appearance, however, remains to be elucidated.

Summary. 1. With the aid of an intragastric buffering technic, albumin and γ-globulin were detected in gastric aspirates of human subjects without gastric disease. 2. The range of albumin concentration in these gastric aspirates was 17-451 μg/ml whereas that for γ-globulin was 33-95 μg/ml. Estimates of the corresponding minimum 24-hour outputs for these 2 proteins were 21-690 mg and 33-384 mg respectively. 3. Saliva aspirated concomitantly with gastric contents also contained these plasma proteins, but not invariably. 4. It is unlikely that the gastric juice proteins derived in significant degree from saliva because of (a) the careful continuous removal of saliva by oral aspiration, and (b) presence of significant amounts of plasma proteins in gastric aspirates of individuals whose saliva did not contain detectable amounts of these proteins.

1. Hollander, F., *Amer. J. Digest. Dis.*, 1934, v1, 319.

2. Henning, N., Kinzlmeier, H., Demling, L., *München med. Wchnschr.*, 1953, v95, 423.

3. Citrin, Y., Sterling, K., Halsted, J. A., *New England J. Med.*, 1957, v257, 906.

4. Hollander F., Horowitz, M. I., *Fed. Proc.*, 1960, v19, 181.

5. Gullberg, R., Olhagen, B., *Nature*, 1959, v184, 1848.

6. Holman, H., Nickel, W. F., Jr., Sleisenger, M. H., *Amer. J. Med.*, 1959, v27, 963.

7. Penner A., Hollander, F., Post, A., *Amer. J. Digest. Dis.*, 1940, v7, 40.

8. Weichselbaum, T. E., *Amer. J. Clin. Path.*, 1946, v16, Tech. Bull. No. 7, 40.

9. Lowry, O. H., Rosebrough, N. J., Farr, L. A., Randall, R. J., *J. Biol. Chem.*, 1951, v193, 265.

10. Wetterfors, J., Gullberg, R., Liljedahl, S.-O, Plantin, L.-O., Birke, G., Olhagen, B., *Acta med. Scand.*, 1960, v168, 347.

11. Glass, G. B. J., Ishimori, A., *Amer. J. Digest. Dis.*, 1961, v6, 103.

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Studies of Fibroblast-Like Cells from the Bone Marrow of Leukemic and Nonleukemic Children.* (27239)

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A number of investigators have cultivated human bone marrow cells *in vitro* as reviewed by Bloom(1), Reisner(2) and Lajtha(3). The technics employed have varied according to the purpose for which the cultures were to be used.

The purpose of the present study was to devise a technic for isolation and serial propagation of bone marrow cells from leukemic and nonleukemic children which would:

(1) Allow for cytologic studies of cells in long-term culture so that unmasking of latent viral agents could be observed.
(2) Provide cultures on which to perform hemadsorption studies to detect the presence of latent viral agents.
(3) Provide normal bone marrow cell cultures which could be used for attempts to isolate cytopathogenic agents from saline suspensions and phenol extracts of human leukemic tissues.

The present report describes a simple technic for cultivation of fibroblast-like cells from human bone marrow and the results of cytologic and hemadsorption studies upon such cells.

Materials and methods. Nutrient medium. The nutrient medium consisted of Eagle's medium(4) supplemented with 20% inactivated calf or human serum, 5% human cord serum, and 5% beef embryo extract.‡ Penicillin,

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‡ All ingredients were obtained from Microbiological Associates, Inc., Bethesda, Md.

streptomycin and nystatin were added to a final concentration of 100 units, 100 μg and 100 units respectively.

Primary isolation. Iliac crest bone marrow was aspirated into syringes wet with 1% versene and 0.5 ml was suspended in 24 ml of nutrient medium. After gentle mixing, 4 ml was pipetted into each of 6 Leighton tubes with coverslips and incubated at 37°C at a 10° slant. After 24 hours, the nutrient medium was replaced, thereby removing the bone marrow cells not attached to the coverslips together with the red blood cells. The cultures were then refed with fresh nutrient medium and incubation continued.

