Swain, in *Mechanisms of Pain and Analgesic Compounds*, R. F. Beers, Jr., and E. G. Basset, Eds. (Raven, New York, 1979), p. 429.
3. C. A. Haertzen, *Psychopharmacology* **18**, 366 (1970); D. R. Jasinski, in *Drug Addiction*, W. R. Martin, Ed. (Springer-Verlag, Berlin, 1977), vol. 1, p. 216.
4. A. S. Keats, J. Telford, in *Drug Design, Advances in Chemistry* (ser. 45) (American Chemical Society, Washington, DC, 1964), p. 170; E. F. Domino, P. Chodoff, G. Corssen, *Clin. Pharmacol. Ther.* **6**, 279 (1965).
5. R. S. Zukin and S. R. Zukin, *Mol. Pharmacol.* **20**, 246 (1981); R. Quirion, R. F. Hammer, M. Herkenham, C. B. Pert, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5881 (1981); S. W. Tam, *ibid.* **79**, 6703 (1983); L. G. Mendelson, V. Kalra, B. G. Johnson, G. A. Kerchner, *J. Pharmacol. Exp. Ther.* **233**, 597 (1985).
6. G. T. Shearman and A. Herz, in *Drug Discrimination: Application in CNS Pharmacology*, F. C. Colpaert and J. L. Slangen, Eds. (Elsevier, Amsterdam, 1982), p. 37.
7. W. R. Martin, C. G. Eades, P. E. Gilbert, J. A. Thompson, *Subst. Alcohol. Actions Misuse* **1**, 269 (1980); D. B. Vaupel, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 1333 (1982); J. J. Teal and S. G. Holtzman, *J. Pharmacol. Exp. Ther.* **212**, 368 (1980).
8. J. J. Teal and S. G. Holtzman, *J. Pharmacol. Exp. Ther.* **215**, 369 (1980); K. T. Brady, R. L. Balster, E. L. May, *Science* **215**, 178 (1982).
9. A. Pfeiffer, S. Braun, K. Mann, K.-D. Meyer, V. Brantl, *J. Clin. Endocrinol. Metabol.* **62**, 181 (1986).
10. MR 2033 Cl: [2α,3(*S**),6α,11(*R**)] (±) 1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-[(tetrahydro-2-furanyl)methyl]-2,6-methano-3-benzazocin-8-ol hydrochloride. H. Merz, K. Stockhaus, H. Wick, *J. Med. Chem.* **18**, 996 (1975); H. Merz and K. Stockhaus, *ibid.* **22**, 1475 (1979); M. Hutchinson, H. W. Kosterlitz, M. Leslie, A. A. Waterfield, L. Terenius, *Br. J. Pharmacol.* **55**, 541 (1975).
11. D. V. Zerssen, in *Assessment of Depression*, N. Sartorius and T. A. Ban, Eds. (Springer-Verlag, Berlin, 1985), p. 270.
12. H. M. Emrich *et al.*, *Pharmakopsychiatr. Neuro-Psychopharmakol.* **10**, 265 (1977); G. Honigfeld, R. D. Gillis, C. J. Klett, in *ECDEU, Assessment Manual for Psychopharmacology*, W. Guy, Ed. (HEW Publ. ADM76-338) (Government Printing Office, Washington, DC, 1976), p. 265.
13. P. L. Wood *et al.*, *J. Pharmacol. Exp. Ther.* **215**, 697 (1980); M. B. Tyers, *Br. J. Pharmacol.* **69**, 503 (1980); G. T. Shearman and A. Herz, *Psychopharmacology* **78**, 63 (1982).
14. A. Pfeiffer, A. Pasi, P. Mehraein, A. Herz, *Neuropeptides* **2**, 89 (1981); ———, *Brain Res.* **248**, 87 (1982); J. Magnan, S. J. Paterson, A. Tavani, H. W. Kosterlitz, *Arch. Pharmacol.* **319**, 197 (1982).
15. R. F. Mucha and A. Herz, *Psychopharmacology* **86**, 274 (1985); R. F. Mucha, M. J. Millan, A. Herz, *ibid.*, p. 281.
16. A. Goldstein, *Physiol. Psychol.* **8**, 126 (1980).
17. H. M. Emrich, P. Vogt, A. Herz, *Ann. N.Y. Acad. Sci.* **398**, 108 (1982); H. M. Emrich, *Psychiatr. Dev.* **2**, 97 (1984); C. Schmauss and H. M. Emrich, in *Handbook of Psychoneuroendocrinology*, C. B. Nemeroff and P. T. Loosen, Eds. (Guilford, New York, in press).
18. The volunteers were healthy male medical doctors or students (mean age 27 ± 1.5 years, $n = 30$) who gave written informed consent to participate in the study. They had no histories of previous major illness and were evaluated as healthy by routine clinical examination.
19. We thank R. Welchering and A. Wendl for help during the experiments and A. Yassourides for statistical advice.

