Toward Development and Production of Human T Cells in Swine for Potential Use in Adoptive T Cell Immunotherapy

Brenda M. Ogle, Ph.D.,^{1,2} Bruce E. Knudsen,¹ Ryuta Nishitai, M.D., Ph.D.,³
Kiyoshi Ogata, M.D.,⁴ and Jeffrey L. Platt, M.D.^{1,5-7}

Immunotherapy and vaccination for cancer or infection are generally approached by administration of antigen or stimulation of antigen-presenting cells or both. These measures may fail if the treated individual lacks T cells specific for the immunogen(s). We tested another strategy—the generation of new T cells from hematopoietic stem cells that might be used for adoptive immunotherapy. To test this concept, we introduced T cell-depleted human bone marrow cells into fetal swine and tested the swine for human T cells at various times after birth. Human T cells were detected in the thymus and blood of the treated swine. These cells were generated *de novo* as they contained human T cell receptor excision circles not present in the T cell-depleted bone marrow. The human T cells were highly diverse and included novel specificities capable of responding to antigen presented by human antigen-presenting cells. Our findings constitute a first step in a new promising approach to immunotherapy in which tumor- or virus-specific T cell clones lacking in an individual might be generated in a surrogate host from hematopoietic stem cells of the individual to be treated.

Introduction

IMMUNOTHERAPY IS WIDELY VIEWED to be a promising approach to the treatment of cancer and chronic viral infections (reviewed by Gilboa,¹ Mocellin *et al.*,² Letvin and Walker,³ and Tramont and Johnston⁴). Immunotherapy is sometimes approached by the delivery of excess antigen that would activate lymphocytes, particularly T cells, which might not be activated naturally by the cancer or virus. As one example, Rosenberg *et al.*⁵ used a synthetic analog of gp100_{209–217}, a human melanoma antigen recognized by T cells, to generate immunity against melanoma cells and thus to treat malignant melanoma. Another approach to immunotherapy involves amplifying the function of antigen-presenting cells. As one example, Salgia *et al.*⁶ engineered autologous tumor cells to secrete granulocyte-macrophage colony stimulating factor (GM-CSF), a potent promoter of the differentiation of dendritic cells,^{7,8} for treatment of non-small-cell lung cancer. Still another approach to immunotherapy involves isolating lymphocytes and/or antigen-presenting cells from the individual to be treated and expanding the population of effector cells or facilitating antigen presentation *ex vivo* and then returning those cells to the individual. As an example of this approach, Slingluff *et al.*⁹ isolated dendritic cells from subjects with gastro-

intestinal malignancies, pulsed the cells with MAGE-3 (a tumor-derived peptide), and returned the pulsed cells to the subjects.

Each of these approaches to immunotherapy has certain advantages; however, each benefits only a fraction of those treated. Treatment with large amounts of gp100_{209–217} peptide enhanced T cell responsiveness (i.e., cytotoxicity) in two of nine and improved the clinical course in only one of the nine subjects.⁵ Treatment with non-small-lung tumor cells engineered to secrete GM-CSF enhanced T cell responsiveness (i.e., delayed-type hypersensitivity) in 18 of 22 but did not improve the clinical course of any of 22 treated subjects.⁶ Treatment with dendritic cells pulsed with MAGE-3 enhanced T cell responsiveness (i.e., cytotoxicity) in four of eight but did not improve the clinical course of any of the eight treated subjects.⁹

While various factors may limit the efficacy of immunotherapy, we reasoned that immunotherapy might fail entirely if the individual to be treated lacks T cells capable of responding to antigen. While healthy individuals may have a broad repertoire of T cells and thus plentiful responder cells, those with cancer or chronic viral infection may not. Further, cancer and virus infections may generate lacunae in the repertoire of potential responder cells.^{10,11} In particular, cancer can also delete potential responding T cells by inducing

¹Transplantation Biology Program and ²Department of Physiology, Mayo Clinic, Rochester, Minnesota.

³Department of Surgery, Kyoto University Hospital, Kyoto, Japan.

⁴Department of Thoracic Surgery, Tokyo Medical University, Tokyo, Japan.

Departments of ⁵Immunology, ⁶Surgery, and ⁷Pediatrics, Mayo Clinic, Rochester, Minnesota.

anergy,¹² and viruses can delete potential responder T cells by directly infecting and destroying the responder cells.^{13–15}

To overcome the limitation imposed by defects in the T cell repertoire, two approaches have been taken—thymus transplantation and generation of new T cells *in vitro*. Markert *et al.*^{16,17} used thymus transplantation to restore the T cell repertoire in subjects with DiGeorge syndrome and acquired immunodeficiency syndrome. New T cells can also be generated *in vitro*.^{18–22} For example, Poznansky *et al.*²² seeded CD34⁺ or AC133⁺ cells on CellFoam coated with thymic stroma. These three-dimensional constructs supported T cell development more efficiently than two-dimensional constructs (~60% of CD45⁺ cells). However, the total number of mature T cells generated was low, and the diversity of these cells unknown.

We reasoned that to the extent that immunotherapy fails because the T cell repertoire of the treated individual lacks responder cells capable of recognizing a virus or a tumor, that problem could be addressed by approaches that would generate new T cells not otherwise present. To achieve that goal we tested whether animals might be used as “incubators” in which new T cells would be generated from human hematopoietic stem cells and whether the T cells so generated could recognize antigen presented by human antigen-presenting cells (APC) and respond specifically to that antigen. Using animals in this way takes advantage of the heightened ability of the young thymus to generate T cells and potentially avoids deletion of useful effector cells during development.

Generating new T cells in animals is limited, however, by at least two barriers. First, the immune system of the animal may reject the human cells. This barrier can be surmounted if hematopoietic stem cells are transferred into the fetus that lacks full adaptive immune function.^{23–25} Second, to the extent that T cell functions are “restricted” by major histocompatibility complexes (MHC), human T cells produced in an animal may not recognize antigen presented by antigen-presenting cells of the human subject. This barrier might be surmounted if T cells were sufficiently cross-reactive. Consistent with this possibility, Nikolic *et al.*²⁶ found that human T cells selected by porcine thymus engrafted in immunodeficient mice cross-react with human MHC and thus may potentially function.

