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Experimental studies of human fetal liver cells – in regard to in utero hematopoietic stem cell transplantation

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ABSTRACT

Successful in utero hematopoietic stem cell transplantation represents a potential step forward in the management of patients with severe inherited hematological, metabolic and immunological disorders. Animal data from a number of species have demonstrated evidence of engraftment, but to date the only effective in utero transplantations (IUTx) in humans have been performed in fetuses with severe immunodeficiencies, suggesting that the fetal immune system play a vital role in the success or failure of in utero transplantation.

To study the capacity of allogenic response of the human fetus in first and second trimesters, mixed lymphocyte culture (MLC) of fetal liver cells (FLC) was performed. FLC were able to recognize and react to allogenic stimulation already from 12 weeks´ gestation and in some cases even earlier. This response varied according to not only gestational ages, but also between fetuses of the same gestational age. In all responding livers there was a tendency for increased responsiveness with increasing gestational age and the number of responding fetuses also increased with gestational age.

Depletion of human leukocyte antigen (HLA) class II+ cells from the stimulator cells resulted in a decreased stimulation index (SI) in a majority of the experiments, indicating that the reactivity is HLA class II dependent. In order to further study the role of CD3+ (T-cells) and CD56+ (NK-cells) cells in the alloreactivity in fetal cells, MLC was performed prior to and after depletion of these cell-subpopulations. The MLC response was reduced either after CD3+ or CD56+ cell depletion from responder cells. Both cell-types may exhibit complementary effects in fetal alloreactivity.

The possibility to decrease colony-forming capacity in FLC was also explored. Such regimens have been discussed as a way to create an advantage for transplanted cells to increase the potential for homing and engraftment after in utero stem cell transplantation. Parvovirus B19 is known to inhibit erythropoietic colony formation in vitro. The hypothesis that recombinant B19 empty capsid proteins (VP1, VP2) could themselves suppress erythropoietic colony formation in the absence of non-structural proteins, was explored. The B19 capsids were shown to inhibit colony formation of BFU-E (70-95%). When B19 capsids were pre-incubated with anti-B19 monoclonal antibody or IgG positive sera, the inhibitory effect on BFU-E was completely blocked. The B19 capsids inhibitory effect could also be reduced by at least 25% by pre-incubating the FLC with anti-P monoclonal antibody.

The sensitivity of fetal hematopoietic stem cells to chemotherapeutic and immunomodulative agents, measured as colony-forming capacity was tested. FLC were incubated with doxorubicin, daunorubicin, antithymocyte-globulin (ATG), Ortoclone OKT-3 and betamethasone. All drugs tested were capable in variable degrees to decrease colony-forming capacity of fetal hematopoietic progenitor cells. However, cytotoxic drugs may adversely affect the fetus. These potential risks needs to be compared to the severity of the disease to be treated and the gains of transplantation.

A case of IUTx with cryopreserved FLC given at 14 weeks by a single intraperitoneal injection to a male fetus with X-linked SCID is reported. Engraftment was detected at 24 weeks, and the fetus had a normal T-cell count at 33 weeks. Complete reconstitution of T-cells was achieved before birth, and the clinical course during the first two years of life has been completely uneventful. Although experience of IUTx in X-linked SCID is still limited, IUTx might be offered as a therapeutic option in cases where the parents are considering termination of an affected fetus identified at prenatal diagnosis.

Key words: In utero transplantation, fetal liver cells, mixed lymphocyte culture, CD3, CD56, HLA class II, parvovirus B19 capsids, corticosteroids, cytotoxic drugs, colony formation.

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To Peter
Johanna and Fredrik

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-V).

I Lindton B, Markling L, Ringdén O, Kjaeldgaard A, Gustafson O, Westgren M. Mixed lymphocyte culture in human fetal liver cells. *Fetal Diagn Ther* 2000;15:71-78.

II Lindton B, Markling L, Ringdén O, Westgren M. In vitro studies of the role of CD3+ and CD56+ cells in fetal liver cell alloreactivity. Submitted.

III Lindton B, Tolvenstam T, Norbeck O, Markling L, Ringdén O, Westgren Broliden K. Recombinant parvovirus B 19 empty capsids inhibit fetal hematopoietic colony formation in vitro. *Fetal Diagn Ther* 2001;16:26-31.

IV Lindton B, Markling L, Ringdén O, Westgren M. In vitro studies of hematopoietic colony-forming capacity of human fetal liver cells at exposure for cytotoxic and immunomodulative drugs. *Fetal Diagn Ther* 2002;17:104-109.

V Westgren M, Ringdén O, Bartmann P, Bui TH, Lindton B, Mattson J, Uzunel M, Zetterquist H Hannsmann M. Prenatal T cell reconstitution after in utero transplantation with fetal liver cells in a patient with X-linked SCID. *Am J Gyneol Obstet* In press.

Cover illustration by Gösta Millberg

CONTENTS

ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
ATG	antithymocyte globulin
BFU-E	burst forming units-erythrocytes
BM	bone marrow
CD	cluster of differentiation
CFU-GEMM	colony forming units-granulocytes, erythrocytes, macrophages, megakaryocytes
CFU-GM	colony forming units-granulocytes, macrophages
cpm	counts per minute
CTL	cytotoxic T-lymphocyte assay
CVS	chorion villi sample
FBS	fetal blood sample
FLC	fetal liver cells
GA	gestational age
GVHD	graft-versus-host disease
HLA	human leukocyte antigens
IUTx	in utero transplantation
MAb	monoclonal antibody
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
NK cell	natural killer cell
NOD/SCID	non-obese-diabetic/severe combined immunodeficiency
OKT-3	Orthoclone, monoclonal antibody against CD3
PBL	peripheral blood lymphocytes
SCID	severe combined immunodeficiency
SI	stimulation index
TCR	T-cell receptor

Introduction

Successful in utero hematopoietic stem cell transplantation may represent a major step forward in the management of patients with severe inherited hematological, metabolic and immunological disorders. The rationale for in utero stem cell transplantation is based on three primary assumptions. First, treatment of certain diseases is only possible in utero because the target disease is lethal for the fetus (e.g. alpha-thalassemia) or the disease results in early fetal and neonatal central nervous system morbidity making postnatal therapy less efficacious (e.g. globoid leukodystrophy). Second, unlike postnatal settings, the immature fetal immune system may be relatively more permissive for the transplantation of human leukocyte antigen (HLA) partially or non-matched donor cells that will ultimately be recognised as "self" by the fetus. Third, fetal growth should create abundant hematopoietic niches or homing sites facilitating engraftment and in vivo expansion of donor cells. In addition, treatment of some diseases, where fetal morbidity does not occur, may be preferable due to improved long-term outcomes (Blazar et al 1998). Furthermore in utero transplantation is easier and less problematic in many ways than post natal hematopoietic stem cell transplantation (SCT), because the uterus provides a natural barrier to infections.

Successful allogenic in utero transplantations of hematopoietic stem cells have been reported in several animal models. The first attempt to perform a human fetal transplantation using fetal bone marrow for severe Rh disease, was reported in 1967 (Davies et al 1967). The first successful transplantation in a human fetus (with Bare lymphocyte syndrome) was reported by Touraine in 1989 (Touraine et al 1989). Since then 39 in utero transplantations have been published or reported at national or international meetings. The transplantations have been performed for a variety of indications, at various gestational ages and with different donor cells. To date, all cases of in utero transplantations performed in fetuses with normal immunological function have clinically failed, and unequivocal engraftment has been demonstrated only in fetuses suffering from severe immuno-deficiency disorders. Because of the failure of in utero stem cell transplantation in the immunolgically intact fetus, the assumption that the fetus is an ideal candidate for stem cell therapy needs to be re-evaluated.