14 November 1985; accepted 15 April 1986

Transplantation of Fetal Hematopoietic Stem Cells in Utero: The Creation of Hematopoietic Chimeras

ALAN W. FLAKE, MICHAEL R. HARRISON, N. SCOTT ADZICK, ESMAIL D. ZANJANI*

Transplantation of normal, immature, fetal hematopoietic cells into a preimmune fetal recipient with a congenital hemoglobinopathy may allow partial reconstitution of normal hemoglobin production without the complications associated with postnatal bone marrow transplantation (immunosuppression and the occurrence of graft versus host disease). In order to test this hypothesis the naturally occurring polymorphism at the β-hemoglobin locus of the sheep was used as a marker for engraftment and hematopoietic chimerism. Intraperitoneal injection of allogeneic fetal stem cells into normal fetal lambs resulted in hematopoietic chimerism in three of four surviving recipients. This chimerism has been sustained for 6 months after birth and 9 months after engraftment, without evidence of graft versus host disease, and without the use of immunosuppressive therapy.

THE RECONSTITUTION IN UTERO OF cell lines that are either deficient or defective with normal cells has considerable theoretical appeal and potential clinical application. Treatment of congenital hematopoietic diseases by the creation of hematopoietic chimerism through stem cell reconstitution with normal cells represents a potential method of alleviating the clinical manifestations of some of these diseases. However, current methods of postnatal transplantation of stem cells are hampered by the complications of immunosuppression and the occurrence of graft versus host disease (GVHD) (*1*). These problems result from the immunocompetence of the recipient or graft or both and thus may be avoidable if grafting is performed before immunocompetence is well established. Transplantation of hematopoietic stem cells derived from an early gestational fetus into an immunologically immature fetal recipient offers such a possibility.

Our goal in this study was to produce an animal model of hematopoietic chimerism by the in utero transplantation of fetal hematopoietic stem cells. We chose sheep for the following reasons: (i) the sheep has two naturally occurring alleles of the β-hemoglobin locus (A and B), which can be used as definitive markers of successful engraftment and chimerism (*2*); (ii) gestation in the pregnant ewe is sufficiently long (term, 145 days) to allow experimental manipulation in the first trimester; (iii) the immune status of the fetal lamb is relatively well documented (*3–5*), permitting accurate selection of preimmune donors and recipients; and (iv) we have extensive experience with fetal surgery in the sheep (*6*). We report here that transplantation of fetal hematopoietic cells into preimmune fetuses resulted in the creation of hematopoietic chimeras. This chimerism has persisted for 6 months after birth and 9 months after engraftment without the occurrence of GVHD.

