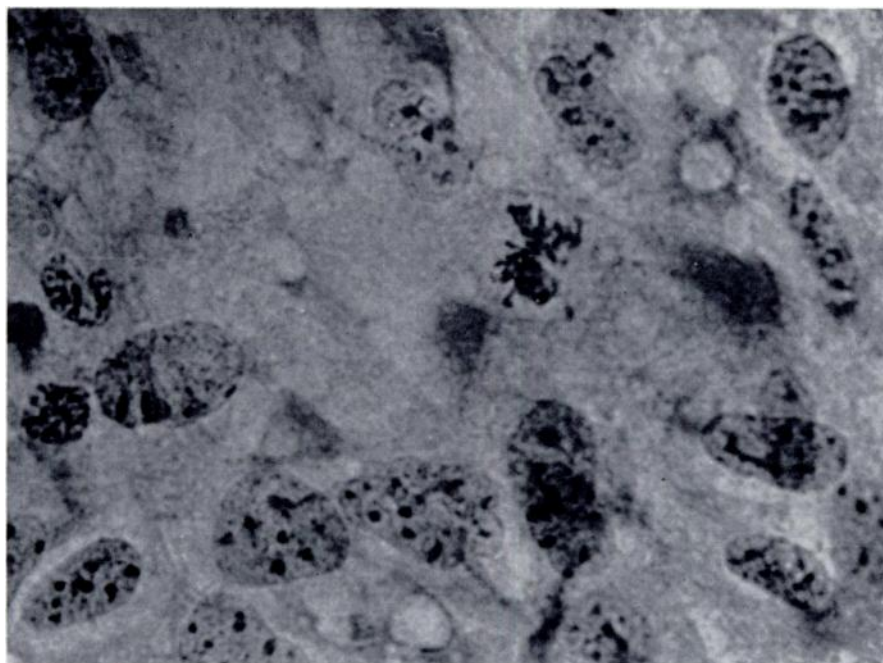


Fig. 1.—Abnormal differentiation of ascorbic acid-depleted leukocytes after 7 days cultivation (400 x).

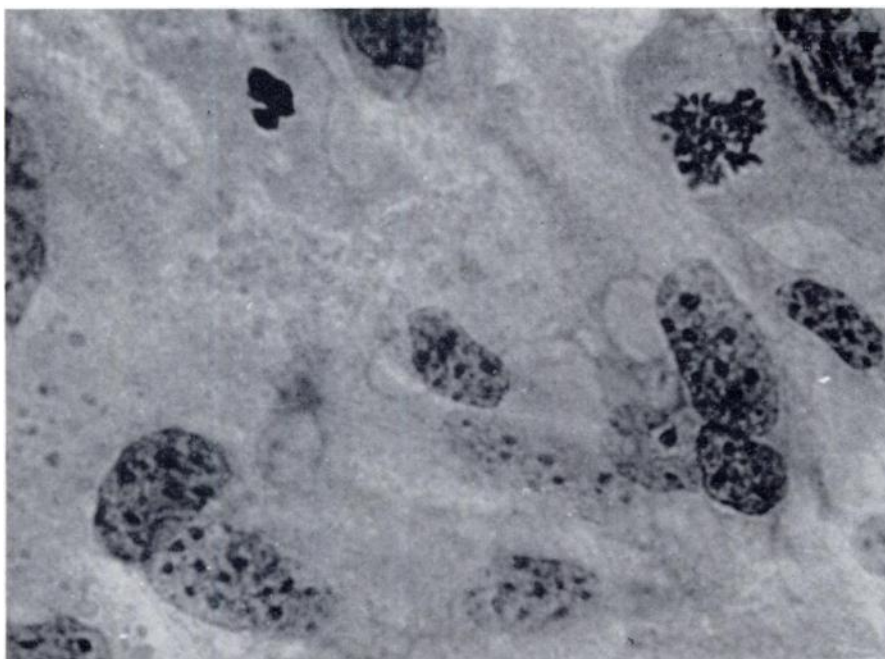


Fig. 2.—Bizarre forms after 10 days cultivation (400 x).

than "normally" seen. They appeared to be growing in a disorganized, non-oriented fashion.

