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# Prenatal Gene Transfer: Scientific, Medical, and Ethical Issues

*A Report of the Recombinant DNA  
Advisory Committee*



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*A Report of the Recombinant DNA  
Advisory Committee*

NOTE: The opinions expressed in the working group papers and speaker manuscripts are those of the authors or working group members only and do not necessarily represent those of the National Institutes of Health.

Sponsored by the Office of Recombinant DNA Activities and the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health

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## Foreword

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As research progresses in the technical practice of gene transfer, the strides we have already made in prenatal medical treatment may soon be overtaken by the possibility of *in utero* therapy for genetic diseases. However, the differences between prenatal surgery and *in utero* gene therapy are not only quantitative, but qualitative, requiring a level of technical expertise, diagnostic acuity, and basic scientific knowledge that we have yet to achieve. Furthermore, in addition to issues of science, prenatal gene therapy raises societal, legal, and ethical issues that we must confront with equal thoroughness and resolve.

In response to these challenges, the Office of Recombinant DNA Activities and the Recombinant DNA Advisory Committee of the National Institutes of Health (NIH) decided to devote the third annual conference on gene therapy policy to an examination of the multiple issues surrounding prenatal gene transfer. We emphasize that this conference—and the report it has produced—is only a beginning. Indeed, perhaps the strongest conclusion we can draw from this effort is the necessity for more research into fetal development, preclinical data, and further discussion to guide future public health policy in this highly complex area of medical research.

This conference should not be construed as an endorsement by NIH of prenatal gene transfer clinical trials. Its primary purpose was to bring together research scientists, clinicians, families, policy makers, and concerned citizens to share expert views and to foster public deliberation. Amid the many controversies explored, however, one area of consensus did emerge, expressed by the unanimous opinion of the Recombinant DNA Advisory Committee: In our present state of knowledge, it is premature to undertake any experiment involving human *in utero* gene transfer. Nevertheless, the potential for such research cannot be denied. In that spirit, the National Institutes of Health will continue to explore the issues raised by this potential application of gene therapy and to provide a forum for its debate.



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## Preface

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Since their establishment in 1997, the NIH Gene Therapy Policy Conferences have aimed to bring public attention to and deliberation on human gene transfer research. The accomplishment of gene transfer in human somatic cells has spurred a new line of research into the possibility of correcting genetic diseases at an earlier stage, through prenatal gene therapy. To date, such research has been limited to nonhuman animals, but the myriad scientific, medical, and ethical questions posed by this approach to the treatment of genetic disease are pressing enough to warrant close examination now. Only last year the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health received two proposals for human prenatal gene transfer, prompting it to seek ways to delineate and consider the issues raised by the potential clinical application of such a technology. Thus prenatal gene transfer became the topic for NIH's third policy conference on gene therapy.

This report presents the findings of three working groups together with highlights of the conference and a concluding statement by the RAC. Each working group considered a specific component of the overall topic of prenatal gene transfer: preclinical research issues (working group I), clinical research issues (working group II), and ethical, legal, and societal issues (working group III). These three components form the core of the report and contain the themes for further discussion.

Principal themes in the area of preclinical research are the efficiency of gene transfer in both *in vivo* and *ex vivo* settings, the expression of genes transferred *in utero*, fetal immune response, and safety of the fetus. Those in clinical research are the present appropriateness and limitations of prenatal gene transfer, determination of clinical endpoints, and the extent of acceptable risk. Finally, the themes involving ethical, legal, and societal issues include ethical permissibility of the research, criteria for candidate diseases, criteria for recruitment and enrollment of participants in the research, design of informed consent, and the ensuring of social justice in the policy-making process, regulation, expenditure of resources, and access to treatment.

The Conference Report and the Consensus Statement of the RAC revisit these themes, providing both a basis and a set of reference points for further discussion.

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# Executive Summary

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## **Introduction**

On July 31, 1998, Drs. W. French Anderson and Esmail Zanjani submitted two preliminary protocols for prenatal gene transfer to the NIH Recombinant DNA Advisory Committee (RAC) to provide a context for discussion and to initiate a national dialogue on the substantive public policy issues raised by prenatal gene transfer research. Following the RAC meeting of September 24–25, 1998, committee members and ad hoc experts were assigned to one or more of three working groups, based on their individual areas of expertise: (1) preclinical research issues; (2) clinical research issues; and (3) ethical, legal, and societal issues; and asked to develop preliminary responses to questions that related specifically to the primary focus of their assigned group.

On January 7–8, 1999, the RAC sponsored a Gene Therapy Policy Conference (GTPC) entitled: Prenatal Gene Transfer: Scientific, Medical, and Ethical Issues. RAC working group and ad hoc participants were brought together to further deliberate the scientific, safety, ethical, legal, and societal implications of prenatal gene transfer research. Following this conference, working group participants were asked to further refine their responses to the assigned questions, incorporate any additional questions that were raised as a result of the conference presentations, identify unresolved issues, and propose “next steps” in the deliberative process.

The working group chairs presented their findings and recommendations, listed below, at the March 11, 1999, RAC meeting, as part of the RAC’s continuing service as a unique public forum for deliberation on the science, safety, and ethics and development of Federal policy for recombinant DNA research.



## Findings

1. In principle, *in utero* somatic cell gene transfer may offer extraordinary potential for the prevention and treatment of serious and life-threatening diseases; however, there are a number of significant technological developments that must be achieved before, and there are insufficient preclinical data to support, the initiation of any clinical trials involving prenatal gene transfer in the U.S. at the present time, irrespective of funding sources.

2. Prenatal gene transfer should only be considered for somatic cells.

3. Prenatal gene transfer should never be performed on a fetus that is expected to develop into a healthy child.

4. In general, candidate diseases for *in utero* gene transfer should be only those diseases that: (i) are associated with serious morbidity and mortality risks for the fetus either *in utero* or postnatally (not simply traits), (ii) do not have an effective postnatal therapy, or have a poor outcome using available postnatal therapies, (iii) are not associated with serious abnormalities that are not corrected by the transferred gene, (iv) can be definitively diagnosed *in utero* and have a well-defined genotype/phenotype relationship, and (v) have an animal model for *in utero* gene transfer that recapitulates the human disease or disorder. However, there may be diseases for which meeting these conditions is not possible. In these cases the investigator should present a strong rationale for proceeding.

## Recommendations

### Technological developments

In anticipation of new scientific advancements in this arena, the RAC presents its consensus regarding the minimal threshold of technological development that should be achieved before any clinical study involving prenatal gene transfer can be initiated.

These technological developments include (but are not limited to):

1. Animal models that recapitulate human diseases or disorders where feasible;

2. Improved gene delivery methods for achieving targeted delivery of transgenes to specific cell types and tissues (for direct *in vivo* applications); however, in some cases nontargeted delivery of genes may suffice;

3. Improved gene delivery methods for achieving efficient gene transduction *in utero*, including efficient transduction when using *ex vivo* transduced donor cells in which a low frequency of gene transfer may be compounded by a low level of donor cell engraftment;

4. Methods that will allow high level gene expression of sufficient duration, that are appropriate for the specific disease or disorder, and that provide effective regulation of gene expression, if necessary;

5. Functional assays that demonstrate resolution of disease phenotype;

6. Assays that will accurately and sensitively assess the production of the functional end product if feasible;

7. Identification of markers (surrogate or direct) that will allow tracking of the early stages of fetal immune competence to clearly define a “window of opportunity” appropriate for *in utero* gene transfer; evaluation of fetal immune response to transduced gene products whenever feasible;

8. Methods to accurately and sensitively assess the potential of interruption or derangement of fetal or postnatal development

9. Methods to assess the potential for insertional mutagenesis;

10. Accurate and sensitive methods to assess the potential for germ cell integration;

11. Methods to accurately assess the pathologic and toxicologic effects of vector administration *in utero*;

12. Improved methods for testing whether genetic mutations correlate with disease phenotype;

13. Laboratory and clinical endpoints that will definitively demonstrate that a clinical phenotype has been reversed by gene transfer *in utero*.

### Preclinical research

The RAC presents its consensus regarding the minimal threshold of preclinical data that should be provided before any clinical study involving prenatal gene transfer can be initiated. These studies include (but are not limited to) the following:

1. Demonstration of efficient gene transfer *in utero* in an animal model that recapitulates the human disease or disorder if feasible. For studies involving the transplantation of *ex vivo* transduced cells, the overall efficiency of gene transfer must be considered as a product of the fraction of treated cells which contain the transgene multiplied by the fraction of transplanted donor cells that engraft. A low level of donor cell engraftment would thus compound a low frequency of gene transfer in donor cells.

2. Demonstration of gene function *in utero* in an animal model that recapitulates the human disease or disorder if feasible using functional assays that assess gene function and resolution of disease phenotype. Specifically, the preclinical studies must demonstrate that the transferred gene is being expressed at the necessary level, for the necessary duration, and that the transferred gene contains the necessary regulation required to achieve therapeutic correction of the specific gene deficit.

3. Demonstration in an animal model that recapitulates the human disease or disorder if feasible that there are no adverse effects of the gene transfer procedure on pre- and postnatal development.

4. For studies involving the administration of viral vectors, demonstration in an animal model that recapitulates the human disease or disorder if feasible that the gene transfer procedure does not pose a significant risk of generating or activating transmissible virus.

5. Demonstration in an animal model that recapitulates the human disease or disorder if feasible that the gene transfer procedure does not pose a significant risk of initiating oncogenic or degenerative processes.

6. Demonstration in an animal model that recapitulates the human disease or disorder if feasible that the gene transfer procedure poses minimal risk of integrating into germ cells of the fetus or pregnant woman.

### **Clinical requirements**

The RAC presents its consensus regarding the minimal clinical requirements that should be incorporated into any clinical study involving prenatal gene transfer.

These requirements include (but are not limited to) the following:

1. Random screening for mutations should not be performed because of the current inability to predict phenotype from genotype; if a mutation is found, it may not be possible to predict accurately the phenotype (severe, mild, or none) of the fetus.

2. Although random genetic screening should not be performed at present, ultrasound screening should be conducted when indicated as a means of identifying some severely affected fetuses, based on the fact that ultrasound findings may have high positive predictive value for the presence of severe morbidity or mortality postnatally.

3. For diseases in which mutations can be identified in a previously affected sibling, fetal genetic testing should be performed.

4. For diseases in which there are no genetic tests available, assays of umbilical venous blood may be required to make an accurate diagnosis.

5. Gene expression (both RNA and protein) in target tissue (if accessible) should be compared with that in normal cells.

6. Immunological testing must be performed at birth to determine the immunological response of the infant to the vector and/or transgene (relevant only for diseases in which T cell function is normal).

7. Postnatal monitoring for unintentional integration of vector sequences must be performed on multiple tissue samples, including (but not limited to) the placenta and target tissue if accessible.

8. Following the gene transfer procedure, maternal blood should be tested periodically during the remainder of the pregnancy and postpartum to monitor for the presence of the vector sequences.

9. Maternal and infant organ function must be periodically evaluated because of concern that inadvertent transfer to unintended tissues or a very high level of

gene transfer to target tissues may have an adverse effect on organ function.

10. Invasive testing *in utero* should have the clearly defined goal of providing the most definitive diagnostic information and should be as minimal as possible, whereas noninvasive testing, i.e., ultrasound, should always be considered (close monitoring of the fetus by ultrasound must be performed with every manipulation), as well as sampling maternal rather than fetal blood (although fetal blood sampling may be necessary for some disease diagnoses).

11. Postnatal blood sampling must be performed periodically after birth to: (i) monitor the state of chimerism, and (ii) determine whether the clinical phenotype has been reversed by gene transfer procedure (if blood tests can be used to assess phenotype).

### **Ethical requirements**

#### **A. Germline integration**

One risk of GTR *in utero* is the possibility of unintended but foreseen germline integration. Information about the nature, probability, and magnitude of inadvertent germline effects anticipated from *in utero* GTR is at present extremely limited. Therefore: there is clear agreement that the prudent course under these circumstances is to consider germline integration undesirable and to minimize the likelihood of its occurrence.

#### **B. Risk-benefit assessment**

*in utero* GTR is only ethically justifiable when the following conditions are fulfilled:

1. The risks of harm to both the fetus and the pregnant woman are minimized.

2. The potential for direct benefit to the fetus is maximized, consistent with the early stage of this research.

#### **C. Informed decision-making**

An optimal informed decision-making process for *in utero* gene transfer should include at least the following components:

1. an initial conversation to maximize the pregnant woman's/couple's ability to consider all the information relevant to the research;

2. adequate education of potential subjects, including an explanation of proposed long-term follow-up of all subjects;

3. securing of the final decision from the pregnant woman under circumstances that reduce the potential for coercive pressures from others;

4. a conversation just prior to initiation of the experimental procedure to discuss the irreversible nature of the procedure and to provide an additional opportunity for withdrawal from the trial.

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**D. Requirements of justice**

1. Creation of a science policy process to ensure that the American public in all its diversity has the opportunity to become involved in the decision to proceed with or forgo this area of research;
2. Exploration of ways to improve equity of access to any proven therapeutic results emanating from this research.

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## Working Group I: Preclinical Research Issues

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The working group on preclinical research issues was assembled to answer questions posed by the National Institutes of Health on a given aspect of the topic of prenatal gene transfer. Group members attended the conference, listened to expert speakers, refined and reexamined their thinking, and fashioned their answers accordingly. The working group paper contained in this report is the result of this process of inquiry, discussion, and reflection.

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# Working Group I: Preclinical Research Issues

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## I. Summary statement

*In utero* gene therapy may prove to be an effective alternative to conventional modes of treatment for some hereditary and acquired diseases. Several well-designed studies in animals have demonstrated expression of transgenes and marker genes following prenatal gene transfer. Nevertheless, a substantial number of critical issues, discussed below, remain to be addressed. Thus, current preclinical data, while encouraging, are insufficient to support clinical trials.

## II. Efficiency of gene transfer in utero

### A. Ex vivo gene transfer

The efficiency of gene transfer into treated cells varies considerably with the specific cells, modes of transfection/transduction, and vectors used. One study has demonstrated that retroviral vectors transduced fetal hematopoietic stem cells more efficiently than adult cells (Ekhterae et al.), although this will vary with different cell types. Numerous clinical studies have already been conducted to evaluate the efficiency of *ex vivo* gene transfer into adult cells (e.g., hematopoietic cells). An important remaining question, therefore, is whether the current results warrant consideration of *ex vivo* transduced cells administered *in utero* as an alternative mode of therapy. A related question is whether neonatal gene transfer would be an appropriate intermediate step between gene transfer in adults and gene transfer *in utero*.

The overall efficiency of gene transfer following infusion of treated hematopoietic stem cells can be considered the product of the fraction of treated cells containing the transgene multiplied by the fraction of infused cells that become engrafted. In this formula, a low level of donor cell engraftment would compound a low frequency of gene transfer into donor cells, rendering the therapy even less effective. The level of engraftment is thus a key component of preclinical feasibility assessments.

*In utero* studies of the function of implanted cells should be based on functional assays. Until that time, such questions can be answered only in the context of newborns (SCIDs, ADA deficiency), and adults. However, the process of engraftment may be very different in the milieu of the developing fetus. Several attempts to rectify thalassemia *in utero* have failed because of inadequate engraftment, expression, or both.

Another concern is the possibility of transplacental migration of transplanted cells.

### B. In vivo gene transfer

Few *in vivo* gene transfer studies have been conducted *in utero* in animal models; thus, the same questions about efficiency exist for *in vivo* as for *ex vivo* gene transfer. However, the *in vivo* setting offers less control over which cells are exposed to vector sequences. The injection of viral vectors into the amniotic fluid or peritoneal cavity of developing fetuses exposes the entire organism to the risks of insertional mutagenesis, oncogenesis, and hence disruption of normal development. The risk of insertional mutagenesis disrupting normal development will remain for retroviral and AAV vectors. For vectors that are expected to remain episomal, such as adenovirus, low frequency of integration cannot be completely ruled out. Lambs born after treatment with retroviral vector have not exhibited any abnormality. However, the number of such animals is very small, and these results therefore cannot be regarded as strong evidence for the absence of potential harm. One way to deal with this problem would be to use vectors that have been modified to target specific cell types and achieve transduction only into targeted cells; transduction into germline cells could potentially be avoided by such targeted transfer. To date, however, no reports dealing specifically with the use of targeted vectors *in utero* have been published. It is not yet clear how specific targets would be identified, let alone specifically trans-

duced, and whether such approaches would rely on specific ligands, receptors, promoters, and the like to effect specific gene delivery and/or expression. Such manipulations could also result in reduced transduction frequencies compared with unmodified vector.

Questions also remain about the study design most effective for establishing lack of transfer to the germline. Consideration must be given to determining (1) which studies should be carried out to assess the potential for incorporation of transfer vectors and inserts into germ cells, (2) the methods and levels of sensitivity needed to give a quantitative estimate of the risk of incorporation, and (3) whether that risk can be avoided by limiting the type and means of vector delivery in future experiments.

### III. Expression of genes transferred *in utero*

While expression of prenatally delivered transgenes has been demonstrated using molecular techniques such as PCR and immunohistochemistry, questions about the level, duration, and regulation of gene expression necessary for correction of specific genetic deficits remain. These will be best answered using animal models that recapitulate human disorders (such as the Jak-3 knock-out mouse), although substantial basic information will also be generated from studies using reporter genes. Well-designed studies should optimally demonstrate effective *in utero* gene transfer with expression of the transgene appropriate for the disorder and functional assays showing resolution of the disease phenotype.

For treatment of some diseases, prenatally delivered genes will have to continue to function well into adult life. Thus, the critical factor of duration of gene expression must be addressed in preclinical studies testing the particular gene, vector, and target tissue in question.

In addition, the design of preclinical studies must take into consideration the circumstances under which regulation of transgene expression would be necessary (e.g., globin disorders), and the mechanism/s that would be used to limit transgene expression to the desired target cell and tissue type.

Given the complexity of the preclinical issues at hand, some thought should be given to those candidate/model diseases currently under consideration for *in utero* gene therapy. Some disorders will require more high-level, regulated, or tissue-specific expression than others. The availability of an animal model for *in utero* gene therapy of a particular condition is also an important component of preclinical studies. However, satisfaction of this requirement alone is not sufficient to make a disease a good candidate for *in vivo* gene therapy. For example, it is unclear whether prenatal gene therapy is appropriate for disorders incompatible with life, such as alpha thalassemia. In such cases preclinical studies should be guided by additional clinical and ethical considerations.

### IV. Immune response

As is the case for adults, the effectiveness of gene therapy *in utero* may be limited by immune responses against either the vector or the transgene product. On the one hand, infusion of transduced allogeneic cells can trigger an immune response; on the other hand, appropriately timed gene transfer could result in the development of tolerance to the gene product, a potential advantage over postnatal gene therapy. Thus, an understanding of the timing of development of immune competence and immune tolerance in the fetus would be likely to improve the effectiveness of *in utero* gene transfer. At present there are no markers, surrogate or direct, that allow tracking of the early stages of development of immune competence. The finding of shifts in fetal immunoglobulin composition may be the first indication of a means to assess fetal immune status. However, some better definition of the stages of fetal immune development is needed, which could possibly determine a "window" for gene transfer.

Preclinical studies in appropriate animal models should therefore address the immune response to specific vectors and gene products during fetal development.

### V. Safety of *in utero* gene therapy

Sources of fetal morbidity can be divided into those resulting from the procedure and those resulting from fetal response to the vector/transgene. The risk of fetal loss as a result of the procedure, and the underlying pathophysiology (preterm delivery, sepsis, fetal resorption, etc.), must be defined in preclinical studies. Delivery of the vector and expression of the transgene may be associated with an interruption or abnormal change in development of the fetus. This must also be defined.

The use of viral vectors allows higher efficiencies than currently seen with other modes of gene transfer. However, there is an inherent risk of wild-type contamination of recombinant vector stocks. The risk to the fetal patient of such contamination may be greater (i.e., have greater consequences) than that to the postnatal patient, and must be defined. *In utero* administration of viral vectors may also pose a greater risk, yet to be assessed, of generating a recombinant virus than administration of such vectors in adults.

Wild-type retroviruses and adeno-associated viruses are known to integrate into the host genome, with potentially mutagenic consequences. Compared with newborns and adults, the fetus may face a greater danger of insertional mutagenesis and its associated risks. Thus, the potential of these events to interrupt or derange development or to cause oncogenesis must be defined in preclinical studies.

Given the seriousness of concerns over the potential for abnormal prenatal and postnatal development, extensive studies of the pathologic and toxicologic effects of *in utero* vector administration are in order, with use of an appropriate animal model.

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## Working Group II: Clinical Research Issues

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The working group on clinical research issues was assembled to answer questions posed by the National Institutes of Health on a given aspect of the topic of prenatal gene transfer. Group members attended the conference, listened to expert speakers, refined and reexamined their thinking, and fashioned their answers accordingly. The working group paper contained in this report is the result of this process of inquiry, discussion, and reflection.

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## Working Group II: Clinical Research Issues

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### **I. Is gene transfer *in utero* appropriate now?**

The consensus of the working group is that gene transfer *in utero* is not appropriate at this time because of the absence of preclinical animal data and postnatal human data showing safety and efficacy.

#### **A. What diseases would be good candidates?**

This discussion must remain theoretical until good preclinical animal and possibly postnatal human data exist to support the use of gene transfer. Any conditions to be treated by *in utero* gene transfer should be serious diseases (not simply traits) with serious morbidity and mortality risks for the fetus either *in utero* or postnatally. The diseases should be those for which available postnatal therapies produce a poor outcome. An additionally important point, however, is that the diseases to be treated should not be associated with serious abnormalities that are not corrected by the transferred gene. For example, with Lesch-Nyhan syndrome, gene transfer should correct or prevent neurological abnormalities, not just biochemical abnormalities in the blood.

#### **1. Limitations related to the accuracy of diagnosis**

Gene transfer should never be performed on a normal fetus; thus accurate diagnosis is of paramount concern. The selection of a disease for prenatal gene transfer requires identification of a disorder in which *in utero* diagnosis is definitive and the genotype/phenotype relationship is well defined. This is the case for both adenosine deaminase deficiency (ADA) and alpha thalassemia, although these diseases are not good candidates for *in utero* therapy for other reasons, discussed below.

At this point it is not reasonable to screen fetuses randomly for mutations to identify patients for prenatal gene therapy. Instead, fetal diagnosis for specific mutations should be confined to families with previously affected children or to couples with a strong family history in a situation in which genotype clearly predicts

phenotype. These families should be willing to undergo prenatal testing to determine the genetic status of the fetus. Adequate genetic counseling, including discussion and explanation of all options, is necessary prior to prenatal genetic testing.

Random screening for mutations is not recommended because of current inability to predict phenotype from genotype. Thus, if a mutation were found, it might not be possible to accurately predict the phenotype (severe, mild, or none) of the fetus. In autosomal recessive disorders such as cystic fibrosis, the presence of one well-established mutation does not indicate that the fetus will have the disease; the second allele must have a known mutation as well. Thus, prenatal testing should be offered to determine the genetic status of the fetus, but random genetic screening is not recommended.

The diagnosis of diseases using fetal tissues can be technically challenging. To ensure accuracy of the diagnosis and to have the highest possible positive predictive value, the testing should be as standardized as possible, optimally with a small number of centers focusing on specific diseases. In some diseases the mutations can be identified in a previously affected sibling. This allows for genetic testing of the fetus using Southern Blot analysis after amniocentesis or PCR-based molecular testing after chorionic villus sampling. For some diseases, such as undefined forms of severe combined immunodeficiency, there is no genetic test available, and flow cytometry of umbilical venous blood is necessary for diagnosis. In other diseases enzymatic testing can be done. Maternal cell contamination must be avoided and its absence verified by an internal contamination control. Parents should be supplied with information regarding the accuracy of the diagnosis.

Although random screening is not recommended, it is likely that ultrasound screening will identify some severely affected fetuses, such as those with alpha thalassemia. If the ultrasound findings have high positive



predictive value for the presence of severe morbidity or mortality postnatally, then prenatal gene transfer could be considered, assuming that prior animal work had shown efficacy. It is necessary to verify that adequate genetic counseling has been provided. This working group makes no recommendation regarding screening of all pregnancies by ultrasound testing.

## **2. Limitations regarding optimal clinical trial design and analysis**

After the diagnosis has been made, all available treatment options, including abortion and prenatal gene transfer, should be explained to the parents. For example, in many immunodeficiency diseases, HLA-identical cells from a sibling or HLA-haploidentical T-cell-depleted bone marrow cells from a parent are an efficacious and safe mode of treatment and would be performed after birth. For diseases in which HLA-identical donors are needed for postnatal therapy (and HLA-half related donors are not sufficient), HLA typing must be performed accurately. The parents can then be counseled about this postnatal treatment option. There was no consensus on whether it is acceptable to proceed with *in utero* gene transfer in situations in which a safe and effective postnatal therapy exists.

### **B. Should diseases be considered if they are fatal *in utero*?**

There was no consensus on whether or not to treat a fetus carrying a disease that is fatal *in utero*. Some argued that financial resources should be applied to diseases in which the fetus will survive birth. In addition, the risk exists for *in utero* fatal diseases, which include alpha thalassemia, that a fetus could receive inadequate treatment and thus survive to birth but be severely damaged and/or in need of lifelong transfusion and iron chelation therapy. Others in the group took the opposing side, advocating treatment for all affected fetuses. There was agreement, however, that compelling animal data would be needed to move forward on diseases that are fatal *in utero*.

### **C. Should diseases be considered for *in utero* gene transfer without prior experiments in postnatal infants?**

The working group again stressed the desirability of accumulating good animal data prior to initiation of human *in utero* gene transfer. However, there was no consensus that an animal model of the disease in question should be a prerequisite for initiation of human studies. Regardless of the availability of such a model, the group agreed that, if at all possible, experiments should initially be conducted on postnatal infants. Three circumstances, however, were envisioned in which *in utero* gene transfer could be contemplated without prior

postnatal experiments: (1) if the disease is fatal *in utero*, (2) if the disease causes irreversible organ damage *in utero*, and (3) if for some diseases, possibly because of the tissues that need to be transduced, there may be technical advantages to *in utero* therapy. An example of the third circumstance would be a disease in which the gene must be inserted into any tissues of the fetus by a retroviral vector. Retroviruses transduce cycling cells, and as the fetus may have more cycling cells than the postnatal infant, the vector might introduce genes more efficiently in fetal than in postnatal tissues. In addition, the group felt that *in utero* gene transfer should still be an option when it is not possible to achieve a satisfactory result by carrying out the transfer postnatally.

## **II. Factors to consider when selecting endpoints for analysis of clinical outcomes**

### **A. Clinical endpoints selected for the prenatal period**

It is unknown whether prenatal endpoints are reliable predictors of ultimate chimerism. At present, postnatal chimerism shortly after gene transfer is not a good indicator of later gene expression. The positive predictive value for a test must be good enough to justify prenatal testing after gene transfer. It is recommended that the extent of chimerism at birth be compared with long-term chimerism. If good correlation is observed, and if a prenatal finding of poor chimerism would affect therapy, then prenatal tests of chimerism could be considered.

### **B. Clinical endpoints selected for the postnatal period**

In the postnatal period tests should be available to demonstrate conclusively that the clinical phenotype has been reversed by gene transfer. Tests should therefore analyze production of the functional end product. Laboratory as well as clinical endpoints should be supplied. Gene expression, both RNA and protein of the target tissue if accessible, should be compared to that in normal cells. Tissues other than that of the target should be examined, as there may be unintended gene transfer to those tissues. Immunological testing should be done to determine the immunological response of the infant to gene transfer.

Postnatal testing should monitor for unintentional germline integration of the vector virus and for general organ function. Testing of the infant should include the examination of accessible tissue samples in addition to the transduced target tissue. The placenta should also be examined for transferred genes. The mother's blood should be tested periodically after gene transfer, during the remainder of the pregnancy, and postpartum, to search for evidence of the vector virus. Permission for autopsy should be sought when the recipient of gene

therapy dies. General organ function should be tested for detrimental effects possibly caused by inadvertent transfer to an unintended tissue or by a very high level of gene transfer.

### **III. What extent of risk is acceptable?**

#### **A. Relative to the pregnant woman**

The pregnant woman faces multiple risks. These include the dangers of carrying a fetus to term (with or without *in utero* gene transfer) in diseases such as alpha thalassemia; the risk of an inadvertent gene transfer to her tissues from the *in utero* procedure; risks from the procedures involved in diagnosis, gene transfer, and monitoring (the major ones being infection and impairment of future ability of the mother to reproduce); and, lastly, the emotional risks. Regarding the latter, it will be difficult for the pregnant woman to avoid feeling that to be a good mother she should submit to this therapy for her fetus. A thorough discussion of these risks is of utmost importance. The mother should not be coerced or pressured into agreeing to *in utero* gene transfer. She should also be given an opportunity to discuss whether her motivation for cooperating derives from a personal commitment to the pregnancy or from a response to society's interests in reproduction. Further, she should realize that the protocol is "experimental."

The mother's consent to gene transfer is absolutely required. The working group did not reach a consensus about the desirability of paternal consent to gene transfer. There was agreement, however, that the father's consent should not lead to gene transfer experiments if the mother does not consent. Some members of the working group, however, felt that the father should be able to veto gene therapy for the fetus even if the mother agrees to it. The rationale for this position comes from the alpha thalassemia protocol, in which incomplete treatment would result in risk to the mother (who could possibly die) and risk to the fetus (of being severely affected for life). If these bad outcomes occurred, the father would be responsible for the affected child and possibly other family members; it would therefore seem unfair to exclude the father's viewpoint in the decision. (Discussion of paternal consent was limited to fathers who are involved with the family.)

#### **1. Inclusion/exclusion criteria for the pregnant woman**

and

#### **2. Monitoring of the pregnant woman (pre- and postpartum)**

The mother must be able to comply with all of the physician's prenatal instructions as well as all of the postnatal monitoring studies. She must consent to blood

sampling as necessary for the study and should agree to consider an autopsy for herself and/or the fetus if death occurs. The importance of this type of examination should be explained.

Maternal blood should be obtained immediately after the gene transfer procedure, and periodic postnatal blood samples should be taken to monitor for evidence of insertion of the viral vector or the transduced genes. Additional examination of the placenta and any other subsequent offspring may also be necessary.

#### **3. Detection and assessment of inadvertent germline transmission**

Although gene transfer procedures must be effectively screened for maternal safety prior to implementation, and death of the mother would be unrelated to the gene transfer, the mother should be asked to agree to an autopsy of her tissues for evidence of gene transfer. Multiple tissues should be examined, including gonadal tissue. However, participation in the *in utero* gene transfer experiment should not be conditional on agreement to autopsy.

#### **B. Relative to the fetus**

##### **1. Inclusion/exclusion criteria for the fetus**

The diagnosis *in utero* must be clear. The genotype/phenotype relationship must be understood as completely as possible. The fetus must be free of other complicating biochemical or anatomical defects. Another exclusion would be HLA-matched related donors available for postnatal therapy (disease dependent restriction).

##### **2. Monitoring (fetal blood sampling) of the fetus (pre- and postpartum)**

Close monitoring of the fetus is required. Ultrasound should be performed with every manipulation. Fetal blood sampling may be necessary for some diagnoses and to monitor the state of chimerism after gene transfer. Safety should be an overriding concern in the decision on whether or not to sample blood prenatally. In the postnatal period, blood samples and tissue samples (as feasible) will be required to monitor whether the clinical phenotype has been reversed by gene transfer.

The risk/benefit ratio is an important consideration along with a concern for minimal risk to the fetus. Assessment of risk should be based on "the minimum extent necessary" to meet the health needs of the fetus. Invasive testing should have the clearly defined goal of providing the most definitive diagnostic information and should be as minimal as possible. Noninvasive testing such as ultrasound should always be considered, as well as sampling maternal rather than fetal blood.

The benefits of an *in utero* gene transfer experiment could vary from none, to barely minimal, to ameliora-

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tion or cure of the genetic disease. There could be a situation in which there was no efficacy for the fetus: The fetus (child) is born with the disease, has a lifetime of illness and suffering, and is worse off than if there had been no intervention and the pregnancy had come to a natural termination with fetal death.

Gene transfer carries substantial risks to the fetus both before and after birth. Prenatal risks include spontaneous abortion after the injection of genetic material or after blood sampling. Blood sampling can also lead to congestive heart failure from decreases in hemoglobin. Immune responses to the inserted gene products in T cell competent patients may result in the destruction of transduced cells or the inadvertent destruction of residual native mutant function. Antibodies from the mother could enter the fetal circulation during blood drawing or gene insertion. Postnatally, there are risks imposed by a successful outcome that would entail a lifetime of scientific and medical follow-up after an experiment to which the fetus (child) had never consented.

### **3. Detection and assessment of inadvertent germline transmission**

Risks include short-term or long-term harm from insertional mutagenesis which could range from severe malformations to activation of oncogenes or other unknown abnormalities. The offspring of the fetus could be affected by expression of too much gene product in normal cells.

### **IV. Summary**

The optimal disease for a clinical trial of *in utero* gene transfer would be one in which the diagnosis can be made with certainty early in pregnancy, giving parents sufficient time to consider this research option. HLA typing should be done to determine the existence of a potential HLA-matched cell donor, if applicable. The disease chosen should have an absolute correlation between the genotype and the resulting phenotype or between uterine and postnatal phenotypes. The *in utero* gene transfer should be safe, with a low likelihood of morbidity caused by insertion of the gene into cells that do not normally express that gene. Data from animal studies should support a level of gene expression conducive to correction of the phenotype rather than merely a slight change. Finally, the mother's life should not be endangered by the gene transfer procedures or by carrying the infant to term.

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## Working Group III: Ethical, Legal, and Societal Issues

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The working group on ethical, legal, and societal issues was assembled to answer questions posed by the National Institutes of Health on a given aspect of the topic of prenatal gene transfer. Group members attended the conference, listened to expert speakers, refined and reexamined their thinking, and fashioned their answers accordingly. The working group paper contained in this report is the result of this process of inquiry, discussion, and reflection.

### **Panelists**

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## Working Group III: Ethical, Legal, and Societal Issues

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### I. Preliminary points

(1) We question whether “prenatal” is the best term to describe this application of gene transfer research (GTR). We have two reasons for this concern:

(a) The term “prenatal” presupposes that a fetus that is a candidate for *in utero* gene transfer research will be intended to come to term. This presupposition implies precluding the option of abortion when the fetus has serious inherited disease. However, a pregnant woman’s decision to participate in GTR should not imply preclusion of this option within the limits permitted by law.

(b) “Prenatal” refers not only to fetuses but also to preimplantation embryos, which fall outside our scope of consideration. The scientific, ethical, legal, and societal issues arising from the application of GTR to embryos following preimplantation diagnosis are significantly different in at least some respects from those arising from the application of GTR to fetuses *in utero*. Our focus, and that of the GTPC, has been limited to the latter.

(2) Our analysis is informed by, though not limited to, existing federal regulations. Changes to Subpart B of the Department of Health and Human Services (DHHS) regulations governing research involving pregnant women and fetuses are expected soon; we do not believe that the changes that have been proposed and discussed will significantly affect our analysis or conclusions.

(3) We take our primary objective in this paper to be the isolation and evaluation of those ethical, legal, and societal concerns that uniquely concern *in utero* GTR. The general ethical issues that arise when a pregnant woman is asked to participate in research that is offered in part because it could potentially benefit her fetus are well recognized. It is important, for instance, to ensure a thorough and thoughtful informed consent process, providing clear information in as unpressured a setting as possible, to offset the possibility that pressure from family, friends, and health care providers might unduly influence the pregnant

woman’s decision. Such pressures could imply that to be a “good mother” a woman must demonstrate a willingness to do anything to help the fetus—even risky things that have only a small chance of success, if any. To illustrate: Much attention has been given recently to a wide variety of surgical and medical interventions *in utero*, many of which are described as last-resort, “heroic treatments” rather than as experimental interventions. The resultant “overselling” of fetal research potentially makes it more difficult to convey balanced and realistic information, especially immediately following a discouraging prenatal diagnosis and in circumstances in which the window of opportunity for intervention appears to be very short. Discussion at the GTPC amply demonstrated this difficulty, even though several presenters called attention to the problem and urged extra care in disclosure and discussion of research interventions in this context.

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The special ethical, legal, and societal concerns raised by GTR in the fetus pertain primarily to unique risks posed by the procedure, its potential for benefit, and the current paucity of data and overwhelming amount of uncertainty regarding relevant scientific information. The following discussion covers five areas: (1) whether, and if so, when, to embark upon *in utero* GTR; (2) what criteria should be used to select the diseases most appropriately targeted for GTR in the fetus; (3) what should be the inclusion/exclusion criteria for recruitment-enrollment; (4) what the concerns regarding informed consent are in this context; and (5) what considerations of justice apply. In each of these five areas, we address issues on which members of the working group have expressed agreement and disagreement, as well as questions that remain largely unexplored.

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## II. Ethical permissibility of embarking on in utero gene transfer research

### A. Animal models and their limits

One difficulty in ascertaining the types and levels of risk of *in utero* GTR stems from the fact that animal models for evaluating the effects of biological products are much less well developed than their counterparts in traditional pharmacology/toxicology. The principles of chemical toxicology studies in animals have developed over several decades, allowing reasonable assumptions to be made about the potential human toxicities of new drugs. Animal testing to predict toxicity in humans for biological products, however, is a nascent field, in which the science is still rudimentary and uncertainties abound. We cannot be sure that toxicities increase with dose for biological products; some toxicities may be threshold events; human immune response to a biological agent, such as a viral vector, may differ enough from that of a given non-human animal to mask potential toxicities. More basically, even the determination of how a potential toxicity might manifest itself is a largely speculative endeavor.

In addition, the ability to compare toxicity studies of biological products is difficult because of differences among gene transfer vectors and the absence of standardized methods for their detection and assessment. Finally, few animal models for diseases in which to test gene transfer have been developed to date.

All of the factors just enumerated combine to complicate decisions to embark on human GTR, especially *in utero*. In light of these considerations, and the low likelihood of reducing this level of uncertainty, we do not believe that it would be ethically permissible to embark upon *in utero* GTR in the near future.

Thus, improvement of gene transfer, with concomitant amelioration of disease, in animal models is a necessary precondition for beginning human *in utero* trials. Considerable preclinical research is needed, with a variety of animal models, potential candidate disorders, and gene delivery methods, so that the risks of harm and the potential for benefit can be assessed as thoroughly as possible before moving to clinical trials. Within this formulation, a significant question remains, namely, how to make the determination that data from animal studies sufficiently demonstrate the safety and feasibility of proceeding to clinical trials. How the data are interpreted and applied is a matter of ethics as well as of science. Our concern is strongest over the problem of irreducible uncertainty in the move from nonhuman to human studies.

### B. Complexity and uncertainty of risk-benefit assessment

As is true for all *in utero* clinical research, *in utero* GTR is not ethically justifiable unless the following conditions

are met: The risks of harm both to the fetus and to the pregnant woman are minimized; the potential for direct benefit to the fetus is maximized, consistent with the early stage of this research; and the possibility of germline effects is minimized. It is, however, extremely challenging to analyze, predict, and measure the risks and benefits of *in utero* GTR because of its high degree of complexity and uncertainty. Many, if not most, of the risks at issue here fall into an indeterminate range: greater than zero, but very difficult to quantify with any further degree of precision.

