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Maternal Immunomodulation of Neonatal Alloantigen Response

Leh Chang

Abstract

MATERNAL IMMUNOMODULATION OF

NEONATAL ALLOANTIGEN RESPONSE

by

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Remarkable success has been achieved in the transplantation of allogeneic cardiac grafts into newborn infants at Loma Linda University Medical Center. The superior graft survival rate documented in these patients has not correlated with the degree of immunosuppression rendered, or the selection of genetically matched donors. However, the clinical success has correlated with the age of the recipient at the time of receiving a transplant. Patients receiving an allograft within the first few weeks of life are unique in that they seem to accept the alloantigens of their cardiac graft while responding aggressively to antigens in their environment. These observations have prompted a search for an explanation of what appears to be a unique state of immunologic privilege in these recipients.

This study was designed to explore matching the genetic material of the donor with the specific recipient, and the donor with the non-inherited maternal antigens to which the recipient was exposed

during gestation. This was accomplished in several ways, including analysis of a) phenotypes of donor and recipient serum-defined class I and II major histocompatibility antigens, b) mismatched donor antigens relative to non-inherited maternal antigens, and c) recipient responses to lymphocyte-defined donor antigens in mixed lymphocyte cultures. When these analyses failed to explain an apparent state of immunologic privilege, immunomodulation of the neonatal immune response was attempted through manipulations of cell-mediated reactions in vitro that were thought to represent possible in vivo phenomena. Through mixed lymhocyte culture experiments, neonatal immune cells were exposed to alloantigens of unrelated individuals (representing organ donors) in the presence of specific maternal alloantigens. Significant immune suppression was observed when maternal cells were cocultured with neonatal cells during alloantigen recognition and processing. This immunomodulation was further augmented by preculturing the neonatal cells with maternal cells for a short period before exposure to the unrelated alloantigens. This data suggests that the non-inherited maternal antigens present on the cocultured maternal cells had down-regulated the neonatal response to unrelated alloantigens. It is possible that the neonatal cells were responding in a manner that had been "programmed" in utero for prevention of harmful reactivity toward maternal tissues.

LOMA LINDA UNIVERSITY

Graduate School

MATERNAL IMMUNOMODULATION OF NEONATAL ALLOANTIGEN RESPONSE

by

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A Thesis in Partial Fulfillment
of the Requirements for the Degree
Master of Science in Microbiology

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Each person whose signature appears below certifies that this thesis in his/her opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.

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AFP	α-fetoprotein
CML	cell-mediated lympholysis
cpm	counts per minute
CSA	cyclosporin
GSR	graft survival rate
HLA	human leukocyte antigen
LLUMC	Loma Linda University Medical Center
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
NIMA	non-inherited maternal antigens
NS	natural suppressor
PGE_2	prostaglandin E_2
PHA	Phytohemagglutinin
RPMI-1640	Roswell Park Memorial Institute-1640
R	responder
SI	stimulation index
sIgA	surface IgA
sIgD	surface IgD
sIgG	surface IgG
sIgM	surface IgM
Sx	stimulator
3P	third party
TLI	total lymphoid irradiation

INTRODUCTION AND BACKGROUND

In 1985, a milestone was reached when a four day old neonate successfully received a transplanted heart at Loma Linda University Medical Center, Loma Linda, CA. Since then, the neonatal cardiac transplant program has rapidly expanded. This new technology provides life for many newborns who would not be able to survive without a transplant. In the experience of transplanting allogeneic cardiac grafts into human newborn, infant and child recipients, significant differences have been noticed in the ability to manage immune reactivity in different age groups (Bailey, *et al.*, 1988; 1989). It has been observed that there are three levels of variable immune responsiveness through which these patients pass: 1) from birth to 30 days, weak alloreactivity 2) from 1 to 3 months, stronger, but manageable reactivity and 3) from 3 months to 2 years, strong responsiveness that is remarkably difficult to control.

The weak immunoreactivity of the neonatal period of life (the first four weeks of life) has allowed for the successful transplantation of heart grafts and is now referred to as the time of natural "immunologic privilege" (Bailey, *et al.*, 1988; 1989). It has been proposed (Nehlsen-Cannarella, *et al.*, 1990a) that natural mechanisms needed to maintain the fetus (an allograft) are simply a "leftover" state from pregnancy.

Many factors involved in the physiology of pregnancy affect the immune system of both the mother and her baby. Several stages of structural development and function of the fetal immune system play a role in what must be a delicate balance between fetus and mother and, subsequently, neonate and environment. In assessing the nature of the immune response in the neonates receiving organ allografts, several aspects of reproductive and neonatal immunology were considered, by asking the following questions:

Is the immune system of the neonate completely developed? (Figure 1)

Humoral immunity:

The immunoglobulin-producing machinery develops at the eighth to tenth week of gestation and immunoglobulin synthesis begins around 20 weeks gestation (Alford, *et al.*, 1974). At about this same time, maternal IgG is beginning to be actively transported across the placental membranes into fetal circulation (Figure 2), an event that has at least two different effects. First, through these passive antibodies, the neonate will be afforded reasonable protection against pathogenic microbes. Second, the immunoglobulin-producing mechanism of the fetus is "switched off" by the presence of maternal antibodies, thus preventing the fetus from producing high titer antibody which could endanger the mother (McCario, *et al.*, 1987; Butler, *et al.*, 1987), and perhaps itself. The newborn has large

Figure 1. Major stages in the ontogeny of the human immune system. The horizontal arrows indicate the beginning and on-going development of the four major systems of the acquired immune response. Although each system has been started early in gestation, none of them are "adult-like" in functional capacity at birth.

Figure 1

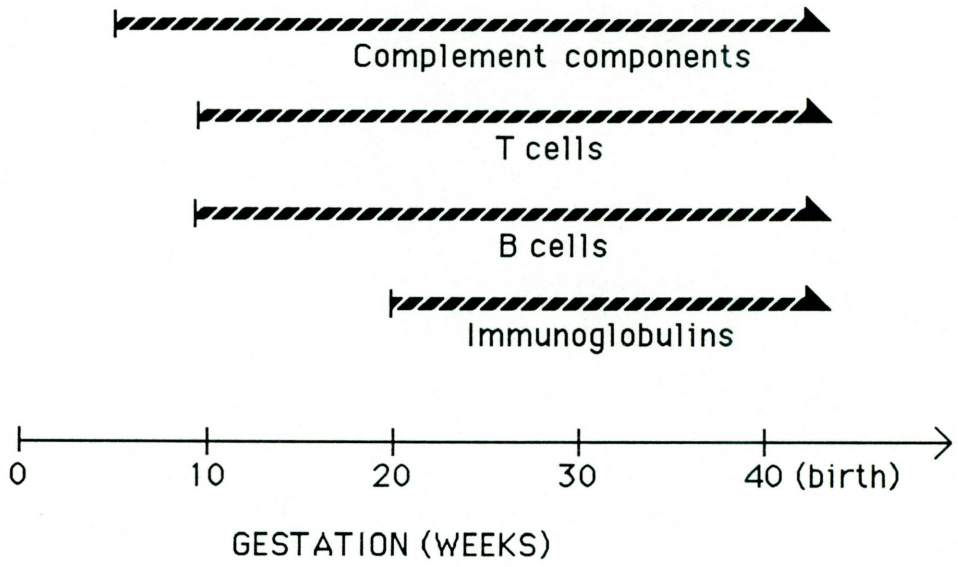
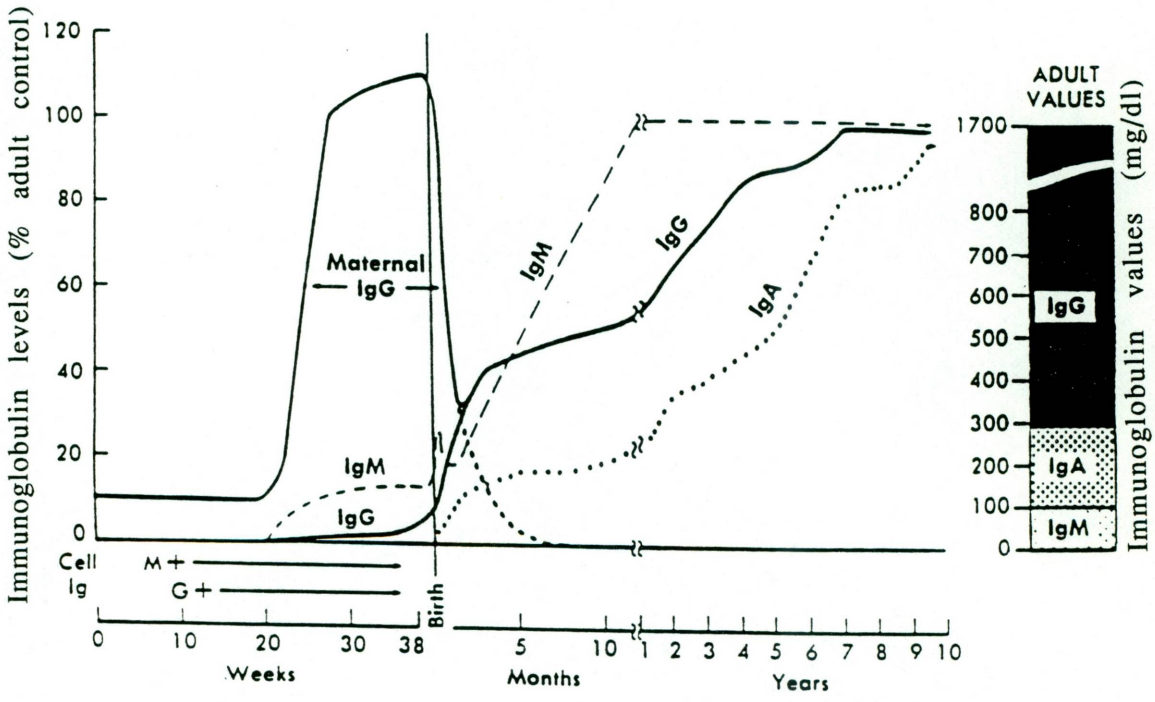

Complement components
T cells
B cells
Immunoglobulins
0
10
20
30
40 (birth)
GESTATION (WEEKS)

Figure 2. Immunoglobulin concentration in fetus, newborn, infant and child.

(From Alford, C.A., Jr.: Pediatr. Clin. North Am. **18**:99, 1971.)


Immunoglobulin levels (% adult control)
120
100
80
60
40
20
0
Maternal IgG
IgM
IgG
IgM
IgG
IgA
Cell Ig
M+
G+
Birth
0
10
20
30
38
Weeks
5
10
1
2
3
4
5
6
7
8
9
10
Months
Years
ADULT VALUES
1700
800
700
600
500
400
300
200
100
0
IgG
IgA
IgM
Immunoglobulin values (mg/dl)

numbers of circulating B cells bearing surface IgM, IgD, IgG, and IgA [sIgM, sIgD, sIgG, and sIgA] (Kamps, *et al.*, 1982), markers of mature B cells and indicators of the capacity to synthesize four of five essential immunoglobulin isotypes. B cells start to express both sIgM and sIgD during the fourth month of gestation and, in addition, subpopulations coexpress either sIgG or sIgA (Anderson, 1987). At the end of gestation, mature cells are present and in place, but immunoglobulin levels are deficient as a result of circulating maternal IgG.

Cellular immunity:

Thymic epithelium appears at approximately the sixth week of gestation and is populated with small lymphocyte-like cells by the ninth to tenth week (Jacoby, *et al.*, 1984). During the eighth week of gestation, circulating precursor stem cells from the fetal liver and bone marrow, lacking T-cell antigens, leave the circulation to enter the thymic micro-environment (LeDouarin, *et al.*, 1975) for the processes of maturation and differentiation. Mature T cells capable of forming spontaneous rosettes with sheep red blood cells by the CD2 receptor are observed after the ninth week of gestation (Asma, *et al.*, 1977). Phytohemagglutinin (PHA)-responsive lymphocytes appear in the spleen and peripheral blood at approximately 14 weeks of gestation (Mumford, *et al.*, 1978).

Cell-mediated immunity matures prior to the end of gestation (Fowler, *et al.*, 1960). As early as 12 week's gestation, T cells can be

activated by antigens represented on the surface of antigen-processing cells (such as macrophages, dendritic cells, and Langerhans cells), and by soluble products of these accessory cells (Hayward, *et al.*, 1977). Interestingly, neonatal spleen cells have up to 1000-fold higher density of alloantigens than adult spleen cells. This strong stimulation may be responsible for inducing clonal depletion, inactivation or activation of suppression (Sherwood, *et al.*, 1989). Fetally-derived placental cells express fetal alloantigens which stimulate maternal T lymphocyte proliferations and lymphokine secretions. This immunostimulation acts to promote the growth of maternal placenta which is beneficial to the fetus (Athanassakis, *et al.*, 1987). Despite the apparent "readiness" of the cell-mediated immune system at the end of gestation, *in vivo* function is relatively depressed by the presence of natural suppressor cells (to be discussed).

Complement:

Complement components can be detected as early as 5-6 weeks of gestation. The concentrations at birth reach approximately 66% of corresponding maternal or pooled adult concentrations. Since complement does not cross the placenta, it has been shown that the lower complement levels in newborns are due to lower hepatic synthesis. Such low concentrations lead to decreased capacity to activate the complement cascade (Cole, 1987), and result in decreased resistance to infection, particularly, bacterial.

Phagocytes:

Neonatal mononuclear phagocytes are equal to adult in both concentration and efficiency. However, newborn macrophages are less responsive to lymphokines than those of adults which render them deficient in their role as antigen presenting cells (Biondi, *et al.*, 1987). Furthermore, neonatal polymorphonuclear leukocytes (PMN) are abnormal in function. The chemotactic response of PMN of neonates is depressed compared with PMN responses of adults, in that they have abnormal critical chemotactic processes. Chemotactic factors bind to specific receptors on the surface of the cell and trigger a series of events which include changes in membrane potential, ion fluxes, transitory early increase in cyclic adenosine monophosphate (cAMP) levels, appearance of microtubules, and polymerization of actin. Although newborn PMN appear to interact normally with chemotatic factors, their rigid cytoskeletal structures are incapable of deforming normally and redistributing adhesion sites or surface receptors following stimulation (Hill, *et al.*, 1987). These and the low levels of complement contribute significantly to the inability of neonates to defend themselves against bacterial microorganisms and must depend on the protection of maternal IgG.

<u>Does the immune system of the neonate have the same composition (proportions) as older individuals?</u>

Newborns fail to produce normal amounts of immunoglobulin to polysaccharide antigens. Their antibody responses are primarily

restricted to the IgM isotype. There is a relatively slow progression, throughout early childhood from IgM to IgG and finally IgA production. The adult levels of immunoglobulins are reached at 1 year for IgM, 5-6 years for IgG, and 10-14 years for IgA (Figure 2). This delay in antibody production is not due to lack of precursor B cells since normal numbers of B and T lymphocytes are present at birth. In addition to the circulating maternal IgG, regulatory imbalances between T-cell-mediated help and suppression, as well as an intrinsic B-cell immaturity, may be responsible for the relative immunodeficiency of the newborn. The gap in the humoral responses of the neonate may also be due to the lack of immunological experience since the population of memory B cells capable of responding to antigen challenge is low in the newborn (Butler, *et al.*, 1987).

There are no substantial differences in the percentages and overall *in vitro* function of T lymphocytes between adults and neonates; at the same time a significant difference has been shown in the distribution of the T-cell subpopulations. While the percentage of CD4+ (helper or suppressor-inducer) cells is similar in neonates and adults, the proportion of CD8+ (suppressor) cells is significantly higher in neonates (Maccario, et. al., 1983). Low helper capacity (measured as the efficiency to induce B-cell immunoglobulin production *in vitro*) of neonatal T lymphocytes has been demonstrated (Hooper, *et al.*, 1986). From this observation, it is concluded that either neonates have fewer CD4+ cells with helper

function, or they have more actively suppressed helper activity (through the large suppressor subset) than adults. Indeed, suppression has been documented to be very active in neonates (Hayward, *et al.*, 1981). Its purpose is probably to inhibit *in vivo* maternal cell proliferation and rejection of the fetus.

A variable percentage of neonatal and cord cells are Tac+ (CD25) (T-cell activation marker, now recognized as the membrane receptor for Interleukin-2 [IL-2R]). In contrast to the low number of Tac+ cells found in normal adult peripheral blood lymphocytes (0.3% $\pm$ 0.7%), 12.3% $\pm$ 6.7% are found in neonates. Since a subset of apparently quiescent lymphocytes as well as all activated lymphocytes are Tac-positive, neonatal Tac+ cells might represent circulating immature T cells, or T cells that have been activated by maternal antigens. The presence of circulating immature lymphocytes is probably also related to the intense traffic from primary lymphoid organs to the periphery, of post-thymic T-cell (Stutman, 1985).

It appears, then, that fetal life has provided the neonate with the right cells but in the wrong proportions; that is, there are too many suppressors. Additionally, helper cell function is somewhat weak, and many of the circulating cells seem to be either immature or activated.

What physiological events take place during pregnancy that may influence early postnatal immune function of neonates?

The placenta:

The placenta is an immunological barrier. It is a highly specialized organ which contains both a fetal and a maternal portion. The fetal portion of the placenta consists of villi (of the *chorion frondosum*.) which are suspended in a space bathed in maternal blood. The maternal portion of the placenta is formed by the *pars functionalis* of the *decidua placentalis*. The fetal blood passes through the placental villi, suspended in the maternal blood flowing through the intervillous space. The two currents are able to exchange materials across the delicate villi walls similar to that done across aveoli and glomeruli.

The placenta is a relatively nonantigenic interface: The major histocompatibility (MHC) antigens (human leukocyte antigen, HLA) are not expressed on the placental surface or on the basement membranes of the trophoblast (Martin, *et al.*, 1977).

The placenta is an immunoadsorbent: Maternal anti-fetal human leukocyte antigen (HLA) antibodies are adsorbed by HLA antigen-positive trophoblasts to help prevent their entering the fetal circulation. These antibodies also bind to free soluble fetal antigen to help prevent further immunization of the mother.

The placenta is a generator of nonspecific immuno-suppressive agents: Known suppressor factors, secreted by human decidual cells, inhibit lymphokine production and lymphocyte activation (Matsui, *et al.*, 1989a, 1989b; Fukuda, *et al.*, 1989).

The placenta is a generator of immune cell blockade: Placental cells have been shown to suppress natural killing activity (Kolb, *et al.*, 1984). Placental adherent cells, class II MHC antigen-positive macrophages, demonstrate suppressive activity in mixed lymphocyte culture (MLC) and cell mediated cytotoxicity (CML) that is not due to the effects of prostaglandin E_2 (Uren, *et al.*, 1990). Human decidual antigen-presenting cells induce the generation of suppressor T cells *in vitro* (Oksenberg, *et al.*, 1988). Placental cells produce significantly greater quantities of γ-interferon, develop considerable lymphokine activated killer activity, and maintain cytotoxic activity in the absence of IL-2 (Chin, *et al.*, 1988).

The mother:

Maternal immune responsiveness is depressed at least at the implantation site to prevent immunological recognition of her fetus, or to delay such response until after birth. The high concentration of steroid hormones probably plays a role at this site since all the pregnancy-associated hormones [such as adrenal corticosteroid, estrogen, progesterone, human chorionic gonadotrophin (HCG), and

human chorionic somatomammotrophin (HCS)] have immunosuppressive properties (Hogarth, 1982).

The fetus:

α-Fetoprotein (AFP), a major protein of fetal origin, found in amnionic fluid and perinatal sera, suppresses T-dependent primary antibody synthesis (Murgita, *et al.*, 1983). AFP is capable of exerting a noncytotoxic immunosuppressive effect on primary and secondary antibody production *in vitro* (Murgita, *et al.*, 1975). AFP can also activate natural suppressor cells (Crainie, *et al.*, 1989). Several groups have reported that prostaglandin E_2 (PGE_2) produced by neoantal lymphocytes, is capable of suppressing maternal mononuclear leukocytes (Johnsen, *et al.*, 1982; 1983; Papadogiannakis, *et al.*, 1986). These substances are finely regulated during pregnancy to maintain the fetal-maternal relationship.

Studies performed in mice have yielded information that is probably applicable to humans. Natural suppressor cells from spleens of newborn mice suppress various lymphocyte activities such as mixed lymphocyte reactions, mitogen responses, cytotoxic T cell development, antibody production, and graft-vs-host reactions (Pavia, *et al.*, 1979). Both T and non-T inhibitory cells, which are capable of suppressing the proliferation of adult and newborn cells reacting against alloantigens, are also found in murine neonatal spleen (Hooper, *et al.*, 1986).

The cumulative effect of the various mechanisms described above result in: 1) a decrease in the maternal immune response and 2) a modification of the quality of the maternal response. These changes normally ensure protection of the conceptus from harmful maternal cellular immune reactivity.

Thus, it has been realized in the most recent years that fetal survival does not depend on the failure of an immune response to develop, but on the unique nature of a very active response. The fetal allograft appears to be able to manipulate its maternal host into an active state of immunologic acceptance instead of rejection. Equally important is the fact that active suppression is observed in the fetus.

Transition from fetal to neonatal life necessitates changing from a predominantly suppressive to aggressive state. Suppressive mechanisms needed to maintain the fetus as a viable entity while *in utero* must be replaced by aggressive immunological functions for protection of the newborn in an alien and hostile environment.

