The University of Manitoba

Human Neonatal Suppressor Lymphocytes

by

Henry J. Cheng

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To Linda & Linda

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ABSTRACT

The objectives of our work were to characterize the human neonatal suppressor cells and to investigate their contributions to the deficient immune responses observed in newborns as well as their effect on adult lymphocyte functions.

Our first study was designed to examine the functional status of neonatal B and T lymphocytes. Using adult lymphocyte antibody response to pokeweed mitogen (PWM), a T cell-dependent B cell polyclonal stimulator as an assay, we showed that neonatal B cells were immature and could produce only a small amount of IgM with virtually no IgG or IgA response despite the presence of adequate helper cells or factors. This was confirmed by the observation that neonatal B cells responded to Epstein-Barr virus (EBV), a T cell-independent polyclonal activator by producing IgM only. Neonatal helper T cells, on the other hand, were functionally mature and produced an adequate amount of helper factors but their helper function was usually masked by an excessive suppressor activity present in the T cell population. This suppressor activity, however, was only effective against adult but not neonatal lymphocytes' response to PWM and EBV suggesting that neonatal lymphocytes are resistant to suppression by their own suppressor cells. Therefore, the deficient immune responses observed in newborns are probably not a direct consequence of their excessive suppressor cell activity. The

target of the neonatal lymphocyte-directed suppression was primarily B cell/monocyte (non-T cell) although helper T cell may also be affected. The suppression was mediated, at least partially, through the production of an inducer of suppressor factor (TisF) by alloantigen- or PWM-stimulated neonatal T cells, which in turn activated the adult suppressor effector cells to inhibit the B cell and possibly helper T cell response.

In the subsequent studies, we proceeded to characterize the neonatal TisF-producing cells and their target, the functional properties of TisF and the suppressor effector cells. With the use of monoclonal antibodies against various T cell surface markers, we found that two populations of neonatal T cells, a radioresistant, $T4^+$, $TQ1^+$ subset and a radioresistant, $T4^+$, $TQ1^-$ subset interacted to produce TisF independent of the monocytes. In the PWM system, TisF suppressed the adult lymphocyte response through the activation of adult suppressor effector T cells and possibly also autologous neonatal suppressor effector T cells both of which were sensitive to irradiation and expressed the T4 phenotype. The action of TisF was antigen-nonspecific and MHC-unrestricted since its influence was not limited to suppressing allogeneic adult lymphocytes' response to PWM but was also effective in inhibiting lymphocyte responses to alloantigen and soluble antigen Candidin. Neonatal $T8^+$ cells, in contrast to adult $T8^+$ cells, exhibited minimal suppressor activity.

Moreover, even after stimulation with Concanavalin A, a mitogen known to activate mainly $T8^+$ suppressor cells, they failed to express any suppressor activity when tested in various assay systems. These results suggest that the excessive suppressor activity of neonatal T cells is originated from their inducer of suppressor cells which produced a factor (TisF) that activates autologous or adult suppressor effector T cells to administer their effect. Furthermore, this strong suppressor activity is mediated predominantly through the $T4^+$ rather than the $T8^+$ suppressor cells which are functionally immature.

LIST OF ABBREVIATIONS

AC:	adherent cell
ADCC:	antibody-dependent cell-mediated cytotoxicity
BSA:	bovine serum albumin
Con A:	concanavalin A
^{51}Cr:	radioactive isotope of chromium, 51
CTSF:	cord T cell-derived suppressor factor
E^{+}:	form rosettes with sheep erythrocytes
EBV:	Epstein-Barr virus
EBV-BA,EBV-BC:	Epstein-Barr virus-transformed adult or cord B cell lines, respectively
FcR:	receptor for the Fc fragment of immunoglobulin
FACS:	fluorescence activated cell sorter
FITC:	fluoresceine isothiocyanate
GVH:	graft-versus-host
HLA:	human lymphocyte antigen
Ig(M,A,G,D,E):	five classes of immunoglobulin
^{125}I:	radioactive isotope of iodine, 125
LPS:	lipopolysaccharide
MHC:	major histocompatibility
Mit-c	mitomycin-c
MLC:	mixed lymphocyte culture
MNC:	mononuclear cells
NK:	natural killer cells

PBL:	peripheral blood lymphocytes
PBS:	phosphate-buffered saline
PGE2:	prostanglandin E2
PHA:	phytohemagglutinin
PNA^+:	has receptor for peanut agglutinin
PWM:	pokeweed mitogen
RIA:	radioimmunoassay
Supa-a:	supernatant from MLC of 2 adult blood
Supc-a:	supernatant from MLC of cord and adult blood
Supc-c:	supernatant from MLC of 2 cord blood
TA:	trophoblast antigens
TCM:	tissue culture medium
Tis:	inducer of suppressor T cells
TisF:	inducer of suppressor T cell factor
TLX antigens:	trophoblast-lymphocyte cross-reactive antigens
TRF:	T helper cell replacing factor

CHAPTER 1

INTRODUCTION AND BACKGROUND

It is well known that newborns are prone to viral and bacterial infections which remain to be one of the leading causes of morbidity and mortality among infants. Undoubtedly, the functional immaturity of lymphocytes and their lack of exposure to antigens are among the main reasons for the newborns' high susceptibility to infection. A number of reports have suggested that active cellular suppressive mechanism may also contribute to the inadequacy of newborn lymphocyte function. Hence, it appears that there exists a paradox in the newborn immune system with its immune response being relatively inadequate in general and yet its immunosuppressive activity being extraordinarily potent. This enhanced suppressor activity may be essential in the protection of the fetus against graft-verses-host reaction by the maternal lymphocytes that managed to traverse the placental barrier.

The present study was undertaken to examine the cellular origin of this newborn and fetal suppressive activity and its effect on the autologous as well as allogeneic lymphocyte functions.

Ontogeny of the Fetal and Newborn Immune System

Development of B Lymphocytes:

Pre-B cells, which contain cytoplasmic μ chain but lack surface immunoglobulin (sIg), were found at 7.5 weeks' gestation in fetal liver and accounted for about 0.2% of nucleated liver cells (Gathings et al 1977). The amount of pre-B cells in liver increased to 1.1% at the 11th week of gestation and by the 14th week of gestation, they

also appeared in bone marrow. B cells first appeared in liver at about the 9th week of gestation and expressed only sIgM (Asma et al 1977; Gathings et al 1977). From the 13th week onward, the majority of B cells expressed both IgM and IgD. Surface IgG-positive and sIgA-positive cells emerged by the 11th and the 12th week of gestation and over 80% of these cells coexpressed sIgM and sIgD (Hayward and Ezer 1974; Gathings et al 1977). This unique feature of triple isotype expression on B cell surface persisted until few months after birth (Conley et al 1980). The frequency of B cells expressing the different immunoglobulin heavy chain isotypes increased as a function of age until adult proportions were reached around the 15th week of gestation (Lawton et al 1972; Hayward and Ezer 1974; Gathings et al 1977). They were found in the highest frequencies in spleen (35%), blood (30%) and lymph nodes (13%) where the relative proportion of cells bearing each isotype was equal to or greater than that found in adult tissues (Gathings et al 1981). Thus, the isotype diversity at the B lymphocyte level appears to be achieved quite early in gestation despite the relative paucity of stimulation from exogenous antigens.

Development of immunoglobulin-secreting plasma cells lags behind that of B cells. IgM-producing plasma cells could be identified by immunofluorescent staining of tissues from most fetuses beyond the 15th week of gestation whereas IgG-producing plasma cells were infrequently seen before the 20th week of gestation and IgA-producing plasma cells have rarely been observed before the 30th week of gestation (Van Furth et al 1965; Gitlin and Biasucci 1969; Lawton et

al 1972; Vossen and Hijmans 1975). This might be partly due to the sheltered intrauterine environment inhabited by fetuses although even in those fetuses that were congenitally infected, their plasma cell responses were still restricted to primarily IgM (Silverstein 1962; Silverstein and Lukes 1962; Alford et al 1964). Serial measurements of serum immunoglobulin correlated well with the results from immunofluorescent studies. IgM was detected in fetal serum as early as the 17th week of gestation with the concentration rising to between 5-20% of adult values at term. The IgM was primarily of fetal rather than of maternal origin since IgM in the maternal circulation does not cross the placental barrier (Githin 1971). Serum IgG, in contrast to IgM, was mostly of maternal origin although using genetic typing, small amounts of fetal IgG was also demonstrable (Martensson and Fudenberg 1965). The other 3 types of antibodies, namely IgA, IgE and IgD were all found to be present in very low levels (Johansson 1968; Brasher and Hartley 1969; Cederqvist et al 1977).

From a number of studies, it was evident that full term or even premature babies can synthesize IgM, IgG and IgA in response to a variety of antigens (Fink et al 1962; Uhr et al 1962; Smith et al 1964). However, their response was generally low compared to adults. For example, neonatal lymphocytes were found practically unable to respond to certain polysaccharides (Gold et al 1975; Cowan et al 1978). Furthermore, the nature of their antibody response also varied from that of adults. In response to thymus-dependent antigens, IgM was the predominant isotype produced by newborn lymphocytes whereas,

in adults, a shift to predominantly the IgG isotype occurred by the second week of immunization.

In vitro assessment of the immunoglobulin-synthesis capacity of the fetus and newborn, with a few exceptions, employed primarily polyclonal activators which could be classified as (1) T cell-dependent, e.g., pokeweek mitogen (PWM); (2) T cell-independent, e.g., Epstein-Barr virus (EBV); (3) relatively T cell-dependent, e.g., water soluble extract from Nocardia opaca and lipopolysaccharide (LPS); and (4) activator containing both T cell-independent and T cell-dependent components, e.g., certain preparations of killed Staphylococcus aureus Cowan 1. In adults, plasma cell production of all 3 major classes of immunoglobulin (IgM, IgG and IgA) could be induced by these various polyclonal activators in vitro. With fetal or newborn lymphocytes, however, little immunoglobulin (mainly the IgM type) synthesis could be induced by PWM (Andersson et al 1981; Miyawaki et al 1981a; Unander et al 1983) or Nocardin opaca (Miyawaki et al 1981a) and virtually only IgM was produced when these lymphocytes were stimulated with EBV (Andersson et al 1981) or LPS (Andersson et al 1981). There was no consensus regarding the type of immunoglobulin produced by Cowan 1 bacteria-stimulated newborn lymphocytes although IgM was reported to be present in significant amount by most investigators (Romagnani et al 1980; Ruuskanen et al 1980; Unander et al 1983). This limited responsiveness of the newborn lymphocytes gradually increased in postnatal life and reached the adult level when the child was between 2-5 years of age (Andersson et al 1981; Miyawaki et al 1981a). The

results from these studies suggest that newborn B lymphocytes are functionally immature with the IgM-producing precursor B cells being more mature than the other isotype-producing precursor B cells. Alternatively, it has been suggested that the deficient newborn B cell response may be a result of active suppression by fetal/newborn T cells or, in the case of the T cell-dependent response, due to a lack of autologous helper T cell function. Indeed, the existence of PWM-activated suppressor T cells has been reported in fetal peripheral blood as early as the 25th-26th week of gestation (Hayward and Kurnick 1981; Durandy et al 1985).

Development of T Lymphocytes:

The first immune-like reactions of the human fetus could be detected with liver cells at about 7-10 weeks' gestation. They exhibited strong proliferative responses when stimulated with allogeneic or xenogeneic cells in mixed lymphocyte cultures (MLC) and were capable of binding antigens (Dwyer and Mackay 1972; Carr et al 1973; Asantila et al 1974; Stites et al 1974). However, both of these functions seemed to be immunologically nonspecific. Thymic epithelium appeared at around the 6th week of gestation and became populated with small lymphocyte-like cells from liver by the 9th to the 10th week of gestation (Papiernik 1970). Thymocytes that expressed receptors for sheep erythrocytes and responded mitotically to phytohaemagglutinin (PHA) were observed by the 9th to the 10th week of gestation at a time when the thymus differentiated histologically into cortex and medulla (Stites et al 1974; Asma et al 1974; August et al 1971). Compared to

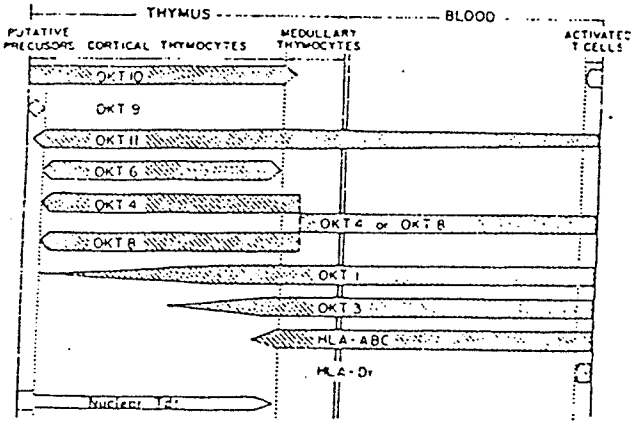
their response to PHA, the response of fetal lymphocytes to another mitogen Concanavalin A (Con A) emerged later and was weaker in intensity (Toivanen et al 1981). No response to Con A stimulation was detectable until 13-14 weeks' gestation for thymocytes and 18 weeks' gestation for splenocytes (Leino et al 1980). The same order of development in the response of fetal lymphocytes to PHA and Con A has been demonstrated in animals such as sheep (Leino 1978), guinea pigs (Merikanto 1979) and mice (Mosier 1974). These findings indicate that Con A and PHA stimulate, at least partially, different T cell subpopulations (Stobo and Paul 1974) and support the concept that during ontogeny, mitogen-responsive cells first appear in the fetal thymus and subsequently migrate to the spleen and then to the pheriphery (Toivanen et al 1981).

The ability of fetal lymphocytes from thymus, spleen and peripheral blood to respond to alloantigen was found to be already present by the 11th to the 15th week of gestation (Ohama and Kajii 1974). The capacity for cell-mediated cytotoxicity against hapten-modified autologous, semiallogeneic or allogeneic target cells, however, remained low throughout fetal life (Olding et al 1974; Granberg et al 1979; Granberg and Hirvonen 1980; Palacios and Andersson 1982a) although several cases of neonatal lymphocytes exhibiting cell-mediated toxicity comparable to the adult level have been reported (Granberg et al 1976; Rayfield et al 1980).

The maturation of lymphocytes within the thymus during fetal and adult life is associated with a series of phenotypic changes which were demonstrated by the use of monoclonal antibodies (Reinherz et al 1980a; Janossy et al 1981; Tidman et al 1981). A schematic representation of the phenotypic changes associated with postnatal T cell differentiation is shown in Figure 1. The most primitive thymic lymphoid cells (Stage I) expressed the T9 (transferrin receptor) and T10 antigens, which are present on progenitor cells of several lineages. They then acquired the receptor for sheep erythrocytes (T11), a thymic-distinct antigen T6 and antigens defined by monoclonal antibodies anti-T4 and anti-T8/T5. These cells, which resided in the thymic cortex, ceased to express the T9 antigen but retained the T10 antigen (Stage II) and accounted for approximately 70% of the total thymic population. With further maturation (Stage III), the cortical thymocytes expressed the T1, T3 and HLA-A,B,C antigens, lost their T6 antigens and migrated to the thymic medulla. Finally, the medullary thymocytes segregated into $T4^+$ and $T8^+$ subsets and lost the T10 antigens. Immunological competency was acquired at this stage but was not fully developed until the thymic lymphocytes entered the circulation and passed to the peripheral lymphoid tissues (Reinherz et al 1979a). T cells expressing T4 antigen largely, though not exclusively, behaved as helper/inducer T cells while those expressing T8 antigen behaved as suppressor/cytotoxic T cells (Reinherz and Schlossman 1981). Similar process of differentiation was apparent in fetal thymus but with 2 exceptions. First, primitive lymphoid cells (Stage I) constituted a larger proportion (12-40% versus 10%) of the

Figure 1: (Adapted from Linch and Levinsky 1983).

FIG. 1. Schematic representation of T-cell differentiation



total cell population in fetal thymus (Janossy et al 1981). Second, compared to adult, fetal thymus had fewer precursor or cortical thymocytes that contained the nuclear enzyme terminal deoxynucleotidyl transferase (Janossy et al 1981).

Development of Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) and Natural Killer (NK) Cells

ADCC activity (by K cells) was readily demonstrable in newborn cord blood when allogeneic Chang human liver cells coated with rabbit anti-Chang antibody were used as target cells (Campbell et al 1974). The mean cytotoxic activity of cord lymphocytes was only slightly lower than that of the adult lymphocytes suggesting that K cell activity is well developed at birth. Similarly, surface-adhering term newborn cord blood monocytes have a fully developed capacity for antibody-dependent cellular toxicity as demonstrated by Milgrom and Shore (1977) who used human group 0, Rh-positive erythrocytes sensitized with human anti-D immunoglobulin and labelled with Cr^{51} as targets. In contrast, Rachelefsky et al (1973), using HLA antibody-coated allogeneic lymphocytes as target cells, observed a significantly lower level of killing by newborn lymphocytes. A more recent report also confirmed low ADCC activity in neonates (Nair et al 1985). Therefore, further investigations are necessary before any conclusions can be reached regarding the ADCC activity of newborn mononuclear cells.

NK cell activity could be seen as early as the 9th week of gestation in fetal liver but was not detected in fetal spleen until the 19th week of gestation (Uksila et al 1983) after which time it gradually increased throughout the prenatal life until term when it reached between 25-60% of the adult level (West et al 1977; Abo et al 1984; Baley and Schacter 1985). The reduced NK activity was a result of a decreased number of NK cells and NK cytotoxic factor as well as diminished binding and killing of the target (K562 cell line) cells (Nair et al 1985). It was believed that elevated estrogen levels during pregnancy contributed to the low NK cell number and activity of newborn lymphocytes (Seaman et al 1978, 1979). Using lymphocyte-specific monoclonal antibodies, Abo and his colleagues reported a decreased absolute and relative number of NK cells among newborn mononuclear cells. Furthermore, a higher proportion of cells having low NK activity was present (Abo et al 1982, 1984; Abo and Balch 1982). None the less, isolated cases of neonatal lymphocytes possessing NK activity comparable to adult had been reported (Uksila et al 1982).

Newborn Monocyte/Macrophage Functions

The ability of fetal and newborn monocytes/macrophages to process antigen, perform phagocytosis, kill pathogens and modulate immune responses has not been clearly delineated. The phagocytic capacity of newborn monocytes was found to be normal for pathogens such as E. coli, Staph. aureus and Strep. pyogenes but deficient for others such as Group B Streptococcus and polystyrene particles (Weston

et al 1976; Schuit and Powell 1980; Marodi et al 1984). Lack of serum opsonic factors in newborn and fetal serum may further reduce their effectiveness in vivo (Forman and Stiehm 1969). Their respones to chemotactic factors has variously been reported to be normal (Pahwa et al 1977) or decreased (Kretschmer et al 1976) and their intracellular killing efficiency also varied depending on the pathogen (Marodi et al 1984). Antigen presentation by newborn monocytes/macrophages was reported to be normal, at least for the antigens ovalbumin (van Tol et al 1983) and tetanus toxoid (Kurnick et al 1978; Hoffman et al 1981).

From the above review together with data from animal studies (reviewed by Murgita and Wigzell 1981), it is quite evident that the developing fetus and newborn remain, throughout the prenatal and much of the neonatal period, relatively immunoincompetent in comparison to the adult. There is good evidence to suggest that the repertoire of antigen-binding cells in the adult and newborn is similar (Dwyer and Mackay 1972; Silverstein and Segal 1975; Klinman and Press 1978) and that these cells arise early in development at a time which may precede the ability of the fetus to mount a specific immune response (Decker and Sercarz 1975; D'Eustachio and Edelman 1975). The process of prenatal and postnatal development from immunological immaturity to full immunocompetency appears to represent a well-ordered sequence of stepwise events leading to the gradual establishment of competency (Sterzl and Silverstein 1967; Silverstein 1977). This notion is borne out by the clinical observations that newborn lymphocytes could only respond to certain types of viruses, bacteria or fungi, and that their

responses to these antigens may be restricted to either the humoral or cellular response with further limitation on the degree of response within each type (Brody et al 1968; Wallach et al 1969; Aase et al 1972; Astor and Frick 1973). Hence, the newborn humoral response to a wide variety of pathogens was found to be limited to the IgM isotype (Miller et al 1969). Although the newborns' relative immunodeficiency is well documented, the contribution, if any, to such a state of immunodeficiency by strong autologous fetal neonatal suppressor cells, which have been described in both murine (reviewed by Murgita and Wigzell 1981) and man (reviewed by Jacoby et al 1984), remains an area of intense investigation.

Immunology of the Fetal-Maternal Relationship

One of the most intriguing phenomena in biological sciences is the coexistence of mother and fetus despite their histoincompatibility. It is an example of successful natural allograft that is superior to any human imitation. Empirically, fetoplacental units are quite successfully accepted by the host, whereas nearly all therapeutic tissue or organ allografts are consistently rejected in the absence of tissue typing and immunosuppressive therapy. A considerable amount of work has been done in the last couple of decades in an attempt to solve this mystery. In order to understand the immunological relationship between mother and her fetus, a priori question of whether the conceptus is immunogenic must first be addressed. It was suggested by Medawar about 2 decades ago that perhaps fetal tissues were not immunogenic and therefore would not

incite a maternal immune response (Medawar 1954). This notion had since been discarded when anti-paternal HLA antibodies were discovered in primiparas in as early as the first trimester (Winchester et al 1975). Both the frequency and the titre of these antibodies increased with the advancement of pregnancy and the female's parity (Doughty and Gelsthorpe 1974). Evaluation of T cell-mediated responses also confirmed maternal sensitization to fetal antigens as shown by the production of migration inhibitory factor by maternal lymphocytes in the presence of fetal or paternal alloantigens (Rocklin et al 1973) and the existence of maternal suppressor T cells that were directed specifically against autologous T cell responses to fetal/paternal antigens (Stankova and Rola-Pleszczynski 1984; Vanderbeeken et al 1984). Additional evidence came from murine studies which showed that oncofetal antigens and minor histocompatibility antigens were present on zygotes and blastomeres (from the 4-cell stage onward), respectively (Hamilton 1983; Heyner 1983). Major histocompatibility antigens, though present, were confined to the inner cell mass of blastocyte or expressed transiently, if at all, on the trophectoderm prior to implantation (Hebner 1983).

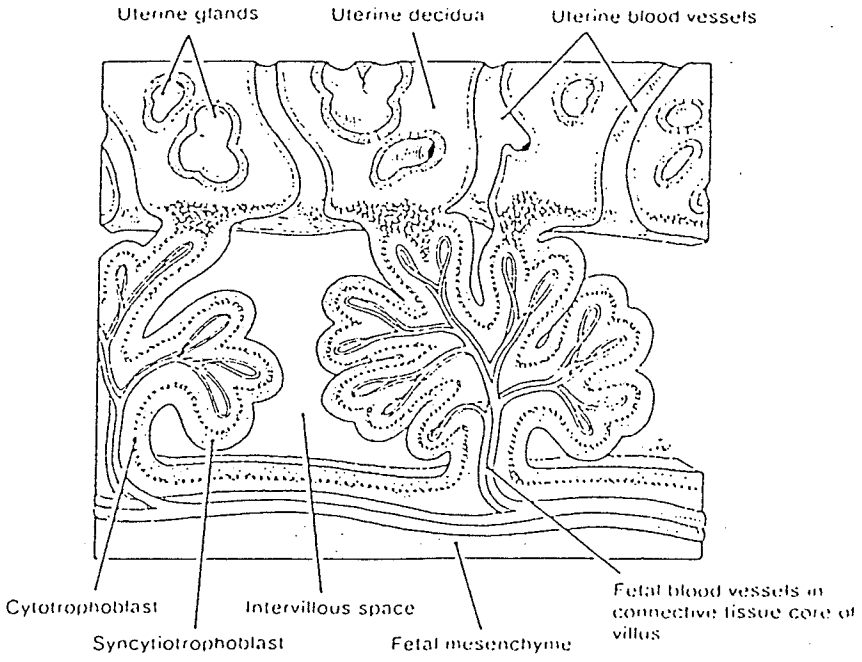
In light of the recent advances in pregnancy immunology, it has become increasingly clear that during pregnancy, several protective immune mechanisms as well as a number of unique cellular and anatomical features of the conceptus enable the host (mother) to accomodate her semi-allogeneic fetal graft in a very efficient manner. Conceptually, these various protective features can be divided into 3

levels: maternal level, fetal-maternal interphase and fetal level. While some of the mechanisms are probably crucial to the success of pregnancy, others assume only secondary or backup roles and work in concert with each other to ensure the survival of the fetus.

Protective Mechanisms at the Fetal-Maternal Interphase:

In higher animals, placenta represents a fetally-derived organ for the exchange of materials between the maternal and the fetal circulation. The two circulations never mix except with occassional cell traffic in either direction. In hemochoriod placentae (e.g., placentae of mouse and human), trophoblast cells of the chorion line the maternal-blood sinusoids and are exposed directly to the maternal immune system. Chorionic villi of the human placenta are lined by 2 layers of trophoblasts: an outer multi-nucleated syncytiotrophoblast layer in contact with maternal blood and an inner cytotrophoblast layer which also extends out of the villi into the endometrial stroma (Fig. 2). The cytotrophoblast layer disappears from the chorionic villi but persists elsewhere in the placental bed and extraplacental chorionic membrane in full-term human placentae. Initially, it was thought that trophoblast cells do not express HLA antigens and are therefore immunologically privileged (Faulk and Temple 1976; Faulk et al 1977). Recently, however, it became evident that although syncytiotrophoblast has neither Class I nor II antigen, cytotrophoblast in the extravillous extensions do express Class I antigens (Sunderland et al 1981a, 1981b). Nevertheless, the fact that pre-sensitized females do not always reject trophoblast indicates its

Figure 2: Chorionic villi of human placenta.


Uterine glands
Uterine decidua
Uterine blood vessels
Cytotrophoblast
Syncytiotrophoblast
Intervillous space
Fetal mesenchyme
Fetal blood vessels in connective tissue core of villus

refractoriness to the effector arm of the maternal immune response (Wegmann et al 1979). Furthermore, it will become obvious in the following review that the presence of MHC antigens on the trophoblast layer may even be beneficial to the survival of the conceptus.

(a) Placenta as an immunoadsorbent

In the human, it was shown that maternal antibodies directed against those paternal transplantation antigens that were also expressed on fetal cells were trapped in the placenta while those that were directed against antigens not expressed on fetal cells entered freely into the fetal circulation (Doughty and Gelsthorpe 1974, 1976; Tongio and Mayer 1975). Similarly, it was demonstrated in murine studies that when radiolabelled intact monoclonal anti-fetal MHC antibodies or their $F(ab')_2$ derivatives were injected into pregnant females, an increased uptake by the placentae of females bearing fetuses that expressed the target MHC antigens was observed. By contrast, fetuses that did not express the target MHC antigens had high radioactivity in their circulations (Chaouat et al 1983). These results indicate that the placenta is capable of adsorbing anti-fetal MHC antibodies and thus of preventing their entry into the fetal circulation. Adsorption of immunoglobulin by placenta could also be achieved nonspecifically through the Fc receptors present on the trophoblast, thus, augmenting its function as an immunoadsorbent (Elson et al 1975; Wood et al 1978). The bound antibodies were rapidly internalized, digested and released as fragments thereby restoring the placental antibody-binding capacity (Chaouat et al

1983). Alternatively, the antibodies were shed from the cell surface in the form of antigen-antibody complexes and were released into the maternal blood with the resultant suppression of the maternal immune response. It is not certain, however, if such a process is operational in humans since immune complexes have not been unequivocally demonstrated in the maternal serum.

The HLA antigen distribution on trophoblast was quite low (Lala et al 1983) and this sparcity of antigen on the trophoblast cell surface served at least 2 purposes. First, it spared the trophoblast cells from complement-mediated cytotoxicity due to inadequate cross-linking of immunoglobulins on the cell surface. Second, it promoted the production of blocking antibodies over that of cytotoxic antibodies (Linscott 1970). Indeed, the majority of antibodies that bound to the murine placenta was found to be the non-complement fixing, IgG1 type (Voisin and Chaouat 1974). Such blocking antibodies may render the placental antigenic sites inaccessible to either cytotoxic antibodies or cytotoxic T cells (Kaliss and Dagg 1964; Lala et al 1983). In addition to these blocking antibodies, uteroglobulin (a product of uterus) and transferrin have also been suggested to modulate immune recognition of the alloantigens present on the placenta (Faulk and Galbraith 1979; Mukherjee et al 1981).

(b) Induction of maternal blocking factors by trophoblast-lymphocyte cross-reactive (TLX) antigens

Faulk and McIntyre (1981) identified 2 types of trophoblast

antigens (TA) that were distinct from the HLA antigens: TA1, which were found on trophoblast and on transformed non-lymphoid cell lines; and TA2, which were shared by trophoblast, normal lymphocytes, kidney and liver tissue. The genes encoding for these TA antigens were proposed to map within the MHC region on chromosome 6. These authors hypothesized that trophoblast antigens functioned as a hapten-carrier unit, where TA1 was the carrier and TA2 was the hapten, to incite the production of blocking factors by maternal lymphocytes. These blocking factors, the nature of which remains to be characterized, in turn impeded the allogeneic recognition of fetal MHC antigens by maternal lymphocytes as illustrated by their ability to inhibit the maternal lymphocyte response to fetal cells in MLC (Faulk and McIntyre 1983; Faulk 1984). They proposed that adequate production of such blocking factors by maternal lymphocytes was essential to the survival of the conceptus; conversely, failure of the maternal lymphocytes to respond to TLX antigens could result in spontaneous abortion (McIntyre et al 1984).

(c) Uterine nonspecific suppressor cell and factor

Clark and his colleagues (1984) reported the existence of a novel type of suppressor cells in the uterine lymph nodes and decidua of pregnant mice. These cells released a soluble factor that could inhibit both the induction and the effector function of maternal cytotoxic T cells in a MHC-unrestricted fashion by blocking the latter's response to interleukin-2 (Clark 1984). These suppressor cells were approximately 7 microns in diameter with a sedimentation rate of 3mm/hr at unit gravity, lacked surface Thy-1 and Lyt markers,

possessed Fc receptors for IgG but were unaffected by anti-asialo-GM1 antibody and complement treatment. They were probably of bone marrow origin (Giozzi et al 1982; Kearns and Lala 1982) and were recruited by signals sent from the implanting trophoblast to the regional lymph nodes and decidua. Their action may be effective only over a short distance and only against those graft rejecting mechanisms that could attack the trophoblast rather than all types of immune mechanisms.

These nonspecific suppressor cells may not be unique to the mouse since similar suppressor activity has also been demonstrated recently in humans (Golander et al 1981; Clark, personal communication) although the cellular origin of this suppressor activity awaits further characterization.

(d) Nonspecific immunoregulatory substances

A wide variety of immunosuppressive substances capable of nonspecifically suppressing maternal immune functions have been reported. These substances were either synthesized or their production was augmented by the fetal-placental unit. It was believed that they may play a role in the protection of the conceptus against maternal rejection. Broadly, these substances can be classified into hormonal and non-hormonal factors. Included in the first group are hormones such as estradiol, progesterone, corticosteroids, human placental lactogen and human chorionic gonadotropin. Although all the steroidal hormones could be shown to exhibit immunosuppressive activity, none of them were immunosuppressive at physiological

concentrations (Rocklin et al 1979; Jacoby et al 1984). Human placental lactogen and purified human chorionic gonadotropin also failed to show any immunosuppressive effect.

Among the non-hormonal agents, alpha fetoprotein (AFP) and prostaglandin E2 (PGE2) have attracted most attention. AFP, a glycopolypeptide of approximately 70,000 daltons is produced primarily in the fetal liver and yolk sac; it could be found in both fetal and maternal serum as well as amniotic fluid. High concentrations of this substance are present in early fetal life with the level reaching its peak at about 14 weeks' gestation followed by a gradual decline until the third trimester (Harris et al 1974). In vitro tests showed that AFP could activate suppressor T cells to inhibit the lymphocyte response to alloantigens and mitogens (Murgita and Wigzell 1981). However, the controversy of whether the immunosuppressive effect of AFP is due to AFP per se or to other components in the AFP preparation such as fetal albumin and other smaller molecules for which AFP merely served as a carrier (Goeken and Thompson 1976) has not been completely resolved.

PGE2 is known to possess a number of immunosuppressive properties when used at physiological concentrations (Goodwin and Webb 1980). A substantial amount of prostaglandins has been shown in the decidua and fetal membranes (Pritchard and MacDonald 1980; Mitchell et al 1982). Since the in vivo effect of prostaglandins is limited to local regions due to its near complete degradation after 1 circulation

through the lungs (Ferreira and Vane 1967), PGE2 becomes an ideal mediator to selectively inhibit the local maternal immune response without jeopardizing her systemic immune defence. More studies are required to substantiate this postulation.

In addition to AFP and PGE2, other non-hormonal factors, presumably of trophoblast origin, that were capable of suppressing T cell responses to mitogens and alloantigens as well as inhibiting cytotoxic T cell-mediated and complement-dependent, antibody-mediated cell killing have also been reported in mice (Chaouat et al 1983) and humans (Main et al 1985).