In general, the degree of risk in GTR has been less precisely categorized than may be necessary for *in utero* GTR. However, it is well recognized that in GTR, the potential for direct benefit that should accompany the intent to benefit subjects has not yet been fulfilled. This raises questions about the likelihood of direct benefit for all subjects enrolled in GTR, and a specific problem of categorization of the level of risk to the fetus in *in utero* GTR. Discussions on the meaning of minimal risk in nearly all clinical research settings has been inadequate, and disagreement about risk assessment is widespread. The application of the concept of “minimal risk” to *in utero* GTR is therefore difficult; however, the assessment of risks for the fetus should not differ substantively from similar assessments of risk for a child or infant. While the possibility of germline transmission initially seems to differentiate fetuses from born subjects, that risk is properly conceived as a risk to future generations rather than a direct risk to the fetus. Assessing the risks and uncertainties of *in utero* GTR must also include those specific to the *in utero* setting, i.e., the risks and uncertainties of preintervention diagnostic testing, of invasive diagnostic, research, and (possibly) adjunctive interventions, and of postintervention testing (invasive testing and interventions that could cause pregnancy loss or other problems.) These risks and uncertainties can be minimized but not eliminated.

Risks of harm unique to *in utero* GTR include the risks of physical harm to the fetus and the pregnant woman from the vector and/or genetic material itself. Some of these harms could materialize immediately (e.g., inflammatory response to viral vector), are probably transient, and probably minimizable with different delivery systems. Others, such as the risk of adverse consequences if vector or genetic material gets to the wrong place, are different in nature, magnitude, and likelihood for the fetus compared with the pregnant woman. Although these risks are generally thought to be low, they are unlikely to materialize for many years. Since it would be difficult to link a late-appearing tumor to *in utero* GTR rather than to another cause, considerable uncertainty about these risks is likely to persist.

The risks of psychosocial harm from being a subject of *in utero* GTR have also been mentioned. These risks are

somewhat analogous to the psychological effects on subjects of much high-technology early-phase clinical research, as well as on persons who suffer from serious chronic disease in general. However, the scrutiny of both fetus and pregnant woman, from prenatal diagnosis through the birth, life, and death of both categories of subjects' future offspring, is unprecedented. Since 1990 there have been many subjects of GTR; some have lived for many years and some may live many years more. Little is known about the effects of long-term scrutiny and medicalization of their lives, and even less about how to minimize adverse effects while maximizing the likelihood of obtaining needed information, e.g., from autopsy.

Evaluating, understanding, and minimizing the risks of harm from *in utero* GTR is clearly a highly disease-specific endeavor. Nonetheless, it should be possible to determine more definitively and to minimize the likelihood of each of the various categories of harm, even though it will be far more difficult, if not impossible, to determine and minimize their magnitude. One means of doing so would be to find ways of encouraging scientific and technical collaboration to ensure the best possible preclinical and clinical trials in a given area. Presumably, the authority to foster such efforts would have to come from research sponsors and funders. It would therefore be optimal if future RAC discussions addressed the feasibility of establishing an approximate risk threshold that could not ethically be exceeded in clinical trials of *in utero* GTR, and explored a way of discussing the remaining irreducible uncertainties.

### C. Germline effects

One risk of GTR *in utero* that requires separate discussion is the possibility of unintended but foreseen germline integration. Integration and expression are always possible outcomes of any injected vector, and present scientific knowledge is inadequate to elaborate on these possibilities with any degree of precision. However, some methods of gene delivery are more likely to pose this risk than others. Given the potential for inadvertent germline integration, several key questions must be addressed and resolved before human *in utero* GTR should proceed: (1) Should any risks of intergenerational genetic harm be tolerated and why or why not? (2) If some level of intergenerational risk is irreducible, what level of is acceptable (for example, can the risks of radiation and chemotherapies serve as adequate analogies?)? (3) Should the successful germline integration of a therapeutic gene be counted as a risk or a benefit of research and why or why not?

If germline integration were to count as a potential benefit, either for subjects or for their future offspring, it would have to be, at minimum, both safe and effective. However, information about the nature, probability, and magnitude of inadvertent germline effects anticipated

from *in utero* GTR is now, and is likely to continue to be, extremely limited. There appears to be clear agreement that the prudent course under these circumstances is to characterize germline integration as undesirable and to minimize it. These risks may have analogies in emerging data on survivors of childhood cancer, specifically the effects of chemotherapy and radiation on fertility and reproduction in this population. More data-gathering is imperative here.

### III. Criteria for selecting candidate diseases

Criteria for determining candidate diseases for *in utero* GTR include (1) severity of the disease, (2) presence or absence of postnatal (or other *in utero*) treatment alternatives, (3) concerns about partial correction, and (4) possibility of inadvertent germline insertion, each of which will be examined below. Prerequisite to all of these criteria are the requirements that the diagnosis of the fetus be certain; that the severity of the effects of disease in the fetus be highly predictable, i.e., that there be tight correlation between genotype and phenotype (so that the efficacy of the intervention can be assessed and the intervention can ultimately provide genuine clinical benefit); and that the intervention be designed to be both minimally intrusive and maximally effective. These prerequisites may require further development of prenatal testing for certain diseases. Because most GTR interventions are currently unsuccessful, it is also important to rule out those (presumably few) diseases in which an unsuccessful intervention would increase the risk to the pregnant woman, as is the case with alpha thalassemia.

#### A. Severity of the disease

We agree that limiting prenatal gene transfer research to diseases that are fatal *in utero* creates a category that is insufficiently useful, particularly in early clinical research (but see section C., below). It seems best to consider the range of diseases not normally fatal *in utero*, in order to be able to maximize the scientific knowledge gained from early trials of *in utero* GTR. It also seems that the best candidate diseases should have *in utero* effects that could potentially be ameliorated by *in utero* treatment (otherwise, postnatal treatment would be possible), but that diseases normally fatal *in utero* and those with neurological effects *in utero* are not especially suitable disease candidates because of the high likelihood that partial correction would cause suffering postnatally.

#### B. Existence of postnatal treatment alternatives

A disease is not an acceptable candidate for *in utero* GTR when a clearly effective postnatal therapy exists, because the additional risks imposed on the pregnant woman and the fetus by *in utero* GTR will always outweigh the benefits of *in utero* as compared with postnatal intervention.

Thus, candidate diseases should have no clearly effective postnatal treatment alternatives. For a disease where no clearly effective postnatal therapy exists, postnatal GTR should be attempted (or ruled out, if scientifically infeasible) before *in utero* GTR is considered.

Of course, many potential candidate diseases do have one or more partially effective treatments, some postnatal, some *in utero*. The best candidate might be a disease for which there is a relatively risky but somewhat effective postnatal treatment, upon which *in utero* GTR could promise to improve.

It could be argued that the availability of any postnatal therapy, even partially effective therapy, makes the decision to go forward with *in utero* GTR less controversial, once safety is demonstrated preclinically, since such therapies can always be offered to subjects after birth. (The problem, raised by the adenosine deaminase deficiency [ADA] GTR protocol, of whether concomitant provision of “standard” treatment is ethically necessary when it is also scientifically confounding must then be addressed.) In contrast, it could be argued that when there is no postnatal treatment that is even partially effective, a very strong case for feasibility and potential effectiveness must also be made. However, this contrast creates an ethically inappropriate dichotomy. The availability and degree of effectiveness of postnatal treatments should not alter the evidence of safety, feasibility, and potential efficacy that must be demonstrated in both preclinical and clinical research. Given that there are some treatments, with varying risk/benefit ratios, for most of the diseases that are potential candidates for *in utero* GTR, the assessment of suitability should take into account all relevant circumstances particular to each disease.

### C. Partial correction

In the case of a disease that is otherwise fatal *in utero*, would the possibility of partial correction make it an acceptable candidate for *in utero* GTR? One view maintains that a disease is not an acceptable candidate for *in utero* GTR when the consequence of no treatment is death of the fetus *in utero* and the intervention might result in compromised survival, thus making a tragic situation even worse. An opposing view maintains that a disease may be an acceptable candidate for *in utero* GTR even though only partial correction may be achieved, because some couples might want any child versus no child at all. This latter view is controversial since in some cases it may conflict with the researcher’s obligation to minimize harm to the fetal subject.

In the case of a disease that has no *in utero* effects, anything less than complete correction would not appear to be an improvement over existing postnatal therapies. Thus, the prospect of partial correction of such a disease through *in utero* gene therapy does not seem justifiable, and therefore neither does *in utero* GTR. In the case of a

disease that does have nonfatal *in utero* effects, partial correction would seem a justifiable goal if it produced some clinical benefit. This category of diseases may include the best candidates for *in utero* GTR, but choosing such a disease would require much more knowledge about the relationship between degree of correction and clinical effects than appears to exist at present.

In summary, good candidate diseases might be those in which partial genetic correction is likely to lead to significant clinical improvement. Poor candidate diseases would seem to be diseases normally fatal *in utero* and those with neurological effects *in utero*, because of the high likelihood that partial correction would cause suffering postnatally. Whether good candidate diseases might also be those in which *in utero* intervention facilitates more effective postnatal intervention would depend on the specifics of such a dual-intervention scheme.

### D. Germline effects

The possibility of inadvertent germline insertion was discussed in the previous section and will not be addressed again here. Whether its likelihood is dependent on choice of disease, choice of vector and delivery method, age of the fetus, or other factors, this topic clearly deserves a more protracted exploration.

### E. Summary comments

In choosing appropriate candidate diseases, it is necessary to consider the potential for development of both scientific knowledge and effective treatments. Thus, it is important to examine the potential for direct benefit to subjects as well as the risks of harm. The potential for direct benefit poses a special difficulty, however. On the one hand, there should be enough of it to warrant clinical trials (in the sense that should the trials produce the desired results, a certain level of effectiveness would be shown). On the other hand, such potential must be carefully described and discussed so that it is not oversold to potential subjects. It would therefore be optimal if future RAC discussions addressed the feasibility of establishing an approximate threshold level of potential benefit that must be met in clinical trials of *in utero* GTR. Could the likelihood of the subject’s receiving direct benefit be estimated based on preclinical data? Could the nature and magnitude (improvement or cure? temporary or permanent?) of any potential benefit, should it materialize, be describable, even if only as a range? Any such information would certainly be highly disease-specific, and a high degree of irreducible uncertainty would of course remain.

Attempting to set even the most approximate thresholds for risk of harm and potential for benefit in *in utero* GTR is difficult, both because of the lack of good analogies and precedents and because this effort is not generally made in other clinical research contexts.



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Attempting to do this could, however, address some fundamental questions about responsible research design that are often in effect abdicated, by reliance on two necessary, but not necessarily sufficient, factors: the inclination and judgment of the individual investigator and the choice of the potential subject or surrogate decision-maker.

#### **IV. Establishing inclusion/exclusion criteria for recruitment-enrollment**

The working group did not address issues in recruitment and enrollment that would be applicable to any comparable clinical research or GTR context. We focused instead on those issues of particular relevance to the context of *in utero* GTR: the role, if any, of the father in enrollment; consent to long-term follow-up; and timing of enrollment.

##### **A. Father of the fetus**

Disagreement on protocols of this kind often focuses on the role of the male partner, with some contending that he should be involved in the enrollment decision and others insisting that he need not be involved. Usually, consensus can be reached that the male partner has an interest in the outcome, but disagreement remains on whether that interest confers a right to be a coequal decision-maker. In effect, the question is whether the male partner should be allowed to veto the pregnant woman's decision to enroll in GTR, since in no case would he be permitted to authorize her enrollment in the absence of her voluntary, informed consent. Ideally, both the woman and the male partner should be involved in the enrollment process. However, disagreement remains over whether the agreement of both parties should be an inclusion criterion for GTR *in utero*. The changing federal regulatory landscape of research involving fetuses and pregnant women is important, but not necessarily morally definitive, in this regard.

##### **B. Staying a subject**

The pregnant woman's willingness to agree, for herself and on behalf of her fetus, to long-term follow-up and consent to autopsy should be an inclusion criterion for entry into these studies, in order to assess germline and mutagenic effects. However, as in all research, if subjects change their minds, their refusal to permit follow-up or autopsy cannot be overridden. *In utero* GTR is like other GTR in this respect, although follow-up and autopsy data have not been systematically obtained in GTR to date and are arguably more important where, as here, the potential for germline effects and other late-appearing effects may be greater. *In utero* GTR may also require follow-up of subsequent offspring of both the pregnant woman and the fetus. Thus, the nature and extent of the follow-up that is scientifically desirable to

assess the relationship between clinical effects and the degree of chimerism in the fetus, the long-term risk of mutagenesis, and any effects transmitted to the pregnant woman or fetus that can be transmitted to the next generation of offspring, appears to be unprecedented. This aspect of research participation, and its potential effects on subjects, has been insufficiently considered. It is nevertheless clear that however important the need for follow-up information in this type of research, it cannot override the subjects' (and their future children's) right to withdraw from participation at any time, including before long-term and intergenerational risks have been assessed.

##### **C. Timing of and preparation for decision-making**

Ideally, for *in utero* GTR there should always be a long period during which information can be provided and the woman/couple can make a decision. This scenario poses some interesting problems. First, it suggests that the ideal situation is that of preconception counseling of couples at risk for producing affected offspring. However, because optimal timing and circumstances are not always possible, the limits of an acceptable time frame must be established to minimize the possibility of rushed decisions fueled by desperation and bad news. Second, appropriate planning implies testing of the couple and prenatal testing of the fetus. Both diagnostic testing and the research intervention itself present risks, uncertainties, and challenges of timing to all concerned. The risks to the fetus and the pregnant woman are dynamic and complicated. Monitoring and assessment of fetal age and development are not exceptionally accurate, and it is relatively unclear when fetal immunity develops to a sufficient extent to interfere with *in utero* GTR. Moreover, if the optimal time for intervention from the pregnant woman's perspective conflicts with the optimal gestational time for intervention on the fetus's behalf, difficulties in weighing the risks and benefits of the research could arise. Finally, as is the case for any *in utero* intervention, the pregnant woman's decision not to have an abortion cannot be used as an inclusion criterion for participation, and she must be told that she can end her participation and decide to have an abortion at any time. She must be free to change her mind about terminating the pregnancy. The subject must be informed that a decision to participate in research could affect the timing of an abortion; however, it is essential to emphasize that the decision about how to proceed in light of these timing considerations must be left to the subject and cannot be constrained by the scientific needs of the protocol.

##### **V. Issues of informed consent**

Here again, the working group addressed the content and process of informed consent only in the context of *in utero* GTR (not including core components of

informed consent in FDA and DHHS regulations and Appendix M, except insofar as they have special application in this context). Quite naturally, an optimal informed consent process should address the questions of uncertainty, safety, and feasibility surrounding *in utero* GTR that must have been answered in order for such research to proceed.

Several special issues of informed consent arise for *in utero* GTR. First, the pregnant woman is volunteering both herself and her fetus, and it may not be clear to the investigator how she is weighing those interests. Second, there is greater risk that pregnant women recruited for *in utero* GTR will not yet have experienced the disease firsthand and will know relatively little about it. Third, the intervention will be irreversible once initiated. Fourth, there are increased risks of germline transformation. Fifth, given the breakneck speed of genetic research, mechanisms need to be in place through which informed consent documents can be revised as new scientifically valid data become available. In some cases, investigators may be required to return to subjects after the intervention to notify them of new findings and perhaps, in some circumstances, to obtain consent for additional follow-up procedures such as ongoing monitoring or testing. These could be necessary if the new data suggest risks to subjects, their offspring, or a wider public. A very careful assessment process is needed to weigh the credibility and seriousness of any such new findings.

### **A. An optimal process for informed consent**

An optimal informed consent process for *in utero* GTR would have the following five components:

(1) Agenda ice-breaking. An initial conversation is needed to maximize the pregnant woman's/couple's ability to consider all the information about the research. Such a conversation should ascertain from the pregnant woman who is a potential subject: (a) her level of knowledge of and experience with the candidate disease; (b) her motivation for volunteering. The same conversation should include a disclosure to the potential subject of: (c) the nontherapeutic nature of research; and (d) reproductive alternatives to the protocol.

(2) Education. Adequate education of potential subjects should include clear exposition of: (a) the purpose of a Phase I trials; (b) the risks of harm and the limited potential for direct benefit, for both pregnant woman and fetus, from the research intervention, including as much information as is reasonably available about the nature, magnitude, and likelihood of both risks of harm and chance of benefit, the range of possible effects, and an honest statement about what is uncertain and unknown; (c) constraints of participation on maternal behavior during pregnancy; (d) follow-up conditions of participation, i.e., the nature and extent of postintervention maternal, fetal, and infant monitoring, autopsy, etc.;

(e) alternatives to participation, including reproductive options, "standard" prenatal and/or postnatal therapies, and other alternatives that should be described as "innovative" or unproven therapies; (f) the right to withdraw, which, it must be explained, persists even after the pregnant woman has undergone the irreversible intervention; (g) confidentiality protections and their limits; and (h) information about pertinent financial aspects of the study, including funding sources, financial interests of the investigators, and financial implications of study participation and follow-up for subjects and their future offspring.

(3) Discussion. A discussion that includes other adults responsible for fetal welfare (father, partner, etc.) should take place, with time for careful consideration and additional consultation as needed. A genetic counselor independent of the research team should be made available to the woman/couple.

(4) Decision-making. The final decision should be secured from the pregnant woman under circumstances that reduce the potential for coercive pressures from others on her decision, without making it difficult for her to consult with support persons, including counselors and family.

(5) No fault bail-out. Another conversation should take place before initiation of an irreversible procedure, providing another opportunity for withdrawal from the trial.

### **B. Summary comments**

It was apparent from presentations and discussion at the GTPC that both the language and circumstances of the offer to participate in *in utero* GTR are likely, without significant oversight, to be confusing and confused at best, and at worst to potentially encourage pregnant women who are potential subjects to incorrectly assume that they are being offered a genetic treatment that is likely to cure their affected fetuses.

Few of the components of an optimal informed consent form and process for *in utero* GTR are unique to this setting. There is considerable agreement, both in the literature and as reflected in the GTPC, on what it takes to do informed consent well in early-phase clinical trials. A recent article in JAMA by Moreno et al. (280 JAMA 1951, 12/9/98, at 1954) recommends that all consent forms for Phase I studies display at the top prominently in bold the following statement: "This medical research project is not expected to benefit you." At present, however, the wording of all of the federal regulations and guidance documents implicitly encourages both investigators and subjects to equate hope with likelihood.

If an appropriate candidate disease and delivery system is chosen for *in utero* GTR, and risk and potential benefit thresholds can be achieved and clinical trials

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begun, the consent form and process should include not only a disclaimer like the one cited above but also careful discussion of both the information and the uncertainty that underlie description of the nature, magnitude, and likelihood of risks of harm and chances for benefit. Given the strength of the desire to view early-phase clinical research as the best or only treatment option for serious disease without effective treatment, this proposal may be controversial. Given the strength of the need for data to be used for developing future effective treatments, this proposal is essential, lest clinical investigators permit their compassion for their subjects to overshadow the primary purpose of early-phase GTR: to gain scientific and medical knowledge. The end result of this misconception about the relationship between early-phase research and therapeutic benefit, for individuals and for the public, has too often been disappointment and loss of trust.

## **VI. Issues of justice**

Provided that the problems of uncertainty, safety, and feasibility have been satisfactorily addressed, several broad societal questions must be considered before proceeding with *in utero* GTR. The first two are questions of procedural justice:

(1) What is the best science policy process to ensure that the American public in all its diversity has the

opportunity to become involved in decisions to proceed with or forgo this research?

(2) Is the current regulatory apparatus adequate to ensure that public concerns about this area of biomedical research are addressed and our science policies enforced?

Two additional questions are matters of distributive justice:

(1) Are there, or should there be, policy processes and criteria through which to address the question of whether it is appropriate to expend resources on this category of research rather than on other health care needs? This complex and important question emerged at the GTPC in the form of the argument that alternatives to *in utero* GTR exist, at least in theory, for couples with a high risk of giving birth to severely affected offspring. However, these alternatives, which include IVF with preimplantation genetic diagnosis, are expensive and of limited availability, potentially making participation in *in utero* GTR desirable by comparison. From the public's perspective, though not for individual couples and their future offspring, the prospect of *in utero* GTR may highlight some major and insufficiently addressed issues of reproductive health policy.

(2) Finally, if this research does go forward, what changes in the health care system will be required to provide the public equitable access to any clinical tools it may yield?

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## Conference Report

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The human body is made up of molecular structures that are controlled by genes which are passed from one generation to another. If the molecular products of the genes are normal, a healthy individual results; if the genes are not normal, the individual has an inherited disease. The nationally sponsored Human Genome project has as its goal the identification of the estimated 100,000 genes that represent the “text” of a given individual. These genes will not only be characterized but also mapped to their location on the human chromosome, the biological structures which organize all genetic material. Although this project is far from complete, enough is known to show that a high level of variation is present in all human genes, offering either unique selective advantages for survival in different environments or unique susceptibility to disease.

Genetic and molecular techniques have been developed that allow these genes to be manipulated and transferred from one individual to another. This ability has led to proposals for the transfer of somatic genes (those which control molecules making up human tissues) from one host to another. Because of concerns about genetic enhancement, somatic (nonreproductive) cell gene transfer, rather than genomic (reproductive) cell gene transfer, remains the goal.

Somatic cell gene transfer offers the promise of treating, and possibly curing, inherited diseases at their fundamental origin—the gene. The primary research objective of the more than 300 gene transfer clinical trials to date has been to prevent or treat disease after birth. Genes producing a normal molecular product have been introduced into patients, ranging in age from newborns to adults, with the aim of conferring on those patients the ability to produce the normal molecular material within their bodies rather than receiving it artificially. Although the efficacy of these early gene transfer studies has yet to be clearly established, short-term safety appears reasonable. Side effects, however, seem to vary depending on the nature of the virus used as vector.

Prenatal gene transfer offers even greater promise than postnatal gene transfer—the ability to treat or cure genetic disorders before the onset of disease. However, prenatal gene transfer research raises significant scientific, medical, ethical, and societal issues that must be addressed before the field can move forward. These issues include: (1) assessing the current state of knowledge about the feasibility, safety, and potential efficacy of prenatal gene transfer; and (2) recognition of and consensus on controversial extensions of this technology into areas that engender ethical and social concerns.

Although hematopoietic stem cell (HSC) transplantation offers successful treatment for some life-threatening conditions, no curative therapies are available for most serious genetic diseases at the present time. Because early prenatal diagnosis is now available for some genetic disorders, additional progress in somatic cell gene transfer research might make it possible to attempt prenatal gene transfer.

Two recent milestones in somatic gene transfer research prompted the organization of a conference on prenatal gene transfer. In July 1998, Drs. W. French Anderson and Esmail Zanjani submitted two preliminary protocols for prenatal gene transfer trials to the NIH Recombinant DNA Advisory Committee (RAC) with the intent of providing a context for the identification of the significant scientific, safety, ethical, and social issues raised by this area of research. That same month Porada et al. published a paper in *Human Gene Therapy* which described the direct intraperitoneal injection of retroviral vectors and vector producer cells into preimmune sheep fetuses—a possible mechanism for prenatal gene transfer. These developments, coupled with the increasing body of literature documenting the ability to transfer bone marrow cells into human fetuses for the treatment of heritable disorders, signaled the need to assemble a broad spectrum of stakeholders (basic scientists, clinicians, ethicists, patients/families, lawyers, biosafety experts, the general public, and proponents/opponents of such applications) to engage in an ongoing public debate on the scientific, ethical, and legal issues raised by prenatal gene transfer research. In doing so the NIH/RAC hoped to facilitate public consensus about the appropriateness of prenatal gene transfer as a long-term goal for clinical investigations and the circumstances necessary for such an intervention to be acceptable.

### **I. Development of the human fetal immune system**

Prenatal introduction of therapeutic genes may present an advantage over postnatal gene therapy by delivering genes before the fetus can immunologically recognize “self.” Since postnatal therapy produces new proteins in neonates after they have become immunologically responsive, these patients may produce antibodies against the new, nonself molecules. In theory, if genes could be delivered to the fetus while it remained immunologically unresponsive, rejection of the transferred foreign antigens would not occur. The neonate would then be tolerant, i.e., immunologically unresponsive to subsequent antigen challenge.

Immature fetal B cells are susceptible to the induction of tolerance. It is thought that between weeks 4 and 6 of human gestation, the stem cells that give rise to B cell lymphopoiesis migrate to the fetal liver. Once in the liver, the development of B cell lymphocyte lineages probably begins between weeks 7 and 8 gestation. By 15 weeks, B cell levels are comparable to those observed postnatally. However, both the fetal and neonatal antibody repertoires are more restricted than their adult counterpart. In particular, the antibody repertoire of normal adults is not observed in first and second trimester tissues. This restricted repertoire lim-

its the range of antibody specificity in the early gestation fetus.

Mechanisms of T cell tolerance fall into two overlapping areas: (1) thymus directed and (2) peripheral. The thymus is the site of early induction of T cell tolerance to “self” antigens. Upon interaction with thymic dendritic cells, thymocytes may undergo negative selection and clonal deletion. Peripheral T cell tolerance encompasses both “self” and exogenous antigens. Despite the potential for development of T cell tolerance *in utero*, antigen specific T cell immunity has been observed following the first and second trimesters in neonates affected with congenital infections (toxoplasmosis, rubella, cytomegalovirus, syphilis, varicella-zoster virus).

Neonates also show substantial cellular immunity to herpes simplex virus infections, although this is delayed in appearance compared with adults. Antibody responses to T cell-dependent protein vaccines are also modestly less when immunization occurs in the first few days after birth as opposed to after one month of age. A tolerance-like state has also been observed when unrelated leukocytes and skin grafts are simultaneously transferred to newborns. Skin grafts demonstrated greater survival in neonates that received fresh blood versus blood that was “stored,” thus suggesting the induction of tolerance in neonates. Once the T cells leave the thymus, they may require further extrathymic maturation upon entering the periphery before full immune competency is acquired, thus explaining decreased T cell competency in neonates. In particular, the CD40 ligand may play a key role in the presentation of antigens to T cells.

### **II. Preclinical research issues**

Besides the potential for antibody production, other limitations of postnatal therapy include the time lag between the development of disease symptoms and the onset of therapy, toxic side effects of therapy, and inefficient transduction. In contrast to postnatal therapies for several diseases, *in utero* gene transfer would not require bone marrow depletion or the use of toxic reagents to prevent graft-versus-host disease (GVHD). In the prenatal setting therapy requires only a small volume of vector, exploiting additional expansion of transduced cells, and has an earlier impact on fetal development as well as potential emotional and societal benefits.

The two approaches being investigated are known as the indirect and the direct. In the indirect approach, blood from the fetus is harvested and transduced. The relative disadvantages of the cellular approach include the complexities of multiple fetal manipulations, the fact that only a fraction of hematopoietic stem cells (HSC) are removed for transduction, the later fetal stage required for manipulations, immunocompetence

of the more mature fetus, and the relatively low gene transfer that has been observed. This last issue motivated investigation of a simpler, direct approach using vectors that in turn necessitated study of additional factors such as safety with respect to gestational age, route of administration, target specificity, vector titer, and use of multiple infusions.

The advantages of the direct approach include the ability to treat early preimmune fetuses and transduction of long-term repopulating cells. When initial experiments were performed in sheep, proviral DNA was found in essentially all tissues of the transduced animals, including brain (both in animals studied at birth and also at 18 months) and testes and ovaries. These studies indicated a broad distribution of the marker gene throughout the body and long-term gene expression in blood cells. The remaining issues to be addressed are the level of expression required for therapy, whether there is truly lifelong persistence of expression, an assessment of the risk to the fetus and to the mother, and the possibility of inadvertent germline alteration (although none was observed in the ovine studies).

The current inefficiency of existing methods for prenatal HSC transplantation may be due to the special environment in which these cells exist. Thus, a number of variables are being studied in 20- to 29-day gestation mice, including route of injection, degree of donor cell proliferation, donor and recipient age, and the number of transplanted cells. Experiments with mouse fetal liver cells suggest that the fetal liver environment into which cells were transplanted stimulated their proliferation. Fetal recipient age influenced the donor cell engraftment, with later (older) donor and older recipient stages yielding higher rates of donor engraftment (though still at low levels). The engraftment of fetal liver HSC was found to be dose dependent, with 5-fluorouracil stimulated fetal liver cells showing reduced engraftment compared with unstimulated/recruited donor cells. In these studies it was evident that transplanted HSC can generate multilineage hematopoiesis and that the fetal environment alters transplanted HSC function. Engraftment via intraperitoneal administration was superior to the transplacental route, and fetal cells were better than bone marrow.

Direct vector-induced transduction also has potential side effects. Any artificially introduced vector carries the potential of distribution beyond the site of administration, including into the blood and possibly the gonads. Therefore, the risk for inadvertent germ cell transduction following *in vivo* administration procedures must be considered. Different delivery systems (e.g., adenovirus, liposome-delivered genes, murine retroviral vectors, AAV, and lentiviral vectors) will have different potentials for germline mutation. Considerations of the nature of each vector (i.e., tropism, efficiency, integration) should provide an estimate of this risk. An adenovirus-mediated

clinical trial of gene therapy for ornithine transcarbamylase deficiency (in which 14 patients have been enrolled), can provide an estimate of such a potential for this vector, which has broad target cell tropism and high efficiency of transduction, but low integration capability. In this case, the probability of causing inadvertent integration into a germline cell can be estimated based upon the cumulative probabilities that: (1) dissemination of vector to gonads is less than 1/10 to 1/104, (2) efficiency of transduction in gonads is less than 1 in 10, (3) the fraction of transduction events in germline cells is less than 1/103, and (4) the chance of an insertion event leading to a birth defect is 1/10 to 1/105. The risk of causing a birth defect is thus approximated to be between 1/109 to 1/1014 for each patient undergoing a single treatment. For Phase I studies and small populations, the risk for germline integration can be quantified and weighed with respect to the potential benefits as illustrated in this case. The implications for Phase III studies and large populations are somewhat more problematic.

In sum, the potential for *in utero* transplantation offers the following possible advantages over postnatal gene transfer therapies:

- The ability to treat potential patients prior to the appearance of the effects of the disease;
- The protected environment of the uterus in terms of potential immune response;
- The possibility of additional treatment after birth;
- The potential for significant therapy-related savings to patients and their families in comparison to other therapies such as organ transplantation.

### III. Clinical research issues

#### A. Candidate diseases

Diseases potentially treatable by prenatal gene therapy include those for which there is presently no effective treatment, in which the pathologic process begins before birth, and which can be diagnosed accurately before birth. Possible target diseases for prenatal gene therapy include neurometabolic disorders, cystic fibrosis, and severe combined immunodeficiencies such as adenosine deaminase (ADA) deficiency, the first disease to be treated by gene therapy.

Neurometabolic genetic disorders have been considered good candidates for prenatal gene therapy because there is no effective treatment for many of them, and because the pathologic process often begins before birth. Importantly, many of these conditions can be diagnosed prenatally, including lysosomal storage disorders, trinucleotide repeat disorders, and several mitochondrial diseases. As an example of the current state of the field, metachromatic leukodystrophy has been studied in animal models and gene transfer has been

demonstrated to correct the metabolic defect in the laboratory, thus making this disorder a suitable candidate for ongoing studies.

The lysosomal storage diseases (LSD) have been paradigms for innovative therapies; the molecular pathology is well understood and ongoing *in vitro* studies, animal models, and human clinical trials have demonstrated the potential for gene therapy. Notably, successful human clinical trials of bone marrow transplantation (BMT) preceded analogous experimental studies in animals. As a consequence, animal models of lysosomal storage disease have become important in developing gene therapy for genetic diseases. Among such conditions,  $\beta$ -glucuronidase (GUSB) deficiency in murine and canine models have figured prominently. In representative experiments, "low GUSB" mice were treated by *in utero* infusion of "high GUSB" cells, and minimal engraftment of donor cells was observed; however, this was not sufficient to suggest that an equivalent level of enzyme from gene therapy would be therapeutic. In another form of cell transplantation, a type of immortalized neural cell has been transplanted into the ventricles of a mouse at birth. Substantial donor cell engraftment provided regional metabolic correction (circumscribed around the engrafted cell) but fell short of systemic CNS correction. Scaling up from mouse to dog has yet to be accomplished.

Cystic fibrosis can be corrected in an animal model by *in utero* gene therapy or replacement of the effector molecule, but the unexpected result of epithelial proliferation currently obviates human clinical application. It was reasoned that direct infusion into amniotic fluid of an adenoviral vector expressing the normal CFTR gene might be feasible and therapeutic. Titers of only 108 cfu/mL administered to the murine knock-out model of cystic fibrosis resulted in a therapeutic response in mutant mice, but decreased survival of normal litter mates. The lethality appeared to be the result of an unexpected effect of CFTR, that being the excessive proliferation of pulmonary epithelium, an unacceptable and potentially lethal problem. This observation suggested that CFTR might participate in lung development by means of a regulatory molecule. Administration of cAMP in the amniotic fluid rescued mutant mice, thus suggesting that CFTR may have a critical role in an cAMP-modulated developmental defect.

The recent experience with postnatal gene therapy in the treatment of ADA deficiency provides additional insight into questions surrounding prenatal gene transfer. In the 3 patients followed for 5-1/2 years, about 1/1,000 mononuclear cells are marked with the ADA transgene. The dose of exogenous PEG-ADA enzyme therapy was stopped 1-1/2 years ago in one patient, but the patient became symptomatic with infections, and enzyme replacement was restarted. During this period,

T cells (CD4) were "stable" in number, but there was an antigen-specific loss of responsiveness to tetanus stimulation. There appears to be transgene expression in dividing, but not resting, T lymphocytes. Thus, the problems are low level of transduction, low amounts of engraftment, and low levels of transgene expression in resting cells. It would seem that resolution of these problems must be achieved as a prerequisite for *in utero ex vivo* gene therapy.

A series of 122 patients with severe combined immunodeficiency disorder (SCID) offers a perspective on the potential for prenatal gene therapy for this disease. Of these cases, some 41% resulted from the IL-2R $\gamma$  defect, and 15% were due to adenosine deaminase (ADA) deficiency. All forms of SCID could be diagnosed if a lymphocyte count was included in screening of newborns. For haploidentical marrow transplantation, recent views suggest that maternal marrow may be superior to paternal marrow owing to the possibility that a small number of the mother's cells may have crossed the placenta and engrafted, thus reducing the risk of graft-versus-host disease. It is speculated that breast feeding may enhance reconstitution of the engrafted allogeneic immune system.

Pediatric patients with SCID also form part of the small group now treated with intrauterine stem cell transplantation to produce dizygotic chimerism, first recognized in cattle in the 1940s. Six patients have received fetal liver stem cells to treat severe genetic disorders and constitute the experience of this research group. Two children suffered from severe immunodeficiencies, three from thalassemia major, and one from Niemann-Pick type A disease. Two received intraperitoneal injection, and four intravenous infusion. Four achieved partial donor engraftment, and two are currently long-term survivors. Such examples demonstrate that *in utero* hematopoietic stem cell transplantation may be a potential means of correcting some genetic disorders. Because there are no foreseen variations in the existing methods that are unlikely to significantly improve the outcome, the focus is on understanding the barriers to effective engraftment which are largely theoretical, such as "niche availability," and competition or prohibition of donor engraftment by preexisting hematopoiesis.

## **B. Current diagnostic techniques**

The major issues for prenatal diagnosis are: provision of accurate and sophisticated testing methods, and the need for a clear relationship between genotype and disease phenotype. Rapid and accurate testing is important, particularly for prenatal diagnosis of genetic disorders, but each gene has unique problems, such as the diversity of mutations. For example, there are many different genes that relate to immunodeficiency. Some

diagnostic laboratories are using relatively archaic methods (linkage analysis); others, more sophisticated functional analyses (e.g., quantification of the common gamma chain); and yet others, the most highly sophisticated molecular genetic analyses, available only in a few research laboratories (e.g., single strand conformational polymorphism analysis, dideoxy fingerprinting, DNA sequencing). None of these approaches is completely sensitive, and the immense range of potential mutations makes it unlikely that any single laboratory will have techniques applicable to all affected individuals.

Establishing the genotype-phenotype relationship or correspondence remains problematic in some but not all cases. In rare circumstances, such as achondroplasia, a unique mutation (glycine at nucleotide 380 changed to an arginine) results in a relatively uniform phenotype. In contrast, more than 750 mutations have been identified in the CFTR gene. These mutations result in cystic fibrosis or, alternatively, sterility due to congenital absence of the vas deferens, and thus produce broad variation in the clinical disease. Similarly, there is great clinical variation in the fragile-X syndrome owing to the size of gene expansion, methylation, and mosaicism. In Gaucher disease even a single mutation results in a range of phenotypes, and cannot be used to predict severity. There are many other examples of great intrafamilial variability due to unknown factors (e.g., Marfan syndrome, neurofibromatosis). Identifying appropriate candidates for *in utero* gene transfer and monitoring the outcome after treatment will be challenging.

### **C. Clinical trial design, conduct, endpoints, and outcome measurement**

Before any attempt is made to perform experiments in humans, animal studies should demonstrate that a new procedure appears to be safe, that transduction (gene transfer) can be accomplished, that the gene will be expressed and found to have some therapeutic effect, and that germline alteration will not occur. To use a given method for *in vivo in utero* gene therapy, the method should show tissue specific transduction or expression, efficient transduction, and a system facilitating expression in resting lymphocytes. Candidate disorders must be diagnosable early enough for the parent to consider all standard therapies and other options (such as termination of the pregnancy), as well as this experimental treatment. In some cases, early prenatal treatment would be preferred to avoid myeloablation and/or immunosuppression for bone marrow transplantation.

Lentiviral vectors may allow these goals to be met. These vectors may have the capability to transduce 30% of nonmitotic hematopoietic stem cells. With an "MND" variant of the same retroviral vector, the aim would be to express the transgene in resting lymphocytes by exploiting human promoters.

Various milestones may be used to accelerate the evaluation of a new treatment. For this purpose, a "surrogate marker" can be defined as a laboratory measurement, sign, or symptom that, if changed by a therapy, would not, in and of itself, be clinically significant enough as a basis to evaluate therapeutic success. Analogously, a "surrogate endpoint" is a predefined change in a surrogate marker that is a primary or secondary outcome of a treatment trial. In terms of efficacy, conditions that are difficult to assess are those that might be heterogeneous, mild or slow in progression, intermittent, or for which the natural history may include several different clinical outcomes. Such surrogate markers may be especially useful for *in utero* treatments; if treatment is undertaken, noninvasive monitoring should be done. To speed the evaluation of a prenatal therapy and minimize the risk to future subjects, permission for an autopsy should be requested in the event of fetal demise or postnatal death.