We report here that human T cells can be produced *de novo* in an adoptive xenogeneic host and that the T cells so generated comprise a concentrated population with a diverse repertoire, including specificities that did not exist in the donor blood. The T cells can recognize and respond to antigen presented in the context of human MHC. This concentrated, diverse sample of T cells should be explored as an approach to immunotherapy and immune reconstitution.

Materials and Methods

See Supplemental Information, available online at www.liebertonline.com/ten.

Results

Human cell detection in swine

To determine whether human T cells can develop in swine, human bone marrow cells depleted of T cells were

TABLE 1. EXPRESSION OF HUMAN RNA SEQUENCES IN CHIMERIC SWINE

Marker	Expression: no. positive (no. tested)			
	1 week	1 month	6 months	18 months
β -2 microglobulin	8 (14)	8 (10)	6 (6)	2 (2)
CD3	8 (14)	8 (10)	6 (6)	2 (2)
Chimera age	Hematopoietic lineage (%CD45)			
	CD3	CD19	CD14	CD16/CD56
6 months	71 $\pm$ 12	21 $\pm$ 9	3 $\pm$ 4	1 $\pm$ 1

transplanted into fetal swine at 40–43 days of gestation and members of litters were studied at various ages after birth. Fourteen injected swine from three separate litters were studied. Human cells were detected by reverse transcriptase (RT)-polymerase chain reaction (RT-PCR) for human β -2 microglobulin mRNA (Table 1) and by *in situ* hybridization for *Alu* (previously reported by Ogle *et al.*²⁷) in peripheral blood and bone marrow of eight swine (approximately 60% of those injected). By both measures, the level of chimerism was greatest 1 week after birth (range 0.01–6%) and declined thereafter as determined by densitometry of RT-PCR bands relative to human:pig mixed controls (i.e., 1:1,1:10, 1:100, 1:1000, and 1:10,000) and by counting the number of *Alu*⁺ cells as a fraction of *Alu*[−] cells (average of 15 fields). Despite this decline, chimerism was maintained at low levels in all swine until sacrifice, in two cases at 18 months of age. To some extent, the decrease in proportion of human cells in the circulation of chimeric swine may reflect dilution in the increasing volume of swine blood; however, the trend does not appear to solely reflect increase in blood volume as normalization for weight does not alter the trend (data not shown).

Identification of human T cells and antigen-presenting cells in chimeric swine

We next asked whether human T cells circulated in the chimeric swine. To address that question, peripheral blood mononuclear cells (PBMC) were tested by RT-PCR for human CD3; the sensitivity for detection was 1 human cell in 10,000 porcine cells (Table 1). Eight of 14 piglets (from three sows) tested had circulating human T cells. Since antigen-presenting cells affect the function of human T cells, we also tested whether these cells were present in peripheral blood of chimeras at 6 months of age. Using assays capable of detecting 1 human cell in 10,000 porcine cells, we determined that T cells made up 71 $\pm$ 12%, B cells (CD19) 21 $\pm$ 9%, macrophages (CD14) 3 $\pm$ 4%, and natural killer cells (CD16, CD56) 1 $\pm$ 1% of CD45⁺ cells of this population in six chimeric pigs. Because we do not know to what extent the populations in blood reflect the total population of these cells, we are reluctant to compare these proportions to those usually observed in human blood (if human B cells or natural killer preferentially reside in spleen the relative numbers of cells in blood of chimeric swine would suggest falsely that these cells were less represented).

FIG. 1. Human cells engrafted in bone marrow of chimeras. BMC were seeded on methylcellulose dishes and incubated at 37°C for 2 weeks. **(A)** Representative colony-forming unit-granulocyte/macrophage (CFU-GM) derived from chimera bone marrow; all colony types were represented in the culture (4×; inset, 40×). To determine whether any of the colonies formed on the treated dishes were derived from human hematopoietic precursors and to determine the fraction of human CD34⁺ cells relative to the total number of CD34⁺ cells of the bone marrow, colonies were removed from plates and pooled. RNA was extracted from pooled colonies and probed for human β -2 microglobulin. **(B)** RT-PCR for human β -2 microglobulin. Chimera 2 and 3 exhibit a detectable level of β -2 microglobulin ranging from 0.1% to 1% of all hematopoietic precursors.

Human cell engraftment in chimeric swine

Because CD3 RNA expression persisted until sacrifice in all swine and in two cases at 18 months, we considered as one explanation that human hematopoietic precursors engrafted in the bone marrow of chimeric swine. To confirm engraftment, we conducted colony-forming assays on bone marrow-derived cells (BMC) of chimeras (Fig. 1). BMC of chimeras were seeded on methylcellulose membranes, and all colonies formed were removed, pooled, and probed for human β -2 microglobulin using semiquantitative RT-PCR. Human CD34⁺ cells accounted for 0.1–1% of all CD34⁺ cells

of chimeras. The level of engraftment is substantial if one considers that human cells were engrafted in a xenogeneic system and that the host did not undergo ablative therapy prior to cellular transplantation.

Localization of human cells in thymus tissue of chimeric swine

The human T cells in chimeric swine could have developed *de novo* or could have been carried into the swine as residual T cells contaminating the T cell-depleted bone marrow (the depleted bone marrow contained 0.1% T cells). To determine whether human T cells might have developed *de novo*, we tested thymus tissue of chimeric swine for human T cells. Six thymus tissues were positive for human CD3 by RT-PCR, suggesting human T cells might be present. To locate these human cells, we performed *in situ* hybridization using an *Alu* probe (blue stain; human specific) and co-reacted the sections with antibodies against CD3 (brown stain; not species-specific). Five of six tissues analyzed contained cells positive both for *Alu* and CD3 (i.e., human T cells); approximately 1 in 2500 thymocytes was dual labeled (15 fields analyzed per thymus; SPOTTM Image Analysis Software; Leica Systems, Bannockburn, IL). Dual-labeled cells were detected in the cortex and medulla (Fig. 2D), some in clusters (Fig. 2C). We also located naïve human T cells in the medulla at a frequency of 1 in 10,000 (CD45RA, 15 fields analyzed per thymus; SPOT Image Analysis Software, Leica Systems; Fig. 2F, G) and confirmed that such cells were not epithelium (cytokeratin; Fig. 2H, I).