Pretyped homozygous AA and BB Dorset-Merino sheep (*Ovis aries*), from separate herds in Minnesota and California, respectively, were used for breeding stock. We transplanted hematopoietic stem cells obtained from the combined livers of 35- to 50-day-old fetal AA sheep (two males and one female) into eight 45- to 65-day-old homozygous BB fetuses by injecting the cells intraperitoneally. Access to the recipient was achieved by exposure of the uterus through a midline laparotomy incision. The layers of the myometrium and chorion were transversely incised, taking care to leave the amnion intact. The fetus was visualized and gently manipulated into an amniotic bubble. Donor cells (1 to 2 ml per fetus) were injected intraperitoneally through a 22-gauge needle. The fetus was returned to the primary amniotic space, and the myometrium was closed in a double layer. The pregnancy was allowed to proceed to term and vaginal delivery.

In order to prepare fetal liver hematopoietic cells, slices of liver were washed [three times in heparinized α-minimum essential medium (MEM) + 10% fetal sheep serum] and passed through a wet screen mesh under a constant stream of α-MEM. The cell suspensions were passed through stainless steel mesh screens of progressively smaller pore sizes until a single cell suspension was obtained (final pore size, 0.2 mm). The mixture was allowed to stand at room temperature for 5 minutes and the top two-thirds of the cell suspension was removed, pelleted by

A. W. Flake, M. R. Harrison, N. S. Adzick, University of California, Department of Surgery, HSE 585, San Francisco, CA 94143.
E. D. Zanjani, Veterans Administration Medical Center, Minneapolis, MN 55417.

*To whom correspondence and reprint requests should be addressed.

centrifugation, and resuspended to the desired concentration in heparinized α-MEM. Each recipient fetus received a single injection of 2×10^8 to 5×10^8 nucleated cells per kilogram of body weight (estimated). Blood samples were obtained within a week of birth and at monthly intervals thereafter on all surviving lambs.

As with all surgical procedures on the early gestational fetus, there was a high perioperative mortality. Four fetuses survived the procedure. Of these, three were born with clear evidence of donor cell engraftment as determined by both hemoglobin (Table 1) and chromosome (Table 2) analyses. The remaining animal (1287), a twin of one of the chimeric lambs (1286), received the same cell suspension as its sibling, but failed to exhibit chimerism by either hemoglobin or chromosome analysis. One of the chimeric lambs (553) died abruptly of sepsis at 1 month of age. Autopsy revealed multiple abscesses in the liver, lungs, and subcutaneous tissue. Histologic examination of the abscesses was suggestive of *Corynebacterium ovis*, a bacterium endemic to the sheep population. Examination of sections of the intestine, skin, liver, and spleen revealed no evidence of GVHD.

The two remaining animals have maintained their chimerism for 6 months after birth without evidence of GVHD. The relative amounts of donor hemoglobin in these two lambs ranged from 14 to 29% of the total hemoglobin present in the host circulation (Table 1). Karyotype analysis of peripheral blood lymphocytes and of the bone marrow hematopoietic cells revealed that the chimerism involved not only the erythroid components but also the donor lymphoid elements. Of the 48 karyotyped cells obtained from the peripheral blood of female animal 1286 at 1 month of age, 13 were male. Karyotype analysis of bone marrow cells from this animal, cultured in the presence of erythropoietin for 3 days and containing more than 92% erythroid and granulocytic cells, revealed 11% male cells.

Bone marrow transplantation has been used to successfully treat many congenital hematologic diseases, including sickle cell disease, thalassemia major, Wiskott-Aldrich syndrome, chronic granulomatous disease, and severe combined immunodeficiency syndrome (*7*). In most cases however, donors with histocompatibility antigens (HLA) identical to those of recipient children are not available, which precludes transplantation. In addition, by the time most of these children are considered for transplantation they have already been compromised by their disease. Recurrent infections, growth retardation, and sensitization by multiple transfusions can reduce the likelihood of a successful outcome. Marrow-conditioning regimens, with radiation and chemotherapy, and chronic immunosuppression to control GVHD are frequently required (*1*). The combination of the disease and its treatment result in a high morbidity and mortality for the pediatric recipient. The possibility of avoiding these problems provides the rationale for therapy in utero.

