*THE IN VIVO LIFE SPAN OF NORMAL AND PRENEOPLASTIC MOUSE MAMMARY GLANDS: A SERIAL TRANSPLANTATION STUDY**

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It is recognized that organismal death of complex metazoan animals cannot usually be ascribed to the large-scale, simultaneous death or degeneration of their constituent cells. Although age-associated degenerative changes can be identified by both morphological and functional criteria, most cells, tissues, and organs of recently deceased animals are alive and in satisfactory condition at the time of natural death.[1–3] One of the fundamental problems in gerontology is therefore to determine whether such somatic cells have a potentially limited or unlimited lifetime if maintained under conditions which are optimal for growth, survival, and function.

Attempts to propagate vertebrate cells serially *in vitro* have yielded conflicting results, although recent work suggests that most diploid cell types proliferate in culture for a finite and relatively small number of cell generations.[4, 5] It is difficult to extend this conclusion to include cells *in vivo* because of the necessarily artificial conditions of culture. The corresponding *in vivo* experiments—serial transfer of normal tissue between isogenic animals—appear simple but have not yielded definitive answers. When normal tissues are transplanted, it is often impossible to distinguish clearly between host and transplant. Even with skin, a favored material for this type of study, it is not known to what extent the graft is invaded by host cells. Transplants placed in unusual locations may be identified but the unnatural environment may not allow normal growth or the full expression of functional potential.[6]

The availability of gland-free mammary fat pads as a transplantation site[7] avoids these complications. Mammary transplants are placed in their natural environment, white fat, which provides ample area for growth and histotypic differentiation.[7, 8] There is no possibility of confusing the epithelial portion of transplant-derived outgrowths with host tissue. Possible effects due to aging of connective tissue elements[1, 9] are avoided because in each generation the transplanted mammary epithelium induces the formation of young, fibrous connective tissue from host mesenchyme.[10]

Serial transplantation is comparatively simple in the case of neoplastic cells, which are often recognizable and which grow in unusual sites. In fact, a convenient working criterion of neoplasia derives from the ability of tumor transplants to grow progressively in isogenic animals. The many tumors that have been perpetuated for periods of years furnish convincing evidence that neoplastic cells have an apparently unlimited life span when propagated *in vivo* under suit-

able conditions. Preneoplastic tissues, such as hyperplastic alveolar nodules in the mouse mammary gland, occupy a position intermediate between normal and frankly malignant types. Nodules have been successfully transplanted,[7] but it has not previously been determined whether they share the ability of tumors to grow for numerous transfer generations extending over long periods of time.

Here we report a series of experiments dealing with the aging of normal and preneoplastic mouse mammary tissues, in which we have used growth rate as a quantitative measure of viability and have defined the aging process in terms of the ability of gland to proliferate when transplanted serially into gland-free mammary fat pads of young, isogenic mice.

Materials and Methods.—Mice: Three strains of inbred mice were used—C57Bl and Balb/c, which are free of the mammary tumor virus (MTV) and which have a very low spontaneous incidence of mammary tumors and of preneoplastic hyperplastic alveolar nodules (nodules), and C3H, which carries MTV and is characterized by a high incidence of mammary tumors and nodules. Mice were obtained from the colony maintained by the Cancer Research Genetics Laboratory. Isogenicity within each strain was checked at 6-month intervals by means of reciprocal second-set skin grafts between randomly selected mice. The ability of hosts to accept these isografts for a period of 100 days was considered evidence of histocompatibility and, with the exception of an occasional technical failure, no rejection was detected.

Tissues and transplantation: At the time of transplantation, recipient female mice 3 weeks of age were anesthetized and the no. 4 mammary fat pads were freed of host gland by a surgical procedure described elsewhere.[7]

Host mice from the preceding generation were anesthetized, the previously transplanted no. 4 fat pads were exposed, and outgrowths were located. The lobuloalveolar nature of nodule outgrowths permitted their identification in either virgin or pregnant hosts. Similarly, normal outgrowths were readily located and measured in pregnant hosts. Normal outgrowths in virgin hosts, however, were composed of simple ducts which could be located only after the hosts had received a single intraperitoneal injection of 0.5 ml of 0.5% trypan blue in distilled water 3–6 hr prior to examination.[11]

Transplants approximately 0.5 mm in diameter were inserted into a small incision in the gland-free fat pad. The host mice were maintained under standard conditions until the next transplantation, which in most experiments followed at 12 weeks but which in some instances varied from 8 to 17 weeks (Fig. 2). When pregnant or lactating hosts were required, males were added to the cages several weeks prior to transplantation.