Detection of newly generated human T cells in chimeric swine

To ascertain whether human T cells in the chimeric swine actually developed *de novo* in swine (rather than having expanded numbers by homeostatic proliferation), we examined blood for human T cell receptor excision circles (TREC), that is, fragments of DNA excised during rearrangement of T cell receptor genes.²⁸ Since TREC are not replicated during mitosis, the quantity of TREC DNA per number of cells should be proportional to the number of T cells that have not undergone mitosis and hence the proportion of recent thymic emigrants. To determine TREC, we designed primers specific for human TREC and conducted real-time PCR on DNA from PBMC of chimeric swine. Six of seven chimeric swine tested 1 month after birth had detectable human TREC (Fig. 3; mean 433 copies of TREC/100 ng DNA). Given the average TREC level for a 1-month-old infant (10,000 TREC/100 ng DNA) and correcting for the fraction of human cells detected in the chimeric swine at this time point (0.1%), one would expect approximately 10 copies of TREC/100 ng of DNA in chimeric swine.^{29–31} The larger than anticipated amount of human TREC in chimeric swine might reflect one of several operative mechanisms. First, the number of human T cells relative to other human blood cells surviving in the chimeric swine might be disproportionately high. Second, thymic export in swine may exceed that of humans and thus a higher proportion of T cells in the periphery (including human T cells) may have TREC. Third, human T cells in chimeric swine might be less apt to proliferate than human T cells in humans. In any case, the detection of human TREC in chimeric swine suggests new human T cells developed in the swine thymus.

Diversity of human T cells in chimeric swine

If swine are to be used as a "system" in which human T cells can be generated, the T cells generated should be sufficiently diverse to provide protective immunity. We therefore asked whether and to what extent human T cells in swine comprise a diverse repertoire. To determine the diversity of human T cells in chimeric swine, we used a technique recently developed in our laboratory for directly measuring diversity of lymphocyte receptor genes.³² The technique uses the oligonucleotide probes present on human gene chip arrays as a "relatively" random probe library to determine diversity of T cell receptor (TCR)-specific RNA of a given T cell compartment. We previously demonstrated that the frequency of hybridization of nucleic acids coding for lymphocyte receptors to the oligonucleotides on a gene chip varies in direct proportion to diversity.³² We also showed that under physiological conditions, diversity of T cells in humans correlates directly with the level of thymic function and correlates with the level of TREC in blood.^{33,34} We focused our analyses on the human α chain of the TCR because porcine β chain of the TCR is quite homologous with human. A primer specific for the human α chain was used to extract TCRV α -specific RNA from PBMC, which was biotinylated and applied to a gene chip. Raw data corresponding to oligo location and hybridization intensity were obtained, and the number of oligo locations with intensity above background (i.e., number of "hits") was summed. Simultaneously, a standard curve (diversity vs. hits) was generated by applying samples with known numbers of different oligonucleotides to gene chips. TCRV α diversity of test samples was extrapolated from the standard curve based on the number of hits. The diversity of α chains of peripheral T cells of three healthy human subjects was 9.8×10^2 , 7.3×10^4 , and 5.1×10^4 (Fig. 4; mean, $4.2 \pm 3.7 \times 10^4$). To our knowledge, this is the first direct quantification of TCR α chain diversity in humans.

Given the fraction of human cells in chimeric swine at this time point (0.1%), one might expect a diversity of human TCRV α of approximately 42 ($42,000 \times 0.1\%$). The diversity of peripheral T cells in three chimeric swine tested was 7.4×10^2 , 3.5×10^3 , and 9.3×10^2 (Fig. 4; mean, $1.7 \pm 1.5 \times 10^3$). Thus, the diversity of human T cell receptor α chains was larger than anticipated. This result could reflect a disproportionate number of human T cells relative to other human blood cells in the chimeric swine. Alternatively, diverse human T cells may emerge from the porcine thymus and survive in the periphery as a consequence of increased cross-reactivity of human T cells with porcine MHC.²⁶ The impact of cross-reactivity between the T cells and antigen-presenting cells of these species may also extend to the periphery where sustained interactions may promote the extended survival of T cells.

Function of human T cells generated in chimeric swine

To determine whether the human T cells generated in swine could contribute to immune responses, we tested the proliferative response of these cells to allogeneic cells in mixed leukocyte cultures. Toward that end, PBMC of four different chimeric swine were labeled with carboxyfluorescein succinimidyl ester (CFSE) and combined with irradiated human and swine cells from various sources. Seventy-two hours later, cultures were stained with anti-human CD4-phycoerythrin. The number of CFSE_{low}/human CD4⁺ cells

was determined by flow cytometry. PBMC of three of four chimeras proliferated in response to allogeneic stimulation; responses of a representative chimera are shown here (Fig. 5A). Human T cells from chimeric swine responded to allogeneic lymphoblastoid cells derived from a healthy individual (6.5% of the cells proliferating; mean of three independent experiments, $4.8 \pm 1.8\%$, $p < 0.05$ compared to serum stimulated chimera PBMC) but not to autologous human lymphoblastoid cells from the chimeric swine ($<1\%$ of cells proliferating; mean $0.9 \pm 0.15\%$, $p > 0.1$). Human T cells responded weakly, if at all to PBMC from an unrelated pig ($\sim 2\%$ of total cells; mean 1.9 ± 0.36 , $p < 0.05$), consistent with the limited *in vitro* responses observed in such xenogeneic mixed cultures.³⁵ As a more sensitive measure, we sorted CFSE_{low} cells/human CD4⁺ cells and probed them for expression of T cell receptor V β by a reverse transcriptase PCR specific for human. Human T cell receptor V β mRNA was detected in the human cells from chimeric pigs that responded to allogeneic (human) antigen-presenting cells but not to syngeneic or xenogeneic antigen-presenting cells (Fig. 5B). The lymphoblastoid cells from chimeric swine did stimulate vigorous proliferative responses by third-party human T cells (data not shown). Thus, the human T cells generated in swine respond vigorously to allogeneic stimulation but exhibit tolerance to "self" based on *in vitro* measures.