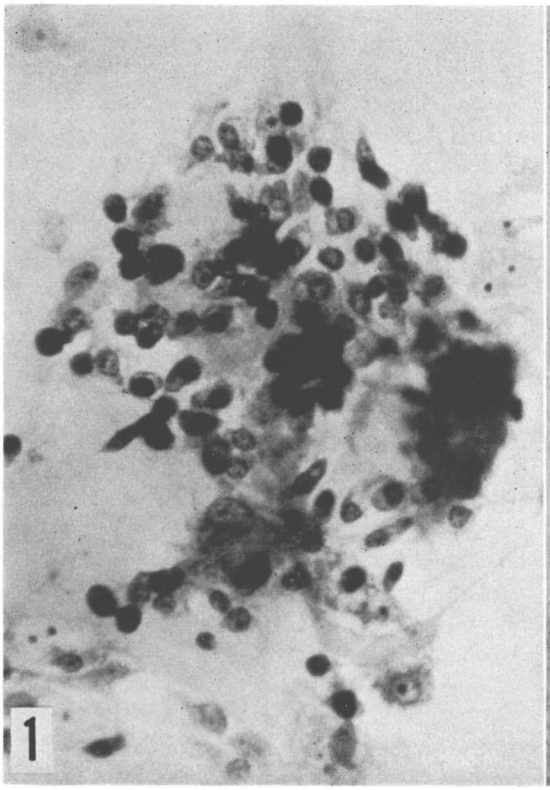
Subcultures. Serial subcultivation of fully grown cultures was made after 5 to 21 days depending upon rate of growth of each culture. The fibroblast-like cells were dispersed from the surface of the glass by replacing the nutrient medium with 2.5 ml of versene-trypsin solution (0.125% versene and 0.125% of trypsin) and incubating at room temperature for 10 minutes. The dispersed cells were sedimented by centrifugation at 1000 rpm for 10 minutes and the cell button was resuspended in nutrient medium and recultivated in Leighton tubes or milk dilution bottles. For mass cultures, the cells were passed from milk dilution bottles into 1 liter Blake bottles or 5 liter Povitsky bottles. Primary cultures and subcultures were refed with fresh nutrient medium every 5 to 7 days.

Staining. When adequate growth had occurred, the coverslips were removed from the Leighton tubes, fixed in absolute methanol and stained with May-Grünwald-Giemsa. In general, stained preparations were made of each passage of every cell line.

Hemadsorption studies. Vogel and Shelakov's technic(5) for demonstrating the presence of hemadsorbing viruses was used in these studies.

Results. A total of 58 fibroblast-like cell lines was isolated from 65 bone marrow aspirates of 43 leukemic, 18 nonleukemic and 4 children with lymphomas (2 Hodgkin's disease and 2 lymphosarcomas). Successful isolation of fibroblast-like cells was similar from nonleukemic bone marrow specimens (17/18), and from leukemic (38/43) and lymphoma specimens (3/4). Of those cultivated successfully in calf serum, there was no significant difference in metabolic rate or mean survival time *in vitro* between 4 nonleukemic cultures (60.5 days) and 14 leukemic bone marrow cell cultures (65.2 days) ($p>0.7$ by the 2-sided "t" test for difference between mean survival times). When cultivated in human serum, mean survival time of 14 nonleukemic cultures was 52.8 days and of 24 leukemic cultures, 85.8 days (significant at $p<0.01$). Mean survival times of leukemic bone marrow cultures in human serum or in calf serum were not significantly different ($p>0.05$), nor were mean survival times of nonleukemic bone marrow cultures in these sera ($p>0.05$). No differences were observed between cultures from nonleukemic and leukemic patients in number of serial transfers that could be made. From both sources, 4 specimens grew only in primary culture. Seven cultures survived one transfer. 23 were maintained for 2-5 serial transfers, 16 for 6-10 transfers, 7 for 11-15 transfers, and 1 for 21 transfers. Thereafter, from both nonleukemic and leukemic patients the cells became swollen, granular and ceased proliferating. The majority of cells ceased proliferating after 1 to 3 months. The cell line that was maintained for 21 subcultures survived 6 months. This line was derived from a leukemic child. None of the fibroblast-like cells transformed to an epithelial-like cell morphology during these studies(6) and none could be maintained in continuous culture as permanent lines. Five cell lines from nonleukemic bone marrow were cultivated in 1 liter Blake bottles. From these mass cultures, large numbers of replicate tube cultures were prepared for use in transmission experiments of saline suspensions and phenol extracts of leukemic tissue. Nine saline suspensions and 12 phenol extracts have been inoculated into these cultures. No viral cytopathology was observed. These data will be reported separately.