### **IV. Ethical considerations in making the transition from bench to bedside**

Although intergenerational research, such as is the case for prenatal gene therapy, poses unique ethical problems, ethical considerations for the transition of any new therapy from the laboratory bench to the clinic must include the following.

- There must be a possibility of benefit to society through increased scientific knowledge, though not necessarily to the individual patient-subject.
- Individual patient-subjects must not be exposed to inappropriate levels of risk.
- The experimental design must reflect standard bioethical principles of respect for persons, beneficence, non-maleficence, and justice.
- A fair subject recruitment process must be in place.
- Informed consent must be obtained.

The unique ethical problem in prenatal gene therapy is the possibility that potential germline effects might transcend the currently acceptable boundaries of research, negating the possibility of further research. Traditionally, ethics has resisted interventions that pose risks to future generations, since these individuals cannot consent. This approach, however, is too absolutist. Given the seriousness of the diseases these protocols intend to treat, unknown risks should not necessarily form a contraindication. There appear to be clear ethical benefits to this therapy both in terms of doing good and increasing choice. The current challenge is to balance these benefits against the potential harms.

Conditions should be selected for intervention based on several criteria. These include the pathophysiology of the disease, the presence of a favorable risk-benefit calculus, the scientific value of the information potentially derived from investigating therapy for a given disease,



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the prevalence of the disease, the presence of a sufficient amount of time between diagnosis of condition and enrollment in study to allow for the informed consent process, and the availability of other treatments.

Obtaining informed consent in this context is uniquely challenging for several reasons. Parents cannot consent for their fetus, but can merely give permission based on the fetus's best medical interests; yet lifelong follow-up may be required for the fetus and its offspring, something to which the parents did not consent. When approaching patients about studies, the language used to describe the study—for instance, “research” or “study” versus “experimentation”—can influence their perceptions and decisions about enrollment. Other issues include the fact that *in utero* gene therapy raises the issue of harm to future generations, as well as the possibility that decisions about enrollment may become inappropriately intertwined with decisions about abortion. In selecting candidates for enrollment in these trials, however, the mother's preferences with respect to abortion should not be a factor; one's choices should not be restricted based on one's preferences in this area.

Public belief in the benefit of scientific investigation is generally strong and, in the setting of prenatal diagnosis, willingness to follow through with any offered investigation or intervention is also strong. But both patients and physicians tend to overestimate the therapeutic benefit of research to the individual patient, particularly in Phase I trials. These features converge to pose unique challenges to parental comprehension and voluntariness in accepting *in utero* interventions. Given the above challenges to voluntary, informed consent, and given that parents do not consent to research for their fetus, but merely give their permission, the institutional review board (IRB) should hold protocols of this kind to very high standards of risk/benefit assessment and possibly should require that there be no effective therapeutic alternatives to the protocol.

Another important and difficult decision in the area of the ethics of prenatal gene transfer is distinguishing whether the fetus is a “patient,” a “subject,” or both. Debates about the legal/moral status of the fetus are unlikely to be resolved in the near future, but determining whether or not the fetus is a patient is necessary in the context of *in utero* interventions and obstetrical care generally. The fetus becomes a patient to whom we are obligated when it is viable, but this is not a clear line. The fetus also becomes a patient, however, when the mother chooses to carry it to term, thus establishing a connection between the currently existing fetus and the future child. While present federal regulations indicate that both the mother and the father must consent to *in utero* interventions, and while ideally the father should be involved in the decision-making

process, there is a clear asymmetry between the father's moral standing as decision-maker and that of the mother, who through her body and her decision not to terminate the pregnancy, is responsible for establishing the link between the fetus and the future child necessary to render the fetus a patient in the first place.

A presentation of the risks and benefits for experimental treatments should address the subject's need for full disclosure, right to privacy, and freedom to refuse or withdraw from treatment. The risks to the pregnancy and the fetus are spontaneous abortion, rupture of membranes, fetal trauma, isoimmunization, viral transmission to fetus, graft-versus-host disease, no engraftment, and partial engraftment (need for postnatal bone marrow transplantation). The greatest risk to the pregnant woman is likely to be intrauterine infection; preterm labor and bleeding are also risks. The risks of the procedures likely depend upon the dose of cells and vector as well as gestational age.

What benefit would be sufficient to justify the risk, and how can this be measured? Unfortunately the current gene therapy technologies may not be able clearly quantify either risk or benefit to respond to the question of whether the risk/benefit ratio is appropriately balanced for a particular *in utero* gene therapy trial. Thus, the existing data and state of gene transfer technologies may suggest that prenatal gene therapy is premature at this time. Perhaps such technologies should be extensively tested and proven postnatally first (i.e., first in adults, then in children, and then *in utero*). Still, some technologies may be appropriate only for the fetus, and may not be available postnatally. Perhaps there are data to be acquired from ongoing *in utero* HSC transplantations. When considering whether an alternative postnatal therapy is available and preferable, one would also want to weigh the complications that might be obviated by a prenatal form of treatment (e.g., the adverse effects of chemotherapy required for conventional bone marrow transplantation). Also, there are some diseases in which damage occurs prenatally, and these diseases might merit intrauterine therapy even though postnatal therapy would never be attempted or has proved unsuccessful at reversing prenatal pathology. Another reason for considering prenatal therapy might be that the fetus is more susceptible to the treatment strategy itself, (i.e., retroviral vectors may be more successful in highly mitotic fetal cells). A very large number of clinical trials have used retroviral vectors with no ill effects. Retroviral vectors may be quite safe and be good gene delivery vehicles for *in utero* gene transfer. Importantly, there is a great deal of relevant data that can be acquired in animal models before considering a specific treatment protocol.

Maternal clinical risks associated with prenatal hematopoietic stem cell transplantation are “procedural”

(i.e., related to making the diagnosis or infusing stem cells) or “infectious.” In practiced hands these risks are exceedingly small. For the fetus the clinical risks are also procedural, infectious, graft-versus-host disease, genetic, experimental design, or failure of transplant. Follow-up fetal blood sampling presents an unnecessary risk.

Tolerance to donor cells remains the major issue. Other species readily accept allogeneic hematopoietic stem cells, yet extrapolation from such animal studies is not successful. Very early (before 12 menstrual weeks) transplants have not yet been done to determine if tolerance can be achieved at that stage. The major impediment to using fetal liver cells is the availability of cells in the United States, but other sources are available (i.e., fetal circulating blood, cord blood cells, and parental marrow). Other variables need to be studied (e.g., screening for infectious agents, fresh/frozen source, isolation of CD34+ fraction, CFU assay results, CD3+ immunophenotypes, single versus serial infusions, disease indication). Postnatal cytokine adjuvant therapy might be useful.

The rights and status of the fetal subject must also be considered. As discussed earlier, when does the fetus become a “patient” and acquire rights and obligations from the medical community? What is meant by “affected” fetus? Are there circumstances in which a “molecular genetic diagnosis” alone might be erroneous? Implicit in consideration of fetal therapy is a sense that the disorder being treated has a substantial disease burden and that there is a good potential for very significant benefit. In the event of the birth of a seriously ill or disabled child following an attempt at prenatal therapy, who would be responsible for the medical care of the seriously affected infant? Presumably, the mother or parents who made the decision for treatment would be responsible, as in the example of a mother who decides to continue a pregnancy involving a nonviable anencephaly (but for which there is a prolonged period of hospitalization of the nonviable product of conception).

Should normal pregnancies be studied as “controls”? The consensus view is that only well-recognized, serious medical conditions should be considered for trials of *in utero* gene transfer. In human experimentation, such as evaluation of new chemotherapy drugs, such “control” studies are not done on normal subjects. Human clinical trials do not undertake treatments with a significant risk where no potential benefit would be realized.

## **V. Domestic and international regulatory perspectives**

Government involvement in a particular field confers the benefits of quality control and regulatory oversight. In the United States, the Food and Drug Administration (FDA) has published guidelines for gene therapy and

cell therapies and has declared its intention to extend this purview to prenatal therapies. The development of such new therapies is reviewed and regulated under the Investigational New Drug (IND) application format, which assures the safety and rights of subjects. During a Phase I study the regulatory process encourages innovation by allowing a maximum level of flexibility while obtaining an initial assessment of the safety of the treatment. In later Phases (II and III), the aim is to assure quality of study design to permit evaluation of effectiveness and to provide an additional evaluation of safety. This process is well-established for the commercial development and licensing of a new drug or medical device.

To protect human research subjects who participate in federally funded projects, such as those supported by the NIH, a separate group of regulations applies. These regulations are promulgated by the Department of Health and Human Services (HHS), and those applying to the fetus and pregnant woman are delineated under 45 CFR 46, subpart B. Such regulations were initially established in 1975 and are subject to proposed revisions (sent out for public comment in 1998) and scheduled for approval in 1999. The actual assessment of compliance with these regulations is vested with the local IRB. Notable in these regulations is the fact that current policy provides for participation of the fetus, which is conferred not only by the mother’s consent but also requires informed consent of the father (with only specified exceptions).

Seventy-five percent of gene therapy clinical trials have been conducted in the United States, but prenatal gene therapy and alteration of the germline have been considered in other countries. In England the Gene Therapy Advisory Committee currently prohibits *in vivo* gene transfer approaches *in utero*, but considers *ex vivo* methods to be under the same regulations as somatic gene therapy procedures done postnatally. In France, protocols which modify the germline are specifically prohibited. In Canada the use of “standards” seems to be the established means of regulating many similar procedures, and will likely provide the framework for regulating gene therapy there. However, some regulatory approaches may fall short in a variety of ways. Strict moratoria and status quo approaches may not work, and “lot release criteria” and manufacturing regulations do not cover all of the ethical and societal issues.

## **VI. Conclusions**

The development of often dramatic phenotypes of transgenic and “knock-out” mice resulting from single-gene alterations has been a major accomplishment in experimental gene transfer. These germline modifications in animals hold promise of a huge increase in our understanding of mammalian biology, and of human molecular

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biology. Fueled by advances from genetically engineered animals and underlying the technical questions related to human prenatal gene therapy are the largely unspoken, yet very challenging, issues surrounding the potential for human “enhancement.” However, the potential for “improving” upon nature by the insertion of a single gene is much less than some might imagine. Natural selection has already “optimized” an organism for its environment: Hundreds and thousands of interacting genes exert their effects on each trait, and single-gene alterations will usually have very little impact.

From the broadest view, prenatal gene therapy presents a complex array of ethical, legal, and societal issues. Some of the questions raised are unique to the situation (e.g., germline modification), while many others are common to other fields of human research and medical practice (e.g., informed consent). While the level of risk is currently perceived as above the acceptable limit, what is the level that must be reached before prenatal intervention would be attempted? In the field of gene therapy, the unknowns about conversion of a virus to a vector are very complex and at present not well understood. These technical issues are currently a major impediment to prenatal gene therapy.

We are not in a position to attempt prenatal gene therapy, and certainly the unknown and potential problems of risk to fetus and mother must be central among the complex questions that require additional consideration. Informed consent will be very important in the discussions of how society might proceed. The IRB has been given some responsibilities in assuring some assessment of risk/benefit decisions, and ensuring informed consent. Perhaps it may be too much to expect the individual to assume the weight of this deci-

sion. However, this is ultimately an individual decision; a mother assumes the responsibility for the fetus. In the future a greater understanding will be needed in several specific areas to determine whether or not prenatal gene therapy might be successful and would be acceptable in a clinical trial. Among the scientific/clinical areas where more knowledge is critically needed are:

- The target cell specificity for the gene delivery vector system and procedures for insertion;
- The specific cells and organs to be targeted;
- The impact of prenatal therapy on the selected disease(s);
- The role of the developing fetal immune system, and the likely need to manipulate immune tolerance;
- The potential for adverse immunologic reactions;
- The level of gene expression required;
- The effects of overexpression of a therapeutic gene;
- The most appropriate animal models for preclinical studies;
- The level of background mutation and teratogenic changes expected in a preclinical animal model, and how such background can influence results.

**Necessary ethical/legal issues include:**

- A clearer understanding of the consent process as it applies to the mother, the father, and the child who is born;
- Deliberation on the potential use of such methods for perceived “enhancements” or abuse for “eugenic” goals;
- Consideration of the broader question of whether germline alteration (inadvertent or intentional) should be even considered as a therapeutic modality because of the potential unknown long-term implications.

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# An Overview of the Development of the Human Immune System during Ontogeny

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The immune system is designed to recognize and accept the existence of domestic cells and molecules and to screen and target foreign cells and molecules as well as altered self components with the aim of neutralizing or eliminating them from the body in order to prevent disease. This task is especially difficult for the fetus. On the one hand, the fetus must lay the groundwork for the challenge that is to come when it emerges from the protection of the womb into a hostile environment. On the other hand, the cells and molecules that comprise the fetus are changing rapidly with new systems making their appearance as the fetus develops. Moreover, the fetus must take care that its immune system does not conflict with that of the mother. In order to work within these constraints, the fetus has chosen to develop its ability to respond to antigen in a controlled and programmed fashion, a process that is not completed until years after birth. The mechanisms used to control the development of the immune response include a delay in building the structures within the lymphoid organs that allow efficient processing of antigen as well as limitations in the diversity of the receptors on the surface of lymphocytes that allow the system to distinguish between “self” and “non-self.” These constraints and experimental evidence in mice that altering the immune response of the fetus can make the adult susceptible to disease must be kept in mind whenever manipulation of the fetus through gene therapy is planned.

## Introduction

The mission of the immune system is to eliminate, neutralize, or block compounds or organisms foreign to the individual, *i.e.*, “non-self” antigens, while avoiding damage or injury to those antigens that appropriately belong to the individual, *i.e.*, “self” antigens. The hazards of generating an inappropriate response can be great. At the extremes, mistaking a dangerous foreign antigen as “self” or mistaking a “self-antigen” as “non-self” can lead to death or disability. Morbidity and mortality can also result from a normal immune response to a beneficial foreign antigen, such as a medicine, a transplant, or a new gene product.

In vertebrates, B and T lymphocytes share responsibility for the identification of “self” and “non-self.” The ability to discriminate between these states is dependent on the individual’s capacity to generate a heterogeneous repertoire of antigen-binding structures, *i.e.*, immunoglobulins and T cell receptors, that are subsequently displayed on the surface of B- and T-lymphocytes.<sup>1</sup> In the B cell, immunoglobulin serves a dual role. In its secreted form, an antibody can function as a guided missile, binding directly to antigen at a distance and interdicting its function. In its cell surface form, membrane bound immunoglobulin enables the B cell itself to bind antigen. In the case of T-independent antigens, such as polysaccharides, binding of antigen to the B cell promotes growth and development of the lymphocyte into a memory cell or into a plasma cell, which can produce and secrete vast quantities of antigen-specific immunoglobulin. In the case of T-dependent antigens, such as proteins, simple recognition of the antigen by the B cell is insufficient to allow triggering of the B cell. In this case, the B cell requires a T cell partner, both of which must respond to the antigen together. Immunoglobulin enhances the ability of B cells to gather antigen, portions of which are bound to Class I and Class II Major Histocompatibility Complex (MHC) proteins. The T cell binds to antigen bound to MHC proteins on the surface of the B cell through its T cell receptor. It then sends a second signal to the B cell, such as the interaction between CD40 and CD40 ligand, which allows the B cell to progress down the developmental pathway to form either a plasma cell or a memory cell.

These delicate interactions between lymphocytes and their antigens are best performed in the appropriate environment. In a primary lymphoid organ, such as the spleen, T cells surround the arteries, monitoring both antigen presenting cells and antigens as they pass into the white pulp. In the marginal zone, mature B cells, especially those that recognize polysaccharides such as the sugars that make up the capsules of *Streptococcus pneumoniae* and *Haemophilus influenzae* and other pathogenic bacteria, lie in wait for antigen. In the lymphoid follicles, B cells responsive to antigen collect and engage in communication with T cells and follicular dendritic cells. In

the germinal centers, B cells alter their immunoglobulins through somatic mutation and through class switching, generating new effector functions and enhancing the affinity of their products.

## Development of the Lymphoid Organs

At the tissue and organ level, the development of the human immune system can be divided into five stages<sup>2</sup> (Fig. 1):

- Stage I:* Lymphocytes form unorganized aggregates of varying cellularity. They are surrounded by a well-defined capsule, but lack cortical and medullary structures.
- Stage II:* Organization of the lymphocyte populations begins. A medulla begins to appear in the form of radially distributed reticular tissue in the central portion of the lymphoid aggregate.
- Stage III:* Organization of the primary structures is completed. The medulla is well demarcated, and there is a distinctive cortex that contains primary lymphoid nodules and follicles.
- Stage IV:* Reaction centers, *e.g.*, germinal centers, appear in the primary lymphoid nodules
- Stage V:* Organization of the primary and secondary structures is complete. In the spleen, the marginal zone has matured.

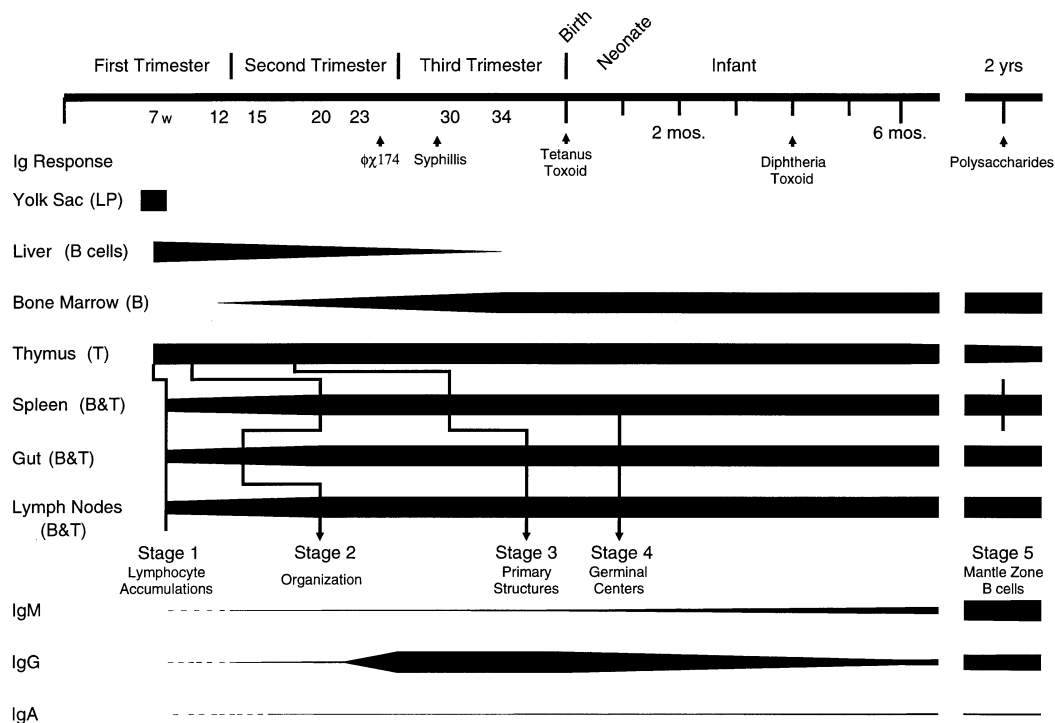
Although lymphocyte precursors can be identified in the yolk sac at 6–7 weeks gestation, lymphocytes are first be detected at 7–8 weeks gestation in the liver,<sup>3</sup> the blood,<sup>4</sup> and the thymus.<sup>5,6</sup> The liver is the primary source of B cells until the 14th week of gestation.<sup>7</sup> Progenitor B cells appear in the bone marrow by 12 weeks gestation.<sup>7</sup> After bone marrow becomes active, fetal liver B cell production begins to decline,<sup>8</sup> ceasing entirely by 34 weeks gestation.<sup>9</sup>

The thymus is the site of T cell production. The thymic primordia can first be detected at 6 weeks gestation.<sup>10</sup> Cells bearing surface proteins characteristic of T cell progenitors are first apparent in the thymus at 7 weeks gestation.<sup>6</sup> By 8 weeks, the thymus is vascularized, and by 10 weeks, the cortico-medullary junction has been established. At 14 weeks, Hassall’s corpuscles are apparent. By 18 weeks, the thymus has an appearance similar to that of a newborn, although the cortex and medulla are of equal size.

The spleen, the primary peripheral lymphoid organ, can be distinguished by 7–8 weeks gestation.<sup>10</sup> By immunofluorescence, both B and T lymphocytes can be seen in the spleen at 15 weeks of age.<sup>4</sup> Beginning at 16 weeks gestation, mature T cells can be detected in the periarteriolar sheath as well as scattered throughout the red pulp.<sup>11</sup> B cells form a loose band adjacent to the T cell region.<sup>12</sup> At 17–18 weeks gestation, follicular dendritic cells (FDC) are evident.<sup>13</sup> Primitive B cell follicles containing a dendritic meshwork formed by FDC are observed from 22 weeks gestation onwards.<sup>13</sup> By 30

FIGURE 1

*Development of the primary lymphoid organs is not complete until two years after birth.*



weeks gestation, mature follicles are present. Germinal centers do not appear until 1 month after birth.<sup>14</sup> Marginal zone B cells are fully mature at 2 years after birth.<sup>15</sup> As the marginal zone is supposed to be the site of the initiation of the immune response to polysaccharide Ag, there is a remarkable coincidence between the first appearance of marginal zone B cells with adult features and the time of acquisition of the ability to mount an immune response to polysaccharides, including encapsulated bacteria.

Development of the secondary lymphoid organs, the gut and the lymph nodes, parallels development of the spleen. In the gut, Peyer's patches and the appendix are apparent by 14 weeks gestation.<sup>10</sup> By 20 weeks gestation, mature lymphocytes can be distinguished.<sup>14</sup> Lymphoid cells are distributed as scattered cells or as small aggregates.<sup>11</sup> Secretory component (SC) becomes readily apparent at 29 weeks of gestation.<sup>16</sup> By 30 weeks gestation, eosinophils are present, and the proportion of lymphocytes is larger. T cells become readily detectable at 32 weeks gestation.<sup>16</sup> Peyer's patches contain well developed follicles by 36 weeks gestation.<sup>14</sup> IgA bearing plasma cells are detected 2 weeks after birth,<sup>16</sup> but are not common until 1 month after birth.<sup>14</sup> Germinal centers can also be detected at this age.<sup>14</sup>

Early lymph nodes can be found as early as 8 weeks gestation in the cervical region, along the posterior wall of the thorax, and in the retroperitoneal region.<sup>10</sup> The

early node is a cellular reticulum interspersed with free cells and blood vessels.<sup>17</sup> By 10 weeks, peribronchial, mediastinal, celiac, pelvic, axillary, inguinal, and popliteal nodes are seen.<sup>10</sup> By 16 weeks gestation, both B and T cells can be detected, but they have not separated into their characteristic areas.<sup>12</sup> By 18 weeks gestation, the lymph nodes are well defined and contain mature lymphocytes, but lack follicles and germinal centers. Over the next four weeks, both T cell areas and tight B cell clusters of primary nodules undergo expansion. By 30 weeks gestation, a hilus, trabeculae, sinusoids, and a peripheral sinus are readily apparent. Follicles are seen in the last month of gestation. Germinal centers begin to appear at one month after birth.<sup>14</sup>

Mononuclear cells can be seen in the tonsils at 14 weeks gestation.<sup>10</sup> Mature lymphocytes are detected at 18 weeks. In the seventh fetal month, the keratinized epithelium forms crypts that serve to divide the lymphoid tissue. Primary follicles are found at 28–30 weeks gestation. Germinal centers develop by the first month after birth. The appearance of plasma cells is delayed until 3–6 months after birth.<sup>14</sup>

It also takes time to reach adult concentrations of immunoglobulin in the blood. Unless challenged by infection, the fetus produces little endogenous antibody. Active transport of maternal IgG across the human placenta begins around 20–21 weeks gestation,<sup>18</sup> but does not reach significant levels until 27–28 weeks gestation. At

FIGURE 2

Two dimensional model of an immunoglobulin molecule containing three constant domains. The top dimer demonstrates the nucleotide structure of the H and L chains, the bottom dimer illustrates the structure of the protein.

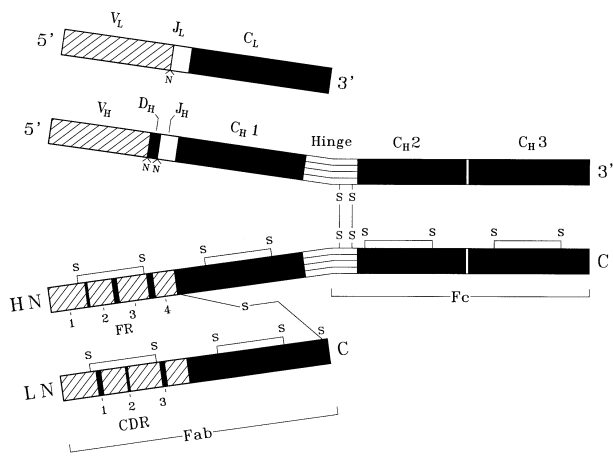
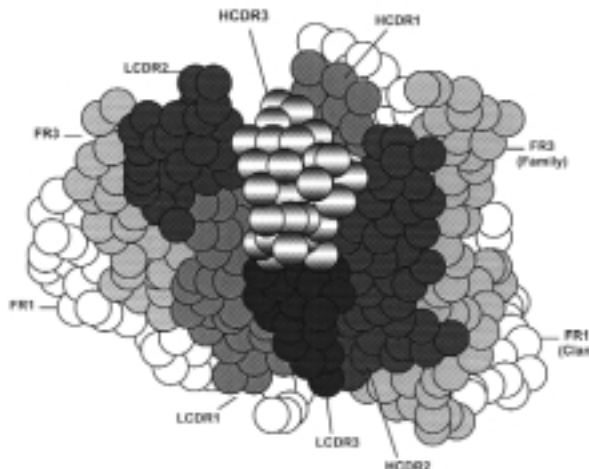


FIGURE 3

The antigen binding site is created by the juxtaposition of the three complementarity determining regions (CDRs) of the H chain and the three CDRs of the light chain. The view is looking into the binding site as an antigen would see the CDRs. The V<sub>H</sub> domain is on the right side. The central location of the CDR3 intervals is readily apparent.



birth, serum IgG levels are comparable to the adult, but then begin to decay as the maternal IgG is catabolized, reaching a nadir at 6 months after birth. Adult levels of IgM are reached by the second year of life, IgG by the sixth year and IgA by the 15th year.<sup>19</sup>

### Immunoglobulin Gene Organization and Structure

In the fetal liver and bone marrow, and in the thymus, B and T lymphocytes generate receptors capable of recognizing a broad range of antigens. The strategies used to generate these receptors were initially dissected in B cells, and will be the focus of this review. At the protein level, immunoglobulins have been shown to be heterodimeric proteins consisting of two heavy (H) and two light (L) chains (Fig. 2).<sup>20</sup> Each H and L chain, like the intact antibody molecule, can be divided structurally and functionally into two domains, one highly variable (V), and one relatively constant (C). The diverse V domain is encoded by the first 110 to 120 amino acids. The remaining half of the L chain and three-quarters of the H chain are termed constant or C domains because their structure is virtually the same for all molecules belonging to a single immunoglobulin class or subclass. These C domains specify effector function, such as complement activation or binding to Fc receptors, adapter proteins that enable other cells to use immunoglobulins as receptors or effector proteins.

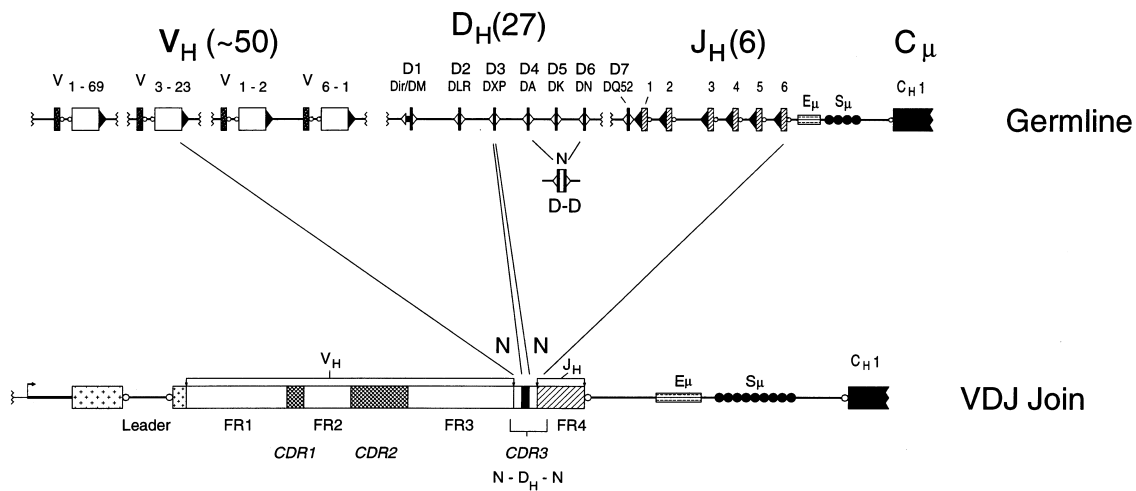
Each V domain can be divided into three hypervariable intervals, termed complementarity determining regions or CDRs, which encode peptide loops that con-

nect the anti-parallel strands of the  $\beta$  pleated sheet structures that form the core of the V domain. The six CDR loops of the H and L chain are juxtaposed to form the antigen binding site, as classically defined (Fig. 3). The three CDR intervals are separated from each other by four framework (FR) intervals that contain relatively conserved sequence.<sup>21,22</sup> These framework regions encode the  $\beta$  pleated sheet scaffolds that support the CDRs and help hold the V<sub>H</sub> and V<sub>L</sub> domains together.

H chains and the two types of L chains,  $\kappa$  and  $\lambda$ , are each encoded by a separate multigene family.<sup>23,24</sup> For each of these chains, more than one gene is required to code for a single immunoglobulin polypeptide.<sup>25</sup> For example, a  $\kappa$  variable domain is created by the joining of two discrete gene segments (V $\kappa$  and J $\kappa$ ), each of which is flanked by a recombination signal sequence (RSS).

Enumerating only those gene segments known to be active, the human  $\kappa$  locus on chromosome 2 contains five J $\kappa$ , 28 V $\kappa$ , and a single C $\kappa$  gene segment.<sup>26</sup> The V $\kappa$  gene segments can be grouped into six different families comprised of gene segments that share greater than 80% sequence similarity with each other.<sup>27</sup> The human  $\lambda$  locus, on chromosome 22, contains four functional C $\lambda$  constant domains two of which are identical in sequence. Each C $\lambda$  gene is accompanied by its own J $\lambda$ , reducing functional diversity to only three J $\lambda$ -C $\lambda$  genes.<sup>28</sup> There are 36 potentially active V $\lambda$  gene segments belonging to ten families and five clans.<sup>29,30</sup> Intriguingly, three of these  $\lambda$  gene segments contribute to more than 50% of all  $\lambda$  light chains.<sup>31</sup>

FIGURE 4  
Generation of an immunoglobulin H chain variable domain.



The human H chain locus, on chromosome 14, contains ~50  $V_H$ , ~27  $D_H$  (D for diversity), and six  $J_H$  gene segments which actively contribute to the repertoire<sup>32</sup> (Fig. 4). The  $D_H$  gene segments can be grouped into seven different families, numbered D1 through D7.<sup>33</sup> The  $V_H$  gene segments can be grouped into seven families (reviewed in<sup>22</sup>). The D7 gene segment, also known as  $D_HQ52$ , is located immediately adjacent to  $J_H1$ . The other  $D_H$  gene segments are located on four ~9 kb repeating structures, each of which contains at least one representative of each of the remaining six families. In the final V domains,  $V_H$  and  $V_L$  gene segments contribute FR1, 2, and 3, CDR1 and 2, and the amino terminal portion of CDR3; the  $D_H$  gene segment forms the middle of HCDR3; and the  $J_H$  and  $J_L$  elements encode the carboxy terminus of CDR3 and FR4 in its entirety (Fig. 4).

Several mechanisms operate to make the third complementarity determining region of the H chain, HCDR3, the most diverse portion of the immunoglobulin molecule. First,  $D_H$  gene segments, which form the center of HCDR3, can rearrange by either inversion or deletion, and the  $D_H$  can be joined to the  $J_H$  in any one of three different reading frames. Thus, each  $D_H$  gene segment effectively encodes six different peptide fragments. Second, there is flexibility in the site of gene segment joining. Third, non-germline encoded nucleotides (N regions) can be inserted between the V and the D, between Ds in D-D rearrangements, and between the D and the J. The random insertion of N nucleotides is attributed to the action of terminal deoxynucleotidyl transferase (TdT).<sup>34</sup> Note that each additional codon created by N region addition multiplies the potential diversity of the repertoire by a factor of twenty.

Located downstream of the human  $VDJ C_\mu$  locus are eight additional C regions ( $C_\delta$ ,  $C_{\gamma_{1,2,3,4}}$ ,  $C_{\alpha_{1,2}}$ , and  $C_\epsilon$ ).

Each of these  $C_H$  genes, except for  $C_\delta$ , is preceded by a region of repetitive DNA termed the switch (S). Through recombination between the  $C_\mu$  switch region and the switch region of one of the other H chain genes, the same VDJ heavy chain variable domain can be juxtaposed to any of the H chain classes.<sup>32</sup> In this way the B cell can tailor both the receptor and the effector ends of the antibody molecule to meet a specific need.

Maturation of the primary response is typically associated with the appearance of higher affinity antibody variants.<sup>35</sup> With T-cell help, the variable domain genes of germinal center lymphocytes can undergo somatic mutation at a rate of up to  $10^{-3}$  changes per base-pair per cell cycle.<sup>36,37</sup> This final mechanism of antibody diversification allows affinity maturation of the antibody repertoire in response to repeated immunization or exposure to antigen. Together, these mechanisms contribute to an antibody repertoire that is theoretically limited only by the total number of B cells present.

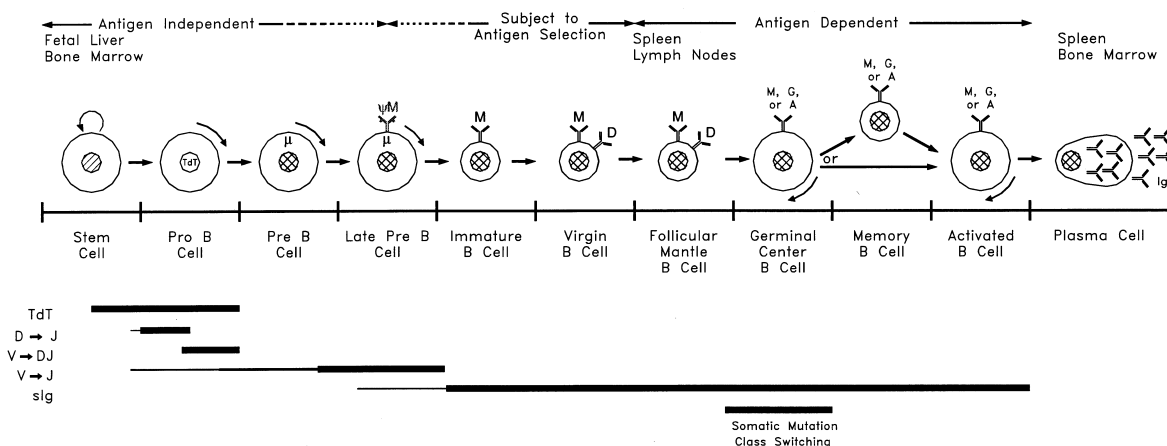
### B Cell Development and the Generation of the Antibody Repertoire

The complexity of the system can best be understood by correlating the molecular events with the development of the B lymphocyte (Fig. 5).<sup>38</sup> In most cases, rearrangement begins in the H chain locus. A  $D_H$  gene segment is joined to a  $J_H$ ; followed by a juxtaposition of a  $V_H$  to the DJ intermediate. Only one in three possible splices will create a VDJ join with  $V_H$  and  $J_H$  in the same reading frame. Failure to generate a translatable H chain on the first chromosome is followed by attempted rearrangement on the second. Subsequent rearrangement of a  $\kappa$  or  $\lambda$  L chain allows expression of a fully functional IgM on the cell surface, which defines the immature B lymphocyte. Alternate splicing of the V domain to the  $C_H1$  exon



FIGURE 5

*B cell development. Shown below the cartoon depicting B cell differentiation are bars depicting the various processes associated with immunoglobulin rearrangement.*



of either the  $C\mu$  or  $C\delta$  genes allows the mature B lymphocyte to express both IgM and IgD on the cell surface. At this stage, exposure to antigen in conjunction with T-cell help can result in class switching and/or differentiation into a plasma cell that can secrete massive quantities of antibody.

**The fetus and infant do not respond to antigen in the same way as an adult.** The ability to respond to specific antigens develops slowly, in a controlled, stepwise fashion during the life of the fetus, the infant, and the child. For the fetus especially, issues of “self” and “non-self” are complicated. During ontogeny, novel self-antigens are expressed, embryonic antigens are suppressed, and the fetal and maternal immune systems must engage in peaceful coexistence.