Experiments of nature in animals

Dizygotic twin cows, marmosets, and goats have provided three experiments of nature suggesting that early in utero hematopoietic stem cell therapy could result in clinically relevant levels of chimerism, reduce or possibly correct disease and induce tolerance. In these species it is assumed that early placental blood vessel anastomosis occurs, allowing mixing of the two fetal circulations (Owen 1945, Picus et al 1985). In dizygotic twin cows, mismatched for the presence of alpha-mannosidosis, the severity of the disease is reduced in the affected cow (Jolly et al 1976). Similar reductions in the severity of storage diseases have been noted in twin cattle with Pompe´s disease (Howell

et al 1991) and in triplet goats with Beta-mannosidosis (Jones et al 1993). Additionally, dizygotic twin cattle demonstrate inter-twin tolerance in mixed lymphocyte culture (MLC) (Emery et al 1980a) and to skin grafts and renal transplants (Emery et al 1980b). Dizygotic twinning in marmosets often results in a high degree of hematopoietic chimerism (28-82% by peripheral blood karyotype). Like twin cattle, in utero induction of tolerance has been demonstrated in twin marmosets, which respond appropriately to allogenic stimulation but not to the cells from their twin using cytotoxic T lymphocyte assays (CTL) (Picus et al 1985).

It is important to note that transplantations through placental anastomosing vessels are different from experimental fetal transplantation. With placental anastomosis the mixing of fetal blood is likely to occur very early in the pregnancy and continuously.

Experiments of nature in humans

While the frequency of hematopoietic chimerism in dizygotic human twins at birth is relatively high (8% for twins and 21% for triplets), the level of chimerism is very low (<<1%) (van Dijk et al 1996). These data are consistent with placental architecture studies showing that there is no significant placental anastomosis between most dizygotic human twins. However, more than 30 cases of opposite sex dizygotic twins have been reported with higher levels of hematopoietic chimerism (Race et al 1975, Bird et al 1980, Hansen et al 1984, Vietor et al 2000, Kuhl-Burmeister et al 2000). Presumably in these cases early placental fusion and anastomosis must have occurred. In some of them it has been demonstrated that the infants are not HLA identical and in two of these cases long-term follow-up has shown stability of the chimerism (Hansen et al 1984, Race et al 1975). In one case chimerism has been permanent, persisting longer than 25 years after birth, with relatively high levels of chimerism in erythroid (5%) and lymphoid (25%) cells (Hansen et al 1984). In a number of other cases absence of reactivity in MLC and acceptance of co-twin skin grafting have demonstrated tolerance between the two twins (Thomsen et al 1977, Vietor et al 2000, Angela et al 1976).

Experimental animal models

Mouse models

In utero stem cell transplantation in mice has been studied in strains with and without lympho-hematopoietic defects, and in syngenic and allogenic models. In utero transplantation in mice with congenital defects of their lymphohematopoietic system (WW, SCID, and NOD-SCID) suggests that when a competitive advantage exists for the donor cells high rates of engraftment and levels of chimerism will be produced. Severely anemic W/W mice (in which cellular defect occurs at the level of the stem cell) transplanted with allogenic fetal liver cells have complete reconstitution of the erythroid lineage in 75% of the animals, while transplantation in mildly affected heterozygous (W/+) and non-affected animals (+/+) resulted in no evidence of engraftment (Fleichman et al 1979). In NOD-SCID mice, with absent T-cells, B-cells and NK cells, in utero transplant studies have demonstrated that 66% of the animals engrafted with levels of chimerism of 25%

in bone marrow and 40% in peripheral blood. When SCID mice with absent T-cell and B-cell lines are transplanted, the rate of engraftment (28%) and level of chimerism (8% in bone marrow and 16% in peripheral blood) was lower than that seen in more severely affected NOD-SCID model.

Allogenic in utero transplantation in fetal mice without stem cell or immune defects has resulted in engraftment in a significant number of animals, but the level of chimerism has been very low (<2%) (Carrier et al 1995, Hajdu et al 1996). Despite the very low levels of chimerism there was evidence of tolerance induction and in beta-thalassemia knockout mice, high levels of donor hemoglobin was noted (Hayashi et al 2000). In mice with evidence of tolerance (durable skin grafts) engraftment could be enhanced with postnatal booster transplantation (Carrier et al 1995, Kim et al 1999, Milner et al 1999).

Ovine and caprine models

The majority of work, evaluating the feasibility of in utero HSC transplantation has been done in the ovine model (Flake et al 1986, Zanjani et al 1995). In the allogenic model, at 0.38 and 0.45 gestation, the proportion of fetuses engrafting varied from 29% (donor cells from T-cell depleted adult bone marrow) to 84% (donor cells from fetal liver). The level of chimerism ranged between 5-14% (Zanjani et al 1997). By increasing the number of T-cells within the donor cell pool the level of chimerism and the risk of GVHD increased in postnatal SCT. Tolerance induction after in utero transplantation shown as reduced response in MLC has also been demonstrated (Zanjani et al 1994).

In contrast, transplantation attempts in genetically defective sheep and goat fetuses have not been as encouraging. When transplanting fetal liver cells into fetal goats affected with beta-D-mannosidosis, the animals were born with low levels of chimerism and there was no clinical effect from the donor cells (Pearce et al 1989). Similar experiments have been carried out in fetal sheep affected with ceroid-lipofuscinosis.(Westlake et al 1995). Monitoring of blood and bone marrow cells showed that an average of 9% of blood cells were of donor origin. No effects were evident in the clinical course of disease, brain weight, or histopathology of organs between transplanted and non-transplanted lambs.

Non-human primate model

Non-human primates have been suggested as a relevant animal model for in utero transplantation(Harrison et al 1989, Shields et al 1995). Harrison reported five rhesus monkeys transplanted by intraperitoneal injection with allogenic fetal liver cells at 0.36 gestation using cryopreserved fetal liver cells (1 x 10^{8-9}/kg). Engraftment was noted in the four survivors with a level of chimerism ranging between 2.4-6.2% in peripheral blood. In utero transplantation using T-cell depleted haploidentical bone marrow cells has been tested by two investigators. (Brent et al 1989,Covan et al 1996). Brent (Brent et al 1989) attempted in utero stem cell transplantation in 22 cynomolugous monkeys between 0.3 and 0.57 gestation using large numbers of allogenic T-cell depleted adult bone marrow (0.2-2.6 x 10^{10}/kg). Eight of the 14 fetuses (57%) transplanted at <0.45 gestation became chimeric. Unfortunately, all of the chimeric animals died in utero and the authors concluded that the fetal death were most likely from GVHD secondary to inadequate T-cell depletion. Prior to the fetal death four of the eight chimeric fetuses were hydropic. One of these hydropic animals was delivered before intrauterine death and GVHD was confirmed histologically.

Cowan (Cowan et al 1996) studied 25 rhesus monkeys using T-cell depleted (CD2) haploidentical adult bone marrow. Engraftment was noted in 8 of 11 survivors. Even with an average CD34+ cell dose of 5

x 10^7/kg the level of engraftment was low (<0.1%). In utero induction of tolerance was also tested in six of the surviving engrafted animals. A reduced response in MLC was noted in four of the animals when tested against the donor as compared to third party stimulators. Further evidence of tolerance induction to solid organ (kidney) grafts was also demonstrated in two of these animals.