Cytologic observations. No cytologic differences were observed between nonleukemic and leukemic cell cultures. Erythroblasts, lymphocytes, megakaryocytes, leukocytes and their precursors became attached to the



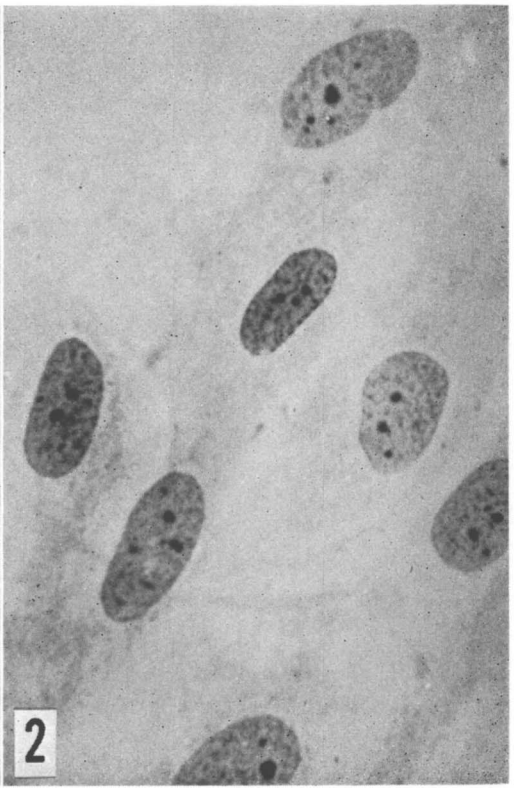


FIG. 1. Normal bone marrow cells after 9 days in primary culture. Note elongation of cytoplasm of some small round cells. 300×.
FIG. 2. Fibroblast-like cells derived from normal bone marrow cells in 2nd passage. 800×.

coverslips within 24 hours and gradually lost their *in vivo* characteristics or disappeared after 3 or 4 days of culture. Large round cells similar to the phase II cells of Berman (6) were noted on the 5th to the 7th day. At about the same period, spindle-shaped cells which appeared fibroblast-like were formed apparently by the elongation of the cytoplasm of small round cells (Fig. 1). By the 10th to the 14th day, a monolayer sheet of fibroblast-like cells covered the entire coverslip and replaced all other cell types (Fig. 2).

After the 3rd passage, the fibroblast-like cells exhibited increasing numbers of cytoplasmic vacuolations and the presence of pinkish bodies (MGG stain) probably due to engulfed media or phagocytized cell fragments.

No evidence of viral cytopathology was noted during the prolonged *in vitro* cultivation in any culture that might have suggested the unmasking of adenoviruses or of salivary gland viruses as has been done from normal adenoid tissue(7,8).

The stained cell cultures showed no multinucleate giant cells, intracytoplasmic or intranuclear inclusions which might have suggested the presence of viral agents(9). Four cultures stained with acridine orange and observed with fluorescence microscopy showed the expected green nuclear chromatin and orange cytoplasm(10,11) but no unusual distribution of nucleic acids.

Hemadsorption studies. No hemadsorption of guinea pig erythrocytes occurred in 14 monolayer cell cultures derived from leukemic bone marrows. Two of 8 nonleukemic bone marrow cell cultures did adsorb guinea pig erythrocytes. The cause of this hemadsorption was undetermined. One of these was derived from a child with a lung abscess and the other from a child with iron deficiency anemia. Hemadsorption studies done

on uninoculated monolayer cell cultures of monkey kidney tissue§ showed a positive test in 29 of 39 batches tested. These observations are in agreement with those of Emery (12) who described hemadsorption in 24 of 44 batches of monkey kidney cell cultures. His studies suggest that this was due to the presence of simian hemadsorbing viruses in the cells. In our studies, no cultures of human fibroblasts§ (0/28 batches) or of primary human amnion cells§ (0/20 batches) showed hemadsorption.

Discussion. The cultural conditions used in these studies obviously favored the appearance of fibroblast-like cells. Whether such cells were derived from hemic or connective tissue has been studied by other investigators but is not clear; however, both monocytes and lymphocytes apparently can transform into fibroblast-like cells(13-16). If our cells were derived from stromal elements, perhaps the purpose of the present study has failed other than to describe a source of human fibroblast-like cells.

Despite his successes in establishing strains of human cell lines from various normal and malignant tissues, Foley(17) failed to establish cell lines from the peripheral blood and bone marrows of leukemic children. He used no feeder layer in his technic. According to Foley's criteria that a cell line is "isolated" after 3 serial passages *in vitro* and "established" after 6 months of continuous propagation, 13 of our cell lines from nonleukemic and 28 from leukemic bone marrows were "isolated" but only one cell line from a leukemic bone marrow was "established". However, this cell line did not survive and become a permanent line. Swim(18) has observed that despite the long list of permanent cell lines, "the cell culture methods currently in general use do not permit the continuous growth of cells from any tissue with a high degree of regularity."

Although our cell cultures derived from leukemic bone marrow survived significantly longer in human serum than did cell cultures from nonleukemic marrow, we do not know the biologic significance of this observation. Discussion of the differences between cells derived from normal and malignant sources has been presented(19).