How is this transition accomplished? As the pregnancy approaches the end of the third trimester, uterine muscle movements increase, and specific pregnancy regulation hormone levels change. The cortisol level rises in the fetus prior to labor which can cause increasing levels of estrogens, prostaglandins, and oxytocin, and a decreasing level of progesterone. This change

triggers a "cascade phenomenon" and sets new feedback points in the maternal host. This hormonal shifting induces labor (Batra, *et al.*, 1976; Challis, *et al.*, 1974; Tulchinsky, *et al.*, 1972). The act of labor includes stress, pain and exercise.

All of these events have the potential to change immune responses by increasing the secretion of corticosteroids, adrenaline and neuropeptides. Stress has been shown to induce complex hormonal responses (Figure 3). It activates the hypothalamus-pituitary-adrenal axis and stimulates the adrenal medulla to release catecholamines (Genazzani, *et al.*, 1989). As a single example, natural killer cell activity can be modulated by these various neuroendocrines (Table 1; Fabris, 1989). These complex hormonal responses interact with other mediators (such as lymphokines) and have been implicated in the role of directing of lymphoid cell traffic and function. Shifting of lymphocyte subsets during acute and chronic exercise has also been demonstrated (Berk, *et al.*, 1986; Nehlsen-Cannarella, *et al.*, 1990b; Nieman, *et al.*, 1990a; 1990b).

Perhaps some or all of these factors are responsible for preparing the fetal immune system for postnatal function. As the mother goes through labor, the changing of hormone and neuropeptide levels may result in active shifting of fetal cell subpopulations out of peripheral circulation into solid lymphoid organs. In this regard, several groups have shown that murine neonatal spleens (Gronvik, *et al.*, 1987), pregnant maternal spleens (Hoskin, *et al.*, 1989), and spleens of total

Figure 3. Stress-induced activation of the neuroendocrine system, which results in release of neuropeptides and hormones, and modulation of the immune response.

(Adapted from Gennazanni, In *Stress, immunity and ageing a role for acetyl-L-carnitine*. 1989)

Figure 3

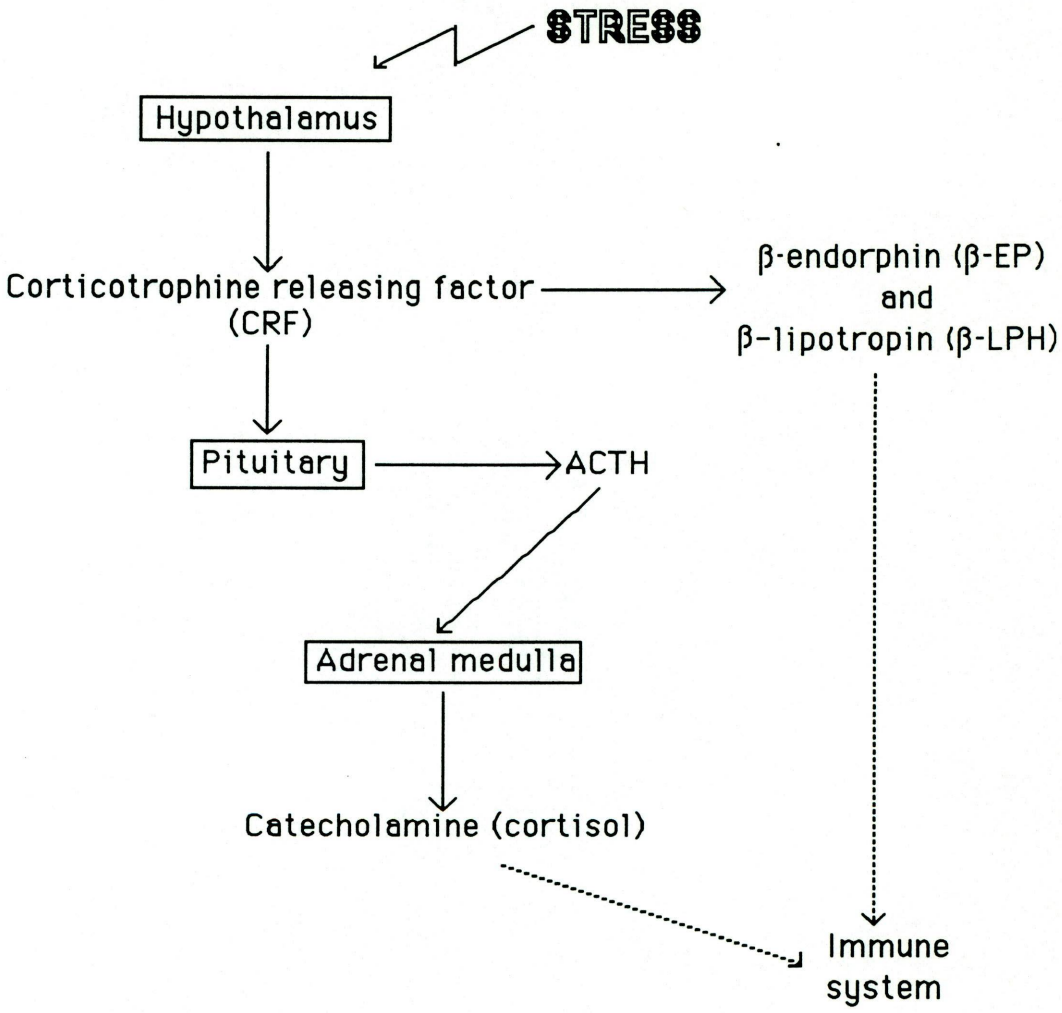

STRESS
Hypothalamus
Corticotrophine releasing factor (CRF)
β-endorphin (β-EP) and β-lipotropin (β-LPH)
Pituitary
ACTH
Adrenal medulla
Catecholamine (cortisol)
Immune system

Table 1

Neuro-Hormonal Modulation of NK Cell Function

Mediators	NK activity
Endorphin	↑
Enkephalin	↑
Epinephrine	↑↓
Vasoactive Intestinal Peptide (VIP)	↑↓
Serotonin	↑
Histamine	↑
Prostaglandins	↑↓
Leukotrienes	↑
GH	↑
TSH	↑
LH	↓
FSH	no effect
Thyroxine	↑↓
Glucocoorticoids	↓
Estrogens Androgens	↓

(Adapted from Fabris, N. In *Immunology of the Neonate*. 1987)

lymphoid irradiated (TLI) adult mice (Schwadron, *et al.*, 1989; Slavin, *et al.*, 1978; Strober, 1986) have high concentrations of non-T natural suppressor (NS) cells. The unexplained presence of newborn-like NS cells in the spleen of TLI-treated adult mice suggests the disappearance, but not depletion, of the NS cell population from the peripheral circulation.

Testing neonatal immunity through function assays performed on blood samples.

It can be concluded from this information that composition of umbilical cord blood appears to be neither completely fetal nor truly neonatal in character. Indeed, some of the cord blood products may be in the process of leaving the circulation and homing-in to tissues where they will reside (or die) postnatally. If this is the case, studies on neonatal blood can not reveal information on the nature of fetal immunity which subsequently affects neonatal immune responses. This depletion of specific cell populations from circulation at the time of birth could explain the discrepancies found between the peripheral blood studies and the enhanced graft survival rate observed in our neonatal allograft recipients.

Since there is a discrepancy between *in vitro* function of neonatal peripheral blood lymphocytes (adult-like aggression) and apparent *in vivo* function (non-aggression toward an allograft), it can be assumed that some extra-circulatory, or circulating non-lymphoid, factor(s) is(are) involved. Although factors such as alpha-fetoprotein

and prostaglandins are circulating in the fetus, they disappear from circulation rapidly after birth, too rapidly to affect transplantation in the late neonatal period. The most probable candidates for mediating neonatal suppression are the splenic (and other organ?) natural suppressor cells that are demonstrable throughout this period of life. Taking into consideration the facts that 1) spleen cells actively process antigen, and 2) maternal cells easily gain access to fetal circulation at the time of placental disruption at parturition (Desai, *et al.*, 1963; Levin, *et al.*, 1989), then it seems plausible that splenic NS cells remain activated in the neonatal period until aggressor cells have cleared the invading maternal cells. It is also possible that the NS cells, which have been in an activated state during fetal life, simply require some time to down-regulate after birth (a process perhaps controlled by the slowly developing lymphokines of the neonate).

The question of interest in this thesis study concerns the nature of the stimulus of NS cell activation. Since the non-inherited maternal antigens (NIMA) are the only nonself antigens to which the fetus is exposed in a normal healthy pregnancy, they are probably the antigenic stimulators of the fetal NS cells, a concept supported by others (Claas, *et al.*, 1988; Jacoby, *et al.*, 1984; Chardonnes, *et al.*, 1980; Owen, *et al.*, 1954). These antigens have been shown to play a special role in hyperimmunized renal transplant patients: they fail to induce antibody production in patients responding to other alloantigens.

With this reasoning in mind, it was hypothesized that the apparent natural suppression observed in young children receiving cardiac allografts within the neonatal period (birth to 30 days) might be a result of, at least in part, suppressor activity of cells that have been activated *in utero* by exposure to non-inherited maternal antigens.

METHODS AND MATERIALS

A. Specimen sources

In the clinical portion of this study, 1-4 mL of heparinized whole blood were collected from each cardiac transplant recipient and donor for routine histocompatibility testing before transplantation. Seven to 10 mL heparinized whole blood were obtained from each transplant recipient's biological mother for major histocompatibility complex (MHC) class I (HLA-A,B,C) and II (HLA-DR, DQ) phenotyping. In the experimental portion, aliquots of 10-15 mL umbilical cord blood were collected in heparinized tubes immediately after delivery from non-complicated normal term pregnancies. Heparinized venous peripheral blood specimens (15 mL) were obtained from the respective mothers within 24 hours post-delivery. Varying quantities of anticoagulated venous whole blood were collected from unrelated healthy donors.

B. Autologous serum

Autologous serum samples were obtained by clotting transplant recipient peripheral blood or normal baby cord blood. To each 10 mL plasma, 20 mg protamine sulfate (Eli Lilly & Co., Indianapolis, IN) and 20 units thrombin (Armour Pharmaceutical company, Kankakee, IL) were added and mixed. After standing at 22°C for 10-20 minutes, the clotted sample was centrifuged at 500 g for 5 minutes. Serum was harvested and complement was inactivated by heating

for 30 minutes at 56°C, and used fresh or stored at -70°C for further use.

C. Lymphocyte separation

Mononuclear lymphocytes were separated by density gradient centrifugation. Whole blood was diluted 1:2 with RPMI 1640 (Irvine Scientific, Irvine, CA), a standard culture medium used in routine histocompatibility testing of human leukocytes. Four mL of Ficoll-Paque density gradient (Pharmacia LKB Biotechnology, Incorporated, Piscataway, NJ) in each 15 mL centrifuge tube was overlaid with approximately 7 mL diluted blood. The tubes were centrifuged at 400g at 22°C for 25 minutes. The media-gradient interface containing the mononuclear cells was transfered into another tube with a Pasteur pipet. The mononuclear cells of each tube were washed twice with 15-mL fresh RPMI 1640, and finally resuspended to a concentration of $1x10^6$ cells/mL in complete medium. The complete medium is RPMI 1640 supplemented with 100 µg/mL penicillin/streptomycin (Sigma Chemical Company, St. Louis, MD), 100 µg/mL L-glutamine (Sigma), and 10% heat-treated complement-inactivated pooled human serum (GIBCO, Grand Island, NY). [Pooled human serum is prescreened in lymphocyte cultures to ascertain its ability to support growth and to assure the absence of cytotoxic antibody.]

D. T and B cell separation using "Dynabeads"

T and B cells were separated from heparinized whole blood (5 mL for each T & B cell separation) by positive selection with the rapid immunomagnetic cell isolation technique. The procedure uses "Dynabeads HLA Cell Prep I" and "HLA Cell Prep II" (Dynal Inc., Great Neck, NY). Dynabeads are microscopic synthetic spheres with metallic cores, coated with murine monoclonal antibody to CD8 (for isolating T cells) or monoclonal antibody specific for the HLA class II DR antigen (for isolating B cells). Cells form rosettes with the specific Dynabead cell prep after a short (3-5 minutes) incubation period. The rosetted cells are subsequently isolated and washed with the help of a magnet placed on the outside wall of the test tube. The isolated cell suspension can be used directly for sensitive and accurate microlymphocytotoxic HLA phenotyping (Vartdal, *et al.*, 1986).

E. HLA phenotyping

MHC Class I and II phenotypes were determined by using the routine two-color fluorescence microlymphocytotoxic assay (Hopkins, *et al.*, 1981) with purified T and B cells (greater than 90% viable; 2-2.5 x 10^6 cells/mL cell concentration). One μL of well-mixed cell suspension was placed into each well of microcytotoxicity HLA typing trays (C-6 Diagnostics, Inc., Meequon, WI) containing one of many antisera to the various HLA specificities. The cells and serum were

mixed by passing a high frequency electric wand (Electrotechnic Products, Inc., Chicago, IL) over the plate to effects mixing by causing vibrations in the fluids within each well. After 30 minutes incubation at room temperature (during which antibodies have the opportunity to bind to their respective antigens), 5 μL of fresh-frozen rabbit serum (containing active complement components) were added to each well. After a further 60-minute incubation at room temperature, the excess complement was removed by flicking the tray upside down quickly. Two μL of acridine orange/ethidium bromide solution (Sigma Chemical Company, St. Louis, MD) and 5 μL of India ink (2.5%; Pelikan, West Germany) were added in succession to the wells of each tray. After centrifuging the tray at 1500 rpm for 1-2 minutes, the cell reactions were read immediately on an inverted fluorescent microscope (Model IM 35, Carl Zeiss, Inc., West Germany).

Acridine orange provides a green color to all living cells while ethidium bromide stains the nuclei of dead cells with a contrasting red color. The India ink provides a physical barrier for eliminating the problem of excess fluorescent dye in the well. The reaction in each well is scored using the International Scoring Scale (Hopkins, *et al.*, 1981)

International Scoring Scale:

Score	% Dead Cells	Interpretation
1	0-10%	Negative--Antigen (Ag) not on cell
2	11-20%	Doubtful--Ag not on cell
4	21-50%	Weak Positive--Ag may cross-reacting
6	51-80%	Positive--Ag on cell
8	81-100%	Strong Positive--Ag on cell

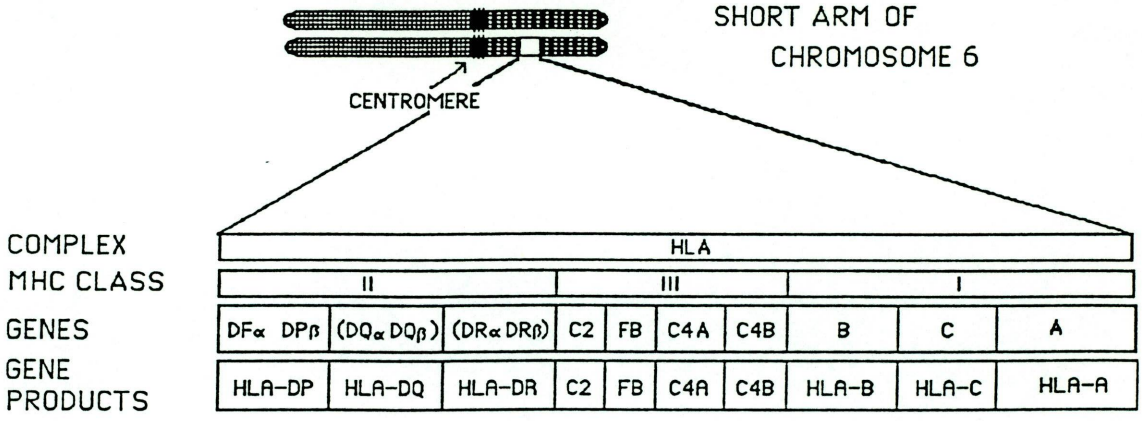
HLA phenotype designations

Human chromosome six contains the genes of the major histocompatibility complex that code for production of HLA markers on cell membrane. There are three loci which are relevant in cardiac transplantation on each of the chromosome six pair: A, B, and DR. The alleles are highly polymorphic and the products of the genes are coexpressed. Thus, each person can express two antigens for each locus; six antigens in total. The antigens of each locus comprise a "series" and they are numbered. When describing an antigen, one designates both the locus and the allele: HLA-A2, -B7, or -DR 6. Table 2 lists the HLA phenotypes in these three series that were recognized as of the 1987 international workshop.

F. Mixed lymphocyte culture

The standard technique of Dupont, *et al.*, 1976 for the allogeneic mixed lymphocyte culture (MLC) reaction was used throughout this study (exceptions noted where indicated). The MLC is an *in vitro* assay which represents the afferent immune response in a primary reaction; it involves lymphocyte recognition of allogeneic class II antigens ("lymphocyte-defined" or LD antigens) that are not definable by routine serotyping ("serum defined" or SD antigen). MLC-induced proliferation is that which would be expected to occur *in vivo* in response to the same antigens (Bach, *et al.*, 1964, 1966). The reaction of the responding cells to disparate antigens on another

Table 2: HUMAN MAJOR HISTOCOMPATIBILITY COMPLEX



HLA-DR	HLA-B			HLA-A
DR1	B5	B44 (12)	Bw62 (15)	A1
DR2	B7	B45 (12)	Bw63 (15)	A2
DR3	B8	Bw46	Bw64 (14)	A3
DR4	B12	Bw47	Bw65 (14)	A9
DR5	B13	Bw48	Bw 67	A20
DRw6	B14	B49 (21)	Bw70	A11
DR7	B15	Bw50 (21)	Bw71 (w70)	Aw19
DRw8	B16	B51	Bw72 (w70)	A23 (9)
DR9	B17	Bw52 (5)	Bw73	A24 (9)
DRw10	B18	Bw53	Bw75 (15)	A25 (10)
DRw11 (5)	B21	Bw54 (w22)	Bw76 (15)	A26 (10)
DRw12 (5)	Bw22	Bw55 (w22)	Bw77 (15)	A28
DRw13 (w6)	B27	Bw56 (w22)		A29 (w19)
DRw14 (w6)	B35	Bw57 (17)	Bw4	A30 (219)
DRw15 (2)	B38 (16)	Bw58 (17)	Bw6	A31 (w19)
DRw16 (2)	B39 (16)	Bw59		A32 (w19)
DRw17 (3)	B40	Bw60 (40)		Aw33 (w19)
DRw18 (3)	Bw41	Bw61 (40)		Aw34 (10)
	Bw42			Aw36
DRw52				Aw43
DRw53				Aw66 (10)
				Aw68 (28)
				Aw69 (28)
				Aw74 (w19)

(Adapted from Histocompatibility Testing 1987; Dupont B. (ed), Springer-Verlag. New York, 1987)

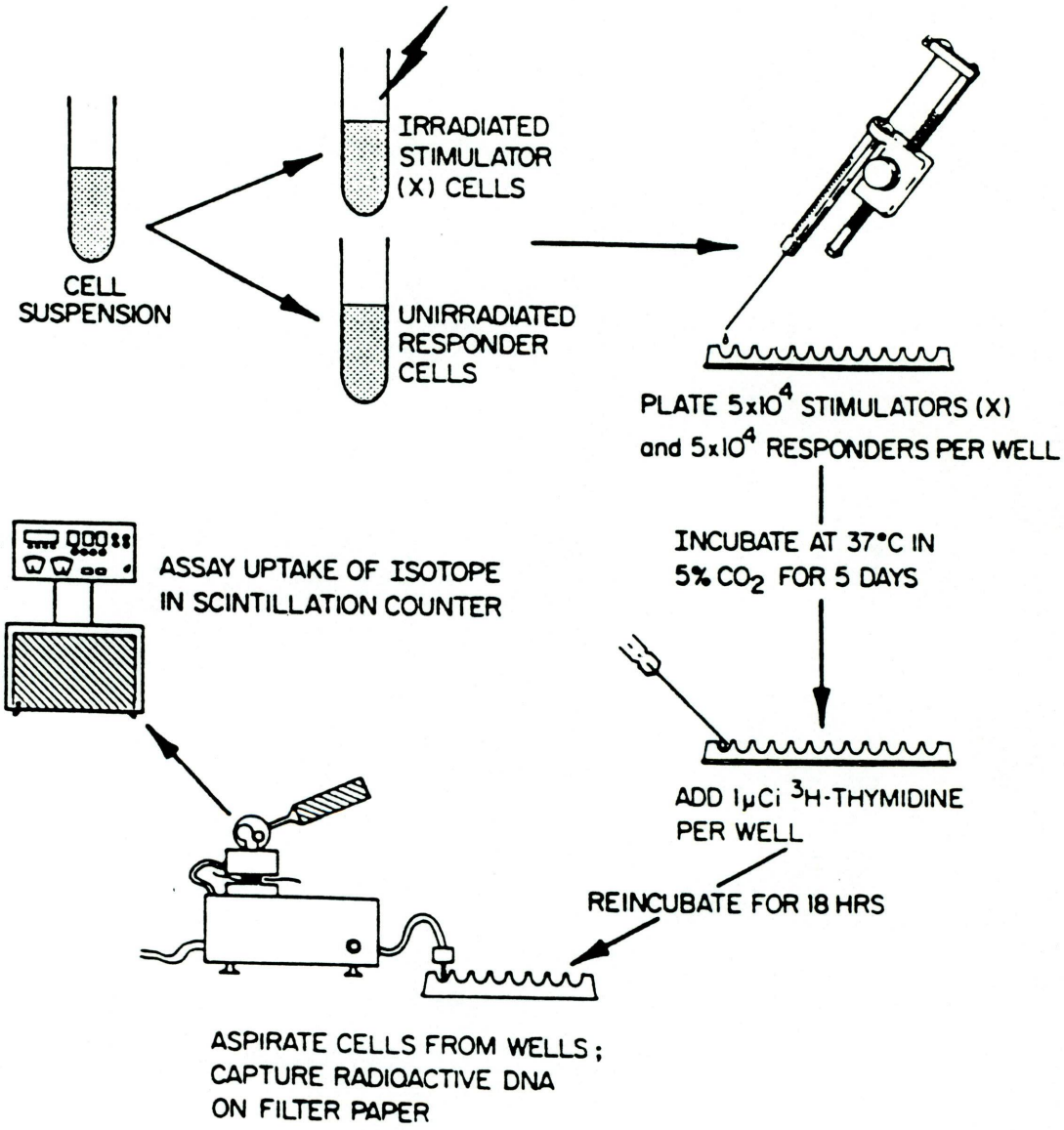
cell population is the formation of lymphoblasts, activated precursor T-cytotoxic cells.