Experiments of nature all suggest that in utero hematopoietic engraftment with clinically relevant results should be feasible. Animal data, from a number of species, have also demonstrated that engraftment occurs after in utero stem cell transplantation and that there is also tolerance induction in some of the engrafted animals. Unfortunately, to date, only in animals (mice) with cellular defects of their stem cells or immune system and sheep has it been possible to repeat the encouraging results noted in the experiments of nature. The fetal sheep model has provided many important insights relevant to in utero stem cell transplantation. Unfortunately they have not been informative for human trials of in utero therapy. The reason for the lack of clinical results in human cases, excluding immunodeficiencies, has not been clearly elucidated. Many questions remain to be answered. We still have little insight into the importance of cell type, immune reactions such as host vs. graft (rejection) and graft vs. host (GVHD) reactions, and alternative methods (non-toxic and target specific conditioning) that may enhance our ability to achieve clinically relevant levels of chimerism after in utero stem cell therapy.

Cell sources

Hematopoietic stem cells have been isolated from fetal liver, umbilical cord blood, adult bone marrow, mobilised peripheral blood and spleen cells. They are best described by their function, the ability to generate mature cells of all lymphohematopoietic lineages and their self-renewal capacity.
There are potential advantages of using fetal liver cells for in utero transplantations. Fetal liver derived stem cells are able to sustain long-term engraftment after in utero transplantation far more readily than adult marrow (Harrison et al 1997), probably owing to their comparatively longer telomeric repeats (Lansdorf et al 1995). They are also primed for developing in the fetal environment, and if retrieved early enough in gestation, they contain no post-thymic immunocompetent T-cells (Roux et al 1996). It is also possible to retrieve sterile fetal liver cells from first trimester abortions (Westgren et al 1994). In our opinion, the major limitations using fetal liver cells are ethical. If postnatal booster therapy is needed after in utero transplantation resulting in low levels of chimerism, the donor cells must be obtained from a renewable source, for instance adult related bone marrow.

Fetal hematopoiesis

The first hematopoietic cells are developed extra-embryonically in the yolk sac in the 5th week of gestation (Bloom et al 1940, Tavian et al 1999). The yolk sac hematopoiesis is restricted to erythropoiesis and only nucleated erythrocytes are produced (Marks et al 1972). At the same time, in gestational week 5, hematopoietic cells are found in the ventral endothelium of dorsal aorta (Tavian et al 1996). These cells exhibited the surface

antigens CD45+CD34+Lin- and in vitro behaviour and gene expression were characteristic for primitive hematopoietic progenitors (Labastie et al 1998). Culturing these hematopoietic cells generated high numbers of CD34+ cells (Tavian et al 1999) and dorsal aortic cells have been suggested to be the primary hematopoietic cells responsible for further fetal hematopoietic development. In fetal liver, hematopoietic cells are found from 6 weeks gestation (Migliaccio et al 1986) and differentiation to the granulocytic and megakaryocytic lineage is observed as well as the erythropoietic cell lineage. From the 7th week of gestation hematopoietic cells are found in spleen with the highest number of CD34+ cells in gestational weeks 14-22. The precise role of the spleen for fetal hematopoiesis is not clear. It has been suggested that the fetal spleen is not normally a hematopoietic organ and that entrapment from fetal blood explains that precursor cells could be found there (Calhoun et al 1996, Wolf et al 1983). In the late second or early third trimester, fetal bone marrow becomes the major hematopoietic organ. (Tavassoli et al 1991).

Already in the second trimester the fetal bone marrow contains high levels of CD 34+ cells (24.6%) compared to 3.1% in adult BM (Michhejda et al 1996, Wu et al 1999). In fetal blood, the frequency of CD34+ cells is higher (6,4% ± 1,3%) in early gestation (17-24 weeks´ GA) than in 37th–41th weeks´ GA (1,3% ± 0,2%) (Shields et al 1998). Fetal stem and progenitor cells are characterised by a high proliferative rate in vitro compared to postnatal bone marrow cells (Opie et al 1998, Thilaganathan et al 1994, Muench et al 1994) The rapid fetal growth suggests that fetal hematopoietic cells needs to expand tremendously. In these rapidly expanding organs it has been assumed that available hematopoietic homing sites are present, allowing donor cells to engraft. However, while fetal hematopoietic environment is expanding at a very rapid rate, the fetus is producing very high levels of hematopoietic progenitor and stem cells that are likely to be competitors to allogenic donor cells. In addition, the homing sites for allogenic donor cells may also affect the degree of engraftment. (Almeida-Porada et al 2000).

Fetal immunology

The early second trimester fetus has been described as preimmune, i.e. unable to mount an immunological response against donor cells (Touraine et al 1989, Crombleholme et al 1991, Zanjani et al 1997). Available published reports of in utero transplantation in humans suggest that this theory needs to be re-evaluated. The only clinically successful in utero transplants have been achieved in severely immunologically compromised fetuses, and thus the fetal immune system likely plays a vital role in the success or failure of in utero transplantation.

The appearance and development of differentiated fetal immune cells such as T- B- and NK-cells occurs at different stages during gestation. Fetal T-cell development is thought to begin with the migration of hematopoietic fetal liver cells to the fetal thymus at approximately 7th-8th week. Shortly after this time period 20-50% of the cells in the fetal thymus express the common T-cell surface antigens CD7 and CD2. VDJ rearrangement and TCR beta transcripts are detectable in first trimester fetal liver cells and these cells are alloreactive against HLA class I molecules (Renda et al 2000). These findings concur with functional studies showing alloreactivity in fetal cells from 9.5 weeks gestation (Stites et al 1974) and cell-mediated lympholysis in human fetal blood lymphocytes, demonstrated in a 16 weeks fetus (Rayfield et al 1981).

Relative to T-cell development, the ontogeny of fetal B-cell development begins slightly later in gestation. In fetal liver as early as in 7-8 weeks gestation, cells expressing early B-cell antigen CD19 and CD20 can be detected using immunofluorecence (Gathings et al 1977). Surface expression of IgM can be noted in weeks 9^{th}–10^{th}. Cells in fetal circulation express common B-cell antigen (CD20) at 14-16 weeks and secretion of IgM has been noted as early as at 15 weeks gestation. IgG secretion is first noted at 20 weeks gestation.

Natural killer (NK) cells are likely to play an important role in early fetal immunological response. NK and T cells share many features and a common intrathymic precursor (Carson et al 1996, Carlyle et al 1997, Phillips et al 1992). From the 9^{th} week of gestation NK cell activity was observed in fetal liver (Uksila et al 1983) and these cells can mediate non MHC-restricted cytotoxicity against NK-sensitive targets (Phillips et al 1992). NK cells function through direct cytotoxicity to foreign antigens, usually virus-infected cells, tumor cells or trophoblastic cell stimulation, as well as antibody-dependent cell mediated cytotoxicity (ADCC). In the fetus NK cells appear early and in high frequency. When expressed as percentage of the total lymphocytes, the proportion of NK-cells in the fetal circulation is 29% at 13 weeks (Thilaganathan et al 1993). As has been suggested for postnatal bone marrow transplantation (Lanier et al 1995), NK cells may play a role in graft failure after in utero transplantation.