Protective Mechanisms at the Maternal Level:

Studies on the functional status of maternal lymphocytes are conflicting. Numerous reports have suggested that the female's immune system entered a hyporesponsive state during pregnancy as determined by her decreased lymphocyte response to antigens, mitogens and alloantigens, lowered proportion of $T4^+$ (inducer/helper) cells and reduced NK activity. However, an equal number of studies have suggested the contrary. In general, the degree of immune hyporesponsiveness was modest at best (Head and Billingham 1982) and may be due to the effect of nonspecific factors in the maternal circulation such as alpha-2-globulins (Stimson 1976) and beta-1-globulins (Towler et al 1976). Alternatively, it may be a downstream reflection of the suppressor activities generated at the fetal-maternal interphase.

Although the general immune responsiveness of pregnant females is not depressed, several specific immune suppressive mechanisms observed in gravid females may have some roles in the downregulation of maternal response against the conceptus. These include: (1) the presence of blocking antibodies directed against the paternal Class II MHC antigens in maternal serum (Rocklin et al 1979); (2) the presence of maternal suppressor T cells that specifically interfered with the autologous T cell response to paternal/fetal antigens (Stanokova and Rola-Pleczczynski 1984; Vanderbeeken et al 1984); and (3) the presence of anti-idiotype antibodies directed against T cell recognition sites for paternal/fetal antigens (Suiu-Foca et al 1983; Singal et al 1984).

Protective Mechanisms at the Fetal Level:

The architectural arrangement of human placenta allows segregation of maternal circulation from fetal circulation by interposing low immunogenic syncytiotrophoblast between the 2 compartments. This protective feature is strengthened by the hemodynamic relationship between the circulations which discourages the entry of maternal cells into the fetal vasculature by virtue of a pressure gradient. Such a barrier between the 2 vascular compartments effectively prevents large scale exchange which would be devastating to the fetus (Scott et al 1973). However, this barrier is not complete and small scale cell leakage does occur. It was found that at least 10% of infants born to mothers who were injected with ^{51}Cr-labelled autologous erythrocytes received substantial amounts (several ml) of maternal blood (Zarou et al 1964). Similarly,

fluorescent-labelled maternal leukocytes were found in as many as two-thirds of newborns (Desai and Creger 1963). Conversely, both fetal erythrocytes and leukocytes were found in the maternal circulation as early as 14-15 weeks' gestation and the quantity increased with the gestational age (Cohen et al 1964; Walknowska et al 1969; Herzenberg et al 1979). In a number of more recent studies where cells bearing XX chromosomes were sought among male newborn lymphocytes that were stimulated to enter mitosis, however, only a few positive results were obtained (Turner et al 1966; Schroder 1974; Adinolfi 1975). The appropriateness of employing such methods to detect maternal cells among fetal/newborn lymphocytes is questionable since fetal/newborn lymphocytes have been shown to inhibit the proliferative response of maternal lymphocytes to mitogens (Olding and Oldstone 1974). Further support of maternal cell transfer to fetal circulation came from analysis of newborns with severe combined immunodeficiency. HLA typing of circulating leukocytes indicated that 25% of newborns have lymphocytes of maternal origin (Pollack et al 1982). The incidence might be even higher if the more sensitive techniques of pre-sorting T cells or prior transformation of B cells with EBV before HLA typing were employed (Geha and Reinherz 1983).

A potential consequence of maternal lymphocyte transplacental passage is graft-versus-host (GVH) disease against the immunologically immature fetus similar to the 'runt' disease induced in newborn rodents by injecting adult lymphocytes into these newborns (Billingham 1968). Extensive animal experimentation has shown that either

adoptive transfer or active anti-paternal immunization of females could lead to 'runt' disease among many of the offspring presumably caused by the effector lymphocytes that had crossed the placental barrier (Beer and Billingham 1973). Observations on animals have also shown that the GVH reaction across weak histocompatibility barrier may lead to a 2- to 4-fold increase in the incidence of lymphoma (Walford 1966). In humans, GVH disease has indeed been observed to occur following isoimmunization (Bastian et al 1984) and exchange or intrauterine transfusion for severe erythroblastosis fetalis (Waiman et al 1969; Parkman et al 1974). Similar to rodents, GVH disease in humans may be responsible for certain types of lymphoma and leukemia in postnatal life (Green et al 1960; Turner et al 1975; Gill 1977). The mechanism responsible for tumor induction in GVH reaction has not been satisfactorily explained. It was believed by some investigators that the GVH reaction caused the release or activation of oncogenic viruses in susceptible individuals leading to malignant transformation of the lymphocytes (Hirsch et al 1970; Cornelius 1972; Gleichmann et al 1972).

Other possible sequelae of the maternal graft-versus-fetal host reaction are auto-allergic diseases in which antibodies directed at 'self' antigens are produced by maternal cell clones (Schwartz 1974) and severe combined immunodeficiency (Bastian et al 1984). It should be emphasized however, that in the majority of newborn severe combined immunodeficiency cases, the presence of maternal lymphocytes in the fetus is probably the result rather than the cause of fetal

immunodeficiency which permit their persistence in the fetus (Pollack et al 1982). It appears that entry of a small number of maternal lymphocytes into the fetal circulation is not uncommon and with the various efficient protective mechanisms in operation, they seldom pose any serious threat to the fetuses. In the event that such mechanisms broke down, GVH disease would ensue and serious complications such as immuno- deficiency, malignancy of the lymphoid system, auto-allergic diseases and spontaneous abortion may develop.

Similar to protection at the fetal-maternal interphase and the maternal level, protection from maternal rejection at the fetal level may also involve a number of mechanisms. For example, it was found that the fetus and newborn have low NK cell number and activity; since NK cells have been implicated in the induction of GVH reaction in humans undergoing allogeneic MHC-matched bone marrow transplant (Lopez et al 1979), the lack of NK cells in the fetus may provide a way of protection against GVH reaction by not providing cells that are crucial to its induction (Siegal 1981). Another possible method of protection against the transplacentally migrated maternal lymphocytes is the presence of both nonspecific and specific immunosuppressive factors. For instance, apoprotein E, a substance that inhibits lymphocyte reaction to mitogens (Curtiss et al 1984) was found to be elevated in fetal blood (Davis et al 1983). Serum IgM with specificity against maternal idiotypes directed at fetal antigens was also reported to be present in newborns. These cytotoxic anti-idiotype antibodies, when in the presence of complement, could

inhibit the maternal T cells' proliferative response to fetal/paternal antigens and suppressed their cytotoxic activity against PHA-induced paternal target cells (Miyagawa et al 1982, 1984).

Perhaps the putative protective mechanism that has generated most interest is the existence of strong suppressor cells in the fetus and newborn which are capable of inhibiting various adult immune functions both in vivo and in vitro.

(a) Murine studies

Spleen cells and thymocytes from neonatal mice were shown to suppress the antibody responses of adult spleen cells to both T cell-dependent and T cell-independent antigens (Mosier and Johnson 1975; Morse et al 1976; Mosier et al 1977; Hardy and Mozes 1978; Dekruyff et al 1980). Although the suppression was antigen nonspecific, T cell-dependent responses were more susceptible to inhibition than T cell-independent responses (Lukenbach et al 1978; Murgita et al 1978) suggesting that either the suppressor cell action was more effective on T cell-dependent than T cell-independent B cells (Lewis et al 1976) or that helper T cells were the primary target of suppression. The mechanism of suppression is not clear although there was some evidence to suggest that the neonatal suppressor cells acted in the early inductive phase of the antibody response (Lukenbach et al 1978) by cell-cell contact rather than through elaboration of soluble factors (Mulder et al 1978). Phenotypic analyses showed that the suppressor cells had a low content of Thy-1 (Lukenbach et al 1978),

resided in the $Lyt1^{+}2^{-}$ subpopulation and expressed both I-A and I-J gene-encoded antigens (Murgita et al 1978; Murgita and Wigzell 1981). Furthermore, they were resistant to hydrocortisone treatment (Lukenbach 1978), sensitive to irradiation or mitomycin C treatment (Mulder et al 1978) and required no priming to exert their suppressor activity (Calkins and Stutman 1978).

The suppressor activity of fetal and newborn lymphocytes was not limited to inhibition of the antibody response but was equally effective on T cell-mediated immune reactions. For example, it was found that embryonic or neonatal liver cells strongly suppressed the adult mixed lymphocyte reaction and cytotoxic T cell activation in an MHC-unrestricted manner (Globerson et al 1975). Injection of neonatal liver cells into sublethally irradiated F1 mice significantly reduced the GVH reaction of the engrafted syngeneic parental spleen cells (Umiel et al 1977; Globerson and Umiel 1978). Spleen cells from fetal or neonatal mice were found to be capable of suppressing local GVH reactions (Skowron-Cendrzak et al 1976), the development of contact sensitivity (Ptak and Skowron-Cendrzak 1977) and in vitro lymphocyte responses to alloantigen and mitogens through the production of soluble factor(s) (Basset et al 1977; Pavia and Stites 1979). These suppressor activities of neonatal spleen cells were independent of glass-adherent cells but could be eliminated by the removal of $Thy\text{-}1^{+}$ cells (Argyris 1978), depletion of $I\text{-}J^{+}$ cells (Argyris and Waltenbaugh 1983) or treatment with heterologous anti-brain associated T cell antigen antiserum and complement (Golub 1971; Pavia and Stites 1979).

Although the above data implied T cells as the source of suppressor activities, the exact nature of fetal and neonatal suppressor cells remains controversial. Rodriguez et al (1979) found a 'naturally occurring' suppressor cell population which did not exhibit the classical macrophage, B, T or NK cell features in the spleens of newborn mice less than 9-10 days of age. These suppressor cells not only possessed the ability to inhibit alloreactivity of newborn and adult cells, but also reduced T cell-dependent and T cell-independent antibody responses of third party adult spleen cells. They were shown to be FcR^-, $Thy\text{-}1^-$, $I\text{-}J^-$, Ia^-, nonadherent to plastic, and they bound to the Ig-anti-Ig column but not to Helix pomatia A hemagglutinin. Another interesting aspect of these suppressor cells is that they were also present in newborns of nu/nu mice, thus strengthening the view that they were not of the T cell lineage. The authors argued that since most studies used one to three week old mice as newborn mice, the 'neonatal' suppressor T cells documented in those studies may not represent the 'true' fetal/neonatal suppressor cells. Their contention was supported by the finding of another group of investigators who demonstrated the existence of a unique type of suppressor cells, which they termed 'natural suppressor' cells, in the spleens of newborn mice and adult mice that had received total lymphoid irradiation (Hertel-Wulff et al 1984). These natural suppressor cells could inhibit mixed lymphocyte reactions and GVH disease in an antigen nonspecific and MHC-unrestricted fashion (King et al 1981; Okada and Strober 1982). Phenotypic analyses showed that they were $Thy\text{-}1^-$ (but became

Thy-1$^+$ in long term cultures), Lyt-1$^-$, Lyt-2$^-$, Ig$^-$, Ia$^-$, H2K$^+$, H2D$^+$, MAC-1$^-$, asialo- GM1$^+$, F4/80$^-$ and 2C2$^-$. Morphologically, they resembled NK cells but functionally, they did not exhibit NK activity in the standard YAC-1 target cell lysis assay. Furthermore, they were usually present only transiently during the immediate postpartum period at a time when NK cell activity was still low (Herberman et al 1979).

Besides T cells and natural suppressor cells, macrophage-like cells or macrophage precursors have also been considered as cells responsible for the suppressor activity observed in neonatal spleen cells. These partially adherent cells were shown to suppress antibody response by the elaboration of prostaglandins (Piguet et al 1981). Others have attributed the neonatal spleen cell-mediated suppression to the non-adherent B lymphocyte-like cells which bore no T cell surface antigens but adhered to Ig-anti-Ig columns and interacted with lectins that were shown to selectively bind B cells.

It is apparent that there are at least 2 subpopulations of suppressor cells in newborn mice: one that possesses T cell characteristics and another that exhibits none of the classical features of macrophage, T, B or NK cells but was related closely to the NK cells lineage by phenotypic and morphologic criteria. The relationships among the latter group (natural suppressor cells), the macrophage-like suppressor cells and the B lymphocyte-like cells await further clarification. One point that becomes evident from these

studies is that the cellular composition of the newborn suppressor cells changes with time in the neonatal period and any further investigation of this dynamic state requires the use of stringent experimental designs to allow fruitful comparisons of results obtained by different laboratories.

(b) Human studies

Olding and his colleagues first observed that human umbilical cord blood lymphocytes can inhibit mitosis of adult lymphocytes (Olding et al 1974; Olding and Oldstone 1974). When equal proportions of mononuclear cells (MNC) from a male newborn and his mother or unrelated female adult were mixed and cultured for 3 days, with or without PHA, 98% of the dividing lymphocytes were found to belong to the newborn as assessed by the fluorescent Y chromosome technique. The low number of adult mitotic cells was not due to cell killing by the neonatal lymphocytes since no increased release of ^{51}Cr from the labelled adult cells were detected (Olding et al 1974). Subsequently, Lawler et al (1975) reported that a similar inhibitory effect by male newborn lymphocytes on maternal cells was also observed in a 2-way MLC. Analysis of the suppressor cells indicated that they were E rosette-positive cells bearing receptors for IgG (Olding and Oldstone 1976; Oldstone et al 1977) and were sensitive to irradiation or mitomycin-C treatment (Lawler et al 1975; Olding et al 1977). They appeared in the fetal circulation as early as the 14th week of gestation (Unander and Olding 1981) and did not seem to inhibit mitosis of other newborn lymphocytes suggesting the selective nature of their suppressor activity (Olding and Oldstone 1976).

With the use of a Marbrook double-chamber culture system in which cord lymphocytes were separated from adult lymphocytes by a cell-impermeable membrane, Olding et al (1977) were able to show that cord lymphocytes suppressed adult lymphocyte mitosis, at least partially, through the production of small molecular weight suppressor factor(s) (less than 10^4 daltons) which diffused across the semi-permeable membrane to the adult lymphocytes (Olding et al 1977; Unander et al 1982). The magnitude of suppression by this suppressor factor, however, was lower than that achieved by the suppressor cells (40-71% versus 90-100%, respectively). The authors speculated that this discrepancy might be due to the short-ranged effect of the factor, the necessity for cell-to-cell contact to facilitate suppression or the requirement for both dialyzable and non-dialyzable substances to accomplish total suppression.

Employing the same double-chamber system, Unander et al (1982) claimed that cord lymphocytes could also inhibit IgM, IgG and IgA production by PHA-stimulated adult lymphocytes. While 90% of cord blood lymphocytes demonstrated suppressor activity in their study, 25% of adult control lymphocytes also exhibited suppressor function. The feature that distinguished these cord suppressor cells from their adult counterpart was the spontaneous nature of their suppressor function which required no prior activation by PHA as was the case for adult suppressor cells. These results should be viewed with caution since the ineffectiveness of PHA as a stimulator for immunoglobulin production has been well documented (Greaves and Roitt 1968; Clement

et al 1983) and yet a significant amount of immunoglobulin synthesis by adult lymphocytes in response to PHA stimulation was observed in this study.

Recently, attempts to identify the cord lymphocyte-originated suppressor factor were undertaken by Johnsen and coworkers (Johnsen et al 1982, 1983a, 1983b, 1983c; Papadogiannakis et al 1985). It was first shown that the addition of either indomethacin (an inhibitor of the cyclooxygenase pathway in arachidonic acid metabolism) or 5, 8, 11, 14-eicosatetraynoic acid (an inhibitor of both the cyclooxygenase and lipooxygenase pathways) to a Marbrook double-chamber containing cord and adult lymphocytes completely abrogated the suppressor effect of cord lymphocytes. A reduction of suppression was also observed when these agents were added to cord-adult lymphocyte cocultures suggesting that the suppressor factor through which cord blood lymphocytes exerted their suppression might be PGE2, a known product of placental tissue, fetal adrenal glands and mononuclear cells (Mitchell et al 1982). Dose-response experiments with indomethacin-treated lymphocytes showed that at physiological concentrations (about 10^{-8}M), exogenous PGE2 inhibited adult but enhanced the cord mononuclear cells proliferative response to PHA. In addition, the binding capacity of cord MNC for PGE2 was found to be only about 30% of maternal MNC. These results, therefore, provided an explanation for the observed difference in susceptibility between cord and adult lymphocytes to the allogeneic cord lymphocyte-derived suppression. It should be mentioned that although PGE2 was the main

mediator of cord lymphocyte-derived suppressor activity, other arachidonic acid metabolites may also be involved (Johnsen et al 1983b).

Unlike adult mononuclear cells in which monocytes were the primary source of prostaglandin synthesis (Goodwin et al 1977; Kurland and Bockman 1978; Goldyne and Stobo 1979), cord MNC-produced PGE2 appeared to be a product of T lymphocytes (Papadogiannakis et al 1985). While depletion of monocytes from cord blood lymphocytes by nylon wool separation (adherent cells constituted less than 0.05% of the remaining cells) did not alter their suppressor activity, supernatants from cord adherent cell cultures were actually found to contain less PGE2 than from adult adherent cell cultures (Fischer et al 1980) thereby confirming the notion that cord T cells were the main provider of PGE2. Quantification of the various arachidonic metabolites produced by unstimulated and stimulated (exposed to PHA or alloantigen) cord T cells failed to discern any difference suggesting that the cord suppressor T cells were either inherently suppressive or may have already been activated in utero (Johnsen et al 1983b).

One of the criticisms of these studies is their reliance on pharmacological agents such as indomethacin and eicosatetraynoic acid, which have actions other than inhibition of prostaglandin synthesis, to demonstrate the involvement of PGE2. A case in point was the inconsistent results obtained when Naprosyn, another cyclooxygenase inhibitor was used instead of indomethacin (Papadogiannakis et al 1985). More direct methods are required to clarify the role of PGE2

and other arachidonic acid metabolites in cord lymphocyte-induced suppression.

Another mitogen that has been used extensively in the study of fetal/newborn suppressor cells is PWM. It was found that the addition of peripheral blood T lymphocytes from newborns or fetuses of as early as the 26th week of gestation to adult mononuclear cells strongly inhibited the latter's antibody response to PWM (Hayward and Lydyard 1978a; Durandy et al 1979, 1985; Miyawaki et al 1979; Tosato et al 1980; Andersson et al 1981; Rodriguez et al 1981; Jacoby and Oldstone 1983). The mechanism of suppression, the exact nature of these neonatal suppressor T cells and their target remains controversial and will be discussed in the next few chapters. It appears that in this system, PGE2 may not be a key factor since addition of indomethacin failed to reverse the suppressor effect of neonatal lymphocytes and perhaps a different subset of cord suppressor cells other than those in the PHA system may be involved.

One disadvantage of using the PWM-induced antibody response assay system to study the target of newborn suppressor cells is the requirement for both macrophage and helper T cells besides B cells to achieve antibody response. Thus, inhibition of antibody response by newborn lymphocytes could be due to suppression on any one or more of these 3 types of cells. To circumvent this problem, antibody response to EBV, a T cell- and monocyte-independent polyclonal B cell activator has been used to examine the target of newborn suppressor cells.

Andersson et al (1981) found that EBV-induced immunoglobulin production by adult lymphocytes could be suppressed by cord T cells but not by allogeneic adult T cells. By contrast, EBV-induced immunoglobulin production by newborn lymphocytes was not suppressible by either adult or newborn T cells. The newborn suppressor T cells were sensitive to irradiation, expressed $T4^+$ phenotype (Andersson et al 1981; Palacios and Andersson 1982b) and were also capable of inhibiting immunoglobulin production by EBV-transformed adult lymphoblastoid B cell lines (Andersson et al 1983). The suppression was believed to be mediated through the elaboration of gamma interferon by newborn T cells. Unfortunately, certain aspects of these findings are inconsistent with observations made by other investigators. First, a considerable number of studies have shown that gamma interferon production by newborn T cells was deficient (Dryson et al 1980; Miyawaki et al 1985; Nair et al 1985; Taylor and Dryson 1985). Second, interferon-mediated suppression was found to be effective against EBV-induced immunoglobulin production by fresh lymphocytes but not by transformed B cell lines (Lotz et al 1985). Therefore, it is quite improbable that the inhibition of the EBV-induced antibody response by newborn T cells was mediated via gamma-interferon. Furthermore, experiments by Tosato et al (1980) showed that neonatal T cells had no suppressive effect on EBV-induced antibody response unless they were also activated by mitogen thereby raising the question of whether neonatal T cells are truly spontaneously suppressive as was suggested by the work of Andersson and his colleagues (Andersson et al 1981, 1983). Re-examination of

the suppressor mechanism of neonatal T cells is necessary to solve these issues.

Besides T cells, monocytes have also been implicated as the source of the strong neonatal suppressor activity. For example, Wolf et al (1977) found that non-activated neonatal monocytes spontaneously produced a soluble factor that could suppress adult lymphocyte responses to alloantigen and PHA. Depletion of adherent cells from neonatal mononuclear cells led to augmented antibody responses while the enrichment of adherent cells led to decreased antibody response (Wilson and Remington 1979; Knutsen and Buckly 1980). These results were taken to suggest that the neonatal monocytes were responsible for the observed potent neonatal suppressor activity. Other investigators, however, either failed to detect any suppressor activity of neonatal monocytes when tested on the adult lymphocyte response to mitogen (Olding and Oldstone 1976; Durandy et al 1979) or could not distinguish the suppressor activity of neonatal monocytes from that of adult monocytes (Andersson et al 1981); and most concluded that the suppressor activity resided in the T cell fraction (Hayward 1981; Miyawaki et al 1981a; Rodriguez et al 1981; Jacoby et al 1983). Furthermore, Durandy et al (1985) reported that neonatal adherent cells actually had less suppressor activity than adult adherent cells possibly due to the down-regulation by their autologous suppressor T cells. Therefore, it appears unlikely that neonatal monocytes are the principal suppressor cells although they may assume a secondary role in the induction or effector phase of the

neonatal T cell-mediated suppression.

With a few exceptions, most investigations of B cell reactivity and T cell regulatory functions of human newborns have been studied by stimulation of cells with polyclonal activators such as PWM, PHA, EBV and Nocardia opaca. These studies primarily dealt with the antigen-nonspecific aspect of the fetal/newborn suppressor cell functions. This is partly due to the technical difficulties encountered in the development of in vitro antigen-specific immune response assays and partly due to the requirement for genetically restricted lymphocyte-monocyte interactions in eliciting antigen-specific response that are difficult to achieve in human studies. Nevertheless, a number of investigators were able to devise appropriate methods to evaluate the antigen-specific suppressor activity of newborn lymphocytes. The results of these studies provided another dimension to our understanding of the functional significance and the mechanism of neonatal lymphocyte-mediated suppression. For instance, Stankova and Rola-Pleszczynski (1984) showed that various subpopulations of newborn lymphocytes were able to specifically inhibit the autologous or maternal lymphocyte reaction to one another thereby providing another protective mechanism against GVH reaction. Using T cell-dependent antigens as stimulators, van Tol et al studied the antibody response of neonatal MNC and found that it was significantly lower than that of adult MNC due to the concurrent induction of both antigen-specific suppressor and antigen-specific helper T cells in the former (van Tol et al 1983, 1984). When

progressively decreasing doses of antigens were used, the antibody response of neonatal MNC increased gradually until it eventually reached the adult level. Furthermore, it was shown that the different dose-response patterns of neonatal and adult MNC was determined by their respective source of monocytes. Thus, if adult monocytes were added to neonatal lymphocytes, an adult type response pattern would result and conversely, if neonatal monocytes were cocultured with adult lymphocytes, a neonatal type response would occur. The authors speculated that this difference in response may be related to the slower antigen catabolic rate of neonatal monocytes which led to an unbalanced activation of T lymphocytes favouring the suppressor faction over the helper faction. The implication of these results, if confirmed by future studies, would be that newborn MNC-mediated antigen-specific and nonspecific suppressor activities involved 2 different types of cells and mechanisms. In the case of antigen-specific suppression, the suppressor signal is originated from neonatal monocytes which exert their action through activation of either adult or neonatal suppressor T cells whereas in the case of antigen-nonspecific suppression, the suppressor signal is generated primarily from neonatal suppressor T cells independent of monocytes. Both types of suppression may be important in the defence against the transplacentally migrated maternal lymphocytes.

CHAPTER 2

IMMUNOREGULATORY FUNCTION OF HUMAN CORD BLOOD LYMPHOCYTES ON IMMUNOGLOBULIN PRODUCTION

Abstract

The functional maturity of human unbilical cord blood B lymphocytes and the immunoregulatory activity of cord T lymphocytes were assessed by measuring the in vitro immunoglobulin (Ig) production by B cells from either cord or adult blood. Supernatants from 48 hr pokeweed mitogen (PWM)-stimulated cord or adult lymphocyte cultures were added to cord or adult B cell cultures in the presence of PWM; a significant amount of immunoglobulin was produced in adult B cell cultures only. Adult B or T cells were then co-cultured with cord T or B cells; a significant amount of immunoglobulin was again found only in adult B, and T cell co-cultures. These results indicate that cord B cells are functionally immature and that cord helper T cell function is adequate but masked by excessive suppressor activity. Indeed, addition of cord T cells but not of allogeneic adult T cells to PWM- or Epstein-Barr virus (EBV)-stimulated adult lymphocyte cultures significantly inhibited their immunoglobulin production confirming cord T cells' increased suppressor activity. The suppressor action of cord T cells was mediated via soluble factor(s) as demonstrated by the suppressor effect of culture supernatants from PWM- or alloantigen- activated cord T cells on the immunoglobulin production by unfrac- tionated adult lymphocytes. In contrast, when these supernatants were added to T cell-depleted adult lymphocyte cultures, enhancement rather than suppression was observed. Taken together, these results suggest that the soluble factor released by cord T lymphocytes exerts its effect through activation of suppressor cells which, in turn, inhibit B cells either directly or through monocytes.

Introduction

The functional status of fetal and neonatal immune systems has been the subject of intense investigations in recent years. A number of distinctive features have been identified. It was shown that in vivo immunoglobulin production was deficient despite normal numbers of circulating B cells (Gathings et al 1977; Hayward and Lydyard 1979). In vitro studies also showed that neonatal B cells failed to secrete an adequate amount of immunoglobulin (compared to adult B cells) upon stimulation by T cell-dependent polyclonal activator PWM (Hayward and Lawton 1977; Oldstone et al 1977; Durandy et al 1979; Miyawaki et al 1979; Morito et al 1979; Tosato et al 1980; Andersson et al 1981) or by the relatively T cell-dependent mitogen Nocardia opaca (Bona et al 1979; Miyawaki et al 1981a). Moreover, the antibody response was found to be restricted to the IgM class when neonatal B cells were stimulated by EBV, a T cell-independent polyclonal activator (Bird and Britton 1979; Andersson et al 1980) and the relatively T cell-dependent mitogen lipopolysaccharide (LPS) (Miller et al 1978; Andersson et al 1981). These observations are generally taken as an indication of cord B cell functional immaturity. Another characteristic of the newborn immune system is the existence of hypereactive suppressor T cells. Olding and his colleagues reported that newborn lymphocytes were capable of suppressing adult lymphocyte responses to alloantigen and phytohemagglutin (PHA) (Olding et al 1974; Olding and Oldstone 1974). The cells responsible for the suppression were shown to be T cells (Oldstone et al 1977). Other investigators extended these observations by showing that cord T cells

could also inhibit immunoglobulin production by adult lymphocytes stimulated with mitogens (Hayward and Lawton 1977; Oldstone et al 1977; Durandy et al 1979; Miyawaki et al 1979; Tosato et al 1980; Andersson et al 1981; Rodriguez et al 1981; Unander et al 1981; Jacoby and Olstone 1983).

Although the inadequate secretion of immunoglobulin by PWM-stimulated neonatal lymphocytes has been well documented, the exact cause of this immunological deficiency has not been resolved. B cell functional immaturity, inadequate helper T cell function (Andersson et al 1981) and excessive suppressor T cell activity (Durandy et al 1979; Morito et al 1979; Tosato et al 1980) have all been implicated. Co-culture of adult B cells and cord T cells in the presence of PWM produced very little immunoglobulin. However, if the cord T cells were pretreated with irradiation, enhancement of immunoglobulin production occurred (Durandy et al 1979; Tosato et al 1980; Hayward 1981; Jacoby and Oldstone 1983) suggesting that cord helper T cell activity was usually masked by radiosensitive suppressor T cells. This observation, however, could not be confirmed by Andersson et al (1981). Furthermore, though cord T cells could suppress the adult B cell response, it remains unclear whether their suppressive action has any effect on the neonatal B cell differentiation. Finally, the target of the neonatal suppressor cells also remains controversial. While Durandy et al (1979) found that suppression required the presence of adult T cells, Andersson et al (1981) showed that adult non-T cells (B cells and monocytes) could be

suppressed directly and Miyawaki et al (1981) demonstrated the need to collaborate with monocytes in order for the neonatal suppressor cells to exert their effect.

To clarify some of these issues, the present study examined the functional characteristics of neonatal B and T cells involved in the production and regulation of immunoglobulin in response to T cell-dependent (PWM) and T cell-independent (EBV) polyclonal B cell activators. The results indicated that: (1) cord B cells are functionally immature though the degree of immaturity differs depending on the stimulator used to elicit the B cell response, (2) helper T cell activity is adequate, and (3) the excessive T cell activity is directed against adult but not cord lymphocytes. More importantly, the data show that the suppressor activity is mediated by soluble factor(s) which exert its effect through the activation of suppressor T cells.

Materials and Methods

Preparation of mononuclear cells (MNC) and isolation of B and T cells. Cord blood was obtained at the time of delivery from normal uncomplicated pregnancies. Immediately after delivery but prior to expulsion of the placenta, cord blood was drained into tubes containing preservative-free heparin. It was then mixed with 5% dextran in normal saline at a ratio of 4:1 for 45 minutes at 37°C to sediment the erythrocytes. The leukocyte rich supernatant was diluted

1:2 in Hank's balanced salt solution (HBSS) (Flow Laboratories) and centrifuged over Ficoll-metrizoate gradient (Pharmacia Fine Chemicals; Nyegaard and Co.) with specific gravity of 1.073 at 900g for 30 minutes. Adult blood donated by healthy volunteers was diluted 1:2 in Hank's solution and centrifuged over Ficoll-metrizoate with specific gravity of 1.077. Mononuclear cells (MNC) in the interphase were collected and washed 3 times in Hank's solution. Further separation of MNC into T and B cells were achieved by rosetting with sheep erythrocytes. Briefly, MNC were suspended in RPMI 1640 and 10% heated-inactivated and absorbed fetal bovine serum (Flow and Gibco Laboratories, respectively) at 3×10^6 cells/ml. To this cell suspension was added 0.1 ml of aminoethylisothiouroniumbromide (Sigma Chemical Co.)-treated sheep erythrocytes (National Biological). The cell suspension was then centrifuged over a Ficoll-metrizoate gradient at 4°C for 30 minutes. Cells collected at the interphase were rosetted again and centrifuged over a Ficoll-metrizoate gradient. After this second rosetting, the cells at the interphase were collected, washed and will be referred to as B cells. The rosette-forming cells obtained after the first centrifugation were re-centrifuged over a Ficoll-metrizoate gradient. The pellet of the rosette-forming cells was then treated with 0.84% ammonium chloride followed by washings in Hank's solution and will be referred to as T cells. In the T cell preparations, 99% of the cells formed rosettes and less than 1% were positive for membrane immunoglobulin as assessed by the membrane fluorescence assay (Landay et al 1983). The non-T cell preparation contained less than 1% rosette forming cells and

30-50% monocytes (nonspecific esterase positive). The viability of these cell preparations was 98-100% by Trypan blue exclusion test.

Lymphocyte culture. Lymphocytes at various concentrations (10^5- 2 x 10^6 cells/ml) were suspended in culture medium consisting of RPMI 1640 and 10% heat-inactivated fetal bovine serum supplemented with 2 mM of glutamine, 100 units of penicillin and 100 µg of streptomycin (all reagents from Gibco Laboratories). They were incubated in 15 x 75 mm round bottom tubes (Falcon Products) at 37°C with 5% CO_2 and 95% humidified air. Pokeweed mitogen (Gibco Laboratories), was used at a final concentration of 1/100. EBV obtained from supernatants of B 95-8 continuous cell culture was used at a final concentration of 50%. All cultures were performed in duplicate.

Production of helper or suppressor activities. Lymphocyte suspensions at a concentration of 3 x 10^6 cells/ml were incubated at 37°C with 5% CO_2 and PWM (1/100) in culture flasks (Falcon Products). In the case of mixed lymphocyte cultures (MLC), lymphocytes from two unrelated subjects were co-cultured. After 48 hours, the cell suspensions were spun and the supernatants were collected, filtered through 0.22 µM Millipore filters and stored at -20°C.

Thymidine uptake. Two hundred microlitres of cell suspension were placed in triplicate into wells of Microtext tissue culture plates (Falcon Products) after various durations of culture. One µCi of

^{3}H-thymidine (Specific activity: 2 Ci/m mol., Amersham Corporation) in 50 µl culture medium was added into each well for 6 to 12 hours. The cells were then collected on glass filter paper by a Mash II cell harvester (Microbiological Ass.) and the radioactivity was counted in scintillation fluid Econofluor (New England Nuclear).