Novel human T cell specificities generated in chimeric swine

Chimeric swine might be used to generate immunity against infectious agents or cancer to which a stem cell donor is tolerant. This therapeutic approach would be possible if the T cells so generated had novel specificities (not detectable in the original donor) and could respond to antigen presented by APC of the donor. To test these questions, we immunized the chimeric swine with parvovirus, pseudorabies, *Mycoplasma hyopneumoniae*, and tetanus toxoid vaccines and measured the human T cell responses that ensued. Four weeks after vaccination, PBMC were obtained from the swine, labeled with CFSE, and incubated with donor APC (lymphoblastoid cells derived from donor bone marrow mononuclear cells as previously described using Epstein-Barr virus³⁶) and vaccine antigens. As a control, donor bone marrow cells were labeled with CFSE and incubated in the same conditions. Four days after incubation, CFSE levels were measured by flow cytometry, and proliferating cells (CFSE_{low}) were isolated and probed for human TCRV α mRNA by semiquantitative RT-PCR. Human T cells from two of three chimeric swine tested proliferated in response to parvovirus, *Mycoplasma hyopneumoniae*, and tetanus toxoid antigens presented by APC of the human donor, while human donor cells did not. Responses of a representative chimera are shown here (Fig. 6A, B). The response to parvovirus, *Mycoplasma hyopneumoniae*, and tetanus by human T cells in chimeric swine could reflect the response of *de novo* sensitized cells or the response of the low frequency of memory T cells transferred. That the T cells in unmanipulated marrow exhibited no response suggests that the former is a better explanation. In addition, while porcine T cells responded to pseudorabies vaccine, human T cells exhibited no detectable proliferation, indicating that the proliferation of human T cells, or in this case lack thereof, is specific for

FIG. 2. Human T cells residing in the thymus of chimeric swine. Human T cell-depleted bone marrow or umbilical cord blood was injected into fetal swine, and the thymi of the chimeras was studied by microscopy at various times after birth. The thymi were tested for the presence of *Alu* (human-specific repeat element; blue staining) by *in situ* hybridization, and expression of CD3 (not species specific; brown staining) by immunohistochemistry. (A) Human thymus tissue (positive control). (B) Porcine thymus tissue (negative control). (C, D) Thymus tissue of two different chimeric swine 6 months after birth. Insets indicate dual-labeled cells. The thymus was also tested for expression of human CD45RA (human specific; brown staining) to detect naïve human T cells. Since this antibody was human specific, *Alu* colocalization was not performed. (E) Human thymus tissue (positive control). (F, G) Thymus tissue of two different chimeric swine 6 months after birth. Insets indicate labeled cells. Thymi were also tested for expression of cytokeratin (not species specific; brown staining) to distinguish epithelial cells from T cells. This antibody did cross-react with swine and so colocalization with *Alu* as in (A–C) was performed. (H) Human thymus tissue (positive control). (I) Thymus tissue of one chimera. Dual-labeled cells were not detected in any tissue section tested. Scale bar, 10 μ m. The procedures used are described in Supplemental Information.

antigens. Thus, T cells generated as reported here might, after transfer back to the donor, exhibit responses not otherwise present in the donor.

Discussion

We report here what we believe can be the first step toward a practical goal of generating new T cells that might contribute to protective immunity against cancer or infectious agents such as viruses. Because these new T cells

originate from the individual to be treated, they would not be eliminated after return to the stem cell donor as would allogeneic T cells. Because these T cells are diverse, more than two orders of magnitude more diverse than the starting population of cells, and because novel clones capable of recognizing antigen presented by human APC were generated, the T cells might recognize cancer or viral antigens to which the stem cell donor is tolerant. Further, we show that the cells can be stimulated by antigen administered to the adoptive host, thus avoiding the impairment of immune

FIG. 3. Production of new human T cells in chimeric swine. Chimeric swine were tested for newly generated human T cells by measuring levels of human TREC in the peripheral blood 6 months after birth. Primers specific for human TREC were used, and reaction was measured by real-time PCR. The dashed line represents baseline signal generated with nonchimeric swine PBMC (Swine PBMC). Levels of TREC in the peripheral blood of an unrelated human (Human PBMC) were consistent with previously published levels³⁰ (2214 ± 565 copies TREC/100 ng DNA). Levels of TREC in the T cell-depleted human bone marrow (Human BM) were nearly undetectable (63 ± 40 copies TREC/100 ng DNA), confirming complete T cell depletion. Human TREC levels in the peripheral blood of chimeric swine (Chimera PBMC) were higher than baseline in six of seven animals tested (433 ± 412 copies TREC/100 ng DNA).

responses seen in those with advanced cancer or chronic viral infection. Of course, efficient generation of immunity might be improved further by increasing the availability and function of antigen-presenting cells of the human donor. For example, one might envision administering antigen-presenting cells freshly obtained from a subject and pulsed with relevant antigen to chimeric pigs as a way of optimizing the T cell response. Still, we show that without further manipulation, the T cells can respond to new viral antigens.

The development and population of the periphery with T cells requires at least three processes: maturation, selection, and proliferation. This communication provides evidence that human hematopoietic stem cells engrafted in swine undergo these processes to generate human T cells. The presence of human cells in the swine thymus, the detection of human TREC, the diversification of T cells and expression of human CD3 and CD45RA, and entry into an immunological reaction all show that human cells were selected, developed, and matured in swine. Since the fraction of human cells in the blood of chimeric swine at 12 months was the same as at 8 weeks, during which time body mass and blood volume expand up to 20-fold, one can conclude that human cells undergo proliferation. While the swine must provide an environment less than what is optimal for the survival of human cells, this expansion suggests that the local environment (i.e., availability of compatible growth factors) is not limiting and perhaps it could be improved.