Our results demonstrate that a significant portion of the recipient fetuses exhibited sustained engraftment of allogeneic donor cells without evidence of GVHD. Sustained engraftment of allogeneic cells in a recipient fetus is critically dependent on fetal immunologic tolerance of the graft. Fetal tolerance resulting in permanent chimerism similar to that reported here has been documented to occur naturally in animal (*8*) and human (*9*) twins with shared placental circulation. Fetal tolerance has been demonstrated experimentally in several animal models (*10–12*). Although transplantation immunity develops early in all mammals of long gestation, there is a period in early gestation when the fetus is uniquely tolerant of foreign antigens (*13*). In the sheep fetus, prolonged survival of allogeneic skin grafts prior to 67 days of gestation has been demonstrated, while grafts placed after 77 days of gestation are rejected vigorously (*5*). In a previous study, we observed chimerism in three of five lambs transplanted at 85 to 105 days of gestation with allogeneic adult bone marrow cells (*14*), which suggested to us that even after the onset of immunocompetence the fetus is uniquely susceptible to tolerance induction. However, these animals died of severe GVHD. These adult-to-fetus transplantation studies demonstrated that, while significant engraftment of adult donor hematopoietic cells occurred in the recipient fetuses, the recognition of the host environment as foreign by the immunocompetent adult cells invariably resulted in the development of GVHD in the chimeric animals.

The absence of GVHD in our two long-term chimeric lambs suggests that the preimmune fetus is also the ideal stem cell donor. Lethally irradiated mice reconstituted with allogeneic fetal stem cells do not develop GVHD (*15*). Similarly, there have been no reports of GVHD in humans transplanted with fetal liver cells from human fetuses less than 14 weeks old (*16*). However, despite this potential advantage, fetal liver stem cells do not readily engraft in postnatal human recipients. It is possible that the relatively high rate (75%) of engraftment reported here is associated with the existence of a more receptive hematopoietic microenvironment in the fetus at an early gestational age than in the postnatal recipient. The developing bone marrow spaces and architecture in recipients at this developmental stage may provide the appropriate environment for the donor fetal cells to follow their natural "homing" and proliferative tendencies (*17*).

Clinical application of this approach will require consideration of several factors. First, the timing of transplantation may be critical to success. The developmental stage at which we were successful in the lamb corresponds to a gestational age of 18 to 20 weeks in the human fetus (*17, 18*). Prenatal diagnosis now allows the diagnosis of thal-

Table 1. Relative distribution of hemoglobins A (donor) and B (recipient) in lambs transplanted in utero. Hemoglobin types were determined on cold peripheral blood red cell lysates by isoelectric focusing (*20*). Percentages of the two hemoglobin types were determined by chromatography on carboxymethylcellulose columns (*21*) for reticulocyte enriched [^{3}H]leucine-labeled peripheral blood lysates or, when required, leucine-labeled bone marrow aspirates.

Animal no.	Hb type	Percentage of type A or B at time					
		1 week	1 month	2 months	3 months	4 months	6 months
1287	A	0	0	0	0	0	
	B	100	100	100	100	100	
553*	A	26					
	B	74					
1286	A	21	29	17	22	24	23
	B	79	71	83	78	76	77
1340	A	16	18	20	17	14	14
	B	84	82	80	83	86	86

*Animal died at 1 month of age of sepsis.

Table 2. Gestational age of the recipient fetuses at transplantation, phenotypic sex, and karyotype of the newborn lamb at 1 week of age. Chromosomal analyses were performed on peripheral blood lymphocytes and cultured bone marrow cells. Results from bone marrow are presented here.