This assay (Figure 4) simply exposes the lymphocytes of an individual under study to lymphocytes of another individual for a period of 5-6 days in culture. The functional cells are called "responders" and the antigen-bearing cells are called "stimulators." These latter cells are irradiated so as to render them incapable of responding to the responders (R), while remaining alive and otherwise intact; they are the stimulators and are designated "Sx." The MLC is quantitated by measuring the amount of radio-labelled nucleotide (tritiated-thymidine) taken up by respondor cells synthesizing new DNA during blastogenesis. In all experiments, mononuclear cells from either peripheral blood of the transplantation recipients, or cord blood specimens from normal births were used as responder cells. Stimulator cells were peripheral blood mononuclear cells from either allograft donors, biological mothers, or unrelated healthy individuals. Stimulator cells are metabolically immobilized by irradiation with 2750 rads from a ^{137}Cesium source (Model 143 Irradiator, J L Shepherd and Associates, San Fernando, CA) to prevent DNA replication when mixed with allogeneic responder cells. One hundred thousand responder cells in 100μL complete media are mixed together with an equal number (and volume) of irradiated stimulator cells in each well of a 96-well round(U)-bottomed tray (Corning 25850, Corning, NY). The cultures are incubated in a humidified mixture of 5% CO_2 in air at 37°C for several (usually 6)

Figure 4. The technique of performing a Mixed Lymphocyte Culture (MLC).

(From LLUMC Histocompatibility Laboratory Procedure Manual, 1989)

Figure 4


IRRADIATED
STIMULATOR
(X) CELLS
CELL
SUSPENSION
UNIRRADIATED
RESPONDER
CELLS
PLATE 5x10^4 STIMULATORS (X)
and 5x10^4 RESPONDERS PER WELL
INCUBATE AT 37°C IN
5% CO2 FOR 5 DAYS
ADD 1μCi 3H-THYMIDINE
PER WELL
REINCUBATE FOR 18 HRS
ASSAY UPTAKE OF ISOTOPE
IN SCINTILLATION COUNTER
ASPIRATE CELLS FROM WELLS;
CAPTURE RADIOACTIVE DNA
ON FILTER PAPER

days, depending on experimental design. Several standard controls were incorporated in each assay: 1) "media only" controls are used to check sterility of the complete media; while 2) "media with responders" are incorporated to measure spontaneous activity of the responding cell population; 3) "irradiated (Rx:Sx)" controls assess the success of blocking metabolic activity by irradiation; 4) the "autologous" controls measure the minimal activity expected from responder cells when cocultured with irradiated cells that are always the responder's own (the negative control) and 5) "pool/unrelated" controls are incorporated to measure the maximal activity expected in the assay (the positive control). In all MLC assays in this study, one of the three positive controls was the cells of one and the same individual (to provide a constant target). Cells were pulsed (radiolabelled) by adding 1 μCi [^{3}H] thymidine (6.7 Ci/mM, Du Pont, Boston, MA) into each culture 12 hours prior to harvesting. Cells were harvested with a multiple well Titertek CELL HARVESTER 530 (Flow Laboratories, Inc., McLee, VA) onto fiberglass filters (Filtermats, Skatron Inc., Sterling, VA). Each filter was placed in a scintillation vial (KIMBLE, Toledo, OH) with 5mL scintillation fluid (Filter Count 6013432, Packard Instrument Company, Inc., Downers Grove, IL). The quantity of radioactivity incorporated into DNA of the responder cells was measured as the "counts per minute" detected over 2 minutes in a liquid scintillation beta counter (Packard Tri-Carb 4530, Packard). Results are expressed as mean

counts per minute of triplicate values, and stimulation index (SI). The SI is calculated according to the formula:

$$\text{Stimulation Index} = \frac{\text{experimental MLC (mean cpm)}}{\text{autologous MLC (mean cpm)}}$$

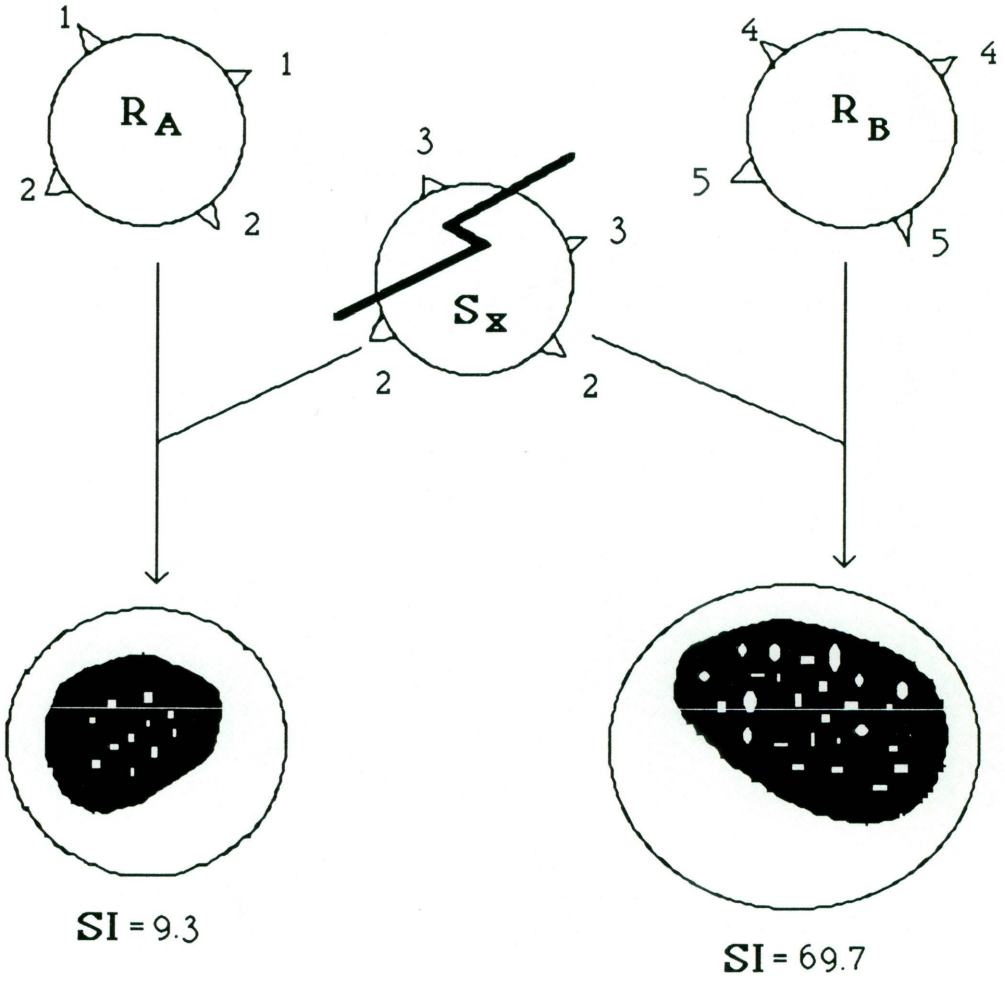
Note: Since the reaction of a responder to any one stimulator depends on two fundamental factors 1) the antigenic disparity in the direction of responder-reaction-to-stimulator, and 2) the intrinsic immunocompetence of the responder, each combination of responder and stimulator in MLC will vary from every other one. For example (see Figure 5), responder A (R_A) will react toward a single stimulator(s) to a different degree than responder B (R_B) because R_A shares an antigen in common with the stimulator while R_B is totally mismatched.

In addition, the cells of a single individual taken from circulation at different times of the day will vary significantly in their response to a single antigen; this is the result of the lymphocytes responding to circadian rhythm. Furthermore, because the immune response is a dynamic system always in a state of fluctuation, MLC reactivity of an individuals' mononuclear cells from day to day will also vary significantly. For these reasons, the data from a MLC response are always expressed (SI) in relation to the negative (autologous) control.

Co-culture MLC assays: In all assays of culturing baby (cord), mother and unrelated individual (third party: "3P") together,

Figure 5. Demonstration of the effect of different degrees of stimulation (different amounts of antigenic disparity relative to the responder) on the MLC reaction of two different responders.

Figure 5.



R_A: Responder A
R_B: Responder B
S_x: Stimulator (irradiated)

100,000 responder (cord) cells were cultured with 50,000 cells of each of the two stimulators (mother and 3P). Thus 200,000 cells in 200 μL culture media were used in each well. Whenever there were only two members represented in each culture well (such as baby and mother, or baby and 3P), then 100,000 cells in 100 μL media of both responders and stimulators were used. In this way, the correct cell concentration was maintained in all situations, and 100,000 responder cells were exposed to an equal number of stimulator cells in each experiment. The only exception was during the period of 12 or 24 hours of "pre-co-culturing" of baby and mother, before the third party was added.

Time study assays: Multiple replicate cultures were prepared for each assay to allow for pulsing and harvesting of triplicate wells at numerous intervals.

Serum for "MLC with autologous serum" assays: Heat-treated complement-inactivated autologous peripheral or cord serum (50 μL) was added to appropriate wells containing responder and stimulator cells. Results of these MLC reactions were compared with similar reactions incorporating only pooled human serum.

G. Statistical analysis

The data were subjected to statistical analysis by both paired and independent student's *t* tests. Statistical tests were considered to be significantly different at the $p \leq 0.05$ level.

Documentation of reproducibility of MLC Procedure: Proficiency testing was accomplished by providing two separate MLC assays with a single pair of whole blood specimens tested in ten replicates each. The results of these 6-day cultures were compared, and the coefficient of variation calculated for all 20 replicates. The C.V. was 11.3%. Up to 15% C.V. is acceptable by clinical labaratory standards.

RESULTS

A. Effect of NIMA on immune reactivity of organ transplant recipients as measured by graft survival rate.

The observation, by the Loma Linda University Medical Center (LLUMC) Pediatric Cardiac Transplant team, that transplantation in neonates is more successful than in older individuals is supported by statistical analysis of graft survival rates (GSR) in these patients.

Figure 6 illustrates the one-year GSR of all cardiac transplants performed at LLUMC. The data are grouped according to the age at which the patients received their grafts (LLUMC statistics). The data reported here are taken from the analysis of all cardiac transplants performed at LLUMC from November 20, 1985 to February 23, 1990. Figure 7 is a plot of the five-year GSR in all patients receiving cardiac transplants throughout the world (statistics of the Sixth Official Report - 1989 of the registry of the International Society for Heart Transplantation; Heck, *et al.*, 1989). It can be seen that within the LLUMC transplant program, the neonatal group (<1 month of age at time of surgery) has a higher (87%) GSR than the older age groups (67% - 80%). The LLUMC neonatal GSR also compares favorably with the international GSR of 78% for all ages combined.

The higher GSR observed for neonates could be attributed to any or all of several factors: 1) neonates may have deficient immunity,

Figure 6. One-year graft survival rate (GSR) of cardiac allografts at LLUMC. The data are segregated into groups based on age of recipient at time of transplantation.
(Adapted from the LLUMC Heart Transplant Survival Statistics of November 20, 1985 - February 23, 1990)

Figure 7. Five-year graft survival rate of international cardiac transplant recipients.
(Reproduced from Heck, C. F., Shumway, S. J., and Kaye, M.P. J. Heart Transplant. 8: 271, 1989)

Figure 6

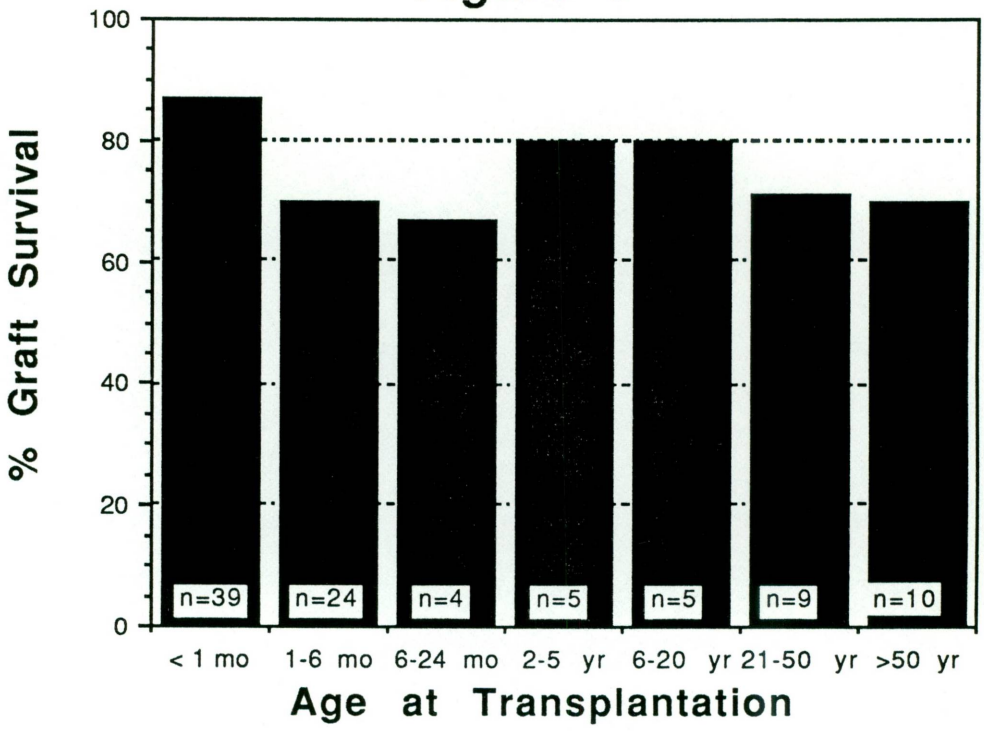
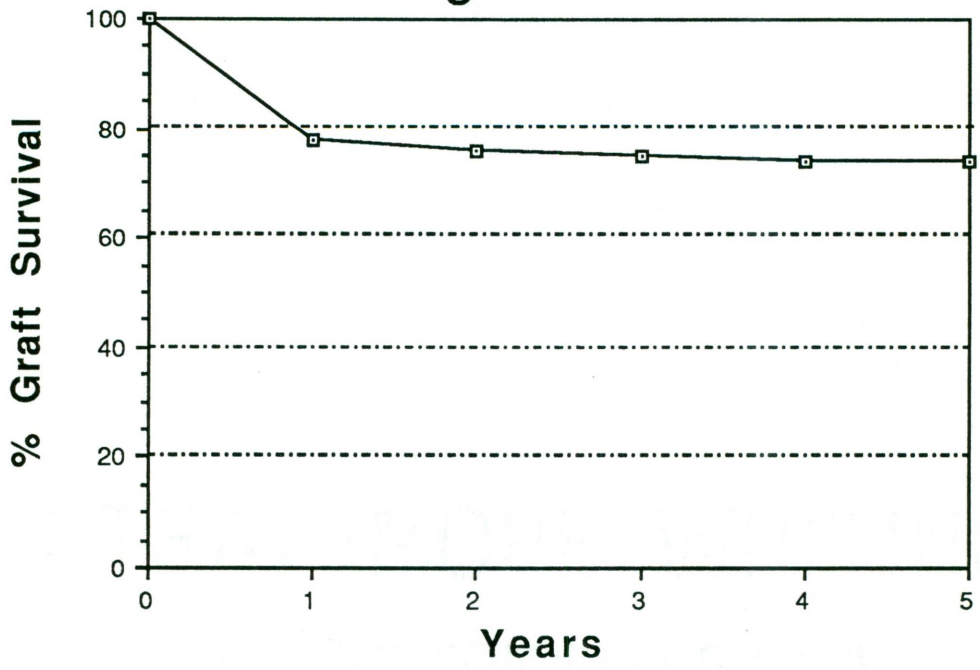


Figure 7



2) immunosuppression may have a greater effect in young than old individuals, and 3) the neonates are usually not presensitized to human leukocyte antigens (HLA), a factor correlating with poor GSR in adults. That neonates are immune deficient is supported by clinical reports on neonatal infection rates. It has been reported that 0.3% to 0.6% of newborns experience bacterial infections, of which 20% to 50% are fatal (Bellanti, *et al.*, 1987) and 0.05% of live births suffer from moderate to severe Herpes Simplex infections (Whitley, 1989). This is an indication of the fact that neonatal immunity is indeed somewhat deficient, at least with regard to microorganisms. Regarding immunosuppression, the allograft responses of all transplant recipients have to be suppressed to prevent allograft rejection. Cyclosporin (CSA), azathiopine (Imuran) and steroid hormones are routinely used as the major immunosuppressive drugs in clinical transplantation. The neonatal cardiac transplant recipients at LLUMC, however, require only minimal doses of CSA and azathioprine to manage rejection processes. Furthermore, only physiological doses of steroid (Prednisone) are used, and administration of this agent is usually discontinued after fourteen days. These low levels of immunosuppression do not result in increased rejection crises and lower GSR. Furthermore, the infection rates and growth patterns of these infant patients appear to be normal because of the limited immunosuppression therapy, particularly the absence of steroid hormones (Baum, *et al.*, 1990; Cutler, *et al.*, 1990; Marxmiller, 1990; Trimm, *et al.*, 1990).

Of importance here, is the fact that the immunosuppression needed to maintain graft viability in these neonates is significantly reduced compared to that required by infants greater than 30 days old at time of transplantation (within the LLUMC program). Indeed, transplantation maintenance immunosuppression in older patients almost always requires double, triple, or quadruple drug therapy. In the neonates, azathioprine is discontinued by one year posttransplantation and CSA has been reduced to more than half the immediate-posttransplantation dose. This ability to substantially reduce immunosuppression is peculiar to neonatal transplant recipients, whether they are human or subhuman (Bailey, *et al.*, 1985).

Lastly, presensitization to HLA is certainly less frequently encountered in neonates (a reflection of less exposure to alloantigens), but when stimulated by foreign antigens in a non-immunosuppressed state, neonates readily become sensitized. For example, a seven day old baby, receiving a heart allograft at LLUMC four days after receiving a transfusion at the referring hospital, developed antibodies in the preoperative period to at least two HLA specificities. One of these specificities was apparently found on the donor heart (unknown at the time of transplantation) and a rapid humoral reaction ensued that irreversibly rejected the graft, resulting in death of the patient.

In spite of the known decrease in neonatal immunity toward infectious organisms, the patients at LLUMC seem capable of mounting significant antiboby titers to both living organisms and vaccines (Marxmiller, 1990), and toward transfused foreign antigens. It has also been noted that these neonates require only minimal immunosuppression during periods of infection, periods notoriously known for inducing substantial immune stimulation through lymphokine release. These facts contradict the common belief that neonates have deficient immune capacity. Thus, in search of other factors that would explain the high GSR in neonates, an investigation of the affect of non-inherited maternal antigens (NIMA) was begun.

The relationships of direct antigen matching and graft survival, and of indirect matching by NIMA (between infant cardiac transplant recipients and their donors) and graft survival are presented here. The method used to determine non-inherited maternal antigens simply involves a) deducing the inherited maternal haplotype, through comparison of the mother's and baby's HLA phenotypes, and b) assuming the remaining antigens as the non-inherited haplotypes. For example:

Mother:	HLA - A 2, 3;	B 7, 35;	DR 1, 7
Child:	HLA - A 2, 23;	B 7, 39;	DR 1, 4
Inherited maternal haplotype:			A2 / B7 / DR1
Non-inherited maternal haplotype:			A3 / B35/ DR7

Major histocompatibility complex class I (HLA-A and B) and II (HLA-DR) antigen phenotypings of both cardiac transplant recipients and donors were performed routinely by the Histocompatibility Laboratory staff at the time of transplantation surgery. In addition, I have performed HLA phenotypings of the recipients' biological mother (under staff supervision) (Table 3). The results of the phenotypings are listed by specificity within the MHC classes (I and II). In this table, antigens that are "matched" (identical) between recipient and donor are indicated in bold numerals. Donor antigens that are matched with NIMA are in Italic numerals.

