at which this might occur in the fetus is not known.

Glucuronidation of acetaminophen in isolated fetal liver cells was not detectable (Table 1). Attempts to demonstrate glucuronidation in human fetal liver microsomes have revealed negligible activity compared to the adult (*15*), which might be ascribed to low concentrations of both uridine diphosphate glucuronic acid (UDPGA) and microsomal glucuronyl transferase. Glucuronidation of many drugs and endogenous substrates is deficient (*16*) or poorly developed in the neonate (*17*). Moreover, sulfation of acetaminophen predominates over glucuronidation in the neonate (*12*). The presence of a sulfate but not a glucuronide conjugate of acetaminophen in human isolated fetal liver cells is in agreement with all these data.

Our results do not suggest that acetaminophen is unsafe during pregnancy when ingested in therapeutic doses for short periods of time. The fetal liver apparently has the ability to detoxify acetaminophen by conjugation with sulfate and GSH. The results do demonstrate that the fetal liver can oxidize acetaminophen to an active metabolite. It is not known whether the quantity of the drug that passes into the fetal circulation when the mother has taken a sublethal dose is large enough to result in damage to the fetal liver, depletion of GSH, and saturation of sulfate conjugation.

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7. Liver microsomes were incubated at a protein concentration of 2 mg/ml in the presence of an NADPH-generating system, GSH (5 m*M*), and acetaminophen (10 m*M*) at 37°C. The reaction was stopped by addition of 3*M* perchloric acid, the mixture was centrifuged, and the supernatant was stored frozen at −20°C until analysis. Formation of the acetaminophen-GSH conjugate by human fetal and adult liver microsomes was linear with time up to 20 minutes and with a microsomal protein concentration up to 4 mg/ml. Human fetal liver cells were isolated by the method of Fry *et al.* (*8*) for rat liver cells with the modification that the cells were allowed to sediment by gravity. Cell viability was examined by exclusion of trypan blue. Viable cells were counted in a Bücher chamber. More than 90 percent of the cells excluded trypan blue. Ultrastructural analysis substantiated the exclusion dye test in demonstrating general criteria for viable cells without signs of anoxic degeneration. Isolated fetal liver cells (7 to 10 × 10^6 cell/ml) in 10 ml of Eagle minimum essential medium (Bio-Cult, Scotland) containing 10 percent fetal calf serum, penicillin (100 unit/ml), streptomycin (100 μg/ml), and acetaminophen (10 m*M*) were incubated at 37°C. At incubation times from 15 to 60 minutes 1 ml of the incubate was removed and handled as described above.
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Angiogenesis in the Mouse Cornea

Abstract. *We have developed a method that permits analysis of neovascular responses in the mouse cornea. Using this method we have demonstrated that both allogeneic lymphocytes and a variety of tumors can induce angiogenesis, but that only the latter appear capable of eliciting secondary capillary sprouting.*

The importance of angiogenesis, or the formation of new blood vessels, is well recognized in a variety of disease processes, in inflammatory reactions, during the course of graft-versus-host reactions, in delayed hypersensitivity responses, and most dramatically as an essential component of tumor growth (*1*). Yet our understanding of the mechanisms underlying such induced neovascularization is limited, largely because no test systems have been developed that permit a critical assessment of the various component contributors to the angiogenesis phenomenon.

The primary test systems for angiogenesis in vivo are neovascular responses induced on the chicken embryo chorioallantoic membrane (CAM) (*2*) and neovascularization induced by im-

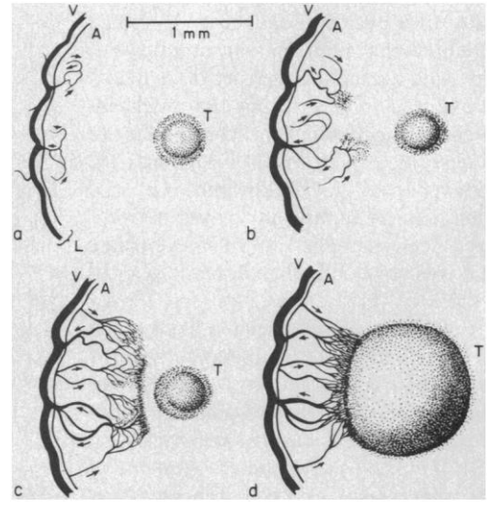

Fig. 1. A sequence of vascular responses in the cornea of albino C57BL/6 mice after implantation of C755 mammary adenocarcinoma fragments. The drawings of the living cornea were made under a stereomicroscope at (a) 2 days, (b) 6 days, (c) 8 days, and (d) 12 days after grafting. Vein (*V*) and artery (*A*) of the limbus (*L*) are shown in relation to the tumor graft (*T*). Arrows indicate the direction of blood flow.