Initially it was thought that the fetus was an immunologic “null,” unable to respond to foreign antigen. In the 1960s, however, it was shown that humans could respond to *in utero* infection with syphilis and toxoplasmosis by the 29th week of gestation.<sup>39</sup> Premature infants weighing as little as 1500 grams (~6 months gestation) were able to respond to vaccination with bacteriophage  $\phi\chi 174$ .<sup>40</sup>

Controversial studies even indicated that infants might be able to respond to some antigens, such as tetanus toxoid, that were given to mothers in the form of a vaccine when the infants were as young as 23 weeks of gestation.<sup>41,42</sup> Long term follow-up of these babies revealed that the antibody response to booster immunization did not differ from control infants. Trans-placental immunization with tetanus toxoid appeared to stimulate the immune system into forming antibody producing cells, but this type of immunization did not appear to induce the formation of long lived memory B cells or to induce tolerance.<sup>43</sup> Intriguingly, this type of

prenatal immunization could not be detected in American women challenged in the same way, raising the possibility that other environmental influences can affect the immune response.<sup>44</sup>

For many other antigens, including many other vaccines, the ability to respond was delayed until beyond the time of birth.<sup>45</sup> For example, children under the age of two are often unable to respond to unmodified bacterial polysaccharide vaccines.<sup>45,46</sup>

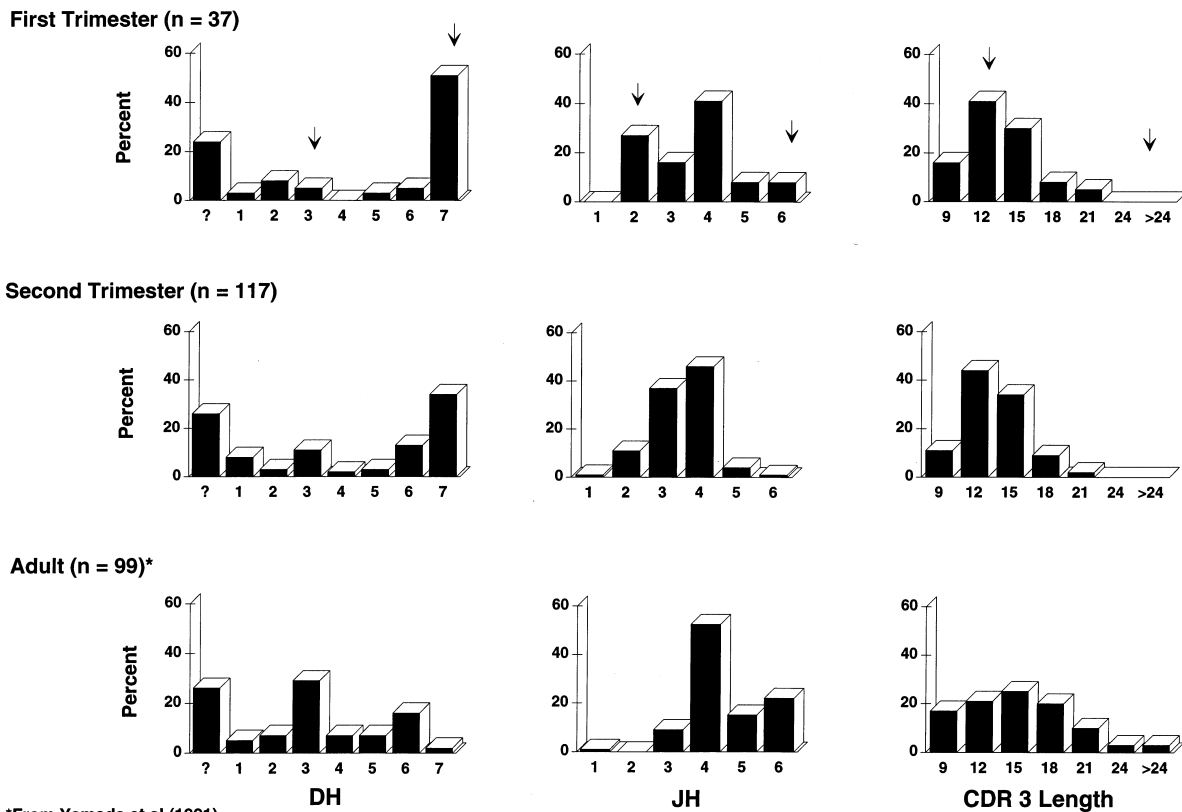
The development of the ability to respond to a given antigen is not random. Studies in sheep<sup>47</sup> and mice<sup>48,49</sup> showed that it was possible to predict when in development the fetus and neonate would respond to specific antigens, and that many mammals appeared to follow a similar developmental program. For example, the response to bacteriophage  $\phi\chi 174$  develops early in sheep, mice and man, whereas both sheep and man are unable to mount a humoral response to diphtheria toxoid until after birth.<sup>39</sup>

**The diversity of the antibody repertoire also develops in a controlled, stepwise fashion.** At first glance, a restriction in the ability to respond to specific antigens seems paradoxical, given that antigen receptor repertoires appear to be generated at random. This paradox was in part resolved when it was shown that immunoglobulin variable gene utilization and HCDR3 diversity also follow an ordered developmental program [reviewed in note 50]. (It should be noted that that restrictions in the T cell receptor repertoire<sup>51</sup> and in the acquisition of mechanisms of antigen presentation and processing also appear to play a role in the delay in responding to specific antigens during ontogeny).

Initial studies showing skewing of the fetal and neonatal repertoire focused on mouse models. Early in

FIGURE 6

HCDR3 diversity during human ontogeny; Top Row: first trimester fetal liver VD<sub>H</sub>JC<sub>μ</sub><sup>+</sup> transcripts. Middle Row: second trimester fetal liver and bone marrow VD<sub>H</sub>JC<sub>μ</sub><sup>+</sup> transcripts. Bottom Row: adult blood VD<sub>H</sub>JC<sub>μ</sub><sup>+</sup> transcripts from the published work of Yamada et al.<sup>67</sup> Left Column: percentage of transcripts that utilize members of the designated D<sub>H</sub> families. A “?” designates transcripts where the D<sub>H</sub> family cannot be determined. The D7 (DQ52) gene segment contributed to 19 of 37 first trimester, 40 of 117 second trimester, and 2 of 99 adult VD<sub>H</sub>JC<sub>μ</sub> transcripts; whereas D3 (DXP) gene segments contributed to 2 of 37, 13 of 117, and 29 of 99 transcripts, respectively ( $p < 0.0001 \chi^2$ ). Middle Column: percentage of transcripts that utilize the designated J<sub>H</sub> gene segment. For each developmental stage, the number of transcripts analyzed is indicated by the value of “n”. Right Column: distribution of the lengths of the CDR 3 intervals of the transcripts [residues 93–102<sup>21,22</sup> divided into 3 residue intervals [e.g., ≤ 9, 10–12, 13–15, 16–18, 19–21, 22–24, and > 24 codons]. The arrows are intended to emphasize the major differences between fetal and adult transcripts.



ontogeny, antibodies were enriched for a small subset of V<sub>H</sub> gene segments belonging to one of 13 V<sub>H</sub> families, the V<sub>H</sub>7182 family.<sup>52,53</sup> In contrast, the V<sub>H</sub>J558 family, which makes up more than 50% of the adult repertoire, contributed little to the repertoire of the fetus.<sup>54,55</sup> HCDR3 diversity was also restricted due to an absence of N addition, forcing use of only germline sequence.<sup>56,57</sup>

These observations would suggest that both species are able to generate similar antigen binding sites at the earliest stages of ontogeny capable of generating a plastic, polyreactive antibody repertoire that can provide a low affinity defense against a wide array of antigens.<sup>58</sup>

This restricted, germline, neonatal repertoire, although unable to respond to some foreign antigens, contains many multi-reactive antibodies that exhibit low affinity binding to many self antigens.<sup>59–61</sup> It has been suggested that this neonatal repertoire of polyreactive

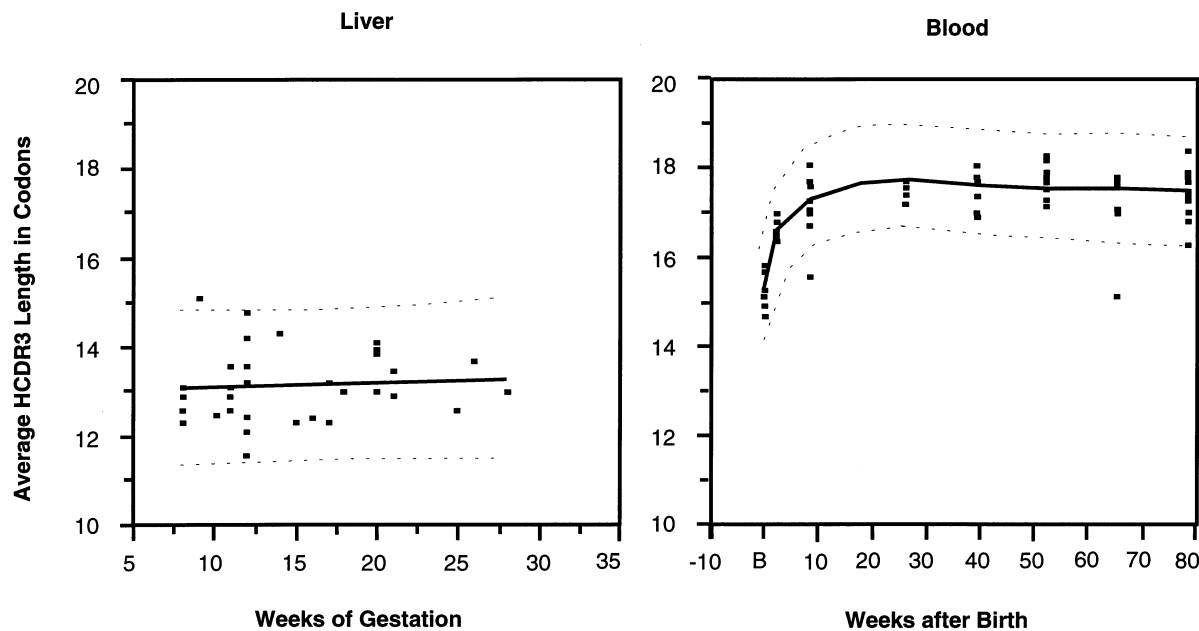
antibodies is the foundation of a “natural” IgM repertoire that may serve as a first line of defense against foreign antigens in general.<sup>58</sup>

Support for the view that the neonatal repertoire plays a unique role in the development of the immune response comes from studies that have manipulated this repertoire. These studies have shown that alteration of the neonatal repertoire can have long-lasting effects. For example, treatment of neonatal mice with antibodies directed against some of these germline encoded antigen binding sites, and hence loss of the B cell that express them, results in the loss of the ability of the **adult** to respond appropriately to some thymus independent antigens, such as phosphorylcholine (PC)<sup>62</sup> and alpha 1-3 dextran (DEX).<sup>63</sup>

There are marked parallels between the human and the mouse fetal repertoires. Both contain antibodies of similar

FIGURE 7

*HCDR3 length distribution during human ontogeny. Shown are average HCDR3 lengths in VDJC $\mu$  transcripts from 34 fetal liver samples ranging in age from 8 to 28 weeks gestation and 72 samples of cord blood and peripheral blood from infants ranging in age from birth to eighteen months of age. The dotted lines denote 5% and 95% confidence limits for the distribution of lengths over time.*



structure and sequence. Both are enriched for polyreactive, self-reactive immunoglobulins [reviewed in note 64].

The human germline  $V_H$  repertoire is considerably smaller and less diverse than the mouse  $V_H$  repertoire, and unlike the mouse, many of the sequences used in fetal life continue to be used at high frequency in the adult.<sup>64</sup> Among these preferentially expressed sequences are gene segments that show a high degree of sequence identity to mouse, frog, and even shark  $V_H$  genes, indicating evolutionary pressure to maintain a desirable protein sequence.<sup>22</sup> One sequence in particular is worthy of mention. The human V3-23 gene segment shares greater than 80% identity at the nucleotide and peptide level with a mouse sequence,  $V_H283$ , a level of identity that is greater than that shared between the “constant” regions of human and mouse IgM. Both share similar framework scaffolds, and both have similar sequence in the CDRs. The similarity in sequence of V gene segments used in fetal life may help explain why both fetal mice and premature human infants are able to respond to similar types of antigens, such as bacteriophage  $\phi\chi174$ .

Perhaps in compensation for the diminished complexity of the germline  $V_H$  repertoire, the diversity of the HCDR3 interval in the human greatly exceeds that of the mouse. At the germline level, humans have two more  $J_H$  gene segments, they have twice as many  $D_H$  gene segments, and these  $D_H$  gene segments are considerably more diverse. At the somatic level, human fetal antibodies, unlike mouse, contain N additions. In the

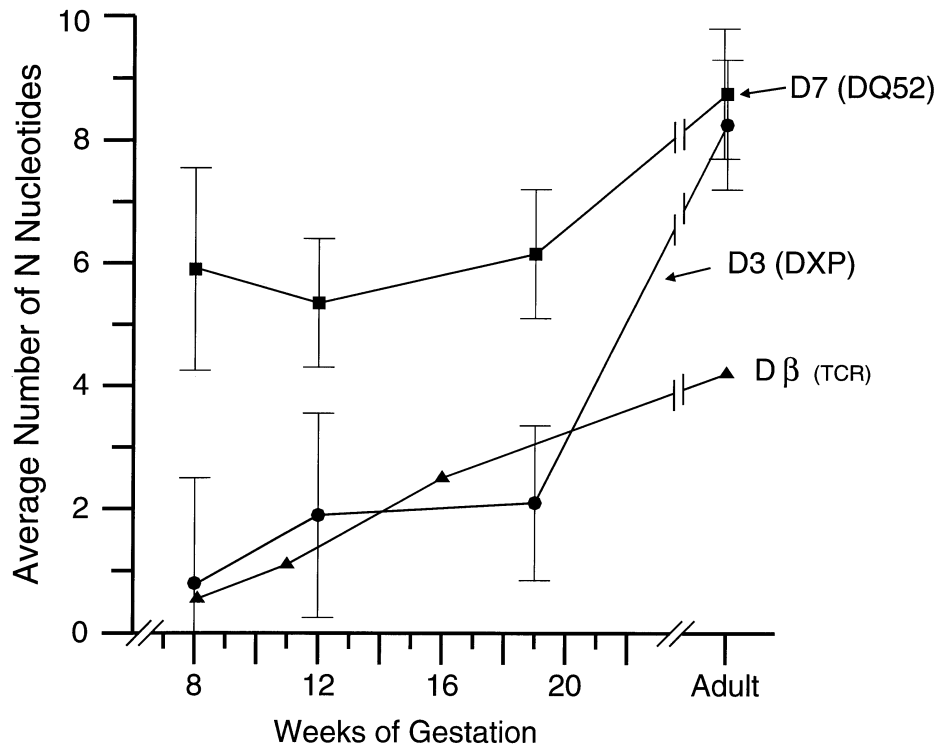
adult, the longest human HCDR3 intervals are more than six codons longer than the longest mouse sequences,<sup>65,66</sup> generating novel antigen binding sites not seen in the mouse.

This emphasis on the diversity of the HCDR3 interval is reflected in the differences seen between the composition of HCDR3 in the fetus versus the adult. Unlike mouse, in the human,  $D_H$  and  $J_H$  utilization differs greatly between the fetus and the adult. For example, the single member of the D7 (DQ52) family, D7-27, contributed to one-half of first trimester, one-third of second trimester, but only one-fiftieth of the adult HCDR3 intervals examined (Fig. 6), whereas the five members of the D3 (DXP) family (D3-3, D3-9, D3-10, D3-16, and D3-22) contributed to one-twentieth of first trimester, one-tenth of second trimester, and nearly one-third of adult sequences. Similarly, among VDJC $\mu$  transcripts,  $J_H4$  was the most commonly used  $J_H$  gene segment at all stages of ontogeny. However,  $J_H2$  was the second most commonly utilized  $J_H$  gene segment among first trimester transcripts,  $J_H3$  was the second most common among second trimester transcripts, and  $J_H6$  was the second most common among adult transcripts.<sup>66</sup>

The average length of HCDR3 length distributions also differed between fetus and adult. By sequence analysis, the average length of HCDR3 was approximately 12 codons in the fetus, and 18 codons in the adult. In order to determine when the length distribution matured, we used an RT-PCR assay to amplify the HCDR3 intervals

FIGURE 8

Patterns of N addition during human ontogeny. Distribution of the extent of N addition in D7- and D3-containing DJ transcripts from normal fetal and adult B lineage cells,<sup>66</sup> and from TCR DJ transcripts from fetal and adult thymus.<sup>51</sup>



from fetal and infant samples. We found that the average HCDR3 length distribution was relatively unchanged in fetal liver of 8 to 28 weeks gestation, was longer, but still immature, in the cord blood, and reached a steady state “adult” average at two months of age (Fig. 7).<sup>66</sup>

It might be presumed that the differential use of  $D_H$  and  $J_H$  gene segments lay behind the change in HCDR3 length between fetus and adult. D3 gene segments are 31 nucleotides long, whereas the D7 gene segment is only 11 nucleotides long. Similarly,  $J_H6$  can encode five more codons than  $J_H4$ . Thus, the absence of long HCDR3 intervals could be attributed to the rare use of long  $D_H$  gene segments, such as D3 and  $J_H6$ , in the fetus. However, these gene segments are used in fetal life, albeit at a lower frequency than in the adult. It might be expected, therefore, that the lengths of HCDR3s that use these longer gene segments would approximate the distribution seen in the adult. Instead, the average length of the CDR3 intervals of fetal D3-containing VDJ transcripts proved nearly identical to those that contained D7.<sup>66</sup>

In order to determine whether genetic or somatic mechanisms underlay this control of HCDR3 length, and thus the structure and diversity of fetal antibodies, we examined DJ rearrangements, the incomplete products of immunoglobulin recombination. Because these

intermediate products are not subject to selection by antigen, DJ rearrangements yield insight into the recombination process. We found that in the fetus most D3-J rearrangements lacked N addition between D and J, whereas D7-J rearrangements had extensive N addition between D and J. In the adult, D7-J and D3-J rearrangements contained a similar quantity of N addition between D and J.

The paucity of N addition in D3-containing DJ joins helped explain why D3-containing HCDR3 intervals were so short in the fetus. A re-analysis of the VDJ sequences revealed that D7-containing VDJ sequences require N addition in order to reach an average length of 12 codons. Thus, HCDR3 intervals are genetically prejudiced to contain certain lengths and structures during fetal life.

However, even though there is a genetic bias towards certain lengths, it is still possible to generate longer HCDR3 interval in the fetus through use of longer  $J_H$  gene segments. Moreover, N addition was present between V and D in both D3 and D7-containing VDJ joins. We then examined the distribution of HCDR3 lengths in fetal and adult bone marrow as a function of B cell development.<sup>66</sup> We found that longer HCDR3 intervals could be detected in pre-B cells, but were no longer apparent in mature B cells,

suggesting that cells expressing these more adult-like antibodies had been selected against at the time of their generation.

### Summary

The ability of the fetus, the infant, and the child to respond to antigen develops in a controlled, programmed fashion. Maturation of the various cellular compartments of the immune system is not complete until the child is two years of age, and immunoglobulins in the blood do not achieve final adult concentrations until puberty. The fetus and the infant express an immature immune response that places it at risk for infection from a variety of organisms, and also makes it tolerant to intervention with exogenous antigen. At present, we cannot predict which antigens will or will not generate an immune response in a fetus, or when that response will appear for a given antigen.

Based upon our present knowledge, we may conclude that in human, as in mouse, diversification of the antibody repertoire appears to follow a strict developmental program. Both genetic and somatic mechanisms are used to restrict the composition of the repertoire. The antibody products of this process are typically of low affinity, but are also multi-reactive. The role of this early repertoire remains unclear, but it appears to be an important one that has been preserved across evolution. In the mouse, experimental elimination of subsets of the neonatal repertoire can alter the immune response of the adult. It is said that the child is the father to the man. It may be that the same holds true for the immune response. If so, vaccination or exposure to certain foreign antigens or infections could contribute to morbidity and mortality in the adult. The possibility of delayed consequences to fetal interventions should be held in mind whenever manipulation of the fetal environment is considered.

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# The Developmental Immunology of Human Fetal and Neonatal T Cells

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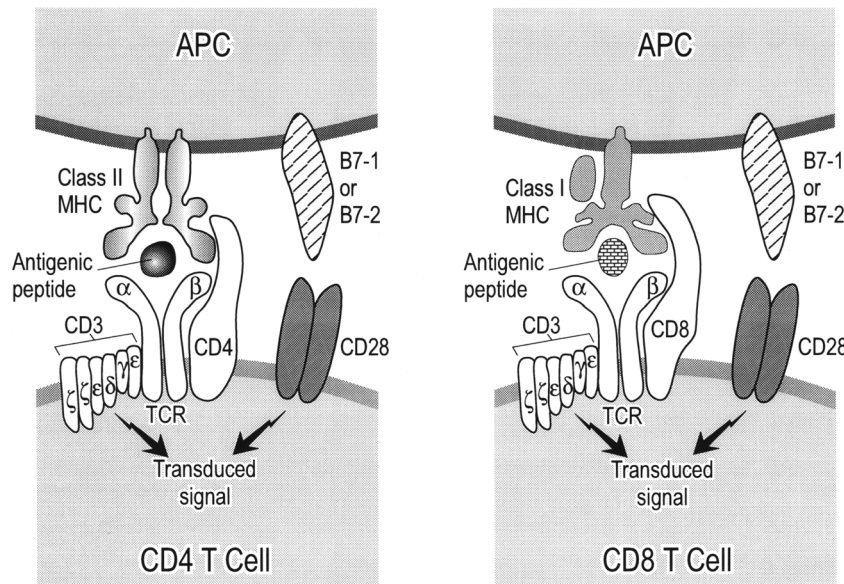
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T cell function of the fetus and neonate, including cell-mediated cytotoxicity, participation in delayed-type hypersensitivity, and help for B cell differentiation, appears to be reduced compared to that of the adult. In vitro studies suggest that selective decreases in T cell production of cytokines, such as CD40-ligand, may contribute to this decreased function. Limitations in the available repertoire of  $\alpha\beta$ -T cell receptors are unlikely to limit immune responses by the fetus from mid-gestation onward, although it is possible they could during earlier development. Following fetal or neonatal infection, the acquisition of detectable T cell-dependent antigen-specific responses is often reduced or delayed in its appearance. The cellular and molecular mechanisms responsible for these decreased and/or delayed responses remain to be defined, but preliminary in vitro results raise the possibility that limitations in dendritic cell function and/or the activation and differentiation of antigenically naïve T cells into effector or memory T cells may be contributory. Despite these limitations in T cell function, many congenitally infected infants are ultimately able to mount antigen-specific T cell responses. This indicates that in most cases there is no durable antigen-specific non-responsiveness following fetal antigen exposure, at least in the context of infection. This suggests that immunogenicity rather than long-term tolerance is the more likely outcome following fetal exposure to foreign antigens, particularly if adequate co-stimulatory signals are present. This could potentially limit the efficacy of some forms of human pre-natal gene therapy, particularly with post-natal aging, when levels of T cell immune function become similar to those of immunocompetent adults.



FIGURE 1

*T cell recognition of antigen and activation. The  $\alpha\beta$ -T cell receptor (TCR) recognizes antigen presented by the antigen presenting cell (APC) in the form of antigenic peptides bound to MHC molecules on the APC surface. Most CD4 T cells recognize peptides bound to class II MHC, while most CD8 T cells recognize peptides bound to class I MHC. This MHC restriction is the result of a thymic selection process, and is due, in part, to an intrinsic affinity of the CD4 and CD8 molecules for the class II and class I MHC molecules, respectively. Once antigen is recognized, the CD3 protein complex, which is invariably associated with the  $\alpha\beta$ -TCR, acts as docking site for tyrosine kinases that transmit activating intracellular signals. Interaction of the T cell CD28 molecule with either B7-1 or B7-2 provides an important co-stimulatory signal to the T cell leading to complete activation, rather than functional inactivation (anergy).*



### Overview of T Cells and Their Immune Functions

T cells bear a highly diverse repertoire of antigen-specific T cell receptors (TCR). TCRs are invariably associated with the CD3 complex, a monomorphic group of proteins that transmit activating signals to the T cell interior following TCR engagement by antigen (Fig. 1). The TCR is a heterodimer consisting of either  $\alpha$  and  $\beta$  chains or of  $\gamma$  and  $\delta$  chains, defining the two major T cell lineages of alpha/beta and gamma/delta cells, respectively. This review will focus on the development and function in the human fetus and neonate of alpha/beta T cells, the predominant T cell lineage in the thymus, peripheral lymphoid organs and circulation. Developmental aspects of human gamma/delta T cells, which are distinct from alpha/beta T cells in their antigen recognition, effector function, and tissue distribution will not be discussed here but have been recently reviewed.<sup>1</sup>

Alpha/beta T cells can be further divided into two major subsets of CD4 and CD8 T cells which predominantly recognize peptide antigens bound to the grooves of either class II or class I MHC molecules, respectively, displayed on antigen presenting cells (APC) (Fig. 1). Most class II MHC-associated peptides are derived from proteins that are internalized by the APC from its own cell membrane or the extracellular space, while class I

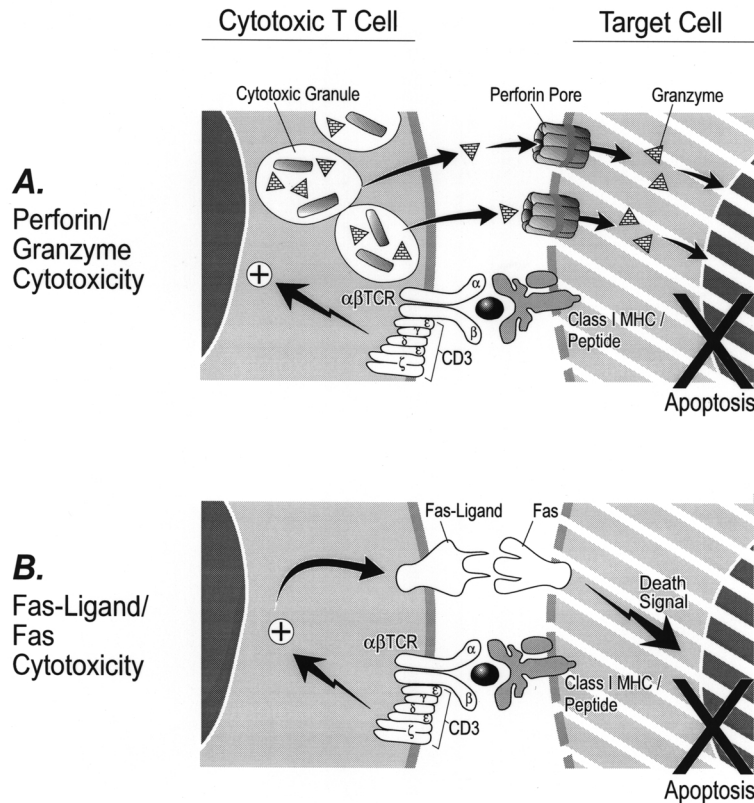
MHC-associated peptides are mainly derived from proteins that are synthesized *de novo* within the APC.

Alpha/beta T cell activation results in expansion and differentiation into effector cells, which regulate multiple aspects of the immune response via their *de novo* production of cytokines and cell-mediated cytotoxic mechanisms. The CD4 subset of alpha/beta T cells is a particularly important source of cytokines. These CD4 T cell-derived cytokines have diverse effects, including enhancing antigen presentation by dendritic cells, promoting B cell activation, memory B and T cell generation, and immunoglobulin isotype switching, and increasing mononuclear phagocyte microbicidal activity. Class II MHC molecules are normally expressed by "professional" APC, such as dendritic cells, mononuclear phagocytes, and B cells, all of which can potentially activate CD4 T cells. Of these, dendritic cells appear to be critical for effective activation of antigenically naive CD4 T cells.

The CD8 subset of effector alpha/beta T cells is important for killing host cells that bear antigenic peptide derived from intracellular pathogens, such as viruses, thus helping to limit the spread of pathogens within the tissues. Cytotoxicity is mediated by the induction of apoptosis of the target cell. This can occur via the secretion of granzyme/perforin proteins by the cytotoxic T

FIGURE 2

Two major mechanisms of antigen-specific class I MHC-restricted T cell-mediated cytotoxicity. Engagement of  $\alpha\beta$ -TCR of CD8 T cells by antigenic peptide bound to class I MHC on the target cell leads to T cell activation and target cell death. A. Cytotoxicity may occur by the extracellular release of the contents of cytotoxic granules from the T cell, including perforins and granzymes. Perforins introduce pores by which granzymes can enter into the target cell leading to the triggering of apoptosis and cell death. B. Activation of T cells results in their surface expression of fas-ligand which engages fas on the target cell, resulting in the delivery of death signal culminating in apoptosis.



cell or by engagement of fas on the target cell by fas-ligand on the cytotoxic T cell (Fig. 2). Class I MHC molecules and associated antigen processing molecules are almost ubiquitously expressed, thus allowing most infected cell types to act as APC and be killed by CD8 T cell-mediated cytotoxicity.

### Generation of the $\alpha\beta$ -TCR Repertoire

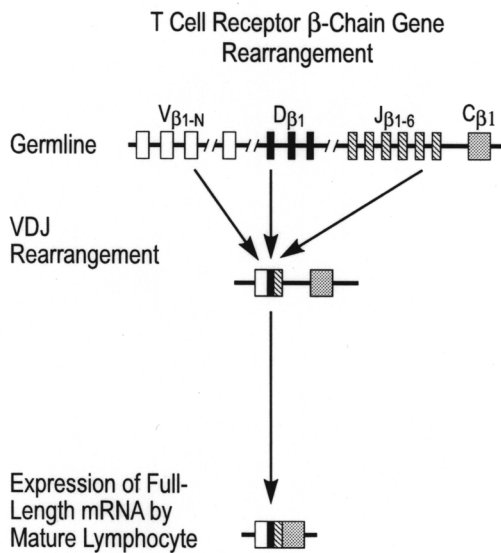
The amino-terminal portion of the TCR- $\alpha$  and - $\beta$  chains is variable and is involved in antigen recognition, while the carboxy-terminal region of each chain is monomorphic or constant (C). The variable portion of the TCR is generated as a result of TCR gene rearrangement of variable (V), diversity (D), and joining (J) segments for the TCR- $\beta$  chain gene, and V and J segments for the TCR- $\alpha$  chain gene. This results in the contiguity of the V(D)J segments within these genetic loci so that they can be transcribed (Fig. 3). This rearrangement process is analogous to that used for the generation of functional immunoglobulin

genes, which is described in detail in the article by Schroeder in this volume.

Differentiation of alpha/beta T-lineage cells begins when the prothymocyte, a bone marrow- or fetal liver-derived cell, enters the subcapsular region of the thymus from the circulation (Fig. 4). The prothymocyte expresses the CD7 surface protein but lacks most of the surface proteins that are characteristic of peripheral T cells, such as CD3, CD4, and CD8. Based on animal studies, the thymus does not appear to have a population of self-replenishing stem cells and probably requires a continual input of prothymocytes to maintain thymocytopoiesis.<sup>2</sup> The thymocyte microenvironment somehow triggers the TCR rearrangement process mediated by the products of recombination activating genes (RAG)-1 and RAG-2 and associated proteins. The TCR- $\beta$  chain gene in its unrearranged state consists of 685 kilobases of DNA on human chromosome 7 and includes 46 potentially functional V gene segments located upstream of two C regions, each associated with one D and six J segments

FIGURE 3

Functional T cell receptor genes are formed by rearrangement in immature lymphocytes. The TCR- $\beta$  chain gene is shown as an example. A similar process is involved with rearrangement of the TCR- $\alpha$ , - $\gamma$  and - $\delta$  chain genes, as well as the immunoglobulin genes. Rearrangement involves the joining of dispersed segments of V (variable), D (diversity), and J (joining) gene segments with the deletion of intervening DNA. This allows expression of a full-length mRNA transcript that can be translated into a functional protein, provided that there are no premature translational stop codons.



(Fig. 3).<sup>3</sup> The D segment first rearranges to a downstream J segment, with the deletion of intervening DNA. This is followed by rearrangement of a V segment to the DJ segment, resulting in a contiguous (VDJC) chain gene segment. If this segment lacks premature translation stop codons, the TCR- $\beta$  chain protein may be expressed on the thymocyte surface in association with a pre-TCR- $\alpha$  chain protein and the CD3 signaling complex.<sup>4</sup> This complex instructs the thymocyte to increase its surface expression of CD4 and CD8, to start rearrangement of the TCR- $\alpha$  chain gene, and to stop rearrangement of the other TCR- $\beta$  chain allele (allelic exclusion). Rearrangement of the TCR- $\alpha$  chain gene then occurs, and involves the joining of V segments directly to J segments, without intervening D segments. Thymocytes in which rearrangement of the TCR- $\beta$  and TCR- $\alpha$  chain genes are both productive (i.e., able to be expressed as full-length protein) express  $\alpha\beta$ -TCR protein on the cell surface in association with CD3, and include the precursors of peripheral alpha/beta T cells.

TCR diversity is generated by the largely random use of V, (D), and J segments in assembling the TCR- $\alpha$  and TCR- $\beta$  chain genes.<sup>5</sup> As in the case of immunoglobulins, the CDR3 region, where the distal portion of the V segment joins the (D)J segment, is a particularly impor-

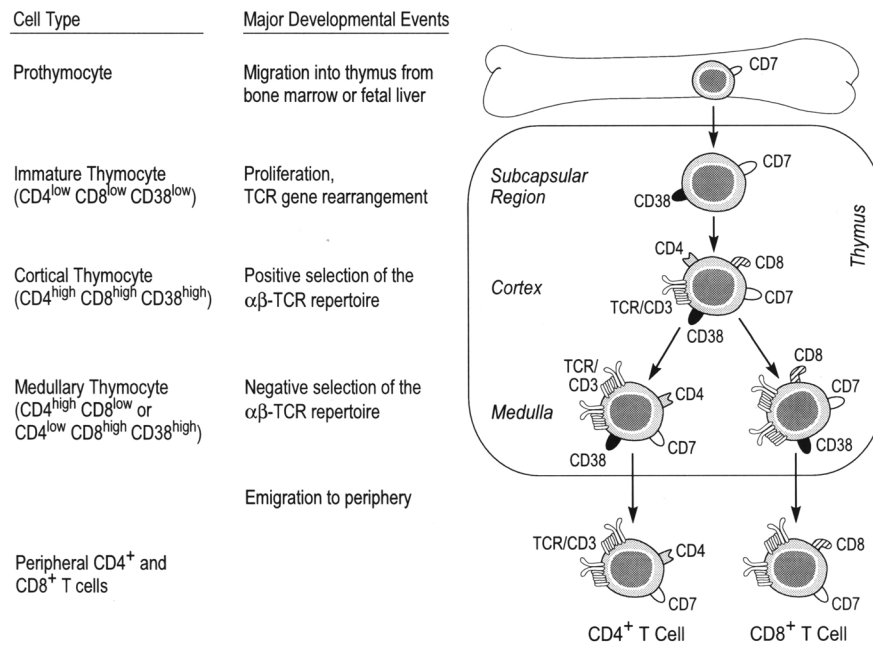
tant source of  $\alpha\beta$ -TCR diversity for antigen recognition. Additional diversity at V(D)J junctions results from imprecision in the cleavage of these segments undergoing rearrangement by the RAG recombinase complex so that variable number of nucleotides are lost, and by the random addition of nucleotides to these segments by the enzyme terminal deoxytransferase (TdT).<sup>6,7</sup> TdT addition is a particularly important mechanism for diversity generation since every three additional nucleotides may encode a codon, potentially increasing repertoire diversity by a factor of twenty. Although these mechanisms together can theoretically generate a highly diverse repertoire, actual diversity is substantially less since particular V, D, and J segments are used less frequently than would be predicted on a random basis.<sup>8</sup> This may reflect differences among segments of the TCR genes in their accessibility to the protein complex involved in the recombination process.<sup>8</sup>

The usage of D and J segments in rearrangement of the TCR- $\beta$  chain gene in the thymus at approximately 8 weeks gestation is less diverse than at 11–13 weeks gestation or subsequently. This restriction is not explained by an effect of surface TCR-based positive or negative selection in the thymus (see below), since it also applies to D-J rearrangements, which are not expressed on the immature thymocyte cell surface.<sup>9,10</sup> The CDR3 region of the TCR- $\beta$  chain transcripts is reduced in length and sequence diversity in the human fetal thymus between 8 and 15 weeks of gestation, most likely due to decreased amounts of the TdT enzyme.<sup>9–11</sup> Since the CDR3 region of the TCR chains is a major determinant of antigen specificity,<sup>5</sup> such decreased CDR3 diversity, particularly in conjunction with restricted V(D)J usage, theoretically could limit recognition of foreign antigens by the first-trimester fetal  $\alpha\beta$ -TCR repertoire, particularly during the first trimester. However any potential “holes” in the  $\alpha\beta$ -TCR repertoire of the human fetus from limitations in CDR3 are likely to be very subtle, particularly after the second trimester, when V segment usage is diverse. This is suggested by the fact that the T cell response to immunization and viral challenge is normal in mice that are completely deficient in TdT as a result of selective gene targeting.<sup>12</sup>

By the second trimester TdT activity and CDR3 length are both increased,<sup>9,10</sup> and  $V_{\alpha}$  and  $V_{\beta}$  segment usage in the thymus and peripheral lymphoid organs is diverse.<sup>10,13–15</sup> The  $\alpha\beta$ -TCR repertoire expressed by cord blood T cells has a diversity of TCR- $\beta$  usage and CDR3 length that is similar to that of antigenically naïve adult T cells, indicating that the functional pre-immune repertoire is fully formed by birth.<sup>16</sup> Repertoire analysis also suggests that there is greater oligoclonal expansion of alpha/beta T cells during the third trimester, particularly after 28 weeks gestation, than in adults, and that these oligoclonal expansions involve a variety of different V segment families.<sup>17</sup> Whether this oligoclonal expan-

FIGURE 4

*Putative stages of human alpha/beta thymocyte development. Prothymocytes from the bone marrow or fetal liver express CD7 and enter the thymus subcapsular region to give rise to progressively mature alpha/beta-TCR thymocytes, defined by their pattern of expression of the  $\alpha\beta$ -TCR/CD3 complex, CD4, CD8, and CD38. TCR- $\alpha$  and TCR- $\beta$  chain genes are rearranged in the subcapsular region, positive selection occurs mainly in the thymic cortex, and negative selection occurs mainly in the medulla. Following these selection processes, medullary thymocytes emigrate into the circulation and colonize the peripheral lymphoid organs as CD4 and CD8 T cells with high levels of the  $\alpha\beta$ -TCR/CD3 complex and lacking CD38 surface expression. However, most peripheral neonatal T cells retain surface expression of CD38.*



sion is antigen driven, e.g., by a response to idiotypes encoded on maternally derived immunoglobulins,<sup>17</sup> or is an antigen-independent process is unknown.

### Thymic-Dependent Generation of Antigenically Naïve Alpha/Beta T Cells

Thymocytes that express  $\alpha\beta$ -TCRs on their surface must sequentially pass two selective processes, positive and negative selection, which test the appropriateness of their TCR receptor specificity (Fig. 4). Positive selection requires that the  $\alpha\beta$ -TCR recognize self-peptides bound to MHC molecules displayed on epithelial cells of the thymic cortex.<sup>18</sup> If the TCR has sufficient affinity for self-peptide/MHC complexes, the thymocyte receives a signal allowing its survival,<sup>19</sup> and if not, the thymocyte dies by apoptosis. Positively selected thymocytes enter the medulla where they undergo a second selection process called negative selection, in which they are eliminated by apoptosis if their TCR has too high an affinity for self-peptide/MHC complexes expressed on medullary dendritic cells.<sup>20</sup> Negative selection helps eliminate alpha/beta T cells with TCRs that could pose a risk of autoimmune reactions, and is an important influence on the final TCR repertoire.<sup>21</sup> Medullary thymo-

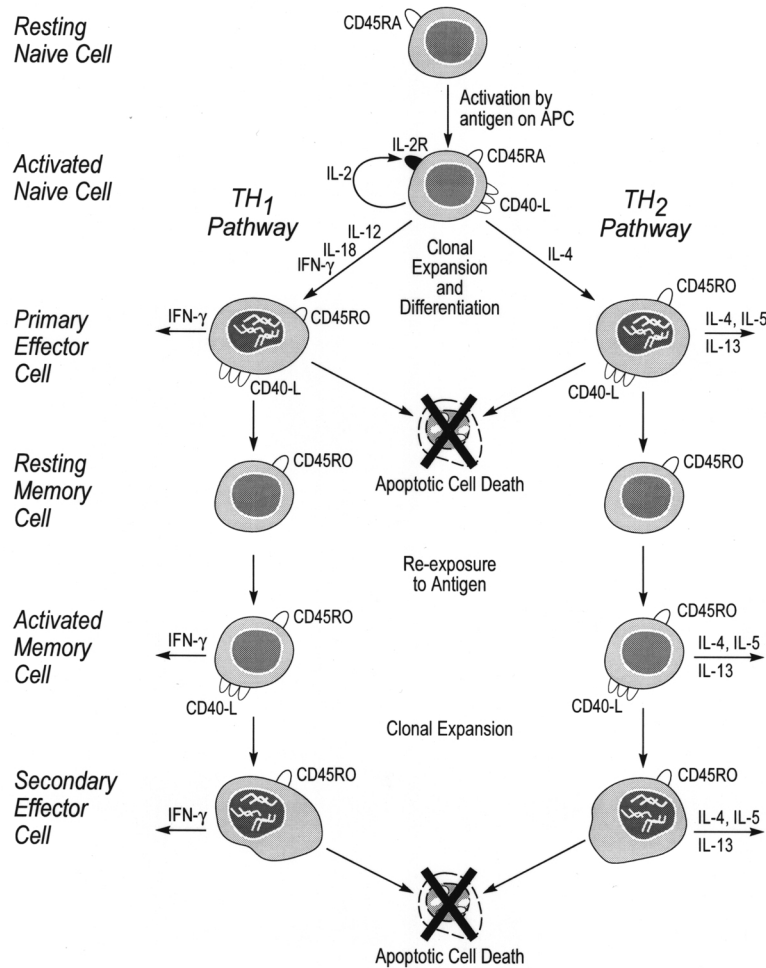
cytes that are not eliminated by negative selection enter into the circulation as antigenically naïve alpha/beta T cells, and preferentially home to the peripheral lymphoid organs (Fig. 4).