Radioimmunoassay (RIA). A solid phase sandwich radioimmunoassay was used to measure the immunoglobulins secreted in the culture supernatant. Briefly, polyvinyl microtitre plates (Dynatech Laboratories) were soaked in 75% acetic acid for 1 hour. They were then washed and the wells were coated with the IgG fraction of sheep antiserum to human IgG, IgM or IgA (50 µg/ml). After overnight incubation, the anti-human immunoglobulin was removed and the remaining active sites were blocked with 10% fetal bovine serum in Hank's solution. Purified human IgG, IgM or IgA were placed in the wells at different concentrations to establish standard curves. The test supernatants from cell cultures were then placed in the wells in duplicate. After overnight incubation at room temperature, the wells were washed with phosphate buffer solution (PBS) and ^{125}I labelled goat anti-human IgG, IgM or IgA were placed in these wells. The wells were then washed with PBS after overnight incubation and counted in a gamma counter. All the immunoglobulins and the specific antibodies were prepared as previously described (Gausset et al 1976).

Epstein-Barr virus (EBV) transformed cell lines. These were obtained by culturing unfractionated mononuclear cells (3 x 10^{5}

cells/ml) isolated from cord or adult blood in the presence of pretitered suspension of EBV (B 95-8) for 48 hours. Cells were then washed and plated in microplate cultures (Falcon Products; 5×10^4 lymphocytes/0.2 ml) in the presence of 10 μg/ml of cyclosporin A. Cultures were examined daily under reverse microscope and growing colonies were grown in 2 ml cultures (Linbro culture plates, Flow Laboratories); cyclosporin A was maintained in the cultures for 2 months. After that time, no T cells could be detected in the cell line by using both the E-rosette assay and the membrane fluorescence test employing OKT3 monoclonal antibody (Landay et al 1983).

Statistical analysis. The statistical significance of differences was determined by a two-tailed t test or 2-way analysis of variance with replicates.

Results

Cord blood B cell immaturity and adequate helper T cell function.

Cord blood lymphocyte functional maturity was first assessed in co-culture studies (Table I). In the presence of allogeneic adult T cells but not cord T cells, adult B cells produced a significant amount of immunoglobulin. By contrast, cord B cells did not respond significantly in the presence of either adult or cord T cells. This lack of response was not due to differences in dose requirement or kinetics since different durations of stimulation with various

Table I. PWM-Induced Ig Production by Adult and Cord B Cells in the Presence of Allogeneic T cells

Cells in Culture	IgG	IgM	IgA
Adult B cells	51.1 ± 2.1	40.0 ± 1.3	20.1 ± 2.7
Adult B cells + Adult T cells	574.0 ± 6.4	423.0 ± 3.0	293.0 ± 3.5
Adult B cells + Cord T cells	48.2 ± 2.7	43.6 ± 2.1	21.3 ± 2.2
Cord B cells	12.2 ± 1.4	11.2 ± 1.3	5.4 ± 1.1
Cord B cells + Adult T cells	21.3 ± 2.7	51.4 ± 2.0	9.8 ± 1.8
Cord B cells + Cord T cells	20.7 ± 2.5	49.3 ± 3.4	10.1 ± 2.3

Eight-day culture of B cells (10^6 cells/ml) and T cells (10^6 cells/ml) in the presence of PWM.

Values are means ± S.D. in ng/ml.

Results from 1 representative experiment out of 5.

concentrations of PWM did not induce significant Ig production in our baseline dose response and kinetics experiments. Similarly, when supernatants containing helper activity from either PWM-stimulated cord or adult MNC cultures were added to B cells, only adult B cells showed a significant response (Table II) suggesting that cord B cells are functionally immature.

As shown in Table II and Table III, supernatants from both PWM-stimulated adult and cord MNC cultures displayed comparable helper activity for adult B cells in terms of IgG production (297 $\pm$ 80 ng vs 413 $\pm$ 68 ng, $p > 0.05$, 2-tailed t test) and B cell proliferation as measured by ^{3}H-thymidine uptake (4575 $\pm$ 1465 cpm vs 2847 $\pm$ 480 cpm, $p > 0.05$, 2-tailed t test).

It is clear from Table III that inadequate Ig production by cord B cells was not due to a lack of B cell proliferation since ^{3}H-thymidine uptake by cord B cells was always higher than adult B cells.

To ensure that the lack of Ig production by cord B cells was not due to suppressor T cells present in the B cell preparation, cord B cells were added to PWM-stimulated adult MNC. No suppression was found in 4 out of 5 such experiments. The result of one representative experiment is shown in Table IV.

Table II. Influence of Supernatants from PWM-Stimulated Adult and Cord Blood MNC Cultures on B Cell IgG Production

Cells in Culture	Supernatants (20%) Added to Culture		
	None	SUP_a [a]	SUP_c [b]
Adult B cells	20.0 ±3.0	297 ±80	413 ±68
Cord B cells	10.0 ±1.4	46 ±7.0	61 ±26

Eight-day cultures of B cells (10^6 cells/ml) in the presence of supernatants and PWM.

Values (ng/ml) represent the means ± S.D. of 5 experiments.

[a] Supernatant from 48 hr culture of PWM stimulated adult MNC.

[b] Supernatant from 48 hr culture of PWM stimulated cord MNC.

Table III. Influence of Supernatants from PWM-Stimulated Adult and Cord Blood MNC Cultures on B Cell ^{3}H-thymidine Uptake

Cells in Culture	Supernatant Added to Culture		
	None	SUP_a [b]	SUP_c [c]
Adult B cells	1,072 ± 158[a]	4,575 ± 1,465[a]	2,847 ± 480[a]
Cord B cells	2,628 ± 563[a]	21,194 ± 1,744[a]	19,437 ± 2,210[a]

^{3}H-thymidine uptake performed on day 5 of PWM stimulated B cell cultures.

Values in c.p.m. and represent means ± S.D. of 5 experiments.

[a] Significant differences ($P < 0.005$, 2 tailed t test) between cord B and adult B cells.

[b] Supernatant from 48 hr culture of PWM stimulated adult MNC.

[c] Supernatant from 48 hr culture of PWM stimulated cord MNC.

Table IV. Effect of Cord Blood Non-rosette-Forming Cells (CB) on IgG Production by PWM-Stimulated Adult MNC

	IgG
Adult MNC (10^6 cells/ml)	100.5 ± 20
Adult MNC + PWM	640 ± 25
CB (1.2×10^6 cells/ml) + PWM	12.8 ± 1.2
Adult MNC + PWM + CB (0.4×10^6 cells/ml)	688 ± 117
Adult MNC + PWM + CB (0.8×10^6 cells/ml)	710 ± 62
Adult MNC + PWM + CB (1.2×10^6 cells/ml)	820 ± 64

Eight-day culture of PWM stimulated adult MNC with various concentrations of non-rosette forming cells (CB).

Values (ng/ml) represent means ± S.D.

Result of 1 out of 5 experiments.

Cord blood T cells suppressed PWM-induced IgG production by adult MNC.

To establish the existence of excessive cord T cell suppressor activity, purified cord T cells were co-cultured with adult MNC in the presence of PWM. Parallel experiments of adult MNC co-cultured with or without (control) allogeneic purified adult T cells were done for comparison. The results showed that (Fig. 1) the addition of cord T cells to adult MNC suppressed IgG production to less than 10% of the control cultures containing MNC alone. In contrast, at a T cell concentration of 0.25×10^6 cells/ml, IgG production was enhanced when allogeneic adult T cells were added. Suppression was observed only when the allogeneic adult T cell concentration was increased. Even then, there was a significant difference between the suppressive effect of adult and cord T cells ($P < 0.01$, 2-way analysis of variance).

Cord T cells suppressed PWM-induced immunoglobulin production by adult MNC via soluble factor(s).

To determine if cord T cell suppressor effect was mediated via soluble factor(s) or through cell contact with allogeneic cells, the effects of supernatants from either PWM- or alloantigen (in MLC)-stimulated T cell cultures on immunoglobulin production by adult MNC were assessed. As shown in Fig. 2, supernatants from PWM-activated cord T cells but not adult T cells suppressed immunoglobulin production. Furthermore, supernatants from cord blood MLC were also capable of suppression while those of cord-adult and adult-adult MLC

Figure 1: Suppressor activity of cord T cells on IgG production. PWM-stimulated adult MNC (10^6/ml) were cultured in the presence of either cord or adult T cells. IgG production was measured on day 8. Results are expressed as a percentage of those from adult MNC alone (control). Values are means ± S.D. of 5 experiments.

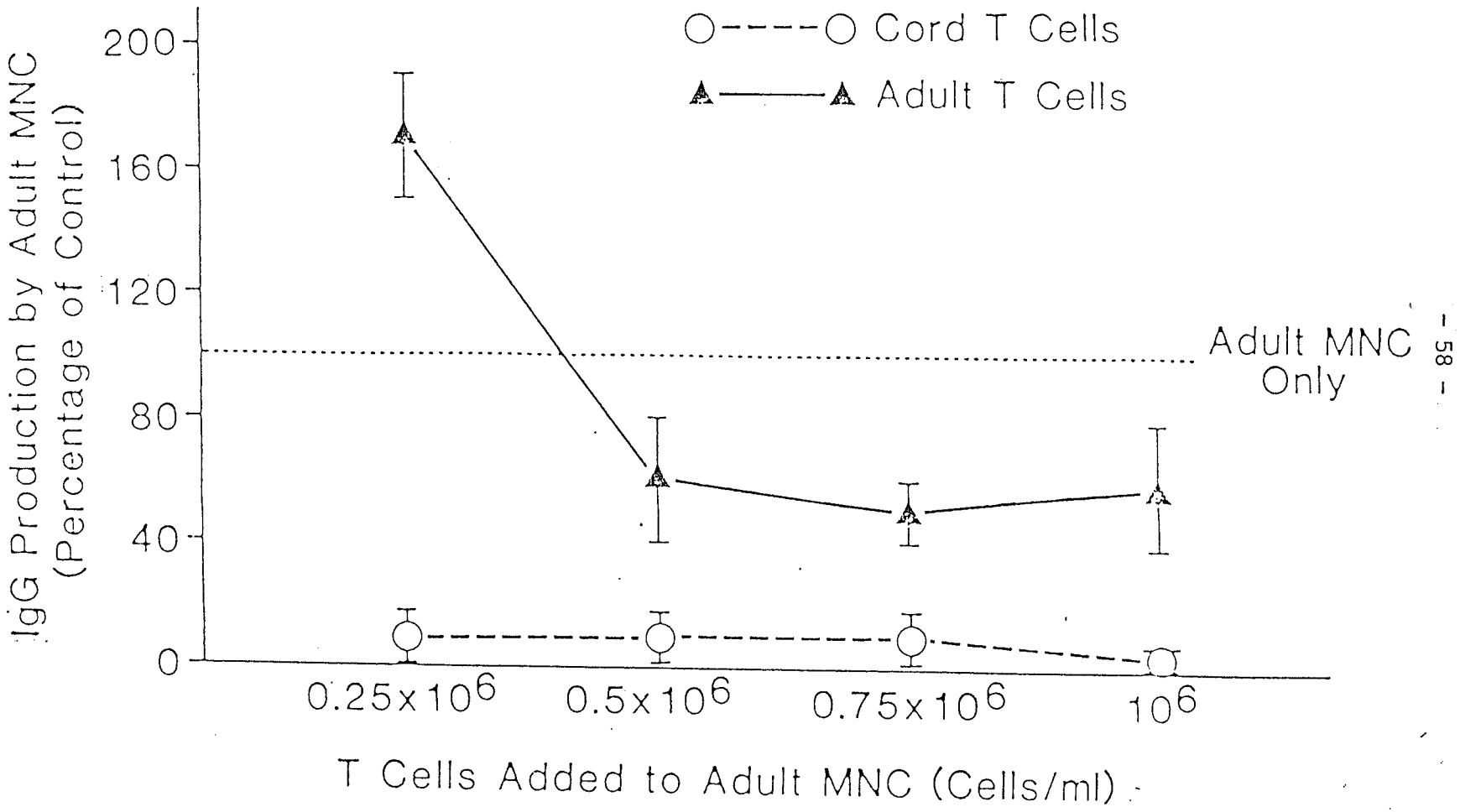
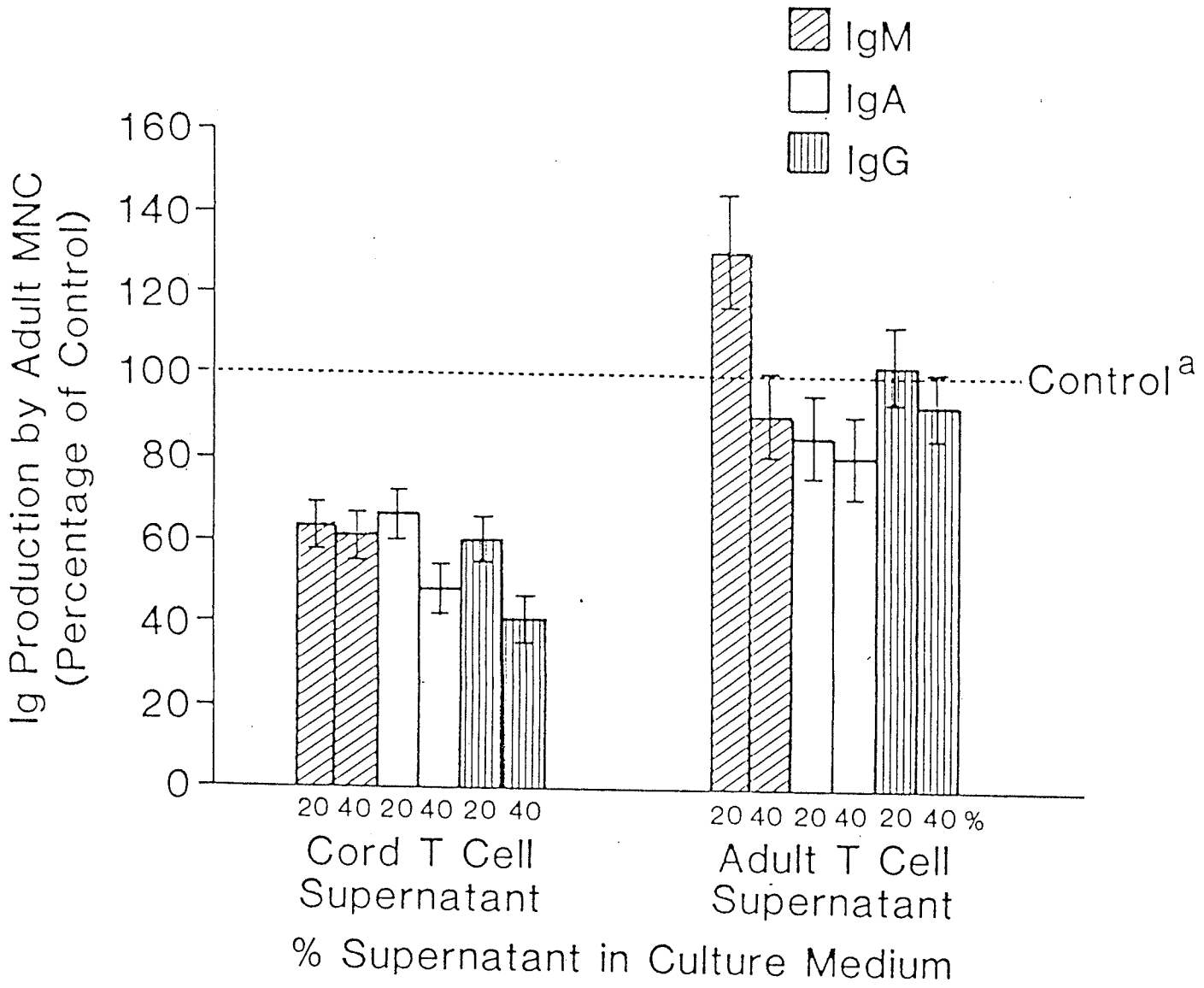

Cord T Cells
Adult T Cells
IgG Production by Adult MNC (Percentage of Control)
200
160
120
80
40
0
Adult MNC Only
0.25x10^6
0.5x10^6
0.75x10^6
10^6
T Cells Added to Adult MNC (Cells/ml)

Figure 2: Effect of supernatants from PWM-stimulated T cells on Ig production by adult MNC (10^6 cells/ml). Results are expressed as percentage of control. Values are mean ± S.D. of 5 experiments.

[a]Adult MNC only.


IgM
IgA
IgG
Ig Production by Adult MNC
(Percentage of Control)
160
140
120
100
80
60
40
20
0
Control[a]
20 40 20 40 20 40
20 40 20 40 20 40 %
Cord T Cell
Supernatant
Adult T Cell
Supernatant
% Supernatant in Culture Medium

has no consistent effect ($P < 0.05$, 2-way analysis of variance) as shown in Fig. 3. These results suggested that cord T cells exerted their suppressor activity through soluble factor(s) present in PWM- or alloantigen-stimulated culture supernatants.

Cord T cell suppressor factor(s) acted on other T cells to exert its suppressor effect.

To determine the target of suppression by cord T cells, i.e., whether suppression was directly on B cells or indirectly through other T cells, the suppressor activity of cord blood MLC supernatants was evaluated using adult B cells as the target instead of unfractionated adult MNC. In contrast to the suppressor effect observed when adult MNC were used as the target (Fig. 3), enhancement was noted when adult B cells were used (Table V). This dual property of suppression and enhancement was also observed in supernatants from PWM-stimulated cord T cell cultures (Table II, Fig. 2). Thus, it appeared that both suppressor and helper factor(s) were present in the supernatants with the former's activity being more prominent and requiring the presence of adult T cells to exert its effect.

Effect of cord T cells on immunoglobulin production by EBV-stimulated B cells.

Cord or adult T cells were added to allogeneic cord or adult B cells in the presence of EBV. Since IgG and IgA synthesis by cord B cells were extremely low, only IgM production by these EBV-stimulated B cells was measured on the 8th day of culture. No suppression on

Figure 3: Effect of MLC supernatants on Ig production by PWM-stimulated adult MNC (10^6 cells/ml). Results are expressed as percentage of control. Values are mean ± S.D. of 8 experiments.

[a]Cord blood MLC supernatant.

[b]Cord-adult blood MLC supernatant.

[c]Adult blood MLC supernatant.

[d]Adult MNC only.

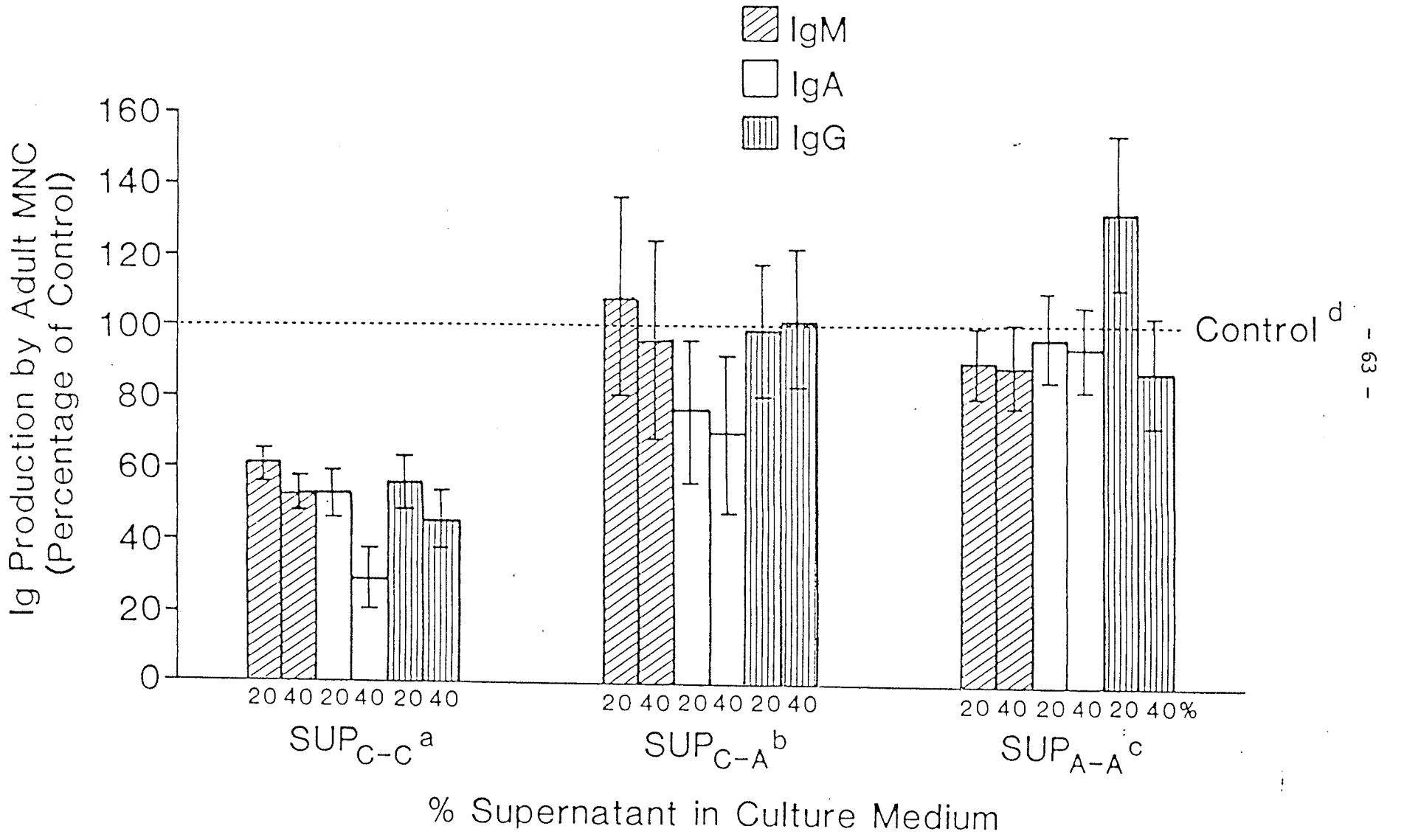

IgM
IgA
IgG
Ig Production by Adult MNC
(Percentage of Control)
160
140
120
100
80
60
40
20
0
Control d
20 40 20 40 20 40
SUP C-C a
20 40 20 40 20 40
SUP C-A b
20 40 20 40 20 40%
SUP A-A c
% Supernatant in Culture Medium

Table V. Effect of MLC Supernatants on the IgG Production by PWM-Stimulated Adult B Cells

Supernatant added	IgG (ng/ml)
None	80 ± 30
Supc-c[a]	380 ± 150
Supc-a[b]	395 ± 55
Supa-a[c]	445 ± 65

Eight-day cultures of B cells (10^6/ml) in the presence of PWM and various supernatants (40%). Values represent mean ± S.D. of 12 experiments.

[a] Supernatant from cord blood MLC.

[b] Supernatant from cord-adult blood MLC.

[c] Supernatant from adult blood MLC.

cord B cell IgM production by either allogeneic cord or adult T cells was observed. By contrast, IgM production by EBV-stimulated adult B cells was inhibited by cord but not allogeneic adult T cells (Fig. 4).

Effect of cord T cells on immunoglobulin production by EBV-transformed cells.

The suppressor effect of cord T cells was also assessed on EBV-transformed adult and cord B cells (EBV-BA and EBV-BC, respectively). These cell lines were co-cultured with either cord or adult T cells for 8 days. The effect of cord and adult T cells on IgM and IgG production by EBV-BA are shown in Figures 5a and 5b. No suppression was observed in either case. Similarly, there was no suppression of IgM production by EBV-BC (Fig. 6). IgG production by EBV-BC was too small (< 5 ng/ml) to be interpreted adequately.

Discussion

Cord blood IgM, IgA, IgD and endogenous IgG levels are low compared to adult with the endogenous IgM rather than IgG as the main immunoglobulin (Johansson 1968; Brasher & Hartley 1969; Cerderqvist et al 1977). These observations suggest that the development of the humoral immune response in the fetus has not reached full maturity at term. Possible explanations for the disparity between cord blood and adult Ig include: B cell functional immaturity, inadequate helper T cell function and excessive suppressor T cell activity. The aim of the

Figure 4: Effect of allogeneic T cells on EBV-stimulated IgM production by adult or cord B cells (10^5/ml). Results are expressed as percentage of control (B cells without T cells). Values are expressed as mean ± S.D. of 4 experiments.

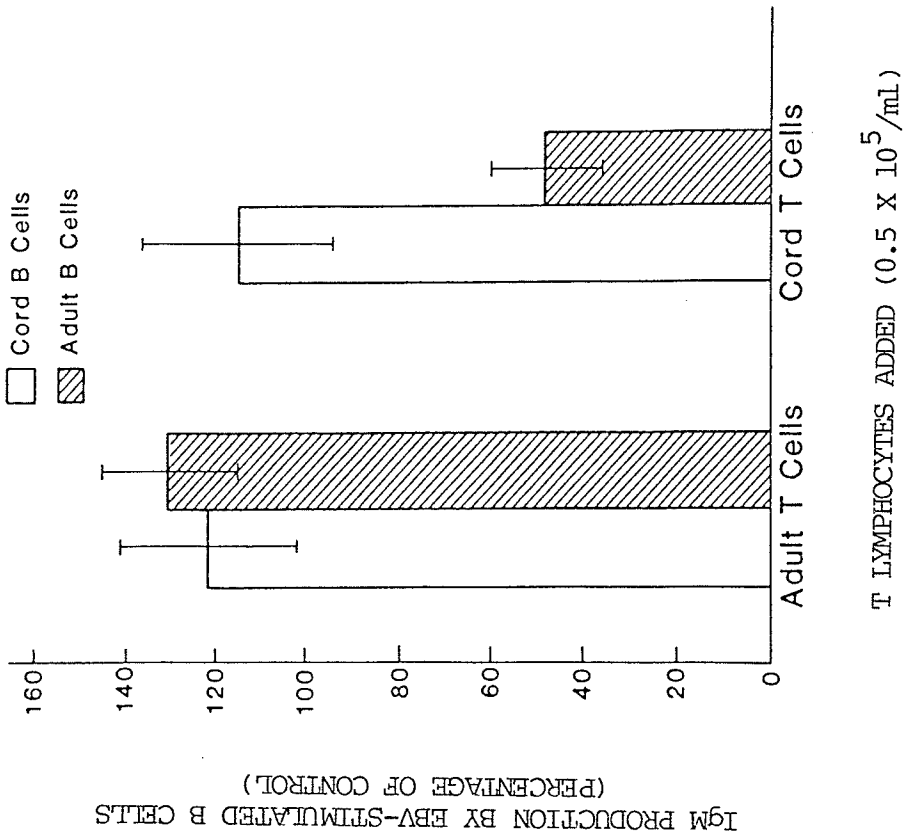

Cord B Cells
Adult B Cells
160
140
120
100
80
60
40
20
0
Adult T Cells
Cord T Cells
IgM PRODUCTION BY EBV-STIMULATED B CELLS
(PERCENTAGE OF CONTROL)
T LYMPHOCYTES ADDED (0.5 X 10^5/mL)

Figure 5a: Effect of allogeneic T cells on IgM production by EBV-transformed adult B cells (10^5 cells/ml). Results are expressed as percentage of control (EBV-transformed B cells only). Values are mean ± S.D. of 4 experiments.

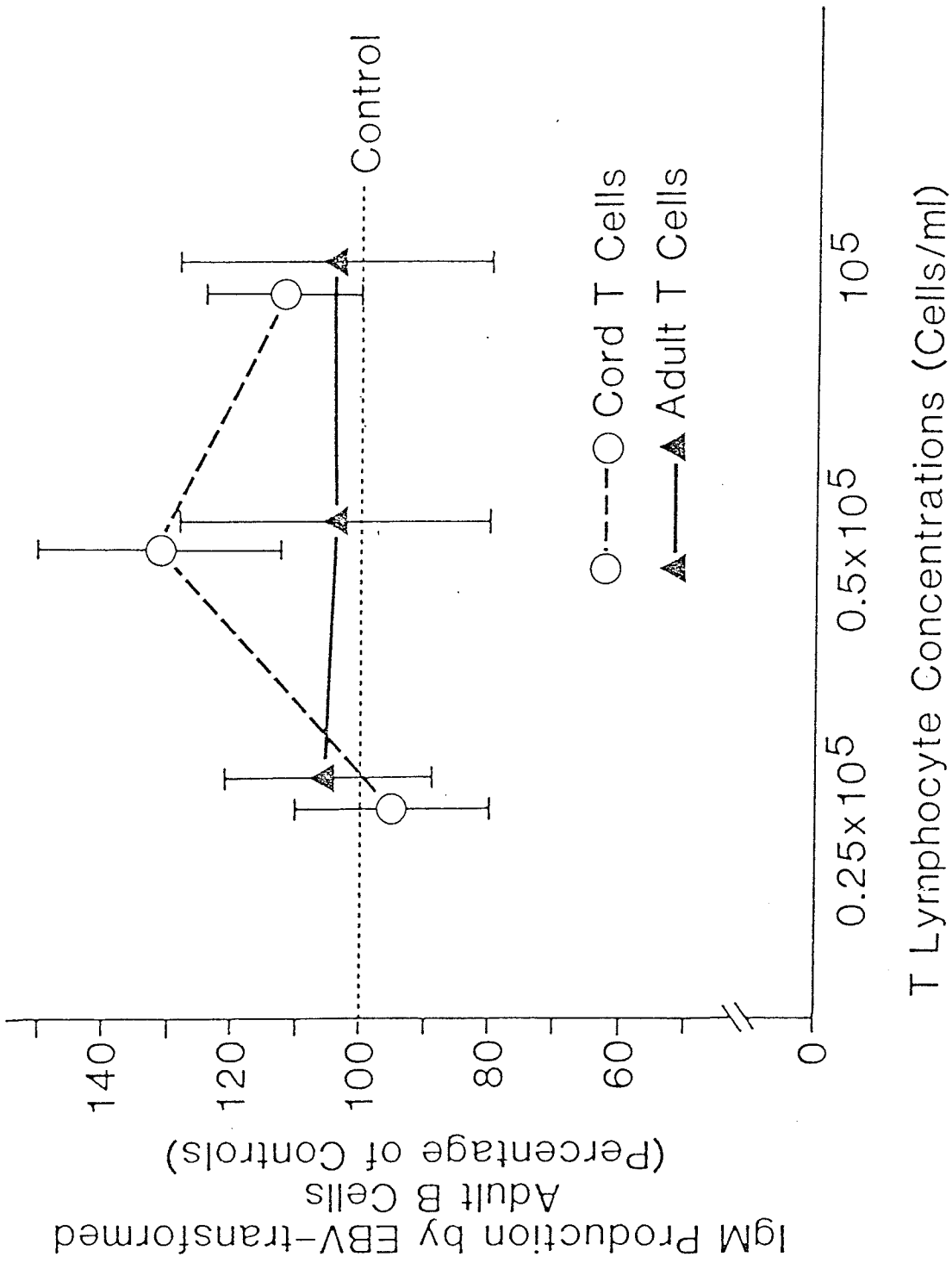

IgM Production by EBV-transformed
Adult B Cells
(Percentage of Controls)
140
120
100
80
60
0
Control
Cord T Cells
Adult T Cells
0.25x10^5
0.5x10^5
10^5
T Lymphocyte Concentrations (Cells/ml)

Figure 5b: Effect of allogeneic T cells on IgG production by EBV-transformed adult B cells (10^5 cells/ml). Results are expressed as percentage of control. Values are mean ± S.D. of 4 experiments.