Analysis of these data revealed no correlations with graft survival rate (Table 4, Figures 8-10). This was interpreted to be attributable to one or more of several reasons. First, too few cases (n=42) have been studied to allow for a significant analysis. Second, there are so many confounding variables that a simple relationship between NIMA on donor tissue and GSR may never be realized. Third, sufficient follow-up time has not accumulated to be able to appreciate any significance to matching NIMA. For example, in renal grafts, it is not until the fifth year posttransplantation that the GSR reflects the HLA match between donor and recipient (Terasaki, *et al.*, 1985). Fourth and most important, it is probable that the mismatched antigens (by definition: antigens on donor tissue not found on recipient tissue) will outweigh any protective effect of NIMA matches. For example, a cell expressing five NIMA and one

Table 3

List of baby, mother and donor HLA phenotypes and NIMA matches in 42 infant cardiac transplant cases

	MHC CLASS I				MHC CLASSII		# MIS-	# NIMA
NAME	HLA-A		HLA-B		HLA-DR		MATCHES	MATCHED
CASE 1 - RECIP	**2**	X	62	X	4	X		
CASE 1 - MOM	2	X	18	62	4	11		
CASE 1 - DONOR	1	**2**	37	44	1	6	5	0
CASE 2 - RECIP	**2**	32	27	35	5	**6**		
CASE 2 - MOM	1	32	51	*57*	*4*	6		
CASE 2 - DONOR	**2**	31	51	*57*	*4*	**6**	4	2
CASE 3 - RECIP	2	3	**8**	27	**3**	7		
CASE 3 - MOM	3	11	27	*35*	2	7		
CASE 3 - DONOR	1	24	**8**	*35*	**3**	5	4	1
CASE 4 - RECIP	1	11	8	35	3	**4**		
CASE 4 - MOM	2	11	27	35	1	4		
CASE 4 -DONOR	24	28	60	70	**4**	9	5	0
CASE 5 - RECIP	2	**24**	7	18	**6**	7		
CASE 5- MOM	2	X	18	*44*	6	7		
CASE 5 - DONOR	**24**	26	*44*	51	**6**	X	4	1
CASE 6 - RECIP	**2**	X	35	44	7	X		
CASE 6 - MOM	2	32	37	44	1	*2*		
CASE 6 - DONOR	**2**	28	7	45	*2*	11	5	1
CASE 7 - PECIP	**1**	25	41	44	1	11		
CASE 7 - MOM	25	32	44	61	1	*7*		
CASE 7 - DONOR	**1**	2	8	13	3	*7*	5	1
CASE 8 - RECIP	30	31	**7**	18	3	**4**		
CASE 8 - MOM	30	31	7	13	4	7		
CASE 8 - DONOR	2	24	**7**	44	2	**4**	4	0
CASE 9 - RECIP	28	31	51	60	4	5		
CASE 9 - MOM	2	31	60	62	4	X		
CASE 9 - DONOR	26	33	7	53	2	7	6	0
CASE 10 - RECIP	2	23	7	51	5	6		
CASE 10 - MOM	2	X	7	51	1	6		
CASE 10 - DONOR	1	X	8	X	3	X	6	0
CASE 11 - RECIP	2	X	44	X	4	8		
CASE 11 - MOM	2	29	44	62	8	X		
CASE 11 - DONOR	3	25	14	35	1	7	6	0
CASE 12 - RECIP	**1**	**2**	**8**	**44**	4	X		
CASE 12 - MOM	2	23	44	X				
CASE 12 - DONOR	**1**	**2**	**8**	**44**	3	5	2	?

Table 3. (CONT.)

	MHC CLASS I				MHC CLASSII		# MIS-	# NIMA
NAME	HLA-A		HLA-B		HLA-DR		MATCHES	MATCHED
CASE 13 - RECIP	1	**2**	8	27	3	11		
CASE 13 - MOM	1	2	27	38	4	5		
CASE 13 - DONOR	**2**	X	18	44	1	2	5	0
CASE 14 - RECIP	**2**	**29**	44	62	4	7		
CASE 14 - MOM	2	29	*42*	44	5	7		
CASE 14 - DONOR	**29**	30	*42*	57	6	11	5	2
CASE 15 - RECIP	**1**	3	8	35	**2**	3		
CASE 15 - MOM	1	38	8	35	2	8		
CASE 15 - DONOR	**1**	23	7	70	**2**	7	4	0
CASE 16 - RECIP	1	X	8	60	4	13		
CASE 16 - MOM	1	X	8	57	4	6		
CASE 16 - DONOR	2	24	62	X	13	X	5	0
CASE 17 - RECIP	3	23	51	70	1	5		
CASE 17 - MOM	2	23	58	70	3	11		
CASE 17 - DONOR	1	28	44	62	4	X	6	0
CASE 18 - RECIP	26	31	49	56	1	8		
CASE 18 - MOM	3	31	39	49	1	2		
CASE 18 - DONOR	2	28	44	60	14	X	6	0
CASE 19 - RECIP	**2**	28	61	62	4	11		
CASE 19 - MOM	2	X	18	62	7	11		
CASE 19 - DONOR	**2**	23	35	51	1	3	5	0
CASE 20 - RECIP	**2**	X	**35**	60	**4**	8		
CASE 20 - MOM	2	X	7	35	2	8		
CASE 20 - DONOR	**2**	3	**35**	44	1	**4**	3	0
CASE 21 - RECIP	**2**	28	7	39	**2**	4		
CASE 21 - MOM	2	X	7	X	2	3		
CASE 21 - DONOR	**2**	25	8	18	**2**	11	4	0
CASE 22 - RECIP	24	31	7	8	3	14		
CASE 22 - MOM	*11*	24	7	27	6	8		
CASE 22 - DONOR	2	*11*	35	62	4	X	6	0
CASE 23 - RECIP	24	31	35	51	1	X		
CASE 23 - MOM	2	31	44	51	1	6		
CASE 23 - DONOR	1	3	7	57	4	7	6	0
CASE 24 - RECIP	11	24	44	55	7	14		
CASE 24 - MOM	2	11	44	62	7	X		
CASE 24 - DONOR	1	26	8	38	3	11	6	0

Table 3. (CONT.)

	MHC CLASS I				MHC CLASSII		# MIS-	# NIMA
NAME	HLA-A		HLA-B		HLA-DR		MATCHES	MATCHED
CASE 25 - RECIP	3	28	7	61	**2**	6		
CASE 25 - MOM	1	3	7	8	2	3		
CASE 25 - DONOR	2	25	18	62	**2**	X	5	0
CASE 26 - RECIP	2	28	8	27	3	12		
CASE 26 - MOM	2	*11*	8	44	3	7		
CASE 26 - DONOR	*11*	25	18	41	2	X	6	1
CASE 27 - RECIP	2	31	38	44	**4**	X		
CASE 27 - MOM	2	X	44	X	4	5		
CASE 27 - DONOR	3	X	35	51	**4**	7	5	0
CASE 28 - RECIP	1	**2**	49	53	2	8		
CASE 28 - MOM	1	23	12	49	2	3		
CASE 28 - DONOR	**2**	3	47	62	4	7	5	0
CASE 29 - RECIP	1	3	8	X	3	X		
CASE 29 - MOM	1	2	8	*44*	3	4		
CASE 29 - DONOR	29	30	7	*44*	2	8	6	1
CASE 30 - RECIP	**23**	**26**	44	X	7	X		
CASE 30 - MOM	1	26	8	44	3	7		
CASE 30 - DONOR	**23**	**26**	7	57	1	10	4	0
CASE 31 - RECIP	1	32	44	58	4	X		
CASE 31 - MOM	1	24	27	58	4	7		
CASE 31 - DONOR	31	33	35	44	1	X	5	0
CASE 32 - RECIP	3	31	35	**60**	1	**2**		
CASE 32 - MOM	3	X	7	35	1	*4*		
CASE 32 - DONOR	11	29	44	**60**	**2**	*4*	4	1
CASE 33 - RECIP	**24**	34	18	44	12	X		
CASE 33 - MOM	1	32	44	62	1	12		
CASE 33 - DONOR	**24**	30	13	63	6	7	5	0
CASE 34 - RECIP	**2**	X	7	**51**	2	6		
CASE 34 - MOM	2	30	18	51	6	*8*		
CASE 34 - DONOR	**2**	3	**51**	60	*8*	9	4	1
CASE 35 - RECIP	**2**	24	35	51	8	X		
CASE 35 - MOM	2	24	35	*62*	*4*	8		
CASE 35 - DONOR	**2**	3	37	*62*	*4*	7	5	2
CASE 36 - RECIP	1	**2**	7	**44**	**2**	6		
CASE 36 - MOM	2	X	7	44	2	12		
CASE 36 - DONOR	**2**	28	**44**	51	**2**	8	3	0

Table 3. (CONT.)

	MHC CLASS I				MHC CLASSII		# MIS-	# NIMA
NAME	HLA-A		HLA-B		HLA-DR		MATCHES	MATCHED
CASE 37 - RECIP	**2**	23	44	70	**4**	7		
CASE 37 - MOM	23	24	7	44	4	7		
CASE 37 - DONOR	1	**2**	13	14	**4**	5	4	0
CASE 38 - RECIP	1	**2**	8	62	3	4		
CASE 38 - MOM	1	31	8	56	3	9		
CASE 38 - DONOR	**2**	24	7	70	8	12	5	0
CASE 39 - RECIP	1	25	7	8	2	3		
CASE 39 - MOM	1	*2*	8	44	3	4		
CASE 39 - DONOR	*2*	24	39	56	5	X	6	1
CASE 40 - RECIP	**1**	26	8	38	3	6		
CASE 40 - MOM	2	26	38	44	4	6		
CASE 40 - DONOR	**1**	24	53	57	7	X	5	0
CASE 41 - RECIP	2	3	57	65	4	**6**		
CASE 41 - MOM	2	24	18	57	4	11		
CASE 41 - DONOR	11	28	35	53	**6**	X	5	0
CASE 42 - RECIP	**2**	X	39	**44**	**4**	X		
CASE 42 - MOM	2	3	35	44	1	4		
CASE 42 - DONOR	**2**	X	**44**	57	**4**	X	3	0

Bold numerals indicate matched HLA between recipient and donor

Italic numerals are the donor matches with NIMA

NOTE:
HLA phenotyping of recipient and donor were performed by LLUMC Histocompatibility Laboratory staff for routine clincial analysis.
HLA phenotyping of the mothers was performed by Leh Chang.
(Class II antigens of case 12 mother cannot be discovered.)

Table 4. Distribution of infant cardiac transplant recipients by number of mismatches (A), matches with class I NIMA (B) or class II NIMA (C), and both class I and II NIMA (D).

A

Donor-Recipient mismatches

Number Mismatches	<30d	31-60d	61-90d	91d-7m
0	0	0	0	0
1	0	0	0	0
2	1	0	0	0
3	1	0	0	1
4	7	1	2	0
5	12	2	0	4
6	4	3	2	2

Age at Time of Transplant

B

Class I matches with NIMA

Number Matches	<30d	31-60d	61-90d	91d-7m
4	0	0	0	0
3	0	0	0	0
2	0	0	0	0
1	4	1	2	1
0	20	5	2	6

Age at Time of Transplant

C

Class II matches with NIMA

Number Matches	<30d	31-60d	61-90d	91d-7m
2	0	0	0	0
1	3	1	1	1
0	21	5	3	6

Age at Time of Transplant

D

Class I and II matches with NIMA

Number Matches	<30d	31-60d	61-90d	91d-7m
6	0	0	0	0
5	0	0	0	0
4	0	0	0	0
3	0	0	0	0
2	3	0	0	0
1	3	2	3	2
0	19	4	1	5

Age at Time of Transplant

Figure 8. The effect of HLA mismatches on the six-month graft survival rate of 42 infant transplant recipients (< 7 months of age at time of cardiac transplantation).

Figure 8

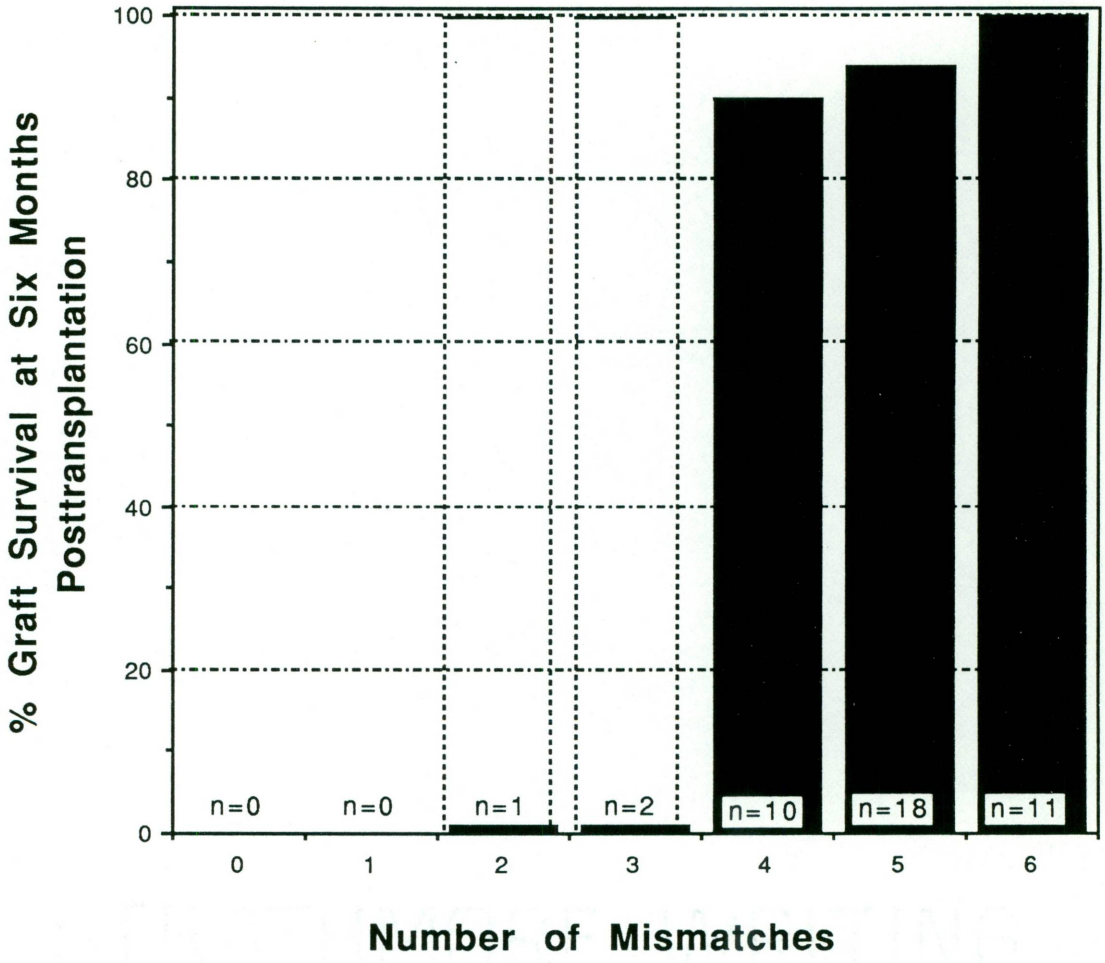

% Graft Survival at Six Months Posttransplantation
100
80
60
40
20
0
n=0
n=0
n=1
n=2
n=10
n=18
n=11
0
1
2
3
4
5
6
Number of Mismatches

Figure 9. The effect of NIMA class I and II matches on the six-month graft survival rate of infant transplant recipients (< 7 months of age at time of cardiac transplantation) (n = 41). (Case 12 not included - DR antigens unknown.)

Figure 9

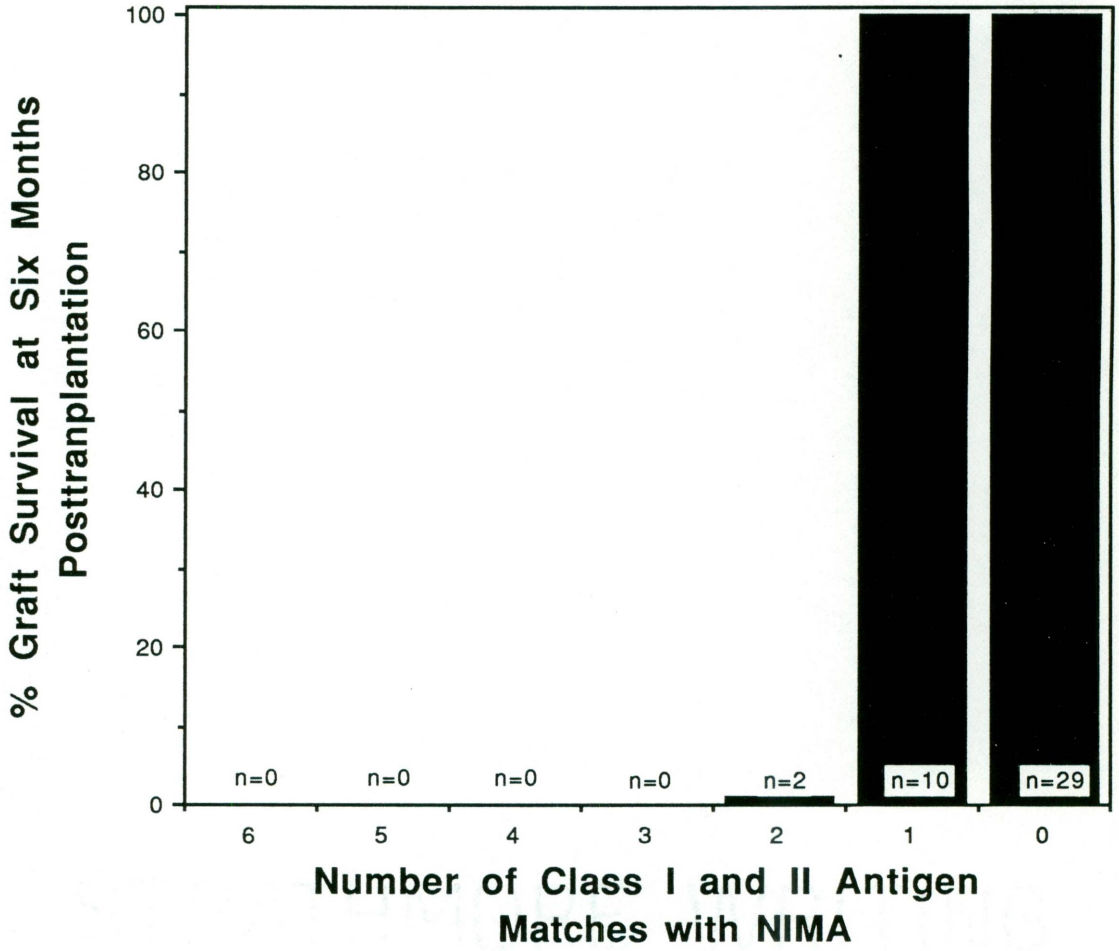

% Graft Survival at Six Months Posttranplantation
100
80
60
40
20
0
n=0
n=0
n=0
n=0
n=2
n=10
n=29
6
5
4
3
2
1
0
Number of Class I and II Antigen Matches with NIMA

Figure 10a. The effect of NIMA class I matches on the six-month graft survival rate of infant transplant recipients (< 7 months of age at time of cardiac transplantation) (n=41).

Figure 10b. The effect of NIMA class II matches on the six-month graft survival rate of infant transplant recipients (< 7 months of age at time of cardiac transplantation) (n=41).

Figure 10a

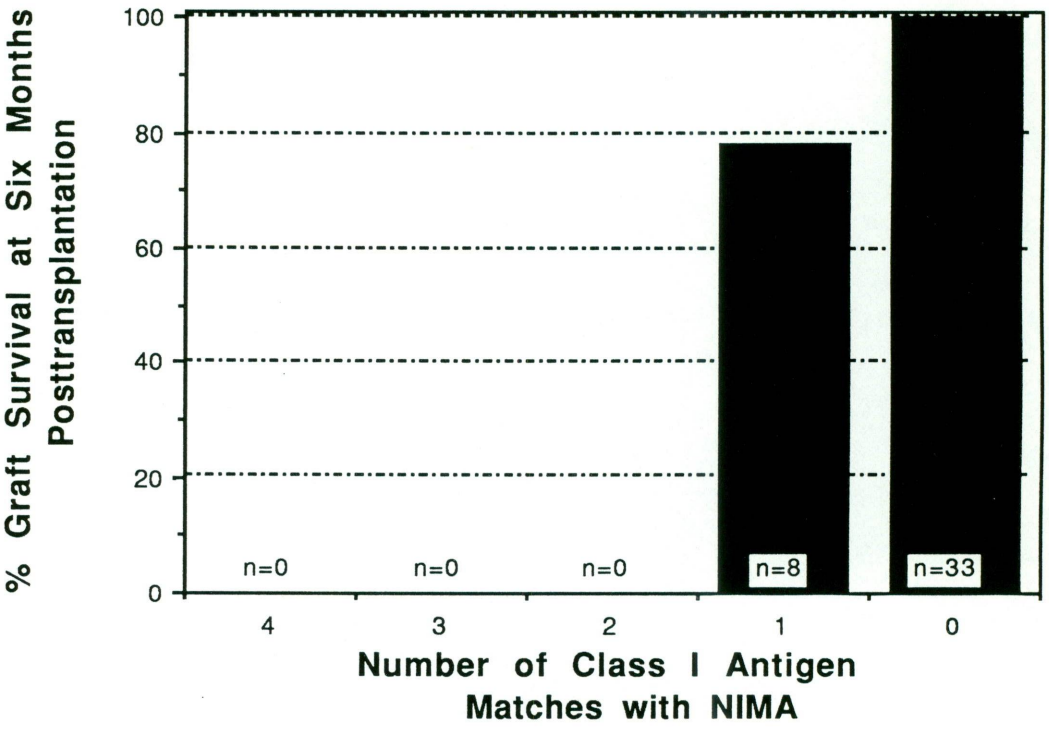
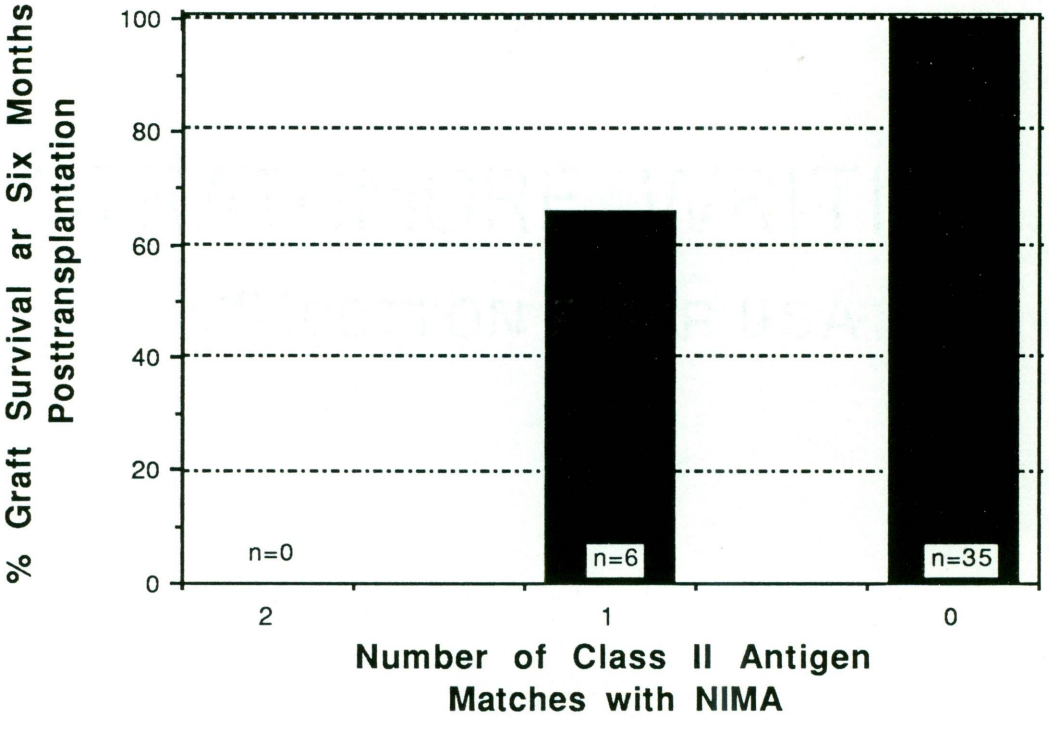


Figure 10b



mismatch can be easily killed by immune attack against the one mismatch.