Evaluation of results: At the close of each transplant generation all no. 4 fat pads and a portion of the hosts' thoracic mammary glands were removed, fixed, extracted in acetone, stained with hematoxylin, and stored in methyl salicylate. The amount of outgrowth was recorded as follows: 0% fat pad filled indicated that no recognizable gland could be located with the dissecting microscope; 1–5% indicated that identifiable mammary tissue was present but had grown only 1–5 times the size of the original transplant. Where more development had occurred, growth was estimated by determining to the nearest 10% the amount of available fat pad occupied by gland.

At each generation the per cent successful transplants and the mean per cent fat pad filled were calculated. The latter figure, which is a measure of growth, was calculated on the basis of successful transplants only (1–100% fat pad filled). Fat pads which showed no evidence of mammary outgrowths were not included in this calculation because these failures reflected technical difficulties—problems associated with locating the donor tissues due to opacity of fat and imperfect vital staining with trypan blue, or difficulties experienced in removing transplants from very small outgrowths.

Results and Discussion.—Serial transplantation of normal gland: Primary transplants of normal mammary gland characteristically grew to fill the cleared

fat pad within 10–12 weeks; these outgrowths could not be distinguished by morphological criteria from the hosts' own mammary glands (Fig. 1*a*). Five lines derived from normal gland were perpetuated by serial transplantation, and growth was determined at each generation.

In all experiments the growth of transplanted tissues was time-dependent, and proliferation declined with tissue age and transplant generation (Fig. 2, Table 1, expts. 1–5). Transplants from later generations often produced identifiable mammary tissue that failed to grow or, if detectable growth occurred, the resulting gland was usually composed of fragile, poorly staining ducts without enlarged end-buds (Fig. 1*b*). The experiments were terminated when tissue for subsequent transplantation could no longer be located. (Expt. 3 was lost in generation 4 due to disease in the animal colony.)

The maximum time that any normal transplant series could be carried was 24 months (expt. 4), a period within the life span of a mouse. It is possible that normal gland could be maintained for far longer periods if the interval between transplantations were lengthened.

Considerable variation in the life span of transplanted glands was found among the five transplant lines, and it may be significant that the two oldest lines (expts. 4 and 5) were transplanted from outgrowths that grew maximally, while the other three lines were always transplanted from outgrowths that displayed average growth for their particular generation. This suggests that epithelial cells of mammary gland may be heterogeneous with respect to their proliferative potential, a possibility which is now under investigation.

The experiments using normal gland were designed to control for several variables which might contribute to the aging process: (1) Infection with MTV appears not to be a determining factor, since the aging process was as evident in experiments using virus-free Balb/c and C57 strains as in the single experiment with infected C3H mice. (2) The hormones of pregnancy or lactation were not able to stimulate growth through unlimited transfers, for an age-related decline in growth rate occurred in lines maintained in both parous and virgin animals. (3) It is unlikely that the aging effect can be ascribed to administration of trypan blue prior to transplantation; in experiments 3 and 4 the dye was not necessary because the gland was transplanted as easily visualized lobules (the most satisfactory control, serial passage of virgin gland without trypan blue, is not possible with present techniques). (4) The effects of tissue damage resulting from the act of transplantation can apparently be discounted since identical transplantation techniques were used in the serial propagation of preneoplastic tissue, and yielded quite different results (see below). (5) Finally, the observed decline in growth rate was found in experiments involving three strains of mice.

Long-term serial transplantation of normal mammary gland has been previously reported only once. Hoshino,[12] using the fat pad transplantation technique, propagated mouse gland for 7 transplant generations and for a period of 3 years and 9.5 months. He found that the number of successful transplants in the terminal generation of each line varied from 72.2 to 14.3 per cent. In our experiments the number of surviving transplants in the final generation was also variable, ranging from 100 per cent success in experiments 1 and 2 to 10 per cent in