The human fetal thymus is first colonized with prothymocytes, probably derived from the fetal liver, at approximately 8.5 weeks of gestation.<sup>22</sup> Shortly thereafter, thymocytes express proteins characteristic for T-lineage cells, including CD3, CD4, CD8, and the TCR- $\alpha$  and TCR- $\beta$  chain proteins.<sup>22</sup> By 12 weeks of gestation, a clear separation between the outer thymic cortex and inner medulla is apparent indicating mature architecture,<sup>23</sup> and by 14 weeks of gestation mature CD4<sup>high</sup>CD8<sup>low</sup> or CD4<sup>low</sup>CD8<sup>high</sup> thymocytes, the precursors of antigenically naïve CD4 and CD8 alpha/beta T cells, are found in the medullary region, indicating that the positive selection process is established (Fig. 4). The thymocyte pattern of expression of a number of other proteins, such as CD38, matches that in the post-natal thymus at this stage of development. Thymic cellularity increases dramatically during the last trimester of gestation, and continues to do so post-natally.

CD4+CD8- and CD4-CD8+ thymocytes entering the circulation eventually colonize the lymph nodes and

FIGURE 5

Differentiation of antigenically naïve and memory CD4 T cells into effector T cells by antigen exposure. Antigenically naïve CD4 T cells express high levels of the CD45RA isoform of a surface protein tyrosine phosphatase. They are activated by antigen presented by APC and express CD40-ligand and IL-2, undergo clonal expansion and differentiation, and switch to expression of the CD45RO surface isoform. Exposure of these expanding T cells to IL-12, IL-18, and IFN- $\gamma$  favors their differentiation into Th1 effector cells that secrete IFN- $\gamma$  but not IL-4, while exposure to IL-4 favors their differentiation into Th2 effector cells that secrete IL-4 but not IFN- $\gamma$ . Most effector cells die by apoptosis, but a few probably persist as memory cells which express high levels of CD45RO. A pathway by which memory T cells are generated directly from antigenically naïve T cells in the absence of an intermediate effector cell stage is also possible.



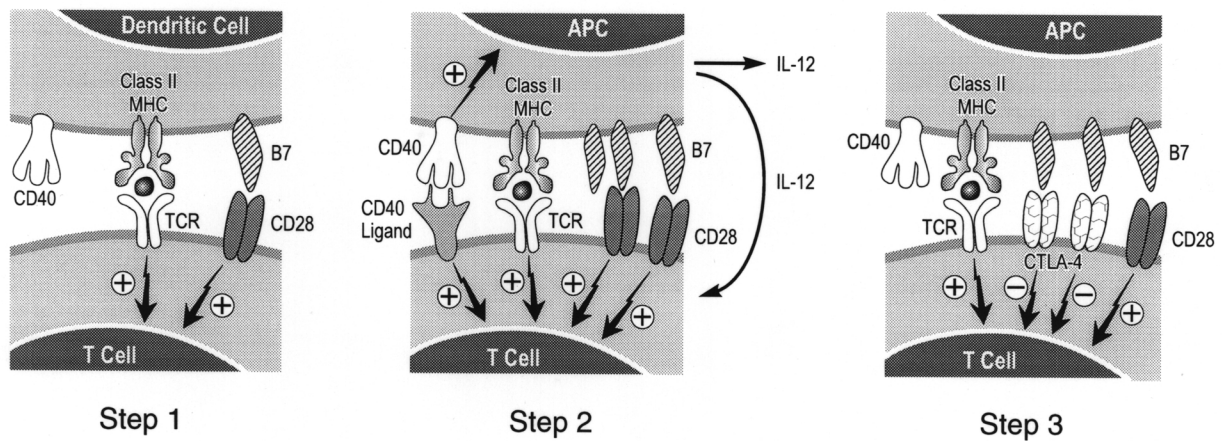
spleen as antigenically naïve CD4 and CD8 T cells, respectively. The long-term survival of peripheral alpha/beta T cells, particularly the antigenically naïve population, may require ongoing engagement of their  $\alpha\beta$ -TCR by self-MHC.<sup>24</sup> By 14 weeks of gestation CD4 and CD8 T cells are found in the human fetal liver and spleen and CD4 T cells are detectable in primary lymph node follicles,<sup>25</sup> demonstrating that thymic emigration is established. The percentage of T cells in the fetal or premature circulation gradually increases during the second and third trimesters of pregnancy through about six months of post-natal age.<sup>26</sup> The ratio of CD4 to CD8 T cells in the circulation is high during fetal life (about 3.5) and gradually declines with age.<sup>27</sup> The levels of expression of the  $\alpha\beta$ -TCR, CD3, CD4, and CD8

proteins on fetal and neonatal alpha/beta T cells are similar to adult T cells (unpublished observations).<sup>28,29</sup> However, unlike adult antigenically naïve T cells, virtually all peripheral fetal and neonatal T cells express the CD38 molecule,<sup>30</sup> which is also found on most fetal and post-natal thymocytes. This suggests that peripheral T cells in the fetus and neonate may represent an immature transitional population.

Most human antigenically naïve T cells can be distinguished from memory T cells that previously been activated by encounters with antigen based on their respective surface expression of either the CD45RA or CD45RO isoforms of CD45, a protein tyrosine phosphatase (Fig. 5).<sup>31,32</sup> Thus, most antigenically naïve T cells have a CD45RA<sup>high</sup>CD45RO<sup>low</sup> surface phenotype,<sup>32,33</sup> while

FIGURE 6

*T cell-APC interactions early during the immune response to peptide antigens. A class II MHC-restricted response by CD4 T cells is shown as an example. Dendritic cells are probably the most important APC for antigenically naïve T cells, and constitutively express B7, CD40 and class II MHC molecules on their cell surface. Engagement of the CD4 T cell by antigenic peptide bound to MHC on the dendritic cells, in conjunction with co-stimulation by B7/CD28 interactions, leads to T cell activation (Step 1). The activated T cell expresses CD40-ligand on its surface, which engages CD40; this increases B7 expression on the dendritic cell enhancing T cell co-stimulation (Step 2). CD40 engagement also activates the dendritic cells to produce cytokines, such as IL-12. IL-12, in turn, promotes the differentiation of T cells into Th1-type effector cells that produce high levels of IFN- $\gamma$  and low amounts of IL-4. CTLA-4 is expressed on the T cells during the later stages of T cell activation. Engagement of CTLA-4 by B7 molecules on the APC delivers negative signals that help terminate T cell activation (Step 3).*



memory T cells are typically CD45RA<sup>low</sup>CD45R0<sup>high</sup>. Circulating T cells in the term and pre-term (22 to 30 weeks gestation) neonate and in the second trimester fetus are predominantly CD45RA<sup>high</sup>CD45R0<sup>low</sup> and lack a CD45R0<sup>high</sup> T cell subpopulation.<sup>34,35</sup> This is consistent with the limited exposure of the fetus to foreign antigens. About 30% of circulating T cells of the term neonate are CD45RA<sup>low</sup>CD45R0<sup>low</sup>, a surface phenotype that is rare or absent in circulating adult T cells. Since these CD45RA<sup>low</sup>CD45R0<sup>low</sup> T cells are functionally similar to neonatal CD45RA<sup>high</sup>CD45R0<sup>low</sup> T cells in their limited capacity to produce cytokines (see below), and become CD45RA<sup>mid</sup>CD45R0<sup>low</sup> T cells with incubation in vitro,<sup>36</sup> they appear to consist mainly of immature recent thymic emigrants that are in transition to becoming antigenically naïve cells. Such extra-thymic maturation probably occurs over a period of days, based on studies in rodents.<sup>37</sup>

### T Cell Activation and Co-Stimulation and the Generation of Effector and Memory Cells

Alpha/beta T cell activation following TCR engagement by antigen/MHC involves the generation of a complex set of intracellular signals. These signals are initiated, in part, by tyrosine kinases associated with cytoplasmic domains of proteins of the CD3 complex (Fig. 1). In addition to recognition of a suitable peptide/MHC complex by the TCR, T cell activation often requires other signals provided by the APC, which are collectively referred to as co-stimulation. Major sources of co-stimu-

lation are due to interactions between the B7 molecules, B7-1 (CD80) and B7-2 (CD86) on the APC, and CD28 on the T cell,<sup>38</sup> and CD40 on the APC with CD40-ligand on the T cell (Fig. 6).<sup>39,40</sup>

The CD28 molecule is constitutively expressed on virtually all antigenically naïve alpha/beta T cells, while CD40-ligand is rapidly expressed (within a few hours) by CD4 T cells following TCR engagement by antigen.<sup>40</sup> CD28 signaling potently augments T cell cytokine production, including the production of IL-2, which helps expand antigen-specific T cells in an autocrine and paracrine fashion. CD28 engagement also increases the percentage of cells that enter into the cell cycle following engagement of the  $\alpha\beta$ -TCR/CD3 complex.<sup>38</sup> CD4 T cell activation is terminated, in part, by the interaction of B71 and B72 on the APC with CTLA-4, a molecule that is mainly expressed on the T cell surface during the later stages of cell activation (Fig. 6).

Co-stimulation appears to be particularly important if the exposure of T cells to antigenic peptide/MHC is relatively limited in terms of dose or of duration (e.g., following immunization with protein). In cases of limited antigen exposure, engagement of the TCR without co-stimulation may not only fail to activate the T cell but, instead, render it anergic.<sup>41</sup> Anergic T cells will not subsequently respond to antigen even when normally adequate co-stimulatory signals are provided by the APC. Anergy is an attractive but controversial model for the maintenance of tolerance by mature T cells to certain

self-antigens, particularly those that may not be expressed at sufficiently high levels in the thymus to induce negative selection.

CD40-ligand is a particularly important positive regulator of the immune response by antigen-activated CD4 T cells, and acts by binding to the CD40 molecule on B cells, mononuclear phagocytes, and dendritic cells.<sup>40</sup> Engagement of CD40 on B cells transduces intracellular signals that promote the expression of antibody isotypes other than IgM and differentiation of the B cell into a memory cell.<sup>40</sup> CD40 signaling by mononuclear phagocytes enhances the microbicidal activity of these cells towards intracellular pathogens. Finally, CD40 signaling in dendritic cells increases their expression of B7 co-stimulatory molecules and the production of certain pro-inflammatory cytokines, such as IL-12 (Fig. 6). Such increased B7 expression may provide additional CD28-dependent co-stimulation to antigenically naïve T cells. In addition, engagement of CD40-ligand on the T cell by CD40 on the APC may also enhance T cell proliferation and cytokine production<sup>28</sup> by so-called “reverse signaling.”

Once antigenically naïve CD4 and CD8 T cells are activated, they undergo clonal expansion and differentiation into effector T cell populations (Fig. 5).<sup>42</sup> Effector T cells are lymphoblasts in the active phases of the cell cycle (i.e., not G<sub>0</sub>). They have a greater capacity than antigenically naïve T cells for the production of many cytokines, including interferon- $\gamma$  and IL-4, and for mediating cell-mediated cytotoxicity. They also have a reduced co-stimulatory requirement and greater tendency to undergo apoptosis than antigenically naïve T cells.<sup>43,44</sup> This apoptotic tendency helps limit effector cell accumulation once they are no longer needed for the immune response.<sup>45</sup> Most human memory CD4 and CD8 T cells as well as CD4 effector cells are CD45RA<sup>low</sup>CD45R0<sup>high</sup> (Fig. 5),<sup>31,32</sup> while human CD8 effector T cells retain a CD45RA<sup>high</sup> surface phenotype, but can be distinguished from antigenically naïve CD8 T cells by their lack of surface expression of the CD27 and CD28 molecules.<sup>46</sup>

### **Fetal And Neonatal T Cell Responses to Polyclonal Stimuli**

Most studies have found that circulating neonatal T cells and adult T cells have similar amounts of proliferation and IL-2 production in response to the mitogenic lectins, bacterial superantigens, or to allogeneic cells.<sup>16,47-52</sup> However, these similar responses may not necessarily apply to protein antigens that are encountered for the first time, i.e., neoantigens. For example, one study using limiting dilution techniques and circulating mononuclear cells from cytomegalovirus (CMV)-non-immune donors found that the frequency of neonatal T cells proliferating to whole inactivated CMV antigen was significantly less than that of adult T cells.<sup>53</sup> Others have found that neonatal peripheral blood

mononuclear cells had decreased antigen-specific T cell proliferation and IL-2 production in response to the protein neoantigen, keyhole limpet hemocyanin, compared to adult cells.<sup>54</sup> The function of circulating dendritic cells may be reduced in the neonate.<sup>55</sup> Since dendritic cells are critical for activation of antigenically naïve T cells by antigen, including *in vitro*,<sup>56</sup> it is plausible that decreased dendritic function in the neonate might compromise the presentation of soluble proteins. However, a reduced function of neonatal or fetal dendritic cells found in the tissues or lymphoid organs has not been documented.

Polyclonally activated neonatal T cells have been reported to have reduced expression of a number of cytokines compared to adult T cells, including interferon- $\gamma$ , IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, and GM-CSF (reviewed in reference 1). In most instances this apparent cytokine deficiency of neonatal CD4 T cells can be accounted for by their antigenically naïve status rather than their developmental immaturity, i.e., neonatal and adult antigenically naïve CD4 T cells both have similar limitations in cytokine production. However, a major exception to this pattern is the production of CD40-ligand: Activated antigenically naïve neonatal CD4 T cells, the predominant circulating T cell population at this age, express substantially less CD40-ligand surface protein and cognate mRNA than adult antigenically naïve CD4 T cells.<sup>57-59</sup> This difference in cytokine production is selective in that IL-2 production and mRNA expression by antigenically naïve neonatal and adult CD4 T cells is similar.<sup>60</sup> Thus, reduced expression of CD40-ligand by neonatal T cells appears to represent a selective and true developmental limitation in cytokine production by an antigenically naïve CD4 T cell population. Decreased CD40-ligand production by neonatal T cells has also been more recently documented in the mouse, suggesting that it may be a general feature of T cells that have recently emigrated from the thymus. Consistent with this idea, human CD4+CD8- thymocytes, the immediate precursors of antigenically naïve CD4 T cells, also have a low capacity to express CD40-ligand.<sup>59</sup>

Given the importance of CD40-ligand in multiple aspects of the immune response,<sup>40</sup> limitations in the production of this cytokine could contribute to decreased antigen-specific immunity mediated by neonatal T effector cells and, as consequence, compromise B cell, mononuclear phagocyte, and dendritic cell function. However, it remains to be shown that reduced CD40-L expression by neonatal T cells applies during physiologic activation (e.g., in response to protein neoantigens), since most studies to date have used pharmacologic stimuli that may not accurately mimic all events in normal T cell activation. It is also unclear if CD40-ligand expression is limited during early fetal ontogeny. One study, in fact, has found that as substantial minority of circulating T cells from the late second trimester through the early third trimester have the capacity to express CD40-ligand

at levels similar to adult T cells.<sup>57</sup> The immunologic function of this distinctive fetal T cell population remains unclear.

### **Co-Stimulation of Fetal and Neonatal T cells**

Neonatal T cells activated using anti-CD3 monoclonal antibodies and mouse cell lines expressing human B7-1 or B7-2 produce IL-2 and proliferate as well as adult T cells.<sup>28</sup> This indicates that CD28-mediated signaling is grossly intact in neonatal T cells. Intact signaling is also suggested by the observation that anti-CD28 monoclonal antibody treatment of neonatal T cells markedly augments their ability to produce IL-2 and proliferate in response to anti-CD2 monoclonal antibody stimulation.<sup>62</sup> However, neonatal T cells differ from adult CD45RA<sup>high</sup> T cells in their tendency to become anergic rather than competent for increased cytokine secretion following priming with bacterial superantigen bound to class II MHC-transfected murine fibroblasts in the absence of human B7 molecules.<sup>51</sup> Superantigens activate T cells by binding to a portion of the TCR- $\beta$  chain outside of the peptide antigen recognition site, but otherwise appear to mimic activation by peptide/MHC complexes in most respects. This tendency appears to be developmentally regulated in that CD4+CD8- thymocytes, the immediate precursors of antigenically naïve CD4 T cells, are also prone to anergy when treated under these conditions.<sup>63,64</sup> These results as well as other studies performed using allogeneic stimulation<sup>64</sup> suggest that neonatal, and presumably, fetal T cells, have a greater tendency than antigenically naïve adult T cells to become anergic, particularly under conditions in which co-stimulation may be limiting.

### **Fetal and Neonatal CD4 T Cell Responses to Specific Antigens**

Pathogen-specific CD4 T cell proliferation and cytokine responses (IL-2 and interferon- $\gamma$ ) to soluble protein antigens are often markedly lower or absent in infants and children who have been congenitally infected [e.g., with syphilis, CMV, varicella-zoster virus (VZV), or *Toxoplasma*] compared to those with post-natally acquired infection.<sup>65-70</sup> This is particularly evident when these infections occur in the first or second trimester. A direct deleterious effect on CD4 T cell development is a possible mechanism for such decreased responses in the case of severe first trimester infections. However, T cells from infants and children with congenital toxoplasmosis retain the ability to respond to alloantigen, mitogen, and, in at least one case, tetanus toxoid.<sup>67</sup> This suggests that these reduced pathogen-specific responses may be more often due to mechanisms that result in antigen-specific unresponsiveness [e.g., antigen-specific anergy, deletion, or ignorance (the failure of the T cell to be initially activated by antigen)].<sup>71</sup> As discussed above, decreases in the TCR repertoire are unlikely to play a role in limiting

these immune responses, particularly in cases occurring from the second trimester onward. Decreased responses may also not apply to all congenital pathogens, since, in one study, most 10-year-old children who were congenitally infected with mumps had delayed-type hypersensitivity reactions to mumps antigen, indicating the persistence of functional mumps-specific memory/effector CD4 T cells.<sup>72</sup>

Limitations in antigen-specific CD4 T cell responses appear to persist even when infections are acquired in the neonate rather than during fetal life. For example, although neonatal infection with HSV results in antigen-specific proliferation and IL-2 and interferon- $\gamma$  production by CD4 T cells, these responses are delayed in their appearance compared to adults with primary HSV infection.<sup>73,74</sup> Infants between 6-12 months-of-age also have moderately lower IL-2 production by CD4 T cells in response to tetanus toxoid than older children and adults.<sup>49</sup> Taken together, this suggests that either antigen-specific memory CD4 T cell generation or function is decreased during early infancy, particularly soon after infection. Whether this reflects limitations in antigen processing and/or in T cell activation and co-stimulation, proliferation, and differentiation remains unclear.

Antigen-specific CD4 T cell function is also a requirement for delayed-type hypersensitivity (DTH) skin test reactivity to cell-free antigens. Skin test reactivity to common antigens such as *Candida*, streptokinase-streptodornase and tetanus toxoid is usually not detectable in neonates.<sup>75-77</sup> This reflects a lack of antigen-specific sensitization because in vitro CD4 T cell recall responses to these antigens is also absent. However, when leukocytes, and presumably antigen-specific CD4 T cells, from sensitized adults are adoptively transferred to neonates, children or adults, only neonates fail to respond to antigen-specific skin tests.<sup>78</sup> This indicates that the neonate may be deficient in other components of the immune system required for DTH, such as monocyte chemotaxis. Diminished skin reactivity after sensitization by infection or vaccination appears to persist post-natally up to a year of age.<sup>79</sup>

Specific antigen reactivity by CD4 T cells can also develop in the fetus by exposure to antigens transferred from the mother.<sup>80</sup> For example, several independent studies suggest that fetal CD4 T cells can become primed to environmental or dietary protein allergens as a result of maternal exposure and transfer to the fetus.<sup>81-84</sup> Interestingly, in one study, protein allergen-specific CD4 T cell proliferation detected at birth appeared to be more common when allergen exposure occurred in the first or second trimester rather than the third trimester of gestation.<sup>84</sup> Whether this reflects decreased maternal-to-fetal transport of antigen during late pregnancy, or is an intrinsic difference between the capacity of early- and late-gestation fetal T cells to be primed *in vivo*, remains unclear. In contrast to fetal sensitization by environmental



protein allergens, specific cellular immunity following maternal vaccination with protein antigens appears to be uncommon. For example, antigen-specific fetal T cell priming to non-viable “killed” vaccines was not detectable following maternal vaccination during the last trimester of pregnancy with tetanus toxoid or influenza.<sup>85</sup> Whether this reflects relatively inefficient maternal-fetal transfer of protein antigens or the associated vaccine adjuvant, and/or intrinsic limitations of the fetus for antigen presentation and T cell priming is unclear.

### Fetal and Neonatal T Cell-Mediated Cytotoxicity and Allograft Rejection

An analysis of the phenotype of second and third trimester fetal T cells following congenital infection with viruses or *Toxoplasma* suggests that CD8 T cells can expand and differentiate into memory/effector cells.<sup>86–88</sup> However, it is uncertain whether these memory/effector-like CD8 T cells are functionally competent. In one case of congenital HIV-1 infection, the expansion of HIV-specific cytotoxic T cells was detected at birth, indicating that the ability of the fetal T cells to be activated by viral antigen and undergo expansion was at least partially intact.<sup>89</sup> However, the CD8 T cell cytotoxic responses to HIV in perinatally infected infants are delayed in their appearance and are more limited than those of adults as far as the antigens that are recognized.<sup>90</sup> Limited studies of viral-specific cytotoxicity in infants with acute respiratory syncytial virus infection (RSV) also suggest that RSV-specific cytotoxicity is more pronounced and frequent in peripheral blood mononuclear cells from infants 6–24 months of age than in cells from 0–5 month old infants,<sup>91</sup> suggesting that the neonate may have a reduced capacity to generate anti-viral CTL populations.

However, neonates, including those that are premature, are capable of rejecting foreign grafts,<sup>92</sup> a process in which alloreactive cytotoxic CD8 T cells play a critical role. Experiments using human-severe combined immunodeficiency (SCID) mouse chimeras also suggest that second trimester human fetal T cells are capable of

becoming cytotoxic effector T cells in response to foreign antigens, and in rejecting solid tissue allografts.<sup>93</sup> Limited clinical observations are also consistent with at least partial function of fetal cytotoxic T cells *in vivo*. For example, transplantation of fetal blood from one unaffected fraternal twin to another with  $\beta$ -thalassemia did not result in marrow engraftment, despite a sharing of similar MHC haplotypes, and instead resulted in a detectable post-natal recipient cytotoxic T cell response against donor leukocytes.<sup>94</sup> A T cell response to alloantigens can also be detected in newborns following *in utero* irradiated red blood cell transfusions from unrelated donors, and these neonates also have a significantly greater percentage of CD45R0<sup>high</sup> T cells than healthy controls.<sup>95,96</sup> Together, these observations support the notion that fetal and neonatal T cells have the capacity to mediate allogeneic responses *in vivo*, including graft rejection. Interestingly, one old study suggests that the infusion of fresh leukocytes into the neonate can induce a state of partial tolerance to skin grafts compared to the rapid rejection that would be expected in adult recipients.<sup>92</sup> Nevertheless, this apparent tolerance is unlikely to be durable, although this was not tested in this study.

### Potential Relevance to Prenatal Gene Therapy

Together, these observations indicate that in most cases there is no durable antigen-specific T cell non-responsiveness following fetal antigen exposure, at least in the context of infection or allografting. Therefore, immunogenicity rather than long-term T cell tolerance would be predicted to be the more likely outcome following fetal antigen exposure, particularly if adequate co-stimulatory signals are present. These findings have important implications, in that the expression of a protein foreign to the fetus might potentially limit the efficacy of human prenatal gene therapy to express non-self proteins. This might become particularly evident during infancy, when most aspects of T cell immune function become similar to that of immunocompetent adults.

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# Immune Tolerance

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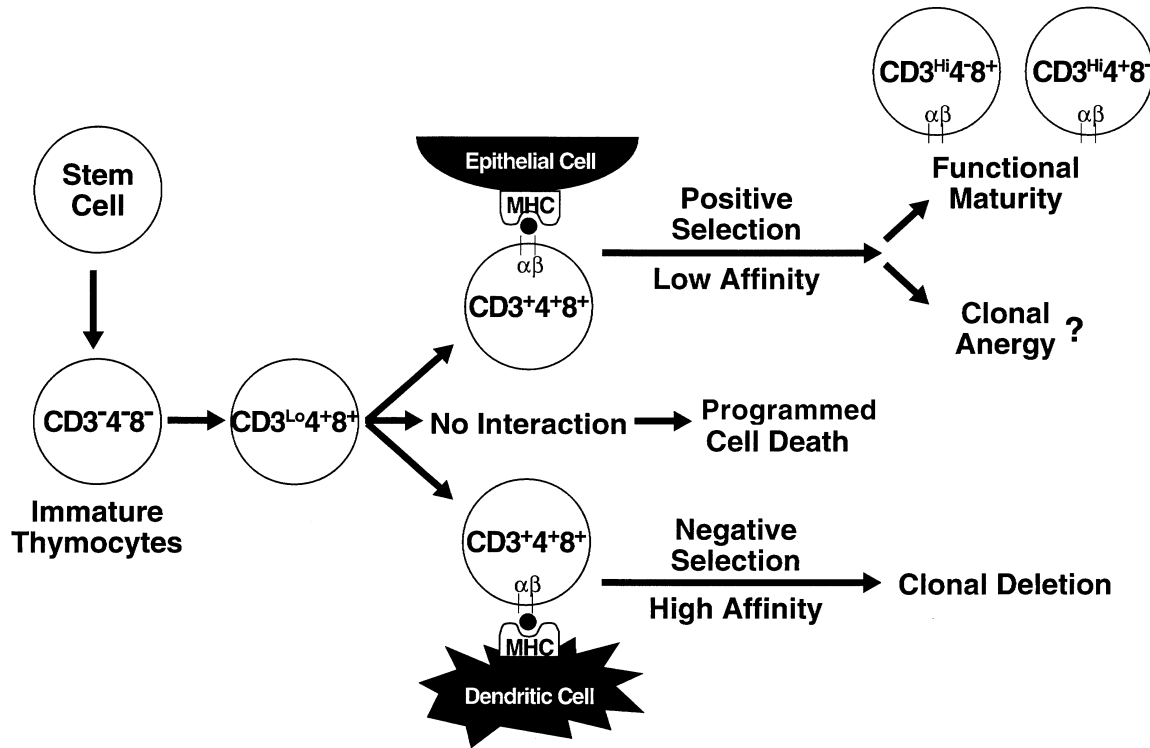
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Immunological tolerance as used in this presentation is defined as an antigen-directed unresponsive state. It is best represented by the unresponsive state that we enjoy to our own body constituents. In T cells it is suggested that tolerance to self antigens is induced primarily during differentiation of precursor T cells in the thymus by a process known as *negative selection*. The maintenance of tolerance in both cell types, as well as the induction of tolerance in T cells to exogenous antigens and selected self antigens, is carried out in the periphery. Unfortunately, in the past, peripheral unresponsiveness (resulting from deletion) has been confused with numerous regulatory events resulting in such phenomena as T cell anergy, polarization of T cell responses, etc. The induction of peripheral tolerance in T cells requires extremely small levels of antigen (either self or exogenous) while induction of tolerance to B cells requires large amounts of antigen. Of importance is that activation of antigen-presenting cells (APC) resulting in release of inflammatory cytokines interferes with the induction of tolerance. Thus, the induction of peripheral tolerance (also possibly that induced in the thymus) results from the avoidance of activation of such APCs and cytokine release. The tolerance induced in either the periphery or through the thymus results in a solid unresponsive state in both CD4<sup>+</sup> T cell subsets. Because of a marked deficiency of APCs, tolerance is more readily induced during neonatal and prenatal life.

FIGURE 1

Selection of mature peripheral T cells. The functional peripheral T cells of both the CD4<sup>+</sup> and CD8<sup>+</sup> phenotype result from negative (deletion) and positive selection in the thymus.



## Introduction

Although immunological tolerance obviously plays a significant role in gene therapy where one must consider a number of factors such as transferred cells, gene products as well as vectors used to introduce the gene DNA, this presentation will be limited to discussion of some of the basic mechanisms involved in the induction and maintenance of tolerance. The major emphasis will be placed on self tolerance, as well as to an *in vivo* model that mimics self tolerance. Immunological tolerance to self antigen as well as that induced to exogenous antigens is antigen-specific and direct antigen-cell (receptor) contact is required for both the induction and maintenance of tolerance. Tolerance to both self and exogenous antigens most likely requires continuous presence of the antigen for a persistent tolerant state.<sup>1</sup> Obviously tolerance can only occur in thymus (T)- and bone marrow (B)-derived cells after such cells have developed to the point where they express their antigen-specific receptors. Although tolerance in T cells to self antigens occurs early in life, in the developmental cells of the thymus, induction of tolerance can be continued and even initiated in T cells in the periphery once they leave the thymus (Table 1). Thus, the main site of tolerance induction to self occurs in the thymus in a particular state in the development of the T cells and in context of the major histocompatibility complex

(MHC).<sup>2</sup> It has been suggested that stem cells arriving from the bone marrow, after expressing the thymus-specific markers, for both the CD4<sup>+</sup> and CD8<sup>+</sup> T cell subset markers as well as antigen-specific receptors, are deleted when they react with a high affinity with the thymus dendritic cells (Fig. 1). On the other hand, when such cells react in a similar manner at low affinity with receptors on the thymus epithelial cells (and MHC), they are positively selected and leave the thymus medullary as CD4<sup>+</sup> and CD8<sup>+</sup> cells.<sup>2</sup>

TABLE 1  
Mechanisms of Induced Tolerance

1. Thymus Directed
  - a. deletion
  - b. early in life
  - c. self (many) antigens
2. Peripheral Tolerance
  - a. deletion
  - b. early as well as adult life
  - c. self as well as exogenous antigens
  - d. maintenance, newly acquired, and many self antigens

## Induction of Tolerance in the Periphery

The induction of tolerance in the periphery most likely plays a major role in the maintenance of tolerance in T cells as well as the induction of tolerance to new antigens that are expressed later in the life of the developing host. Furthermore, peripheral tolerance offers a mechanism that may have some benefit to the host, e.g., controlling autoimmune induction and progression. Models of peripheral tolerance to exogenous antigens have also contributed considerably to the understanding of the mechanisms of self-nonsel recognition. Since even in neonatal life peripheral tolerance probably plays a significant role in self tolerance, information obtained with these experimental models plays an important role in understanding self tolerance. Unfortunately, tolerance to exogenous antigens during either neonatal or adult life is difficult to induce and depends on very selective conditions.<sup>3</sup> The most important of these conditions is the nature of the antigen. Antigens such as intact cells, viruses, bacteria antigens and even complex proteins such as hemocyanin which are both readily taken up by, and activate, antigen-presenting cells (APC), do not induce an unresponsive state that mimics self tolerance. Similarly, antigens such as ovalbumin which are rapidly excreted through the kidney are also difficult to induce an unresponsive state.<sup>4</sup> Although the induction of tolerance to such antigen has been at times reported, such "tolerance" is rather a reflection of polarization of the immune response in one but not the other CD4<sup>+</sup> T cell subset (reviewed in 5). On the other hand, tolerance can readily be induced into serum protein antigens, certain organ-specific antigens (e.g., thyroglobulin), and peptide antigens. These antigens can be presented in a non-aggressive fashion to the immune system which avoids activation of APC. Tolerance can readily be induced both in neonatal and adult animals to serum protein antigens which are devoid of either APC aggravating complexes or endotoxins.<sup>1,6</sup> Furthermore, tolerance to allelic cells under conditions that result in a chimeric state can be induced in mice during early life.<sup>7</sup> The classical model of peripheral tolerance is that induced in adult mice with monomeric preparations of mammalian gamma globulins. For example, deaggregated preparation (monomeric) of human gamma globulin (DHGG) possesses a number of characteristics which favor its induction of tolerance in the periphery of both neonatal and adult animals (Table 2). The primary property of this protein is its ability upon injection to equilibrate between inter- and extravascular fluid spaces, to persist in these spaces with a half-life of over 7 days,<sup>1</sup> and to avoid activation of APCs.<sup>5</sup>

## Tolerance in T and B Cells

Although immunological tolerance to DHGG can be induced in both T and B cells, both the duration of the unresponsive state (once induced) and the level of tolerance required for induction dramatically differ. A large

TABLE 2  
*Peripheral Tolerance Induced to Human Gamma Globulin in Mice*

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1. Mimics self tolerance
2. Not down regulation
3. Both CD4 T cell subsets and B cells
4. Long lasting
5. T cell proliferation, cytokine release, T helper and antibody
6. Thymus independent
7. Tolerogenic (monomeric) and immunogenic (aggregated) forms

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amount of antigen is required for the induction of tolerance in B cells, whereas quite small amounts of antigen are required for the induction and maintenance of tolerance in the T helper cells.<sup>8</sup> This observation was also made with similar proteins by others,<sup>9</sup> and suggests that when antigen is present in relatively small levels in body fluid, tolerance may only be induced and maintained in T cells, whereas B cells may remain competent.<sup>2</sup> Examples of such split tolerance have been seen with self antigens such as thyroglobulin (reviewed in 2). Thus, the possibility that one can circumvent T cell tolerance and activate B cell autoimmunity has been demonstrated with several self antigens (reviewed in 1).

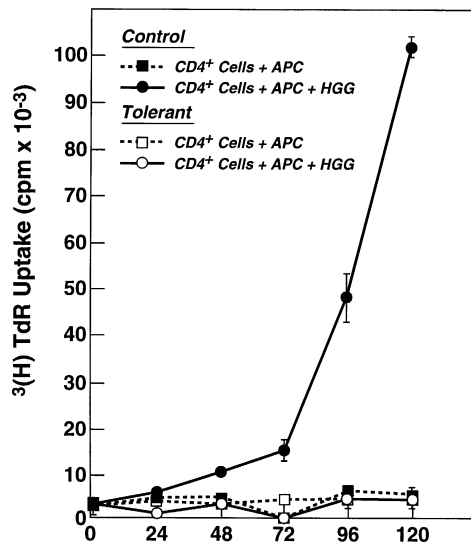
A single injection of DHGG into adult mice results in a virtually complete unresponsive state in the CD4<sup>+</sup> T cells of relatively long duration (Fig. 2).<sup>10</sup> Tolerance occurs in both the CD4<sup>+</sup> subsets (Th1 and Th2) as evidenced both by the failure of CD4<sup>+</sup> cells from tolerized and subsequently immunized mice to produce either IL-2 (Th1-like) or IL-4 (Th2-like) or cytokine mRNA upon in vitro stimulation.<sup>10-12</sup> Tolerance is more readily induced in T cells during neonatal life (reviewed in 3) and thus in some cases tolerance to certain antigens which cannot be induced in adults can readily be induced in neonatal mice.

## Interference with Tolerance Induction

The ability to interfere with the induction of tolerance to DHGG in adult mice by both inducers of cytokine release and cytokines themselves has allowed considerable insight into the cellular sites and mechanisms of T cell tolerance.<sup>5</sup> It is well documented that LPS and other inducers of cytokine release interfere with the induction of tolerance accompanied by the injection of mice with DHGG.<sup>13</sup> The ability of IL-1 and TNF $\alpha$  to interfere with the induction of tolerance, along with the ability of aggregated HGG but not DHGG to activate APCs (release of cytokines), supports the suggestion that avoidance of APC activation and cytokine release favors the induction of tolerance to DHGG (Table 3). Further support of this assumption is the ability of APCs to aggressively degrade AHGG, but not DHGG. Furthermore, the dose response for the

FIGURE 2

HGG-specific proliferative response in CD4<sup>+</sup>-enriched lymph node cells from control and tolerant mice. CD4<sup>+</sup>-enriched T cells were harvested from lymph nodes of mice injected with HGG in CFA 9 d previously (control) or in similarly injected mice that had received 2.5 mg DHGG i.p. 15 d before immunization (tolerant). Cultures were harvested at the times indicated after a 6-h pulse with 1  $\mu$ Ci [<sup>3</sup>H]TdR. [Reproduced from Romball & Weigle, *The Journal of Experimental Medicine*, 1993, vol. 178, pp. 1637-1644, by copyright permission of The Rockefeller University Press.]



induction of tolerance in both subsets is the same.<sup>14</sup> It appears that the peptides required for the presentation of tolerogenic DHGG to antigen-presenting cells results from the normal non-aggressive catabolism of DHGG.