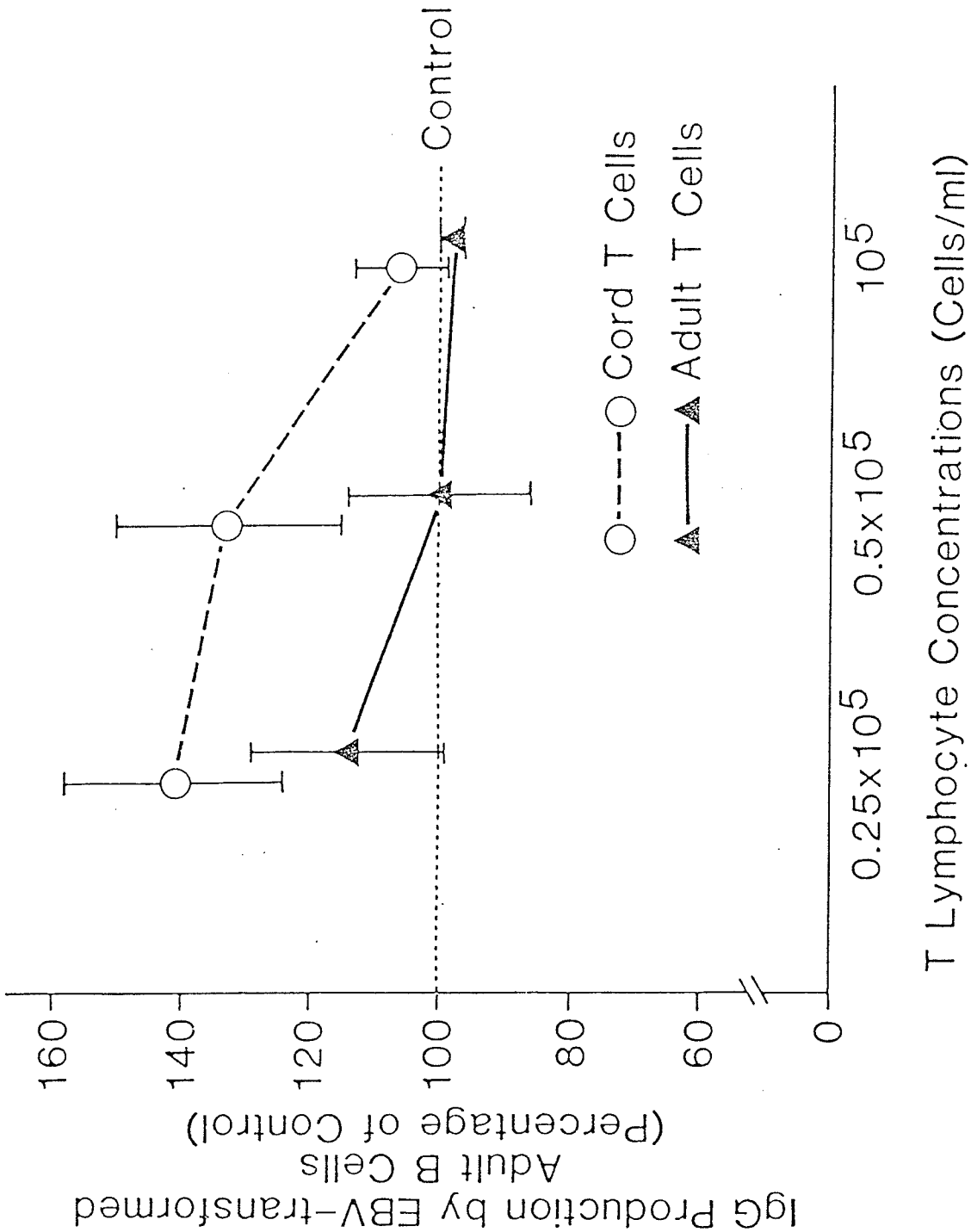
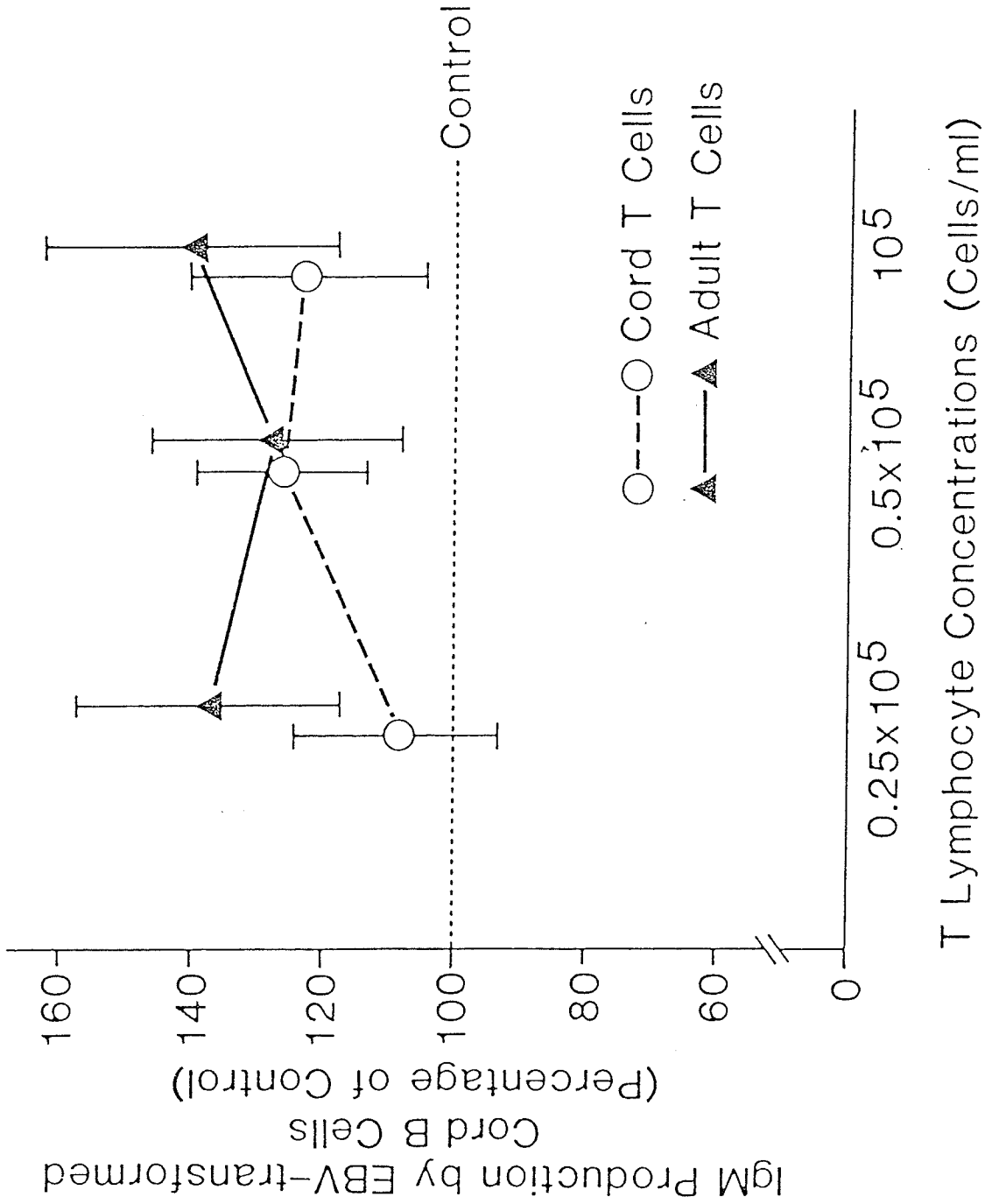

IgG Production by EBV-transformed
Adult B Cells
(Percentage of Control)
160
140
120
100
80
60
0
Control
Cord T Cells
Adult T Cells
0.25x10^5
0.5x10^5
10^5
T Lymphocyte Concentrations (Cells/ml)

Figure 6: Effect of allogeneic T cells on IgM production by EBV-transformed cord B cells (10^5 cells/ml). Results are expressed as percentage of control. Values are mean ± S.D. of 4 experiments.


IgM Production by EBV-transformed
Cord B Cells
(Percentage of Control)
160
140
120
100
80
60
0
Control
Cord T Cells
Adult T Cells
0.25x10^5
0.5x10^5
10^5
T Lymphocyte Concentrations (Cells/ml)

present study was to investigate the cord B cell response and the immunoregulatory role of cord T cells. Our data confirm previous observations that cord blood B cells are functionally immature (Hayward & Lawton 1977; Morito et al 1979; Tosato et al 1980; Andersson et al 1981; Gathings et al 1981; Miyawaki et al 1981a). Indeed, when cord B cells were co-cultured with adult T cells capable of providing helper activity to adult B cells, low levels of IgM and only minute amounts of IgG and IgA were produced when stimulated with PWM (Table I); similarly, cord B cells did not produce a significant amount of IgG in the presence of T helper factors (Table II). Upon stimulation with EBV, comparable amounts of IgM were found in cord and adult B cell cultures; IgG and IgA production, however, was barely detectable in cord B cell cultures. These results illustrate the immaturity of IgG- and IgA-producing precursor B cells in cord blood and the incongruous state of maturity of IgM-producing precursor B cells. The data are consistent with previous reports that B cell maturation follows a sequential order with the IgM response reaching adult levels first followed by IgG and IgA responses (Andersson et al 1981; Miyawaki et al 1981a).

In mice, it has been shown that the immaturity of neonatal B cells is characterized by a lack of the receptor for T helper cell replacing factor (TRF) (Schimpl et al 1982) which accounts for the poor response to T cell-dependent antigens. In the present study, cord B cell proliferation, measured by ^{3}H-thymidine uptake, was higher than that of adult B cells when stimulated with PWM in the

presence of supernatants containing helper factors thereby suggesting that B cell functional inadequacy is not due to a lack of B cell proliferation (Table III) but rather to a lack of response to TRF. The high B cell proliferation was either due to the presence of dividing pre-B cells (Phillips and Melchers 1976) or sheep erythrocyte rosette-negative immature T cells present in the cord B cell preparation (to be discussed later). Hence, highly purified B cell preparations obtained, for example, by monoclonal antibody labelling and fluorescence activated cell sorter separation, have to be employed to test this possibility.

When cord T cells were co-cultured with adult B cells, no Ig production was observed (Table I), this might be due to either a lack of cord T cell helper activity or excessive cord T cell suppressor activity. The first possibility is unlikely since both supernatants from PWM-stimulated cord lymphocyte cultures and cord blood MLC provided helper activity to adult B lymphocytes (Table II and V). This is in keeping with previous observations of a normal number of helper T cells in cord blood as determined by their reactivity with monoclonal antibodies (OKT4, Leu 3) specific for the T helper inducer subset of T cells (Hayward 1981; Siegal 1981; Yachie et al 1981). It should be mentioned that not all cord lymphocyte or adult lymphocyte culture supernatants contained helper factors. Approximately 70% of cord lymphocyte and 90% of adult lymphocyte supernatants contained helper factors suggesting that some neonatal lymphocytes are functionally less mature than others. Also, in accord with previous

studies (Durandy et al 1979; Tosato et al 1980), our data show that cord T cells suppressed PWM-induced Ig production by adult MNC (Fig. 1) in a isotype nonspecific manner (data not shown), thus favouring the possibility of excessive cord T cell suppressor activity. Such suppression, however, appears to be ineffective against cord lymphocytes since co-cultures of cord B cells with allogeneic cord T cells produced a similar quantity, albeit a small amount, of IgM compared to co-cultures with allogeneic adult T cells. Furthermore, cord T cells failed to suppress EBV-activated cord B cell IgM production (Fig. 4).

It has been described that cord MNC has a lower antigen dose-requirement for optimal response compared to adult MNC and if the optimal dose for the adult was used to stimulate cord MNC, preferential activation of suppressor cells may result (van Tol et al 1983). This, however, cannot account for the strong suppressor activity seen in the PWM system since suppressor activity was observed in PWM-stimulated cord T cell culture supernatants over a wide range of PWM concentrations in our initial dose-response experiments.

Separation of T from non-T cells was done in this study by rosetting T cells with aminoethylisothiourniumbromide-treated sheep erythrocytes. This method is useful for separating adult T cells from non-T cells as virtually all adult T cells have receptors for sheep erythrocytes. In cord blood, however, a sizable subpopulation of T cells do not express or have low affinity receptors for sheep

erythrocytes (Ferrari et al 1981). Indeed, it was shown that these non-rosette forming T cells consist mainly of $OKT8^+$, $OKT3^-$ and PNA^+ (peanut agglutinin) cells representing probably immature T cells (Maccario et al 1983). Therefore, the cord B cell fractions employed in the present study not only contained monocytes but also immature T cells. In order to rule out the possibility that these monocytes and immature T cells were causing the suppression of immunoglobulin production by both autologous cord and adult B cells, non-rosette-forming cord mononuclear cells were co-cultured with adult unfractionated MNC. The result indicates that there was no suppressor activity in these T cell-depleted preparations (Table IV). Thus, the inability of cord B cell to produce immunoglobulin in the presence of supernatants from PWM-stimulated adult MNC cultures, cannot be attributed to the non-rosette-forming T cells or monocytes present in the cord B cell preparation but to the functional immaturity of cord B cells.

In contrast to their ability to inhibit EBV-stimulated adult B cell immunoglobulin production (Fig. 4), cord T cells were unable to inhibit the immunoglobulin production of either EBV-transformed adult or cord B cell lines (Fig. 5,6). This may be related to the EBV-transformed cells' increased resistance to suppression (Lotz et al 1985). Interestingly, Andersson et al (1981) demonstrated that cord T cells were capable of suppressing EBV-transformed cells. The cord T cell: transformed cell ratios they used were higher than those used in the present study.

Culture supernatants of cord lymphocytes that were stimulated with either PWM or allogeneic cord lymphocytes (MLC) suppressed immunoglobulin synthesis by unfractionated adult MNC (Fig. 2,3). Further analysis showed that these same supernatants actually enhanced immunoglobulin production in the cultures of purified adult B cells (Table II, V). The latter preparations differed from the former by their lack of T cells. The most obvious explanation for this seemingly paradoxical finding is that cord T cells or supernatants of activated cord T cell cultures exerted their suppressor effect via induction of adult suppressor T cells. In other words, cord blood suppressor cells are in fact T inducer of suppressor cells (Tis), the existence of which has been established in both mouse (Tada 1984) and human (Thomas et al 1979; Morimoto et al 1981; Uytdehaag et al 1981). Without the autologous suppressor T cells, as in the case of purified adult B cell preparations, cord Tis were incapable of suppressing adult B cell immunoglobulin production. Hence, only the helper effect was observed when supernatants of activated cord T cells were added to purified adult B cell cultures (Table II, V).

Interestingly, suppressor activity was observed in supernatants from cord blood MLC but not consistently in cord-adult MLC supernatants (Fig. 3). A plausible explanation would be that the suppressor activity generated by cord lymphocytes in cord-adult MLC supernatants was not strong enough to negate the helper activity generated by both the adult and cord T helper cells. Furthermore, if adult lymphocytes were the sole target of TisF, TisF produced in

cord-adult TLC would have been taken up by the adult MNC leading to a decreased availability of TisF in the cord-adult MLC supernatant. The net result is an unpredictable pattern of suppression in some cases and enhancement in others.

Closer examination of the results in Figures 2, 3 and Tables II and V showed that supernatants from PWM-stimulated cord blood lymphocytes or MLC suppressed immunoglobulin production by adult MNC despite the presence of helper factors in these supernatants. Similarly, culturing adult B (non-T) cells with cord T cells in the presence of PWM did not result in significant immunoglobulin production despite adequate help provided by cord T cells (Table I, II). These results strongly suggest that the targets of suppressor T cells are B cells and/or monocytes. This notion is strengthened by the observation that cord T cells suppressed the IgM production of adult B cells when the latter were stimulated by EBV, a T-independent activator (Fig. 4). Nevertheless, selective inhibition of the adult but not cord helper T cell function by cord suppressor T cells, cannot be excluded.

Although the presence of excessive suppressor T cell function in the fetus and newborn is generally accepted, the nature of the suppressor cells remains elusive. Oldstone et al (1977) observed that the suppressor cells carried receptors for IgG but Moriya et al (1977) showed that the suppressor activity of cord blood T cells was mediated by a subpopulation lacking receptors for IgG. Durandy et al (1979)

found suppressor activity in both IgG receptor positive and negative cells while Hayward and Lydyard (1978) reported that suppression was confined to T cells with IgM receptors. Since these cell surface receptors could be modulated by circulating immune complexes and their expression on cell surface may merely be reflection of a particular stage of cellular maturation or activation (Moretta et al 1981a, 1981b), the conflicting findings mentioned above are not unexpected. Monoclonal antibodies against other cell surface markers have been employed to characterize the cord blood T cells. Siegal (1981) found that Leu-2^+ (suppressor/cytotoxic) and Leu-3^+ (helper/inducer) T cell distribution are quite similar to adult with the latter representing a relatively high proportion. Hayward (1981) and Yachie et al (1981) found an adequate proportion of OKT4^+ (inducer/helper) cells but low numbers of OKT8^+ (suppressor/cytotoxic) cells in newborns. These data are certainly in keeping with our view that cord helper T cell function is adequate and that the suppressor activity of cord blood lymphocytes is due to the excessive number or function of Tis (OKT4^+) cells rather than increased suppressor T cells or activity.

It was demonstrated that adult T cells acquired Ia surface antigen upon stimulation with mitogens, soluble antigens or allogeneic cells (Evans et al 1978; Ko et al 1979; Reinherz et al 1979d) and that in the case of PWM stimulation, Tis were found to belong to the OKT4^+ and Ia^+ T cell subset (Yachie et al 1982). In contrast, only a small number of cord T cells express Ia antigen before or after

PWM stimulation (Ceuppens et al 1981; Miyawaki et al 1982) despite their excessive suppressor activity (Durandy et al 1979; Tosato et al 1980; Miyawaki et al 1981a). These data suggest that cord Tis are either incapable of expressing Ia antigen or belong to a different subpopulation of T cells from adult Tis. The use of other cell surface markers such as TQ1 and JRA (identified by its reaction with antiserum from patients with juvenile rheumatoid arthritis) which were reported to be associated with Tis (Morimoto et al 1981; Reinherz et al 1982) may be helpful in characterizing cord Tis.

CHAPTER 3

HUMAN CORD BLOOD SUPPRESSOR T LYMPHOCYTES. I. TARGET OF INDUCER OF SUPPRESSOR CELLS

Abstract

The present study examined the characteristics of the cord suppressor T cells and their targets and compared them with those of the inducer of suppressor T cell factor (TisF). TisF from culture supernatants of alloantigen-activated cord lymphocytes was used as a source of suppression. Adult lymphocyte responses to soluble antigen Candidin, alloantigens and Pokeweed mitogen (PWM) were all suppressed. These results suggest that TisF acts primarily on T cells in an antigen nonspecific fashion. Furthermore, pre-culture T cell irradiation, but not $T8^+$-cell depletion of adult lymphocytes abrogated the suppressive effect of TisF on the PWM-stimulated IgG production suggesting, that the target of TisF belongs to the radiosensitive $T8^-(T4^+)$ T cell subset. When different cord T cell subsets were tested for suppressor activity, only radiosensitive $T4^+$ cells were capable of suppressing IgG production by adult lymphocytes. Neither pre-culture $T8^+$ cell depletion nor irradiation of adult T cells affected the suppression. Taken together, these results suggest that the suppressor activity of cord T cells originated from the inducer of suppressor cells which activate radiosensitive $T4^+$ suppressor effector cells through TisF. In turn, these effector cells act on either radioresistant $T4^+$ helper T cells or B cells/monocytes.

Introduction

In mammalian pregnancy, the fetal graft successfully coexists with its host (mother) despite their histoincompatibility. Recent development in this field indicates that a number of complex immunological mechanisms, among which fetal suppressor cells may be one, are responsible for the success of this intriguing phenomenon. In 1974, Olding et al (Olding and Oldstone 1974; Olding et al 1974) reported the presence of human neonatal suppressor T lymphocytes capable of suppressing maternal lymphocyte proliferative responses to alloantigens and phytohemagglutinin (PHA). They suggested that these suppressor cells may play a role in the protection of the fetus against maternal rejection during pregnancy. Subsequently, other investigators confirmed and extended these findings by showing that neonatal suppressor cells also inhibit immunoglobulin production by adult lymphocytes (Hayward and Lawton 1977; Morito et al 1979; Miyawaki et al 1981b; Unander et al 1982; Cheng et al 1984). However, the exact nature and target of these neonatal suppressor cells, their mode of action and functional significance are far from clear. While most investigators agree that the neonatal suppressor cells are radiosensitive (Hayward and Lydyard 1979; Durandy et al 1979; Tosato et al 1980; Andersson et al 1981), their exact phenotype remains controversial. For example, using monoclonal antibodies against human T cell surface antigens, Hayward (1981) and Rodriquez et al (1981) showed that the suppressor cells express the T8 phenotype. Anderson

et al (1983) on the other hand, reported that both $T8^+$ and $T4^+$ cells were capable of suppression while other investigators found that the major neonatal suppressor cell population resided in the $T4^+$ fraction (Yachie et al 1981; Jacoby and Oldstone 1983). Earlier studies which attempted to characterize these neonatal suppressor cells based on their Fc receptors failed to show consistent results (Oldstone et al 1977; Hayward and Lydyard 1978; Durandy et al 1979; Miyawaki et al 1981a). The precise mode of action and target of suppression by the neonatal suppressor cells are also unsettled. Although the target of suppression was considered to be adult T cells (Durandy et al 1979), direct inhibition of non-T cells (Andersson et al 1981) and suppression through collaboration with monocytes (Miyawaki et al 1981a) have also been cited. It appears that suppression is mediated, at least partially, through soluble factors (Olding et al 1977; Nagaoki et al 1980; Miyawaki et al 1981b), the nature of which remains to be completely elucidated. Recent studies strongly suggested that prostaglandin E2 may be one of them (Johnsen et al 1982, 1983b).

A previous study (see Chapter 2) from this laboratory suggested that neonatal T lymphocytes produced an inducer of suppressor factor(s) (TisF) which interacted with other T lymphocytes (suppressor effector cells) to inhibit the humoral response of adult lymphocytes to PWM, a T cell dependent B cell activator, in an isotype nonspecific fashion (Cheng et al 1984). This TisF was generated when neonatal

(cord blood) lymphocytes were activated by alloantigens in a 2-way mixed lymphocyte culture (MLC). Control culture supernatants from adult-adult 2-way MLC did not contain such a factor (Cheng et al 1984). The aim of the present study is to investigate the nature and target of neonatal suppressor cell activity by employing both TisF and neonatal T lymphocytes as sources of suppression. The data indicate that TisF acts primarily on a radiosensitive $T4^+$ lymphocyte subpopulation. This is in contrast to the neonatal suppressor T cells which are radiosensitive, $T4^+$ and act on radioresistant $T4^+$ cells or B cells/monocytes.

Materials and Methods

Preparation of peripheral blood lymphocytes (PBL) and isolation of B and T cells

Cord blood was obtained at the time of delivery from normal uncomplicated pregnancies. Immediately after delivery but prior to expulsion of the placenta, cord blood was drained into tubes containing preservative-free heparin. It was then mixed with 5% dextran in normal saline at a ratio of 4:1 for 45 min at 37°C to sediment the erythrocytes. The leukocyte rich supernatant was diluted 1:2 in Hank's balanced salt solution (HBSS) (Flow Laboratories) and centrifuged over a Ficoll-metrizoate gradient (Pharmacia Fine Chemicals; Nyegaard and Co.) with a specific gravity of 1.073 at 900g

for 30 min. Adult blood donated by healthy volunteers was diluted 1:2 in Hank's solution and centrifuged over a Ficoll-metrizoate with a specific gravity of 1.077. PBL in the interphase were collected and washed 3 times in Hank's solution. Further separation of MNC into T and B cells was achieved by rosetting with sheep erythrocytes. Briefly, PBL were suspended in RPMI 1640 and 10% heated-inactivated and absorbed fetal bovine serum (Flow and Gibco Laboratories, respectively) at 3 x 10^6 cells/ml. To this cell suspension was added 0.1 ml of aminoethyl- isothiouroniumbromide (Sigma Chemical Co.)-treated sheep erythrocytes (National Biological). The cell suspension was then centrifuged over a Ficoll-metrizoate gradient at 4°C for 30 min. Cells collected at the interphase were rosetted again and centrifuged over a Ficoll-metrizoate gradient. After this second rosetting, the cells at the interphase were collected, washed and will be referred to as B cells. The rosette-forming cells obtained after the first centrifugation were re-centrifuged over a Ficoll-metizoate gradient. The pellet of the rosette-forming cells was then treated with 0.84% ammonium chloride followed by washings in Hank's solution and will be referred to as T cells. In the T cell preparations, 99% of the cells formed rosettes and less than 1% were positive for membrane immunoglobulin as assessed by membrane fluorescence assay (Landay et al 1983). The non-T cell preparation contained less than 1% rosette forming cells and 30-50% monocytes (nonspecific esterase positive). The viability of these cell preparations was 98-100% by the Trypan blue exclusion test.

Selection of T cell subsets with T8 and T4 monoclonal antibodies

FITC conjugated monoclonal antibodies FITC-T8 and FITC-T4 specific for human T cell subsets were purchased from Coulter Immunology. An aliquot of 10^7 unfractionated lymphocytes or T cells was incubated with 50 µl of FITC-T8 or FITC-T4 in a final volume of 2 ml of tissue culture medium (TCM) for 30 min at 4°C. TCM consisted of RPMI 1640, 10% heat inactivated fetal bovine serum supplemented with 2 mM of L-glutamine, 100 units of penicillin and 100 µg of streptomycin (all from Gibco Laboratories). The cells were then washed in TCM and sorted into fluorescence positive and negative cells by Fluorescence Activated Cell Sorter (FACS, EPICS V, Coulter Electronics) under sterile conditions. Since $T4^+$ and $T8^+$ cells belong to 2 mutually exclusive subsets, the fluorescence positive cells were considered as positively selected cells for the specific subset identified by the corresponding monoclonal antibody. Conversely, the fluorescence negative cells were considered as negatively selected cells. Analysis of the sorted cells indicated that the positively selected cells consisted of about >95% fluorescence positive cells while the negatively selected cells consisted of 1-5% fluorescence-positive cells. Sorted cells were washed in TCM prior to culture.

In some experiments cells were negatively selected by an indirect rosette technique (Mills et al 1983). Briefly, 0.3 mg of affinity purified sheep anti-mouse antibody (prepared by Dr. Delespesse's laboratory) was coupled to 300 µl of packed ox

erythrocytes with $CrCl.6H_2O$ (300 µl at 1 mg/ml). After incubation for 10 min at 37°C, the ox erythrocytes were washed and resuspended as a 2% cell suspension in RPMI 1640. Adult lymphocytes (10^7 cells/2 ml) were incubated with 50 µl of OKT8 monoclonal antibody (Ortho Diagnostics) for 30 min at 4°C. After washing, the cells were suspended in TCM to a concentration of 2×10^6 cells/ml. An equal volume of the 2% ox erythrocyte suspension was added to the cells, centrifuged for 5 min at 200g, 4°C and then put on ice for 90 min. Thereafter, the cells were centrifuged over Ficoll-metrizoate at 400g, 4°C for 30 min. Non-rosette forming cells (T8 depleted cells) were collected from interphase, washed and suspended in TCM. Analysis of the non-rosette forming cells by indirect immunofluorescence employing OKT8 and FITC goat anti-mouse antibody (Coulter Immunology) revealed less than 4% $T8^+$ cells. Viability of cells from all the above preparations was 97-100% by the Trypan blue exclusion test.

Production of TisF

Although both cord-cord and cord-adult 2-way MLC were capable of producing TisF, the TisF activity in cord-adult MLC supernatants could not be consistently observed (Cheng et al 1984; Chapter 2). Hence, only supernatants from cord-cord MLC were used as a source of TisF. Briefly, 1.5×10^6 cells/ml of PBL from each subject were cultured in a flask (Falcon Products) at 37°C with 5% CO_2 and 95% humidified air for 48 hr. The cell suspensions were spun, supernatants collected, filtered through 0.22 µm Millipore filter and stored at

-20°C. Such supernatants would be referred to as Supc-c. Preliminary studies indicated that 48 hr was the optimal incubation period for the production of TisF. Longer incubation led to the production of suppressor factor (probably suppressor effector factor) or accumulation of metabolic waste, the effect of which could not be abrogated by irradiation of target T cells as was the case of TisF (Fig. 1). Kinetic experiments showed that TisF had to be added to the assay culture within 24 hrs to be effective (Fig. 2). Titration experiments showed that Supc-c's suppressive effect on lymphocyte function was dose dependent (Fig.3,4) and accordingly, a final concentration of 20% which provided near maximum suppressor activity was used in the present study.

Lymphocyte Culture

Lymphocytes (10^6) were suspended in 1 ml of TCM, placed in 15 x 75 mm round bottom tubes (Falcon Products), incubated at 37°C with 5% CO_2 and 95% humidified air for 6-7 days. PWM (Gibco Laboratories) and Candidin (Halab) were used at final concentrations of 1/100 and 20 µg/ml, respectively. In some experiments, T cells were irradiated with 2500 rad (Cobalt-60) before being added to cultures. For MLC, 10^6 cells/ml from each individual were placed in flat bottom wells of 1.7 x 1.6 cm tissue culture plates (Linbro, Flow Laboratories) for 5 days. All cultures were performed in duplicate or triplicate.

Figure 1: Effect of MLC culture duration on the production of TisF. Supc-c from 24 hr, 48 hr or 72 hr MLC cultures were added to PWM-stimulated adult B and T cell cultures (0.5 x 10^6 cells/ml from each) at a final concentration of 40%. Hatched bars and open bars represent the IgG production in cultures in which the adult T cells did or did not receive 2500 rads, respectively. Results are expressed as a percentage of those cultures that had no Supc-c added (control). Values are means ± S.D. of 4 experiments.

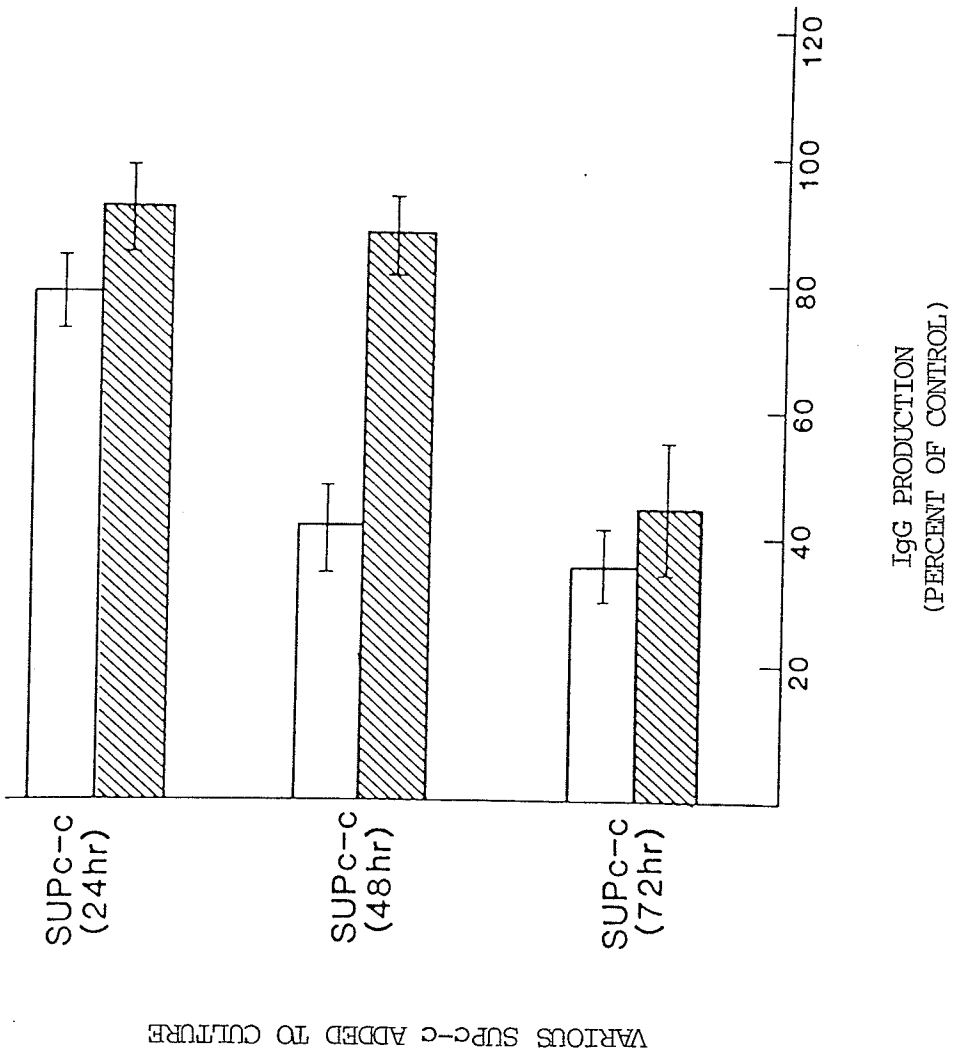

120
100
80
60
40
20
IgG PRODUCTION
(PERCENT OF CONTROL)
SUPc-c
(24hr)
SUPc-c
(48hr)
SUPc-c
(72hr)
VARIOUS SUPc-c ADDED TO CULTURE

Figure 2: Suppressor effect of Supc-c on the PWM-stimulated IgG production by adult PBL as a function of time. Supc-c (20%) was added to adult PBL (10^6 cells/ml) culture at various time intervals. Results are expressed as percentage of those without Supc-c added (control). Values are mean ± S.D. of 3 experiments.