Having exhausted the various possibilities of explaining extended GSR through correlation with direct and indirect HLA matching, functional assays were designed to test donor-specific cell-mediated alloreactivity of the neonatal recipients.

B. Mixed Lymphocyte Culture Reactions of Peripheral Blood Lymphocytes from Neonates

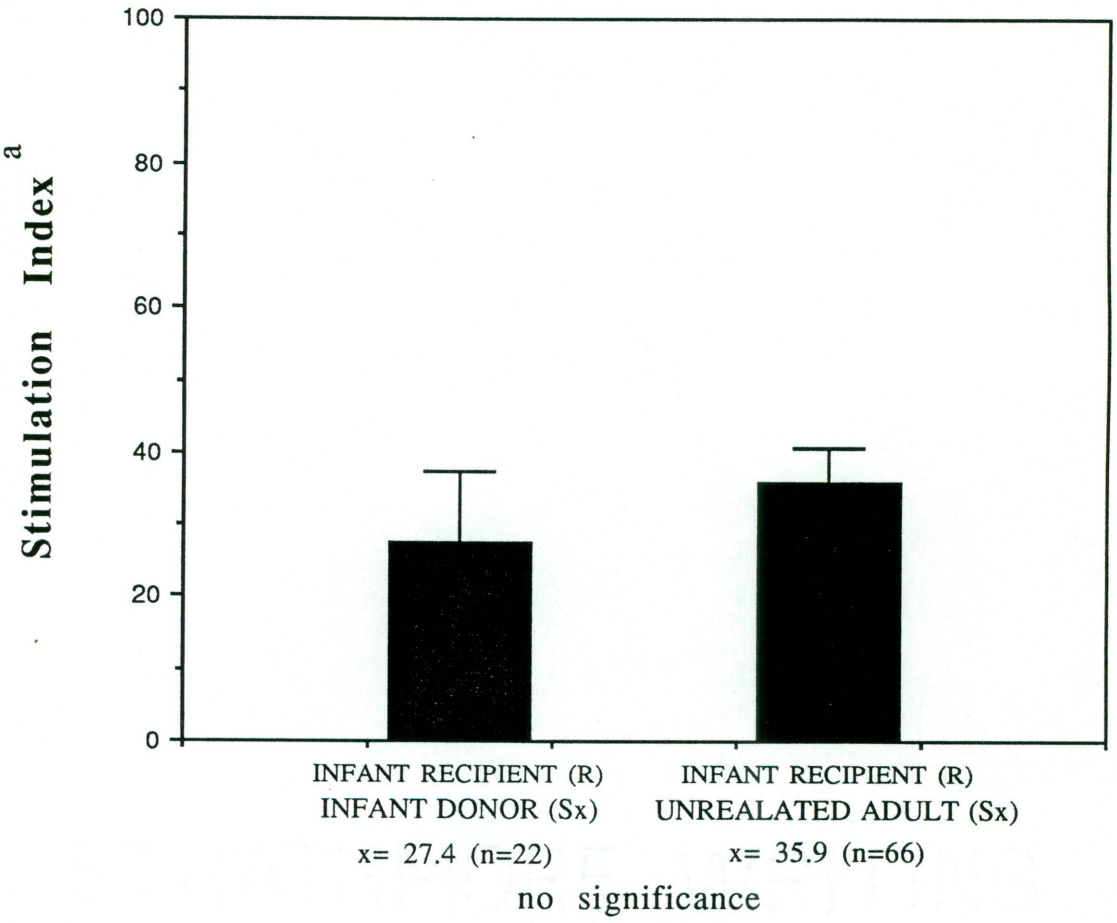
Venous blood specimens were obtained from each transplant recipient and his donor at the time of transplantation, before administration of immunosuppression and general anesthesia.

Figure 11 shows the MLC results of 22 neonatal transplant recipients to their respective infant allograft donors (n=22) and 3 each of unrelated adult controls (n=66) (55% of the 22 MLC were performed by Leh Chang). The stimulation index (SI) for each sample is calculated as the ratio of a) the responder toward antigens on the stimulator (donor or unrelated control) mononuclear cells, to b) the response towards autologous cells.

When these results were reviewed, an increase in the radiolabel-uptake in the autologous controls was noted to be higher than adult autologous reactions (Figure 12). As mentioned in the Methods and Materials section, the autologous control is used to measure the minimal activity expected from the responder cells; it is measured by

Figure 11. Mixed lymphocyte culture reactions of isolated infant peripheral blood mononuclear cells toward unrelated infant (cardiac donor) and adult (standard control) cells. Results are expressed as mean stimulation index ± S.E.

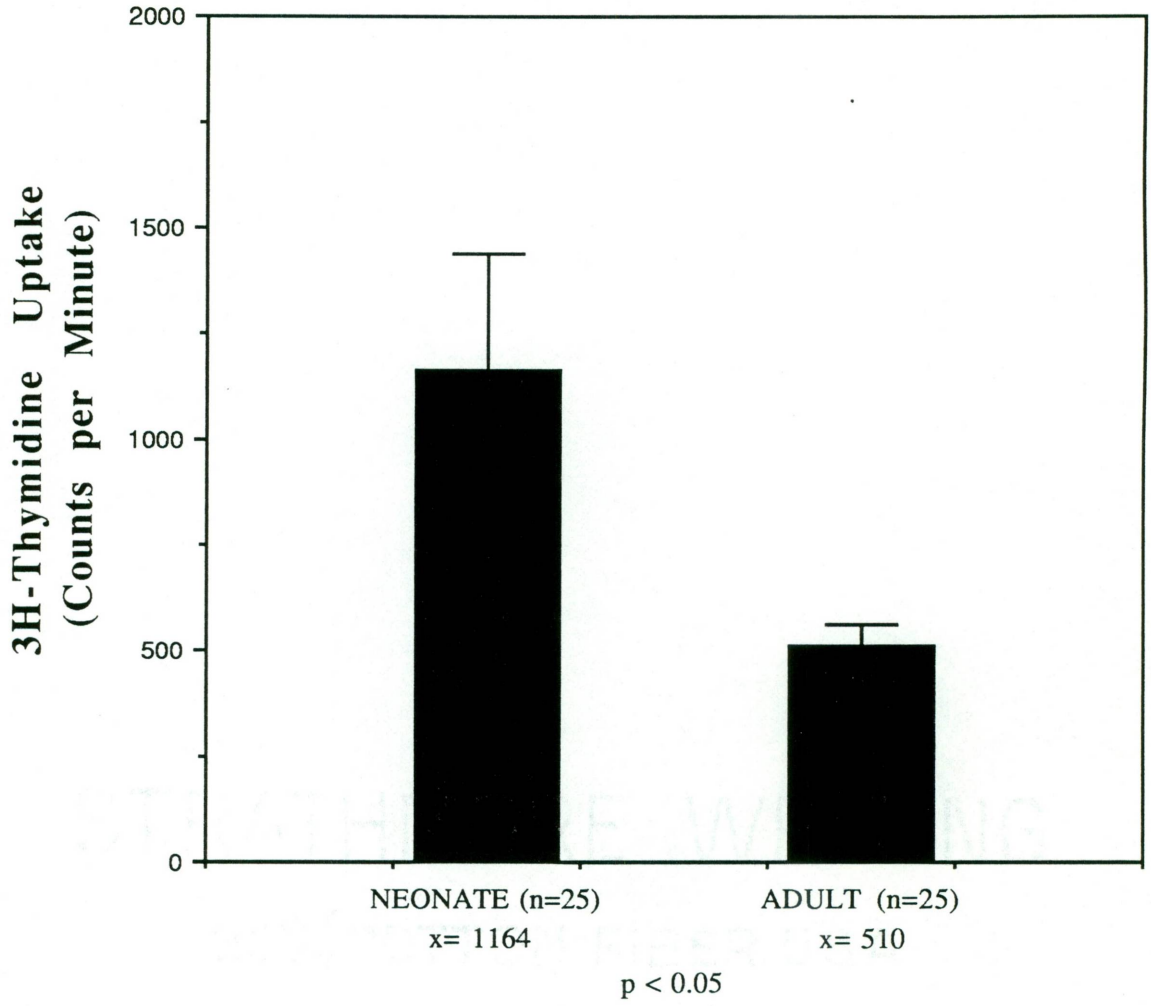
Figure 11



a: Stimulation Index = $\frac{\text{experimental cpm}}{\text{autologous cpm}}$

Figure 12. Comparison of levels of ^{3}H-thymidine uptake in neonate and adult cells in autologous control cultures. Results are expressed as mean counts per minute $\pm$ S.E.

Figure 12


3H-Thymidine Uptake
(Counts per Minute)
2000
1500
1000
500
0
NEONATE (n=25)
x= 1164
ADULT (n=25)
x= 510
p < 0.05

testing the responder against himself. Furthermore, it is against this autologous response that all the other responses are measured (by definition, the SI). In any MLC, the responder T cells are being stimulated by the class II antigens on B and non-T cells of the stimulator population (Kuntz, *et al.*, 1976). It has been reported that neonatal blood contains greater absolute numbers and higher percentages of non-T cells than adult blood (Hooper, *et al.*, 1986; Murgita, *et al.*, 1983; Stutman, 1985) Therefore, the increase in activity noted in the neonatal samples could be due to the increase in the number of cells expressing class II antigens. However, stimulation by maternal antigens before birth, and the effect of neuroendocrine hormonal changes occurring during parturition must also be considered as possibly affecting stimulation of the responders (Batra, *et al.*, 1976; Challis, *et al.*, 1974; Tulchinsky, *et al.*, 1972).

To test this hypothesis, neonatal cells were simply incubated with ^{3}H-thymidine for two hours and the radiolabelling of these cells was quantitated. In this way, spontaneous blastogenesis (SB) can be measured, and used to indicate the degree of *in vivo* stimulation occurring before the blood specimen was obtained. The spontaneous blastogenesis of these neonate samples were always greater than adult cells measured in the same manner [neonate: 500-1500 counts per minute (cpm) versus adult: 360 cpm (mean of >1400 SB assays at LLUMC)].

As illustrated in Figure 11, the mean SI of infant recipient cells reacting to infant donor cells (27.4 $\pm$ 9.8) is not statistically different than that when reacting to normal adult controls (35.9 $\pm$ 4.8). The results also indicate that the class II antigen matches in the recipient and donor pairs appear to be just as poorly-matched for class II lymphocyte-defined antigens as for class I and II serum-defined antigens. Since the infant cells are capable of reacting strongly and appropriately to the infant donor cells (stimulators), the MLC reactivity does not correlate with the *in vivo* observations. That is, if the infant cardiac transplant recipients are not reacting to their donor grafts (the clinical observation), one would expect to see a reduction in the stimulation indexes of infant recipients toward infant donor cells in the MLC. This is not the case. In fact, it has been observed that in the general population receiving kidney allografts at older ages, the MLC does not always reflect or predict *in vivo* phenomena (Streilein, *et al.*, 1989). It is assumed that this discrepancy is due to the problem that occurs when testing is done on only circulating cells (which represent only part of the immune system) and that, furthermore, they are removed from all contact with their natural blood environment and other organ systems.

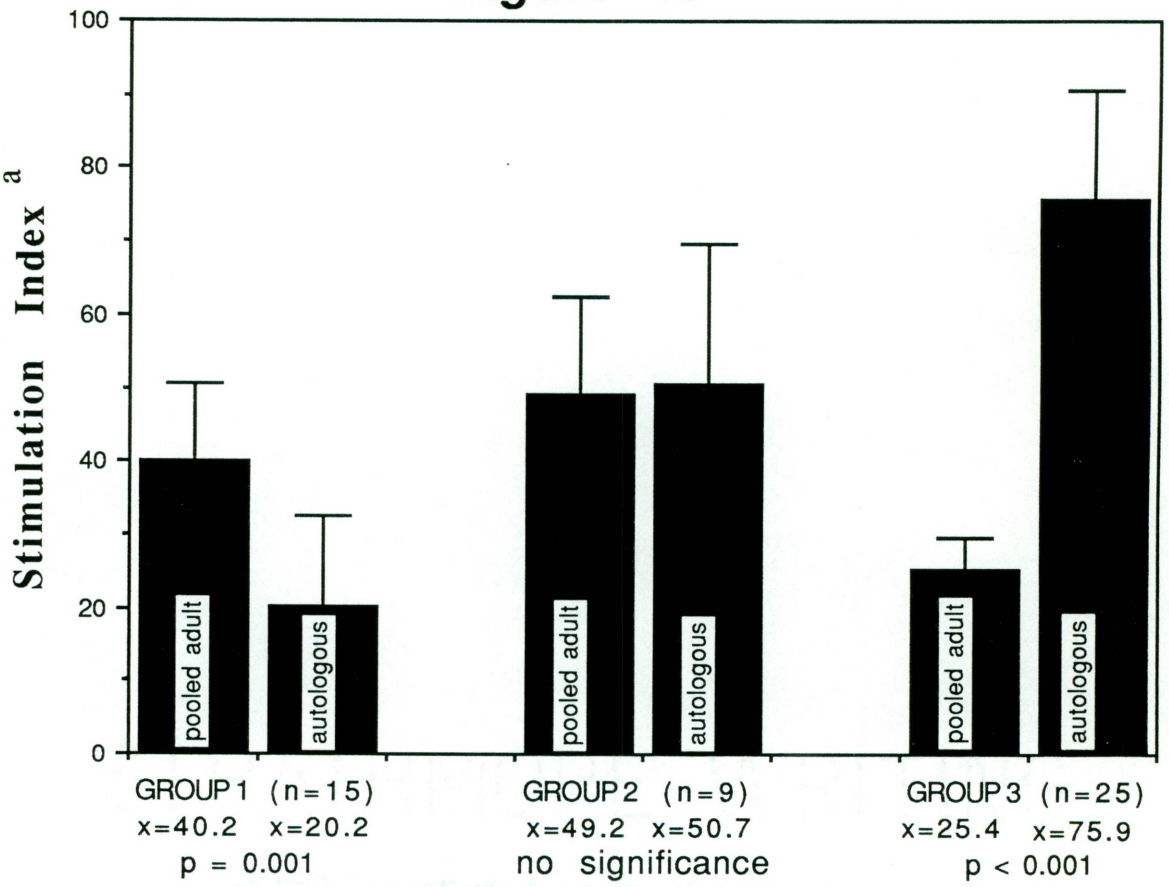
If isolating the cells from peripheral blood was effectively eliminating some circulating factor(s) that was contributing to *in vivo* suppression, then it was reasoned that the addition of peripheral autologous serum to the MLC assay might result in more closely

imitating the *in vivo* natural environment. To test this hypothesis, MLC assays were set up to evaluate the effect of adding heat-treated, complement-inactivated autologous serum (experimental) as compared to the addition of pooled adult serum (control). Since each well of the culture plate contains 200,000 cells, 50 μL of either autologous or pooled adult serum was added (because 200,000 lymphocytes are normally found in 50 μL of whole blood). In addition, since it was observed (Figure 11) that infant donor cells are slightly weaker stimulators than adult cells in MLC reactions, only adult cells were used to stimulate the responders in this section of the study.

The addition of autologous serum to the MLC yielded inconsistent results; both suppressive and stimulatory actions were observed. Using the definitions of non-reactivity and reactivity from clinical MLC methodology (Dupont, *et al.*, 1976), the results of each assay seemed to fit into one of three apparent groups: 1) >20% inhibition, 2) modification within $\pm$ 20%, or 3) >20% augmentation (Figure 13). Although statistical analysis (paired t-test) shows that a significant change has been induced by the addition of autologous serum to cultures of groups 1 and 3, but not group 2, the differences cannot be explained easily. In group 1, the autologous serum inhibited the reaction, indicating the presence of suppressor factor(s). Yet, the assays falling into the second and third groups seem to indicate that either no or insufficient suppressor factor exists, or that there is a stimulatory factor(s) that further activates the cells. How

Figure 13. Effect of adding autologous serum to infant (cardiac recipient) isolated peripheral blood mononuclear cells in mixed lymphocyte culture against unrelated adults. Results are expressed as mean stimulation index ± S.E.

Figure 13



GROUP 1: MLC reactivity inhibited > 20% of MLC with no serum
GROUP 2: MLC reactivity not changed (-20% to + 20%) from MLC with no serum
GROUP 3: MLC reactivity augmented > 20% of MLC with no serum

a: Stimulation Index = $\frac{\text{experimental cpm}}{\text{autologous cpm}}$

or why these events are taking place cannot be answered in this setting. What can be deduced is that these results do not correlate consistently with the clinical observation of an apparent natural suppression, and that the assay design used herein does not accurately reflect that observation.

It appeared from these studies that there were at least two problems with the MLC assay. First, as already mentioned earlier, isolated peripheral blood cells cannot represent the total immune system as it functions *in vivo*. Second, cells derived from the neonatal period of life may not be able to yield information on the nature of fetal immunity. It appeared that cord blood, already known to contain cells found in fetal but not neonatal circulation, was a good source of material for this study. Not only could cord blood samples provide large numbers of cells, but also cells that were qualitatively and quantitatively closer to representing the fetal condition than neonatal peripheral blood. Therefore, it was decided that umbilical cord blood should be used hereafter, as it could offer a better model for this study.

C. Mixed Lymphocyte Culture Reactions of Cord Blood Cells and Demonstration of Natural Suppression Inducible by Maternal Cells

In consultation with E. Sakala, M.D., a proposal was prepared for obtaining fresh cord blood and related maternal venous blood. Only term babies (38-42 weeks gestation) from normal pregnancies,

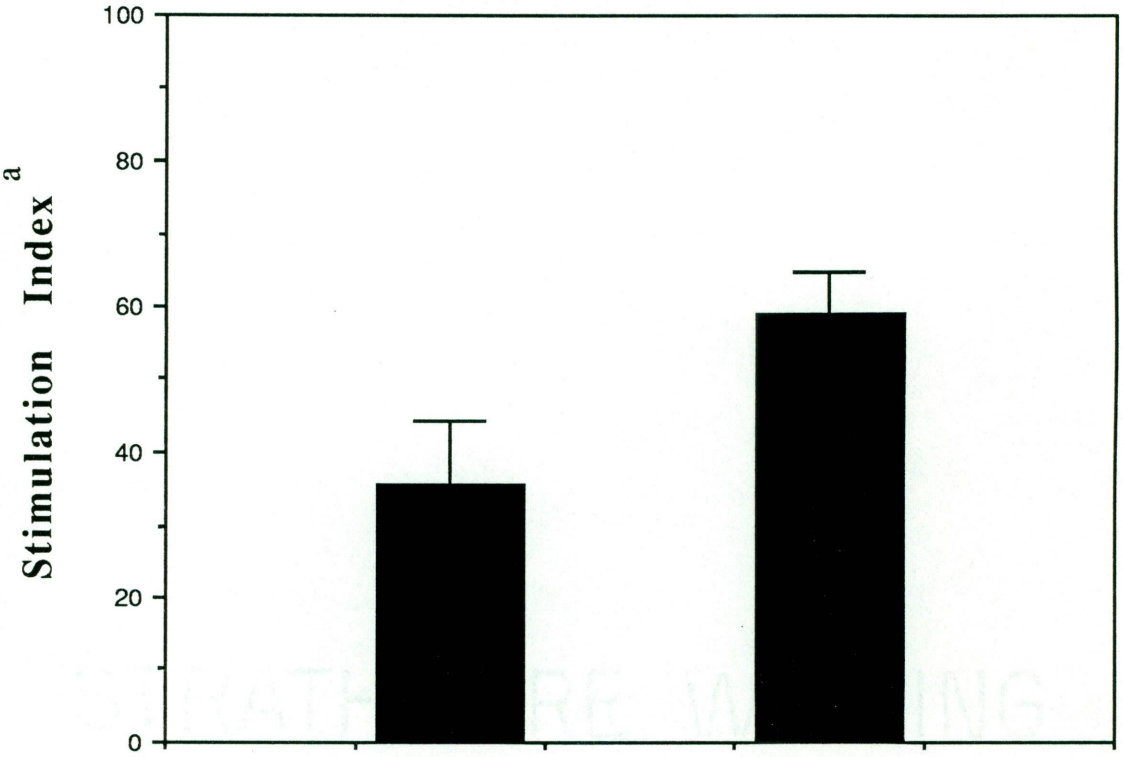
delivered either vaginally or by cesarean section, were included in the protocol. This proposal was submitted to, and accepted by, the LLU Institutional Review Board. (All assays performed by Leh Chang.)