The ability of LPS and IL-1 to differentially activate CD4<sup>+</sup> T cell subsets further defines the mechanism of tolerance induction to DHGG.<sup>5,14</sup> Although both LPS and IL-1 given along with DHGG activate T helper cell function and antibody production in vitro, CD4<sup>+</sup> cell proliferation is minimal.<sup>14</sup> It appears most likely that optimal expansion of the CD4<sup>+</sup> cells had already occurred *in vivo*. Further evidence for T cell involvement is the interference of tolerance in the release of Th1- and Th2-like lymphokines following immunization. Mice not treated, but immunized, produce both types of lymphokines from in vitro stimulation of the CD4<sup>+</sup> cells (Table 4). CD4<sup>+</sup> cells from DHGG-treated, and those immunized, produced no lymphokines upon challenge. CD4<sup>+</sup> cells from DHGG/LPS-injected mice produce both Th1- and Th2-like lymphokines, while such cells from DHGG/IL-1-treated mice produce only Th2-like lymphokines. Furthermore, immunized mice previously injected with LPS along with DHGG produce both IgG2a (Th1-like) and IgG1 (Th2-like) antibodies, whereas mice injected with IL-1 along with DHGG

TABLE 3

The Ability of Various Factors to Cause IL-1 Availability and Interfere with the Induction of Tolerance

Factor	Release of Cytokines	Interference with Induction of Tolerance
LPS	+	+
8-Bromoguanosine	+	+
Indomethacin	+	+
IL-1	+	+
AHGG	+	+
DHGG	-	-

8-Bromoguanosine—lymphocyte of accessory cell activator  
Indomethacin—enhances IL-1 release

produce only IgG1 antibodies. These results suggest that LPS inhibits the induction of tolerance in both subsets, whereas IL-1 inhibits the induction of tolerance in only the Th2 subset. Although IL-12 is an initiator of Th1-like responses, the injection of IL-12 along with DHGG does not interfere with the induction of tolerance in either subset.<sup>15</sup>

The best interpretation of the above data is that tolerance induced to monomeric preparations of HGG, occur in both the Th1 and Th2 CD4<sup>+</sup> T cell subsets. That certain cytokines interfere with the induction of tolerance and that aggregated HGG, but not monomeric HGG is aggressively processed by antigen-presenting cells suggests that tolerance results from the failure of DHGG to activate APCs and cause the release of cytokines. It appears that the CD4<sup>+</sup> precursor T cell is first activated by peptides released as the result of normal catabolism of DHGG. The activation of the precursor T cells normally results in the generation of both CD4<sup>+</sup> subsets, but in the absence of cytokine production (in the tolerized host) neither subset can be expanded. Tolerance to subsequent challenge with immunogen results from an as yet unknown intracellular mechanism. IL-1, probably along with IL-4, may be responsible for the expansion of the Th2 subset. Although IL-12 is likely to be involved in expansion of the Th1 cells, other cytokines are probably also required.

TABLE 4

Interference with Induction of Tolerance to DHGG

Agent	T Cell Proliferation	T Cell Help	Cytokine		IgG	
			IL-2	IL-4	IgG2a	IgG1
HGG/CFA	+	+	+	+	+	+
DHGG	0	0	0	0	0	0
DHGG/LPS	0	+	+	+	+	+
DHGG/IL-1	0	+	0	+	0	+
DHGG/IL-12	0	0	0	0	0	0



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## *In Utero* Transfer of *cftr*

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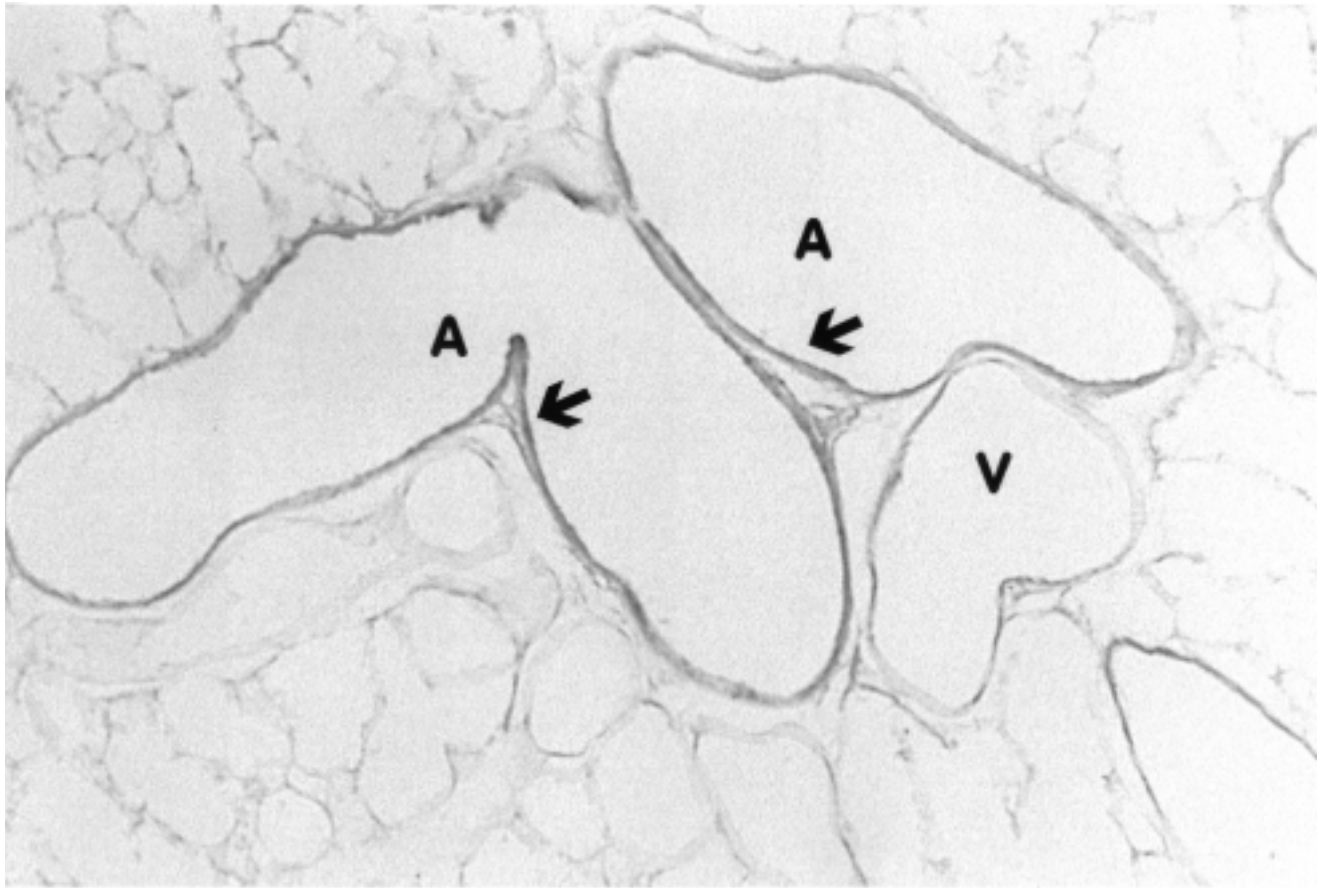
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*In utero* adenoviral-mediated transfer of genes via the amniotic fluid results in sustained high-efficiency expression in the fetus. Transfer to the developing lung and intestine is accomplished by direct injection of a replication-defective adenovirus into individual amniotic sacs. At the time of infection, rodent fetal development is comparable to that of a 10–20 week gestation human and the targeted epithelial cells of the airways and intestine are undifferentiated. When the developing epithelial cells of the lung and intestine are targeted with an adenovirus containing *cftr*, the gene responsible for Cystic Fibrosis (CF), permanent changes occur in these organs. The *in utero* therapy completely reverses the lethal phenotype of the CF (*cftr*<sup>-/-</sup>) mouse and produces phenotypic and functional changes in the lung and intestine. These developmental changes remain past the expression of the *cftr* transgene. Survival statistics from the progeny of *cftr*<sup>+/-</sup> matings following *in utero* *cftr* treatment demonstrated an increased mortality in the *cftr*<sup>+/+</sup> pups, indicating that overexpression during development was lethal. The lungs of these pups revealed accelerated secretory cell proliferation which inhibited normal lung development and caused structural hypoplasia. The sum of these changes indicated that CFTR participated in growth and differentiation via a regulatory molecule such as extracellular ATP, known to be increased by CFTR expression. Administration of ATP in the amniotic fluid rescued the CF (*cftr*<sup>-/-</sup>) mice and established the link between CFTR gene expression and Cystic Fibrosis as an ATP-modulated developmental defect.

FIGURE 1

*Lac Z* expression in rat lung 13 days following in utero adenovirus gene transfer. Beta-galactosidase expression is highest in cells lining the airways (A), minimal in parenchymal cells, and absent in endothelial cells lining the vessels (V).



The transfer of genes into the developing fetus via the amniotic fluid is attractive because it is minimally invasive and provides ready availability to the developing lungs and intestines. Infection in this manner also limits exposure to the gonads and reduces the risk of germline modification. Several groups, including ourselves, have shown high-efficiency adenoviral-mediated transfer into the fetus (Ballard, 1995; Sekhon and Larson, 1995; Douar et al., 1997).

Direct injection of a replication-defective adenovirus encoding *lacZ* into individual amniotic sacs of rodent fetuses resulted in sustained expression with no inflammatory response (Sekhon and Larson, 1995). Gene transfer was performed at a stage of lung and intestinal development comparable to that of a 10–20 week gestation human, and targeted the somatic stem cells of the developing epithelium. These animals delivered normally and the introduction of this biologically inert transgene showed no adverse effect on viability. Lung and intestinal development was unchanged following infection with recombinant adenovirus containing *lac Z*, establishing adenoviral-mediated transfer as safe and effective in the

developing rodent fetus if the transgene was administered at an appropriate gestational age.

At the time of infection, the rodent airways were lined with multipotential columnar and cuboidal stem cells; further differentiation and further growth occurred after the infection. Figure 1 shows the high levels of  $\beta$ -galactosidase expression in the bronchi of a seven-day-old rat injected with  $10^8$  pfu/ml amniotic fluid with a replication defective adenoviral vector (AD5.CMVlacZ) at 16 days gestation. Although there are isolated cells expressing  $\beta$ -galactosidase in the periphery of the infected lungs (alveoli), maximum expression is in the cells lining the airways. These data are consistent with a centripetal pattern of normal lung growth described by lung developmental biologists (Ten Have-Opbroek, 1981) and illustrated in Figure 2. Figure 2A demonstrates a targeted airway lined with multipotential stem cells. These cells are present at the time of injection and are infected with the adenovirus. At the time of the infection approximately one third of the cells in the fetal lung are undergoing mitosis; this is mainly occurring in the distal airways and developing airspaces.

FIGURE 2

Mammalian lung development. Panel on left shows primitive airway cells targeted by adenovirus; panel on right shows where growth has occurred following gene transfer.

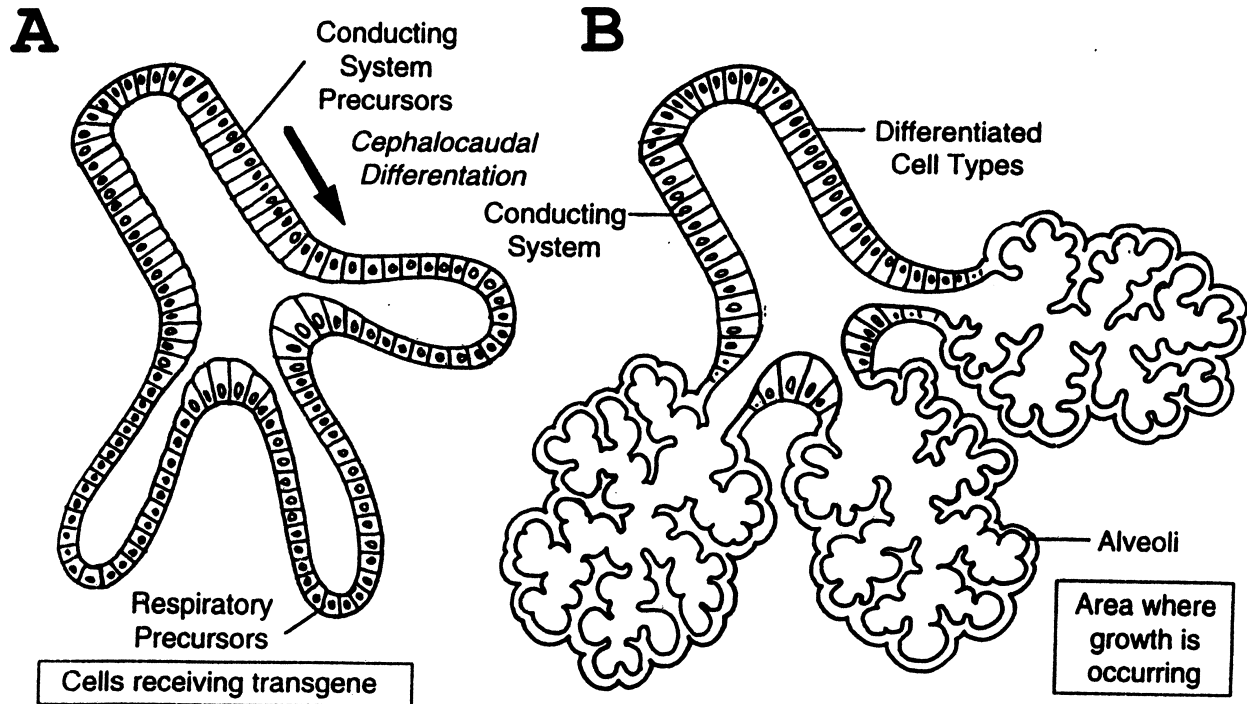


Figure 2B illustrates where the lung growth occurs between the time of infection and inspection (2 weeks following). Proliferation occurs in the lung periphery (with alveolarization) and subsequently this increased cell population expresses less transgenic protein per cell.

The effectiveness of gene transfer is highly dependent on gestation age. Treatment at later times in gestation (19 days) results in significant inflammation and limited transfer to the lung pulmonary epithelium. Transfer efficiency is high when developmental timing is correct, and higher viral concentrations are unnecessary. The fetus is immune tolerant, but will mount a response if challenged excessively (McCray et al., 1995) or later as the immune system matures. The importance of ideal timing with amniotic fluid transfer has been recently confirmed and supported by another laboratory. Douar et al.'s (1997) recent study confirms our findings that efficient infection of the pulmonary fetal airways is dependent on the developmental stage of the fetus; they also confirmed that the ideal time point for infection in the mouse is 15–16 days (corresponding to 16–17 days in the rat fetus or 10–20 weeks in the human fetus).

Cystic Fibrosis (CF) has been a major target for corrective gene therapy and an ideal candidate for *in utero* gene therapy targeting the developing lung. The gene responsible for CF, the Cystic Fibrosis Transmembrane Conductance Regulator (*cftr*) gene, was cloned without

the prior knowledge of the structure and function of the protein (Riordan et al., 1989). Structural analysis suggested that it was a cAMP-regulated chloride channel. Reports examining CFTR's chloride channel function and its possible relation to disease, however, have overshadowed observations of the protein's numerous regulatory abilities. These include regulation of other secretory channels (Guggino et al., 1995), mediation of vesicular trafficking (Bradbury et al., 1992), and alteration of glycosylation (Kube et al., 1995; Weyer et al., 1995).

CFTR expression is greater in the fetal lung than in the adult lung. CFTR mRNA and protein show temporal and tissue-specific distribution during development (McGrath et al., 1993; Tizzano et al., 1994; Gaillard et al., 1994; McCray et al., 1992; Harris et al., 1991). CFTR distribution follows the cephalocaudal pattern of maturation and differentiation of epithelial cells of the developing airways in the lung (Gaillard et al., 1994). Mammalian lung development begins proximally with the large conducting airways and proliferates distally. Once the proliferation has diminished differentiation occurs in this same cephalocaudal pattern (Ten Have-Opbroek, 1981; Kauffman, 1980). The cellular distributions of CFTR protein and mRNA change with the degree of differentiation of these epithelial cells. CFTR is diffusely expressed in immature cells and as the cells become differentiated, the localization of CFTR mRNA and protein shifts to a more apical distrib-

ution similar to an adult lung (McGrath et al., 1993; Gaillard et al., 1994). This suggests that the role of CFTR changes with the differentiated state of the cell.

When the developing epithelial cells of the lung and intestine are targeted with an adenovirus containing *dtr*, the gene responsible for Cystic Fibrosis, permanent changes occur in these organs. We demonstrated the complete reversal of the lethal phenotype of the CF (*dtr*<sup>-/-</sup>) mouse following *in utero* gene therapy as well as phenotypic changes in the targeted tissues of lung and intestine. Following transfer with a first generation adenovirus vector carrying the *dtr* gene (Av1CF2), low levels of *dtr* were detectable in the fetal gut up to 72 hours but not after birth. Although the transgene was gone and no traces of the cAMP-regulated chloride channel were found, knockout mice survived for over one year of age. Their litter mates treated with an *lacZ* transgene did not survive into adulthood (>45 days of age). These results suggest that the rescue of the knockout mouse is due to a requirement of CFTR for normal lung and intestine epithelial development.

Intestine from the *in utero dtr*-treated *dtr*<sup>-/-</sup> mice did not develop the intestinal pathology of crypt dilation and goblet cell hypertrophy that proves fatal for the untreated knockout mice (Snouwaert et al., 1992). The intestinal crypt cells in the untreated knockout mice were deficient in both intracellular uptake of Calcium Green-AM and purinergic receptors. Both of these deficiencies were partially corrected in the rescued knockout mice, and the restored cells were clonally distributed along the villi (Cohen et al., 1998).

The phenotypic changes in the lungs included epithelial airway cells. Clara cells, which are the predominant secretory cell in the terminal airways, showed marked differences in ultrastructure in animals treated *in utero* with *dtr* (Cohen et al., 1998). Clara cells in *dtr*<sup>-/-</sup> mice treated *in utero* with *dtr* showed significant increases in vesicle number and dilated smooth endoplasmic reticulum. Untreated knockout mice had an increase in secreted glycoconjugates containing  $\alpha(2,6)$ -sialic acid and fucose (both implicated in the pathogenesis of CF) when compared to control animals. Lectin histochemistry located these glycoconjugates to the airway epithelial cell surface. The *in utero* treated knockouts had an increase in this material as well, but it was contained within intracellular vesicles. These data demonstrated that *dtr* restored regulated secretion in both the lung and intestine and was required for normal differentiation of secretory cell populations. Thus, rescue from the lethal CF phenotype is characterized by correction of the dysfunctional secretory cell pathology and physiology, without permanent replacement of the cAMP-dependent chloride channel (Larson et al., 1997; Cohen et al., 1998).

The developmental effects of *dtr* occurred in other species in addition to the mouse (Morrow et al., 1998). *In utero* delivery and subsequent over expression of

CFTR at the same gestation in Sprague-Dawley rats resulted in permanent phenotypic and functional changes in the lung and intestinal epithelium. When 45–90 day old adult rats treated *in utero* with the *dtr* transgene were challenged with a lethal dose of *Pseudomonas*, enhanced resistance to *Pseudomonas* was observed.

In addition to the phenotypic changes described above, we found further evidence of the developmental effects of *dtr in utero*. This became apparent when we examined offspring of heterozygote/heterozygote matings used to produce knockout animals.

Adult animals from heterozygote/heterozygote matings treated *in utero* at 15–16 days gestation with *dtr* were genotyped to evaluate the effect of the transgene expression on genotype-specific survival. Uniform correction of a lethal autosomal recessive defect such as CF would result in a normal adult population distribution, with an expected Mendelian ratio of 1:2:1.

The population ratios of adult survivors (>75 days) following *in utero* treatment with *dtr* varied significantly from expected. Only 8% of the surviving adult population was *dtr*<sup>+/+</sup> (expected percentage of 25%;  $p < 0.001$ ). The intrauterine treatment of fetuses with *dtr* resulted in twice as many surviving knockouts as treated homozygous normal genotypes. Thus, transient expression of *dtr* during development reversed the lethal phenotype in the *dtr*<sup>-/-</sup> mice but was lethal to the *dtr*<sup>+/+</sup> mice.

Mice dying during the perinatal period were genotyped when possible. The predominant genotype of mice dying within 72 hours following birth and that had received *in utero dtr* treatment was *dtr*<sup>+/+</sup> (71%). This high perinatal mortality explained the decreased proportion of treated homozygous normal mice surviving to adulthood. Although perinatal mortality occurred in the control (Ad5.CMV*lacZ*-infected) mice, the genotype distribution of the deceased pups suggested a more random effect. Comparison of survivors of litters that received adenovirus containing *lac Z in utero* and litters that delivered without surgical intervention showed that the surgical procedure itself had no impact on the homozygous normal perinatal mortality. Therefore, the large perinatal loss seen in the *dtr*<sup>+/+</sup> population treated *in utero* with *dtr* was specifically due to the overexpression of the gene during development.

Previous work with the reporter gene *lac Z* demonstrated that injection of adenovirus into the amniotic fluid targeted the fetal intestine and the lung specifically (Sekhon and Larson, 1995). It would be expected that these would be the primary organs to reflect toxicity. To evaluate the possible toxic effects of *in utero dtr* gene therapy on the lungs and intestines, animals homozygous for the normal *dtr* allele were bred, and the fetuses were treated at 15–16 days gestation with either Ad5.CMV*lacZ*, serving as control, or Av1CF2. Only *dtr*<sup>+/+</sup> mice were used because these animals exhibited the greatest sensitivity to CFTR *in utero* toxicity. Treated mice were evaluated

TABLE 1  
Proliferative Effects of In Utero *ctr* Treatment in *ctr+/+* Mice

Parameter	Changes with <i>in utero ctr</i>	p value
Body weight	Decreased	<.001
Lung weight/body weight ratio	Increased	.031
µg DNA/mg lung (indicator of cell number)	Increased	.008
Volume proportion of saccular air	Decreased	.025
Volume proportion of air-exchanging parenchyma	Increased	.001

References: Wigglesworth et al., 1991; Cooney and Thurlbeck, 1985

at 21 days gestation, immediately prior to birth, because of the high mortality in the perinatal period. Both the lung and intestine were evaluated, but cyanosis suggested that respiratory insufficiency was the primary cause of death.

Somatic and organ growth in the *in utero ctr*-treated *ctr+/+* mice varied markedly from the *in utero lacZ*-treated *ctr+/+* mice. The body weights of the *in utero ctr*-treated *ctr+/+* mice were significantly decreased compared to the control animals (Table 1). Absolute lung weights were also significantly decreased. Despite the lung weight decrease, the lung weight/body weight ratios in the *in utero ctr*-treated *ctr+/+* mice were significantly increased. The intestine weight and intestine weight to body weight ratios were slightly increased in the *in utero ctr*-treated *ctr+/+* mice, but the differences did not approach significance. These data suggested that lung growth was either spared or accelerated following *in utero ctr* gene therapy in the *ctr+/+* mice and their somatic growth suffered.

DNA content was measured in the lung and intestine as an indicator of cell number (Wigglesworth et al., 1991). There was a significant increase in the amount of DNA per mg of lung weight of the *in utero ctr*-treated *ctr* mice (Table 1). These findings concurred with the increase in lung weight/body weight ratio in the same group and suggested that the increase in the lung weight/body weight ratio was due to increased cellularity in these lungs. The DNA content in the intestines between the two groups showed no significant difference, and this corresponded to the unchanged intestine weight between the two groups.

When growth alterations such as hyperplasia occur, morphometry can delineate the specific changes in different tissue compartments of the lung. Morphometric analysis was used to demonstrate these specific changes in lung growth. The percentage of saccular air in the ani-

TABLE 2  
Parenchymal Cell Differentiation Following In utero *ctr* Treatment

Cellular Changes	Change with Maturation	Change with <i>ctr</i>	References
Lamellar bodies			
Volume proportion	Increases	Increased p = .008	Snyder and Magliato, 1991 Williams and Mason, 1977
Glycogen			
Volume proportion	Decreases	Decreased p < .001	Snyder and Magliato, 1991 Brandsma et al., 1993
Percentage of Undifferentiated Epithelial cells			
	Decrease	Absent p < .001	Alcorn et al., 1981
Percentage of Type I and Type II Epithelial Cells			
	Increase	Increased p = .01	Alcorn et al., 1981

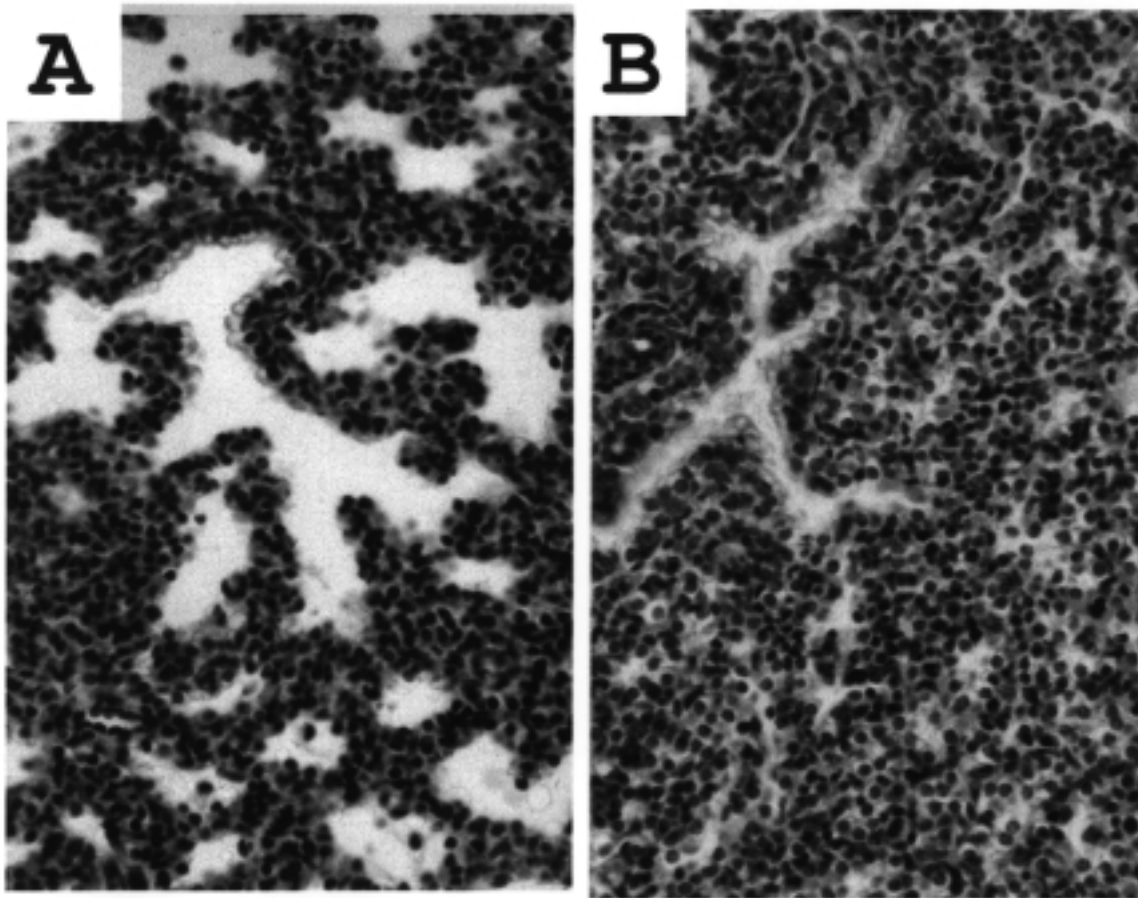
mals treated with *ctr* was significantly decreased and the percentage of saccular wall was significantly increased (Table 1). Normally, as the lung develops, the volume density of air increases and the mesenchyme and epithelia become more attenuated. The epithelial proliferation that occurred as a result of the *ctr* did not allow this attenuation to happen. The volume proportion of the remaining lung structures (airways and vessels) was unchanged between the two groups. At the time of *in utero ctr* therapy, the large conducting airways were already formed and most of the proliferation was occurring in the developing respiratory part of the lung, i.e., the distal tubules which would develop into saccules and eventually alveoli. The morphometric analysis established that *ctr* had its most visible effect on the cells that were rapidly proliferating at the time of infection.

Figure 3A illustrates the normal lung of a (*ctr+/+*) animal treated *in utero* with *lacZ* immediately prior to birth. The epithelium is beginning to attenuate and the air-exchanging interface is increasing. Figure 3B demonstrates the lung of a homozygous normal animal treated *in utero* with *ctr*. So much proliferation has occurred that the air exchanging interface is compromised and would not be able to support life after birth.

In addition to proliferation, ultrastructural examination of the epithelial cells revealed more differentiation in the *ctr*-treated animals compared to the control litters (Table 2). There was an increase in type I and type II cell epithelium and an absence of undifferentiated cells.

FIGURE 3

Effect of *in utero* CFTR on development of mouse lung. Panel A-untreated mouse parenchyma; Panel B-treated mouse parenchyma.



The predominant assumption in CF is that the pathogenesis is due to the immediate loss of a cAMP-regulated chloride channel and its affect on mucosal salt or fluid concentration. It is reasoned that these mucosal surface alterations result in defective barrier immunity. The rescue and developmental changes in the CF (*cftr*<sup>-/-</sup>) mice suggest that continuing function of CFTR is unnecessary and that CFTR participates in the growth and differentiation of the secretory epithelium. We hypothesized that CFTR regulated development via an effector molecule. Examination of the literature revealed that in addition to its role as a chloride channel, CFTR is known to increase extracellular ATP (Devidas and Guggino, 1997). Extracellular ATP has been shown to regulate proliferation and differentiation in a number of developing organs (Neary et al., 1998;

Ishikawa et al., 1997). Finally, the differentiation marker most affected by *in utero* *cftr* involved stimulation of purinergic receptors that would react with ATP. Given these data, the effect of *in utero* ATP on survival of *cftr*<sup>-/-</sup> mice was examined.

Administration of ATP in the amniotic fluid rescued the CF (*cftr*<sup>-/-</sup>) mice. Mice treated at 15–16 days gestation showed increased survival into adulthood (>45 days). Survival was significantly increased ( $p=0.003$ ) over controls treated with saline.

*In utero* CFTR appears to modulate differentiation and proliferation of secretory cells in the lungs and intestines of both the rat and mouse. The molecular mechanism for this rescue appears to be modulation of ATP concentrations either by direct stimulation of ATP secretion or by modification of ATP metabolism in the fetus.

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# Ethical Questions Related to the Prospect of *In Utero* Gene Transfer Experiments

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The prospect of human *in utero* gene transfer experiments raises some unique ethical questions. While many of these questions have been raised previously, a set of them seem particularly salient, including issues related to predicting safety of the experiments, causing 'incidental' germ line effects, selecting the target condition(s) for initial experiments with human subjects, challenges to obtaining valid informed consent, and considerations related to experimental protocols involving abortion. The complexities and uncertainties associated with these issues suggests the need for continued public discourse and prospective review of proposed research prior to pursuing the possibility of conducting such experiments with human subjects.

The theoretical simplicity of replacing single defective genes to correct serious human disease is scientifically appealing. However, while clinical experiments with gene therapy have met with limited success, its promise of efficacy has not been met. One limitation of previous attempts at gene therapy has been inadequate uptake of the new gene. Therefore, scientists have recently proposed the possibility of human experiments of *in utero* gene transfer, hypothesizing that fetal cells might more readily assimilate new genes than do adult somatic cells. As inviting as this may seem at first glance, such trials raise a set of ethical questions that warrant attention before moving forward. In this report, I will describe briefly two “pre-protocols” for *in utero* gene transfer experiments that were prepared by French Anderson and his colleagues in part to generate discussion about such experiments. I then discuss some of the relevant ethical issues raised by them.

### **Two Proposals for Human *in Utero* Gene Transfer Experiments**

Although there have been attempts at treating severe combined immunodeficiency (SCID) with gene therapy, success has been limited in part because of poor uptake of the corrective gene. Consequently, Anderson proposes to inject retrovirus incorporating the corrective gene into the fetus, anticipating uptake by fetal stem cells. Nonetheless, this approach runs the risk of “incidental” effects on the germ line.<sup>1</sup> Of relevance, stem cell transplantation can be effective in treating children born with SCID.<sup>2</sup>

The second proposal involves an intervention for fetuses with alpha-thalassemia, a disorder typically fatal *in utero* that seems to result in increased maternal risk the longer the fetus is carried. In this pre-protocol, Anderson proposes to remove cells from affected fetuses, transfect them with a retrovirus holding the corrective gene, and then return them to the fetus.<sup>3</sup> Although this approach may minimize the likelihood of germ line effects compared to the direct injection of retrovirus, the risks to pregnant women of carrying an affected fetus to term, and whether such a child brought to term would suffer in some way as a result of the intervention, are unknown.

### **Ethical Considerations**

Although all research with human subjects is accompanied by an array of ethical issues, the prospect of human *in utero* gene transfer experiments evoke some of these issues in a unique fashion. While there is some overlap in these issues to those related to *in utero* fetal experiments such as fetal stem-cell transplantation<sup>4</sup>, others are distinct.<sup>5-7</sup> For example, Fletcher and Richter address ethical questions related to: “(i) how the pre-viable fetus becomes a ‘patient,’ (ii) benefits and risks to the fetus and pregnant woman, (iii) voluntary informed consent and privacy, (iv) selection of subjects, (v) harm to germ line cells, and (vi) public oversight of fetal gene

therapy.”<sup>6</sup> In addition, the Recombinant DNA Advisory Committee (RAC) has formed working groups to examine in detail *in utero* gene transfer experiments. The work of the Preclinical Research Issues group and the Clinical Research Issues group helps to consolidate information about safety and appropriateness of such human experiments. The Ethical, Legal and Societal Issues group is addressing ethical questions related to gene transfer research (GTR) including: “(1) whether, and if so, when, to embark upon *in utero* GTR; (2) what criteria should be used to select the diseases most appropriately targeted for GTR in the fetus; (3) what should be the inclusion/exclusion criteria for recruitment-enrollment; (4) what are the concerns regarding informed consent in this context; (5) what considerations of justice apply.”<sup>8</sup>

While these questions remain important, in this report I elaborate on some of the issues that seem particularly salient, including issues related to predicting safety of the experiments, causing ‘incidental’ germ line effects, selecting the target condition(s) for initial experiments with human subjects, challenges to obtaining valid informed consent, and considerations related to experimental protocols involving abortion.

**Predicting safety of the experiments.** While the efficacy of *in utero* gene transfer can not be known until adequate studies in humans have been conducted, the safety of these interventions must be assessed carefully, not only in Phase I experiments designed to test toxicity, but also in preclinical studies. The substantial risks that might be posed to human subjects argues for the need for broad scientific agreement about the likely risks associated with a proposed intervention. In assessing the appropriateness of moving forward with a Phase I experiment of *in utero* gene transfer in humans, it is essential to consider the risks to all parties who might be potentially affected by the experimental intervention. Therefore, at minimum, risks to the pregnant woman, the fetus and future generations must be considered.<sup>8</sup> Moreover, to inform the ethical analysis, the risk assessment will need to be quite nuanced. At least three dimensions of risk need to be considered: the nature of the risk, its magnitude, and its likelihood of harm.

Determining safety also involves questions related to the accuracy of prenatal testing as well as the adequacy and appropriateness of data from preclinical animal studies. Accurate prenatal testing is needed to ensure that only affected fetuses are exposed to experimental interventions.<sup>8</sup> Lacking appropriate experience with human subjects from which to make determinations of safety, adequate and appropriate animal studies must precede human experimentation. In turn, data from these animal studies must be convincing with regard to safety as well as demonstrating that the experimental hypothesis is tenable. With potential effects on the pregnant woman, the fetus and future generations, animal models must be able

to assess safety for each. There needs to be good evidence that pregnant women are not harmed as a result of fetal manipulation, that the fetus will not be harmed as a consequence of the gene transfer, and that the germ line is not affected. Discussion among scientists at the Gene Therapy Policy Conference suggested that current animal models seem to be inadequate to answer these questions. For example, pregnant sheep may be more tolerant of manipulation than pregnant women, making it difficult to predict the likelihood of harm. Moreover, in the alpha-thalassemia pre-protocol, it is difficult to determine if gene transfer will result in maternal harm as a result of carrying the pregnancy and it is possible that although the fetus may survive to birth, its condition may be devastating. Finally, the extent to which the germ line is affected is unclear, both on empirical and conceptual grounds. That is, there seems to be uncertainty regarding what animal experiments would be needed to ensure that *in utero* gene transfer would not adversely affect the germ line and subsequent generations.

**Causing 'incidental' germ line effects.** One consequence of *in utero* gene transfer may be the "incidental" effect on the germ line. The term "incidental" has been used since investigators do not intend to manipulate the germ line, yet see it as a possible consequence of the proposed intervention. This potential consequence raises substantial reasons for caution. Until somewhat recently, there has been consensus that gene transfer experiments are ethically appropriate only if they avoided transgressing two moral boundaries. First, gene transfer should only be employed in attempts to treat serious human diseases and not traits. Second, gene transfer experiments should be directed at somatic, not germ line, cells. Proposals for *in utero* gene transfer that involve incidental germ line effects challenge this second boundary. While some scholars have opined that holding this second line may no longer make sense, it seems fair to say that there is a need for more deliberation about the acceptability of research involving germ line manipulation.<sup>9-12</sup>

Mark Lappé has suggested that incidental germ line effects associated with somatic cell therapy can be acceptable.<sup>13</sup> However, at present it seems difficult to justify *in utero* germ line experiments that involve the possibility of incidental gene transfer. While Lappé's argument relies in part on the rule of double effect, in which otherwise unethical acts may be employed as long as the intent of the agent is good (for example, the justification of using narcotic analgesics at the end of life, even though doing so may result in a shortening of life since they also can suppress respiration) the rule requires that four conditions be met: (1) the act must be good; (2) the agent only intends the good effect; (3) the bad effect is not a means of bringing about the good effect; and (4) the good must outweigh the bad.<sup>14</sup> However, in considering the acceptability of *in utero* gene transfer experiments that involve the possibility of incidental germ line

effects, applying the rule of double effect would seem to be inappropriate because of the last condition, sometimes referred to as proportionality. That is, the criterion of proportionality cannot be assessed since the good and the bad associated with these experiments are incalculable. To assume that an early experimental intervention is necessarily therapeutically good, conflates the nature of early phase research and treatment. Further, as discussed above, it is impossible to calculate the bad effects given current pre-clinical studies. Moseley has also argued that the rule of double effect fails, but does so on the grounds of intentionality rather than proportionality.<sup>15</sup> Thus, protocols that have the potential for affecting the germ line ought to be avoided in favor of those with an intrinsically safer design.

**Selecting the target condition(s) for initial experiments with human subjects.** There is a set of ethical questions related to selecting the target disease for initial human experiments. Relevant considerations include: (1) the risk/benefit calculus in the proposed experiment, (2) the prevalence of the condition and scientific value of the experiment, (3) how potential subjects will be identified, and (4) the availability of effective treatments for the target conditions.

Under the ethical principle of beneficence, there must be a favorable risk/benefit calculus for proceeding with a proposed experiment.<sup>16</sup> This measure is difficult to assess in the proposed *in utero* gene transfer experiments.<sup>6</sup> For example, in the alpha-thalassemia pre-protocol, if the fetus survives until birth this would be considered a benefit, provided it does not have to endure a life-time of suffering. Again no reliable data are available in this case to make an accurate assessment of the risk/benefit calculus.

Related to determining if the risk/benefit calculus is favorable, is the ethical acceptability of a Phase I experiment involving two research subjects, the pregnant woman and the fetus. This is a tricky area since current US federal regulations, following the deliberations of the National Commission for the Protection of Human Subjects in Biomedical and Behavioral Research, seem to invoke a therapeutic paradox of permissibility. Let me explain. The regulations preclude research in this setting "Except where the purpose of the activity is to meet the health needs of the mother or the particular fetus, the risk to the fetus is minimal . . ." <sup>17</sup> However, while some patients in Phase I experiments derive direct health benefits, by definition these experiments are intended to test toxicity, not efficacy. Consequently, justifying such research under the current regulatory scheme is difficult. This is not to say that the research ought not to be done, but in order to promote an honest examination and portrayal of the issues at hand, we may need to rethink the current way we justify such experiments.

