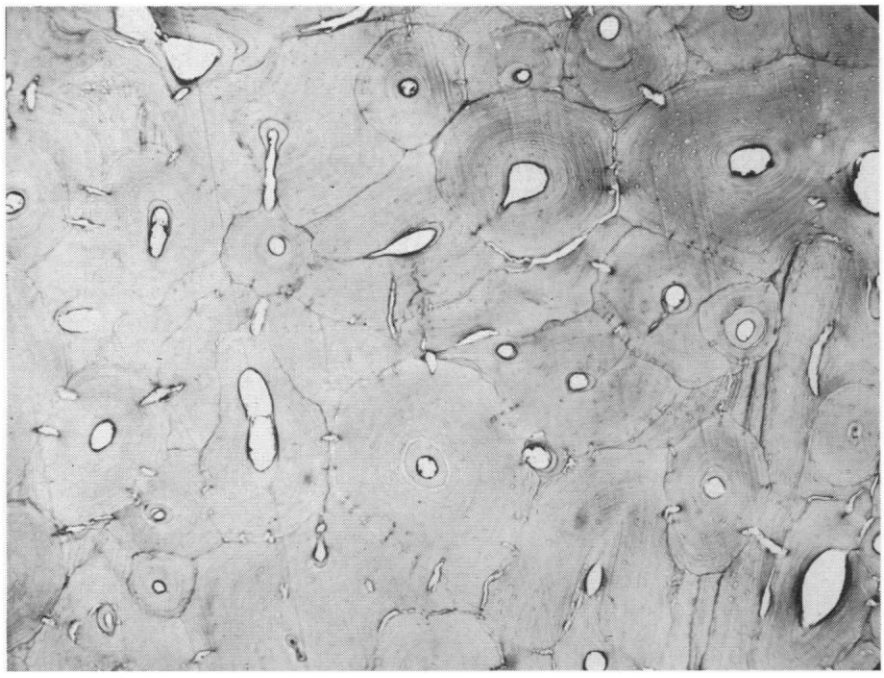

Fig. 1. Cross section of compact mammoth bone, decalcified and stained with hematoxylin and eosin. (× about 47)

in. in size, was a piece from a long bone —femur, tibia, or the like. On exposure of the marrow cavity, the spongy tissue was found to be infiltrated with dirt, hair, and a few small pebbles. The outer surface resembled fresh bone in that it was smooth and shiny. It had the characteristic odor of fresh bone but was yellowish brown in color.

Longitudinal and transverse slices of the specimen, about 3 mm thick, were decalcified, cut at 17 μ, and stained with hematoxylin and eosin (Fig. 1). By comparing our sections with Amprino's picture and description (*2*) of fresh elephant bone, we were able to identify our specimen conclusively as belonging to the genus *Elephas*. The fiber bundles are relatively thin and in orderly disposition. The vascular canals are transversely situated, as in the femur and humerus of the elephant. The fragment is probably from the more central portion of the diaphysis —that is, it is well removed from the epiphyseal plates, as indicated by the osteons of different sizes and shapes. From the descriptions of Amprino we were able to conclude that the fragment is from an immature adult.

The routine histology of the sections thus confirmed our initial observation that the fragment had all the appearances of a fresh bone. No foreign material was observed in the sections. There was no evidence of loss by leaching, and the typical bone pattern was remarkably intact. These facts support the hypothesis that glacier preservation constitutes as inert an environment for biological material as can be found under natural conditions (*3*).

As a further check on the condition of the mammoth bone, we analyzed the bone for total nitrogen and acid-extractable carbonate. The mean value for nitrogen, by the Kjeldahl method, was 4.2 percent by weight; for the carbonate, 3.4 percent carbon dioxide. These values fall definitely within the range to be expected in fresh, compact mammalian bone.

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References

1. L. S. Quackenbush, *Bull. Am. Museum Nat. Hist.* **26**, 87 (1909).
2. R. Amprino and G. Godina, *Pontif. Acad. Sci. Commentationes* 11, No. 9 (1947).
3. R. F. Heizer and S. F. Cook, *Univ. Calif. (Berkeley) Publs. Anthropol. Records* **12**, No. 2 (1949).

