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13. We thank B. Forget for providing the plasmids JW101 and JW102 and C. Palmer for manuscript preparation. Supported by research funds from the Veterans Administration and a grant from the Mississippi affiliate of the American Heart Association.

29 April 1982; revised 9 August 1982

Angiogenesis Induced by "Normal" Human Breast Tissue: A Probable Marker for Precancer

Abstract. *Normal human breast lobules, freshly isolated by precision microdissection of tissue stained with methylene blue chloride, were assayed for their ability to induce neovascularization (angiogenesis) in rabbit irises. Histologically, normal lobules from cancerous breasts induced angiogenesis twice as often as lobules from noncancerous breasts, suggesting that preneoplastic transformation is diffuse.*

The ability to evoke blood vessel formation (angiogenesis) is a common property of malignant tissue, including that of the breast (*1*). Without the benefit of neovascularization, tumors can grow only to spheroids 1 to 2 mm in diameter, the limit of nutriment diffusion (*2*). Most cancers and epithelial hyperplasias of the breast are believed to originate in the hormone-sensitive lobules located on the branching duct system that drains secretions to the nipple (Fig. 1A) (*3*, *4*). Although normal tissues are rarely angiogenic when transplanted to a suitable host (*5*), we now report that normal-appearing lobules from cancerous breasts are significantly more angiogenic than lobules from noncancerous breasts (*6*).

Multiple random samples were obtained 3 cm or more from the invasive ductal carcinoma in mastectomy specimens from 26 patients and from benign breast tissue in biopsy material from 34 patients. About 12 hours or less after surgery the excised tissues were cut into 2-mm-thick slices and submerged in methylene blue chloride (1 mg per 100 ml) at 4°C for 90 minutes (*7*). The blue-stained lobules (Fig. 1B) were then separated from the surrounding collagenous stroma under a dissecting microscope.

The lobules were transplanted onto the irises of New Zealand White female rabbits (2 kg) under anesthesia (*8*). The anterior chamber of selected eyes had been drained, causing the cornea to collapse. This pressed the transplant against the iris, where it adhered. Seventy-two percent of the lobules were ~ 1 mm in diameter and were transplanted in toto; of the bigger lobules, 1-mm fragments were transplanted. Since the excised breast tissues were originally handled without strict aseptic technique, they were rinsed in four batches of sterile saline before being cut into smaller pieces. Otherwise, aseptic technique was used throughout. The irises were observed daily for 5 days with a magnifying lens for the appearance of delicate capillaries growing toward the transplant (Fig. 1, C and D). On day 5 the rabbits were killed and the preparations were fixed and processed for light microscopic examination (Fig. 1E).

As shown in Table 1, 44 of the 155 normal-appearing lobules from cancerous breasts were angiogenic by day 5 (28 percent), compared to 46 of 307 lobules from noncancerous breasts (15 percent) ($P < .001$). Angiogenesis was observed sooner in lobules from cancer patients. By day 3, 21 percent of the lobules from cancerous breasts had elicited new vessel formation, compared to only 7 percent of the lobules from noncancerous breasts ($P < .0001$).

Lobules in older women tend to be smaller than those in younger women. Therefore, the data were analyzed to evaluate the effect of age and lobule size on angiogenesis. The analysis showed no significant effect (Table 2).

Usually the mastectomy specimens arrived at the laboratory in aseptic condition and the tissue used in the experiments was removed with sterile techniques. Therefore, the reported differences in angiogenicity between the two types of specimens cannot be attributed to bacterial contamination. Furthermore, the transplants did not display an acute inflammatory response.

To determine whether differential delays between excision and transplantation played a role in the observed differ-

Table 1. Angiogenicity of histologically normal lobules from cancerous and noncancerous breasts. Yates' correction for continuity was applied in making the statistical comparisons.

Breast type	Number of lobules eliciting angiogenesis	
	Three days after transplantation	Five days after transplantation
Cancerous	32 of 155 (21 percent)	44 of 155 (28 percent)
Noncancerous	21 of 307 (7 percent)*	46 of 307 (15 percent)†

*Significantly different from corresponding value for cancerous breast lobules [χ^2 (1) = 17.992, $P < .0001$]. †χ^2 (1) = 10.958, $P < .001$.

Table 2. Effects of patient age and lobule size on the angiogenicity of histologically normal lobules from cancerous and noncancerous breasts. Values in parentheses are percentages. Differences are not statistically significant.