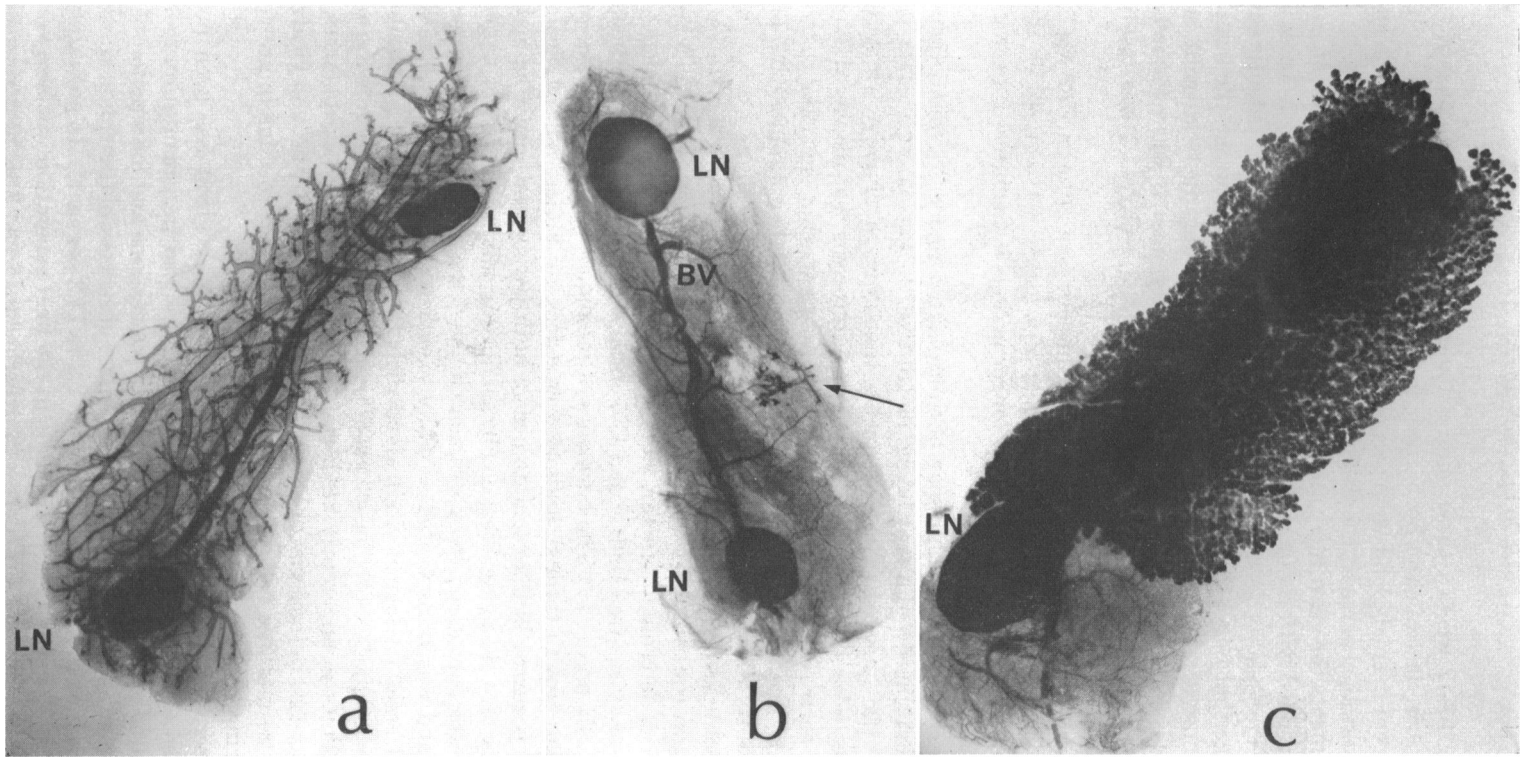

FIG. 1.—Typical outgrowths derived from serially transplanted mammary tissues. Number 4 fat pads were removed 12 weeks after transplantation and stained with hematoxylin. *LN*, lymph node; *BV*, blood vessel; all photomicrographs ×6.

(*a*) Normal outgrowth taken from virgin host showing maximum ductal development; transplant generation 1, recorded as 100% fat pad-filled.

(*b*) Small, normal-appearing outgrowth (*arrow*) which has occupied only a fraction of the available fat; generation 4, recorded as 10% fat pad-filled.

(*c*) Preneoplastic outgrowth taken from a virgin host; note extensive lobuloalveolar development; generation 31, recorded as 70% fat pad-filled.