This report is the first to show *in situ* evidence of human T cells in the porcine thymus and that mature human T cells were exported from the porcine thymus. Significant levels of human TREC were detected in animals 1 month after birth. Of course, the presence of human TREC can reflect relative lack of T cell proliferation. However, since the human cells administered to fetal pigs were depleted of T cells and had no detectable TREC, the presence definitely proves that new human T cells were produced in swine. Moreover, the human T cells found in chimeric swine did undergo some degree of proliferation since T cell numbers were maintained with time and corresponding increases in blood volume. Others have studied the fate of human bone marrow cells engrafted in swine,^{37–39} sheep,^{25,40–42} and mice.^{43–46} These studies suggested that human T cells were generated in the animals given human bone marrow. However, proof that human T cells developed *de novo* in the xenogeneic thymus was not reported, and the possibility that the human T cells were actually transferred with the original bone marrow was not excluded. Some conducting similar experiments have found evidence of human thymocytes. Fujiki *et al.*³⁹ grafted human bone marrow-derived stem cells into swine at 40–50 days gestation and found cells expressing human CD3 in the thymus, but did not provide evidence of human T cell emigration from the thymus. Nikolic *et al.*²⁶ grafted human fetal liver fragments and swine fetal thymus fragments under the kidney capsule of SCID mice and found that human thymocytes taken from the mice could recognize swine MHC and participate in responses to MHC-mismatched but not MHC-matched porcine peripheral blood lymphocytes. While these findings and studies of phenotype suggest that some human T cells were produced in the swine, whether these

FIG. 4. Diversity of human T cells of chimeric swine. Diversity of human TCRV α was measured using a gene chip method described in the Supplemental Information. The dashed line represents baseline signal generated with nonchimeric swine PBMC (Swine PBMC). TCRV α diversity of T cells of healthy human PBMC (Human PBMC) was $4.2 \pm 3.7 \times 10^4$. TCRV α diversity of T cell-depleted bone marrow of the graft (Human BM) was nearly baseline (33 ± 49). TCRV α diversity of T cells of chimeric swine (Chimera PBMC) was two orders of magnitude greater than that of the T cell-depleted bone marrow of the graft ($1.7 \pm 1.5 \times 10^3$).

FIG. 5. Contribution of human T cells of chimeric swine to cellular immune responses. PBMC from four different chimeric swine were labeled with CFSE and incubated with irradiated lymphoblastoid cells generated from blood of an unrelated human subject (allogeneic LCL), lymphoblastoid cells derived from the corresponding chimeric swine (syngeneic LCL), and mononuclear cells from unrelated swine (xenogeneic MC). After 4 days, responding cells were labeled with a phycoerythrin (PE)-conjugated antibody against human CD4. PBMC of three of four chimeras proliferated in response to allogeneic stimulation; responses of a representative chimera are shown here. Proliferating cells (CFSE_{low}) were isolated and probed for human TCRV α mRNA. Human T cells of chimeric swine proliferated in response to unrelated human LCL but did not proliferate in response to LCL from the human donor or in the absence of stimulus as determined by (A) flow cytometry and (B) RT-PCR.

cells were ultimately exported from the thymus and whether the exported cells could function in the periphery were not investigated. Our detection of significant levels of human TREC does indicate that human T cells underwent development from human T cell precursors in chimeric swine.

Our work offers evidence, we think compelling, that human T cells underwent selection in the thymus of chimeric swine. Selection promotes maturation of a diverse repertoire of T cells that can recognize and respond to foreign antigens, in the form of foreign peptides associated with MHC, but only minimally with autologous antigens, presented in the same way. The most definitive test for selection in the thymus is the generation of TREC; hence, the finding of TREC in peripheral human T cells in chimeric swine (but not in the T cell-depleted human bone marrow administered) shows that selection of human T cells occurred *de novo*. Further evidence that selection of new T cells occurred can be inferred from the diversity of the human TCR in the chimeric swine. Using a recently developed method for measuring TCR diversity,³² we provide direct evidence that the diversity of human T cells in chimeric pigs exceeds the diversity of the few T cells that escaped depletion (and inoculated in the fetal swine). Hence, human TCR specificities were generated *de novo* in the chimeric swine and these specificities after expansion might be exploited for therapeutic purposes.

The finding of a relatively small but nonetheless diverse population of human T cells in chimeric swine has one potential advantage of importance. The diverse population of human T cells was "concentrated" in that diversity per unit number of human T cells was greater in chimeric swine than would be expected in human blood. The concentrated sample of human T cells in chimeras may reflect a decreased rate of survival of mature human T cells compared to mature

swine cells in the periphery. Concentration of human T cells in chimeric swine may reflect the limited ability of human cells to respond to porcine growth factors and cytokines. For example, in a model of full hematopoietic chimerism, porcine interleukin-2 (IL-2) induced very limited proliferation of human lymphocytes, while it functioned well on porcine lymphocytes.⁴⁷ Although concentration of the T cell compartment probably limits the responses of human T cells in chimeric swine, it provides an important advantage if the T cells are to be transferred into a human subject. The mean TCRV α chain diversity per milliliter of peripheral blood of chimeric swine was 1.7×10^3 , and the mean TCRV α chain diversity per milliliter of normal human blood is 4.2×10^4 . Assuming the work of Arstila *et al.*⁴⁸ estimating human α chain diversity is correct, one could restore human TCR diversity by administering as little as 100 mL of blood from chimeric swine (or the T cells contained therein). Even if Arstila *et al.* are wrong by an order of magnitude, one pig would provide enough cells in their blood to restore diversity. This illustrates the power of the concentrated sample of human T cells. Clearly one prefers to transfer a diverse, concentrated sample of T cells rather than the much larger sample that would be needed if each clone were represented redundantly. While one might argue that a next step should be taken to devise strategies for expanding the number of human T cells in chimeric swine, we believe it would be better for the concentrated sample of T cells to expand homeostatically in the "treated" human subject than in the pig in which the T cells were generated.

If human T cells can develop in swine, then there is reason to think that the T cells so generated will differ from the T cells that would develop in the person from whom hematopoietic stem cells were obtained. This difference will reflect

FIG. 6. Novel specificities of human T cells of chimeric swine. Three chimeric swine were vaccinated with antigens listed in the figure. Approximately 8 weeks later PBMC from chimeras and BMC from the human donor were labeled with CFSE and mixed with listed antigens plus APC of the human donor (lymphoblastoid cells derived from donor bone marrow mononuclear cells as previously described using Epstein-Barr virus³⁶) or an unrelated individual. PBMC of two of three chimeras tested were responsive to stimulation by vaccines; responses of a representative chimera are shown here. CFSE_{low} (proliferating) cells were isolated and probed for TCRV α mRNA. Human T cells of chimeric swine respond to antigens of parvovirus, *Mycoplasma hyopneumoniae*, and tetanus toxoid antigens after vaccination, while human donor cells did not proliferate in response to these antigens. (A) RT-PCR for human TCRV α . (B) Densitometry image comparing responses to antigens. Densitometry of gel bands was conducted using The Discovery Series™ Quantity One® software. The procedures used for RT-PCR analyses are described in Supplemental Information.

in part the intrinsic limitation of the thymus of the person to select and not to delete suitable T cells. It may also reflect the action of the tumor or infectious agent on the survival of mature T cells,^{10–15} as discussed in the introduction to this communication. It is conceivable that the ability of the newly developed T cells to mount a protective immune response might be increased if the swine in which the human T cells are generated were immunized with tumor cells or with cells

containing the infectious agent (as we have shown here with porcine vaccines). In addition, although we have shown that newly generated T cells can respond to antigen presented by donor APC, an amplified response might be possible if antigen-presenting cells of the donor were made more readily available at the time of stem cell transplantation.