Cord blood was obtained at the time of delivery (kindly provided by E. Sakala, M.D.); and peripheral blood from the biological mother was collected within 24 hours of delivery. Mixed lymphocyte culture assays were conducted that included isolated mononuclear cells of cord blood as responder and peripheral mononuclear cells of either the biological mother or unrelated adult as stimulator.

Before starting these MLC assays, a small study was carried out to determine the ability of cord cells to respond to foreign antigen. To test this, the reactions of cord cells were compared with those of healthy adult cells when either were cultured against unrelated adult stimulators. The results of these assays, depicted in Figure 14, indicate that umbilical cord cells are significantly ($p < 0.05$) less capable of responding to alloantigens in MLC reactions than adult cells. Nonetheless, they are capable of responding to the same degree as normal adults reacting to one-haplotype mismatched relatives (siblings, parents or progeny; SI = 20-40; LLUMC Histocompatibility MLC data). This apparent suppression of cord cell alloreactivity indicates either the presence of suppressor cells, or a degree of intrinsic inability of cord lymphocyts to react strongly.

Figure 14. Demonstration of the immunocompetence of isolated cord blood mononuclear cells compared to similar cells of adults by reacting them in the mixed lymphocyte culture against an unrelated individual. Results are expressed as mean stimulation index ± S.E.

Figure 14



x= 35.5 (n=25)

x= 58.9 (n=133)

$p < 0.05$

a: Stimulation Index = $\frac{\text{experimental cpm}}{\text{autologous cpm}}$

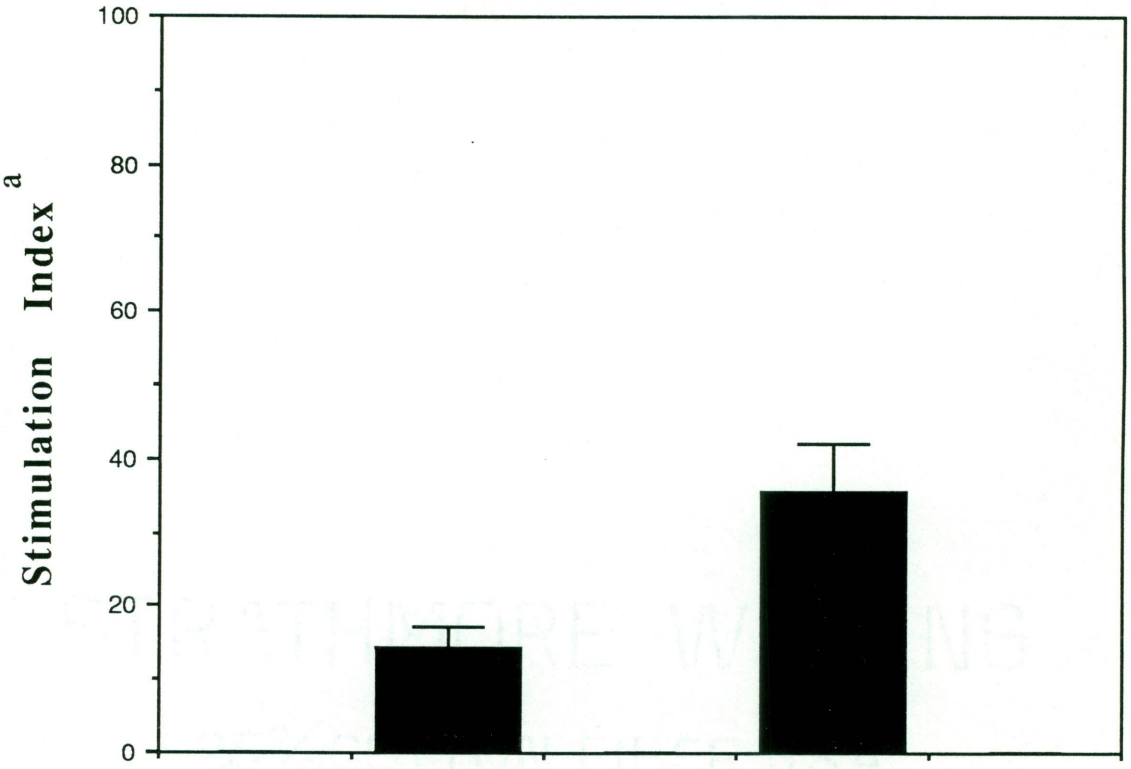
Having ascertained that cord cells are functionally capable of responding (albeit less strong than neonatal or adult), their response to maternal cells was tested and compared with their reaction to unrelated individuals (third party). Since the cord cells share half of the antigens with the maternal stimulators, the response to maternal cells was expected to be reduced as compared to that against third party. As expected, cord cells responded much less vigorously (<50%) to maternal than to third party cells (Figure 15). Although mother and child share antigens (and, therefore, the response toward the mother is diminished), the possibility of suppression induced by maternal cells in these cultures cannot be ruled out.

Recalling that neonatal cells expressed higher background activity (autologous control values and spontaneous blastogenesis, Figure 12) than adult cells, special attention was given to measuring the spontaneous activity of these cord specimens. Indeed, a substantial increase was also noted in the cord blood autologous control values (Figure 16). Again, the background activity of these cells was assessed further with the spontaneous blastogenesis assay, and this revealed higher spontaneous activity in the cord cells than in neonatal cells (1000-2000 cpm versus 500-1500 cpm ^{3}H-Thymidine incorporation, respectively).

Since the products within cord blood are quite different than in neonatal peripheral blood, the "MLC with autologous serum" experiments were repeated using autologous cord serum. The results

Figure 15. Demonstration of the immunocompetence of isolated cord blood mononuclear cells reacting to antigens of either maternal cells or third party cells, tested by mixed lymphocyte culture. Results are expressed as mean stimulation index $\pm$ S.E.

Figure 15

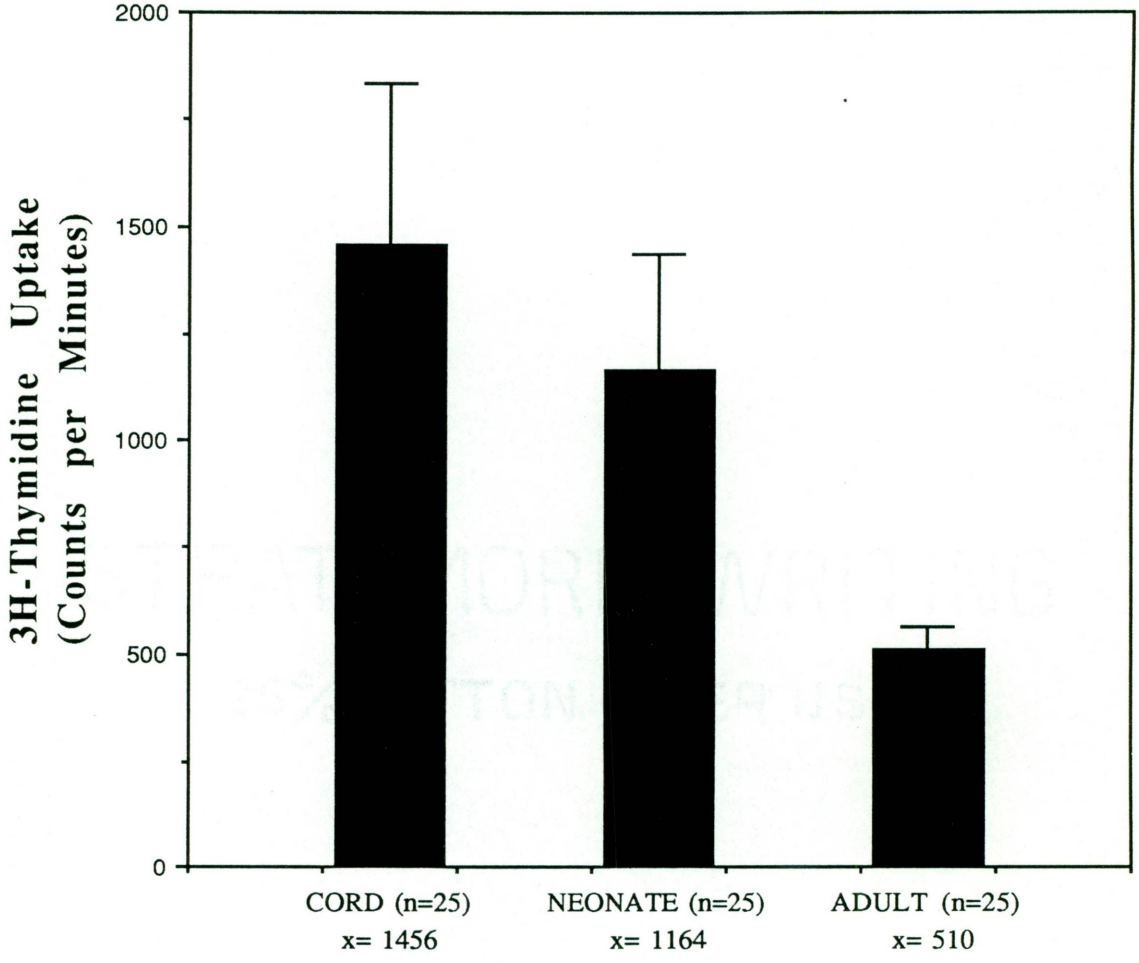


$p < 0.01$ (n=25)

a: Stimulation Index = $\frac{\text{experimental cpm}}{\text{autologous cpm}}$

Figure 16. Comparison of levels of ^{3}H-thymidine uptake in cord, neonate and adult cells in autologous control cultures. Results are expressed as mean counts per minute $\pm$ S.E.

Figure 16

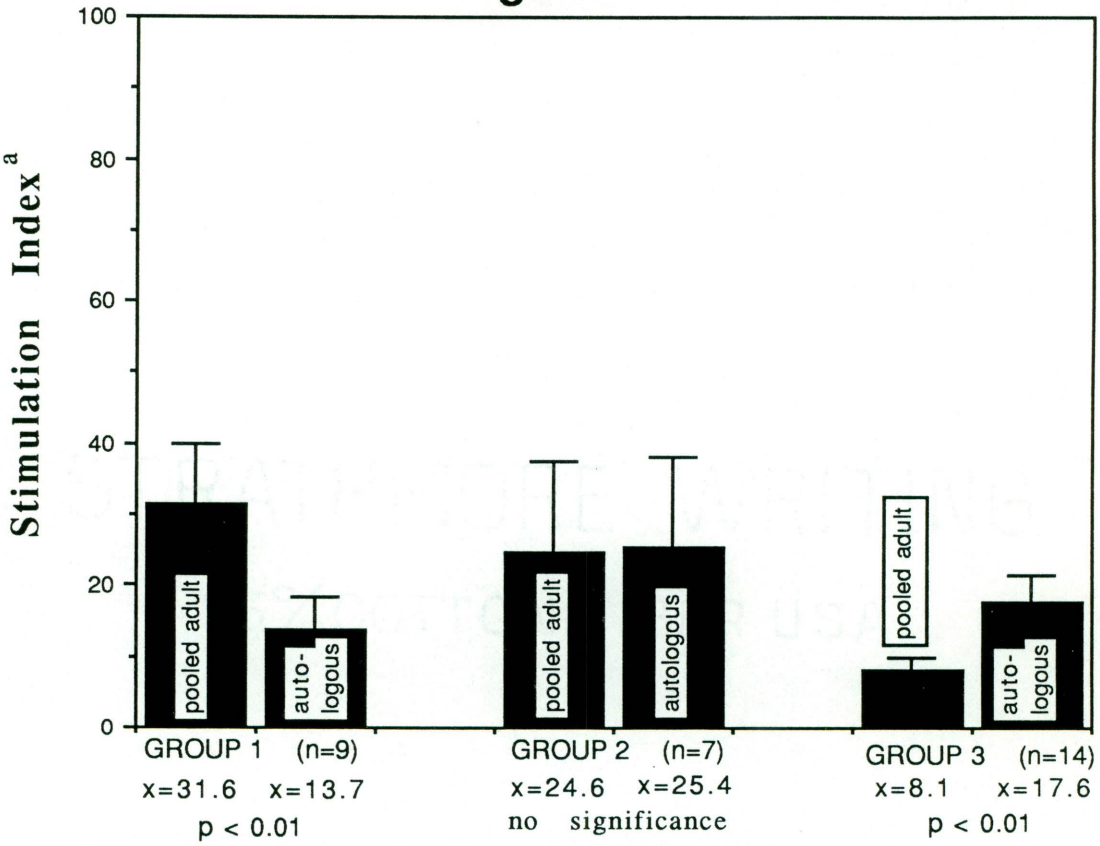

3H-Thymidine Uptake
(Counts per Minutes)
2000
1500
1000
500
0
CORD (n=25)
x= 1456
NEONATE (n=25)
x= 1164
ADULT (n=25)
x= 510

of this effort are illustrated in Figure 17. The data from these assays were somewhat similar to those from culturing neonatal cells with autologous peripheral serum (Figure 13), but the overall responses of cord cells were noted to be substantially lower than those of the neonatal cells. Since the order of activity of autologous controls has been observed to be cord $\geq$ neonate > adult (Figure 16), and the order of overall MLC responsiveness to be cord = neonate < adult (compare Figure 11 with 14), then the low responses in these cord blood cells can not be assigned to an inability of cord cells to react. Rather, it may be due to the fact that the stimulation index reflects the amount of stimulation expressed by the responder to a stimulator above and beyond the background stimulation observed in the autologous cultures. In other words, if the total capacity of a cell to react to allogeneic cells is considered to be 100%, and the reaction expressed in the autologous culture is 80%, then there is only a 20% allowance for further reactivity.

Seeking a demonstration of suppressive activity by these young cells in the presence of maternal antigens, the cord blood cells of fourteen babies were cocultured together with a combination of maternal and third party cells (equal parts of each stimulator population) for six days. The mean SI of these 3-member cocultures was 7.1 units lower than cord with third party alone (Figure 18), a small but significant decrease. Since cord cells in these experiments were being exposed to both unrelated (third party) and maternal cells simultaneously, it was reasoned that any maternally-induced

Figure 17. Effect of adding autologous cord serum to isolated cord blood mononuclear cells in mixed lymphocyte culture with unrelated individual. Results are expressed as mean stimulation index $\pm$ S.E.

Figure 17

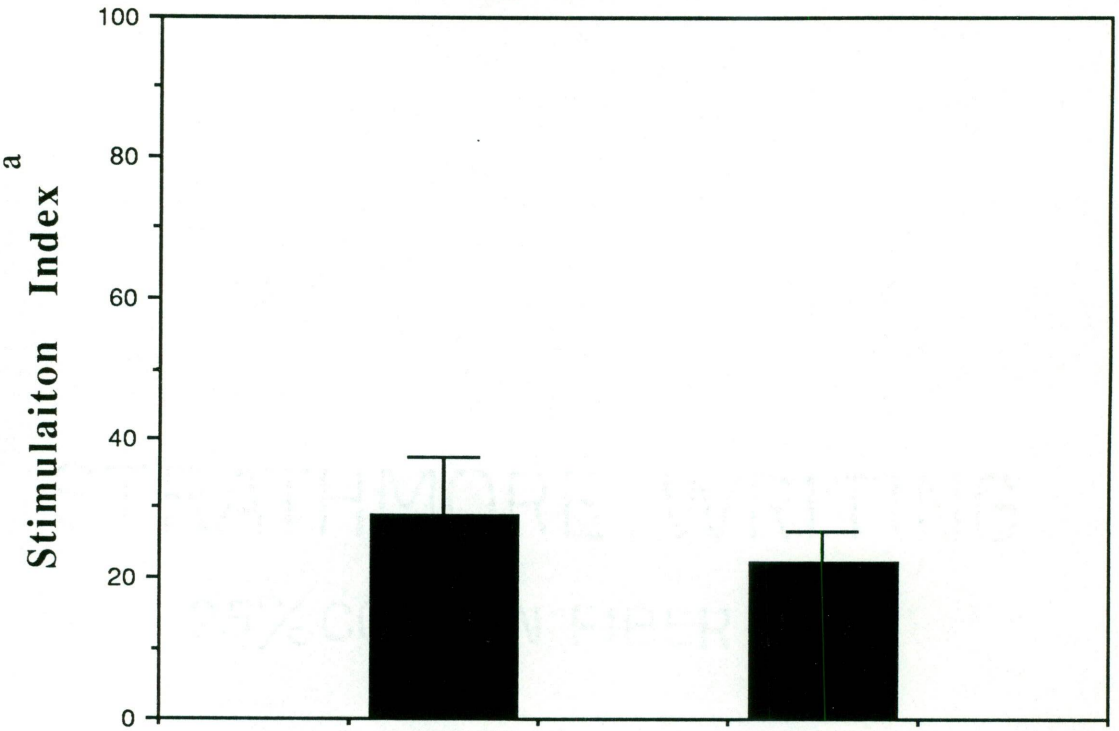


GROUP 1: MLC reactivity inhibited > 20% of MLC with no serum
GROUP 2: MLC reactivity not changed (-20% to +20%) from MLC with no serum
GROUP 3: MLC reactivity augmented >20% of MLC with no serum

a: stimulation index = $\frac{\text{experimental cpm}}{\text{autologous cpm}}$

Figure 18. Demonstration of the immunocompetence of isolated cord blood mononuclear cells reacting to antigens of unrelated cells compared to 3-member (cord against maternal plus 3P) cocultures, tested by mixed lymphocyte culture reactions. Results are expressed as mean stimulation index $\pm$ S.E.

Figure 18



$p < 0.05$ (n=14)

a: Stimulation Index = $\frac{\text{experimental cpm}}{\text{autologous cpm}}$

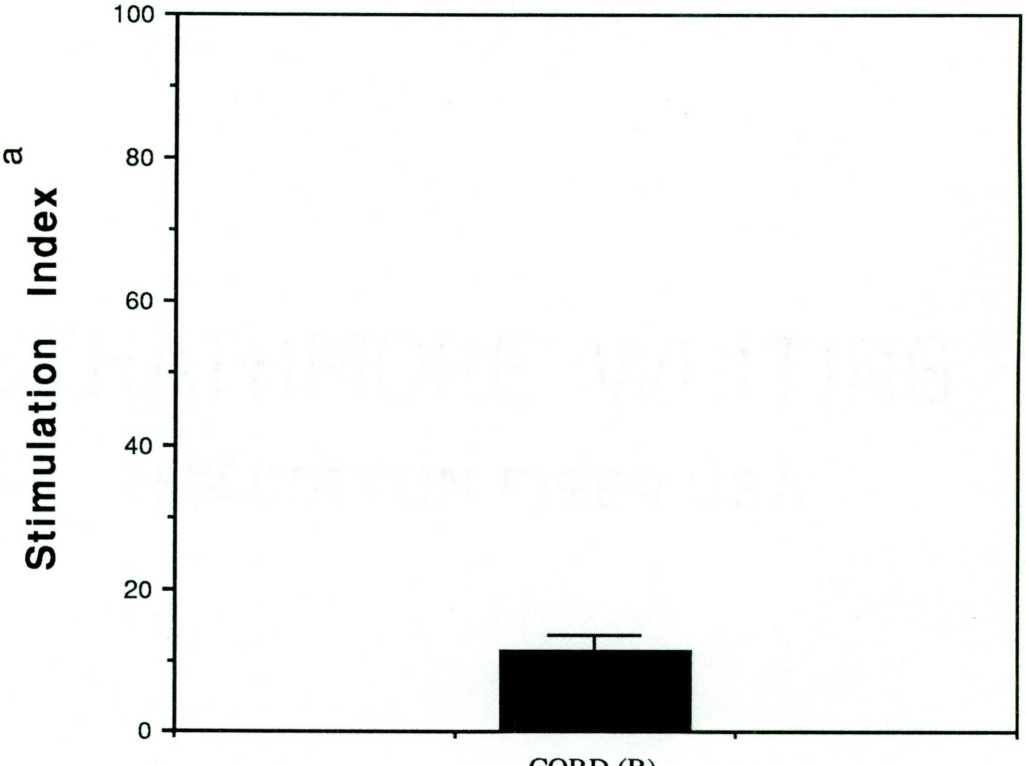
modulation of the cord cell response could be obscured by simultaneously stimulating with third party. In other words, immunomodulation by maternal cells could have been lost or at least diminished by reacting all three members together from the beginning of the culture. Therefore, it was proposed that a greater suppression may be demonstrable through preincubation of cord with maternal cells before adding unrelated cells. This was done by preincubating mother-baby pairs for 24 hours before adding third party stimulators. Indeed, preincubation of cord and mother's cells before the addition of unrelated 3P cells resulted in strong suppression (by 60%, $p=0.015$; compare Figure 18 with 19) of the cord cell responses.

It was realized, however, that the cultures needed to be maintained for seven instead of six days to accommodate the additional 24 hours of precoculturing. Thus, it was necessary to assess whether seven-day cultures were yielding similar information to the six-day cultures. Figure 20 reveals the results of this comparison, and the results clearly demonstrate that the additional 24 hours of culture did not affect the results of cord blood cells reacting to unrelated cells. This finding is supported by the work of others (Dubey, et al., 1986).

