PROCEEDINGS OF THE SOCIETY OF EXPERIMENTAL BIOLOGY AND MEDICINE 151, 307-309 (1976)

Leukemia Allotransplants in Canine Fetuses: Influence of Host Age and Immune Responsiveness[1] (39198)

MOSHE SHIFRINE AND BERNARD J. BRYANT[2]

(Introduced by C. Stormont)

Radiobiology Laboratory, School of Veterinary Medicine, University of California, Davis, California 95616

To ascertain the utility of a fetal allotransplant system in our studies of ^{90}Sr-induced canine leukemia, preliminary transplants of a myelomonocytic cell line were passaged in Beagle fetuses (8). The application of this method to studies of cancer immunology and immunotherapy awaits an experimental exclusion of the possibility that the grafts induce tolerance in the developing immune system by inhibiting the production of allospecific factors. The data reported relate our experience with the passaging of leukemia cells in canine fetuses and some of the immunologic sequelae.

Materials and Methods. Purebred Beagle dams in the final trimester of gestation were laparotomized under anesthesia and both uterine horns were exposed. The fetuses were injected ip through the uterine wall with inocula containing various radionuclide markers for subsequent identification by whole body gamma spectrometry (11). After surgical reconstitution, the dams progressed to normal whelping at term (62 to 63 days gestation).

Three cellular preparations were injected: sheep red blood cells (SRBC) (10^9 washed cells/ml), myelomonocytic leukemia cells (MMLC) (1-7 $\times$ 10^8 viable cells/0.1 ml), and MMLC X-irradiated with 2500 R at 250 kVp (irradiated MMLC) (2 $\times$ 10^7 viable cells/0.1 ml). Cell viability was measured in a standard trypan blue exclusion test. X-Irradiation of MMLC in culture prior to grafting inhibits subsequent mitoses but does not kill MMLC (unpublished data). The MMLC utilized in all procedures were derived from the stock of ^{90}Sr-induced canine MMLC that we used in the first fetal transplants (8).These cells had been passaged in culture for different lengths of time without loss of leukemogenicity or apparent alteration of the karyotype to the 13th passage (unpublished data).

Allografts of MMLC were performed on nine litters at ages ranging from gestation Day 43 to postnatal Day 1. The pups were monitored for evidence of disease in routine clinical examinations (8).

Pups of two other litters were inoculated on gestation Days 52 or 56 with tissue culture medium (controls) or with SRBC, irradiated MMLC, or both cell suspensions. Each pup was challenged with 1.7 $\times$ 10^8 proliferating MMLC injected ip on postnatal Day 1 and with 10^9 SRBC iv on postnatal Day 7. Titers of SRBC hemagglutinins in postnatal Day 7 prechallenge sera and in postnatal Day 14 sera were determined by a microhemagglutination method using 1% SRBC suspension and heat-inactivated serum. These 10 pups were monitored in clinical examinations for more than 8 months.

A final litter was inoculated on gestation Day 50 with 1.5 $\times$ 10^7 proliferating MMLC, and assays were done on postnatal Day 10 for peripheral blood lymphocyte reactivity to MMLC (mitomycin-treated) (4) and to phytohemagglutinin (PHA-M, Difco). Tumor cell cultures were initiated as mixtures of 2 $\times$ 10^6 mitomycined MMLC/5 $\times$ 10^5 blood lymphocytes in 2-ml culture. One-way tumor cell cultures, PHA cultures, and nonstimulated lymphocyte cultures were incubated for 6 days and assayed in triplicate by [^{14}C]thymidine uptake (4). At 21 days of age the pups received multiple dorsal intradermal injections of 3.6 $\times$ 10^6 proliferating MMLC/0.1 ml. The injection sites were examined daily for gross signs of engraftment.

Results and discussion. The 19 Beagle pups that developed myelomonocytic leuke-

[1] This work was supported by the U.S. Energy Research and Development Administration.

[2] Current address: Dept. of Pathology, School of Medicine, Wayne State University, Detroit, Michigan 48201.

mia after inoculation with proliferating MMLC on gestation Days 43 to 53 (Table I) presented clinical symptoms (enlarged lymph nodes, solid tumors, and hematologic abnormalities; ref. 8) within 8–71 days postpartum. Cells from passage 7 at a dosage of 2.5–3 × 10^7 cells per fetus induced leukemia in all inoculated fetuses at 43 days gestation but did not induce leukemia in any of the dogs inoculated at Day 1 postnatally. The disposition to leukemia apparently declined markedly with increasing fetal age at the time of grafting.

Successful leukemogenic transplants could have grown because of fetal immunoinsufficiency or because they were tolerogenic in the developing immune system. Results in Table II show that irradiated MMLC (viable but nonproliferating) failed to induce tolerance in four Beagle fetuses to a neonatal challenge with MMLC. Allograft rejection was indicated by the pups' asymptomatology for 8 months. SRBC pretreatment in four fetuses likewise failed to produce tolerance to a neonatal challenge with SRBC.