It is important to consider the limitations and potential toxicities of the approach we propose. One limitation to this approach is the possibility that human T cells capable of recognizing critical peptides associated with self-MHC may not be produced during the period of human T cell development in swine. If the specificity needed is rare, we can imagine adapting the system, by administration of a selecting peptide, to promote selection of the desired T cells. However, because assembly of TCR is stochastic, repeated efforts may be needed to generate suitable effector T cells.

One potential toxicity of this approach is graft versus host disease (GVHD) or autoimmunity caused by T cells generated in a foreign host. Martin *et al.*⁴⁹ found that T cells generated in a major histocompatibility complex class II molecule DM knockout mouse can react with autologous MHC from DM++ mice and thus are “auto-reactive” and thus theoretically capable of inciting graft versus host disease; however, GVHD and autoimmunity were not demonstrated. In addition, Zhao *et al.*⁵⁰ found that nude mice grafted with fetal and porcine thymic and liver fragments, and so capable of generating murine T cells, were 60% more likely to develop autoimmunity; however, nude mice are generally susceptible to autoimmunity when given lymphocytes by adoptive transfer. Although human T cells selected on a swine thymus might recognize antigens of the individual who provided the hematopoietic stem cells, frank autoimmunity should be prevented by central deletion during development in the swine. Consistent with this possibility, human T cells generated in swine do not respond *in vitro* to cells of the donor but do respond to third-party cells. However, if tolerance to the stem cell source was incomplete, it might still ensue through peripheral mechanisms after the T cells were returned to the stem cell donor. Of course it is possible that in some cases, tolerance might not be acquired and autoimmunity might occur. Then, one would have to weigh the consequences of autoimmunity against the consequences of the disease for which the person was undergoing treatment. If that disease was a lethal viral infection or cancer, the treated person might welcome some manifestations of autoimmunity at the cost of effective therapy.

Still another potential toxicity of this approach is the transfer of porcine endogenous retrovirus. Surveys of hundreds of human subjects who received a xenotransplants from swine have failed to document even one case in which that virus passed from swine to human^{51–54}; however, we recently found that porcine endogenous retrovirus can transfer when cells fuse and DNA is resorted.^{27,55} Whether cell fusion can generate infectious retroviruses, however, is still not clear.

Acknowledgments

We thank Kim Butters, Karen Lien, and Andrea McConico for technical assistance. This work was supported by grants from the National Institutes of Health (HL52297, HL79067, and AI57358).