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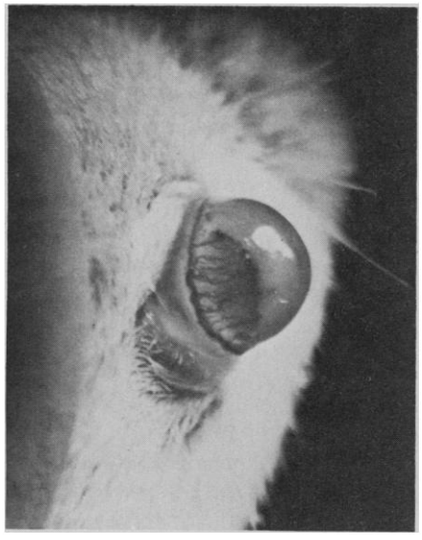

Fig. 2. Photograph of the living cornea of a BALB/c mouse bearing an S180 sarcoma graft.

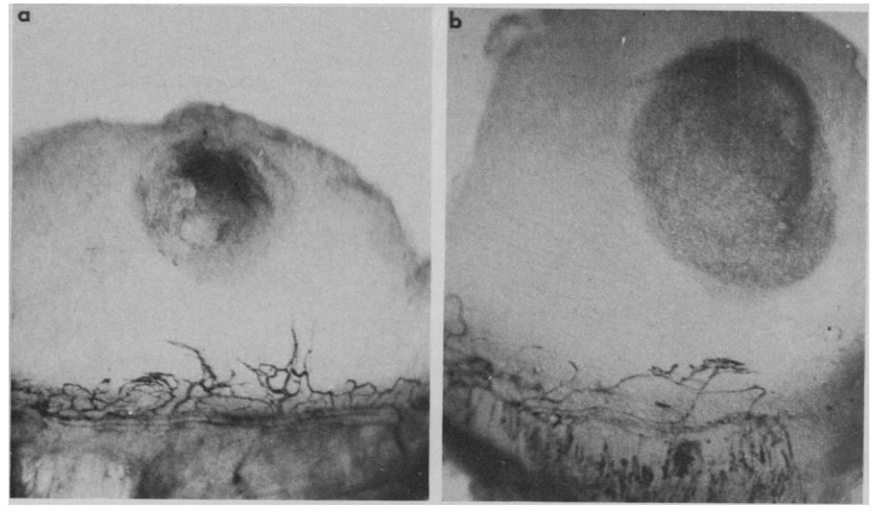

Fig. 3. Photograph of India ink–perfused corneas bearing lymph node or tumor graft. A contrasting pattern of distal capillaries can be seen. (a) BALB/c lymph node grafted into F_1 (BALB/c × A/J) host, 7 days after grafting and (b) C755 mammary tumor grafted into syngeneic C57BL/6J albino host, 10 days after grafting.

plantation of test tissues into the rabbit cornea (*3*). In both instances the test is usually carried out in the absence of genetically characterized host animals and usually involves xenogeneic donor tissue or cells. Test systems with endothelial cell cultures in vitro have not been fully validated as adequate models for angiogenesis, and to date also involve xenogeneic test systems (*4*).

It seemed to us of critical importance to develop angiogenesis assays for the mouse since this would permit us to utilize the wealth of information that has been obtained for inbred mouse lines and mutants, as well as to take advantage of the extensive data that are available for murine tumors of a wide variety of types and origins. To date the only assay system available for the mouse has been the intradermal assay developed in our laboratory (*5*), but this assay does not readily enable us to distinguish between neovascularization and other vascular changes, nor is it amenable either to sequential manipulation or to continuous monitoring. Clearly, the corneal assay is ideal in that it is conducted in vivo, yet all vessels originate from a distance, the limbal region, thus permitting monitoring and measurement of the progress of the vascular reactions. We have now developed a method of corneal implantation in the mouse that can be used as a means of assessing angiogenesis reactions induced by tumor cells and by semi-allogeneic lymphocytes.