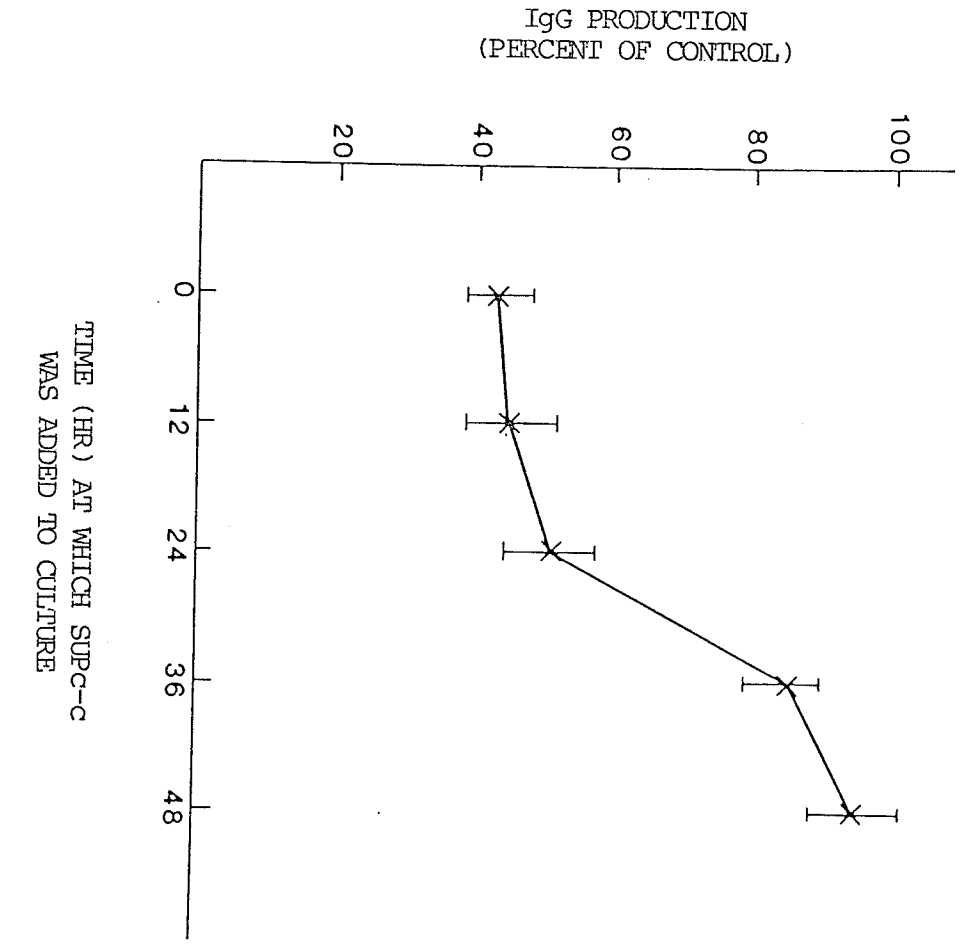

IgG PRODUCTION
(PERCENT OF CONTROL)
20
40
60
80
100
0
12
24
36
48
TIME (HR) AT WHICH SUPc-c
WAS ADDED TO CULTURE

Figure 3: Effect of various concentrations of Supc-c on the IgG production by PWM-stimulated adult peripheral blood lymphocytes (10^6 cells/ml). Results represent mean ± S.D. of 3 experiments.

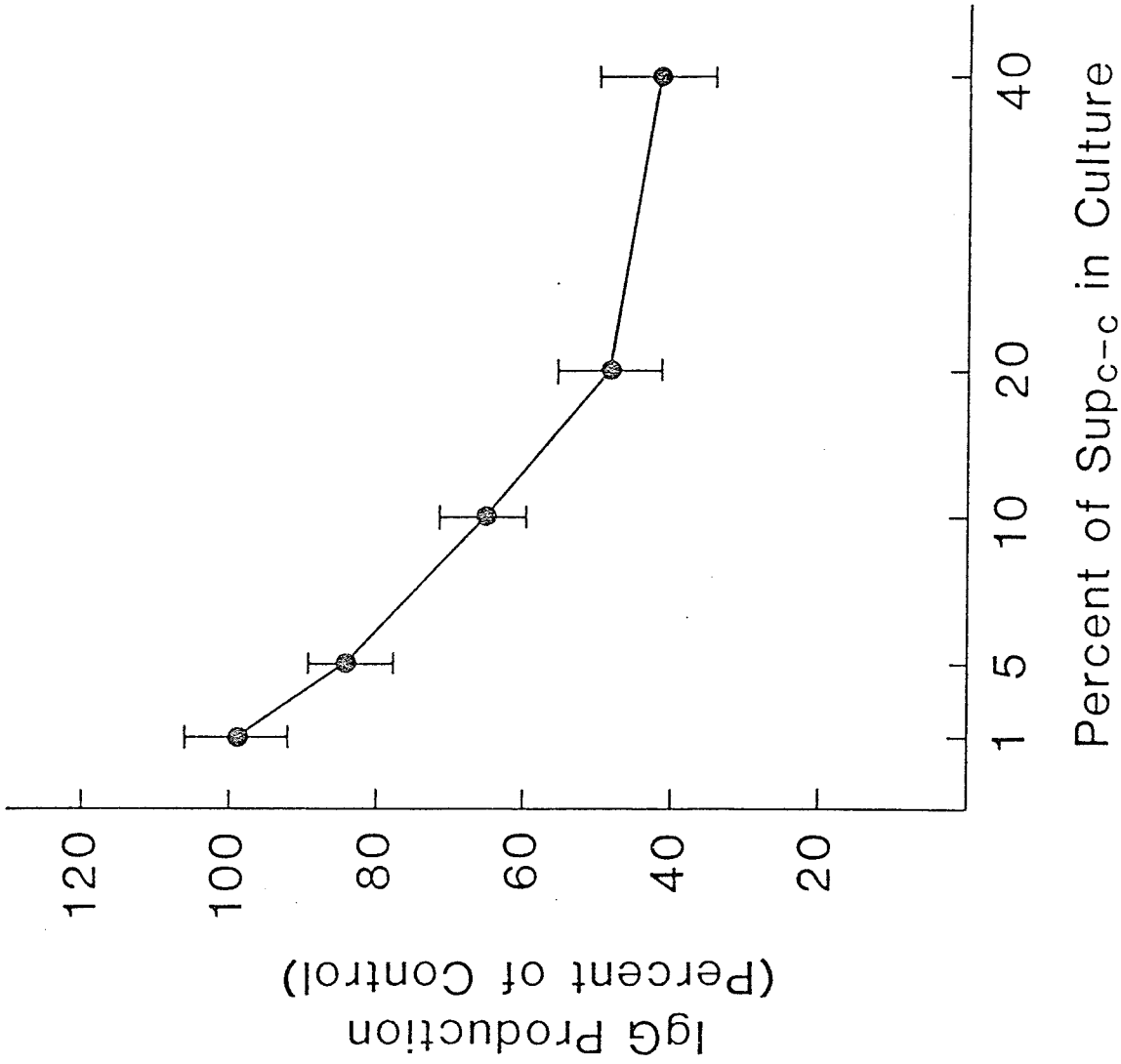
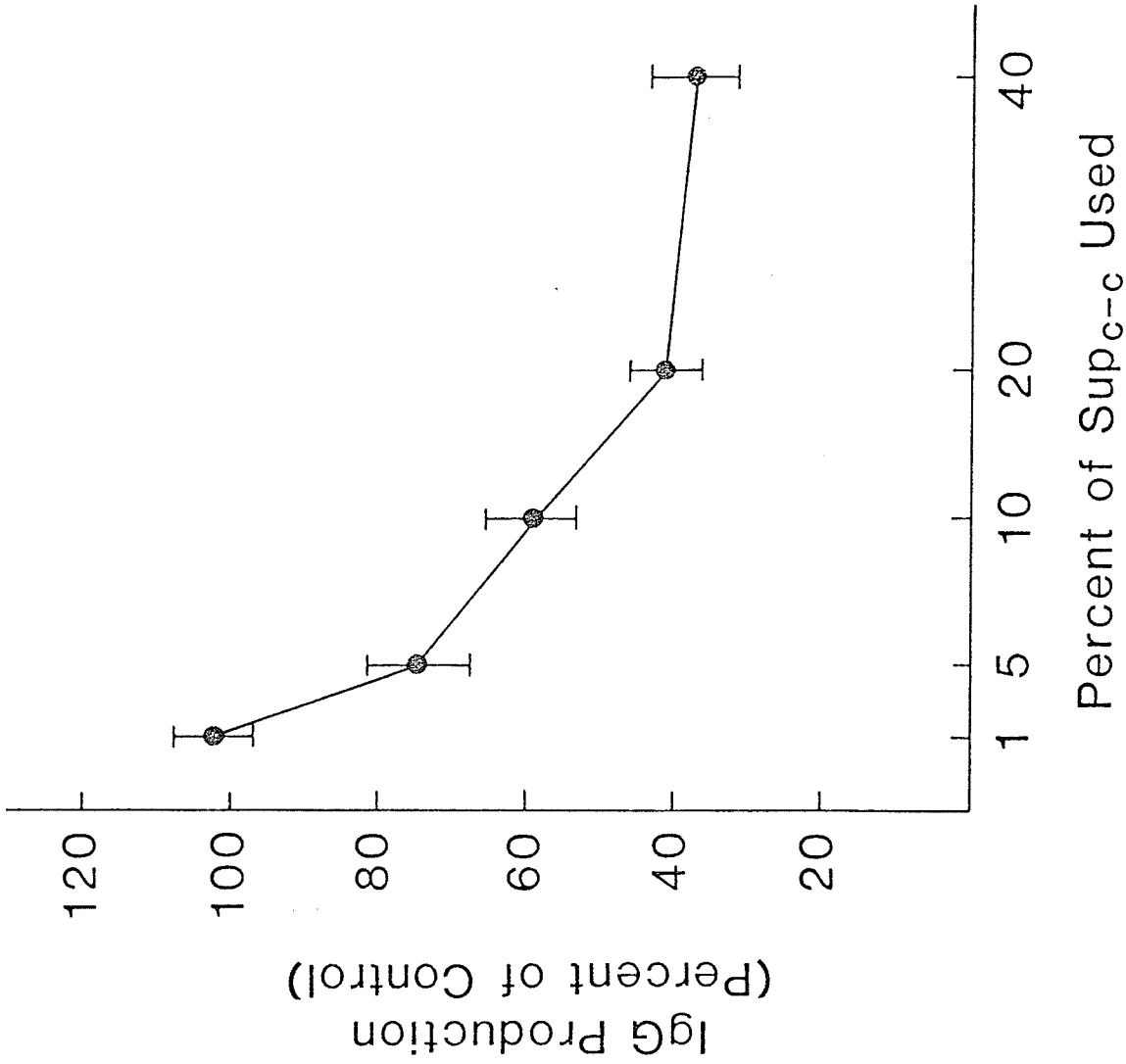

IgG Production
(Percent of Control)
120
100
80
60
40
20
1
5
10
20
40
Percent of Supc-c in Culture

Figure 4: Effect of Supc-c pretreated adult T cells (0.3×10^6 cells/ml) on the IgG production by PWM-stimulated adult peripheral blood lymphocytes (PBL; 10^6 cells/ml). T cells, obtained from PBL by adherent cell depletion and rosette formation with sheep erythrocytes were treated with various concentrations of Supc-c for 24 hr prior to culturing with fresh PBL. Results represent means ± S.D. of 3 experiments.


IgG Production
(Percent of Control)
120
100
80
60
40
20
1
5
10
20
40
Percent of Supc-c Used

^{3}H-Thymidine Uptake

Two hundred microlitres of cell suspension were placed in triplicate into wells of Microtitre tissue culture plates (Falcon Products) after various duration of culture. One μCi of ^{3}H-thymidine (Specific activity: 2 Ci/m mol., Amersham Corporation) in 50 μl culture medium was added into each well for 6 to 12 hrs. The cells were then collected on glass filter paper by a Mash II cell harvester (Microbiological Ass.) and the radioactivity was counted in Econofluor scintillation fluid (New England Nuclear).

Radioimmunoassay (RIA)

A solid phase sandwich radioimmunoassay was used to measure the immunoglobulin secreted in the culture supernatant. Briefly, polyvinyl microtitre plates (Dynatech) Laboratories) were soaked in 75% acetic acid for 1 hr. They were then washed and the wells were coated with the IgG fraction of sheep antiserum to human IgG (50 μg/ml). After overnight incubation, the anti-human immunoglobulin was removed and the remaining active sites were blocked with 10% fetal bovine serum in Hank's solution. Purified human IgG was placed in the wells at different concentrations to establish standard curves. The test supernatants from cell cultures were then placed in the wells in duplicate. After overnight incubation at room temperature, the wells were washed with phosphate buffer solution (PBS) and ^{125}I labelled

sheep anti-human IgG was placed in these wells. The wells were then washed with PBS after overnight incubation and counted in a gamma counter.

Statistical Analysis

One-way or two-way analysis of variance were used for the calculation of statistical significance of differences. P values of less than 0.05 were taken as significant.

Results

Characteristics of cord blood suppressor T Cells

The nature of cord blood suppressor T cells were first delineated by a radiosensitivity study (Table I). Adult B cells were stimulated with PWM in the presence of either allogeneic adult or cord T cells. As expected, adult B cells did not produce a significant amount of IgG when co-cultured with cord T cells (see Chapter 2). However, when cord T cells were pre-irradiated with 2500 rads, they provided substantial helper activity to adult B cells. These data were interpreted as indicating that cord suppressor T cells were radiosensitive.

Table I. Radiosensitivity of Fetal Suppressor T Cells

Cells in Culture	IgG (ng/ml)[b]
Adult B cells + adult T cells	155 ± 50[b]
Adult B cells + adult T cells + PWM	1675 ± 355[c]
Adult B cells + adult T cells (R)[a] + PWM	2250 ± 410[c]
Adult B cells + cord T cells	95 ± 35
Adult B cells + cord T cells + PWM	105 ± 35
Adult B cells + cord T cells (R) + PWM	1600 ± 405[c]

0.5×10^6 B cells and 0.5×10^6 allogeneic T cells were co-cultured for 7 days. IgG production was measured by RIA.

[a] T cells irradiated with 2500 rads before culture.

[b] Mean ± SD of 4 experiments.

[c] Not significantly different from each other.

The phenotype of cord suppressor T cells was examined by employing monoclonal antibodies T4 and T8. Cord unfractionated T cells, $T4^+$ cells and $T8^+$ cells were added to adult PBL and their effect on IgG production was examined. The cord T cell: adult PBL ratio used in the study was 0.3:1 because it was shown that significant suppression occurred even at this ratio (Cheng et al 1984). As shown in Table II and III, IgG production by PWM stimulated adult lymphocytes was suppressed by cord T cells but not by adult T cells. Major suppressor activity of the cord T cells resided in the $T8^-$ (negative selection), $T4^+$ (positive selection) subpopulation. These results indicated that cord blood suppressor T cells were $T4^+$.

Target of cord blood suppressor T Cells

To determine if cord suppressor T cells act through adult suppressor ($T8^+$) cells to exert their effect, adult lymphocytes depleted of $T8^+$ cells were used to assay cord suppressor T cell activity. $T8^+$cell depletion was achieved by the indirect rosette technique or FACS sorting. The results (Table IV) indicated that cord T cells were capable of suppressing IgG production by adult lymphocytes despite depletion of $T8^+$ cells from adult lymphocytes. Additional experiments using cord $T4^+$ cells showed similar suppression (data not shown). Irradiation of adult T cells (2500 rads) prior to culture also did not affect the cord T cells suppressor effect (Table V). These results suggested that cord suppressor T

Table II. Phenotype of Fetal Suppressor T Cells

Cells in Culture	IgG (ng/ml)	% of Control[c]
Adult PBL (background)	140 ± 30[b]	0
Adult PBL + PWM (control)	1185 ± 310	100
Adult PBL + PWM + adult T cells	1040 ± 330	86
Adult PBL + PWM + cord T cells	430 ± 165	28[d]
Adult PBL + PWM + cord $T8^+$ cells[a]	880 ± 280	71
Adult PBL + PWM + cord $T8^-$ cells[a]	440 ± 110	29[d]

10^6 adult peripheral blood lymphocytes (PBL) were co-cultured with 0.3×10^6 allogeneic adult or cord T cells for 7 days. IgG production was measured by RIA.

[a]Cord T cells were incubated with FITC-T8 and the positive cells were then separated from the negative cells by FACS.

[b]Mean ± SD of 3 experiments.

[c]Values are expressed as percentage of control (line 2). Background values (line 1) were subtracted in the calculation.

[d]Significantly different from the control ($P < 0.05$).

Table III. Phenotype of Fetal Suppressor T Cells

Cells in Culture	IgG (ng/ml)	% of Control[c]
Adult PBL (background)	160 ± 35[b]	0
Adult PBL + PWM (control)	1135 ± 315	100
Adult PBL + PWM + adult T cells	980 ± 345	84
Adult PBL + PWM + cord T cells	435 ± 180	28[d]
Adult PBL + PWM + cord $T4^+$ cells[a]	450 ± 195	30[d]
Adult PBL + PWM + cord $T4^-$ cells[a]	875 ± 300	73

10^6_6 adult peripheral blood lymphocytes were co-cultured with 0.3 x 10^6 allogeneic adult or cord T cells for 7 days. IgG production was measured by RIA.

[a]Cord T cells were incubated with FITC-T4 and the positive cells were then separated from the negative cells by FACS.

[b]Mean ± SD of 3 experiments.

[c]Values are expressed as percentage of control (line 2). Background values (line 1) were subtracted in the calculation.

[d]Significantly different from the control ($P < 0.05$).

Table IV. Phenotype of the Target of Fetal Suppressor T Cells

Cells in Culture	IgG (ng/ml)	% of Control[c]
Adult PBL (background)	115 ± 35[b]	0
Adult PBL ($T8^-$)[a,c] (background)	140 ± 30	100
Adult PBL + PWM (control)	1275 ± 310	0
Adult PBL ($T8^-$) + PWM (control)	1450 ± 370	100
Adult PBL + PWM + adult T cells	1410 ± 350	112
Adult PBL + PWM + cord T cells	515 ± 250	34[d]
Adult PBL ($T8^-$) + PWM + adult T cells	1400 ± 330	96
Adult PBL ($T8^-$) + PWM + cord T cells	505 ± 205	29[d]

10^6 adult PBL or $T8^+$ cell-depleted ($T8^-$) adult PBL were co-cultured with 0.3 x 10^6 allogeneic adult or cord T cells. After 7 days, IgG production was measured by RIA.

[a] Depletion of $T8^+$ cells was by either the indirect rosette technique or FACS.

[b] Mean ± SD of 7 experiments.

[c] Values are expressed as percentage of their respective controls (line 4 or 5). Appropriate background values (line 1 or 2) were subtracted in the calculation.

[d] Significantly different from the control ($P < 0.01$).

Table V. Radiosensitivity of the Target of Fetal Suppressor T Cells

Cells in Culture	IgG (ng/ml)	% of Control[c]
Adult B cells + adult T cells (background)	85 ± 55	0
Adult B cells + adult T cells (R)[a] (background)	150 ± 50	0
Adult B cells + adult T cells + PWM (control)	1820 ± 310	100
Adult B cells + adult T cells (R) + PWM (control)	2380 ± 345	100
Adult B cells + adult T cells + PWM + cord T cells	705 ± 255	36[d]
Adult B cells + adult T cells (R) + PWM + cord T cells	950 ± 250	36[d]

0.5×10^6 adult B cells and 0.5×10^6 of adult T cells stimulated with PWM were cultured with or without 0.3×10^6 cord T cells for 7 days. IgG production was measured by RIA.

[a] Adult T cells irradiated with 2500 rads before culture.

[b] Mean ± SD of 4 experiments.

[c] Values are expressed as percentage of their respective controls (line 4 or 5) with their appropriate background values (line 1 or 2) subtracted in the calculation.

[d] Significantly different from the control ($P < 0.01$).

cells did not require the adult suppressor T cell ($T8^+$) subset to inhibit the IgG production by adult lymphocytes but that they acted on radioresistant $T4^+$ helper T cells or directly on B cells/monocytes.

Suppressor properties of Supc-c

In our previous study, it was shown that supernatant from cord blood MLC (Supc-c) contained a factor which suppressed immunoglobulin synthesis by adult lymphocytes stimulated with PWM (Cheng et al 1984; Chapter 2). Furthermore, the presence of adult T cells was required for the suppression to occur, implying that the factor in Supc-c was an inducer of suppressor T cell factor. In this study, the suppressor properties of Supc-c were further assessed.

Lymphocyte proliferative responses to Candidin and alloantigens (in MLC) were used to assess the effect of Supc-c on adult T cell reactions. Supc-c significantly inhibited ($P < 0.01$ in both systems) lymphocyte proliferative response to Candidin (Table VI) and in MLC (Fig. 5). It is worthy to note that cord lymphocyte response in MLC was not suppressed by Supc-c. To determine if the target of Supc-c was the same as that of cord suppressor T cells, the phenotyping and radiosensitivity experiments described above were repeated but using Supc-c as a source of suppression instead. The phenotype of the target adult T cells was first determined by culturing $T8^+$ cell-depleted adult lymphocytes in the prescence of PWM. As shown in Figure 6, $T8^+$ cell depletion by either the indirect rosette

Table VI. Effect of SUPc-c on Adult PBL Proliferative Response to Candidin

Expt.	-[a]	Candidin	Candidin + SUPc-c
1	7,451 ± 301[b]	17,520 ± 1,047	8,099 ± 400
2	8,531 ± 345	15,321 ± 1,120	9,013 ± 537
3	7,043 ± 293	16,347 ± 893	10,514 ± 445
4	9,117 ± 400	17,835 ± 997	11,077 ± 819
5	6,978 ± 233	15,483 ± 591	9,374 ± 712

10^6 adult peripheral blood lymphocytes stimulated with Candidin with or without the presence of SUPc-c for 6 days at the end of which ^{3}H-thymidine was measured.

[a] Background ^{3}H-thymidine uptake by adult PBL (alone).

[b] ^{3}H-thymidine incorporation (c.p.m.); mean ± SD of triplicates.

Figure 5: Effect of Supc-c on adult and cord blood lymphocyte proliferative responses in 2-way MLC measured by ^{3}H-thymidine uptake. On the horizontal axis, $adult_1$-$adult_2$ and $cord_1$-$cord_2$ represent adult and cord blood MLC, respectively; adult and cord represent background ^{3}H-thymidine uptake by adult and cord lymphocytes, respectively. Results are expressed as the mean ± S.D. of 10 experiments.

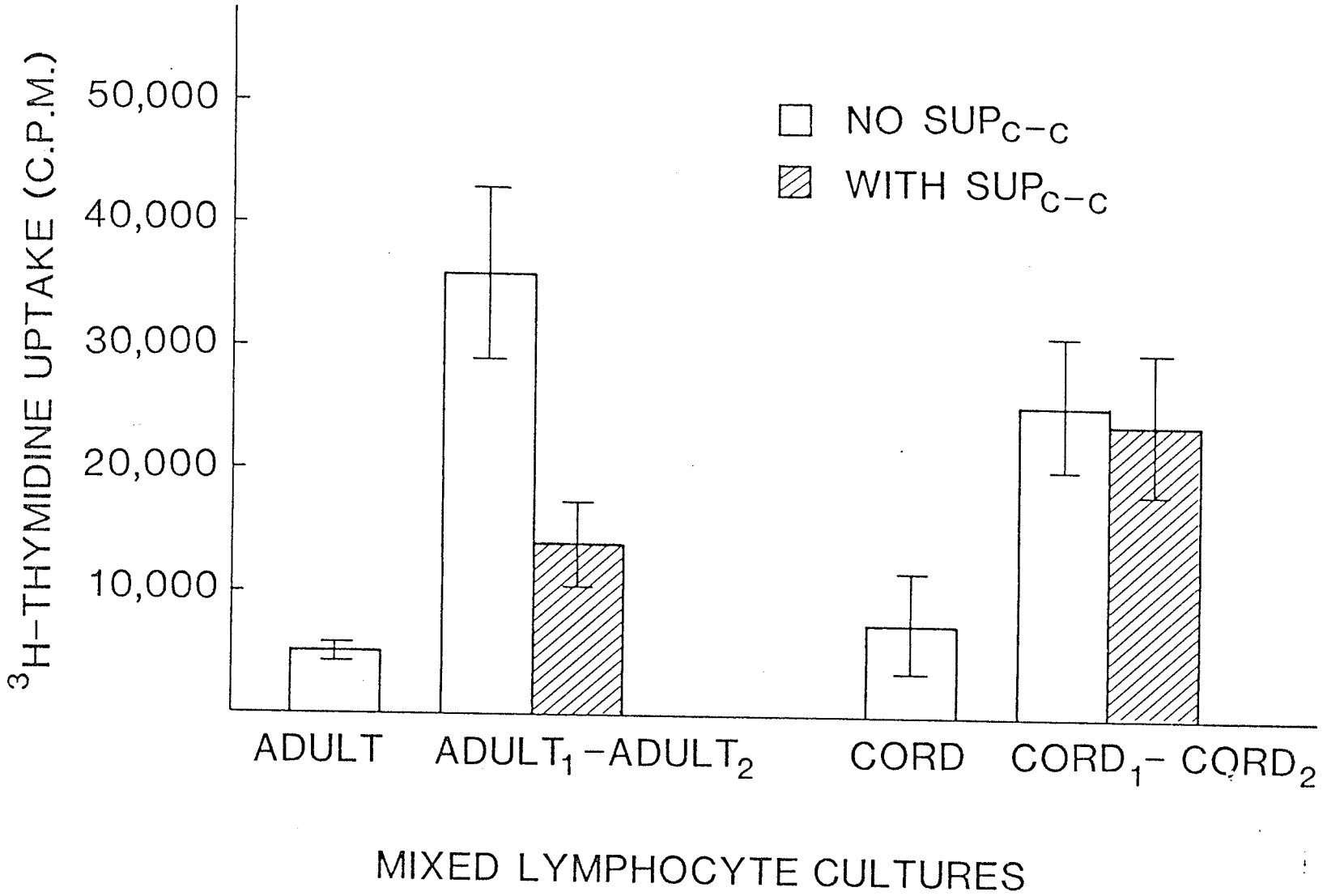
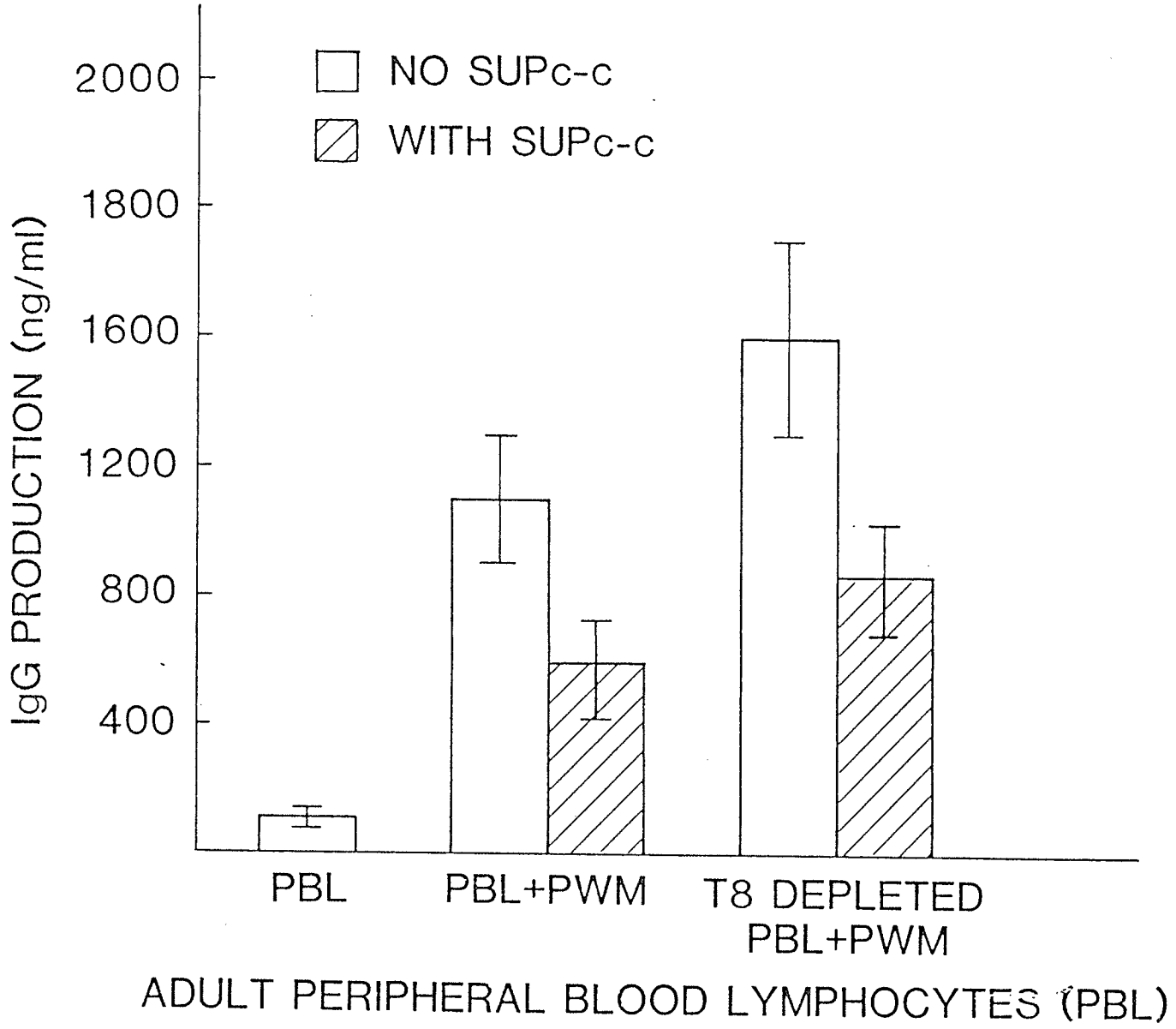

^{3}H-THYMIDINE UPTAKE (C.P.M.)
50,000
40,000
30,000
20,000
10,000
NO SUP$_{C-C}$
WITH SUP$_{C-C}$
ADULT
ADULT$_1$ – ADULT$_2$
CORD
CORD$_1$ – CORD$_2$
MIXED LYMPHOCYTE CULTURES

Figure 6: Effect of Supc-c on IgG production by PWM-stimulated $T8^+$ cell-depleted adult peripheral blood lymphocytes (PBL). Values are expressed as mean ± S.D. of 6 experiments.


NO SUPc-c
WITH SUPc-c
IgG PRODUCTION (ng/ml)
2000
1800
1600
1200
800
400
PBL
PBL+PWM
T8 DEPLETED PBL+PWM
ADULT PERIPHERAL BLOOD LYMPHOCYTES (PBL)

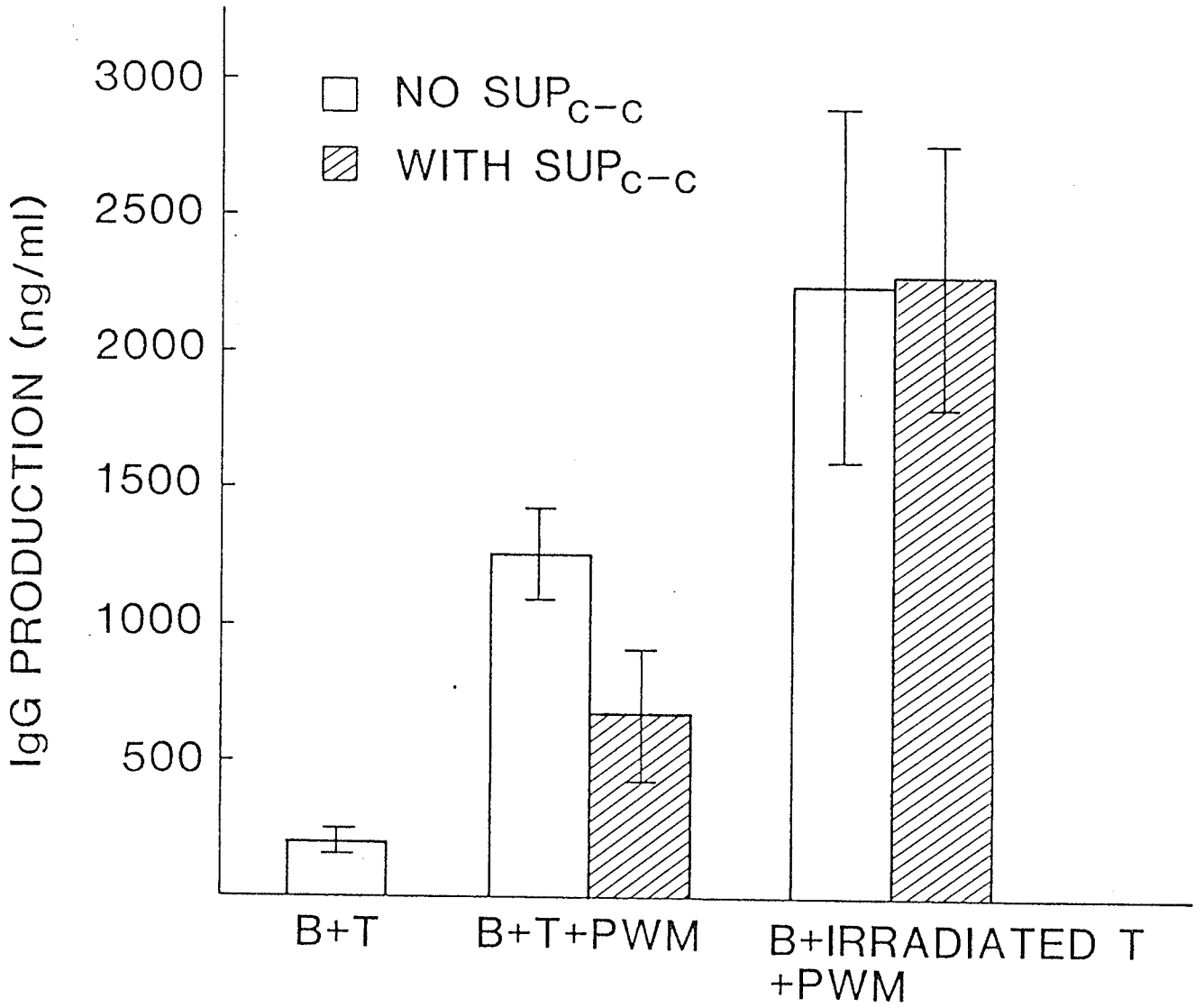
technique or cell sorting, prior to culture, did not alter the suppressive effect of Supc-c.