Should human trials of *in utero* gene transfer become a reality, the selection of the disease(s) in which these

experiments are conducted will pose both risks and benefits for the subjects of this research as well as others similarly affected.<sup>6</sup> Further, any such experiment will have substantial cost that will likely be in large part borne by society. Justice requires fairness in the distribution of these risks and benefits.<sup>18</sup> Accordingly, consideration needs to be given to a variety of disease characteristics,<sup>8</sup> including the prevalence of the disease (for instance, promising results in an experiment involving a high prevalence condition might redound in benefit to a larger portion of society than an experiment in a low prevalence condition), the severity of the condition, the availability of other therapies, and the scientific value of the experiment such that the results might somehow benefit those with other diseases besides the target disease.<sup>6</sup>

There are other questions related to justice when considering how potential subjects will be identified because prenatal diagnosis for the proposed target diseases is not currently widely available. Consequently, it would be unfair if potential subjects are identified only by those who receive prenatal testing simply because they can afford to pay for it. Alternatively, if potential subjects are identified based on those with a strong family history of the disease they may be highly vulnerable to suggestions that a 'cure' is available. While routine prenatal screening might obviate some of these difficulties, doing such screening itself is associated with a set of ethical questions including the specter of false results based simply upon the test characteristics, knowing how to use this information correctly, and potential discrimination in insurance, housing, education, and employment. Given the problems associated with each of these approaches, it is clear that whatever mechanism is employed will need to be accompanied by appropriate protections to minimize such untoward effects.

Finally, the existence of effective therapies is also relevant in determining the appropriate selection of a target disease as well as the validity of the informed consent process. On the one hand, if no therapy exists for a candidate target disease, it may be easier to justify the selection of that disease. Nonetheless, such a situation undoubtedly challenges the informed consent process because decision makers might believe that they have no real choice. On the other hand, the existence of an effective therapy might facilitate obtaining valid informed consent yet the appropriateness of exposing the relevant parties to risk is less certain. However, the risks, benefits, acceptability, and efficacy of the existing treatment option also influence this assessment. Regardless, when potentially effective therapies are available as is certainly true for ADA-SCID (post-natal transplants)<sup>2</sup> and possibly for alpha-thalassemia (*in utero* transfusion),<sup>19,20</sup> this needs to be made explicit in the informed consent process.

**Challenges to obtaining valid informed consent.** While the required elements of informed consent for research are clear, obtaining meaningful informed

consent for Phase I experiments involving patients is commonly difficult due to the very understandable hope that patients may place in physicians, investigators and experimental treatments.<sup>21</sup> Protocols for *in utero* gene transfer experiments makes obtaining valid informed consent an even more formidable task.<sup>6</sup> For instance, the duration of time between diagnosing a fetal disease and the timing of an experimental intervention will obviously play a role in how long the decision-making process might be. Given the substantial uncertainties of initial experiments with *in utero* gene transfer, measures should be taken to maximize the amount of time available for this decision to be made.<sup>8</sup> In addition, as discussed above, it is likely that such experiments might be justified by invoking a therapeutic paradox of permissibility. Given this, to minimize the chance that decisions about undergoing *in utero* gene transfer experiments are not confounded by a therapeutic misconception whereby subjects believe that the intervention is going to provide effective therapy, it is critical that investigators communicate realistically about the experimental nature of the intervention.<sup>22,23</sup> Among other things, this involves using terms such as "research" or "experiment" rather than "study" or "trial," since the connotations of the former two terms are for patients more consonant with the nature of *in utero* gene transfer experiments.<sup>24</sup>

While the term "informed consent" is appropriate when considering the pregnant woman being asked to participate in an *in utero* gene transfer experiment, it seems inappropriate and conceptually ill-suited when considering the fetus and future generations. While the term "informed permission" may more accurately describe the task parents are asked to perform regarding their fetus, the implications are unclear of this permission for a yet unborn person who by virtue of being the subject of an *in utero* gene transfer experiment may very well be expected to remain a subject once born. Further, there is a clear need to examine the suitability of obtaining permission for subsequent generations that may need to be followed if there is a lingering risk of germ line effects.<sup>9,13</sup> In both cases, the right to withdraw from experimental interventions, a core component in the ethics of research with human subjects, is threatened.

**Considerations related to experimental protocols involving abortion.** Due to the difficulties in determining risks and benefits of *in utero* gene transfer, at the RAC meeting in which the pre-proposals were introduced, Anderson suggested that these interventions might be tried in pregnant women who have already made a decision to abort their fetus. While a complete examination of this topic is beyond the scope of this report, the National Commission did considerable work in this area. In summary, the Commission argued that decisions to abort should not be confounded by the decision to participate in such protocols.<sup>25</sup> In addition, even though a prior decision to

abort may have been made, it is possible that a woman might change her mind following the intervention. Therefore, even in protocols such as this, the risk to the fetus must be minimal.

### Concluding Comments

A range of moral questions accompanies the prospect of human *in utero* gene transfer experiments that need to be addressed before initiating this sort of experimentation with human subjects. This will require work in science and in ethics. There is a clear need for consensus in the scientific community about the appropriate animal data that are needed to ensure the safety of human subjects. If these scientific issues can be resolved, careful

consideration needs to be given to the target diseases for possible *in utero* transfer experiments, our willingness to trespass the moral boundaries of potential germ line effects and acceptable experimental fetal interventions, and approaches to obtaining informed permission for fetuses and future generations. In the meantime, continued public discourse and prospective review of this research should be encouraged.

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# Considerations for Future Paths of Prenatal Hematopoietic Stem Cell Transplantation

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Both fetal and maternal risks of prenatal hematopoietic stem cell transplantation to correct genetic defects should be considered and minimized. The fetal risks include: (1) procedural caused injury or abortion, (2) infection introduced by either the procedure or the cells transplanted, (3) GVHD, (4) genetic disease introduced by the transplanted cells, and (5) failure of the procedure, leaving a surviving sick child. The maternal risks include: (1) procedural caused bleeding or bowel puncture and, (2) infection introduced by the specimen. The path to successful prenatal stem cell transplantation consists of a number of trails that must be followed. These include: (1) the time of attempted transplantation, (2) the source of the cells to be transplanted, (3) the number of cells to be transplanted, (4) the possibility that serial injections of the fetus to utilize “progressively opening niches in the fetal bone marrow” might be of value, (5) the diseases for which prenatal transplantation should be considered, (6) the possibility of postnatal therapies that may enhance the proportion of successfully transplanted cells, and (7) that involving donor specific tolerance induced by the prenatal transplantation allowing therapeutic postnatal allogeneic transplantation from the same source of donor cells.

FIGURE 1  
LMP Weeks of Attempted Prenatal Transplants

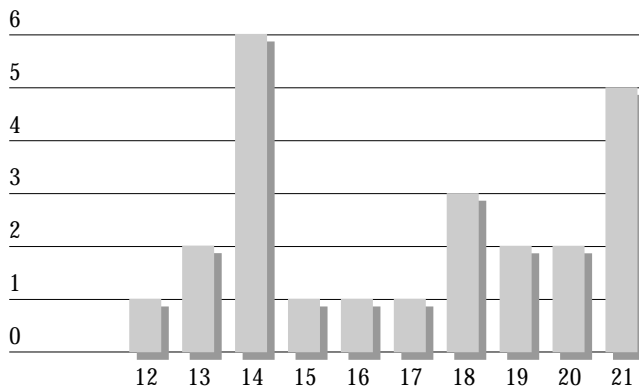
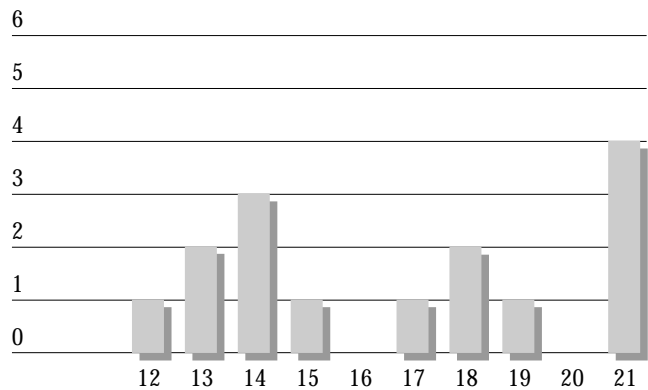


FIGURE 2  
LMP Weeks of Continuing Non-SCIDA Attempted Transplants



The first consideration for the future of prenatal stem cell transplantation is minimizing any risk to either the mother or fetus. The maternal risks include: (1) procedural caused bleeding or bowel puncture, (2) isoimmunization (e.g., Rh), and (3) infection introduced by the specimen. In practiced hands the risk of a transabdominal needle placed under ultrasound guidance piercing either a major vessel or the bowel should be infinitesimally low. Appropriate technique plus the use of anti-D gamma globulin (RhoGam) will negate the risk of isoimmunization. Proper sterile technique should obviate the risk of infection, so that, in toto, maternal risks should be quite low.

The fetal risks include: (1) procedural caused injury or abortion, (2) infection introduced by either the procedure or the cells transplanted, (3) GVHD, (4) genetic disease introduced by the transplanted cells, (5) an error in experimental design, and (6) failure of the procedure, leaving a surviving sick child. Here also, operator experience should minimize the risk of procedural problems including infection while appropriate screening of the donor should minimize the risk of cell mediated fetal infection (e.g., HIV, hepatitis, etc.). The risk of GVHD can be lessened by removal of CD 3+ cells and any number of techniques appear to be suitable for this purpose. It seems obvious that one should be sure that the donor cells do not carry the same genetic disorder that is being treated by transplantation and that the donor cells should be tested, at minimum, for that genetic state. Consideration should be given to whether there is a set of genetic tests that should be performed on the stem cell donor, similar to those done on sperm donors, to assure the absence of certain common genetic abnormalities. An error in experimental design led to the therapeutic abortion of two fetuses after prenatal stem cell transplantation resulted in no donor cells in the recipient fetus' peripheral blood 5–6 weeks later (Diukman and Golbus, 1992). We now know that demonstrable donor

cells will probably not be present until delivery even in cases of successful transplantation, and that fetal blood sampling to evaluate the transplant is inappropriate. Other such errors in experimental design are likely until the factors influencing success in prenatal stem cell transplantation are known. The final risk of any prenatal therapeutic procedure is that it will fail and the parents will deliver a child with a chronic debilitating disease. This is a risk inherent to any experimental prenatal therapeutic procedure and one which must be thoroughly discussed with the parents and be acceptable to them.

The path to successful prenatal stem cell transplantation consists of a number of trails that must be followed. The first of these regards the time of attempted transplantation. The period of fetal immunological immaturity during which allogeneic transplantation would be possible cannot be reliably inferred from animal data. The sheep accepts allogeneic cells through 43% of its gestation (Zanjani, Lim, McGlave et al., 1982), the rhesus monkey through 48% of its gestation (Harrison, Slotnick, Crombleholme et al., 1989), and some mouse strains even postnatally (Sellers and Polani, 1966). The human clinical trials to date include 24 cases (Fig. 1) (Jones, Bui, Anderson et al., 1996; Flake and Zanjani, 1997). In analyzing these cases the three spontaneous abortions, two therapeutic abortions, and four cases done for an indication of SCID (in which there is no fetal immunologic maturation) should be excluded from consideration. The remaining 15 fetuses were transplanted at 12–34 menstrual weeks (Fig. 2) with no evidence of long-term engraftment in any case. This indicates that even 13–14 menstrual weeks may be too late, and that the immunologically normal fetus already has the capability at this time to reject the transplanted cells. This assumes that this is the reason for the failures and not some other factor such as an inadequate number of CD 34+ cells to produce a successful transplant. However, in “practiced hands” it is possible to safely perform intraperitoneal

injections in a fetus of 10–11 menstrual weeks of age and future clinical trials should limit themselves to this period or slightly later. It is probably possible, albeit with increased fetal risk, to achieve fetal intravascular injections as early as nine menstrual weeks. One consequence of such early treatment is that the parents will have a very short time between a CVS diagnosis of a fetal abnormality and having to make a decision about experimental therapy.

The second trail concerns the source of the cells to be transplanted. Sources to be considered include: (1) fetal liver with or without fetal thymus cells from abortus specimens, (2) fetal circulating cells obtained by fetal blood sampling in the second trimester, (3) cord blood cells, and (4) parental marrow. The use of fetal liver cells is least practical because the normal methods of pregnancy termination lead to a high rate of bacterial or fungal contamination of the fetal tissues (Rice, Hedrick, Flake et al., 1993). Fetal blood sampling in the second trimester of pregnancy can obtain 1–5 cc. of blood which may be sufficient for gene transfection but is inadequate for transplantation purposes. Cord blood cells from pre-term and term deliveries appear to have proliferative and engraftment advantages over adult cells (Lansdorp, Dragowska, and Mayani, 1993) without the high rate of infection of fetal tissues. Experienced collectors obtain 30–300 cc of blood with a mean of 115 cc, sufficient for both a prenatal transplant and a postnatal “boost” should it be necessary (Flomenberg and Keever, 1992). The source of transplantable cells which is most readily available is parental marrow. The argument for using the father is that he will not be pregnant if it is necessary to do a later second transplant, while the argument for using the mother is that fathers are more likely to leave the family units and that with the maternal sample the question of cell transferred infection is moot. The choice should be individualized for each family depending on its dynamics. For any of these possible samples there are questions to answer as to whether the samples must be fresh or may be stored (frozen), whether the samples should be positively selected for CD 34+ cells, whether additional selection against CD 3+ cells is necessary, whether pretreatment of the cells with cytokines (and which ones) would enhance engraftment, whether stromal elements should be transplanted along with the stem cells, and for which cell transmitted infectious agents there should be screening. Some of these questions might be answered using models of xenotransplantation of human cells to fetal sheep and monkeys.

The next trail concerns the number of cells to be transplanted. The standard has been to consider this in terms of the number of nucleated cells per kilogram of recipient. It is not clear that because this may be appropriate for the child or adult patient, it is appropriate for the 5–20 gram weight fetus. Whether the number of CD 34+ cells or the number of CFU in the sample might be

a better measure of what is being transplanted should be considered. A standard of what measurements should be made on the sample to be transplanted should be agreed upon and followed by all centers working in this field so that attempts at different institutions would be more comparable.

The fourth trail involves the possibility that serial injections, rather than a single injection, of the fetus to utilize “progressively opening niches in the fetal bone marrow” might be of value. Whether this is in fact true, and what interval of injections is optimal, could be addressed using xenotransplantation of human cells into fetal sheep and monkeys.

The next trail, one that is sure to engender disagreement, concerns the diseases for which prenatal transplantation should be considered. Should the indication be diseases such as SCID for which postnatal transplantation offers a relatively easy and certain second chance for therapy, or is that an argument that such diseases should be excluded because there is already a curative postnatal therapy available? Or should the indication be diseases such as the thalassemias for which postnatal therapy involves significant risks to the patient and, therefore, the potential gain for the fetus from the experimental prenatal treatment is much greater? What about diseases such as alpha-thalassemia which would be lethal if untreated prenatally and for which prenatal therapy in the form of transfusions will be required along with the transplant? In addition to the ethical aspects, might the transfusions interfere with the engraftment? A number of the attempts at prenatal transplantation have involved fetuses with diseases with a neurological component. This author feels that it is inappropriate to include such diseases as indications unless there is animal experimental data and human postnatal transplant data for the specific disease state indicating that such therapy will also alleviate the neurological component of the disease in question. Lastly, there are issues of genotype-phenotype correlation that vary greatly for different diseases and must be considered for the disorder in question. Would it be best to limit initial attempts at prenatal stem cell transplantation to couples who have already rejected abortion as an option and have accepted the possibility of having a child affected with a specific genetic disorder? Clearly, these decisions will require input from many areas of expertise and argue for the inclusion of, at least, pediatric immunologists, pediatric hematologists, pediatric neurologists, perinatologists, neonatologists, medical geneticists, and medical ethicists on the team planning and performing prenatal hematopoietic stem cell transplants.

The sixth trail involves the possibility of postnatal therapies that may enhance the proportion of successfully transplanted cells or even of non-apparent transplanted cells. Note is made of experiments in which mice apparently unsuccessfully prenatally transplanted had donor



cells called forth from the marrow by postnatal treatment of the recipients with various cytokines (Carrier, Lee, Busch et al., 1997). Whether this applies to species other than mice, which cytokines would work best, how long the effect would last, whether the effect is repeatable, and how to make the response clinically significant are all issues to be experimentally addressed.

The last trail to be mentioned is that involving donor specific tolerance induced in the recipient by the prenatal transplantation, allowing therapeutic postnatal allogeneic engraftment from the same source of donor cells. Generally, the requirement for such tolerance has been the existence of micro-chimerism after the prenatal transplantation (Zanjani, Ruthven, Ruthven et al., 1994; Hadju, Tanigawara, McLean et al., 1996; Kim, Shaaban, Yang et al., 1998). The ongoing chimerism produces an ongoing presence of the allogeneic antigens required for the ongoing tolerance. The question of why some but not all prenatally transplanted chimeric animals (and patients) are tolerant but others are not, and what factors govern this phenomenon needs to be explored. How can we maximize the chances of such donor specific toler-

ance? Is there an optimum time for the postnatal transplantation? Zanjani and co-workers (1994) "boosted" their chimeric lambs at 3 weeks after birth and obtained 21–86% increases in donor cells in the circulation, while Hayward et al. (1998) "boosted" their alpha-thalassemic micro-chimeric newborn at 3 months of age with no increase in chimerism noted. Is this a time difference or a species difference? Should there be additional therapy such as antibodies against the recipient HLA type to encourage the take of the postnatal transplant? This technique resulted in chimerism "boosted" to more than 50% of peripheral red blood cells which was sustained for a prolonged period (Hadju et al., 1996). Should cytokines be utilized and, if so, when? The potential for donor specific tolerance, in fact, may be the real promise of prenatal stem cell transplantation to correct genetic defects.

Lastly, if one is allowed to dream, could a fetus exposed *in utero* to a mixture of HLA antigens become tolerant so that the resulting person would be a "universal recipient" for any transplants required during his/her lifetime? The path is laid out, it is only required that we proceed step by thoughtful step along it.

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# The Correlation Between Genotype and Phenotype

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Human gene therapy, first attempted in the 1970s by Stanfield Rogers,<sup>1,2</sup> may soon become a reality. Although ideally the goal of gene therapy would be replacement of the mutant gene with its normal copy, realistically most research efforts have focused on delivering a normal gene sequence to tissues in which gene mutation has resulted in absence of protein product.<sup>3</sup> It is thus likely that autosomal recessive disorders will be targeted first, although dominant disorders in which the abnormal protein product can be overwhelmed by its normal counterpart might also qualify. Other criteria for antenatal gene transfer will likely be that the candidate disease is severely debilitating and/or lethal, that it has a prenatal onset and irreversible sequelae, that no satisfactory postnatal therapy exists, that the responsible gene has been identified and completely characterized, and that a suitable vector is available.<sup>4,5,6,7</sup> Another very important requirement is that an accurate phenotype can be predicted from the fetal genotype, either alone or in combination with other diagnostic tests; such prediction will be essential both to select appropriate subjects for therapy, and to determine whether the post-transfer phenotype is the result of the therapy or simply represents phenotypic variation. While such phenotype prediction is possible for certain genetic disorders, it is not possible for many others. The determinants of phenotype, and genotype-phenotype correlation in general, are only now beginning to be understood. Many factors, including other genes or gene products, environmental influences, and gender, racial, or ethnic differences are likely to influence phenotype. Considerable phenotypic variation also results from the variety of mutations that can disable each gene; an affected carrier may express none, reduced amounts, or altered versions of the gene product, with corresponding variation in clinical effect. Although data illuminating the relationship of genotype and phenotype in many severe, molecularly characterized diseases are scant, some factors contributing to either good or poor correlation are known.

## Factors Making Phenotype Prediction More Accurate

**Single or limited number of mutations cause disease.** Phenotype prediction is easier when a single or limited number of mutations is associated with the disease. In this situation, it is more likely that the mutation(s) will result in the same alteration in gene product in every case. If other genes or gene products cannot compensate for this alteration, and if environmental or other factors are not influential, we would expect the same phenotypic changes in all individuals carrying the mutation. Examples of genetic conditions known to be caused by only one or a few well-documented mutations are rare. **Achondroplasia**, the most common non-lethal skeletal dysplasia with a birth incidence of 3.7/100,000, is one example. The gene responsible for achondroplasia is the fibroblast growth receptor 3 gene, which has been mapped to 4p16.3.<sup>8</sup> Almost all cases have a G-A transition at nucleotide 1138, and the remainder have a G-C substitution at the same location.<sup>9</sup> The altered gene product results in defective endochondral bone formation, leading to short, bowed long bones. Although this condition is autosomal dominant, 80% of cases occur as the result of a new mutation.<sup>8</sup> These new cases are not usually identified until late in pregnancy, as the altered growth is not obvious sonographically before the third trimester. Early antenatal therapy would thus be a possibility only for the 20% of cases that result from an inherited mutation.

**Factors influencing protein function are limited.** Achondroplasia is unusual, in that very few if any other genetic conditions have been identified in which the phenotype is solely the result of a single mutation. Although other diseases caused by only one or a few mutations are known, in most cases the phenotype is influenced by additional genes or environmental factors. The hemoglobinopathies illustrate this point. **Sickle cell anemia** is a severe hematologic disease in which abnormal hemoglobin structure results in impaired oxygen binding and reduced oxygen delivery to tissues. Hemoglobin is a tetramer, consisting of two alpha globin chains and two beta globin chains. In all cases, sickle cell (SS) hemoglobin has a single amino acid substitution in the beta chain, resulting from a T-A substitution at codon 6 of both beta globin genes.<sup>10</sup> All individuals carrying this mutation have a similar phenotype, characterized by red cell sickling and the inability to increase oxygen delivery to the tissues at times of hypoxic stress, resulting in hemolysis, entrapment of sickled red cells in the small vessels of major organs or structures (e.g. kidney, hand) and severe pain crises. However, red cells containing SS hemoglobin can be rendered somewhat resistant to sickling if they also carry

fetal hemoglobin. Fetal hemoglobin, consisting of two alpha chains and two gamma chains, is the predominant hemoglobin of fetal life but ordinarily accounts for less than 1% of total hemoglobin in the adult. Because fetal hemoglobin has a greater oxygen affinity than adult hemoglobin, red cells containing appreciable amounts of fetal hemoglobin are relatively resistant to irreversible sickling. Thus, individuals carrying the SS beta globin gene mutation have a milder phenotype if their red cells contain higher than normal levels of fetal hemoglobin.<sup>10</sup>

The genetic or other factors leading to persistence of fetal hemoglobin are currently unknown. However, the geographic origin of the SS mutation may be influential. It is generally believed that the SS mutation arose independently in several geographic regions, and individuals whose SS mutation originated in Senegal (as opposed to Benin or the Central African Republic) generally have elevated levels of fetal hemoglobin and thus a milder phenotype.<sup>11</sup> The geographic origin of each SS mutation can be determined by evaluation of polymorphisms associated with the beta globin gene. Haplotype analysis may therefore make it possible to predict whether a fetus carrying the SS gene is likely to have a less severe disease course.

**Siblings are similarly affected.** It is likely that many families investigating antenatal gene transfer will do so because they already have an affected child. Antenatal phenotype prediction is more accurate if siblings carrying the same mutation are similarly affected, although this is unfortunately not always the case. Diseases resulting from complete absence of a gene product, for which no genetic or metabolic compensation exists, are most likely to produce similar phenotypes in all carriers. **Duchenne muscular dystrophy** is such a disease.

Duchenne muscular dystrophy results from gene disruption at the Xp21 locus, causing a quantitative abnormality in dystrophin production, and thus all dystrophin-associated proteins.<sup>12,13</sup> The majority of Duchenne's patients (92%) produce no dystrophin at all. This deficiency disrupts muscle cell membranes, leading to an influx of calcium, mitochondrial overload, and cell death. Affected individuals have proximal limb muscle weakness, progression to other organs, a drop of 20 IQ points, and death before age 20. The dystrophin gene is 2.5 Mb in length, and the rod-shaped protein product, consisting of 3,685 amino acids, has four domains. Deletions in the amino terminal domain I result in severe dystrophin deficiency and a severe phenotype, while deletions or duplications in the central rod domain result in more variation in dystrophin levels and thus a more variable phenotype.<sup>14</sup> Although many different mutations have been described, siblings inheriting the affected gene tend to inherit the same mutation, thus allowing antenatal phenotype prediction. However, rare cases of siblings inheriting two different mutations, and families which include members with both Duchenne and Becker mus-

cular dystrophy (an allelic but milder disease caused by *qualitative* changes in dystrophin) have been reported.<sup>15</sup> Any uncertainty about phenotype resulting from antenatal DNA studies, however, may be dispelled by the availability of second trimester fetal muscle biopsy. Direct evaluation of fetal muscle dystrophin levels allows accurate phenotype prediction, and thus the possible identification of candidates for antenatal gene therapy.<sup>16</sup>

Another example of a disease caused by absence of a gene product, for which no genetic or metabolic compensation exists, is **severe combined immunodeficiency (SCID)**. SCID is actually a heterogeneous group of diseases all having in common a profound deficiency of both cellular and humoral immunity, and autosomal or X-linked recessive inheritance. Approximately 25% of cases result from a complete deficiency of adenosine deaminase, a catalytic enzyme coded by a gene at chromosome 20q13.1.<sup>17</sup> Although the exact mechanism of immune deficiency is unknown, it is likely that absence of this crucial enzyme leads to incapacitation of lymphocytes as the result of build up of toxic substrates and alterations in cAMP and ATP levels. In 80 to 90% of cases, the immunodeficiency is evident shortly after birth, while 10 to 15% of cases have a later onset (6 months to several years), probably as the result of residual enzyme activity.<sup>18</sup> Most siblings inheriting the disease are similarly affected, and although some phenotypic diversity has been observed (thought to be due to environmental influences), the clinical manifestations are consistently severe.<sup>19</sup> However, antenatal gene transfer in such cases would likely be controversial because successful postnatal therapy is available. The immune compromise characterizing adenosine deaminase deficiency facilitates hematopoietic stem cell transplantation, and obviates the need for chemotherapeutic conditioning or prophylaxis against graft versus host disease.<sup>20</sup> Successful engraftment is likely if the donor is HLA identical, or even haploidentical. Prospective candidates for either ante- or postnatal therapy can be readily identified either by molecular methods or by measuring enzyme levels in amniocytes or chorionic villus.

### Factors Making Phenotype Prediction Difficult

**The abnormal gene produces multiple clinical effects of varying severity.** Unfortunately, many more factors make phenotype prediction difficult than make it easy. Few genes act in isolation to produce clinical effects, and in most cases, the myriad influences of other factors are impossible to delineate. Even when all or most of the clinical characteristics of a disease can be attributed solely to mutation of the gene in question, different mutations within that gene may produce markedly different results. An example of this phenomenon is found in cystic fibrosis. **Cystic fibrosis (CF)** is an autosomal recessive disorder, which in its classic form is marked by abnormal

sweat chloride levels, chronic pulmonary disease, pancreatic insufficiency, liver disease, and obstructive azospermia in males.<sup>21</sup> Some individuals inheriting two CF mutations have an atypical presentation, such as pulmonary disease associated with pancreatic sufficiency, or have only isolated features such as pancreatitis, liver disease, nasal polyps, or congenital absence of the vas deferens.<sup>22,23,24,25</sup> This range of clinical expression likely reflects both the degree to which protein function is changed by the mutation, and variation in susceptibility to environmental factors associated with the altered protein. Not only can the clinical presentation vary widely, but improvements in medical care of CF patients (such as earlier diagnosis, better management of meconium ileus, improved dietary therapy including pancreatic enzyme supplementation, the use of antipseudomonal antibiotics, and routine physiotherapy) have so greatly improved survival that the life expectancy of a child born with CF today is at least 40 years.<sup>26</sup> Antenatal gene therapy for CF would probably be considered only if were possible to identify those fetuses who were destined to have the most severe form of the disease.

The CF gene is located on the long arm of chromosome 7. This gene encodes a protein of 1480 amino acid residues that regulates epithelial cell chloride channel function (the cystic fibrosis conductance transmembrane regulator, or CFTR); mutations anywhere in this large gene can result in features of CF, and over 750 mutations have been described thus far. The most common mutation in caucasians of North European ancestry,  $\Delta F508$ , and the most common mutation in individuals of Ashkenazi Jewish heritage, W1282X, are associated with the classic, severe CF phenotype.<sup>27,28</sup> Antenatal identification of a fetus homozygous for  $\Delta F508$  or W1282X would allow selection of such fetuses for possible prenatal gene therapy. However, because the  $\Delta F508$  and W1282X mutations may not be the most common mutations in other ethnic groups,<sup>29</sup> and because it is difficult to predict clinical outcome in individuals inheriting other, less common CF mutations (especially if there are no affected relatives), antenatal phenotype prediction will likely not be possible for a substantial proportion of cases. This problem has been brought to the forefront by a recent NIH consensus conference advocating offering antenatal CF screening to pregnant women with no family history of CF.<sup>29</sup>

Efforts to establish genotype-phenotype correlation in CF have generally taken one of two approaches: Patients with very similar clinical status are studied to see if they carry the same CF mutations, or individuals with identical mutations are evaluated to determine if their clinical status is similar. Both approaches have produced valuable data. For example, it has been demonstrated that several CF mutations are fairly consistently associated with pancreatic insufficiency ( $\Delta F508$ , G542X, 1717G-A, N1303K), pan-

creatic sufficiency (R117H, R334W, T3381, R347P, etc.), and congenital absence of the vas deferens ( $\Delta$ F508, R117H, 5Tvar, 7Tvar).<sup>25,30,31</sup> However, because 95% of the morbidity and mortality of CF is attributable to pulmonary disease, much effort has focused on predicting pulmonary function in CF patients.<sup>30,31</sup> Aside from the  $\Delta$ F508 mutation, very poor genotype-phenotype correlation exists for this trait. Phenotype may be indirectly predicted by assessing pancreatic function, because the major determinant of pulmonary deterioration is the onset of pseudomonas colonization, and such colonization correlates with pancreatic insufficiency (which may be predicted by the specific CF mutation identified). However, there are case reports of individuals inheriting identical CF mutations who nonetheless have very different degrees of pulmonary pathology, suggesting that environmental influences or possibly varying treatment regimens play a role.<sup>25</sup> Prediction of liver disease or diabetes mellitus, age at diagnosis, weight/height ratio, or sweat chloride levels does not appear to be possible by any means.

**The mutation is not the same in all cells.** Prenatal genetic testing, which would be required prior to antenatal gene transfer, typically involves analysis of amniocytes (sloughed fetal epithelial and mucosal cells), chorionic villus, or, in the more mature fetus, blood cells or tissue (e.g., muscle). The assumption is that the DNA in all fetal cells and tissues is identical, thus allowing accurate assessment of the whole organism by evaluating only a few cells from one tissue. Unfortunately, this assumption is not always correct. The phenomenon of somatic mosaicism, in which two or more populations of cells exist in the same organism or tissue, is now recognized as a cause of phenotypic variation. Fragile X is an example of a disease in which somatic mosaicism plays an important role in determining phenotype.

**Fragile X** is the most common heritable form of mental retardation, affecting approximately 1 in 1000 males and 1 in 2000 females.<sup>32</sup> It is characterized by mild to profound mental retardation, attention deficit/hyperactivity, features of autism, and specific dysmorphic features. Fragile X appears to be caused by the expansion of a region of trinucleotide repeats in the 5' untranslated region of exon 1 of the FMR1 gene. If the number of repeats exceeds a critical number, the FMR1 gene will be inactivated by methylation and the individual inheriting the inactivated gene will likely exhibit the full Fragile X phenotype.<sup>33,34</sup> In contrast to the tenets of Mendelian inheritance, however, this gene is not transmitted unchanged from generation to generation. Instead, the number of triplet repeats and thus gene size can increase when transmitted by the mother, but remains stable when transmitted by the father. Although the precise timing of the expansion is unknown, it is theorized to occur during maternal meiosis in the majority of cases. Normal individuals have fewer than 54 repeats, individuals with a

“premutation” have 55 to 199 repeats, and affected individuals have  $\geq 200$  repeats.<sup>32</sup> Thus only female premutation carriers are at risk to pass on an expanded, inactivated gene and to have affected offspring.

Despite the fact that the ability to determine the number of repeats and the methylation status of the gene with some accuracy allows the identification of premutation carriers, it is not currently possible to precisely determine the risk of expansion with each conception (although several sources suggest that the risk of expansion is virtually 100% if the premutation contains more than 100 repeats).<sup>35</sup> Once identified, some premutation carriers may therefore request prenatal diagnosis to determine whether or not their fetus inherited an expanded gene. Unfortunately, although the FMR1 gene can be evaluated in amniocytes and chorionic villus, phenotype prediction in a fetus carrying the full mutation is still imprecise because of several phenomena. For example, approximately 30% of females inheriting the Fragile X mutation will be intellectually normal because of favorable X-inactivation, in which the X chromosome carrying the gene expansion is selectively inactivated in the majority of cells.<sup>36</sup> It is not possible to assess the eventual distribution of X-inactivation in all tissues antenatally. Wide phenotypic variation also occurs in males inheriting the gene, due to mosaicism for the number of triplet repeats (size of the gene).<sup>32,37</sup> Mosaicism of the degree of methylation (inactivation) has also been reported in male carriers, indicating the lack of an obligatory relationship between mutation size and methylation status.<sup>38,39</sup> The existence of monozygotic twins discordant for both expansion size and methylation status, and the identification of individuals carrying different size genes in different tissues, indicate that transition to the full mutation can occur postzygotically, another phenomenon that cannot be assessed with prenatal diagnosis.<sup>40,41,42</sup> Although currently the only therapeutic option after prenatal diagnosis is pregnancy termination, identification and sequencing of the gene raises the possibility of eventual antenatal gene transfer. In the case of Fragile X, however, antenatal evaluation of a single fetal tissue may not allow accurate phenotype prediction. In addition, it would likely be difficult if not impossible to determine whether a phenotype milder than predicted by the mutation size in amniocytes was due to antenatal gene transfer or to spontaneous mosaicism.

**A specific mutation may not predict the degree of residual protein activity.** The correlation of one or a few mutations with a single disease entity raises the possibility of accurate antenatal detection of affected individuals. In some cases, however, a small number of mutations result in such a wide range of phenotypic variation that accurate predictions cannot be made on the basis of molecular analysis. Gaucher's disease illustrates this point.

**Gaucher's disease** results from a deficiency of glucocerebrosidase, a lysosomal enzyme required for the breakdown of glycosylceramide and other glycosphingolipids.<sup>43</sup> In the absence of adequate amounts of glucocerebrosidase, glycosylceramide accumulates in the lysosomes of reticuloendothelial cells. This accumulation results eventually in massive enlargement and thus decompensation of the bone marrow (leading to aseptic necrosis of the femoral heads, infarcts, fractures, and pain crises), spleen (with resultant thrombocytopenia), liver (causing fibrosis, abnormal liver function, and right to left pulmonary shunting), and other organs. Severe neurologic deterioration characterizes two forms of Gaucher's disease, and in its severest form can result in death within the first two years of life. Although rare in the general population, the gene frequency in the Ashkenazi Jewish population is 1:17, thus allowing identification of a high-risk group suitable for carrier screening, prenatal diagnosis, and possible antenatal gene transfer.<sup>43</sup>

The Gaucher's gene is located on chromosome 1q21, and has been well characterized. Despite the fact that over 30 mutations are known to occur in this gene (the majority being missense mutations that result in an inefficient or unstable enzyme), a panel of only 7 mutations accounts for more than 96% of mutations in the Ashkenazi Jewish population.<sup>44</sup> Carrier screening and prenatal diagnosis are therefore possible. The difficulty lies in phenotype prediction. There are three forms of Gaucher's disease, displaying a broad range of pathology. Type I Gaucher's disease, the most common form of the disease accounting for 99% of cases, is so mild that fewer than 20% of homozygotes are symptomatic enough to come to medical attention.<sup>44</sup> Type I disease is associated with the mutation 1226G in the majority. Type II disease, called the acute neuronopathic form because of onset at only 3 months of age and death by 9 months, and Type III disease, the subacute neuronopathic form, with childhood onset and slow progression, are usually associated with a different mutation, 1448C.

Unfortunately, the relationship between specific mutations and disease severity is not completely understood. Although the majority of individuals homozygous for the 1226G mutation are predicted to have mild disease (or even to be completely asymptomatic), some patients homozygous for 1226G have severe disease with onset in adolescence.<sup>45</sup> Conversely, although the 1448C mutation usually results in severe neuronopathic disease, individuals who are homozygous for this mutation but are completely symptom free have been reported.<sup>45</sup> Types II and III Gaucher's have been linked to the same mutation, and all three types have occurred in the same family.<sup>46</sup> The prognostic uncertainty associated with Gaucher's mutation analysis is such that inclusion of Gaucher's in population screening programs ("Jewish genetic disease carrier screening") is controversial.<sup>47</sup> Accurate prenatal identification of individuals with a severe phenotype and deter-

mination of whether the ultimate phenotype results from genetic variation or antenatal gene transfer would therefore be impossible.

**Siblings may not be similarly affected.** It is likely that many, if not all, couples seeking antenatal gene therapy will do so because they already have a severely affected child. Such couples currently are offered prenatal diagnosis, usually molecular, and decide whether or not to continue the pregnancy under the assumption that all offspring carrying the same mutation(s) will be similarly affected. While this assumption is valid for certain diseases (e.g., Duchenne muscular dystrophy), it is not valid for many others. These diseases or syndromes, in which individuals inheriting the gene mutation display none or a wide range of symptoms, severity, and age of onset, are said to have either reduced penetrance or variable expressivity. "Penetrance" indicates whether or not the gene will be expressed (in any way) if it is present; "expressivity" describes the degree to which it is expressed. The factor or factors affecting penetrance and expression are usually unknown, but are generally thought to include the genotype at other loci, exogenous or environmental influences, and stochastic factors.

**Retinitis pigmentosa (RP)** is a condition which illustrates reduced penetrance. The term "retinitis pigmentosa" applies to a group of diseases characterized by progressive visual loss, night blindness, and abnormal electro-oculogram. Because the diagnosis has traditionally relied on a clinical evaluation (the appearance of the retina), and different gene mutations/diseases can produce the same retinal appearance, classification of the various forms of RP has been confusing. In many cases RP occurs as part of a recognized syndrome (e.g., Stickler, Waardenburg, or Usher syndromes), and thus may result from the interaction of several genes, the influence of environmental factors, or a combination of both. RP also occurs as an isolated condition, however, with all forms of inheritance reported. The frequent occurrence of an individual with RP in a family with no other affected members has led to the assumption that most cases are autosomal recessive. However, it is now known that many such cases actually result from an autosomal dominant mutation with incomplete penetrance (the proband's carrier parent displays no clinical features).<sup>48</sup> Variable expressivity is also common; one report of first degree relatives carrying identical 3-base pair deletions of the peripherin gene documented a 32-year disparity in the onset of symptoms and a wide range of severity;<sup>49</sup> another report describes sisters carrying an identical RP-associated mutation who were so disparate that one sister was blind and the other could still see well enough to drive at night.<sup>50</sup> Even after careful pedigree analysis, and with molecular testing, it is not possible to accurately counsel individuals within the same family carrying identical RP-associated mutations regarding the

features of RP (if any) they will exhibit. Genetic conditions of this type would thus not be amenable to either prenatal diagnosis or antenatal gene transfer.