The inbred mice we used included BALB/c, C57BL/6, albino C57BL/6, and F_1 (BALB/c × A/J). The tumors used were the C755 mammary adenocarcinoma of C57BL/6 origin and the S180 sarcoma originally obtained from BALB/c mice. The intracorneal grafting procedure was modified from that described by Gimbrone *et al.* (*4*). Young mice (6 to 12 weeks old) served as host animals. Mice were anesthetized with tribromoethanol. The eye was irrigated with antibiotics (500 units of penicillin and 500 μg of streptomycin per milliliter of phosphate-buffered saline). By means of a Beaver No. 65 surgical blade a transverse incision was made centrally, penetrating about halfway through the cornea. The corneal pocket was made by inserting a malleable iris spatula, with the pocket reaching to within 1 mm of the limbus. Tissue fragments (0.5 mm^3) were then inserted into the pocket.

The progress of vascularization of tumor implants is shown in Fig. 1. After initial looping of limbal capillaries, enlargement and extension of the loops into the cornea occurred. Subsequently, vascular sprouts appeared at the apices of the loops, a vascular network developed with the differentiation of arterial and venous branches, and vessels continued to grow toward the tumor implant. The development of blood vessels in the mouse eye, as well as the progressive growth of tumor grafts following vascularization, was comparable to that previously described for the rabbit eye (*3*). All five of the S180 sarcomas and 14 out of 22 C755 mammary tumors became vascularized and entered the logarithmic growth phase (Fig. 2); the others failed to initiate vascular reactions and did not develop. None of the control grafts (six mammary gland grafts and nine syngeneic lymph node grafts) induced angiogenesis.

We also examined the angiogenesis reaction resulting from implantation of immunocompetent, semi-allogeneic lymph node fragments. Prominent vasodilation in the limbal region immediately adjacent to the graft (day 2) was followed, in all eight animals, by the extension of new vascular loops into the cornea. The vascular reaction was greatest by day 4 (Fig. 3) and persisted up to 10 days before gradually receding. Unlike what was observed for tumor-induced angiogenesis (TIA), however, lymphocyte-induced angiogenesis (LIA) (*5*) did not manifest itself by the subsequent formation of capillary sprouts from the apices of capillary loops, and lymph node fragments did not become fully vascularized.

In these experiments we were able to distinguish between LIA and TIA (Fig. 1 and Fig. 3); in both instances angiogenesis was initiated, but the second phase of angiogenesis, involving distal sprouting of capillaries, occurred only in the tumor grafts. It would be interesting to determine the pattern of angiogenesis induced by hyperplastic pretumorous lesions (*6*): Would capillary sprouting mark the transition from benign to malignant growth potential?

By using the mouse corneal assay procedure it should be possible to examine the effect of specific immunization against tumor and transplantation antigens, the effect of natural killer cell activity, the function of blocking factors, and the role of strain differences in susceptibility to tumor growth.

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Imbalance in X-Chromosome Expression: Evidence for a Human X-Linked Gene Affecting Growth of Hemopoietic Cells

Abstract. *In each of six family members who were heterozygous at the X-linked locus for glucose-6-phosphate dehydrogenase, only one or the other of the two alleles at that locus was almost exclusively expressed. The data are consistent with evidence that X-chromosome inactivation is a random process that may be followed by selection for one of the two resulting cell types on the basis of an unknown gene, which is located on the X chromosome and which can affect the rate of proliferation of hemopoietic cells in humans.*

The structural gene for glucose-6-phosphate dehydrogenase (G6PD) is located on the X chromosome in humans (*1*). It is generally accepted that inactivation of the X chromosome occurs at random early in embryonic life (*2*). Only one allele is active in each somatic cell of women heterozygous at the *Gd* locus (*3*); this results in two populations of somatic cells. The factors determining the ratio between the two cell populations in various tissues are not yet fully understood (*4*). A marked deviation from a 1:1 ratio of the two cell types in adults is regarded as infrequent (*5*). This situation, in which one X chromosome is expressed to the almost complete exclusion of the other, will be referred to as an extremely unbalanced mosaic phenotype. There are two ways that this phenotype might be produced in a particular tissue: (i) by chance alone, if inactivation were to occur earlier than normal and were to affect the same chromosome (paternal or maternal X) in most cells of the whole embryo or of the primordial cell pool of that tissue, and (ii) by selection of one cell population over the other. An example of the latter mechanism was found to operate for the deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) in heterozygotes in whom erythroid cells consisted almost exclusively of the normal type, whereas in fibroblasts the expected two cell populations, HGPRT(+) and HGPRT(−), could be recognized (*6*).

We report that an extremely unbalanced mosaic phenotype can be demonstrated in heterozygotes for qualitative variants of G6PD. The data from one family suggest that at least one locus on the X chromosome (other than loci for G6PD or HGPRT) can affect the rate of proliferation of hemopoietic cells.