Lobule source	Number of lobules eliciting angiogenesis			
	Age of patient		Lobule size	
	< 50 years	> 50 years	≤ 1 mm	> 1 mm
Cancerous breasts	26 of 95 (27)	18 of 60 (30)	30 of 112 (26)	14 of 43 (32)
Noncancerous breasts	43 of 279 (15)	3 of 28 (10)	33 of 224 (14)	13 of 83 (15)

0036-8075/82/1015-0293$01.00/0

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ences in angiogenesis, the recorded delays for 49 angiogenic transplants were compared statistically with those for 280 nonangiogenic transplants. For angiogenic and nonangiogenic transplants the mean delays were 9.1 and 10.2 hours, respectively. From the combined variances, the probability of a difference of 1.1 hours occurring by chance can be estimated as greater than .86; thus the difference is not significant and we may conclude that delays between excision and transplantation had no effect on the results.

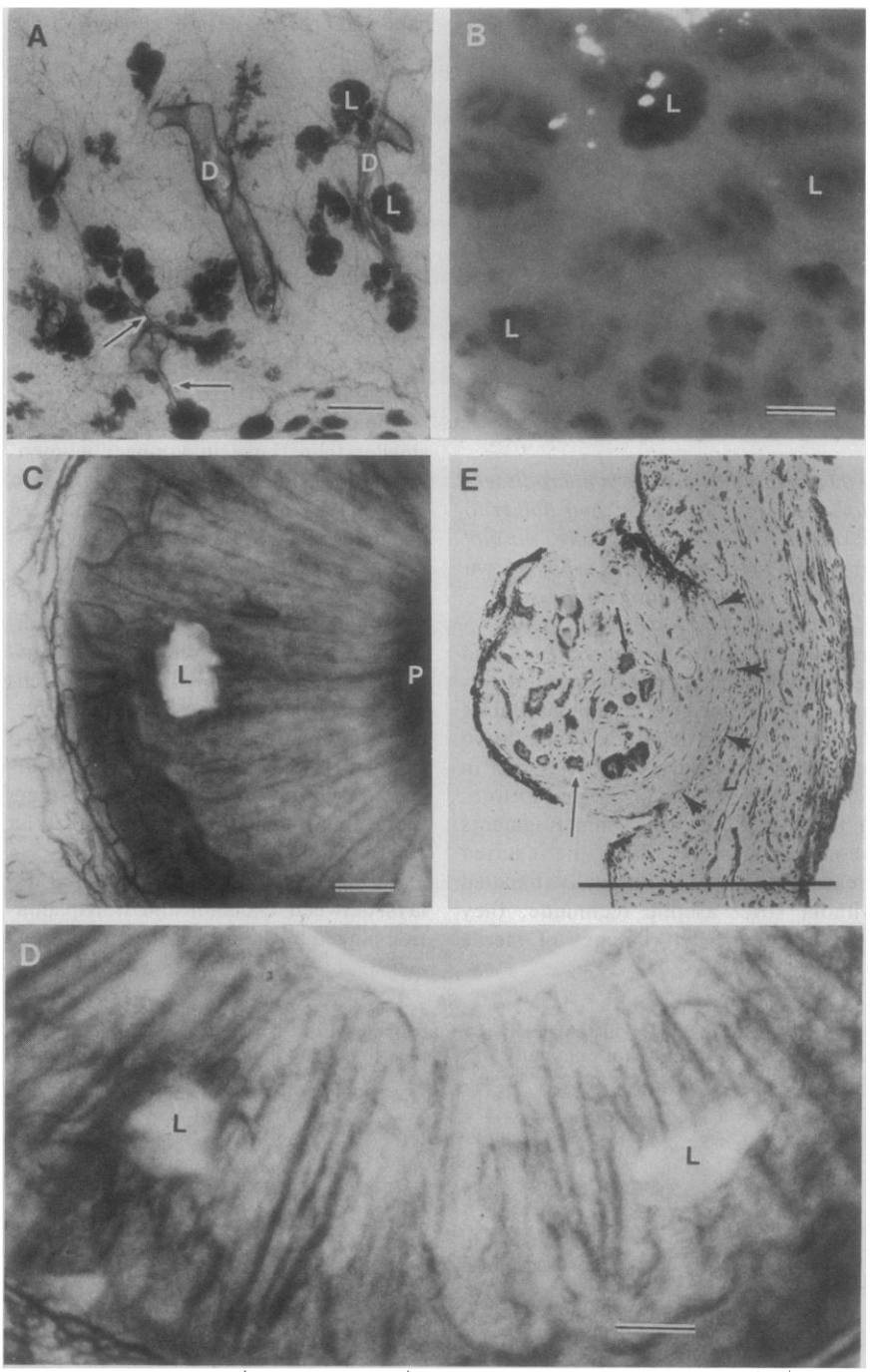

Fig. 1. (A) Section of Formalin-fixed human breast tissue (thickness, 2 mm) stained with hematoxylin. Lobules (*L*) are clustered around ducts (*D*). Arrows point to terminal ducts that drain individual lobules (×6). (B) Section of fresh tissue stained with methylene blue chloride. The cellular lobules are stained because the cells have imbibed the dye. (×10). (C) Lobule transplanted 5 days earlier, showing a halo of delicate vessels developing around it. The pupil (*P*) of the living rabbit is also visible (×8). (D) Two histologically normal lobules transplanted onto rabbit iris. Vessels surround the transplanted lobule on the left; the lobule on the right is not angiogenic (×10). Scale bars in (A) through (D), 1 mm. (E) Section of transplanted lobule and underlying iris (thickness, 7 μm) stained with hematoxylin and eosin. Arrows point to delicate ductules and arrowheads mark the border between lobule and iris. The surface of the lobule was inked before embedding to facilitate identification (×35). Scale bar, 1 mm.

Hyperplastic alveolar nodules (HAN) in the mammary glands of C3H mice, a strain with a high incidence of breast cancer, have been found to be precancerous by syngeneic homotransplantation (*9*). Thirty percent of HAN's tested are angiogenic on the rabbit iris (*10*). A HAN cell line with a low incidence of tumor induction (D1) induces angiogenesis at a lower rate (32 percent) than a HAN line with a high incidence of tumor induction (D2) (76 percent) (*10*). In mouse mammary papillomas the capacity to induce angiogenesis precedes visible malignant transformation (*11*). Hyperplastic