erably greater degree of multilayered growth. These colonies stained very darkly and could be recognized with the unaided eye against the lighter-staining background cells (Figs. 1*A* and 1*B*). When cultures containing one or more of these dense colonies were replated and the cells fixed before a confluent layer was formed, two distinct kinds of colonies were obtained. One is typical of PY-3T3-31 (Fig. 1*C*, left); the other, seen only in plates infected with SV40 (Fig. 1*C*, right), is much denser and has sharper borders. Such SV40-transformed colonies when recloned gave rise only to dense colonies with sharp borders, and cell lines derived from such clones were able to grow to a saturation density greater than 10^6 cells per cm^2.

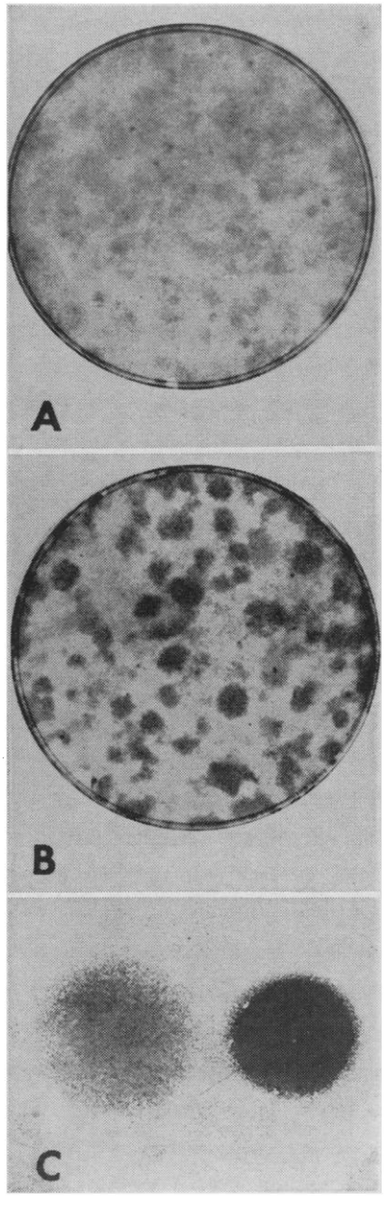

Fig. 1. Morphology of PY-3T3-31 cultures transformed by SV40. *A*, Saturation density PY-3T3-31, 12 days after inoculation of 1000 cells. *B*, Colonies transformed by SV40 12 days after inoculation of 1000 cells from a virus-infected culture. These colonies can be easily distinguished against the background of PY-3T3-31. They are very numerous, as the parent culture was allowed to remain at high density for several days after infection, in order to increase selectively the number of SV40-transformed cells. *C*, Discrete colonies from an SV40-infected PY-3T3-31 culture, 10 days after plating 100 cells. The dense colony on the right is SV40-transformed. All cultures were fixed with formalin and stained with hematoxylin.

The frequency of transformation of PY-3T3-31 cells by SV40 can be measured quantitatively in the same manner as described for transformation of 3T3 cells by SV40 (*3*, *6*). With SV40 at a titer that gives transformation frequencies of from 2 to 3 percent with 3T3 or clonal lines derived from 3T3 (*7*), we obtained transformation frequencies of PY-3T3-31 of from 0.02 to 0.03 percent (Table 1). Plates of PY-3T3-31 inoculated with 100 cells and read before confluence was reached showed no SV40 colonies out of a total of 2400 counted, a value consistent with a greatly reduced transformation frequency of PY-3T3-31. Similar values were obtained with line PY-3T3-11.

The production of different types of polyoma-transformed cells, some that form thin colonies and have a relatively low saturation density and others that form thick colonies and have a high saturation density, has been described by Vogt and Dulbecco (*8*) and Stanners, Till, and Siminovitch (*9*) using hamster cells and Weisberg (*10*) using mouse cells. While some of their clonally isolated lines of "thin" polyoma-transformed cells gave rise spontaneously during serial cultivation to "thick" colonies (*8*, *9*), PY-3T3-31 and PY-3T3-11 do not appear to contain thick colony-forming cells, at least under our culture conditions.