The most serious objection to the interpretation against induction of fetal tolerance is the possible inadequacy of cellular dosage. To test this possibility, proliferating MMLC (0.5–1.5 × 10^7 cells) were given to four fetuses on gestation Day 50. Although this cell dosage and fetal age combination could permit the inoculum to expand into a large clone and bring about high-zone tolerance, postnatal testing of peripheral blood lymphocyte reactivity to MMLC and intradermal MMLC allograft rejection indicated no such tolerance development.

The lymphocyte stimulation indices (mitogen-treated/control mean cpm ratios) for peripheral blood lymphocyte cultures initiated on postnatal Day 10 were 2.3, 5.4, 13.2, and 17.0 with one-way stimulation (MMLC), and 3.5, 3.7, 3.3, and 12.3, for the four pups, respectively, with PHA stimulation. All pups responded to MMLC with vigor comparable to that elicited by PHA;

TABLE I. TRANSPLANTATION OF CANINE MYELOMONOCYTIC LEUKEMIA IN BEAGLE FETUSES AND NEONATES.

Age at inoculation	Number of viable MMLC[a] (× 10^{-7})	Passage number	Leukemic dogs/Fetuses inoculated	"Takes" (%)
Gestation day				
43	2.5 or 20	7, 7[b]	8/8	100
46	60	2	2/3	66
50	1.0 to 25	1, 2, 4, 5[c]	8/12	66
53	14	3	1/4	25
Postnatal day				
1	3	7	0/4	0

[a] Myelomonocytic leukemia cells.
[b] Two litters were inoculated on gestation Day 43.
[c] Four litters were inoculated on gestation Day 50.

TABLE II. HEMAGGLUTININS AND ASYMPTOMATIC SURVIVAL IN BEAGLE PUPS TREATED WITH SHEEP RED BLOOD CELLS (SRBC) AND MYELOMONOCYTIC LEUKEMIA CELLS (MMLC) AFTER FETAL PRETREATMENT WITH SRBC AND IRRADIATED MMLC.

Pups per treatment group	Fetal inoculum[a]	Postnatal inoculum[b]		SRBC titers[c] Day 14	Health status, 8 months
		Day 1	Day 7		
Gestation Day 52					
3	Medium control	MMLC	SRBC	8, 8, 4	Aleukemic
1	Irradiated MMLC and SRBC	MMLC	SRBC	8	Aleukemic
Gestation Day 56					
3	Irradiated MMLC	MMLC	SRBC	256, 64, 32	Aleukemic
3	SRBC	MMLC	SRBC	256, 64, 32	Aleukemic

[a] 1.7 × 10^7 Irradiated MMLC (11th passage) and/or 7 × 10^8 SRBC/fetus.
[b] 1.65 × 10^8 Proliferating MMLC (11th passage) or 7 × 10^9 SRBC/pup.
[c] Day 7 (prechallenge) titers were negative.

but whether these were "full" or "partial" MMLC responses is not known because of the lack of a non-MMLC control group. Such responses nonetheless show that induced blood lymphocyte tolerance was at best incomplete.

The pups also rejected the multiple intradermal MMLC allografts, which were initiated at 21 days of age. The grafts presented within 1 to 2 days as small, discrete nodules at the injection sites. Microaggregates of medium- and large-sized mononuclear cells were found in Day 3 biopsy samples. However, all such evidence of engraftment had disappeared by Days 5 and 6. The rapidity of this presumptive rejection in comparison to skin grafts (7) implies prior sensitization, not tolerance.

These results, albeit limited, tend to deemphasize the explanation of tolerogenicity to leukemia growth in successful fetal allografting. Data for fetal lambs (5, 10) suggest that the sequential appearance of immune responsiveness to specific antigens represents a multifactorial maturation process within the developing immune system, not a variably diminishing tolerogenesis induced by specific antigens. Experimental tolerance has not been reported in any large mammalian species, and the only acknowledged natural nonself tolerance system in such species is that expressed in dizygotic twinning in cattle (6) and marmosets (2).

The apparent failure of gestation Day 50 leukemia allografts to induce full tolerance in pups suggests that leukemias can develop in the face of a hostile immune system. What, then, might be the factors disposing toward "takes" of leukemia allografts?

The intrathymic differentiation of PHA-responsive cells in Beagle concepti begins with a rapid concomitant expansion of thymocyte populations on gestation Days 50 to 55 (3, 4). Organized lymphatic tissues then begin to appear (3) and allograft rejection becomes vigorous. Humoral antibody synthesis remains deficient to a variety of nonpathogenic stimuli until after birth (9). These correlations suggest that the degree of fetal immunoinsufficiency at the time of allografting may decisively influence the tumorigenic capability of leukemia allotransplants.

Summary. Myelomonocytic leukemia cells allografted prenatally in some developing purebred Beagle fetuses elicited tumors postnatally in some of the pups. The later in gestation that the inoculum was delivered, the less was the disposition to eventual leukemia, indicating that fetal host age at the time of grafting and tumorigenesis are correlated. The leukemogenic transplants may have grown because of fetal immunoinsufficiency or because they were tolerogenic in the developing immune system. Limited immunologic data for exclusion of the latter possibility are reported.

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Received September 16, 1975. P.S.E.B.M. 1976, Vol. 151.