The control mononuclear leukocytes from the normal non-deficient guinea pigs did not undergo this abnormal pattern of cellular development, but after five to seven days underwent differentiation to normally appearing fibroblasts in scorbutic and normal hosts (fig. 3). In several instances, the scorbutic host guinea pigs lived beyond 10 days following implantation of the chambers. In these longer lived animals, the control leukocytes developed additional changes in nuclear morphology resembling those found in the deficient leukocytes, which apparently resulted from the eventual depletion of their ascorbic acid content.

Interesting alterations in cellular morphology were found in the four scorbutic host guinea pigs which received ascorbic acid therapy. Concomitant with the recovery of the host animals from scurvy, the abnormal cells were not found in the chambers, but were replaced by fibroblasts having normal morphology (fig. 4).

In chambers from two of these animals, peculiar morphologic forms were found on the second day after treatment, consisting of cells having marked nucleolar enlargement (fig. 5). The finding of these changes in binucleated cells suggested that they represented degeneration forms of the disturbed ascorbic acid-deficient cells. At the seventh and 14th days after treatment, the remaining chambers were found to contain typical fibroblast forms.

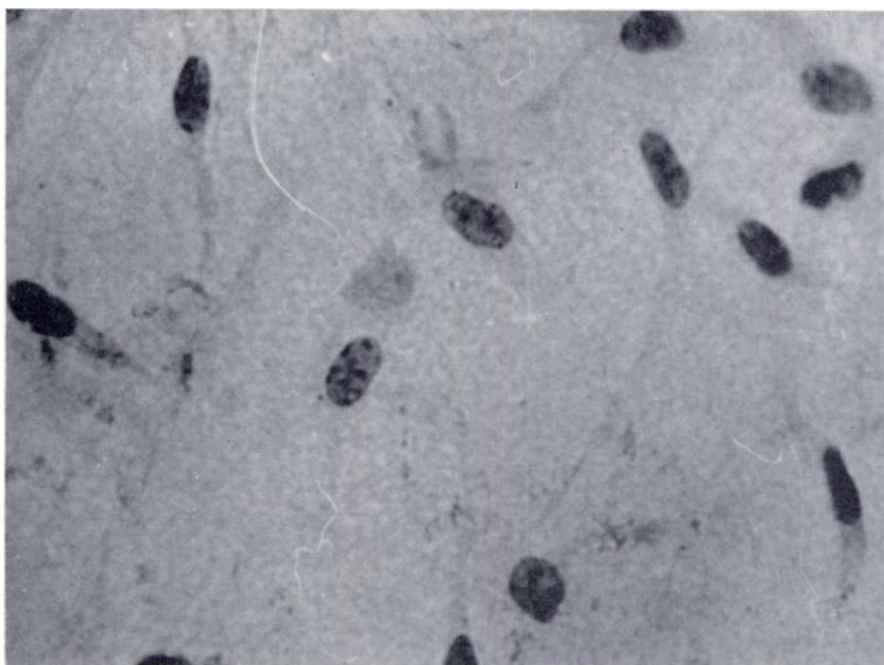


Fig. 3.—Early fibroblastic differentiation of normal leukocytes at 10 days (400 x).