There is now evidence that the viral genome, or some portion of it, persists in transformed cells even in cases where the intact virus can no longer be demonstrated (*11*). Since both polyoma- and SV40-transformed cells contain new and specific cellular antigens (*12*), the doubly transformed cells described here might be expected to contain both antigens. If this is so, the presence of new genetic material from one oncogenic virus does not fully prevent the cell from being transformed again by a second virus, though it may be responsible for the greatly reduced susceptibility to SV40 transformation of the polyoma lines as compared with 3T3.

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16 November 1964

Transplantation Immunity of Gestational Origin in Infant Rats

Abstract. *Comparison of the survival times of homografts of BN skin on 3-day-old Lewis rats born of mothers of the same isogenic strain with those of BN grafts on infant Lewis hosts that had developed in an F_1 (Lewis × BN) hybrid, gave no evidence of maternally induced tolerance as a result of development in an antigenically alien environment. On the contrary, the significantly shorter median survival time of the grafts on the hybrid-derived Lewis group suggests that sensitization had occurred as a consequence of natural exposure during gestation to small numbers of maternal cells.*

Inoculation of fetal or infant mammals with living homologous tissue cells may induce tolerance of foreign transplantation antigens. The degree to which the animals are rendered unresponsive depends upon the species, their genetic disparity, the timing and route of inoculation, the dosage of cells, and other variables (*1*).

Consequently, in outbred popula-

tions, some individuals may display a diminished capacity to react against homografts of maternal skin as a result of natural prenatal "leakage" or exchange of cells across the placental barrier (*2*). In man, fetal-maternal exchange of red cells is fairly common, and evidence is accumulating that such an exchange applies to leucocytes and platelets as well. At present there is only indirect evidence of the occurrence of similar transplacental exchanges in other species (*3*).

Appropriately controlled skin grafting on mice, rabbits, and cattle has not produced evidence for maternally induced tolerance, though equivocal results have been reported for guinea pigs and man (*2, 3, 4*). Experiments have been made to increase the permeability of the placenta to cells. The administration of hyaluronidase or histamine to pregnant female rabbits (*5, 6*), or the x-irradiation of the gravid uterine horns of female rats (*7*), results in the induction of tolerance of maternal skin homografts in some of the offspring. An inherent shortcoming of outbred animals for these experiments is one's inability to distinguish between chance genetic compatibilities and compatibilities which are immunologically procured.

A possible explanation of the failure to demonstrate that maternally induced tolerance occurs naturally is that the tests were too exacting. Challenge of the offspring when a few weeks or months of age with grafts of their mother's skin may have failed to reveal feeble degrees of tolerance of short duration.

The availability of a sensitive test for tolerance, applicable to infant rats of one particular combination of strains, stimulated us to reinvestigate the influence of a genetically alien uterine environment on the immunological responsiveness of the offspring.

The basis of our test is that 3-day-old Lewis rats reject thin homografts of ear skin from adult BN donors as rapidly as adult Lewis hosts do (9). The median survival time of BN skin homografts on 3-day-old Lewis rats is 10.2 ± 0.8 days [standard deviation (S.D.) 1.18 days]; that of BN grafts on adult Lewis hosts is 10.0 ± 0.5 days (S.D. 1.17 days). With this antigenic system the 3-day-old Lewis rat behaves as if it has attained immunological maturity. If newborn (0- to 24-hour) Lewis rats are injected intravenously with low dosages of BN marrow cells and challenged with BN skin on the 3rd day after birth, then prolongations of graft survival, attributable to tolerance, are detectable. However, no such prolongations are observed if test grafting is postponed until the subjects have grown up. For example, test grafting at 3 days reveals that as few as 10^6 BN bone marrow cells induce some degree of tolerance (Table 1), whereas inoculation with at least 5×10^6 marrow cells is required if even a feeble degree of tolerance is to be detected by test grafting when the hosts are 8 weeks of age.