Hemolyzates were prepared and analyzed for G6PD by starch-gel (*7*) and cellogel (*8*) electrophoresis (*9*). In Nigeria, where the present study was carried out, the commonest type of heterozygote has the AB phenotype (*10, 11*). The relative proportions of the two G6PD types can be estimated semiquantitatively by visual inspection of gels; cases with a marked excess of either the A or B band have been reported (*12*). In addition, several G6PD variants can be easily resolved and more accurately quantitated by chromatography on DEAE-Sephadex columns (*13*), from which each enzyme has been found to elute at a characteristic and highly reproducible concentration of KCl (*14–16*). This technique shows that the relative amounts of two allelic G6PD types present in heterozygotes exhibit, in a number of cases, a large imbalance in favor of the mutant or of the normal enzyme type (Fig. 1).

To prove that at least some of such extreme phenotypes are caused by somatic cell selection, we studied a Nigerian family in detail (Fig. 2). The propositus, III-5, was a patient with homozygous sickle-cell anemia who had a slow-moving type of G6PD, the Ilesha variant (*17*). Upon testing 11 members in three generations of the family, one additional hemizygote and five heterozygotes for the Ilesha variant were found. In all five of them, the ratio between the two enzyme types was markedly different from unity. There was an approximately tenfold excess of G6PD Ilesha in four (I-2, II-4, III-2, and III-3) and a more than tenfold excess of G6PD B in the fifth (II-3) heterozygote.

In three members (II-4, III-2, and III-3), the white blood cells were also examined. Their G6PD phenotype was similar to the red cell phenotype. In member III-3, a repeat examination after 1 year showed that the ratio between G6PD B and G6PD Ilesha was stable, and chromosome studies, including G-banding, revealed a normal karyotype.

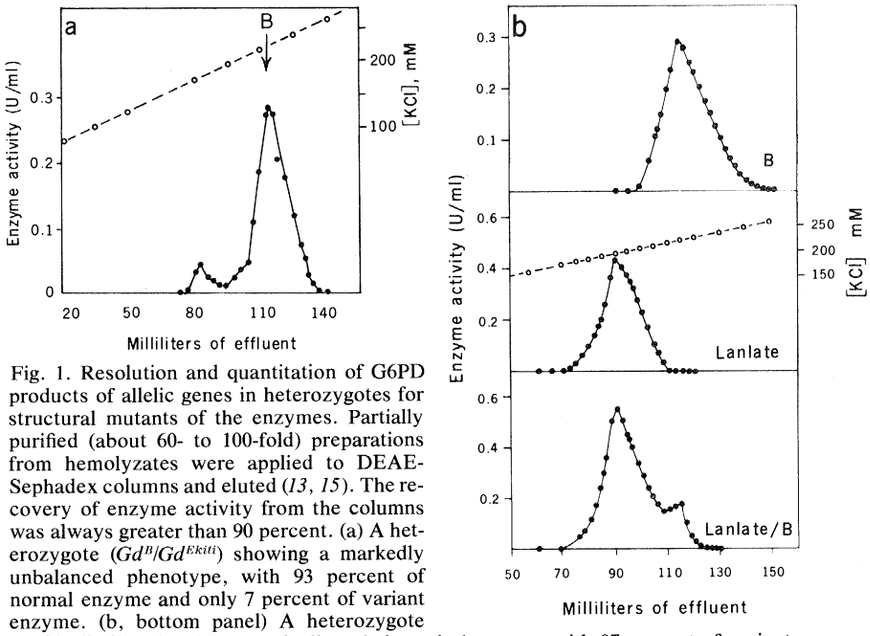

Fig. 1. Resolution and quantitation of G6PD products of allelic genes in heterozygotes for structural mutants of the enzymes. Partially purified (about 60- to 100-fold) preparations from hemolyzates were applied to DEAE-Sephadex columns and eluted (*13, 15*). The recovery of enzyme activity from the columns was always greater than 90 percent. (a) A heterozygote (Gd^B/Gd^{Ekiti}) showing a markedly unbalanced phenotype, with 93 percent of normal enzyme and only 7 percent of variant enzyme. (b, bottom panel) A heterozygote ($Gd^B/Gd^{Lanlate}$) showing a markedly unbalanced phenotype with 97 percent of variant enzyme and only 3 percent of normal enzyme. The upper two panels show control runs of G6PD type B and type Lanlate alone. The B band in the $Gd^B/Gd^{Lanlate}$ heterozygote could not be seen on starch gel. Characterization of these variants has been published (*15*).

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