The seven-day precoculture MLC was then compared with the normal six-day MLC of cord blood with unrelated 3P. The question

Figure 19. The results of culturing cord mononuclear cells with third party after preculturing with maternal for twenty-four hours. Results are expressed as the mean stimulation index ± S.E.

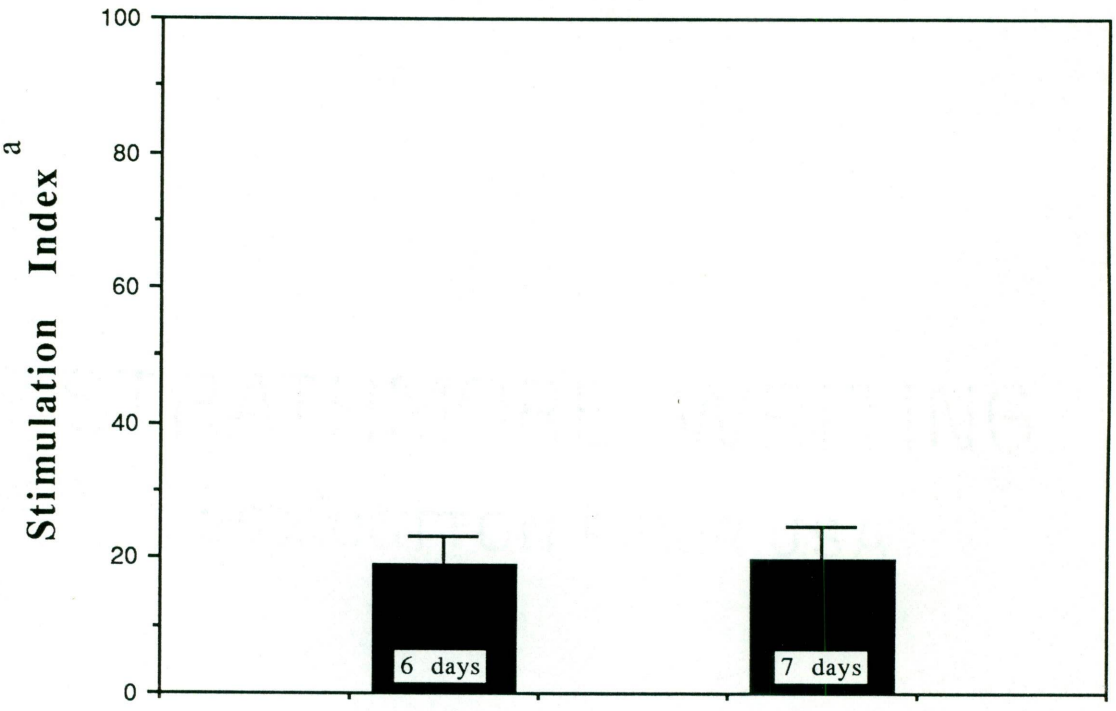
Figure 19



a: Stimulation Index = $\frac{\text{experimenttal cpm}}{\text{autologous cpm}}$

Figure 20. Comparison of six- and seven-day 3-member mixed lymphocyte cocultures (MLC). Results are expressed as the mean stimulation index $\pm$ S.E.

Figure 20



(n = 11)

a: Stimulation Index = $\frac{\text{experimental cpm}}{\text{autologous cpm}}$

was: how much time is needed for maternal cells to induce modulation of cord cell reactivity toward third party antigens?

A pilot study was performed to find the optimal preincubation time. As seen in Figure 21, twelve hours preincubation gave the strongest suppression, although six, twelve and twenty-four hours all resulted in significant suppression. In future coculture experiments, a precoculturing time of twelve hours was chosen because it was assumed that 6 hours may not always be enough, and 24 hours was considered undesireable in that the 3P cells needed to be stored fresh (not frozen) during that period.

Ten experiments were performed using the 12-hour precoculturing of cord with maternal cells before adding third party cells. In each of the ten experiments, a significant ($p<0.05$) suppression was observed in the cultures in which maternal cells were preincubated for 12 hours before the addition of unrelated stimulators (Figure 22), as compared to simultaneous coculturing of all three members.

While the observed suppression in these MLC reactions might be the result of cord cell modulation by maternal antigens (probably NIMA), it might also be the result of the cord cells being consumed in a secondary reaction before the day of harvest. That is, if the fetus is presensitized to maternal antigens <u>in utero</u>, then reexposure to maternal cells *in vitro* may result in a rapid proliferation in a secondary immune reaction occurring within the first 72 hours of

Figure 21. Results of pilot study to determine the optimal preincubation time needed for maternal cells to modulate cord cell reactivity to third party cells in mixed lymphocyte cocultures.

Figure 21

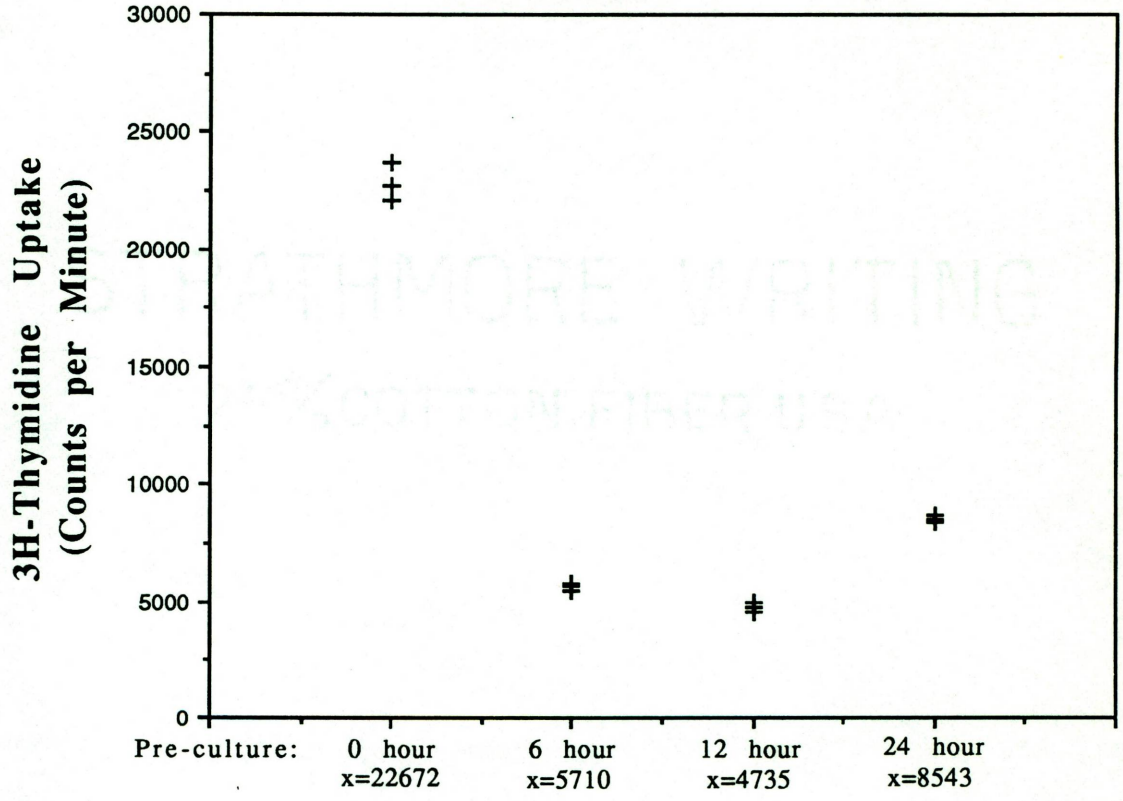
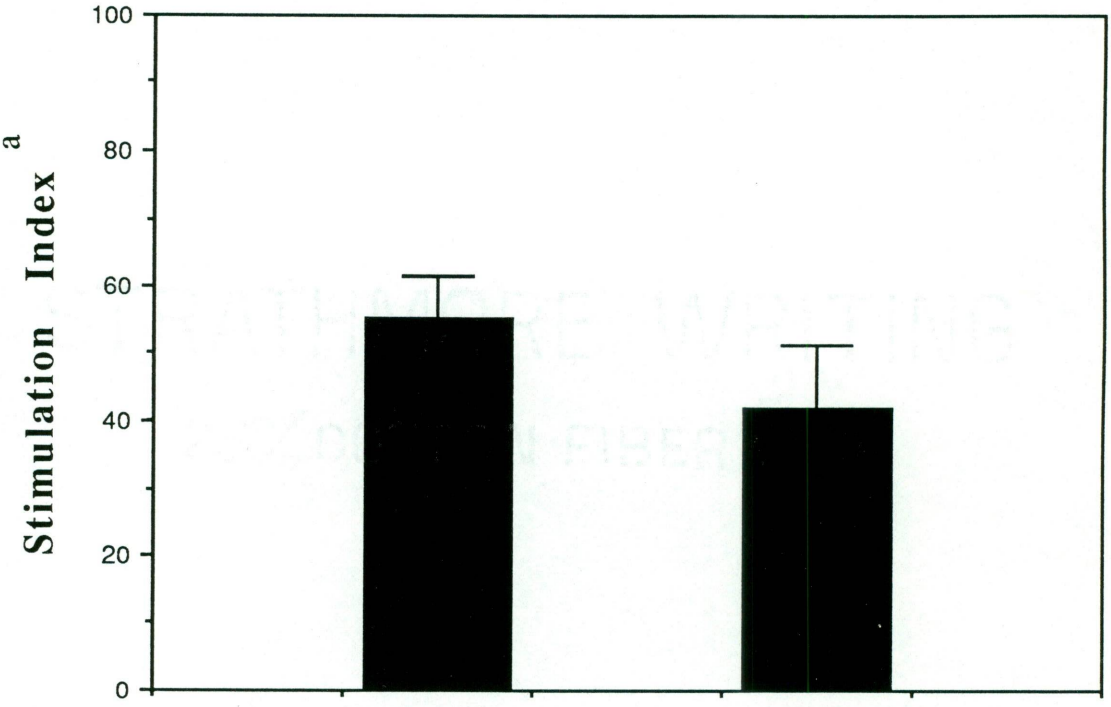

3H-Thymidine Uptake
(Counts per Minute)
30000
25000
20000
15000
10000
5000
0
Pre-culture:
0 hour
x=22672
6 hour
x=5710
12 hour
x=4735
24 hour
x=8543

Figure 22. The modulation effect of maternal cells on cord cell reactivity to third party alloantigens. Maternal cells were pre-cultured with related cord cells for twelve hours before third party was added to 3-member mixed lymphocyte cultures. Results are expressed as the mean stimulation index ± S.E.

Figure 22



$P < 0.05$ (n=10)

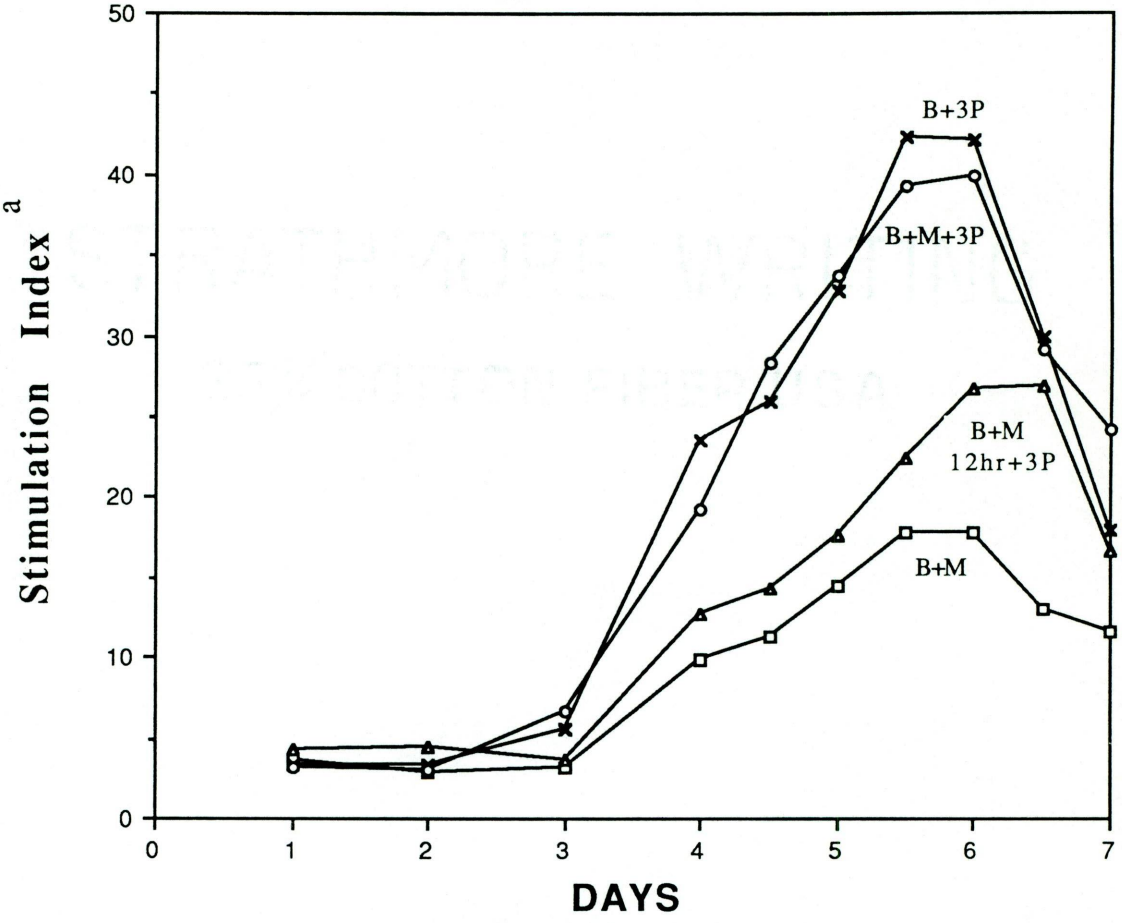
a: Stimulation Index = $\frac{\text{experimental cpm}}{\text{autologous cpm}}$

culture. This, in turn, would result in the depletion of cord cells capable of responding in a primary fashion, and a diminished MLC reaction on day six or seven.

To test this hypothesis (that presensitized baby cells will react rapidly and deplete themselves within 72 hours), multiple replicate pre-cocultures were produced and harvested at six- or twelve-hour intervals to determine the dynamics of the entire 7-day reaction. Figure 23 illustrates the results of testing 10 baby-mother precocultures. The MLC reactivities of the cord cells have been measured throughout the seven days, and the results plotted. Absence of an early peak (notably within the first 72-hour period of each assay) in all ten experiments, proved that the suppression observed at 6-7 days was not due to depletion of the baby's cells early in the culture period. That is, there was no "hidden" secondary response against maternal antigens within the culture period.

Figure 23. Dynamics of mixed lymphocyte cultures in which two or three members were cocultured. Results are expresed as the mean stimulation index.

Figure 23



a: Stimulation Index = $\frac{\text{experimental cpm}}{\text{autologous cpm}}$

DISCUSSION

The hypothesis of this study was that the apparent natural suppression observed in young children receiving cardiac allografts within the first few weeks of life is due, at least in part, to suppressor activity of cells that have been activated by exposure to non-inherited maternal antigens during gestation.

These unique allograft recipients experience few or no rejection episodes in spite of the fact that they are receiving remarkably low doses of immunosuppression. In addition, they are capable of responding to infectious organisms and donor-specific antigens. These apparent discrepancies are not due to immunodeficiencies, or tolerance to donor antigens.

If one assumes that these children are more sensitive than older individuals to the effects of pharmacological immunosuppressive agents, an increase in both frequency and severity of infections should be expected. This assumption is not supported clinically. Furthermore, blood cell counts (whole, differential leukocyte, and lymphocyte subsets), and *in vitro* cell function tests have proved to be the same as those of normal individuals at that age.

It was obvious from reviewing the donor and recipient HLA phenotypes that these children received poorly-matched organ allografts, a feature normally avoided in routine organ transplantation (Terasaki, *et al.*, 1985). Only transplantation of well-

matched organs permit recipients to accept allografts without strong immunosuppression with the attending increased susceptibility to infection.

It seemed that some form of naturally-occurring suppression, perhaps from feto-maternal immune interactions, may be underlying the favorable graft and patient survival rates, and by all indication, the responsible factor(s) seemed to be non-circulating. To assure the survival of the fetus, natural suppressor mechanisms actively counter the aggressive fetal and maternal immunoreactivity during gestation. Both suppressor cells (Hooper, *et al.*, 1986; Hoskin, *et al.*, 1989; Stankova, *et al.*, 1984;) and soluble suppressor factors (Matsui, *et al.*,1989a, 1989b; Papadogiannakis, *et al.*, 1984; Johnsen, 1982; Murgita, *et al.*, 1975) are thought to participate in this activity.

In the light of the report of Claas and colleagues in 1988, concerning the effect of fetal exposure to non-inherited maternal antigens on immune responsiveness throughout a lifetime, it was proposed here that perhaps the good graft survival rate might be related to matching of the donor mismatched antigens with the recipient's NIMA. Following this reasoning, a diligent search was made, first, to disclose the identity of the NIMA in each of the forty-two cardiac allograft recipients and, second, to compare these NIMA with the specific donor antigens (Table 3). Third, an attempt was made to correlate a) direct HLA matches between donor and

recipient, and b) indirect matches between recipient's NIMA and donor antigens, with the graft survival rate (Figure 9 and 10).

It was determined that, in forty-two transplants, none were well-matched. In fact, 39 (93%) of them were very poorly-matched (4 or more mismatches; Figure 8). Also, only two cases had 2 NIMA, and ten had 1 NIMA in common with the donor mismatches. From these investigations it was realized that, even if NIMA that are present on donor tissue could lead to prolonged graft survival, this phenomenon could not explain the twenty-nine cases lacking NIMA in the donor phenotypes.

This lack of correlation between NIMA and donor organ survival prompted a study in which donor-specific cell-mediated responsiveness was evaluated through mixed lymphocyte cultures. Reactivity against the donor was compared to that against an unrelated adult; no significant difference was seen between recipient responses toward infant donor cells and against adult tissue (Figure 11). On the assumption that circulating soluble factor(s) rather than cells may be involved, autologous serum was added to such cultures, but results failed to explain the favorable graft survival rate. That is, MLC reactivity failed to be consistently suppressed by autologous serum (Figure 13); only fifteen (39%) of the forty-two cases were suppressed. In contrast, 51% were actually stimulated by the serum, a surprising and unexplained result.

Realizing at this time that the most probable agents of suppression in these children may be the natural suppressor cells found in fetal circulation (Olding, *et al.*, 1974a; 1974b), cord blood (Olding, *et al.*, 1976; Oldstone, *et al.*, 1977), and neonatal spleen (Gronvik, *et al.*, 1987), it was decided that cord blood should be used for this study. In this way, blood samples could provide cells that partly represented both fetal circulation and neonatal spleen (both of which were practically unattainable in the human model), and supply the cells that may be the responsible agents of the natural suppression observed in our cardiac allograft recipients. In addition, through an IRB-approved study, blood cells of the biological mother could be obtained for use in studying the effect of NIMA on infant immune cell function relative to donor-specific reactivity.

Before proceeding with the proposed cord blood studies, it was first ascertained that cord mononuclear cells were capable of alloreactivity. Although this reactivity was significantly ($p < 0.05$) less than that of adult cells to the same stimulating alloantigens, the response was equivalent to reactions observed between relatives sharing half of their genetic material (one haplotype matched and one haplotype mismatched).

When the cord cells were tested for their response to maternal antigens, not surprisingly, they reacted significantly less as compared with reactions to unrelated adults. These reactions could be interpreted in one of two ways: either the reaction was diminished

only because of the obligatory haplotype match that exists between parent and child, or also because of suppression induced by the non-inherited maternal antigens. The possibility that the maternal cells were providing soluble suppressor factors (which could down-regulate cord cell functions), was eliminated by using a large dose of gamma irradiation. Soluble suppressor factors cannot be synthesized or released from cells receiving greater than 1000 rads (Engleman, *et al.*, 1978). In these studies, 2750 rads were delivered to all stimulator cells, rendering them metabolically incapacitated but physically intact for appropriate presentation of cell membrane antigens.

Although cord cells failed to react as strongly as neonatal or adult cells in these assays, the reactions were stronger than would be expected in light of the superior graft survival rate observed in the infant transplant recipients. Therefore, the effect of adding autologous cord serum was tested, with the same result as that found in the neonatal peripheral blood experiments.

Having failed to discover sufficient evidence of soluble factor-mediated suppression in cord blood, demonstration of a cell-mediated component was sought. It was reasoned that, if NIMA (expressed on maternal cells) were the cause of fetal immune stimulation, they might also cause the activation of natural suppressor cell activity in fetal circulation and neonatal spleen. Theoretically, this information would provide a better understanding

of feto-maternal compatibility and about subsequent postnatal immune reactivity. For practical reasons, it might help explain the high GSR in the infant patients. It is known that maternal cells gain access to fetal circulation during gestation, and again at parturition during separation of the placenta. If maternal cells are removed from circulation in a normal manner, they should be filtered out by the spleen, the same organ in which the natural suppressor cells are sequestered. This would allow the NS cells to be further stimulated by the NIMA. Furthermore, in clinical transplantation it has been reported that dendritic cells (that express class II HLA) migrate out of the transplanted organ into recipient circulation where they are filtered out by the spleen (Larsen, *et al.*, 1990). This concept of three different individuals juxtaposition within the same lymphoid compartment, the spleen, provided the basis for the remainder of this study.