Ideally one would like to be able to test infant Lewis animals that had developed in a BN maternal environment. Although this could be effected either by transfer of fertilized eggs or by orthotopic ovarian homotransplantation into tolerant animals (*10*), the following alternative and simpler procedure was adopted since it achieves the same end (Fig. 1). Young BN × Lewis F_1 hybrid females were ovariectomized and concomitantly grafted orthotopically (*11*) with ovaries from Lewis donors which are genetically compatible. These hybrid hosts were then mated with Lewis males. The Lewis offspring resulting from these foster pregnancies should be in every respect similar to offspring of Lewis × Lewis matings except for the fact that, as a consequence of their development in the F_1 milieu, they may have been exposed to BN transplantation isoantigens through transplacental cellular exchange with their foster mothers. If enough maternal leucocytes gain access to these fetuses, then the induction of some degree of tolerance of test grafts of BN skin might be expected.

Since 50 percent of the progeny resulting from the fertilization by Lewis sperms of any ova deriving from re-

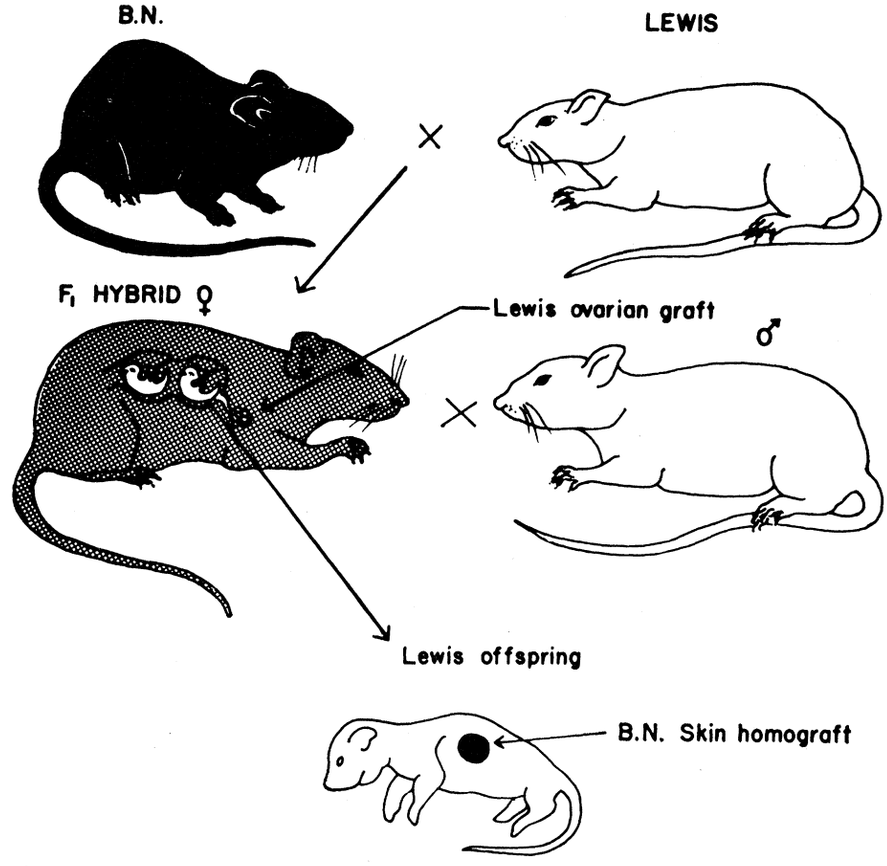

Fig. 1. Experimental design for determining whether there is a natural exposure of rats to maternal transplantation antigens. Lewis ovaries were grafted orthotopically to Lewis × BN F_1 hybrid females. Animals bearing these grafts were then mated with Lewis males, producing a population of Lewis animals which were in every respect similar to Lewis-derived Lewis animals except for the fact that they had developed in an F_1 hybrid uterine environment and consequently may have been exposed to BN transplantation antigens. From their reactivity to BN test skin grafts, transplanted when they were 3 days old, it was possible to determine whether the immunological reactivity of these hybrid-derived Lewis animals had been altered.

Table 1. Survival times of BN skin homografts transplanted to 3-day-old Lewis hosts. ME, maternal environment.