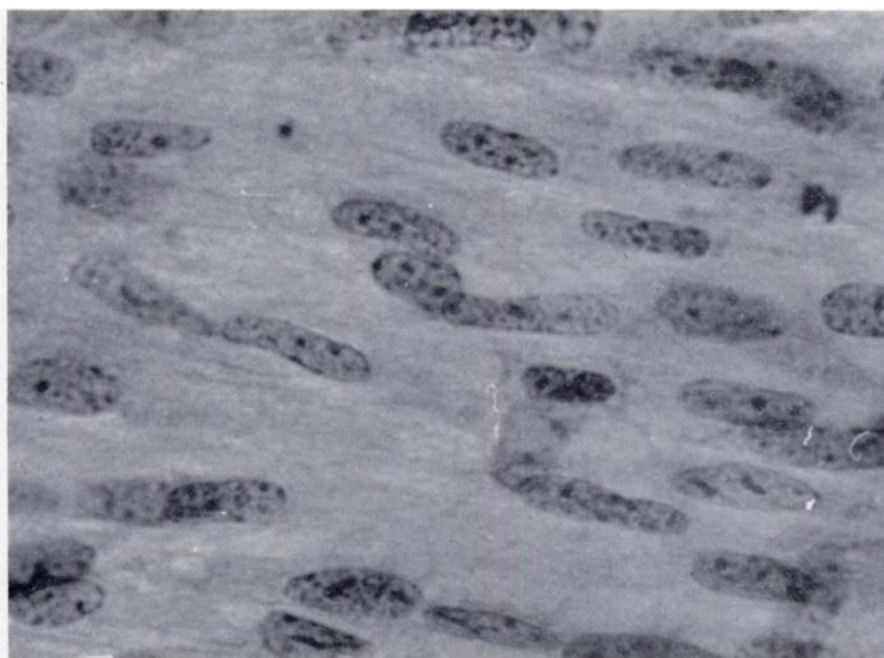


Fig. 4.—Fibroblastic differentiation at 14 days after treatment with ascorbic acid (400 x).

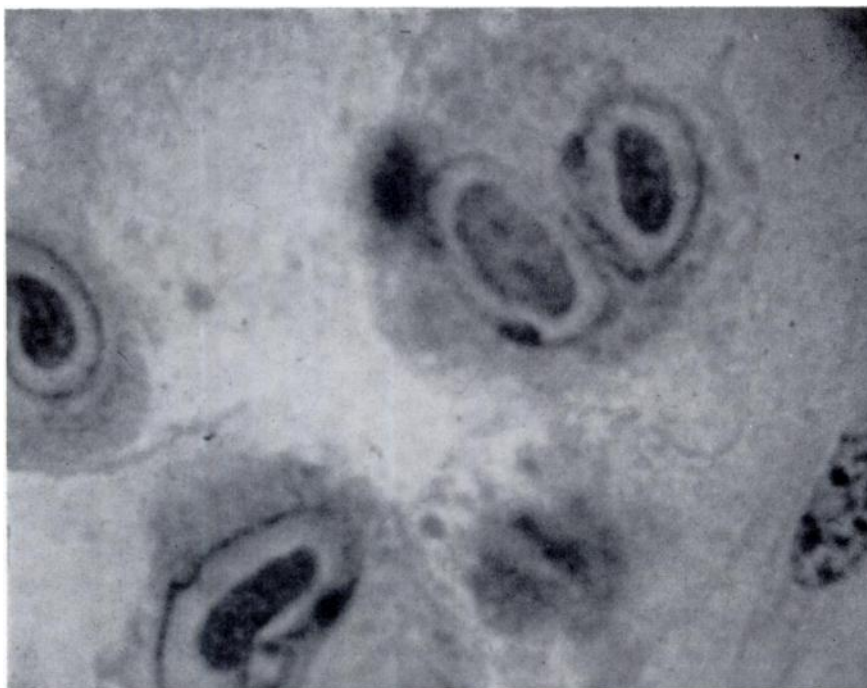


Fig. 5.—Bizarre cellular forms with giant nucleoli found two days after treatment with ascorbic acid (800 x).

DISCUSSION

These studies demonstrate that in the absence of ascorbic acid, severe morphologic disturbances in the differentiation of mononuclear leukocytes to fibroblasts result, which can be corrected by the administration of ascorbic acid to the host guinea pigs. The findings were not altogether unexpected, since from a functional standpoint, ascorbic acid has been shown to be necessary for phagocytosis and macrophage activity.^{4,5} Although it is generally acknowledged that the connective tissues are affected in scurvy, disagreement exists as to the primary site of action of ascorbic acid, i.e., on the intercellular collagenous matrix or directly upon the function of the connective tissue cells.⁶⁻⁹ It is presently held by most investigators that the site is extracellular, although recent studies by Gould¹⁰ suggest a cellular defect. Immaturity of fibroblasts and odontoblasts have been frequently reported in histologic studies of scorbutic tissues, but these changes do not resemble the extreme forms found in the present studies. Possibly the growth of the cells over the surfaces of the Millipore membranes results in more readily detectable cytologic changes than seen in standard histologic tissue preparations. The absence of morphologic abnormalities in the non vitamin-C deficient leukocytes during the 10-day period of vitamin-C deficiency indicates that the changes are not a result of the environmental conditions peculiar to the diffusion chamber, but rather are due to a basic nutritional deficiency of ascorbic acid.