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Aggregate Replication, a Factor in the Growth of Cancer

Abstract. Microscopically, most clinical carcinomas appear as aggregates of neoplastic cells in a matrix of connective tissue. As the tumor grows, increasing numbers of nests of cells are seen. Studies in which matrix tissue culture techniques are used implicate factors intrinsic to the aggregate in the continuing propagation of new nests of cells.

The growth of cancer is commonly measured in terms of an increase in the number of various structural units. The cell is traditionally the primary unit of interest. Improved techniques of optical and biochemical resolution have recently provided opportunities for considering replication of subcellular structures down to the size of large molecules. Another kind of structural unit commonly occurring in cancer is the nest or aggregate of cancer cells. Most carcinomas are made up of such nests of tumor cells scattered in the connective tissue of the host. Microscopically this feature is seen most clearly with low magnifications of about 100.

As the cancer grows, increasing numbers of nests of cells are seen. Similar aggregates are usually seen in local and distant metastases. Although the appearance of these aggregates of cells is used in the histogenic classification of tumors, aggregate multiplication as a quality in the growth of cancer appears to have gone unnoticed until very recently (*1*). Inadequacy of techniques and an absence of suitable experimental material may have been mainly responsible for this apparent oversight.

The general problem of aggregate replication has been studied in this laboratory during the past year, primarily from the point of view of this phenomenon as a factor in the spread of cancer. To provide a workable approach, the question "How does cancer spread?" has been rephrased so that it reads "How do groups of cancer cells multiply?" For convenience in initiating our study, we contrived an oversimplification, involving the idea of intrinsic and extrinsic factors in aggregate replication. Is the capacity to form new groups of cells contained within preexisting groups of tumor cells so that aggregate replication proceeds without the intervention of stromal tissue (intrinsic factors)? Do stromal cells participate in the replication of nests of tumor cells (extrinsic factors)?

This is a preliminary report describing some of our observations on the intrinsic capacity of nests of neoplastic cells to form new nests (*2*). We have found that the cultivation of continuous lines of human cells with matrix tissue culture techniques provides a convenient way to examine this phenomenon.

The sponge matrix tissue culture method is as follows. For a dense inoculum, a slice of cellulose sponge is wiped over the wall of a stock tube containing cells, or a particle of Gelfoam bearing cells is placed on a slice of cellulose sponge. The sponge is then cemented to the wall of a tube with a clotting mixture consisting of one drop of chick plasma and two drops of 20-percent chick embryo extract. Dilute suspensions of cells are prepared directly in the clotting mixture.The cells are suspended in the embryo extract just before preparation of the coagulum, or are

added to the clotting mixture in a fourth drop of conditioned medium. The clotting mixture is rapidly irrigated through a rectangular slice of cellulose sponge (*3*). After clot formation, a nutrient consisting of 40-percent human serum in Eagle's basal medium is added. The medium is replenished as indicated by changes in *p*H and by the microscopic appearance of the cells. The cell suspensions have varied in population; the smallest practicable concentration of cells has been 2000 cells per cubic milliliter of chick embryo extract.

The following observations have been made. Individual cells that can be readily seen in the living culture in many interstices of the sponge have been followed with day-to-day microscopic observations. Over a period of several days, through progressive cellular divisions, globular aggregates of cells are seen in the coagulum. As the aggregates increase in size they give rise to short, tortuous, branching processes and to long, cordlike, multicellular structures. Eventually the interstices are peppered with aggregates of varying size.

Where cells reach the free surface of the plasma clot, the aggregate morphology is replaced by sheetlike growth of cells. Where cells reach the interfaces between plasma clot and sponge trabeculae within the depths of the sponge, the quality of the growth in the living culture is obscured.

After fixation and the preparation of serial histologic sections, aggregates of varying size are seen in the sponge at all levels. In some of the interstices the spherical nodules of cells compress and distort one another (see Fig. 1). Each group of cells, however, generally retains its identity. The structure of the intercellular membranes within an aggregate, as contrasted with the intercellular membranes between contiguous aggregates, is of interest. In routine histologic preparations, the intercellular membranes between the aggregates appear to be more substantial than those between cells within an aggregate. Coalescence between aggregates appears to take place primarily when the centers of contiguous aggregates undergo necrosis. Aggregate formation has been observed in sponge matrix for several cell lines, including D-189, Chang's Conjunctiva, and strain HeLa.