epithelial lesions from human breasts evoke an angiogenic response in 30 percent of the cases (*12*). When sarcomas are induced in mice by the implantation of plastic cover slips, angiogenic factor can be demonstrated in the stimulated tissues months before any histological evidence of transformation is noted (*13*). These findings suggest that angiogenicity not only identifies cell populations at risk for neoplastic transformation but also precedes histological evidence of hyperplasia or neoplasia. Our data strongly support these conclusions.

In a previous study, Gullino and coworkers (*12*) found that only 3 percent of normal lobule fragments from noncancerous breasts and 7 percent of normal lobule fragments from cancerous breasts elicited neovascularization. This lower incidence of angiogenicity may have been due to the transplantation of most of the lobules as multiple fragments. In the present study lobules were transplanted in toto unless they were larger than 1 mm in diameter; thus there was more epithelium in each transplant. This probably accounted for the greater incidence of angiogenicity.

In most research on neoplastic transformation in mammary tissue, lobules selected for study have showed some morphological change. Such visibily altered lobules constitute only a small fraction of the 5000 to 10,000 normal lobules present in the premenopausal breast. Our data suggest that preneoplastic transformation is diffuse, precedes morphological changes, and involves at least one-fourth of all the lobules present. Thus more lobule tissue is available for study than has been realized, and it is in this morphologically normal tissue that the origins of malignancy are more likely to be found.

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14. We thank J. C. Holmes for computer programming, J. V. Edwards and S. J. Stowell for histology slides, and I. Fierro for preparation of the manuscript. Supported by contract N01-CB-84316 from the Breast Cancer Task Force of the National Cancer Institute.

28 April 1982; revised 16 July 1982

Destruction of *Trypanosoma cruzi* by Natural Killer Cells

Abstract. *Mice infected with* Trypanosoma cruzi *or stimulated with poly(inosinic · cytodylic acid) were found to possess splenic and peritoneal exudate cells with enhanced cytotoxic activity against epimastigote and trypomastigote forms of* Trypanosoma cruzi. *By use of specific alloantiserums it was determined that the effector cells responsible for this cytotoxic activity were typical natural killer cells.*