Animal no.	Gestational age (days)	Phenotype	XY (%)	XX (%)
1287	62	F	0	100
553	55	M	90	10
1286	62	F	11	89
1340	55	M	88	12

assemia and sickle cell disease by the early second trimester (*19*), a time when intrauterine transfusion is technically feasible. Second, the optimal dose of donor stem cells remains to be defined. It is generally accepted that even partial reconstitution of normal hemoglobin production in patients with hemoglobinopathies may alleviate the clinical manifestations of these disorders. The donor hemoglobin levels of 14 to 29% achieved in this study are impressive when one considers that only a fraction of the 2×10^8 to 5×10^8 nucleated cells injected per kilogram of body weight were actually hematopoietic stem cells. Higher concentrations of stem cells in a similar volume of injected material should be well tolerated by the fetus, and may result in greater engraftment. Finally, our findings in this sheep model may not be applicable to the human fetus. Similar studies in a primate model are needed.

The successful achievement of hematopoietic chimerism in a large animal model by transplantation of fetal stem cells in utero suggests that this approach may provide an alternative to termination of pregnancy when, on prenatal diagnosis, a fetus is found to have a congenital hematopoietic disease.

REFERENCES AND NOTES

1. N. Kamani and C. S. August, *Med. Clin. North Am.* **68**, 657 (1984).
2. M. H. Blunt and T. H. J. Huisman, in *The Blood of Sheep: Composition and Function*, T. H. J. Huisman, Ed. (Springer-Verlag, New York, 1975), pp. 155–187.
3. A. M. Silverstein, R. A. Prendergast, K. L. Kraner, *Science* **142**, 1172 (1963).
4. A. M. Silverstein, J. W. Uhr, K. L. Kraner, *J. Exp. Med.* **117**, 799 (1963).
5. A. M. Silverstein, R. A. Prendergast, K. L. Kraner, *ibid.* **119**, 955 (1964).
6. M. R. Harrison, M. S. Golbus, R. A. Filly, *The Unborn Patient: Diagnosis and Treatment* (Grune and Stratton, New York, 1984).
7. E. D. Thomas, *Transplant. Proc.* **17**, 428 (1985).
8. R. D. Owen, *Science* **102**, 400 (1945).
9. I. O. Szymanski, C. A. Tilley, M. C. Crooksteon, *J. Med. Genet.* **14**, 279 (1977).
10. R. E. Billingham, L. Brent, P. B. Medawar, *Nature (London)* **172**, 603 (1953).
11. R. M. Binns, *ibid.* **214**, 179 (1967).
12. K. A. Porter, *ibid.* **200**, 789 (1960).
13. J. Soloman, *Foetal and Neonatal Immunology* (North-Holland, Amsterdam, 1971), pp. 234–306.
14. E. D. Zanjani *et al.*, *Nature (London)* **295**, 244 (1982).
15. D. E. Uphoff, *J. Natl. Cancer Inst.* **20**, 625 (1958).
16. R. J. O'Reilly, N. Kappoor, D. Kirkpatrick, in *Primary Immunodeficiencies*, M. Seligman and W. H. Hitzig, Eds. (Elsevier/North-Holland, New York, 1980), pp. 419–447.
17. D. Metcalf and M. A. S. Moore, in *Frontiers of Biology-Hemopoietic Cells*, A. Neuberger and E. L. Tatum, Eds. (North-Holland, Amsterdam, 1971), pp. 172–271.
18. N. Mukhopadhyay *et al.*, *Clin. Immunol. Immunopathol.* **10**, 59 (1978).
19. J. M. Old *et al.*, *Lancet* **1982-II**, 1413 (1982).
20. P. G. Righetti *et al.*, *J. Biochem. Biophys. Methods* **1**, 45 (1979).
21. J. B. Clegg, M. A. Naughton, D. J. Weatherall, *J. Mol. Biol.* **19**, 91 (1966).

22. Supported in part by Veterans Administration and by NIH grants from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases AM-24027, AM-30914, and AM-29890.