References

- Gilboa, E. The promise of cancer vaccines. *Nat Rev Cancer* **4**, 401, 2004.
- Mocellin, S., Mandruzzato, S., Bronte, V., Lise, M., and Nitti, D. Part I: vaccines for solid tumours. *Lancet Oncol* **5**, 681, 2004.
- Letvin, N.L., and Walker, B.D. Immunopathogenesis and immunotherapy in AIDS virus infections. *Nat Med* **9**, 861, 2003.
- Tramont, E.C., and Johnston, M.I. Progress in the development of an HIV vaccine. *Expert Opin Emerg Drugs* **8**, 37, 2003.
- Rosenberg, S.A., Yang, J.C., Schwartzentruber, D.J., Hwu, P., Marincola, F.M., Topalian, S.L., Restifo, N.P., Dudley, M.E., Schwarz, S.L., Spiess, P.J., Wunderlich, J.R., Parkhurst, M.R., Kawakami, Y., Seipp, C.A., Einhorn, J.H., and White, D.E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med* **4**, 321, 1998.
- Salgia, R., Lynch, T., Skarin, A., Lucca, J., Lynch, C., Jung, K., Hodi, F.S., Jaklitsch, M., Mentzer, S., Swanson, S., Lukanich, J., Bueno, R., Wain, J., Mathisen, D., Wright, C., Fidias, P., Donahue, D., Clift, S., Hardy, S., Neuberger, D., Mulligan, R., Webb, I., Sugarbaker, D., Mihm, M., and Dranoff, G. Vaccination with irradiated autologous tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor augments antitumor immunity in some patients with metastatic non-small-cell lung carcinoma. *J Clin Oncol* **21**, 624, 2003.
- Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* **176**, 1693, 1992.
- Caux, C., Dezutter-Dambuyant, C., Schmitt, D., and Banchereau, J. GM-CSF and TNF- α cooperate in the generation of dendritic Langerhans cells. *Nature* **360**, 258, 1992.
- Slingluff, C.L., Jr., Petroni, G.R., Yamshchikov, G.V., Barnd, D.L., Eastham, S., Galavotti, H., Patterson, J.W., Deacon, D.H., Hibbitts, S., Teates, D., Neese, P.Y., Grosh, W.W., Chianese-Bullock, K.A., Woodson, E.M., Wiernasz, C.J., Merrill, P., Gibson, J., Ross, M., and Engelhard, V.H. Clinical and immunologic results of a randomized phase II trial of vaccination using four melanoma peptides either administered in granulocyte-macrophage colony-stimulating factor in adjuvant or pulsed on dendritic cells. *J Clin Oncol* **21**, 4016, 2003.
- Zhou, G., Lu, Z., McCadden, J.D., Levitsky, H.I., and Marsden, A.L. Reciprocal changes in tumor antigenicity and antigen-specific T cell function during tumor progression. *J Exp Med* **200**, 1581, 2004.
- Killian, M.S., Monteiro, J., Matud, J., Hultin, L.E., Hausner, M.A., Yang, O.O., Gregersen, P.K., Detels, R., Giorgi, J.V., and Jamieson, B.D. Persistent alterations in the T-cell repertoires of HIV-1-infected and at-risk uninfected men. *AIDS* **18**, 161, 2004.
- Staveley-O'Carroll, K., Sotomayor, E., Montgomery, J., Borrello, I., Hwang, L., Fein, S., Pardoll, D., and Levitsky, H. Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. *Proc Natl Acad Sci USA* **95**, 1178, 1998.
- Casella, C.R., and Finkel, T.H. Mechanisms of lymphocyte killing by HIV. *Curr Opin Hematol* **4**, 24, 1997.
- McMichael, A.J., and Rowland-Jones, S.L. Cellular immune responses to HIV. *Nature* **410**, 980, 2001.
- Kostense, S., Raaphorst, F.M., Joling, J., Notermans, D.W., Prins, J.M., Danner, S.A., Reiss, P., Lange, J.M., Teale, J.M., and Miedema, F. T cell expansions in lymph nodes and peripheral blood in HIV-1-infected individuals: effect of antiretroviral therapy. *AIDS* **15**, 1097, 2001.
- Markert, M.L., Sarzotti, M., Ozaki, D.A., Sempowski, G.D., Rhein, M.E., Hale, L.P., Le Deist, F., Alexieff, M.J., Li, J., Hauser, E.R., Haynes, B.F., Rice, H.E., Skinner, M.A., Mahaffey, S.M., Jagers, J., Stein, L.D., and Mill, M.R. Thymus transplantation in complete DiGeorge syndrome: immunologic and safety evaluations in 12 patients. *Blood* **102**, 1121, 2003.
- Markert, M.L., Alexieff, M.J., Li, J., Sarzotti, M., Ozaki, D.A., Devlin, B.H., Sedlak, D.A., Sempowski, G.D., Hale, L.P., Rice, H.E., Mahaffey, S.M., and Skinner, M.A. Postnatal thymus transplantation with immunosuppression as treatment for DiGeorge syndrome. *Blood* **104**, 2574, 2004.
- Freedman, A.R., Zhu, H., Levine, J.D., Kalams, S., and Scadden, D.T. Generation of human T lymphocytes from bone marrow CD34+ cells *in vitro*. *Nat Med* **2**, 46, 1996.
- Pawelec, G., Muller, R., Rehbein, A., Hahnel, K., and Ziegler, B.L. Extrathymic T cell differentiation *in vitro* from human CD34+ stem cells. *J Leukoc Biol* **64**, 733, 1998.
- Fisher, A.G., Larsson, L., Goff, L.K., Restall, D.E., Happerfield, L., and Merckenschlager, M. Human thymocyte development in mouse organ cultures. *Int Immunol* **2**, 571, 1990.
- Plum, J., De Smedt, M., Defresne, M.P., Leclercq, G., and Vandekerckhove, B. Human CD34+ fetal liver stem cells differentiate to T cells in a mouse thymic microenvironment. *Blood* **84**, 1587, 1994.
- Poznansky, M.C., Evans, R.H., Foxall, R.B., Olszak, I.T., Piascik, A.H., Hartman, K.E., Brander, C., Meyer, T.H., Pykett, M.J., Chabner, K.T., Kalams, S.A., Rosenzweig, M., and Scadden, D.T. Efficient generation of human T cells from a tissue-engineered thymic organoid. *Nat Biotechnol* **18**, 729, 2000.
- Rothkotter, H.J., Sowa, E., and Pabst, R. The pig as a model of developmental immunology. *Hum Exp Toxicol* **21**, 533, 2002.
- Tran, N.D., Porada, C.D., Almeida-Porada, G., Glimp, H.A., Anderson, W.F., and Zanjani, E.D. Induction of stable pre-natal tolerance to beta-galactosidase by *in utero* gene transfer into preimmune sheep fetuses. *Blood* **97**, 3417, 2001.
- Zanjani, E.D., Pallavicini, M.G., Ascensao, J.L., Flake, A.W., Langlois, R.G., Reitsma, M., MacKintosh, F.R., Stutes, D., Harrison, M.R., and Tavassoli, M. Engraftment and long-term expression of human fetal hemopoietic stem cells in sheep following transplantation *in utero*. *J Clin Invest* **89**, 1178, 1992.
- Nikolic, B., Gardner, J.P., Scadden, D.T., Arn, J.S., Sachs, D.H., and Sykes, M. Normal development in porcine thymus grafts and specific tolerance of human T cells to porcine donor MHC. *J Immunol* **162**, 3402, 1999.
- Ogle, B.M., Butters, K.B., Plummer, T.B., Ring, K.R., Knudsen, B., Litzow, M.R., Cascalho, M., and Platt, J.L. Spontaneous fusion of cells between species yields trans-differentiation and retroviral *in vivo*. *FASEB J* **18**, 548, 2004.
- Hazenbergh, M.D., Verschuren, M.C., Hamann, D., Miedema, F., and van Dongen, J.J. T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. *J Mol Med* **79**, 631, 2001.
- Steffens, C.M., Al-Harhi, L., Shott, S., Yogeve, R., and Landay, A. Evaluation of thymopoiesis using T cell receptor