An example of a syndrome characterized by variable expressivity is neurofibromatosis. **Neurofibromatosis type 1 (NF1)** is an autosomal dominant disorder caused by mutations in the NF gene, located at 17q11.2, and encoding a protein called neurofibromin. Individuals inheriting an NF mutation can have any combination of features including café-au-lait spots, freckling, lisch nodules, neurofibromas, skeletal dysplasia, intellectual handicap ranging from mild learning difficulties to severe retardation, epilepsy, optic gliomas and other central nervous system tumors, sarcomas, and other rare pathologies. The majority of NF1 mutations detected thus far are thought to truncate the gene product.<sup>51</sup> Genotype/phenotype correlation is poor, with the exception of cases in which the entire gene is deleted. Thus, even though it is possible to identify NF1 mutations antenatally, it is not possible to predict phenotype even in affected siblings. Numerous NF families have come to light only after one severely affected member is diagnosed, when careful evaluation of first degree relatives makes it apparent that other family members are also (very mildly) affected. The inability to predict phenotype after molecular diagnosis has limited the enthusiasm for prenatal diagnosis, and would likely exclude this disease from consideration of antenatal gene transfer.

**Marfan syndrome**, likewise, includes such diverse clinical features that for many years researchers thought it resulted from mutations at several separate loci.<sup>52</sup> It is now known that the characteristic abnormalities involv-

ing three separate organ systems (skeletal, cardiovascular, ocular) all result from mutations in the fibrillin-1 gene on chromosome 15q21.1.<sup>53</sup> The phenotype of a carrier typically includes some combination of ocular abnormalities (myopia, ectopia lentis), skeletal disproportion (tall stature, scoliosis, arachnodactyly) and cardiac malformations (mitral valve abnormalities, aortic aneurysm and dissection). However, both the degree of pathology and the age at first manifestation vary widely, even among family members. Although a relatively large proportion of Marfan syndrome cases result from new mutations, very careful examination of the parents of the proband indicates that approximately 70–85% are familial.<sup>54</sup> Although the gene can be identified prenatally (and has been analyzed for the purpose of preimplantation diagnosis), the ultimate phenotype cannot be predicted solely on the basis of laboratory analysis.

**Gene function may be modified by gender, other genes, or epigenetic changes.** The influence of other genes, hormonal and biochemical status, the environment, and other epigenetic factors on phenotype is complex and probably impossible to sort out. At present, none of these factors can be measured, nor can their potential influence on phenotype be estimated.

Even when the importance of environmental factors is known (for example, the correlation between exposure to pulmonary infections/infectious agents and both poor respiratory function and poor prognosis in cystic fibrosis) it is not possible to predict future exposures for a fetus undergoing prenatal diagnosis. Thus, to a greater or lesser degree, some portion of phenotype can never be predicted.

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## Assessment of Fetal and Maternal Risk

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The risks or potential risks of *in utero* gene therapy include 1) risks to the mother or fetus associated with invasive prenatal diagnostic or therapeutic procedures; 2) potential risks of adverse sequelae to the fetus or the mother specific to gene therapy, some of which represent potential risk to the germ line of the fetal patient and, therefore, a future generation; and 3) risks attendant to the informed consent process which involve exchange of information between patient and caregiver in clinical medicine, or the patient and investigator in clinical research trials. *In utero* gene therapy protocols will entail different risks dependent upon multiple factors including the gestational age of the recipient, what cells or vectors are used, and at what dose. Each protocol will need to be individually addressed in terms of risk assessment.

There are several ways that the risk or potential risk of *in utero* gene therapy may be categorized: 1) There are risks to the mother or fetus attendant to all invasive prenatal diagnostic or therapeutic procedures; 2) there are potential risks of adverse sequelae to the fetus or the mother specific to gene therapy, some of which represent potential risk to the germ line of the fetal patient and, therefore, a future generation; and 3) there are those risks which we deal with daily in clinical medicine and in clinical research trials, which involve exchange of information between patient and caregiver or patient and investigator, that is, the informed consent process which includes full disclosure of alternatives, the right to voluntary participation, or to withdraw from a protocol, and the right to privacy.

All invasive prenatal diagnostic procedures (amniocentesis, chorionic villus sampling, fetal blood sampling, intrauterine transfusion, etc.) have associated risks: spontaneous abortion, or fetal demise if later in gestation; rupture of membranes; preterm labor or contractions; infection to fetus and/or mother; bleeding of the fetus or, rarely, the mother; fetal trauma; and for Rh negative unsensitized patients, isoimmunization.

Probably the greatest risk to the pregnant woman is that of intrauterine infection. While rare, it is a very serious complication. It is of utmost importance that care be taken by the obstetrician performing the procedure as well as by the laboratory personnel preparing the samples to assure *sterility* before anything is injected into the pregnant uterus. Indeed, the pregnant uterus is hallowed ground.

Additional risks of *in utero* allogeneic human stem cell transplantation, i.e., hematopoietic stem cell transplantation, include 1) viral transmission to the fetus, when you harvest the donor bone marrow, e.g., hepatitis, cytomegalovirus, etc.; 2) graft versus host disease; 3) the risk of no engraftment to the child once he/she is born; and 4) very importantly, the risk of partial engraftment, which would have an unknown effect on the disease and may dictate the need for a postnatal bone marrow transplantation. One might well consider the example of hemoglobin Bart's ( $\alpha$ -thalassemia), in which the natural history is *in utero* demise from hydrops fetalis, and for which the issue of *in utero* therapy has been raised at this conference.  $\alpha$ -thalassemia represents a case in point should only partial treatment of fetus occur. What might we expect for that fetus' life after birth—that child's life?

There are many questions which will dictate what risks are going to be involved in all of the types of gene therapy being discussed at this conference. For *in utero* hematopoietic stem cell transplantation, we do not know to what tissues these donor cells will migrate. We believe it will be dose-dependent. We are fairly sure it will be gestational age-dependent and it will probably be dependent upon donor cell types, which may vary more in the future.

For direct gene transfer we are, likewise, unsure of what cells are going to be transduced. We believe this will be dose-dependent. We know it will be gestational age-dependent because if we are using retroviral vectors that integrate with mitotically active cells or are transduced in mitotically active cells, the fetus varies greatly in its mitotic activity during gestation. And, obviously, it will also be vector-dependent.

For *ex vivo* gene transfer, what cells are going to be used? If you are going to use the fetus' hepatocytes, as have been used in some animal studies, that obviously entails a different risk to the fetus than other protocols in terms of procurement of the fetal hepatocytes, i.e., before reinjection and gene transfer back into that patient—that fetal patient. What vectors to use will confer differing risks regarding into what tissues these vectors may integrate. What are the risks from transduction or expression of the genes under investigation by cells not intended for disease correction? This is a key question in terms of risk assessment. If we think about the developing human fetus, most cells continue to proliferate rapidly in the second trimester albeit at a slower rate than in the first trimester. Therefore, retroviral vectors may integrate much more fully in the first trimester but will still be able to integrate fully into many tissues in the second trimester, and in the third trimester to some extent as well.

There needs to be special consideration for the gonads, particularly for retroviral vectors because mitotic activity implies transduceability. First, let us think about the fetal ovary. The oogonia proliferate like crazy by mitosis during early fetal life, and the proliferation slows but it is not halted until 20 weeks of gestation, at which point the number of primary oocytes reaches its maximum. The fetal testis is a different question; there is less known about it—certainly, less written about it. We do know that primordial germ cells proliferate by mitosis into prospermatogonia—some call them prespermatogonia—until the early fetal period, and thereafter the mitotic activity may slow, but I certainly do not know the answer as to precisely how late in gestation this mitotic activity of the human testes' germ cells may continue. Those who believe the testes are immune from this may be talking about adults, and not fetuses. Fetuses are different.

As for the mother, and potential reproductive risks to her in the future in terms of retroviral vectors and what maternal tissues may be susceptible to transduction, one needs to consider what tissues actively divide in pregnancy. The uterus is mitotically active; it grows during pregnancy, especially during the first part of pregnancy, in the first half of pregnancy. We know also that decidua is mitotically active; we can obtain direct metaphase preps from maternal decidua if we inadvertently use it from a chorionic villus sample, i.e., decidual cells are undergoing active mitosis. The maternal bone marrow we know is mitotically active; erythropoiesis is particularly active in the bone marrow during pregnancy. The breasts appar-

ently undergo ductal proliferation which involves mitosis during pregnancy. Thus, these sites—the uterus, decidua, bone marrow, and the breast—would be sites we might want to study carefully for inadvertent transduction in the mother.

Let us turn now to some of the critical factors we have discussed at our institution regarding *in utero* bone marrow transplantation, all of which involve risk assessment. We feel that the questions that have to be addressed for successful *in utero* bone marrow transplantation involve 1) the gestational age of the recipient; we felt that most of the procedures previously reported had been performed too late in gestation; 2) the dose of the cells infused; we felt it was too small; 3) the optimal source of the donor cells; and 4) the route of administration—the safest route may furthermore be dictated by the gestational age.

How might each of these items affect the risk for *in utero* gene therapy protocols? First, clearly the risk will be dependent on gestational age. Let us look at this procedurally (technically). If one tries to do an intraperitoneal injection on a very early fetus say at eight weeks of gestation, the head is in the way. Because of this, and the small size of the fetus, it is technically more difficult. The fetuses are more fragile, and the risk of trauma is greater. The needle is much, much bigger, relatively speaking, to the very early fetal patient versus a larger fetus, such as our first fetus who underwent *in utero* bone marrow transplantation at our institution at 13½ weeks. At 13½ gestational weeks, the fetus has become more stretched out. A safe intraperitoneal injection into the fetal abdomen becomes quite feasible because the fetus is no longer curled up, and the head is smaller relative to the rest of the body.

Fetal blood sampling also has risks that are different depending on gestational age. The earlier you go the more difficult it is. In cordocentesis, you are sampling blood or injecting cells at the umbilical cord's insertion at the placental site. At 20 weeks, the umbilical cord is fairly small. Later in gestation, when the umbilical cord is bigger, cordocentesis is, quite simply, technically easier. There has been a study by Orlandi that looked at risks of cordocentesis by gestational age; in the mid-trimester cordocentesis had a higher risk of fetal loss attributable to its being technically more difficult: 5.2% versus 2.5% for cases performed at >19 weeks of gestation.

A numeric figure that many cite for fetal blood sampling in general comes from data from a large registry that indicated a risk of fetal loss of 1.2% per procedure. This risk, however, may be higher at 17 weeks when you are e.g., procuring blood cells for an  $\alpha$ -thalassemia gene therapy protocol. Fetal blood sampling can also be performed via the intrahepatic vein. While it may sound more risky, in practice, it may actually be less risky than by cordocentesis.

Second, the risk of *in utero* gene therapy, including bone marrow transplantation, is dependent on dose. This was brought home to us very dramatically by our first case of hematopoietic stem cell transplantation for Krabbe disease. Dr. Flake has shown us animal data indicating that engraftment is dependent on cell dose. Incidentally, I would echo Dr. Golbus' plea that when we discuss cell dosage for *in utero* hematopoietic stem cell transplants, we talk in terms of CD34<sup>+</sup> cells per kilogram. When we injected the cells in our first case, we used 5x10<sup>9</sup> CD34<sup>+</sup> cells per kilogram, a dose, as mentioned, that was greater than others had used *in utero* bone marrow transplantation attempts in humans. It is also greater than the dose of donor cells used in previous *in utero* animal studies, and than that used in an adult bone marrow transplantation, which is 5x10<sup>8</sup> CD34<sup>+</sup> cells per kilogram. The result in our first fetal patient was marked over-engraftment. The fetal liver was over 95% donor in origin, the donor being the father in our protocol. The spleen was 50% donor in origin. There were myeloperoxidase positive cells in many organs of this fetus. The sites were akin to severe Rh disease in which erythropoiesis is seen in many tissues of the body. For instance, in our fetus' epicardium of the heart, which happened to be one of the tissues that was best preserved (since this fetus died *in utero*), the cells which heavily infiltrated the epicardium were myeloperoxidase positive, indicating that they were myeloid and not lymphoid in origin. These cells were also found in the kidney where they formed perivascular infiltrates. It appeared, in fact, that the fetus died of massive over-engraftment with secondary clogging of the blood vessels.

Subsequently, we performed *in utero* bone marrow transplantation on two fetuses, also affected with Krabbe disease, at a lesser dose, at 5x10<sup>8</sup> CD34<sup>+</sup> cells per kilogram. These two fetuses, while not engrafted, were born at full term and appeared normal. We therefore believe that CD34<sup>+</sup> cell dose is a very important consideration for future studies. An optimal dose of donor cells may lie somewhere between 5x10<sup>8</sup> and 5x10<sup>9</sup> CD34<sup>+</sup> cells per kilogram.

Third, the risk is going to be dependent on the source of cells, or the vector, or the gene used. Obviously, if you are going to need to procure fetal hepatocytes, which is feasible for autologous *ex vivo* gene therapy, it is going to be a riskier procedure than if you took fetal blood for autologous *ex vivo* gene therapy. The source of cells is probably going to also vary in terms of where engraftment occurs following *in utero* hematopoietic stem cell transplantation in part because e.g., fetal liver has more proliferative capacity than cord blood and than adult bone marrow.

Lastly, the risk will depend on the route of administration. Intraperitoneal injection *in utero* has less procedure-related risk than intravascular injection, and certainly amniocentesis (as per Dr. Larson's talk) would entail even

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less of a procedure-related risk. Intraamniotic injection, in other words, would confer the least risk of all.

The bottom line is that the gene therapy for each protocol, for each disease, may vary and the risks are going to vary accordingly. Each protocol will require individual consideration in terms of risk assessment.

What diseases should be addressed initially?

Historically, the initial trials of any prenatal diagnostic procedure of unknown risk were limited to patients at high risk for having an affected child. This was true of the early trials of amniocentesis, at which time we did not use ultrasound guidance, and of chorionic villus sampling; we began offering these novel prenatal diagnostic procedures to women who were age 40 or more and then later dropped to maternal age 37 years or more, or to patients who were at a 25% risk for a recessive disorder in their fetus. It might seem prudent, then, to begin clinical trials of *in utero* gene therapies of unknown risk with severe congenital disorders for which alternative postnatal corrective therapy is absent or minimally effective, or available therapy confers a high morbidity and mortality.

Risk assessment by the patient is often very different from risk assessment by the investigator despite every attempt to provide informed consent and nondirective counseling. The informed consent process for the initial clinical trials of *in utero* gene therapy must clarify the main question to be answered, for instance, for *in utero* hematopoietic stem cell transplantation, can engraftment occur? Similarly, for gene therapy, can transduction occur? We do not know if it can even happen yet. And can it occur safely? How much engraftment and then how stable the engraftment would be amongst the *secondary* questions for investigations of *in utero* transplantation to address only after some level of engraftment had already been found.

However, the patient is going to hope for a cure; patients who might be best considered for these trials are those who would not consider the option of pregnancy termination for an affected fetus and who are prepared to deal with an affected child in the event of a protocol's failure to cure. But then, why would you have chorionic villus sampling if you are not going to consider the option of pregnancy termination? For some couples, the information gained from having a prenatal diagnosis by a chorionic villus sampling can prepare the parents for the birth psychologically. For others, it can dictate the par-

ents' choice of a hospital center, as for some genetic disorders for which medical therapy of the affected neonate will be necessary immediately following delivery; as well, an affected newborn may need the availability of intensive care facilities which are not present in all community hospitals. Other patients may opt for prenatal HLA typing through chorionic villus sampling for the prospect of early postnatal bone marrow transplantation if the fetus is affected, e.g., for globoid cell leukodystrophy.

Patients are going to want a normal baby. They are going to hope for a cure. In this respect, there are some analogies to Phase I oncology trials we might consider. According to Bridget Leventhal's textbook on research methods and clinical oncology, cancer patients agree to participate in Phase I trials for two reasons: 1) the possibility of therapeutic benefit even if they realize that that possibility is small; and 2) the altruistic wish to help others. These reasons are not incompatible with a Phase I trial which involves a dose escalation study often entailing a minimally effective dose of a drug; it is, at the same time, ethically justifiable to administer new anticancer drugs to human beings only if they are being given with some therapeutic intent.

How does an investigator discuss the risks and benefits when the risks and benefits are unknown? This often presents as a dilemma to clinical investigators. And how to balance the counseling between the positive reasons as to why this therapy has been selected for a clinical trial in the first place and the lack of knowledge about potential efficacy and side effects in humans? In this respect, an independent counselor is worth considering during the informed consent process, and may be valuable. For gene therapy, an ideal person might be a genetic counselor who is well experienced in counseling issues.

One final aspect of risk assessment in gene therapy trials involves another individual, and that is the father. The father is involved in the risk assessment and decision-making in most prenatal diagnostic counseling sessions. He does sit in a different seat from the mother or the fetus. For the procurement of e.g., hematopoietic stem cells, if he is going to be the donor, his risks from bone marrow harvest are well-defined, but he does face a risk nonetheless. Even if a particular protocol entails no medical risks to him, however, he, too, will generally be included in the informed consent process that will accompany *in utero* gene therapy trials.

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# Ethical Issues in Gene Transfer Research

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Proposals for human gene transfer on fetuses raise important ethical issues. These include the nature and content of the informed consent process for such research, study-design assessment of potentially serious morbidity that could result, the obligation to provide prenatal diagnosis, selection criteria related to acceptability of abortion, science by press conference, germ-line risk, and public oversight. We address these issues with particular reference to the concept of the fetus as a patient.

The emergence of the potential for gene transfer in human fetuses raises a number of ethical concerns.<sup>1-9</sup> In this paper we address some of those concerns, with particular reference to the concept of the fetus as a patient. We provide analyses of the informed consent process, the assessment in study design of morbidity that could result from gene therapy, prenatal diagnosis, selection criteria, science by press conference, germ-line risk, and public oversight.

### **The Fetus as a Patient**

The authors have argued elsewhere for the clinical relevance and application of the concept of the fetus as a patient.<sup>10</sup> This concept should not be understood in terms of the independent moral status of the fetus, i.e., some feature(s) of the fetus that, independently of other entities, including the pregnant woman, physician, and the state, generates obligations of others to it, because all attempts to establish such status are doomed to failure. This failure results from the irreconcilable differences among philosophical and theological methods that have been deployed over the centuries of debate about the independent moral status of the fetus. Instead, the moral status of the fetus depends on whether it is reliably expected later to achieve the relatively unambiguous moral status of becoming a child and, still later, the more unambiguous moral status of being a person. The fetus is a patient when reliable links exist between it and its later achieving moral status as a child and then person.

The first link between a fetus and its later achieving moral status as a child and then person being presented to a physician is viability, the ability of the fetus to exist *ex utero* with the levels of technological support required to supplant immature or impaired anatomy and physiology through the neonatal period and into the second year of life, times at which no one disputes that childhood and then personhood exist. Viability is *not* an intrinsic characteristic of the fetus, but a function of both biology and technology. In developed countries, fetal viability occurs at approximately the 24th week of gestational age, as determined by reliable ultrasound dating.<sup>11</sup>

The second link between a fetus and its later achieving moral status as a child and then person is being presented to a physician and the decision of the pregnant woman to continue a previable pregnancy to viability and thus to term. That is, the only link between a previable fetus and its later achieving moral status as a child and then person is the pregnant woman's autonomy, exercised in the decision not to terminate her pregnancy and to present the fetus (and herself) to the physician, because technological factors do not exist that can sustain the fetus *ex utero*.

### **Informed Consent for Research on Fetal Gene Transfer**

Gene transfer technology will be introduced into the clinical setting as human subjects research. Recent studies

of the experience of subjects of research have shown that patients are not always aware when they are subjects of research.<sup>12</sup> It has been suggested that the language used during the consent process may contribute to this disturbing lack of understanding.<sup>12</sup> In our judgment, the use of the word "therapy" should be avoided in order to prevent this lack of understanding. Phrases such as "innovative therapy," "gene therapy," and "experimental therapy" therefore should not be used in consent forms or in discussions with pregnant women about their participation in gene transfer research. Instead the consent form and these discussions should be explicit about the fact that the clinical application of gene therapy to the fetus at this time is research or experimentation.

It is never obligatory for an individual who can consent for himself or herself to consent to become a subject for research. Nor is it obligatory for a surrogate, such as a parent, to consent for a patient not capable of participating in the consent process, e.g., an infant or very young child, to become a subject of research.<sup>13</sup> It therefore follows that no pregnant woman is obligated to consent to gene therapy research on her fetus, even when the fetus is a patient. This is because no surrogate is obligated to give consent to such a patient becoming a subject of research. The consent process should make this moral fact very clear to pregnant women and to others who might be involved with them in the consent process.

Institutional review boards should scrutinize consent forms and procedures to require efforts on the part of investigators to prevent coercion of a woman's decision by internal factors such as unreasoning desperation, and external factors such as partners and family members. These recommendations parallel the ethically justified practice of protecting women from subtle coercion in decisions about using assisted reproduction technologies.

Gene therapy research is new, and the informed consent process should be structured with this fact in mind. We recommend therefore that the consent process should begin with the research team inviting the pregnant woman to state what she understands about the fetus' diagnosis, available alternatives for managing that diagnosis, and the benefits and risks of those alternatives. If there is no intervention currently available she should be asked what she understands the fetal prognosis to be. This will be very important for aiding women to understand the distinction between gene transfer for uniformly lethal conditions and gene transfer for conditions that result in serious morbidity. The research team should be attentive to factual errors and incompleteness in the woman's fund of knowledge. Educating her about the protocol should begin by making sure her initial fund of knowledge is accurate, thus laying a solid intellectual foundation for the rest of the consent process.

That process should continue with an explanation of the fetus' genetic condition and how the gene transfer research is designed to address that condition. She



should be given information about the results of animal studies, especially about documented benefits and risks identified in such studies. She should also be informed about the unknown risk that transferred genes could malfunction in unpredictable ways (the law of unintended consequences).

She should then be assisted to identify her relevant values and beliefs. This can be accomplished in a nondirective fashion by asking what is important to her about this pregnancy, about having children, and about having children with potentially severe health problems. She can then be asked to assess the offered gene therapy research on the basis of her values and beliefs, thus enhancing her autonomy in the consent process.

Throughout the consent process and in the consent form, the options of abortion and nonintervention should be presented as entirely acceptable to the research team. We make this recommendation to reinforce the nondirective character of the informed consent process for gene therapy research.

Current federal regulations require paternal consent,<sup>14</sup> although, at the time we write, it is expected this regulation will be repealed. On the account we have given of the fetus as a patient, the father of the fetus does not determine whether the fetus is a patient. There is an obvious moral asymmetry between the father and the pregnant woman during pregnancy such that his role in decisions about interventions on the fetus should be a function of the pregnant woman's autonomy.

### **Assessment of Potential Fetal and Neonatal Morbidity in Study Design**

Gene transfer research will be used especially to try to reduce the mortality of uniformly lethal conditions, such as alpha thalassemia. The traditional logic of beneficence that drives such research has been that every reduction of mortality from such conditions is worth whatever morbidity might result for survivors. In the clinical setting, especially in critical care, the traditional logic of beneficence has been appropriately challenged when morbidities eliminate or greatly impair developmental capacity of survivors. As McCormick put it over 25 years ago, when critical care results in all of the patient's energies being used in an irreversible struggle to survive, critical care intervention can be stopped.<sup>15</sup> The moral lessons for gene transfer research are twofold. First, if animal studies reduce mortality but survivors are left with devastating morbidity, then human trials should not be started until animal outcomes improve. Second, human trials should include, as a stopping rule, high rates of occurrence of devastating fetal morbidity.

### **Prenatal Diagnosis after Gene Transfer**

Recall that the previsible fetus is a patient solely as a function of the pregnant woman's autonomy. For gene transfer on previsible fetuses, the exercise of such auton-

omy is greatly restricted in the absence of prenatal diagnosis to determine the effectiveness of the gene transfer. In particular, some women may want to terminate a pregnancy before viability, when there is no laboratory evidence of successful transfer. In our view, therefore, offering prenatal diagnosis should be required by IRBs for gene therapy interventions with previsible fetuses. The consent process should include careful explanation about the potential for false negative and false positive results.

### **Selection Criteria Based on Abortion Preference**

It is an accepted feature of study design in general that clinical trials should be conducted in such a way as to control for the idiosyncratic effects of patients' preferences on results. This, for example, justifies a double-blind study design.

For gene transfer research this general rule of study design raises significant ethical issues. On the one hand, to get the cleanest results one would not want any pregnancies in which gene transfer occurred to result in elective abortions. On the other, it would be desirable to prevent adverse outcomes of gene transfer through abortion in a study population of women who would accept this option.

To address the first problem, one would exclude women who indicated any willingness to consider elective abortion. To address the second problem, one would exclude women who were opposed to abortion. Both solutions share a common and disabling ethical problem: They decide for the woman whether the previsible fetus is a patient, thus unjustifiably overriding her autonomy in favor of research considerations, a paternalistic abuse of research subjects.

To avoid this unacceptable ethical problem there should be no exclusion criteria for fetal gene transfer based on willingness to countenance elective abortion. Study designs would therefore have to include elective abortion and birth of adversely affected infants as hard endpoints.

### **Science by Press Conference**

Gene transfer research is bound to attract a great deal of public concern and attention, especially in print and electronic media. Moreover, institutions that sponsor this research will be desirous of publicizing such research as a way to bring prestige to the institution. These pressures, we fear, could combine to create a very powerful incentive to bypass the rigors of scientific investigation, in particular the intellectual and clinical ethical obligations to report the results of research in the peer-reviewed literature. Consistent with the accepting journals' policies, press conferences are acceptable. This approach prevents the deleterious phenomenon of "science by press conference."

Anecdotal reports by grateful parents of a healthy newborn do not count as evidence for the efficacy and

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safety of fetal gene transfer. It follows that press conferences meeting the stipulations above should not involve parents and their names should not be released. Parents are free to release private information about themselves to the media. Institutional publicity independent of parents will help maintain the crucial distinction between scientific investigation of experimental intervention and anecdotal reports of benefit or harm.

### **Germ-Line Risks**

Fletcher and Richter have raised the important ethical concern for germ-line harm that could result from the unknown harms of gene transfer.<sup>1</sup> They propose that somatic cell gene transfer research “ought not be approved unless investigation in animal studies shows that the vector does not convey copies of exogenous genetic material into sex cells of fetuses.”<sup>1</sup> This, in our view, is a prudent recommendation designed to prevent unnecessary harm to future generations. As fetal gene transfer research matures, this position may need to be reconsid-

ered, especially when it is reliably thought that germ-line benefits convincingly outweigh germ-line harms. Any attempt to address this question will be controversial.

### **Public Oversight**

Fletcher and Richter also propose that a public body be mandated to “oversee” gene transfer research, at least for the near future.<sup>1</sup> They express confidence that the NIH’s Recombinant DNA Advisory Committee could effectively play this role. They argue that this public policy response would “continue the tradition of scientific and ethical restraint in the introduction of human gene therapy in medicine.”<sup>1</sup> Given the fractious debate about abortion in American society, such public oversight will help increase confidence among the public that gene transfer research, while unavoidably controversial, is accountable to society. The scientific community should welcome such public scrutiny as a way to build and sustain public trust in ethically controversial scientific research.

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# Recombinant DNA Advisory Committee Consensus

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The RAC continues to explore the issues raised by the potential of *in utero* gene transfer research. However, at present, the members unanimously agree that it is premature to undertake any human *in utero* gene transfer experiment.

## **Rationale**

Significant additional preclinical and clinical studies addressing vector transduction efficacy, biodistribution, and toxicity are required before a human *in utero* gene transfer protocol should proceed. In addition, a more thorough understanding of the ontogeny of human organ systems, such as the immune and nervous systems, is needed to better define the potential efficacy and risks of human *in utero* gene transfer. Prerequisites for considering any specific human *in utero* gene transfer procedure include an understanding of the pathophysiology of the candidate disease and a demonstrable advantage to the *in utero* approach. Once the above criteria are met, the committee would be willing to consider well rationalized *in utero* gene transfer protocols.

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## Conclusions

This conference should not be considered as an endorsement by the NIH of prenatal gene transfer research. Rather, this conference was an initial step in an ongoing process of active public deliberation among scientists, clinicians, families, policy makers, individuals, and groups of concerned citizens to gather expert views and solicit public opinion regarding the substantive public policy issues raised by prenatal gene transfer research. It is anticipated that these deliberations will ultimately lead to the development of Federal policy in this arena. In doing so, the NIH and the Recombinant DNA Advisory Committee (RAC) continue to serve as a unique public forum for the discussion of science, safety, and ethics of recombinant DNA research.

At present, there are insufficient preclinical data to support the initiation of clinical trials involving prenatal gene transfer. A substantial number of critical scientific, safety, ethical, legal, and social issues must be addressed before clinical trials proceed in this arena. These issues include (but are not limited to):

- Efficiency of gene transfer to target cells;
- Specificity of delivery to target cells;
- Level, duration, and regulation of gene expression;
- Appropriate disease candidates;
- Fetal immune response to transgene products and/or vectors;
- Emergence of fetal immune tolerance;
- Effects of gene transfer on pre- and postnatal development;

- Possibility of generation and activation of transmissible vector or virus;
- Possibility of initiating oncogenic or degenerative processes;
- Limitations related to the accuracy of disease diagnosis;
- Implications of diagnostic limitations on the design and conduct of clinical trials;
- Elements of optimal clinical trial design and analysis;
- Definition of clinical endpoints for the analysis of clinical outcomes;
- Potential risk to the fetus and acceptable level of risk to the fetus in human experimentation;
- Potential risk to the pregnant woman;
- Inclusion and exclusion criteria for the pregnant woman;
- Inclusion criteria for the fetus;
- Pre- and postpregnancy monitoring of the mother;
- Pre- and postpartum monitoring of the fetus/child;
- Detection and assessment of inadvertent germline transmission;
- Ethical issues specific to the fetus;
- Ethical issues specific to the pregnant woman;
- Patient recruitment/enrollment processes;
- Informed consent issues;
- Societal issues; and
- Legal issues.

## Next Steps

The RAC will continue to deliberate these issues at future meetings and is charged with the responsibility of recommending guidance on this topic.

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# Conference Agenda

National Institutes of Health  
**Prenatal Gene Transfer: Scientific, Medical, and Ethical Issues**  
Third Gene Therapy Policy Conference  
January 7-8, 1999

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## **Conference Co-Chairs:**

Jon Gordon, M.D., Ph.D., Mt. Sinai School of Medicine  
Rebecca H. Buckley, M.D., Duke University Medical Center  
M. Louise Markert, M.D., Ph.D., Duke University Medical Center  
Alexander Capron, LL.B., University of Southern California

## *Thursday, January 7, 1999*

- 9:00 a.m.      **Introductory Remarks**  
Lana Skirboll, Ph.D., NIH, Office of Science Policy
- 9:05 a.m.      **Keynote Address**  
Robertson Parkman, M.D., Children's Hospital of Los Angeles

### **SESSION I: RATIONALE AND PRECLINICAL STUDIES**

- 9:20 a.m.      **Chair Opening Remarks**  
Jon Gordon, M.D., Ph.D., Mt. Sinai School of Medicine
- Development of the Human Fetal Immune System**
- 9:25 a.m.      **The Diversity of the Antibody Repertoire is  
Constrained by Genetic and Somatic Mechanisms  
Until Two Months After Birth**  
Harry W. Schroeder Jr., M.D., Ph.D., University of Alabama at Birmingham
- 9:45 a.m.      **Ontogeny of Human Fetal B Lymphocytes**  
Richard A. Insel, M.D., University of Rochester
- 10:05 a.m.      **Human Fetal T Cells: A Transitional Lymphocyte Population**  
David B. Lewis, M.D., Stanford University Medical Center
- 10:25 a.m.      **Break**
- Preclinical Studies:**  
**Animal Models of Stem Cell Transplantation and Gene Transfer**
- 10:40 a.m.      **In Utero Gene Transfer and Expression**  
Esmail D. Zanjani, Ph.D., University of Nevada

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- 11:00 a.m.      **Fetal Hematopoietic Stem Cells**  
Maria Pallavicini, Ph.D., University of California at San Francisco
- 11:20 a.m.      **Immune Tolerance**  
William O. Weigle, Ph.D., The Scripps Research Institute
- 11:40 a.m.      **Lunch**
- 12:40 p.m.      **Prenatal Gene Transfer in Animal Models of Lysosomal Storage Diseases**  
John H. Wolfe, V.M.D., Ph.D., University of Pennsylvania
- 1:00 p.m.        **Induced Development Therapeutics for Cystic Fibrosis - The Effect of Gene Dosage**  
Janet Larson, M.D., Alton Ochsner Medical Foundation
- 1:20 p.m.        **Challenges Posed by Inadvertent Germline Gene Transfer**  
James M. Wilson, M.D., Ph.D., University of Pennsylvania
- 1:40 p.m.        **Ethical Encounters in Making the Transition from Bench to Bedside**  
Jeremy Sugarman, M.D., M.P.H., M.A., Duke University Medical Center
- 2:00 p.m.        **Questions and Discussion**
- 2:15 p.m.        **Working Group I Discussion: Preclinical Research Issues**
- 3:20 p.m.        **Public Comments**
- 3:30 p.m.        **Break**

**SESSION II: RELEVANT CLINICAL EXPERIENCE IN HEMATOPOIETIC STEM CELL TRANSPLANTATION AND POSTNATAL GENE TRANSFER**

- 3:50 p.m.        **Chair Opening Remarks**  
M. Louise Markert, M.D., Ph.D., Duke University Medical Center
- 3:55 p.m.        **Successful In Utero Stem Cell Transplants into Human Fetuses: How They Can Pave the Way for In Utero Gene Therapy**  
Jean-Louis Touraine, M.D., Ph.D., Claude Bernard University
- 4:15 p.m.        **In Utero Hematopoietic Stem Cell Transplantation: Current Clinical Status and Challenges for the Future**  
Alan W. Flake, M.D., Children's Hospital of Philadelphia
- 4:35 p.m.        **Experience with Neonatal Hematopoietic Stem Cell Transplantation**  
Rebecca H. Buckley, M.D., Duke University Medical Center
- 4:55 p.m.        **Considerations for Future Paths of Prenatal Hematopoietic Stem Cell Transplantation**  
Mitchell S. Golbus, M.D., University of California at San Francisco
- 5:15 p.m.        **Is the Time Right for In Utero Gene Therapy?**  
Robertson Parkman, M.D., Children's Hospital of Los Angeles
- 5:35 p.m.        **Questions and Discussion**
- 6:00 p.m.        **Adjournment**

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*Friday, January 8, 1999*

**SESSION III: PRENATAL GENETIC DIAGNOSTICS: LIMITATIONS AND IMPLICATIONS**

- 8:30 a.m.      **Chair Opening Remarks**  
Benjamin Wilfond, M.D., NIH, NHGRI
- 8:35 a.m.      **Current Diagnostic Techniques**  
Jennifer Puck, M.D., NIH, NHGRI
- 8:55 a.m.      **Genotype and Phenotype**  
Katharine Wenstrom, M.D., University of Alabama at Birmingham
- 9:15 a.m.      **Ethical Issues In Prenatal Decision-Making**  
Benjamin Wilfond, M.D., NIH, NHGRI
- 9:35 a.m.      **Questions and Discussion**
- 9:55 a.m.      **Break**

**SESSION IV: ISSUES IN CLINICAL TRIAL DESIGN AND CONDUCT**

- 10:15 a.m.     **Chair Opening Remarks**  
Rebecca H. Buckley, M.D., Duke University Medical Center
- 10:20 a.m.     **Prenatal Diagnosis of Neurometabolic Disorders:  
Policy Issues Relating to Gene Therapy**  
Hugo W. Moser, M.D., Johns Hopkins University
- 10:40 a.m.     **Clinical Endpoints and Measurement of Outcome**  
David Feigal, M.D., Food and Drug Administration
- 11:00 a.m.     **Assessment of Fetal and Maternal Risk**  
Karin Blakemore, M.D., Johns Hopkins University
- 11:20 a.m.     **Ethical Issues in Gene Transfer Experiments on Human Fetuses**  
Frank A. Chervenak, M.D., The New York Hospital-Cornell Medical Center
- 11:40 a.m.     **Questions and Discussion**
- 12:00 p.m.     **Lunch**
- 1:00 p.m.      **Working Group II Discussion: Clinical Research Issues**
- 2:05 p.m.      **Public Comments**

**SESSION V: REGULATORY AND INTERNATIONAL PERSPECTIVES**

- 2:15 p.m.      **Chair Opening Remarks**  
Chair, Robert J. Levine, M.D., Yale University
- Regulatory Perspectives**
- 2:20 p.m.      **FDA Oversight of Prenatal Therapies**  
Jill Warner, J.D., Food and Drug Administration
- 2:35 p.m.      **Federal Regulations and Protection of Human Research Subjects**  
Melody H. Lin, Ph.D., NIH, Office for Protection from Research Risk



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### **International Perspectives**

- 2:45 p.m. André LaPrairie, BSc, CTBS, Health Canada
- 2:55 p.m. Françoise Touraine Moulin, Ph.D., Embassy of France in the United States
- 3:05 p.m. Jayne Spink, Ph.D., Gene Therapy Advisory Committee Secretariat, United Kingdom
- 3:15 p.m. **Break**
- 3:30 p.m. **Roundtable Discussion**
- Moderator:*  
Alexander Capron, LL.B., University of Southern California
- Panelists:*
- André LaPrairie, BSc, CTBS, Health Canada  
Melody H. Lin, Ph.D., NIH, Office for Protection from Research Risk  
Françoise Touraine Moulin, Ph.D., Embassy of France in the United States  
Mary K. Pendergast, J.D., LL.M., Elan Corporation  
Jayne Spink, Ph.D., Gene Therapy Advisory Committee Secretariat, United Kingdom  
Jill Warner, J.D., Food and Drug Administration
- 3:45 p.m. **Questions and Discussion**
- 3:55 p.m. **Working Group III Discussion: Ethical, Legal, and Societal Issues**
- 4:50 p.m. **Public Comments**

### **CONFERENCE CONCLUSIONS**

- 5:00 p.m. Jon Gordon, M.D., Ph.D., Mt. Sinai School of Medicine
- 5:05 p.m. Rebecca H. Buckley, M.D., Duke University Medical Center
- 5:10 p.m. M. Louise Markert, M.D., Ph.D., Duke University Medical Center
- 5:15 p.m. Alexander Capron, LL.B., University of Southern California

### **NEXT STEPS**

- 5:20 p.m. Claudia Mickelson, Ph.D., Chair, Recombinant DNA Advisory Committee
- 5:30 p.m. **Adjournment**



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