In contrast to the results of those experiments which tested the target of cord suppressor T cells (Table V), pre-culture irradiation of adult T cells completely abrogated the suppressor activity of Supc-c on IgG production by adult lymphocytes (Fig. 7). These results indicated that the target of Supc-c was different from those of cord suppressor T cells. The former being radiosensitive, $T8^-$ ($T4^+$) T cells while the latter was radioresistant, $T8^-$ ($T4^+$) T cells or B cells/monocytes.

Discussion

It was thought that the human peripheral blood T lymphocyte subset expressing the T4 surface marker contained helper/inducer cells while that expressing the T8 surface marker contained cytotoxic/suppressor cells and these two T cell subsets were mutually exclusive (Reinherz et al 1979b,c; 1980b,c,d). More recent studies, however, revealed a more complex relationship between cell surface antigenic markers and the functional activities of various lymphocyte subsets. For example, Thomas et al (1981, 1982) found a population of radiosensitive suppressor cells that bear T4 and T17 surface antigens.

Figure 7: Effect of Supc-c on IgG production by PWM-stimulated adult B and irradiated T lymphocytes. Irradiation dose was 2500 rads. Results are expressed as the mean ± S.D. of 8 experiments.


3000
2500
2000
1500
1000
500
IgG PRODUCTION (ng/ml)
NO SUP C-C
WITH SUP C-C
B+T
B+T+PWM
B+IRRADIATED T
+PWM
CO-CULTURES OF ADULT B AND T LYMPHOCYTES

Using a suppressor factor from a human T cell hybridoma, Grillot-Courvalin and coworkers (1983) demonstrated that radiosensitive T4 cells could be induced to suppress PWM elicited B cell differentiation. Furthermore, both alloantigen-activated $T4^+$ and $T8^+$ cells were found capable of suppressing B cell differentiation (Goeken and Melton 1983). Therefore, it appears that $T4^+$ cells are a functionally heterogeneous group and it is not too surprising to find that cord suppressor T cells belong to the $T4^+$ rather than the $T8^+$ subset which is generally regarded as having cytotoxic/suppressor cell activities (Table II, III).

Griffiths-Chu et al (1984), employing a double labelling technique found a significant population of T cells that expressed both $T4^+$ and $T8^+$ phenotypes. This finding, however, is not consistent with those of other investigators who detected very little overlap between these two subsets (Maccario et al 1983; Foa et al 1984). Nonetheless, the possibility, of a $T4^+/T8^+$ T cell subset being responsible for the cord blood lymphocyte suppressor activity is small since positive and negative selection with both T4 and T8 monoclonal antibodies were used in the present study.

It is clear from Table IV and V that cord suppressor T cell action on adult lymphocyte IgG production was independent of adult T8 or radiosensitive T cells and the cells are, therefore, unlikely to be the typical inducer of suppressor cells which inhibit immune responses

through the activation of the radiosensitive, $T8^+$ suppressor effector cells (Thomas et al 1980, 1981; Morimoto et al 1981, 1983). Rather, they are suppressor effector cells that act directly on radioresistant, $T4^+$ helper cells or B cells/monocytes. This is consistent with the data presented in Chapter 2 which indicated that non-T cells (B cells and monocytes) are the immediate target of cord T cell-directed suppression. Nevertheless, the possibility of a $T4^+$ suppressor inducer-$T4^+$ suppressor effector circuit existing within the cord T cell population cannot be excluded and is indeed suggested by our present findings (Fig. 6 and 7).

The mechanism of suppression by cord T cells has been an area of considerable interest. A number of investigators demonstrated that the suppressor activity of cord T cells was mediated, at least partially, through a soluble factor(s). The nature of this soluble factor(s), however, varied depending on the assay system and method used (Olding et al 1977; Nagaoki et al 1980; Fischer et al 1981; Miyawaki et al 1981b; Durandy et al 1982; Johnsen et al 1982; Jacoby et al 1984). Perhaps the divergent observations concerning the nature of the soluble factor underscores the complexity of the cord lymphocyte suppressor cell network which may involve multiple factors produced by different participating lymphocyte and monocyte subsets.

As reported previously, cord lymphocytes, when activated by alloantigens, produced a suppressor factor capable of inhibiting

immunoglobulin synthesis by PWM stimulated adult lymphocytes (Chapter 2). However, interaction with T cells was required for the suppressor activity to manifest itself suggesting that the factor was an inducer of suppressor factor (TisF). The present study further characterized the functional activity of this TisF. First, TisF was found to be antigen nonspecific since its suppressor effect in the PWM system could be extended to 2 other T cell-dependent antigen systems: Candidin and alloantigens as shown in Table VI and Figure 5. Interesting is the observation that Supc-c did not suppress cord blood T cell response in MLC (Fig. 3), whereas it strongly inhibited adult T cell response. Similar observations of resistance of neonatal cells to suppressor signals have been documented in other assay systems (Dwyer and Johnson 1983; Johnsen et al 1983b). From a teleological standpoint, it would be logical that the strong suppressor activity of fetal lymphocytes is directed only against maternal (adult) lymphocytes which could be potentially harmful to the fetus, and not against their own functionally immature immune system. The exact reason for the cord lymphocytes' relative resistance to suppression is not yet clear. Johnsen et al (1982) using prostaglandin inhibitors, indirectly showed that prostaglandin was involved in the suppression of maternal lymphocytes by cord lymphocytes. When exogenous prostaglandin E2 was used to suppress lymphocyte responses to PHA, the dose requirement for cord lymphocyte suppression was 100 times higher than for adult lymphocytes (Johnsen and Olding 1983). Andersson et al (1983) reported that Epstein-Barr virus-induced immunoglobulin

synthesis by adult B cells, but not cord B cells, could be suppressed by $T4^+$ suppressor cells. The suppression was claimed to be mediated through gamma type interferon and the insensitivity of cord lymphocytes to this interferon accounted for their resistance to suppression. These studies suggest that the relative resistance of cord lymphocytes to suppression may be related to their lack of sensitivity to various immunoregulatory mediators. Another plausible explanation could be the existence of strong contrasuppressor cells in neonates which negate the effect of suppressor cells. Skowron-Cendrzak et al (1983) demonstrated potent contrasuppressor cell activity in newborn mice which became evident from 1-2 days after birth and persisted until 2 months of age. Indirect evidence implying the existence of contrasuppressor cells in humans has also emerged recently (Lehner 1983; Thomas et al 1984). These cells are believed to express the same phenotype (T8) as suppressor/cytotoxic cells. Perhaps some of the contradictory observations reported in the literature regarding the functional status of cord blood $T8^+$ lymphocytes might be related to the emergence of strong contrasuppressor cell activity at the perinatal period. As a result, some newborn lymphocytes contained only the suppressor cells while others had both contrasuppressor and suppressor cells in the same $T8^+$ subset thus rendering the experimental results unpredictable depending on the relative potency of these two functionally diagonal populations. Inherent in this hypothesis is the requirement that neonatal contrasuppressor cells are effective in conferring resistance

only to the neonatal lymphocytes. Such restriction, however, has not been observed, at least in the murine studies. Clarification of the role of contrasuppressor cells in cord blood awaits further investigation and may promote our understanding of the intricate nature of the fetal suppressor cell circuit.

The target of Supc-c were $T8^-$ ($T4^+$) radiosensitive T cells rather than the $T8^+$ suppressor effector cells as originally expected (Fig. 6,7). They bore similar characteristics to the fetal suppressor effector cells which were also $T4^+$ and radiosensitive (Table I-III). Therefore, it is very probable that TisF acts on the same subset of T lymphocytes be it of adult or cord blood origin. It is of interest to note that an antigen specific TisF obtained within 24 hr of antigenic stimulation has been identified and the target of this TisF was a radiosensitive suppressor effector T cell subpopulation (Uytdehaag et al 1979). The present results support the hypothesis that the strong suppressor property of neonatal T lymphocytes may be secondary to the potent inducer of suppressor cells and the $T4^+$, radiosensitive suppressor effector cells present in neonate. Upon stimulation, the inducer of suppressor cells activate the suppressor effector cells which, in turn, act on either the radioresistant $T4^+$ helper cells or directly on B cells or both. These data also confirms Jacoby et al's (1984) postulation that dominant suppressor T cells in cord blood belong to the radiosensitive $T4^+$ subset instead of the radiosensitive $T8^+$ subset as in the case of adult lymphocytes.

CHAPTER 4

HUMAN CORD BLOOD SUPPRESSOR T LYMPHOCYTES. II. CHARACTERIZATION OF INDUCER OF SUPPRESSOR CELLS

Abstract

Previously, we reported an antigen non-specific inducer of T suppressor cell factor (TisF) produced by cord blood mononuclear cells (MNC) in 48 hr, 2-way mixed lymphocyte cultures (MLC). The target of this factor was a radiosensitive, $T4^+$ ($T8^-$) adult suppressor T cell subset or B cell/monocytes. The cellular origin of this TisF was examined in the present study. IgG production by pokeweed mitogen (PWM)-stimulated adult MNC was used as an assay for TisF activity. It was found that TisF-producing cells formed rosettes with sheep erythrocytes (E^+) and were independent of adherent cells (AC) in the production of TisF. They were resistent to irradiation (2500 rads) and phenotypic characterization with T cell-reactive monoclonal antibodies indicated that they resided in the $T8^-$ ($T4^+$) population. Furthermore, both $TQ1^-$ and $TQ1^+$ cells were required for the production of TisF activity since such activity could not be reconstituted by supernatants from $TQ1^-$ MLC and/or $TQ1^+$ MLC. These results indicate that the production of TisF is dependent upon interactions between radioresistant E^+, $T8^-$, $TQ1^-$ and radioresistant E^+, $T8^-$, $TQ1^+$ cells.

Introduction

Human newborn umbilical cord blood T lymphocytes are capable of suppressing PWM-induced immunoglobulin production by adult lymphocytes (Hayward and Lawton 1977; Oldstone et al 1977; Durandy et al 1979; Morito et al 1979; Tosato et al 1980) as well as the latter's proliferative response to phytohemagglutinin (PHA) and alloantigen (Olding et al 1977; Oldstone et al 1977). The suppression is mediated, at least partially, through soluble factor(s) the nature of which is dependent upon the culture system employed (Miyawaki et al 1981a, 1981b; Johnsen et al 1982, 1983b; Andersson 1983; Papadogiannakis et al 1985). We have previously reported that cord T cells produced an inducer of suppressor factor(s) (TisF) upon stimulation by alloantigen in supernatants of 48 hr, 2-way mixed lymphocyte cultures (Cheng et al 1984; Chapter 2). Control supernatants from adult MLC did not contain such factor. Addition of this factor to adult lymphocyte culture inhibited the PWM-induced immunoglobulin production in an isotype nonspecific fashion (Cheng et al 1984). Furthermore, its action was not antigen-specific since adult lymphocyte responses to soluble antigen Candidin and alloantigen (in MLC) were also inhibited by this factor. The target of TisF was either the radiosensitive, $T4^+$ adult T cell subset or B cells/monocytes (Cheng et al 1985, Chapter 3). The suppressor effect of TisF appeared to be specific for adult lymphocytes since the addition of such a factor to cord blood MLC had no demonstrable

effect. The characteristics and phenotype of the cells that produced TisF was examined in the present study.

Materials and Methods

Lymphocyte preparation

Procedures for the preparation of MNC was described in Chapter 2. A T cell enriched fraction was prepared by rosetting MNC with aminoethylisothiouronium bromide hydrobromide (Sigma, St. Louis, MO) treated sheep erythrocytes and centrifuged over a Ficoll-metrizoate gradient. The rosette forming (E^+) cells were treated with 0.84% NH_4Cl for lysis of erythrocytes and will be referred to as T cells. Cells in the interphase were subjected to another cycle of rosette formation and centrifugation. These cells will be referred to as non-T cells which contained < 1% rosette forming cells and 30-50% monocytes (nonspecific-esterase positive). Adherent cells (AC) in the E^+ preparation were depleted by applying 3×10^7 cells in 10 ml of culture medium to Petri dishes (15 x 100 mm, Fisher, Pittsburgh, PA) for 60 min at 37°C. Culture medium consisted of RPMI 1640, 10% heat-inactivated fetal bovine serum, 2 mM of L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin (all from Gibco, Grand Island, NY). Non-adherent cells were collected and plated on a fresh plate for further depletion of AC. Indirect immunofluorescence indicated that such preparations contained < 1% surface

immunoglobulin positive cells, < 1% Leu M2 positive cells (Becton Dickinson, Mountain View, CA) and nonspecific esterase staining showed < 2% positive cells.

Selection of T cell subsets with monoclonal antibodies

Panning method: A modified panning method described by Wysocki and Sato (1978) was used to separate $T8^+$ from $T8^-$ T cells. Briefly, monoclonal antibody OKT8 (Ortho, Raritan, NJ) was added to 20 x 10^6/ml of unfractionated cord T cells at a final dilution of 1:80. The cells were incubated at 4°C for 30 min with gentle agitation every 10 min, washed and suspended in phosphate buffered saline and 3% bovine serum albumin (PBS - 3% BSA) at 8 x 10^6 cells/ml. An aliquot of 2.5 ml of this cell suspension was applied to a 60 x 15 mm polystyrene Petri dish (Falcon, Oxnard, CA) which had been precoated with 45 µg/ml of goat anti-mouse IgG (Calbiochem, La Jolla, CA) and 120 µg/ml of normal goat IgG (Sigma). The cells were incubated at 4°C for 30 min, gently swirled and then incubated for another 30 min. The non-adherent cells were re-panned on a second plate coated with 45 µg/ml of goat anti-mouse IgG for further depletion of $T8^+$ cells while the adherent cells were removed by repeated gentle pipetting. Cells were washed twice in PBS - 3% BSA before culture. Analysis by indirect immunofluorescence showed that the adherent cells contained > 97% $T8^+$ cells and the non-adherent cells, after 2 pannings,

contained < 5% $T8^+$ cells. The viability of both populations was > 95% as determined by trypan blue exclusion test.

Cell sorting by the fluorescence activated cell sorter (FACS): Initial attempts to separate $TQ1^+$ from $TQ1^-$ cells by the panning method was unsuccessful due to either a low number or affinity of cell receptors for monoclonal antibody TQ1. Analysis by FACS showed that cells which reacted with monoclonal antibody TQ1 exhibited low fluorescence when stained with FITC-goat anti-mouse IgG (Coulter, Hialeah, FL). Consequently, separation of $TQ1^+$ from $TQ1^-$ cells was performed by FACS (Epics V, Coulter). Briefly, 10^7 cord T cells were incubated with 50 µl of monoclonal antibody TQ1 (Coulter) in 2 ml of culture medium at 4°C for 30 min. The cells were then washed and stained with FITC-goat anti-mouse IgG at a final dilution of 1:30. After 30 min of incubation at 4°C, the cells were washed and sorted into fluorescence positive and negative cells by FACS under sterile conditions. Sorted cells were washed prior to culture and their viability was > 95% as determined by trypan blue exclusion test. Analysis of the sorted cells by FACS indicated that > 95% of the positively selected ($TQ1^+$) cells were fluorescence positive while 1-5% of the negatively selected ($TQ1^-$) cells were fluorescence negative.

Production of TisF

Although both cord blood 2-way MLC and cord-adult 2-way MLC supernatants contained TisF, the former produced more consistent TisF activity (Cheng et al 1984) and was used as a source of TisF in the present study. Briefly, 1.5 x 10^6/ml of cord MNC or non-T and various autologous T cell preparations (1:1 ratio) from each of the two individuals were cultured in a culture flask (Falcon) at 37°C with 5% CO_2 and 95% humidified air for 48 hr. The cell suspensions were spun, the supernatants collected, filtered through a 0.22 μm Millipore filter and stored at -20°C. Supernatants from 2-way MLC of cord MNC or non-T and autologous T cells (Supc-c) served as controls against which TisF activity of supernatants from 2-way MLC of various non-T and T cell preparations were compared. Kinetic studies showed that 48 hr was the optimal period for the production of TisF and dose response studies indicated that the suppressor effect of TisF was dose dependent and when used at 20% final dilution demonstrated significant suppression (Cheng et al 1985; Chapter 3).

Assay for TisF activity

TisF activity of various MLC supernatants was measured by adding these supernatants, at a final concentration of 20%, to adult MNC (10^6/ml) or non-T and T cells (5 x 10^5/ml from each), in round bottom tubes (Falcon) for 7 days in the presence of PWM (final dilution 1:100, Gibco). All cultures were performed in duplicate. IgG production in these cultures was measured by a solid phase

sandwich radioimmunoassay previously described (Cheng et al 1984). Since TisF activity could be abolished by irradiating the target adult T cells with 2500 rads (Cheng et al 1984; Chapter 3), parallel cultures consisting of non-T and irradiated T cells plus various test MLC supernatants were performed to ensure that suppression by these supernatants was due to TisF rather than nonspecific effects such as insufficient nutrients or metabolic waste.

Statistical analysis

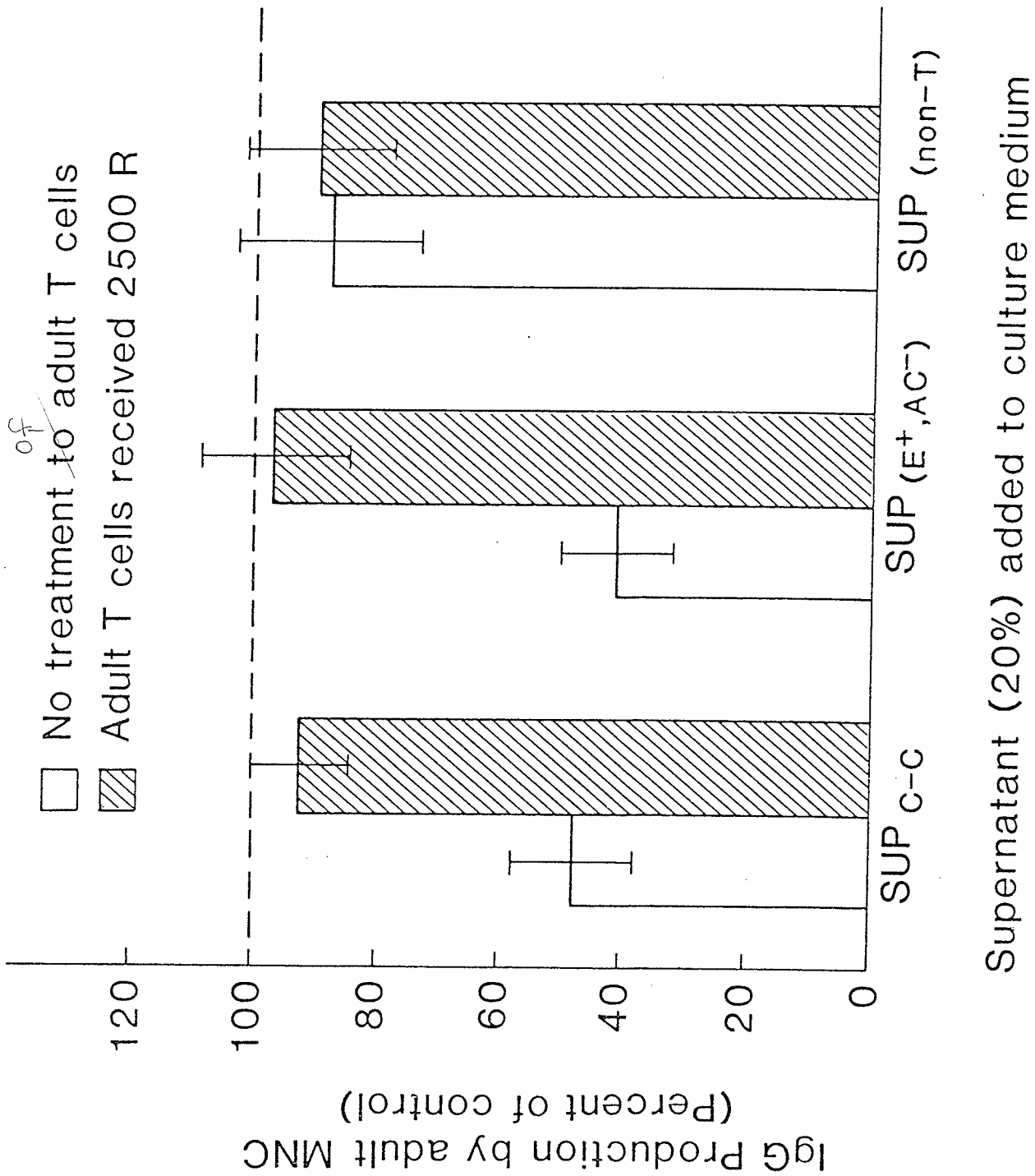
Two-way analysis of variance was employed. P values < 0.05 were taken as significant.

Results

TisF was produced by rosette forming (E^+), adherent cell (AC)-depleted cord T lymphocytes

Mononuclear cells (MNC), T cell enriched (E^+, AC-depleted) lymphocytes or non-T cells from 2 umbilical cord blood samples were co-cultured in 2-way MLC. After 48 hr, supernatants from these cultures were collected and assessed for TisF activity. As shown in Fig. 1, only supernatants from cord MNC and T cell enriched MLC (Supc-c and Sup (E^+, AC^-)) suppressed PWM-induced IgG production ($P < 0.05$). This suppression was not due to a nonspecific effect since irradiation of the target adult T cells completely abrogated the suppression.

Figure 1: Effects of various cord blood 2-way MLC supernatants on the PWM-induced IgG production by adult non-T and T cells (5×10^5 each) with (hatched bars) or without (open bars) irradiation. Results are expressed as percent of control cultures (no supernatant added). Supernatants from cord MNC, E^+-AC depleted cell and non-T cell MLC are represented by Supc-c, Sup (E^+, AC^-) and Sup (non-T), respectively. Values are mean ± S.D. of 6 experiments.


IgG Production by adult MNC
(Percent of control)
120
100
80
60
40
20
0
No treatment of adult T cells
Adult T cells received 2500 R
SUP C-C
SUP (E+,AC-)
SUP (non-T)
Supernatant (20%) added to culture medium

TisF producing cells were radiosensitive and $T8^-$

The radiosensitivity of the TisF producing cells was examined by irradiating T cells from 2 cord blood samples with 2500 rads, recombining them with their autologous non-T cells at a 1:1 ratio and co-culturing in a 2-way MLC. TisF activity from such cultures was compared with control Supc-c from cultures consisting of non-irradiated T and autologous non-T cells from the 2 cord blood samples. No effect on the generation of TisF activity was demonstrable by prior irradiation of cord T cells (Fig. 2) indicating that TisF producing cells are radioresistant.

The phenotype of the TisF producing cells was then assessed by the use of monoclonal antibody OKT8. Cord T cells were first separated into $T8^+$ and $T8^-$ fractions, these fractions were then reconstituted with their autologous non-T cells at 1:1 ratio and co-cultured with similarly prepared allogeneic cells in a 2-way MLC. TisF activity of the culture supernatants from these various cultures was then measured. No TisF activity was detected in supernatants from MLC cultures containing $T8^+$ cells while $T8^+$ cell depletion showed no effect on TisF production by cord lymphocytes suggesting that TisF-producing cells were $T8^-$ (Fig. 3).

Production of TisF required both $TQ1^-$ and $TQ1^+$ T lymphocytes

Monoclonal antibody TQ1 was used to separate cord T cells into $TQ1^-$ and $TQ1^+$ cells. Analysis by FACS indicated that the $TQ1^-$

Figure 2: Effect of cord T cell irradiation on TisF production in MLC (Supc-c). TisF activity was tested on PWM-induced IgG production by adult non-T and T cells (5×10^5 each) with (hatched bars) or without (open bars) irradiation. Results are expressed as percent of control cultures (no supernatant added). Mean ± S.D. of 8 experiments are shown.

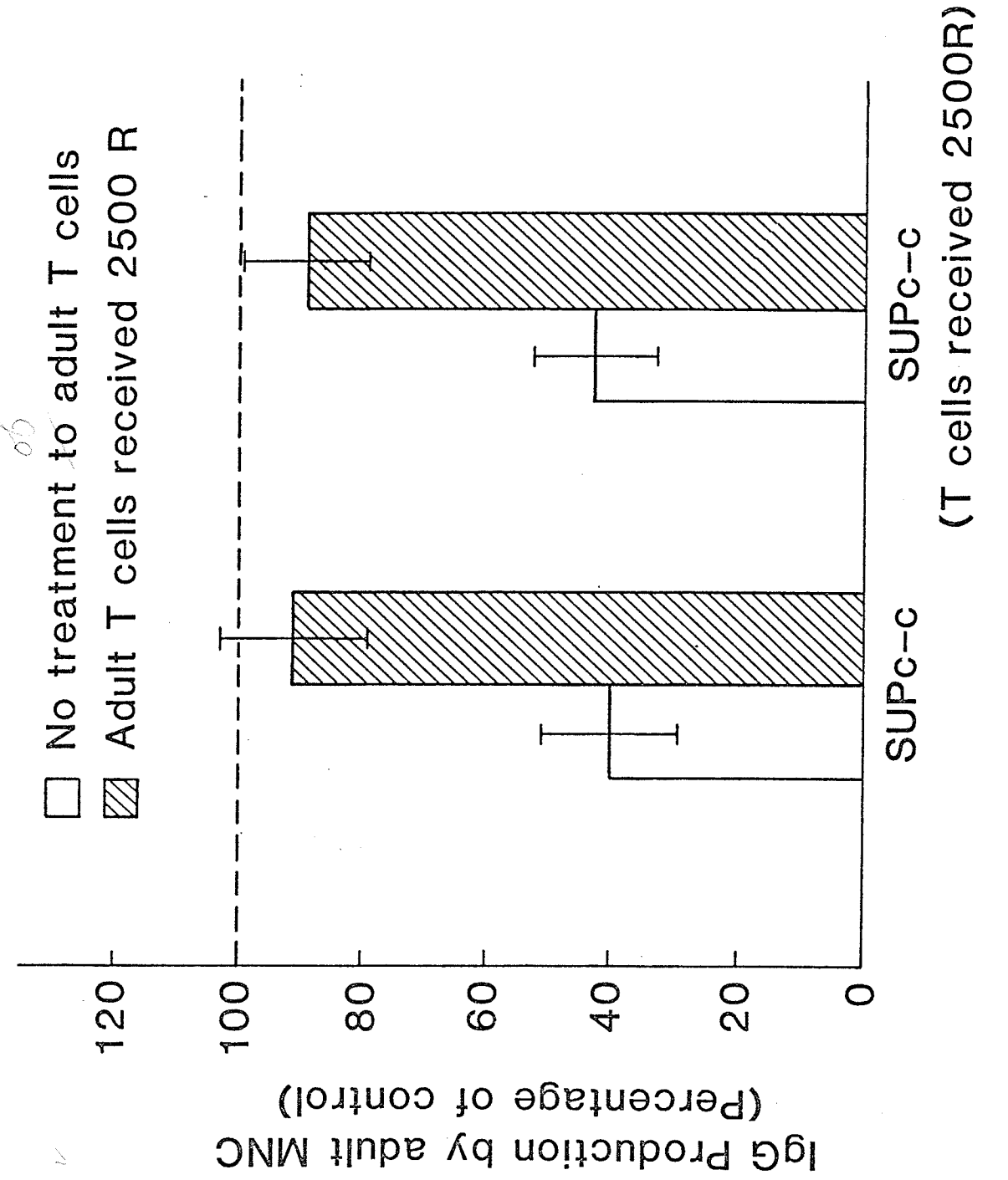
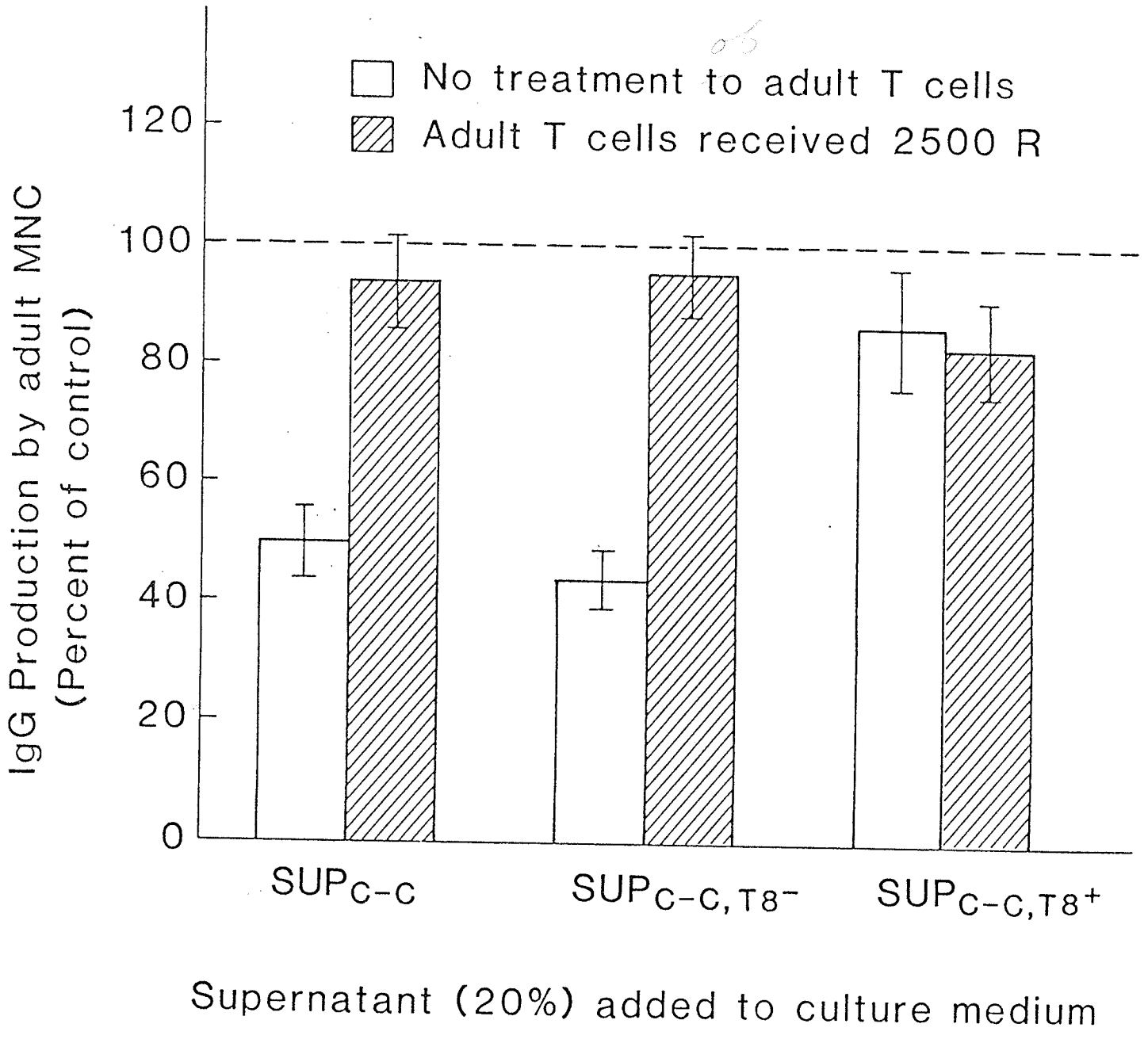

IgG Production by adult MNC
(Percentage of control)
120
100
80
60
40
20
0
No treatment to adult T cells
Adult T cells received 2500 R
SUPc-c
SUPc-c
(T cells received 2500R)
Supernatant (20%) added to culture medium

Figure 3: Phenotype of TisF producing cord T cells. Two-way MLC supernatants of cord non-T and unfractionated T cells (Supc-c), T8-depleted cells (Supc-c, $T8^-$) or T8 cells (Supc-c, $T8^+$) at 1:1 ratio from 2 cord blood samples were assayed for TisF activity on PWM-stimulated IgG production by adult non-T and T cells with (hatched bars) and without (open bars) irradiation. Results are expressed as a percent of control cultures (no supernatant added). Mean ± S.D. of 5 experiments are shown.


No treatment to adult T cells
Adult T cells received 2500 R
IgG Production by adult MNC
(Percent of control)
120
100
80
60
40
20
0
SUPC-C
SUPC-C,T8-
SUPC-C,T8+
Supernatant (20%) added to culture medium

and $TQ1^+$ ratio in the cord T cell-enriched fraction (E^+, AC-depleted) was between 1:2 to 1:3. $TQ1^-$ or $TQ1^+$ cells were recombined with autologous non-T cells at a 1:1 ratio and cultured in a 2-way MLC. It was found that neither $TQ1^-$ nor $TQ1^+$ cells provided TisF in their respective 2-way MLC: [Supc-c($TQ1^-$) and Supc-c($TQ1^+$)]; but when both were used, the resultant MLC supernatant Supc-c ($TQ1^-$ + $TQ1^+$) exhibited TisF activity (Table 1).