HLA

In postnatal bone marrow transplantation HLA identity between recipient and donor is of major importance for successful engraftment and manageable risks of GVHD (Ringdén et al 1993). The rate of both graft failure and GVHD is directly related to the degree of HLA mismatch (Beatty et al 1985, Anasetti et al 1989, Jankowski et al 1997). The few experiments of nature (marmosets and cows) and the ovine model of in utero transplantations have demonstrated that successful engraftment with clinically relevant levels of chimerism can occur even when significant major histocompatibility antigen barriers appear to exist. Based on this data, it is presumed that successful engraftment will occur with less than perfect HLA-matching, presumably due to the fetal immune system and induction of tolerance (Zanjani et al 1997, Crombleholme et al 1990, Crombleholme et al 1991, Diukmann et al 1992, Hajdu et al 1993, Touraine et al 1990). Unfortunately, the levels of chimerism noted in animal models have not been repeated in human in utero stem cell transplantation in absence of immunodeficiencies. To date, the role of HLA matching for engraftment and levels of chimerism for in utero transplantation has not been addressed.

Tolerance induction

Induction of tolerance has been demonstrated in mice (Carrier et al 1995) sheep (Zanjani et al 1994) and non-human primates (Cowan et al 1996). Furthermore increased hematopoietic chimerism has been demonstrated in mice and sheep after postnatal booster grafts. Two cases of human intrauterine transplantation have been reported where donor specific tolerance was tested. In both of these cases a reduced

response to the donor was noted in MLC and in CTL (Thilaganathan et al 1993, Hayward et al 1998).

Cells trafficking placenta

It is well established that trafficking of cells between fetal and maternal circulation occurs in human pregnancies (Lo et al 1996). Fetal cells could be detected in maternal circulation (Nelson et al 1998) and used as a non-invasive method for diagnosis of fetal chromosomal anomalies (Bianci et al 2000). In the opposite direction, maternal cells can traffic placenta to the fetus. It is well known that in fetuses with inherited severe immunological disorders such as SCID, engraftment of maternal cells can occur and the children suffer from GVHD at birth (Sottini et al 1995, Muller et al 2001). Even in normal pregnancies maternal cells could be found in fetal circulation (Lo et al 1998, Scaradavon et al 1996, Lo et al 2000, Artlett et al 2000). How the fetus react to these maternal cells and inactivate them are not known, but it is likely that such cells are rejected in immunologically normal fetuses.

Human in utero transplantation

39 cases of allogenic in utero hematopoietic stem cell transplantation in humans have been either published or presented at national and international meetings. The timing of the transplantation, source of donor cells and the target disease have varied. Detectable engraftment has been reported in 13 of the 39 cases. In all cases, with the exception of the severe immunodeficiencies, the level of chimerism has been very low and had no impact on the course of the disease being treated. In four successful well-documented cases, of in utero therapy of X-linked SCID, the infants were born with functional T-cells, reducing the risk of postnatal opportunistic infection. In one case, tested for B-cell function, the infant showed near normal immunoglobulin (IgM, IgG) response (Flake et al 1999). The success of in utero stem cell therapy for SCID will have to be evaluated against the results obtained by either postnatal hematopoietic stem cell transplantation (Buckley et al 2000), postnatal gene therapy (Cavazzana-Calvo et al 2000, Cavazzana-Calvo et al 2001) or possibly in the future, in utero gene therapy (Billings 1999, Schneider et al 1999).

It is unclear if the failures of most in utero allogenic hematopoietic stem cell transplantations in humans are related to fetal immune function and HLA incompatibility, lack of potential niches and advantage for the donor cells, the types and numbers of stem cells transplanted or the inability of donor cells to home and engraft. The following studies were undertaken to further explore the fetal immune function and to study the possibility to create space for donor cells for in utero stem cell transplantation.

Aims of the study

- To investigate if fetal liver cells have the capacity to mount an allogenic response in vitro in different gestational ages
- To explore the possibility to detect a clear gestational age where the fetus is unable to respond to allogenic stimulation
- To study the effects of different drugs on FLC colony forming capacity
- To study the effects of parvovirus B19´s empty capsid on FLC colony forming capacity
- To investigate the role of T- and NK-cell populations in allogenic response in FLC and to study if the response is HLA class II restricted
- To evaluate the efficacy of in utero transplantation of hematopoietic stem cells to resuscitate T-lineage cells in a fetus with X-linked SCID

Materials and methods

Fetal liver cells (I-V)

Human fetuses were obtained from therapeutic abortions where the patients had volunteered to donate fetal tissue. The ethics committee at Huddinge University Hospital had approved the studies (Dnr:91:157, Dnr:233/98, Dnr:363/98). Gestational age (given as menstruational age) was 7-18 weeks estimated according to specific anatomical markers (England et al 1988) in fetuses < 12 weeks and by ultrasound biparietal diameter measurement in fetuses > 12 weeks (Persson et al 1986). The abortions were performed in pregnancies with an obvious healthy fetus where no structural or chromosomal anomalies were found.

The abortions were performed with vacuum aspirations after osmotic cervical dilation with Laminaria® in the first group (< 12 weeks) under general anesthesia. After the vagina had been cleaned with antiseptic solution, the cervix was dilated with Hegar 10. A plastic cannula (10 mm in diameter) was inserted and vacuum was produced using a 60 ml syringe connected to the cannula by a rubber tube. When the fetus or fetal fragments appeared in the plastic cannula, the aspiration was stopped and the fetal tissue was placed in sterile tubes.

In the second group (> 12 weeks), cervical dilation was performed with Laminaria® on day 1 followed by vaginal administration of gemeprost (16,16-dimethyl-trans-D2 PGE1 methyl ester) on day 2 at every 4th hour until the fetus was delivered. Median time of abortion with this method has been shown to

be 15-18 hours after the first administration (Thong et al 1992).

The fetal livers were dissected under sterile conditions, using stereoscopic microscopy, and the tissue was washed three times in RPMI 1640 and put in sterile tubes. The fetuses > 12 weeks were all delivered intact and dissected within a time range of 20 min to 2 hours after delivery. The intact abdomen and thorax was opened and liver and in some cases thymus were kept intact and removed under sterile conditions and then washed in RPMI 1640.

The tissue was then disintegrated by passage through a 40 µm nylon mesh to form a single cell suspension. The nucleated cells were counted and diluted with RPMI 1640 medium supplemented with 10 % pooled human AB-serum, antibiotics (1%) and glutamine (1%) to give a final cell concentration of 1 x 10^6 cells/ml. Prior to storage in the cell-bank for in utero transplantations (IV) the fetal karyotype and ABO blood group are determined. The women who donated fetal liver tissue at abortion were serologically screened for syphilis, toxoplasmosis, rubella, HIV 1, cytomegalovirus, hepatitis B and C, parvovirus and herpes simplex type l and 2.

Bone marrow cells, peripheral blood lymphocytes and cord blood (I-IV)

Samples of adult bone marrow cells (BM) and of peripheral blood lymphocytes (PBL) were drawn from healthy donors after informed consent was obtained. Cord blood samples were obtained after informed consent immediately after vaginal delivery from normal births. The suspensions of BM were heparinized and diluted in 0.9 % NaCl solution and separated on Lymphoprep (Nycomed Pharma, Oslo, Norway) for gradient centrifugation at 2000 rpm for 20 min. Cells were carefully removed with a Pasteur pipette, washed twice in NaCl solution and centrifuged twice for 5 min at 1500 rpm. The cells were resuspended, counted and diluted to a concentration of 1 x 10^6 cells/ml in RPMI 1640 supplemented as described above. PBL and cord blood cells were prepared in the same way with Lymphoprep for gradient centrifugation.