The severe morphologic disturbances in the differentiation of mononuclear leukocytes to fibroblasts are in many respects reminiscent of those associated with malignancy. It has been reported that the buffy coat leukocytes from patients with leukemias and other blood dyscrasias are markedly deficient in ascorbic acid.¹¹ Since the "mesenchymal" mononuclear of blood are considered by many investigators¹² to be capable of differentiation to hematopoietic as well as to connective tissues, it is interesting to speculate as to the relationship of ascorbic acid deficiency and the impairment of cellular differentiation seen in these conditions.

The present studies demonstrate a direct cellular role of ascorbic acid in the differentiation of "mesenchymal" mononuclear leukocytes of blood to fibroblasts.

SUMMARY

Studies were made to evaluate the influence of ascorbic acid upon the differentiation of mononuclear leukocytes to fibroblasts when cultivated in diffusion chambers, *in vivo*. Ascorbic acid-depleted leukocytes grown in ascorbic acid-deficient host guinea pigs developed into abnormal cellular forms characterized by nuclear enlargement, multipolar mitoses, and giant forms. These changes could be reversed by treatment of the host guinea pigs with ascorbic acid. The findings indicate a direct cellular role of ascorbic acid in the differentiation of mononuclear leukocytes to fibroblasts.

SUMMARIO IN INTERLINGUA

Esseva effectuate studios pro evaluar le influentia de acido ascorbic super le differentiation de leucocytos mononucleari ad in fibroblastos quando illos es culturata *in vivo* in cameras de diffusion. Leucocytos a depletion de acido ascorbic, crescente in porcos de India a carentia de acido ascorbic, se disveloppava ad in anormal formas cellular per allargamento nucleari, mitoses multipolar, e formas gigante. Iste alterationes poteva esser revertite per tractar le porcos de India hospite con acido ascorbic. Le constatationes indica un directe rolo cellular pro acido ascorbic in le differentiation de leucocytos mononucleari ad in fibroblastos.

ACKNOWLEDGMENTS

This research was supported in part by research grants CY-3422 and C-5485 from the Division of Research Grants, National Institutes of Health, United States Public Health Service and by a gift from Mr. and Mrs. Winston S. Cowgill in memory of Charles Arthur Christenson. The author expresses his appreciation to Miss Mary Davis for her technical assistance in the conduct of these studies.

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1961 18: 310-316

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Intracellular ascorbic acid was depleted greater than 96% during cell culture and was rapidly reaccumulated after addition of physiologic...^Â Ascorbic acid (vitamin C) was found in isolated human mononuclear leukocytes and their purified components in millimolar concentration. Intracellular ascorbic acid was depleted greater than 96% during cell culture and was rapidly reaccumulated after addition of physiologic concentrations of ascorbic acid to the extracellular medium. Purified cells maintained concentration gradients of ascorbic acid as large as 100-fold across the plasma membrane. Purified cells maintained concentration gradients of ascorbic acid as large as 100-fold across the plasma membrane. The ability to vary intracellular ascorbic acid concentrations over such a wide range makes it possible for the first time in these cells to study ascorbic acid function in direct relationship to intracellular concentration.^Â Ascorbic acid (vitamin C) was found in isolated human mononuclear leukocytes and their purified components in millimolar concentration. Intracellular ascorbic acid was depleted greater than 96% during cell culture and was rapidly reaccumulated after addition of physiologic concentrations of ascorbic acid to the extracellular medium. Purified cells maintained concentration gradients of ascorbic acid as large as 100-fold across the plasma membrane. Peripheral blood mononuclear leukocytes (PBMLs) from untreated patients with immune-mediated neuropathies such as GBS or CIDP, or patients with chronic neuropathic pain can be infused across the BNB model at predefined flow rates or shear forces that mimic estimated capillary hemodynamics in peripheral nerves.^Â In vivo models of leukocyte trafficking at the blood-nerve barrier. There are currently no models that permit direct assessment of leukocyte trafficking across the BNB in real time in vivo.^Â A limitation of single beam two-photon IVM is the requirement of higher laser power to enhance sensitivity and detection speed which results in sample degradation.