The validity of this logic was tested by reacting cord blood cells against unrelated alloantigens in the presence of maternal cells (3-member MLC). In comparison with cord cell reactivity to unrelated cells alone (routine 2-member MLC), the reaction of cord cells in the 3-member MLC was significantly inhibited. This reduction in cord cell alloreactivity by coculturing with maternal cells was consistently demonstrated in all experiments (Figure 18).

Nonetheless, the residual activity in the 3-member cocultures (SI=22.1) was greater than that seen to occur in cultures between

well-matched related individuals (SI $\leq$ 10; data from LLUMC Renal Transplant Program). Assuming that the exposure of the responder cell population simultaneously to both maternal and third party cells may be defeating any modulation by maternal cells before stimulation by third party alloantigens, the experimental design was changed to provide a pre-culture period of baby with mother.

Previously initiated exposure to maternal cells resulted in remarkable down-regulation of baby cord cell reactivity to unrelated stimulators (Figure 19). Furthermore, this immunomodulation was shown to be independent of the constraints of an additional 24 hours of incubation (Figure 20) and possible responder cell depletion through a rapid secondary immune response occurring in the early culture period (Figure 23).

In summary, the results of this study can be interpreted as follows:

a) The favorable graft survival rate of neonatal cardiac transplant recipients cannot be the result of direct HLA matching of donor with recipient.

b) The favorable graft survival rate cannot be the result of donor HLA mismatches coinciding with recipient non-inherited maternal antigens.

c) Maternal cells can effectively modulate the alloreactivity of related baby cells to alloantigens of an unrelated third party in mixed lymphocyte cultures.

d) The down-regulation observed at the end of six or seven days is not due to responder cell depletion through secondary reactivity to maternal antigens.

e) The modulation by maternal cells was not due to extended incubation periods.

In speculating on the meaning of these results in the overall scheme of neonatal organ transplantation, the following scenario was developed that demands elucidation through future investigation: fetal natural suppressor cells are activated by exposure to non-inherited maternal antigens. These freely circulating fetal cells are routed to the spleen at the time of birth, perhaps through neuroendocrine direction, where they remain in an activated state for a short time (<30 days). Maternal cells that have crossed into fetal circulation would be removed by the neonatal spleen (a normal function) and the resulting juxtaposition of maternal cells with splenic cells may result in further stimulation of NS cells. Pharmacologic immunosuppressants administered at the time of transplantation may enhance this process in that cyclosporine specifically affects T-helper cells function while sparing suppressor function. An allograft introduced at this time would most probably be favored, or at least ignored, by the host's immune system.

REFERENCES

Alford, C.A., Wu, L.Y.F., and Blanco, A. Developmental humoral immunity and congenital infections in man. In *The immune system and infectious diseases.* Neter, J. and Milgram, F., eds, Karger, Basel, 1974, pp.42.

Anderson, U. Regulation of antibody synthesis in the neonate. In *Immunology of the Neonate.* Burgio, G.R., Hanson, L.A., and Ugazio, A.G., eds. New York, Springer-Verlag, 1987, pp.37.

Asma, G.E.M., Schmit, H.R.E., and Hijmans, W. The determination of numbers of T and B lymphocytes in the blood of children and adults by the direct immunofluorescence technique. Clin. Exp. Immunol. 29, 236, 1977.

Athanassakis, I., Bleackley, R.C., Peatkau, V., Guilbert, L., Barr, P. J., and Wegmann, T.G. The immunostimulatory effect of T cells and T cell lymphokines on murine fetally derived placental cells. J. Immuno. 138, 37, 1987.

Bach, F. and Hirschhorn, K. Lymphocyte Interaction: a potential histocompatibility test *in vitro.* Science 143, 813, 1964.

Bach, F. and Voynow, N.K. One-way stimulation in mixed leukocyte cultures. Science, 153, 545, 1966.

Bailey, L.L., Assaad, A.N., Trimm, F., Nehlsen-Cannarella, S.L., Kanakriyeh, M.S., Haas, G.S., and Jacobson, J.G. Orthotopic transplantation during early infancy as therapy for incurable congenital heart disease. Ann. Surg. 208, 279, 1988.

Bailey, L.L., Nehlsen-Cannarella, S.L., Concepcion, W., and Jolley, W.B. Baboon-to-human cardic xenotransplantation in a neonate. J. Am. Med. Asso. 254, 3321, 1985.

Bailey, L.L., Wood, M., Razzouk, A., Arsdell, G.V., and Gundry, S. Heart transplantation during the first 12 years of life. Arch. Surg. 124, 1221, 1989.

Batra, S., Benqtsson, L.P., Grundsell, H., and Sjoberg, N.O. Levels of free and protein-bound progesterone in plasma during late pregnancy. J. Clin. Endocrinol. Metab. 42, 1041, 1976.

Baum, M., Rincon, D., and Bailey, L.L. Growth patterns in infant heart transplant patients. Loma Linda International Conference on Pediatric Heart Transplantation. Palm Desert, CA. March 11-14, 1990. (J. Heart Transplantation, In Press.)

Bellanti, J.A. and Jeligs, B.J. Immunity and infection in the neonate. In *Immunology of the Neonate*. Burgio, G.R., Hanson, L.A., and Ugazio, A.G., eds. New York, Springer-Verlag, 1987, pp.135.

Berk, L.S., Nieman, D., Tan, S.A., Nehlsen-Cannarella, S., Kramer, J., Eby, W.C. and Owens, M. Lymphocyte subset changes during acute maximal exercise. Med. and Sci. in Sports and Exercise, 18, 706, 1986.

Biondi, A., Poli, G., Parravicini, C., Molinari, A., and Mantovani, A. Mononuclear phagocyte function in the perinatal period, In *Immunology of the Neonate*. Burgio, G.R., Hanson, L.A., and Ugazio, A.G., eds. New York, Springer-Verlag, 1987, pp.59.

Butler, J.L., Suzuki, T., Kubagawa, H., and Cooper, M.D. Humoral immunity in the human neonate. In *Immunology of the Neonate*. Burgio, G.R., Hanson, L.A., and Ugazio, A.G., eds. New York, Springer-Verlag, 1987, pp.27.

Challis, J.R.G., Davies, I.J., Benirschke, K., Hendrickx, A.G., and Ryan, K.J. The concentrations of progesterone, estrone and estradiol-17β in the peripheral plasma of the rhesus monkey during the

final third of gestation, and after the induction of abortion with $PGE_{2\alpha}$. Endocrinology 95, 547, 1974.

Chardonnens, X. and Jeannet, M. Immunobiology of pregnancy: evidence for a fetal immune response against the mother (?) Tissue Antigens 15, 401, 1980.

Chin, T.W., Ank, B.J., Strom, S.R., and Stiehm, E.R. Enhanced interferon production and lymphokine-activated cytotoxicity of human placental cells. Cell. Immunol. 113, 1, 1988.

Claas, F,H.J., Gijbels, Y., Velden-de Munck, J.V.D., and vanRood, J.J. Induction of B cell unresponsiveness to non-inherited maternal HLA antigens during fetal life. Science 241, 1815, 1988.

Cole, F.S. Complement function in the neonate. In *Immunology of the Neonate*. Burgio, G.R., Hanson, L.A., and Ugazio, A.G., eds. New York, Springer-Verlag, 1987, pp.76.

Crainie, M., Semeluk, A., Lee, K.C., and Wegmann, T. Requlation of constitutive and lymphokine-induced Ia expression by murine α-fetoprotein. Cell. Immunol. 118,41, 1989.

Cutler D. and Marxmiller, J. Acute infections and immunizations in pediatric heart transplant recipients. Loma Linda International Conference on Pediatric Heart Transplantation. Palm Desert, CA. March 11-14, 1990. (J. Heart Transplantation, In Press.)

Desai, R.G. and Creger, W.P. Maternofetal passage of leukocytes and platelets in man. Blood 21, 665, 1963.

Dubey, D.P., Yunis, I., and Yunis, E.J. Cellular typing: mixed lymphocyte response and cell-mediated lympholysis. In *Manual of clinical laboratory immunology*. Rose, N.R., Friedman, H., and Fahey, J.L. eds., American Sociely for Microbiology, Washinton, D.C. 1986. pp. 847.

Dupont, B., Hansen, J.A., and Yunis, E.J. Human mixed-lymphocyte culture reaction: Genetics, specificity, and biological implications. In *Advances in Immunology*; Academic Press, New York; 1976, pp. 107.

Engleman, E.G., McMichael, A.J., and McDevitt, H.O. A suppressor T cell of the mixed lymphocyte reaction in man specific for the stimulating alloantigen. J. Exp. Med. 147, 137, 1978.

Fabris, N. Neuroendocrine-immune system interaction: a sepial remark for neuroendocrine influence on peripheral immune functions. In *Stress, immunity nad ageing a role for acetyl-L-carnitine*. Simone, C.E. and Martelli, E.A., eds. Amsterdam, Elsevier Science Publishers B. V. 1989, pp. 37.

Fowler, R., Shubet, W.K., and West, C.D. Acquired partial tolerance to homologous skin grafts in the human infant at birth. Ann. NY Acad. Sci. 87,403, 1960.

Fukuda, A., Mori, Ts., Mori, Et., Tatsumi, H., Kanzaki, H., and Mori, Ta. Effects of the supernatants of mixed lymphocyte cultures and decidual cell line cultures on mouse embryo development *in vitro*. J. in vitro Fertili. and Embryo Transfer. 6, 59, 1989.

Genazzani, A.R. Petraglia, F., Monzani, A., Fabbri, G., Ramundo, B.D., Domenica, P.D., Facchinetti, F. and Volpe, A. Stress and hormones in humans. In *Stress, immunity and ageing a role for acetyl-L-carnitine*. Simone, C.E. and Martelli, E.A., eds. Amsterdam, Elsevier Science Publishers B. V. 1989, pp. 67.

Gronvik, K.O., Hoskin, D.W., and Murgita, R.A. Monoclonal antibodies against murine neonatal and pregnancy-associated natural suppressor cells induce resorption of the fetus. Scand. J. Immunol. 25, 533, 1987.

Hayward, A.R. and Kurnick, J. Newborn T cell suppression: early appearance, maintenance in culture, and lack of growth factor suppression. J. Immunol. 89, 194, 1981.

Hayward, A.R., and Lawton, A.R. Induction of plasma cell differentiation of human fetal lymphocytes: evidence for functional immaturity of T and B cells. J. Immunol. 119, 1213,1977.

Heck, C.F., Shumway, S.J., and Kaye, M.P. The registry of the international society for heart transplantation: sixth official report-1989. J. Heart Transplant. 8, 271, 1989.

Hill, H.R. and Sacchi, F. Mechanisms os abnormal neutrophil function in the human neonate: prospects for therapy. In *Immunology of the Neonate*. Burgio, G.R., Hanson, L.A., and Ugazio, A.G., eds. New York, Springer-Verlag, 1987, pp.67.

Hogarth, P.J. Chapter 5. Immunological implacations of pregnancy. In *Immunological aspects of mammalian reproduction.* Praeger Publishers, New York, 1982, pp. 83.

Hooper, D.C., Hoskin, D.W., Gronvik, K.-O., Murgita, R.A. Murine neonatal spleen contains natural T and non-T suppressor cells capable of inhibiting adult alloreactive and newborn autoreactive T-cell proliferation. Cell. Immunol. 99, 461, 1986.

Hopkins K.A. and MacQueen J.M. The basic microlymphocytotoxicity technique. In *AACHT Procedure Manual* II-1-1-II-1-9, 1981.

Hoskin, D.W., Gronvik, K.-O., Hooper, D.C., Reilly, B.D., and Murgita, R.A. Altered immune response patterns in murine syngeneic pregnancy: presence of natural null suppressor cells in maternal spleen identifiable by monoclonal antibodies. Cell. Immunol. 120, 42, 1989.

Jacoby, D.R., Olding, L.B., and Oldstone, M.B.A. Imunologic Regulation of fetal-maternal balance. Ad. Immunol. 35, 157, 1984.

Johnsen, S-A and Olding, L.B. Differences in binding sites for prostaglandin E_2 on mononuclear leukocytes from human newborns and from their mothers. Scand. J. Immunol. 17, 389, 1983.

Johnsen, S-A., Olding, L.B., Westberg, N.G., and Wilhelmsson, L. Strong suppression by mononuclear leukocytes from human newborns on maternal leukocytes: mediation by prostaglandins. Clin. Immunol. Immunopha. 23, 606, 1982.

Kamps, W.A. and Cooper, M.D. Microenvironmental studies of pre-B and B cell development in human and mouse fetuses. J. Immunol. 129, 526, 1982.

Kolb, J.P., Chaouat, G., and Chassoux, D. Immunoactive products of placenta. III. Suppression of natural killing activity. J. Immunol. 132, 2305, 1984.

Kuntz, M.M., Innes, J.B., and Weksler, M.E. Lymphocyte transformation induced by autologous cells IV. human T-lymphcyte proliferation induced by autologous or allogeneic non-T lymphocytes. J. Exp. Med. 143, 1042, 1976.

Larsen, C.P., Morris, P.J., and Austyn, J.M. Migration of dendritic leukocytes from cardiac allografts into the host spleens. A novel pathway for initiation of rejection. J. Exp. Med. 171, 307, 1990.

LeDouarin, N.M. and Jotereau, F.V. Tracing of cells of the avian thymus through embryonic life in interspecific chimeras. J. Exp. Med. 142, 17, 1975.

Levin, D. and Gershon, H. Antigen presentation by neonatal murine spleen cells. Cell. Immunol. 120, 132, 1989.

Loma Linda University Medical Center Heart Transplant Survival Statistics, 23, Feb. 1990.

Loma Linda University Medical Center Histocompatibility Laboratory procedure manual, V2-13, 1989.

Maccario, R., Nespoli, L., Mingrat, G., Vitiello, A., Ugazio, A.G., Burgio, A.G. Lymphocyte subpopulations in the neonate: identification of an immature subset of OKT8-positive, OKT3-negative cells. J. Immunol. 130. 1120, 1983.

Martin, B.J., Spicer, S.S., and Smythe, N.M. Cytochemical studies of the maternal surface of the syncytiotrophoblast of human early and term placentae. Anat. Res. 178, 769, 1977.

Marxmiller, J. Life-threatening infections in pediatric heart transplantation. Loma Linda International Conference on Pediatric Heart Transplantation. Palm Desert, CA. March 11-14, 1990. (J. Heart Transplantation, In Press.)

Matsui, S., Yashimura, N., and Oka, T. Characterization and analysis of soluble suppressor factor from early human decidual cells. Transplantation 47, 678, 1989a.

Matsui, S., Yashimura, N., and Oka, T. Immunochemical characterization of the suppressor factor from early human decidual cells. Transplantation 48, 651, 1989b.

McCario, R. and Burgio, G.R. T and NK lymphocyte subpopulations in the neonate. In *Immunology of the Neonate*. Burgio, G.R., Hanson, L.A., and Ugazio, A.G., eds. New York, Springer-Verlag, 1987, pp.120.

Mumford, D.M., Sung, J.S., Wallis, J.D., and Kaufman, R.H. The lymphocyte transformation response of fetal hemolymphatic tissue to mitogens and antigens. Pedric. Res. 12, 171, 1978.

Murgita, R.A. and Tomasi, T.B. Suppression of the immune response by α-fetoprotein. I. The effect of mouse α-fetoprotein on the primary and secondary antibody response. J. Exp. Med. 141, 269, 1975.

Murgita, R.A., Peck, A.B., and Wigzell, H. Chapter 15: Immunosuppressive elements in the fetal and neonatal environment. In *Immunology of reproduction.* Wegmann, T.G., Gill, T.J. eds. New York: Oxford University Press, 1983.

Nehlsen-Cannarella, S.L., Buckert, L., Seltman, K., Chang, L., Mitzelfelt, D. Unique newborn immune response. Loma Linda International Conference on Pediatric Heart Transplantation, Palm Desert, CA. March 11-14, 1990a. (J. Heart Transplantation, In Press.)

Nehlsen-Cannarella, S.L., Nieman, D.C., Balk-Lamberton, A.J., Karkoff, P.A., Chritton, D.B.W., Gusewitch, G., and Lee, J.W. The effects of moderate exercise training on immune response 2. Lymphocyte subpopulations, immunoglobulins, and acute upper respiratory tract infections. Med. and Sci. in Sports and Exercise. In press. 1990b.

Nieman, D.C. and Nehlsen-Cannarella, S.L. The acute and chronic effects of exercise on immunoglobulins. Sports Med. In press. 1990a.

Nieman, D.C., Nehlsen-Cannarella, S.L., Markoff, P.A., Balk-Lamberton, A.J., Yang, H., Chritton, D.B.W., Lee, J.W., and Arabatzis, K. The effects of moderate exercise training on natural killer cells and

acute upper respiratory tract infections. International J. Sports Med. In press. 1990b.

Oksenberg, J.R., Mor-Yosef, S., Ezra, Y., and Brautbar, C. Antigen presenting cells in human decidual tissue: III. Role of accessory cells in the activation of suppressor cells. Am. J. Reprod. Immuno. 16, 151, 1988.

Oldong, L.B. and Oldstone, M.B.A. Lymphocytes from human newgorns abrogate mitosis of the mother's lymphocytes. Nature, 249, 101, 1974a.

Oldong, L.B. and Oldstone, M.B.A. Thymux-derived peripheral lymphocytes from human newborns infibit division of their mothers' lymphocytes. J. Immunol. 116, 682, 1976.

Oldong, L.B., Benirschke, K., and Oldstone, M.B.A. Inhibition of mitosis of lymphocytes from human adults by lymphocytes from human newborns. Clin. Immunol. Immunopathol. 3, 179, 1974b.

Oldstone, M.B.A., Tishon, L.A., and Moretta, L. Active thymus-derived suppressor lymphocytes in human cord blood. Nature 269, 333, 1977.

Owen, R.D., Wood, H.R., Foord, A.G., Sturgeon, P., and Baldwin, L.G. Evidence for actively acquired tolance to Rh antigens. Proc. Natl. Acad. Sci. *USA* 40, 420, 1954.

Papadogiannakis, N., Johnsen, S.A., and Olding, L.B. A prostaglandin-mediated suppressive activity of cord as compared to maternal or other adult adherent cells in OKT3 antibody-induced proliferation. Cell. Immunol. 101, 51, 1986.

Papadogiannakis, N., Johnsen, S.A., and Olding, L.B. Strong prostaglandin associated suppression of the proliferation of

human maternal lymphocytes by neonatal lymphocytes linked to T versus T cell interations and differential PGE_2 sensitivity. Clin. Exp. Immunol. 61, 125, 1984.

Pavia, C.S. and Stites, D.P. Immunosuppressive activity of murine newborn spleen cells. Cell. Immunol. 42, 48, 1979.

Schwadron, R.B., Palathumpat, V., and Srober, S. Natural suppressor cells derived from adult spleen and thymus. Transplantation 48,107, 1989.

Sherwood, R.A. and Brent, L. Antigen Presentation by Neonatal Spleen cells: A Novel Finding. Transplant Proc. 21(1), 644, 1989.

Slavin, S., Reitz, B., Bieber, C.P., Kaplan, H.S., and Strober, S. Transplantation Tolerance in Adult Rats using Total Lymphoid Irradiation: Permanent Survival of Skin, Heart, and Marrow Allografts. J. Exp. Med. 147, 700, 1978.

Stankova, J. and Rola-Pleszczynski, M. Suppressor cells in the human-maternal-fetal relationship. J. Repr. Immunol. 6, 49, 1984.

Streilein, J.W., Strome, P., and Wood, P.J. Failure of *in vitro* assays to predict assurately the existence of neonatally induced H-2 tolerance. Transplantation 48, 630, 1989.

Strober, S. Total Lymphoid Irradiation: basic and clinical studies in transplantation immunity. In *Transplantation: Approaches to graft rejection.* Alan R. Liss, Inc. 1986. p.263.

Stutman, O. Ontogeny of T cells. Clin. in Immunol. allergy 5,191,1985.

Terasaki, P.I., Toyotome, A. Mickey, M.R., Cicciarelli, J., Iwaki, Y., Cecka, M., and Tiwari,J. Patient, graft, and functional survival rates: an overview. In Clinical kidney transplants 1985.

Terasaki, P.I. (ed), UCLA Tissue Typing Laboratory, Los Angeles, 1985. pp. 1-26.

Trimm, F. Ashwal, S. Rincon, D. Breyer, B., åkamine, M., and Bailey, L. Neurodevelopmental outcome in infant heart transplantation: the first four years. Loma Linda International Conference on Pediatric Heart Transplantation. Palm Desert, CA. March 11-14, 1990. (J. Heart Transplantation, In Press.)

Tulchinsky, D., Hobel, C.J., Yeager, E., and Marshall, J.R. Plasma estrone, estradiol, estriol, progesterone and 17-hydroxy-progesterone in human pregnancy. I. Normal pregnancy. Am. J. Obstet. Gynecol. 112, 1095, 1972.

Uren, S.J. and Boyle, W. Class II MLC Antigen-Positive Macrophages from Human Placentae Suppress Strong MLC and CML Reactions. Cell. Immunol. 125, 235, 1990.

Vartdal, F., Gaudernack, G., Funderid, S., Bratlie, A., Lea, T., Ugelstad, J., and Thorsby, E. HLA class I and II typing using cells positivly selected from blood by immunomagnetic isolation - a fast and reliable technique. Tissue Antigens 28, 301, 1986.

Whitley, R.J. Herpes Simplex Viruses. In *Virology*. Field, B.N. and Knipe, D.M. eds. Raven Press, New York. 1989. pp.1863.