Cryopreservation (I-II, IV-V)

Washed and counted cells were re-suspended (10-20 x 10^6 cells/ml) in a cryopreservation medium consisting of RPMI 1640 medium supplemented with 10 % pooled human AB serum, antibiotics (1%) and glutamine (1%) medium, and 10 % DMSO. Before cryopreservation in liquid nitrogen, the cell suspension was divided to 2-ml aliquots in cryo tubes, which were frozen stepwise over 24 h. Immediately before use, cells were thawed for 1 min in a 37 °C water-bath, suspended in supplemented RPMI 1640 medium (see above) and washed three times in the same medium. The cells are counted, their viability evaluated with eosin, and for transplantation, concentrated to a volume of 1 ml.

Mixed lymphocyte culture (MLC) (I,II)

Cell suspensions from two different cell populations were co-cultured. One of the populations was irradiated with 20 Gy according to a standard protocol (”one-way MLC”). The cell suspensions, 100 µl, from each of the two populations were incubated in 96 well plates, in triplicate, in 0.2-ml vials (Falcon-3040, USA) for 6 days. On day 5, for DNA-incorporation 3H-thymidine was added at a final concentration of 20 µCi/ml. After a further 24 h the cells were harvested by using a semiautomatic harvesting machine Skatron A/S, Lierbyen, Norway). Radioactivity was determined as counts per minute (cpm) by use of an Intertechnique beta-counter (Nanoteknik, Stockholm, Sweden).

MLC is a method used to detect HLA class II differences. Reactivity in MLC is correlated with risk for rejection and graft survival after intrafamilial renal transplantation (Ringdén et al 1976).

Lymphocytes of donor and recipient are co-cultured, and either of donor or recipient cells are pretreated with irradiation. When the two cell lines are co-cultured, evidence of DNA synthesis indicates stimulation of growth of the untreated cell line (responder) by the pretreated cell line (stimulator). The radioactivity in cpm indicating stimulation of each tested cell combination was analyzed. Background cpm for each cell type was achieved using the same method, but with cells co-cultured with irradiated cells from the same individual. All cpm-values are means of triplicate cultures. Stimulation index (SI) was calculated as stimulation cpm divided by background cpm. SI was considered positive if SI > 3. SI is normally considered negative if SI < 2 (Ringdén et al 1976) but in order not to overestimate the results, SI > 3 was used as a limit for positive responses.

In paper I fetal transplantations was simulated having the fetal liver and thymic cells as recipient (responder) cells stimulated with irradiated different cell types as fetal liver cells from a different fetus (donor) or adult PBL or BM-cells. The capability to generate cell proliferation, as a response to allogenic stimulation, was measured. In addition, the ability of fetal donor (stimulator) cells to stimulate other cells was also analyzed. The cells were tested either fresh or previously frozen.

In paper II MLC were performed using FLC from 10 fetal livers (gestational age 13-18 weeks) and PBL from 10 donors, stimulated with an irradiated FLC-pool or PBL prior to and after extraction of HLA class II+ cells from the stimulator cells and prior to and after extraction of CD3+ and CD56+ cells from the responder cells. As stimulator FLC, a pool of three irradiated fetal livers of gestational age 6-11 weeks was used for each experiment.

Extraction of cell subpopulations (II)

Immunomagnetic Dynabeads precoated with mouse monoclonal antibodies (mAb) specific to HLA class II epitope and specific to CD3 respectively, (Dynabeads® M-450, Dynal, Oslo, Norway) were added to cell suspensions of FLC and PBL and incubated for 30 min in 4°C on a sample mixer. Cells attached to the beads were then removed, using a magnetic particle concentrator (Dynal MPC, Dynal, Oslo). To remove the CD56+ cells, a mouse mAb specific to CD56 epitope (Becton Dickinson Labware, Lincoln Park, NJ) was added and incubated for 20 min in 4° C on a sample mixer and after washing 3 times, incubated with sheep anti-mouse Ig G Dynabeads M450 (Dynal, Oslo, Norway) as described above and removed with MPC. An evaluation of the effectiveness of the extraction of the different cell types in a fetal liver of 16 weeks gestation revealed that HLA class II+ cells were reduced from 17 to 1.4 %, CD3+ cells from 10 to 0.8 % and CD56+ cells from 11 to 1%.

Immunologic Reagents (III)

Recombinant B19 parvovirus empty capsid particles were used. These capsids were produced in a recombinant baculovirus insect cell (Spodofera frugiperda) expression system, previously described (Kajigaya et al 1991). Recombinant human papillomavirus (HPV6) and cottontail rabbit papillomavirus (CRPV) capsids had been produced in similar baculovirus insect cell systems. Anti-B19 monoclonal antibody (MAB8292) was mouse IgM (Chemicon, Malmö, Sweden). Anti-P_1 (Seraclone) monoclonal antibody (Labdesign, Stockholm, Sweden) and anti-P monoclonal antibody (CLB-ery-2) (von dem Borne et al 1986) were mouse Ig M. B19 Ig G positive (Ig M-negative) sera was obtained from two asymptomatic individuals in our laboratory.

Incubation with virus capsids, pre-incubation with antibodies prior to colony formation (III)

Virus capsids were diluted in buffer (20 mM Tris, 0.5 M NaCl, pH 8.5), and 30 µl of each

dilution was added to 25 x 10^3 fetal cells (50 x 10^3 for postnatal cells) in 100 µl of culture medium and incubated for 1 h at 4° C. The mixtures were then transferred to incubation dishes, and culture medium was added to a final volume of 0.5 ml per well. The cells were incubated for 11 days in a humidified atmosphere at 5% CO_2 . In additional experiments, either B19 capsids or fetal cells were pre-incubated with antibodies prior to the incubation of the cells for 11 days:

(1) 25 µl of either B19 IgG positive serum or anti-B19 monoclonal antibody (MAB8292) was pre-incubated with 25 µl of B19 capsids for 2 h at 4 ° C. The mixture was then added to cells as described above.
(2) 25 µl of either anti-P (CLB-ery-2) or anti-P_1 (Seraclone) monoclonal antibody was added to 25 x 10^3 cells suspended in 100 µl of medium. Cells and monoclonal antibody were pre-incubated for 1 h at 4° C. The mixtures were washed twice in cold culture medium prior to adding B19 capsids to the cells as described above.

Incubation with cytotoxic drugs, betamethasone, OKT-3 and ATG prior to colony formation (IV)

Fetal liver cells were diluted in RPMI with 10% pooled human AB-serum, antibiotics (1%) and glutamine (1%) to a concentration of 5 x 10^5 cells/ml. The cells were then incubated with daunorubicin in 0.05, 0.1, 0.5, 1, 2 and 4 µM for 1 hour at 20°C and thereafter washed twice in RPMI. Incubations with doxorubicin were performed in final concentrations of 0.05, 0.1, 0.2, 0.5 and 1 µM for 3 hours at 20°C, with ATG (antithymocyte globulin, Fresenius AG, Germany) in 4, 40, 400 and 800 µg/ml and with OKT-3 (monoclonal antibody against CD3, Orthoclone®, Jensen-Cilag, USA) in 5, 10, 50, 100 and 200µg/ml in final concentrations, the incubation time being 30 min. The cells were then washed twice in RPMI. Betamethasone was added in final concentrations of 0.2, 2, 20, 40 and 80 µg/ml and incubated continuously.