To determine if interaction between $TQ1^-$ and $TQ1^+$ cells was actually required or it was sufficient to merely provide factors produced by both $TQ1^-$ and $TQ1^+$ cells to obtain TisF activity, the effect of adding both Supc-c ($TQ1^-$) and Supc-c ($TQ1^+$) to the assay culture was assessed. As shown in Table 2, TisF activity could not be reconstituted by adding both supernatants suggesting that interaction between $TQ1^-$ and $TQ1^+$ cells was essential for the production of TisF.

Discussion

Cord blood MNC is a heterogeneous group of cells in which several types of cells may possess immunoregulatory activity and are possible candidates for the production of TisF. The present data indicate that the cells which produced TisF formed rosettes with sheep erythrocytes and belonged to the $T8^-$ ($T4^+$) subset (Fig. 1 and 3). Therefore, it seems unlikely that they are the relatively immature

Table 1. Phenotype of TisF-Producing cells

Culture Composition	IgG(ng/ml) Expt.1	Expt.2	Expt.3
Non-T + T	115	150	105
Non-T + T + PWM	1415	1200	1385
Non-T + T + PWM + Supc-c	685	640	700
Non-T + T + PWM + Supc-c ($TQ1^-$)	1285	1300	1385
Non-T + T + PWM + Supc-c ($TQ1^+$)	1335	1095	1515
Non-T + T + PWM + Supc-c ($TQ1^-$,$TQ1^+$)	700	595	710
Non-T + T(R)	150	180	165
Non-T + T(R) + PWM	1840	1800	2215
Non-T + T(R) + PWM + Supc-c	1720	1805	2175
Non-T + T(R) + PWM + Supc-c ($TQ1^-$)	1735	1790	2095
Non-T + T(R) + PWM + Supc-c ($TQ1^+$)	1695	1775	2155
Non-T + T(R) + PWM + Supc-c ($TQ1^-$,$TQ1^+$)	1805	1695	2210

Adult non-T and T cells (0.5×10^6 each) were stimulated with PWM for 7 days in the presence of Supc-c (20%) from various cord lymphocyte preparation. IgG production was then measured. Supc-c: from cord MNC; Supc-c ($TQ1^-$): from cord non-T and $TQ1^-$ cells; Supc-c ($TQ1^+$): from cord non-T and $TQ1^+$ cells; Supc-c ($TQ1^- + TQ1^+$): from cord non-T, $TQ1^-$ and $TQ1^+$ cells (2:1:1).

[a] T cells received 2500 rads before culture.

Table 2. Effect of Supc-c ($TQ1^-$) and Supc-c ($TQ1^+$) on PWM Induced Adult MNC IgG Production

Culture Composition	IgG(ng/ml) Expt. 1	Expt. 2	Expt. 3
Adult MNC	240	195	205
Adult MNC + PWM	1575	1670	1305
Adult MNC + PWM + Supc-c	780	750	680
Adult MNC + PWM + Supc-c ($TQ1^-$)	1435	1575	1300
Adult MNC + PWM + Supc-c ($TQ1^+$)	1395	1660	1450
Adult MNC + PWM + Supc-c ($TQ1^-$) + Supc-c ($TQ1^+$)	1550	1700	1400

Adult MNC (10^6) were stimulated with PWM for 7 days after which the IgG production was measured. Various Supc-c were added at a final concentration of 20%. Supc-c: from cord MNC; Sup ($TQ1^-$): from cord non-T and $TQ1^-$ cells; and Sup ($TQ1^+$): from cord non-T and $TQ1^+$ cells.

$T8^+$, $T3^-$, E^-, PNA^+ (peanut agglutinin) (Maccario et al 1983) or the $T4^+/T8^+$ (Solinger 1985) cells that have been reported to be present in significant number in fetal and newborn peripheral blood. Monocytes also did not appear to be the producer of TisF since supernatants from non-T MLC, which contained 30-50% of monocytes, did not demonstrate any TisF activity and AC depletion did not alter the TisF production by E^+ cells (Fig. 1). Nevertheless, an indirect role played by monocytes could not be completely ruled out since a minute number of monocytes (< 2%) remained in the E^+, AC-depleted T enriched fraction. Furthermore, a possible role of monocytes in the ultimate expression of TisF-induced suppression, i.e., their role in the induction and/or effector function of the target radiosensitive, $T4^+$ suppressor cells, remains to be defined. In this respect, our preliminary data showed that the TisF effect on adult MNC immunoglobulin production could be significantly reduced if adherent cells were removed from adult MNC suggesting monocytes' involvement in the effector phase of the TisF-induced suppression.

It has been reported by a number of investigators including ourselves that cord suppressor T cells which inhibit PWM-induced immunoglobulin production by adult MNC are radiosensitive (Hayward and Lydyard 1978; Durandy et al 1979; Tosato et al 1980; Jacoby and Oldstone 1983; Cheng et al 1985) and $T4^+$ (Yachie et al 1981; Jacoby and Oldstone 1983; Cheng et al 1985). Our previous study showed that TisF-induced suppression is mediated through adult suppressor cells

that possessed the same radiosensitivity and phenotype as the cord suppressor cells (Cheng et al 1985) suggesting that TisF may act on either the cord or adult radiosensitive, $T4^+$ target suppressor cells to exert its effect. Since irradiation of cord T cells completely abrogated their suppression activity in the adult MNC assay culture, it was expected that TisF producing cells were also radiosensitive. Otherwise, cord T cells would be able to maintain their suppression through activation of radiosensitive $T4^+$ adult target cells by TisF despite the inactivation of cord target cells by irradiation. This prediction, however, was not confirmed by the present study which demonstrated the radioresistant nature of the TisF producing cells. This apparent discrepancy may be due to differences in potency, dose requirement and kinetics between the adult and cord suppressor cells' response to TisF. In our baseline kinetic experiments, it was found that TisF had to be added to adult MNC cultures within 24 hr to be effective. In contrast, optimal production of TisF in cord MLC was not achieved until 48 hr of culture (Chapter 3). Hence, if the TisF-dependent adult target suppressor cells were less potent and required more TisF to be activated, the amount of TisF generated by cord T cells within the first 24 hr may be sufficient to activate only the cord, but not adult TisF-dependent suppressor cells. Thus, irradiation of cord T cells prior to their addition to the adult MNC culture could eliminate their suppressor effect by inactivation of the TisF target cord T cells. Furthermore, the difference in potency between adult and cord target suppressor cells could also explain the

weaker suppression achieved by TisF compared to cord T cells (50-60% versus 70-90%, respectively). Alternatively, the alloantigen-induced TisF and its adult target suppressor cells may be part of a different suppressor circuit from the radiosensitive $T4^+$ cord suppressor cells which were shown to be inducible directly by PWM (Miyawaki 1981b).

The radioresistant nature of the TisF producing cells suggested that these cells may have already been activated (Schrek 1961; Lowenthal and Harris 1985) and the presence of alloantigen (in MLC) merely served to enhance TisF production. This is supported by our observation that between 30-40% of cord blood MNC samples produced TisF without alloantigen stimulation in autologous MLC.

Monoclonal antibody TQ1 was reported to react with 70-85% of $T4^+$ lymphocytes, 50% of $T8^+$ lymphocytes and together with monoclonal antibody T4, defined a population of T cells ($T4^+$, $TQ1^+$) that included the majority of $T4^+$ lymphocytes that were reactive to serum from patients with juvenile rheumatoid arthritis (JRA) (Reinherz et al 1982). This $T4^+$, JRA^+ subset contained the inducer of suppressor cell population in both PWM and antigen systems (Morimoto et al 1983). We took advantage of this property of the monoclonal antibody TQ1 to further characterize our TisF-producing cells which were shown to be $T8^-$ ($T4^+$). Both $TQ1^-$ and $TQ1^+$ cells were found to be necessary for the production of TisF (Table 1). Collaboration between these 2 subsets was evidently necessary since

recombination of factors produced by each subset independently could not restore the TisF activity. Further studies are necessary to elucidate the nature of interactions between these 2 subpopulations. It is of interest to note that using antigen KLH, Morimoto et al (1984) found that maximal activation of the antigen specific, radiosensitive $T8^+$ adult suppressor cells required both $TQ1^-$ and $TQ1^+$ cells. The use of more specific monoclonal antibodies for the inducer of suppressor cell population such as the recently reported anti-2H4 (Morimoto et al 1985) may further characterize the nature of TisF producing cells.

Using different stimulators and assay systems, a number of immunoregulatory factors such as prostaglandin E2 (Johnsen et al 1982, 1983b; Durandy et al 1985; Papadogiannakis et al 1985) and cord T cell-derived suppressor factor (CTSF) (Miyawaki et al 1981a, 1981b) have been identified and are believed to be the messengers for cord T cell-mediated suppression. Unfortunately, little is known about the phenotype of these factor producing cells. Further investigations such as biochemical characterization of TisF and phenotypic analysis of the various immunoregulatory factor-producing cells are necessary to understand the relationships among the various modes of cord T cell-induced suppression.

CHAPTER 5

EVALUATION OF THE FUNCTIONAL MATURITY OF NEWBORN $T8^+$ SUPPRESSOR CELLS AND THE RESISTANCE OF NEW BORN LYMPHOCYTES TO SUPPRESSION

Abstract

Concanavalin A (Con A) was used to study the suppressor function of human umbilical cord blood T cells. The suppressor activity and the phenotype of Con A-activated adult and cord T cells were compared using allogeneic adult mononuclear cell (MNC) responses to pokeweed mitogen (PWM), phytohemagglutinin (PHA) and alloantigen as indicator culture systems. It was found that Con A-activated adult suppressor T cells were predominantly $T8^+$ cells and suppressed PWM-induced IgG production by adult lymphocytes, as well as PHA- and alloantigen-induced proliferation of, allogeneic adult MNC. Con A-activated cord T cells, however, exhibited no significant suppression in any of the 3 indicator systems. Furthermore, using Con A-activated adult $T8^+$ cells as a source of suppression, the proliferative response of cord lymphocytes to PHA and alloantigen were as susceptible to suppression as those of adult lymphocytes. These results, together with previous findings that PWM-induced cord suppressor T cells were predominantly $T4^+$ and that cord lymphocytes were resistant to $T4^+$ cell-mediated suppression suggest that fetus/newborn lymphocytes achieved their selective inhibition on adult lymphocytes through the $T4^+$ suppressor cell circuit.

Introduction

The existence of strong suppressor T lymphocyte activity in the fetus and newborn is well documented in both mice (Murgita and Wigzell 1981) and humans (Jacoby et al 1984). These lymphocytes are capable of inhibiting the proliferative responses of adult lymphocytes to PHA and alloantigen (Olding et al 1977) as well as the mitogen induced adult B cell differentiation (Hayward and Lawton 1977; Unander et al 1982). In spite of the large number of studies, however, the exact nature, target and mode of action of these suppressor T cells remain unclear other than the fact that they are radiosensitive (Durandy et al 1979) and mediate their suppression, at least partially, via soluble factor(s) (Olding et al 1977; Nagaoki et al 1980). Early attempts to characterize these cells based on their expression of Fc receptors failed to yield consistent results since they had variously been reported to reside in the $Fc\mu R^+$ (Hayward and Lydyard 1978), $Fc\gamma R^+$ (Oldstone et al 1977) and/or $Fc_\gamma R^-$ (Durandy et al 1979; Moriya et al 1979) subpopulation of T cells.

More recent studies, including our own, which employed monoclonal antibodies directed against T cell surface antigens showed that, in contrast to adult, newborn suppressor cells are primarily of the $T4^+$ phenotype with little suppressor activity exhibited by the $T8^+$ cells (Yachie et al 1981; Jacoby and Oldstone 1983; Cheng et al 1985). This finding, together with the observation that newborn

cytotoxic T ($T8^+$) cells have diminished activities (Granberg and Hirvonen 1980) suggest that newborn $T8^+$ cells may be functionally immature. This notion, however, is not without contention since a number of investigators found that newborn suppressor cells belonged to the $T8^+$ subset (Hayward 1981; Rodriguez et al 1981). These conflicting data were generated in studies employing PWM exclusively as stimulator which is known to stimulate both the $T4^+$ and $T8^+$ suppressor cells (Thomas et al 1981). Therefore, the use of another stimulator which can activate a more defined subset of suppressor cells may prove helpful in resolving the controversy.

In the present study, Con A was used to activate newborn and adult suppressor T cells (Hubert et al 1976). The phenotype of these suppressor cells and their activities were compared by assessing their effect on fresh allogeneic adult lymphocyte responses to PWM, PHA and alloantigens. Furthermore, since there was some evidence to suggest that cord lymphocytes are resistant to suppression (Olding et al 1977; Andersson et al 1983; Johnsen et al 1983; Cheng et al 1985), their susceptibility to Con A-induced suppressor activity was also compared with that of the adult lymphocytes.

Materials and Methods

Preparation of mononuclear cells (MNC) and isolation of B and T cells:

Cord blood was obtained at the time of delivery from normal uncomplicated pregnancies. Immediately after delivery but prior to expulsion of the placenta, cord blood was drained into tubes containing preservative-free heparin. It was then mixed with 5% dextran in normal saline at a ratio of 4:1 for 45 min at 37°C to sediment the erythrocytes. The leukocyte rich supernatant was diluted 1:2 in Hank's balanced salt solution (HBSS) (Flow Laboratories) and centrifuged over Ficoll-metrizoate gradient (Pharmacia Fine Chemicals; Nyegaard and Co.) with specific gravity of 1.073 at 900g for 30 minutes. Adult blood donated by healthy volunteers was diluted 1:2 in Hank's solution and centrifuged over Ficoll-metrizoate with specific gravity of 1.077. Mononucelar cells (MNC) in the interphase were collected and washed 3 times in Hank's solution. Further separation of MNC into T and B cells were achieved by rosetting with sheep erythrocytes. Briefly, MNC were suspended in RPMI 1640 and 10% heated-inactivated and absorbed fetal bovine serum (Flow and Gibco Laboratories, respectively) at 3×10^6 cells/ml. To this cell suspension was added 0.1 ml of aminoethylisothiouroniumbromide (Sigma Chemical Co.)-treated sheep erythrocytes (National Biological). The cell suspension was then centrifuged over Ficoll-metrizoate gradient at 4°C for 30 minutes. Cells collected at the interphase were

rosetted again and centrifuged over a Ficoll-metrizoate gradient. After this second rosetting, the cells at the interphase were collected, washed and will be referred to as B cells. The rosette-forming cells obtained after the first centrifugation were re-centrifuged over a Ficoll-metrizoate gradient. The pellet of the rosette-forming cells was then treated with 0.84% ammonium chloride followed by washings in Hank's solution and will be referred to as T cells. In the T cell preparations, 99% of the cells formed rosettes and less than 1% were positive for membrane immunoglobulin as assessed by membrane fluorescence assay (Landay et al 1983). The non-T cell preparation contained less than 1% rosette forming cells and 30-50% monocytes (nonspecific esterase positive). The viability of these cell preparations was 98-100% by Trypan blue exclusion test.

Separation of T cells into $T8^+$ and $T8^-$ cells:

A modification of the panning method described by Wysocki & Sato (1978) was used. See Chapter 4 for details.

Con A-activation of T cells:

Unfractionated T cells (2×10^6/ml) were cultured with or without 25 µg/ml of Con A (Pharmacia) in tissue culture flasks for 48 hr at 37°C with 5% CO_2-95% humidified air. Cultures that had no Con A served as a control for Con A-activated T cell cultures. Cells were then washed in 0.3M of alpha-methyl-D-mannoside (Sigma) and treated with 50 µg/ml of mitomycin-c (mit-c) (Sigma) for 30 min at

37°C. After washing in culture medium, they were tested for their suppressor activity in various assay systems.

Assays for suppressor cell activity:

(a) PWM system: Adult MNC (10^6) (target cells) and various numbers of allogeneic T cells (test cells) were cultured in 1 ml of PWM-containing (Gibco, 1:100 dilution) culture medium for 7 days in 15 x 75 mm round-bottom tubes (Falcon). The suppressor effect of the test cells was assessed by measuring adult MNC IgG production using a solid phase sandwich radioimmunoassay described previously in Chapter 2. All cultures were performed in duplicate.

(b) PHA system: Equal numbers (10^5 cells) of target MNC and mit-c treated autologous T cells (control) or allogeneic test T cells were co-cultured, in quadruplicate, in round-bottomed wells of Linbro plates (Flow). PHA-P (Difco, Detroit, MI), at a final dilution of 1:8000 which represented a suboptimal dose, and the response which was more susceptible to suppression, was added into each well. The suppressor effect on the target cell proliferative response was measured by ^{3}H-thymidine uptake after 72 hr. The cells were pulsed with 1 μCi of ^{3}H-thymidine (sp.act. 2 Ci/m mole; Amersham, Arlington Heights, IL) for 8 hr prior to harvesting by a cell harvester (Cambridge Technology, Cambridge, MA). Radioactivity (cpm) of the cells was counted in scintillation fluid Econofluor (New England Nucleus, Boston, MA).

(c) Mixed lymphocyte culture (MLC): Responder MNC (5×10^4) (target cells), 10^5 mit-c treated allogeneic adult stimulator MNC and 5×10^4 mit-c treated adult stimulator T cells (served as control) or allogeneic test T cells were co-cultured, in quadruplicate, in Linbro plate wells. On the fifth day, cell proliferation was measured by ^{3}H-thymidine uptake.

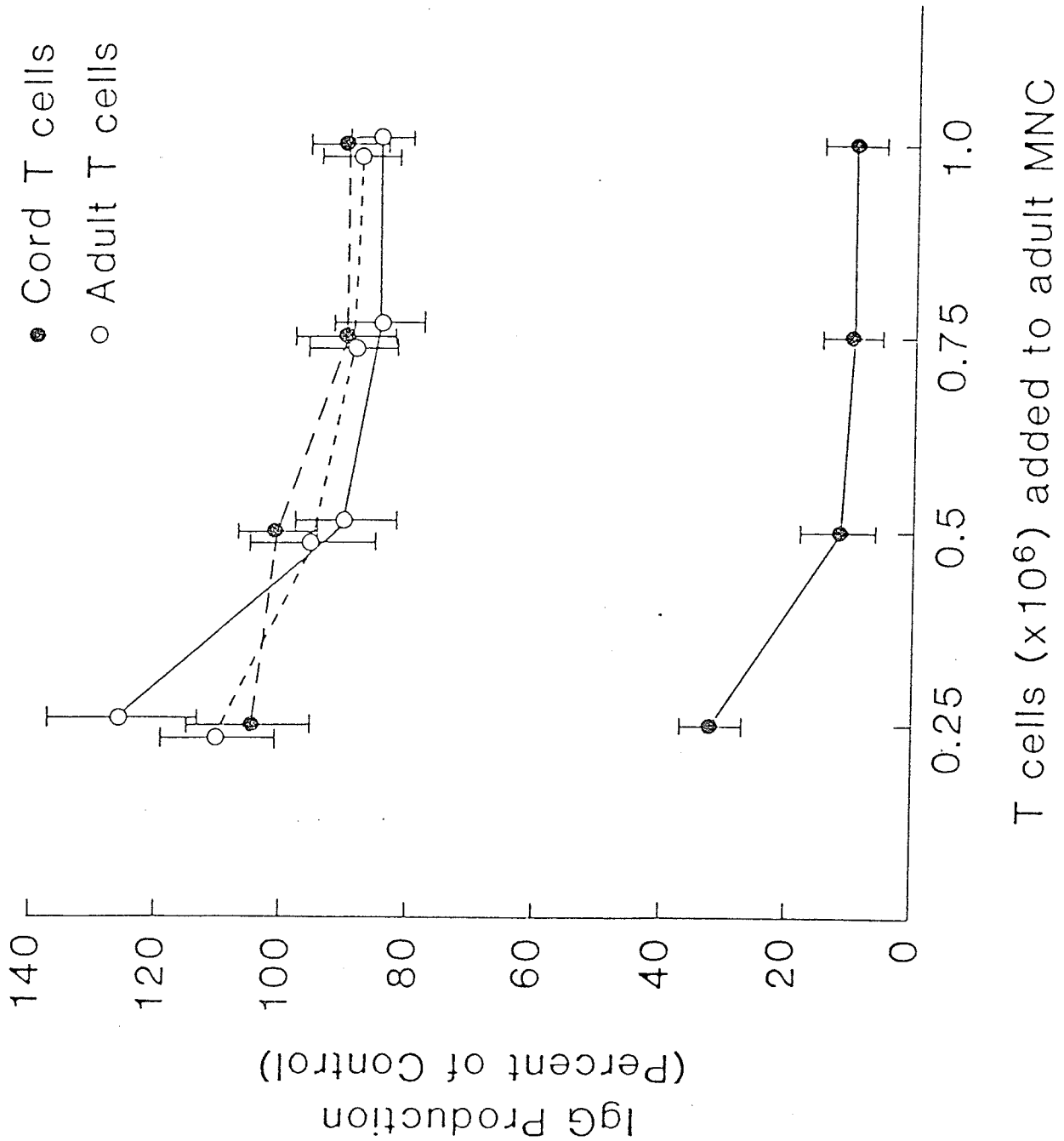
Statistical analysis

One way analysis of variance was used for the calculation of the statistical significance of differences. P values < 0.05 were taken as significant.

Results

The indicator systems used for the evaluation of Con A-induced suppressor cells contained either PWM, PHA or alloantigen, all of which are capable of triggering strong suppressor activity in cord blood lymphocytes (Olding et al 1977; Nagaoki et al 1980). It is, therefore, important to eliminate the effect from these stimuli when assessing Con A-induced suppressor activity of cord blood lymphocytes. This could be achieved by pretreating cord T cells with mit-c prior to the addition of these cells to the indicator culture. As shown in Fig. 1, mit-c treated cord T cells lost their suppressive effect on PWM-induced IgG production by adult MNC. Similar treatment also

Figure 1: Effect of allogeneic T cells on the PWM-induced IgG production by adult MNC (10^6). Results are expressed as percent of control (adult MNC alone). Broken lines represent mit-c treated T cells and solid lines represent untreated T cells. Values are mean ± S.D. of 3 experiments.


● Cord T cells
○ Adult T cells
IgG Production
(Percent of Control)
140
120
100
80
60
40
20
0
0.25
0.5
0.75
1.0
T cells (x10⁶) added to adult MNC

rendered them non-suppressive on the adult lymphocyte proliferative response to PHA and alloantigens in MLC (Table 1; line 2 and line 6).

First, the suppressor activities generated by Con A-stimulated adult and cord T cells on PWM-induced IgG production were examined. Adult T cells, when stimulated with Con A for 48 hr, suppressed the allogeneic adult MNC response to PWM. The suppressor activity resided mainly in the $T8^+$ subpopulation (Fig. 2) with little suppression demonstrated by $T8^-$ cells ($p > 0.2$). Cord T cells, on the other hand, showed no significant suppression despite Con A stimulation ($p > 0.2$) as illustrated in Fig. 2.

In order to decide if the absence of Con A inducible suppressor activity in cord T cells was restricted to the PWM system, Con A-activated T cells were also tested in the PHA and MLC systems. Similar to the results observed in PWM system, the suppressor activity of Con A-activated adult T cells was found to reside primarily in the $T8^+$ subset while Con A-activated cord T cells exhibited little suppression on the allogeneic adult lymphocyte response to PHA and alloantigens (Table 1).

To determine if cord blood lymphocytes were more resistant to Con A-induced suppressor activity, similar experiments were performed, except cord blood lymphocytes were used as target of suppression. As

Table 1. Effect of Con A-activated Adult and Cord T Cells on Adult MNC Response to PHA and Alloantigens (in MLC)

Cells added to indicator culture[a]	^{3}H-thymidine uptake (cpm) by adult MNC Indicator Culture PHA	(%)[b]	MLC	(%)[b]
Control T[c]	47,912		21,170	
Adult T	45,037	(94)	18,630	(88)
Adult T (Con A)	24,435	(51)[d]	6,554	(31)[d]
Adult $T8^-$ (Con A)	42,642	(89)	17,571	(83)
Adult $T8^+$ (Con A)	24,914	(52)[d]	5,920	(28)[d]
Cord T	48,870	(102)	20,747	(98)
Cord T (Con A)	42,163	(88)	22,652	(107)
Cord $T8^-$ (Con A)	41,683	(87)	21,593	(102)
Cord $T8^+$ (Con A)	43,600	(91)	21,382	(101)

Result of 1 representative experiment of 4.

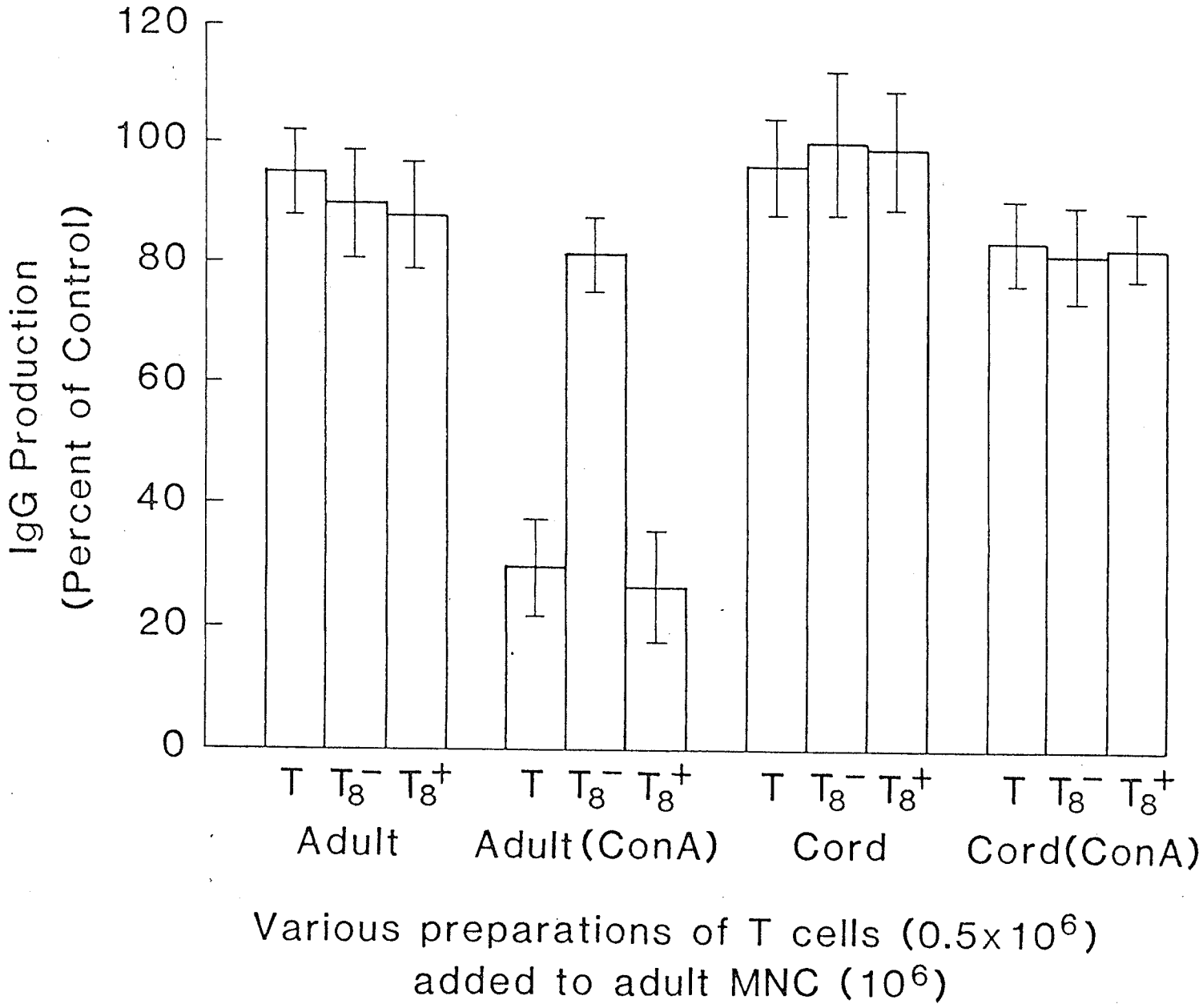
[a] Allogeneic adult or cord T cells were precultured for 48 hr with or without Con A, then treated with mit-c, separated into $T8^-$ and $T8^+$ cells and added to the indicator cultures consisting of adult MNC stimulated with either PHA or alloantigen (in MLC).

[b] Percent of control cultures in which control T cells were added.

[c] Control T cells were either T cells autologous to the responder MNC (in PHA culture) or to the stimulator MNC (in MLC). All control T cells were pretreated with mit-c.

[d] $P < 0.05$ compared to control cultures.

Figure 2: Suppressor activity and phenotype of Con A-activated T cells. Adult or cord T cells were cultured with or without Con A, then treated with mit-c and separated into $T8^-$ and $T8^+$ fractions. These cells were added to allogeneic adult MNC. PWM activated IgG production was measured and expressed as percent of control (adult MNC only). Mean ± S.D. of 4 experiments are shown.


IgG Production
(Percent of Control)
120
100
80
60
40
20
0
T
T8−
T8+
Adult
Adult(ConA)
Cord
Cord(ConA)
Various preparations of T cells (0.5x10^6)
added to adult MNC (10^6)

shown in Table 2, Con A-induced adult, but not cord, T cells suppressed the proliferative response of cord MNC to PHA and alloantigens. Furthermore, the magnitude of suppression was comparable to that on adult lymphocyte response.

Discussion

One of the possible explanations that has been suggested for the successful co-existence of the fetal graft and its maternal host is the presence of strong suppressor T cells in the fetus and newborn which inhibit the immune response of the mother. Such suppressor T cells were not only documented in vitro but had also been shown to prevent graft versus host reactions in vivo (Skowron-Cendrzak and Ptak 1976). The exact nature of these suppressor T cells remains elusive. In the PWM system, we and others have shown that, unlike adult T cells, cord suppressor T cells were predominantly $T4^+$ rather than $T8^+$ (Cheng et al 1985). This is not entirely unexpected since $T4^+$ lymphocytes are not homogeneous and contain several functional subsets such as helper, suppressor-inducer and suppressor cells (Thomas et al 1981; Morimoto et al 1981). Perhaps the strong suppression exhibited by cord $T4^+$ cells represents the utilization, in the fetus and newborn, of a usually weak $T4^+$ suppressor cell circuit which gradually shifts to the adult type $T8^+$ suppressor cell circuit in the postnatal period as suggested by Jacoby et al (1984).

Table 2. Effect of Con A-activated Adult and Cord T Cells on Cord MNC Response to PHA and Alloantigens (in MLC)

Cells added to indicator culture[a]	^{3}H-thymidine uptake (cpm) by cord MNC Indicator Culture			
	PHA	(%)[b]	MLC	(%)[b]
Control T[c]	72,068		38,574	
Adult T	65,582	(91)	40,503	(105)
Adult T (Con A)	37,475	(52)	15,815	(41)[d]
Adult $T8^-$ (Con A)	63,420	(88)	34,717	(90)
Adult $T8^+$ (Con A)	38,196	(53)	13,115	(34)[d]
Cord T	64,141	(89)	34,602	(90)
Cord T (Con A)	69,185	(96)	34,330	(89)

Result of 1 representative experiment of 4.

[a] Allogeneic adult or cord T cells were precultured for 48 hr with or without Con A, then treated with mit-c, separated into $T8^-$ and $T8^+$ cells and added to the indicator cultures consisting of cord MNC stimulated with either PHA or alloantigen (in MLC).

[b] Percent of control cultures in which control T cells were added.

[c] Control T cells were either T cells autologous to the responder MNC (in PHA culture) or to the stimulator MNC (in MLC). All control T cells were pretreated with mit-c.

[d] $P < 0.05$ compared to control cultures.

The lack of cord $T8^+$ cell suppressor activity prompted our investigation of the functional maturity of these lymphocytes. It has been reported that Con A-induced suppressor cells possessed the $T8^+$ phenotype and their induction was largely independent of either $T4^+$ cells or monocytes (Reinherz et al 1980b; Davidsen and Kristensen 1984). Hence, it provides us a vehicle to study cord $T8^+$ suppressor cell function irrespective of the stage of maturation of cord $T4^+$ cells or monocytes. Our result confirmed those from the PWM studies that cord $T8^+$ cells were not inducible to provide significant suppressor activity by mitogens. This lack of suppressor activity is not due to their inability to respond to Con A, their different dose requirement for Con A or different kinetics since in the preliminary experiments no significant suppression was demonstrable regardless of the dose or duration of stimulation despite their good proliferative response. In this study, Con A-induced suppressor activity was found mainly in the adult $T8^+$ subset, although $T8^-$ cells also showed some degree of suppression albeit statistically insignificant (Fig. 2, Table 1 & 2). This may be due to contamination of the $T8^-$ fraction by a small number of $T8^+$ cells or activation of a small subset of $T4^+$ suppressor cells. It should be pointed out that recently, contrasuppressor cells capable of rendering helper cells resistant to suppression have been described in mice and in humans. In man, these cells possessed the T8 phenotype (Lehner 1983; Thomas et al 1984) and in the murine system they had their strongest activity in the newborn period (Skowron-Cendrzak et al 1983). Therefore, it is conceivable

that this contrasuppressor cell activity also peaked in the perinatal period in humans and negated the suppressor effect of the $T8^+$ cells. Further investigation is needed to clarify the relationship between contrasuppressor and suppressor cells in the newborn.