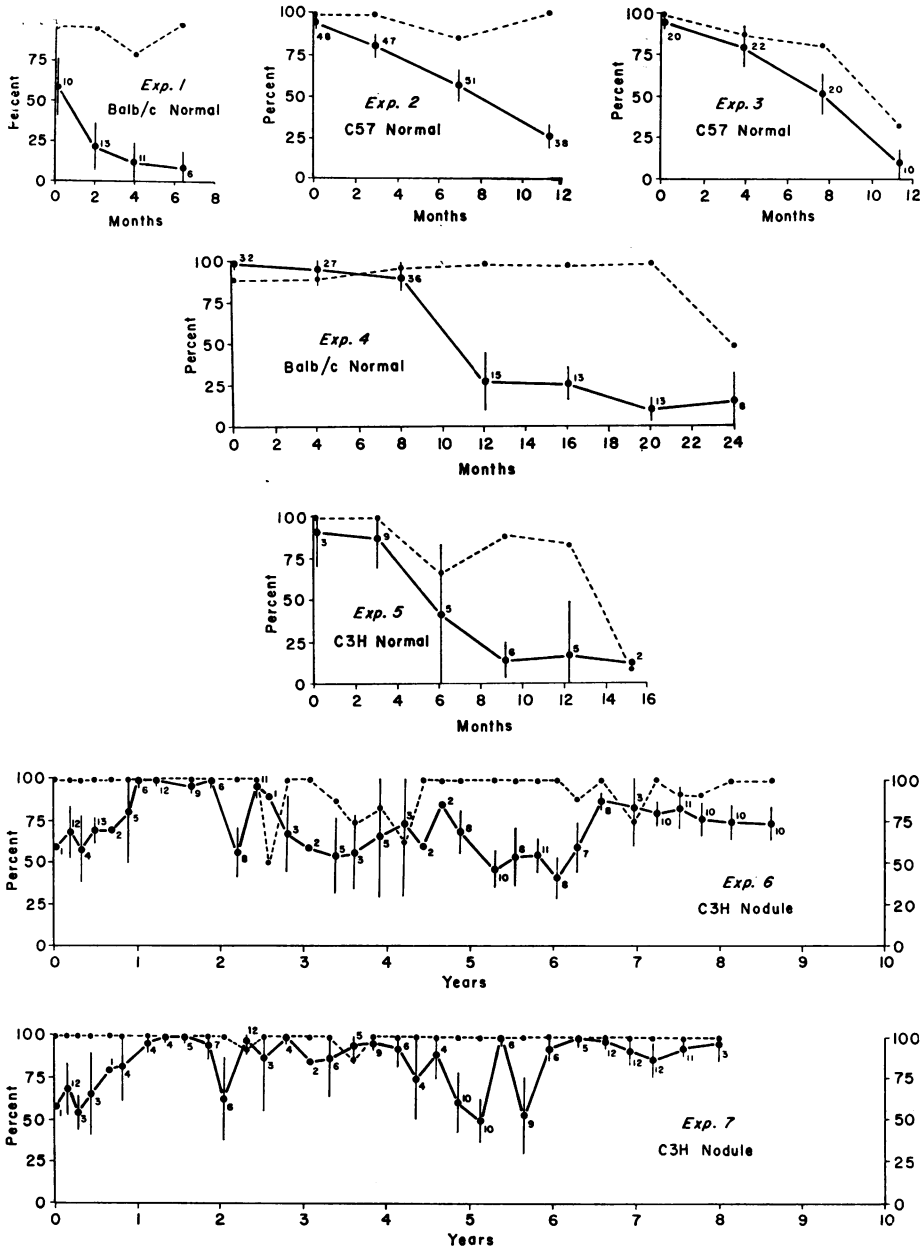

FIG. 2.—Serial transplantation of mouse mammary glands. Five transplant series using normal gland (expts. 1–5) and two using preneoplastic gland (expts. 6 and 7) are shown. ●—●, Growth rate and the mean per cent of available fat pad filled by the transplants (calculated on the basis of successful transplants only); ●—●, per cent successful transplants. Each point denotes a transplant generation, and small numbers beside points refer to the number of successful transplants in each generation. Vertical lines indicate 95% confidence intervals and are omitted from points which represent the mean of fewer than three outgrowths. Additional details concerning these experiments are given in Table 1.