Colony formation (III-IV)

A commercial kit "Stem cell CFU kit" (GIBCO BRL, Life Technology Inc., N.Y., USA) was used to study colony formation. The components included are Iscove´s modified Dulbecco´s medium, modified fetal bovine serum, methylcellulose, 2-mercaptoethanol, conditioned medium and erythropoietin. This semi-solid system mimics the extracellular matrix produced by stromal cells. Cells were cultured at 37°, in 5% CO_2 and humidified atmosphere for 10 days after drug incubation on day 1. Colonies were identified as BFU-E (burst-forming units-erythroid cells) with densely packed hemoglobinised cells, CFU-GM (colony-forming units-granulocytes, macrophages) with arrangements of non-hemoglobinised cells, and CFU-GEMM (colony-forming units-granulocytes, erythrocytes, macrophages, megakaryocytes) with hemoglobinised cells and small and large peripheral cells.

In paper III all assay sets included triplicates of each serum, monoclonal antibody or capsid dilution. Triplicates of medium control (no added antibody) and triplicates of capsid control (no added antibody) were included in each experiment. As fresh human cells were used, on each occasion from different donors, the total numbers of colony counts varied between experiments and are, therefore, given in the figure legends. A B19 capsid concentration of 7 µg/ml repeatedly reduced colony counts by at least 50% as compared with the medium control. Appropriate controls (´capsids only´), tested on the same cells at the same time, are also shown in the figures. Mean values ± SD of colony counts based on triplicates in one representative experiment are given. Interassay variation did not exceed ±10%. Colony counts in the medium control were considered to be 100% in the respective experiments. In paper III all assay

sets included triplicates of cell solutions for each drug concentration and triplicate from the same fetus or bone marrow with no drug added as a control.

Diagnoses of X-linked SCID prior to transplantation (V)

DNA from the chorion villi sample (CVS) revealed a hemizygous Gln 188 stop mutation in position 576 in the gene for the common γ chain of the IL2RG, identical with the one previously found in the carrier mother and her affected son. Mutation analysis was performed using standard techniques described previously (Niemela et al 2000, Fugmann et al 1998). The CVS showed a normal 46,XY karyotype.

Fetal blood sampling and transplantation procedure (V)

Fetal blood sample (FBS) was carried out with the usual technique, as originally described by Daffos (Daffos et al 1984). The transplantation injection was performed with a 22-gauge spinal needle under ultrasonographic guidance. After injecting saline to ensure a free position of the needle tip, 1 ml of fetal liver cell suspension was injected into the peritoneal cavity of the fetus.

Engraftment monitoring and immunological monitoring (V)

HLA-A and –B typing was performed serologically, using commercially available antibodies (Biotest Ag, Dreieich, Germany) to subgroups of HLA class I cell surface molecules. Genomic HLA class II antigens were determined using commercially available primer pairs (Olerup SSP, Stockholm, Sweden) and the PCR-SSP method, defining DRB1-5 and DQB1 alleles (Olerup et al 1992).

The mixed chimerism of various cell lines (CD45, CD19, CD3 and CD56) was determined by PCR amplification of variable number of tandem repeats (VNTRs) as described elsewhere (Zetterquist et al 2000). In brief, pretransplant DNA samples from the patient (obtained from the chorionic villus biopsy) and donor (fetal liver cells) were screened with six different VNTR-specific primer pairs in the polymerase chain reaction to obtain at least one informative locus for subsequent estimations of admixture of fetal leukocytes. Posttransplantation mixed chimerism analysis was performed using fetal blood leukocytes at 24 and 33 weeks of gestation as DNA substrate. The mixed chimerism analysis at 6 months of age was performed with cell lysate DNA obtained from CD19+, CD3+, CD56+, and CD45+ immunomagnetic bead-separated leukocytes as previously described (Zetterquist et al 2000). After thermal cycling, PCR products were separated on precasted polyacrylamide gels and visualized by silver staining, using automated procedures (Pharmacia Biotech, Uppsala, Sweden). The donor-recipient mixed chimerism was determined by a visual comparison with a standard donor-recipient dilution series.

T-, B- and NK-cells were quantified by cytofluorography, with the use of murine monoclonal antibodies against lineage-specific surface molecules. Serum IgG, IgG subclasses, IgA and IgM were quantified by nephelometry. Anti-diphtheria, anti-tetanus and anti-HIB antibodies were measured with ELISA (Kabelitz et al 1985, Bartmann et al 1993). Peripheral blood lymphocytes (PBL) were cultured in 0.2 ml microcultures, using RPMI 1640 medium with the addition of ^{3}H-thymidine 24 hours before harvesting and determination of cpm in a liquid scintillation counter. Lymphocytes were stimulated *in vitro* with phytohaemagglutinin (PHA Wellcome Ltd, UK) and monoclonal antibody OKT3, and the cells were harvested after three days in culture. Following stimulation with tetanus, purified protein derived from tubercle bacilli, Candidin and CMV-positive and negative antigens, lymphocytes were harvested after five days. Lymphocytes were also stimulated with

irradiated lymphocytes from a pool of healthy donors in mixed lymphocyte cultures and harvested on day 5.

Statistical analyses
The differences in colony-forming capacity after incubation with different drug concentrations were compared with the student's t-test. P-values less than 0.05 were regarded as significant.

Results and discussion

Capacity of fetal cells to recognize and respond to allogenic cells

The aims of the first paper were to study if fetal cells in first and second trimester have a capacity to allogenic response. FLC from 47 fetuses and thymic cells from 13 fetuses were tested in MLC. Fetal liver cells were capable to recognise and react to allogenic stimulation already from 12 weeks gestation and in some cases even earlier. In fetuses with a gestational age 12-18 weeks, 10 of 18 (56%) had a SI > 3 in FLC, when stimulated with fetal liver cells. Eleven of 16 (68%) had a SI > 3 when stimulated with adult cells. Clear responses among more immature fetuses of 7-12 weeks occurred less frequently where 4 of 29 (14%) had a SI > 3 when fetal liver was used as stimulator and 5 of 20 (25%) with adult stimulator cells. The response in fetal liver cells showed wide variations in different gestational ages, but also between different individuals of the same gestational age. In all responding livers there was a tendency for increased responsiveness with increasing gestational age and the number of responding fetuses also increased with gestational age. Of the 13 experiments with thymic cells 5 had an SI>3 when stimulated with FLC and 7 when stimulated with PBL. The gestational age of the thymic cells was 12-18 weeks. If the fetal liver cells did not respond, a response could be seen in thymus or vice versa. MLC reactivity in thymocytes exceeded the fetal liver reactivity in fetuses of 13-14 weeks. Later, however, in fetuses of 14.5-15 weeks, most cases showed a decreased reactivity in the thymic cells compared to fetal liver cells. This first study indicates that some fetuses are able to respond to allogenic stimulation already during the first trimester and that the responding capacity varies between different individuals and is enhanced with increasing gestational age. It was not possible to detect a certain gestational age limit where the fetus was not able to respond. Of the fetuses in the 13^{th}-17^{th} gestational weeks, a majority, 78 %, had an SI > 3 in either FLC or FTC, when stimulated with fetal or adult cells, as compared to 24 % of those in the 7^{th}–12^{th} gestational weeks. Clear-cut responders could be demonstrated already from the 9^{th} week of gestation.