- excision circles (TRECs): differential correlation between adult and pediatric TRECs and naive phenotypes. *Clin Immunol* **97**, 95, 2000.
30. Douek, D.C., Vescio, R.A., Betts, M.R., Brenchley, J.M., Hill, B.J., Zhang, L., Berenson, J.R., Collins, R.H., and Koup, R.A. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. *Lancet* **355**, 1875, 2000.
 31. Douek, D.C., and Koup, R.A. Evidence for thymic function in the elderly. *Vaccine* **18**, 1638, 2000.
 32. Ogle, B.M., Cascalho, M., Joao, C.M., Taylor, W.R., West, L.J., and Platt, J.L. Direct measurement of lymphocyte receptor diversity. *Nucleic Acids Res* **31**, e139, 2003.
 33. Ogle, B.M., West, L.J., Driscoll, D.J., Strome, S.E., Razonable, R.R., Paya, C.V., Cascalho, M., and Platt, J.L. Effacing of the T cell compartment by cardiac transplantation in infancy. *J Immunol* **176**, 1962, 2006.
 34. AbuAttieh, M., Rebrovich, M., Wettstein, P.J., Vuk-Pavlovic, Z., Limper, A.H., Platt, J.L., and Cascalho, M. Fitness of cell-mediated immunity independent of repertoire diversity. *J Immunol* **178**, 2950, 2007.
 35. Cascalho, M., and Platt, J.L. The immunological barrier to xenotransplantation. *Immunity* **14**, 437, 2001.
 36. Jantschkeff, P., Herrmann, R., Spagnoli, G., Reuter, J., Mehtali, M., Courtney, M., and Rochlitz, C. Gene therapy with cytokine-transfected xenogenic cells (Vero-IL-2) in patients with metastatic solid tumors: mechanism(s) of elimination of the transgene-carrying cells. *Cancer Immunol Immunother* **48**, 321, 1999.
 37. Beschorner, W.E., Qian, Z., Mattei, P., Hess, A.D., Colombani, P.M., Sciscione, A.C., Khouzami, A., Blakemore, K.J., and Burdick, J.F. Induction of human chimerism and functional suppressor cells in fetal pigs: feasibility of surrogate tolerogenesis for xenotransplantation. *Transplant Proc* **28**, 648, 1996.
 38. Beschorner, W., Sudan, D., Yang, T., Radio, S., Stammers, A., Johnson, N., Shearon, C., Kuszynski, C., Dixon, R.S., Matamoros, A., and Langnas, A. Surrogate tolerogenesis: possible pretransplant induction of accommodation of pig xenografts. *Transplant Proc* **32**, 994, 2000.
 39. Fujiki, Y., Fukawa, K., Kameyama, K., Kudo, O., Onodera, M., Nakamura, Y., Yagami, K., Shiina, Y., Hamada, H., Shibuya, A., and Nakauchi, H. Successful multilineage engraftment of human cord blood cells in pigs after *in utero* transplantation. *Transplantation* **75**, 916, 2003.
 40. Zanjani, E.D., Flake, A.W., Rice, H., Hedrick, M., and Tavassoli, M. Long-term repopulating ability of xenogeneic transplanted human fetal liver hematopoietic stem cells in sheep. *J Clin Invest* **93**, 1051, 1994.
 41. Srouf, E.F., Zanjani, E.D., Brandt, J.E., Leemhuis, T., Briddell, R.A., Heerema, N.A., and Hoffman, R. Sustained human hematopoiesis in sheep transplanted *in utero* during early gestation with fractionated adult human bone marrow cells. *Blood* **79**, 1404, 1992.
 42. Srouf, E.F., Zanjani, E.D., Cornetta, K., Traycoff, C.M., Flake, A.W., Hedrick, M., Brandt, J.E., Leemhuis, T., and Hoffman, R. Persistence of human multilineage, self-renewing lymphohematopoietic stem cells in chimeric sheep. *Blood* **82**, 3333, 1993.
 43. Turner, C.W., Archer, D.R., Wong, J., Yeager, A.M., and Fleming, W.H. *In utero* transplantation of human fetal hematopoietic cells in NOD/SCID mice. *Br J Haematol* **103**, 326, 1998.
 44. Ohishi, K., Varnum-Finney, B., and Bernstein, I.D. Delta-1 enhances marrow and thymus repopulating ability of human CD34(+)CD38(-) cord blood cells. *J Clin Invest* **110**, 1165, 2002.
 45. Shultz, L.D., Lyons, B.L., Burzenski, L.M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S.D., King, M., Mangada, J., Greiner, D.L., and Handgretinger, R. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* **174**, 6477, 2005.
 46. Ishikawa, F., Yasukawa, M., Lyons, B., Yoshida, S., Miyamoto, T., Yoshimoto, G., Watanabe, T., Akashi, K., Shultz, L.D., and Harada, M. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. *Blood* **106**, 1565, 2005.
 47. Zhang, B., Ge, B., Xia, X., Liu, J., Sun, L., Rao, E., and Zhao, Y. Compatibility of porcine and human interleukin 2: implications for xenotransplantation. *Xenotransplantation* **13**, 423, 2006.
 48. Arstila, T.P., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J., and Kourilsky, P. A direct estimate of the human alphabeta T cell receptor diversity. *Science* **286**, 958, 1999.
 49. Martin, W.D., Hicks, G.G., Mendiratta, S.K., Leva, H.I., Ruley, H.E., and van Kaer, L. H2-M mutant mice are defective in the peptide loading of class II molecules, antigen presentation, and T cell repertoire selection. *Cell* **84**, 543, 1996.
 50. Zhao, Y., Rodriguez-Barbosa, J.I., Shimizu, A., Sachs, D.H., and Sykes, M. Despite efficient intrathymic negative selection of host-reactive T cells, autoimmune disease may develop in porcine thymus-grafted athymic mice: evidence for failure of regulatory mechanisms suppressing autoimmunity. *Transplantation* **75**, 1832, 2003.
 51. Heneine, W., Tibell, A., Switzer, W.M., Sandstrom, P., Rosales, G.V., Mathews, A., Korsgren, O., Chapman, L.E., Folks, T.M., and Groth, C.G. No evidence of infection with porcine endogenous retrovirus in recipients of porcine islet-cell xenografts. *Lancet* **352**, 695, 1998.
 52. Paradis, K., Langford, G., Long, Z., Heneine, W., Sandstrom, P., Switzer, W.M., Chapman, L.E., Lockey, C., and Onions, D. Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. *Science* **285**, 1236, 1999.
 53. Elliott, R.B., Escobar, L., Garkavenko, O., Croxson, M.C., Schroeder, B.A., McGregor, M., Ferguson, G., Beckman, N., and Ferguson, S. No evidence of infection with porcine endogenous retrovirus in recipients of encapsulated porcine islet xenografts. *Cell Transplant* **9**, 895, 2000.
 54. Dinsmore, J.H., Manhart, C., Raineri, R., Jacoby, D.B., and Moore, A. No evidence for infection of human cells with porcine endogenous retrovirus (PERV) after exposure to porcine fetal neuronal cells. *Transplantation* **70**, 1382, 2000.
 55. Ogle, B.M., Cascalho, M., and Platt, J.L. Biological implications of cell fusion. *Nat Rev Mol Cell Biol* **6**, 567, 2005.

Address reprint requests to:

Jeffrey L. Platt, M.D.

University of Michigan

Biomedical Sciences Research Building

109 Zina Pitcher Place

Ann Arbor, MI 48109-2200

E-mail: plattjl@umich.edu

Received: February 20, 2008

Accepted: July 8, 2008

Online Publication Date: September 29, 2008