Trypanosoma cruzi is the obligate intracellular protozoan parasite that causes American trypanosomiasis (Chagas' disease). The parasite infects diverse species of mammals in addition to man, and elicits both humoral and cell-mediated immune responses. These immune responses are generally effective in protecting the host against lethal acute infections. Humans who survive the acute infection may develop chronic disease and frequently display various cardiopathies or enlargement of the hollow viscera (megasyndrome). The nature of the specific or nonspecific immune mechanisms that protect hosts from *T. cruzi* are not understood, although it appears that several interacting mechanisms collectively contribute to protective, but not complete, immunity (*1*). Assays in vitro show that direct destruction of the extracellular stage of the parasite (trypomastigote) is effected by antibody-dependent, complement-mediated lysis and antibody-dependent, cellular cytotoxicity involving granulocytes, lymphocytes, and monocytes (*1*). Cytotoxic lymphocytes from infected experimental animals or humans can detect and destroy *T. cruzi*–infected host cells (*2*, *3*). We have shown previously (*4*) that mice infected with *T. cruzi* exhibit augmented levels of apparently nonspecific killer cell activity against allogeneic tumor cells and that two distinct populations of killer cells are induced at separate times during the course of acute infection. Within 48 hours of the initiation of infection with *T. cruzi* (10^2 to 5×10^4 trypomastigotes, injected intraperitoneally) in mice, a significant increase occurs in natural killer (NK) cell activity in cells from the spleen or peritoneal cavity (*5*). This early phase of cytotoxic activity is followed at 16 to 19 days after infection by the expression of another phase of increased killer cell activity mediated by Thy 1^+, NK 1^- effector cells against several tumor cell lines (*6*). The significance of these observations on immunity to *T. cruzi* was not determined in our earlier work. In the studies reported here, we examined the ability of NK cells from two strains of mice, which differ in their susceptibility to *T. cruzi*, to destroy epimastigotes and trypomastigotes (from blood or from infected fibroblast cultures).

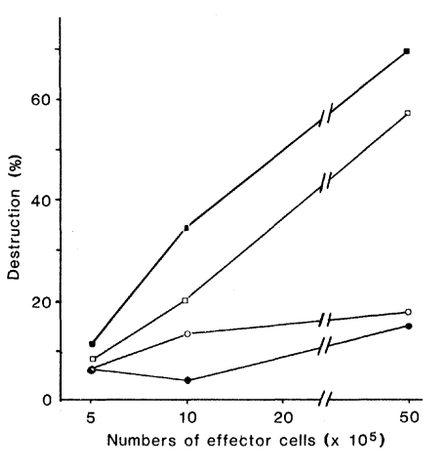

Fig. 1. Cytotoxicity assays with epimastigotes of *T. cruzi*. Spleen cells were obtained from normal (○, ●) or poly(I · C)–stimulated (□, ■) mice. Cells that were unfractioned (○, □) or nonadherent (●, ■) to plastic (primarily lymphocytes) were incubated with the parasites for 6 hours and then evaluated for their cytotoxic activity. Various concentrations of effector cells were tested with 2×10^4 parasites (see Table 1). Similar results were obtained when peritoneal exudate cells were used as effectors (data not shown).

Spleen cells and peritoneal exudate cells were removed from normal C57B1/6 or C3H(He)Dub female mice (8 to 12 weeks of age), from mice stimulated 18 to 20 hours previously with poly(inosinic · cytodylic acid) [poly(I · C), 100 μg in 0.1 ml of phosphate-buffered saline, injected intraperitoneally], or from mice infected with *T. cruzi* for 48 hours as previously described (*4*). Treatment of mice with either poly(I · C) or *T. cruzi* augments NK cell activity in various lymphoid organs as well as the peritoneal cavity of mice (*5*, *7*). We placed these cells in RPMI-1640 medium (supplemented with fetal bovine serum, 10 percent) containing epimastigotes (from axenic cultures in liver-infusion tryptose broth) or blood-form trypomastigotes [derived from infected mice as described in (*4*)] and determined their ability to destroy the parasites. As shown in Table 1, the ability to destroy both the trypomastigotes and epimastigotes was increased in cells from mice stimulated with poly(I · C) or infected with *T. cruzi*. The stimulation of cytotoxic activity by infection with *T. cruzi* was similar to that stimulated by poly(I · C), and both the highly susceptible C3H and the resistant C57B1/6 mice were stimulated to approximately the same degree (Table 1).

The assay system used to determine the destruction of *T. cruzi* is dependent upon the difference in numbers of parasites at the beginning and the end of the incubation period. It was therefore possible that the observed reduction in numbers of parasites (percentage destruction) was due to the phagocytic activity of macrophages in the cell populations. To test this possibility we conducted experiments with spleen and peritoneal exudate cells that had been depleted of adherent macrophages prior to their use in the cytotoxicity assays. On a cell to cell basis, cells that were nonadherent to plastic (predominantly lymphocytes) from poly(I · C)–stimulated mice were as active, or more so, as the unfractionated cells (Fig. 1). These results also demonstrated that increased numbers of effector cells mediated increased destruction of the parasite, as would be expected for active cytotoxic cells.

We studied the kinetics of the destruction of *T. cruzi* by incubating effector cells with epimastigotes for 3, 6, and 18 hours before assessing cytotoxic activity. The numbers of viable parasites cultured with cells from poly(I · C)–stimulated mice at the end of these periods

0036-8075/82/1015-0295$01.00/0

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