MLC was used as a functional test of the capacity to respond to allogenic stimulation. It was not possible to state, in this first study, if the reaction in the MLC is an HLA-restricted recognition by T-cell receptors on mature T-cells or an alternative type of reaction. A possible candidate may be NK cells since it has been shown that freshly isolated fetal liver NK cells can mediate MHC-unrestricted cytotoxicity against sensitive target cells (Phillips et al 1992). In order to further study the role of CD3+ (T-cells) and CD56+ (NK-cells) cells in the alloreactivity in fetal cells, the study in paper II was undertaken. MLC was performed prior to and after extraction of HLA class II+ cells from the stimulator population and

prior to and after extraction of CD3+ and CD56+ cells from the responder cells.

In MLC with FLC as responder cells with an initial positive SI, extraction of class II+ cells from the stimulator cells resulted in a decreased SI in 5 of 7 experiments using PBL as stimulators and in 4 of 5 experiments using FLC as stimulators. In similar studies with PBL as responders, extraction of HLA class II+ cells from PBL stimulators led to a decline in 8 of 9 experiments and in 6 of 7 experiments with FLC stimulators. These results indicate that the reactivity, seen in fetal cells, as well as for PBL is HLA class II dependent. The failure to achieve engraftment of fetal donor cells transplanted in utero to fetuses with hemoglobinopathies in contrast to fetuses with severe combined immune deficiencies also suggests that the fetus is immunocompetent and can mount an alloresponse (Westgren et al 1996).

In an attempt to further study the type of mediator cells, responsible for the reactivity, we performed extraction of fetal responder cells with T-cell- (CD3) and NK-cell markers (CD56). From the first trimester the amount of CD3+ cells in fetal liver increase from 2-5% (Ek et al 1994) to 9-11% in the second trimester. Extraction of CD3+ cells led to varying responses as measured by SI. All 3 cases with relatively high initial SI (SI=7-15) exhibit a decrease in SI, whereas some fetuses (3 of 5) with low initial SI (SI<7) demonstrated a marked increase in SI. Also 3 of 4 fetuses with extremely high initial SI (SI>20) exhibit an increased SI. We speculate that the varying response of T-cell extraction might reflect different phenotypes of T-lymphocytes. In those with a low initial SI it is possibly that the low activity reflects T cell mediated suppression and consequently extraction led to an increase in SI. In fetuses with a medium-high initial SI indicating high T-cell alloreactivity we could demonstrate a decreased reactivity after T-cell depletion. This was also observed in the control experiments using adult responder cells. This may suggest that fetal liver cells contain a varying degree of alloreactive T cells. Previous studies have indicated that mature VDJ TCR β chain transcript are present in fetal liver already in the first trimester and T-cells with TCR (T-cell receptor) αβ+, CD8+ phenotype, which indicate postthymic T-cells are found in 16 weeks fetuses (Renda et al, 2000). In fetal livers with extremely high SI response in MLC an increase in SI was seen in 3 of 4 experiments after CD3 depletion. In these experiments background values were low (<300cpm) indicating a low degree of initial proliferation in these fetal liver cells. MLC was definitely positive and this response increased after T-cell- depletion in 3 of 4 cases indicating the presence of a T suppressor cell population. These cells may be responsible also for the low background activity.

NK and T cells share many features and share a common intratymic precursor (Carson et al 1996, Carlyle et al 1997, Phillips et al 1992). The number of NK cells in fetal circulation decrease exponentially with gestational age and it has been claimed that these cells play a critical role in host defence in early pregnancy (Thilaganathan et al 1993). From the 9th week of gestation, NK cell activity was observed in fetal liver in 1983 by Uksila (Uksila et al 1983), and the cells can mediate non MHC-restricted cytotoxicity against NK-sensitive targets (Phillips et al 1992). Extraction of CD56+ cells in the present study led to a decline in SI in 5 of 7 experiment using PBL as stimulators and in 4 of 5 experiments with FLC as stimulators. Thus, the present study lends support to the opinion that NK cells are capable of generating an immune response.

Limitations of the MLC method

MLC is a functional method where cells of responder (recipient) and stimulator (donor) are co-cultured, and stimulator cells are pre-treated with irradiation to prevent DNA synthesis while maintaining HLA class II antigen expression. When the cells are co-cultured, evidence of DNA synthesis indicates stimulation of growth of the untreated cells by the pre-treated cells if HLA class II incompatibility exists between the two cell populations (Ringdén et al 1976). The result is presented as SI, which is calculated as activity after stimulation divided by baseline activity. An inherent problem using this method in fetal liver cells is the highly varying background activity of fetal liver cells. Most fetuses exhibit a high background activity probably due to a high differentiation capacity. The high background makes it difficult to detect a relative change in stimulation and this will lead to a low SI. Conversely a low background activity will lead to a relatively larger change in SI.

Another concern when evaluating the present results is the possible contamination of fetal tissue with maternal cells. When performing the first trimester abortions the aspiration was stopped when the fetus was in the plastic cannula and this should probably minimize the risk for maternal contamination. In the second trimester abortions a more regular dissection is performed with opening of the sterile abdominal cavity and the risk for contamination should be minimal, if existing at all. In addition, all fetal livers were thoroughly rinsed in RPMI 1640 solution. Thus, even though the risk of contamination can be considered minimal, it should nevertheless be kept in mind when dealing with aborted tissue.

Inhibition of fetal liver cell colony forming capacity

In paper III and IV the possibility to decrease colony-forming capacity in FLC was explored. Such regimens have been discussed as a way to create space for transplanted cells to increase the possibility for homing and engraftment for in-utero stem cell transplantation.

Parvovirus B19 is known to inhibit erythropoietic colony formation in vitro (Mortimer et al 1983). It has been suggested that the B19 nonstructural protein induces apoptosis in erythroid lineage cells (Moffatt et al 1998). In paper III, the possibility that recombinant B19 empty capsid proteins (VP1, VP2) could themselves suppress erythropoietic colony formation in the absence of non-structural proteins, was explored. The B19 capsids were shown to inhibit colony formation of BFU-E (70-95%) but structurally similar recombinant papillomavirus-capsids (cottontail rabbit papillomavirus and human papillomavirus type 6) had no effect on colony formation. When B19 capsids were pre-incubated with anti-B19 monoclonal antibody (MAB8292) prior to adding the mixture to the FLC colony assay the inhibitory effect on BFU-E was completely blocked. The same effect was observed when IgG positive sera from asymptomatic individuals were added to the B19 capsids. The B19 capsids inhibitory effect could also be reduced by at least 25% by pre-incubating the FLC with anti-P monoclonal antibody (CLB-ery-2) prior to incubation with B19 capsid. This suggests that the effect is fully or partly mediated by binding to the P-antigen and these effects are similar to those reported when native virus

was added to erythroid progenitor cells (Brown et al 1993). When a monoclonal antibody against the P_1-antigen, not involved in the binding of parvovirus B19 to the cell surface was used, there was no protection. The inhibitory effect of B19 capsids on colony formation was also tested on cord blood and adult bone marrow stem cells and was comparable to FLC.

The mechanism by which B19 capsids inhibit colony formation needs to be explained. It is possible that by binding to the P antigen receptor, and possible internalization of the capsids, a signal is triggered, which ultimately leads to a lytic effect. The inhibitory effect of B19 capsids on colony formation could possibly be a strategy to inhibit hematopoiesis in recipient stem cells prior to hematopoietic stem cell transplantation. Furthermore, it would be possible to pretreat donor cells with anti-P monoclonal antibodies to protect them from suppression by the capsids and thereby giving these cells a more favorable status.