No. of BN marrow cells injected at birth*	No. of grafts alive on day												Median survival time (days)	S.D. (days)
	0	6	7	8	9	10	11	12	13	14	15	16		
						ME: Lewis								
None	40	40	39	37	27	25	9	8	1	0			10.0±0.5	1.17
						ME: Lewis								
10^6	19	19	19	16	15	12	12	11	11	11	10	8†		
						ME: Lewis × *BN* F_1								
None	44	44	41	30	15	5	2	0					8.5±0.4	1.16
						Lewis								
10^5	32	32	28	24	12	5	1	1	1	1	1	0	8.6±0.4	1.16

*All injections made intravenously. † All of these grafts were rejected by the 22nd postoperative day.

sidual fragments of indigenous F_1 ovaries should be of albino phenotype, the genotype of all albino animals born of ovarian graft recipients was confirmed at the conclusion of the tests. This entailed transplanting skin from all the progeny to Lewis hosts. Compatibility of these grafts indicated that only Lewis-strain transplantation antigens were present in the donors and, by implication, that they must have been of Lewis genotype.

Seventeen litters, comprising 72 rats, were delivered by hybrid females bearing orthotopic Lewis ovaries. Of these, 44 rats were successfully challenged with BN skin. Contrary to what was anticipated, none of these grafts outlived similar grafts of BN skin transplanted to 3-day-old Lewis rats born of Lewis mothers, which constituted the controls (Table 1). Indeed, the median survival time of the homografts on the rats which had developed in the F_1 environment (8.4 ± 0.4 days) was significantly shorter than the median survival time of grafts on Lewis rats born of Lewis mothers (10.0 ± 0.5 days; $.01>P>.001$). From this it is inferred that in some way maturation within the F_1 environment resulted in a sensitization of the offspring to BN antigens, instead of the induction of tolerance. The alternative, that a postpartum difference, such as the suckling on F_1 milk instead of on Lewis milk, was responsible for these findings seemed most improbable.

In the light of evidence that exposure of newborn mice to very small numbers of homologous cells—too few to cause tolerance—may elicit a weak though transient sensitivity, our finding suggests that the placental barrier is indeed compromised by cells of maternal origin, but in relatively small numbers. Some idea of the number of cells that may have entered the fetuses from their F_1 mothers may be gained from the observation that the median survival time of BN skin on 3-day-old Lewis hosts injected at birth with 100,000 BN marrow cells is 8.6 ± 0.4 days (Table 1).

Attempts were made to detect humoral antibodies in the young rats at intervals of 3, 7, 10, and 14 days after they had rejected their test grafts. Serum samples from panels of 3 to 4 animals were pooled and tested for agglutinins and cytotoxins (*12*). The failure to detect antibodies was not unexpected, since minimum immunization of even adult Lewis rats by inoculation of 10^6 BN lymphoid cells or by means of a single BN skin graft produce antibodies of low titer.

Our findings lend support to the thesis that some exchange of cells between mother and fetus must be a common event. The results are consistent with the belief that maternally induced tolerance may occur in some circumstances. As mentioned already, the response of the fetus to foreign cells will depend upon the time of their ingress, their number, and their genetic relationship to the host insofar as histocompatibility factors are concerned. Where there are differences with respect to strong histocompatibility factors, and where the cell numbers are small as in the experiments reported here, then a weak immune response can be anticipated. But where there are only minor histoincompatibilities, or where there has been infiltration by large numbers of maternal cells, tolerance may result. Either result may be transient and escape detection by conventional test grafting in adult life. This may account for Jones and Krohn's (*10*) failure to detect any difference in the response of CBA mice, originating from orthotopic CBA ovarian homografts in specifically tolerant A-strain females, to A-strain skin grafts. However, Moulton, Stimpfling, and Storer (*13*), in a study of differences at the H-2 locus, reported accelerated rejection of maternal-skin homografts by mice irradiated on the 15th day of gestation.

Our interpretation may likewise apply to Najarian and Dixon's observation (*14*) that neonatal rabbits from a heterogeneous population reject skin homografts significantly faster than adult rabbits. Furthermore, Taylor's (*15*) recent evidence that exposure *in utero* of Rh-negative females to cells of their Rh-positive mothers is responsible for their exhibiting, as adults, an immune response equivalent to the sensitizing stimulus of at least one Rh-positive pregnancy also is in accord with our observations.

The apparent ability of fetuses to build up a prompt immune response on exposure to small numbers of maternal cells may help to explain the rarity with which malignant cells—especially leukemic cells—of maternal origin become overtly established in infants (*3*).

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27 October 1964