TABLE 1. *Serial transplantation of mouse mammary gland.*

Mice					Transplants			
Expt.	Strain	Age at transplantation (wks)	Reproductive state of host*	Trypan blue used†	Tissue	Growth rate of donor outgrowth	Total time carried	Total generations carried
1	Balb/c	12–20	LP	No	Lobule	Average	6 mos	4
2	C57	3	LP	Yes	Lobule	Average	11 mos	4
3	C57	3	V	Yes	Duct	Average	11 mos	4
4	Balb/c	3	V	Yes	Duct	Fast	24 mos	7
5	C3H	3	LP	No	Lobule	Fast	12 mos	6
6	C3H	3	V	No	Nodule	Fast	8.5 yr	34
7	C3H	3	V	No	Nodule	Fast	8 yr	32

* V = virgin; LP = lactating or pregnant.
† See *Materials and Methods.*

experiment 5. It appears that variation in survival reflects the technical difficulties associated with locating and transplanting a particular outgrowth, and is not necessarily related to growth (Fig. 2). Hoshino interpreted his results, which were based principally upon survival, as suggesting the capability of mammary parenchyma to continue indefinitely *in vivo* under favorable circumstances. Our results, in which transplantability is measured by growth rate, are interpreted as indicating a relatively short life span even under optimum conditions.

The few reported attempts to propagate serially *in vivo* other normal cells or tissues tend to support the findings reported here, although in *Drosophila* selected imaginal disc tissues can be transplanted for long periods.[13] In the case of mice, Krohn found that skin grafts became progressively smaller with repeated transfers.[14, 15] The ability of normal hematopoietic cells derived from marrow to proliferate *in vivo* also appears to be limited under the conditions of test.[16, 17] Similar results were obtained with cells from fetal liver, with the exception of two variant, undifferentiated lines which continued to proliferate during repeated serial transfer.[18] Experiments using hematopoietic cells required the use of heavily irradiated mice, and it is not known to what extent a decline in viability would take place under normal physiological conditions.

Serial transplantation of preneoplastic gland: Two lines of nonmalignant, preneoplastic mammary gland were serially propagated; both were originally derived from a single nodule found in an old, multiparous C3H female. The morphology of these outgrowths was variable,[19] but all were characterized by the presence of lobuloalveolar differentiation in the hormonal milieu of virgin hosts (Fig. 1*c*). The outgrowths occasionally gave rise to frank mammary carcinomas, which were not subsequently transplanted.

Under conditions of serial transfer identical to those described for propagation of normal gland, both preneoplastic lines survived and grew for longer than 8 years and for more than 30 transplant generations (Fig. 2, Table 1, expts. 6 and 7). Both lines were proliferating vigorously when the experiments were terminated. We conclude that for practical purposes these results indicate an indefinite life span *in vivo.*

From *in vitro* studies, Hayflick[4, 5] has proposed that both permanent cell lines and transplantable tumors are capable of unlimited cell multiplication, while temporary cell strains and normal *in vivo* somatic cells both have a finite capacity for cell replication. In this scheme the transition from limited to unlimited growth potential is coincident with the transformation from normal to neoplastic. Our finding that normal mammary gland has a limited life span *in vivo* is in agreement with this formulation, but the ability of nonmalignant, preneoplastic cells to grow without apparent limit constitutes an exception.

It is important to emphasize that mammary nodules and the outgrowths derived from them differ from malignant neoplasms in a fundamental respect: the proliferation of preneoplastic gland is fat-pad-dependent and its growth is limited by the amount of unoccupied fat available. Hyperplastic outgrowths are therefore subject to similar homeostatic control mechanisms that limit the expansion of normal gland *in situ*.[20] However, if unlimited space and substrate are provided by serial transplantation into gland-free fat pads, preneoplastic gland is capable of unlimited proliferation. In this respect it resembles mammary tumors but differs from normal gland, whose life span under similar conditions is limited.

Summary.—The *in vivo* life span of normal and preneoplastic mammary gland was studied by serial transplantation in gland-free mammary fat pads of young, isogenic female mice. The growth of transplants at each generation was used as an index of aging. The growth rate of normal gland declined with time and the oldest transplant line was lost after 7 generations and 2 years of serial passage. In contrast, the growth of preneoplastic gland was not time-dependent and two transplant lines were growing vigorously after more than 30 generations and 8 years in passage. It is concluded that normal mammary gland has a limited ability to proliferate *in vivo* even under favorable conditions, but that preneoplastic gland, like mammary tumors, has an apparently unlimited life span when similarly propagated.

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