Paper IV is a preliminary in-vitro study of the sensitivity in fetal hematopoietic stem cells, derived from fetal livers, to chemotherapeutic and to immunomodulative agents, measured as colony-forming capacity. The cytotoxic drugs daunorubicin and doxorubicin were studied. As expected a clear dose dependent inhibition of hematopoietic colony formation was seen. 0.1 μM doxorubicin reduced the colony-forming capacity of BFU-E by 25% ($p<0.05$), CFU-GM by 52% ($p<0.05$) and CFU-GEMM by 31% ($p<0.05$). The reduction after daunorubicin incubation was significant when 0.5 μM were used, BFU-E by 44% ($p<0.05$), CFU-GM by 59% ($p<0.05$) and BFU-GEMM by 80% ($p<0.05$). However, the use of cytotoxic drugs may adversely affect the normal development of the fetus. Furthermore, they may have a potential risk of inducing cancer.

Unlike the above-mentioned cytotoxic drugs, corticosteroids have been used extensively in human pregnancies. When given to the mother, they accelerate anatomic, biochemical and physiological maturation of the fetal lung and, during the last 30 years, thousands of women whose pregnancies were expected to end prematurely have been treated. Furthermore, in congenital adrenal hyperplasia, mothers are given high doses of glucocorticoids to suppress the fetal adrenal gland already in the first trimester. The risk of antenatal corticosteroid exposure has been regarded as tolerable, and if it proves effective as an immunosuppressive agent, betamethasone may be a realistic alternative at fetal transplantations. The results in paper IV show that betamethasone is an effective inhibitor of hematopoietic colony-forming capacity. A concentration of 0.2 mg/ml caused a 41% reduction in mean BFU-E in FLC ($p<0.05$), 39% reduction in CFU-GM ($p<0.05$) and 52% in CFU-GEMM ($p<0.05$). Fetal hematopoietic progenitor cells from different individuals seem to differ in sensitivity to betamethasone. No significant difference between adult bone marrow cells and fetal liver cells was demonstrated (ns).

These findings with a marked decrease in colony-forming capacity of fetal hematopoietic stem cells could raise concern regarding antenatal corticosteroid fetal exposure. However the effective concentrations used in the present experiment are 10-100 times higher than those used for conventional treatment of adrenal genital hyperplasia. These concentrations cause a more than 50% reduction in the proliferative capacity of different hematopoietic cell lines. An effect on immunological function after antenatal betamethasone treatment has been suggested of Murthy and Moya. They reported a reduction in PHA-induced lymphocyte proliferation and Il-2 production both in the mother and in the offspring in mice treated with betamethasone (Murthy et al 1994). Several recent studies have stressed that more research about the safety of antenatal corticosteroids is needed (Quinlivan et al 1998), and our findings support this view.

Anti-CD3 monoclonal antibody (Orthoclone OKT-3) is a potent T-cell inhibitor that has been used to treat acute rejection (Ortho Multicenter Transplant Study Group 1985, Palmer et al 1992). Antithymocyte globulin (ATG) is an immune serum that strongly suppresses activated lymphocytes. Like OKT-3, the drug has been used to treat or prevent graft rejection and GVHD. In paper IV, colony-forming capacity in fetal liver, incubated with different doses of OKT-3 or ATG was studied. Pre-incubation with 5μg/ml OKT-3 reduced colony formation of BFU-E 39% ($p<0.05$), CFU-GM 14% (ns) and CFU-GEMM 65% ($p<0.05$). A further reduction in the colony-forming capacity occurred with increasing doses, except high doses of OKT-3 (100 μg/ml) that seemed to stimulate colony formation. This might be a similar effect as the known lymphocyte stimulating effect of OKT-3 (Palmer et al 1992). Pre-incubation with 4μg/ml ATG reduced colony formation of BFU-E 50% ($p<0.05$), CFU-GM 60% ($p<0.05$) and CFU-GEMM 15% ($p<0.05$).

To our knowledge this is the first study to explore the possibility, that different cytotoxic and immunosuppressive drugs could decrease colony-forming capacity of fetal hematopoietic progenitor cells. If this strategy will become an alternative at fetal stem cell transplantations is highly questionable and further studies are required to elucidate the potential use and especially the safety of these drugs. Available information does not permit any quantitative conclusions regarding the effects on the fetus of these drugs. One can really question whether one will ever purposefully expose the human fetus to the cytotoxic drugs. A combination of steroids and ATG may be a possibility and may be least harmful for the fetus. The possible side-effects of treatment also needs to be compred to the fetal disorder, many of which are life-threatening. Interestingly, Shields, in studies on non-human primates, recently demonstrated a significant higher rate of chimerism after ATG and betamethasone treatment (Shields et al 2001).

Successful in utero transplantation in a fetus with X-linked SCID

In paper V we report a case of in utero transplantation (IUTx) with cryopreserved fetal liver cells given at 14 weeks by a single intraperitoneal injection to a male fetus with X-linked SCID. Engraftment was detected at 24 weeks, and the fetus had a normal T-cell count at 33 weeks. Information about fetal T-cells in normal pregnancies is limited, but comparison with available data suggests a severely reduced number at 24 weeks. The rapid increase between the 24th and 34th weeks lends support to the hypothesis that a limited number of donor cells may fully reconstitute specific defects of host lineage development during prenatal life. In agreement with previous reports on IUTx in X-SCID fetuses, a selective T-and NK-cell reconstitution was achieved, whereas B-cells remained autologous. Complete reconstitution of T-cells was achieved before birth, and the clinical course during the first two years of life has been completely uneventful. Although experience of IUTx in X-linked SCID is still limited, we find the outcome of this procedure encouraging, and regard it as a valuable adjunct to postnatal transplantation and, more recently, gene therapy. In our opinion IUTx can be offered as a therapeutic option in cases where the parents are considering termination of an affected fetus identified at prenatal diagnosis. More recently, normal donor B-cell function has also appeared.

Conclusions

• Fetal liver cells can respond to allogenic stimulation in MLC. The response increased with gestational age and the number of responding fetuses also increased with gestational age (GA). From 12th week of GA a majority of the fetuses have this capacity, but clear response could be seen in some fetuses already from the 9th week of gestation.

• It was not possible to detect a certain gestational age were the fetus was unable to react to allogenic stimulation.

• MLC responses of fetal liver cells decreased after depletion of cells expressing HLA class II.

• The positive MLC response seen in fetal liver cells could be reduced either after CD3+ or CD56+ cell extraction.

• Parvovirus B19´s empty capsid could reduce FLC´s colony-forming capacity 70-95%. Pre-incubation of the B19 capsids with Mab to the virus or with B19 IgG+ human sera reduced the inhibitory effect. By pre-incubating the target cells with Mab to the P-antigen the inhibitory effect could also be reduced.

• The cytotoxic agents doxorubicin and daunorubicin led to a decrease in FLC´s colony-forming capacity as did the immunomodulative drugs OKT-3 and ATG. Betamethasone also induced a dose dependent inhibition of colony-forming capacity.

• A successful T-cell reconstitution after in utero transplantation with fetal liver cells was performed in a fetus with X-linked SCID. IUTx might be offered as a therapeutic option in cases where the parents are considering termination of a fetus with severe immunodeficiency identified at prenatal diagnosis.

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