13 January 1986; accepted 6 June 1986

Amplification of an Esterase Gene Is Responsible for Insecticide Resistance in a California *Culex* Mosquito

CLAUDE MOUCHÈS, NICOLE PASTEUR, JEAN B. BERGÉ, OLIVIER HYRIEN, MICHEL RAYMOND, BRUNO ROBERT DE SAINT VINCENT, MONIQUE DE SILVESTRI, GEORGE P. GEORGHIOU

An esterase gene from the mosquito *Culex quinquefasciatus* that is responsible for resistance to a variety of organophosphorus (OP) insecticides was cloned in λgt11 phage. This gene was used to investigate the genetic mechanism of the high production of the esterase B1 it encodes in OP-resistant *Culex quinquefasciatus* Say (Tem-R strain) from California. Adults of the Tem-R strain were found to possess at least 250 times more copies of the gene than adults of a susceptible strain (S-Lab). The finding that selection by pesticides may result in the amplification of genes encoding detoxifying enzymes in whole, normally developed, reproducing insects emphasizes the biological importance of this mechanism and opens new areas of investigation in pesticide resistance management.

ALTHOUGH THE DEVELOPMENT OF resistance to chemical insecticides in arthropod pests constitutes a worldwide economic problem (*1*), there is virtually no information on the modifications that occur at the gene level in the process of acquiring resistance. Genes have been identified and sometimes mapped in studying the physiological or enzymological modifications in resistant insects. Hypotheses concerning genomic changes include structural changes as well as overproduction of either the target molecules or the detoxifying enzymes. For example, the modification of acetylcholinesterase encountered in some pests resistant to organophosphates (OP) and carbamates is believed to be a consequence of point mutation (*2*).

Here we show that overproduction of a detoxifying esterase is the result of gene amplification in *Culex quinquefasciatus* Say. In this mosquito, and in many other species of arthropods, OP resistance arises because one or several detoxifying esterases have become highly active and thus prevent the toxicant from reaching its target at lethal concentrations (*3*). Recently, one of these enzymes (esterase B1) was purified to homogeneity from the Californian strain Tem-R (*4*) and an antiserum raised against the single 67-kD structural polypeptide subunit it contains. The antiserum enabled us to estimate that esterase B1 is 500-fold more abundant in OP-resistant Tem-R than in susceptible (S-Lab strain) mosquitoes (*5*). In the present study, we have cloned a complementary DNA (cDNA) to messenger RNA (mRNA) of esterase B1 and we show that the corresponding genomic fragment is amplified at least 250-fold in resistant Tem-R.

We first verified that the in vitro translation products of total polyadenylated [poly(A)$^+$] RNA's isolated from larvae of Tem-R mosquitoes contain a 67-kD polypeptide comigrating with purified esterase B1 (Fig. 1A, lane 2) that could be specifically precipitated by its antiserum (Fig. 1B, lane 1). RNA's from Tem-R larvae were then utilized to prepare cDNA's. These cDNA's were introduced into the single Eco RI restriction site of the expression phage λgt11 and plated on an *Escherichia coli* Y1090 host. One recombinant phage, λgt-est, contained in the *lacZ* gene a cDNA insert coding for a 19-kD amino acid sequence immunoreactive with antiserum to esterase B1. This cDNA insert (700 bp) was purified and shown to be able to select by hybridization an mRNA that codes for esterase B1, as judged by in vitro translation (Fig. 1C) (*6*).

We therefore used λgt-est DNA as a probe to detect the esterase B1 gene in genomic DNA of OP-resistant (Tem-R) and -susceptible (S-Lab) adult mosquitoes. Equal quantities of genomic DNA from both types of mosquito were digested to completion with Eco RI and the fragments

C. Mouchès, J. B. Bergé, M. de Silvestri, Institut National de la Recherche Agronomique, Station de Recherches de Nématologie et de Génétique Moléculaire des Invertébrés, B.P. 2078, 06606 Antibes, France.
N. Pasteur and M. Raymond, Institut des Sciences de l'Evolution (UA 327 du CNRS), Université de Montpellier II, Place Eugène Bataillon, 34060 Montpellier, France.
O. Hyrien and B. Robert de Saint Vincent, Institut Pasteur, Laboratoire de Génétique Somatique, 28 Rue du Docteur Roux, 75724 Paris, France.
G. P. Georghiou, Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside CA 92521.