The umbilical cord matrix tissue culture method is as follows. Pieces of frozen-thawed human umbilical cord have been used as a matrix to support the growth of inocula of strain D-189 and of Chang's Conjunctiva. Cell suspensions have been injected into the pieces of cord, and particulate explants of Gelfoam bearing these cell lines have been inserted into the pieces of cord with the aid of a trocar. The fragments of cord are kept in roller tubes and fed on the medium described above, as well as on other mixtures less rich in serum. In the living culture the pieces of umbilical cord are opaque, and no growth can be recognized. The frequency with which the medium must be replenished as the culture ages (as indicated by changes in *p*H) is the only index in the living culture of increased growth. It is an effective one.

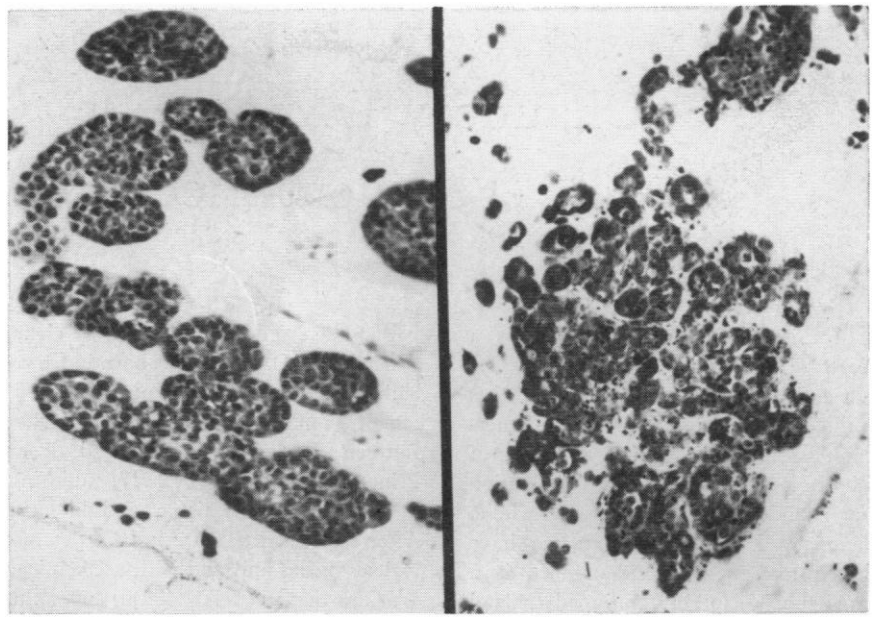

Fig. 1. (Left) Histologic section of sponge matrix culture of strain D-189. Aggregates have formed and given rise to secondary aggregates. Even when aggregates are in contact with and compress one another, they retain their identity. (Right) Histologic section of strain D-189 in umbilical cord matrix. The cells are arranged primarily in small nests or aggregates, although a few individual cells are seen.

Growth of cells within the substance of the cord, as observed on histologic section, is seen primarily in the form of multicellular aggregates, either as spherical nests or as anastamosing, serpentine, branching cords. Some cells appear singly; most of the cells, however, are found to be present in a pattern of aggregation (see Fig. 1).

This preliminary report is presented to call attention to an interesting, potentially important topic that appears to have been largely skipped over in considerations of the growth of cancer. Nests of "transformed" human cells that resemble carcinoma have been found to give rise to other nests in matrix tissue culture. The process does not require the presence of living stromal cells. We are currently considering the extent to which patterns of aggregate replication may vary, in different cell lines and with changes in the composition of the nutrient medium. The extent to which host stromal cells may play a role in the formation of new nests of cells will also be explored.

A broad area for future investigation concerns the molecular events that are associated with aggregate replication and the response of this phenomenon of growth to pharmacologic agents. The manipulation of aggregate replication by chemical agents may provide a means of augmenting the efficacy of currently available chemotherapeutic approaches that concentrate on the cellular level.

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References and Notes

1. J. Leighton, *J. Natl. Cancer Inst.* **19**, 607 (1957).
2. This study was supported by a research grant from the National Cancer Institute, U.S. Public Health Service. This report is publication No. 174 from the department of pathology, University of Pittsburgh School of Medicine.
3. J. Leighton, *An Introduction to Cell and Tissue Culture* (Burgess, Minneapolis, 1955), pp. 36–40.

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