We found that mit-c treatment blocked the induction of cord suppressor T cells by PWM (Fig. 1), PHA and alloantigens (Table 1). This suggests that proliferation is required for the induction of cord suppressor T cells and is consistent with Hayward's finding that cord T cell suppression could be prevented by deoxyguanosine, a DNA synthesis inhibitor (Hayward 1981). In contrast, Dwyer & Johnson (1983) reported the presence of "spontaneous" suppressor activity in both cord and adult MNC which was resistant to mit-c treatment. Such a discrepancy may be due to the different culture conditions used and/or possible in utero activation of cord suppressor T cells as suggested by the data of Williams & Korsmeyer (1978) and Unander et al (1982). Nevertheless, the fact remains that there was no demonstrable Con A-induced cord T cell suppressor activity whether or not the "spontaneous" suppressor activity was observed in our system.

Teleologically, it would be advantageous for fetal suppressor T cells to selectively suppress maternal lymphocyte function without affecting its own. Such selective suppression does occur as demonstrated by the discriminatory inhibition of adult but not allogeneic cord T cell proliferation in a 2-way MLC and their response

to PHA (Olding et al 1977). Furthermore, Andersson et al (1983) reported that IgM production by Epstein-Barr virus-driven adult B cells but not cord B cells was suppressible by $T4^+$ suppressor cells. Employing yet another approach, we found that cord T cells produced an antigen- nonspecific and MHC-unrestricted suppressor inducer factor upon stimulation with alloantigens which suppressed the adult but not the cord lymphocyte response in MLC. The target of this factor were $T4^+$ cells (Cheng et al 1985). The present study suggests that the selective inhibition by cord T cells is not due to an intrinsic resistance of cord lymphocytes to suppression since Con A-activated adult $T8^+$ cells suppressed adult and cord lymphocyte responses to the same extent. Based on these data and the finding that PWM-activated cord suppressor T cells belonged to the $T4^+$ subset, it is of interest to speculate that cord lymphocytes are resistant only to $T4^+$ cell mediated suppression. Thus, the utilization of $T4^+$ suppressor cells instead of $T8^+$ cells provides cord lymphocytes a method to selectively inhibit adult immune responses. This may explain why prostaglandin E2, which activates predominantly $T8^+$ suppressor cells (Chouib et al 1984), inhibited only adult T cell responses to PHA at physiological concentrations (Johnsen et al 1983b).

CHAPTER 6

GENERAL DISCUSSION

In this section, results presented in the previous chapters will be related in a cohesive fashion. Certain observations that have not been adequately addressed will also be discussed.

The objectives of our work were to characterize the fetal/neonatal suppressor cells and to evaluate their effect on adult as well as neonatal lymphocyte function by in vitro methods. It was hoped that the results from such studies may increase our understanding of the modus operandi of these suppressor cells which are thought to (1) play a role in the protection of the fetus against maternal lymphocytes and (2) contribute to the relatively deficient functional state of fetal/newborn lymphocytes.

In this study, cord suppressor cell activity was examined by assessing its effect on the autologous or allogeneic lymphocyte response to mitogens or third party alloantigens. These assays evaluated primarily the antigen-nonspecific rather than the antigen-specific type of suppression which might involve different cell subpopulations with different modes of action (van Tol et al 1983; Stanokova and Rola-Pleszczynski 1984). The decision for studying the antigen-nonspecific suppressor activity of neonatal lymphocytes was based on the following reasons. First, the antigen-specific response required monocyte-T lymphocyte MHC linkage which is difficult to achieve in human experimentation due to the limited accessibility of specimens. Second, antigen-nonspecific type of

suppression has the theoretical advantage of eliminating the need for selective immunosuppression of maternal responses to a potentially large number of paternal histocompatibility antigens by the fetus. Furthermore, antigen-nonspecific suppression can be induced by both antigen-specific and antigen-nonspecific (polyclonal) lymphocyte activation (Fischer et al 1983) suggesting that this form of suppression may be of more biological importance in the fetal-maternal immunologic relationship.

To determine the role of neonatal suppressor cells in the causation of the relatively deficient state of the neonatal immune response, we first examined the functional status of B and helper T cells by stimulating various combinations of these cells with their respective adult counterparts with PWM or EBV. The results indicate that neonatal B cells are functionally immature with the IgM-producing precursor B cells being at a relatively more advanced stage of maturation and consequently, more responsive to stimuli than the other immunoglobulin isotype-producing precursor cells. The exact cause of the deficient B cell response is not certain although a lack of receptors for T helper cell replacing factor (TRF) on neonatal B cells (demonstrated in murine neonatal lymphocytes) may provide a partial explanation for their inability to respond, at least to the T cell-dependent B cell stimulators (Schimpl A et al 1982). This could also explain our observation that the B cell proliferative response to PWM was not impaired despite their deficient immunoglobulin

production. An interesting paper by Miller et al (1984) which claimed that neonatal B cells could be induced to respond to PWM if interleukin-1 was simultaneously added also lends support to the notion that TRF receptor deficiency may be a cause of neonatal B cell immaturity since interleukin-1 has been shown to induce TRF receptors on B cells (Leibson et al 1981).

Culture supernatants of mitogen- or alloantigen-activated lymphocytes from the majority (70%) of cord blood specimens were found to contain adequate helper factors (Chapter 2). When cord lymphocytes were challenged with T cell-dependent antigens, normal helper T cell function was observed (van Tol et al 1983). Other investigators have also shown that mitogen-stimulated cord T cells produced interleukin-2 comparable in amount to that by adult T cells (Hayward and Kurnick 1981; Paganelli 1981; Miyawaki et al 1985). These results indicate that neonatal helper T cells are, by and large, functionally mature. However, since not all newborn T cells exhibited helper activity in our experiments, the rates of T cell maturation are probably different among individuals. Perhaps the conflicting reports concerning cord helper T cell function in the literature simply reflect the asynchronous state of maturation of these cord helper T cells.

It could be argued that the observed helper T cell activity of cord blood lymphocytes might have been produced by the contaminating maternal T cells. This possibility is unlikely since the amount of

contamination is usually not enough to account for the significant helper activity generated by cord lymphocytes (Zarou et al 1964). More importantly, maternal lymphocytes are usually suppressed by fetal suppressor cells in mixed cultures (Olding et al 1974) and are therefore unable to provide substantial help.

Studies in mice have shown that the poor antibody response by newborn lymphocytes was partly due to an excessive endogenous suppressor T cell activity which not only inhibited the helper T cell function but also T cell-independent B cell responses (Mosier and Johnson 1975). This has also been assumed to be the case in humans by most investigators. Careful examination of the data from human studies, however, revealed little evidence that would confirm this assumption. On the other hand, several groups including ourselves, have provided evidence to suggest that cord lymphocytes are quite resistant to their strong suppressor T cell action. For example, the antibody responses of PWM-stimulated cord B cells were comparable regardless of whether adult T cells or cord T cells (which contained strong suppressor T cells) were employed to provide helper activity (Olding et al 1977; Cheng et al 1984). Also, when cord T cells were used to suppress B cell antibody response to EBV, only adult B cells were found to be susceptible to inhibition (Andersson et al 1981; Cheng et al 1984). These results, together with those reported by Olding and his colleagues that cord T cells could selectively inhibit adult but not allogeneic cord T cell proliferation (Olding and

Oldstone 1976; Olding et al 1977) all point to the refractory nature of cord lymphocytes to the neonatal T cell-mediated suppression.

Although we favour the view that the cord B cells' deficient immune response is due to their inherent functional immaturity rather than their inactivation by autologous suppressor T cells, it could be argued that cord B cells might have already been inactivated in utero by the autologous suppressor T cells and therefore were not amenable to further suppression by these cells. Longitudinal study of lymphocyte functions of infants and children, however, showed that neonatal suppressor T cell activity disappeared long before B cell responses reached adult levels, thus, making the first view more plausible.

Initially, when suppressor factor(s) was found in the alloantigen- and PWM-activated cord T cell culture supernatants, we presumed that it was a suppressor effector factor through which the suppressor function of cord T cells was carried out. Therefore, we proceeded to study the properties of this factor by examining its antigen specificity and target. Since the factors in the alloantigen- and PWM-activated T cell culture supernatants may not be identical, we elected to study the former because the condition in which it was produced mimicked the in vivo event where fetal lymphocytes are challenged by those maternal lymphocytes that have crossed the placental barrier into the fetal circulation. It was found that this

factor suppressed adult lymphocytes in an MHC-unrestricted and antigen-nonspecific manner as illustrated by its inhibitory effect on allogeneic adult lymphocyte responses to alloantigens (in MLC), Candidin and PWM. Its suppressor effect was only apparent when T cells were also present indicating that it is an inducer of suppressor factor (TisF) rather than a suppressor effector factor. Furthermore, the target of this factor was a radiosensitive and $T4^+$ suppressor cell subpopulation.

At least 2 populations of T lymphocytes were involved in the production of TisF. Both were radioresistant and expressed T4 phenotype but only one was $TQ1^+$. It appears that TisF production was independent of monocytes since adherent cell-depletion from the cord T cells did not alter their TisF production. Between 30-40% of our cord lymphocyte preparations were capable of producing detectable quantities of TisF in 48 hr culture supernatants without stimulation from PWM or alloantigen. This finding suggests that TisF-producing cells may have already been activated in utero and is consistent with the radioresistant character of these TisF-producing cells, a property usually observed in activated lymphocytes (Schrek 1961; Lowenthal et al 1985). Hence, in vivo, these TisF-producing cells may function as memory cells after the initial exposure to allogeneic cells; upon subsequent encounter, they would activate the suppressor effector T cells, through production of TisF, to inhibit the invading allogeneic lymphocytes.

A schematic representation of the interactions among the various cell types in the neonatal suppressor T cell circuitry is shown in Figure 1. It should be emphasized that although both adult and cord suppressor effector cells are considered to be targets of TisF, only the former has actually been shown to be the target in our study. Cord suppressor effector T cells were only assumed to be the targets of TisF based on their similarities to the adult suppressor effector T cells (i.e., they were both radioresistant and possessed the $T4^+$ phenotype). The possibility remains that cord suppressor effector T cells may act independently of TisF and could be part of a different suppressive mechanism of cord lymphocytes. This could explain our observation that TisF-induced suppression was always weaker than cord T cell-induced suppression (50-60% versus 70-90%, respectively). Alternatively, the different magnitude of suppression exerted by TisF compared to cord T cells could be attributed to the difference in potency and dose-response requirement for TisF between adult and cord suppressor T cell as discussed in Chapter 4. In order to determine which of the 2 possibilities is correct, examination of the activation requirement of cord suppressor effector T cells in the absence of TisF-producing cells will be necessary. Unfortunately, no simple method of separating these 2 subpopulations is available at the present. Perhaps with the availability of other T cell markers, it may be possible to identify and isolate one factor from the other in the near future and enable us to resolve this issue.

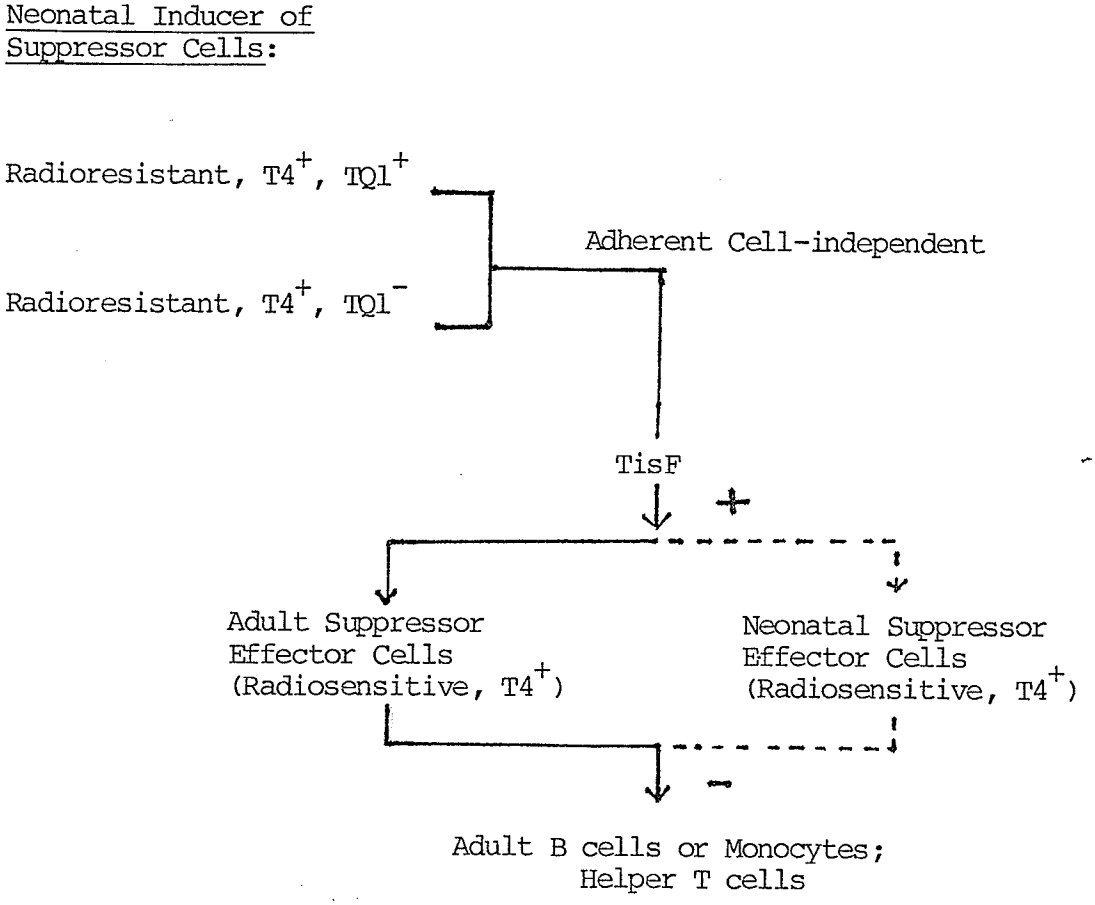


Figure 1. A proposed model of neonatal suppressor cell circuit.

The ultimate target of cord lymphocyte-mediated suppression, be it through the activation of adult or cord suppressor effector T cells, remains to be determined. When alloantigen- or PWM-activated cord lymphocyte culture supernatants were added to adult lymphocytes, their response to PWM was suppressed in spite of helper factors being present in these supernatants. Similarly, when adult B cells were cocultured with cord T cells, little immunoglobulin production was observed despite the adequate helper function of cord T cells. Furthermore, when adult B cells were directly stimulated by EBV, a T cell-independent stimulator, their response was also suppressed when cord T cells were added. These results indicate that adult non-T cells (B cells/monocytes) are the prime targets of cord T cell-mediated suppression. The question of whether adult helper T cells are also susceptible to cord T cell inhibition cannot be answered directly from the data available. However, in view of the suppressor effect of TisF on adult lymphocyte responses to alloantigen and Candidin (Chapter 3) as well as cord T cells' ability to suppress adult lymphocyte responses to PHA and alloantigen (Olding et al 1974), all of which are primarily T cell stimulators, it is safe to assume that adult helper T cells are also amenable to cord lymphocyte-mediated suppression. In mice, it has been shown that the adult spleen cell antibody response to T cell-dependent antigens is more sensitive to suppression by neonatal lymphocytes than its response to T cell-independent antigens (Luckenbach et al 1978; Murgita et al 1978). This phenomenon can be interpreted to suggest that both adult

B cells and helper T cells are targets of suppression by the neonatal lymphocytes. Therefore, in the case of the antibody response to T cell-dependent antigens, because both B cells and helper T cells were suppressible by neonatal lymphocytes, the degree of suppression was naturally stronger than in the case of T cell-independent B cell responses. Further investigation employing quantitative analysis of helper factor production by adult T cells under the influence of TisF may help to determine the susceptibility of adult helper T cells to neonatal T lymphocyte-mediated suppression more directly.

In our experiments, mononuclear cells that did not form rosettes with sheep erythrocytes were considered as B cells. Since these preparations contained 30-50% of monocytes the direct suppressive effect of TisF-activated adult or cord suppressor effector T cells on adult "B" cells observed in our experiments may actually require monocytes as intermediaries. Evidence in the literature regarding the role of monocytes in cord lymphocyte-mediated suppression is conflicting. While some investigators found that adherent cell-depletion from both cord and adult lymphocytes did not affect the formers' suppressor effect on the latter (Andersson et al 1983; Jacoby and Oldstone 1983), others have shown that adherent cells were necessary for the effector phase of cord T cell-directed suppressor activity (Miyawaki et al 1981b). Preliminary data from our experiments using TisF as a source of suppression support the results

of the latter group since the suppressor effect of TisF on PWM-activated adult lymphocytes could be substantially decreased with prior depletion of adherent cells from the adult lymphocytes. No doubt the different degrees of adherent cell-depletion achieved in the above studies was a deciding factor in determining the outcome of these experiments.

A striking feature of the cord T cell-mediated suppression is the prominent suppressor activity exhibited by cells bearing the T4 phenotype rather than those bearing the T8 phenotype. In adults, both $T8^+$ and $T4^+$, radiosensitive suppressor cells are present in the peripheral blood with the former being more potent than the latter (Thomas et al 1980). The reverse was observed in the neonate (Chapter 3). An analogous phenomenon has also been reported in mice in which newborn suppressor T cells were identified as Lyl^+2^- cells (Murgita et al 1978) bearing I-A and I-J gene-encoded products (Murgita and Wigzell 1981) rather than the Lyl^-2^+ suppressor cells detected in adults. This indicates that the newborn suppressor T cells represented either an inducer of suppressor cell subset or a specialized suppressor effector cell subpopulation which bore the marker of inducer T cells. Our study suggests that in human neonates, both the inducer of suppressors and the suppressor effector T cells are $T4^+$. Furthermore, it appears that the utilization of $T4^+$ suppressor effector cells by neonatal lymphocytes may be partly due to a compensatory mechanism for the functional immaturity of $T8^+$ cells

as evidenced by the low cytotoxic T cell ($T8^+$) activity (Granberg and Hirvonen 1980; Palacios and Andersson 1982a) and deficient Con A-induced suppressor cell ($T8^+$) activity (Chapter 5) of neonatal T cells.

It has been suggested that the reason for the failed neonatal suppressor cell induction by Con A was because the Con A-inducible neonatal suppressor T cells have already been 'spontaneously' activated (Durandy et al 1979; Dwyer and Johnson 1982). Phenotypic analysis of the neonatal suppressor cells and the Con A-induced suppressor cells, however, does not support this suggestion since the former was found to be in the T4 subset while the latter was almost exclusively $T8^+$ (Chapter 5). Functional immaturity of neonatal monocytes as a possible cause of cord suppressor T cell unresponsiveness to Con A induction is also unlikely because it has been shown that the role of monocytes in mitogen-induced T cell responses is primarily that of facilitation in the form of interleukin-1 supplementation (Katzen et al 1985; Oudrhiri et al 1985; Roosnek et al 1985a, 1985b) which is adequate in newborns (Ziabinger et al 1983). Our observation of the comparable proliferative responses to Con A by adult and cord T cells further strengthens the notion that the deficient suppressor activity of Con A-stimulated cord lymphocytes was not due to their inability to respond to the mitogen; rather, it was due to the functional immaturity of their $T8^+$ suppressor cells. Other immunoregulatory agents which were reported

to activate $T8^+$ suppressor cells such as PGE2 (Gualde et al 1982; Chouaib et al 1984; Rocklin et al 1985) and histamine (Beer and Rocklin 1984) may be helpful in future studies to verify our hypothesis.

Based on the data from our study and those in the literature, it would appear that fetal suppressor lymphocyte function is carried out mainly by the $T4^+$ suppressor cells with a gradual shift to the $T8^+$ suppressor cell-predominated adult pattern in the postnatal life. The significance of this $T4^+$ suppressor cell dominance is not clear although it would be interesting to speculate that this arrangement may offer selective protection of the fetal lymphocytes from the autologous T cell suppressor activity. In order to mount a T cell-dependent antigen-specific immune response, at least 3 T cell surface structures namely, T3, Ti (clonotypic) and T4 or T8 molecules are necessary. T3 and Ti molecules form a T3-Ti complex which serves as an antigen receptor while T4 or T8 molecule acts as an associative recognition structure which facilitates T cell interaction with the non-polymorphic regions of Class II or Class I MHC gene products, respectively, on its target cells. Thus, the $T4^+$ helper cell recognizes antigens that are presented by macrophage/monocyte in the context of Class II MHC gene-encoded products on the macrophage while the $T8^+$ suppressor cell interacts with helper T cell through its Class I MHC gene-encoded molecules (Romain and Schlossman 1980; Damle

et al 1985). In the case of the fetus and newborn, Class II antigen expression on monocytes and lymphocytes is substantially lower than that on the adult cells (Yokoi et al 1982; van Tol et al 1984; Durandy et al 1985). This is especially so for activated T cells which have only 5-6% of them expressing Class II antigens. Since cord suppressor T cells are predominantly $T4^+$ cells that interact more efficiently with cells expressing Class II MHC gene-encoded antigens, the paucity of these antigens on cord lymphocytes may render them resistant to $T4^+$ cell-mediated suppression. Another possible explanation for the dominance of $T4^+$ cells in cord T cell-mediated suppression is that $T4^+$ suppressor cells may exert their suppression through the elaboration of immunoregulatory factors different from those of $T8^+$ suppressor cells. These factors may have differential effects on adult and cord lymphocytes leading to preferential inhibition of adult lymphocyte function without affecting cord lymphocyte immune response.

We are aware of the reports which showed $T8^+$ cells as the predominant suppressor cells in cord blood (Hayward and Lydyard 1978; Rodriguez et al 1981; van Tol et al 1983; Durandy et al 1985). Others, including ourselves, could not substantiate these findings (Yachie et al 1981; Andersson et al 1983; Jacoby and Oldstone 1983; Cheng et al 1985). This difference is difficult to reconcile based on technical grounds alone and could be due to the presence of contrasuppressor cells in the $T8^+$ subset of cord lymphocytes which counteracted the $T8^+$ suppressor cells thus making the overall effect

of $T8^+$ cells unpredictable. Inherent in this hypothesis are the assumptions that contrasuppressor cells can only protect lymphocytes from $T8^+$ cell-mediated but not $T4^+$ cell-mediated suppression and in the absence of contrasuppressor cells, $T8^+$ cell-mediated suppression prevails with resulting inactivation of the autologous $T4^+$ suppressor cells. Alternatively, a shift in the expression of T cell surface markers from T4 to T8 may explain the conflicting results regarding the phenotype of cord suppressor T cells. Indeed, such a phenomenon has been observed in adult $T4^+$ lymphocytes and cultured lymphoblasts (Birch et al 1982; Burns et al 1982; Goodwin 1984; Packmann et al 1985).

At this point, it is worthwhile to digress momentarily to review the literature on contrasuppressor cells. The existence of the contrasuppressor cell circuit was originally proposed by Gershon and coworkers in the mouse. Using antibody responses to sheep erythrocytes (a T cell-dependent antigen) as a test system, they showed that $Ly2^+$, $I\text{-}J^+$ T cells (contrasuppressor inducer), with the help of cells that expressed $Ly1^+2^+$, $I\text{-}J^+$, Qal^+ phenotype, activated a third subset of T cells to confer resistance to helper T cells against suppressor T cell action. This last population of T cells possessed the $Ly1^+$, $I\text{-}J^+$ phenotype, could bind Vicia villosa lectin, had minimal helper function and was termed contrasuppressor cells by the investigators (Gershon et al 1981; Green et al 1981; Yamauchi et al 1981). Subsequently, contrasuppressor cells were also

demonstrated in the contact hypersensitivity response (Ptak et al 1981), the graft versus host reaction (Skowron-Cendrzak et al 1983) and the T cell-independent B cell antibody response (Braley-Mullen 1984). Ontogenic development study showed that the contrasuppressor activity was especially potent in the neonatal period, when the contrasuppressor cells emerged from the thymus between 1-2 days postpartum; the high level activity lasted for about 2 weeks before declining to a lower level (Skowron-Cendrzak et al 1983). It was thought that the contrasuppressor cells may play a role in the protection of the neonates against the immunosuppressive effect of autologous suppressor cells. With the use of histamine receptor agonists, Gershon's group also reported that contrasuppressor cells could be distinguished from the suppressor cells by their differences in sensitivity to stimulation by H1 and H2 agonists (Siegel et al 1982).

In contrast to the mouse, the evidence for the existence of contrasuppressor cells is not as clear in humans. Lehner et al, employing a rather complex system, postulated the existence of contrasuppressor cells to explain their experimental observations (Lehner 1983; Lehner and Jones 1984; Lehner et al 1985a, 1985b). Their putative contrasuppressor cells belonged to the $T8^+$ subset, were rich in Ia antigens and bound to Vicia villosa lectin. In addition, they also possessed the function of antigen presentation to $T4^+$ helper cells. Using a different experimental system, Thomas et al (1984) found that when peripheral blood lymphocytes were stimulated

by PWM for 60-70 hr, the activated radioresistant, $T8^+$ lymphocytes exhibited dual functions. If these $T8^+$ cells were added to fresh lymphocytes consisting of B cells and an optimal number of $T4^+$ cells (to provide help), suppression of B cell response to PWM would ensue. Otherwise, if the $T8^+$ cells were added to fresh B cells with suboptimal numbers of $T4^+$ cells, enhancement of the B cell response would occur. These results were interpreted by the authors to suggest that in the presence of a low level $T4^+$ signal, the contrasuppressor circuit predominated and the $T8^+$ contrasuppressor inducer cells would activate the $T4^+$ contrasuppressor cells to negate the suppressor cell activity.

In view of the above data, one has to concede that, as yet, no solid proof for the presence of contrasuppressor cells exists in humans. We had attempted to detect the contrasuppressor activity, based on the work in the mouse (Siegel et al 1982), by preferentially stimulating the putative contrasuppressor and suppressor cells with H1 and H2 agonists, respectively, but were unable to achieve consistent results. Trials with H1 and H2 receptor blockers also failed to produce meaningful results. Perhaps, the contrasuppressor cell effect observed by various investigators was related to the ability of these activated T cells to induce fresh T cell proliferation resulting in the generation of helper T cell factors (Chiorazzi et al 1979; Yu et al 1980). Certainly more work is required to (1) ascertain the existence of contrasuppressor cells in humans; (2) characterize these

contrasuppressor cells; (3) examine their possible interactions, if any, with the fetal/neonatal suppressor cells.

A considerable number of studies have been undertaken to determine the functional status of neonatal helper and suppressor cell activity by examining the relative proportion of $T4^+$ and $T8^+$ cells based on the assumption that $T4^+$ cells represented inducer/helper cells and $T8^+$ cells represented suppressor/cytotoxic cells. Unfortunately, the results varied from study to study and, in general, did not show any significant difference from those of adults (Rodriguez et al 1981; Yachie et al 1981; Jacoby and Oldstone 1983; Johnson and Dwyer 1983; Maccario et al 1983; Zola et al 1983; Foa et al 1984; Griffith-Chu et al 1984). Considering the functional heterogeneity of the cells in these 2 subsets, however, it is doubtful if such an approach will yield much valuable information on the functional status of the neonatal lymphocytes. Nevertheless, several interesting observations did emerge from these phenotypic studies. It was shown that a significant number of cord T cells expressed T6, a common thymocyte antigen (Griffith-Chu et al 1984) and T10, an antigen found on thymocytes and activated T cells (Foa et al 1984). In immature fetuses, as much as 25% of peripheral blood T cells coexpressed T4 and T8 antigens; this proportion gradually decreased with gestation until term when only a small proportion of $T4^+/T8^+$ cells remained (Griffith-Chu et al 1984; Solinger 1985). Another interesting observation was that a sizeable number of neonatal T cells

did not express receptors for sheep erythrocytes ($T11^-$); they were $T8^+$, $T3^-$ and PNA^+ (bind peanut agglutinin) (Ferrari et al 1981; Maccario et al 1983). These observations indicate that a considerable number of cord T lymphocytes express unique patterns of surface antigen distribution some of which are hallmarks of immature lymphocytes. Whether these cells are truly immature T cells with no functional significance or represent specialized T cells that have distinct immunoregulatory capabilities (e.g., natural suppressor cells) (Arai et al 1983; Clement et al 1984) awaits further investigation. At any rate, since our data indicate that both the neonatal inducer of suppressor and suppressor effector cells were E^+ ($T11^+$), $T4^+$ and $T8^-$, they are unlikely to be the E^-, $T8^+$, $T3^-$, PNA^+ or the $T4^+$, $T8^+$ cells described in the literature although the possibility of them expressing T6 and/or T10 antigens cannot be excluded. If that is the case, monoclonal antibodies against T6, T10, 2H4 (specific for adult inducer of suppressor T cells) and Leu 8 (an antigen closely related to TQ1) antigens may be used to further characterize and differentiate the 2 subsets of inducer of suppressor cells ($TQ1^+$ and $TQ1^-$) from the suppressor effector cells. This will, in turn, enable us to dissect these various subsets and study their mode of action and activation both individually and in association with each other.

In our study, cord inducer of suppressor cells were shown to activate the suppressor effector cells through the elaboration of a

soluble factor TisF. Other investigators have also described the involvement of immunoregulatory factors in cord T cell-mediated suppression. A promising candidate is PGE2, which is known to have the immunoregulatory properties (Goodwin and Webb 1980). The original study of Olding et al (1977) indicated that cord T cells suppressed adult lymphocyte proliferative response to PHA through the release of a low molecular weight factor. Subsequently, Johnsen et al (1982) showed that the cord T cell-mediated suppression could be blocked by indomethacin suggesting that the suppressor factor may be PGE2. This gave impetus to further experimentation along the same path culminating in a small clinical study (consisting of 9 mothers and 3 newborns) in which lymphocytes from pregnant women who were in premature labour and were given indomethacin, and their offsprings were tested for suppressor activity (Durandy et al 1985). Not one of the 3 newborns who received indomethacin in the prenatal period showed discernible suppressor activity by their lymphocytes. By contrast, control newborn lymphocytes that were not exposed to indomethacin in utero but were treated with indomethacin in vitro exhibited strong suppressor activity. These results suggest that PGE2's role in cord T cell-mediated suppression is in the induction phase. Furthermore, analysis of arachidonic acid metabolites produced by cord lymphocytes revealed that activation by PHA or alloantigen had virtually no effect on the production of metabolites implying that cord suppressor T cells had already been activated in utero and required no further activation (Johnson et al 1983b). Therefore, it is apparent that PGE2 and the

TisF described by us resembles one another in that they both acted at the inductive phase of cord T cell-mediated suppression and that they were both produced by cells that had been activated in utero. However, they also differ from each other in one aspect: while TisF could significantly inhibit PWM-induced adult B cell response, PGE2 had no suppressive effect on such a response (Morito et al 1979; Durandy et al 1985) and, therefore, the 2 factors are unlikely to be identical. It is conceivable that cord T cells exert their suppressor effect through the elaboration of several factors and each has its own distinct mechanism of suppression and, depending on the assay system, one will be more prominent than the other. Alternatively, the putative significance of PGE2 in cord T cell-mediated suppression may be merely an artifact since most studies employed prostaglandin synthetase inhibitors which had actions of their own. In addition, the more direct approach of measuring arachidonic acid metabolite production by cord lymphocytes showed no difference compared to their maternal control. Regardless of what role PGE2 has in the generation of T cell-mediated suppression, it undoubtedly plays a part in the downregulation of Ia antigen expression on cord B cells, monocytes and activated T cells (Yokoi et al 1982; van Tol et al 1984; Durandy et al 1985) and possibly also the cord suppressor T cell response to Con A stimulation (Goodwin 1980).

Another immunoregulatory factor that is relevant to our discussion is the cord T cell-derived suppressor factor (CTSF)

obtained by culturing cord T cells in the presence of PWM (Nagaoki et al 1980; Miyawaki et al 1981a, 1981b). The factor was dialyzable, had a molecular weight between 8,000-20,000 daltons, was stable at 56°C for 30 min but sensitive to heating at 80°C for 10 min. It was capable of suppressing the PWM-stimulated adult B cell response but in contrast to the TisF, acted on B cells through the activation of monocytes and, therefore, appeared to be a product of suppressor effector cells.

Clearly, further studies such as phenotypic characterization of PGE2- and CTSF-producing cells as well as biochemical analysis of TisF are crucial to the advancement of our understanding of the relationships among these different immunoregulatory factors and the various cell types involved in the generation and execution of neonatal T cell-originated suppression. Although biochemical analysis of TisF was not performed in the present study as this constitutes a major project by itself, the functional characterization of the various lymphocyte subsets that was undertaken provides us with a framework for future investigation of the neonatal suppressor T cell circuit.

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