In addition, although none of our lines became permanent, at least our cell cultures have not been contaminated with the permanent cell lines carried in this laboratory(20). Recently, Berg and Rosenthal(16) were successful in cultivating fibroblastic cells from the marrows of leukemic and nonleukemic children by using a feeder layer of human amnion cells. However, their attempts to cultivate bone marrow cells directly on glass were "infrequently successful." Among other possibilities, the success of our technic compared to theirs perhaps could be explained by the fact that we diluted 0.5 ml of bone marrow to inoculate 6 cultures while they diluted 1-3 ml of bone marrow to inoculate 60 cultures.

A number of investigators have attempted to isolate viral agents from human leukemic tissues(16,21-23). Recently, Hanshaw(24) has isolated salivary gland virus from the urine of 3 children with leukemia and one with Hodgkin's disease. In addition, one of the leukemic children who shed SGV concurrently excreted an adenovirus. One might wonder if these agents could be unmasked from kidney cell cultures of leukemic children. During the present studies of cell cultures derived from bone marrow, no known or unknown human virus was unmasked.

Summary. Human bone marrow aspirates were suspended in nutrient media and dispensed into Leighton tubes with coverslips but no feeder layer. Fibroblast-like cell sheets were cultivated from 17 of 18 (94%) nonleukemic children and from 42 of 47 (89%) children with leukemia or lymphomas. Such cell cultures could be maintained in serial subcultivation for 1 to 21 passages. No differences in morphology or rate of metabolism *in vitro* were observed between nonleukemic and leukemic cultures. Leukemic cell cultures survived significantly longer than nonleukemic cultures when cultivated in human serum. None became permanent cell lines. Unstained and stained preparations of every passage of each cell line failed to reveal the unmasking of any latent viral agent. Leu-

§ Obtained from Microbiological Associates, Inc., Bethesda, Md.

kemic cell cultures failed to hemadsorb guinea pig erythrocytes. The technic described provides a simple method for cultivation of human fibroblast-like cells on coverslips without a feeder layer. Such preparations may be useful in hematological or microbiological studies where cytological observations are important.

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1. Bloom, W., *Handbook of Hematology,* Paul B. Hoeber, Inc., New York, 1938, v2, 1469.

2. Reisner, E. H., Jr., *Ann. N. Y. Acad. Sci.,* 1959, v77, 487.

3. Lajtha, L. G., *Brit. Med. Bull.,* 1959, v15, 47.

4. Eagle, H., *J. Exp. Med.,* 1955, v102, 595.

5. Vogel, G., Shelakov, A., *Science,* 1957, v126, 358.

6. Berman, L., Stulberg, C. S., Ruddle, F. H., *Blood,* 1955, v10, 896.

7. Rowe, W. P., Huebner, R. J., Gilmore, L. K., Parrot, R. H., Ward, T. G., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 570.

8. Rowe W. P., Hartley, J. W., Waterman, S., Turner, H. C., Huebner, R. J., *ibid.,* 1956, v92, 418.

9. Pinkerton, H., *Ann. N. Y. Acad. Sci.,* 1959, v81, 207.

10. Kornfield, H. J., Werder, A. A., *Cancer,* 1960, v13, 458.

11. Von Bertalanffy, L., Masin, M., Martin, F., *ibid.,* 1958, v11, 873.

12. Emery, J. B., York, C. J., *Virology,* 1960, v11, 313.

13. Osgood, E. E., Brooke, J. H., *Blood,* 1955, v10, 1010.

14. Berman, L., Stulberg, C. S., *ibid.,* 1958, v13, 1149.

15. Petrakis, N. L., Davis, M., Lucia, S. P., *ibid.,* 1961, v17, 109.

16. Berg, R. B., Rosenthal, M. S., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 614.

17. Foley, G. E., Drolet, B. P., McCarthy, R. E., Goulet, K. A., Dokos, J. M., Filler, D. A., *Cancer Res.,* 1960, v20, 930.

18. Swim, H. E., *Ann. Rev. Microbiol.,* 1959, v13, 141.

19. McAllister, R. M., Conference on Biology of Connective Tissue Cells, Princeton, 1961 (in press).

20. Rothfels, K. H., Axelrad, A. A., Siminovitch, L., McCullock, E. A., Parker, R. C., *Proc. 3rd Canad. Cancer Conf.,* 1959, 189.

21. Syverton, J. T., Ross, J. D., *Am. J. Med.,* 1960, v28, 683.

22. Sykes, J. A., Dmochowski, L., Shullenberger, C. C., Howe, C. D., *Proc. Am. Asso. Cancer Res.,* 1961, v3, 271.

23. Stewart, S. E., Irwin, M., *Cancer Res.,* 1960, v20, 766.

24. Hanshaw, J. B., Weller, T. H., *J. Pediatrics,* 1961, v58, 305.

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